HOW DO METABOLISM, ANGIOGENESIS, AND HYPOXIA MODULATE RESISTANCE?

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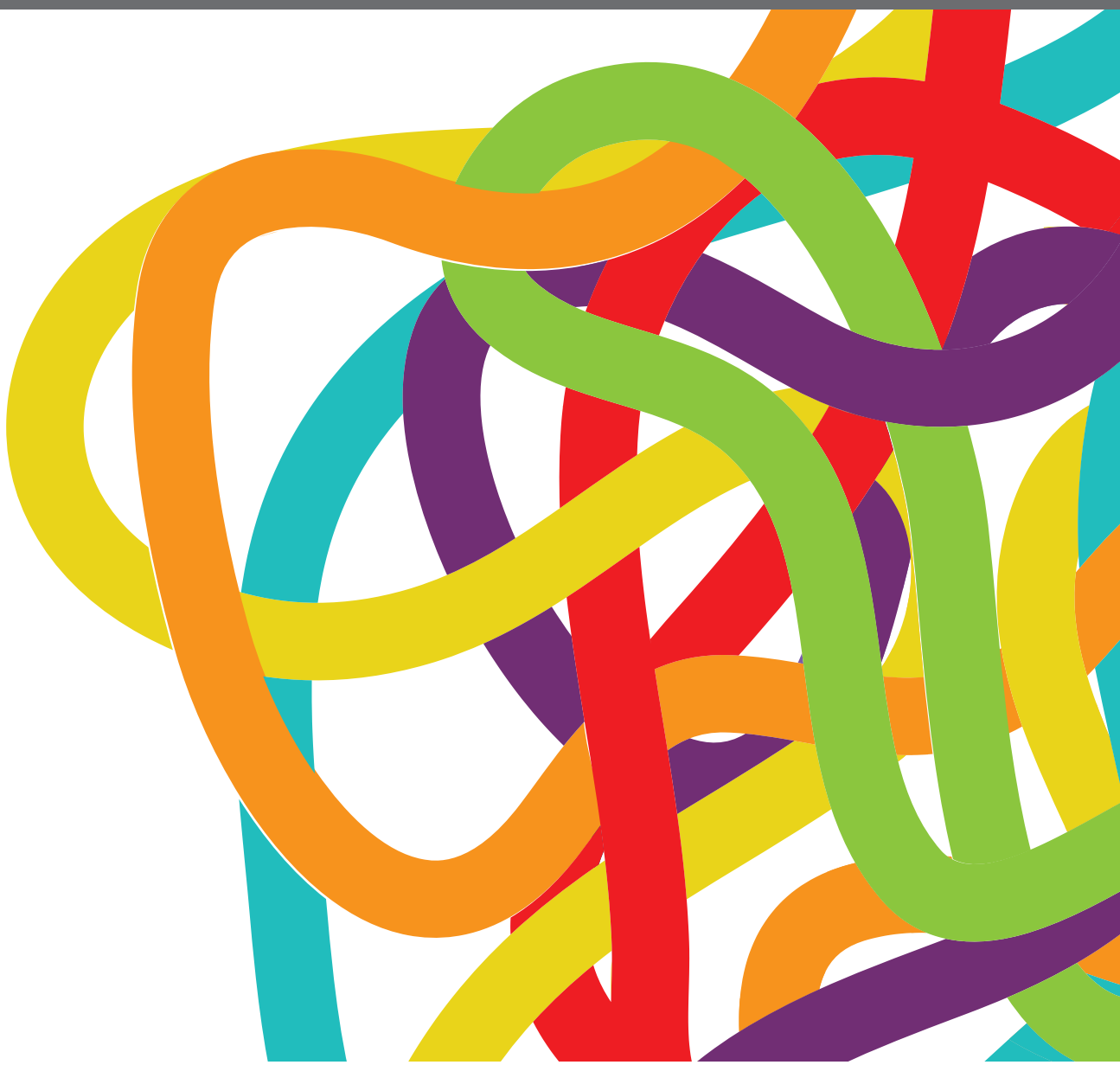

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HOW DO METABOLISM, ANGIOGENESIS, AND HYPOXIA MODULATE RESISTANCE?

Topic Editors:
Matilde Esther LLeonart, Vall d'Hebron Research Institute (VHIR), Spain
Josep Castellvi, Vall d'Hebron University Hospital, Spain
Hiroshi Kondoh, Kyoto University, Japan

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EDITORIAL
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Editorial: How Do Metabolism, Angiogenesis, and Hypoxia Modulate Resistance?

Hiroshi Kondoh[1], *Josep Castellvi*[2] *and Matilde Esther LLeonart*[3,4]*

[1] Geriatric Unit, Kyoto University Hospital, Kyoto, Japan, [2] Pathology Department, Vall d'Hebron Hospital, Barcelona, Spain, [3] Biomedical Research in Cancer Stem Cells Group, Vall d'Hebron Research Institute (VHIR), Barcelona, Spain, [4] Spanish Biomedical Research Network Centre in Oncology, CIBERONC, Madrid, Spain

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Editorial on the Research Topic

How Do Metabolism, Angiogenesis, and Hypoxia Modulate Resistance?

Metabolic alterations were among the first discovered hallmarks of cancer. They were first described 90 years ago when Otto Warburg realized that cancer cells in culture had a relatively increased metabolic rate (the Warburg hypothesis). It has been proposed that the drastic changes seen in cancer metabolism are in part attributed to mutations in the mtDNA, metabolic reprogramming, or mitochondrial dysfunction. However, novel players in cancer metabolism are emerging. In this regard, the review of Fernández et al. describes how lipidic alterations impact cancer prognosis and response to treatment. For example, it has been described that obesity increases the risk of cancer death, possibly due to the consequences of lipid accumulation throughout a lifetime. Lipid accumulation changes the microenvironment and produces chronic inflammation by increasing several cytokines. While the levels of genetic or epigenetic modifications diverge in different cancer types, all cancer cells adapt to drastic microenvironmental conditions. This adaptation entails metabolic reprogramming to cope with scarce nutrients and oxygen. Lipid metabolism sustains cancer initiation and contributes to cancer progression and therapy resistance. The role of lipids has been underestimated, as they have largely been considered scaffolds of biological membranes. In recent decades, the role of lipids in cancer has emerged in parallel to the characterization of lipids as essential components of cell signaling, redox homeostasis control, and energy sources (i.e., ß-fatty acid oxidation).

Moreover, while *de novo* synthesis of fatty acids and cholesterol is restricted to the liver and adipocytes in normal cells, cancer cells can synthesize such components. This altered lipid metabolism affects key steps involved in the metastatic process, like migration, invasion, and angiogenesis, and can also be associated with prognosis. Moreover, Fernández et al. provide a list of preclinical and clinical studies with bioactive compounds from natural sources to target lipid metabolism and associated risk factors in cancer.

Tumor adaptation to hypoxia is another important aspect that modulates resistance in cancer. Hypoxia is a forced situation where oxygen levels are different from normal physiological conditions. Hypoxia occurs in higher or minor levels in most cancers, if not all. The detection of hypoxic areas by clinical imaging would improve cancer chemotherapeutic treatments and optimal radiotherapy planning. The technique of positron emission tomography (PET) measures cancer metabolism and cellular proliferation, but it can also measure blood flow and oxygen use. PET can

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Michael P. Lisanti,
University of Salford Manchester,
United Kingdom

****Correspondence:***
Matilde Esther LLeonart
matilde.lleonart@vhir.org

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identify patients who would be good candidates for molecularly targeted chemotherapies and can be used to monitor response to these personalized therapies. The primary PET radiotracers for this are ^{18}F-fluorodeoxyglucose (for observation of abnormal energy metabolism) and ^{18}F-fluorothymidine (for evaluation of cell proliferation). In clinical trials, the current candidate of PET tracers for hypoxia is ^{18}F-labeled fluoromisonidazole. In this context, Lu et al. aimed to design a novel nitroimidazole derivative to detect hypoxic regions in tumors, which is important to predict resistance to therapy. These authors observed that Al^{18}F-NOTA-NI is a novel nitroimidazole tracer. The major advantage of this new tracer is the quick elimination from normal tissue and its retention in hypoxic cancer tissue. Therefore, it has great potential in the planning of new and efficient therapies.

To identify the new molecular markers of hypoxia with translational relevance, Zhang et al. describe the increase in the expression of the non-coding RNA Inc-NEAT1 under hypoxic conditions in the hepatocellular carcinoma model (HCC), which is among the tumors with the worst life expectancies. Using *in vitro* (i.e., RNA immunoprecipitation and luciferase reporter assays) and *in vivo* (mice model) approaches, Zhang et al. characterize the role of the miR-199a-3p/uridine-cytidine kinase 2 (UCK2) axis and its functional association with Inc-NEAT1. These authors propose that the coordinated pathway that involves IncRNA-NEAT1, miR-99a-3p, and UCK2 upregulation in HCC is a potential signaling cascade and contributes to HCC progression under hypoxic conditions, making it a suitable drug target.

On the other hand, cancer stem cells (CSCs) are the most representative cell type resistant to radio and chemotherapies. Espinosa-Sánchez et al. performed an exhaustive review of various canonical and non-canonical CSC pathways. In addition to the pathways associated with the Yamanaka factors, such as Wnt signaling, the Notch pathway, or Sonic Hedgehog; they also added novel signaling players with a relevant role in resistance, which includes the Hippo pathway, NF-KB signaling, and Toll-like receptors. An overview of the current chemotherapeutic drugs against each specific gene comprised in these signaling routes is shown. Furthermore, these authors propose that cross-talk among different CSC pathways drives the resistance to single pathway inhibitors, enabling CSCs to maintain their CSC phenotype. This aspect should be considered in CSC-directed therapies.

To identify possible cancer markers at the preventive level, Feliciano et al. reported a microRNAs signature (miR-125b, miR-29c, miR-16, miR-1260, and miR-451) in the serum of breast cancer patients that can distinguish women with cancer from healthy individuals. Most of the microRNAs included in the genetic signature (predictor) are related to stemness and resistance. This is the case for miR-125b, miR-29c, miR-16, and miR-451, which have been associated with resistance in various cancer types and cellular models. Interestingly, the predictor described by Feliciano et al. was able to detect the risk of 11 healthy women to potentially develop breast cancer in the future. Moreover, to corroborate the expression of microRNAs with protein expression levels in serum (proteomic study), the low expression of miR-16 was correlated with elevated levels of the CD44 protein. Feliciano et al. describe the stem-related marker CD44, so far identified in the serum of triple-negative breast cancer patients (the subtype of breast cancer with high mortality), as a marker present in the other subtypes of breast cancer with less aggressiveness (luminal breast cancer A or B). Detecting resistance/stem markers in serum will help predict cancer in healthy individuals and identify which patient subgroups are at most risk of recurrences.

The success of personalized therapy for cancer patients has attracted much attention. Autophagy has a decisive role in several cellular functions, and its dysregulation is associated with cancer progression, tumor-stroma interactions, CSC maintenance, and resistance to therapy. A growing body of evidence shows that autophagy is a key regulator in the tumor microenvironment and cellular drug response. Two extensive review articles have focused on the function of autophagy in cancer resistance. Alvarez-Meythaler et al. reinforce the role of chemotherapeutic treatment failure as the main cause of tumor resistance. If the tumor cell population is extremely heterogeneous, certain cells would not respond properly to standard chemotherapeutical treatments. Tumor heterogeneity comprises different tumor cell subpopulations and the interactions with stroma and other immune or fibroblast cell types. Alvarez-Meythaler et al. propose a model by which autophagy-directed therapies can be determinant to avoid the propagation of resistant cell variants when applied in the first-line therapy in combination with standard chemotherapeutic treatments.

Chavez-Dominguez et al. describe the dual role of autophagy in cancer as a tumor suppressor or oncogenic mechanism. They attributed the dual autophagy function mainly to the evolution of the tumor (tumor stage). Chavez-Dominguez et al. claim that in early tumorigenesis, autophagy acts as a tumor suppressor mechanism aiming to destroy those molecules or organelles defective in proliferative cells. While in advanced tumors – especially when the metastatic spread has reached stage III or IV– autophagy acts as an oncogenic driver. In this case, the interaction of several pathways induced by hypoxia, metabolism, and tumor microenvironment contribute to burst autophagy.

Another aspect to consider is that the tumor microenvironment modulates tumor evolution and growth and can also actively participate in conditioning the therapeutic response. This is because – depending on the cell types involved and the cyto- and chemokines released into the extracellular medium – a pre-metastatic niche can emerge in the tumor microenvironment. In this sense, Benavente et al. propose novel strategies to enhance the antitumor immune response, with a special focus on radiotherapy. Strategies to enhance tumor perfusion can increase tumor immunogenicity. Angiogenesis, desmoplasia, and inflammation promote leakage and compression of tumor vessels. Vascular normalization strengthens the vessel wall by reducing intercellular gaps and improving perfusion. Decompression of blood vessels by

depleting cancer-associated fibroblasts or extracellular matrix reperfuses the vessel and increments perfusion. Overall, reprogramming the tumor microenvironment to an immunomodulatory state augments antitumor immunity. This can be especially relevant to optimize treatment immunogenicity, improving patient outcomes.

Lastly, there have been major advances in massive sequencing technology, known as next-generation sequencing (NGS). Fernández-Rozadilla et al. describe how, with its ultra-high throughput, speed, and scalability, NGS allows researchers to scrutinize genetic and gene expression information at an unprecedented level. Fernández-Rozadilla et al. highlight the importance of NGS in the revolution of biomedical research. NGS was developed just over a decade ago. Since then, it has been used not only for the diagnosis and prognosis of tumors but also to identify the best chemotherapy treatments.

Overall, although additional basic and clinical research is needed to identify additional regulatory proteins and how they interact to contribute to cancer resistance, in the medium-short term, we expect the FDA to approve new drugs targeting the revised pathways described in this special issue.

AUTHOR CONTRIBUTIONS

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ORIGINAL RESEARCH
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Hypoxia-Induced lncRNA-NEAT1 Sustains the Growth of Hepatocellular Carcinoma via Regulation of miR-199a-3p/UCK2

Qiangnu Zhang[1,2†], *Qian Cheng*[3†], *Mengting Xia*[4], *Xiaotao Huang*[4], *Xiaoyan He*[5] *and Juan Liao*[3*]

[1] Department of Hepatobiliary and Pancreas Surgery, The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University, Guangzhou, China, [2] Integrated Chinese and Western Medicine Postdoctoral Research Station, Jinan University, Guangzhou, China, [3] Department of Gastroenterology, West China School of Public Health and West China Fourth Hospital, Sichuan University, Chengdu, China, [4] North Sichuan Medical College, Nanchong, China, [5] Department of Pathology, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou, China

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Edited by:
Hiroshi Kondoh,
Kyoto University, Japan

Reviewed by:
Maria Louca,
University of Cyprus, Cyprus
Dhiraj Kumar,
University of Texas MD Anderson Cancer Center, United States

***Correspondence:**
Juan Liao
juanliao@scu.edu.cn

[†]These authors have contributed equally to this work

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Objective: The long noncoding RNA (lncRNA) nuclear paraspeckle assembly transcript 1 (NEAT1) has emerged as a novel player in hepatocellular carcinoma (HCC). Hypoxia is a common characteristic of the microenvironment of HCC. This study aimed to investigate whether lncRNA-NEAT1 is induced by hypoxia in HCC, and the mechanism that underlies LncRNA-NEAT1 function.

Methods: The expression changes of lncRNA-NEAT1 in HCC cell lines under hypoxic conditions were examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The regulatory effect of HIF-1α on lncRNA-NEAT1 was confirmed with chromatin immunoprecipitation (ChIP) and luciferase reporter assays. The function of lncRNA-NEAT1 on HCC cell growth under hypoxic conditions was determined by CCK-8 assay and flow cytometry. lncRNA -NEAT1 was predicted to serve as a competing endogenous RNA (ceRNA) within microRNA (miRNA)/mRNA axes based on microarray data, public HCC-related datasets and integrative bioinformatics analysis, and the miR-199a-3p/UCK2 axis was selected and validated by qRT-PCR, western blotting, RNA immunoprecipitation, and luciferase reporter analyses. The role of miR-199a-3p/UCK2 in HCC and its functional association with lncRNA-NEAT1 were assessed both *in vitro* and *in vivo*.

Results: LncRNA-NEAT1 expression was significantly induced by hypoxia in SNU-182 and HUH7 cells. HIF-1α was shown to regulate lncRNA-NEAT1 transcription. Under hypoxic conditions, lncRNA-NEAT1 maintained the growth of HCC cells and inhibited apoptosis and cell cycle arrest. LncRNA-NEAT1 was predicted to regulate a panel of HCC-associated miRNA-mRNA pairs consisting of 8 miRNAs and 13 mRNAs. LncRNA-NEAT1 was shown to function as a ceRNA of miR-199a-3p/UCK2 both in HCC cells under hypoxic conditions and in an animal model.

Conclusion: LncRNA-NEAT1 is a hypoxia-responsive lncRNA in HCC cell lines Insilico evidence suggested that LncRNA-NEAT1 may sustainthe growth of HCC cells by

regulating HCC-associated mRNAs that interact with tumor-suppressive miRNAs. The lncRNA-NEAT1/miR-199a-3p/UCK2 pathway may contribute to the progression of HCC cell lines in a hypoxic microenvironment and therefore may represent a novel therapeutic target for HCC.

Keywords: hepatocellular carcinoma, LncRNA-NEAT1, microRNA, UCK2, hypoxia

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed primary cancers of the liver and the leading cause of cancer-related mortality worldwide, accounting for more than 780,000 deaths in 2018 (1). Although progress has been made in recent years in diagnostic, treatment, and screening technologies, HCC is often diagnosed at advanced stages, and in most cases, advanced HCC patients miss the best surgical window (2). Unfortunately, the effects of chemoradiotherapy, targeted therapy, and immunotherapy against HCC are rather limited, with dismal survival rates for most HCC patients (3). Therefore, there is an urgent need to identify novel targets and molecular markers for the diagnosis and treatment of HCC. Concerted efforts are needed to better understand the pathophysiological mechanisms underlying the development and progression of HCC.

Abnormal vascular networks surrounding solid tumors and the requirement of excess oxygen for rapid growth of cancer cells can lead to hypoxia, which is a common characteristic of the microenvironment of solid tumors (4). At the cellular level, hypoxia induces angiopoiesis, metabolic reprogramming, epithelial–mesenchymal transition, remodeling of extracellular mechanisms, stemness, and immune escape. Clinically, hypoxia contributes to the aggressive clinical characteristics of HCC and resistance to both radiotherapy and chemotherapy (5, 6). Genes, especially those that are regulated by hypoxia inducible factors (HIFs), as well as signal transduction related to the hypoxic microenvironment, have been a common focus over the last decade. Nevertheless, it is still a challenge to treat HCC by targeting hypoxia.

Considerable evidence suggests that hypoxia regulates long non-coding RNA (lncRNAs) that involved in the onset and progression of various cancers. Hypoxia responsive lncRNAs may play clinical roles on patients' progression and prognosis by regulating proliferation, migration, invasion, and therapy resistance of cancer cells (7, 8). For instance, Deng et al. found that lncRNA-BX111887 transcription is induced by HIF-1α in response to hypoxia, which enhances the proliferation and invasion of pancreatic cancer cells (9). LncRNA-MALAT1 expression is also dramatically increased in HCC cells in response to hypoxic conditions, whereas knock-down of MALAT1 counteracts the tumor-promoting effect of hypoxia (10). Thus, elucidation of the roles of lncRNAs under hypoxic conditions is crucial to better understand the onset, features, and poor clinical outcome of HCC.

The lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) has been reported as a novel player in the onset and progression of HCC. Overexpression of NEAT1 drives HCC progression and has been correlated with the poor prognosis of patients (11, 12). Furthermore, Choudhry et al. demonstrated that lncRNA-NEAT1 activation in response to hypoxia promotes the survival of breast cancer cells (13). However, the activation of lncRNA-NEAT1 in response to hypoxia has not been elucidated in HCC, and lncRNA-NEAT1-regulated downstream pathways are not well-established. Therefore, in the present study, we investigated the response of lncRNA-NEAT1 to hypoxia and revealed its mechanism, which involves transcriptional regulation by HIF-1α. Potential miRNA and mRNA targets of lncRNA-NEAT1 were selected and filtered by an integrative bioinformatics analysis approach that was based on numerous HCC-related datasets. Moreover, candidate miRNA and mRNA targets were validated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Culture and Hypoxic Conditions

HCC cells (SNU-182 and HUH7) were obtained from the American Type Culture Collection (Manassas, VA, USA) and routinely cultured in Roswell Park Memorial Institute 1640 (for SNU-182 cells) or Dulbecco's Modified Eagle's Medium (for HUH7 cells) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Hyclone Laboratories, Inc., South Logan, UT, USA) under an atmosphere of 5% CO_2 at 37°C. As a model for hypoxia, SNU-182 and HUH7 cells were cultured under an atmosphere of 1% O_2/5% CO_2/94% N_2 for 24 h. we also treated cells with $CoCl_2$, a hypoxia mimetic agent (14), to simulated hypoxia. The $CoCl_2$ treatment performed under normoxic condition.

Cell Transfection

Cells were transfected with the plasmid pcDNA3.1-NEAT1 to up-regulate lncRNA-NEAT1 expression, while the plasmid pcDNA3.1-UCK2 was used to overexpress UCK2. The empty plasmid pcDNA3.1 served as a negative transfection control. All plasmid were obtained from GenePharma(Shanghai, China). Small interfering RNA for lncRNA-NEAT1 (siRNA-NEAT1) UCK2 (siRNA-UCK2) and HIF-1α (siRNA- HIF-1α) were used to silence their expression (Genepharma, Shanghai, China). To upregulate candidate miRNAs, miR-mimics were obtained. AllStars Negative Control siRNA was used to transfect cells with siRNA and miR-mimics (Qiagen, Hilden, Germany). Prior to experimentation, the cells were transfected for 24 or 48 h. To obtain stably transfected SNU-182 cells, lentiviral vectors were prepared by Genechem Company (Shanghai, China) and used to deliver lncRNA-NEAT1 (Lv-NEAT1), miR-199a-3p (Lv-miR-199a-3p), and shRNA-UCK2 (Lv-shRNA-UCK2).

Successful transfection was confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis after 48 h (**Supplementary Figure 1**).

qRT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA), quantified, and reverse transcribed into complementary DNA (cDNA) using PrimeScript™ RT Master Mix and the PrimeScript™ RT Reagent Kit (Takara Bio, Inc., Shiga, Japan). Then, the cDNA samples were analyzed using the SYBR® Premix Ex Taq™ II Kit (Takara Bio, Inc., Shiga, Japan) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. For miRNA analysis, cDNAs were generated and amplified using the Mir-X™ miRNA First-Strand Synthesis and qRT-PCR TB Green® Kit (Takara Bio, Inc., Shiga, Japan). U6 was used as reference for miRNA analysis. The PCR protocol was conducted in accordance with the manufacturer's instructions using the primers shown in **Supplementary Table 1**.

Western Blot Analysis

Total protein samples were prepared in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease inhibitor cocktail. Samples with equal amounts of protein were loaded into the wells of a 12% polyacrylamide gel and separated by electrophoresis. Then, the protein bands were transferred onto nitrocellulose blotting membranes, which were blocked with 5% fat-free milk and incubated with primary antibodies (Abcam, Cambridge, UK) against UCK2 (dilution, 1:1000) and GAPDH (1:2000) for 12 h at 4°C. After incubation with IRDye® secondary antibody (LI-COR Biosciences, Lincoln, NE, USA), the protein bands were imaged using the Odyssey® Infrared Imaging System (LI-COR Biosciences).

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP analysis was conducted using the ab500 ChIP Assay KIT with anti-HIF-1α antibody (Abcam, Cambridge, UK) in accordance with the manufacturer's instructions. Immunoglobulin G (IgG) served as a control. The resulting DNA fragments were amplified by PCR with the primers listed in **Supplementary Table 1**. The PCR products were analyzed by electrophoresis.

RNA Immunoprecipitation (RIP) Assay

RIP analysis was performed using the Imprint® RNA Immunoprecipitation Kit (Sigma-Aldrich Corporation, St. Louis, MO, USA) in accordance with the manufacturer's instructions, with an antibody against argonaute-2 (AGO2, Abcam, Cambridge, UK). IgG served as a control. The purified RNAs were then subjected to qRT-PCR analysis.

Luciferase Reporter Assay

The 3′ untranslated region of the wild-type (WT) or mutant lncRNA-NEAT1 (or UCK2) sequence was inserted into the psiCHECK-2 luciferase reporter vector (Promega Corporation, Madison, WI, USA). After 48 h of co-transfection with the luciferase reporter vector and miR-199a-3p mimic (or AllStars Negative Control), the cells were lysed, and luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega Corporation). *Renilla* luciferase activity was normalized to firefly luciferase activity. SNU-182 cells or HIF-1α knock-down cells were transfected with luciferase reporter vectors containing the WT or mutant putative hypoxia response element (HRE) sequence (ACGTGC) and then treated with $CoCl_2$ for 24 h. A similar luciferase reporter assay was performed to assess the effect of HIF-1α on the promoter of lncRNA-NEAT1.

Proliferation Analysis

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Sigma-Aldrich Corporation). In brief, 1×10^4 cells were seeded into the wells of 96-well plates and cultured for 24 h. Then, adherent cells were cultured under normoxic or hypoxic conditions. After 24, 48, or 72 h of culture, the cells were incubated with 10% CCK-8 reagent at 37°C for 1 h. Cell viability was determined by measuring the absorbency at 450 nm. The relative proliferation rate was calculated as the cell viability at 24, 48, or 72 h/cell viability at 0 h. The viability of untreated adherent cells was assessed at 0 h.

Flow Cytometry Analysis

At 48 h after transfection or 24 h under hypoxic conditions, the cells were harvested and washed with phosphate-buffered saline. The proportion of apoptotic cells was determined by flow cytometry with an Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, Shanghai, China). For cell cycle analysis, harvested cells were fixed with 70% cold ethanol for 12 h and then treated with propidium iodide for 30 min. The proportion of apoptotic cells and the cell cycle distribution were measured by flow cytometry using a FACSCalibur™ Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using the FlowJo™ platform for flow cytometry analysis (version 10; FlowJo LLC, Ashland, OR, USA).

Microarray Analysis

SNU-182 cells were transfected with pcDNA3.1-NEAT1. After 48 h, RNA was collected and analyzed by Agilent Whole human genome Microarray and Human miRNA Microarray, Release 21.0 (Agilent Technologies, Santa Clara, CA, USA). The differentially expressed mRNAs and microRNAs (fold change>1.5 and $P < 0.05$, control *vs.* pcDNA3.1-NEAT1 transfection) were identified using R with Limma package.

Animal Tumor Model

Twelve BALB/c nude mice, aged 4–6 weeks, were purchased from Biolite Biological Engineering Co., Ltd. (Nanjing, China) and subcutaneously injected with SNU-182 cells stably transfected with Lv-NEAT1, Lv-miR-199a-3p, Lv- shRNA-UCK2, or Lv-control (5×10^6 cells/mouse, $n = 3$ for each group). Tumor diameters were measured every 3 days. The tumor volumes were calculated as $0.5 \times (\text{length} \times \text{width}^2)$. All mice were sacrificed on day 24, and the tumors were resected and weighed. All animal experiments were performed in accordance with the guidelines of

the Research Animal Care Committee of Zhengzhou University (Zhengzhou, Henan, China).

Statistical Analysis

Statistical analysis was performed with R software (version 3.5.3; https://www.r-project.org/). Normally distributed data are presented as the mean ± standard deviation. Non-normally distributed data are presented as median values. The *t*-test was used to identify significant differences between two sets of normally distributed data, while one-way analysis of variance was used identify differences among multiple groups. Non-normally distributed data were analyzed using the Mann–Whitney *U*-test. The significance of survival data was determined using the log-rank test. A probability (p) value of <0.05 was considered statistically significant.

Bioinformatics Analysis

Liver cancer transcriptome profiling data were downloaded from The Cancer Genome Atlas (TCGA) database (https://www.cancergenome.nih.gov). The expression profile data of one miRNA (GSE36915) and 7 mRNAs (GSE14520, GSE22058, GSE25097, GSE36376, GSE45436, GSE64041, and GSE76427) of HCC patients were retrieved from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). Differentially expressed genes and miRNAs were extracted from each dataset with the Limma package of R software (version 3.5.3). The RobustRankAggreg package of R was used for integrated analysis of the expression profile data of the 7 mRNAs. Survival analysis was performed using the "survival" package, and data visualization was performed using the "ggplot2" package of R. The open-source online software (ENCORI v1.0) provided by Encyclopedia of RNA Interactome database (http://starbase.sysu.edu.cn/index.php) was used to predict lncRNA-NEAT1/miRNA interactions. miRWalk 2.0 online software (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) and TargetScan web server (http://www.targetscan.org/vert_72/) were used to predict miRNA-target interactions and the binding sites of candidate miRNAs and mRNAs. Gene ontology enrichment analysis were performed using online tools provided by DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/). microRNA pathway analyses were conducted using mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr/).

RESULTS

LncRNA-NEAT1 Is Induced by Hypoxia via HIF-1α in HCC Cells

To evaluate the response of lncRNA-NEAT1 to hypoxia, we cultured SNU-182 and HUH7 cells under hypoxic conditions (1% O_2) or treated them with the hypoxia mimetic agent $CoCl_2$. As shown in **Figures 1A,B**, both hypoxic conditions and $CoCl_2$ significantly increased lncRNA-NEAT1 expression in SNU-182 cells; however, knock-down of HIF-1α suppressed the response of lncRNA-NEAT1 to hypoxia. Additionally, inspection of the TCGA-LIHC database revealed that HIF-1α expression positively correlates with the lnc-NEAT1 level in HCC tissues (**Figure 1C**). Therefore, we speculated that transcription of lncRNA-NEAT1 might be regulated by HIF-1α. Inspection of the genomic sequence showed that there is an HRE (ACGTGC) in the lncRNA-NEAT1 3' upstream region that is predicted to bind HIF-1α (**Figure 1D**, left). We further validated the binding of HIF-1α to the lncRNA-NEAT1 promoter by ChIP assay in SNU-182 and HUH7 cells (**Figure 1D**, right). Additionally, dual-luciferase reporter assays verified that $CoCl_2$ increases the luciferase activity in cells transfected with plasmids containing the WT HRE sequence, but not a mutant sequence (**Figure 1E**). On the other hand, knock-down of HIF-1α reduced the luciferase activity in $CoCl_2$-treated cells for HRE-WT but not HRE-MUT reporter plasmids (**Figure 1F**). These data indicate that transcriptional upregulation of lncRNA-NEAT1 in HCC cells under hypoxic conditions is mediated by HIF-1α.

LncRNA-NEAT1 Sustains the Growth of HCC Cells Under Hypoxic Conditions

To assess the function of lncRNA-NEAT1 in HCC cells in under hypoxic conditions, we evaluated the effect of lncRNA-NEAT1 overexpression and knock-down. Overexpression of lncRNA-NEAT1 had no effect on the viability of SNU-182 cells under normoxic conditions; however, knock-down of lncRNA-NEAT1 inhibited cell viability (**Figure 2A**, left panel). Furthermore, under hypoxic conditions, cells transfected with pcDNA3.1-NEAT1 had higher proliferation rates, while knock-down of lncRNA-NEAT1 inhibited proliferation (**Figure 2A**, right panel). Consistently, knock-down of lncRNA-NEAT1 induced apoptosis of SNU-182 cells, while after 24 h of exposure to hypoxic conditions, siRNA-NEAT1 treatment increased the proportion of apoptotic cells, which was reduced by lncRNA-NEAT1 overexpression (**Figure 2B**). Similarly, overexpression of lncRNA-NEAT1 had no effect on the cell cycle under normoxic conditions. However, lncRNA-NEAT1 knock-down induced G1 arrest after hypoxia treatment for 24 h, and overexpression of lncRNA-NEAT1 antagonized this effect (**Figure 2C**). These results were confirmed in HUH7 cells (**Supplementary Figure 2**). Therefore, these findings suggest that lnc-NEAT1 increases the proliferation rate in HCC cells under hypoxic conditions by decreasing the apoptotic rate and promoting G1 arrest.

Identification of a Panel of HCC-Associated miRNAs and mRNAs That lncRNA-NEAT1 May Regulate as Competing Endogenous RNAs (ceRNAs)

Next, we investigated the mechanisms underlying the effects of lncRNA-NEAT1 in inducing cell growth under hypoxic conditions. LncRNAs are known to regulate mRNAs by sponging miRNAs as ceRNAs. Firstly, we used microarray analysis to identify the differentially expressed microRNAs and mRNAs ($>$ 1.5-fold with $P < 0.05$, compared with negative transfection control) after pcDNA3.1-NEAT1 transfection in SNU-182 cells. 63 microRNA were significantly down-regulated and 414 mRNA significantly up-regulated after lncRNA-NEAT1 overexpression (**Supplementary Tables 2, 3**). Next, we built a ceRNA network of potential lncRNA-NEAT1/microRNAs/mRNAs combinations

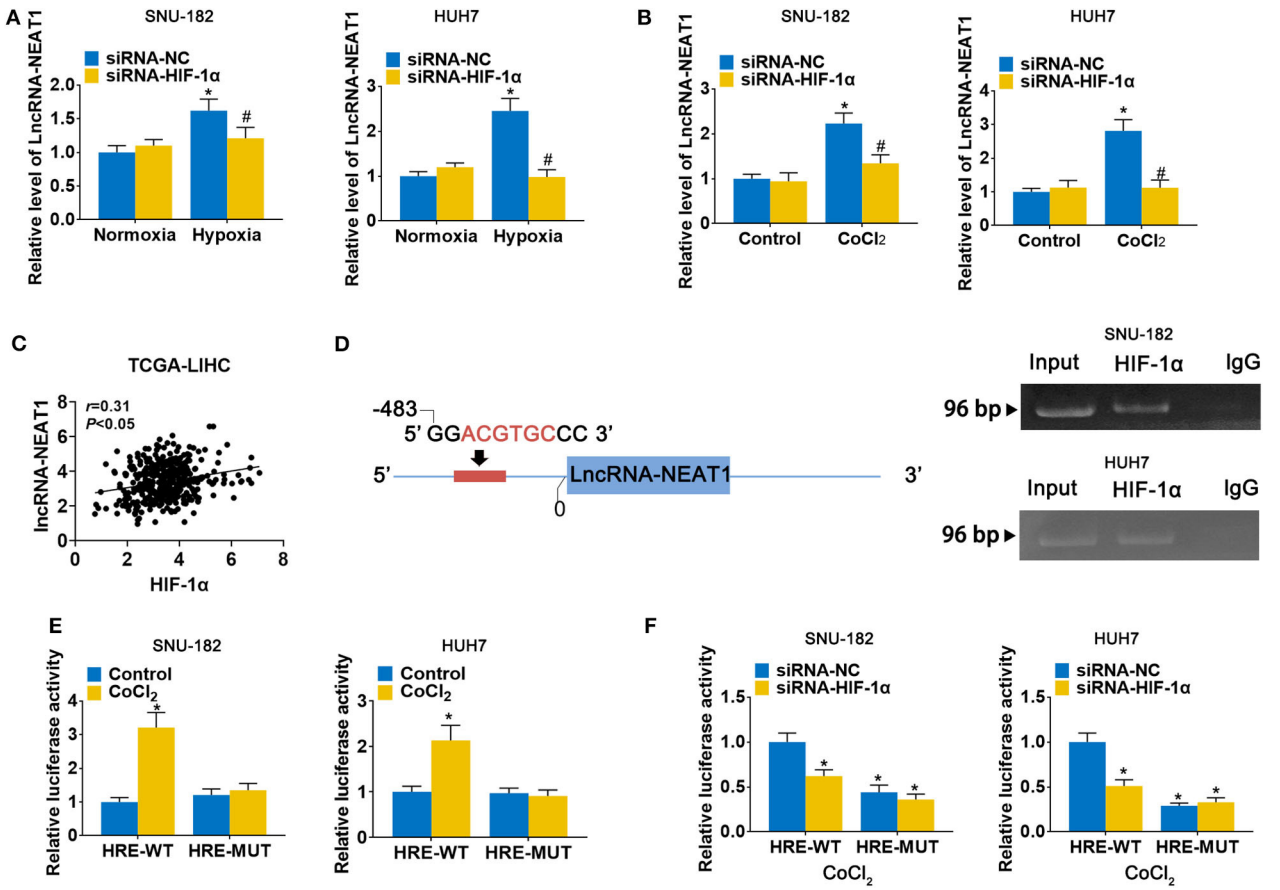

FIGURE 1 | LncRNA-NEAT1 expression is enhanced by hypoxia via transcriptional regulation of HIF-1α in HCC cells. **(A,B)** HIF-1α knock-down and control SNU-182 and HUH7 cells were cultured for 24 h under normoxic vs. hypoxic conditions (*Panel a*) or without and with $CoCl_2$ treatment (*Panel b*). Changes in the expression level of lncRNA-NEAT1 were detected by qRT-PCR. **(C)** The correlation of HIF-1α with lnc-NEAT1 in tumor tissues from TCGA-LIHC is shown. **(D)** *Left:* A putative HRE (ACGTGC) was identified in the promoter of lncRNA-NEAT1. *Right:* Binding of HIF-1α to the HRE (ACGTGC) was validated by ChIP assay in SNU-182 and HUH7 cells. HIF-1α antibody or IgG was added to the reaction. DNA fragments were amplified and analyzed by qRT-PCR with specific primers. **(E)** SNU-182 and HUH7 cells were transfected with a luciferase reporter containing the WT or mutant putative HRE (ACGTGC) sequence. Cells were treated with $CoCl_2$ for 24 h, where indicated, and relative luciferase activity was detected. **(F)** HIF-1α knock-down SNU-182 and HUH7 cells were transfected with a luciferase reporter containing the WT or mutant putative HRE (ACGTGC) sequence. Cells were treated with $CoCl_2$ for 24 h, and relative luciferase activity was detected. In *Panels a and b*, $^{*}P < 0.05$ compared with normoxia condition or control. $^{\#}P < 0.05$ compared with siRNA-negative control (siRNA-NC). In *Panels e and f*, $^{*}P < 0.05$ compared with normoxia control or siRNA-NC.

based on the microarray data and bioinformatics predictions (see **Supplementary Figure 2** for workflow). CLIP-Seq analysis data derived from online software provided by Encyclopedia of RNA Interactome database were used to identify miRNAs from our microarray analysis containing 7-mer or 8-mer seed matches that are predicted to bind to lncRNA-NEAT1. To further select HCC-associated miRNAs, the miRNAs were filtered with the use of the following criteria: (a) down-regulation by more than 1.5-fold ($P < 0.05$) in HCC tissues according to the GSE36915 dataset; and (b) reported in the literature as cancer suppressors in HCC. Eight candidate miRNAs conforming to these criteria were identified (miR-144-5p, miR-129-5p, miR-199a-3p, miR-214-5p, miR-483-3p, miR-486-5p, miR-542-3p, and miR-582-5p). Next, the targeted mRNAs of these 8 candidate miRNAs were predicted using integrated data from TargetScan and miRWalk 2.0. In total, 780 genes were predicted as target mRNAs of the 8 candidate miRNAs. We compared this list of 780 genes with the set of differentially expressed mRNAs measured in pcDNA3.1-NEAT1 transfected cells. To identify HCC-related genes in the overlapping mRNA list, results were further filtered using the expression profiles results obtained from 7 independent HCC-related public datasets (GSE14520, GSE22058, GSE25097, GSE36376, GSE45436, GSE64041, and GSE76427). An integrated list of differentially expressed genes (HCC-RRA-list) was obtained using the robust rank aggregation (RRA) algorithm with genes that were significantly up- or down-regulated (> 1.2-fold with $P < 0.05$, tumor tissue *vs.* non-tumor tissue) in all 7 HCC-related datasets. Thirteen genes in the overlapping mRNA list were also in the HCC-RRA-list and were up-regulated in HCC tissues. Finally, a potential HCC-associated lncRNA-miRNA-mRNA regulatory flow network

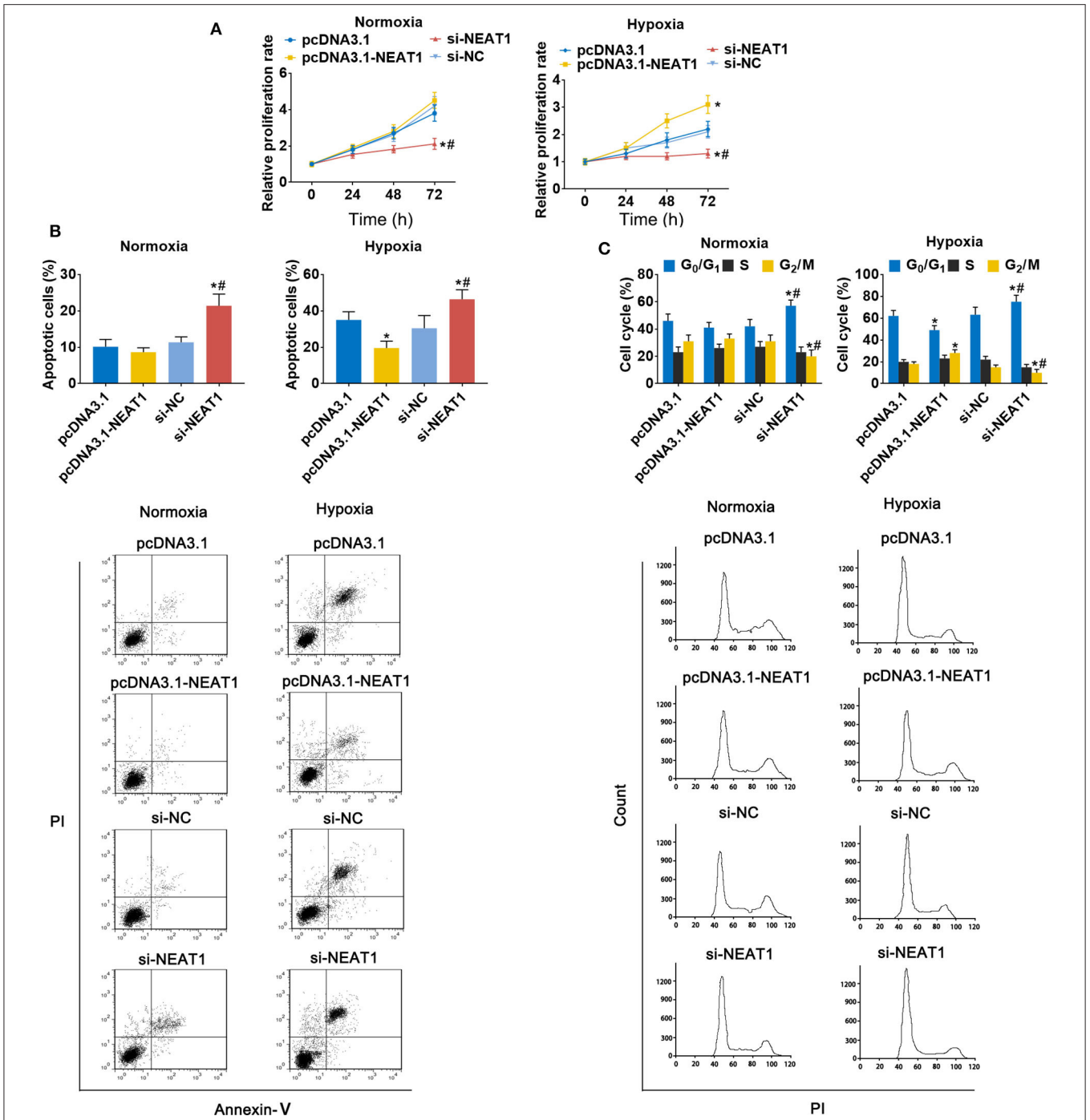

FIGURE 2 | LncRNA-NEAT1 sustains the growth of SNU-182 cells under hypoxic conditions. **(A)** The expression of lncRNA-NEAT1 was modulated by transfection with the pcDNA3.1-NEAT1 vector or siRNA-NEAT1. After transfection for 24 h, cells were cultured under hypoxic or normoxic conditions. Cell viability was detected using CCK-8 assay and used to estimate proliferation. **(B,C)** The roles of lncRNA-NEAT1 on apoptosis (*Panel b*) and the cell cycle (*Panel c*) in SNU-182 cells under hypoxic and normoxic conditions were assessed by flow cytometry. $^{*}P < 0.05$ compared with transfection negative control. $^{\#}P < 0.05$ compared with pcDNA3.1-NEAT1.

was assembled, comprised of lncRNA-NEAT1, 8 candidate miRNAs, and 13 candidate genes (**Figure 3A**). To better understand the potential biological function of the candidate miRNAs and mRNAs, functional enrichment analysis based on Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway databases was performed. As

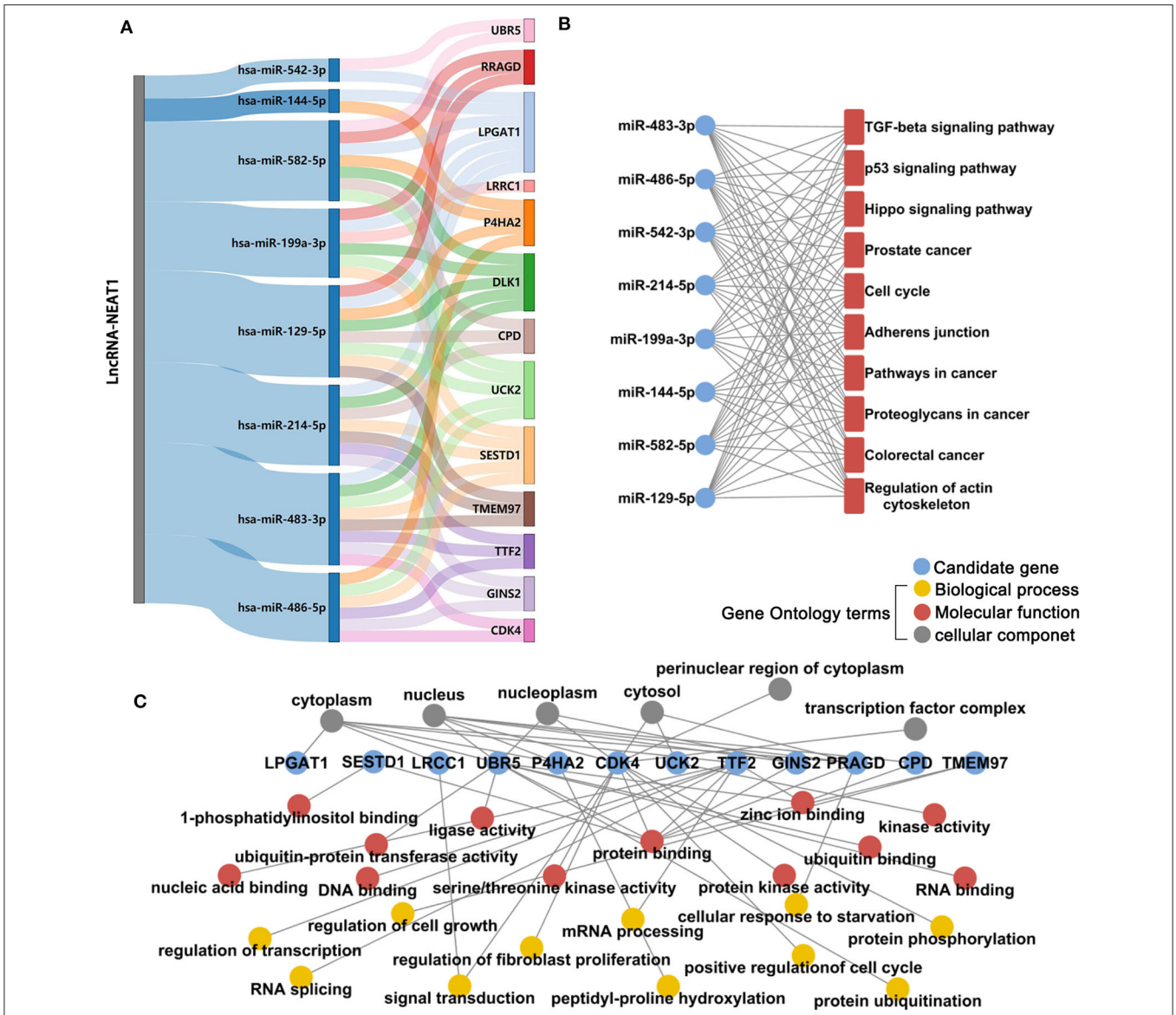

FIGURE 3 | Microarray and integrative bioinformatics analysis of HCC-associated miRNA-mRNA pairs that are potentially regulated by lncRNA-NEAT1. **(A)** Differentially expressed miRNAs and mRNAs in lnc-NEAT1 knock-down SNU-182cells were identified by microarray analysis. According to the ceRNA theory, miRNAs that may be sponged by lncRNA-NEAT1 and their possible target mRNAs were also predicted by bioinformatics analysis and filtered according to their expression patterns in 7 public HCC-related datasets. Candidate miRNA-mRNA pairs that passed each of these filters are shown in the Sankey diagram. The miRNAs that are shown are predicted to act as suppressors in HCC. **(B)** KEGG pathway enrichment analysis for the 8 candidate microRNA's target genes. **(C)** GO enrichment analysis for the 13 candidate genes. GO-biological process terms, -molecular function terms and -cellular component terms are shown.

shown in **Figure 3B**, the 8 candidate miRNAs were mainly associated with the typical tumor-associated pathways (e.g., cell cycle, p53 signaling pathway, pathway in cancers). The enriched GO-biological process terms, -molecular function terms and -cellular component terms are shown in **Figure 3C**. Among them, potential terms (e.g., "regulation of cell growth," "positive regulation of cell cycle," and "cellular response to starvation") further support a role for these miRNAs in tumor-related functions.

To further verify the potential role of the 8 miRNAs and 13 mRNAs in HCC, we evaluated their expression patterns reported in public databases. The fold changes (tumor tissue *vs.* non-tumor tissue) of the 8 candidate miRNAs in the GSE36915 and The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) datasets are shown in **Figure 4A**. Except for miR-129-5p, which was not included in the TCGA-LIHC dataset, all of the other candidate miRNAs were consistently down-regulated in HCC tissues in the GSE36915 and TCGA-LIHC datasets. Furthermore, all 13 genes were up-regulated in HCC tumor tissues compared with normal tissue ($>$ 1.5-fold with $P <$ 0.05), as confirmed in the TCGA-LIHC dataset (**Figure 4B**). The integrative fold changes of the 13 candidate genes among the

7 HCC-related public datasets are shown in **Figure 4C** (tumor tissue *vs*. non-tumor tissue). We further evaluated the potential roles of these genes in HCC prognosis. In the GSE14520 dataset, 7 of 13 candidate genes (UCK2, LRCC1, GINS2, CDK4, LPGAT1, UBR5, and DLK1) are risk factors for poor survival, while according to the TCGA-LIHC dataset, high expression of 7 of the 13 candidate genes (UCK2, LRRC1, TTF2, GINS2, CDK4, CPD, and SESTD1) predict poor survival of HCC patients (**Figure 4D**). These data indicate that lncRNA-NEAT1 may play a role in the progression of HCC through a panel of HCC-related miRNAs and mRNAs associated with HCC.

LncRNA-NEAT1 Regulates UCK2 by Sponging miR-199a-3p

Next, we sought to validate the predicted candidate lncRNA-NEAT1-miRNA-mRNA regulatory patterns. Among the 4 genes (UCK2, LRCC1, GINS2, and CDK4) that were identified to be associated with poor survival of HCC patients in both the GSE14520 and TCGA-LIHC datasets, UCK2 had the greatest number of predicted interactions with NEAT1-targeted miRNAs (miR-199a-3p, miR-483-3p, miR-486-5p, miR-582-5p, and miR-129-5p) and, thus, can be regarded as a potential hub mediator. Furthermore, UCK2 was found to be an impressive risk factor for survival (hazard ratio $= 2.2$, $P < 0.01$ in the GSE14520 dataset; hazard ratio $= 2.1$, $P < 0.01$ in the TCGA-LIHC dataset). Therefore, we sought to further validate the functional interaction of UCK2, along with miR-199a-3p, miR-483-3p, miR-486-5p, miR-582-5p, and miR-129-5p, with lncRNA-NEAT1. As shown in **Figure 5A**, only miR-199a-3p mimic influenced UCK2 mRNA expression in SNU-182 and HUH7 cells. The inhibitory effect of miR-199a-3p on UCK2 was confirmed by western blotting (**Figure 5B**). Furthermore, overexpression of lncRNA-NEAT1 was found to suppress the expression of miR-199a-3p (**Figure 5C**) and enhance UCK2 mRNA and protein expression (**Figures 5D,E**), while lncRNA-NEAT1 knock-down had the opposite effect. The role of miR-199a-3p in mediating the effect of lncRNA-NEAT1 on UCK2 expression was further verified by luciferase reporter assay results, which demonstrated that miR-199a-3p decreases luciferase activity in cells transfected with reporter plasmids containing either the NEAT1-WT or UCK2-WT sequences that are predicted to be binding sites for miR-199a-3p, but not corresponding mutant sequences (**Figures 5F,G**). In addition, the results of RIP assays demonstrated that lncRNA-NEAT1 and miR-199a-3p were both contained in complexes that were pulled down with AGO2 antibody (**Figure 5H**), which suggests that lncRNA-NEAT1 resides within RNA-induced silencing complexes that are involved in miRNA processing. Taken together, these data indicate that lncRNA-NEAT1 regulates UCK2 by sponging miR-199a-3p as a ceRNA.

LncRNA-NEAT1 Sustains the Growth of HCC Cells Under Hypoxic Conditions via the Regulation of miR-199a-3p/UCK2

To further evaluate the role of the lnc-NEAT1/miR-199a-3p/UCK2 axis in lnc-NEAT-1-mediated growth promotion, we repeated the MTT assays in cells transfect with UCK2 overexpression vector or miR-199a-3p mimic. Under normoxic conditions, UCK2 overexpression significantly enhanced cell proliferation, while miR-199a-3p overexpression produced opposite results. Furthermore, under hypoxic condition, UCK2 knock-down or miR-199a-3p overexpression significantly neutralized the sustaining effect of lncRNA-NEAT1 on cell proliferation (**Figure 6A**). The converse trend was observed in our evaluation of cell apoptosis, for which miR-199a-3p-mimic caused an increase in apoptosis under normoxic conditions, and si-UCK2 and miR-199a-3p neutralized the reduction in apoptosis levels mediated by lncRNA-NEAT1 overexpression under hypoxic conditions (**Figure 6B**). A similar trend was also observed for cell cycle arrest (**Figure 6C**), and these results were confirmed in HUH7 cells (**Supplementary Figure 4**), which suggests that UCK2 and miR-199a-3p have critical roles in lncRNA-NEAT1-induced HCC cell promotion under conditions of hypoxia.

LncRNA-NEAT1 Promotes HCC Tumor Growth Through miR-199a-3p/UCK2 *in vivo*

To determine whether lnc-NEAT contributes to HCC by a similar mechanism *in vivo*, we evaluated the effect of lncRNA-NEAT1, sh-UCK2 and miR-199a-3p expression in xenografted mouse tumors from SNU-182 cells. LncRNA-NEAT1 promoted the development of xenografted tumors (**Figure 7A**), which was evidenced by larger tumor volumes (**Figure 7B**) and higher tumor weights (**Figure 7C**). However, co-transfection of either miR-199a-3p mimic or sh-UCK2 inhibited these promotive effects of lncRNA-NEAT1. Thus, these findings support the role of the lnc-NEAT1/miR-199a-3p/UCK2 axis in HCC tumor growth *in vivo*.

DISCUSSION

Hypoxia-regulated lncRNAs play pivotal roles in the development of various cancers, including HCC, gastric cancer, and pancreatic cancer, by regulating cellular proliferation, invasion, metastasis, metabolism, and autophagy (15). As an example, Zhao et al. found that lncRNA-MALAT1 is significantly overexpressed in HCC cells under hypoxic conditions, whereas knock-down of MALAT1 weakened the promotive effect of hypoxia on cellular proliferation, migration, and invasion (10). Zhang et al. reported that lncRNA-PCGEM1 is induced in GC cells under hypoxic conditions and acts as an oncogenic factor (16), while up-regulation of lncRNA-BX111 in response to hypoxia promotes metastasis and progression of pancreatic cancer (9). In the present study, we confirmed the response of lncRNA-NEAT1 to hypoxia and demonstrated that lncRNA-NEAT1 is transcriptionally regulated by HIF-1α in HCC cells. Integrated analysis of public HCC-related datasets was performed to select a group of HCC-associated miRNA-mRNA pairs that could potentially be modulated by lncRNA-NEAT1 in a ceRNA-related manner. Moreover, the regulatory effects of lncRNA-NEAT1 on the miR-199a-3p/UCK2 axis in HCC were validated both *in vitro* and *in vivo*.

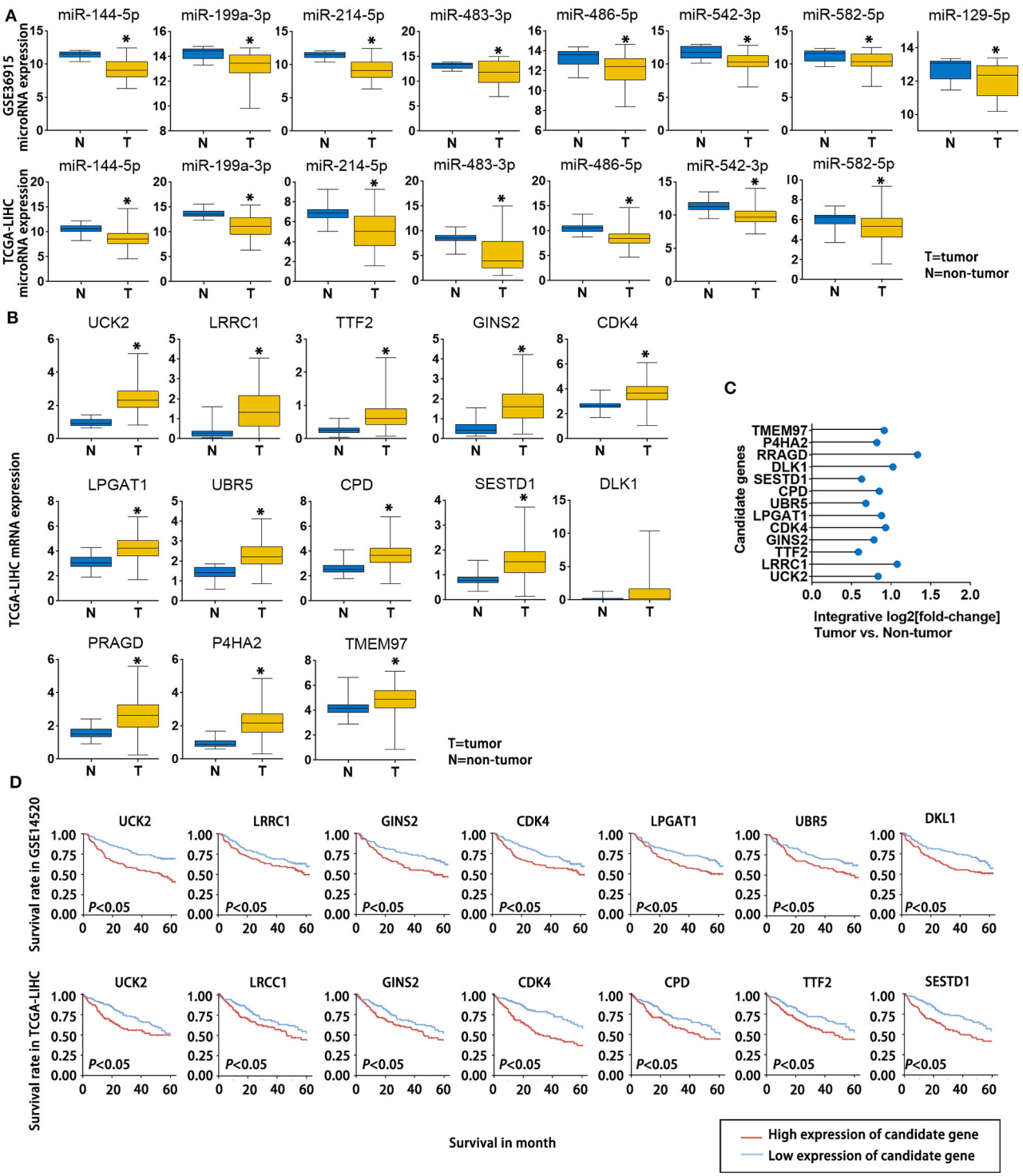

FIGURE 4 | Expression changes and survival relevance of HCC-associated miRNAs and mRNAs that are potentially regulated by lncRNA-NEAT1. **(A)** Expression differences of 8 candidate miRNAs between tumor and non-tumor tissues. Data are from the GSE36915 and TCGA-LIHC datasets. In GSE36915 n(Tumor) = 72, n (non-tumor) = 21. In TCGA-LIHC n(Tumor) = 375, n (non-tumor) = 50. **(B)** Expression difference of 13 candidate mRNAs between tumor and non-tumor tissues in the TCGA-LIHC dataset. n(Tumor) = 375, n (non-tumor) = 50. **(C)** Integrative fold change of 13 candidate mRNAs in tumor tissues as compared with non-tumor tissue in 7 independent HCC datasets obtained from the Gene Expression Omnibus database were calculated by the Robust Rank Aggregation method. **(D)** The associations of the 13 candidate genes with survival are shown using Kaplan–Meier survival curves based on data from the GSE14520 and TCGA-LIHC datasets. In GSE14520, n (High expression of candidate gene) = 123, n (low expression of candidate gene) = 124. In TCGA-LIHC, n (High expression of candidate gene) = 182, n (low expression of candidate gene) = 182.*$P < 0.05$ compared with normal tissue.

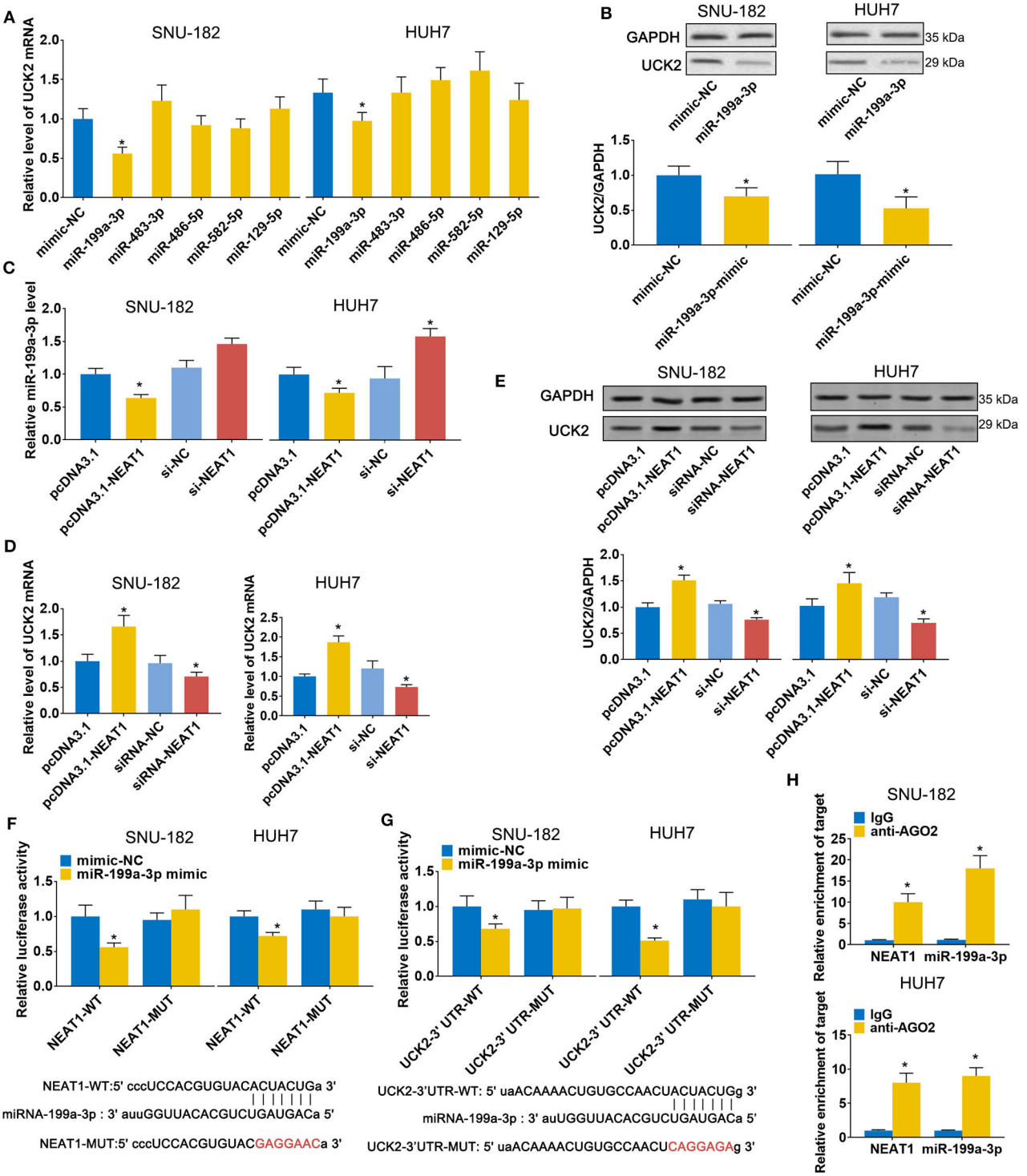

FIGURE 5 | LncRNA-NEAT1 regulates UCK2 by sponging miR-199a-3p in HCC cells. **(A,B)** SNU-182 and HUH7 cells were transfected with miR-mimics of miR-199a-3p, miR-483-3p, miR-486-5p, miR-582-5p, or miR-129-5p. After 48 h, the mRNA and protein expression changes of UCK2 were determined by qRT-PCR (*Panel a*) and western blot analyses (*Panel b*). **(C)** SNU-182 and HUH7 cells were transfected with pcDNA3.1-NEAT1, siRNA-NEAT1, or their respective controls. After 48 h of transfection, changes in miR-199a-3p expression levels were detected by qRT-PCR. **(D,E)** SNU-182 and HUH7 cells were transfected with pcDNA3.1-NEAT1, siRNA-NEAT1, or their respective controls. After 48 h of transfection, changes in UCK2 mRNA (*Panel d*) and protein expression levels (*Panel e*) were determined by qRT-PCR and western blot analyses. **(F,G)** The binding of lncRNA-NEAT1 to miR-199a-3p (*Panel f*) and miR-199a-3p to the 3' untranslated region of UCK2 (*Panel g*) were verified by luciferase reporter assay. Wild-type and Mutant sequences of NEAT1 or UCK2 3' UTR are shown at the bottom. **(H)** RIP assay was performed to further confirm whether lncRNA-NEAT1 regulates miR-199a-3p as a ceRNA. Cell lysates collected from SNU-182 and HUH7 cells were incubated with antibodies against AGO2 or IgG. Enrichment of lncRNA-NEAT1 and miR-199a-3p in purified RNA was detected by qRT-PCR. In *Panels a-g*, $^*P < 0.05$ compared with transfection negative control. In *Panel h*, $^*P < 0.05$ compared with IgG control.

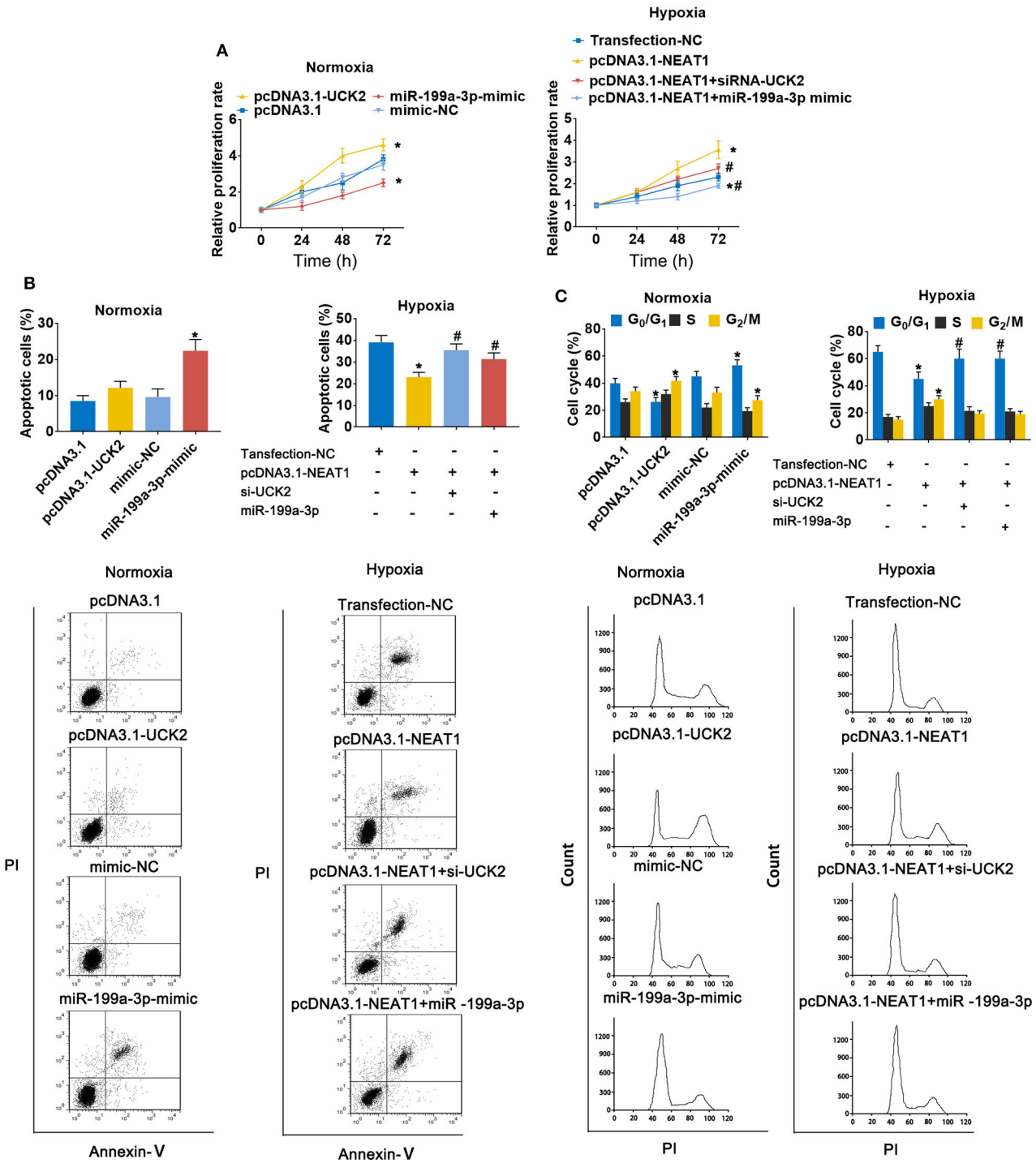

FIGURE 6 | LncRNA-NEAT1 sustains the growth of SNU-182 cells under hypoxic conditions by regulating miR-199a-3p/UCK2. **(A)** The expression levels of miR-199a-3p and UCK2 in SNU-182 were up-regulated by transfection under normoxic conditions; or miR-199a-3p or siRNA-UCK2 were co-transfected with pcDNA3.1-NEAT1 in SNU-182 cells under hypoxic conditions. The cell proliferation changes were determined by CCK-8 assay. **(B,C)** The effects of miR-199a-3p and UCK2 on apoptosis (*Panel b*) and the cycle (*Panel c*) of SNU-182 cells were determined by flow cytometry. $^{*}P < 0.05$ compared with transfection negative control. $^{\#}P < 0.05$ compared with pcDNA3.1-NEAT1.

LncRNA-NEAT1 has been established as a target for the diagnosis and treatment of various solid tumors. Elevated expression of lncRNA-NEAT1 drives tumor initiation and progression by regulating cellular growth, migration, invasiveness, epithelial-to-mesenchymal transition, and stemness (17, 18). Furthermore, lncRNA-NEAT1 has been reported to

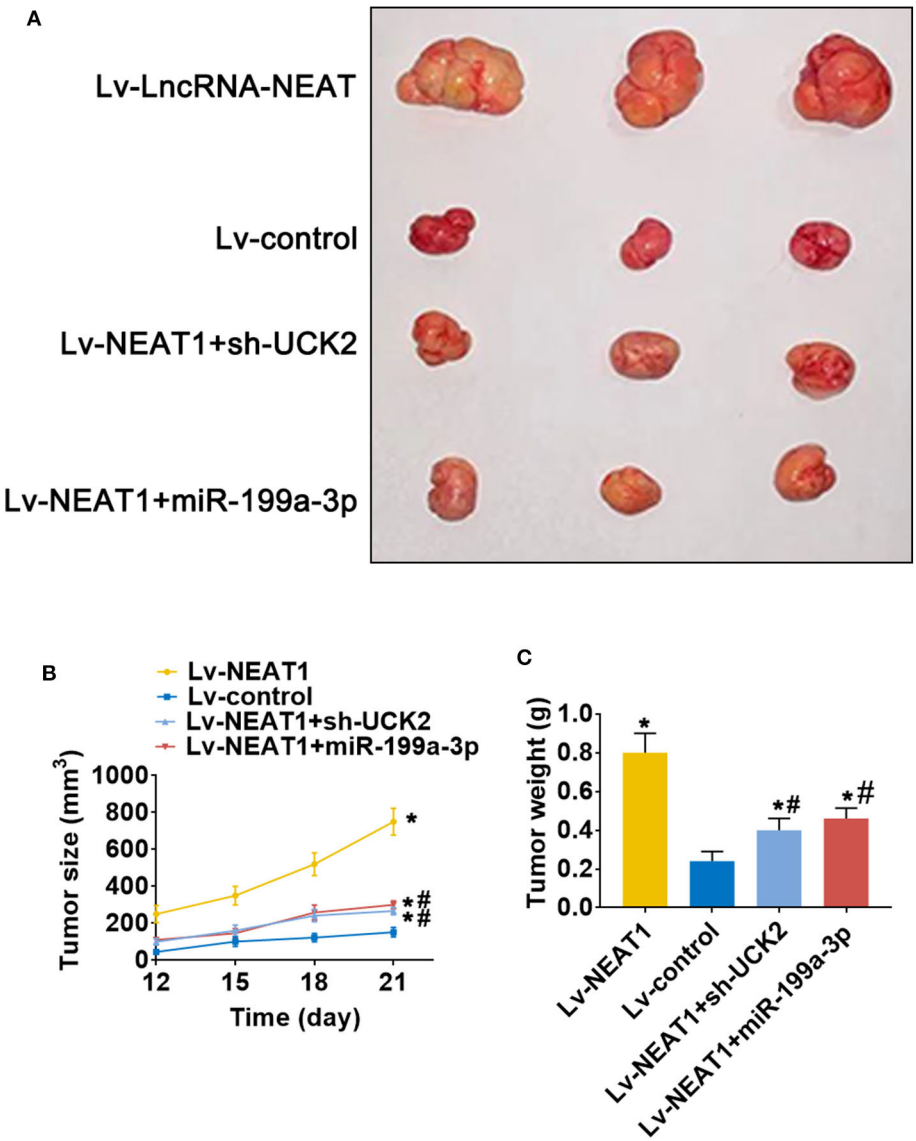

FIGURE 7 | LncRNA-NEAT1 promotes HCC tumor growth through miR-199a-3p/UCK2 *in vivo*. **(A)** SNU-182 cells transfected with Lv-NEAT1, Lv-NEAT1 + Lv-miR-199a-3p, or Lv-NEAT1 + Lv-siRNA-UCK2 were subcutaneously injected into nude mice. After 3 weeks, the tumor formation was examined. **(B)** Tumor diameters were measured every 3 days. **(C)** Tumor weights were measured on day 21 post injection. $^{*}P < 0.05$ compared with transfection negative control. $^{\#}P < 0.05$ compared with Lv-NEAT1.

affect the sensitivity to sorafenib and radiotherapy, as well as immune escape in HCC *in vitro*. Consistent with this role, aberrant expression of lncRNA-NEAT1 has been demonstrated in HCC and is associated with poor survival of HCC patients (11, 19–21). Liu et al. demonstrated that high expression of lncRNA-NEAT1 in a Chinese population is an independent risk factor for poor survival of patients with HCC (22). Furthermore, Ling et al. evaluated the expression level and clinical relevance of lncRNA-NEAT1 in HCC based on data from TCGA-LIHC and other HCC datasets from the Oncomine database, and found that lncRNA-NEAT1 is consistently up-regulated in HCC tumor tissues, though in the TCGA-LIHC dataset, lncRNA-NEAT1 was not significantly associated with overall patient survival but was significantly correlated to distant metastasis. Combined with data from *in vitro* experiments showing that knock-down of lncRNA-NEAT1 inhibits proliferation and induces apoptosis, Ling et al. suggested that lncRNA-NEAT1 promotes deterioration in HCC (23). In the present study, we focused more on the up- and downstream regulatory mechanisms of lncRNA-NEAT1 in HCC rather, than on its tumor-promoting role under conventional conditions, and our results, therefore, may be increase the mechanistic understanding of lncRNA-NEAT1 in each of these prior investigations.

In general, the abnormal expression of lncRNA-NEAT1 in cancer cells is known to be caused by genetic alterations, transcription factors, DNA methylation, miRNAs, and

RNA-binding proteins (24). Emerging evidence indicates that hypoxia can modulate the expression of lncRNAs, including lncRNA-NEAT1. Up-regulation of lncRNA-NEAT1 has been found in breast cancer cells under hypoxic conditions (13) and in cardiomyocytes (25), further indicating that lncRNA-NEAT1 may be a hypoxia-responsive lncRNA. However, the response of lncRNA-NEAT1 to hypoxia in HCC has not been well-elucidated. We investigated changes in lncRNA-NEAT1 expression levels in HCC cells under hypoxic conditions (1% O_2) or treatment with the hypoxia mimetic $CoCl_2$. Both the 3.7-kb poly-adenylated NEAT1-1 and the 23-kb non-adenylated NEAT1-2 are upregulated by hypoxia (13). However, NEAT1_1 is a highly conserved and abundant poly-adenylated transcript, which is much more abundant than the longer NEAT1_2 isoform (26). Hence in our study we focused on NEAT1_1. Our data verify that lncRNA-NEAT1 is induced by hypoxia in HCC cells. HIF-1α has been established as a predominant transcriptional regulator in response to hypoxia with the ability to binding to HREs and enhance expression of target genes, including lncRNAs (27). By accessing the JASPAR database, we identified a potential putative HIF-1α-related HRE (5'-ACGTGC-3') located in the promoter of lncRNA-NEAT1. Knock-down of HIF-1α eliminated the response of lncRNA-NEAT1 to hypoxia. Furthermore, the results of the ChIP and luciferase reporter assays supported the binding of HIF-1α to the promoter of lncRNA-NEAT1, suggesting that lncRNA-NEAT1 is transcriptionally induced by HIF-1α. These results provide evidence for the role of HIF-1α and the HRE in the lncRNA-NEAT1 promoter as a mechanism that regulates lncRNA-NEAT1 expression under conditions of hypoxia.

We further demonstrated that overexpression of lncRNA-NEAT1 does not promote proliferation of HCC cells under normoxic conditions, possibly because the overactive proliferative properties of tumor cells under normal conditions may obscure the effect of lncRNA-NEAT1 overexpression by the "ceiling effect." In addition, the endogenous lncRNA–NEAT1 in HUH7 and SNU-182 cells may be redundant, so in the absence of stress (such as hypoxia), overexpression of lncRNA-NEAT1 cannot promote cell proliferation without limitation. Under hypoxic conditions, the proliferation of HCC cells is inhibited, which provides an opportunity for lncRNA-NEAT1 to function.

As expected, knock-down of lncRNA-NEAT1 inhibited the growth of HCC cells, which was evident both under normoxic and hypoxic conditions. Moreover, acute hypoxia inhibited growth and promoted apoptosis and cell cycle arrest of HCC cells, and HCC cells overexpressing lncRNA-NEAT1 grew relatively faster with less apoptosis and G1 phase arrest. Therefore, these results suggest that increased lncRNA-NEAT1 levels sustain the growth of HCC cells under hypoxic conditions.

LncRNAs regulate gene expression in cancers through distinct mechanisms. For instance, lncRNAs may regulate target genes by specific recruitment of transcriptional activators or suppressors; by acting as decoys that bind to and block transcription factors from target genes; or by recruiting chromatin-remodeling complexes as scaffolding proteins, thereby affecting target genes (28). In tumor biology, lncRNAs primarily serve as ceRNAs that sponge tumor–promotive or tumor-suppressive miRNAs. Sponged miRNAs lose their regulatory effect on target mRNAs, which ultimately influences tumor progression (29). Thus, we employed microarray analysis and a series of advanced online bioinformatics tools to identify potential miRNA-mRNA pairs that may interact with lncRNA-NEAT1 according to a ceRNA mechanism. The candidate miRNA-mRNA pairs were filtered according to their reported suppressive functions and predicted interactions with tumor-related genes, as well as by their down-regulated expression patterns in HCC. To obtain more evidence for the roles of candidate miRNAs in HCC, we performed pathway annotation analysis of predicted miRNA targets based on the KEGG database. The candidate miRNAs were determined to be involved in multiple tumor-related pathways. To filter HCC-related mRNA targets of lncRNA-NEAT1-miRNAs, 7 independent datasets from HCC patients with different backgrounds were used for integrated analysis using the RRA method, which strengthened the evidence. Eight candidate mRNAs were up-regulated in HCC tissues in the 7 datasets. To better explore the potential biological functions of the candidate mRNAs, GO enrichment analysis was performed. Enriched GO-terms, such as "regulation of cell growth" and "positive regulation of cell cycle," provided a potential explain of how lncRNA-NEAT1 may sustain growth of HCC cells by regulating the candidate mRNAs identified in our study. Though we could not perform a detailed analysis of all candidate miRNA-mRNA pairs in the present study, we selected UCK2, a hub target gene and impressive prognosis risk factor of HCC, for confirmation. The miRNAs that potentially regulate UCK2, including miR-199a-3p, miR-483-3p, miR-486-5p, miR-582-5p, and miR-129-5p, were considered. However, definite interactions were verified only for the lncRNA-NEAT1-miR-199a-3p-UCK2 axis. The results of ChIP and luciferase analyses confirmed the binding of lncRNA-NEAT1/miR-199a-3p and miR-199a-3p/UCK2, thus providing a downstream mechanism that may regulate lncRNA-NEAT1 function.

Previous studies have reported that miR-199a-3p acts as a tumor suppressor via various mechanisms in HCC. For instance, miR-199a-3p inhibits tumor growth in an animal model of HCC by modulating the mTOR pathway (30). Giovannini et al. suggested that miR-199a-3p down-regulation is a common characteristic of HCC and that miR-199a-3p regulates E-cadherin expression through Notch1 (31), Jia et al. reported that miR-199a-3p represses tumorigenesis in HCC by targeting HIF-1α (32). Fornati et al. showed that miR-199a-3p modulates the cell cycle of HCC cells and sensitizes these cells to hypoxia-induced apoptosis (33). In the present study, the suppressive role of miR-199a-3p was confirmed and expression changes of lncRNA-NEAT1 were shown to induce alteration of miR-199a-3p in HCC cells. Furthermore, luciferase reporter and RIP assay demonstrated that lncRNA-NEAT1 sponges miR-199a-3p, which is consistent with our other bioinformatics and experimental data.

Functionally, UCK2 is a pyrimidine ribonucleotide kinase that catalyzes phosphorylation of uridine to uridine monophosphate and cytidine to cytidine monophosphate. Overexpression of UCK2 is regarded as an indicator of unfavorable prognosis in various cancers, including HCC, pancreatic cancer, breast cancer,

and lung cancer (34–37). However, few studies have revealed the detailed mechanisms underlying the regulation of UCK2. Zhou et al. found that UCK2 promotes metastasis via up-regulation of MMP2/9 expression and activation of STAT3 signaling (38). The upstream mechanisms of UCK2, especially those involved with lncRNA/miRNA, had not been clarified prior to this study. Therefore, we confirmed the growth-promotive effect of UCK2 in HCC cells and demonstrated that UCK2 is regulated by lncRNA-NEAT1/miR-199a-3p. Most importantly, lncRNA-NEAT1 was shown to function under hypoxic conditions partly through miR-199a-3p/UCK2. Moreover, an animal model was used to further explore the role and regulatory relationship of lncRNA-NEAT1/miR-199a-3p/UCK2. As a limitation of this study, some miRNAs and mRNAs that may be also controlled by lncRNA-NEAT1 were not validated so that we could focus our efforts on validating the lncRNA-NEAT1/miR-199a-3p/UCK2 axis. These miRNAs or mRNAs should be investigated in future studies.

In conclusion, we identified lncRNA-NEAT1 as a hypoxia-responsive lncRNA in HCC cell lines *in vitro*. Based on *in silico* data, we suggested that lncRNA-NEAT1 sustains the growth of HCC cells under hypoxic conditions. LncRNA-NEAT1 may regulate a panel of HCC-associated mRNAs by interacting with tumor-suppressive miRNAs in HCC. The roles of lncRNA-NEAT1/miR-199a-3p/UCK2 were validated HUH7 and SNU-182 cells, which indicated that lncRNA-NEAT1 and its downstream miRNAs/mRNAs may contribute to the progression of HCC cells in hypoxic microenvironments and, therefore, are potential targets for novel therapeutic strategies for HCC.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the Cancer Genome Atlas (TCGA) database (https://www.cancergenome.nih.gov). The expression profile data of one miRNA (GSE36915) and 7 mRNAs (GSE14520, GSE22058, GSE25097, GSE36376, GSE45436, GSE64041, and GSE76427) of HCC patients were retrieved from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/).

ETHICS STATEMENT

The animal study was reviewed and approved by Research Animal Care Committee Zhengzhou University.

AUTHOR CONTRIBUTIONS

QZ and JL: study design. QC, MX, XHe, and XHu: acquisition of data. QZ and QC: analysis and interpretation of data. QZ and QC: drafting of the manuscript. QZ and QC statistical analysis. JL: funding and study supervision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00998/full#supplementary-material

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REVIEW
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Therapeutic Targeting of Signaling Pathways Related to Cancer Stemness

Asunción Espinosa-Sánchez[1,2†], *Elisa Suárez-Martínez*[1,2†], *Laura Sánchez-Díaz*[1,2†] *and Amancio Carnero*[1,2*]

[1] *Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, Universidad de Sevilla, Consejo Superior de Investigaciones Científicas, Seville, Spain,* [2] *CIBER de Cancer, Madrid, Spain*

The theory of cancer stem cells (CSCs) proposes that the different cells within a tumor, as well as metastasis deriving from it, are originated from a single subpopulation of cells with self-renewal and differentiation capacities. These cancer stem cells are supposed to be critical for tumor expansion and metastasis, tumor relapse and resistance to conventional therapies, such as chemo- and radiotherapy. The acquisition of these abilities has been attributed to the activation of alternative pathways, for instance, WNT, NOTCH, SHH, PI3K, Hippo, or NF-κB pathways, that regulate detoxification mechanisms; increase the metabolic rate; induce resistance to apoptotic, autophagic, and senescence pathways; promote the overexpression of drug transporter proteins; and activate specific stem cell transcription factors. The elimination of CSCs is an important goal in cancer therapeutic approaches because it could decrease relapses and metastatic dissemination, which are main causes of mortality in oncology patients. In this work, we discuss the role of these signaling pathways in CSCs along with their therapeutic potential.

Keywords: CSC, cancer, EMT, stem cell-like pathways, therapy

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Parames C. Sil,
Bose Institute, India
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***Correspondence:**
Amancio Carnero
acarnero@us.es;
acarnero-ibis@us.es

† These authors have contributed equally to this work

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INTRODUCTION

The understanding on the tumorigenesis process has been explained during decades according to the clonal evolution model. This model postulates that all cells within a tumor contribute to the maintenance of the tumor at different levels (1). In the cell, a number of genetic and epigenetic changes accumulate during time, and, by selection, the most aggressive cancer cells drive tumor progression (1, 2). Therefore, any cancer cell can become highly malignant, contributing to metastases and the resistance against therapies (2). However, currently, the cancer stem cell (CSC) model proposes a more suitable explanation to cancer complexity. The tumors contain a subset of different tumor cells, called cancer stem cells that are crucial for tumor initiation, progression, and recurrence (3, 4). These CSCs, through self-renewal and differentiation, are critical for the generation of most tumor cell types contributing to tumor heterogeneity. However, the rest of mature cells compose the bulk of the tumor, but are not responsible for the tumor generation. Therefore, tumor resistance to therapies and metastases are the direct result of these CSCs (5).

CSCs, or "tumor-initiating cells," have the ability to self-renew and differentiate as normal stem cells. However, the mechanisms that regulate these processes are deregulated; therefore, CSCs continuously expand and produce differentiated progeny (5–7). Furthermore, CSCs can form new form tumors when grown into animals, but normal stem cells are unable to do (8, 9). CSCs compose a small population of cells within a tumor, share similar surface markers with normal stem cells (10, 11) and share common signaling pathways with normal stem cells (12, 13).

The size of the CSC pool is different in each tumor (14–16) and show, in general, a good correlation with agresivity and response, as well as patient survival (17, 18). CSCs show great heterogeneity, and may be different between grades and/or stages of the same type of tumors. The different pools of CSCs usually coexist in the same tumor, even within the same microenvironment niche. Interestingly, CSC different pools may communicathe physiologically among them thorugh phenotype interconversion (10, 19–21). CSCs are a "status" rather than a fixed "category" of cells. CSCs and non-CSCs can also interconvert in dynamic equilibrium. Non-CSCs can acquire CSC properties through de-differentiation (8, 22–24), and in this process, either genetic or epigenetic alterations, as well as microenvironment may be involved (25–28). Therefore, the CSCs model should be considered to be bidirectional, switching between stem and mature cells within the tumor (8, 22–24). Numerous studies support the concept that mature tumor cells (non-CSCs) are respond to cancer therapy, while CSCs are resistant to treatment [(29) and references therein]. In tumor stem cells (CSCs), different clonal evolution at the genetic and epigenetic levels generate distinct tumorigenic potential and heterogeneity which greatly influences disease progression and response to treatment (27, 29). On the other hand, the dynamic equilibrium between CSCs and differentiated non-CSCs adds another level of complexity. The potential for non-CSCs to revert to CSCs due to genetic, epigenetic, or microenvironment alterations that confer phenotypic plasticity to the tumor cell population is a strong driving force of tumor evolution and resistance to cancer therapy. Moreover, exposure of differentiated cells to therapeutic doses of radiation or many therapeutic compounds increases the CSC pool. To explain this CSC plasticity through cell maturation and dedifferentiating processes, it has been described that acquiring new genetic mutations, epigenetic changes, or microenviromental conditions that are able to activate the epithelial mesenchymal transition (EMT) induces the mature non-CSCs to dedifferentiate and acquire the CSC phenotype. Thus, activating TGF-β activates the EMT transcription factors Twist or Snail, and/or hypoxic conditions seem to influence dedifferentiation processes greatly, activating the SC pathways in tumor mature cells, leading to new CSCs (21, 30–33). Therefore, the phenotypic plasticity inducing conversions between mature non-CSCs and CSCs influences tumor evolution and clinical management.

The capability of one cell type to acquire the phenotype of another or of differentiated somatic and stem cells to interconvert states is denominated *cellular plasticity* (22, 34). This plasticity may explain the altered gene expression found in different tumor types resembling cell lineages that differ from the true progenitors (22, 35–38). Indeed, the inherent plasticity of stem cell pathways such as Wnt, Notch or Hedgehog, can be modified suggesting that these pathways may be relevant for anticancer research (5, 34, 39–41).

These and other results suggest that some oncogenic signals are able to induce CSCs. These signals are accompanied by an increase in resistance to chemotherapeutic treatments (35, 36) and, in some cases, radiotherapy (42, 43). Therefore, we must take into account the processes involved in the activation of stemness pathways and tumor evolution and evaluate how their influences affect therapy to effectively eliminate a tumor (**Figure 1**).

Therefore, cancer cells can move from stem to differentiated states, and viceversa, in response to therapy, transcription changes or signaling in the microenvironment (20, 44, 45). Moreover, inside a single tumor, CSCs can coexist in more than one metabolic and/or pluripotency state. CSCs from breast cancer, for example, can be found in different mesenchymal- and epithelial-like states (24, 46). The transition between these states has been reported to be regulated by epigenetic alterations (47). Phenotypic plasticity contributes to the complexity of the cancer ecosystem and represents a major challenge for tumor eradication since it actively contributes to tumor cell survival and metastasis.

CSC cells present many mechanisms for therapy resistance, such as high-level of drug efflux pumps, reactive oxygen species scavengers, antiapoptotic proteins, DNA repair efficient mechanisms, interactions with the protective microenvironment (37, 48–51) or exosomes loaded with proteins of non-coding RNA prone to modify the environment to favor metastasis (51–54). On the other hand, similar to normal stem cells, CSCs are known to be slow cycling in many tumors and are maintained in the G0 phase (55).

Epigenetic mechanisms may mediate therapeutic resistance in CSCs in many different ways (27, 35, 43, 51, 56–59). The silencing of the epigenome is also involved in maintaining plasticity and the transition of mature tumor non-CSCs to CSCs, as reported for the transition of metabolic states in renal tumor cells by the inactivation of MYBBP1a and the activation of MYB (60–62). For example, epigenetic demethylation of MAP17 driving the resistance against some targeted therapies was observed in lung adenocarcinoma (43). Additionally, studying lung cancer, Sharma and coworkers reported that a reversible drug-tolerant state of EGFR TKi therapy was obtained by chromatin alterations induced by histone demethylase activity (63). These and other results established that CSCs can regulate epigenetic factors to maintain their pool and overcome targeted therapies. However, the reversible nature of these epigenetic alterations suggests that inhibitors of the pathways modifying these epigenetic regulators may hold promise as relevant clinical therapeutic targets, either alone or in combination.

Thus, the CSC hierarchical model explains the failure of treatment and tumor recurrence and promises new targets for anticancer drug discovery. This article does not pretend to be an exhaustive review of all CSC pathways related to plasticity and/or therapeutic approaches. We summarize some evolving treatment strategies related to these pathways with the aim of shedding new light on current therapy development with promising new anticancer agents. Other CSC-related signaling pathways more commonly studied, not reviewed here, but relevant to stemness include MYB, TGF-β, JAK-STAT, FGFs, PI3K, or MEK. Targeting these pathways has been shown to exert anti-CSC effects, and promising agents are currently under investigation, as recently reviewed elsewhere (30, 36, 37, 47, 48, 51, 62, 64–74).

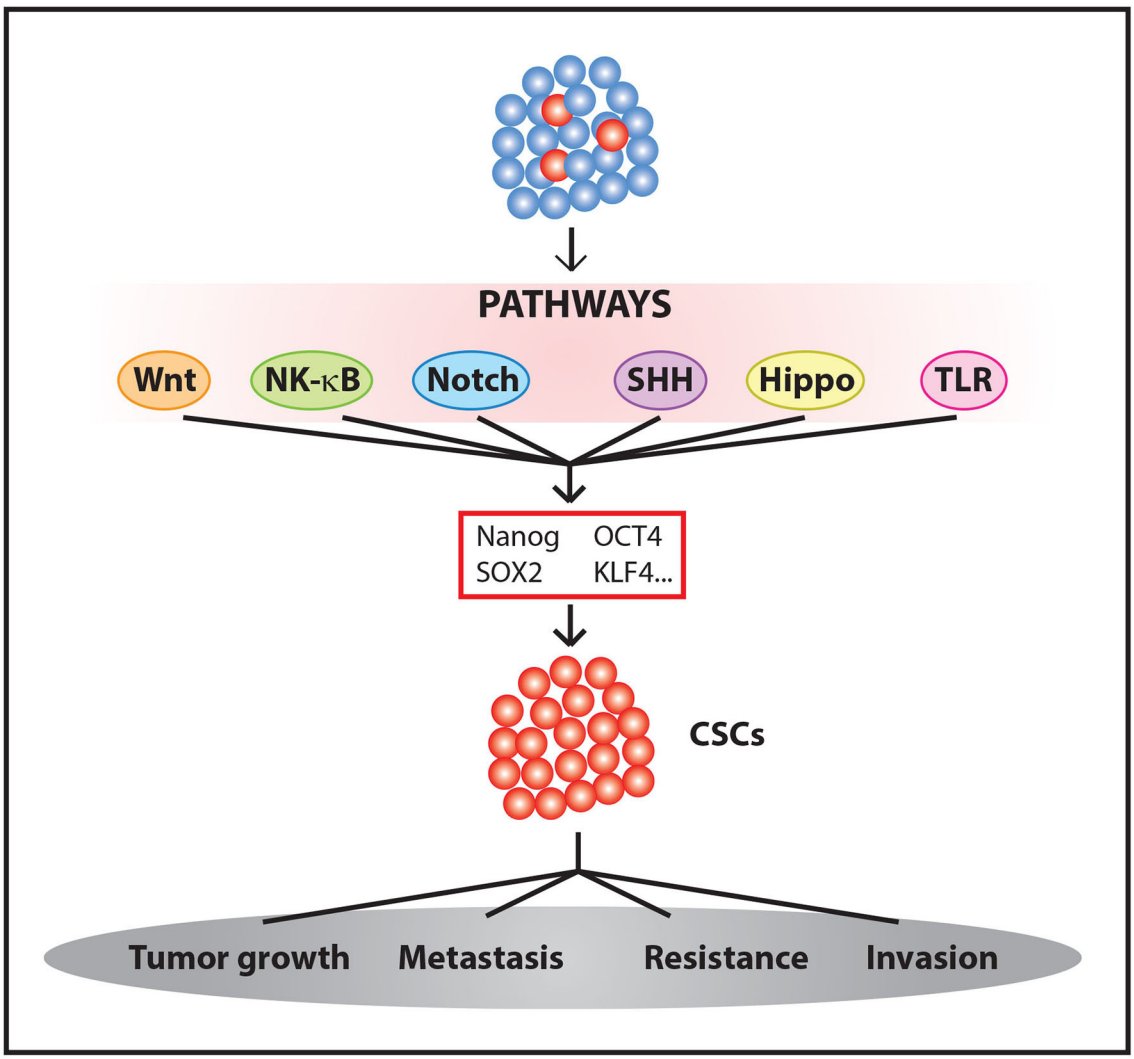

FIGURE 1 | Generation and maintenance CSCs. The activation of different signaling pathways leads to Yamanaka factors expression among other genes, promoting the enrichment of CSC populations within the tumor.

WNT PATHWAY

The Wnt pathway is involved in embryonic development and homeostasis. Moreover, this signaling pathway regulates cell proliferation and survival (75). This pathway has two different signaling pathways: canonical (β-catenin dependent) and non-canonical (β-catenin-independent).

According to canonical signaling, Wnt is secreted and binds to Frizzled receptors and/or the low-density lipoprotein-related protein (LRP) 5 and 6 coreceptors. When R-spondin ligands bind to Lgrs, the action of the Wnt pathway is enhanced. In the presence of a Wnt ligand, Wnt interacts with Frizzled and/or LRP5/6 (76). This situation generates a cascade in which the phosphorylation of the cytoplasmic domain in LRP, the recruitment of scaffolding protein Disheveled (Dvl) and the hijack of GSK-3b and Axin are realized, with the last molecule produced through tankyrases. Thus, β-catenin is available for translocation into the nucleus, where it binds to lymphoid enhancer factor (LEF)/T cell factor (TCF) transcription factors and activates target gene transcription (76). In the absence of a Wnt ligand, the level of intracellular β-catenin is very low due to the action of the proteasome degradation complex; this complex is composed of scaffolding protein Axin and adenomatous polyposis coli (APC). Moreover, the kinase proteins glycogen synthase kinase-3b (GSK-3b) and casein kinase 1a (Ck1a) phosphorylate β-catenin triggering its ubiquitination (76) (**Figure 2**).

In non-canonical signaling, transduction proceeds in some different ways; for example, in the Wnt/Ca^{2+} pathway. The interaction between Wnt5A and Frizzled FZD2 receptors activates Dvl. Moreover, it promotes the release of Ca^{2+} into the cytosol as facilitated by FZD2, which cleaves guanine nucleotide-binding protein (G protein). Ca^{2+} activates CaMKII_AD (Ca^{2+}/calmodulin-dependent protein kinase II) and suppresses

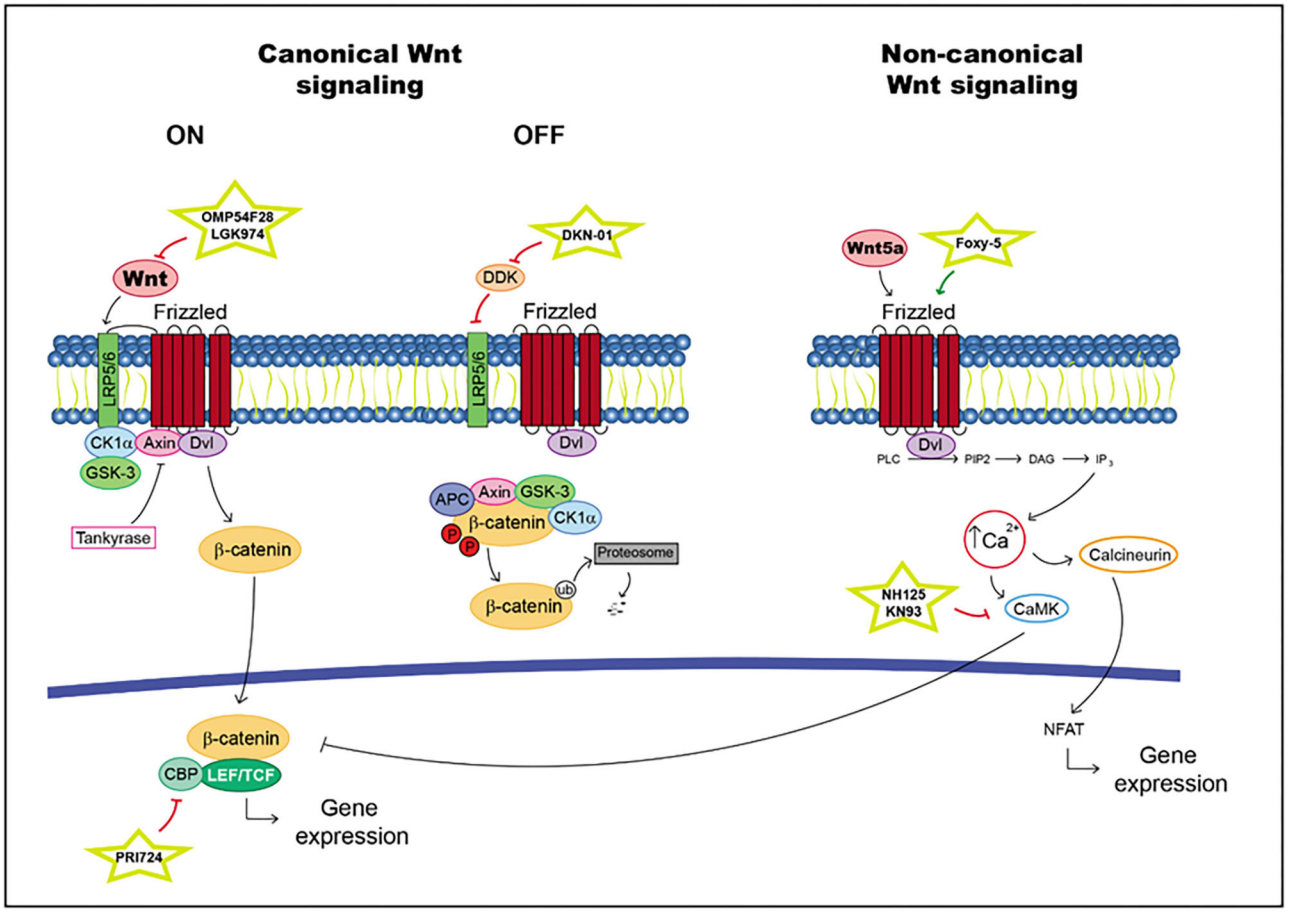

FIGURE 2 | Wnt pathway including targets and drugs that could be employed to inhibit it. **(Left)** canonical Wnt signaling. **(Right)** non-canonical Wnt signaling.

the canonical Wnt pathway. This situation increases the differentiation of cells in the neuronal system (77–80) (**Figure 2**).

Cancer Stem Cells

Some articles suggest that the role of Wnt in the regulation of CSCs differs depending on the types of cancer, such as blood, intestine, lung, mammary, gland, nervous system, skin, and urinary tract. During the development and maintenance of CSCs, the most common alteration is the hyperactivation of Wnt signaling. In skin cancer, the role of the Wnt pathway was discovered using a reporter mouse strain. It was observed that the genetic deletion of β-catenin produced induced tumor regression in chemically induced skin tumors through a decrease in the pool of CD34+ stem cells (81). In human acute leukemia, the crucial role of Wnt signaling activation in cell self-renewal capacity and drug-resistant properties has been suggested (82). Another article reported that the overexpression of miR-582-3p causes Wnt signaling activation by targeting multiple negative regulators of the Wnt pathway, such as AXIN2, DKK3, and SFRP1. Therefore, miR-582-3p promotes tumorigenesis in NSCLC and CSCs *in vitro* (83). Wnt enhancer R-Spondin-2 (RSPO2) is highly expressed in subpopulations with high intrinsic Wnt activity and with properties indicative of CSCs. Therefore, the employment of promoter inhibitors such as RSPO2 was proposed to block stemness-promoting pathways (84). In metastatic colorectal cancer, an increase in the level of progastrin, a tumor-promoting peptide essential for the self-renewal of colon CSCs, was observed, implicating β-catenin/TCF4 as a direct target gene. Therefore, therapy employing antibodies directed against progastrin was proposed for patients with metastatic colorectal cancer K-RAS-mutations (85). A recent article suggested a role for SOX8 in cancer stem cell properties and therapy resistance. It reported that SOX8 conferred chemoresistance and enhanced stemness properties. Additionally, SOX8 mediated EMT via the FZD7-mediated Wnt/β-catenin pathway (86).

Therapeutic Targets and Drugs

A large number of mutations in the Wnt pathway have been identified. Above all, aberrations in the expression of Wnt ligands, Frizzled receptors, β-catenin and APC were discovered in many different tumors (87–89). According to these results, this pathway has been researched in recent years to identify potential therapeutic targets. Some drugs that may be potential

therapeutics to be employed in the treatment of different tumors are detailed.

A fusion protein called OMP-54F28, ipafricept (OncoMed Pharmaceuticals), competes for the FZD8 receptor and antagonizes Wnt signaling. Preclinical models with this drug have shown reduced tumor growth as a single drug in combination with other chemotherapeutic agents (90). OMP-54F28 was studied in phase Ib clinical trials for different types of cancer (ClinicalTrials.gov Identifiers: NCT02050178, NCT02069145, and NCT02092363). In ovarian cancer patients, ipafricept was well-tolerated in combination with sequential carboplatin/paclitaxel, but it caused bone toxicity at efficacy doses; therefore its use is limited (91).

Another drug of interest is SM08502 (Samumed LLC), which is a potent CDC-like kinase (CLK) inhibitor. Moreover, CLKs play important roles in the integral cellular mechanism of mRNA splicing to induce the retention of introns in DVL2, TCF7, ERBB2, and LRP5 and exon skipping in LEF1 and TCF7L2 (92). SM08502 inhibits the growth of gastrointestinal tumors in xenograft mouse models (93). The molecule was recently found in phase 1 (ClinicalTrials.gov Identifier: NCT03355066) to show safety, tolerability and efficacy in patients with advanced solid tumors.

On the other hand, the combination of gedatolisib (a pan-class I PI3K/mTOR isoform inhibitor) and PTK7-ADC (an antibody-drug conjugate used against the cell-surface PTK7 protein) has a double synergistic effect, whereby gedatolisib increases the expression of PTK7-ADC and an auristatin payload causes an effect similar to that of gedatolisib. Currently, this combination is in s phase I trial for patients with metastatic triple-negative breast cancer because the PI3K pathway is dysregulated in most cells of this type of cancer (94).

The inhibition of porcupine, an O-acyltransferase required for Wnt activation and secretion, induced by LGK974 (Novartis, Basel, Switzerland, https://www.novartis.com), reduced the phosphorylation of the LRP6 receptor and the expression of WNT target genes in preclinical models. Furthermore, *in vivo* studies of LGK974 have demonstrated an antitumoral response in different types of cancer (95). Phase I evaluation of LGK974 alone and in combination with PDR001 (spartalizumab, an anti-PD-1 antibody) is ongoing (ClinicalTrials.gov Identifier: NCT01351103) in patients with lobular or triple negative breast cancer, melanoma or pancreatic cancer (96).

A monoclonal antibody against dickkopf WNT signaling pathway inhibitor 1 (DKK1) and DKN-01 was discovered. The high expression of DKK1 may be a predictive biomarker for effective treatment with DKN-01 and pembrolizumab in gastroesophageal adenocarcinoma (97). A phase 2 study is assessing this molecule as a monotherapy or in combination with paclitaxel in patients with endometrial, uterine or ovarian cancer (ClinicalTrials.gov Identifier: NCT03395080).

PRI-724 (PRISM BioLab) is a small molecule that inhibits the interaction between β-catenin and CBP in the nucleus. Thus, the downregulation of genes involved in symmetric non-differentiation division and increased p300/β-catenin binding were produced. This situation promotes stem cell differentiation and increases the sensitivity of cytotoxic drugs (98). Phase IIb of this drug in combination with gemcitabine in patients with advanced pancreatic adenocarcinoma (APC) has been completed, and the results showed the necessity for more work to find useful predictive and PD markers.

Aberrant activation of signaling from Wnt pathway usually is associated with stem-like phenotypes and EMT, which induce resistance to endocrine therapy. Plumbagin inhibited colony formation and mammosphere formation and decreased the number of cancer stem cell markers by inhibiting the Wnt signaling pathway. Plumbagin also reduced Wnt-dependent genes as well as β-catenin. Moreover, treatment of orthotopic xenografts with Plumbagin inhibited tumor growth, angiogenesis and metastasis without significant adverse effects on body weight or blood coagulation; therefore, Plumbagin may be useful to treat endocrine-resistant breast cancer (99).

On the other hand, the inhibition of the non-canonical Wnt pathway can be achieved through different drugs. Foxy-5 is a mimetic Wnt5a molecule that binds to and activates the Frizzled 2/5 receptor. Increased Wnt-5a signaling may inhibit endothelial tumor cell migration and invasion. This effect may decrease the metastasis of susceptible tumor cells (100). A phase 2 evaluation of it as a neoadjuvant therapy will be performed for subjects with colon cancer who express low levels of Wnt-5a (ClinicalTrials.gov Identifier: NCT03883802). Moreover, other molecules, such as NH125, which is more selective as an eEF-2 kinase inhibitor that is as an PKC, PKA, or CaMKII inhibitor (101), and KN93, which is a selective inhibitor of CaMKII (102), may be potential drugs to regulate the non-canonical Wnt pathway (**Table 1**).

NF-κB PATHWAY

The NF-κB pathway is a complex and pathway that has been studied mainly for its controversial roles in inflammation and immune responses. This signaling also plays an important role in cellular proliferation, survival and differentiation (103–105).

The inflammatory effects on cancer development have been studied, above all the modulation of inflammation-associated cancer through the transcription factor NF-κB (106). The excessive innate immunity activation and growth caused by NF-κB are involved in tumor development and progression (107). Moreover, the activation of NF-κB could be caused for genetic alterations such as amplification, mutations, or deletions in different cancer cells. NF-κB binds to promoters of genes such as IL1B, TNF, and IL6 which cause the secretion of cytokines and chemokines (108). For this reason, it is suggested that the exposure to proinflammatory stimuli in tumor microenvironment or genetic alterations in components of IKK—NF-κB pathway could cause the activation of NF-κB in cancer.

The NF-κB family of transcription factors includes five different proteins: p105/p50, p100/p52, p65 (RelA), c-Rel, and RelB, which are inactivated in the cytoplasm upon binding to IκB proteins (109). Two signals are differentiated: canonical and non-canonical.

TABLE 1 | Overview of drugs targeting CSC pathways.

Pathway	Agent	Mechanism of action	Model	Clinical trial phase
Wnt pathway	OMP-54F28	Wnt antagonist	Solid tumors	Phase Ib
	SM08502	CDC-like kinase (CLK) inhibitor	Advanced solid tumors	Phase I
	Gedatolisib (1) and PTK7-ADC (2)	(1) PI3K/mTOR inhibitor (2) PTK7 protein antagonist	Metastatic triple-negative breast cancer	Phase I
	LGK974	Porcupine inhibitor	Melanoma, breast cancer, and pancreatic cancer	Phase I
	DKN-01	DKK1 inhibitor	Endometrial, uterine, and ovarian cancer	Phase II
	PRI-724	CBP inhibitor	Advanced pancreatic adenocarcinoma	Phase IIb
	Plumbagina	Wnt ligands inhibitor	Endocrine resistant breast cancer	Preclinical
	Foxy-5	Mimetic Wnt5a molecule	Colon cancer	Phase II
	NH125	eEF-2 kinase inhibitor	Solid tumors	Preclinical
	KN93	CaMKII inhibitor	Solid tumors	Preclinical
NFkB pathway	Thalidomide	TNF-a inhibitor	Solid tumors	Phase II
	Bortezomib	Proteosome inhibitor	Myeloma, lymphoma, and acute leukemia	Phase III
	BMS-345541	IKKb and IkB protein inhibitor	Lung cancer	Preclinical
	Bardoxolone methyl (RTA-402)	IKKb and JAK1 inhibitor	Pancreatic adenocarcinoma and lymphomas	Phase I
	IMD-0354 and KRT1853	IKKb inhibitor	Lung cancer	Preclinical
	PS1145	IKK inhibitor	Prostate cancer and nasopharyngeal carcinoma	Preclinical
Notch pathway	PF-03084014	γ-secretase inhibitor (GSI)	Desmoid tumors	Phase II/III
	BMS-906024	γ-secretase inhibitor (GSI)	T-ALL relapses and T cell lymphoblastic lymphoma	Phase I
			Adenoid cystic adenocarcinoma	Phase II
	MK-0752	γ-secretase inhibitor (GSI)	Recurrent central nervous system tumors, advanced breast cancer, and T-ALL	Phase I
	MK-0752 + docetaxel/gemcitabine	γ-secretase inhibitor (GSI)	Locally advanced or metastatic breast and pancreatic cancer, respectively	Phase I/II
	RO4929097	γ-secretase inhibitor (GSI)	Renal cell carcinoma, metastatic pancreatic cancer, and non-small cell lung cancer relapses	Phase II
	RO4929097 + temsirolimus/ Cediranib/gemcitabine/ Bevacizumab	γ-secretase inhibitor (GSI)	Solid tumors	Phase I
	Enoticumab	DLL-4 monoclonal antibody	Advanced solid cancers (ovarian carcinoma)	Phase I
	Demcizumab + gemcitabine	DLL-4 monoclonal antibody	Pancreatic cancer	Phase I
	Demcizumab + FOLFIRI	DLL-4 monoclonal antibody	Colorectal cancer	Phase I
	Demcizumab + Carboplatin and pemetrexed	DLL-4 monoclonal antibody	Non-squamous NSCLC	Phase II
	Tarextumab	Notch2, 3 monoclonal antibody	Epithelial cancers (breas, small cell lung, ovarian, and pancreatic cancers)	Phase I/II
	Brontictuzumab	Notch1 monoclonal antibody	Hematological malignances and advanced solid tumors	Phase I
	AMG 757	DLL-3 Bi-specific T cell engager	Neuroendocrine carcinomas (small cell lung cancers)	Phase I
	AMG 119	DLL-3 Chimeric antigen receptor (CAR) T cell	Neuroendocrine carcinomas (small cell lung cancers)	Phase I
	Rovalpituzumab tesirine	DLL-3 antibody-drug conjugate (ADC)	Neuroendocrine carcinomas (small cell lung cancers)	Phase II
	CB-103	Pan-Notch inhibitor against transcriptional complex	Advanced-stage solid tumors and hematological malignancies	Phase I/IIa
Sonic Hedgehog pathway	Vismodegib	SMO inhibitor	Metastatic basal cell carcinoma (BCC) or recurrent locally advanced BCC	Approved by FDA 2012 + clinical trials in other tumors (breast cancer)

(Continued)

TABLE 1 | Continued

Pathway	Agent	Mechanism of action	Model	Clinical trial phase
	Sonidegib	SMO inhibitor	Recurrent locally advanced BCC	Approved by FDA 2015
	Saridegib	SMO inhibitor	Chondrosarcoma, head and neck, pancreatic, adenocarcinoma, BCC, myelofbrosis	Phase II
	BMS-833923	SMO inhibitor	Basal cell nevus syndrome chronic myeloid leukemia	Phase II
	Glasdegib	SMO inhibitor	Acute myeloid leukemia, other cancers	Phase II
	Taladegib	SMO inhibitor	Various carcinomas and sarcomas	Phase II
	TAK-441	SMO inhibitor	BCC, advanced non-hematologic malignancies	Phase I
	Arsenic trioxide	GLI1, 2 inhibitor	Acute promyelocytic leukemia	Approved by FDA 2000 + phase I–IV clinical trial in other tumors
	Genistein	GLI1 inhibitor	Different solid tumors (gastric, prostate, breast) and hematological malignancies	Phase I/II
	5,00E+01	SHH monoclonal antibody	Various cancer types	Preclinical
	RU-SKI43	SHHat enzyme inhibitor	Various cancer types	Preclinical
Hippo pathway	Verteporfin	YAP-TEAD interaction inhibitor	Metastatic breast cancer, pancreatic tumors, prostate cancer	Phase I–II
	CA3	YAP-TEAD interaction inhibitor	Esophageal adenocarcinoma	Preclinical
	Flufenamic acid	YAP-TEAD interaction inhibitor	Metastatic breast tumor and hepatocellular carcinoma	Preclinical
	Chloromethyl ketone	YAP-TEAD interaction inhibitor	Various cancer types	Preclinical
	Super-TDU	YAP-TEAD interaction inhibitor	Pancreatic, gastric, and colorrectal cancer	Preclinical
	CT-707	YAP post-transcriptional modifications	Non-small cell lung	Phase I
	C19	MST agonist	Melanoma, breast cancer, colon cancer, and neuroblastoma	Preclinical
	BET inhibitors	Epigenetic modulation	Solid and hematological malignancies	Phase I–II
	Panobinostat	Epigenetic modulation	Solid and hematological malignancies	Phase I–IV
TLR pathway	Bacillus Calmette-Guerin (BCG)	TLR2/4 agonist	Carcinoma *in situ* or muscle non-invasive cancer of the urinary bladder	FDA approved in 1998 + Clinical trials (melanoma, colorectal and lung cancer…)
	CADI-05	TLR2 agonist	Advanced melanoma	Phase I–II
	Monophosphoryl lipid A (MPLA)	TLR4 agonist	Adjuvant in vaccines of human papillomavirus (HPV)-associated cervical cancer	FDA approved + Phase I–IV in other tumors
	Glucopyranosyl lipid A	TLR4 agonist	Skin and colorectal cancer, sarcoma, lymphoma	Phase I–II
	AS15	TLR4 agonist	Various cancer types	Phase I–III
	Poly (I:C)	TLR3 agonist	Various cancer types	Phase I–II
	Poly-ICLC (Hiltonol®)	TLR3 agonist	Solid tumors	Phase I–II
	Rintatolimod (Ampligen®)	TLR3 agonist	Fallopian tube, ovarian, colorectal, prostate, and brain tumors	Phase I–II
	Entolimod (CBLB502)	TLR5 agonist	Local or metastatic malignancies	Phase I-II
	Imiquimod	TLR7/8 agonist	Superficial basal cell carcinoma	FDA approved + Phase I–IV in other tumors

(Continued)

TABLE 1 | Continued

Pathway	Agent	Mechanism of action	Model	Clinical trial phase
	Resiquimod	TLR7/8 agonist	Skin tumors and vaccine adjuvant	Phase I–II
	852A	TLR7 agonist	Melanoma, breast, ovarian, endometrial, cervical and esophageal cancer, and hematological malignancies	Phase I–II
	VTX-2337	TLR8 agonist	Solid and hematological maliganancies	Phase I–II
	Agatolimod (CpG7909)	TLR9 agonist	Vaccine adjuvant in solid and hematological malignancies	Phase I–II
	SD-101	TLR9 agonist	Various solid tumors and lymphoma	Phase I–II
	E5564	TLR4 antagonist	Leukemia	Phase I
	CRX-526	TLR4 antagonist	Colon cancer	Preclinical
	OPN305	TLR2 antagonist antibody	Myelodysplastic syndrome	Phase I–II

LPS and proinflammatory cytokines, such as IL-1, Toll-like receptors (TLRs) and tumor necrosis factor alpha (TNF-α), activate the NF-κB signaling pathway. The recruitment of a receptor proximal adaptor protein facilitates the phosphorylation and activation of IκB kinase (IKK) protein complex, which subsequently initiates the phosphorylation of IκB proteins. IκB proteins are degraded, allowing NF-κB translocation into the nucleus to activate specific target genes. This pathway can activate antiapoptotic factors and cytokines as well as proliferation factors (109).

The non-canonical brand of the NF-κB pathway may activated by different factors such as the receptor activator of NF-κB (RANK) and CD40, leading to the stabilization of NF-κB-inducing kinase (NIK). This molecule activates IKKα dimers, promoting p100 phosphorylation and cleavage into p52. The activated RelB/p52 dimer can be translocated into the nucleus regulating specific target gene transcription (109, 110) (**Figure 3**).

Cancer Stem Cells

The link between inflammation and cancer stem cells (CSCs) is found during tumorigenesis and disease progression (111, 112). The involvement of the NF-κB pathway in CSCs was discovered in primary AML samples, in which the DNA in CD34+ cells bind with NF-κB. However, this binding was not observed in normal hematopoietic stem cells (113). In adult murine neurogenesis, TLR2 and TLR4 were found to have opposite functions in adult neural stem/progenitor cell (NPC) proliferation and differentiation. Moreover, the inhibition of the LRPs impacted the self-renewal and the cell fate decision of NPCs (114). A MEC-targeted inducible transgenic inhibitor of NF-κB was developed in mammary oncomice, and it inhibited breast tumor stem cell markers and expanded NANOG and SOX2 expression *in vivo* and *in vitro* (115).

Chronic inflammation may be responsible for the accumulation of proinflammatory cytokines, which increase the action of NF-κB. This situation promotes a tumorigenic microenvironment in colon cancer (116). In the basal-like subtype of triple-negative breast cancer, high levels of activated NF-κB pathway components were observed. Inflammatory cytokines or dysregulated NIK expression induced this activation and caused the upregulation of JAG1 expression in normal cancer cells. NOTCH signaling was stimulated in cancer stem cells and induced the expansion of CSC populations (117). The upregulation of NIK caused a decrease in the CSC population, and its reduction led to the results that were opposite those found for breast cancer (118). In addition, mammary stem and progenitor cells were expanded by constitutive RANK signaling, and the overexpression of this activator increased tumorigenesis properties (119).

IKKα and its activator, NF-κB-inducing kinase, were critical for the expansion of tumor-initiating cells (TICs). IKKα is translocated into the nucleus, where it phosphorylates the cyclin-dependent kinase (CDK) inhibitor p27/Kip1 and promotes its nuclear export or exclusion (120).

Therapeutic Targets and Drugs

To determine the efficacy of cancer treatment through the inhibition of the NF-κB pathway, various problems have been found: low efficacy of NF-κB inhibitors in addition to lymphoma and leukemia (121), immunosuppression after long-term systemic administration of these drugs (122) and the appearance of short-term drug resistance (123, 124). However, NF-κB inhibitors can be useful in combination with other chemotherapies, as most anticancer agents can activate this pathway, protecting cancer cells from apoptosis (125).

TNF-α is a target that can be employed to inhibit the NF-kB pathway. Drugs such as thalidomide and its derivatives act in this way (126, 127). However, thalidomide does not show good results in combination with gemcitabine or irinotecan chemotherapy, and its use increases the risk of thrombotic events (128).

On the other hand, bortezomib is an innovative drug with remarkable preclinical and clinical antitumor activity in different types of cancer. The mechanism of action consists of proteasome inhibition, which causes inactivation of the canonical NF-κB pathway (129, 130). Additionally, the prolonged inhibition of this pathway accelerates chemical lung carcinogenesis by perpetuating carcinogen-induced inflammation (131). Currently, many phase III clinical trials are in the recruitment stage for patients with myeloma, lymphoma or acute leukemia who will be treated with bortezomib in combination with other

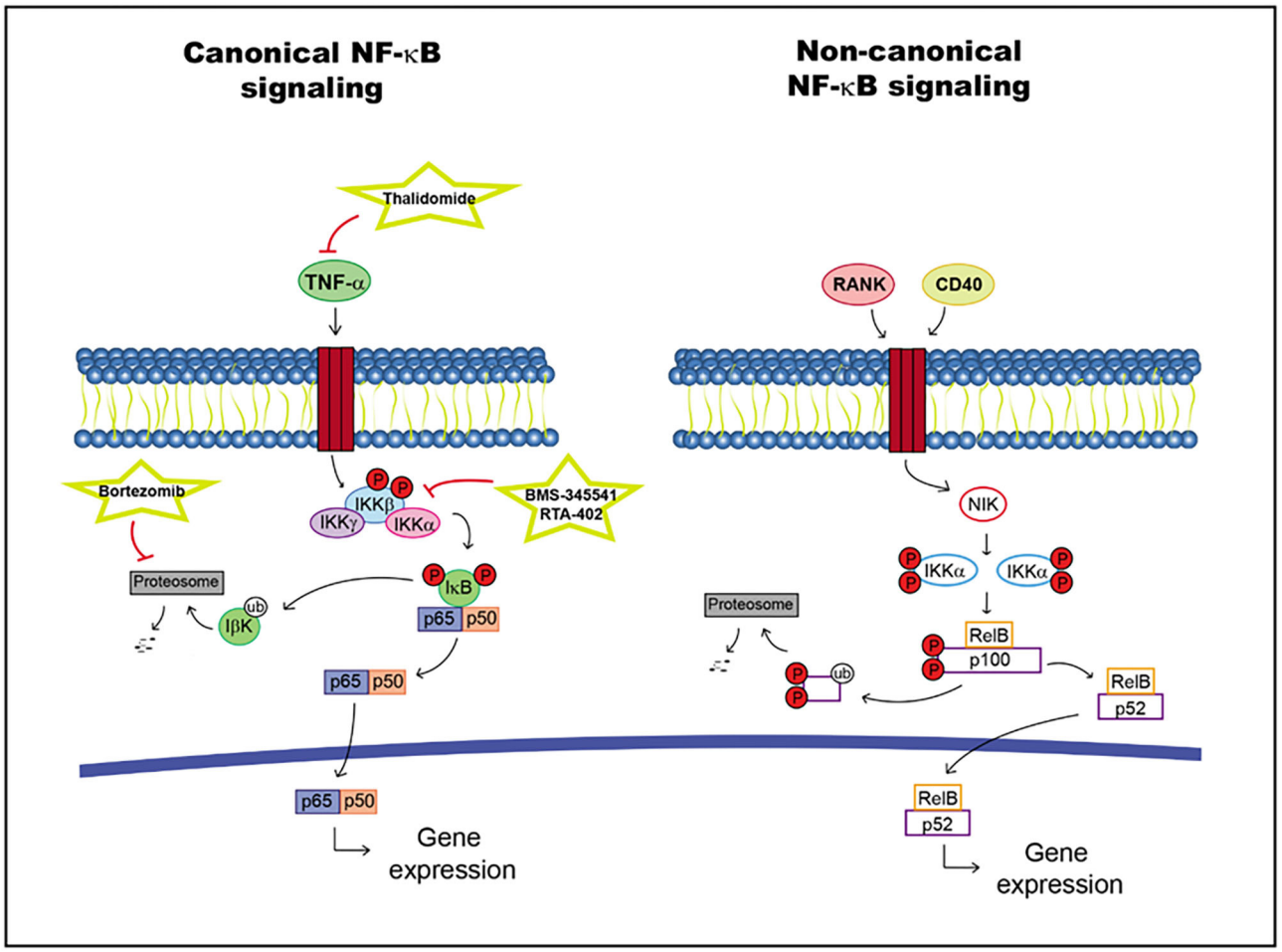

FIGURE 3 | NF-κB pathway including targets and drugs that could be employed to inhibit it. **(Left)** canonical NF-κB signaling. **(Right)** non-canonical NF-κB signaling.

anticancer agents, and the results will be compared to those of conventional treatments.

There is another type of drug that inhibits IKKβ and IκB protein, BMS-345541, which causes the reduction of stemness, self-renewal and migration capacity in lung cancer (132). Another drug in this group, bardoxolone methyl (RTA-402), is a triterpenoid and antioxidant inflammation modulator that inhibits IKKβ and JAK1. Phase I studies have been conducted for various types of cancer: pancreatic adenocarcinoma (133), solid tumors and lymphomas (134). Additionally, IMD-0354 and KRT1853 are IKKβ inhibitors and block IκBα phosphorylation, inactivating its translocation into the nucleus and the activation of NF-κB (135). In lung cancer, it was discovered that these drugs can be potential anticancer agents through suppression of cancer cell invasion, proliferation, and survival (136). In addition, PS1145 reduced the growth of tumorigenic prostate cancer (137) and nasopharyngeal carcinoma (PNC) cell lines (138). Moreover, in the most recent study, it was observed that NF-κBp65 and KLF4 upregulation was involved in drug resistance (138).

These drug groups only partially inhibit NF-κB signaling because IKKα can also activate this pathway. Therefore, inhibitors of IKKα and IKKβ need to be found to generate better effects (139) (**Table 1**).

NOTCH PATHWAY

The Notch pathway is an evolutionarily conserved signaling route involved in a variety of developmental and homeostatic processes, such as proliferation, stem cell maintenance, cell fate specification, differentiation, or angiogenesis, despite the apparent simplicity of its signaling network. The effects of activating this signaling pathway are very diverse, depending on the signal dose and cell context (140–144). Its deregulation contributes to a wide range of disorders and diseases, such as congenital afflictions, viral infections, and/or different types of cancer (142).

The Notch signaling pathway is composed of Notch receptors and Notch ligands, as well as different proteins that serve as posttranslational modifiers, but there is not an amplification cascade induced by different proteins in well-known signaling pathways (142, 145).

There are four Notch receptors encoded by different genes: Notch1–4. The general structure of Notch receptors consists of a single-pass transmembrane protein composed of different protein modules, with some differences: Notch3 and 4 have a shorter extracellular domain and lack the intracellular transcription-activating region, and Notch4 lacks the Notch cytokine response domain. Notch1 and Notch2 are expressed in many tissues during development and in adults, whereas Notch3 is expressed mostly in vascular smooth muscle and pericytes, and Notch4 is expressed in the endothelium (142, 145–149).

There are four Notch ligands in humans: Dll1 and Dll4, from the Delta family of ligands, and Jag1 and Jag2, from the Serrate family. There is a Dll3 ligand, but it is not able to activate Notch receptors. These ligands are also single-pass transmembrane proteins. The expression pattern of these ligands is less established than that of the Notch receptor ligands (142, 145, 150, 151).

Notch receptor activation begins with the binding of a Notch ligand expressed in a neighboring cell upon the release of the Notch receptor negative regulatory region (NRR). The Notch ligand, which has been previously ubiquitinated in the cytoplasmic tail by E3 ligases Neuralized or Mind Bomb to trigger endocytosis, induces a conformational change in the receptor, exposing cleavage sites for ADAM metalloproteases to access to cleave the extracellular receptor domain. Then, the transmembrane domain is cleaved by γ-secretase, releasing the Notch intracellular domain (NICD). The NICD is translocated to the nucleus and, together with the DNA-binding factor RBPJ and coactivators in the Mastermind-like (MAML) family, it forms the Notch transcription complex (NTC). NTC binds to Notch regulatory elements (NREs), and transcriptional coregulators are recruited, activating the transcription of Notch target genes, such as *HES* and *HEY* (3, 142, 145, 152, 153) (**Figure 4**).

The Notch signaling pathway is highly regulated, and its target genes vary according to different epigenetic contexts, such as different cell subtypes, changing the cellular outcomes. Indeed, NICD function is influenced by other signaling routes and undergoes many posttranslational modifications at different sites, with a variety of consequences, which have not yet been fully elucidated. Notch receptor expression is also closely regulated in normal cells. Notch1 upregulation is crucial in the early stages of T cell development, whereas its downregulation in later stages is important to prevent the transformation in this lineage. Furthermore, there are other levels of regulation, such as inhibitory feedback loops or RBPJ binding to transcriptional repressors, explaining why deregulation of this pathway can lead to different pathologies, such as cancer development (142, 145, 153, 154).

Cancer Stem Cells

Loss of balance in the Notch signaling pathway can lead to tumor formation. Indeed, Notch has been implicated in all of the hallmarks of cancers, including cell growth and survival, the EMT, angiogenesis, and/or metastasis. The most frequent alterations have been found in Notch receptor genes. Notch might act as an oncogene or a tumor suppressor, depending on the context and/or the tumor type (140, 142, 145, 149, 155). Interestingly, in some cancers, such as head and neck squamous cell carcinoma (HNSCC), Notch seems to have a bimodal role, as it might function as an oncogene or a tumor suppressor gene (149).

The Notch pathway can be deregulated by mutational activation or inactivation, overexpression, posttranslational modifications or epigenetic alterations (156). In T cell acute lymphoblastic leukemia, translocation 7; 9 produces a fusion gene consisting of the end of Notch1 and enhancer elements or the TCRβ gene, causing a the removal of the NRR receptor activation inhibitor, and ligand-independent activation of the Notch pathway. Other tumors are characterized by mutations in the Notch PEST domain (C-terminal domain), such as B cell tumors (chronic lymphocytic leukemia, splenic marginal zone lymphoma, etc.) or basal-like breast cancer (142, 157). In these cases, among others, such as cancer of the pancreas, prostate, and lung, hepatocellular cancer, esophageal tumors, and HNSCC (149), Notch functions as an oncogene. On the other hand, several tumors present with mutations in the Notch N-terminal domain, producing a loss of function, such as squamous cell carcinomas (skin, head and neck, esophagus and lung) and small cell lung cancers, urothelial carcinomas, and low-grade gliomas; in these cases, Notch acts as a tumor suppressor (142, 149).

Notch has also been associated with stemness maintenance, important in adult organisms and in pathologies such as cancer. In several cell types, Notch activation seems to maintain or promote expansion of stem cell pools, especially in solid tumors such as glioblastoma, hepatocellular carcinoma, ovarian cancer, breast cancer, and HNSCC. Notch inhibitors can decrease stemness marker expression and sensitize tumor cells to chemo- and radiotherapy (142, 149, 153, 155, 157–160).

Notch signaling mediates the interactions between cancer stem cells and tumor microenvironment cells (endothelial, immune, and mesenchymal cells). The Notch pathway is activated in tumor cells by ligands localized in the vascular niche. Specifically, Jag1 is the main ligand that plays this role. For example, in B cell lymphomas, this interaction induces aggressive behavior and resistance to chemotherapy. In other tumors, Jag1 promotes cancer stem cell self-renewal, proliferation and stemness maintenance. Jag1 can eventually activate the transcription of several genes, such as Klf4 in head and neck cancer cell lines, inducing a stem cell phenotype and chemotherapy resistance (145).

Therapeutic Targets and Drugs

Among the signaling pathways that are activated in cancer cells, the Notch signaling pathway is among the most upregulated, and it is implicated in cancer metastasis, angiogenesis and CSC self-renewal, making it is an important target in cancer therapy (144).

There are two groups of inhibitors that have been developed to target the Notch signaling pathway: γ-secretase inhibitors (GSIs) and monoclonal antibodies against Notch ligand-receptor interactions (mAbs). These therapeutic strategies can be effective in combination with conventional therapies to treat cancer patients with promising results. Furthermore,

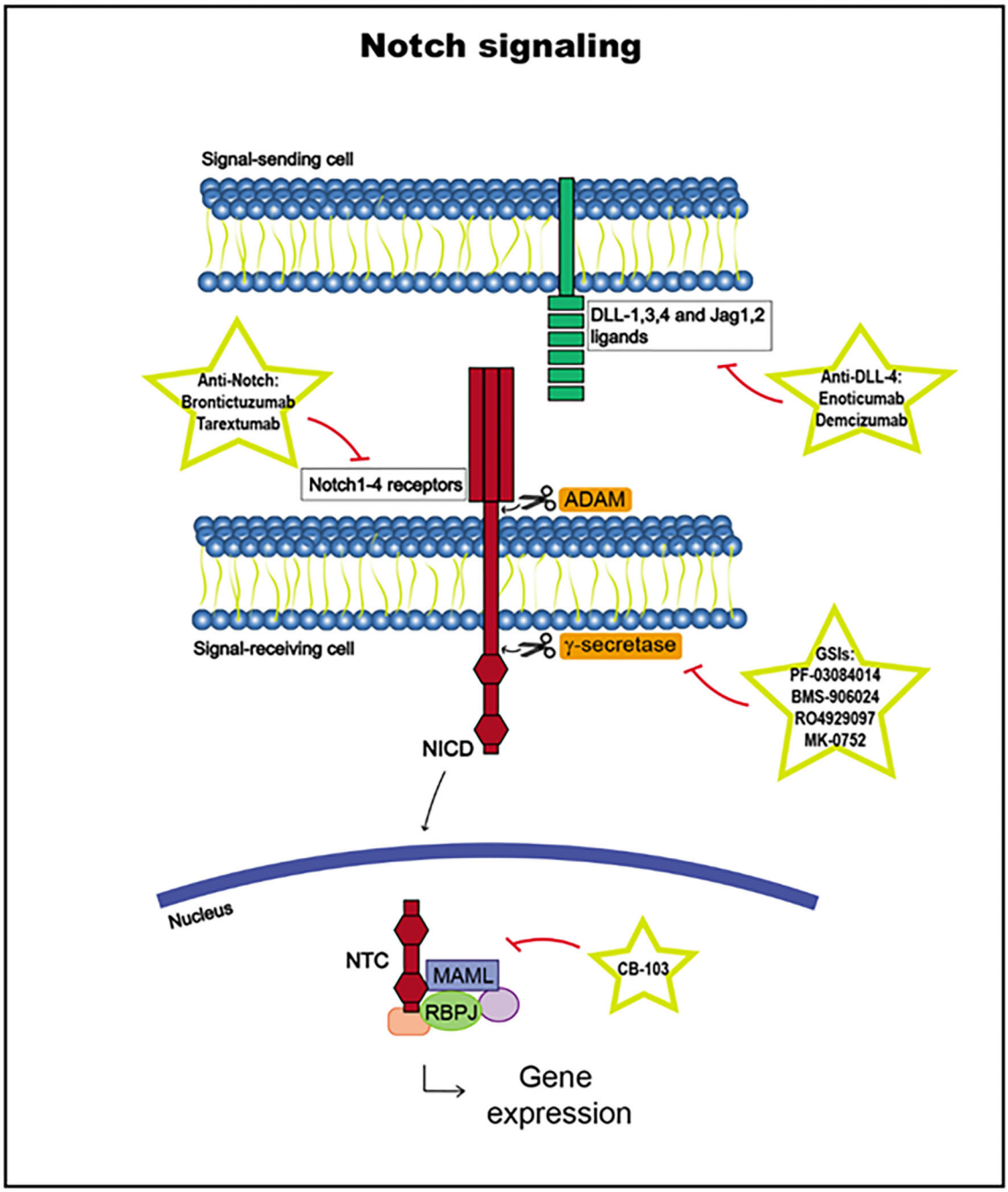

FIGURE 4 | Notch signaling pathway, including targets and drugs that could be employed to inhibit it.

GSIs and mAbs have been demonstrated to be useful as single agents in the early stage of several tumors, such as thyroid, lung, intracranial and colorectal cancers, and sarcomas. However, Notch signaling is also crucial for normal tissue homeostasis; therefore, its inhibition can induce serious side effects, especially GSIs, such as gastrointestinal toxicity, diarrhea, hepatotoxicity, and nephrotoxicity (3, 140, 144, 149, 161). For this reason, these inhibitors are dose limiting and are administered intermittently (144).

γ-Secretase Inhibitors (GSIs)

γ-Secretase is the enzyme critical for releasing the Notch intracellular domain (NICD) and activating the signaling pathway. Therefore, this enzyme may offer promising results as an inhibiter of the Notch pathway. GSIs constituted the first class of inhibitors developed to be used in cancer patients, and more than 100 GSIs have been developed to date (162).

There are many GSIs currently in clinical trials in several cancer types, as single agents or in combination. For example,

PF-03084014 is a small reversible and non-competitive GSI that is being studied alone or in combination in several cancer subtypes (163, 164), but currently, its clinical development has focused on desmoid tumors (phase II/III) (165). BMS-906024 is being studied in relapsed T-ALL and T cell lymphoblastic lymphoma (phase I) (166), as well as adenoid cystic adenocarcinoma (phase II), with promising results (3). Another GSI, MK-0752, is a non-competitive oral inhibitor that is being tested for use against recurrent central nervous system tumors, advanced breast cancer and T-ALL (phase I) and in combination with docetaxel or gemcitabine for use in locally advanced or metastatic breast and pancreatic cancer, respectively (phase I/II) (3, 144, 167, 168). RO4929097 has been studied as a single agent in renal cell carcinoma, metastatic pancreatic cancer, and non-small cell lung cancer relapses (phase II) and in combination with temsirolimus, cediranib, gemcitabine, or bevacizumab in different solid tumors (phase I) (3, 144, 169–171).

All these GSIs have been demonstrated to promote the inhibition of the Notch pathway, tumor growth, angiogenesis, stem cell marker expression, and metastasis, etc. (3, 140, 144). However, some of them, such as MK-0752 or RO4929097, are not being developed for use in active clinical trials because of the limited efficacy observed (3) (**Table 1**).

Monoclonal Antibodies (mAbs)

Several monoclonal antibodies have been developed to inhibit Notch signaling as in inhibitors of ligands (DLL-4) and receptors (Notch1–3). They impair ligand-receptor interactions or extracellular domain conformational changes, which are crucial for exposing Notch receptor cleavage sites (140, 144).

DLL-4 is one of the ligands that binds Notch receptors and is involved in growth control, stem cell renewal and development. The deletion of its gene has lethal consequences in the vasculature, and its overexpression has been found in cancer cells and tumor vasculature. Furthermore, DLL-4 inhibition impairs the formation of functional capillaries, leading to aberrant angiogenesis and promoting direct effects that inhibit Notch signaling in tumor cells (172–175).

Enoticumab is a humanized mAb against DLL-4 that seems to have a reasonable safety profile and efficacy in advanced solid cancers, such as ovarian carcinoma (phase I) but is no longer in clinical development. Demcizumab is a DLL-4 antibody that is being studied with gemcitabine in pancreatic cancer, with FOLFIRI in colorectal cancer (phase I) and with carboplatin and pemetrexed in non-squamous NSCLC (phase II), but it did not improve efficacy; therefore, it not in clinical development (3, 140, 144).

Monoclonal antibodies against Notch receptors have also been studied. There are two groups of monoclonal antibodies: those directed to the NRR domain, preventing the conformational change necessary to activate Notch signaling, and those targeted to the EGF-repeat region in Notch receptors to impede the ligand-receptor interaction. Both groups promote substantial downregulation of Notch1 signaling (161, 176).

Tarextumab is a humanized monoclonal antibody against Notch2 and 3 that inhibits Notch signaling as a single agent or in combination with chemotherapeutic agents in epithelial cancers, such as breast, small cell lung, ovarian, and pancreatic cancers (phase I/II). It can inhibit tumor cell proliferation, reduce the number of CSCs and prolong the time before tumors recur after chemotherapy. However, the clinical trials did not show survival improvement; therefore, clinical development has been discontinued (3, 140, 144). Brontictuzumab is an anti-Notch1 mAb that is being studied in hematological malignances and advanced solid tumors (phase I) (177, 178) (**Table 1**).

Other Therapeutic Targets

The DLL-3 Notch ligand is highly expressed in neuroendocrine carcinomas, such as most small cell lung cancers. DLL-3 can promote tumorigenesis through Notch signaling pathway inhibition (in contrast to DLL-4). Therefore, DLL-3 is being studied as a therapeutic target in these cancers. Some other approaches include co-administering AMG 757, a bispecific T cell engager; AMG 119, a chimeric antigen receptor (CAR) T cell (phase I); or as an anti-DLL3 antibody-drug conjugate (ADC), rovalpituzumab tesirine (phase II) (3, 179, 180).

Another strategy involves inhibiting the formation of the Notch transcriptional complex, which acts downstream of abnormal Notch receptor activation. CB-103 is an oral pan-Notch inhibitor that belongs to a novel class of small molecules that target the Notch transcriptional complex, inhibiting the expression of target genes. CB-103 is being studied in phase I/IIa clinical trials for patients with advanced-stage solid tumors or hematological malignancies (181) (**Table 1**).

SONIC HEDGEHOG PATHWAY

The Sonic Hedgehog pathway plays a role in embryogenesis and brain development. In adults, it is usually inhibited, although it participates in the maintenance of somatic stem cells and pluripotent cells of many organs, tissue repair, and regeneration of several epithelial cells (182–186). Furthermore, there is evidence that the Hedgehog pathway (Hh) is deregulated in various cancer types, such as pancreatic, gastric, prostate, and esophageal cancer (187–189). The activation of the Hh signaling pathway may have a variety of effects involving cell proliferation, cell fate determination, the epithelial-to-mesenchymal transition, and cell motility or adhesion. Therefore, the deregulated activation of this pathway may lead to the development of tumors or resistance to treatment (190).

There are two different mechanisms by which Hh signaling activated: ligand-receptor binding, known as the canonical pathway, or as a consequence of the activation of another downstream member of the signaling pathway, known as the non-canonical pathway (191).

Canonical

The main receptor in the Hh signaling pathway is Patched (Ptch1), which localized to the base of the primary cilia (PCs), structures that protrude from the cell membrane to sense a variety of stimuli (192). The main ligand that binds Ptch1 is Sonic Hedgehog (SHh), but there are two other ligands: Indian Hedgehog (IHh) and Desert Hedgehog (DHh).

In the absence of Hh binding, Ptch1 does not translocate with the transmembrane protein Smoothened (SMO) to the PCs. When Hh binds to Ptch1, Ptch1 is internalized and degraded, causing the accumulation of SMO in the PC, and as a consequence, the signaling cascade is initiated through a complex formed by kinesin protein (Kif7), suppressor of fused (SUFU) and full-length glioma-associated oncogene (GliFL). This pathway eventually leads to the translocation of the Gli transcription factor to the nucleus.

There are three Gli proteins: Gli1, Gli2, and Gli3. Gli1 is a transcriptional activator, whereas Gli2 and Gli3 could act as activators or repressors. However, Gli2 acts mainly as an activator, and Gli3 acts as a repressor.

SUFU usually inhibits Gli transcription factor translocation to the nucleus by direct binding. SUFU activates the phosphorylation by glycogen synthase kinase 3 beta (GSK3β), casein kinase I (CK1), and protein kinase A (PKA) and promotes the recruitment of β-transducin repeat-containing protein (β-TrCP), which causes the transformation of Gli2 and Gli3 into the Gli repressors Gli2R and Gli3R, which are translocated to the nucleus to inhibit the transcription of Hh pathway target genes (186, 190, 193) (**Figure 5**).

When the signaling pathway is activated by the Hh ligand, the accumulation of SMO leads to the hyperphosphorylation of SUFU, releasing Gli proteins, which translocate to the nucleus and activate the transcription of the target genes Ptch1 and Gli1 (186, 193) (**Figure 5**).

Non-canonical

The non-canonical signaling pathway is independent of Gli proteins but is not yet fully understood. In the type I non-canonical pathway, SMO appears to be critical for modulating Ca^{2+} and the actin cytoskeleton, whereas SMO does not participate in the type II non-canonical signaling pathway, which may enable cell proliferation and survival (194).

Cancer Stem Cells

The cancer stem cell (CSC) model explains tumor heterogeneity through the presence of a small group of cells with unlimited self-renewal capacity and the potential to regenerate all cell types in the entire tumor. They seem to be critical for therapy resistance and relapses. The Hh signaling pathway, as well as other signaling pathways, has been implicated in the maintenance of CSCs (190, 195, 196).

The mechanisms by which the Hh signaling pathway can be activated are mutations in Hh signaling members or the deregulation of Hh ligand release. Furthermore, the Hh signaling pathway may participate in the formation of CSCs.

A variety of members of the Hh pathway have been found to be affected by mutations in human cancers, such as the inactivating mutations of Patch1 or SUFU or the activating alterations of SMO, Gli1, and Gli2, which lead to signaling pathway activation independently of ligand binding. Patch1 loss of heterozygosity is usually found in Gorlin syndrome or nervoid basal cell carcinoma syndrome. Furthermore, alterations to the Hh pathway in combination with tumor suppressor mutations are able to generate other sporadic tumors, such as skin, medulloblastoma, gastric, and rhabdomyosarcoma tumors (190, 197–199).

In other cases, the Hh signaling pathway is stimulated by a deregulation in ligand release, which could be autocrine (from the tumor cell to itself, as it seems to occur in gliomas), paracrine (from the tumor cell to the stroma or from the stroma to the tumor cell, as it may happen in multiple myeloma, which has been studied to a lesser extent) or both, as it might take place in esophageal or gastric cancers (182, 190, 195, 196, 199). It is thought that tumor cells can release ligands that may stimulate the Hh signaling pathway in stromal cells via paracrine signaling and then promote a supportive microenvironment for the tumor (190).

Hh signaling pathways have been demonstrated to participate in the formation and maintenance of cancer stem cells in a variety of tumors, such as hematological malignancies and gastric, pancreatic, prostate, and lung cancers. The Hh signaling pathway is able to activate the transcription of key genes that contribute to the stem cell phenotype, such as Nanog, Oct4, Sox2, and Bmi1. Targeting this pathway in CSCs may be a promising strategy to reduce tumor growth, relapse, and metastasis (200–202).

Therapeutic Targets and Drugs

Tumors that present with mutations in signaling components may be ameliorated by some Hh pathway inhibitors, but the efficacy of these treatments is dependent on the level of alterations in the signaling pathway. In addition, ligand-dependent tumors may be treated by inhibitors of the Hh signaling cascade that are directed against any of its signaling components, regardless of the level in the route (190).

Among all proteins that may take part in the signaling cascade, SMO and Gli transcription factors are the main targets on which current research is focused. Although there are currently many SMO inhibitors, spontaneous mutations can arise as a consequence of the treatment, which may cause drug resistance (203).

SMO Inhibitors

Cyclopamine, a natural alkaloid from *Veratrum californicum*, was the first SMO inhibitor, but the significant side effects in mice did not allow it to be used in humans. Vismodegib, a second-generation cyclopamine derivative approved by the FDA in 2012, is being used in metastatic basal cell carcinoma (BCC) treatment and in recurrent locally advanced BCC, which are not candidates for surgery or radiotherapy. More than 85% of BCC patients have constitutive activation of the SHH pathway, most of which are due to a mutation in PCHT1. Vismodegib binds to SMO and inhibits its function, but continuous exposure can induce mutations in SMO, promoting drug resistance. Furthermore, vismodegib is being studied in many clinical trials in a variety of human cancers, such as breast cancer (204–206).

Sonidegib is another SMO antagonist, approved in 2015 by the FDA, that is used in the treatment of patients with locally advanced BCC that recurred disease after surgery or radiotherapy or patients who are not able to receive surgery or radiation treatments. Sonidegib, similar to vismodegib, binds SMO in

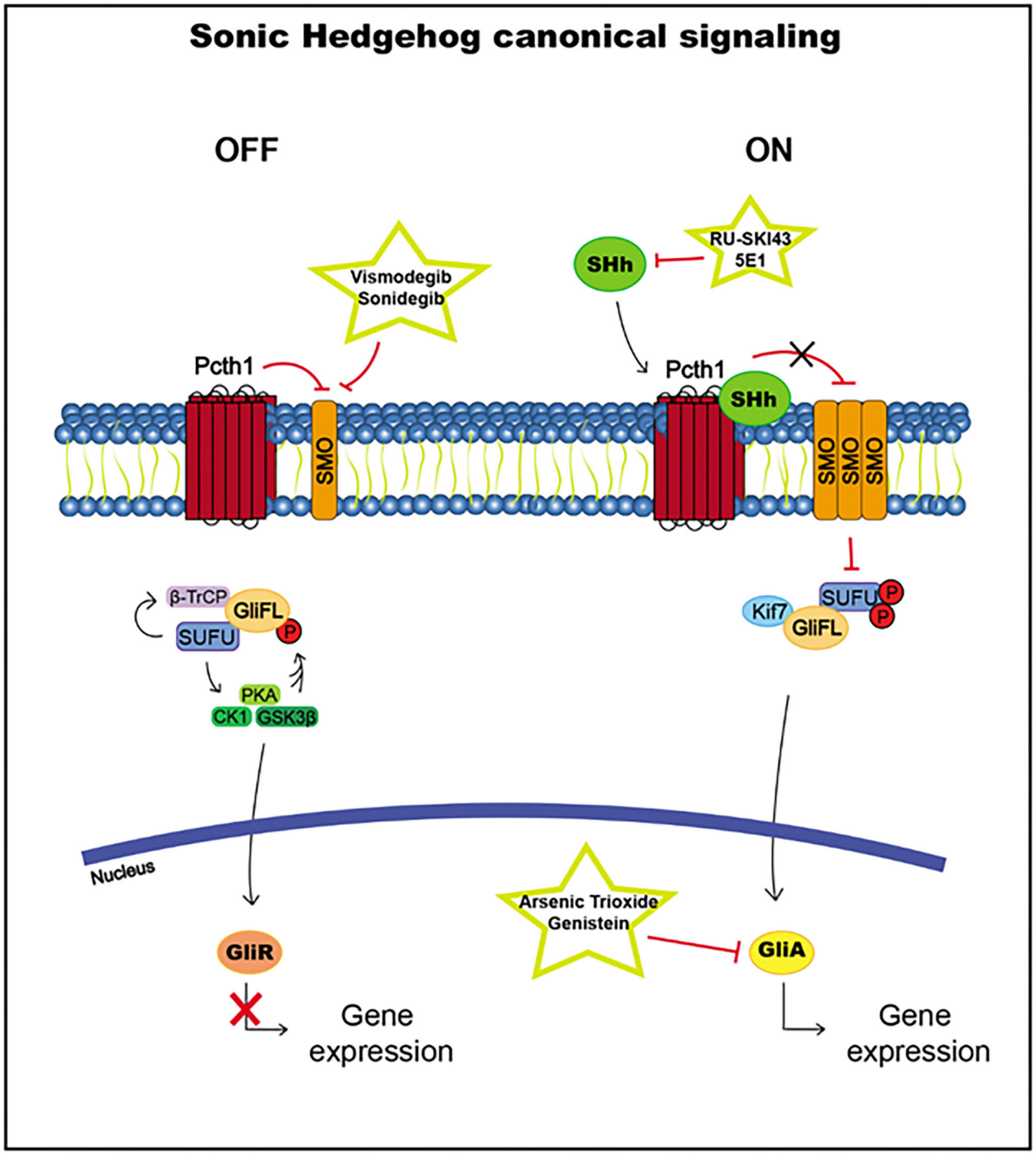

FIGURE 5 | Sonic Hedgehog canonical signaling, including targets and drugs that could be employed to inhibit it. **(Left)** inactive signaling pathway. **(Right)** active signaling pathway.

the "drug binding pocket," and mutations at this site lead to resistance (205, 206). Sonidegib and Vismodegib are teratogen, as other Sonic Hedgehog pathway targeting drugs, due to the role of this signaling route in embryogenesis (206).

There are other drugs that are currently in clinical trials for different cancer types, such as Saridegib (phase II), BMS-833923 (phase II), glasdegib (phase II), taladegib (phase II), or TAK-441 (phase I), and they have shown promising results in preclinical

models (205, 206). TAK-441 has shown efficacy in cells that have developed adaptive mutations in SMO; therefore, it may be relevant for patients with drug resistance (206) (**Table 1**).

GLI Inhibitors

Arsenic trioxide, a Gli inhibitor approved by the FDA for the treatment of acute promyelocytic leukemia, binds directly to Gli1 and Gli2, among other actions, causing the inhibition of their activity (205, 206). It has been shown to affect the viability of cancer stem cells in pancreatic and prostate cancer (in the cancer-initiating cells). This drug is being studied in phase I–IV clinical trials for solid tumors and hematological malignancies. Arsenic trioxide could imply some cardiac side effects such as QT interval elongation or tachycardia, as well as circulatory, gastrointestinal and respiratory disorders (205–207).

Genistein (phase I and II), an isoflavone isolated from *Genista tinctoria*, has been shown to inhibit Gli1, causing the growth suppression of different tumors and the cancer stem niche (206, 208) (**Table 1**).

SHh Inhibitors

Inhibitors of SHh, which is the most important ligand, have not yet been approved for use in the clinic, but they have been shown to effectively inhibit the SHh pathway in mice. Some of these are SHh monoclonal antibody 5E1, or RU-SKI43, which inhibits the SHHat enzyme, critical for catalyzing the binding of palmitate to SHh, one of the final steps in SHh synthesis (205, 206) (**Table 1**).

Other Therapeutic Targets

A truncated isoform of the Gli1 transcription factor, which indicates a gain of function, has been discovered: tGli1. This isoform has been detected exclusively in tumor cells, not in normal cells, and promotes tumorigenesis to a greater extent than does Gli1, which makes it a promising drug target. There is no drug directed specifically against tGli1, but it may be possible to target the genes activated by tGli1, such as CD24, VEGF-A, VEGFR2, or HPA1, and inhibit their expression. A phase I clinical trial is testing a CD24 monoclonal antibody. Furthermore, some heparanase (HPA1) inhibitors, such as PI-88 or PG545, are being studied as antiangiogenic anticancer drugs (PI-88 in phase III and PG545 in phase I). Finally, there are several antiangiogenic drugs approved by the FDA and directed against VEGF-A and VEGFR, such as bevacizumab, Ziv-aflibercept, and sorafenib (205).

HIPPO PATHWAY

The highly evolutionarily conserved Hippo signaling pathway regulates biological processes, such as survival, differentiation, cellular proliferation, fate determination, organ size, or tissue homeostasis (209). The core pathway consists of a kinase cassette that is composed of MST1/2 and LATS1/2 (210). In addition to MST1/2, MAP4K, and TAOK also directly phosphorylate LATS1/2 (211–214). NF2 is critical for pathway activation through the phosphorylation of MST1/2. The major target of the Hippo pathway is YAP and its paralog transcriptional coactivator TAZ. Phosphorylation of YAP and TAZ leads to their sequestration in the cytoplasm by 14-3-3 proteins and ubiquitination-dependent proteosomal degradation (215). In the nucleus, YAP/TAZ it can bind and regulate a family of sequence-specific transcription factors called TEA DNA-binding proteins (TEAD1–4) that modulate genes such as CTGF, CRY61, BIRC5, ANKRD1, and AXL, involved in proliferation and survival. In addition to TEADs, the YAP/TAZ complex also cooperates with RUNX1 and 2, TBX5, and SMADs, among others (216–219) (**Figure 6**). High TEAD expression levels have been correlated with poor clinical outcomes, and therefore, it can serve as a prognostic marker in many solid tumor types (220–231).

Cancer Stem Cells

Hippo pathway effectors activated by YAP/TAZ have been shown to induce cancer stem cell (CSC) properties in a wide range of human cancers, including osteosarcoma, glioblastoma, and chemoresistant breast cancer (232, 233). Moreover, breast cancer tissues with a high content of CSCs show a gene expression profile that overlaps with YAP/TAZ-induced gene expression, and breast CSCs with a CD44+/CD24– phenotype have a relatively high expression of TAZ (234).

YAP/TAZ are known to promote other properties of CSCs, such as the epithelial-to-mesenchymal transition (EMT) and metastasis, via activation of TEAD transcription factors in different tumors, including breast cancer and melanoma (235–241). In the context of these tumors, TEAD activation leads to the disruption of cell–cell junctions, mesenchymal gene expression, increased cell migration, anoikis resistance and cell invasion. TEAD specifically regulates genes such as ZEB1, ZEB2, DNp63, and Slug, which induce an increase in the progression and metastatic potential of tumors such as squamous cell carcinoma, breast cancer, and small-cell lung carcinoma (NSCLC) (242–244). Interestingly, YAP expression was critical for the progression of various KRAS-driven cancers, and YAP/KRAS converged on FOS to promote the EMT, which contributed to oncogenic KRAS oncogenic addiction (245).

It has also been proven that the activation of YAP/TAZ confers resistance to chemotherapy in cancer cells, in part because of the CSC characteristics acquired by the cells. YAP/TAZ has been linked to castration resistance in prostate cancer and paclitaxel and doxorubicin resistance in breast cancer (234, 246, 247). Moreover, different studies suggest that Hippo pathway activation promotes cancer cell survival in the presence of DNA-damaging agents such as UV exposure, radiation, cisplatin, Taxol, and fluorouracil (5-FU) in a wide number of tumor types (248–251).

YAP/TAZ, in conjunction with their target genes of secreted ligands, also promote resistance to targeted therapies, such as the drugs targeting RAF and MEK and BRAF inhibitors and receptor tyrosine inhibitors, such as gefitinib (252–258).

The relevance of the Hippo pathway in cancer and, more specifically, in the biology of CSCs has already been demonstrated in multiple publications. Therefore, the Hippo pathway is currently being studied as an interesting target for use in developing targeted therapies for different types of tumors. There are some approved compounds able to regulate this pathway; however, they have shown to have some bioavailability

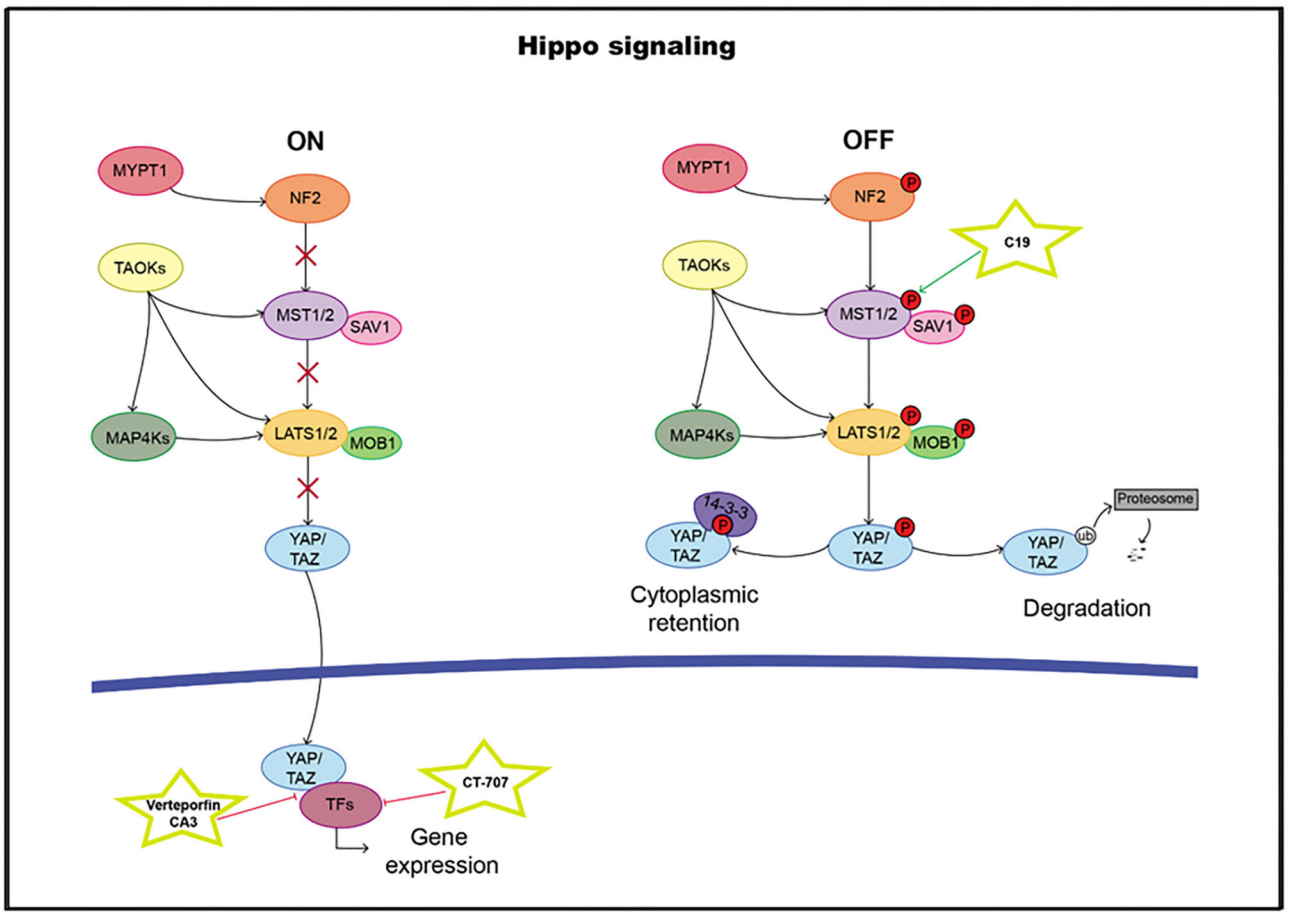

FIGURE 6 | Hippo pathway including targets and drugs that could be employed to inhibit it. **(Left)** active signaling pathway. **(Right)** inactive signaling pathway.

or toxicity problems. Consequently, extensive effort is being made in the research of new drugs to address these issues.

Therapeutic Targets and Drugs

Regarding therapeutic opportunities for the Hippo pathway, YAP inhibition has been shown to produce promising therapeutic effects in different types of tumors, such as NSCLC, breast cancer and colorectal cancer (259–262). Additionally, the regulation of upstream or downstream molecules of this pathway also show antitumoral effects. However, the inhibition of the upstream regulators of YAP might serve to increase transcriptional activity and thus be counterproductive. This result makes the inhibition of the YAP/TAZ interaction and the suppression of their binding to their targets the most appealing strategies.

Verteporfin, an FDA-approved drug, was identified to inhibit the interaction between YAP and TEAD (263, 264). Therefore, it can decrease the expression of Hippo target genes; however, it has shown low solubility and stability, unfavorable pharmacokinetics, and rapid clearance; complicating its clinical use. Moreover, this compound lacks tumor specificity and have serious Hippo-independent effects, and thus, may originate adverse effects in healthy tissues (265–267). In addition, its mode of inhibition and mechanism of interaction with YAP remain unknown. Considering all, verteporfin might not be the most promising drug, nevertheless, some studies have shown that the loading of verteporfin into microparticles improves its specificity and pharmacokinetics, making it more suitable as a treatment (267–269). Similarly, the CA3 compound has been reported as a modulator of YAP/TEAD transcriptional activity through the inhibition of YAP, but it has drawbacks similar to those of verteporfin (270, 271).

Another possibility for YAP/TEAD inhibition is the lipid pocket at the core of the TEAD family, which is essential for TEAD folding, stability, and YAP binding (272–274). Some compounds targeting this domain in cell assays, such as flufenamic acid or chloromethyl ketone moieties, have been shown to inhibit cell proliferation and several Hippo pathway responsive genes. However, the underlying mechanism remains unclear because, in some cases, YAP/TAZ binding remains unchanged (275, 276). In recent studies, a cyclic YAP-like peptide has been shown to block the YAP-TEAD interaction through competition with endogenous YAP (277). However, this peptide has not yet been converted into a cellularly active compound. "Super-TDU," a peptide mimicking VGLL4, has also

been reported to compete with YAP binding to TEADs. Its mechanism is based on the binding of vestigial-like proteins (VGLL) to TEADs, and it has led to the reduced growth of gastric cells *in vitro* and *in vivo* (278). All these antagonistic compounds offer promising strategies; however, their clinical viability and applicability remain unproven.

Due to YAP druggability problems, it has been proposed to target its posttranscriptional modifications, which are critical for its oncogenic properties. In this context, some already approved drugs, including metformin, statins, dasatinib, pazopanib, and nicotinamide (NAM), and some agents being tested in clinical trials, such as the multikinase inhibitor CT-707, have been reported to decrease YAP activation through the regulation of posttranscriptional modifications (279, 280). These findings suggest that they might be plausible cancer therapies, although many more studies are needed.

Another potential approach to inhibiting the Hippo pathway is through the activation of MST and LATS kinases, which phosphorylate and inactivate YAP (281). The small-molecule compound C19 has shown the ability to activate MST and therefore to suppress melanoma cell growth in a mouse xenograft model (282). Moreover, metformin and statins have been known to activate LATS and inhibit YAP activity (283). However, their applicability as cancer therapeutic agents is still controversial and requires more clinical trials (284, 285).

Finally, the downregulation of Hippo pathway regulators is also possible at the transcriptional level. The combination of some epigenetic modulators, including I-BET151 and panobinostat, has been shown to induce the synergistic downregulation of the AKT and Hippo pathways in melanoma cell lines without binding to the cytoplasmic proteins of these pathways (286). Nevertheless, compounds regulating the epigenome show some drawbacks, for instance, off-target effects due to their lack of specificity, low stability and sustainability, and significant toxicity in normal cells (287).

Altogether, there are significant advances in the field of developing targeted therapies for the Hippo pathway. They could be a priceless weapon in the fight against cancer; nevertheless, we need more research in this area. It is important to better understand the mechanism of action of these compounds and to develop clinical trials to test their secondary effects and their applicability in cancer patients (**Table 1**).

TLR PATHWAY

Toll-like receptors (TLRs) belong to the pathogen recognition receptor (PRR) group in the innate immune system. They recognize exogenous ligands from invading pathogens (pathogen-associated molecular patterns or PAMPs) and endogenous ligands released from damaged host cells (damage-associated molecular patterns or DAMPs) (288–290). Ten TLRs are found in both human immune cells (T-cell and B-cell subsets, macrophages, and dendritic cells) and non-immune cells (epithelial cells and fibroblasts). Some of these TLRs are localized in intracellular vesicles, for instance, TLR3, TLR7, TLR8, and TLR9, while others are localized on the cell surface (291). TLRs are type I glycoproteins that share some common structural domains: an extracellular domain containing multiple leucine-rich repeats that enable the recognition of the ligand, a transmembrane region, and a highly conserved intracellular Toll-interleukin 1 (IL-1) receptor domain (TIR) necessary for signal transduction (292–294).

When a ligand binds to the extracellular domain of a TLR, it induces a conformational change in the receptor allowing for its homodimerization, and therefore, the binding of their TIR domains and the recruitment of different intracellular adaptor molecules. These adaptor proteins include Myd88, TRIF/TICAM-1, TIRAP/Mal, TIRP/TRAM, and SRAM (295, 296). All TLRs except TLR3 initiate signaling through MyD88 (the classical inflammatory pathway), forming a Myd88/IRAK1/IRAK4/TRAF6 axis that activates TAK1. This pathway triggers the activation of transcription factors, such as NF-κB, AP-1, and IFN regulatory factors (IRFs) (297–300). On the other hand, TLR3 and TLR4 can induce signaling through TRIF/TIRAM adaptor molecules instead of Myd88, leading to the activation of IRFs (301). All these activated transcription factors are translocated to the nucleus and interact with their target genes, including inflammatory cytokines, chemokines, and type I interferon (IFNs) (**Figure 7**).

Alterations in TLR signaling can have both antitumoral and protumoral effects on carcinogenesis and tumor progression. These effects depend on the TLR class, the cell type and the signaling pathway that is triggered in those cells. For instance, TLR agonists have been used as adjuvants in anticancer vaccines to stimulate immune cells to fight the tumor (302, 303). However, TLR expression in some cancer and immune cells is related to the activation of genes related to tumor progression and thus to tumor growth (304). Due to this double action of TLR signaling, it is important to study the role of each type of TLR in cancer individually and to always consider the origin of the tumor being investigated. Additionally, various cells in the tumor and its microenvironment can have different TLR expression patterns, and therefore, they will react differently to TLR modulation (305, 306).

Cancer Stem Cells

Despite their involvement in immunity against tumor invasion, the TLR signaling pathway with cancer stem cell properties have been found in different tumor types. For example, TLR2 activation in epithelial ovarian cancer has been shown to enhance a proinflammatory environment and thus to increase cell self-renewal through the upregulation of stem cell-associated genes (307). Similarly, targeting TLR3 with an agonist in breast cancer cells led to the expression of stem-associated genes, including OCT3/4, NANOG, and SOX2, because of the activation of β-catenin and NF-κB signaling pathways (308). Additionally, in murine models of hepatocellular carcinoma, TLR4 expression was associated with stem-like properties, including the invasion and migration of cells. TLR4

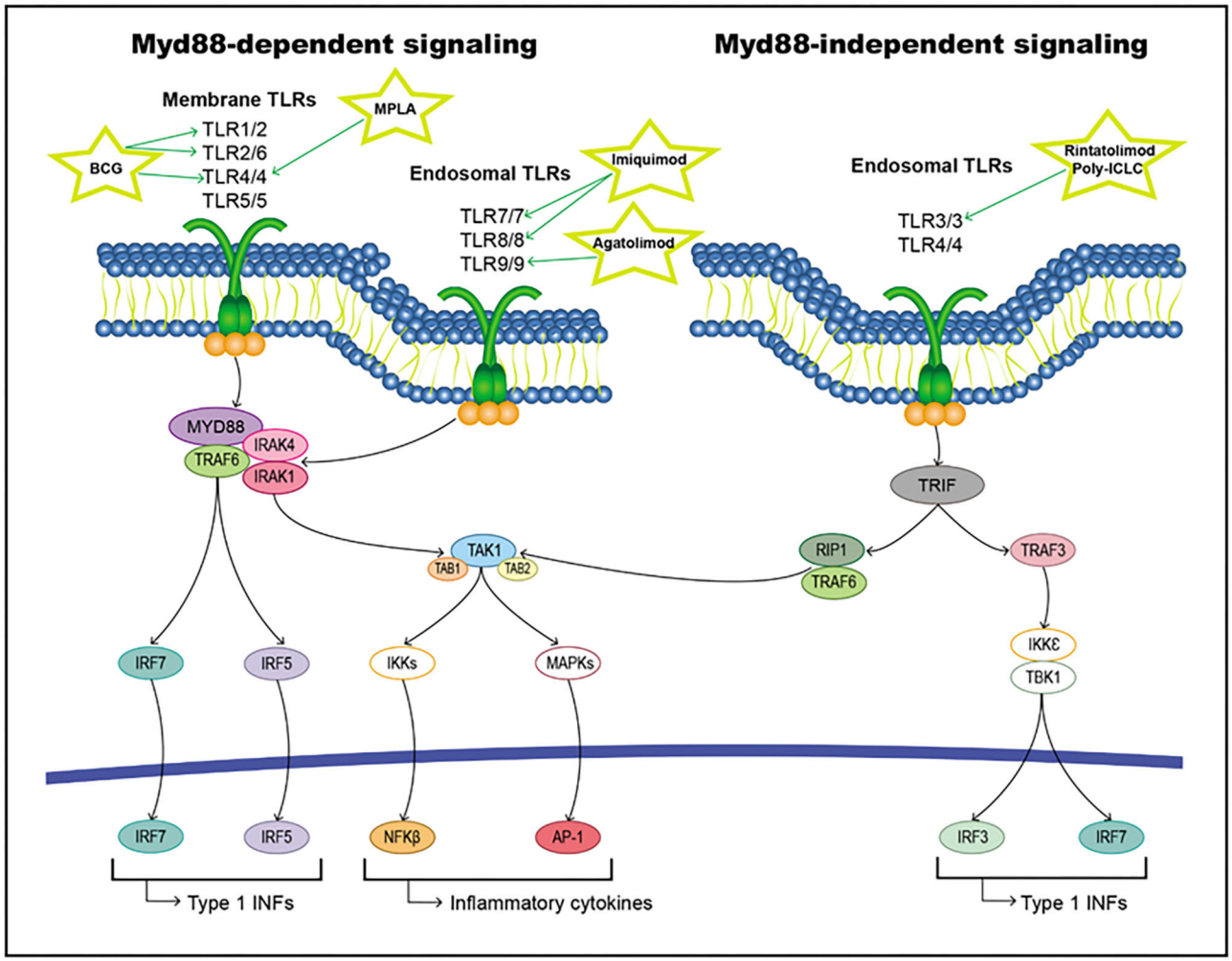

FIGURE 7 | TLRs pathway including targets and drugs that could be employed to inhibit it. **(Left)** Myd88-dependent signaling. **(Right)** Myd88-independent signaling.

stimulation is suggested to enhance the emergence of stem-like cancer cells through Nanog and STAT3, which cooperate to activate the metastasis regulator TWIST1 (309). Accordingly, there was a strong correlation between TLR4 expression and poor prognosis for patients (310, 311). Moreover, in breast cancer, TLR4 activation has resulted in enhanced stemness and tumorigenicity of cells. It has also been linked to poor prognosis and an increased relapse rate in patients (312). Nevertheless, the downregulation of TLR4 in glioblastoma CSCs has been observed. This result may be considered a mechanism by which these CSCs escape immune surveillance and is thought to be related to an increase in retinoblastoma-binding protein 5 (305).

In the case of TLR7, CD133+ cells in colorectal cancer patients showed increased expression of this marker, which was associated with a poor prognosis (313). Similarly, it has been suggested that TLR9 is able to promote the stem-like prostate cancer cell phenotype through NF-κB and STAT3 upregulation of stem-related genes, including NKX3.1, KLF-4, BMI-1, and COL1A1 (314). It has also been demonstrated that the upregulation of TLR9 in glioma cancer stem-like cells is able to activate STAT3 and thus maintain the quiescent state of tumor-repopulating cells (315).

All this information suggests a feedback loop of inflammation and stemness in tumor cells. The presence of extrinsic stimuli in the tumor microenvironment can activate proinflammatory pathways, for instance, TLR signaling. This activation leads to the upregulation of genes related to stemness and the epithelial-to-mesenchymal transition (EMT), which are able to induce and maintain the dedifferentiation of cells, converting them to CSCs (115, 316–319). On the other hand, CSCs constitutively exhibit deregulation in the expression of NF-kB, increasing the levels of inflammation within the tumor (320). This positive feedback loop might promote malignancy and resistance to treatments. Therefore, targeting TLR signaling may be a promising strategy for reducing CSC expansion and, thus, tumor progression.

Therapeutic Targets and Drugs

Targeting TLRs represents a promising strategy for tumor immunotherapy due to their ability to activate innate immunity and even stimulate adaptative responses for long-lasting defense against tumor antigens. Furthermore, in some cases, the activation of certain TLRs, such as TLR2 or TLR4, can have direct antitumoral effects on cancer cells. These TLRs have been used in different approaches; for example, they have been used as adjuvants for cancer vaccines or in combination with radiotherapy, chemotherapy and other immunotherapies. However, when targeting this pathway, it is important to consider that the same TLR can have different behavior, either protumoral or antitumoral, depending on the tumor type.

Treatments Based on TLR Agonism

There are multiple cancer treatments based on activating the immune response through different TLR receptors. A large number of compounds have been developed and used as monotherapy or in combination with other strategies to treat diverse types of tumors. Some molecules targeting this pathway have already been approved by the Food and Drug Administration (FDA) and are currently being used to treat patients. Many more are being tested in preclinical and clinical trials and might be in the clinic in the near future.

Bacillus Calmette-Guerin (BCG) is an FDA-approved treatment that activates TLR2/4 through its cell wall and TLR9 through its bacterial DNA (321, 322). It is the standard of care for patients with carcinoma *in situ* or urinary bladder cancer that has not invaded muscle (323). However, it still shows a 50% risk of failure (324) and the difficulty of its manufacturing process and its increasing demand have created a shortage of this treatment (325). Agents similar to BCG and their combination with other therapies are being tested in multiple clinical trials for different tumor types, such as colorectal cancer (326, 327), melanoma (328–330), and small cell lung cancer (331). Other bacteria that activate TLR2, such as *Mycobacterium indicus pranii* (Immuvac, CADI-05), are also being studied. CADI-05 is an approved treatment for leprosy, and interestingly, it has been shown to reduce myeloma and thymoma tumor size in murine models (332, 333). In addition, monophoryl lipid A (MPLA), currently used as a synthetic adjuvant in vaccines, is a derivative of lipid A and can stimulate TLR4. Some of its analogs are being tested as adjuvants for cancer vaccines, for instance, glucopyranosyl lipid A for skin cancer (334) and AS15 (a combination of MPLA with other immune stimulators) in distinct types of tumors (335, 336). These type of combinations might show a slight increase of mild side effects probability, specially, local injection site reactions (336, 337).

In the case of TLR3, Poly (I:C) is a TLR3 ligand that functions as a potent adjuvant for cancer vaccines (338); however, due to its fast degradation, new alternative agonists are being investigated. Some Poly(I:C) derivatives are promising, for instance, poly-ICLC (Hiltonol®) for solid tumors (339) and rintatolimod (Ampligen®) for fallopian tube, ovarian and brain tumors (NCT03734692 and NCT01312389).

Targeting TLR5 has shown promising effects in murine models, in which flagellin and different nanoparticles have shown antitumoral effects (340–342). A *Salmonella* flagellin derivative, entolimod (CBLB502), is being tested in clinical trials against advanced local and metastatic malignancies (343). Additionally, it has demonstrated radioprotective effects in animal models (344).

The TLR7/8 agonist imiquimod has been approved by the FDA and is currently being used for treating superficial basal cell carcinoma (345, 346). It has also shown efficacy in the treatment of different cutaneous tumors (347–349), and it is being tested in several clinical trials for skin and other malignancies, such as glioma and breast and prostate cancer (350, 351) (NCT01792505, NCT00899574, and NCT02234921). It might cause some local inflammatory reactions and systemic symptoms, including muscle aches, fatigue, and nausea, but, in general terms, it is well-tolerated (352). Resiquimod is another TLR7/8 agonist and has shown a more intense immune response than imiquimod (353). It is being explored in precancerous and malignant skin tumors (354, 355) and in multiple clinical trials as a vaccine adjuvant. Additionally, 852A, a TLR7 agonist, and VTX-2337, a TLR8 agonist, are being examined in clinical trials against different tumor types, such as ovarian, breast, cervical, endometrial, and head and neck cancers (NCT00319748 and NCT01334177).

Finally, CpG oligodeoxynucleotides are agonists of TLR9 and are being tested in several tumor types and in some clinical trials (356–359). For example, Agatolimod (CpG 7909) and is being studied as a vaccine adjuvant and as a monotherapy for various solid and hematological malignancies (360–362). Although the tolerability and safety of TLR9 ligands in monotherapy have been proven in numerous clinical trials, they have shown scarce antitumoral efficacy (363). The combination with other immune modulating compounds can greatly improve CpG ODNs-based strategies. Hence, Agatolimod is also being tested in combination with monophosphoryl lipid A and MAGE-A3 (a melanoma antigen) in phase II clinical analysis (NCT00085189). Furthermore, an alternative TLR9 agonist, SD-101, has been shown to overcome resistance to checkpoint inhibitors and is being investigated in association with these inhibitors in ongoing clinical trials (NCT02521870). With these types of drugs the primary adverse effects are also related to immunostimulation or systemic-flu like symptoms (364). Nevertheless, they could lead to autoimmune disorders if used as long-term treatments (365) (**Table 1**).

Treatments Based on TLR Antagonism

In some cellular locations, TLR antagonism, not TLR activation, is needed. For this purpose, inhibiting treatments are being tested, and some have entered phase I and II clinical trials. Molecules derived from LPS, such as E5564 and CRX-526, and antibodies targeting TLRs, such as OPN305, are able to inhibit TLR signaling and reduce inflammation, but they are not currently being used against cancer (366–369) (NCT02363491). Recently, various studies have shown that the blockade of TLR4 might have antitumoral effects in ovarian, breast and prostate

cancer (370–372). In another possible approach, bacteria, and/or gut microbiota are modulated by using probiotics or antibiotics. This approach has been shown to reduce TLR tumor-promoting signals and inflammation in several studies (373–376). However, the use of this technique in cancer is still being developed (**Table 1**).

Future Treatment Opportunities Targeting the TLR Pathway

Immunomodulation in cancer is currently being intensely researched, with some approved innovations showing substantially positive results, such as antibodies targeting T cell-mediated immune checkpoints, adoptive cellular therapies, or cancer vaccines. In this context, numerous trials are combining these novel treatments with TLR agonists to improve their activity (377–380).

Another possible approach using TLR as a clinical cancer treatment is based on targeting its downstream modulators. The MYD88 protein is involved in surface and endosomal TLR pathways and thus is considered an attractive candidate. In addition, some studies suggest that it may be involved in oncogenesis (381) and related to the clinical response to ibrutinib (382, 383). Similarly, IRAK4 is a downstream mediator of the pathway that links multiple TLRs to NF-κB activation. Constitutive activation or increased expression of this gene has been reported for different tumor types and is correlated with chemoresistance (384, 385). Other proteins, including TRAF6 and NF-κB, have been shown to stimulate bortezomib activity in preclinical models (386).

Finally, TLR might serve as a protective agent in cancer treatment. TLR5 agonists have shown chemoprotective and radioprotective effects in numerous studies (344, 387–389). Furthermore, they have been demonstrated to improve the outcome of bone marrow allotransplant and decrease the incidence of graft-vs.-host disease (GVHD) (390, 391).

In summary, TLR-targeted therapies for cancer are currently being successfully used, however, many opportunities remain to develop new compounds to provide better treatment for patients and to minimize the secondary effects of current strategies (**Table 1**).

OXIDATIVE STRESS AND CSCs

The generation of reactive oxygen species (ROS) in a high concentration is detrimental for the cell, due to the fact that oxidative stress promotes DNA, RNA, proteins, and lipids modifications. It is well-known that cancer cells, in consequence of its active metabolism and altered oncogenic and tumor suppressor signaling pathways, are usually characterized by a high ROS level. However, cancer cells are able to adapt and proliferate, becoming resistant to oxidative stress (392–394).

Oxidative stress has been associated with a variety of cancer-related effects: cellular proliferation, apoptosis evasion, invasion, metastasis, or angiogenesis. Different signaling pathways mentioned in this review are implicated in these ROS production outcomes. For instance, ROS could regulate EMT through activation of NFkB signaling pathway, or metastasis through Wnt signaling cascade stimulation, among other signaling routes (392, 395–398).

Aerobic glycolysis is energetically more efficient than anaerobic one, but leads to the generation of a big amount of ROS. Tumor cells accomplish adapting to oxidative stress switching aerobic glycolysis for anaerobic one, independently of oxygen available in the microenvironment. This phenomenon is called Warburg effect, and leads to a low ROS level formation, accompanied by a redox potential increase, through NADPH production (399–402). Furthermore, cancer cells possess higher antioxidant efficiency than non-tumoral cells. In order to counteract the less ATP generation in anaerobic glycolysis, tumor cells considerably increase glucose intake. It has been shown that cancer cells also activate the pentose phosphate pathway (PPP) with a prolonged ROS exposure, in order to acquire NADPH and nucleotides for DNA synthesis and repair (402–406).

Cancer stem cells seem to possess a lower ROS level than non-tumoral stem cells, due to different mechanisms useful to reduce oxidative stress and maintain stem cell properties (404, 407–409). However, there are controversial aspects in the metabolism regulation of CSCs. While several studies show that CSCs often present a high glycolytic metabolism, which preserves them from oxidative stress damaging, others highlight that they could be more dependent on oxidative phosphorylation. Furthermore, CSC could switch from anaerobic glycolysis to PPP in order to obtain a stronger antioxidant potential to maintain stemness. Also, increased PPP has been associated with drug resistance (403, 405, 406, 408, 410, 411).

Due to the dual consequences of an augmented ROS levels, the oxidative stress targeted therapies are under discussion. On the one hand, several well-known anticancer treatments increase ROS levels in cancer cells, in order to reach a threshold which causes the cell death, such as radiation, arsenic trioxide, 5-fluorouracil, or paclitaxel. Another strategies try to decrease ROS scavengers in cancer cells. On the other hand, as it has been mentioned above, ROS is related to several cancer-promoting effects, thus it might be advantageous to reduce ROS levels or increase antioxidant molecules, in order to restore de redox balance in the cell. For these reasons, the efficacy of these anticancer strategies depends on the ROS level in the tumor type and the capacity of the tumor to modulate its metabolism routes (404, 409, 412–419).

CONCLUSIONS

Currently, many observations have indicated the association between the CSC content and clinicopathological characteristics of tumors upon diagnosis. Additionally, CSC populations are resistant to conventional therapies aimed at the bulk of proliferative cells, and they can be enriched in a posttreatment setting. Therefore, targeting stemness pathways may provide promising strategies to actively eradicate tumors and metastasis. There are many pathways that regulate the CSC phenotype and its pluripotency state, providing mechanistic support for acquired drug resistance, including altered metabolism and oxidation states; phenotypic plasticity; increased membrane

extrusion pumps and receptors; altered apoptosis, autophagy, and/or cell cycle regulation; different DNA damage, and/or repair responses; epigenetic regulation and/or differential secretion of proteins or non-coding RNA. Some of these stemness pathways have been reviewed here, and their targeting approaches have been described. However, data on their proper response to the inhibitors and accounts of the extent to which they have antitumor efficacy are scarce. In most cases, the inhibitor activity depends on the extent of the molecular dependence on the specific targeted pathway in the CSCs or its ability to avoid targeting mature cells or normal stem cells to prevent undesirable toxicity.

Importantly, the stemness pathways are interconnected to regulate the CSC phenotype and the transition among different pluripotency states. This cross talk among pathways may drive the resistance to single pathway inhibitors and maintain the CSC phenotype. For example, cross talk between PI3K and Notch may contribute to the resistance to therapeutic PI3K inhibitors of breast cancer (420). Additionally, PIM inhibition might not have the same effect as AKT on MEK targets, which may not be effected by PIM single inhibitors *in vivo*. This phenomenon may explain in part the negative results obtained in clinical trials with single-agent therapy.

Other important reason for tumor resistance is based on advanced tumors, in general, containing expanded polyclonal CSC populations, which render them resistant to therapies against single CSC signaling pathways. Therefore, ongoing clinical trials using molecular biomarkers may be used to overcome these challenges. Moreover, the incorporation of CSC-based functional assays (for example, 3D organoids, pluripotency assays) may provide a better view of CSCs and therefore of tumor, eradication using these pathway-based therapeutic strategies.

Despite all of the challenges, some inhibitors for these pathways are currently used as a standard of care for patients, and many others are being tested in phases III and IV clinical trials. A huge effort is being made in order to develop more specific and less toxic compounds to target every single pathway known to be involved in CSC establishment and maintenance. Different drugs, antibodies, vaccines and, even, immunotherapeutic approaches are being assayed for this purpose. Therefore, although more research is needed to undercover additional regulatory elements, we expect to see new molecules targeting these pathways approved by the FDA in the following years.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Double-Edge Sword of Autophagy in Cancer: From Tumor Suppression to Pro-tumor Activity

Rodolfo Chavez-Dominguez[1,2], *Mario Perez-Medina*[1,3], *Jose S. Lopez-Gonzalez*[1], *Miriam Galicia-Velasco*[1] *and Dolores Aguilar-Cazares*[1]*

[1] Departamento de Enfermedades Cronico-Degenerativas, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosio Villegas", Mexico City, Mexico, [2] Posgrado en Ciencias Biologicas, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico, [3] Laboratorio de Quimioterapia Experimental, Departamento de Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico

During tumorigenesis, cancer cells are exposed to a wide variety of intrinsic and extrinsic stresses that challenge homeostasis and growth. Cancer cells display activation of distinct mechanisms for adaptation and growth even in the presence of stress. Autophagy is a catabolic mechanism that aides in the degradation of damaged intracellular material and metabolite recycling. This activity helps meet metabolic needs during nutrient deprivation, genotoxic stress, growth factor withdrawal and hypoxia. However, autophagy plays a paradoxical role in tumorigenesis, depending on the stage of tumor development. Early in tumorigenesis, autophagy is a tumor suppressor via degradation of potentially oncogenic molecules. However, in advanced stages, autophagy promotes the survival of tumor cells by ameliorating stress in the microenvironment. These roles of autophagy are intricate due to their interconnection with other distinct cellular pathways. In this review, we present a broad view of the participation of autophagy in distinct phases of tumor development. Moreover, autophagy participation in important cellular processes such as cell death, metabolic reprogramming, metastasis, immune evasion and treatment resistance that all contribute to tumor development, is reviewed. Finally, the contribution of the hypoxic and nutrient deficient tumor microenvironment in regulation of autophagy and these hallmarks for the development of more aggressive tumors is discussed.

Keywords: autophagy, cell death, metabolic reprograming, metastasis, carcinogenesis, tumor microenvironment, immune evasion, chemotherapy and targeted therapy resistance

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Hiroshi Kondoh,
Kyoto University, Japan

Reviewed by:
Sumit Sahni,
Royal North Shore Hospital, Australia
Cesar Cardenas,
Universidad Mayor, Chile

***Correspondence:**
Dolores Aguilar-Cazares
doloresaguilarcazares@yahoo.com.mx

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INTRODUCTION

Eukaryotic cells, over their lifespan, are continuously exposed to a variety of physical, chemical, and biological stresses that result in homeostatic imbalance. However, cells are equipped with a set of intracellular defense mechanisms to neutralize and adapt to such stress. Macroautophagy, hereafter referred to as autophagy, is an adaptation mechanism to preserve cellular integrity and viability. Intracellular content, including proteins, organelles and portions of cytoplasm, are sequestered in double-membrane structures, called auto phagosomes, that are delivered to lysosomes for degradation of their content (1). Autophagy is strictly regulated by a variety of genes termed autophagy-related genes (ATG). Autophagy in the absence of stress is active at basal levels to

degrade damaged cellular components and recycle nutrients to preserve the energetic state of the cell (2). However, in response to stresses, such as nutrient deprivation, hypoxia, genotoxic stress, accumulation of misfolded proteins, inhibition of protein synthesis or presence of pathogens, autophagy is upregulated to maintain cellular homeostasis (1).

Autophagy is dysregulated in distinct pathological conditions, such as infection, aging, neurological disorders and cancer. Autophagy in cancer cells is considered a double-edged sword since, in initial stages of tumorigenesis, it may act as a tumor suppressor by degrading potentially harmful agents or damaged organelles, thus avoiding the spread of damage including DNA alterations (3). However, in advanced stages of tumor development, autophagy is a tumor-promoting mechanism because of its ability to sustain tumor viability in stressful microenvironments. Besides this tumor-promoting activity, autophagy makes a notable contribution to resistance to distinct types of therapy, representing a serious obstacle for successful treatment (4).

According to Hanahan and Weinberg, tumor cells exhibit eight particular characteristics, called as hallmarks of cancer, that include sustained proliferation, evasion of growth suppressing signals, replicative immortality, angiogenesis, immune escape, evasion of cell death, metabolic reprogramming and activation of invasion and metastasis (5). Recent reports demonstrate that autophagy is associated with some of these hallmarks. For example, autophagy and apoptosis are typically considered as opposite pathways, yet under specific biological circumstances, they act in a cooperative fashion for cell demise.

Little is known concerning crosstalk between these pathways in the early stages of cancer development, but an increasing body of evidence suggests that under stressful conditions associated with cancer, autophagy and apoptosis cooperate to limit the growth of incipient tumor cells. Kitanaka et al. reported that autophagy participates in spontaneous regression of high expressing-RAS neuroblastoma. Dying cells during regression do not exhibit morphological and biochemical signs of apoptosis, suggesting that autophagy may serve as an additional mechanism for cell death (6).

Nutrient demand is increased as tumors develop to sustain cell proliferation. Moreover, the uncontrolled proliferation of cells leads to critical fluctuations in the availability of nutrients. Tumor cells display reprogrammed metabolism adapted to stress induced by decreased supplies of essential nutrients. Additionally, some metabolites derived from metabolic reprogramming, activate autophagy to increase recycling of nutrients and sustain tumor viability. Autophagy thus provides tumor cells with metabolic plasticity to tumor cells due to the diversity of substrates degraded (7). The role of autophagy in epithelial to mesenchymal transition as well as during metastasis will also be discussed. Autophagy participates in promoting cell survival against stressful conditions elicited along with these processes (8).

In this review, we will discuss the role of autophagy during tumor development, from early to late stages of tumor growth. Moreover, crosstalk between autophagy and apoptosis, metabolic reprogramming, and metastasis will be examined. Further, the emerging role of autophagy as an immune evasion mechanism is considered. Finally, the repercussions of autophagy in resistance to distinct cancer treatments are assessed.

REGULATION OF AUTOPHAGY

The mammalian autophagic process can be divided in three phases: phagophore formation, elongation of isolation membranes, and maturation. Under optimal physiological conditions, the nutrient sensor mammalian target of rapamycin (mTOR) interacts with Unc-51-like kinase 1/2 (ULK 1/2) complex, composed of ULK 1/2 kinases, Atg13, Atg101, and FIP200 proteins. mTOR phosphorylates ULK 1/2, causing inhibition of its kinase activity. However, under stress, such as starvation, ULK 1/2 is activated by the kinase of AMP (AMPK). AMPK functions as a monitor of intracellular energy levels by sensing AMP/ATP ratio. During starvation, intracellular levels of AMP increase leading to AMPK activation (9). AMPK regulates activation of ULK 1/2 by direct and indirect mechanisms. The direct mechanism is due to AMPK-mediated phosphorylation of ULK-1 at serine residues 467, 555, and 638, resulting in ULK activation (10). Mutational-directed loss of these residues in ULK-1 in human osteosarcoma U-2 OS cells and mouse embryonic fibroblast (MEF) inhibits autophagy. This loss leads to accumulation of damaged mitochondria (10). The indirect regulation of ULK 1/2 occurs via suppression of mTOR activity. In this sense, AMPK downregulates mTOR by phosphorylation of the tuberous sclerosis complex 2 (TSC2), which is an mTOR inhibitor, or by phosphorylation of the regulatory associated protein of mTOR (Raptor) (11, 12). These post-translational modifications promote mTOR dissociation from the ULK 1/2 complex leading to activation of ULK 1/2 kinase, which phosphorylates Atg13 and FIP200 (1, 11).

When ULK-1, located in the nascent phagophore, activates class III phosphatidylinositol 3-kinase (PI3K) VPS34, conversion of phosphatidylinositol to phosphatidylinositol 3-phosphate is promoted by the VPS34, Beclin-1, VPS15, Atg14, and p150 complex (13, 14). The activity of this PI3K complex is modulated in two ways: ultraviolet irradiation resistance-associated gene and BAX-interacting factor 1 (Bif-1) favoring its activity. Conversely, members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, or the run domain Beclin-1 interacting cysteine-rich containing protein (Rubicon), have a negative effect on the activity of the complex (1). In the latter case, Bcl-2 proteins interact with the BH3-binding region of Beclin-1 that prevents their interaction with VPS34, thus inhibiting autophagy. Transgenic mice with Beclin-1 gene mutations in its BH3-binding region show higher levels of basal autophagy in distinct tissues compared to wild type mice (15) (See **Figure 1**, left panel).

The next step, the elongation of isolation membranes, is regulated by two ubiquitin-like conjugation systems: Atg5-Atg12 and LC3 pathways. The Atg5-Atg12 complex is formed by Atg12 activation by Atg7 and transfer to Atg10 before conjugation with Atg5. Finally, the complex Atg5-Atg12 is non-covalently conjugated to Atg16 to form the complex Atg5-Atg12-Atg16 that displays E3 ligase activity (16). Conversely,

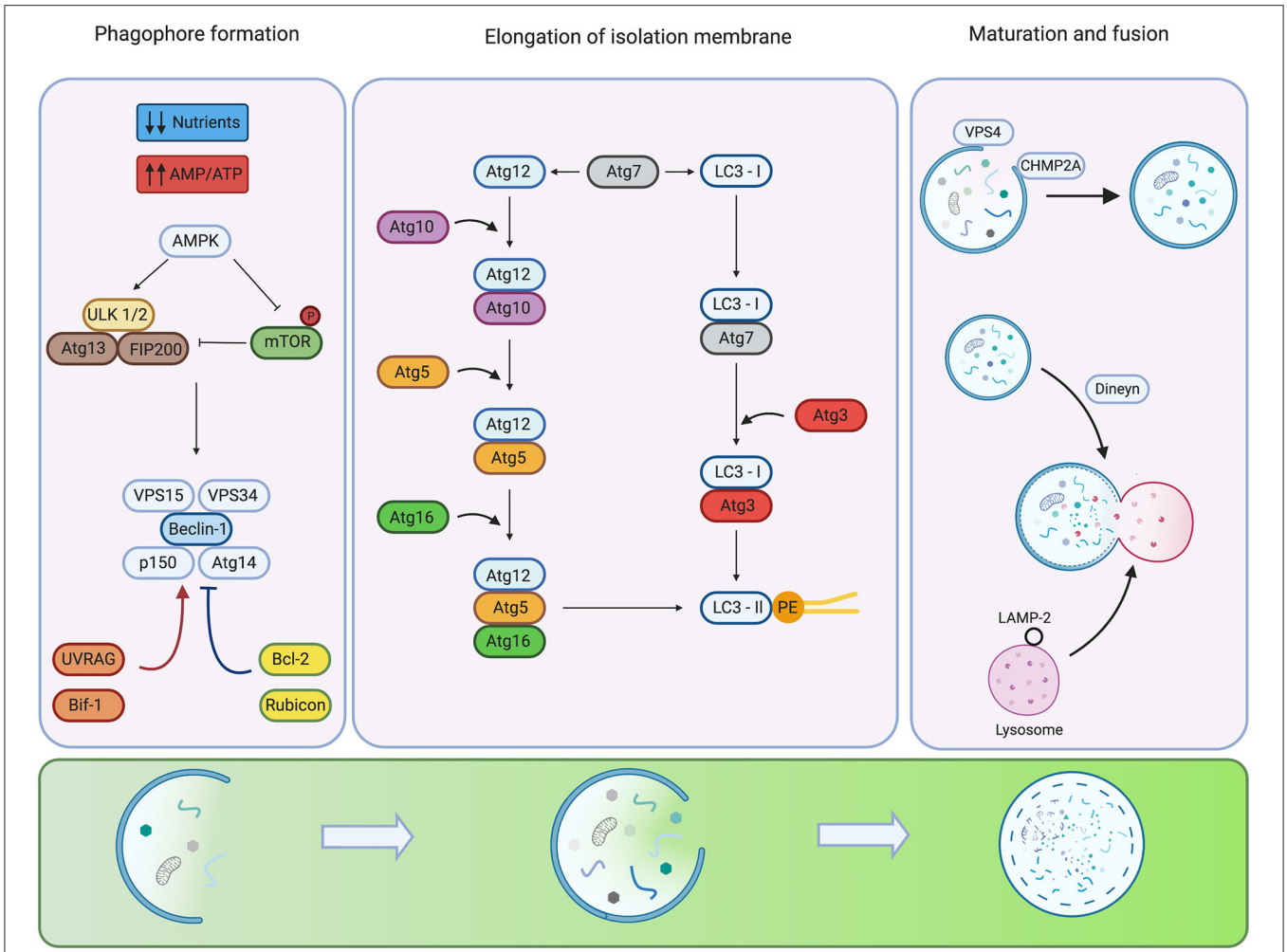

FIGURE 1 | Regulation of the mammalian autophagy. Under low nutrient conditions or starvation, the energy sensor AMPK detects alterations in energy pools (AMP/ATP ratio) inhibiting autophagy repressor mTOR and activating ULK1/2 complex. For phagophore formation, ULK1/2 complex activates the Beclin-1 P13K-class III complex. Additional systems activate (red arrows) or inhibit (blue arrows) the activity and assembly of the complex. The elongation of the isolation membrane requires the participation of two ubiquitin-like conjugation systems. The formation of the Atg12-Atg5-Atg16 complex involves the activity of Atg7 and Atg10. The LC3 requires the participation of Atg4 to hydrolyze LC3 into LC3-I, Atg3 as well as Atg7 for conjugation of LC3-II to pohosphatidyletanolamine (PE). Phagophore closure is regulated by members of ESCRT, CHMP2A VPS4. In the late steps of maturation and fusion, Dynein participates in the mobilization of auto phagosomes. The fusion of auto phagosomes with lysosomes is mediated by members of the SNARE family. Created by BioRender.com.

the LC3 pathway begins with the C-terminus cleavage of LC3 by the protease, Atg4B, to generate the soluble form, LC3-I. LC3-I is then conjugated to phosphatidylethanolamine (PE) by Atg7, Atg3 and the Atg5-Atg12-Atg16 complex, producing the LC3-II conjugated form (1) (See **Figure 1**, mid-panel). Some proteins, such as p62 (also known as sequestosome-1), NBR1 and NIX harbor an LC3-interacting region (LIR) which facilitates the recognition of ubiquitylated proteins or specific organelle membranes to selectively deliver cargo to auto phagosomes (17, 18). Although Atg5 and Atg7 are crucial molecules for autophagy, recent studies show that autophagy can be induced by etoposide in Atg5 or Atg7-deficient MEF (19). This Atg5/Atg7 independent form of autophagy is termed "alternative autophagy". The elongation and closure of the isolation membrane in this alternative pathway are mediated by fusion of endosomal membranes with *trans*-Golgi, and depends on the activity of Rab9 GTPase that replaces Atg5/Atg7 of the canonical pathway (19).

For phagophore closure in the canonical pathway, participation of members of the endosomal sorting complex required for transport (ESCRT), mainly CHMP2A and the vacuolar protein sorting-associated-4 (VPS4), is required (20). CHMP2A is translocated to the edge of phagophore structures in this process to promote closure of the membranes. Also, VPS4 locates on the outer leaf of nascent autophagosomal membranes to promote disassembly of ESCRT molecules in an ATP-dependent manner (20) (See **Figure 1**, right panel). Experiments carried out in U-2 OS cells demonstrate that genetic

inhibition of CHMP2A or VPS4 impairs phagophore closure, preventing the formation of nascent auto phagosomes and causing late fusion with lysosomes (20).

Finally, in the maturation step of auto phagosomes, LC3-II located in the outer autophagosomal membrane is delipidated, and auto phagosomes fuse with lysosomes to form auto phagolysosomes, leading to degradation of auto phagosome content by several hydrolytic enzymes (1). Auto phagosome-lysosome fusion is mainly regulated by soluble NSF attachment protein receptors (SNAREs), specifically Qa-SNARE, syntaxin 17, Qbc-SNARE and lysosomal R-SNARE (21). Also, small GTPase Rab7 and the homotypic fusion and protein sorting participate in auto phagolysosome formation (22) (See **Figure 1**, right panel).

AUTOPHAGY AND APOPTOSIS CROSSTALK IN CANCER

Autophagy and apoptosis represent two self-regulatory mechanisms by which cells respond to different types of stresses and death stimuli to maintain homeostasis. Apoptosis is a type of regulated cell death related to the elimination of cells and tissues during embryonic development and also in the removal of damaged cells in adult organisms, thus limiting their proliferation (23). Apoptosis is classified in two mechanisms depending on the type and the source of stress. The intrinsic pathway of apoptosis is activated by intracellular stressors such as DNA damage, endoplasmic reticulum stress, accumulation of reactive oxygen species (ROS), and mitotic defects (23). In contrast, the extrinsic pathway is triggered by extracellular stress and is sensed by distinct death receptors expressed on cell surfaces. Such factors include tumor necrosis factor receptor 1A (TNFR1A) and Fas cell surface receptor (FAS). Activation of extrinsic pathway requires the formation of the death-inducing signaling complex which in turn requires association with TNFRSF1A associated via death domain (TRADD) and Fas-associated via dead domain (FADD) to TNFR or FAS, respectively (23). Both pathways converge in the induction of permeability in the mitochondrial outer membrane, releasing a wide variety of apoptogenic molecules leading to cellular disassembly.

Although autophagy and apoptosis act antagonistically, under specific biological conditions, their crosstalk can lead to cooperation for cellular demise. Currently, accurate molecular interactions of apoptosis-autophagy crosstalk in cancer remain unclear. In the present section, we discuss the participation of key regulatory molecules shared between processes and their impact on cancer, focusing on early stages of tumor development.

As previously mentioned, Beclin-1 is an important protein in the early stages of autophagy. Several studies demonstrate that autophagy may serve as a tumor suppressor. *beclin 1* $^{+/-}$ mice show a higher incidence of spontaneous lymphomas and carcinomas in lung, liver, and mammary tissue (24). Moreover, Beclin-1 is monoallelically deleted or epigenetically silenced in 50–70% of human breast, prostate and ovarian cancer (4, 25, 26). These findings suggest that Beclin-1 is important for the development of cancer and may serve as a tumor suppressor. Loss of Beclin-1 blocks activation of autophagy, and thus precludes its cytoprotective role. This impairment of degradation of potentially carcinogenic agents or damaged organelles leads to the spreading of damage inside cells and increases the risk of cancer development. In this sense, autophagy is proposed as the "guardian of the genome" since it helps mitigate DNA damage (3). Monoallelic loss of *beclin-1* gene in a mouse model of breast cancer led to increased signs of DNA damage and activity of repair systems, therefore increasing the chance for introduction of mutation and thus the risk of tumorigenesis (27). Besides autophagy, Beclin-1 is implicated in apoptotic cell death, representing a node of crosstalk between these mechanisms (28). *In vitro* experiments show that Beclin-1 overexpression in gastric cancer and glioblastoma cell lines induces apoptosis upon exposure to cytotoxic agents (29, 30). These pro-apoptotic properties of Beclin-1 might be explained by two mechanisms. First, as Beclin-1 interacts through its BH3-only domain with Bcl-2 anti-apoptotic molecules, Beclin-1 overexpression may release pro-apoptotic molecules such as BAX and BAK from Bcl-2 to promote intrinsic apoptosis (**Figure 2**, right panel). Additionally, caspase-mediated cleavage of Beclin-1 promotes apoptosis. Withdrawal of serum in Ba/F3 murine pro-B cell lines promotes autophagy. However, sustained depletion of growth factors induces apoptosis with activation of caspases which cleave Beclin-1, rendering distinct fragments. The C-terminal fragment moves into mitochondria and introduces and provokes the release of pro-apoptotic molecules, such as cytochrome-c and HtrA2/Omi (31) (**Figure 2**, right panel). It is possible that in early stages of carcinogenesis, loss of Beclin-1 affects autophagy induction, and also impacts apoptosis regulation, especially in cells with molecular alterations in apoptotic genes.

Members of the Atg5-Atg12-Atg16 complex are also involved in the interplay between autophagy and apoptosis. This complex, as previously mentioned, is part of an ubiquitin-like conjugation system active in the elongation phase of autophagy. Specifically, some findings relate Atg12 protein to apoptotic cell death. Atg12 harbors a BH3-like domain within its structure and physically interacts with anti-apoptotic Bcl-2 molecules such as Mcl-1 and Bcl-2 (32). This interaction may release pro-apoptotic molecules to induce intrinsic apoptosis. For example, Atg12 expression is regulated by distinct transcription factors, such as factors in the forkhead homebox transcription factor family (FOXO) that are induced by different stressors (33). Atg12 is overexpressed after different carcinogenic insults, suggesting that it might participate in autophagy and apoptosis induction in the early stages of carcinogenesis (34). In 2018, Yoo et al. transfected rat intestinal epithelial cells with oncogenic H-RAS and observed that Atg12 was downregulated in these cells due to increased proteasomal degradation, mediated by MAPK activation. In addition, this same group demonstrated that ectopic expression of Atg12 in oncogenic-RAS intestinal epithelial cells resulted in decreased clonogenicity and increased cell death by apoptosis (35). Although increased expression of Atg12 has been found in certain solid tumors, in the early stages of carcinogenesis it might participate in the induction of autophagy also in activation of apoptosis.

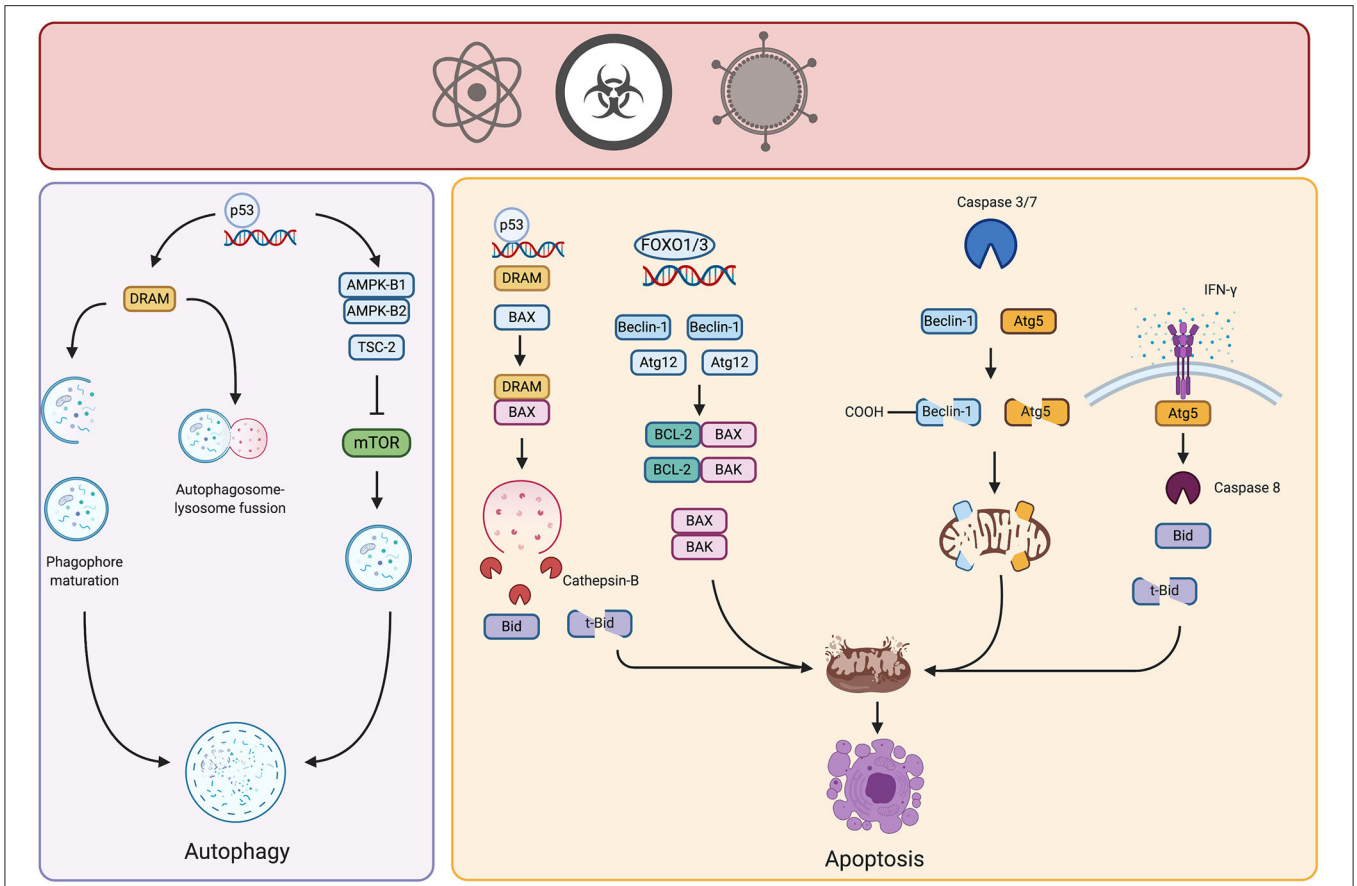

FIGURE 2 | Crosstalk of autophagy and apoptosis in cancer. Potential carcinogenic agents induce distinct types of stress in cell, triggering autophagy or apoptosis. Under certain threshold of damage, stress-responsive transcription factors such as p53 or FOXO promote the upregulation of genes involved in control and activation of autophagy, thereby neutralizing the damage. However, if the carcinogenic stimulus persists and damage is above threshold, autophagic proteins interact with pro- or anti- apoptotic molecules triggering intrinsic or extrinsic apoptosis, therefore limiting the growth of incipient tumor cells. Created by BioRender.com.

In vitro studies using HeLa cells indicate that IFN-γ treated cells die by apoptosis preceded by autophagy. Cell death is dependent on expression and interaction of Atg5 and FADD (36) (**Figure 2**, right panel). Although precise molecular mechanisms remain elusive; the extrinsic pathway of apoptosis is presumably activated. We propose a similar phenomenon in the early stages of carcinogenesis, especially considering the participation of immune response. Immunoediting theory suggests that, during the elimination phase, immune cells remove incipient tumor cells through different mechanisms, involving the release of some cytokines such as IFN-γ (37). Accumulation of this cytokine could lead to the elimination of nascent tumor cells. Moreover, similar to Beclin-1, Atg5 is cleaved by calpain rendering fragments that localize in the mitochondria and promote the release of pro-apoptotic molecules (28).

Another important molecule participating in the crosstalk between autophagy and apoptosis is the BH3-only protein, BIM. BIM interacts with other pro-apoptotic members of the Bcl-2 family during apoptosis to induce the release of apoptogenic molecules from mitochondria, thereby activating the intrinsic pathway (38). BIM is present in cells in three splice variants: BIM-short (BIM_S), BIM-long (BIM_L), and BIM-extra-long (BIM_{EL}) (39). BIM_S and BIM_{EL} participate in apoptosis induction, BIM_L displays an important role in autophagy. In IL-7 cultured T-lymphocytes, BIM_L localizes in mature lysosomes through interaction with dynein (39). BIM_L silencing was not reported, however, lack of BIM_L may affect fusion of lysosomes with phagosomes and subsequent degradation of contents. BIM polymorphisms are detected in lung cancer patients (40). We propose that participation of BIM in cancer is crucial since its loss in early stages of carcinogenesis impairs both apoptosis and autophagy, leading to the emergence of tumors.

Another key modulator of autophagy and apoptosis is the tumor suppressor protein TP53, hereafter referred to as p53. p53 is an intracellular sensor of stress caused by genotoxic agents or activation of oncogenes (41). Under non-stressed conditions, p53 is degraded in the cytoplasm by the E3-ubiquitin ligase MDM2. Nonetheless, the cytoplasmic pool of p53 downregulates autophagy by physical interaction with FIP200, thereby inhibiting ULK-1/2 complex activation (42, 43). However, different cellular insults cause stabilization of this

protein and localization in the nucleus. In turn, p53 presence in the nucleus leads to upregulation of transcription of distinct genes involved in cell cycle control, repair of damaged DNA, apoptosis and autophagy (41). p53, activated by genotoxic stress, induces autophagy by upregulation of AMPK, thus increasing expression of its β-1 and β-2 subunits and TSC-2, leading to mTOR inhibition, as discussed above (44). In addition, animal models show that the absence of Atg7 induces pancreatic neoplasia without progression to an aggressive phenotype in mice expressing mutated K-RAS. However, the concomitant loss of p53 leads to development of more aggressive pancreatic tumors. Further, p53 activated cell cycle arrest and apoptosis during early stages of tumor development in defective autophagy cells, limits tumor growth (45). These findings suggest that autophagy protects cells from the damage induced by oncogenic signals. Additionally, whether autophagy is defective, p53 limits tumor development by arresting or eliminating incipient tumor cells.

Nuclear p53 also regulates the transcription of the damage-regulated autophagy modulator (DRAM) that represents another point of crosstalk between autophagy and apoptosis. In A549 lung cancer cell lines, soon after exposure to mitochondrial inhibitors or genotoxic agents, DRAM was localized in lysosomes, regulating the process of autophagy in a p53-dependent manner (46). Specifically, DRAM participates in LC3-I to LC3-II conversion, lysosomal acidification, and degradation (46) (**Figure 2**, right panel). However, sustained stress promotes participation of DRAM in apoptosis, a phenomenon again dependent on p53. Further investigation in lung and cervical cancer cell lines revealed that DRAM regulates apoptosis by disrupting Bcl-2/BAX interaction, interacting with BAX and directing it to lysosomes, where BAX promotes the release of cathepsin-B. Once cathepsin-B is in cytosol, cleaves Bid into t-Bid provoking the release of apoptogenic molecules from mitochondria (47) (**Figure 2**, right panel). In ovarian cancer, DRAM is downregulated in cell lines and tumor samples of advanced stages, highlighting its participation as a tumor suppressor gene (48). Evidence is poor for participation of DRAM in cancer onset, and we propose that is important in autophagy-dependent clearance of damaged organelles elicited by potentially carcinogenic stimuli sensed by p53, hence, preserving cellular viability. Nonetheless, if carcinogenic stimuli persist or damage is above certain threshold, DRAM might participate in the induction of apoptosis of incipient cancer cells.

Thus, according to the experimental findings and propositions, during early stages of tumor development autophagy and apoptosis cooperate to prevent damage elicited by carcinogenic stimuli or eliminate damaged cells. However, more experimental evidence is required to demonstrate the precise molecular mechanisms governing the crosstalk between these processes during tumor development.

Notably, crosstalk between autophagy and apoptosis in cancer is not steady during tumor progression. Instead, it is modified by intracellular and extracellular perturbations affecting both processes. As tumors evolve, extracellular perturbations caused by a limited influx of nutrients and oxygen modify uptake and metabolism of nutrients and production of intermediary metabolites. Some of these metabolites regulate autophagy activation. Thus, autophagy can be activated via extracellular perturbations, inhibiting cell death, and sustaining cell viability.

AUTOPHAGY IN CANCER METABOLIC REPROGRAMING

The ability of cells to adapt to stress requires diverse changes in cellular processes, including metabolic pathways. Autophagy is a principal pathway for adaptive metabolic response, an important survival process.

Tumor cells reorganize metabolic pathways to supply ATP, building blocks for macromolecule biosynthesis, and redox molecules required to cell proliferation, invasion, migration, and other processes essential for malignancy, including chemo resistance (49). Consequently, the current research focus on metabolic reprogramming on the development and progression of human cancers reflects these hallmarks of cancer (5, 50).

Otto Heinrich Warburg was the first author to identify changes in the metabolism of tumor cells; he demonstrated that cancer cells avidly consume glucose and excrete high amounts of lactate when oxygen is present. He concluded that tumor cells increase glucose consumption and lactate production because of mitochondrial function (51). This effect was termed the Warburg effect, or aerobic glycolysis (52).

In normal cells, mitochondria oxidize glucose in the presence of oxygen to obtain ATP via the tricarboxylic acid cycle (TCA) and electron transport chain. In the absence of oxygen, the glucose molecule is converted to lactate by lactate dehydrogenase using NADH+, to ensure ATP production and evade glycolysis inhibition. The Warburg effect was initially considered a disadvantage for cancer cells, considering that the amount of ATP produced by the glycolytic pathway much less in comparison to mitochondrial ATP production (53). Nevertheless, glycolysis is the fastest way that cells obtain ATP from the glucose breakdown, and occurs independently of oxygen. Tumor growth is unorganized and the tumor microenvironment is poorly oxygenated; hence, glycolysis allows cancer cells to proliferate even in hypoxic conditions (54). Additionally, this metabolic pathway provides building blocks necessary for other metabolic pathways, such as the synthesis of fatty acids, nucleotides and serine (55, 56).

The Warburg effect is a metabolic adaptation associated with cell transformation that requires oncogene activation, such as RAS, AKT (57), and MYC (58), and the inhibition of tumor suppressors, such as p53 (59, 60). MYC and RAS activation impair decarboxylation of pyruvate, leading to reduce acetyl-CoA production, an essential metabolite in TCA cycle (61). Moreover, in RAS transformed cells, acetyl-CoA production is affected by inhibition of β-oxidation of fatty acids (62). Further, uptake of glucose and glutamine in MYC transformed cells is enhanced along with glycolysis and glutaminolysis (1).

Autophagy supports broad metabolic plasticity to tumor cells, providing biomolecules to almost all carbon metabolic pathways, based on the diversity of substrates degraded (63, 64). For example, the breakdown of several carbohydrates into monosaccharides can fuel glycolysis, and proteins break down

into amino acids or degradation of lipids in fatty acids provides substrates necessary for the TCA cycle. This process is essential for metabolic reprogramming (64, 65). Autophagy in tumor cells is closely associated with oncogenic activators and tumor suppressors. RAS activation induces autophagy via PI3K/mTOR, Rac1/JNK, Raf-1/ERK pathways, in addition to the Warburg effect discussed above (63, 66, 67).

Uncontrolled proliferation of malignant cells causes loss of tissue architecture. This structural tissue alteration promotes dysfunctional distribution of nutrients, growth factors, and oxygen within a tumor. Deficient formation of vasculature in the tumor supports the development of heterogeneous tumor microenvironments that differ depending on tumor region (5). The concentration of oxygen is a crucial parameter affected by the heterogeneous nature of tumors. Regions exist where oxygen concentration is <2% within the tumor, therefore, generating a hypoxic zone (68). These hypoxic conditions trigger cellular mechanisms to maintain homeostasis. Hypoxia-inducible factor 1 (HIF-1) is a primary transcriptional regulator during hypoxic conditions. HIF-1 is a complex of two subunits, α and β. The α subunit is degraded under normoxic conditions (oxygen-rich) (69, 70). However, during hypoxia ubiquitylation of the α subunit is decreased, promoting HIF-1 stability. HIF-1 binds to hypoxia-responsive element DNA sequences, facilitating a metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis (70). In tumor cells, HIF-1 upregulates expression of over 80 genes that are critical in glucose metabolism, cell survival, tumor angiogenesis, invasion, and metastasis, independent of oxygen concentration (71). In hypoxia or starvation, HIF-1 stimulates AMPK and subsequently induces autophagy via BINP3/Beclin-1 or by mTOR inhibition (72). Further, in hypoxia, HIF-1 stimulates transcription of regulated in development and DNA damage response 1 (REDD1) that activates the TSC1/2 complex, thereby inhibiting mTOR activity and promoting autophagy (73). HIF-1 also promotes the transcription of the gene encoding the Bcl-2/adenovirus E1 19 kDa protein-interacting protein 3 (BNIP3) that induces mitochondrial autophagy (mitophagy) by releasing Beclin-1 from Bcl-2 family members, therefore inducing autophagy (69).

However, glycolysis is strictly regulated. The hexokinase (HK) family in mammalian cells catalyzes the conversion of glucose to glucose 6-phosphate (G6P), representing the first rate-limiting step in glycolysis and other metabolic pathways such as pentose phosphate and gluconeogenesis (74). Phosphofructokinase (PFK) is another regulatory enzyme essential in regulating glycolysis. High levels of ATP allosterically inhibit the enzyme, decreasing affinity to fructose 6-phosphate. Thus, ATP/AMP ratio is an essential regulator of PFK. If ATP/AMP ratio is reduced, enzyme activity is increased. In addition, pH also regulates PFK activity. The inhibition of PFK by excessive accumulations of H^+ prevents the formation and release of lactic acid, which avoids a precipitous drop in blood pH (acidosis) (55).

Nonetheless, overexpression or specific mutations in cancer cells in HK proteins is associated with poor prognosis (75). Specifically, mutations in the catalytic site of PFK enzyme are promoted in the oncogenic process. In glioblastomas, AKT is degraded by polyubiquitylation leading to increased PFK activity, and consequent increase glycolysis, cell proliferation, and tumor growth (76).

Some tumor cells generally express high levels of isoform M2 pyruvate kinase (PKM2) and low levels of isoform M1 of pyruvate kinase (PKM1), a specific regulatory enzyme of glycolysis. Overexpression of PKM1 promotes glycolysis and inhibits mitochondrial oxidative phosphorylation. When PKM2 was knocked out in cancer cells, the PI3K/AKT/mTOR pathway and autophagy were inhibited, thereby leading to a decreased proliferation and inhibition of the invasive phenotype (77). Use of stable isotope tracers (e.g., ^{13}C), is currently employed for mapping metabolic pathways. Using this experimental strategy, it is possible to trace the fate of biosynthetic fuels through analysis of downstream isotope enrichment of labeled nutrients. Experiments in cancer patients confirmed that (i) glucose is metabolized through glycolysis and the mitochondrial TCA cycle and (ii) a significant fraction of the acetyl-CoA used in the TCA cycle is not derived from blood-borne glucose (78–80). This information casts doubt on the glycolysis dependency in tumor cells. Besides, accumulating evidence suggests that mitochondrial metabolism is required in tumor cells and is crucial for tumorigenesis, treatment resistance, migration, and metastasis. Some tumors overexpress critical metabolic enzymes and pathways associated with the mitochondrial metabolism. Progression in these tumors is driven by oncogenes and is associated with poor prognosis (52, 74, 81). For example, several cancer mutations in TCA cycle-associated enzymes, such as succinate dehydrogenase, fumarate hydratase, and isocitrate dehydrogenase, contribute to mitochondrial dysfunction during tumorigenesis (82, 83). Autophagy in this case might be essential for providing substrates for anaplerotic reactions, such as amino acids through protein degradation or lipids through turnover, to sustain mitochondrial metabolism (61). Most glucose is consumed by glycolysis, and glutamine becomes the primary substrate for the mitochondrial TCA cycle and generation of fatty acids and NADPH. Autophagy supports necessary metabolic rearrangements which makes cells highly dependent on autophagy for survival.

Metabolites, oxygen concentration, and oncogenes all regulate the initiation of auto phagosome formation, and regulation of autophagy is finely balanced by the integration of these signals. Autophagy is strongly induced in response to nutrient starvation, primarily controlled by mTOR (65).

Glutamine is the most abundant free amino acid and becomes physiologically essential in conditions of high proliferation. Glutaminolysis is the pathway that cells employ to transform glutamine to α-ketoglutarate, an irreversible reaction catalyzed by glutaminase (GLS) and glutamate dehydrogenase. In cancer cells, increased consumption of glutamine has been linked to regulation of oncogenes like MYC. Overexpression of MYC correlates with expression of cellular transporter of glutamine, SLC1A5, and enhances glutamine consumption in cancer cells (84, 85).

Glutaminolysis is proposed as an essential metabolic pathway in tumor cells that supplies carbon for anaplerotic pathways, such as TCA (86, 87). Proliferating cancer cells require high quantities of fatty acids and lipids to generate new membranes.

Citrate is diverted from the TCA cycle to sustain fatty acid synthesis, causing TCA cycle disruption, and compelling cancer cells to consume alternative nutrients to reestablish the TCA cycle (87, 88). Hence, glutamine stimulates the production of α-ketoglutarate, reconstituting the TCA cycle. In addition, glutamate produced by GLS is necessary for the synthesis of glutathione (GSH), an intracellular antioxidant that contributes to mitigation of oxidative stress in proliferating cells (88, 89).

α-ketoglutarate, induces translocation of mTORC1 in the lysosome, increasing phosphorylation of ribosomal protein S6 kinase (S6K) and inhibiting the formation of the ULK-1/ATG13/FIP200 complex resulting in inhibition of autophagy (86). However, in cancer cells this link between mTORC1 and glutaminolysis acts in both directions. Starvation leads to the activation of forkhead box O3 (FOXO3), which in turn, increases the expression of glutamate-ammonia ligase, the enzyme that resynthesizes glutamine from glutamate. The increase in glutamine synthesis abolishes the production of α-ketoglutarate from glutaminolysis, and thus inhibits mTORC1 and enhances autophagy (86, 90).

However, the interaction between glutaminolysis and mTORC1/autophagy seems to be more complex. α-ketoglutarate might activate mTORC1 and inhibit autophagy through an alternative mechanism involving acetyl-CoA synthesis and protein acetylation (91). Further, despite the inhibitory effect of glutaminolysis on autophagy, a by-product of glutaminolysis, ammonium, has a dual role in autophagy, activating this process at low concentrations and inhibiting it at higher concentrations (92).

Reprogramming of glucose and amino acid metabolism is accompanied by alterations in lipid metabolism in tumor cells to meet energy demands for sustaining viability and proliferation (**Figure 3**).

Lipids represent a wide variety of molecules, including sterols, triacylglycerols, and phospholipids. When energy supplies are plentiful, lipids are stored in cells as lipid droplets (LD) to avoid the accumulation of fatty acids in the cytosol (93). However, starvation promotes degradation of lipids stored in LD into fatty acids that are then metabolized by β-oxidation to obtain large amounts of ATP. Two primary metabolic pathways for lipid degradation within LD: neutral lipolysis and autophagic degradation. Neutral lipolysis involves the breakdown of lipids into fatty acids by cytosolic lipases which function under neutral pH environments (94). In contrast, autophagic degradation of LD (termed lipophagy) involves sequestration of portions or entire LD into auto phagosomes with subsequent degradation in lysosomes by acidic lipases (95). Lipophagy was firstly detected and studied in hepatocytes of starved mice (96). More recently, the process was shown in starved adipocytes, neurons and immune cells (96, 97).

Lipophagy is also strictly regulated by a variety of transcription factors that respond to nutrient status, such as the nuclear receptors of farnesoid X receptors, master regulator of lysosomal biogenesis transcription factor EB (TFEB), TFE3, members of the FOXO family and CCAAT enhancer binding protein α (C/EBP-α) (94, 98). The precise molecular mechanism of lipophagy is not clear. It is initiated by recognition of LD mediated by p62, NBR1, and NDP52, which display LIR domains and interact with LC3-II present in phagophores (94).

The role of lipophagy in cancer is still unknown, since some studies report a positive effect in tumor progression and others a negative impact. In 2015, Lu et al. reported that increased expression of C/EBP-α correlated with poor prognosis in patients with hepatocellular carcinoma (98). Hepatocarcinoma cell lines deprived of glucose and glutamine overexpress C/EBP-α and avoid cell death owing to increased lipid catabolism. Further, fatty acid β-oxidation or autophagy inhibition, induced cell death after nutrient deprivation, suggesting that lipophagy protects tumor cells from starvation (98). However, contrasting results were obtained in lung and hepatic tissue of knockout lysosomal acid lipase (LAL) mice that develop more tumors than wild type counterparts and display major susceptibility to metastasis. Further, the absence of LAL was associated with increased release of tumor-promoting cytokines (99, 100). In this case, it seems that lipophagy could act as a tumor suppressor in the early stages of tumor development, and in advanced stages, in which environmental and metabolic alterations are present, lipophagy may promote tumor progression. More studies are required to test this hypothesis.

The metabolic implications of this process are profound and multifaceted. First, autophagy-mediated degradation and recycling of cell substrates supports metabolism and promotes survival and tumor growth. Second, activation of autophagy in response to cancer therapy potentially leads to tumors resistance to conventional chemotherapy.

THE INTERPLAY OF AUTOPHAGY AND METASTASIS

Metastasis is a specific process of tumor aggressiveness, and most cancer patients die as a result of metastasis. Metastasis is a response to the challenge of metabolic alterations and tumor microenvironment (101). The unfavorable conditions in this microenvironment, such as hypoxia and lack of nutrients that occur during uncontrolled cell proliferation contribute to the development of metastasis (102). Clear evidence exists of migration of tumor cells at early stages of tumor development, but the metastatic process is associated with advanced stages of tumors. Autophagy plays an essential role in the metastasis cascade (8).

The steps of this cascade are the invasion of tumor cells into the primary site, the intravasation, and survival of the tumor cells in blood or lymph, and finally, extravasation and colonization by tumor cells at a distant site. Studies on the role of autophagy during the metastatic process are contradictory. Autophagy is reported to stop tumor cell metastasis (103, 104), but other authors suggest that autophagy favors metastasis (105, 106). Molecules involved in autophagic process are upregulated during metastasis. The LC3B protein is increased in lymph nodes of breast cancer patients compared to the primary tumor, and the expression of LC3B increases in advanced stages of disease (107). LC3B also increases in metastases of melanoma and hepatocellular carcinoma compared to primary

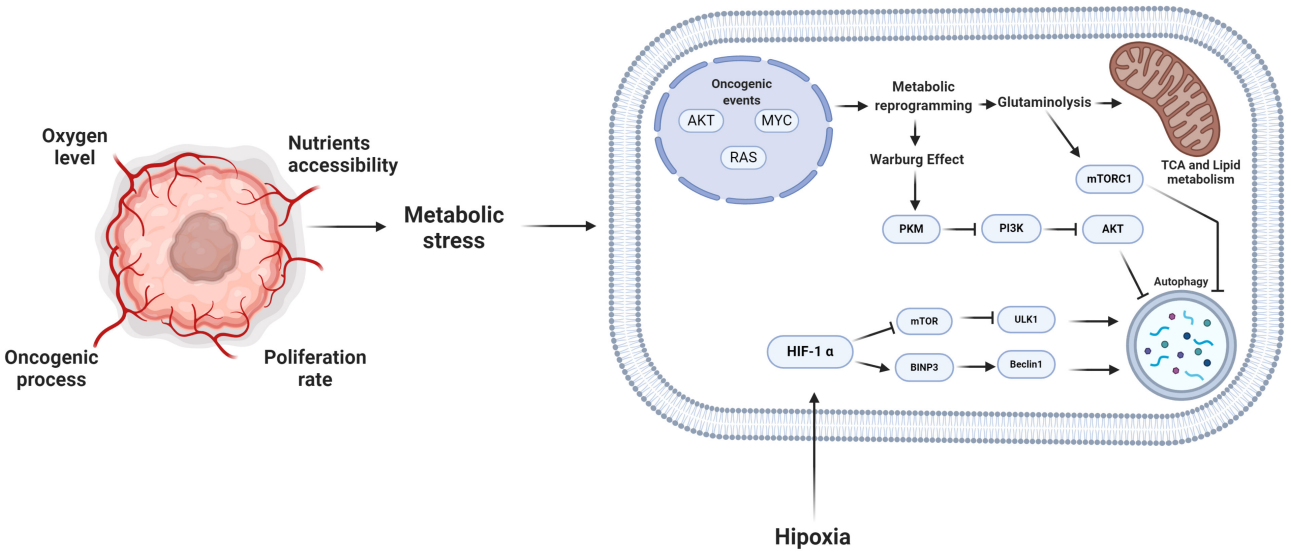

FIGURE 3 | Metabolic stress and autophagy. During the oncogenic process, the proliferation rate and the microenvironmental conditions promote that the tumor cells reprogram their metabolism. Consequently, autophagy plays an essential role in this reprogramming, providing different substrates to feed the pathways of tumor cells. However, the induction of autophagy depends on the stimuli to which the cell is subjected, the alteration of oncogenes such as MYC or RAS, the autophagy process is inhibited and during some microenvironmental tumor conditions such as hypoxia, autophagy is promoted. Created by BioRender.com.

tumors (108). Expression of autophagic molecules DRAM1 and p62 in glioblastoma correlates with a poor prognosis (109). Other molecules with oncogenic activity, such as long non-coding RNA (lncRNA) MALAT1 in pancreatic cancer, increase autophagy during the metastatic process (110). Blocking the expression of PD-L2 in osteosarcoma inhibits LC3-II and Beclin-1, impeding the ability of tumor cells to invade surrounding tissue (111). Annexin-A1 protein inhibits autophagy by activating the AKT pathway, which inhibits ERK-1/2 in nasopharyngeal carcinoma (112).

As previously mentioned, hypoxia is an autophagic-inducing factor, but may also promote autophagy and cell migration. IncRNA CPS1-IT1 in colorectal carcinoma suppresses expression of HIF-1α and decreases epithelium-mesenchymal transition (EMT). Autophagy was observed in this study (113). Levels of BNIP3, PI3KC3, and LC3-II were increased in a model of $CoCl_2$-induced hypoxia in cholangiocarcinoma. $CoCl_2$ at 100 μM, accelerated cell migration due to upregulation of the metastasis marker, phosphorylated focal adhesion kinase (pFAK) (114).

Soluble factors in the tumor microenvironment, secreted in an autocrine or paracrine manner by the tumor cells, trigger metastasis, and autophagy (115). One such factor is transforming growth factor (TGF)-β. Exposure to TGF-β in non-small cell lung carcinoma cell lines, induced autophagy and EMT (116). Autophagy and EMT are initiated in a TGF-β dependent manner in starved hepatocellular carcinoma cells (117).

The metastasis process begins with tumor cell invasion at the primary site and is coupled with EMT. Neoplastic cells lose adhesion and contact with other cells because of the EMT program (118). Loss of adhesion and activation of EMT trigger cell death stimuli that are avoided by activation of autophagy (119). Autophagy is reported to be mainly involved in promoting cancer cell motility. Tumor cells must evade anoikis, a type of programmed cell death that occurs when a cell detaches from the extracellular matrix. This process of cell death is mediated by apoptosis. Tumor cells can evade anoikis by activating autophagy (120). Another mechanism involving autophagy during cell motility is the degradation of adhesion molecules, such as paxillin in auto phagosomes (8).

Autophagy and Anoikis

Interaction between cells and extracellular matrices (ECM) requires complex bonds called focal adhesions (FA) (121). These junctions connect the cytoskeleton of epithelial cells with components of the ECM through integrins. On the extracellular side, integrins bind to ECM components, such as collagen, fibronectin, vitronectin, and laminin (122). While in the interior of the cell, the integrins bind to the cytoskeleton by means of a protein complex formed by talin, vinculin, paxillin, zyxin, and α-actin (121, 123). FA is regulated by the focal adhesion kinase (FAK)-Src, which is part of this complex. FA bond composition varies among tissues and recognizes components of ECM, changes in the cell surface, and physiological and mechanical stress. Dissociation of FA from ECM leads to cell death by apoptosis in a process called anoikis (124). The disruption of integrins interactions with ECM activates FAK-Src, which suppresses survival signals such as ERK, PTEN, and NF-kB (125). Lack of cell adhesion activates Bid and Bim, pro-apoptotic molecules that promote the assembly of BAX-BAK oligomers on the outer mitochondrial membrane, activating the intrinsic apoptosis pathway (125). Death by anoikis might also occur via the extrinsic pathway since the loss of adhesion leads

to downregulation of FLIP and increased expression of Fas and FasL (125).

The multi-functionality of FA allows detection of reduced integrin signaling that occurs after tumor cell detachment to the ECM. The signal of cell detachment is translated as a signal of metabolic stress, activating pathways such as PI3K-AKT, which has a fundamental role in the regulation of integrins by growth factors such as epidermal growth factor and TGF-β. These signals mediate a cellular survival response and inhibit pro-apoptotic proteins such as Bad, caspase-9, and glycogen synthase kinase 3b, among others (104, 126).

Tumor cells are remarkably resistant to anoikis, which favors cell motility and metastasis. Autophagy is the primary mechanism of resistance to anoikis in cancer (125). Fung et al. (127), showed in a 3D oncogenesis model of breast epithelial cultures, that cell shedding from ECM induces autophagy and tumor cell survival. In hepatocellular carcinoma cells, cell detachment from the ECM produced inactivation of the mTORC1 complex and activation of autophagy, evading anoikis. BNIP3 was upregulated by the ERK/HIF-1α pathway in this study, leading to autophagy (128). Also, astrocyte elevated gene 1 (AEG-1) protein has a high correlation with metastasis in hepatocarcinoma. AEG-1 induces resistance to anoikis by activating autophagy (129). Another molecule that induces resistance to anoikis by activating autophagy is miR-30a. By inhibiting this miRNA, a decrease in Beclin-1 and Atg5 was observed, as well as an increase in cell death (130) (**Figure 4**).

Autophagy and FA

As previously mentioned, cell-ECM attachments are essential for cell homeostasis. During cell migration, FA is involved in generating tension and traction necessary for cell motility. FA at the front of a cell is employed to anchor the cell to ECM, generating tension required to move the cell. At the rear of the cell, FA must be disassembled to producing advancing movement of the cell. This mechanical movement is termed FA turnover (121, 131, 132).

The metabolic stress produced by the lack of oxygen and nutrients in the tumor and the tumor microenvironment activates cellular motility. Autophagy participates in FA turnover in this context, by degrading paxillin in auto phagosomes and disrupting FA. Sharifi et al. found that inhibiting autophagy suppresses metastasis to the lungs and liver without affecting tumor cell proliferation in a metastatic 4T1 mouse model of breast cancer (8, 133). Paxillin in breast cancer and melanoma metastasis serves as FA scaffolding and contains a LIR. FAK-Src phosphorylates this domain in Y40, and paxillin is activated by LC3B and degraded via autophagy (134). Paxillin is recruited via the c-Cbl cargo receptor and LC3 (135). Finally, endothelial cells around the tumor secrete large amounts of the chemokine CCL5 that induces autophagy in tumor cells that display suppressed androgen receptors in a castration-resistant prostate cancer model. These authors reported co-localization of paxillin in auto phagosomes in metastatic tumor cells, indicating that paxillin is degraded via autophagy, favoring the disassembly of FA and cell motility (136) (**Figure 4**).

Autophagy During Colonization

The last step in the metastasis cascade is the colonization of host secondary organs. At this point, metastatic cells show EMT, detachment from ECM, intravasation and extravasation. Metastatic cells must reprogram their metabolism to cope with stress induced by metastasis processes.

Colonization represents a final challenging step for metastatic cells since target organs exhibit distinct environmental conditions from the primary tumor. Moreover, organs display varying environmental and metabolic conditions and exhibit distinct ECM composition, oxygen abundance and nutrient disposition (137).

When reaching host organs, metastatic cells encounter these distinct and hostile microenvironments. Cells do not adapt to these adverse environmental conditions, may enter into a state of dormancy. These dormant cancer cells remain clinically undetectable and progress, causing tumor relapse, and organ failure. Signals responsible for triggering tumor outgrowth and colonization of secondary organs remain unknown, the participation of ECM components and aspects of tumor microenvironments likely play essential roles. Dormant cells are characterized by a reversible growth arrest in G_0-G_1 cell cycle phases, reduced metabolism and a stem-cell-like phenotype (138, 139). To survive to this stage, dormant cells activate autophagy. Recent findings of Green et al. showed that autophagy inhibition in dormant breast cancer cells of mice decreased their viability, potential to growth and ability to form lung metastases *in vitro* and *in vivo* (140).

When metastatic cells are able to adapt to distinct environmental conditions, cells display a highly flexible metabolism that allows for colonization and formation of secondary tumor foci.

For example, metastatic cells attempting to invade and colonize lungs must adapt to the acute oxidative environment of these organs. To cope with oxidative toxicity, metastatic cells upregulate the expression of molecules controlling endogenous antioxidant responses, such as glutathione peroxidase 1, superoxide dismutase and peroxiredoxins (141, 142). If these antioxidant defense mechanisms are not sufficient, oxidative damage is generated in organelles. A growing body of evidence shows that accumulation of ROS triggers autophagy through distinct signaling pathways such as inhibition of PI3K-AKT-mTOR, and activation of AMPK and MAPK (143). ROS-activated autophagy promotes degradation of damaged material or organelles (143). In 2013, Peng et al. demonstrated *in vivo* that lung metastases of hepatocellular carcinoma cells exhibit higher levels of autophagy than primary tumors (108). In addition, the same group demonstrated that genetic inhibition of autophagy of highly metastatic hepatocellular carcinoma cells blocked lung colonization potential without changing EMT activation, invasion and migration (144). These findings do not provide information about the redox state of metastatic cells in intact and inhibited autophagy, but autophagy could, in theory,

be important for protecting cells against oxidative damage in the lungs.

Another example is the colonization of the liver. The liver is characterized into zones with a varying oxygen gradient and high glucose concentrations, therefore showing hypoxic regions enriched with glucose. In this way, any metastatic cell seeking to establish in the liver must be able to adapt to hypoxic and glucose-rich conditions. Several reports demonstrate that, under hypoxia, HIF-1 upregulates transcription of distinct genes involved in glucose metabolism including, but not limited to, glucose transporters and the enzymes, hexokinase 1/2, lactate dehydrogenase (LDH), enolase 1 and pyruvate dehydrogenase kinase 1 (PDK-1) (145). PDK-1 is a negative regulator of pyruvate dehydrogenase complex, thus reducing the entry of pyruvate to TCA cycle, decreasing mitochondrial activity, and promoting glycolytic metabolism. Kim et al. reported that hypoxia-induced transcriptional upregulation of PDK-1 ensures the glycolytic synthesis of ATP, mitigation of hypoxic ROS production and inhibition of apoptosis (146). Dupuy et al. reported that liver metastases upregulate their glycolytic activity under hypoxia by enhancing the activity of the PDK-1 (147). PDK-1 also regulates autophagy in other cellular settings. Quin et al. reported in acute myeloid leukemia cells that PDK-1 associates with ULK-1 promoting its activation and leading to induction of autophagy (148). Mariño et al. reported, in starved human osteosarcoma cells and in mouse heart tissue, that genetic or pharmacological inhibition of PDK genes resulted in autophagy inhibition (149). Participation of PDK-1 in autophagy induction during liver colonization by metastatic cells has not been studied, and we propose that besides promoting metabolic reprograming, PDK-1 also promotes autophagy as an adaptation mechanism to encourage the survival and colonization of liver by metastatic cells.

INVOLVEMENT OF AUTOPHAGY IN TUMOR IMMUNE EVASION

In the previous sections, we discussed the evolution of tumor microenvironments and how they sustain most hallmarks of cancer such as tumor growth, metabolic reprogramming, and cell death evasion, invasion and metastasis (5). In this sense, cellular components of the tumor microenvironment like endothelial cells, pericytes, cancer-associated fibroblasts and tumor-infiltrating immune cells play a key role in tumor growth (5).

The immune response is implicated as a key factor during tumor development. According to the cancer immunoediting theory, during early stages of tumor development, the immune system recognizes nascent tumor cells expressing neoantigens on major histocompatibility complex (MHC) molecules, thereby promoting tumor elimination mediated by natural killer (NK) cells or cytotoxic lymphocytes (CTL) (150). However, immune-mediated elimination also represents a selective pressure, and highly immunogenic tumor cells are eliminated while less immunogenic tumor cells survive, avoiding immune recognition and destruction, a feature established as a hallmark of cancer (5, 150). Distinct immune evasion mechanisms have been reported. For instance, decreased expression of death receptors; development of an immunosuppressive microenvironment through release of cytokines, such as TGF-β and IL-10, and recruitment of immunosuppressive cells (150). Emerging evidence also demonstrates that autophagy plays a key role in protecting tumor cells against immune-mediated elimination. In the present section, we discuss the participation of autophagy as an immune evasion strategy, focusing on NK and CTL-mediated elimination.

Autophagy is induced in response to adverse conditions elicited by the tumor microenvironment, such as nutrient deprivation and hypoxia. Tumor cells activate autophagy to help meet energy demands and sustain viability and proliferation. Additionally, in 2009 Noman et al. reported that hypoxic conditions impaired elimination of non-small cell lung carcinoma cells by autologous CTL (151). They found that stabilization of HIF-1α and increased phosphorylation of the signal transducer and activator of transcription 3 (pSTAT3), in tumor cells, were associated with evasion of immune surveillance. Further studies performed by this group demonstrate that hypoxia-induced autophagy is responsible for this phenomenon since pharmacologic or genetic inhibition of autophagy in hypoxic conditions restored susceptibility of tumor cells by CTL elimination (152). Further, inhibition of autophagy during hypoxia promoted pSTAT3 degradation in proteasome in a p62-dependent manner. Autophagy degrades p62 and consequently enhances the accumulation of pSTAT3. However, the mechanism by which hypoxia promotes the dissociation of pSTAT3 from p62 remains unclear. Molecular mechanisms are not completely studied, but STAT3 activation by hypoxia-induced autophagy in tumor cells could, in theory, help in escaping CTL-mediated elimination, since this transcription factor controls the expression of anti-apoptotic genes (153) (See **Figure 5**, upper panel).

Autophagy is also implicated in decreased susceptibility of tumor cells to elimination by NK cells. Baginska et al. reported, in MCF-7 breast cancer cells, that hypoxia-induced autophagy blocked NK cell-mediated lysis of tumor cells (154). In this study, recognition of tumor cells by NK cells and NK cell degranulation were not affected by hypoxia. Instead, tumor cells sequestered granzyme B and perforin granules inside auto phagosomes for subsequent degradation. These findings are supported using *in vivo* models, in which tumor growth of melanoma or breast cancer cells in C57BL/6 or BALB/c mice was reduced in autophagy-deficient tumor cells (154). Results obtained in this work led us to propose that a similar mechanism of cytoprotection elicited by autophagy could be responsible for impaired elimination of tumor cells by CTLs, such as granzyme B and perforin that are also present in CTLs (150) (See **Figure 5**, upper panel).

Collectively, these findings support the notion that tumor microenvironment has a critical role in tumor development since hypoxic conditions promote the activation of autophagy to protect cells against elimination by innate or adaptive immune cells.

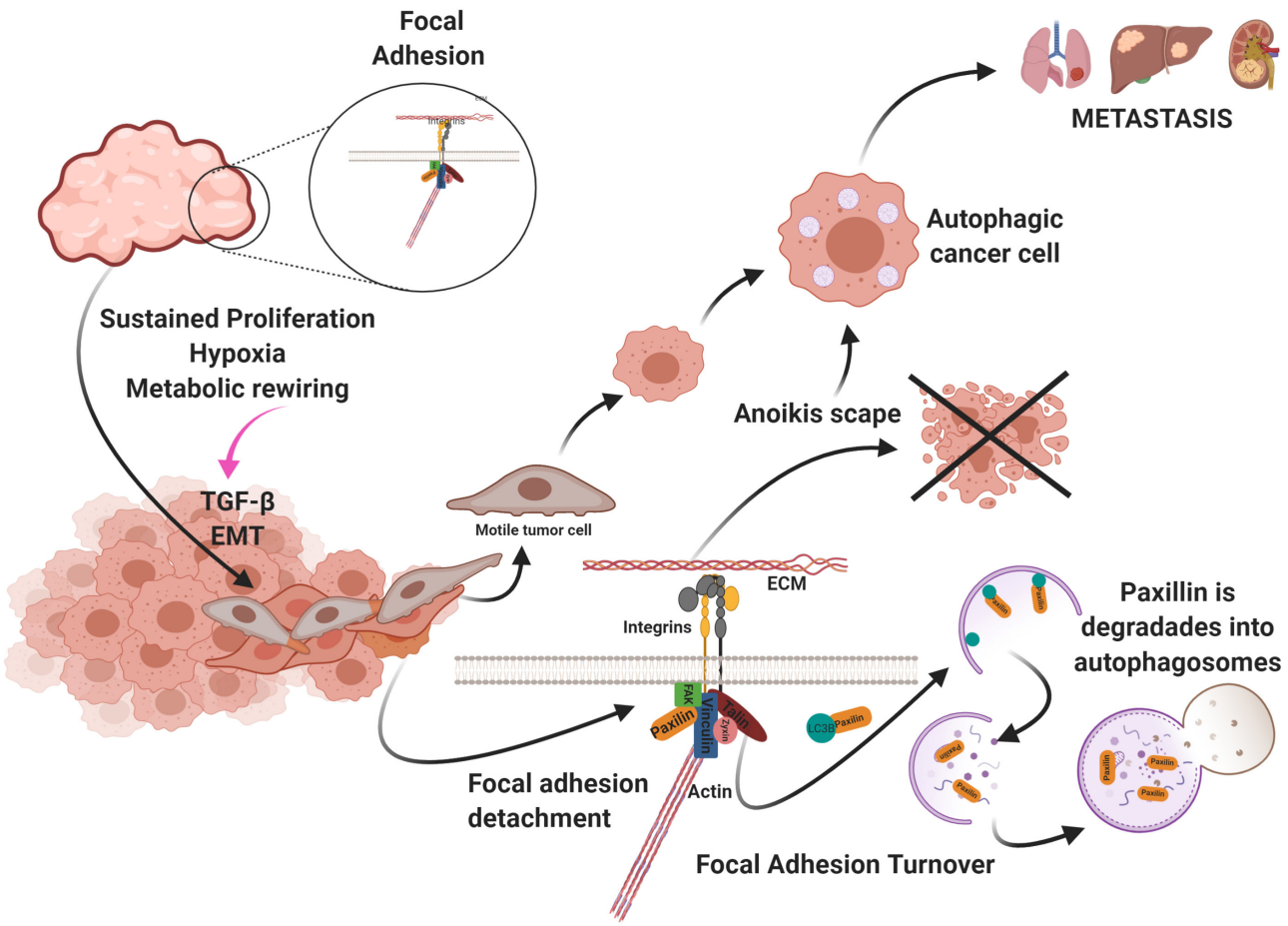

FIGURE 4 | Uncontrolled cell proliferation produces a high demand for oxygen and nutrients. As a result, the tumor becomes hypoxic and starved. These metabolic changes generate the activation of the epithelium-mesenchymal transition (EMT) program and the presence in the environment of factors that promotes metastasis and autophagy such as TGF-β. Autophagy participates in two ways favoring cellular migration: (a) avoiding anoikis and (b) in the turnover of the focal adhesion. Created by BioRender.com.

A main aspect during CTL-mediated elimination of tumor cells is the interaction between MHC-I molecules, harboring tumoral neoantigens, and TCR on surface of primed CTL (150). However, tumor cells develop distinct evasion mechanisms to limit this interaction. For example, mutations in the beta-2 microglobulin coding gene or deletions in genes involved in antigen processing are responsible for downregulation of MHC-l molecules (155, 156). Current evidence demonstrates, in pancreatic ductal adenocarcinoma cell lines, that autophagy promotes degradation of MHC-l molecules, therefore reducing their surface expression (157). In this study, MHC-l molecules are targeted for selective autophagic degradation mediated by NBR1. Pharmacologic or genetic inhibition of autophagy increased surface expression of MHC-I molecules and restored susceptibility of pancreatic tumor cells for elimination by CTLs. An increased number of infiltrating CTLs and reduced tumor volume were found using a genetically engineered mouse model (157). Also, concomitant inhibition of autophagy by expression of mutated ATG4B in cancer cells and systemic administration of chloroquine improved efficacy of dual immune checkpoint therapy. This work reveals new insights in the participation of autophagy as an immune evasion strategy, yet some questions remain.

First, MHC-l molecules were degraded by selective autophagy and neither by LC3-associated phagocytosis nor LC3-associated endocytosis, and we speculate that this degradative process occurs during biogenesis in the endoplasmic reticulum. Therefore, NBR1 could interact with MHC-l molecules or their chaperones (calnexin, calreticulin, ERp57), to mediate selective degradation of the endoplasmic reticulum (158) (See **Figure 5**, lower panel). However, more studies are required to test this possibility.

Second, results were obtained in non-stressful conditions, in which basal levels of autophagy in tumor cells were associated with degradation of MHC-l molecules. However, study during hypoxia, nutrient starvation or other micro environmental stress could determine if these alterations enhance degradation of these and other surface molecules.

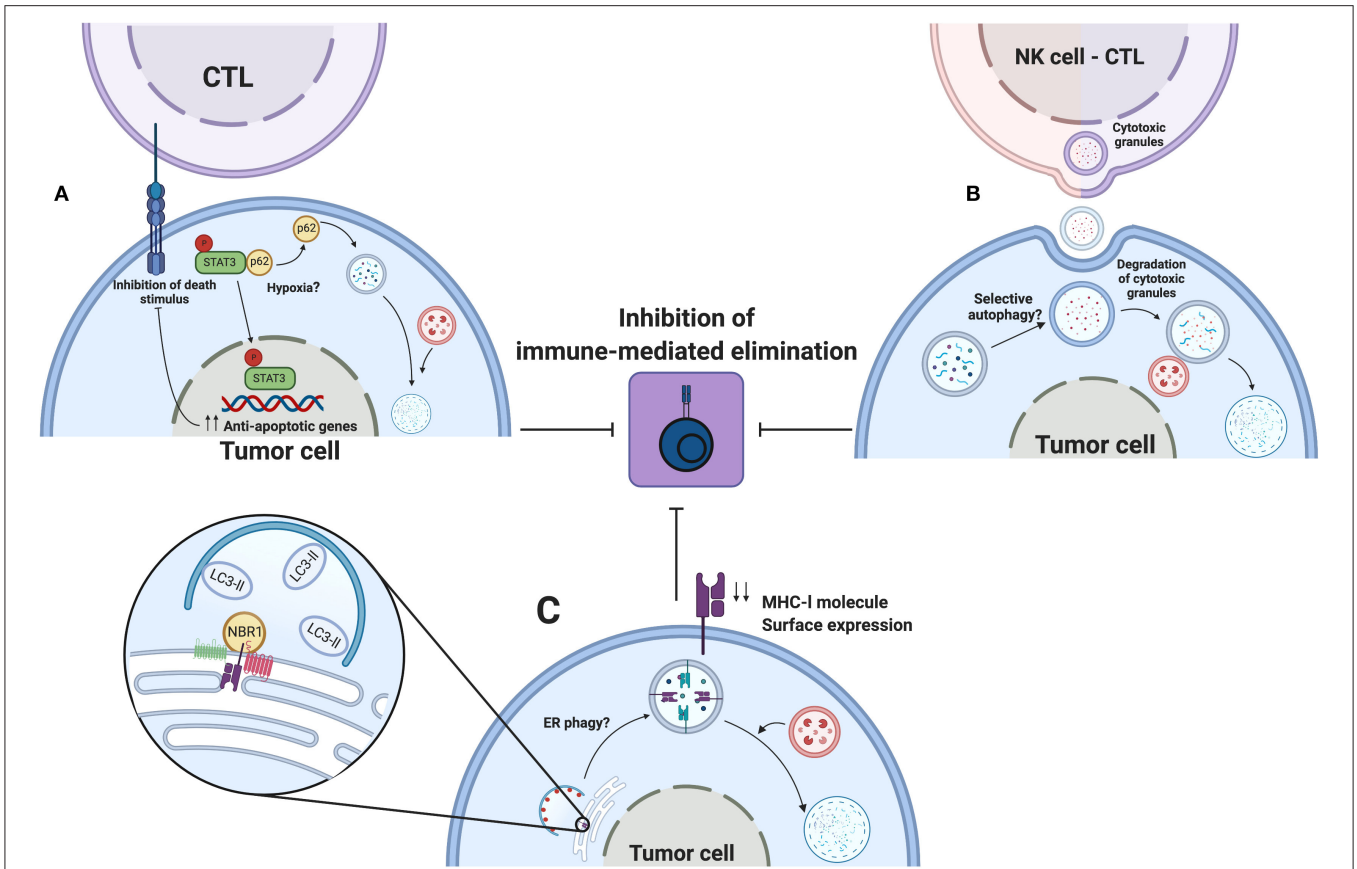

FIGURE 5 | Autophagy as an immune evasion mechanism. Autophagy induced by environmental stress such as hypoxia promotes the escape to CTL or NK mediated elimination of tumors cells. **(A)** Hypoxia, by an undefined mechanism, releases pSTAT3 from p62, thereby degrading p62 by autophagy and favoring pSTAT3 nuclear localization to up-regulate transcription of antiapoptotic genes. **(B)** During hypoxia, tumor cells activate autophagy to sequester and degrade cytotoxic granules released by NK cells or CTLs, thus impeding the elimination of tumor cells. **(C)** Selective ER phagy might participate in degradation of MHC-I molecules during their biogenesis. NBR1 associate to MHC-I molecules or their chaperones in ER. Decreased surface expression of MHC-I molecules leads to impaired recognition by innate or adaptive immune cells, leading to escape for immune-mediated elimination. Created by BioRender.com.

Autophagy has a pivotal role in the late stages of tumor development, assisting with immune evasion. This finding points for inhibition of autophagy as a therapeutic alternative to treat tumors.

ROLE OF AUTOPHAGY IN CHEMOTHERAPY AND TARGET THERAPY RESISTANCE

Chemoresistance is the leading challenge anti-tumor therapy, mainly in advanced stages of cancer. Several mechanisms for chemoresistance are recognized, including autophagy. Stress produced by chemotherapy induces autophagy as a cytoprotective mechanism, allowing the tumor cells to resist chemotherapeutic treatment (159, 160).

Cis-diamminedichloroplatinum (II) (cisplatin) is a platinum-based compound approved since the 1970s for the treatment of various neoplasms, such as bladder, ovarian, lung, head and neck, testicular, and others (161). Cisplatin induces autophagy through increased expression of BECN1 in bladder cell lines, which promotes resistance of these cells to the drug (162). Overexpression of thioredoxin-related protein of 14 kDa (TRP14) in ovarian cancer cell lines decreases sensitivity to cisplatin. TRP14 induced autophagy by activating AMPK and inhibiting mTOR and p70S6K. When TRP14 expression was inhibited using shRNA, sensitivity to cisplatin was markedly increased (163). Lung adenocarcinoma cell line A-549/DDP is resistant to cisplatin, and expression of tripartite motif-containing proteins (TRIM)-65 is enhanced along with LC3-II expression. When TRIM65 is inhibited by shRNA in cell lines and in a mouse xenograft model, the cisplatin-induced apoptosis increased, associated with reduction of ATG5, ATG7, and Beclin1 mRNAs levels (164). The LncRNA-small nucleolar RNA host gene 14 (SNHG14) is an antisense sequence of the ubiquitin-protein ligase. In colorectal cancer biopsies, high expression of SNHG14 and ATG14 was observed. In the same work, SNHG14 inhibited miR-186, which blocked ATG14 expression in cisplatin-resistant colorectal cancer cell lines. The authors concluded that SNHG14 induced autophagy and cisplatin resistance by

inhibiting miR-186 (165). In another study, cisplatin resistance was related to autophagy by inhibiting the expression of Bcl-2 associated athanogene 3 (BAG3) in cisplatin-resistant ovarian epithelial cancer SKOV3 cells. Autophagy inhibition in SKOV3 cells increased sensitivity to cisplatin (166). In osteosarcoma cell lines, the heat shock chaperone molecule HSP90AA1 is overexpressed when cells are treated with cisplatin, doxorubicin, and methotrexate. Treatments induce autophagy through the PI3K/Akt/mTOR signaling pathway, resulting in resistance to chemotherapy. When HSP90AA1 was inhibited, autophagy was blocked and sensitivity to chemotherapy was enhanced (167). These studies demonstrate that autophagy acts as a cytoprotective mechanism against cytotoxic agents.

Food Drug Administration (FDA)-approved targeted therapy is classified in two groups, monoclonal antibodies, and small inhibitor molecules. These compounds block the growth of tumor cells by interfering with specific and essential molecules required for tumor development (168).

In the breast cancer cell line, MCF-7, which is estrogen receptor-positive and resistant to 4-hydroxytamoxifen (4-OHT), inhibition of autophagy with siRNAs for Atg5 and Beclin-1 increased sensitivity to tamoxifen (169). Further, exposure of MCF-7 cells to 4-OHT induced autophagy in 95% of the cells, yet only 15–20% exhibited markers associated with active cell death II (ACDII). When cells were treated with 4-OHT and 3-methyladenin (3-MA), an inhibitor of auto phagosome formation, or siRNA for Beclin-1, the cells showed sensitivity to 4-OHT (170).

Autophagy is usually a mechanism of resistance for targeted therapy, but contradictory results are reported. Cetuximab is a monoclonal antibody approved by the FDA that inhibits epidermal growth factor receptor (EGFR). Exposure of A431 human vulvar squamous carcinoma, DiFi colorectal carcinoma, HN5, and FaDu head and neck carcinomas cells to cetuximab, elicited diverse responses. In DiFi cells, cetuximab induced cytoprotective autophagy, which was inhibited with chloroquine, thus activating cell death. In A431 cells, cetuximab induced a slight apoptotic response, which was potentiated with an autophagy inhibitor such as chloroquine or activator such as rapamycin. Finally, in HN5 and FaDu cells, cetuximab induced a cytostatic effect. By exposing these cells to a combination of cetuximab and rapamycin, cell death was induced (171).

The antitumoral compounds erlotinib and gefitinib are first-generation tyrosine kinase inhibitors (TKI's) that target cells harboring EGFR-activating mutations, causing growth inhibition and cell death. However, these TKI's trigger cytoprotective autophagy. Cell lines, such as HeLa-R30, are resistant to erlotinib, yet do not display autophagy. When these cells were treated with erlotinib and rapamycin, cell death was increased. The depletion of ATG7 with siRNA restored erlotinib resistance, suggesting that defects in autophagy might be a mechanism of resistance (172). Osimertinib (OSI), is a third-generation EGFR TKI that has been approved for the treatment of NSCLC patients harboring EGFR T790M mutation. Exposure of NSCLC cell lines H-1975, HCC827, and A-549 to OSI induced ROS, which in turn activates autophagy leading to decreased cell viability. Thus, ROS inhibition decreased autophagy and apoptosis in NSCLC cell lines (173).

Autophagy, as a response to treatment, is diverse. Cytotoxic autophagy is characterized by promotion of cell death associated with apoptosis and reduced sensitivity to treatment when it is inhibited (159). Rituximab-monomethyl auristatin E (MMAE) treatment, induced cell death by autophagy in B cell lymphoma by inactivating the AKT/mTOR pathway. Cell death was stimulated with exposure to rapamycin and was inhibited with chloroquine (174). Oridonin is an active diterpenoid compound isolated from *Rabdosia rubescens*. Colorectal carcinoma lines HT-29, HCT116, SW480, and S620 exposed to oridonin showed autophagic cell death due to metabolic imbalance characterized by a dramatic inhibition of glucose uptake without reduction of ATP levels. In this setting, tumor cells become autophagy-dependent to meet energetic and nutritional demands to sustain viability, causing autophagic cell death (175). Brefeldin A is a lactone that inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus. In colorectal carcinoma cell lines and xenograft tumor models, brefeldin A produced stress at the endoplasmic reticulum level by increasing regulation and interaction of binding immunoglobulin protein (Bip) with AKT, which activated autophagic cell death (176). In other study, when folate receptor was blocked using a monoclonal antibody MORAB-003 (farletuzumab) in ovarian cancer cells and in an orthotopic mouse models tumor growth was inhibited due to autophagic cell death. When MORAB-003 was combined with hydroxychloroquine, the inhibition of tumor growth was reversed (177).

Chloroquine and hydroxychloroquine are the only autophagy inhibitors approved by the FDA for clinical use (178), comprehensive reviews are examining the role of various compounds and biological molecules in the regulation of autophagy and various ATG genes (160, 176, 179–181). Clinical trials are underway in which inhibitors of autophagy are administered in combination with chemotherapy or targeted therapy (182, 183). However, because of dissimilar participation of autophagy as a cytoprotective or cytotoxic mechanism, biomarkers related to these scenarios must be identified to predict treatment response.

CONCLUDING REMARKS

The role of autophagy in several stages of tumor development is reviewed. Metabolic status through distinct stages of tumor impacts in tumor suppressor or tumor-promoting roles of autophagy is discussed. In incipient tumors, nutrient, and oxygen supply is sufficient and do not represent environmental stress; therefore, autophagy acts as an intrinsic cytotoxic response suppressing tumor development. However, as tumor grows metabolic requirements are increased to sustain high proliferation rates. Autophagy provides reduced carbon to maintain the energy demand and support survival of tumor cells in hostile microenvironments. In advanced stages of tumor development, the hypoxic, and starvation conditions generate signals promoting tumor invasion and metastasis. Autophagy

helps cells evade anoikis and promote focal adhesion turnover favoring cell motility and metastasis. Additionally, autophagy serves as an immune evasion strategy in cancer advanced stages. In these settings, autophagy might promote resistance to chemotherapy or targeted therapy in most scenarios.

We consider autophagy and cancer metabolism parts of an overall process. For this reason, it is necessary to consider the metabolic status of tumor for use of autophagy inhibitors as a therapeutic strategy for impacting clinical outcomes.

AUTHOR CONTRIBUTIONS

DA-C, RC-D, and MP-M organized the entire manuscript, wrote the draft, and revised the last version of the manuscript. RC-D and JL-G wrote the autophagy, apoptosis crosstalk, and involvement of autophagy in tumor immune evasion. MP-M and DA-C wrote the autophagy and cancer metabolic reprograming section. DA-C and MG-V wrote the interplay of autophagy in metastasis. RC-D and JL-G wrote involvement of autophagy in tumor immune evasion. DA-C and JL-G wrote autophagy in chemotherapy and target therapy resistance. **Figures 1**, **2**, **5** were designed by RC-D. **Figure 3** was designed by MP-M and **Figure 4** by DA-C. All authors contributed to the article and approved the submitted version.

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REVIEW
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Autophagy Takes Center Stage as a Possible Cancer Hallmark

*Jose G. Alvarez-Meythaler[1], Yoelsis Garcia-Mayea[1], Cristina Mir[1], Hiroshi Kondoh[2] and Matilde E. LLeonart[1,3]**

[1] Biomedical Research in Cancer Stem Cells Laboratory, Vall d'Hebron Research Institute (VHIR), Barcelona, Spain, [2] Geriatric Unit, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [3] Spanish Biomedical Research Network Center in Oncology, CIBERONC, Barcelona, Spain

Cancer remains one of the leading causes of death worldwide, despite significant advances in cancer research and improvements in anticancer therapies. One of the major obstacles to curing cancer is the difficulty of achieving the complete annihilation of resistant cancer cells. The resistance of cancer cells may not only be due to intrinsic factors or factors acquired during the evolution of the tumor but may also be caused by chemotherapeutic treatment failure. Conversely, autophagy is a conserved cellular process in which intracellular components, such as damaged organelles, aggregated or misfolded proteins and macromolecules, are degraded or recycled to maintain cellular homeostasis. Importantly, autophagy is an essential mechanism that plays a key role in tumor initiation and progression. Depending on the cellular context and microenvironmental conditions, autophagy acts as a double-edged sword, playing a role in inducing apoptosis or promoting cell survival. In this review, we propose several scenarios in which autophagy could contribute to cell survival or cell death. Moreover, a special focus on novel promising targets and therapeutic strategies based on autophagic resistant cells is presented.

Keywords: autophagy, cancer, therapy, resistance, protective autophagy

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Donat Kögel,
Frankfurt University, Germany

***Correspondence:**
Matilde E. LLeonart
matilde.lleonart@vhir.org

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INTRODUCTION

Autophagy is a conserved catabolic process that sequesters and degrades intracellular components in double-membraned compartments known as autophagosomes, playing a key role in homeostasis maintenance (1). The recycling capabilities of this process prevent the accumulation of damaged proteins and organelles that can generate cell toxicity; therefore, autophagy functions as an internal quality control system (2). Autophagy is tightly regulated and normally induced in response to different intrinsic and extrinsic signals, such as starvation, growth factor deficiency, hypoxia, and many other types of stress (3). In normal conditions, the functions of autophagy comprise cell survival control to regulate homeostasis. However, in cancer cells, autophagy is frequently deregulated in and becomes important in tumorigenesis (4). Moreover, autophagy plays a pivotal role in some cancer hallmarks, including cell survival, cell death, deregulation of metabolism, modulation of the immune response, epithelial–to-mesenchymal-transition (EMT) process, cancer stem cell (CSC) promotion, and multidrug resistance (MDR) (**Figure 1**).

This paradoxical dual role in stimulating cell survival or promoting cell death is still under investigation in cancer at clinical and molecular levels (5). Deciphering in which genetic background and under which circumstances autophagy stimulates or eliminates cancer cells may

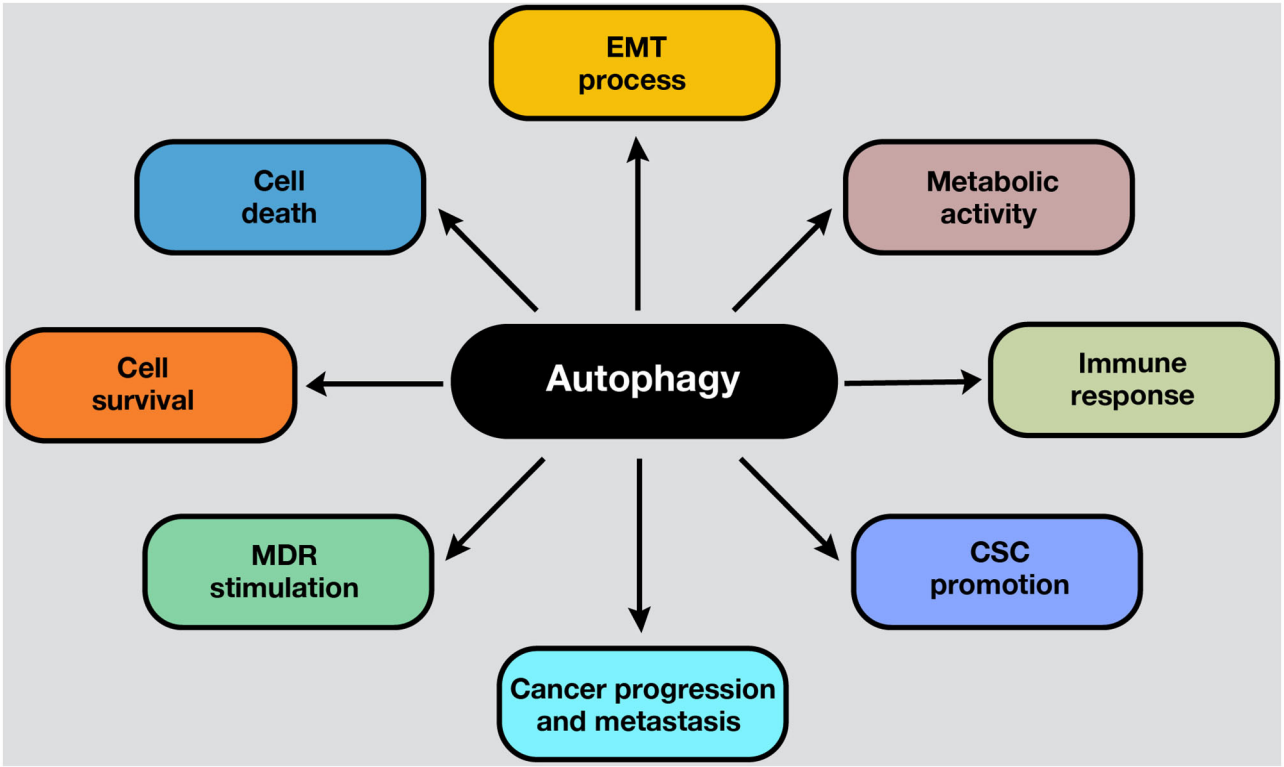

FIGURE 1 | Roles of autophagy in cancer. Autophagy mechanisms are involved in several hallmarks in cancer cells. Autophagy can lead to cell survival or death, depending on the presence, duration, and intensity of the stimulus in which it develops. In addition, autophagy can modulate the EMT phenotype after the adaptation to hypoxia. Moreover, the metabolic switch of cancer cells into aerobic glycolysis (i.e., the Warburg effect) is sustained by autophagy activation, ensuring energetic requirements, and metabolic homeostasis. On the other hand, the activation of autophagy process influences the suppression or activation of antitumor immune response, depending on the stage, genetic, and microenvironmental conditions. For example, in response to chemotherapy, autophagy-competent cancer cells attracted dendritic cells, and T lymphocytes to the tumor, activating the immune response. Moreover, autophagy activation maintains the CSC phenotype and functions inside the tumor. Also, an upregulated autophagic activity are involved in cancer progression and metastasis. Furthermore, autophagic machinery triggered by anticancer drugs may facilitate multiple drug resistance in cancer cells and tumor survival. All these processes depend on the cell type, genetic background, and the microenvironment stimulus in the tumors.

facilitate the development of specific therapeutic strategies. Also, many studies associate autophagy with drug resistance (6). In this review, we discuss how the role of autophagy associated with cell survival and drug resistance might determine an effective therapeutic approach against, particularly aggressive tumors.

MECHANISMS OF AUTOPHAGY

While the molecular mechanisms that govern autophagy in normal and cancer cells have not been thoroughly elucidated, several pathways are involved in each case. It is known that the central pathway governing autophagy is led by PI3K/AKT/mTOR signaling (7). Strikingly, this pathway is one of the most altered pathways in cancer (8, 9). The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase, part of the mTOR complex 1 (mTORC1), in which different stimuli converge, including autophagy-stimulating signals (nutrient or growth factor deprivation, hypoxia, oxidative stress, or protein aggregation) (10). The activation of mTOR by growth factors exerts a negative effect on autophagy, inhibiting the autophagy process (11, 12). The process of autophagy is divided into five phases: initiation, phagophore nucleation, elongation and autophagosome formation, autophagosome-lysosome fusion, and cargo degradation, where autophagy-related genes (ATGs) play an important role in the entire pathway (13). In the initiation phase, mTORC1 is inactivated in response to these autophagy signals, and consequently, the Unc-51-like kinase 1 (ULK1 or ATG1) complex, which consists of ULK1, ULK2, ATG13, RB1-inducible coiled-coil protein 1 (RB1CC1 or FIP200) and ATG101, is activated. This complex stimulates phagophore nucleation, activating, by phosphorylation, the components of class III phosphatidylinositol 3-kinase (class III PI3K or PI3KC3) complex, which consists of vacuolar protein sorting 34 (VPS34), ATG14, activating molecule in Beclin-1-regulated autophagy (AMBRA1), general vesicular transport factor (p115), UV radiation resistance-associated gene protein (UVRAG or p63) and Beclin-1, with the last protein acting as the scaffold. This complex activates local phosphatidylinositol-3-phosphate (PI3P) production at the endoplasmic reticulum (ER), specifically in an ER structures named the omegasome (14). Then, PI3P associates

with different members of the WD-repeat protein interacting with phosphoinositides (WIPI) protein family (15). Bcl-2 is a key control protein of autophagy that interacts with Beclin-1 at the Bcl-2-homology 3 (BH3) domain to inhibit its pro-autophagic activity. Bcl-2 reduces the interaction of Beclin-1 with VPS34 and UVRAG (10). However, Beclin-1 has also autophagy-independent functions; for example, it has been described to act as a negative regulator in the execution of necroptosis (16).

Elongation of the phagophore is controlled by two ubiquitin-like protein systems. First, ATG7 and ATG10 regulate the synthesis of the ATG12-ATG5-ATG16L1 complex. WIPI proteins, specifically WIPI2, bind ATG16L1 directly, anchoring the ATG12-ATG5-ATG16L1 complex to the phagophore. This complex enhances the second system, in which ATG4B, ATG7, and ATG3 act coordinately to cleave the precursors of protein light chain 3 (LC3)-like proteins and conjugate them to phosphatidylethanolamine (PE) present in the membrane. Also, γ-aminobutyric acid receptor-associated protein (GABARAP) conjugates with PE and, as a result, is incorporated into the rising autophagosome. LC3 and GABARAP give the autophagosome the capability to attach autophagic substrates targeted by selective autophagy receptors (SARs), such as sequestosome-1 (p62/SQSTM1), before membrane sealing and complete autophagosome formation (15, 17). Ultimately, microtubule proteins facilitate autophagosome transport to the lysosomes. SNARE proteins, including syntaxin 17 (STX17) and vesicle-associated membrane protein 8 (VAMP8), facilitate autophagosome-lysosome fusion. Autolysosomal contents are degraded due to the acidic lysosomal hydrolases, and the recovered nutrients are released back and recycled by the cell, using them in new metabolic processes (10, 13, 18).

Besides, key oncogenes inhibit autophagy, such as AKT or $p21^{Cip1}$, while tumor suppressor genes activate it, such as PTEN, p53, and TSC1/TSC2 (19). AMPK, a protein that maintains metabolic homeostasis, is crucial for determining the destiny of autophagy. AMPK induces autophagy by phosphorylation of mTORC1, part of the mTOR pathway, and the autophagy-related complexes ULK1 and PI3KC3. Also, AMPK regulates autophagy indirectly through several transcription factors and coactivators, such as DAP1, p300, TFE/MITF, and FOXO3 (20). Proteins involved in the different phases of the autophagic process are shown in **Figure 2**. In the following section, the roles of autophagy in different scenarios will be discussed.

SELECTIVE AUTOPHAGY

Selective autophagy is that type of autophagy that is specifically aimed at a specific cellular organelle. Selective autophagy is committed to preserving intracellular homeostasis by eliminating specific substrates in the autophagosome through recognition of

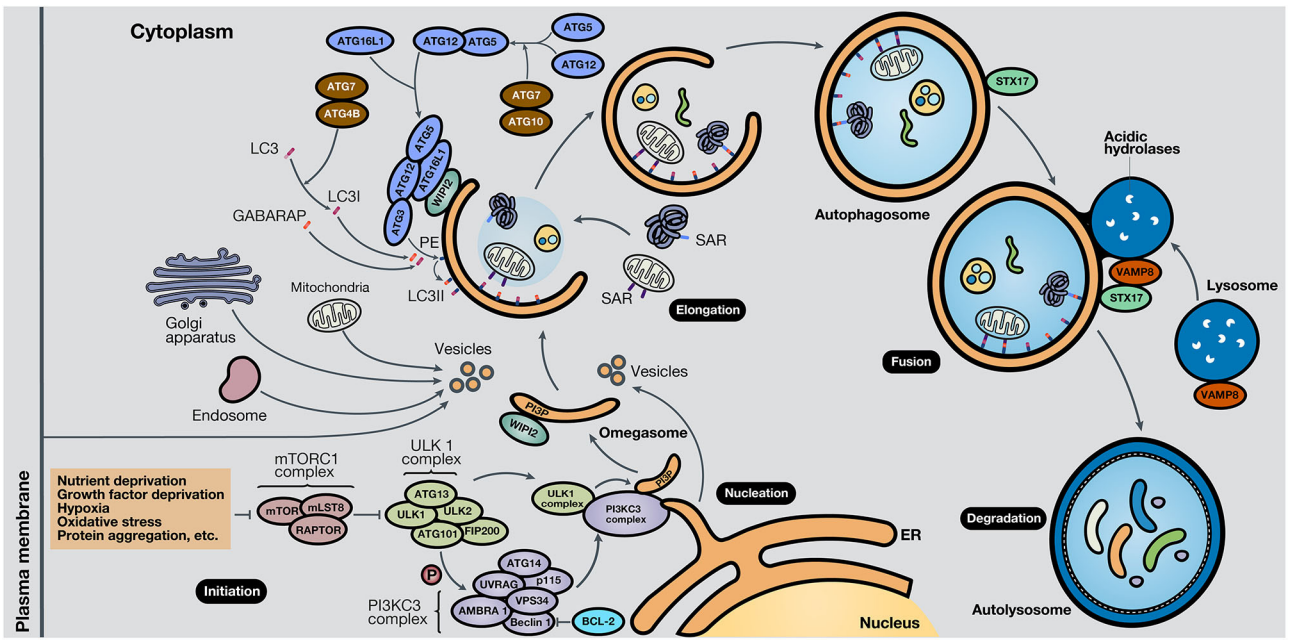

FIGURE 2 | Schematic representation of the autophagy process. The autophagy mechanism consists of five phases. In the initiation phase, mTORC1 is inactivated due to autophagy-stimulating signals, liberating the repression of the ULK1 complex. During the nucleation phase, the ULK1 complex phosphorylates the PI3KC3 complex, which induces phagophore formation in the omegasome, through the production of PI3P and association with WIPI protein family members, commonly WIPI2. In the elongation phase, two ubiquitin-like protein systems, ATG12-ATG5-ATG16L1 and ATG4B-ATG7-ATG3, mediate the activation of LC3 into LC3I, lipidation with PE to form LC3II, and subsequent anchoring to the phagophore. GABARAP also conjugates with PE and attaches to the membrane. LC3 and GABARAP mediate the sequestration of autophagic substrates marked with SARs, such as p62/SQSTM1, before phagophore closure and total autophagosome development. During the fusion phase, STX17 and VAMP8, present in the autophagosome and lysosome, respectively, interact and stimulate autolysosome formation. Finally, in the degradation phase, acidic lysosomal hydrolases degrade autophagic cargo, generating nutrients that are released to the cytoplasm and reused by the cell. ER, endoplasmic reticulum.

specific receptors. Contrary to the bulk degradation process of unspecific autophagy, the objective of selective autophagy is to maintain cell homeostasis by maintaining the number of integral organelles, including mitochondria (mitophagy), ribosomes (ribophagy), aggregated proteins (aggrephagy), peroxisomes (pexophagy), lysosomes (lysophagy) or invading pathogens (21, 22). Many research findings related to selective autophagy receptors (SARs) have demonstrated that autophagy can be directed against a specific cargo. Examples of SARs, such as p62/SQSTM1, NBR1, TOLLIP, BNIP3L/NIX, and Cue5, show the mechanisms behind the formation of autophagosomes for selective autophagy (23, 24).

The cargo receptor p62/SQSTM1 is one of most extensively studied receptors and modulates selective autophagy due to its mediation in the degradation of ubiquitinated material, such as protein aggregates, mitochondria, peroxisomes, lysosomes, or intracellular bacteria (22). For example, the binding of the bacterial type III effector protein HopQ to vimentin provokes the degradation of vimentin through p62/SQSTM1-dependent selective autophagy (25). Moreover, it has been demonstrated that constant p62 levels, due to autophagy defects, were enough to alter NF-κB regulation and gene expression, thereby stimulating tumor generation (26). Another selective cargo receptor is the nuclear receptor coactivator 4 (NCOA4), which is involved in selective autophagy of ferritin, called ferritinophagy, which is activated during low levels of intracellular iron (27).

AUTOPHAGY-MEDIATED CELL DEATH

Although it was identified as an initial function of autophagy, currently, autophagic cell death is a process that occurs less frequently in cancer cells than protective autophagy. Autophagic cell death is characterized by cytoplasmic vacuolization, accumulation, and assembling of autophagosomes labeled by LC3, and elimination of cell organelles via autolysosomes. However, the criteria to differentiate autophagic cell death from other types of cell death accompanied by autophagy are still controversial (28, 29). Although several studies suggest that an uncontrolled and nonspecific overactivation of autophagy induces cell death, other studies emphasize that the selective removal of autophagy substrates is a key factor in cell death promotion (30, 31).

Autophagic cell death—described in mammalian development, other less complex organisms, and cancer cells—can be suppressed by pharmacological or genetic inhibition or induced by specific cancer drugs (30, 32). As an example, kaempferol, a flavonoid with anticancer properties, was shown to induce autophagic cell death in gastric cancer through IRE1/JNK/CHOP signaling pathway activation, and the suppression of kaempferol-induced autophagy restores cancer cell survival (33). RY10-4, an analog version of proto-apigenone, promotes ACD by inactivation of the AKT/mTOR pathway in the breast cancer cell line MCF-7, and the inhibition of autophagy through genetic and chemical approaches extends cancer cell viability (34). Another novel anticancer drug, designated ABTL0812, which is already in preclinical trials, induces ER stress-mediated cytotoxic autophagy by increasing dihydroceramide levels in cancer cells of several models, including lung and pancreatic cancer (35). In ovarian cancer cells, activation of oncogenic H-Ras activates autophagy mechanisms, upregulating BH3-only protein Noxa and Beclin-1 and triggering cell death. Silencing of ATG5, ATG7, Beclin-1, or Noxa expression reduces autophagy and increases survival (36).

Autosis, considered a form of autophagic cell death, is regulated by Na^+, K^+-ATPase in the presence of Tat-Beclin-1 and Tat-vFLIP α2, Beclin-1-derived peptides, or starvation (37). Recently, treatment with Tat-Beclin-1 and Tat-vFLIP-α2 peptides showed to induce autosis as a strategy to selectively kill HIV-infected macrophage and resting memory $CD4^+$ T cells, avoiding reactivation of virus (38, 39). Autosis is characterized by a dependence of Na^+, K^+-ATPase pump, an enhanced cell-substrate adherence, a dilated, fragmented, and finally disappeared endoplasmic reticulum, and an initial nuclear membrane convolution with a subsequent focal ballooning of the perinuclear space (37). Autosis is not entirely regulated by autophagy markers nor controlled by apoptotic and necrotic markers, although autosis is induced with a high level of autophagic activity (40). However, a recent study demonstrated the interaction of Beclin-1 and Na^+, K^+-ATPase, whose interaction and autotic death process increase during pathological and physiological stress conditions, and decrease by cardiac glycosides, inhibitors of Na^+, K^+-ATPase (41). Also, autosis can be interrupted by knockout of the autophagy-related genes ATG13 and ATG14 or by blocking treatments of autophagosomal assembly (42).

AUTOPHAGY AND OTHER TYPES OF CELL DEATH

Autophagy and Apoptosis

Apoptosis, a programmed cell death widely studied in cell biology, is a highly controlled process that mediates the efficient and orderly elimination of damaged cells. In the body, the balance between apoptosis and proliferation is crucial to ensure homeostasis (43). Apoptosis induces morphological changes such as cell membrane asymmetry and blebbing, protein cleavage, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and phagocytic recognition (44, 45). At the molecular level, the adequate regulation of apoptosis involves several signaling pathways that control biological responses such as embryonic development, cell renewal, and external factors (e.g., radiation, chemicals), which produce DNA damage. As a result, a complete process of apoptosis implicates the interactions of many proteins, signal transducers, and signaling pathways (44). The balance between anti- and pro-apoptotic proteins is essential to decide if the apoptosis ultimately occurs. Evasion of apoptosis encourages cancer initiation and tumor progression and facilitates the emergence of resistant variants with great metastatic potential (43, 45).

Many studies indicate that autophagy and apoptosis are closely interconnected because of their regulation by effector

proteins, pathways, and intracellular locations. For example, autophagy boosts apoptosis by degrading a negative regulator of the Fas (CD95/Apo-1) ligand, but it can also protect it by modifying levels of the Bcl-2 family members. Besides, autophagy is activated by several apoptotic stimuli, and both occur after cellular stress (46). Thus, it is expected that autophagy and apoptosis in certain circumstances cooperate in cancer progression. However, the interplay of both processes is complex due to the double-edged sword function of autophagy, stimulating apoptosis, or cell survival (47). In most cases, autophagy precedes apoptosis under stress conditions. For example, low-stress conditions stimulate autophagy as a way to cope and adapt to this scenario. However, if the stress event crosses a threshold of time and/or intensity, apoptosis is activated (48). Some proteins have a dual role in apoptosis and autophagy. For example, Beclin-1 binds to Bcl-2, forming a complex at normal conditions, resulting in the inhibition of autophagy, without losing anti-apoptotic capacities of Bcl-2 (49) (**Figure 3A**). Bcl-2 is a mitochondrial membrane protein belonging to the Bcl-2 family, which consists of ∼25 proapoptotic (e.g., Bax, Bak, and PUMA) and antiapoptotic (e.g., Bcl-2, Bcl-X_L, and MCL-1) protein family members (50). Bcl-2 promotes anti-apoptotic functions through the interaction with Bax, which repress the Mitochondrial Outer Membrane Permeabilization (MOMP) (51), and the subsequent release of proteins, such as cytochrome c (cyt-c), high-temperature requirement protein A (HtrA2/Omi), and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis (IAP)-binding protein with low pI (Smac/DIABLO) to the cytosol (52). In cancer cells under starvation, C-Jun N-terminal protein kinase 1 (JNK1) becomes activated and phosphorylates Bcl-2, disrupting the Bcl-2/Beclin-1 complex and promoting autophagy due to the

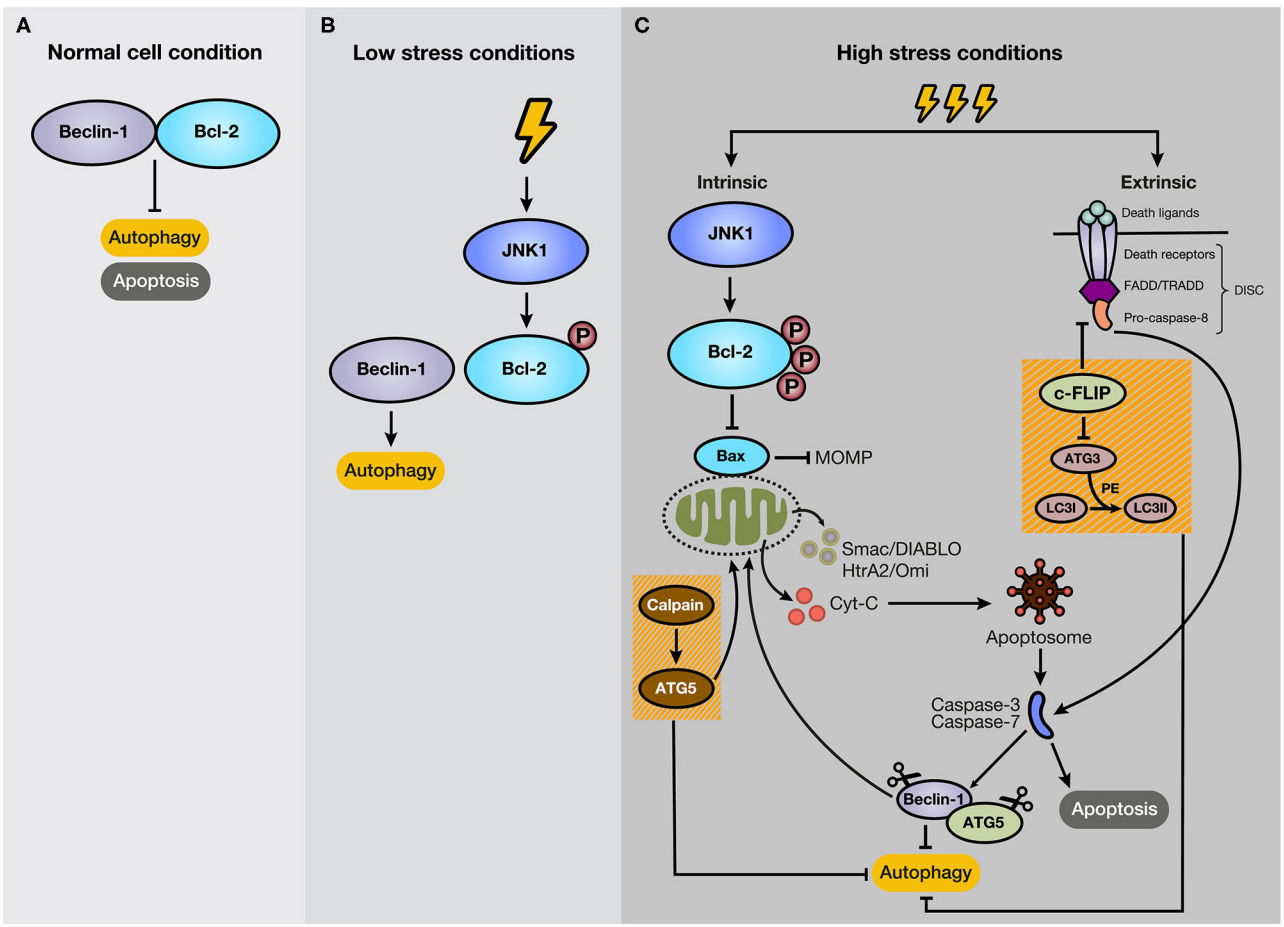

FIGURE 3 | Mechanism of crosstalk between autophagy and apoptosis. **(A)** Under normal cellular conditions, Beclin-1 binds to Bcl-2, keeping autophagy and apoptosis inactivated. **(B)** However, if the cell experiences low-level stress conditions (e.g., nutrient deprivation), JNK1 phosphorylates Bcl-2, disturbing Bcl-2/Beclin-1 union. As a result, isolated Beclin-1 activates the autophagic pathway. **(C)** However, if the stressful stimulus crosses a threshold of time, JNK1 promotes Bcl-2 hyperphosphorylation, inducing its dissociation with Bax and the subsequent activation of the intrinsic apoptotic pathway. In addition, c-FLIP, a suppressor of extrinsic apoptosis, also inhibits autophagy through interaction with ATG3, reducing LC3 lipidation. Moreover, caspase activation mediates autophagy-related proteins, such as Beclin-1 and ATG5. Additionally, the C-terminal fragment generated by caspase-mediated cleavage of Beclin-1 translocates to the mitochondrial membrane and stimulates intrinsic apoptosis. Furthermore, ATG5, after cleavage by calpains, suppresses autophagy activity and induces apoptosis.

activation of core autophagic components by isolated Beclin-1 as a response for cell protection (47, 53) (**Figure 3B**). However, if the starvation is prolonged, JNK1 induces hyperphosphorylation of Bcl-2 that generates its dissociation with Bax and apoptosis stimulation (54) (**Figure 3C**). Therefore, Bcl-2 and Beclin-1 interaction represent a significant mechanism for regulating the switch between autophagy and apoptosis. As an example of other members of Bcl-2 family, BNIP3 and NIX are also implicated in the stimulation of autophagy, and specifically mitophagy, due to a BH3 domain in their structure, apart from their role as pro-apoptotic proteins (55, 56). The augmentation of reactive oxygen species (ROS) production and the competition for Bcl-2 binding with Beclin-1, with consequent Beclin-1 liberation, are strategies that BNIP3 and NIX can apply to induce autophagy (56). Moreover, BNIP3 and NIX regulates mitophagy through HIF-1α/BNIP3 signaling pathway, which promotes a decrease of ROS production and plays a protective role during hypoxia (57–59).

Beyond their autophagic functions, many autophagy-related proteins have a pivotal role in apoptosis. For example, non-conjugated forms of ATG5 and ATG12 induce apoptosis under stress conditions. ATG12 directly binds to Bcl-2 family members, including the antiapoptotic proteins Bcl-2 and MCL-1, independent of its interaction with ATG5 or ATG3 (60). ATG5 is cleaved by calpains, suppressing its autophagy activity (**Figure 3C**). Also, the N-terminal fragment of ATG5 translocates to mitochondria and induces the release of cyt-c, leading to the activation of effector caspases and apoptosis (61). Some studies indicate that overexpression of ATG5 sensitizes tumor cells to chemotherapy, and knockout of this protein increases tumor cell resistance to chemotherapeutic drugs (62, 63).

Additionally, some key apoptotic proteins also participate in the regulation of autophagy. For example, FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP) is an anti-apoptotic protein that suppresses extrinsic apoptosis (62). Ligation of dead receptors, such as type 1 TNF receptor (TNFR1), Fas, and TRAIL, at their extracellular domain generates recruitment of specific procaspases (−8 and sometimes −10) and adaptor proteins to the cytosolic domain, such as Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD), forming a multiproteic structure called death-inducing signaling complex (DISC) (64). Due to the death effector domain (DED) present in its structure, c-FLIP interferes with the interaction of dead receptors, and adaptor proteins (50). Besides, FLIP suppress autophagy through blockage of LC3 lipidation by competitive interaction with ATG3 (**Figure 3C**). In contrast, if the autophagic process is initiated, the FLIP and ATG3 interaction is substantially reduced (65).

Autophagy usually becomes regulated due to cleavage of essential proteins in the autophagic process by caspases (48). Caspases (cysteinyl, aspartate-specific proteases) comprise a family of cysteine proteases that mediate the molecular process of apoptosis and participate actively in the initiation and execution pathways (66). Caspases are crucial proteins in the apoptosis process and are involved as apoptotic initiators (caspase-2,−8,−9, and−10) and executors (caspase-3,−6, and−7) of cell death (67). Essential autophagy proteins, such as Beclin-1, ATG3, ATG5, and ATG7, are cleaved by caspase-3,−7, and−8, destroying their autophagic function (68). Also, caspase-mediated cleavage of Beclin-1 produces a C-terminal fragment that translocates to mitochondria and boosts intrinsic apoptosis (69, 70) (**Figure 3C**). However, although caspase cleavage of ATG4, principally ATG4D by caspase-3 (71), induces cytotoxicity through its movement to the mitochondria, this autophagy-related protein also induces the autophagy pathway (29), demonstrating a complex interaction with very fine regulation determined by the levels of apoptotic and anti-apoptotic proteins present in the cells.

Autophagy and Necroptosis

Necroptosis was discovered as a new form of strictly regulated programmed cell death with characteristics of necrosis (72). Escape from necroptosis via loss of RIPK3 expression is a feature of some cancers. Moreover, downregulation of necroptosis mediators such as RIPK3 and MLKL in tumors suggests an escape mechanism from necroptosis in cancer (73). Necroptosis is principally controlled by receptor-interacting protein kinase 1 (RIP1 or RIPK1), RIPK3, and mixed lineage kinase domain-like pseudokinase (MLKL), and its activation is mediated by death receptors, mainly TNFR1 (74). Death receptor binding with its ligand, tumor necrosis factor α (TNFα), promotes the recruitment of RIPK1, TRADD, a cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2), and TRAF5, forming pro-survival complex I. RIPK1, which is polyubiquitinated in complex I, assembles complex IIa after deubiquitination, formed by RIPK1, RIPK3, TRADD, FADD, and caspase-8. Complex IIa mediates caspase-8 activation and subsequent apoptosis. However, if caspase-8 is inhibited, RIPK1 recruits RIPK3, forming complex IIb that, after their phosphorylation, activates the necroptosis pathway through the establishment of a necrosome (75). Then, RIPK3 phosphorylates MLKL, promoting its oligomerization and translocation to the plasma membrane, which boosts membrane permeabilization due to phospholipid disturbance. This stimulation of membrane permeability, resulting in cytokine and chemokine release, causes an immune response that provokes inflammation and determines the outcome of apoptosis or necroptosis (76).

Necroptosis and autophagy maintain a close and complex interplay, considering that both processes can be activated sequentially or at the same time, activating or suppressing one with the other, and with the same or contrary purposes of cell survival or death (77). Regularly, autophagy is activated to restore levels of energy, saving cells that would otherwise undergo necroptosis due to ATP deficiency (78). Besides, autophagy is induced by necroptosis as a reaction to high levels of reactive oxygen species (ROS) produced, eliminating critically damaged cell structures, ensuring homeostasis, and ultimately avoiding necroptotic cell death (79). Phosphorylation of VSP34 and Beclin-1 by protein kinase D1 (PKD) and death-associated protein kinase (DAPK), respectively, to stimulate autophagosomal formation are two examples of autophagy activation mechanisms against oxidative stress, with subsequent necroptosis suppression (77). Another example is the induction

of necroptosis signaling by poly(ADP-ribose) polymerase-1 (PARP-1) overactivation, which provokes ATP depletion and consequent autophagy pathway activation through the LKB1-AMPK-mTOR pathway to ensure cell survival (80). Therefore, autophagy inhibition during low cell energy availability could generate a metabolic crisis that promotes necroptosis activation (78).

Several studies highlight the caspase-8/RIPK1 interaction as crucial in the regulation of the autophagy pathway and the interplay between autophagy, necrosis, and apoptosis (74). For example, caspase-8 is triggered inside autophagosomal membranes in some cases and acts as a platform and eliminates inhibitors of apoptosis, promoting apoptosis (81). Also, activated caspase-8 cleaves RIPK1, reinforcing apoptotic vs. necroptosis signaling. However, in a MAP3K7 deletion context, autophagy changes the death cell mode toward necroptosis, recruiting and scaffolding RIPK1 via p62/SQSTM1 to the autophagosome. As a result, the necrosome becomes more selectively and quickly activated (82).

Moreover, necroptosis and autophagy can be activated in parallel to boost cell death (78). For example, some investigations with zVAD, a general caspase and apoptosis inhibitor, evidenced a stimulation of necroptosis and autophagy by this peptide after TNFα stimulation, characterized by the formation of autophagosomal vacuoles (77). However, the cell death response can be suppressed by downregulation of RIPK1, ATG7, or Beclin-1 expression (83). Thus, these autophagic genes participate actively in the control of necroptosis-mediated cell death.

Autophagy and Pyroptosis

Pyroptosis is a regulated cell death accompanied by a proinflammatory response. Various microbial infections and internal damage-associated signals, such as dysfunctional mitochondria, induce the assembly of inflammasome, a multiprotein complex that promotes the activation of inflammatory caspases (-1,−4,−5, and−11), which mediate the pyroptotic signaling pathway (84). These non-apoptotic caspases play two important roles in pyroptosis activation. First, inflammatory caspases activate the inflammatory cytokines interleukin 1β (IL-1β) and IL-18 (75). Second, caspases activate Gasdermin D (GSDMD), a pyroptotic protein that, after caspase-mediated cleavage of its N-terminal fragment (GSDMD-N), moves toward the inner plasmatic membrane by generating porosity and permeabilization (85). It results in an uncontrolled flow of ions and water, causing cell lysis, cell death, and subsequent release of additional cytokines in the extracellular microenvironment (86). Pyroptosis, contrary to apoptosis and other types of cell death, is characterized by maintaining nuclear integrity, without DNA fragmentation, but showing signs of nuclear condensation and cell swelling (75, 85).

The autophagy mechanism plays an important role in the suppression of pyroptosis by inactivation of the inflammasome (87). To avoid the pyroptotic pathway, autophagy applies two strategies. First, autophagy sequestrates inflammasome inducers such as ROS, bacteria, and critical damaged mitochondria that, after ubiquitination for recognition, are delivered to autophagosomes for degradation (24). Second, autophagic machinery recognizes overactivated components of the inflammasome, especially NLR family pyrin domain-containing protein 3 (NLRP3) and Absent In Melanoma 2 (AIM2), which are specifically recognized by the autophagy receptor p62/SQSTM1, transported and destroyed via the autophagosome (88, 89). Both strategies limit the activation and release of the proinflammatory cytokines IL1β and IL-18, reducing inflammation and pyroptosis signaling (87, 89).

Autophagy and Ferroptosis

Ferroptosis is a novel type of programmed cell death characterized by iron and lipidic ROS/peroxides accumulation (29). It has been proposed that cancer cells from different tissues show different degrees of ferroptosis sensitivity. Even so, some authors have shown that ferroptotic reagents can induce cancer cell death that could be rescued by ferroptosis inhibitors (90). This iron- and oxidative-mediated cell death is activated through excessive levels of iron production by Fenton reaction and through the loss of balance in ROS production and cell glutathione (GSH)-dependent antioxidants, which protect cells from lipid peroxidation (85). Glutathione peroxidase 4 (GPX4) is a crucial enzyme for the elimination of lipid ROS continuously generated by the cell. Its inhibition can induce ferroptosis even with normal levels of the cofactor GSH (91). Besides, depletion of GSH or its precursor, cysteine (Cys), constitutes an indirect way to activate ferroptosis (92). Ferroptosis is characterized, contrary to other regulated cell death mechanisms, by cell membrane integrity, normal nucleus size, and dense small mitochondria (76).

Recent studies have described a direct contribution of autophagy in ferroptosis initiation, arguing the presence of a specific autophagic cell death called ferritinophagy (91). After Cys suppression, autophagy is activated to sequester and degrade ferritin, a cell iron storage protein, by the selective autophagy cargo receptor NCOA4, inducing ROS accumulation and the consequent ferroptotic cell death. Inhibition of the expression of autophagic proteins such as ATG5, ATG7, and NCOA4 reduces ferritin elimination, iron levels, lipid peroxidation, and ferroptosis activation (93). Furthermore, autophagy pathway activation by Tat-Beclin-1, a direct autophagic-mechanism inducer, selectively promotes ferroptotic cell death in tumor cells (94). Other studies demonstrate that ferroptosis stimulation also induces autophagy, evidenced by an intensification in the conversion of mature LC3 and autolysosome assembly (95), demonstrating a close interplay between both signaling mechanisms.

AUTOPHAGY AND EMT

The epithelial-to-mesenchymal transition (EMT) is a key process involved in the genetic, biochemical, and phenotypic changes that epithelial cells experience to convert them to mesenchymal cells, a cellular type with greater versatility and plasticity (96). Further, has been discovered that the reverse process, designated mesenchymal-to-epithelial transition (MET), is also crucial in the metastatic process. When a cell undergoes EMT, it loses its basal polarity to acquire a fibroblast-like morphology (97).

EMT is important to allow migratory properties to the cancer cells, facilitating their entry into the bloodstream. Further, to find a niche in any tissue, the cancer cells need to exit from the circulation by experiencing the MET process to acquire epithelial properties to nest in the tissues and establish metastatic niches. The most important signals to stimulate EMT occur in the microenvironment. For example, hypoxia, which appears in certain parts of the tumor that are oxygen-deprived, generates EMT through by the activation of HIF-1α, which in turn stimulates inflammatory cytokines (e.g., TNFα or IL-6), which contribute to EMT induction (98, 99). Moreover, hypoxia induces high levels of ROS in cancer cells, which leads autophagy stimulation (100, 101). Conversely, autophagy also increases the EMT phenotype after the adaptation to hypoxia (102).

The effect of autophagy on EMT appears controversial and depends on the type of stimulus, the cell genetic background, and the cell type. Different cytokines or microenvironmental conditions that stimulate EMT can provoke opposite reactions in autophagy (97). For example, salvianolic acid B, an active component of a Chinese natural product, suppresses EMT in a renal fibrosis animal model by induction of autophagy, mediated by silent information regulator 1 (Sirt1) (103). Imprinted gene pleckstrin homology-like domain family A member 2 (PHLDA2) is upregulated in colorectal cancer, and its knockout stimulates autophagy via the PI3K/AKT pathway, reducing cell proliferation, invasion, migration, and EMT process (104). In gastric cancer, forkhead box K1 (FOXK1) is a transcription factor involved in cancer development. The inhibition of FOXK1 in an acidic microenvironment triggers autophagy and reverses EMT in gastric cancer cells (105). However, in bladder cancer cells, it has been demonstrated that starvation conditions promote autophagy, which boosts the EMT process through TGF-β1/Smad3 signaling, enhancing cell invasion and migration (106). Moreover, knockdown of the autophagy-related protein DNA damage-regulated autophagy modulator 1 (DRAM1) reduces the migrative and invasive capabilities of hepatoblastoma cells, inactivating autophagy, and EMT (107).

AUTOPHAGY AND METABOLISM

It has been assumed that malignant cells have a hyperactivation of metabolic activities that increase ROS levels. However, the known Warburg effect described one century ago in cancer cells, is based upon the use of glycolysis, even in the presence of oxygen, to avoid the OXPHOS respiration through the mitochondria and, consequently, high ROS accumulation (108, 109). The high use of glycolysis generates huge concentrations of lactic acid released in the microenvironment. It has been suggested that not only the avoidance of ROS accumulation gives an extra survival capacity to cancer cells but also the lactic acid acidifying the microenvironment (110). For example, it has been described that in melanoma cells, glucose-deprivation stress induces autophagic cell death, but this is inhibited by the large concentrations of lactic acid in the microenvironment (111).

Autophagy can be activated by ROS through diverse signaling pathways, such as ROS-FOXO3-LC3/BNIP3, ROS-NRF2-P62, ROS-HIF1-BNIP3/NIX, and ROS-TIGAR; as a result, autophagy suppresses ROS-promoted damage by eliminating oxidized substance, keeping cellular homeostasis (112, 113). In cancer, autophagy also regulates tumor homeostasis, preventing the accumulation of ROS generated by the hyperactivation of metabolism (114). On the other hand, in principle, autophagy counteracts the metabolic switch followed by malignant transformation by eliminating deteriorated mitochondria to sustain the maximum bioenergetic needs and preserve the physiological, metabolic homeostasis. ROS has been described to oxidize ATG4, resulting in the formation of autophagosomes and autophagy (115). This process occurs in cadmium-mediated cell proliferation, migration, and invasion in pulmonary adenocarcinoma cells (116).

Moreover, ATG12 has been shown to control mitochondrial biogenesis and metabolic pathways such as glycolysis, tricarboxylic acid cycle, and β-oxidation in cancer cells (117). Additionally, tyrosine kinase signaling by hepatocyte growth factor (HGF) and its receptor tyrosine kinase (MET/HGFR) is hyperactivated in numerous cancers, inducing proliferation, invasion, and metastasis. In liver cancer, HGF/MET pathway activation provokes the Warburg effect and glutaminolysis, mediating cancer cell development. However, targeting MET to suppress kinase activation triggers the autophagy pathway to ensure cell growth and survival (118). In nasopharyngeal carcinoma, the Epstein-Barr virus latent membrane protein 1 (LMP1) can promote tumor development by its transference inside extracellular vesicles released by fibroblasts, boosting their transformation into cancer-associated fibroblasts (CAFs) via the NF-κB pathway. As a result, CAFs activate autophagy machinery and mediate a metabolic switch from OXPHOS to glycolysis to generate energy-rich nutrients for cancer cells, which enhance their OXPHOS metabolic activity, in a process called the Reverse Warburg Effect (RWE) (119).

AUTOPHAGY AND THE IMMUNE SYSTEM

Autophagy participates actively in the regulation of the immune system, playing significant roles in the activation, differentiation, and survival of immune cells such as T and B cells, monocytes, macrophages, natural killer (NK)-cells, and dendritic cells. Thereby, the autophagic process modulates innate and adaptative immunity (120, 121). Also, autophagy controls the production and release of cytokines, such as IL-1, IL-18, Type I IFN, and TNF-α. Apart from immune cells, immune components, such as cytokines and immunoglobulins, influence the activation and suppression of autophagic processes. It has been described that IL-1, IL-2, IL-6, IFN-γ, TNF-α, and TGF-β1 are stimulators and IL-4, IL-10, and IL-13 are inhibitors of the autophagy process (122). For example, natural secretion of IL-17 and IL-22 by γδ T cells can be regulated by IL-1-dependent autophagy activation (123). Moreover, in antigen donor cells, upon severe stress exposure (which might be prolonged in time), cell death will take place, causing autophagy-mediated antigen release and stimulation of immune and inflammatory responses (124).

Several studies indicate that a piece of active autophagic machinery produces tumor-specific antigens in tumor cells which, after their release due to antigen donor cells, boost antitumor effects by enhancing antigen presentation and subsequent T cell activation (120). Autophagy induces liberation of more ATPs as a required signal to stimulate recruitment of antigen-presenting cells (APCs) and tumor sensitivity to cytotoxic T lymphocytes (124). Also, inhibition of autophagy by targeted drugs or genetic deficiencies in autophagy-related genes such as ATG5, ATG7, ATG12, Beclin-1, and VPS34 reduces ATP release and hinders the recruitment of required immune cells from boosting antitumor immune responses (125). Furthermore, radiotherapy- or chemotherapy-induced autophagy mediates the release of the mannose-6-phosphate receptor (MPR) without its natural ligand from the autophagosome and its movement back to the cell surface, promoting T cell activation after granzyme B binding (126). Autophagy has also been involved in antigen processing for major histocompatibility complex class I (MHC-I) and II (MHC-II) presentation, including cross-presentation. To give an example, alpha-tocopheryloxyacetic acid (α-TEA), derived from vitamin E, promotes autophagy-controlled cross-presentation of tumor antigens in lung cancer cells to the immune system, mainly antigen-specific cytotoxic T lymphocytes (127).

Moreover, autophagy can suppress immune effector mechanisms against tumors. For example, the hypoxia condition triggers autophagy machinery in lung cancer cells, which suppresses T cell antitumoral activity through phosphorylation of STAT3 and subsequent HIF-1α signaling pathway activation (128). Tumor susceptibility to the cytotoxic effect and tumor cell lysis of T lymphocytes are restored through hydroxychloroquine (HCQ)-mediated autophagy inhibition or knockdown of ATG and Beclin-1 genes (125, 129). Moreover, hypoxia-induced autophagy also interrupts the anticancer killing activity of NK-cells by selective degradation of NK-derived granzyme B, which can be reversed after autophagy inhibition by targeting Beclin-1 (130, 131). On the other hand, tumor-associated macrophages (TAMs) are key components of the immune system and the main drivers of inflammatory microenvironment inside tumor and cancer progression (132). According to a recent study in metastatic ovarian cancer, TAMs that specifically express T-cell immunoglobulin and mucin domain-containing 4 (TIM4) showed high oxidative phosphorylation and adapted mitophagy to mitigate oxidative stress (133). Besides, genetic deficiency of autophagy protein FIP200 ensued in Tim-4^{+} TAM loss via ROS-mediated apoptosis increasing T cell-immunity and tumor inhibition *in vivo* (133).

Therefore, autophagy activation can induce antitumor immune responses but can also mediate inhibition of immune cell activity against tumors to allow cancer cells to escape from the immune system. Overall, autophagy has a context-dependent function as an activator and inhibitor of the immune response in cancer cells, which might be crucial in current immunotherapies.

AUTOPHAGY AND NON-CODING RNAs

Non-coding RNAs (ncRNAs) comprise 98% of the human genome, and their biological functions consist of chromatin and epigenetic modifications, regulation of gene expression, transcription, mRNA splicing, regulation of protein localization and activity, and apoptosis, among others (134). These regulatory RNAs are classified into two groups: long ncRNAs (lncRNAs), larger than 200 nucleotides, and small ncRNAs, which mainly comprise microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), circular RNA (circRNAs), and piwi-interacting RNAs (piRNAs) (135). The role of ncRNAs in cancer cells has been associated with many physiological and pathological processes, such as proliferation, differentiation, migration, invasion, metastasis, and drug resistance (136).

Recent studies have described the mechanisms of several ncRNAs in the regulation of the autophagy process in tumor cells (137). For instance, circNRIP1 was proven to modulate the autophagy and cancer cell metabolism switch into the Warburg effect by alteration of AKT1 expression and, consequently, the AKT/mTOR pathway, which induces tumor development and metastasis in gastric cancer (138). Moreover, miRNA-133a-3p suppresses tumor growth, and the development of metastatic lesions in gastric cancer, inhibiting autophagy-mediated glutaminolysis by targeting GABARAPL1 (a GABARAP subfamily) and ATG13 (139). Additionally, miR-142-3p was demonstrated to target ATG5 and ATG16L1, causing the inhibition of autophagy, producing an increased sensitization of hepatocellular carcinoma cells to sorafenib (140). Also, miR-519a sensitizes glioblastoma cells to temozolomide by the activation of autophagy via the STAT3 pathway, which generates Bcl-2/Beclin-1 complex dissociation and resultant autophagy-mediated apoptosis (141). There are many other miRNAs, such as miR-124, miR-144, miR-224-3p miR-301a/b, and miR-21, involved in the alteration of autophagy in many cancer cell types, either activating or inhibiting, which influence tumor resistance to conventional therapy (142–145). Additionally, lncRNAs control autophagy mainly by directly or indirectly regulating ATG expression (146). As an example, knockdown in colorectal cancer cells of homeobox transcript antisense intergenic RNA (HOTAIR), a lncRNA that has been widely studied, induces upregulation of miR-93 and a downregulation of ATG12, resulting in a blockage of autophagy and the induction of apoptotic cell death (147). In hepatocellular carcinoma, the lncRNAs phosphatase and tensin homolog pseudogene 1 (PTENP1) activate autophagy, interacting with miR-17, miR-19b, and miR-20a, denying their targeting of the autophagy genes ULK1, ATG7 and p62/SQSTM1, and the tumor suppressor PTEN. As a result, the overexpression of PTENP1 reduces tumor size, restrains proliferation, suppresses angiogenesis, and induces cancer cell apoptosis (148). Also, highly upregulated lncRNA in hepatocellular carcinoma cells diminishes their sensitivity to chemotherapeutic drugs by autophagy triggering, mediated by suppressing silent information regulator 1 (Sirt1) (149). Other lncRNAs, such as XIST, BLACAT1, and MEG3, also

play a pivotal role in the regulation of autophagy processes in different types of tumors, which modulate cancer progression and chemotherapeutic resistance (150–152).

AUTOPHAGY AND CSCs (CANCER STEM CELLS)

The cancer stem cell hypothesis proposes that many cancer types originate from cancer cells with stemness-like characteristics, known as cancer stem cells (CSCs) (153). CSCs are a subpopulation of cancer cells that possess the abilities of differentiation, tumor initiation, pluripotency, and self-renewal capabilities, being able to reconstruct the original tumor by themselves. CSCs are the cell type most representative of resistance to conventional anticancer therapies (including radiation and chemotherapy) in comparison to other cells that constitute the tumor (154). These features confer CSCs the abilities of tumor relapse and metastasis dissemination. Besides, CSCs show the capacity to grow under serum starvation, forming spheres in 3D conditions, maintaining high aldehyde dehydrogenase (ALDH) activity while showing cell cycle dysregulation (155). Moreover, under the term CSCs, there is a large heterogeneous population of different CSCs with different degrees of malignancy (156).

Many studies underline the crucial role of the autophagy mechanism in the maintenance of CSC homeostasis, features, and functions inside tumor and cancer progression (157). CSCs use autophagy to reinforce their resilience against microenvironmental stress conditions, such as starvation and hypoxia, promoting their survival to preserve their stemness phenotype (155). It has been proposed that through TGF-β1 inducing EMT in CSCs; it is autophagy activation that enables them to invade the circulation. In breast cancer, the inhibition of the autophagy-related proteins Beclin-1, ATG12, and LC3 reduces the stemness-like phenotype, reinforcing that the activation of protective autophagy supports the maintenance of the breast CSC population (158, 159). In the same line of evidence, autophagy inhibition by knockdown of ATG5 and ATG7 drastically decreases the stemness features of colorectal CSCs, evidenced by a reduction in the expression levels of stemness markers (OCT4, SOX2, and NANOG), increased cellular senescence, and the decline of cell proliferative capacities in CSCs in tumors (160). As would be expected, enhancement of the autophagy pathway in colon cancer induces resistance to anticancer therapies and an increase in the stemness-like phenotype (161). In glioblastoma, the autophagy regulator p62/SQSTM1 and DNA damage-regulated autophagy modulator 1 (DRAM1) is highly expressed in CSCs and control their migrative and invasive capacities (162, 163).

Additionally, some studies associate pluripotency-related factors with autophagy activation (55). For example, in non–small lung carcinoma, melanoma, and breast cancer, NANOG induces autophagy under hypoxia conditions in CSCs by direct regulation of BNIP3, a protein that interacts with Bcl-2 and mediates the disruption of the Bcl-2/Beclin-1 interaction (164), promoting tumor cell immune resistance (165). Furthermore, SOX2 induces autophagy through enhancement of ATG10 gene expression in colon cancer cells (165). These results corroborate that autophagy is an essential process involved in stem-like phenotype maintenance and tumor resistance to treatment in CSCs.

AUTOPHAGY IN STRESS RESPONSES, CANCER PROGRESSION, AND METASTASIS

It is broadly known that a basal level of autophagy is present in all cell types that naturally occurs. In contrast, an increase of the autophagy pathway or the autophagy flux accounts when cells are exposed to certain levels of stress (166). The autophagic stress response consists of two parts: a very rapid increment (minutes or hours after exposure to the stressor) in the autophagic flux through post-translational modifications, and a long term autophagic response consisting in the activation of stress-responsive transcription programs, being transcription factors such as p53, NF-κB, and STAT3 relevant in regulation of the autophagy facing stressful conditions (167).

As we analyze before, autophagy activity can be tumor suppressive or promoting depending on the scenario, such as nutrient availability, microenvironment influence, immune response, and among others (168). Genomic analysis of human cancers has identified that oncogenic events involving classical oncogenes and tumor suppressor genes have a key role in autophagy including PI3K, AKT1, PTEN, proteins of the Bcl-2 family, among others (169). However, functional evaluation of autophagy at the clinical level is demanding because the autophagic flux is not possible to measure in tumor samples of patients (168, 170). Even so, different studies corroborate that autophagy is upregulated in different types of cancer since progression to metastasis, and expression of several autophagy markers has been correlated with poor outcomes (171). As an example, the identification of a novel autophagy associated-gene signature can predict the prognosis of cancer patients with hepatocellular carcinoma. Such five genes are HDAC1, RHEB, ATIC, SPNS1, and SQSTM1, that were associated with overall survival in hepatocellular carcinoma patients (172). Of interest, the expression of autophagy-related genes was correlated with drug sensitivity in hepatocellular carcinoma cell lines (172).

Autophagic activity plays the primary role in the regulation of the different metastatic phases, including invasion, intravasation, survival inside the circulation, extravasation, survival, and growth in the second site; and also in the diverse mechanisms involved in metastasis, such as focal adhesion, integrin trafficking, cytoskeleton remodeling, anoikis resistance, detachment from the extracellular matrix, EMT, and tumor-stromal interaction (170, 173). Although it is challenging to determine autophagy flux in tumor patients, surrogate markers, such as LC3, have found a correlation between increased levels of autophagy and metastasis generation in varied types of cancer (112, 174, 175). Moreover, novel proteins related to metastasis have been shown to have a role in autophagy. For example, Nuclear protein 1 (NUPR1) is a molecule regulated in response

to stress, that has been implied in the progression of many cancers including of breast, pancreas, brain, and thyroid, in the development of metastasis (176, 177). NUPR1, initially associated with the rescue of cells to doxorubicin-induced genotoxic stress, has been shown to have a multifaceted role, including involvement in autophagy (178). Chaperones represent other examples. BAG3, a multifunctional HSP70 co-chaperone, exerts various physiological functions, including stress response and apoptosis, and oncopathological roles such as cell adhesion, metastasis, angiogenesis, stimulation of autophagy flux, and others. Also, BAG3 interacts with HSP70 and LC3 delivering polyubiquitinated proteins to the autophagy pathway. (179).

AUTOPHAGY AND CANCER CELL RESISTANCE

Intriguingly, different chemotherapeutic drugs may exert opposite effects on autophagy, resulting in cell death or cell survival. Autophagy in cancer cells during stress might emerge spontaneously due to gene mutations/epigenetic modifications or due to an imbalance of the cellular capacity to control its growth during adverse conditions. Moreover, ribosomal stress, ER stress, or the unfolded protein response (UPR) can trigger autophagy (180). In the last decade, the role of autophagy has been reinforced as a protective mechanism to mediate cell survival during chemotherapy, conferring MDR (181). For example, ATP-binding cassette (ABC) transporters, specifically ABCB1, also known as multidrug resistance protein 1 (MDR1), have been associated with MDR against a wide variety of chemotherapeutic agents (6). The expression in ABCB1 is positively correlated with autophagic-related genes Beclin-1, LC3, Rictor, and poor outcome survival of colorectal cancer patients (182), highlighting an association between autophagy triggering and MDR. It has been demonstrated that resistance to FGFR1-targeted therapy promotes autophagy via the TAK1/AMPK pathway (183).

Furthermore, several studies proved that autophagy stimulated by anti-cancer drugs probably enable the development of multiple resistance feature against epirubicin, paclitaxel, tamoxifen or herceptin, through inhibition of apoptosis in breast cancer cells (184). Besides, miR-495-3p was found to regulate autophagy and, consequently, MDR by its interaction with the GRP78/mTOR axis in gastric cancer (185). Another study showed that autophagy develops a protective function in multi-drug resistant ovarian cancer cells mediated by vincristine, and the inhibition of autophagy resensitizes tumors cells to vincristine and restore its killing effects (186). Our group demonstrated that the overexpression of PTOV1, induced resistance in cells through autophagy activation, a fact appreciated in head and neck squamous carcinoma cell lines. Also, we observed that both in cell lines and head and neck cancer patients resistant to cisplatin, they overexpressed markers of autophagy and PTOV1. Of interest is that some of these markers had prognostic value

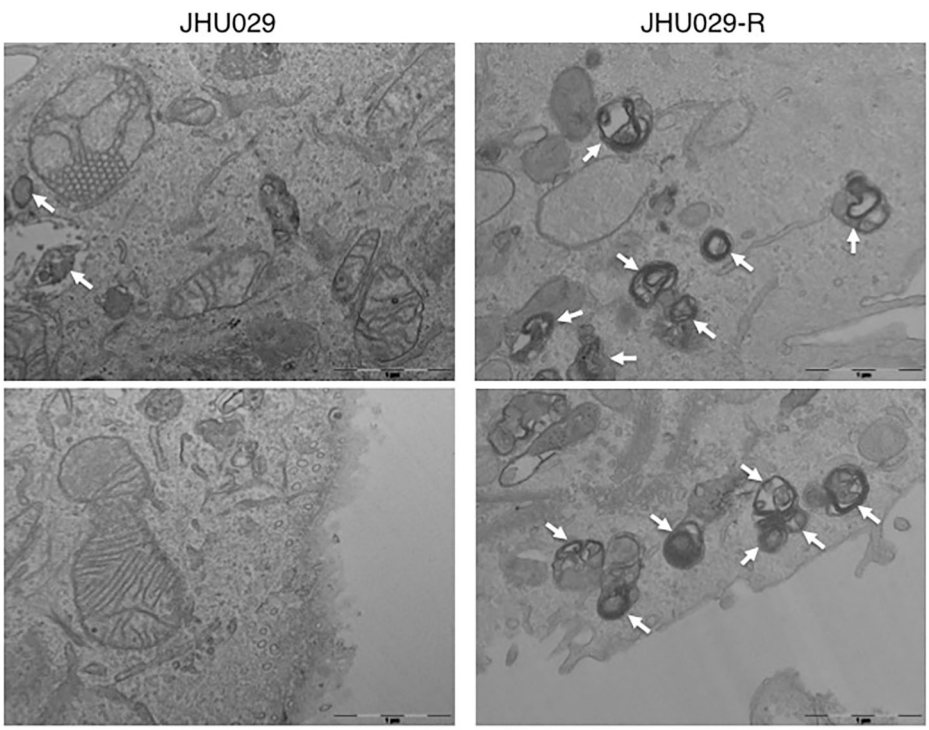

FIGURE 4 | Autophagy vesicles in cancer cells sensitive and resistant to chemotherapy. TEM images showing the presence of autophagy vesicles in JHU029 cell lines derived from laryngeal cancer, sensitive, and resistant (R) to cisplatin (see arrows). As was demonstrated by our group (187), cancer cells resistant to chemotherapy generate more autophagy vesicles than sensitive ones, which is correlated with the resistant phenotype developed.

when correlated with clinical variables (**Figure 4**). Besides, we suggested that the acquisition of resistance to cisplatin is related to the development of 5-fluorouracil resistance, supporting the presence of a common regulatory resistance pathway (187).

Moreover, it has been described that cisplatin or 5-fluorouracil promotes cytoprotective autophagy through upregulation of Beclin-1 in bladder cancer cells (188), an effect that has also been reported in other tumor cells, such as laryngeal, ovarian, esophageal, and colon cancer models (189–191). Upregulation of another autophagy-related gene, ATG7, also induces autophagy after treatment with cisplatin or 5-fluorouracil in esophageal cancer cells (192, 193). Of interest, the chemotherapeutic agents' cisplatin, temozolomide, and daunorubicin have been seen to stimulate protective autophagy by upregulation of the extracellular signal-regulated kinase (ERK) pathway, a mechanism observed in non-small cell lung cancer, ovarian cancer, glioma, and myeloid leukemia (194–198). Also, autophagy stimulation via AMPK, promoted by the chemotherapeutic agents' temozolomide, 5-fluorouracil, and docetaxel, confers resistance in different tumor types, such as prostate cancer, gastric cancer, and glioma (199–201). Besides, JNK upregulation induces autophagy-mediated chemoresistance to 5-fluorouracil in colorectal cancer cells (202).

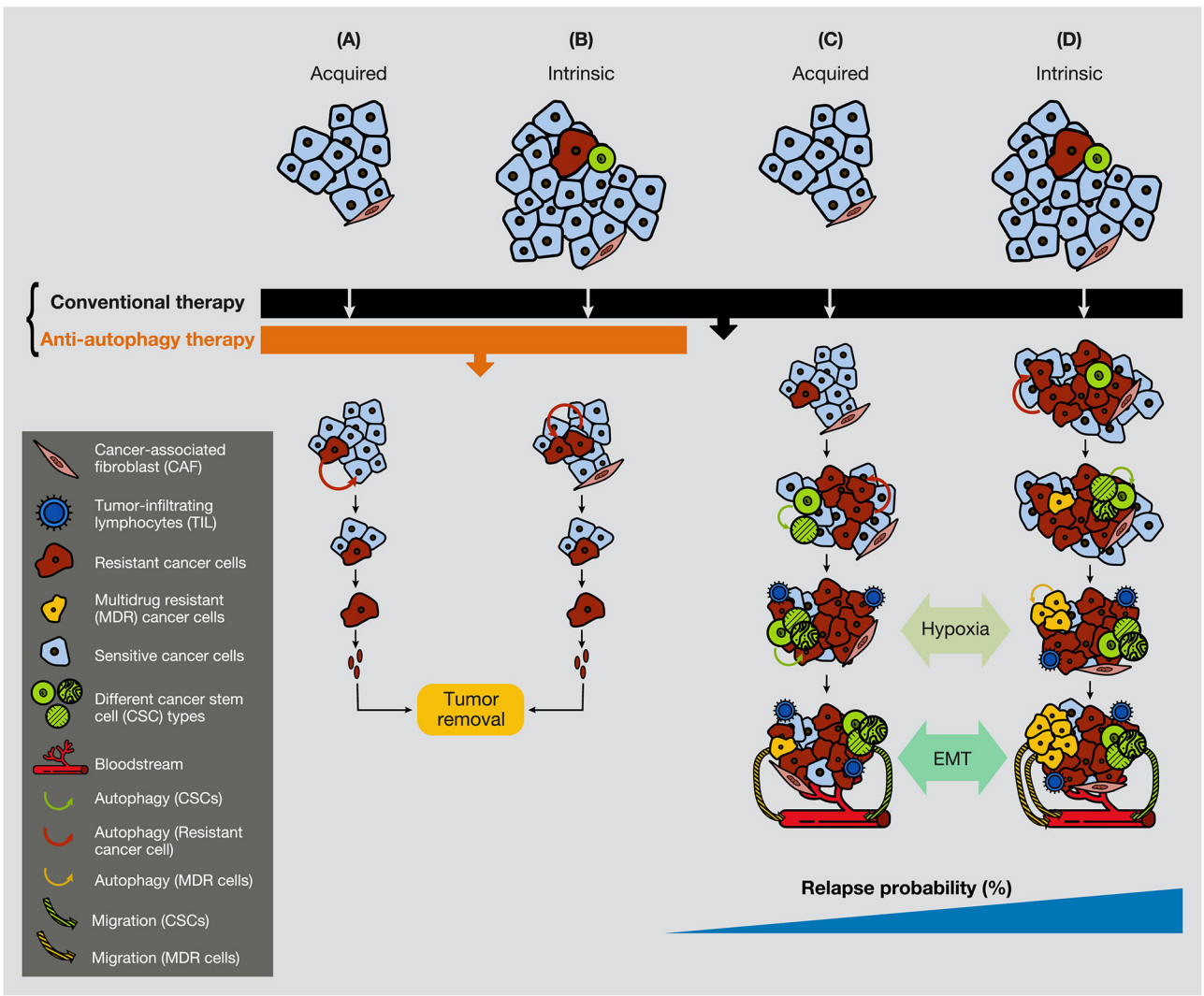

FIGURE 5 | Anti-autophagic therapy can be efficient in the initial stages of tumors in the presence of resistance or CSCs. In the figure are depicted several scenarios where acquired or intrinsic resistance is already present in malignant tumors. **(A)** Acquired resistance appears consequently to the conventional treatment therapy, but it cannot progress due to the anti-autophagic therapy (e.g., hydroxychloroquine). **(B)** If resistant cells are present in the tumors, they cannot grow expansively because of the anti-autophagic therapy. **(C)** Acquired resistance appears consequently to the conventional chemotherapeutic treatment. Resistant cells survive largely supported by the activation of autophagy, either from surrounding cancer cells or from CAFs. **(D)** While conventional chemotherapeutic treatment annihilates the bulk of cancer cells, it favors the spread of resistant cells, including the CSCs. At a certain time of tumor development, the hypoxic conditions enable EMT process, allowing cancer cell plasticity to enter into the circulation. CAF, cancer-associated fibroblast; TIL, tumor-infiltrating lymphocyte; CC, cancer cell.

Furthermore, chemotherapeutic inhibition of the mTOR pathway induces an autophagy process that, in sensitive cells and after long-exposure treatment, provokes cell death. However, if cancer cells have already reached some degree of resistance, the inhibition of mTOR can also generate JNK activation, P-ERK upregulation, and Bcl-2/Bcl-xL phosphorylation in the breast, gastric and esophageal cancer, with subsequent activation of protective autophagy (203–205). Overall, while conventional chemotherapy treatment is used extensively against malignant tumors and is efficient against the bulk of cancer cells, a certain small population of cancer cells (resistant cells and CSCs) activates autophagy to survives therapy. **Figure 5** illustrates that a small quantity of resistant cells or CSCs present in tumors can be annihilated if a combination of conventional therapy and anti-autophagic therapy is applied from the beginning of the treatment (**Figure 5**).

AUTOPHAGY AS A TARGET FOR THERAPEUTIC PURPOSES: INHIBITION OR STIMULATION?

The protective function of autophagy in healthy cells in response to soft or severe stimuli, such as starvation or hypoxia, acts as a protective mechanism to ensure cell survival, and if healthy cells cannot restore the damage, they will die by apoptosis (48). Thereby, autophagy activation acts as a tumor suppressor mechanism, preventing tumor initiation by maintaining metabolic homeostasis and suppressing genomic instability (**Figures 6A,B**). We propose a model where, if a severe stimulus occurs in cancer cells, such as chemotherapy or radiotherapy, protective autophagy emerges in most cases when apoptosis is defective (a common feature of cancer cells) (**Figure 6C**). Nevertheless, if the stress exceeds a threshold incompatible with cellular life, autophagy activation can mediate

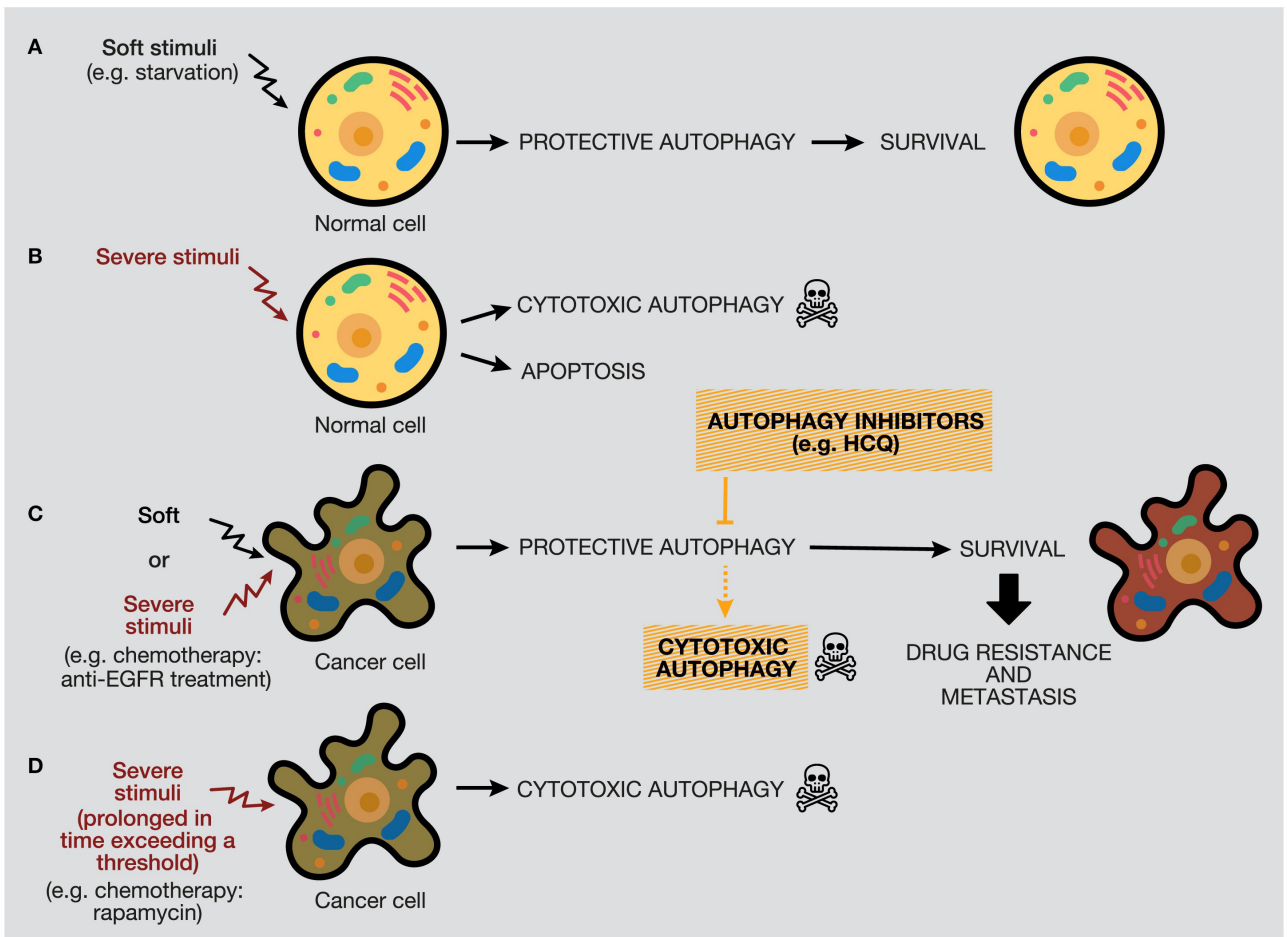

FIGURE 6 | Distinctive responses of normal and cancer cells to different stimuli. The autophagy response depends on the intensity and duration of the stimuli (external or internal), highlighting a threshold in the autophagy mechanism that would determine cellular outcome. **(A)** Normal cells under soft stimuli, such as starvation, will ensure their survival through protective autophagy. **(B)** Severe stimuli on normal cells can induce cytotoxic autophagy or apoptosis. **(C)** In contrast, soft or severe stimuli over cancer cells (for example, anti-EGFR treatments) will provoke protective autophagy, which will confer survival properties, drug resistance, and metastasis. In this case, the use of autophagy inhibitors would provoke cell death by autophagy or occasionally by apoptosis. **(D)** Last, severe stimuli able to reach a threshold can increase cytotoxic autophagy.

cell death depending on the genetic background, tumor evolution, and microenvironment (181). However, as mentioned above, the lack of consensus of whether there is a cytotoxic form of autophagy or stimulating autophagy in several tumor models under some circumstances just promotes or facilitates another type of cell death is a complex field (40, 206).

By one side, some anticancer treatments trigger autophagy as a death executioner, causing a cytotoxic effect that ends in autophagic cell death (30). This therapeutic strategy can be suitable in tumors with deficiencies in the activation of the apoptotic programmed cell death pathway, such as tumor cells lacking functional p53 (207). According to our model, a long-term autophagy activation strategy, generated by chemotherapy, radiotherapy, or targeted drugs, can be used to generate cytotoxic autophagy and cancer cell death (**Figure 6D**). For example, long-term treatment of PtAcacDMS, a novel platinum-based drug, triggers both apoptosis and autophagy, resulting in cell death in neuroblastoma cells (208). However, stimulation of autophagic cell death by different targeted drugs has been considered by researchers as an attractive alternative to treat some tumors that show some signs of apoptosis. The most representative example is the inhibition of the mTOR pathway as the main method to trigger autophagy in preclinical and clinical studies. For example, rapamycin is a selective inhibitor of mTORC1 and causes activation of autophagy (209). In several studies, rapamycin has been demonstrated to suppress cancer proliferation and induce autophagic cell death in different cancer models, such as neuroblastoma, osteosarcoma, and sarcoma (210–212). Rapamycin analogs (rapalogs), such as temsirolimus and everolimus, also inhibit the mTOR pathway in renal cancer and breast cancer, among others (213, 214). Other types of autophagy activators include the BH3 mimetics (e.g.,

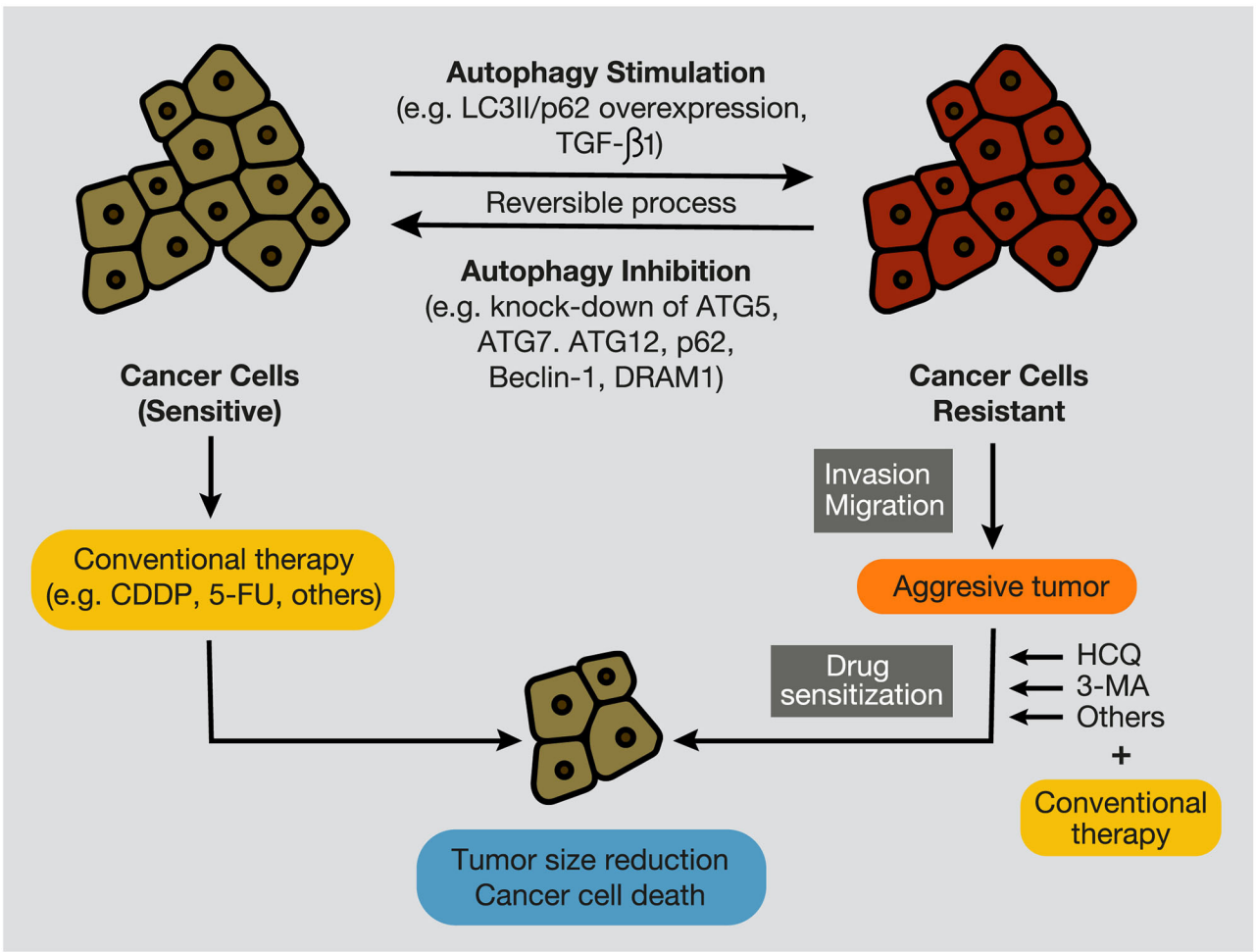

FIGURE 7 | Autophagy stimulation and inhibition in cancer cells. During tumor development, autophagy inhibition, by targeting autophagy-related proteins, such as ATG7, Beclin-1, p62/SQSTM1, and DRAM1, promotes the sensitization of cancer cells to conventional anticancer treatments, such as chemotherapeutic agents, including CDDP and 5-FU. In contrast, autophagy stimulation, evidenced by overexpression of LC3II and p62/SQSTM1, and by high levels of TGF-β1, provokes cancer cell resistance to therapies (e.g., chemotherapy and radiation), development of an aggressive phenotype, and increment of migratory and invasive capacities. In this case, several autophagy inhibitors, such as CQ, HCQ, or 3-MA, can re-sensitize resistant tumors and promote tumor regression and cancer cell death. CQ, chloroquine; HCQ, hydroxychloroquine; 3-MA, 3-methyladenine; CDDP, cisplatin; 5-FU, 5- fluorouracil.

TABLE 1 | Anticancer drugs that inhibit autophagy used in combination with chemotherapy.

Autophagy inhibitor	Function	Tumor type	Chemotherapeutic	(Pre-) clinical phase	NCT number	Ref
Chloroquine	Inhibits acidification of lysosome and autophagosome-lysosome formation	Glioblastoma, Gliosarcoma	Temozolomide	I	NCT04397679	–
		Glioma, Gliomatosis Cerebri	Temozolomide	III	NCT03243461	–
		Glioblastoma, Astrocytoma	Temozolomide	II	NCT02432417	–
		Glioblastoma	Temozolomide	I	NCT02378532	–
		Solid Tumors	Carboplatin, Gemcitabine	I	NCT02071537	–
		Pancreatic Cancer	Gemcitabine	I	NCT01777477	(227)
		Multiple Myeloma	Cyclophosphamide	II	NCT01438177	–
		Glioblastoma	Not specified	III	NCT00224978	(228)
Hydroxy Chloroquine	Inhibits acidification of lysosome and autophagosome-lysosome formation	Osteosarcoma	Docetaxel, Gemcitabine	I/II	NCT03598595	–
		Pancreatic Cancer	Gemcitabine, Nab-Paclitaxel	II	NCT03344172	–
		Small Cell Lung Cancer	Gemcitabine, Carboplatin	II	NCT02722369	–
		Acute Myeloid Leukemia	Mitoxantrone, Etoposide	I	NCT02631252	–
		Pancreatic Cancer	Gemcitabine, Abraxane	II	NCT01978184	–
		Multiple Myeloma	Cyclophosphamide	I	NCT01689987	–
		Non-Small Cell Lung Cancer	Paclitaxel, Carboplatin	II	NCT01649947	–
		Pancreatic Cancer	Gemcitabine, Nab-Paclitaxel	I/II	NCT01506973	(229)
		Pancreatic Cancer	Capecitabine and Radiation	II	NCT01494155	–
		Colorectal Cancer	Oxaliplatin, 5-fluorouracil	I/II	NCT01206530	–
		Pancreatic Cancer	Gemcitabine	I/II	NCT01128296	(230)
		Colorectal Cancer	Capecitabine, Oxaliplatin	II	NCT01006369	–
		Prostate Cancer	Docetaxel	II	NCT00786682	–
		Breast Cancer	Ixabepilone	I/II	NCT00765765	–
		Non-Small Cell Lung Cancer	Carboplatin, Paclitaxel	I/II	NCT00728845	–
		Solid Tumors	Temozolomide	I	NCT00714181	–
		Glioblastoma	Temozolomide	I/II	NCT00486603	(231)
Wortmannin	Inhibits PI3KC3 complex and autophagosome formation.	Lung cancer, Prostate cancer	Docetaxel	Pre-clinical	NA	(232)
		Ovarian cancer	Cisplatin	Pre-clinical	NA	(233)
3-Methyl Adenine (3-MA)	Inhibits PI3KC3 complex and autophagosome formation.	Non-Small Cell Lung Cancer	Camptothecin	Pre-clinical	NA	(234)
		Colon cancer	Oxaliplatin	Pre-clinical	NA	(235)
Spautin-1	Enhances Beclin1 ubiquitination and prevent PI3KC3 complex formation	Melanoma	Cisplatin	Pre-clinical	NA	(236)
		Osteosarcoma cells	Doxorubicin	Pre-clinical	NA	(237)
LY294002	Inhibits PI3KC3 complex and autophagosome formation.	Oesophageal squamous cell carcinoma	5-fluorouracil	Pre-clinical	NA	(238)
Resveratrol	Regulates S6K1, inhibit ROS/ERK pathway	Glioma	Temozolomide	Pre-clinical	NA	(197)
		Ehrlich ascitic carcinoma	Doxorubicin	Pre-clinical	NA	(239)
4-Acetylantroquinonol B	Downregulation of ATG-7 and ATG-5	Ovarian cancer	Cisplatin	Pre-clinical	NA	(240)

(–) information available at www.clinicaltrials.gov, NA: Not Apply.

gossypol, obatoclax), which provoke Bcl-2/Beclin-1 complex dissociation through interaction with Bcl-2 (215, 216). Histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid (e.g., SAHA or Vorinostat), have also been implied in the activation of apoptosis and autophagy by inactivation of the PI3K/AKT/mTOR pathway (217, 218). Also, natural compounds have demonstrated antitumor properties through autophagy stimulation. For example, curcumin promotes autophagy-mediated cell death at high doses by the oxidative stress pathway. However, at low concentrations of curcumin, autophagy mediates cell protection through AMPK and ER stress pathways, evidencing a dual effect of curcumin, depending on the duration and concentration administered (219).

It is frequently observed that tumor cells activate autophagy to protect themselves from the stress caused by anticancer treatments, such as chemotherapy, radiotherapy, targeted therapy, or immunotherapy (220, 221). This activation occurs under prolonged treatments, due to the waiting time for patients to recover between different chemotherapy sessions; this process can also be considerably accelerated if the therapies are not effective. Therefore, upregulation of autophagy due to chemotherapeutic treatment in some cancer types (e.g., pancreatic cancer) or due to specific genetic conditions (e.g., Ras gene family mutations) promote drug resistance, which permits tumor recurrence, invasion, and metastatic development (3, 222). Our group has suggested that for particularly aggressive tumors, which might contain many mutations at the genetic and non-genetic levels, the activation of autophagy is a mechanism used by cancer cells to acquire an MDR phenotype (223). In this scenario, the inhibition of the autophagic process concomitantly to conventional therapy may be the appropriate strategy. The lysosomal inhibitors chloroquine (CQ) and its analog, HCQ, are the most extensive autophagy inhibitors in research and clinical studies (224). CQ and HCQ suppress autophagy via alteration of lysosomal pH and inactivation of acidic hydrolases, resulting in blocking of autophagolysosomal formation, accumulation of autophagosomes, and inactivation of autophagic degradation (157, 224, 225).

Moreover, the inhibition of the PI3KC3 complex is another strategy to inhibit autophagy in cancer cells (207). Wortmannin, 3-Methyl Adenine (3-MA), Spautin-1, and LY294002 have shown promising results in preclinical studies in coadministration with chemotherapeutics such as docetaxel, cisplatin, doxorubicin, or 5-fluorouracil (**Figure 7**). Resveratrol, which controls S6K1 and inhibits the ROS/ERK pathway, and 4-Acetylantroquinonol B, which reduces ATG-7 and ATG-5 expression, are two compounds used to reduce the autophagic process with promising preclinical outcomes. Moreover, HCQ treatment has been used to treat resistant cells to radiotherapy through *in silico*-designed nanoparticles for autophagy inhibition (226). Many studies in preclinical models (tumor cell lines and animal models) have demonstrated that CQ and HCQ induces cancer cell killing through treatment alone or in combination with targeted agents, radiotherapy, or chemotherapy (1). Besides, CQ and HCQ have been part, and are currently part, of several clinical trials in cotreatment with chemotherapeutics of different types of cancer, including glioblastoma, multiple myeloma, small and non-small cell lung, colorectal, pancreatic, prostate, and breast cancers (**Table 1**). Although clinical results to autophagy inhibition by CQ or HCQ has not been as consistent as seen in preclinical studies until now, the overall results published in clinical trials have proved their safe use as cancer therapy and their commitment to the biological target. Therefore, these autophagy inhibitors continue being used in active clinical trials in cotreatment with target therapy and chemotherapeutic drugs, including the International Cooperative Phase III Trial (HIT-HGG-2013) in Glioma and Gliomatosis Cerebri of temozolomide in cotreatment with valproic acid or CQ (NCT03243461).

Overall, our model proposes that if acquired or intrinsic resistance is present at the initial stages of a tumor, it is possible to eradicate aggressive resistant cells by applying an autophagy inhibitory therapy from the beginning of the treatment concomitantly to conventional therapy. Personalized medicine to predict the status of autophagy (activated or defective) in cancer cells and the presence of specific markers able to predict the resistance or sensitization of cancer cells are key factors for predicting and choosing the best treatment for cancer patients. The incorporation of molecular (e.g., next-generation sequencing) and pathological (assessment of the overexpression of autophagy-related proteins or determination of the lymphocyte infiltration of tumors) techniques would improve the focus toward the most appropriate therapy.

CONCLUSIONS

- The need to use, in general terms, high doses of conventional therapy to achieve therapeutic effects is the cause of the severe side effects of chemotherapies. As a result, chemotherapy sessions must be spaced to let patients recover from the side effects. This time-lapse is exploited by tumor cells to recover, proliferate, develop drug resistance, and create metastases responsible for most cancer deaths.
- How to tackle the acquisition of therapy resistance by tumors represents one of the most important challenges in cancer.
- Autophagy seems to favor cancer cells to acquire resistance; however, autophagy has a context-reliant function in cancer.
- Anti-autophagic treatments (e.g., HCQ) are very tolerable for patients and rarely cause severe side effects.
- It is of crucial importance that an effective treatment should be given to each cancer patient as the first therapeutic choice. Personalized medicine includes (a) the culturing of patient biopsies using spheroids, organoids, or mouse models to advance the benefits of a particular treatment and (b) the identification of genetic alterations by next-generation sequencing, which would point out specific drugs for particular mutations.

AUTHOR CONTRIBUTIONS

JA-M wrote the manuscript, YG-M, CM, and HK revised critically the manuscript, ML wrote the manuscript, revised it critically, and coordinated the work. All authors contributed to the article and approved the submitted version.

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REVIEW
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Therapy-Induced Modulation of the Tumor Microenvironment: New Opportunities for Cancer Therapies

Sergi Benavente[1]*, *Almudena Sánchez-García*[2], *Silvia Naches*[3], *Matilde Esther LLeonart*[2,4] and *Juan Lorente*[3]

[1] Radiation Oncology Department, Vall d'Hebron University Hospital, Barcelona, Spain, [2] Biomedical Research in Cancer Stem Cells Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain, [3] Otorhinolaryngology Department, Vall d'Hebron University Hospital, Universitat Autònoma de Barcelona, Barcelona, Spain, [4] Spanish Biomedical Research Network Centre in Oncology, CIBERONC, Barcelona, Spain

Advances in immunotherapy have achieved remarkable clinical outcomes in tumors with low curability, but their effects are limited, and increasing evidence has implicated tumoral and non-tumoral components of the tumor microenvironment as critical mediators of cancer progression. At the same time, the clinical successes achieved with minimally invasive and optically-guided surgery and image-guided and ablative radiation strategies have been successfully implemented in clinical care. More effective, localized and safer treatments have fueled strong research interest in radioimmunotherapy, which has shown the potential immunomodulatory effects of ionizing radiation. However, increasingly more observations suggest that immunosuppressive changes, metabolic remodeling, and angiogenic responses in the local tumor microenvironment play a central role in tumor recurrence. In this review, we address challenges to identify responders vs. non-responders to the immune checkpoint blockade, discuss recent developments in combinations of immunotherapy and radiotherapy for clinical evaluation, and consider the clinical impact of immunosuppressive changes in the tumor microenvironment in the context of surgery and radiation. Since the therapy-induced modulation of the tumor microenvironment presents a multiplicity of forms, we propose that overcoming microenvironment related resistance can become clinically relevant and represents a novel strategy to optimize treatment immunogenicity and improve patient outcome.

Keywords: radiotherapy, immunotherapy, tumor microenvironment, surgery, cancer

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***Correspondence:**
Sergi Benavente
sbenavente@vhebron.net

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INTRODUCTION

Cancer treatment modalities vary considerably depending on stage and location, however surgical excision and radiation therapy are an integral part of treatment for most solid tumors. In an era of exceptionally dynamic evolution of knowledge, some recently published clinical studies have reshaped the role of surgery such as neoadjuvant immunotherapy combinations leading to less invasive surgery for advanced melanoma, antiangiogenics as an alternative to immediate surgery in renal cell carcinoma or upfront treatments making surgery possible for more patients with pancreatic cancer (1). Most therapeutic combinations in clinical trials are based on knowledge of resistance mechanisms and recently immunotherapy, which has revolutionized the clinical management of multiple tumors, has been included in multiple clinical trials which are mainly

based on T cell and pursue a maintained antitumor immune response. Accumulating evidence suggests that conditioning the tumor microenvironment (TME) toward an immunomodulatory state may have a major impact on cancer outcome (2, 3). However, the TME comprises all the non-malignant cellular and non-cellular components of the tumor, including the immune system, blood cells, endothelial cells, fat cells, and the stroma. The tumor stroma is a critical component of the TME with cancer-promoting capacity as part of the response to treatments and leads to cancer resistance. For example, immunosuppressive cytokine secretion and metabolic alterations strongly participate in the suppression of host immune responses against tumor cells facilitating tumor proliferation. Extensive work exploring the interactions between cancer cells and the TME has been done but the advancements still require a better understanding of the potential targets before implementation in conceptual antitumor strategies. In this regard, recent advances resulting in more effective and localized radiation treatments (stereotactic radiosurgery and stereotactic body radiotherapy, SRS/SBRT) can achieve an effective alteration and ablation of tumor stromal tissue, which can be a singular advantage against tumoral immune evasion [reviewed in (4)]. In addition, technological developments have led to minimally invasive surgery with evident clinical benefits in terms of less invasiveness, excellent outcomes, and a shorter hospital stay (5).

In this review, we address challenges to identify responders vs. non-responders to the immune checkpoint blockade (ICB), discuss recent developments in combinations of immunotherapy and radiotherapy for clinical evaluation, and consider the clinical impact of immunosuppressive changes in the TME in the context of surgery and radiation. Overcoming microenvironment related resistance may have a fundamental impact on treatment efficacy and patient outcome.

Abbreviations: 4-1BB, tumor necrosis factor receptor superfamily member 9, CD137; A2AR, adenosine receptor A2; APC, antigen-presenting cells; *ATM*, ataxia telangiectasia mutated; ATP, adenosine triphosphate; CAFs, cancer-associated fibroblasts; CCL2, CC chemokine receptor 2; CD28, cluster of differentiation 28; CD39, cluster of differentiation 39; CD73, cluster of differentiation 73, ecto-5′-nucleotidase; CD80, cluster of differentiation 80; CD86, cluster of differentiation 86; cGAS, cyclic GMP-AMP synthase; CHK1, checkpoint kinase 1; CSF-1, colony stimulating factor 1; CSF-1R, colony stimulating factor 1 receptor; CTLA-4, CTL antigen 4; CXCL1, C-X-C motif chemokine ligand 1; CXCL2, C-X-C motif chemokine ligand 2; CXCL10, C-X-C motif chemokine ligand 10; CXCL16, C-X-C motif chemokine ligand 16; CXCR4, C-X-C chemokine receptor type 4; DAMPs, damage-associated molecular patterns; DC, dendritic cells; DDR, DNA damage response; DNA-PK, DNA-dependent protein kinase; ECM, extracellular matrix; HIF-1α, hypoxia-inducible factor-1α; HLA, human leukocyte antigen; HMGB1, high mobility group box 1; HSP90, heat shock protein 90; ICB, immune checkpoint blockade; IFN, interferon; IFN-γ, interferon gamma; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-13, interleukin 13; IL-35, interleukin 35; irAEs, immune-related adverse events; *KPNA2*, karyopherin subunit alpha 2; LAG3, lymphocyte-activation gene-3; LUM, LUM imaging system (Lumicell Inc.); LUM015, cathepsin activatable fluorescent probe; MDSCs, myeloid-derived suppressor cells; MHC-I, major histocompatibility complex-I; NF-κβ, nuclear factor-kappa beta; NK, natural killer; nMOFs, nanoscale metal-organic frameworks; NP, nanoparticles; NSCLC, non-small cell lung cancer; OX-40, tumor necrosis factor receptor superfamily member 4, CD134; PARP, poly(ADP-ribose) polymerase; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; ROS, reactive oxygen species; SBRT, stereotactic body radiotherapy; SDF-1, stromal cell-derived factor-1; SRS, stereotactic radiosurgery; STING, stimulator of interferon genes; TAMs, tumor-associated macrophages; TGFβ, transforming growth factor β; Th1, T helper type 1; Th2, T helper type 2; TIGIT, T cell immunoglobulin and ITIM domain; TIM-3, T cell immunoglobulin and mucin domain-3; TLR, toll-like receptors; TLR3, toll-like receptor 3; TLR7/8, toll-like receptors 7/8; TLR9, toll-like receptor 9; TMB, tumor mutational burden; TME, tumor microenvironment; TNFα, tumor necrosis factor α; Treg, regulatory T cells; Trex1, three-prime repair exonuclease 1; VEGF, vascular endothelial growth factor.

CHARACTERIZING THE IMMUNE FUNCTION IN THE RESPONSE TO CHECKPOINT INHIBITOR IMMUNOTHERAPY

Combinatorial Approaches to Treat Differences in the Immune Contexture of the TME

Immunotherapeutic approaches have transformed treatment and outcomes for some solid tumors, in particular, melanoma and non-small cell lung cancer (NSCLC), but do not benefit the majority of patients with cancer and have failed to induce broadly durable responses. Immunotherapy with ICB uses monoclonal antibodies that target the inhibitory proteins CTL antigen 4 (CTLA-4) or programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) on T cells or cancer cells to unleash the immune response. However, response rates vary widely and predictive factors of response to ICB remain elusive. It has been suggested that PD-L1 expression, high tumor mutational burden (TMB) which is highly influenced by the epitopes displayed in the human leukocyte antigen (HLA) genes of a tumor, and the presence of $CD8^+$ T cells are prognostic of clinical response to treatment with ICB (6).

The distinction between hot, altered (excluded and immunosuppressed) and cold tumors, based on the cytotoxic T cell landscape within a tumor, establishes the important role of the TME but only a thorough profiling of the TME can analyze the complexity of the tumors and provide dynamic information about the complex networks operating in the TME to guide clinical decisions (7, 8). Combining immune and genomic data has revealed six immune subtypes across 33 different cancer types including immune (macrophage or lymphocyte signatures, Th1:Th2 cell ratio, expression of immunomodulatory genes) and non-immune parameters (intratumoral heterogeneity, aneuploidy, neoantigen load, overall cell proliferation, and patients' prognosis) (9). It has been proposed that an integrative view of the multi-omics experimental platforms and computational power is required to identify signatures of immune response with improved predictive power (10).

It has been clearly established that $CD8^+$ T cells are the ultimate effectors of tumor rejection and the strongest predictor of ICB response across tumor types. Significantly, the functional variability of tumor-infiltrating T cells can influence their cytotoxicity. Subsets with reactivation of dysfunctional $CD8^+$, memory-like $CD8^+TCF7^+$, $CD103^+$ tumor-resident $CD8^+$, and $Tcf1^+PD\text{-}1^+$ $CD8^+$ with stem-like properties T cells have shown durable responses. $CD4^+$ T cell subpopulations that play a critical role in immunotherapy include $CD4^+$ Th1 cells that generate

functional $CD8^+$ T cell responses, $CD4^+FoxP3^+$ regulatory T cells (T_{reg}) generally associated with suppression of antitumor immune responses in several cancers although responses to CTLA-4 blockade have been shown, and $CD4^+FoxP3^-PD\text{-}1^{Hi}$ ($4PD\text{-}1^{Hi}$) T cells can indicate a negative prognosis when there is persistence after PD-1 blockade (6). Emerging factors associated with ICB response include B cells and tertiary lymphoid structures (11, 12). As for innate immune populations, $BDCA\text{-}3^+CLEC9A^+$ dendritic cells (DC) and XCL1-producing NK cells are linked to ICB response (13).

ICB is most efficacious in tumors with a high degree of T cell infiltration (hot tumors), such as melanomas and NSCLC. Alternative combinations include other checkpoint molecules, such as T cell immunoglobulin and mucin domain-3 (TIM-3), lymphocyte-activation gene-3 (LAG3), and T cell immunoglobulin and ITIM domain (TIGIT) in the case of T cell exhaustion; or co-stimulatory checkpoint proteins, including OX-40, CD28, and 4-1BB ligand receptor to enhance T cell expansion or effector functions. Preliminary results also suggest a potential role of microbiome modulation. On the other hand, immune cold tumors, including pancreatic and prostate cancers, are not well-infiltrated by immune cells. Therefore, research efforts have focused on making cold tumors hot by increasing immune infiltration and activity, such as vascular normalization, increasing the neoantigen burden, oncolytic therapy, vaccines, adoptive T cell therapy, T cell immunomodulators, and radiotherapy. Clinical strategies in immune-altered tumors have an impact on T cell trafficking, inhibition of hypoxia-associated pathways, and the immune suppressive microenvironment (14).

As more combinations of immunotherapeutic strategies reach the clinical arena, two clinical challenges become more relevant. Checkpoint disruption leads to a wide range of inflammatory toxicities grouped as immune-related adverse events (irAEs). The majority occur in barrier tissues (gastrointestinal or pulmonary mucosa, skin) or in endocrine glands. Although many are mild, they can carry considerable morbidity, lead to reduced treatment dosage and/or duration, and on occasions may be fatal (e.g., in patients with pre-existing autoimmunity) (15, 16). On the other hand, it has been suggested that irAEs could help select responders to ICB in bladder cancer (17). Secondly, some patients experience an acceleration of tumor growth kinetics with poor survival called hyperprogression which, at present, remains difficult to characterize (18, 19).

The composition of the TME is dynamic and evolves during ICB treatment. It has been suggested that the TME evolves differently between responders and non-responders. Of interest, stronger differences were found early on-treatment than before the ICB based on the differences in the densities of $CD4^+$ or $CD8^+$ T cells and the expression of PD-1/PD-L1 after two or three anti-PD-1 doses than at baseline (20, 21). Another interesting feature is that PD-1 blockade can induce clonal replacement preferentially of exhausted $CD8^+$ T cells, meaning that T cells present at baseline may show reduced proliferation and that the response to ICB could be due to T cell clones that enter the tumor during the course of treatment (22).

Clinical relevance of distinctions in the immune contexture mainly based on the cytotoxic landscape of T cells in tumors has been established although the potential of analyzing dynamics and plasticity of TME networks will offer more powerful stratification systems between responders and non-responders.

Interactions Within the TME

Interactions between malignant and non-malignant cells create the TME (**Figure 1**). Non-malignant cells are usually highly dynamic and display tumor-promoting capabilities. Major non-malignant cell types found in the TME are immune cells, vasculature and lymphatic vessels, and fibroblasts. Cell communication is accomplished by a network of cytokines, chemokines, and diverse metabolites that reacts to changes in the physical and chemical characteristics of the tissue (23). Cancer treatment effects induce a variety of mechanisms which lead to T cell exclusion and avoidance of their cytotoxic function (24) that ultimately shift the balance of stromal cell phenotypes in the TME toward an immunosuppressive state. These pro-tumorigenic responses to therapy can induce local and/or systemic changes that underlie tumor recurrence and treatment resistance.

In a broad sense, the mechanisms leading to a pro-tumorigenic microenvironment can be grouped into three categories: immune cell regulation, metabolic reprogramming, and hypoxia (4). The biochemical and physical properties of the TME undergo substantial changes during tumor evolution and treatment determined by the increased demand for blood vessels to endure tumor growth, which requires an adequate supply of oxygen and nutrients delivered through the blood vasculature. The resulting abnormal vessels are leaky and compressed which can induce a dense stromal reaction and reduction of blood flow that promotes hypoperfusion. The TME then becomes hypoxic with enhanced potential for tumor progression in multiple ways. In this situation, hypoxia reduces immune cell activity and the TME acquires an immunosuppressive phenotype (25). Hence, better understanding and reprogramming of these components may greatly influence cancer outcome.

Clinically, this may significantly limit cancer treatment efficacy and represent a shift in our understanding of tumor progression and resistance. Major emphasis has been placed on advancing clinical applications that strengthen the effectiveness of immunotherapies, leading to rapid regulatory approval of ICB combined with targeted therapies and/or chemotherapy in large numbers of patients with cancer, facilitating their incorporation into clinical practice. However, in spite of the extensive use of surgery and radiation strategies in cancer, as a definitive strategy in early or moderately-advanced stages of cancer, as part of a multimodal strategy in advanced loco-regional disease and, more recently in selected cases of oligometastatic disease, there is very limited understanding of the biological changes in the TME induced by local treatments.

RADIOIMMUNOTHERAPY COMBINATIONS

Radioimmunotherapy is an area of extensive research due to the potential immunomodulatory effects of ionizing radiation and has established a new paradigm in which radiation is as efficient as its capacity to elicit tumor-targeting immune responses (2).

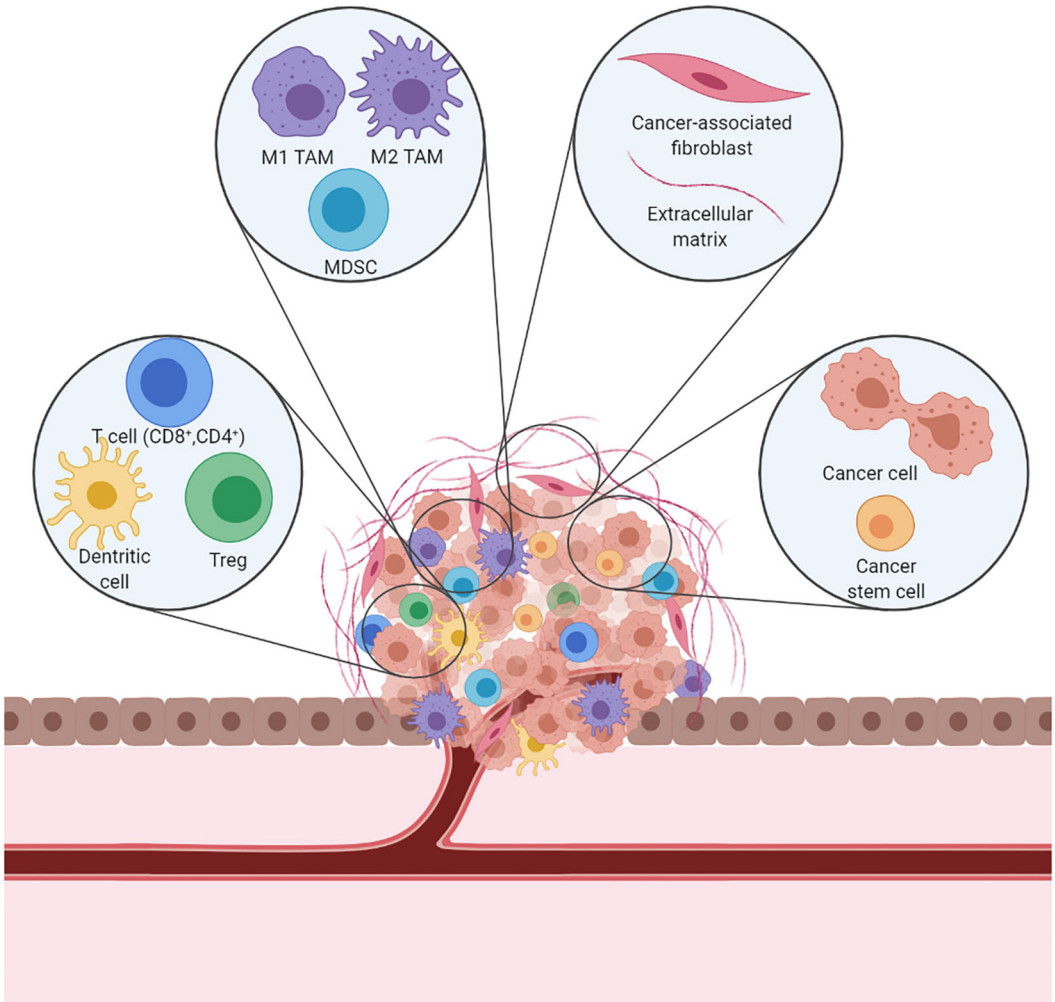

FIGURE 1 | A schematic representation of the immunosuppressive TME. In a tumor, cancer cells coexist with immune cells, fibroblasts, and blood vessels to form the TME. Cancer cells can alter the microenvironment and promote cancer growth and dissemination.

Ionizing radiation is able to induce immunogenic cell death, a form of cell death that promotes a T cell-based immune response against antigens derived from dying cells, enhances antigen presentation, and activates cytotoxic T cells. Cytosolic DNA from dying cells function as neoantigens that are highly immunogenic. Radiation induces the release of danger signals, including calreticulin, high mobility group box 1 (HMGB1), and adenosine triphosphate (ATP), which are collectively known as damage-associated molecular patterns (DAMPs), and support the recruitment and maturation of antigen-presenting cells (APC), migrate to lymph nodes, and prime a cytotoxic T cell-dependent immune response.

Critical to the immunogenicity of radiotherapy is the fragmentation of nuclear DNA from the DNA damage response (DDR) of radiation, shuttled to the cytoplasm where it activates cyclic GMP-AMP synthase/stimulator of interferon genes (cGAS/STING) pathways and induces transcription of the IFN-stimulated genes. The cytoplasmic three-prime repair exonuclease 1 (Trex1), induced by radiation, is a negative regulator of this pathway. The release of IFN type I from APC supports antigen uptake by Batf3^{+} DC and cross-presentation of tumor antigens to CD8^{+} T cells. Activated CD8^{+} T cells are recruited to the irradiated tumor site by cytokines upregulated by radiation (CCL2, CXCL1, CXCL10, and CXCL16). In addition, radiation enhances the expression of major histocompatibility complex-I (MHC-I) antigens on cancer cells that favor antigen presentation (4).

DNA Damage Response Following Radiation and Exposure of Neoantigens

Tumor cell-intrinsic events driven by DNA damage are central to the immunomodulatory effects of radiotherapy. Radiation-induced DNA damage alters gene transcription and

modulates the expression of tumor neoantigens, resulting in activation of innate and/or adaptive antitumor immune response (6, 26, 27). The finding that a patient with metastatic, treatment refractory NSCLC who responded to ipilimumab plus radiotherapy was carrying a mutation in a *KPNA2* gene, upregulated in expression by radiation; tumor-specific T cell clones were developed in peripheral blood shortly after completion of radiotherapy and the first dose of ipilimumab to a metastatic site and remained elevated while the patient achieved a complete response in all of the non-irradiated lesions supports the hypothesis of *in situ* tumor vaccination (28).

Identification of genetic determinants of radiotherapeutic efficacy has remained elusive but a recent report identifies genetic *ATM* inactivation to be strongly associated with clinical benefit from radiotherapy. The identification of a radiosensitive phenotype across multiple cancer types inaugurates the possibility of further testing in prospective clinical trials and progress in personalized radiation strategies. For example, patients with metastatic tumors harboring a somatic *ATM* mutation may receive a reduced dose of radiation with the goal of reducing toxicity and maintaining tumor control (Pitter et al., accepted).

Defects in DDR have been exploited for drug development as radiosensitizers including poly(ADP-ribose) polymerase (PARP), checkpoint kinase 1 (CHK1), DNA-dependent protein kinase (DNA-PK), or the chaperone HSP90 inhibitors. Radiation damage in the context of defective DDR pathways generates micronuclei in cancer cells that activate cGAS/STING pathways and propagate an inflammatory response that can enhance radiation effects. Adding ICB to the immunomodulation induced by DDR inhibitors plus radiotherapy is a new area of clinical research that can provide additional insights into the immunomodulatory effects of radiation given that DDR inhibitors can enhance the immunostimulatory effects of radiation while ICB can target the immunosuppressive radiation effects (27).

Central Role of Dendritic Cell Maturation in Radiation-Induced Immunological Response

DC are a sparsely distributed immunological component of the TME with high biological heterogeneity that play a central role in linking innate and adaptive immune responses. Therefore, DC are a key element in the immunostimulatory effect of radiotherapy. It has been recently reported that poorly radioimmunogenic murine tumors fail to activate DC following treatment, and that it could be successfully reverted with an exogenous adjuvant, resulting in tumor cures (29). Therefore, it could be hypothesized that in patients with a poor TME, the combination of radiation with adjuvants that promote DC maturation or target the immunosuppressive TME can improve tumor control.

Toll-like receptors (TLR) signaling pathways activate innate immunity and regulate adaptive immune responses. Preclinical evidence suggests that TLR-agonists targeting TLR3, TLR 7/8 or TLR9 in combination with radiotherapy can enhance antitumor immunity with long-term tumor control. Mechanistically, TLR can enhance DC-mediated cross-presentation and activation of T cells. Novel formulations of TLR agonists with reduced toxicity and precise and image-guided radiation techniques are favorable aspects for this strategy (30, 31).

Addressing the Evasive Objective of Durable Responses of Radiation-Immunotherapy Combinations

Studies on resistance to ICB reveal a complex and rapidly evolving network of mechanisms of immune resistance specific to each host and tumor (32). The absence of biomarkers that identify the different types of resistance obliges the use of empirical approaches to target them.

The immunogenicity of radiation has been approached with two different strategies, one that emphasizes the local interaction of radiotherapy and the immune system where the majority of clinical knowledge has been accumulated, and a second strategy where focal radiation elicits systemic disease control (abscopal effect) known as *in situ* tumor vaccination that has attracted a lot of attention. The basis for combining ICB with radiotherapy stems from the fact that radiation upregulates PD-L1, which leads to $CD8^+$ T cell exhaustion. In addition, many tumors devoid of T cells at baseline (and secondary lack of PD-L1 expression on effector T cells) could benefit from the radiation-induced increase in PD-L1 and the combination (33). In the case of CTLA-4, upon radiation, it is recruited to the membrane of activated T cells and binds to the ligands CD80 and CD86, expressed on DC and other APC, thereby attenuating T cell activation (34).

Tumor burden has been regarded as a surrogate for ICB effectivity based on clinical observations that adjuvant ipilimumab in resected stage III melanomas obtains major benefits in recurrence-free survival and overall survival (48.3 and 65.4% at 5 years, respectively) (35), and locally advanced NSCLC treated with definitive chemoradiation followed by adjuvant durvalumab in the PACIFIC trial with an impressive prolongation of time to death or distant metastasis from 16.2 to 28.3 months and a favorable toxicity profile (36). Moreover, in patients that do respond to ICB, failure frequently occurs in sites of previous disease, with 60% of failures in anti-PD-1/PD-L1 treated NSCLC and 39% of failures in anti-PD-1 treated melanoma (37, 38). Although it is not specific criteria, the best outcome with ablative radiation in oligometastatic clinical trials has been obtained in patients with low tumor burden and as local consolidation (39, 40).

While the majority of clinical studies have targeted a single metastatic site, abscopal responses are relatively rare, and mainly in melanoma and NSCLC (41). Improved outcomes have been obtained in several phase 2 clinical trials using local consolidation with ablative doses of radiation in the oligometastatic setting (39, 40, 42) while ongoing phase 3 trials are investigating whether this approach may lead to improve overall survival in a subset of patients with

limited metastatic disease (NCT02417662, NCT03137771, NCT02364557, NCT03862911, and NCT03721341).

It has been recently reported that tumor-resident $CD8^+$ T cells play a significant role in mediating the immune effects of radiotherapy. Even if proliferation decreases after radiation, their functionality, measured as production of IFN-γ, augments, and mediates the early antitumoral effect of local SBRT doses. Nonetheless, as newly infiltrating $CD8^+$ T cells play a key role in antitumor immunity, that may also be the case with radiation-induced immunogenicity (43). If radiation could increase the population of pro-immunogenic T cell subtypes within the local TME, it would enhance the response to ICB. This hypothesis raises the possibility that targeting multiple metastatic sites with SBRT to achieve complete cytoreduction in the metastatic setting may become clinically relevant (44). Moreover, the irradiation of each visible metastasis addresses the challenge of heterogeneity by attempting to convert each target into an *in situ* vaccine (45). Clinical support data comes from a phase 2 clinical trial in NSCLC with up to 4 metastatic sites (93% had <2 metastases), which underwent locally ablative treatment with metastasectomy or multi-site SBRT followed by pembrolizumab, with median survival of 19.1 months (vs. 6.6 months in historical controls) and favorable toxicity profile (46).

Research has been very controversial with variations in dose or fraction for radiation delivery in the metastatic setting, where the role of treatment parameters such as duration (more or <7 days), fraction size (1.8–3 to 8–30 Gy) and scheduling (single or multiple fractions) are largely unknown. While a short course (1–5 fractions) of high dose radiation can be safely administered and is able to elicit an immunogenic response that can benefit from the addition of ICB, the predominance of the immunosuppressive effects of radiation may limit the effectiveness of ablative doses of radiation, especially if single fractions are used (47, 48). Nonetheless, the immune context of the tumor type or even the metastatic organ may require a different dose and/or fractionation to elicit an immunogenic response. This possibility offers the potential to reduce the dose and volumes of radiation and still prove efficacious. In the PACIFIC trial (36), immunotherapy was administered sequentially (i.e., following chemoradiation) with a good toxicity profile but data on toxicity of concomitant radiation and immunotherapy in the clinical setting is scarce. Yet the biological context remains to be proven that would favor multiple rounds of high-end ablative dose schedules in oligometastatic patient as advocated by some groups (49). Another concept of potential clinical relevance that has been put forward is the possibility that the immunomulatory effect of low-dose radiation for stromal modulation could favor T cell infiltration and enhance the immune response (47, 50).

The next generation of clinical trials addressing radiotherapy-immunotherapy combinations will have to include immunological read-outs with proper endpoints for immune monitoring as well as the identification of immune biomarkers that optimize the selection of treatment strategies (31, 51).

CLINICAL IMPLICATIONS OF TECHNOLOGICAL DEVELOPMENTS

Surgery and radiation remain strong curative modalities for treatment of established solid tumors but treatment failure continues to be a significant problem. The best established role of surgical oncology is the complete removal of the tumor, with an additional strong foundation to question the elective treatment of uninvolved regional lymph nodes in a large variety of tumor types and resection of metastatic disease which is increasingly offered to selected patients with indolent oligometastatic disease (52). Critical to all of them is securing negative surgical margins.

Less invasive technologies and advances in imaging leading to minimally invasive and robot-assisted surgeries are revolutionizing surgical care (5). Likewise, advanced image guidance and motion management strategies are shaping new therapeutic radiation strategies enabling the safe administration of ablative doses of radiation (2). Advanced imaging is fundamental and uniquely placed to serve both margin negativity rates and future radiation strategies.

Surgical margin positivity rate (cancer cells at the edge of tumor resection) has not significantly improved in recent decades and when it does occur prognosis is significantly affected in many tumor types. Margin positivity rates across all types of cancer range from 15 to 60% (53). A recent report on positive surgical margins in the ten most common solid cancers has identified oral cavity cancer with the highest rate with up to 25% of cases, no change over time, with significant effects on tumor relapse and overall survival. For advanced disease, the rates ranged between 20.9% (breast) and 65.5% (prostate) with related worse outcome in seven tumor types (54). Although not a true resistance type, we propose the term "margin-missing" effect to characterize this situation which leads to treatment failure and resistance.

Fluorescence-guided surgery, which allows intraoperative visualization of tumors, is an evolving image-guided surgical strategy to help differentiate tumor cells from normal surrounding tissues in real time. Near-infrared fluorescence imaging has a higher tumor to background ratio, high tissue penetration (5–10 mm), and little interference from intrinsic fluorescence. Indocyanine green is the most widely used probe in fluorescence-guided surgery although tumor detectability is not very good and optical technology is still evolving (55).

More than 50% of patients with cancer receive radiotherapy, which defines its leading role in cancer management, in particular for several locally advanced solid tumors. The latest developments in radiotherapy have swiftly enabled local dose escalation making it possible to deliver high doses of radiation with incredibly high anatomical precision and reduced risk of long-term adverse effects. As a consequence, relevant clinical benefit has been achieved in a variety of cancer types such as prostate, gynecologic, breast, head and neck cancers, and brain and lung metastases (2). However, no significant advance has occurred in the past 30 years in the development of strategies that enhance radiation effects. On the other hand, due to the recognition that the immune system can strongly contribute

to therapeutic responses to radiation, radioimmunotherapy has become an intensive area of research.

The current challenge in near-infrared fluorescence-guided surgery is to design probes with high selectivity for tumors and clear visualization, referred to as smart probes, which are only activated at the tumor site (turn-on probes). There was a recent report about the design, synthesis, and characterization of three novel polymeric turn-on nanoprobes that are activated at the tumor site by cysteine cathepsins (highly expressed in multiple tumor types) showing a stable and well-defined signal from the tumor during the whole surgical procedure in orthotopic breast cancer and melanoma models resulting in less tumor recurrence and prolonged survival compared with standard commercial probes (56). This is a significant lead toward real-time image-guided tumor margin assessment during surgical oncology.

Emerging approaches seek to integrate analytical tools with optical technology to help improve the decision-making of fluorescence-guided surgery to reduce margin positivity rates. For example, combinations of fluorescence-guided surgery have been made with mass spectrometry (57), Raman spectroscopy (58), and hyper spectral imaging (59).

The most clinically advanced nanoprobe is LUM015 (a pegylated cathepsin-activatable probe) which is undergoing eight clinical trials, including a pivotal phase 3 study (60). The phase 3 trial is a multicenter study with the primary objective of assessing the ability of LUM015 and LUM fluorescence-guided surgery system to detect residual tumors in 250 breast cancer patients undergoing lumpectomy (NCT03686215).

CLINICAL IMPLICATIONS OF THE IMMUNOSUPPRESSIVE ENVIRONMENT

It has been traditionally assumed that recurrent tumors arise from transformed neoplastic clones that are more resistant to oncological therapies, however, an early experience challenged this view and hypothesized that primary and recurrent tumors of equal size did have different microenvironments that explained their response to therapies. The study found that while small primary tumors had a healthy population of antitumor effector $CD8^+$ T lymphocytes, recurrent tumors had an immunosuppressive condition consisting in expanded populations of tumor-associated macrophages (TAMs), T_{reg} cells, and pro-tumoral cytokines that inhibited cytotoxic $CD8^+$ T lymphocytes. These changes were also identified in regional draining lymph nodes. Disruption of these immunosuppressive pathways restored the efficacy of the tumor vaccine in recurrent tumors, as if they were primary tumors (61).

Research in preclinical models has shown that a syringeable immunomodulatory multidomain nanogel (iGel) containing gemcitabine, imiquimod, and clodronate locally applied as a postsurgical treatment is able to deplete immunosuppressive cells from the TME (myeloid-derived suppressor cells (MDSCs), M2 macrophages, and T_{reg} cells), increase immunogenicity, and induce immunogenic cell death. Indeed, it generates systemic antitumor immunity and a memory T cell that significantly inhibits tumor recurrence and lung metastases. Reprogramming the immunosuppressive TME also converts tumors not responding to ICB to responding ones (62). This platform may serve to reshape immunosuppressive TME and synergize with other therapies.

Recent clinical data in melanoma and NSCLC have shown that response to ICB in individual patients with metastasis vary depending on the anatomical location of the metastasis, untangling the importance of the local TME in antitumor immunity. Of interest, tissue specific response to immune checkpoint inhibition depends on the cancer type, which implies that responsive and non-responsive sites are different among patients with NSCLC or melanoma (63, 64). These heterogeneous responses are an evident clinical problem, since patients with responses to ICB in all lesions survive longer than those with response in some of the lesions (65). Potential mechanisms include myeloid cell exclusion and alteration of T cell activation in response to tumor growth and local factors, but this will require unraveling a very complex network of interactions for differential responsiveness across different tissue sites of tumor deposits.

Regulatory T Cells

T_{reg} cells are a small subset of circulating $CD4^+$ T cells with potent suppressive functions with a central role in regulating immune responses and maintaining self-tolerance although they also impede antitumor immunity. In contrast with circulating T_{reg} cells, intratumoral T_{reg} cells maintain an active configuration, suggesting that antigen stimulation may play an important role in the activation and accumulation of T_{reg} cells in the TME. The immunosuppression mediated by T_{reg} cells is mainly mediated by the release of anti-inflammatory cytokines including IL-10 and transforming growth factor β (TGFβ), facilitating proliferation of $CD4^+$ T cells to T_{reg} cells, while suppressing proliferation to $CD8^+$ T cells and NK cells. In addition, T_{reg} cells can also reprogram macrophages to the M2 phenotype (via IL-4, IL-10, and IL-13) and favor MDSCs infiltration (via IL-10 and IL-35) (66).

Immunological cell death induced by radiation upregulates or releases DAMPs, including ATP, with further recruitment and activation of DC to initiate the antitumor immune response but ATP is rapidly catabolized in the TME into adenosine by the enzymes CD39 and CD73. Local accumulation of extracellular adenosine suppresses DC and $CD8^+$ T cells and promotes proliferation of T_{reg} cells, increases the expression of CTLA-4 and adenosine receptor A2 (A2AR) on T_{reg} cells, and enhances the polarization of macrophages to the M2 phenotype. Radiation can also induce conversion of ATP to adenosine through the induction of reactive oxygen species (ROS) and TGFβ. Thus, targeting of A2AR, CD73, and TGFβ may reduce resistance to immunotherapy in the radiotherapy setting (33). Blockade of CD73 plus radiotherapy restored radiation-induced DC infiltration of tumors in a poor immunogenic setting, and the addition of CTLA-4 blockade improved control of non-irradiated lung metastases in murine models. These findings set the stage for clinical testing CD73 in patients who carry cGAS/STING tumors or show upregulation of soluble CD73 following radiotherapy to determine if CD73 blockade can enhance responses to ICB (67).

T_{reg} cells also express PD-1 at a low level in the blood and at a high level in tumors, promoting the suppressive activity of PD-1-expressing T_{reg} cells upon antibody-mediated PD-1 blockade (68). Recently reported, ∼10% of cancer patients treated with anti-PD-1 antibody develop hyperprogressive disease, characterized by rapid cancer progression. T_{reg}-specific depletion prior to, or combined with, an anti-PD-1 antibody may prevent hyperprogressive disease and enhance the effectiveness of anti-PD-1 therapy (69).

Macrophages

TAMs account for the largest fraction of the myeloid infiltrate in the majority of solid tumors. The tumor-associated macrophage compartment is highly dynamic in time (during tumor progression and response to treatment) and space (at different tumor sites) through an extensive remodeling of energy metabolism. In addition, the tumor-associated macrophage compartment is highly heterogeneous both within and across tumors in response to environmental changes ranging from a pro-inflammatory (M1) to an anti-inflammatory (M2) state. However, the M1/M2 phenotypes represent the extremes of a continuum and the plasticity of these cells makes therapeutic targeting challenging. Solid experimental evidence informs that the crosstalk between TAMs and the immune cells facilitates an immunosuppressive environment by supporting angiogenesis and extracellular matrix (ECM) remodeling, promoting active recruitment of T_{reg} cells, and expression of PD-L1, paving the way for metastatic development (70). M2 polarization is mostly mediated by growth factors and cytokines secreted by cancer cells that reach M2 cells via exosomes (71). Intriguingly, ontogeny can influence the functional profile of TAMs, i.e., tissue-resident vs. circulating macrophages, such that they can have opposing functions depending on the tumor type (72). Based on these findings, it has been speculated that macrophage origins may be important in determining the permissiveness of an organ to metastatic growth.

Preliminary studies have evaluated the influence of radiation in macrophage polarization. Macrophages are highly radioresistant due to high production of anti-oxidative molecules such as manganese superoxide dismutase by a mechanism depending on tumor necrosis factor α (TNFα) signaling and nuclear factor-κβ (NFκβ) activation (73). Early studies established that radiation exposure recruited bone marrow-derived $CD11b^+$ monocytes/macrophages to irradiated sites (74, 75) and related it to the transcription factor hypoxia-inducible factor-1α (HIF-1α) and effectors stromal cell-derived factor-1 (SDF-1) and C-X-C chemokine receptor type 4 (CXCR4) (76, 77). Therapy can polarize macrophages to the M2 phenotype with very high levels of proangiogenic molecules through the treatment-induced expression of colony stimulating factor 1 (CSF-1), the ligand for the colony stimulating factor 1 receptor (CSF-1R) on macrophages, which can be prevented by CSF-1R antagonists and enhance radiation effects (78, 79).

Ongoing research efforts are directed toward the alteration of the macrophage phenotype to attenuate immunosuppression and improve antitumor immunity (80). Current approaches aim to shift M2 cells to M1 by targeting secreted immunosuppressive factors released by cancer cells and cells in the TME (**Figure 2**).

Preclinical studies suggest that macrophage manipulation to avoid recruitment or prevent M2 polarization produce a significant enhancement of the radiation effect irregardless of the tumor model [reviewed in (81)]. The increase in radiosensitivity with this strategy has been attributed to blockade of vasculogenesis. If angiogenesis supports the formation of tumor blood vessels from the sprouting of local vessels, tumors can also develop or repair blood vessels from circulating proangiogenic cells mainly from the bone marrow, which is known as vasculogenesis (82). This effect could be exploited in radiation treatments, namely if the increase in hypoxia that occurs at the end of radiation through recruitment of circulating proangiogenic cells to rescue damaged tumor vasculature and promote tumor recurrence can be reversed. A first-in-human clinical trial of glioblastoma examined the effects of CXCR4 blockade through a 4-week continuous infusion of plerixafor, a small molecule CXCR4 inhibitor, at the end of irradiation in newly diagnosed glioblastoma and showed high efficacy and local control with an excellent median survival time of 21.3 months. Unexpectedly, a high proportion of patients had out-of-field recurrences with local tumor control which deserves further evaluation (83).

A relevant aspect that remains unanswered is whether the effect of blocking the CXCR4 pathway could be more pronounced with ablative doses of radiation which seems likely since greater vascular damage would be expected. Furthermore, it is of interest to know if CXCR4 blockade can enhance tumor immunity. Very limited information suggests that T cell exclusion from cancer cell deposits secondary to SDF-1 could be overcome by inhibiting the CXCR4 axis, improving the effect of checkpoint inhibitors or stroma normalizing strategies in pancreatic cancer (84, 85) and triple-negative breast cancer (86) models.

Pre-metastatic Niche and Exosomes

In addition to TAMs, radiation also recruits MDSCs in the irradiated tumors by tumor-secreted factors like SDF-1. MDSCs encompass a heterogeneous population of polymorphonuclear MDSCs and monocytic MDSCs which inhibit the activity of $CD8^+$ T cells. Moreover, MDSCs play a prominent role in the establishment of the pre-metastatic niche, promote angiogenesis and facilitate the development of metastasis (87).

Tumors induce the formation of microenvironments in distant sites that support future metastatic tumor growth before their arrival at these sites, known as pre-metastatic niches. Tumor-secreted factors and tumor-shed extracellular vesicles promote a sequence of events that start with vascular leakiness, and are followed by alteration of local cells in the TME, recruitment of MDSCs, and finally attraction of circulating tumor cells (88). Following seeding in a secondary organ, cancer cells interact with their environment to create the metastatic niche. The microenvironment in pre-metastatic niches is immunosuppressive and MDSCs are the main cellular component, however, migration of MDSCs into pre-metastatic niches and subsequent activation is not well-characterized. More than 100 different immunosuppressive tumor-secreted proteins

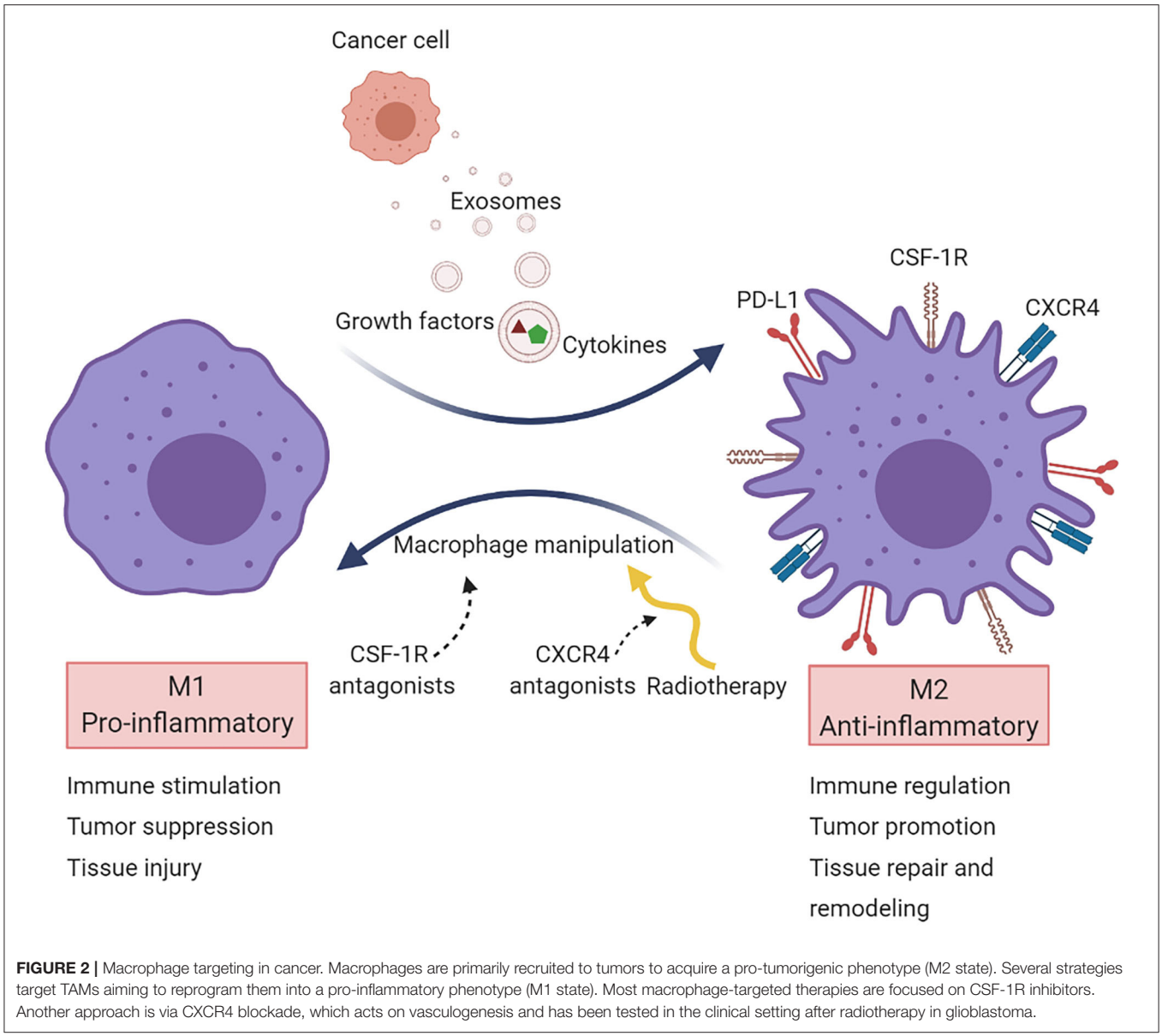

FIGURE 2 | Macrophage targeting in cancer. Macrophages are primarily recruited to tumors to acquire a pro-tumorigenic phenotype (M2 state). Several strategies target TAMs aiming to reprogram them into a pro-inflammatory phenotype (M1 state). Most macrophage-targeted therapies are focused on CSF-1R inhibitors. Another approach is via CXCR4 blockade, which acts on vasculogenesis and has been tested in the clinical setting after radiotherapy in glioblastoma.

have been identified (89). Fibronectin accumulates and anchors to collagen in the ECM to facilitate the adherence of circulating tumor cells through high affinity of membrane integrins (90). The vascular changes allow for uptake of tumor-secreted exosomes by cancer-associated fibroblasts (CAFs) in the local stroma, which contributes to the formation of a tumor-associated desmoplastic stroma, characteristic of many carcinomas (91) (**Figure 3**). Exosomes are extracellular vesicles released by exocytosis and essential to intercellular communication. They can contain genetic material, proteins, and lipids; they can be found in all body fluids and are considered to be major drivers of pre-metastatic niche formation (92). Measurement of exosomal microRNA has been shown to accurately reflect tumor progression in several cancer types (93, 94) as well as dropping levels of exosomal microRNA after surgery indicate that the resected tumor was the main source of exosomal release (95). However, in animal models of abdominal cancer surgery can induce increased levels of ROS, that may downregulate tight junctions in the endothelium and peritoneum, form intercellular gaps and expose the underlying ECM; which can promote integrin-binding of circulating tumor cells during surgery, and result in an excess of liver metastases in a colorectal cancer model (96).

Research in animal models of breast cancer known to produce immunosuppressive MDSCs in the spleen and lungs, has shown that surgical resection of the primary tumor decreased levels of MDSCs in the spleen but persisted in the lungs for 2 weeks after resection, indicative of a pro-metastatic environment. Post-surgical treatment with gemcitabine depleted lung MDSCs and decreased posterior metastatic disease (97).

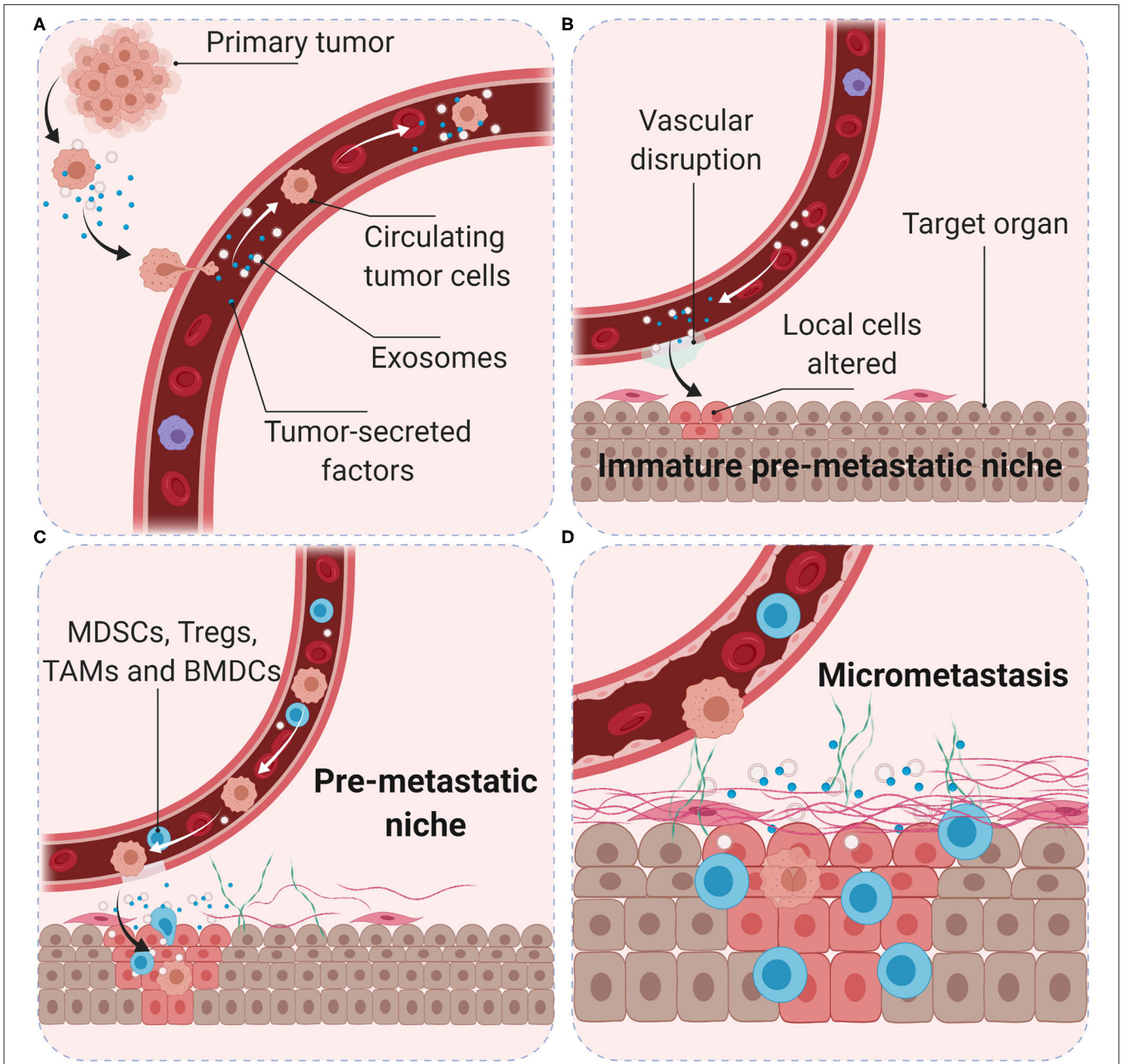

FIGURE 3 | Role of the pre-metastatic niche in cancer metastasis. Primary tumor cells produce soluble factors and exosomes **(A)** to trigger the formation of an immature pre-metastatic niche in the target organ **(B)**. Primary tumor conditions (hypoxia, acidity, and interstitial pressure) promote tumor cell migration into the blood vessels. Tumor-secreted factors and exosomes mobilize bone marrow-derived cells (such as CD11b+ myeloid cells) and suppressive immune cells (such as MDSCs, T_{reg}, and TAMs) to target organs **(C)**. Interactions with local stroma, hypoxia and active ECM remodeling may create a niche with suitable microenvironment conditions for tumor cell colonization **(D)**. Surgery, inflammation, and immunosuppression may increase the number and survival of circulating tumor cells and favor the development of metastasis.

Accumulating evidence indicates that exposure to radiation induces the release of exosomes (98–100) that could contribute to radioresistance but additional mechanistic understanding to define potential interventions is lacking. The potential role of exosomes has also been explored as biomarkers of disease outcome in head and neck cancer patients treated with cetuximab, radiation, and ipilimumab; exosomes were isolated from plasma and the molecular cargo contents (derived from T_{reg} cells) could separate patients who remained free 2 years after treatment from those who did not (101).

An important aspect required to characterize extracellular vesicles is the development of highly specific detection

techniques. Since the distribution of extracellular vesicles in the TME depends on the cellular function, it is highly necessary to visualize them in freshly resected tissues. There was a recent report about an intraoperative optical imaging system that was able to provide rich details and molecular contrast thanks to a label-free multimodal nonlinear optical technology in human breast cancer showing good correlation with stained histological slides. The enriched areas with extracellular vesicles in the microenvironment correlated with macroscopic tissue deposits as well as increasing distance from tumor to margin (102).

A recent publication has shown that after surgical removal of resected primary lung, breast, and esophageal cancer, low-dose adjuvant therapy with epigenetic therapy can disable the pre-metastatic niche and inhibit the formation of lung metastases by avoiding the trafficking of MDSCs and promoting their differentiation into a macrophage-like phenotype (103). These preclinical findings represent a novel paradigm to be tested in clinical trials.

Cancer-Associated Fibroblasts

Fibroblasts, the major cell type in the TME, are critical determinants of cellular crosstalk (104). CAFs, a subpopulation of activated fibroblasts, are difficult to identify and in practice, are described as any mesenchymal cell that lacks lineage markers for epithelial cells, endothelial cells, and leukocytes. CAFs are proliferative, migratory, and highly secretory cells that promote extensive tissue remodeling which influences the physical and chemical properties of the tumor and increases the ECM stiffness, which promotes malignancy in experimental models. An extensive range of functions have also been attributed to CAFs, including secretion of growth factors, cytokines, and exosomes that promote tumor growth and alter treatment responses. The principal effect of CAFs is considered to be immunosuppressive with IL-6, SDF-1, and TGFβ as well-established mediators (105). These CAFs contribute to a rigid matrix that creates a physical barrier that leads to vessel compression and reduces diffusion of therapeutic agents to cancer cells which are particularly relevant for colorectal and pancreatic cancer (106, 107). CAFs are also effective in the remodeling of the tumor vasculature through the secretion of vascular endothelial growth factor (VEGF), fibroblast growth factor, and IL-6 to enhance angiogenesis (108, 109) (**Figure 4**).

Emerging evidence implicates CAFs in immune escape and resistance to immunotherapy but not all subpopulations seem to have the same functions. A comprehensive identification of specific subsets of CAFs and their function is needed to become a viable targeting option (110). Currently, several preclinical strategies that target specific subsets of CAFs are under development (109).

Two promising strategies are normalization of activated CAFs, which intends to revert the activated state into a quiescent state or to induce them to acquire tumor-suppressor phenotypes (111), and targeting CAF-derived ECM proteins, either their production or degradation to alleviate the ECM stiffness (109). Reprogramming of CAFs to enhance immune responses, normalizing their ECM, is being investigated through the addition of vitamin D analogs (known to convert them into a quiescent state) to ICB in pancreatic cancer, and through TGFβ blockade combined with immune checkpoint inhibition in multiple tumor types (111).

CLINICAL IMPLICATIONS OF METABOLIC REMODELING

Metabolic crosstalk across all cellular compartments is responsible for homeostasis and evolution of the TME. All cells of the TME, both malignant and non-malignant, compete for nutrients and oxygen, which are generally limited, especially in a stiffened and poorly vascularized TME, or secondary to the accumulation of the excessive production of metabolites by cancer cells. Additional aspects that influence how the TME reacts include immune-related substances released by cancer and/or immune cells, mechanical forces in the ECM, and reactions to treatment (112).

Although the metabolic pathways are shared between cellular compartments of the TME, the singularity of the reaction of stromal cells to energy demands is crucial. TAMs and CAFs are recruited to the tumor bed and activated in response to different stressful situations, such as limited nutrient disposal, hypoxia, and oxidative stress, attracted by cytokines such as TGFβ and CXCL2 or ROS from cancer cells [reviewed in (113)]. In such complex interactions, metabolites can serve different roles such as being a source of energy or communicate signals between different cellular compartments, and metabolism byproducts can favor an immunosuppressive phenotype. CAFs can rapidly adapt to these poor conditions through glycolysis and fatty acid oxidation in mitochondria. This increased consumption of glucose is coupled with extensive lactate secretion, which acidifies the TME and facilitates the activation of TAMs (114). The result of this swift metabolic adaptation of CAFs is the secretion of ECM-remodeling enzymes that promote fibrosis and further limit the availability of nutrients and oxygen, establishing a dynamic circuit in which lactate accumulation, glucose deprivation, and hypoxic conditions stimulate the recruitment and activation of additional stromal cells (113). Hypoxia supports the stabilization of the transcription factor HIF-1α to foster glycolysis. In this setting, HIF-1α also mediates CAF-secretion of proangiogenic factors such as VEGF, and hypoxia contributes to tumor progression by stimulating CAFs to secrete immunomodulatory molecules, growth factors, antioxidants, and ECM-remodeling enzymes. Taken together, the response of CAFs under poor nutritional conditions promotes tumor progression through engagement of endothelial cells. In addition, altered metabolism of cancer cells can create a gradient of metabolites around the tumor that can signal the distance to blood vessels and tailor the secretion of VEGF to match the tumor spatial organization and optimize the angiogenic response (115), and the metabolic switch in the TME may add to the disrupted immune cell metabolism (80).

Amino acids synthesize nucleotides and are also intermediate metabolites that contribute to other bioenergetic pathways. Glutamine is an abundant nutrient that provides carbon and nitrogen for pathways that contribute to energy formation, redox,

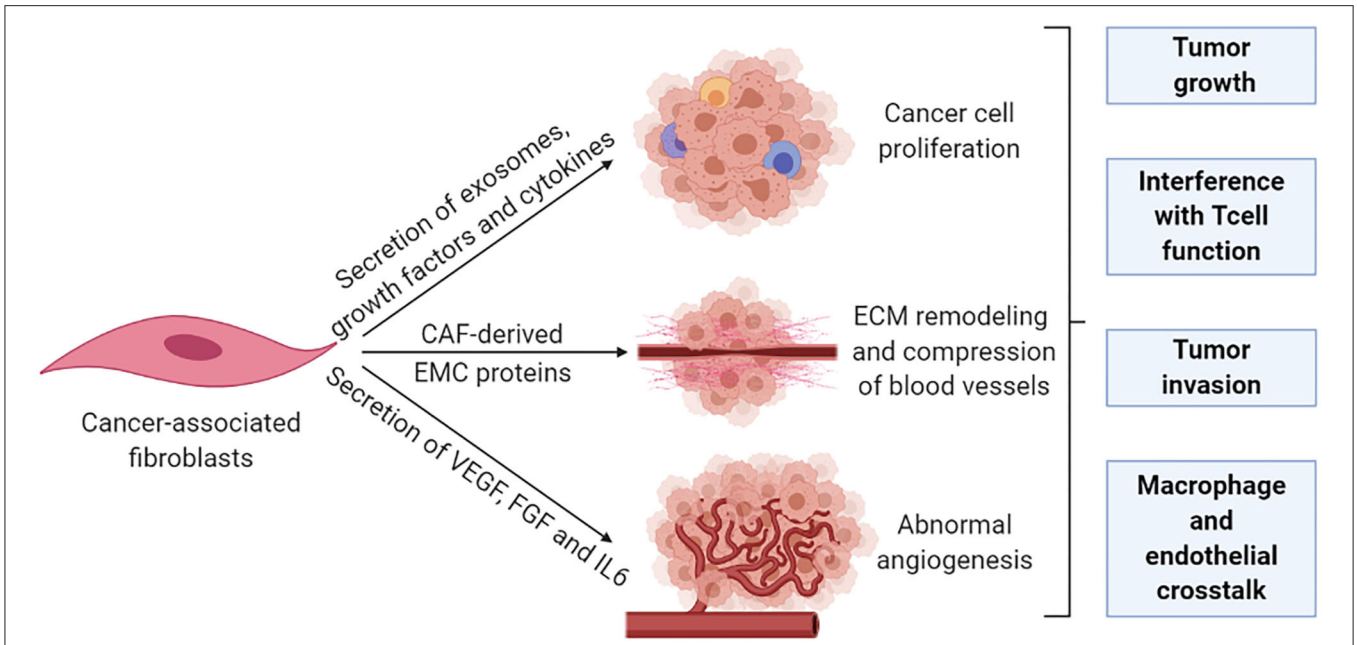

FIGURE 4 | Cancer-associated fibroblasts remodel the tumor stroma. The pro-tumorigenic functions of CAFs are generally associated with their highly secretory activity. Secretory functions and matrix remodeling contribute to tumor invasion and angiogenesis. In addition, secreted soluble factors also contribute to immune reprogramming and tumor growth. Metabolic remodeling by CAFs supports an immunosuppressive microenvironment and promotes tumor growth.

homeostasis, macromolecular synthesis and signaling for cancer-cell growth of particular relevance in hypoxic conditions (116).

Endothelial cells form the lining of blood vessels and lymphatics and require glycolysis for proliferation and migration during angiogenesis. As the tumor grows, new vessels are required to supply the tumor with nutrients and oxygen and the endothelial cells increase their synthetic and energetic demands. Sprouting, the formation of new vessels, is a well-known mechanism in the angiogenic process and an area of intensive research. Although endothelial metabolism has been mainly described as glycolytic, recent findings suggest that mitochondrial oxidative phosphorylation is also required for endothelial cell proliferation during angiogenesis (117).

The interplay between metabolic remodeling and immune regulation in cancer is an active area of investigation. Preclinical models in glioblastoma have identified that tryptophan and adenosine metabolism result in accumulation of T_{reg} cells and M2 macrophages, contributing to an immuno-suppressive phenotype. Future studies will need to define the role of the intermediary metabolites of these pathways to determine their therapeutic function (118).

Preclinical results with the prodrug JHU083, a glutamine antagonist that targets glutaminase and a broad range of glutamine-requiring enzymes, provide a strong and differentiated metabolic response in which cancer cells stop growth, through depletion of glutamine pathways and impairment of glucose uptake, and in addition stimulates T cell functionality, even with persistent antitumor memory (119). Disengaging the metabolism of cancer cells and that of T cells is an evolving therapeutic concept.

A link has been recently proposed between ECM stiffness and metabolic transformation that facilitates tumor progression. It was found that through metabolic crosstalk between CAFs and cancer cells, aspartate secreted by CAFs maintains cancer cell proliferation while glutamate secreted by cancer cells balances the redox state of CAFs to promote ECM remodeling. This amino acid exchange among glutamate and aspartate offers new targeting options for both stromal and cancer cells (120).

CLINICAL IMPLICATIONS OF ANGIOGENIC RESPONSES

An abnormal vasculature is a paramount characteristic of solid tumors, with suboptimal function resulting from a leaky and immature vessel network (via overexpression of proangiogenic molecules such as VEGF), and compression of these anomalous vessels by physical forces (via TME cells and the ECM molecules they produce) (121). The resulting hypoxia enforces the stimulation of immune checkpoints and infiltration of immunosuppressive cells in the TME (122). Specifically, hypoxia up-regulates immune checkpoints, reprograms TAMs to an M2 state, may influence the efficacy of antigen presentation by DC, and affects the function of T cells, while hypoperfusion stiffens the TME that becomes a physical barrier to T cell infiltration into the tumor (123).

An emerging field of interest investigates the synergy of immune-vascular interactions to promote an antitumor effect (124). The objective of this strategy is to induce vascular normalization that needs to be coupled to vessel

decompression (to avoid vessel collapse). Restoring vessel function by normalizing tumor stroma has been evaluated in preclinical models through targeting angiotensin signaling with anti-hypertensive agents (125) or inhibiting SDF-1/CXCR4 (86) which can target CAFs and collagen/hyaluronan to decompress tumor vessels and improve perfusion and effect of the ICB. Vascular normalization can be achieved with antiangiogenic agents to improve tumor perfusion and treatment delivery, but it is dose- and time-dependent, making outcome predictions for combinations of antiangiogenics, stroma normalization and immune therapies difficult to optimize (126) (**Figure 5**).

Successful clinical evidence that the combination of ICB with antiangiogenic drugs has been recently reported in lung (127), renal (128, 129), and endometrial (130) cancer. However, the potential to improve the treatment outcome of this approach is under evaluation in an ongoing clinical trial, which tests the role of adding losartan (an antihypertensive angiotensin inhibitor) to chemo-radiation (delivered via SBRT) and nivolumab in pancreatic cancer patients (NCT03563248).

Apparently, any method that improves tumor perfusion is likely to enhance immunotherapy. It has been proposed that strategies that normalize the stroma would be more beneficial in tumors with abundant compressed vessels, while vascular normalization should improve perfusion in tumors with leaky vessels (131), and the combination when both co-exist. However, addressing the cause of hypoperfusion and identifying the normalization window for each tumor is challenging (126).

Since tumor perfusion is key for the efficacy of immunotherapy, perfusion markers could be used as markers for immunotherapy prediction (132).

FUTURE PERSPECTIVES

While recent studies have improved our understanding of mechanisms supporting immune resistance, we still have an incomplete view of how the TME works as a whole. We propose that advancements in cancer metabolism and nanotechnology represent promising areas of research that have the potential to significantly improve our understanding of immune escape in nutrient- and oxygen-poor environments which may lead to opportunities for therapeutic intervention.

A comprehensive understanding of the metabolic needs of cancer cells has been achieved during this past decade. Significantly, metabolic signatures and hypoxia within the TME impact the immune function. The fact that these findings have been translated into actionable anticancer targets provides the

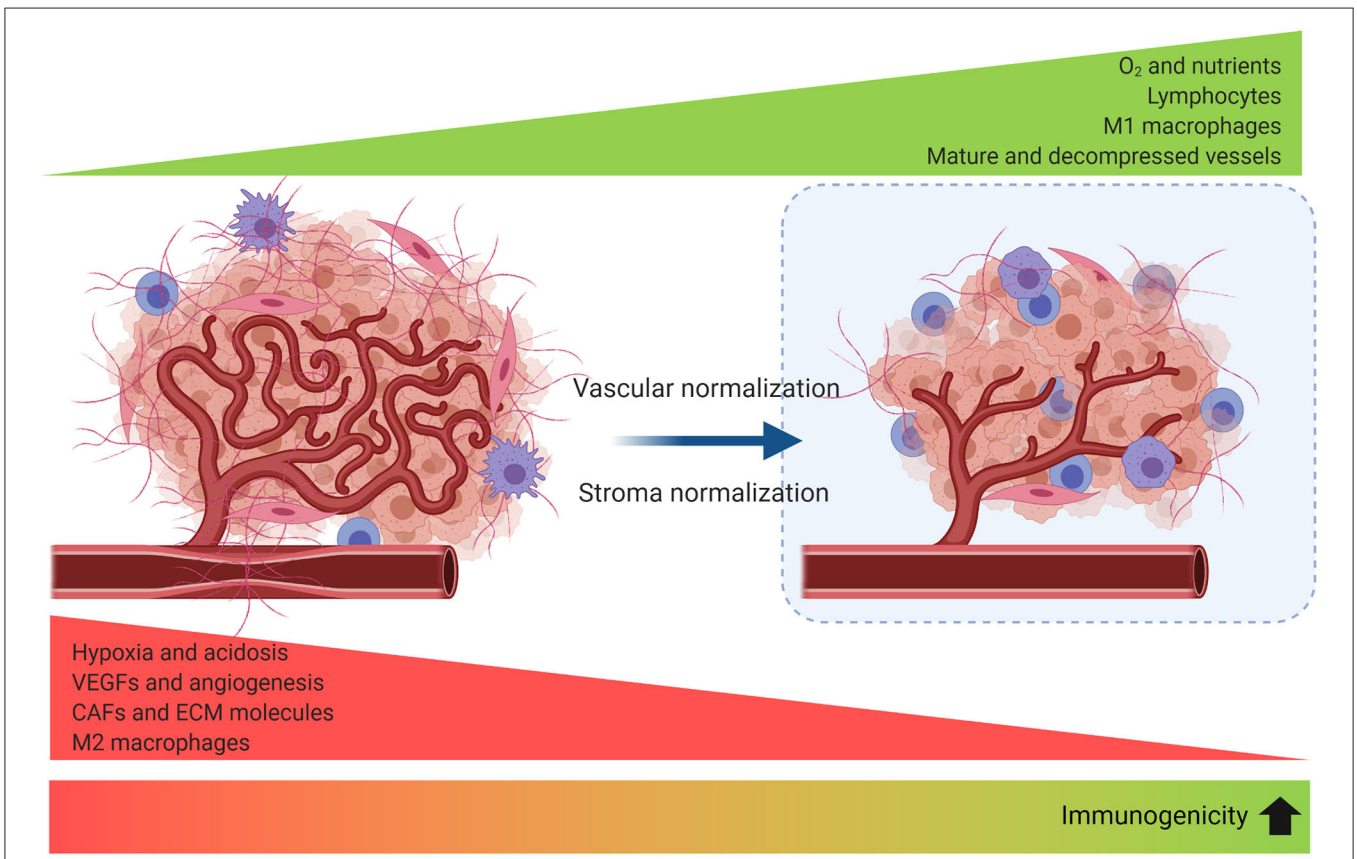

FIGURE 5 | Strategies to improve tumor perfusion increase tumor immunogenicity. Angiogenesis, desmoplasia, and inflammation promote leaky and compressed tumor vessels. Vascular normalization strengthens the vessel wall reducing intercellular gaps and improving perfusion. Blood vessel decompression by depletion of CAFs or ECM reperfuses the vessel and augments perfusion. As a result, reprogramming of the TME to an immunomodulatory state enhances antitumor immunity.

basis for a metabolic characterization of the TME to identify novel targets and signatures in the future. Indeed, better technologies to investigate cancer metabolism at the single-cell level without disrupting the tissue will be required to achieve a deeper understanding of the role of metabolism in cancer.

Advancements in nanotechnology have been effectively developed in cancer therapy. Innovative nanomedicines can use the conditions and characteristics of the TME to deliver therapeutics with increased precision, while providing for signal outputs that allow to follow their effects in real time. Likewise, recent advances in nanotechnology have broadened opportunities for the development of radiosensitizers in synergy with other treatment modalities. We highlight recent progress of nanotechnology between radiotherapy and immunotherapy.

Metabolic Rewiring of the TME

The complex interplay between cellular crosstalk, interactions in the ECM and the biochemical environment within a tumor has an impact on the metabolic phenotype and polarization of immune cells. Thus, the concerted actions of different immune subsets suppress or promote growth. Solid tumors have a dynamic oxygen supply with hypoxic regions where interactions among immune cells are not well understood. Untangling these interactions might offer new potential for response prediction. Tumor infiltrating lymphocytes are at a metabolic disadvantage within the TME since tumor cells impede their access to nutrients needed for activation and acidify the TME through lactate accumulation, favoring a T_{reg} phenotype (133).

Targeting specific metabolic alterations shared by tumor cells and tumor promoting immune populations in the TME is a new strategy under evaluation. Preclinical research has focused on fatty acid metabolism as a source of metabolic plasticity in cancer cells (134), carbon metabolism to stimulate antitumor activity of macrophages (135), or targeting metabolism of ferroptosis (a form of death that relies on ROS) in tumors (136), among others. Strategies that reduce immunomodulatory metabolites are also under evaluation, which include altering the acidic microenvironment, blocking the thryptophan metabolism pathway, inhibition of adenosine within the TME (33), or avoiding lactate accumulation in the TME (137).

A coordinated approach, which takes into account tumor types and tumor biology with detailed molecular links between cancer genotypes and metabolic dependency in a longitudinal fashion, will be best suited to detect the patient populations that are most likely to benefit from metabolism-targeted therapies.

Nanoparticle-Mediated Immunogenic Cell Death

Nanoparticles (NP) have been increasingly studied for radiosensitization. The combination of hafnium oxide NP (NBTXR3, a high-Z nanomaterial with high-level electron density that increases energy dose deposit within cells) plus radiation vs. radiation alone has recently demonstrated meaningful clinical benefit in locally advanced soft tissue sarcoma by doubling pathologic response rates (16 vs. 8%) (138). Significantly, recent research has reported that radiation-activated hafnium oxide NP can augment tumor infiltrates of $CD8^+$ T cells and generate an antitumor immune response, with systemic effect on untreated tumors on the same animals in a murine model of colon cancer (139).

Newly designed hafnium-based nanoscale metal-organic frameworks (nMOFs) have demonstrated effective radioenhancement for low-dose radiation in preclinical models. The combination of nMOF-mediated radiotherapy and PD-L1 blockade extended the local therapeutic effects of radiation to distant tumors via systemic antitumor immunity. This powerful platform can minimize toxic effects by lowering the administered dose of radiation; it can be redesigned for rational tuning and can significantly strengthen the effect of immunotherapy for treatment of non-immunogenic tumors (140, 141).

CONCLUSION

Evolution in the technological delivery of radiation and precision surgery parallels the rapid progress in immune biology that identifies novel strategies to enhance the antitumor immune response. In this setting, alterations in the TME could become especially relevant to optimize treatment immunogenicity and enhance patient outcome.

Defining the individual response of tumors to surgery and radiation offers the possibility to design innovative treatment strategies and re-adapt treatment to new emerging targets. This could have a major impact since it potentially represents a novel way to enhance local and systemic treatments.

AUTHOR CONTRIBUTIONS

The structure of the manuscript was compiled by all authors. SB and ML wrote the first draft. AS-G and SB assembled the figures. All authors reviewed and approved the final version of the manuscript.

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REVIEW
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Alterations of Lipid Metabolism in Cancer: Implications in Prognosis and Treatment

Lara P. Fernández†, Marta Gómez de Cedrón*† and Ana Ramírez de Molina**

Precision Nutrition and Cancer Program, Molecular Oncology Group, IMDEA Food Institute, Campus of International Excellence (CEI) University Autonomous of Madrid (UAM) + CSIC, Madrid, Spain

Cancer remains the second leading cause of mortality worldwide. In the course of this multistage and multifactorial disease, a set of alterations takes place, with genetic and environmental factors modulating tumorigenesis and disease progression. Metabolic alterations of tumors are well-recognized and are considered as one of the hallmarks of cancer. Cancer cells adapt their metabolic competences in order to efficiently supply their novel demands of energy to sustain cell proliferation and metastasis. At present, there is a growing interest in understanding the metabolic switch that occurs during tumorigenesis. Together with the Warburg effect and the increased glutaminolysis, lipid metabolism has emerged as essential for tumor development and progression. Indeed, several investigations have demonstrated the consequences of lipid metabolism alterations in cell migration, invasion, and angiogenesis, three basic steps occurring during metastasis. In addition, obesity and associated metabolic alterations have been shown to augment the risk of cancer and to worsen its prognosis. Consequently, an extensive collection of tumorigenic steps has been shown to be modulated by lipid metabolism, not only affecting the growth of primary tumors, but also mediating progression and metastasis. Besides, key enzymes involved in lipid-metabolic pathways have been associated with cancer survival and have been proposed as prognosis biomarkers of cancer. In this review, we will analyze the impact of obesity and related tumor microenviroment alterations as modifiable risk factors in cancer, focusing on the lipid alterations co-occurring during tumorigenesis. The value of precision technologies and its application to target lipid metabolism in cancer will also be discussed. The degree to which lipid alterations, together with current therapies and intake of specific dietary components, affect risk of cancer is now under investigation, and innovative therapeutic or preventive applications must be explored.

Keywords: lipid metabolism, cancer prognosis, tumor microenviroment (TME), obesity, cancer risk, precision medicine, precision nutrition

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Elisabetta Benedetti, University of L'Aquila, Italy
Krishna Beer Singh, University of Pittsburgh, United States

***Correspondence:**
Ana Ramírez de Molina
ana.ramirez@imdea.org
Marta Gómez de Cedrón
marta.gomezdecedron@imdea.org
Lara P. Fernández
lara.fernandez@imdea.org

†These authors have contributed equally to this work

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INTRODUCTION

Cancer is a significant public health problem and is the second leading cause of death globally (1). The World Health Organization (WHO) has indicated that lung, prostate, colorectal (CRC), stomach, and liver cancers are among the most frequent types of cancer in men, whereas breast, CRC, lung, cervical, and thyroid cancers are the most frequent among women. Together with the genetic alterations, environmental factors orchestrate the multifactorial and multistage characteristics of cancer, modulating the expression of both tumor suppressor genes and oncogenes.

One of the hallmarks of cancer is the abnormal regulation of cellular metabolism (2). Tumor cells exhibit high rates of aerobic glycolysis and an increased anabolism to support growth, proliferation, and survival. Consequently, metabolism-related pathways have acquired enormous relevance in cancer research. Together with the Warburg effect and the increased glutaminolysis, lipid metabolism plays a key role in cancer metabolic reprogramming (3). Lipids, a highly diverse class of biological molecules, exert three main functions in the cells. First, they are employed for energy storage, principally as triacylglycerol esters and steryl esters, in lipid droplets (LDs). In addition, lipids are structural components of cellular membranes, and they also operate as metabolic signaling messengers (4). The sterol regulatory element-binding proteins (SREBPs) are transcription factors that coordinate and regulate the synthesis of lipids. They act in response to upstream signaling networks and to the intracellular nutrient status, to regulate the expression of enzymes involved in cholesterol and fatty acid (FA) synthesis and uptake (5).

Together with genetic alterations mediating the metabolic reprogramming in a cell autonomous manner, cancer progression and dissemination also depend on the availability of nutrients and oxygen at the tumor microenvironment. Tumors communicate with the surrounding microenvironment, which includes fibroblasts, adipocytes, immune cells, endothelial cells, and components of the extracellular matrix—to support cancer proliferation and dissemination (6).

Furthermore, key lipid metabolism genes have been proposed as prognostic biomarkers in several types of cancer associated with tumor recurrence and/or survival (7, 8). Indeed, the role of lipid metabolism alterations in tumor cell migration, invasion, and angiogenesis has been clearly demonstrated (9–11).

The technical improvement and development of "omics" approaches, together with the availability of large public accessible databases, have redefined current strategies of cancer research (12) allowing to reanalyze, recapitulate, and update our knowledge of the relevance of lipid metabolism–related genes in cancer. Genomics and transcriptomics are being applied for precision medicine purposes in cancer. The design, validation, and use of polygenetic scores open a window of new opportunities to integrate "omics" technologies into clinical advice. Moreover, proteomics, metabolomics, lipidomics, and metagenomics will complete the full scenario (13). Additionally, clinical trials combining current chemotherapies with natural bioactive compounds toward altered lipid metabolism represent a promising strategy to improve cancer treatment (14).

In this review, we will discuss about the role of lipid metabolism alterations in cancer. We will explore their mechanism of action and their oncologic implications. Moreover, we will analyze current reports and knowledge of lipid metabolism biomarkers in the most frequent types of cancer. Finally, we will investigate their emergent use in precision medicine and precision nutrition strategies.

IMPACT OF OBESITY IN CANCER

In recent years, it has demonstrated that cancer malignancy not only relays on the genetic factors—oncogenic and tumor suppressor alterations—from patients, but also on environmental factors associated with lifestyle (15). In this regard, it has been shown that up to one-third of cancer deaths could be prevented by modifying environmental factors related to lifestyle such as physical activity and diet, alcohol consumption, and smoking. Unhealthy diets—high consumption of saturated FAs or high-glucose-content beverages—are also associated with the development of systemic metabolic alterations including obesity, insulin resistance, and metabolic syndrome, among others. Obesity, which is defined as a high body weight with excessive adipose tissue accumulation, can be considered as a chronic, multifactorial, and proinflammatory disease (6, 16). Obesity is a risk factor for several chronic diseases including type 2 diabetes mellitus, cardiovascular diseases, hepatic steatosis, and cancer initiation and progression (17, 18). In fact, the overall risk of cancer death is around 1.5- to 1.6-fold in individuals with a body mass index higher than 40 kg/m^2 (19). The main types of cancer where obesity has been found associated with are prostate cancer (20), postmenstrual endometrial (21), breast cancer (22), ovary (23), bladder (24), liver (25), colon (26), and pancreas (22). During obesity, adipocytes accumulate in locations not classically associated with adipose tissue. Fat accumulation in ectopic sites is classified as central adipose tissue with systemic effects and locally accumulated adipose tissue supporting tumor microenvironment. The central adipose tissue leads to alterations in the levels of steroidal sex hormones, decreased insulin sensitivity, and low-grade inflammation (27), and it has been associated mainly with CRC (27) and breast cancer (6, 28). In addition, visceral depots of adipose tissue may provoke alterations in the cellular composition of cells surrounding the tumor microenvironment contributing to tumor cell proliferation and dissemination such as in the case of tumors located close to adipose tissues, such as breast, ovary, or colon tumors (6, 29).

The effects of tumor cells at the tumor microenvironment has been also found to associate with drug resistance (30). Cancer-associated adipocytes present metabolic features that sustain tumor progression and dissemination, because of the release of FAs and proinflammatory mediators, which contribute to support the surrounding tumor microenvironment (6). Thus, ovarian cancer partially relies on lipids provided by adipocytes at the tumor microenvironment (29, 31). Moreover, the hyperplasia and hypertrophy of adipose tissue diminish the levels of oxygen available, promoting angiogenesis, which may contribute to tumor dissemination (32). In this regard, breast, gastric, and colon cancers preferentially grow in adipocyte-enriched environments. In addition, excess of adipose tissue induces low chronic inflammation augmenting the circulating levels of proinflammatory interleukins (IL-6 and IL-8), tumor necrosis factor α, vascular endothelial growth factor (VEGF), and prostaglandins and leukotrienes, which have protumorigenic effects. Arachidonic acid (AA) is the main precursor of proinflammatory lipid mediators,

such as prostaglandins, thromboxanes, and leukotrienes, which promote proliferation, cell survival, and dissemination of cancer cells. Inflammatory prostaglandins, such as prostaglandin E_2 produced by COX2 (cyclooxygenase 2), activate epidermal growth factor receptor cell signaling to promote angiogenesis and the expression of matrix metalloproteases in colon cancer (33). Prostaglandins have been shown to inhibit the antitumor immune response by diminishing the activation of cytotoxic $CD8^+$ T lymphocytes and the infiltration of natural killer cells and dendritic cells to the tumor (34). In this regard, COX2 inhibitors have been demonstrated to augment the response to immune checkpoint inhibitors in melanomas (35, 36).

In addition, it has been described that obese individuals present an altered gut microbiota and disrupted intestinal epithelium barrier. Dysbiosis is associated with microbial diversity together with an increase in proinflammatory species. Intestinal dysbiosis has been associated with gastric, CRC, and esophageal cancers (37, 38). Thus, the design of microbiota-targeting therapies is now considered as a feasible strategy in the clinic.

Because of the important metabolic link between obesity and the tumorigenic process (**Figure 1**), effective control of the nutritional and metabolic status of individuals (control of glucose, lipid levels, blood pressure, and chronic inflammation) might represent a specific and mechanistic approach to prevent and/or ameliorate cancer progression. In this scenario, precision nutrition has emerged as a complementary therapeutic tool in the management of metabolic alterations associated with cancer prognosis. Personalized nutrition compiles nutrigenetics (genetic variants and epigenetic signatures), deep phenotyping, and a wide spectrum of data concerning metabolic personalization through omics technologies—transcriptomics, metabolomics, lipidomics, and metagenomics. Importantly, nutritional interventions based on the knowledge of how nutrients and bioactive dietary compounds interact with the genome, metabolism, microbiome, etc., at the molecular level, represent an effective tool to fight against metabolic alterations.

LIPID METABOLIC REPROGRAMMING OF ONCOGENIC PATHWAYS IN CANCER

Cancer cells present metabolic alterations to provide the additional requirements of energy and metabolites for cancer cell proliferation and dissemination (2). Enormeous diversity exists between the different types of cancer, and even within the same tumor. Moreover, cancer cells are characterized by the continuous capacity to adapt to changes in the levels of nutrients and oxygen at the tumor microenvironment (6). The altered tumor metabolism depends not only on the cell autonomous genetic alterations, but also on additional factors including diet, food behavior, exercise, and microbiome. All these factors together will determine the biology of the developing tumor (39) (**Figure 1**).

One of the most frequent metabolic alterations observed in cancer is the increased of the glycolytic pathway, independently of the oxygen levels (Warburg effect) (40). Aerobic glycolysis in cancer is coupled to increase glutamine metabolism for the anaplerosis of intermediated of the tricarboxylic acid (TCA) cycle (41). In addition, different studies including *in vitro*, preclinical, and clinical trials have demonstrated the relevance of lipid metabolism to sustain cancer initiation and progression (6). The inhibition of lipid metabolic enzymes has been shown to induce tumor regression, to inhibit the metastatic spread, and/or to avoid drug resistance. Lipids not only are structural components of biological membranes, but also provide energy by means of β-FA oxidation (β-FAO), control the redox homeostasis, and act as signaling molecules affecting a plethora of crucial processes in cancer including proliferation, migration, invasion, transformation, tumor microenvironment reshaping, and/or modulation of inflammation (42). Cholesterol is a key component of the cell membranes affecting its fluidity, stabilizing specific areas (lipid rafts) to transduce intracellular cell signaling pathways (43), and being precursor of steroidal hormones (44). In addition, lipids are also signaling molecules such as proinflammatory prostaglandins or tromboxanes—synthesized from omega-6 AA (45), or anti-inflammatory omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid, which availability depends on lipids provided from diet.

Herein, we describe potential strategies to target the altered lipid metabolism in cancer. In addition, as the uptake of high levels of saturated FAs from diet is a risk factor in several types of cancers, strategies to diminish lipolysis and promotion of healthy diets should also be considered.

Activation of *de novo* Lipogenesis and Cholesterogenesis

Lipid metabolism alterations affect not only tumor cell proliferation, but also dissemination and resistance to chemotherapeutic drugs (46). Most of adult tissues obtain FAs, cholesterol, and lipids from diet; meanwhile, *de novo* synthesis of FAs and cholesterol is restricted to the liver and adipocytes. Tumors frequently present the capability to activate the *de novo* synthesis of cholesterol and FAs (47) making them more independent from externally provided lipids (48, 49). Importantly, targeting enzymes associated with *de novo* lipogenesis and/or the mevalonate pathway has been shown to inhibit tumor growth (6, 50).

FAs are synthesized from cytoplasmic acetyl-CoA (AcCoA), generated from citrate produced from glucose, glutamine, or acetate (48). ATP-citrate lyase (ACLY) generates AcCoA and oxaloacetate (OAA) from citrate (48, 51). AcCoA carboxylases (ACC1/2) carboxylase AcCoA to form malonyl-CoA. Subsequent condensation steps, catalyzed by FA synthase (FASN), forms the 16-carbon saturated FA palmitate. Palmitate is then elongated by FA elongases (ELOVL) and desaturated by stearoyl-CoA desaturase (*SCD1*) or FA desaturases (FADS) to form other nonessential FAs, such as the 18-carbon monounsaturated FA (MUFA) oleate (C18:1) (**Figure 2**).

Many enzymes implicated in *de novo* synthesis of FAs and cholesterol have been proposed as biomarkers for prognosis in specific types of cancer. FASN is found upregulated in prostate and breast cancer (47, 52), and ACLY has been shown to support

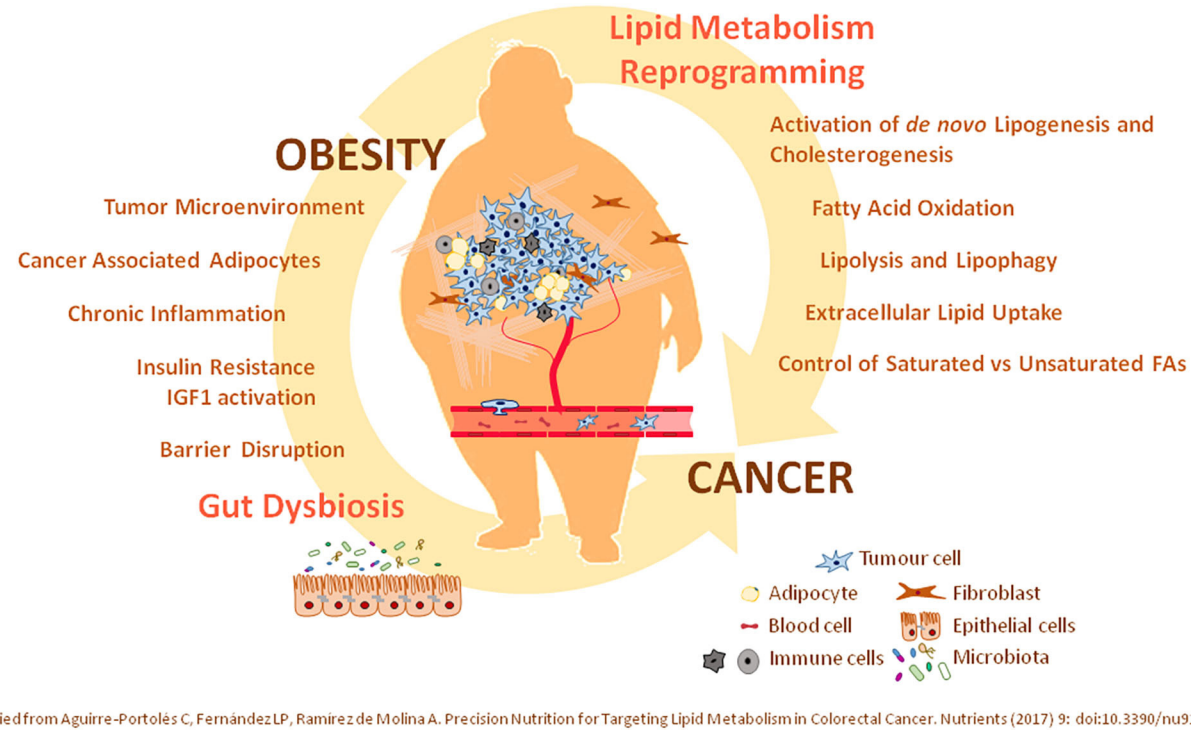

FIGURE 1 | Relevance of lipid metabolism alterations in cancer. Illustrated is the crucial role of (i) oncogenic mutations supporting the lipid metabolism reprogramming in cancer, together with (ii) systemic lipid metabolic alterations associated with obesity—as an environmental modifiable risk factor. Precision interventions should include therapeutic clinical drugs targeting identified lipid metabolism molecular targets together with nutritional interventions—bioactive compounds, diet-derived ingredients—considering the nutritional and metabolic status of patients. T2DM, type 2 diabetes mellitus; IR, Insulin Resistance; TME, tumor microenviroment; CAAs, cancer-associated adipocytes; FAO, fatty acid oxidation; FA, fatty acid.

tumor formation and transformation (51). Inhibition of several enzymes of *de novo* lipogenesis, such as FASN, and ACC1 and ACC2, has been tested in different cancer models showing their relevance on tumor growth inhibition (53).

Similarly, inhibition of hydroxymethylglutaryl-CoA (HMGCoA) reductase (HMGCR), by statins, leads to inhibition of cell proliferation of breast cancer cells (54) and tumor regression in several preclinical mouse models, and it is being tested in clinical trials (43). The overexpression of enzymes of the mevalonate pathway has been proposed as biomarkers of poor prognosis in breast cancer (55). Cholesterol is generated by the mevalonate pathway, by condensation of two AcCoA molecules to form 3-HMGCoA, which is then reduced to form mevalonate, and then isoprenoid farnesyl-pyrophosphate. Several studies have shown that targeting the synthesis of cholesterol inhibits cancer cell proliferation and transformation (56).

De novo synthesis of FAs and cholesterogenesis are transcriptionally regulated by SREBPs, which are downstream oncogenic pathways including PI3K/Akt (57) and c-Myc (47) (**Figure 2**).

The SREBP family includes three transcription factors: SREBP1a and SREBP1c, which are derived from *SREBF1* gene by alternative splicing (58), and SREBP2, which is encoded by *SREBF2* gene. SREBPs are bound to the endoplasmic reticulum (ER) as inactive precursors (59). When the intracellular levels of cholesterol are high, insulin-induced genes interact with SREBP-cleavage–activating proteins (SCAPs) to retain SREBP inactive precursors attached to the ER. When cholesterol levels are low, SCAPs facilitate the translocation SREBPs to the Golgi apparatus to be further processed releasing the active forms (56). SREBP1 promotes the expression of lipogenic genes; meanwhile, SREBP2 regulates the expression of genes involved in the synthesis, uptake, and efflux of cholesterol. Nevertheless, SREBP1 and SREBP2 have overlapping activities. Both SREBP1 and SREBP2 are found overexpressed in several cancers. Regulation of the intracellular content of cholesterol has also been shown crucial for cancer cell survival. The ATP-binding cassette transporter (ABCA1) controls the efflux of cholesterol to ApoA-coated lipoproteins (57). Recently, it has been demonstrated that activation of p53 increases the retrograde transport of cholesterol from the plasma membrane to the ER, to prevent SREBP2 maturation (60). In addition, cholesterol levels are fine tune regulated by microRNA33—encoded by an intron within the *SREBF2* gene (51)—which targets ABCA1. In addition, the esterification of cholesterol for storage in LDs, by sterol *O*-acyltransferase 1 (ACAT1), has been shown to augment the survival in prostate cancer (61).

Fatty Acid Oxidation in Cancer

In addition to *de novo* synthesis of FAs and cholesterol, the mobilization of intracellular FAs for FAO at mitochondria is crucial for cancer survival and dissemination. It is well-known

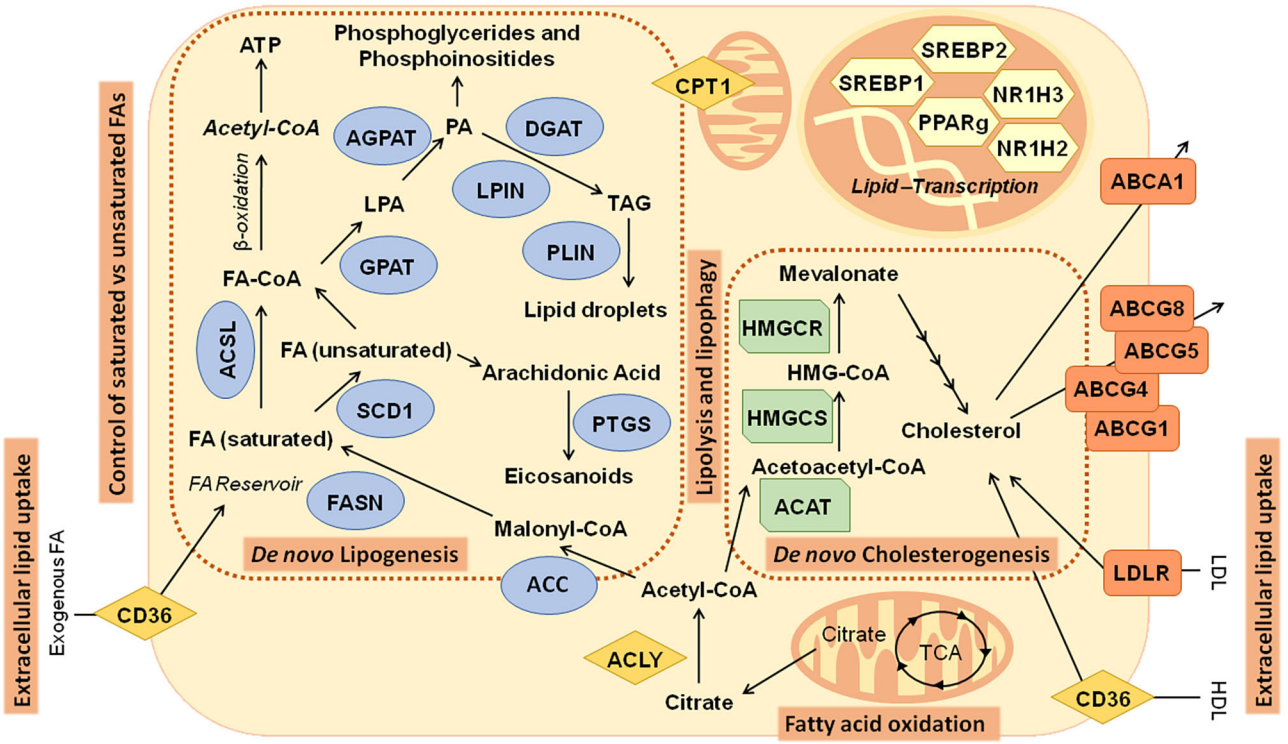

FIGURE 2 | Main metabolic pathways related to lipid metabolism in cancer: Illustration of pathways and genes implicated in *de novo* lipogenesis—fatty acids and cholesterol biosynthesis. ABCA1, ATP-binding cassette subfamily A member 1; ABCG1, ATP-binding cassette subfamily G member 1; ABCG4, ATP-binding cassette subfamily G member 4; ABCG5, ATP-binding cassette subfamily G member 5; ABCG8, ATP-binding cassette subfamily G member 8; ACAT, acetyl-CoA acetyltransferase; ACC, acetyl- CoA carboxylase; ACLY, ATP citrate lyase; ACSL, acyl-CoA synthetase long chain; AGPAT, 1-acylglycerol-3-phosphate *O*-acyltransferase; CD36, CD36 molecule; CPT1, carnitine palmitoyltransferase; DGAT, diacylglycerol *O*-acyltransferase; FA, Fatty acids; FASN, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; HDL, high-density lipoprotein; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPIN, Lipin; NR1H2, nuclear receptor subfamily 1 group H member 2; NR1H3, nuclear receptor subfamily 1 group H member 3; PLIN, perilipin; PPARγ, peroxisome proliferator-activated receptor γ; PTGS, prostaglandin-endoperoxide synthase; SCD1, stearoyl-CoA desaturase; SREBP1, Sterol regulatory element binding transcription factor 1; SREBP2, sterol regulatory element binding transcription factor 2; TCA, tricarboxylic acid cycle.

that tumor cells present higher levels of reactive oxygen species (ROS) than not tumor cells, which allow them to activate prosurvival and epithelial-to-mesenchymal transition programs to support cancer progression and dissemination. Nevertheless, excessive ROS may promote apoptotic cell death. It has been demonstrated that enzymes implicated in the mobilization of intracellular neutral lipids provide metabolic flexibility to increase the levels of FAs for oxidation at mitochondria. In the FAO pathway, acyl-CoAs are cyclically dehydrogenated, hydrated, and decarboxylated, resulting in the progressive shortening of the FA, together with the production of NADH and $FADH_2$ and AcCoA. NADH and $FADH_2$ will be used for ATP production in the electron transport chain, and AcCoA can enter the Krebs cycle. AcCoA together with OAA gives rise to citrate, which after being exported to cytoplasm, can enter two metabolic pathways to produce cytosolic NADPH (62).

Enhanced mitochondrial β-oxidation of FAs has been described in pancreatic cancer (63, 64) and in metastatic breast cancer (65). FAO not only provides energy when glucose becomes limiting, but it also contributes to a better control of the oxidative stress, by augmenting the intracellular levels of NADPH (66). Increased FAO augments survival in leukemia and gliomas by counteracting the metabolic oxidative stress. Moreover, FAO has been shown crucial for the survival of cells from solid tumors when undergoing loss of attachment, which triggers *anoikis* or cell death due to oxidative stress (67, 68).

In addition, FAO is also influenced by the tumor microenvironment such as in the case of ovarian cancers, which preferentially metastasizes to the omentum enriched in adipocytes, which provides lipids for ATP and NADPH production to control metabolic stress during metastasis.

Regulation of FA Storage and Intracellular FA Mobilization (Lipolysis and Lipophagy)

De novo synthesis of FAs in cancer cells is coupled to additional processes to accommodate the increase in the intracellular lipid content, to preserve the homeostasis between lipid storage and lipid mobilization (69). FAs from *de novo* lipogenesis are accumulated into neutral lipids (stored in LDs) and phospholipids (in membranes). LDs are complex and dynamic

organelles consisting of a neutral lipid core surrounded by a phospholipid monolayer and a complex proteome associated. LDs itself have been proposed as novel diagnostic biomarkers for glioblastoma. It has been demonstrated that while they are not detectable in low-grade gliomas or normal brain tissues, they are common in glioblastoma, the most lethal brain tumor (70). Among the LD-associated proteins, there are enzymes of the sterol biosynthetic pathway, the acyl-CoA metabolism (ACSLs), and triacylglycerol (TAG) biosynthesis. Structural proteins, such as perilipins (PLINs) or caveolins, are critical for the integrity of LDs to avoid collapse and to protect them from lipolysis (**Figure 2**). Cancer cells present higher amounts of LDs than normal cells (71). Increased expression of PLIN2 has been shown to favor the accumulation of LDs (72), contributing to a better control of the ER stress, to increase the protection against ROS, and to augment the resistance to therapeutic drugs in cancer cells. On the contrary, PLIN2 depletion significantly attenuated the proliferation of colon cancer cells (73), supporting the LD-associated proteins as potential druggable targets for cancer treatment (11).

The increase in *de novo* synthesis of FAs in cancer cells requires efficient and complementary lipolytic mechanisms to accommodate the intracellular lipid content. Thus, lipolysis allows the stored lipids to be available for the synthesis of phospholipids and lipid signaling mediators and/or to increase the levels of ATP or NADPH when required. Several enzymes involved in lipolysis—adipose TAG lipase (ATGL), hormone-sensitive lipase (HSL), monoacylglycerol lipase (MAGL)—have been described to promote tumorigenesis (74). In this sense, ATGL knockdown in HCT116 CRC cells reduced cell proliferation (75). Increased levels of MAGL are associated with aggressive cancer types such as melanoma and ovarian and breast cancer (74), and inhibition of MAGL suppresses cancer cell migration, invasion, and survival (76). Recently, it has been demonstrated that glioblastomas, which acquire large amounts of free FAs, upregulate diacylglycerol-acyltransferase 1 (DGAT1) to store the excess FAs into triglycerides and LDs (77). Inhibition of DGAT1 disrupted lipid homeostasis, resulting in increased levels of ROS leading to apoptotic cell death.

In addition, a specific function of autophagy associated with the regulation of the intracellular lipid content—lipophagy—has been described to augment resistance to cell death in cancer (78).

Extracellular Lipid Uptake

In addition, similar to normal cells, cancer cells can uptake exogenous lipids when *de novo* lipogenesis is inhibited. Upregulation of cell surface receptors, such as cluster of differentiation 36 (CD36) (**Figure 2**), has been found to augment metastasis (79, 80). CD36 inhibition diminished tumor growth and metastasis in preclinical models of prostate cancer (80). Moreover, the expression of low-density lipoprotein receptor (LDLR) for the internalization of low-density lipoproteins (LDLs) has been found upregulated in renal cell carcinoma (RCC) cells (81). FA-binding proteins (FABPs) contribute to augment the lipid uptake, as well as the intracellular lipid trafficking in cancer cells (82). In breast cancer and glioblastoma cell lines, it has been shown that the uptake of extracellular FAs during hypoxia is sustained by the upregulation of FABP3 and FABP7; meanwhile, FABP5 increases cell proliferation and growth in prostate cancer (83).

Control of Saturated vs. Unsaturated FAs

Depending on the source of FAs, *de novo* lipogenesis or extracellular lipid uptake, the levels of saturated FAs incorporated in the phospholipids of cell membranes are different. The lipogenic pathway increases the saturation level of cell membranes with saturated and MUFAs (84), which are less sensitive to suffer lipid peroxidation compared to polyunsaturated acyl chains (PUFAs) mainly obtained from diet. This way, *de novo* lipogenesis contributes to augment the resistance to oxidative stress and chemotherapy in cancer cells (85).

Nevertheless, excessive accumulation of saturated FAs in the cell membranes can lead to lipotoxicity. In this regard, SCD1 inhibition induces ER stress and apoptosis in cancer cells and diminishes the tumor growth in xenografts models of colon and lung cancers (86). During tumor growth, inner parts of the tumors are faced to hypoxia and reduced nutrient availability. Tumors have developed different strategies to balance the levels of saturated vs. unsaturated FAs. Thus, tumors anticipate lipotoxicity by augmenting the uptake of MUFAs/PUFAs from plasma, which are further stored into LDs or incorporated into phospholipids at the cell membranes. As SCD1 activity requires oxygen, during hypoxia some tumors rely on the activity of DGATs to incorporate MUFAs into TG, which are further accumulated into LDs (**Figure 2**). In addition, tumors balance, via the Lands cycle, the levels of saturated vs. unsaturated FAs in the phospholipids at the cell membranes. Recently, a process known as ferroptosis has been described associated with high levels of MUFA/PUFAs in the phospholipids of cell membranes, which induce cell death by means of their oxidation through the Fenton pathway. Long-chain FA acyl CoA synthetases (ACSLs)—implicated in the long chain FA activation—may control ferroptosis, as distinct isoforms use distinct substrates. Meanwhile, ACSL4 has PUFAS as main substrates such as AA, ACSL3 can activate both MUFAs and PUFAs, allowing a better control of the excessive accumulation of PUFAs in phospholipids (87). In addition, ACSL3 allows a better control of FA distribution between LD storage or β-FAO, providing a better control of the oxidative stress (42).

LIPID METABOLISM ALTERATIONS AND CANCER PROGNOSIS

Alterations of lipid metabolism genes are found in many tumor types, predominantly, but not exclusively, because lipid metabolism can modulate different cellular processes that go from plasmatic and organelle membrane organization and plasticity (88, 89), substrate supply for ATP synthesis, (62) and intracellular cell signaling activation (90). Cancer tissues display abnormal activation of *de novo* lipogenesis and cholesterogenesis (91). Extremely proliferative cancer cells exhibit an intense lipid and cholesterol avidity, which they satisfy by increasing

TABLE 1 | Prognostic value of lipid metabolism–related genes.

Prognostic value	Literature								The Cancer Genome Atlas (TCGA)-The Protein Atlas																
	LC		CRC		BC		PC		LC	CRC	BC	PC	CC	EC	G	HNC	LC	M	OC	PC	RC	SC	TC	ThC	UC
Fatty acid–related pathways																									
Fatty acid synthesis																									
ACLY	■	(61, 102, 103)	■	(104)									■				■								
pACC	■	(105)	■	(39)																					
ACACA																	■								
ACACB			■	(106)																■	■				
FASN	■	(107)	■	(36, 108–112)	■	(113–115)	■	(116–119)					■								■				
ACSL1			■	(7–9, 120, 121)																	■				
ACSL3	■	(+)							■								■								
ACSL4			■	(7–9, 120, 121)	■	(122)																			■
ACSL5					■	(121, 123)									■										
ACSL6																									
SCD1	■	(124)	■	(7, 125)	■	(126)															■				■
FADS1	■	(127)																			■			■	■
FADS2																					■				■
FADS3																■					■				
FADS4																					■				
FADS6																									
FADS7														■			■				■				■
FADS8																					■				■
PTGS1																					■				■
PTGS2	■	(128–130)	■	(131)	■	(132)	■	(133)													■				
GPAT1																					■				
GPAT2																									
GPAT3																					■				■
GPAT4												■		■					■						■
AGPAT1	■	(7, 8)									■			■											
AGPAT2																									
AGPAT3													■								■				
AGPAT4													■								■				
AGPAT5										■							■								
LPIN1	■	(134)			■	(135)	■	(136)																	
LPIN2																■									
LPIN3														■					■		■				

(Continued)

TABLE 1 | Continued

Prognostic value	Literature								The Cancer Genome Atlas (TCGA)-The Protein Atlas																	
	LC		CRC		BC		PC		LC	CRC	BC	PC	CC	EC	G	HNC	LC	M	OC	PC	RC	SC	TC	ThC	UC
PLIN1		(137)				(138)																			
PLIN2						(139)																			
PLIN3																									
PLIN4																									
PLIN5																									
DGAT1																									
DGAT2						(140)																			
Fatty acids–related transportation																									
CD36				(141)		(141)		(141)																	
CPT1A						(142, 143)																			
CPT1B																									
CPT1C																									
Cholesterol-related pathways																									
Cholesterol synthesis																									
ACAT1								(144, 145)																	
ACAT2																									
HMGCS1																									
HMGCS2				(146)				(147)																	
HMGCR				(148)		(149, 150)																			
Cholesterol-related transportation																									
ABCA1				(7, 8, 151)																					
ABCG1																									
ABCG4		(152)																							
ABCG5				(153)																					
ABCG8																									
LDLR				(154)																					
Lipid transcription																									
Transcription factors																									
SREBP1						(155)																			
SREBP2				(156)				(157)																	
PPARγ		(158, 159)		(160)		(161, 162)		(163)																	

(Continued)

TABLE 1 | Continued

Prognostic value	Literature							The Cancer Genome Atlas (TCGA)-The Protein Atlas															
	LC		CRC		BC		PC	LC	CRC	BC	PC	CC	EC	G	HNC	LC	M	OC	PC	RC	SC	TC	ThC	UC
NR1H2				(156)																				
NR1H3		(164)		(156)																				

Favorable
Unfavorable

Gene prognostic value reported in the literature in most frequent types of cancer according to the World Health Organization (WHO) together with gene prognostic value using data from The Cancer Genome Atlas (TCGA) and visualized using The Pathology Atlas online tool. Abbreviations: LC, lung cancer; CRC, colorectal cancer; BC, breast cancer; PC, prostate cancer; CC, cervical cancer; EC, endometrial cancer; G, glioma; HNC, head and neck cancer; LC, liver cancer; M, melanoma; OC, ovarian cancer; PC, pancreatic cancer; RN, renal cancer; SC, stomach cancer; TC, testis cancer; ThC, thyroid cancer; UC, urothelial cancer; ACLY, ATP citrate lyase; pACC, phospo acetyl-CoA carboxylase; ACACA, acetyl-CoA carboxylase A; ACACB, acetyl-CoA carboxylase B; FASN, fatty acid synthase; ACSL1, acyl-CoA synthetase long chain family member 1; ACSL3, acyl-CoA synthetase long chain family member 3; ACSL4, acyl-CoA synthetase long chain family member 4; ACSL5, acyl-CoA synthetase long chain family member 5; ACSL6, acyl-CoA synthetase long chain family member 6; SCD1, stearoyl-CoA desaturase1; FADS1, fatty acid desaturase 1; FADS2, fatty acid desaturase 2; FADS3, fatty acid desaturase 3; FADS4, fatty acid desaturase 4; FADS6, fatty acid desaturase 6; FADS7, fatty acid desaturase 7; FADS8, fatty acid desaturase 8; PTGS1, prostaglandin-endoperoxide synthase 1; PTGS2, prostaglandin-endoperoxide synthase 2; GPAT1, glycerol-3-phosphate acyltransferase 1; GPAT2, glycerol-3-phosphate acyltransferase 2; GPAT3, glycerol-3-phosphate acyltransferase 3; GPAT4, glycerol-3-phosphate acyltransferase 4; AGPAT1, 1-acylglycerol-3-phosphate O-acyltransferase 1; AGPAT2, 1-acylglycerol-3-phosphate O-acyltransferase 2; AGPAT3, 1-acylglycerol-3-phosphate O-acyltransferase 3; AGPAT4, 1-acylglycerol-3-phosphate O-acyltransferase 4; AGPAT5, 1-acylglycerol-3-phosphate O-acyltransferase 5; LPIN1, lipin 1; LPIN2, lipin 2; LPIN3, lipin 3; PLIN1, perilipin 1; PLIN2, perilipin 2; PLIN3, perilipin 3; PLIN4, perilipin 4; PLIN5, perilipin 5; DGAT1, diacylglycerol O-acyltransferase 1; DGAT2, diacylglycerol O-acyltransferase 2; CD36, CD36 molecule; CPT1A, carnitine palmitoyltransferase 1A; CPT1B, carnitine palmitoyltransferase 1B; CPT1C, carnitine palmitoyltransferase 1C; ACAT1, acetyl-CoA acetyltransferase 1; ACAT2, acetyl-CoA acetyltransferase 2; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; ABCA1, ATP-binding cassette subfamily A member 1; ABCG1, ATP-binding cassette subfamily G member 1; ABCG4, ATP-binding cassette subfamily G member 4; ABCG5, ATP-binding cassette subfamily G member 5; ABCG8, ATP-binding cassette subfamily G member 8; LDLR, low-density lipoprotein receptor; SREBP1, Sterol regulatory element binding transcription factor 1; SREBP2, Sterol regulatory element binding transcription factor 2; PPARγ, peroxisome proliferator-activated receptor γ; NR1H2, nuclear receptor subfamily 1 group H member 2; NR1H3, nuclear receptor subfamily 1 group H member 3. (+) Unpublished results.

the uptake of dietary or exogenous lipids and lipoproteins or activating lipogenesis or cholesterol synthesis (3). Importantly, this aberrant lipid metabolism does not only influence the primary tumor, but the exogenous lipids produced by tumor microenvironment can also influence malignancy (14, 92–95). Besides, three basic steps during metastasis: migration (96), invasion (9, 10) and angiogenesis (97, 98), are affected by lipid metabolism regulation (11).

Nowadays, there are increasing evidences of the role of lipid metabolism alterations as biomarkers of cancer prognosis and survival. Here, we are going to review previous knowledge on the prognostic value of lipid-related genes that belong to FAs and cholesterol pathways (**Figure 2**) in the most frequent types of cancer according to the WHO: lung, CRC, breast, and prostate.

Furthermore, "omics" data publicly available in huge searchable databases facilitate addressing specific medical issues in thousands of patients. Remarkably, The Cancer Genome Atlas (TCGA) gene expression dataset (https://www.cancer.gov/tcga) and The Human Protein Atlas website together with The Pathology Atlas online tool (https://www.proteinatlas.org/humanproteome/pathology), which contains mRNA data from TCGA study and protein expression data from different forms of human cancer (99–101), allowing us to obtain a global view of the putative implications of lipid metabolism–related genes in cancer prognosis. Data from TCGA visualized using The Pathology Atlas online tool, are summarized in **Table 1**.

Fatty Acid–Related Alterations as Biomarkers of Cancer Prognosis and Survival

De novo FA biosynthesis occurs in the cellular cytoplasm. FAs originate from acetyl-coenzyme A, which is mostly provided by citrate produced by the TCA cycle. Switch of citrate into AcCoA is catalyzed by ATP citrate lyase (ACLY) (**Figure 2**). Consequently, ACLY is a key enzyme connecting carbohydrate to lipid metabolism by producing AcCoA from citrate for both FA and cholesterol synthesis (61). Several studies have associated ACLY expression in tumor tissues with worse prognosis. ACLY overexpression correlated with stage, differentiation grade, and a poorer prognosis in non–small cell lung cancer (NSCLC) (61). Besides, in combination with the glucose transporter *GLUT1, ACLY* was also an independent prognostic factor for overall survival (OS) in node-negative patients with NSCLC (102). However, one study reports that young NSCLC patients overexpressing *ACLY* had longer OS, in contrast to older patients where overexpression of ACLY appears to predict the opposite prognosis (103). *ACLY* also facilitates colon cancer cell metastasis, and high expression levels of *ACLY* and Catenin β1 (*CTNNB1*) protein were positively correlated with metastasis of colon cancer (104). Data from TCGA showed *ACLY* as a putative unfavorable marker of cervical and liver cancer (**Table 1**).

At the genomic level, single nucleotide polymorphisms (SNPs) in *ACLY* gene have been described as independent cancer prognostic markers in Asiatic populations. SNP rs9912300 in *ACLY* gene was significantly associated with OS in lung cancer patients (165). rs9912300 and rs2304497, both functional *ACLY*

SNPs, exhibited a significant association with risks of death and recurrence in patients with advanced stages of colon cancer (166).

The following step of FA biosynthesis involves the activation of AcCoA to malonyl-CoA, which is catalyzed by AcCoA carboxylase (ACC) (**Figure 2**). ACC is a complex multifunctional enzyme system. There are two ACC forms, α (ACACA) and β (ACACB), encoded by two different genes. High phospho-acetylCoA carboxylase (pACC) was an independent marker for prediction of better survival in lung adenocarcinoma patients (105), and low pACC levels detected by immunohistochemistry were associated both with worse OS and progression-free survival in advanced stage CRC (167). In the same line, gene expression analysis reported that patients with upregulation of six of these hub genes (genes with high correlation in candidate modules) (*ACACB*, acyl-CoA dehydrogenase medium chain, adiponectin, *C1Q* and collagen domain containing, acyl-CoA synthetase short-chain family member 2, phosphoenolpyruvate carboxykinase 1 and PLIN1) displayed improved breast cancer prognosis (106). In TCGA dataset, *ACACA* gene expression is an unfavorable risk factor for liver cancer, whereas *ACACB* is a favorable prognostic factor for both renal and pancreas tumors (**Table 1**). Finally, it has been described in prostate cancer that genetic alterations of *ACACA*, *FASN*, and *SREBF1* predicted worse overall patient survival (168).

Malonyl-CoA is coupled to the multifunctional enzyme FASN. Repeated cycles of acetyl group's condensation produce the primary FA palmitate that can suffer separate elongation and/or unsaturation cycles to yield other FA molecules (169) (**Figure 2**). FASN is the key enzyme necessary for the *de novo* synthesis of long-chain FAs. FASN has been found overexpressed in nearly all of cancer tissues, and its expression is associated with a poorer prognosis.

One study reported that *FASN* gene expression was higher in the adjacent non-cancer tissue than in the NSCLC tissue, but authors concluded that it was a weaker predictor of shorter patient survival (170). However, a correlation analysis between expression levels of CD276 (B7-H3) and FASN exhibited a positive correlation with poor prognosis in clinical lung cancer tissues (107).

FASN levels were clearly upregulated in CRC tissues with high expression of FASN significantly associated with lymph node metastasis (108), liver metastasis (109), TNM (tumor, node, metastasis) stage, and poor prognosis (36). Moreover, a significant association was shown between FASN and VEGF expression, suggesting the involvement of FAS in tumor angiogenesis (110). Interestingly, one study reported that, among non-obese patients with colon cancer, tumoral FASN overexpression is associated with better survival, while among moderately overweight or obese patients, FASN overexpression may predict a poorer outcome (111). Furthermore, a panel of five genes including *FASN* (*ACOT8/ACSL5/FASN/HMGBCS2/SCD1*) has been reported to display a improved prognostic performance than validated clinical risk scales, and it is applicable for early discovery of CRC and tumor recurrence (112). Finally, FASN levels in serum were also examined in CRC patients, where it was associated with tumor stage (171), and high FASN levels are considered as a promising independent predictor of CRC with advanced phases, late clinical stages, and shorter survival (172).

FASN is associated with poor prognosis in breast and prostate cancer, and its inhibition is selectively cytotoxic to human cancer cells (113). FASN was found overexpressed in most of the triple-negative breast cancer (TNBC) patients but not always correlated with OS or disease-free survival. High FASN was significantly associated with positive node status (114). A greater part of clinically HER2-positive tumors was achieved as FASN overexpressors. Reclassification of HER2-positive breast tumors based on FASN gene expression predicted a significantly inferior relapse-free and distant metastasis-free survival in $HER2^{+}/FASN^{+}$ patients (115).

A substantial subset of prostatic cancers displays clearly elevated expression of immunohistochemically detectable FASN, a feature that has been associated with poorer prognosis (116–119). Furthermore, high expression level of FASN resulted in a significantly poor prognosis of pancreatic cancer (173), and data from TCGA study suggest that *FASN* expression could be a marker of bad outcome in cervical and renal cancer (**Table 1**).

In addition, several genetic changes in *FASN* gene have been associated with cancer prognosis. Two SNPs rs4246444 and rs4485435 were significantly associated with the recurrence of NSCLC (165). Finally, as it has been previously mentioned in prostate cancer that genetic alterations of *FASN* together with *ACACA* and *SREBF1* predicted worse prognosis (168).

Then, FAs are activated with CoA by fatty acyl-CoA synthetases (ACSLs) (**Figure 2**), which is essential for phospholipid and triglyceride synthesis and lipid modification of proteins in addition to for FA β-oxidation (169).

Family of long-chain acyl-CoA synthetases has been extensively proposed as putative prognostic biomarkers of cancer. *ACSL3* is up-regulated in lung cancer compared to the healthy lung tissue (174), and recently, an association with *ACSL3* expression, NSCLC prognosis, and the efficacy of statins treatment has been discovered (L. P. Fernandez et al., unpublished results). *ACSL3* was also found to be overexpressed in estrogen receptor–negative breast cancer (175) and prostate cancer (176). *ACSL1* and *ACSL4* overexpression was associated with a poor clinical outcome in stage II CRC patients (7–9, 120, 121). In addition, *ACSL4* is considered a biomarker for liver and breast cancers (122, 177). By contrast, downregulation of *ACSL5* in breast cancer was associated with a poorer prognosis (121, 123). There have not been reported associations between *ACSL6* and cancer survival (178).

An *in silico* study (121) also suggested that high *ACSL1* expression was associated with worse outcome in lung cancer patients, and *ACSL3* overexpression was associated with worse survival in patients with melanoma. In contrast, high *ACSL3* expression predicted a better prognosis in ovarian cancer. In the same study, *ACSL4* overexpression predicted bad prognosis in CRC, but good prognosis in breast, brain, and lung cancers. High expression of *ACSL5* predicted good prognosis in breast, ovarian, and lung cancers. Finally, low *ACSL6* predicted a worse prognosis in acute myeloid leukemia. *In silico* analysis of TCGA data (**Table 1**) suggested that *ACSL1*, *ACSL4*, and *ACSL5* are associated with favorable outcome in renal, urothelial, and

endometrial cancers, respectively, whereas *ACSL3* expression predicts poor survival in lung and liver tumors.

Genetically, a 3′-UTR polymorphism in *ACSL1* is associated with *ACSL1* expression levels and poor clinical outcome in CRC patients (14, 120). Patients carrying the *ACSL1* rs8086 T/T genotype had significantly reduced disease-free survival compared with patients carrying the C/T or C/C genotype, with 3-fold higher risk of recurrences. T/T genotype for rs8086 is correlated with worse clinical outcome and simultaneously associates with high *ACSL1* mRNA levels (14, 120).

Stearoyl CoA desaturase 1 (*SCD1*) catalyzes the rate-limiting step in the synthesis of MUFAs that are the main components of tissue lipids. *SCD1* has been associated with tumor development, late stage, and reduced survival in lung adenocarcinoma (124). Together with other three lipid metabolism–related genes (*ABCA1*, *ACSL1*, and *AGPAT1*), *SCD1* expression separated stage II colon cancer patients with a 5-fold higher risk of relapse (7). Moreover, positive associations between *SCD1* expression and CRC patient clinical status and the expression of cancer stem cell–related genes (*WNT* and *NOTCH* signaling) were found based on TCGA data analysis (125). In the same line, high *SCD1* expression is associated with shorter survival in breast cancer patients (126). **Table 1** shows that *SCD1* is an unfavorable marker of survival in renal and urothelial cancer in TCGA tumors. Other desaturases have also been analyzed as prognostic markers, and, for example, reduced expression of *FADS1* suggests pessimistic prognosis for NSCLC patients (127).

Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first step in the production of almost all membrane phospholipids. GPAT transfers an acyl group from acyl-CoA or acyl-ACP at the sn-1 or-2 position of glycerol 3-phosphate originating lysophosphatidic acids (LPAs) (179). LPA is a substrate for synthesis of numerous important glycerolipid intermediates, such as storage lipids, extracellular lipid polyesters, and membrane lipids (**Figure 2**). Four GPATs have been discovered; nevertheless, only GPAT1 (GPAM) has been related to cancer outcome. High GPAT1 expression has been associated with reduced OS in ovarian cancer (180). Data from TCGA suggested that *GPAT1* could be a favorable prognostic marker in renal cancer, while *GPAT3* is a putative biomarker of good prognosis in renal cancer in contrast to urothelial cancer. Finally, *GPAT4* expression could have a risk effect in ovarian and endometrial cancers, and a protective one in prostate and urothelial cancer (**Table 1**).

LPA is further metabolized to phosphatidic acid (PA) by AGPATs (1-acylglycerol-3-phosphate *O*-acyltransferases) (**Figure 2**). AGPAT1 belongs to previously mentioned transcriptional signature where combined analysis of four genes, *ABCA1*, *ACSL1*, *AGPAT1*, and *SCD1*, is associated with higher risk of relapse in stage II CRC patients (7). Furthermore, individuals with upregulation of *AGPAT1* expression have an increased risk of CRC recurrence, independently of tumor stage (8). Expression of *AGPAT2* was significantly related to decreased OS as well as to shorter progression-free survival in ovarian cancer patients younger than 60 years (181). When we consider tumors from TCGA study, several associations were found (**Table 1**). *AGPAT3* is a marker of good prognosis in renal cancer and predicts bad outcome in cervical cancer. High expression levels of *AGPAT4* may be associated with poor prognosis in cervical and renal cancers, whereas *AGPAT5* is an unfavorable prognostic marker in liver cancer and a favorable one in CRC.

Then PA is converted to diacylglycerol (DAG) by LPIN, a PA phosphatase. Three LPIN isoforms have been described. LPIN1 is upregulated in lung adenocarcinoma tumor tissues, and high LPIN1 expression was correlated with poor prognosis of patients with lung adenocarcinoma (134). In breast cancer, previous results seem to indicate that the high LPIN expression is related to a good prognosis (135). However, in basal-like TNBC, high LPIN1 expression correlates with the poor prognosis of these patients (136). In TCGA dataset analysis, *LPIN2* appears as a favorable prognostic marker in head and neck cancers, while *LPIN3* could be an unfavorable biomarker of endometrial, ovarian, and renal tumors (**Table 1**).

The final step in triacylglycerols synthesis is catalyzed by DGAT, which esterifies the DAG with a FA. Two human DGAT isoforms have been described (182). The expression of DGAT2 in HER2-positive breast cancer was decreased and was closely related to patient prognosis (140). However, data from TCGA reported *DGAT2* as an unfavorable prognostic factor for endometrial cancer (**Table 1**).

Subsequently, TAGs could be stored in LDs, and PLINs, an LD surface family of proteins, are necessary for optimal lipid storage and FA release. There are multiple PLIN proteins encoded by mRNA splice variants of a single PLIN gene. PLIN1 expression in lung adenocarcinoma is associated with apocrine-like features and poor clinical prognosis (137). In contrast, *PLIN1* mRNA expression is significantly downregulated in human breast cancer. The reduced expression of PLIN1 is an independent predictor of OS in estrogen receptor–positive and luminal A-subtype breast cancer patients (138). Also in breast cancer, low expression of PLIN2 was associated with favorable prognosis (139). The prognostic effects of PLINs in several types of cancer from TCGA analysis are multiple and very diverse (**Table 1**).

Eicosanoids are biologically active metabolites of AA and are produced by cyclooxygenases 1 and 2 (COX1 and COX2) [also known as prostaglandin-endoperoxide synthase 1 and 2 (PTGS1 and PTGS2)]. They are overexpressed in a variety of malignant tumors. It has been reported that the mRNA levels of *COX-1* and *COX-2* in lung cancer patients were significantly higher than in normal patients (183). However, another study reports that in tumor cells COX-2 rather than COX-1 expression may account for the variable prostanoid production seen in NSCLC (128). It is clear that multivariate analysis showed that tumoral *COX-2* mRNA expression and lymph node status were the most important independent prognostic predictors for NSCLC survival and disease relapse (129). Elevated COX-2 expression in tumors was significantly associated with lower survival in NSCLC and might be useful in identifying patients who would benefit from additional therapies for managing their disease (130).

The same tendency was observed in CRC, where elevated COX-2 expression, but not that of COX-1, was significantly associated with reduced survival and recognized as an independent prognostic factor (131). However, it has been

reported that COX-1 and COX-2 expression is highly variable in Dukes' C tumors, and changes in COX-1 expression may be of importance in CRC (184).

COX-2 expression level and its prognostic value are also a matter of debate in breast cancer (185). Nevertheless, at least eight immunohistochemical reports have explored expression of COX-2 in a total of 2,392 primary breast carcinomas, of which 40% were found to be COX-2 positive (132). At least, four studies have detected that overexpression of COX-2 is linked to poor prognosis in breast cancer. These studies provide the basis for further estimation of a possible therapeutic effect of COX inhibitors in therapy of breast cancer.

In prostate cancer, a subset of Chinese patients with high-COX-2 expression showed minor disease-free and OS rates than those with low COX-2 expression. In this work, univariate and multivariate analyses suggested that the status of COX-2 protein expression was an independent prognostic factor for patients' survival (133).

Data from TCGA showed *COX-1* and *COX-2* as unfavorable markers of renal cancer, whereas only *COX-1* was a risk biomarker of urothelial cancer (**Table 1**).

Chronic inflammation is a recognized risk factor for CRC, and polymorphisms in genes regulating inflammatory processes appear to modify the risk of neoplasia and the efficacy of non-steroidal anti-inflammatory drugs in CRC chemoprevention. *COX-1* polymorphism G213G was significantly associated with an increased CRC (186). Finally, another study reports four *COX-1* variants that were associated with CRC survival. rs1213266 was associated with approximately 50% lower CRC mortality. Three other variants, including L237M, resulted in significantly elevated CRC mortality risk (187).

Proteins related to FAs transportation are also relevant as cancer biomarkers. Carnitine palmitoyltransferase, CPT1A, is a protein that is responsible for the translocation of FAs from the cytosol to the mitochondrial matrix, where FA oxidation occurs. Associations of shorter disease-free survival with CPT1A positivity in invasive lobular carcinoma of the breast have been found (142).

Another study recognized a gene expression signature composed of 19 genes associated with FAO that was significantly associated with breast cancer patient survival. These 19 genes are referred to as the "fatty acid oxidation (FAO)" signature. Included in this signature were genes that have previously been identified as the core components of the FA β-oxidation pathway, such as *CPT1A*. Moreover, the expression of CPT1A was elevated in estrogen receptor–positive, compared to estrogen receptor–negative tumors and cell lines (143). Data from TCGA clearly confirm a *CPT1A* association with poor prognosis in breast cancer, whereas *CPT1A* is a marker of good prognosis in renal cancer and *CPT1C* in pancreas (**Table 1**).

Other relevant FA transporter is CD36. CD36, a scavenger receptor expressed in multiple cell types, mediates lipid uptake, immunological recognition, inflammation, molecular adhesion, and apoptosis. CD36 has been continually proposed as a prognostic marker in diverse cancers, mostly of epithelial origin (breast, prostate, ovary, and colon) and also for hepatic carcinoma and gliomas (141). Through systematic analysis of the multiple omics data from TCGA, it has been found that the most widely altered lipid metabolism pathways in pan-cancer are FA metabolism, AA metabolism, cholesterol metabolism, and peroxisome proliferator-activated receptor (PPAR) signaling. Genes related to lipid metabolism and immune response that were associated with poor prognosis were discovered including *CD36* (188).

Cholesterol-Related Alterations as Biomarkers of Cancer Prognosis and Survival

First step of cholesterol or mevalonate pathway is catalyzed by acetyl-coenzyme A ACAT1 (**Figure 2**). ACAT1 is a mitochondrial enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of AcCoA. An increased expression of ACAT1 in intratumor cholesteryl ester–rich breast tumors was reported (189). Also it has been proposed that ACAT1 expression could serve as a potential prognostic marker in prostate cancer, specifically in differentiating indolent and aggressive forms of cancer (144, 145). Data from TCGA suggest that *ACAT1* is a marker of good prognosis in liver and renal tumors. Interestingly, isoform 2 (*ACAT2*) is a marker of good prognosis in CRCs, whereas in endometrial and renal tumors, *ACAT2* has the opposite effect (**Table 1**).

Next step in cholesterol synthesis is mediated by 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS). This enzyme, with two isoforms, condenses AcCoA with acetoacetyl-CoA to form HMG-CoA, which is the substrate for HMG-CoA reductase. *HMGCS2* expression is associated with reduced clinical prognosis and outcomes in patients with CRC and oral squamous cell carcinoma. It has been suggested that HMGCS2 may act as a helpful prognostic marker and essential target for potential therapeutic strategies against advanced cancer (146). Also, it has been described that HMGCS2 works as a tumor suppressor and has a prognostic impact in prostate cancer, capable of predicting the risk of biochemical recurrence (147). However, in TCGA population, both isoforms are favorable makers of renal cancer. Besides, *HMGCS2* determines good prognosis in ovarian and liver cancer (**Table 1**).

HMGCR is the rate-limiting enzyme of the mevalonate pathway (**Figure 2**). HMG-CoA reductase expression in CRC and breast cancer correlates with favorable clinicopathological characteristics and an improved clinical outcome (148–150). Besides, *HMCGR* expression is a predictor of response to tamoxifen in breast cancer (190) and also may predict patient response to radiotherapy in ductal carcinoma *in situ* (191). In TCGA subset, *HMGCR* also is a good prognosis marker of renal tumors (**Table 1**). Statins, lipid-lowering compounds commonly used in cardiovascular disease, are competitive inhibitors of HMGCR. The value of HMGCR as a predictor of response to neoadjuvant or adjuvant statin treatment in cancer was also studied (192).

Once that cholesterol is synthesized, there are several cholesterol transporter proteins that play key roles in cholesterol and phospholipids homeostasis. The ATP-binding cassette transporter ABCA1 is a transmembrane protein responsible

for the reverse cholesterol transport from the inner cell to circulatory system. *ABCA1* is significantly overexpressed in patients of all stages of CRC, and its overexpression gives proliferative advantages together with caveolin-1–dependent increased migratory and invasive capacities (151). Individuals with upregulation of *ABCA1* expression have an improved risk of CRC recurrence and OS independently of tumor stage (8). *ABCA1* also forms part of the metabolic-signature ColoLipidGene able to precisely stratify stage II CRC with 5-fold higher risk of relapse (7). Moreover, the presence of tumoral genetic variants located in *ABCA1* coding region seems to be associated with CRC risk of death (8). In other tumor types, ABCA1 expression was related to positive lymph nodes, but not significantly associated with tumor recurrence or breast cancer–specific survival (193).

Together with ABCA1, ATP-binding cassette G1 (ABCG1) also initiates and propagates cellular cholesterol efflux. Several genetic variants in *ABCG1* have been associated with survival of NSCLC patients (194). Moreover, *ABCG1* expression seems to be a favorable prognostic marker of renal cancer in data from TCGA (**Table 1**).

Other members of the family are the ATP-binding cassettes G4, G5, and G8. High ABCG4 expression has been associated with poor prognosis in NSCLC patients treated with cisplatin-based chemotherapy (152). ABCG5 positivity in tumor buds have been proposed as an indicator of poor prognosis in node-negative CRC patients (153), whereas in TCGA tumors, *ABCG5* seems to have a favorable effect in liver prognosis (**Table 1**).

While cellular cholesterol efflux is mainly performed via ABCA1, cholesterol uptake is principally executed via the LDLR. The prognostic value of LDLR expression was analyzed in CRC where authors found that the absence of LDLR predicts a shorter survival (154). In the same line, lower LDLR expression was an independent prognostic factor associated with longer survival in patients with small cell lung cancer (195). By contrast, TCGA data suggest that *LDLR* could be a bad prognostic marker of pancreatic, renal, and urothelial cancers (**Table 1**).

Lipid-Related Transcription Factor Alterations as Biomarkers of Cancer Prognosis and Survival

Five are the main transcription factors that regulate the expression of mediators of lipid metabolism: SREBP1, SREBP2, PPARγ, NR1H3, and NR1H2. Sterol regulatory element-binding protein 1 (SREBP1) is a known transcription factor of lipogenic genes, which plays important roles in regulating *de novo* lipogenesis. *SREBP1* is overexpressed and strongly associated with worse clinical outcomes in breast cancer (155). Moreover, SREBP1 also seems to have an essential role in pancreatic cancer, regulating tumorigenesis and being associated with bad prognosis (196). However, data from TCGA propose *SREBP1* as a favorable prognostic marker in pancreatic and endometrial cancers (**Table 1**).

The combined expression of sterol regulatory element-binding protein 2 (*SREBP2*) together with *HMGCR*, *NR1H3*, and *NR1H2* genes was associated with poor CRC clinical outcome

TABLE 2 | Preclinical and clinical studies with main drugs evaluated to target the altered lipid metabolism in cancer.

Target	Drug	Type of cancer	Preclinical/clinical trial	
FASN	Cerulenin	Breast Cancer		(48)
		Ovarian Cancer		(201)
	C75	Renal Cancer		(59)
		Breast Cancer		(53)
		Lung Cancer		(43)
	Orlistat	Melanoma		(57, 202)
		Prostate Cancer		(86)
	Fasnall	Breast Cancer		(87)
	C93	NSCLC		(42, 43)
	C247	Breast Cancer		(44)
	TV3166	CRC		(45)
	TVB-2640	NSCLC	NCT03808558	(56)
		TNBC	NCT03179904	(56)
		HG Astrocytoma	NCT03032484	(203)
		Ovarian, Breast Cancer	NCT02223247	(204)
	Triclosan	Breast		(58, 60)
ACLY	SB-204990	NSCLC, Prostate, Ovarian		(51)
		NSCLC		(61)
ACC1/2	ND-630 (GS-0976)	NASH		(71)
	TOFA	HNSCC		(205)
		Ovarian		(33)
	ND-654	HCC		(34)
	GS-0976	NASH		(36)
			NCT02856555	(35)
	ND-646	NSCL		(206)
SCD1	CVT-12	HCC		(207)
	SSI-4	HCC		(208)
	Betulinic acid	CRC		(209)
		GBC		(210)
	MF-438	NSCLC		(211)
	A939572	NSCLC		(212)
		ccRCC		(213)
		Prostate		(213)
CPT1A	Etomoxir	Leukemia		(214)
	Ranolazine	Prostate Cancer		(215)
		Glioblastoma		(216)
	Etomoxir, Ranolazine, Perhexiline	Prostate Cancer		(217)
	Perhexiline	CLL		(218)
		Breast Cancer		(219, 220)
SREBP	Betulin	HCC		(221)
		Melanoma		(222)
	Fatostatin	Prostate		(223, 224)
		Glioma		(225)
		HCC		(226)

(Continued)

TABLE 2 | Continued

Target	Drug	Type of cancer	Preclinical/clinical trial	
LXR	T0901317/GW3965	BPDCN		(227)
	LXR623 and GW3965	Colon/Glioblastoma		(228)
	GW3965	Glioma		(229)
ACAT1	Avasimive	Prostate/Colon Cancer		(230)
		GBM		(231)
		CML		(232)
CD36	FA6.152	Oral Cancer		(80)
		Prostate Cancer		(233)
HMGCR	Fluvastatin	Prostate	NCT01992042	(234)
			NCT00608595	
	Simvastatin	CRC	NCT00994903	(235)
		NSCLC	NCT00452244	(236)
MAGL	URB602	Colon		(237)
PTGS2	Celecoxib	Lung Cancer		(238)
		Ovarian Cancer (HFD)		(239)
		NSCLC	NCT00046839	(+)
		PDAC	NCT01111591	(240)
		Prostate cancer	NCT00073970	(+)
		Early CRC	NCT00608595	(+)
PPARG	VSP-17	Breast Cancer		(241)
FABP4	BMS309403	HCC		(242)
		Prostate Cancer		(243)
	FABP5	SBFI26	CRPC	(244)

(+) Unpublished results.

independent of lymph node metastasis, distant metastasis, and advanced stage (156). Besides, expression of SREBP-2 was elevated in advanced pathologic grade and metastatic prostate cancer and significantly associated with poor clinical outcomes (157).

The PPARγ is a nuclear receptor that controls expression of mediators of lipid metabolism but also the inflammatory response. Additionally, it has been demonstrated that PPAR b/d and a isotypes also have important roles in FAO, FA storage, and cholesterogenesis (197).

Decreased expression of PPARγ has been observed in many tumor types. In this sense, reduced PPARγ expression within the tumor is associated with poor prognosis in lung cancer patients (158, 159). In the same line, tumor expression of PPARγ is independently associated with increased survival of CRC patients (160). Also in patients with breast and prostate cancer, PPARγ is a marker of better prognosis and is associated with better survival (161–163). Importantly, one study reports that cytoplasmic PPARγ expression appeared as an independent marker of poor prognosis in primary breast cancers (198). TCGA analysis proposed PPARγ as a favorable prognostic marker for renal and urothelial cancers (**Table 1**).

Finally, several studies have also evaluated the association between PPARγ genetic variants and the risk of CRC (199). In patients with stages II/III CRC, polymorphism rs1801282 in PPARγ was significantly associated with tumor recurrence (200).

NR1H3 and *NR1H2* encode for liver X receptor (LXR) α and LXR β, respectively. They are intimately related nuclear receptors that react to elevated levels of intracellular cholesterol by enhancing transcription of genes that control cholesterol efflux and FA biosynthesis. *NR1H3* expression was significantly correlated to better survival in completely resected stages II and III NSCLC patients (164). Moreover, one study reports that *NR1H3* and *NR1H2* belong to a transcription signature associated with poor CRC clinical outcome independent of lymph node metastasis, distant metastasis, and advanced stage (156). This result is validated in TCGA dataset (**Table 1**) where *NR1H2* was also associated with CRC poor prognosis.

TARGETING THE ALTERED LIPID METABOLISM IN CANCER

Because of the essential role of FAs for cancer cell proliferation and progression, drugs to target lipogenic enzymes and/or transcription factors regulating the intracellular lipid homeostasis are considering as promising therapeutic strategies against cancer.

Different drugs have been already evaluated to target (i) lipogenic enzymes (FASN, ACLY, ACC); (ii) the exogenous lipid uptake (LXR, CD36, FABP4/5); (iii) inflammatory signaling pathways (PTGS2); (iv) regulation of intracellular lipid homeostasis (PPARγ, CPT1a, lipin2, HSL, MAGAT, DAGAT…); and/or (v) saturated vs. unsaturated FAs. Their efficacy has been demonstrated in numerous models of cancer, including *in vitro* preclinical and clinical studies.

In **Table 2**, we summarize main drugs evaluated in preclinical and clinical studies. Nevertheless, although the results of these studies are encouraging, side effects due to the many different regulatory mechanisms of lipid metabolism are still a big challenge.

Recently, there is growing interest on complementary approaches by means of dietary interventions for cancer treatment. The success of such interventions requires a deep knowledge of the metabolic requirements of tumors, considering the nutritional status of the individuals—obesity, metabolic syndrome and/or insulin resistance, among others—and the genetic susceptibilities to metabolic alterations. Moreover, the knowledge of the molecular targets and mechanism of action of dietary ingredients will be crucial to apply these approaches with the conventional chemotherapy in order to improve the responses to the clinical treatments and the well-being of patients.

Precision nutrition should be considered at three levels: (1) nutritional guidelines based on age, gender, and other sociocultural factors; (2) individualized recommendations after refined phenotyping; and a (3) genetic-nutrition based on genetic variants with high penetrance and on the response to nutritional interventions (6).

The improvement of the "omics" sciences, including transcriptomics, proteomics, metabolomics, lipidomics, and metagenomics, provides a more complete scenario for

TABLE 3 | Preclinical and clinical studies with bioactive compounds from natural sources to target the altered lipid metabolism and/or associated risk factors (mainly obesity and T2DM) in cancer.

Family	Bioactive compounds	Molecular targets, metabolic effects	Preclinical/clinical trials	References
Polyphenols				
Flavonoids	Gallic acid and its derivatives EGCG, gallate, ethyl gallate, gallocatechin gallate, methyl gallate, propyl gallate, theaflavin-3-gallate	↑AMPK, FAO, thermogenesis		(258)
		↓antiobesity		(259)
		↓Cholesterol, LDL	NCT02147041	(260)
		↓lipogenesis, ↓PPARG, LXR, ↑AMPK		(261, 262)
		↑AMPK, SIRT, PGC1a, FAO, UCP1, CYp7a1		(263)
		↓dyslipidemia		(264)
		↓dyslipidemia	NCT02627898	(265)
		↑FAO, ↓antiobesity	NCT02381145	(266)
		↓HOMAIR, T2DM	Human study	(267)
	Citrus flavonoids			
	Nobilettin	↓HSL, ACC, ↑AMPK, CPT1a, ACOX1, FAO		(268)
	Naringenin	↑PPARα, CPT-1, UCP-2, FAO, ↓SREBP1c, 3HMGCR, hepatic steatosis		(269–272)
	Tangeretin	↑PPARα, FAO		(273)
	Hesperetin	↑PPARα, PPARγ, AMPK, FAO, ↓lipogenesis		(274)
	Baicalin	↓SREBP-1c, FASN, ACC		(275)
	Hispidulin	↑PPARα, CPT1α ↑Acat1, Acad1, HMGCS2		(276, 277)
	Mangiferin	↓inflammation, T2DM, steatosis, ACC, DGAT2, ↑ FAO(CPT1a)		
	Dihydromyricetin	↓hepatic steatosis	ChiCTRTRC12002377	(278)
	Berberin	↓hepatic steatosis, TG and cholesterol levels	NCT00633282	(279)
	Luteolin	↑FAO, ↓lipogenesis, cholesterogenesis, HMGCS1	NCT00633282	(280)
	Quercetin	↓ CYP2E1, inflammation, obesity, T2DM		(281, 282)
Stilbenos	Resveratrol	↓ steatosis, adipogenesis, SREBP1c, lipin1, ACC, ↑AMPK, SIRT1, FAO	(283–285)	
Curcuminoids	Curcumin	↓steatosis, adipogenesis, SREBP1c, FASN, SCD1, GPAT-1, ↑1AMPK, FAO	(286, 287)	
Phenolic acids	Ellagic acid	↓steatosis, Insulin resistance		(288)
Terpenoids				
	Carnosol	↓hyperglycemia, inflammation, lipogenesis, anticancer		(289, 290)
	Betulinic acid	↓SCD, steatosis, lipogenesis		(209)
	Ursolic acid	↑AMPK, FAO, ↓lipogenesis		(291)
	Ginsenoside	↑AMPK, perilipin, FAO		(292–294)
	Licopene	↓inflammation	ISRCTN99660610	(295)

personalized nutritional interventions (13, 245). The main challenge is to define tumor heterogeneities, which can be originated by genomic, epigenomic, transcriptomic, and immune variability. This will lead to patients' stratification for personalized treatments in the clinics (246).

Nutrigenetics aims to study the effect of genetic variants on the dietary response and the risk of several diseases. For example, SNPs in the *CD36* gene associate with dyslipidemia when high amounts of fats are consumed (247). In addition, dietary ingredients affect cancer risk and progression affecting gene expression. Nutrigenomics considers the effect of diet-derived ingredients on gene expression and, consequently, on the proteome and metabolome.

Dietary ingredients and nutrients from natural sources, such as epigallocatechin-3-gallate, curcumin, sulforaphane, and genistein, have been shown to have anticancer properties regulating the expression of genes related to cancer. Polyphenols contribute to the prevention of obesity through the modulation of genes implicated in adipogenesis, lipolysis, and FAO (248–251).

Importantly, in the frame of precision nutrition, dietary interventions might also provide systemic responses affecting the antitumoral response of the immune system, as well as the reduction of low-grade chronic inflammation, dyslipidemia, insulin resistance, and/or obesity.

The direct association of diet with obesity and dysbiosis requires further research to understand the impact of diet on cancer prognosis. High intake of saturated FAs increases the expression of genes related to inflammation, insulin resistance, and/or hepatic steatosis. In contrast, Mediterranean diet downregulates the expression of genes related to oxidative stress, inflammation, and/or insulin signaling (252, 253). Importantly, high levels of triglycerides and LDLs have been associated with CRC prognosis and distant metastasis. Cholesterol in high-fat diets associates with colorectal tumorigenesis (254). Ceramide sphingolipids have been shown to be antitumoral in combination with tamoxifen (255). Phosphatidylcholine is increased in CRC cells. Increased intake of MUFAs is associated with reduce inflammation in CRC cancer (256). Energy-restricted diets supplemented with EPA and α-lipoic acid increase the expression of FAO genes, diminishing the expression of genes related to *de novo* lipogenesis and inflammation (257) (**Table 3**).

Importantly, the efficacy of fasting cycles or cycles of fasting mimicking diets in dampening tumor development has already been established (296), and the implementation of other dietary approaches for cancer therapy is likely to take a similar approach.

CONCLUDING REMARKS

Metabolic alterations of tumors have been well-recognized as one of the hallmarks of cancer. At present, several investigations have demonstrated the consequences of lipid metabolism deregulation in cancer not only sustain tumor growth but also promote cell migration, invasion, and angiogenesis. In this review, we have discussed about the main lipid metabolism alterations found in cancer by describing their mechanism of action and their oncologic implications. Importantly, we emphasize the crucial role of the aberrant lipid metabolism not only affecting the primary tumors but also shaping the tumor microenvironment to promote malignancy and dissemination. Moreover, we have explored the available public data bases containing mRNA data (TCGA) and protein expression data (The Human Protein Atlas) to obtain a global view of the putative implications of lipid metabolism–related genes in cancer prognosis of the most frequent types of cancer according to the WHO: lung, CRC, breast, and prostate cancers.

We also highlight the relevance of "omics" technologies, including genomic and transcriptomic data, considering the phenotypic metabolic status (mainly obesity) to define lipid metabolic scores to be integrated into the clinical advice. Thus, the use of this knowledge will allow a better stratification of patients, which will be translated into improvements on the OS and well-being of the patients. In the frame of precision medicine, new clinical trials integrating classical chemotherapies with precision nutrition–based strategies—bioactive products and diet derived nutrients—will provide an unquestionable line of research in cancer treatment.

AUTHOR CONTRIBUTIONS

LPF and MGC wrote the paper. AR performed the critical revision of the article. All authors conceptually designed the manuscript.

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Five microRNAs in Serum Are Able to Differentiate Breast Cancer Patients From Healthy Individuals

*Andrea Feliciano[1], Lucila González[1], Yoelsis Garcia-Mayea[1], Cristina Mir[1], Mireia Artola[2], Nieves Barragán[2], Remedios Martín[2], Anna Altés[2], Josep Castellvi[1], Sergi Benavente[1], Santiago Ramón y Cajal[1], Martín Espinosa-Bravo[3], Javier Cortés[4,5], Isabel T. Rubio[6] and Matilde E. LLeonart[1,7]**

[1] Biomedical Research in Cancer Stem Cells Group, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain, [2] Primary Care Center CAP-Vallcarca-Sant Gervasi, Barcelona, Spain, [3] Breast Pathology Unit, Vall d'Hebron University Hospital, Barcelona, Spain, [4] Institute of Breast Cancer, Quiron Group, Barcelona, Spain, [5] Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain, [6] Breast Surgical Oncology, University of Navarra Clinic, Madrid, Spain, [7] Spanish Biomedical Research Network Center in Oncology, Madrid, Spain

Breast cancer is the cancer with the most incidence and mortality in women. microRNAs are emerging as novel prognosis/diagnostic tools. Our aim was to identify a serum microRNA signature useful to predict cancer development. We focused on studying the expression levels of 30 microRNAs in the serum of 96 breast cancer patients *vs.* 92 control individuals. Bioinformatic studies provide a microRNA signature, designated as a predictor, based on the expression levels of five microRNAs. Then, we tested the predictor in a group of 60 randomly chosen women. Lastly, a proteomic study unveiled the overexpression and downregulation of proteins differently expressed in the serum of breast cancer patients *vs.* that of control individuals. Twenty-six microRNAs differentiate cancer tissue from healthy tissue, and 16 microRNAs differentiate the serum of cancer patients from that of the control group. The tissue expression of miR-99a, miR-497, miR-362, and miR-1274, and the serum levels of miR-141 correlated with patient survival. Moreover, the predictor consisting of miR-125b, miR-29c, miR-16, miR-1260, and miR-451 was able to differentiate breast cancer patients from controls. The predictor was validated in 20 new cases of breast cancer patients and tested in 60 volunteer women, assigning 11 out of 60 women to the cancer group. An association of low levels of miR-16 with a high content of CD44 protein in serum was found. Circulating microRNAs in serum can represent biomarkers for cancer prediction. Their clinical relevance and the potential use of the predictor here described are discussed.

Keywords: breast cancer, serum, microRNAs, prognosis, diagnosis

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***Correspondence:**
Matilde E. LLeonart
matilde.lleonart@vhir.org

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INTRODUCTION

Breast cancer is one of the most frequent carcinomas and the second leading cause of death in women (1). Specifically, in the United States and Europe, about 1 in 8 women (12.5%) will develop invasive breast cancer over the course of their life. Therefore, comprehensive research should be devoted to cancer prevention in order to scale down these numbers and reach higher life expectancy in affected patients, lower mortality rates, and decline socio-economical burdens due to the high cost of chemotherapeutical treatments.

Currently there is no precise model to estimate breast cancer risk. Most of the predictor models consider clinical factors, including the density of breast tissue, biopsy history, and several clinical parameters. However, such models are not informative at an individual level. Predictive tests (i.e., Oncotype DX, Prosigna, MammaPrint) based on the status of genetic and non-genetic factors in cancer tissue have proven their prognostic and predictive ability in a personalized way (2). Currently, the liquid biopsy is being used to establish the biomarkers that are able to predict or envisage a potential future cancer development risk (3).

microRNAs are key factors in oncogenesis because they contribute to the modulation of key oncogenic and tumor suppressor proteins. In particular, microRNA expression profiling can be used to classify human cancer (4). On the other hand, recent evidence suggests that microRNAs are very stable molecules in serum and that they have been established as biomarkers for some cancer types (5). Interestingly, the level of certain microRNAs in combination with known tumor markers (e.g., CEA or CA15-3) improves sensitivity to breast cancer detection (6). Thereby routine monitoring of circulating microRNAs can result in significant benefits for the prognosis, diagnosis, and breast cancer treatment (7).

Previously, we identified a molecular signature based on 35 microRNAs that vary significantly in normal tissue *vs.* cancer tissue in breast cancer patients (8). According to our previous work and literature search, we selected 30 cancer-related microRNAs that could be potentially detected in serum (miR-96, miR-451, miR-155, miR-195, miR-200c, miR-106b, miR-141, miR-21, miR-486, miR-16, miR-125b, miR-99a, miR-497, miR-191, miR-145, miR-100, miR-144, miR-382, miR-29c, miR-10b, miR-133a, miR-1260, miR-1274a, miR-1274b, miR-133b, miR-92, miR-376c, miR-411, miR-299, and miR-215) (8–11). In the present study, we compared the expression of 30 microRNAs in tumor *vs.* normal tissue and serum from 96 breast cancer patients (in comparison with control serum). Through statistical and bioinformatic studies, we determined a predictor, comprised by five microRNAs, that categorize an individual in the control group or breast cancer group. The potential benefit of this classifier and its validation for breast cancer prediction is discussed.

MATERIALS AND METHODS

Patients and Controls

This study comprises 96 breast cancer patients. For each patient, we had samples of cancer tissue (CANtum), normal tissue (CANnorm), and serum (CANse). For comparison purposes, we had serum from 92 control individuals (CTLse). The method to select the control group established the following criteria: 20- to 80-year-old women, non-smokers, non-drinkers, no evidence of breast cancer in their family history, and healthy women that have had no cancer episodes in the past. For the validation study, we had additional serum from 20 breast cancer patients. Finally, for the test study, we had serum from 60 volunteer women where no selection criteria were applied. The pathological and clinical characteristics of the patients include the presence of estrogen receptor (ER), progesterone receptor (PR), Ki-67 expression, p53, tumor grade determined by tumor heterogeneity (low, medium, and high), tumor stage determined by the size of the tumor and its infiltrating capacity to neighboring local areas (T1b, T1c, or T2), subtype of breast cancer (molecular classification), presence of metastasis, disease-free survival, and overall survival. All patients included in the study were recruited from the Vall d'Hebron Hospital and selected for primary breast cancer. Patients were not treated with radio- or chemotherapy before sample collection. Control individuals were recruited from the Castilla-La Mancha Blood Bank and the Government of Catalonia Blood and Tissue Bank. Volunteer women came from the Primary Care Center (CAP-Vallcarca Sant Gervasi). The study was conducted in accordance with the instructions and requirements stated in the Declaration of Helsinki international standards for studies and approved by the Ethics Committee of Vall d'Hebron Hospital (CEIC). Informed consent was obtained from the patients to participate, analyze, and publish their data.

Sample Collection

Serum was collected from each patient prior surgery. Hemolytic sera (representing 5%) were discarded from the study. Summing up, blood sample was obtained and centrifuged at 1,300 rpm for 10 min and the supernatant fraction (serum) was collected and stored at −80°C. The collection and pre-processing of the cancer samples vs. the healthy ones were treated with the same technical conditions. Normal and tumor tissue were collected from the surgery room and stored at −80°C before RNA extraction. Hematoxylin and eosin staining of the slides from frozen biopsies was validated histologically to ensure that the tissue area had an adequate tumor density (>80%). RNA was isolated with a MirVana kit (Ambion® Life Technologies) according to the manufacturer's instructions. The RNA concentration from tissue was quantified using the Nanodrop-2000 UV-Vis Spectometer (Fisher Scientific) and its quality was determined by the Bioanalyser (RIN ratio> 8).

On the other hand, to verify that in RNA extractions from sera, there was enough RNA to analyze the 30 microRNAs considered in this study, each sample was amplified using RNU and cel-miR-39-3p probes individually using quantitative real-time qRT-PCR (data not shown).

qRT-PCR

The reverse transcription was performed on 10 ng of RNA using specific primers for the 30 selected microRNAs, including endogenous control RNU6 (ID 00973) and the exogenous control cel-miR-39-3p (ID 000200) with the TaqMan commercial kit microRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, CA, USA) as described (8). The pre-amplification reaction was carried out on 5 μl of cDNA product using a pool containing the specific preamplification primers for each microRNA with the TaqMan® PreAmp Master Mix 2×

Abbreviations: CANnorm, normal tissue; CANtum, cancer tissue; CANse, cancer serum; Ct, cycle threshold; CTLse, control individual serum; dCt, Ct reference gene – Ct gene of interest; Down, downregulated; FC, Fold change; qRT-PCR, Quantitative Real-time PCR; RIN, RNA integrity number; Up, upregulated.

solution (Applied Biosystems, Life Technologies, CA, USA). Reactions were performed in the Veriti™ Thermal Cycler Assays thermocycler (Applied Biosystems, Life Technologies, CA, USA). The study of each microRNA levels was conducted by triplicate. The references used for each microRNA are the following: mir-125b-5p (ID 000449), mir-99a-5p (ID 000435), mir-100-5p (ID 000437), mir-497-5p (ID 001043), mir-1274b (ID 002884), mir-106b-5p (ID 000442), mir-1260a (ID 002896), mir-141-3p (ID 000463), mir-96-5p (ID 000186), mir-21-5p (ID 000397), miR-1274a (ID 002883), mir-145-5p (ID 002278), mir-299-5p (000600), mir-376c-3p (ID 002122), mir-451a (ID 001141), mir-486-5p (ID 001278), cel-miR-39-3p (ID 000200), U6, miR-16-5p (ID 000391), mir-195-5p (ID 000494), mir-191-5p (ID 002299), mir-215-5p (ID 000518), mir-382-5p (ID 000572), mir-411-5p (ID 001610), mir-10b-5p (ID 002218), mir-155-5p (ID 002623), mir-200c-3p (ID 002300), mir-144-5p (ID 002148), mir-92a-3p (ID 000431), mir-133a-3p (ID 002246), mir-133b (ID 002247), mir29c-3p (ID 000587), and miR-362 (ID478058). **Supplementary Tables 1, 2** show the raw qRT-PCR data for the indicated microRNAs in tissue and serum samples, respectively. **Supplementary Tables 3, 4** show the qRT-PCR results for the indicated microRNAs in tissue and serum samples respectively upon normalization. The probes cel-miR-39-3p and RNU6 were used as internal controls, both to monitor the efficiency of RNA isolation and subsequent retrotranscription and to normalize possible variations between samples during RNA isolation. Although RNU6 is used as one of the most frequent endogenous controls to study profiling microRNA in cell and tissue samples, it is not a suitable endogenous control to study the expression of serum microRNAs (12). Therefore, in order to compare the data, results were normalized using the quantile method (using the normalized CtData function of the R package HTqRTPCR) including all patients per sample type.

Proteomic Study

Serum from 70 breast cancer patients and 70 controls was studied at protein level. Each sample was depleted individually using the Pierce™ Abundant Protein Depletion Spin Columns kit (ref. 13434319, Thermo Scientific™) according to the manufacturer's instructions. This kit eliminates ∼95% of 12 abundant proteins in serum (α1-Acid Glycoprotein, Fibrinogen, α1-Antitrypsin, Haptoglobin, α2-Macroglubulin, IgA, Albumin, IgG, Apolipoprotein A-I, IgM, Apolipoprotein A-II, and Transferrin), allowing the identification of other proteins in the samples. The quantitative study of proteins was performed through Tandem Mass Tag marking as previously described (13). Then, samples were grouped by pools (nine cancer pools and nine control pools) for sequencing. Each pool (80 μg of protein) was composed of equivalent amounts of seven samples of each type (cancer or control). Sequencing was performed by quantitative liquid chromatography tandem mass spectrometry using an LTQ-Orbitrap XL instrument as described above (14).

Statistical Analysis

The study has been conducted using Leave-One-Out Cross Validation (LOOCV) as cross-validation technique, thus ensuring greater robustness in the results obtained (15).

Mann–Whitney *U*-test was used to identify microRNAs differently expressed between patients and controls. Benjamini–Hochberg's false discovery rate (FDR) method was used to correct for multiple testing. The analysis to select the differently expressed microRNAs has been based on the fitting of a linear model.

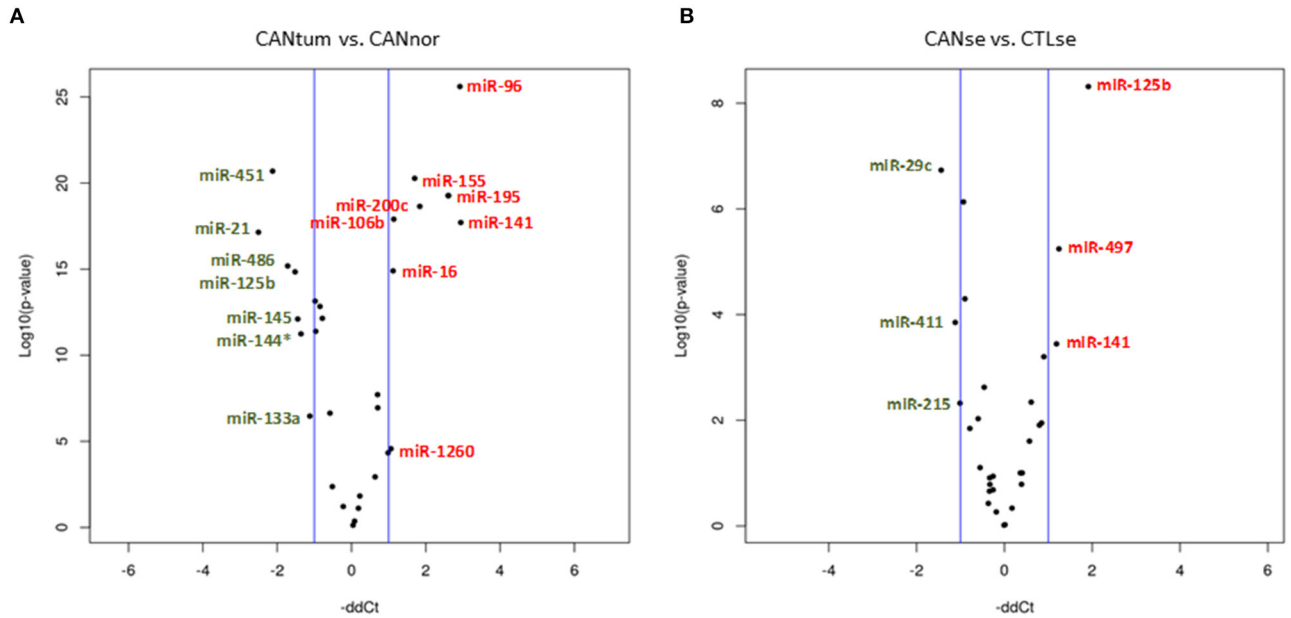
For the predictor, we considered that the best classification method was CART (Classification and Regression Trees) (16). The statistical analyses have been performed using ExpressionSuite (Life Technologies, CA, USA) (R version 3.5.1, copyright© 2018, Foundation for Statistical Computing, Vienna, Austria) and the libraries developed for microRNA-target analysis by the Bioconductor Project (www.bioconductor.org). Regarding the validation of the microRNA expression with the pathological characteristics of the patients, ANOVA and *t*-test methods were used (SPSS v9.3). A statistical analysis to determine differential proteins and peptides was performed using DanteR software (http://omics.pnl.gov/software/danter). $p < 0.05$ were considered significant.

RESULTS

Tumor-Associated microRNAs in Breast Cancer

For the selection of the microRNAs studied here, they were selected: (a) the 17 most significantly deregulated microRNAs in breast cancer based on our previous work (miR-21, miR-96, miR-141, miR-1274a, miR-1260, miR-1274b, miR-106b, miR-299, miR-486, miR-376c, miR-497, miR-195, miR-100, miR-145, miR-99a, miR-451, and miR-125b) and (b) the potentially relevant microRNAs in the serum of breast cancer patients (miR-155, miR-200, miR-16, miR-191, miR-144, miR-382, miR-29c, miR-10b, miR-133a, miR-133b, miR-92, miR-411, and miR-215) (8–11, 17). The following microRNAs were studied in serum and cancer tissue in comparison with control individuals: miR-21, miR-96, miR-141, miR-1274a, miR-1260, miR-106b, miR-1274b, miR-299, miR-376c, miR-497, miR-195, miR-100, miR-145, miR-99a, miR-451, miR-125b miR-486, miR-16 (only serum), miR-191, miR-215, miR-382, miR-411, miR-106, miR-155, miR-200c, miR-144, miR-92a, miR-133a, miR-133b, miR-29c, and miR-362 (only tissue) (8).

Supplementary Table 5 shows the 26 microRNAs differently expressed when comparing tumor tissue with normal tissue in 96 breast cancer patients and 92 control individuals ($p < 0.05$). The volcano plot shows the most relevant microRNAs (**Figure 1A**) ($p < 0.01$). **Supplementary Table 6** shows that 16 microRNAs (out of 30 initially selected) are significantly deregulated when comparing the serum from cancer patients *vs.* the serum from control individuals. The volcano plot shows the top significant microRNAs (**Figure 1B**). The miR-125b and RNU6 levels were validated by another approach based on the manual performance of the Assays-on-Demand Taqman Gene Expression Assays according to the procedure previously described (data not shown) (18). In order to check if the microRNAs expressed in the tumor reflect the same trend in the serum of breast cancer patients, we compared significant



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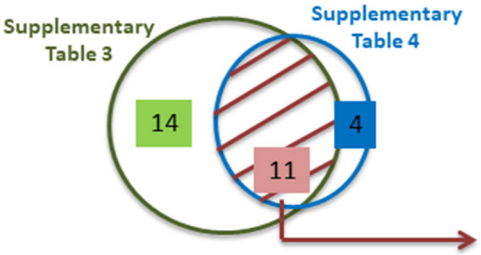

microRNA	CANtum/CANnor	CANse/CTLse	R (Pearson)	95% CI	p value
miR-125b	Down	Up	-0.129	(-0.324, 0.077)	0.217
miR-29c	Up	Down	-0.036	(-0.238, 0.169)	0.735
miR-497	Down	Up	0.417	(0.233, 0.572)	0
miR-191	Down	Down	-0.047	(-0.249, 0.158)	0.652
miR-141	Up	Up	0.168	(-0.037, 0.359)	0.108
miR-133a	Down	Up	0.163	(-0.043, 0.355)	0.12
miR-155	Up	Down	0.119	(-0.087, 0.315)	0.255
miR-100	Down	Up	-0.094	(-0.292, 0.112)	0.37
miR-1260	Up	Down	0.056	(-0.324, 0.077)	0.217
miR-133b	Down	Up	0.809	(0.725, 0.869)	0
miR-96	Up	Up	0.217	(0.014, 0.403)	0.036

FIGURE 1 | MicroRNAs expression in breast cancer. **(A)** Volcano plots indicating the top deregulated microRNAs when cancer tissue *vs.* normal tissue was compared (green, downregulated microRNAs; red, upregulated microRNAs). **(B)** Volcano plots indicating the top deregulated microRNAs when the serum of cancer patients was compared with a group of control serum. **(C)** Left side: Eleven commonly deregulated microRNAs when tissue and serum samples were compared. Right side: Table showing the potential association between the expression of 11 microRNAs in tissue and serum. It can be observed that three microRNAs (miR-497, miR-133b, and miR-96) have a statistically significant correlation coefficient (*R*) for a 95% confidence interval (CI) ($p < 0.05$). As indicated in the table, the microRNA values of the cancer tissue are relativized to normal tissue and the microRNA values of the cancer sera are relativized to the control sera. Up, upregulated; Down, downregulated.

microRNAs in the tumor tissue and serum in all patients. Eleven out of 16 significant microRNAs were deregulated in both samples: tumor tissue of cancer biopsies and serum (**Figure 1C**, **Supplementary Tables 5, 6**). Three microRNAs, miR-191, miR-141, and miR-96, followed the same trend when the tumor and serum of cancer patients were compared (**Figure 1C**).

Pathological and Clinic Characteristics of the Tumors

The pathological characteristics of the patients are shown (**Supplementary Table 7**). **Supplementary Figure 1** shows the serum microRNAs that correlate with tumor stage. **Supplementary Figure 2** shows the tumor microRNAs that correlate with tumor grade. **Supplementary Figure 3** shows the tumor microRNAs that correlate with tumor stage. We found that the expression of miR-99a, miR-497, miR-62, and miR-1274a correlated with overall survival (**Figure 2A**). In addition, miR-362 and miR-133b expression correlated with disease-free survival (**Figure 2A**). In addition, we found that high miR-141 expression in the serum of breast cancer patients correlated with better survival (**Figure 2B**). There is a lack of correlation regarding the studied microRNAs with the molecular classification of tumors (19).

Construction of a Predictor

The experimental design of the study is summarized in **Figure 3**. In order to establish a microRNA signature designated here as predictor, statistical and bioinformatic studies were performed in the serum from 92 control women and 96 breast cancer patients. Accordingly, the minimal number of microRNAs able to predict whether a serum sample should be categorized as

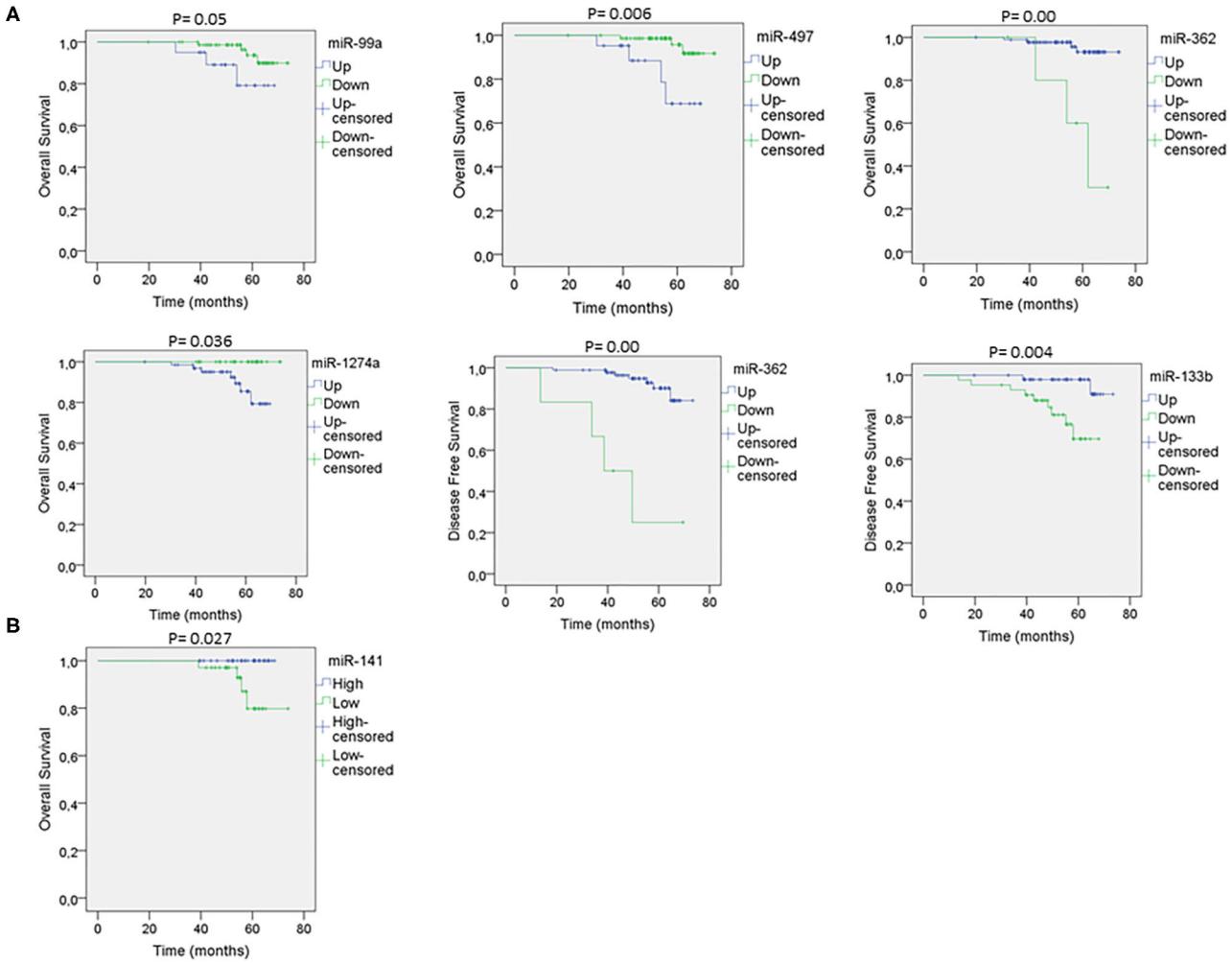

FIGURE 2 | Prognosis-related microRNAs. **(A)** Kaplan–Meier curves showing that miR-99a, miR-497, miR-362, and miR-1274a levels in the tissue sample correlate with survival and/or disease-free survival. Up, upregulated; Down, downregulated. **(B)** Kaplan–Meier curve showing that miR-141 levels in the serum correlate with survival. Low, low expression; High, high expression.

control or cancer was reduced to five: miR-125b, miR-29c, miR-16, miR-1260, and miR-451 (**Figure 3**). The proposed microRNA signature that derives exclusively from serum samples has the following percentages of accuracy, sensitivity, and specificity: 90.43, 90.62, and 90.22%, respectively (**Figure 3**). The internal classification error was 9.26%.

Later on, in an external validation phase, the predictor was used to verify the status of the serum from 20 additional cancer patients plus 60 serum samples from a group of volunteer women taken randomly to be tested by the predictor. **Supplementary Table 8** shows the raw qRT-PCR data for the indicated microRNAs in serum samples. **Supplementary Table 9** shows the qRT-PCR results for the indicated microRNAs in serum samples upon normalization. All serum samples were confirmed as cancer patients (**Figure 3**). Eleven out of 60 samples were classified as cancer patients (**Figure 3**). The percentages of accuracy, sensitivity, and specificity of this later study are 86.25, 100, and 81.67%, respectively (**Figure 3**). The internal classification error was 5.45%.

Proteomic Study

A total of 110 significantly deregulated proteins were found when comparing the serum of cancer patients *vs.* the serum of healthy individuals (**Supplementary Table 10**). Thirty-five proteins were selected as the top differently expressed ones between cancer *vs.* normal serum using a fold change (FC) ratio above 1.2 or below 0.8 (**Figure 4A**). By using the multiMiR Bioconductor's package, microRNA–gene target interactions were explored (20). The search for validated targets was performed across miRecords, miRBase, and TarBase databases. A total of 3,947 validated unique target genes were found to the 16 microRNAs deregulated in serum (data not shown). CD44 protein (upregulated in the serum pools from breast cancer patients *vs.* the pools from the control group patients) was found in the list of the 3,947 validated

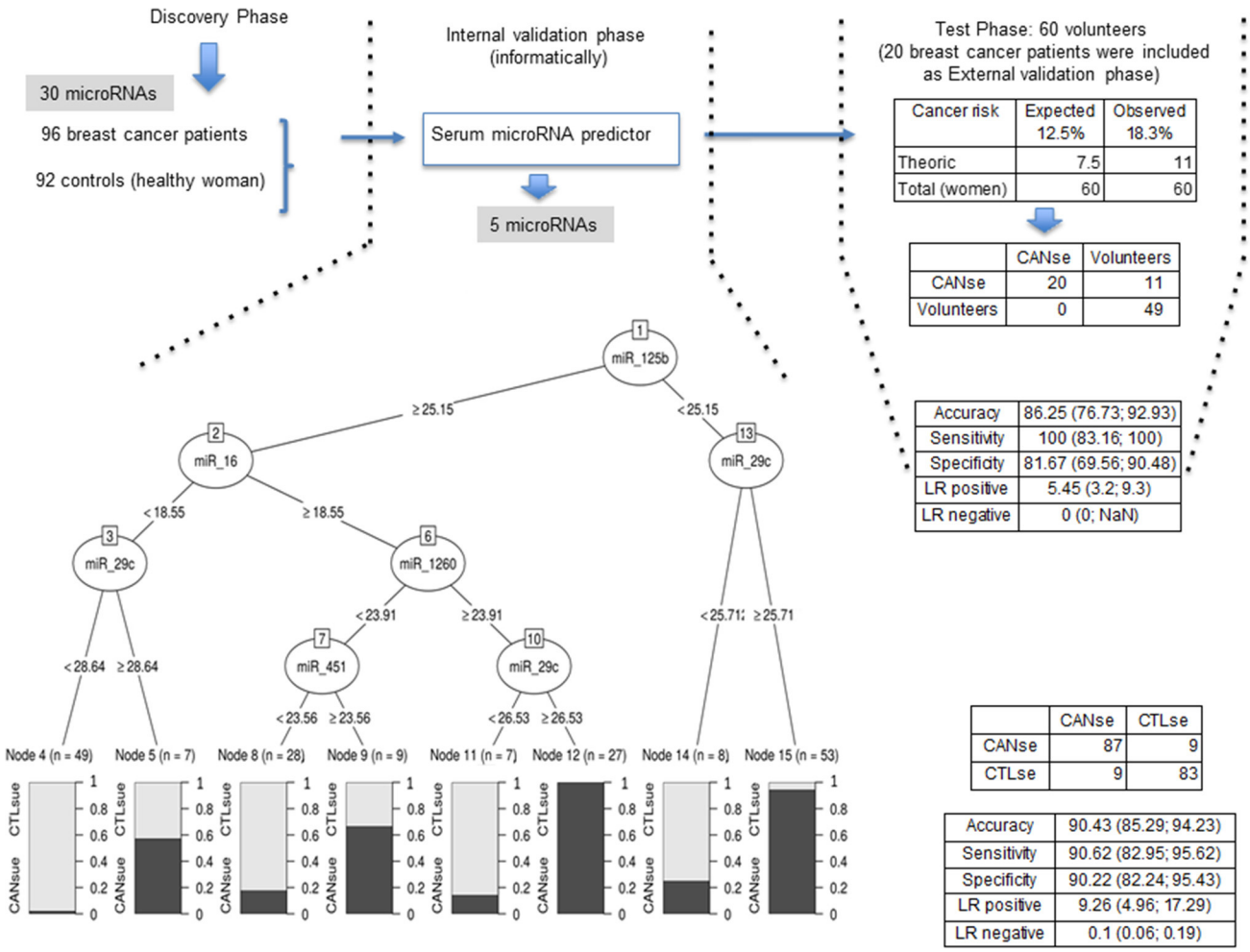

FIGURE 3 | Flow chart of the analysis design in the present study. The expression change-based method pipeline is described (left). The interval validation phase provides 5 microRNAs revealed by the predictor (middle). The validation and test phase comprises 20 patients and apparently 60 healthy women (right). Indicated values of accuracy, sensitivity, and specificity are shown for each phase.

targets. CD44 inversely correlates with miR-16 expression, which appears downregulated in the serum from cancer patients in comparison with controls (**Supplementary Table 10**, **Figure 4B**). The 35 proteins were classified accordingly to their involvement in different regulatory pathways (**Figure 4C**). Among them, CST3 (Cystatin C) seems to be involved in the modulation of different pathways (**Figure 4D**).

DISCUSSION

The final purpose of this research is to establish a microRNA signature associated with breast cancer to determine molecular evidence of cancer that will lead to future cancer development in serum samples. Firstly, we found 26 microRNAs significantly deregulated in the cancer *vs.* the healthy tissue from 96 breast cancer patients. Our results corroborate previous studies showing upregulation of miR-96, miR-200c, and miR-141, and downregulation of miR-145, miR-99a, and miR-125b in breast cancer tissue (8, 21–24). Secondly, we found that 16 out of 30 microRNAs were significantly deregulated in the serum of cancer patients *vs.* the serum of the control group. Interestingly, in serum of breast cancer patients, downregulation of miR-411, miR-376c, miR-16, and miR-155 (9, 17) and upregulation of miR-125b, miR-1260, and miR-96 had been previously described, confirming the validation of our results (9, 17, 21, 25). Some of these 16 microRNAs have been associated with breast cancer diagnosis including miR-125b, miR-191, miR-411, miR-155, and miR-215 (26, 27). In particular, 11 deregulated microRNAs were found in the serum and tissue of breast cancer patients (**Figure 1C**). Most of them are contrarily overexpressed among both types of samples, that is, although we found 11 deregulated microRNAs that are common to serum and tissue, their expression (either upregulated or downregulated) was inversely correlated when comparing serum and tissue. The fact that the expression of a

A

Top deregulated proteins in serum	Protein ID
highly similar to von Willebrand factor	B4DMS3
HP	P00738
HBB	P68871
HBA1	E9M4D4
C4A	A0A0G2JPR0
TMSB4Y TB4Y	O14604
KRT9	P35527
highly similar to complement factor H	A8K5T0
highly similar to Proteoglycan-4	B7Z4R8
CST3	P01034
B2M	P61769
similar to Cholesteryl ester transfer protein	B4DMZ5
highly similar to Galectin-3-binding protein	B4DVE1
Sulfhydryl oxidase	A8K477
LYZ	F8VV32
DF	Q6FHW3
APOE	J9ZVQ3
CD44	C1PHC3
KRT2 KRT2A KRT2E	P35908
F11	Q9UEG0
β2-GP1	D9IWP9
APOA2	P02652
highly similar to Angiotensinogen	B4E1B3
SHBG hCG_42018	I3L145
Vitamin D-binding protein	P02774
highly similar to Insulin-like growth factor-bindin	B0AZL7
SERPIN A6	B2R9F2
TF PRO1400	P02787
PON1 PON	P27169
IGHG2	P01859
highly similar to Fetuin-B	B7Z8T3
Ig mu chain C region	A0A087WYJ9
HPR	P00739
SERPIN A1	P01009
CPAMD6	P20742

B

Protein ID	Gene	Prot. Ratio	Protein adj. p value	microRNA	microRNA FC	microRNA adj. p value
PIPHC3	CD44	1.24	0	miR-16	0.52	0

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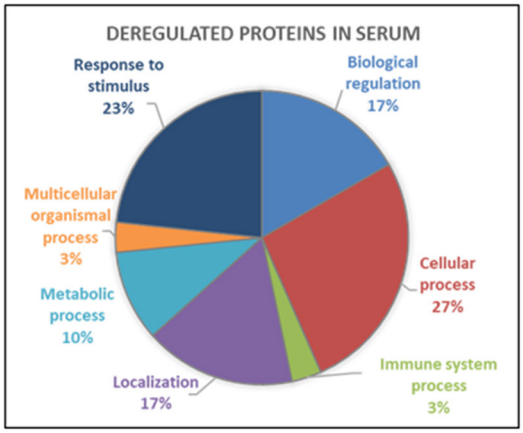

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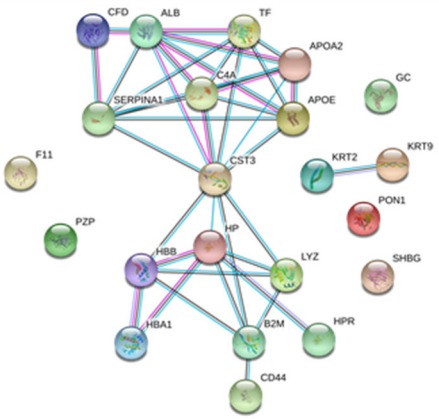

FIGURE 4 | Proteins deregulated in the serum of breast cancer patients. **(A)** Proteins significantly deregulated in the serum pools of breast cancer patients *vs.* controls (upregulated proteins are indicated in red and those that are downregulated are indicated in green). **(B)** microRNAs and validated target proteins found in serum revealed miR-16 and CD44. FC, fold change. **(C)** Different molecular pathways involving significantly deregulated proteins. **(D)** Interactions of CST3 with other proteins which might have a relevant role in cancer.

specific microRNA in different sample types can have inverse implications in prognosis/diagnosis, has already been described as well as microRNA deregulation in the opposite direction when comparing their expression in serum *vs.* tissue (21, 28–30). For example, miR-125b, known to be downregulated in breast cancer tissue (8, 31), is upregulated in the bloodstream of breast cancer patients (21, 25, 32). Possible explanations include (i) extracellular and cellular microRNAs profiles differ, and freely circulating microRNA might not reflect their abundance in cancer cells (33); (ii) the total level of free microRNAs in the bloodstream might be masked by certain microRNAs present into exosomes (34).

In relation with the use of microRNAs as biomarkers, it has been suggested that the association of miR-99a in breast cancer tissue with survival differs depending on the molecular subtype (35). Our study corroborates the fact that high levels of miR-1274a are associated with worse prognosis and proposes two novel microRNAs associated with survival in breast cancer: miR-497 and miR-362 (36). Apart from miR-362, miR-133b correlates with disease-free survival, the latter already been described as a diagnostic marker in breast cancer (28). Interestingly, serum levels of miR-125b and miR-29c (the top 2 in order of significance; **Figure 1C**) were associated to tumor stage. Moreover, high levels of miR-141 in serum were correlated with better survival. Contrary to our results, Debel et al. found that miR-141 expression in serum was associated with shorter brain metastases (37).

Lastly, despite the growing interest in assessing predictive cancer models based on microRNA signatures, most of the reported studies need to be further evaluated in larger cohorts of breast cancer patients (21, 24, 38). In this study, we identified a predictor (based on the following microRNAs: miR-125b, miR-29c, miR-16, miR-1260, and miR-451), capable of differentiating the serum of breast cancer patients from that of control

individuals with ∼90% of accuracy, sensitivity, and specificity. The fact that the predictor model includes microRNAs less statistically significant such as miR-16 and miR-1260 than other more deregulated microRNAs is because the predictors work by combining different variables in a unique model to maximize discrimination between groups. The advantage of using a combination of variables is that predictive ability is obtained from the combination of this precise set of variables. That is, although some variable may show a small difference between groups, it may be the case that its contribution is different from other variables, so that including this variable in the model results in an increase of its global predictive capability. In a second phase, the predictor was validated and tested in 20 additional breast cancer cases plus 60 volunteer women, respectively. While the 20 patients were correctly categorized, the predictor included 11 out of 60 women into the cancer group. Although the theoretical breast cancer risk in the overall women population of Europe and United States is 12.5%, according to our predictor, we found a percentage of 18.3% women that will develop cancer in the future. This percentage (18.3%) represents an increase of ∼1.5 over the expected values. A possible explanation of this high incidence could be the fact that, unlike the control group, this group of 60 women were not selected by any criteria; therefore, they could have a higher risk of developing breast cancer than the control group. It would be interesting to determine the health condition of those 60 women in the following 5–10 years with the purpose of establishing the validation of our predictor in the future.

On the other hand, differently expressed proteins in the serum of breast cancer patients *vs.* controls have been described (39). The deregulated proteins found in the pools of cancer *vs.* control serum samples—PEDF, IGKC, CD44, and CST3—have been previously reported (39–41). High levels of CD44 in serum are an independent prognosis indicator in primary breast cancer, since it correlates with overall survival and disease-free survival (42). Interestingly, we found that lower expression of miR-16 in the serum of cancer patients correlated with high expression of its CD44 target protein. Our results reinforce the potential relevance of CD44 as a potential marker of breast cancer as well as propose other proteins that might play key roles as biomarkers such as CST3, which needs to be extensively and individually studied in the serum of large series of patients (40).

Liquid biopsy (i.e., serum) is gaining importance in the clinical practice as novel biomarkers (i.e., microRNAs and proteins) are being considered to monitor healthy individuals. We hope that the results here reported open new avenues for future cancer prevention and diagnosis.

Overall, while much effort is being devoted to cancer predictive methods, it is not yet possible to detect cancer before the appearance of the first clinical symptoms. A molecular signature based on the detection in serum of five microRNAs capable of differentiating breast cancer patients from healthy individuals was found. The clinical application of the molecular signature herein described will be determined in large women's cohorts.

New microRNAs detected in serum and biopsy from breast cancer patients have been discovered. An association of low levels of miR-16 with a higher content of CD44 protein in serum was identified. This suggests the prognosis value of CD44 protein in serum as a potential marker of breast cancer. Collectively, our results support the fact that microRNA detection in serum can represent a viable predictive method applicable to breast cancer.

DATA AVAILABILITY STATEMENT

The information has been made public and accessible in the repository https://figshare.com/ with the updated information in the new document "Supplementary materials" provided.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of Vall d'Hebron Hospital (CEIC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF, LG, and ML conceived and designed the experiments. AF, LG, YG-M, and CM performed the experiments and designed the graphs. JCa and SB performed the statistical study. JCo, IR, NB, RM, and AA collected and organized the patients' information. AF, ME-B, SR, IR, JCa, and ML analyzed and interpreted the data. MA contributed to technical support. AF, YG-M, CM, NB, RM, AA, IR, SR, ME-B, JCo, and JCa contributed to scientific support. ML wrote the paper and conducted the study supervision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.586268/full#supplementary-material

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REVIEW
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Tumor Profiling at the Service of Cancer Therapy

Ceres Fernandez-Rozadilla[1], Ana Rita Simões[1], Matilde E. Lleonart[2,3], Amancio Carnero[3,4] and Ángel Carracedo[1,5]**

[1] Grupo de Medicina Xenómica (USC), Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain, [2] Biomedical Research in Cancer Stem Cells, Vall d'Hebron Research Institute (VHIR), Barcelona, Spain, [3] Spanish Biomedical Research Network Centre in Oncology, CIBERONC, Madrid, Spain, [4] Instituto de Biomedicina de Sevilla, IBIS, Hospital Universitario Virgen del Rocío, Universidad de Sevilla, Consejo Superior de Investigaciones Científicas, Seville, Spain, [5] Grupo de Medicina Xenómica (USC), Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain

Cancer treatment options have evolved significantly in the past few years. From the initial surgical procedures, to the latest next-generation technologies, we are now in the position to analyze and understand tumors in a one-by-one basis and use that to our advantage to provide with individualized treatment options that may increase patient survival. In this review, we will focus on how tumor profiling has evolved over the past decades to deliver more efficient and personalized treatment options, and how novel technologies can help us envisage the future of precision oncology toward a better management and, ultimately, increased survival.

Keywords: cancer treatment, omics, profiling, immunotherapy, personalized medicine, precision oncology

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****Correspondence:***
Ceres Fernandez-Rozadilla
ceres.fernandez.rozadilla@gmail.com
Ángel Carracedo
angel.carracedo@usc.es

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INTRODUCTION

Classically, choice of therapy depends mainly on location and grade of the primary tumor (as ascertained by histology), as well as the stage of the disease. Types of therapies are classically subdivided into surgery, chemotherapy, radiotherapy, hormonal therapy and immunotherapy, although the boundaries between these categories are sometimes blurry. Typically, localized tumors will be selected for resection *via* surgical procedures, which may be coupled with preceding (neoadjuvant) therapy to shrink the tumor prior to removal, and/or followed by adjuvant therapy to reduce the chances of relapse.

Cancer treatments have suffered a considerable revolution in the past few years owing to the recent development of high-throughput omic technologies. These have constituted the flourishing of targeted therapies, which can drive the final hurdle from histologic treatments to individualized treatments that attack each tumor precisely based on its very own molecular features. In this review, we summarize the road so far, from the earliest treatments to current strategies and what lies beyond.

CANCER THERAPY

It has been known for many years now that cancer cells have particular features that make them different to normal cells of the same tissue. Arguably, one of the most remarkable ones is the fact that tumor cells can obtain their energy through glycolysis instead of oxidative phosphorilation,

even in the presence of oxygen. This feature is known as the *Warburg effect* after the clinician who discovered it (1), and is one of the biological capabilities acquired during the multistep development of human tumors, also known as the *hallmarks of cancer* (2, 3). The Warburg effect has meaningful implications in cancer cell metabolism, thereby allowing these cells to gain a selective advantage when competing for shared and limited energy resources, which results in them proliferating more rapidly (4). The same happens to the other features, which must occur at a certain point in time over the course of cancer development. Hence, targeting one or several of these features is key for cancer therapeutic intervention [**Figure 1** - (3)].

Cancer therapeutic approaches were initially based on surgical removal of the tumor, with radiotherapy moving on quickly in the early 1900s (5). By the 1930s, the field was starting to point toward novel strategies, based on the findings on tumor biology provided by Warburg himself and others, and by the 1930s, Paul Ehrlich who coined the term "chemotherapy" to describe the use of chemical compounds to fight cancer. By 1946, the first alkylating agent was approved as a chemotherapeutic agent (6), and since then, several other agents have been used in the fight against cancer. The common feature that all of these chemotherapeutics share is their use of these particular properties of cancer cells to destroy them. For instance, alkylating agents such as the platins (carboplatin, cisplatin, and oxaliplatin) and topoisomerase inhibitors like irinotecan produce DNA damage; alkaloids such as paclitaxel and docetaxel disrupt cell division and, antimetabolites like 5-fluorouracil, gemcitabine or methotrexate work by inhibiting cell division (7–9) (**Table 1**).

In general, traditional chemotherapeutic agents are mainly cytotoxic (also coined cytostatic), which means they interfere with and stop cell division. This is primarily aimed to target highly-proliferating cells, such as neoplastic ones. Cytotoxic agents may be used alone (monotherapy) or in combination with other therapies, and up to today, still constitute the backbone of cancer treatment (10). Nevertheless, there are two main problems with cytotoxic therapies: response (or sometimes resistance) and toxicity. For the former, response rates to standard cytotoxic chemotherapy are varied and depend greatly on tumor site and stage. For instance, it is well known that advanced pancreatic tumors only present response rates of about 20% to classical treatments with gemcitabine and nab-paclitaxel (11, 12). Moreover, the development of secondary resistance (refractory response after an initial responsive period) is also common, and is one of the major causes of failure of cancer treatment. For the latter, the fact that cytotoxic agents target rapidly dividing cells may also affect other normal cell types, such as the bone marrow, hair follicles or digestive tract, thereby resulting in the development of adverse drug reactions that may result in discontinuation in the administration of the drug, and therefore, may compromise its curative purpose (13, 14).

TARGETED THERAPIES

Small Molecules

Regardless of all hallmarks acquired by tumor cells, cancer is ultimately a genetic disease caused by genomic mutations in

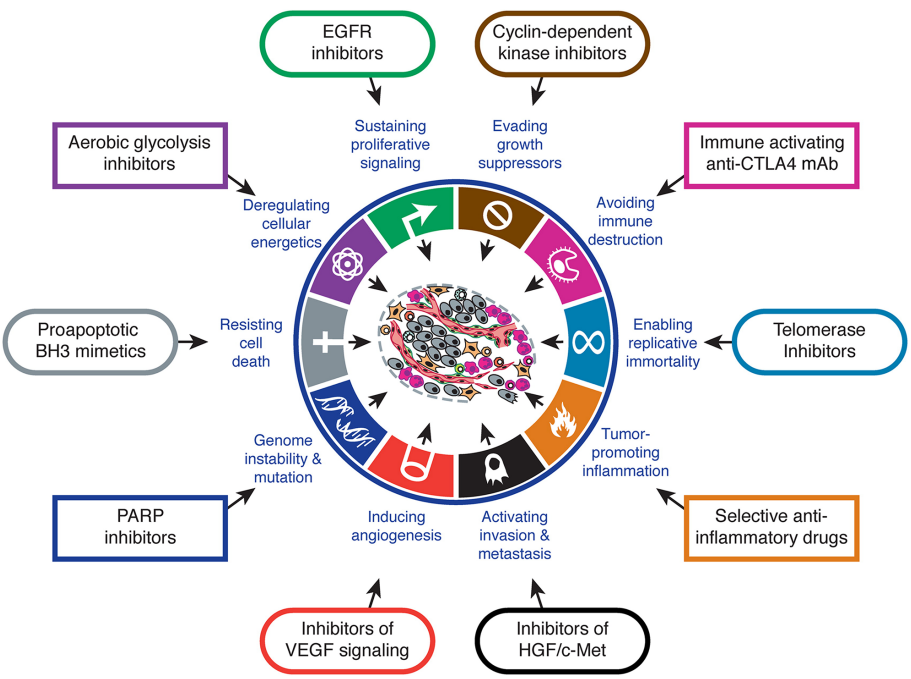


FIGURE 1 | Hallmarks of cancer and therapeutic implications. Adapted from (3).

TABLE 1 | Commonly used chemotherapeutic agents.

Types of chemotherapy	Subtype	Examples
Alkylating agents	Oxazsaphosphorines	cyclophosphamide, ifosfamide
	Nitrogen mustards	busulfan, chlorambucil, melphalan
	Hydrazine	temozolomide
	Platinum-based agents	cisplatin, carboplatin, oxaliplatin
Antimetabolites	Pyrimidine antagonists	cytarabine, 5-fluorouracil, gemcitabine, capecitabine
	Purine antagonists	fludarabine
	Purine analogs	6-mercaptopurine, azathioprine, cladribine
	Antifolates	methotrexate, pemetrexed, pralatrexate
	Ribonucleotide reductase inhibitors	hydroxyurea
Topoisomerase inhibitors	Topoisomerase I inhibitors	irinotecan, topotecan
	Topoisomerase II inhibitors	etoposide; teniposide; anthracyclines, e.g., idarubicin, daunorubicin, doxorubicin
Mitotic spindle inhibitors	Taxanes	docetaxel, paclitaxel
	Vinca alkaloids	vincristine, vinblastine
Other	Enzymes	l-asparaginase
	Tyrosine kinase inhibitors	imatinib and erlotinib
	Antibiotics	bleomycin, actinomycin D, anthracyclines
	Proteasome inhibitors	bortezomib
	Autophagy inhibitors	hydroxychloroquine

genes that allow them to obtain a selective advantage, whether that is in terms of faster proliferation, nutrient acquisition or blood vessel formation (15). Research on oncogenes and tumor suppressor genes (the two main types of genes in which mutations drive cancer development) has been strongly active since the first description of a cancer-causing mutation (16–18).

Generic cytotoxic drugs are not enough to target these changes specifically, and therefore the identification of these driver mutational events launched for the first time the possibility to test for and treat against specific mutations appearing in particular genes and tumors, and hence, provided the basis for targeted cancer therapies. Indeed, it has been described that the Warburg effect is possibly an early event in oncogenesis that is an immediate consequence of an initial oncogenic mutation, such as that of *KRAS* in pancreatic cancer or *BRAF* in melanoma, and may occur in early stage lesions as well (19, 20).

Targeted therapies work primarily by attacking deregulated proteins that support survival of cancer cells (21, 22). There is quite a variety of small molecules to target these proteins, but arguably, kinase inhibitors have been the most successful. We know that in many tumors, signaling pathways regulated by protein kinases are the frequent targets of somatic mutations, and indeed of the more than 100 oncogenes known, many encode kinases (23). These kinases may be led to aberrant function by several mutational processes, including genomic rearrangements, gain-of-function mutations, or overexpression and/or gene amplification, which ultimately result in the loss of regulatory constraints and a constitutive activation of the protein.

The first targeted therapy directed against a specific genetic abnormality was imatinib, a tyrosin-kinase inhibitor (TKI) that inhibits proliferation of BCR-ABL-expressing hematopoietic cells by specifically targeting the constitutively active fusion protein produced by the reciprocal translocation of chromosomes 9 and 22 (t(9;22)(q34;q11)) (24, 25). The list of targeted therapies has rapidly expanded ever since its discovery, and a selection of the most commonly used targeted therapies and their corresponding molecular changes is represented on **Table 2**. Albeit the explosion of targeted therapies, these small-molecule approaches have been more favorable for cancers like lung, colorectal, breast, lymphoma and leukemia, as they focus on particular molecular changes unique to a specific cancer, whereas other cancer types such as pancreatic or upper gastrointestinal tumors have experienced less progress in targeted drug therapy development.

Immunomodulation and Immunotherapy

Another important hallmark of cancer is that, for a tumor to arise, it must evade the strict control to which malfunctioning cells are subject by the immune system (3). Although it is still unclear whether this immune evasion happens as a passive or active process (or possibly even both), it is however certain that at some or other point tumors acquire the ability to surpass the control of the immune system. This observation gave rise to the field that utilizes the artificial stimulation of the immune system to treat cancer: immunotherapy. Immunotherapy has become such an important part of cancer therapy in the past few decades that it was merited with the Nobel Prize on Physiology or Medicine to James P. Allison and Tasuku Honjo for their discovery of cancer therapy by inhibition of negative immune regulation.

There are two main types of immunotherapies: passive immunotherapy, which consists in the blocking of cell surface receptors that are specific to tumor cells, and active immunotherapy, that aims to stimulate the patient´s immune system to reactivate the fight against cancer cells (30, 31). For the former, monoclonal antibodies (mAbs) have been the main strategy. These antibodies are produced specifically to block cell surface receptors that are present (ideally) exclusively on tumor cells and tumor-promoting molecules. They recognize a tumor antigen and cause cell death through various mechanisms, including apoptosis or indirect elimination by recruitment of immune cells with cytotoxic properties, or by activation of the

TABLE 2 | List of common small molecule therapies (26–29).

Target	Drug	Tumor type
BCR-ABL	imatinib; dasatinib; nilotinib; bosutinib; regorafenib; ponatinib	CML; ALL; GIST; CRC
PDGFR	imatinib; dasatinib; nilotinib; sunitinib; sorafenib; regorafenib; erdafitinib; lenvatinib; pazopanib	ALL; CML; GIST; RCC; pNET; HCC; thyroid cancer; CRC; UC; RCC; soft tissue sarcoma
EGFR	afatinib; gefitinib; osimertinib; vandetanib; erlotinib; lapatinib; dacomitinib; neratinib	NSCLC; PDAC; medullary thyroid cancer; BrCA
FGFR	erdafitinib; lenvatinib; pazopanib	UC; thyroid cancer, HCC; RCC; soft tissue sarcoma
HER	afatinib; osimertinib; neratinib; lapatinib	NSCLC; BrCA
CDK 4/6	ribociclib; abemaciclib; palbociclib	BrCA
C-KIT	imatinib; dasatinib; nilotinib; sunitinib; sorafenib; regorafenib; erdafitinib; lenvatinib; cabozantinib; pazopanib	CML; ALL; GIST; HCC; pNET; RCC; thyroid cancer; CRC; UC; soft tissue sarcoma
SCF	imatinib	CML; ALL; GIST
SRC	dasatinib: bosutinib; vandetanib	ALL; CML; medullary thyroid cancer
CSF	nilotinib; sunitinib; erdafitinib	CML; GIST; RCC; pNET; UC
DDR	nilotinib; regorafenib	CML; CRC
C-MET	crizotinib; cabozantinib	NSCLC; HCC; RCC
VEGFR	sunitinib; sorafenib axitinib;, vandetanib; regorafenib; erdafitinib; lenvatinib; cabozantinib; pazopanib	RCC; HCC; medullary thyroid cancer; GIST; pNET; thyroid cancer; CRC; UC; soft tissue sarcoma
RET	vandetanib; sunitinib; regorafenib; sorafenib; erdafitinib; alectinib; lenvatinib; cabozantinib	Medullary thyroid cancer; GIST; RCC; pNET; CRC; HCC; thyroid cancer; UC; NSCLC
TIE2	vandetanib; regorafenib; cabozantinib	Medullary thyroid cancer; CRC; RCC; HCC
RAF	vemurafenib; sorafenib; regorafenib; encorafenib; dabrafenib	Melanoma; HCC; RCC; thyroid cancer; CRC
PARP	olaparib; rucaparib; talazoparib; niraparib	ovarian cancer; BrCA
TRK	larotrectinib; regorafenib; entrectinib; cabozantinib; lorlatinib	solid tumors; CRC; NSCLC; HCC; RCC
BTK	ibrutinib	MCL; CLL; SLL
MEK	cobimetinib; binimetinib; trametinib	melanoma
FTL	sorafenib; sunitinib; erdafitinib; brigatinib; cabozantinib; gilteritinib	HCC; RCC; thyroid cancer; GIST; pNET; UC; NSCLC; AML
ROS1	entrectinib; crizotinib; brigatinib; lorlatinib; ceritinib; cabozantinib	solid tumors; NSCLC; RCC; HCC
ALK	entrectinib; alectinib; crizotinib; brigatinib; lorlatinib; ceritinib	solid tumors; NSCLC
IGF-1R	brigatinib; ceritinib	NSCLC
IDH1	ivosidenib; enasidenib	AML
26S proteasome	bortezomib; carfilzomib; marizomib	multiple myeloma; MCL
PI3KCA	alpelisib	BrCA
PI3K	duvelisib; copanlisib	CLL, SLL; Follicular lymphoma

CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; pNET, pancreatic neuroendocrine tumors; NSCLC, non-small-cell lung cancer; PDAC, Pancreatic ductal adenocarcinoma; UC, urothelial cancer; HCC, hepatocellular carcinoma; RCC, renal cell carcinoma; MCL, Mantle cell lymphoma; CLL, Chronic lymphocytic leukemia; SLL, Small lymphocytic lymphoma; AML, acute myeloid leukemia; BrCA, breast cancer; CRC, colorectal cancer.

complement cascade. Examples of these mAbs are those directed toward the vascular endothelial growth factor (VEGF), interleukins or the macrophage migration inhibitory factor (MIF) (31–34).

A special type of mAbs that has claimed great benefits in patient survival over the past ten years are immune checkpoint inhibitors (ICIs) (35). Their success radicates in the fact that may be directed to the tumor cells but also to T cells, to reinstate recognition of tumor cells by the immune system, thereby relaunching an immune response. The main three ICIs used to data have been CTLA-4, PD-1 and PD-L1 inhibitors (36) (**Figure 2** - www.cancer.gov).

As for small molecules, immunotherapy has also become an important tool in the development of targeted anticancer therapies, and there are nowadays numerous mAbs to treat various types of cancers, with numbers rapidly increasing (**Table 3**). Among these, rituximab (anti-CD20 mAb used in the treatment of non-Hodgkin lymphoma) is possibly the most extensively used. However, over 2900 clinical trials have been reported on the use of mAbs in cancer, and many others are currently ongoing in cancer patients (ClinicalTrials.gov).

Active immunotherapy is composed of CAR-T antibodies, which are harvested, modified T cells from the patient that are genetically altered to specifically recognize cancer cells when infused back into the patient. The features and implications of CAR-T technologies are extensive and far beyond the scope of this review, but comprehensive reviews can be found in (41–44).

Molecular Testing for Targeted Therapies

Because targeted therapies are particularly directed toward the specific changes present in a given tumor's cells, clinical molecular pathology analysis has therefore become an indispensable laboratory tool that can be used to characterize tumor biology and to drive therapeutic decisions (45). This is known as pharmacodiagnostics and aims to determine whether a patient will successfully respond to a given therapy, and is therefore an intrinsic part of personalized medicine approaches. The indications for molecular testing in the most prevalent tumor types are summarized in **Table 4**.

Classical detection methods in cancer pathology include gold standard techniques in molecular biology: immunohistochemistry (IHC): as for the case of p16 staining for

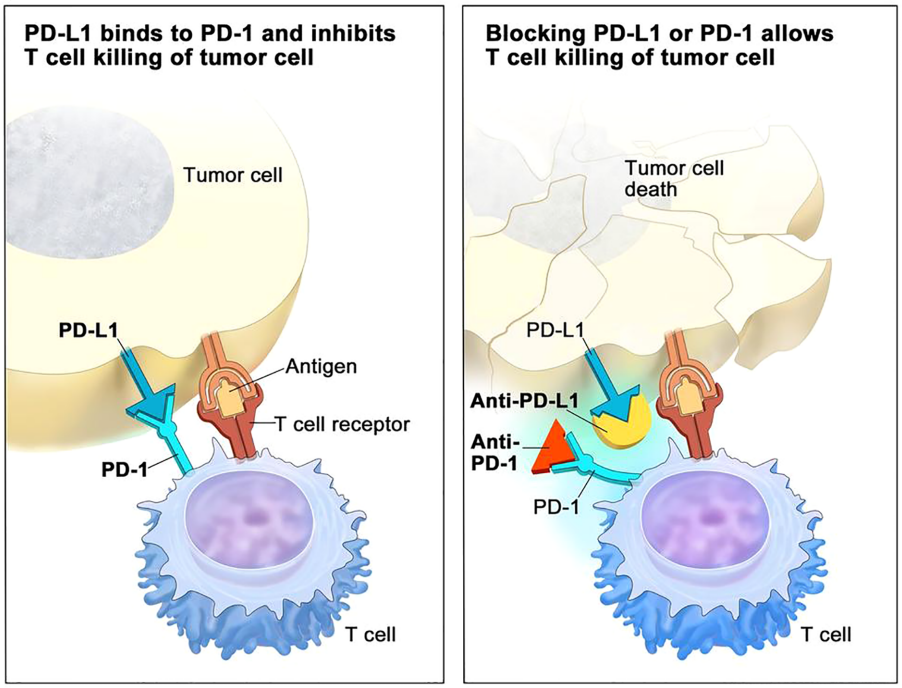


FIGURE 2 | PD-1/PDL-1 immune checkpoint inhibitor (ICI) mechanisms of action in immunotherapy (adapted from NCI – www.cancer.gov).

TABLE 3 | List of common monoclonal antibody (mAb) therapies (27, 37–40).

Target	Drug	Tumor type
HER2	adotrastuzumab; trastuzumab; pertuzumab	BrCA
EGFR	cetuximab; panitumumab; necitumumab	CRC, HNSCC; NSCLC; PDAC; glioma; Squamous NSCLC
VEGFR	ramucirumab	gastric cancer; NSCLC
VEGF	bevacizumab	CRC; NSCLC; BrCA; Glioblastoma; RCC
CD-20	rituximab; ofatumumab; ibritumomab; tositumomab; obinutuzumab	Non-Hodgkin lymphoma; CLL; follicular lymphoma
CD-22	inotuzumab	ALL
CD-52	alemtuzumab	CLL
CD-33	gemtuzumab	AML
CD-30	brentuximab	Hodgkin lymphoma; anaplastic large cell lymphoma
CD19/CD3	blinatumomab	ALL
CD38	daratumumab	multiple myeloma
CTLA-4	ipilimumab	melanoma; RCC
PD-1	nivolumab	melanoma; NSCLC; SCLC; RCC; UC; Hodgkin lymphoma; HNSCC; MSI-H/dMMR CRC; HCC
PD-L1	atezolizumab; avelumab; cemiplimab; pembrolizumab; durvalumab	UC; NSCLC; BrCA; RCC; CSCC; melanoma; NSCLC; HNSCC; Hodgkin lymphoma; MSI-H cancer; gastric cancer; cervical cancer; HCC; MCC
RANKL	denosumab	giant cell tumor of the bone
GD2	dinutuximab	pediatric neuroblastoma
PDGFR	olaratumab	soft tissue sarcoma
SLAMF7	elotuzumab	multiple myeloma

BrCA, breast cancer; CRC, colorectal cancer; HNSCC, Head and neck squamous cell carcinoma; NSCLC, non-small-cell lung cancer; PDAC, Pancreatic ductal adenocarcinoma; RCC, renal cell carcinoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; UC, urothelial cancer; MCL, Mantle cell lymphoma; MSI-H, microsatellite instability-high; dMMR, mismatch repair deficient; MCC, Merkel cell carcinoma; CSCC, cutaneous squamous cell carcinoma.

HPV infection in FFPE tissues (49); fluorescent in-situ hybridisation, (FISH) to detect chromosomal rearrangements in hematological malignancies (50, 51), PCR or Sanger sequencing for point mutations (52).

These tests are currently essential to classify tumors and decide on treatment strategies. For example, breast tumor biology has historically been classified based on immunohistochemical (IHC) staining of proliferation proteins (Ki-67), hormone receptor status

TABLE 4 | Molecular testing for each tumor type (46–48).

Tumor type	Gene	Change	Treatment	Drugs (examples)
NSCLC	EGFR	mutation	TKI	gefinitib; erlotinib; afatinib, dacomitinib; osimertinib
	ALK	translocation	TKI	crizotinib, ceritinib, alectinib, lorlatinib, brigatinib
	ROS1	translocation	TKI	crizotinib, entrectinib
	PD-L1	protein expression	PD-1 blocking antibody	pembrolizumab
	KRAS	mutation	TKIs	
	BRAF	mutation	kinase inhibitors	vemurafenib; dabrafenib
	HER2	mutation	kinase inhibitors	afatinib; osimertinib
	MET	amplification, mutation	kinase inhibitors	crizotinib; cabozantinib
	RET	fusion, rearrangement	kinase inhibitors	alectinib
Melanoma	BRAF	mutation	kinase inhibitors	dabrafenib; trametinib, vemurafenib; cobimetinib, encorafenib; binimetinib
	KIT	mutation	kinase inhibitor	imatinib
GIST	KIT	mutation	kinase inhibitors	imatinib
	PDGFR	mutation	kinase inhibitors	imatinib
	HER2	gene amplification	HER receptor antagonists	trastuzumab
	PD-L1	expression	PD-1 blocking antibody	pembrolizumab
Pancreatic cancer	BRCA1/2	mutation	PARP-inhibitors	olaparib
CRC	KRAS/NRAS	mutation	EGFR antagonists	cetuximab; panitumumab
	BRAF	mutation	EGFR antagonist	cetuximab; panitumumab
	MSI-H or dMMR	expression	PD-1 blocking antibody	nivolumab; ipilimumab
BrCa	HER2	amplification	HER2-targeted therapy	trastuzumab; lapatinib; pertuzumab
	BRCA1/2	mutation	PARP inhibitors	olaparib, talazoparib, rucaparib
	PI3KCA	mutation	kinase inhibitors	alpelisib
OvCa	BRCA1/2	mutation	PARP-inhibitors	olaparib, talazoparib, rucaparib
	ATM			
	BRiP1			
	CHEK2			
	PALB2			
	RAD51C			
	RAD51D			
Sarcoma	MDM2, CDK4	amplification	CDK4/CDK6 inhibitors	palbociclib
	IDH1/IDH2	mutation	IDH1 inhibitor	ivosidenib
Melanoma	BRAF	mutation	kinase inhibitors	vemurafenib; encorafenib; dabrafenib
	KIT	mutation	kinase inhibitors	imatinib; nilotinib
Head and Neck Cancer	PD-L1	protein expression	PD-1-blocking antibody	pembrolizumab
Solid tumors	MMR/MSI	expression	PD-1-blocking antibody	pembrolizumab
	TRK	fusion	kinase inhibitors	entrectinib; larotrectinib
Chronic myeloid leukemia	BCR/ABL	fusion	kinase inhibitors	imatinib, dasatinib, nilotinib, bosutinib, ponatinib
	PI3K	mutation	kinase inhibitors	duvelisib
Acute myeloid leukemia	IDH1/2	mutation	IDH1 inhibitors	ivosidenib, enasidenib
	FLT3	mutation	kinase inhibitors	gilteritinib
Follicular lymphoma	PI3K	mutation	kinase inhibitors	copanlisib
Urothelial cancer	FGFR2/3	mutation, fusion	kinase inhibitors	erdafitinib

List of molecular tests currently indicated for the most prevalent tumor types.

(estrogen receptor alpha (ER), progesterone receptor (PR) and/or androgen receptor (AR), and the presence/absence of specific cytokeratins (CK). Therapeutic strategies are based on this histological classification and Ki-67 assays have additional prognostic value (53).

TUMOR PROFILING TO GUIDE CANCER THERAPY

Targeted therapies provided the first evidence that treating a tumor based on its molecular features could result in better patient outcome in terms of increased survival. However, molecular testing based on the features provided on **Table 4** is clearly insufficient, particularly for underrepresented tumors that tend to have worse prognosis, such as pancreatic or endometrial cancers. Therefore, there have been extensive efforts to upgrade our molecular knowledge on cancer to a more comprehensive view of each individual cancer. Tumor profiling constitutes the pinnacle of these efforts, where we aim to classify neoplasms into subgroups that give us information about how the cancer has evolved, how it can be better treated, and how we should direct drug design strategies to treat them. Several approaches to tumor profiling have been undertaken in the past few years that will be discussed below.

Genomics

The publication of the human genome sequence in 2003 (54) and the implementation of next-generation sequencing technologies since the turn of the century has allowed for our knowledge on germline and somatic tumor genomics to increase tremendously in the past two decades. This is particularly relevant in the

context of targeted cancer treatments, where we aim to achieve better outcomes by treating tumors with drugs that are specifically matched to their molecular features. Whole-exome and whole-genome cancer sequencing initiatives like The Cancer Genome Atlas (TGGA) (cancergenome.nih.gov/) and the International Cancer Genome Consortium (icgc.org) have sequenced hundreds of cancers across 38 tumor types to provide the most comprehensive cancer genome database to date (55).

The benefits of these initiatives have been unprecedented and multiple. Firstly, we have been able to identify a much larger proportion of cancer driver mutations. These are changes that give the tumor cell a selective advantage in its microenvironment, through either increasing its survival or reproduction. Driver mutations tend to cause clonal expansions and are the fundamental first step of cancer development. Therefore, identifying them is key for the design of targeted therapies that can stop cancer growth and spreading. Driver mutations happen preferentially in oncogenes and tumor suppressor genes, and hence the list of cancer genes has increased exponentially in the past few years (56–59). Moreover, because whole-genome cancer sequencing provides a more comprehensive assessment of the mutational spectrum, we can assess not only point mutations on a large scale, but also other genomic features that can be relevant drivers, such as mutations in non-coding regions (60–62), CNVs and structural variations (63).

Secondly, because cancer is highly heritable (64), candidate driver genes may also be identified by NGS of the patient´s germline DNA following Knudson´s two hit hypothesis (18). Since somatic mutation analysis inherently requires the sequencing of the matching normal tissue, this can be used to advance into the description of germline variants that confer cancer predisposition (65). Germline pathogenic mutational events may have important consequences for cancer treatment, as it has been proven that both germline and somatic mutations in the homologous recombination genes *BRCA1*, *BRCA2*, *PALB2* (also termed "BRCAness") respond well to treatment with poly-ADP ribose polymerase (PARP) inhibitors. This is true for several cancer types, including breast, ovarian, prostate and pancreatic tumors (66, 67). Another example is that of tumors arising from germline and/or somatic mutations in polymerases ϵ and δ (*POLE* and *POLD1* genes), which have an indication for treatment with immunotherapy (68).

In any case, the determination of both germline and somatic mutation events leading up to cancer has great consequence for the establishment of actionable mutations. A study performed on 2,520 pairs of primary and metastatic tissue tumors found that 62% of patients presented with genetic variants that could be used to stratify patients toward either approved therapies or those in clinical trials (69). Moreover, half of the patients with a predicted candidate actionable event (31% of total) contained a biomarker with a predicted sensitivity to a drug at level A (approved anti-cancer drugs) and lacked any known resistance biomarkers for the same drug. Hence, big efforts are being made at current to categorize somatic mutation variants into likely actionable mutations in order to advance in the design of novel anticancer drugs (70, 71).

All of these key features of genomic high-throughput sequencing are ultimately key for tumor profiling, and have made it possible to gain a much better insight into the molecular and genomic features of different tumor types. This has been particularly relevant for those with less available therapeutic options (72, 73). Relevant developments in novel targeted therapies that have sprung thereof are for instance the treatment of tumors with *ARID1A* mutations and dasatinib (74, 75); among many others.

Epigenomics

Carcinogenesis has been shown to be accompanied by widespread DNA methylation changes in the tumor cell, that are usually visible as a globally hypomethylated genome mimicking a stem cell phenotype (76). These changes in the methylation patterns of tumor cells can appear as a result of genomic mutations or constitute neoplastic drivers by themselves (77). For instance, the germline or somatic methylation of the *MLH1* gene promoter is a well-known epigenetic driver event. Many of these epigenetic changes occur early in tumorigenesis and are highly pervasive across a tumor type. Therefore, intensive studies have been performed to elucidate the methylatory landscape of several cancer types, including breast (78), lung (79), prostate (80), or CLL (81), among others (82).

Interestingly, for CRC, a redistribution of methylation sites has been observed, where there is focal hypermethylation of CpG islands on tumor suppressor genes. This phenomenon is called the CpG island methylator phenotype (CIMP) (83), and it has since been discovered in multiple other tumor types, including bladder, breast, gastric or pancreatic cancers (84–88). The CIMP phenotype has been linked to multiple genetic causes, including at least the *BRAF* V600E mutation, or pathogenic mutations in the *IDH1* gene (89, 90).

Because epigenetic alterations are reversible, they can be a substrate for therapy development as well as influence the choice of treatment. DNA methylation differences have been observed, for instance, between radio-sensitive and radio-resistant cell lines (91). Moreover, methylation of the *MGMT* promoter in gliomas is a useful predictor of the responsiveness of the tumors to alkylating agents (92). Differential methylation has also been associated with increased risk of recurrence in NSCLC and breast cancers (93, 94), and methylation of a CpG in the transcription factor *FOXP1* is predictive of response to ICB in NSCLC patients (95). In the case of CIMP tumors, it has been noticed that it almost invariably results in hypermethylation of the *MLH1* promoter, which in turn provokes a MSI phenotype, and has been shown to correlate with response to immune checkpoint inhibitors (88, 96).

Nevertheless, data about the relationship between drug response and the epigenomic variations is still scarce mainly because the epigenome is highly variable between individuals, and hence therapeutic choice based on methylation profiling is rare. Alternatively, other epigenomic events (histone modifications, chromosome remodeling or RNA editing) have also been explored to a smaller extent that could also potentially identify druggable pathways (97).

Transcriptomics

The study of the genome provides only a steady-state view of highly dynamic molecular populations, and the information reflected in the genome may not be relevant if, for instance, the gene product is not expressed in the tissue-of-origin of the tumor. RNA sequencing (RNA-seq) has also been a relevant source of knowledge to inform on tumor profiling and therapeutic management. As sequencing methods became more cost-efficient, there have been large-scale molecular profiling efforts that have inspected RNA-seq in tumors (TCGA) and in normal tissues [the Genotype-Tissue Expression project – GTEx – (98)]. Transcriptome profiling presents several advantages over genomics and epigenomics studies: first, it is the most reliable way to detect gene fusions *ad hoc* and at a great scale, which may be particularly relevant in some types of tumors (99); secondly, gene expression signatures can be derived to infer prognostic and predictive information, and they allow for refinement of disease subclassification beyond what can be achieved by currently validated biomarkers (100); thirdly, it can give us information not only from the tumor, but also from the microenvironment, including cell composition derivations using deconvolution strategies [and these may be especially relevant for targeted immunotherapy strategies (101)]; fourth, gene expression analyses can be done reliably on a single-cell basis, to target, for instance, activated pathways in cancer stem cells (102). Lastly, transcriptome quantification can summarize the effects of known and unknown driver (epi)genomic events into measurable phenotypes, and therefore, it has the potential to link tumor genotypes to their phenotypic consequences (103).

Hence, transcriptomics has been essential in the road toward a more efficient tumor molecular profiling. There are now four fully established CRC consensus molecular subtypes (CMS) based on transcriptomic profiling: CMS1 (MSI), CMS2 (canonical), CMS3 (metabolic) and CMS4 (mesenchymal). Given the consistent classification, it would be then advisable to devise therapeutic strategies based on these molecular profiles, and indeed, CMS1 tumors are indicated to receive immunotherapy with ICIs, such as pembrolizumab or nivolumab. For the other categories however, studies are still underway to design novel targeted treatment options (104) [**Figure 3** - (105)].

Transcriptomics is also essential in breast cancer, where more than 15 years ago, profiling of breast tumors revealed a gene set whose expression varied significantly between tumors 500 gene set revealed 5 gene expression profiles, which were labeled as luminal A, luminal B, basal-like, HER2+, and normal-like, a classification that is used to this day [**Figure 4** - (106)]. This classification has been also relevant for treatment guidance in early-stage breast cancer (107), to predict response to immune checkpoint blockade therapy (108, 109) and to prognosis (110, 111).

Small RNAs

Moreover, transcriptomic studies are not restricted to mRNAs that are protein-coding. Other sources of RNA molecules, such as micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) have also been studied extensively in the context of tumor profiling. For instance, it is well known that miRNAs are widely dysregulated in cancers, and that they may also act as signaling molecules between cancer cells and others in the tumor microenvironment (22, 62, 112), whereas lncRNAs have been involved in cancer immunity, cancer metabolism and metastasis (113, 114). Small RNAs have some advantages over protein-coding RNAs: they are more stable (which is ideal for use in formalin fixed specimens); they can be routinely assessed sensitively and accurately with high-throughput technologies; they can be used as proxies for the mutational status of known, and possibly unknown gene drivers (115); and they may also be assessed in a circulatory setting (as we will explain later for liquid biopsy approaches). Hence, they have been helpful as a source for biomarkers for cancer risk stratification (116), outcome prediction (117) and classification of histological subtypes (118, 119). Nevertheless, their potential is yet to be fully exploited in the years to come, to establish reliable biomarkers to monitor treatment response and guidance of therapeutic strategies.

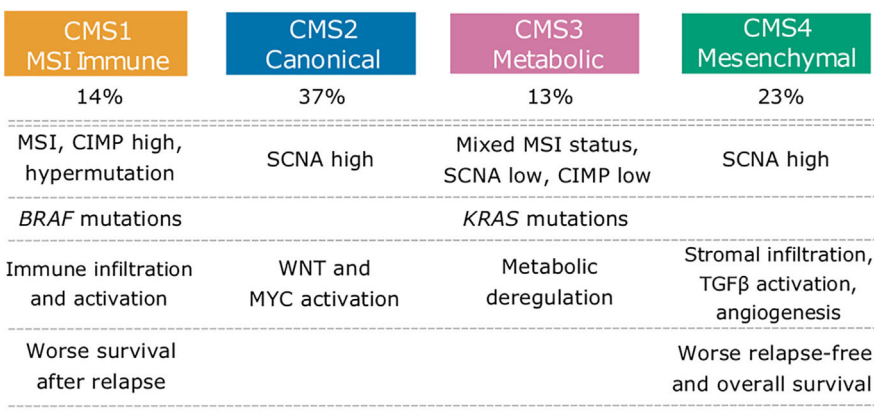
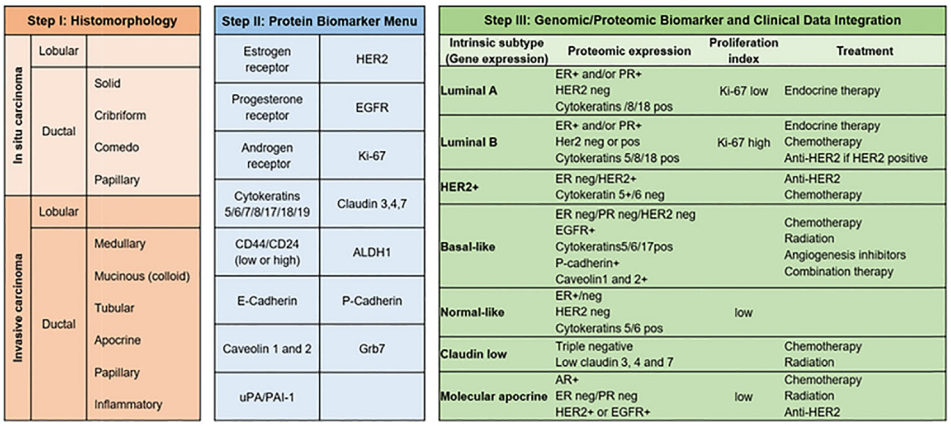


FIGURE 3 | Colorectal cancer consensus molecular subtypes (CMS). CMS showing their main molecular features. Adapted from (105). This subtyping has influence on choice of therapeutic strategy and also has prognostic value at the therapeutic level. CMS1 cancers have an indication to be treated with immunotherapy and better prognosis than CMS2-4.



Step I: Histomorphology		
In situ carcinoma	Lobular	
	Ductal	Solid
		Cribriform
		Comedo
		Papillary
Invasive carcinoma	Lobular	
	Ductal	Medullary
		Mucinous (colloid)
		Tubular
		Apocrine
		Papillary
		Inflammatory

Step II: Protein Biomarker Menu	
Estrogen receptor	HER2
Progesterone receptor	EGFR
Androgen receptor	Ki-67
Cytokeratins 5/6/7/8/17/18/19	Claudin 3,4,7
CD44/CD24 (low or high)	ALDH1
E-Cadherin	P-Cadherin
Caveolin 1 and 2	Grb7
uPA/PAI-1	

Step III: Genomic/Proteomic Biomarker and Clinical Data Integration			
Intrinsic subtype (Gene expression)	Proteomic expression	Proliferation index	Treatment
Luminal A	ER+ and/or PR+ HER2 neg Cytokeratins /8/18 pos	Ki-67 low	Endocrine therapy
Luminal B	ER+ and/or PR+ Her2 neg or pos Cytokeratins 5/8/18 pos	Ki-67 high	Endocrine therapy Chemotherapy Anti-HER2 if HER2 positive
HER2+	ER neg/HER2+ Cytokeratin 5+/6 neg		Anti-HER2 Chemotherapy
Basal-like	ER neg/PR neg/HER2 neg EGFR+ Cytokeratins5/6/17pos P-cadherin+ Caveolin1 and 2+		Chemotherapy Radiation Angiogenesis inhibitors Combination therapy
Normal-like	ER+/neg HER2 neg Cytokeratins 5/6 pos	low	
Claudin low	Triple negative Low claudin 3, 4 and 7		Chemotherapy Radiation
Molecular apocrine	AR+ ER neg/PR neg HER2+ or EGFR+	low	Chemotherapy Radiation Anti-HER2

FIGURE 4 | Breast cancer subtypes. From morphology, to immunohistochemistry and transcriptomic classifications (106), breast cancer subtypes have also a deep influence on therapeutic strategy. As reflected in the figure, Luminal A tumors will be treated with endocrine therapy and have the best prognosis among all subtypes, whereas triple negative cancers have the worst prognosis.

Proteomics

Proteins represent the clear majority of therapeutic drug targets in cancer This is due to the fact that many driver mutations result in the structural modification of proteins, and the fact that these particles can also act as signaling molecules with other cell types in the tumor microenvironment (120, 121). Also, the fact that global transcript levels may poorly reflect global protein-level data, makes it of obvious importance to study the changes in proteins occurring in tumor cells that can drive carcinogenesis and influence disease phenotype, aggressiveness, immune escape, and therapeutic response (122).

Thus, the novel proteomic methods that have evolved in the past few years (imaging mass spectrometry, single-cell proteomics, preanalytical sample processing, such as laser microdissection), could be uniquely positioned for the study of cellular populations beyond tumor cells themselves (123). High-throughput proteomic assessments from reverse-phase protein arrays (RPPA) have been undertaken by TCGA and others in >4000 tumor samples across 11 cancer types (124). Additionally, more comprehensive unbiased approaches using liquid chromatography–tandem mass spectrometry (LC–MS/MS) quantitative analyses have also been performed in the past few years (125–127). These technologies have the potential to identify molecular subtypes and associated pathway features that might be otherwise missed using (epi)genomics and transcriptomics, and indeed have been able to describe ten pan-cancer subtypes across tumor lineages (128). Proteomic studies have also produced significant advances on drug target identification and therapy management, including the observation that treatment with avasimibe, an inhibitor of SOAT1, markedly reduced the size of early-stage hepatocellular carcinomas (129).

Tumor Microenvironment

Apart from tumor cells, several other cell types are essentially relevant to cancer development and behavior. Potential targets in the TME include the extracellular matrix, cancer-associated fibroblasts (CAFs), immune cells such as infiltrating T-cells (TILs) and macrophages (TAMs). These cells participate in a variety of processes related to tumorigenesis, from immune evasion to angiogenesis promotion (130–132). Indeed, current therapeutics such as bevacizumab already target the TME specifically by inhibiting neoangiogenesis, and the reactivation of lymphocytes is the basis for current immunotherapeutic approaches.

A positive correlation has been observed between the increasing number of diverse cellular subpopulations and patient outcome, which greatly impacts on disease prognosis. For instance, type, density and location of infiltrating T-cells within colorectal tumors can greatly predict disease outcome (133, 134), and the presence of a dense stromal compartment around pancreatic tumors dramatically affects antitumoral therapies in pancreatic tumors (135). One advantage of therapies targeting the microenvironment is that these non-tumor cells are presumably genetically stable, which is in contrast to tumor cells that can accumulate adaptive mutations and rapidly acquire drug resistance (136).

Furthermore, apart from analyzing the heterogeneous contribution of each cell type to a tumor, these extrinsic cell types are also susceptible to profiling by omics approaches that can help refine current therapeutic approaches. For instance, TME profiling may be useful to sub-stratify tumor types classified by other omics (137). Additionally, omics such as RNA-seq or methylation can implement tissue deconvolution strategies that can quantify the contribution of each cell type to the tumor phenotype, and hence help identify novel therapeutic pathways.

Microbiome

The human microbiome community has fundamental implications in health and disease. In cancer, it has been estimated that 20% of tumors worldwide are microbially driven (138). This includes examples such as HPV-related cervical,

oropharyngeal and anal cancers, or *Helicobacter pilorii*-resultant gastric carcinomas, but actually the interaction between the gut normal microbiome and cancer is far more complicated (139). Some cancers appear to be critically dependent on their resident microbiome to continue to subsist and evade the immune system, as in the case of *Fusiobacterium nucleatum* in colorectal cancer (122). This and other studies have revealed causal mechanisms for both microbes within tumors and microbes in other host niches, that can mediate tumor growth through direct and immunological mechanisms (140, 141).

Furthermore, the gut microbiota can define key aspects of drug metabolism, pharmacokinetics, effect and toxicity, since the rate of absorption and bioavailability of many oral drugs depends on their exposure in the gut to both host and bacterial enzymes before entering the circulation. The microbiota can also regulate inflammation and adaptive immunity responses, which in turn can affect cancer immune therapies (142), and tumor profiling has shown to date that distinct gut microbiome patterns associate with consensus molecular subtypes of colorectal cancer (143).

As for examples on the influence of the microbiome in therapy outcomes, mice harboring members of the *Gammaproteobacteria* family in their gut have restored therapeutic effect of gembitacine if concomitant antibiotic ciprofloxacin was administered during treatment (144), and CTLA-4 blockade has been shown to depend on microbiomic response in murine isograft models (145). Hence, the application of novel omic technologies can be very useful in assessing the relevance of these interactions, and the possibilities to address better therapeutic and prognostic value may inevitably pass through microbiome profiling (146).

Pan-Omics, Big Data, and Data Integration

Clearly, the advantages offered by novel omic approaches are large, and will surely have important repercussions in the development of novel therapeutics in the future. Surely, the availability of these technologies and its affordable costs have also resulted in relevant efforts to combine the different data sources. Emerging systems for better data integration (including *Big Data* approaches) have been focused on filling the gap between generating large volumes of data and our understanding of biology to reproduce the complexity within biological systems.

For instance, the PCAWG consortium has produced data on 20,000 samples from 33 tumor types that includes whole-genome sequencing, DNA methylation, mRNA transcriptomics, miRNA and protein arrays. With this comprehensive overview, they have been able to reveal that tumor clustering across these tumor types is defined primarily by cell-of-origin (147). This has important repercussions in that perhaps the molecular similarities among histologically or anatomically related cancer types could be a basis for pan-cancer therapeutic strategies and drug development, instead of our current arbitrary decisions based on location only. The validity of this argument has to some extent already been validated by the FDA-recommendation to indicate immunotherapy on all MSI cancers, regardless of site, and clearly it will suggest future directions for exploiting clinical actionability in therapeutics.

Another mentionable effort includes the large panel of comprehensively characterized human cancer cell lines (Cancer Cell Line Encyclopedia - CCLE). The CCLE characterized 1,072 tumor lines to include genetic, RNA splicing, DNA methylation, histone H3 modification, microRNA expression and reverse-phase protein array data. Downstream from these analyses, they also performed data integration with functional characterizations such as drug-sensitivity, short hairpin RNA knockdown and CRISPR-Cas9 knockouts to reveal potential targets for cancer drugs and associated biomarkers. The data is publicly available and could provide a resource for the acceleration of cancer research using *ex vivo* models (148).

EFFICIENCY OF TARGETED THERAPIES: RESPONSE AND RESISTANCE

Choice of therapy is usually undertaken at the moment of diagnosis and throughout disease progression. Apart from the histological information available when the tumor is biopsied, there are two very important factors that determine the therapeutic strategy: response and resistance. Both of those could be aided by omic tumor profiling strategies that could determine the behavior of the tumor prior to drug administration, but are also dynamic processes over the course of treatment (particularly as for the development of secondary resistances). Several examples have been already mentioned in the paper with regards to how tumor profiling at diagnosis can help us define the individuals that will best benefit from a given treatment, with the most prominent likely being the administration of anti-EGFR drugs to *KRAS* wild-type only patients in bowel cancer (149). Currently, further interesting studies are being made on patients exhibiting exceptional responses to systemic therapy, that may provide with unprecedented insights into cancer biology and treatment tailoring (150).

The case for resistance, however, is more complicated, as it is a multi-factorial phenomenon: it summarizes the innate and/or acquired ability of cancer cells to evade the effects of chemotherapeutics and is one of the most pressing issues in cancer therapy. Chemotherapy resistance can arise due to several host or tumor-related factors (151). Resistance can arise at the macroscopic level, based on human organ and/or tissue function, particularly ADME (Absorption, Distribution, Metabolism, and Excretion of drugs) proteins, or at the microscopic level: microenvironmental resistance (changes in pH, glucose or oxygen availability, or changes in TME cell-type composition), or as result of evolutionary resistance. Examples for molecular changes associated with the development of resistance are, for instance, the apparition of the *EGFR* T790M resistance mutation in NSCLC (152), c-MET mutations and loss of anti-VEGF agent effectiveness (153), or therapy resistance mediated by lncRNA inhibition (154), and surely the further is known about the biological features of extensive tumor datasets the more clues we will have to the molecular changes underpinning drug response and resistance.

LIQUID BIOPSY

One of the main limitations of tumor profiling to guide therapy comes from the fact that the information we may obtain on neoplastic features is strictly limited by our capacity to obtain tumor samples. In clinical practice, tumor biopsies are taken routinely for diagnosis, but depending on tumor location and accessibility, obtaining a sample representative enough is somewhat complicated, invasive (even with patients with good health status) and costly. Moreover, biopsies are usually taken from the primary tumor, whereas samples obtained from the metastases are often scarce and additionally overlooked for the purpose of treatment decisions.

In the past couple of decades, novel approaches have arisen to detect tumor products from bodily fluids, including the blood, urine or saliva. These are the so-called liquid biopsy procedures, and include the analysis of circulating tumor cells (CTCs), circulating tumor nucleic acids (ctDNA and ctRNA) or tumor exosomes, among others (155–157). Liquid biopsies provide with several advantages over classical approaches, mostly springing from the fact that they are much less invasive and more affordable. Firstly, they provide an unbiased overview of the tumor molecular features, because they are *a priori* not dependent on how well the biopsy is taken. Secondly, they can inform on primary tumor as well as on secondary growths. This is quite relevant when current therapeutic strategies focus mostly on the primary tumor, whereas the majority of cancer mortality is derived from the consequences of tumor spreading. The possibility to obtain data from the metastases as well could implicate a shift into how we design therapeutic approaches to treat cancer patients. Thirdly, they can provide with a dynamic view on tumor evolution and behavior, because they can be taken sequentially over the course of disease and treatment (i.e. monitoring minimal residual disease). This means steady information that can guide monitoring and therapeutic strategies and that could potentially improve overall survival rates.

These considerable advantages have started to take over in the clinics, and there are now FDA-validated blood tests to detect *EGFR* mutations as a first approach to NSCLC treatment (158). Moreover, some studies based on blood biomarker detection have shown presence of resistance variants even before relapse was evident by imaging diagnostics in a few cancer types already (159, 160). Additionally, ctDNA sequencing in colorectal and breast cancer patients can allow for the detection of chromosome copy number and structural alterations that are therapeutically relevant (161, 162), or *HER2* amplifications in patients with gastric cancer treated with trastuzumab (163). Moreover, ctDNA sequencing has also been shown to prove invaluable in order to monitor the evolution of *KRAS* of secondary resistance mutations (164).

TUMOR HETEROGENEITY AND EVOLUTION, AND ITS IMPACT IN TUMOR PROFILING

As we have mentioned before, NGS technologies have constituted an important leap in our understanding of cancer, particularly in our search for better, more individualized therapies. However, they are not exempt of limitations, some of which we have already mentioned in the previous sections. There is also another factor to take into account that has considerable impact on treatment response, and that derives from the intrinsic and heterogeneous features of the tumor: clonality and evolution of the tumor. Heterogeneity obviously has great impact on treatment decision and disease progression (165). This evolution is marked by the competition of different clones that arouse from the original cell and acquired the necessary driver mutations to fulfill the hallmarks of cancer and gave rise to the tumor. In this situation, the different clones compete against each other for resources such as nutrients and oxygen, and this provokes an accelerated evolution in terms of the tumor's intrinsic diversity and heterogeneity (166).

The first implication for this delicately balanced environment is that, when defining targeted therapies for treatment, we have to take into account the clonality of the targeted mutations. At the moment, treatment decisions are based on the rough presence/absence of the mutation. This relies on the sensitivity of the technologies used for detection, but no formal studies have been made to determine if, for instance, the same responses and increases in survival are observed with patients with 1% or 30% mutated allele fractions for a given variant. Because current NGS technologies can give us a more quantifiable overview of how representative a mutation is within a tumor, trials should be designed to define the actionability level of the detected mutations (what is the optimal percentage rate of a mutation in order for it to produce observable endpoint results)?. Secondly, treatment administration profoundly changes the tumor clonality landscape. In other words, when a patient starts therapy, the tumor suffers from accelerated selective pressure, which significantly changes the fitness of each clone and drives heterogeneity variations. This may eradicate major clones that are sensitive, thereby leaving the chance for other opportunistic, and perhaps previously less adaptive clones to flourish, whereas novel mutations may also arise to overcome sensitivity to treatment. This situation applies to all types of chemotherapy, but even more so to targeted treatments, which may well contribute to vanish the responsive clone population at first instance, but may result in the development of resistance once the targeted clones disappear. This phenomenon has been studied extensively by evolutionary geneticists (167–170), and some models have already been produced by liquid biopsy approaches, where each tumor is treated sequentially depending on the dynamic clone proportions that arise over treatment (171, 172) [**Figure 5** - (173)].

FUTURE PERSPECTIVES AND THE ROAD TO CLINICAL IMPLEMENTATION

Tumor profiling has the potential to radically change the way we treat cancer. Novel omic technologies, as we have shown in this review, have so far provided us with a considerable amount of information, some of which has already been translated into more efficient therapeutic strategies. Nevertheless, there is a lot of

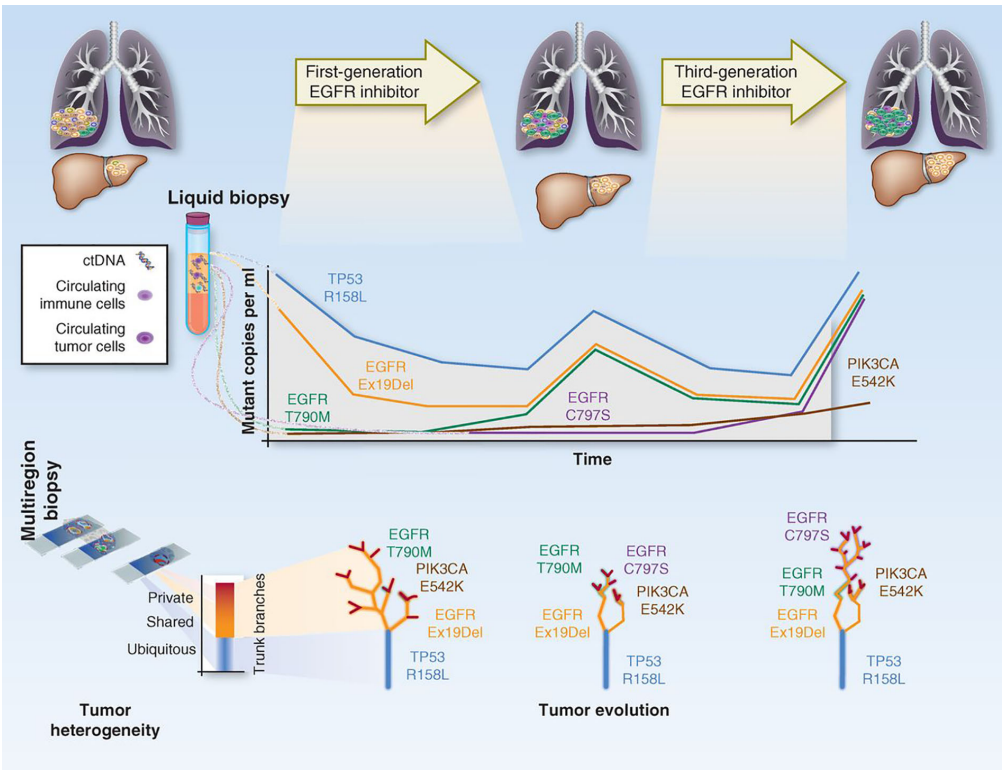


FIGURE 5 | Depiction on how tumor clonal evolution can be integrated into therapy. The figure represents how the tracking of the different clones/mutations can be used to predict treatment response and guide therapeutic decisions. Adapted from (173).

potential to improve and there are several fronts in which profiling could considerably advance therapeutic management and outcome.

For instance, there has already been a shift between classical therapeutic designs decided based on organ and histology toward treatments based on molecular features: immunotherapy is now indicated for all MSI or TMB-high (>=10mutations/Mb) cancers regardless of location, and patients with BRCA-positive tumors of the breast, ovary, prostate and pancreas are treated with PARP inhibitors (35). Because therapy will tend toward a location-agnostic approach, so should diagnostic procedures also. NGS pan-cancer panels have shown to be efficient in detecting actionable mutations in up to 50% of the patients, while allowing for a higher throughput and a quicker turnover than one-by-one IHC approaches (174). Additionally, as often happens with research, although we are at a point where the technologies are vastly available, our capacity to interpret the vast amount of data is limited and overwhelming, and this often results in scientific production failing to translate into clinical practice. Some tools to mine the genomic available info (such as cBio Portal, TCGA database) are already in place, and some will surely become available in the next few years that could help us interpret and integrate the results obtained by omic profiling (175, 176). Moreover, most of the studies on profiling of tumors have been done *a posteriori*, which has given us a lot of insight about tumor molecular features, but may be insufficient to target patients prospectively under the limitations imposed by sample retrieval in the actual clinics. Comprehensive efforts need to be made to protocolize molecular profiling procedures and utilize the data to design meaningful clinical trials that can fill the last step toward clinical implementation of these profiles. Some of these trials have already started to happen, showing great promise in their outcome (177). Another important field of development will be that of broad-spectrum and combined treatments. Synergistic approaches where more than one cancer hallmark is targeted as indicated by the tumor's own features (anti-angiogenetic factors + different mutation-specific treatments, immunotherapy…) will be key to subdue tumor growth and allow for a better patient prognosis (178).

Overall, cancer molecular profiling will surely revolutionize the way we understand, manage and produce drugs for cancer treatment, and it will be an invaluable tool toward our goal of precision medicine and personalized medicine approaches that guarantee increased patient survival in the near future.

AUTHOR CONTRIBUTIONS

CF-R, AS, ML, ÁC, and AC participated in the drafting and revision of this review. All authors contributed to the article and approved the submitted version.

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Synthesis and Preliminary Evaluation of a Novel ^{18}F-Labeled 2-Nitroimidazole Derivative for Hypoxia Imaging

Jing Lu[1,2], Chi Zhang[1], Xi Yang[3,4], Xi-Juan Yao[1], Qun Zhang[2] and Xin-Chen Sun[1]**

[1] Department of Radiation Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China, [2] Department of Health Promotion Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China, [3] Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Fudan University, Shanghai, China, [4] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China

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***Correspondence:**
Qun Zhang
jsph_jkglzx@163.com
Xin-Chen Sun
sunxinchen@njmu.edu.cn

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Objective: Hypoxia is prevalent in tumors and plays a pivotal role in resistance to chemoradiotherapy. ^{18}F-MISO (^{18}F-labeled fluoromisonidazole) is currently the preferred choice of PET hypoxia tracers in clinical practice, but has severe disadvantages involving complex labeling methods and low efficient imaging due to lipophilicity. We aimed to design a novel nitroimidazole derivative labeled by ^{18}F *via* a chelation technique to detect hypoxic regions and provide a basis for planning radiotherapy.

Materials and Methods: First, we synthesized a 2-nitroimidazole precursor, 2-[4-(carboxymethyl)-7-[2-(2-(2-nitro-^{1}H-imidazol-1-yl)acetamido)ethyl]-1,4,7-triazanonan-1-yl]acetic acid (NOTA-NI). For ^{18}F-labeling, a ^{18}F solution was reacted with a mixture of $AlCl_3$ and NOTA-NI at pH 3.5 and 100°C for 20 min, and the radiochemical purity and stability were evaluated. Biological behaviors of Al^{18}F-NOTA-NI were analyzed by an uptake study in ECA109 normoxic and hypoxic cells, and a biodistribution study and microPET imaging in ECA109 xenografted mice.

Results: Al^{18}F-NOTA-NI required a straightforward and efficient labeling procedure compared with ^{18}F-MISO. The uptake values were distinctly higher in hypoxic tumor cells. Animal studies revealed that the imaging agent was principally excreted *via* the kidneys. Due to hydrophilicity, the radioactivities in blood and muscle were decreased, and we could clearly distinguish xenografted tumors from para-carcinoma tissue by PET imaging.

Conclusions: The nitroimidazole tracer Al^{18}F-NOTA-NI steadily accumulated in hypoxic areas in tumors and was rapidly eliminated from normal tissue. It appears to be a promising candidate for hypoxia imaging with high sensitivity and resolution.

Keywords: PET tracer, hypoxia, 2-nitroimidazole, chemoradiotherapy resistance, ^{18}F

INTRODUCTION

Hypoxia associated with tumor resistance to chemoradiotherapy has been reported to occur in more than 60% of solid tumors. The unlimited proliferation of tumor cells and the abnormal distribution of neoplasm vasculature give rise to oxygen supply-demand disequilibrium. Hypoxia appears in tumor cells that are beyond the range of effective oxygen diffusion (about 100-200 μm from nutrient vessels) (1). A hypoxic microenvironment induces a series of self-protection mechanisms, such as apoptosis inhibition, cell-cycle arrest, increased anaerobic glycolysis and tumor angiogenesis (2). Furthermore, hypoxia, an independent prognostic risk factor, triggers resistance to radiotherapy, such that the lethal radiation dose in hypoxic cells is 3-fold higher than that in normoxic cells, which contributes to recurrence and metastasis by reactivating quiescent cells (3–5). Re-oxygenation should play a crucial role in individualized anti-tumor treatment. Hypoxia imaging by positron emission tomography (PET) combined with computed tomography (CT) or magnetic resonance (MR) is a non-invasive method that could be used to measure the level of hypoxia in tumor areas and outline the biological target volume (BTV) to boost the radiation dose. Furthermore, the variance in the standardized uptake value (SUV) of hypoxic regions before and after treatment could be used to evaluate the treatment efficacy and predict the prognosis.

Although many PET tracers that are specifically designed to detect hypoxia have been developed, including nitroimidazole labeled by ^{18}F, ^{68}Ga or ASTM combined with a Cu (^{62}Cu or ^{64}Cu) (6–10), ^{18}F-MISO (^{18}F-labeled fluoromisonidazole) is currently the most common hypoxia imaging agent used in a clinical setting. Nitroimidazole may undergo single-electron reduction by xanthine oxidase. In normoxic cells, the nitro group can be reoxidized and washed out. However, under hypoxic conditions, it is further reduced to form highly reactive intermediates that can bind to cellular macromolecules and be irreversibly trapped in hypoxic cells (11). Due to its lipophilicity, ^{18}F-MISO rapidly penetrates both tumors and normal tissues to provide a robust and reproducible signal, but this also causes slow clearance kinetics and a low target-to-background ratio and contrast (12, 13). Furthermore, ^{18}F-labeling requires time-consuming reactions such as repeated evaporations, radiolabeling, purification and complex synthesis conditions that include a high temperature and the presence of a base catalyst.

We aimed to synthesize a novel hypoxia imaging agent to overcome these drawbacks. McBride et al. reported a high-efficiency labeling method in which ^{18}F is first attached to aluminum as Al^{18}F, which is then bound to a chelate attached to a foundational structure (14). This one-pot process requires only 15 min without a heating evaporation step, and has been used for labeling with positron-emitting nuclides like ^{18}F and ^{68}Ga (15, 16). We used this proven method to design a PET hypoxia tracer with a stable structure and high hydrophilicity, where 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) as a chelate joins Al^{18}F and nitroimidazole.

MATERIALS AND METHODS

Synthesis of NOTA-NI

1) *tert-butyl 2-(2-nitro-^{1}H-imidazol-1-yl)acetate (II)*

A mixture of 2-nitro-^{1}H-imidazole (I, 1 g, 8.8 mmol), tert-butyl 2-bromoacetate (1.8 g, 9.2 mmol) and potassium carbonate (1.3 g, 8.8 mmol) in dry acetonitrile (20 ml) was refluxed under nitrogen for 3h. The reaction mixture was filtered, and the filtrate was evaporated. The residue was purified by recrystallization using ethyl acetate/hexane to give the product as a white solid. Yield: 1.4 g (71%).

2) *N-(2-bromoethyl)-2-(2-nitro-^{1}H-imidazol-1-yl)acetamide (IV)*

A solution of compound II (1.32 g, 5.8 mmol) in CH_2Cl_2 (39 ml) and Tallow Fatty Acid (TFA, 17 ml) was stirred at room temperature for 3.5 h. After the reaction was completed (as determined by thin layer chromatography (TLC)), the filtrate was evaporated, and the residue was used in the next step without purification. A mixture of 2-(2-nitro-^{1}H-imidazol-1-yl)acetic acid (III), 2-bromoethanamine hydrobromide (1.1 g, 5 mmol), 2-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 2 g, 5.2 mmol) and N,N-diisopropylethylamine (DIEA, 5 ml) in dry CH_2Cl_2 (200 ml) and dry N,N-dimethylformamide (DMF, 20 ml) was stirred at room temperature overnight. After the reaction was completed (as determined by TLC), the filtrate was evaporated, and the residue was dissolved in 50 ml of water. The aqueous layer was extracted with ethyl acetate (50 ml × 3). The organic layers were collected and washed with 5% aqueous K_2CO_3, 5% aqueous HCl and saturated brine. The organic layer was dried over $MgSO_4$. The crude product was purified by silica gel column chromatography (petroleum/ethyl acetate = 1:2) to give the pure product as a solid. Yield: 0.52 g (32%).

3) *1,4-bis(tert-butoxycarbonylmethyl)-1,4,7-triazanonane (VI)*

A solution of tert-butyl bromoacetate (21.4 g, 55mmol) in $CHCl_3$ (100 ml) was slowly added to triazacyclononane (V, 6.5 g, 50 mmol) in $CHCl_3$ (50 ml) over 1 h using a syringe pump. The resulting mixture was stirred at room temperature for 24 h. The reaction mixture was then filtered, and the filtrate was evaporated. The residue was treated with water (30 ml), and the resulting solution was adjusted to pH 3 using 1 M HCl and washed with ether (20 ml × 3). The aqueous layer was then adjusted to pH 11 using 1 M NaOH and extracted with CH_2Cl_2 (20 ml × 3). The organic layers were collected and evaporated, and dried to obtain a crude product. Hexane (15 ml) was added to give the disubstituted product as a solid. Yield: 10.9 g (61%).

4) *tert-butyl 2-{4-[2-(tert-butoxy)-2-oxoethyl]-7-[2-(2-(2-nitro-^{1}H-imidazol-1-yl)acetamido)ethyl]-1,4,7-triazanonan-1-yl} acetate (VII)*

A solution of compound IV (665 mg, 2.4 mmol) in dry acetonitrile (7 ml) was added dropwise to a mixture of compound VI (715 mg, 2mmol) and potassium carbonate (331 mg, 2.4 mmol) in dry acetonitrile (10 ml). The reaction was stirred at room temperature for 24 h. The mixture was filtered, and the filtrate was evaporated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 9:1) to give the purified product as an oil. Yield: 354 mg (32%).

5) *2-[4-(carboxymethyl)-7-[2-(2-(2-nitro-^{1}H-imidazol-1-yl) acetamido)ethyl]-1,4,7- triazanonan-1-yl]acetic acid (NOTA-NI)* (**Figure 1A**).

A solution of compound VII (354 mg, 0.64 mmol) in trifluoroacetic acid (2 ml) was stirred at room temperature for 4.5 h. The reaction mixture was concentrated to dryness in vacuo and treated with ether (10 ml), and the ether layer was decanted. The residue was dissolved in water (2 ml) and washed with $CHCl_3$ (2-5 ml). The aqueous layer was concentrated to dryness in vacuo to provide the pure product (254 mg, 90%). ^{1}H NMR (D_2O, 500 MHz): δ 7.37 (d, 1H, J = 1.5 Hz), 7.16 (d, 1H, J = 1.5 Hz), 5.36 (s, 1H), 5.18 (s, 2H), 3.75 (s, 4H), 3.65 (t, 2H, J = 6.0 Hz), 3.51 - 3.53 (m, 4H), 3.45 (t, 2H, J = 6.0 Hz), 3.35 (m, 4H), 3.18 (s, 4H). ^{13}C NMR (MeOD, 500 MHz): δ 172.8, 167.3, 145.1, 128.0, 127.1, 54.9, 54.3, 51.6, 50.7, 49.9, 35.4.

^{18}F-Labeling

$H_2{}^{18}O$ was bombarded by protons in a cyclotron to produce a ^{18}F aqueous solution that was collected under a positive pressure of helium. The radiation dose of fluoride ions was 20 mCi measured by a CRC-15R activity meter (CAPINTEC, Florham Park, NJ).

A stock solution of $AlCl_3$ (0.005mg) prepared by dissolving $AlCl_3{\cdot}6H_2O$ in AcONa buffer (pH 3.5) and Glacial acetic acid (10 μL) was added to 0.1 mg of the synthesized NOTA-NI in acetonitrile (200 μL), and specific amounts of glacial acetic acid, aluminum trichloride, and acetonitrile were added. The prepared ^{18}F solution (50 μL) was then added to the solution of a precursor. The reaction mixture was placed on a 100°C heating block for 20 min. Labeled compounds were passed through an Alumina-N light cartridge (prewashed with 10 ml of normal saline) to remove unlabeled Al^{18}F, and washed with normal saline (5 ml). The collected labeled products were heated to evaporate the rest of the solution and redissolved in saline. The radioactive product was analyzed by analytical HPLC and no impurity was found (**Figure 1B**).

Stability Study

The stability of the above Al^{18}F-NOTA-NI in phosphate-buffered solution (PBS) at room temperature and in human serum at 37°C was analyzed by instant thin-layer chromatography-silica gel (iTLC-SG) [80% methyl cyanide (MeCN) in water] at approximately 0, 3 and 6 h.

In Vitro Cell Uptake Study

Cell uptake studies were carried out using the ECA109 cell line (esophageal squamous cell carcinoma, ESCC). The cells were seeded into 24-well plates at a density of 1×10^5 cells per well before overnight incubation in RPMI-1640 culture medium enriched with 10% fetal bovine serum, and then preincubated under normoxic or hypoxic (1.0 ± 0.1% pO_2) conditions for 4 h. In the test group, Al^{18}F-NOTA−NI was added to the wells (about 0.15 MBq/well) and cells were incubated for 10, 30, 60, 90 and 120 min (n = 3 per time point). The hypoxia tracer of control group was ^{18}F-MISO (**Figure 1C**) with the same dosage. Wells were then washed with RPMI-1640, and cells were isolated by tryptic digestion and dissolved in PBS. The cell uptake value was measured using a γ counter.

Biodistribution Study in Xenografted Mice

ECA109 cells were grown in a normal environment. Cells were harvested after treatment with trypsin and washed with 10 ml of PBS by centrifugation (3000 rpm). Four-week-old female BALB/c mice were each injected subcutaneously with 2×10^5/0.1 ml cancer cells in the right shoulder. At 14 days after the induction of tumor xenografts, all mice had developed a solid tumor mass (weight approximately 1.0 g) in which hypoxia essentially built up. Each xenografted mouse in the test group was administered Al^{18}F-NOTA-NI (1.85 MBq/0.1 ml) *via* a tail vein, while each one in the control group was injected ^{18}F-MISO with the same dosage. Mice were sacrificed by decapitation at 20, 60 and 120 min after radiotracer administration (n = 3 per time point). Tumor, muscle and significant organs were excised and weighed, and blood samples were taken. Counts were obtained using a γ counter. The radioactivity contents of representative organs are expressed as percentages of injected dose per gram of tissue (% ID/g). Results are shown as the mean and SD for four animals. The study was approved by the ethics committee of Nanjing Medical University (IACUC-1712027).

MicroPET Study of Xenografted Mice

MicroPET scans were performed using an Inveon microPET scanner (Siemens Medical Solutions, Erlangen, Germany). Al^{18}F-NOTA-NI (3.7 MBq/0.1 ml) was administered *via* tail vein injection to xenografted mice after isoflurane anesthesia. Pimonidazole (80 mg/kg), a kind of classical hypoxia probe, was simultaneously injected *via* a tail vein. Two-hour dynamic imaging was performed by acquiring 12 × 10-min frames from

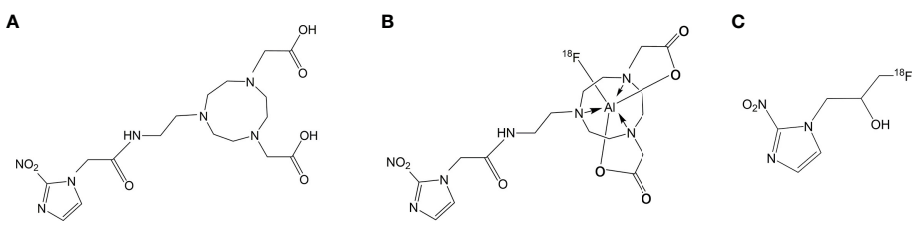

FIGURE 1 | **(A)** Structures of NOTA-NI. **(B)** Structures of Al^{18}F-NOTA-NI. **(C)** Structures of ^{18}F-MISO.

the start of tracer injection to measure time-activity curves (TACs) (n = 4). Ten minutes of static microPET images were acquired at 30, 60, 120 and 240 min after injection (n = 4 per time point). Image reconstruction was performed by Fourier rebinning using an ordered subsets expectation maximization (OSEM) algorithm without attenuation or scatter correction. Regions of interest (ROIs) over the tumor, normal tissue, and major organs were drawn on decay-corrected whole-body coronal images. The radioactivity concentration was obtained from mean pixel values within the multiple ROI volumes. Imaging ROI-derived % ID/g was calculated by dividing the ROIs by the administered activity. Subsequently, animals were sacrifice and tumors were excised, snap frozen, and embedded in ornithine carbamyl transferase. Sections of 8-μm thickness were prepared for fluorescein isothiocyanate (FITC) studies to verify the intratumoral hypoxia. Sections were exposed to FITC-conjugated murine antipimonidazole monoclonal antibody diluted 1:25 for one hour at room temperature, and then imaged again with the markers visualized by red fluorescence.

Statistical Analysis

Quantitative data are expressed as mean ± SD. Means were compared using one-way ANOVA and Student's t-test. For all hypothesis testing, we used two-sided *p*-values <0.05. The results were compared with the data obtained using ^{18}F-MISO under the same experimental conditions.

RESULTS

Physicochemical Evaluation

The chemical structure of NOTA-NI is described in the Methods and the synthetic process is summarized in **Figure 2**. The reaction time for precursor labeling was shortened to 20 min with only one step under 100°C heating (labeling rate, 52.6 ± 3.7%). Al^{18}F-NOTA-NI (after purification by HPLC) is a colorless transparent liquid that is significantly more hydrophilic (logP: -0.952 ± 0.034, pH 7.4) than ^{18}F-MISO (logP: -0.353 ± 0.016, pH 7.4) to reduce the duration of concentration in normoxic cells. The specific activity of either the novel compound or ^{18}F-MISO was 50 GBq/μmol and radiochemical purity was more than 95%. In the *in vitro* stability study, more than 99% of the radiochemical purity of Al^{18}F-NOTA-NI in PBS was retained for 6 h (**Figure 3**). Meanwhile, in human serum at 37°C, more than 95, 85, and 80% of the radiochemical purity was retained after incubation for 0, 3 and 6 h, respectively. This good stability ensures adequate transportation and detection *in vivo*. Considering ^{18}F is a nuclide with the short half-life, we suggest that new imaging agents should be applied in four hours after labeled by ^{18}F.

Cell Uptake Study

Cell uptake *in vitro* was studied using the cell line ECA109. As shown in **Figure 4**, the uptake rate of Al^{18}F-NOTA-NI under hypoxic conditions gradually reached a peak value of 3.46 ± 0.56% at 120 minutes after administration, which was 2.27-fold higher than that under normoxia. The hypoxic-to-normoxic uptake ratio of Al^{18}F-NOTA-NI was increased by 1.53-fold compared to that of ^{18}F-MISO ($P < 0.05$) (**Table 1**).

Biodistribution Study

For both Al^{18}F-NOTA-NI and ^{18}F-MISO, a biodistribution study was performed at different time points (20, 60 and 120 min) after intravenous injection of these labeled derivatives into mice bearing ECA109 xenografts (**Figures 5A, B**). **Figure 5C** and **Table 2** show the uptake values in tumor, blood, muscle and other normal tissues as well as the tumor-to-blood (T/B) and tumor-to-muscle (T/M) uptake ratios for Al^{18}F-NOTA-NI and ^{18}F-MISO. For Al^{18}F-NOTA-NI, the highest uptake was observed in the kidneys (17.90 ± 0.81%ID/g) at 20 min post-injection (p.i.), and this value rapidly decreased to 4.50 ± 0.89% ID/g at 120 min, indicating that this drug is principally excreted *via* the kidneys. The next highest initial uptake was in blood, and again the activity rapidly decreased over time (7.38 ± 0.14%ID/g at 20 min and 1.01 ± 0.44%ID/g at 120 min). Initial uptake in the liver was similar to that in blood (5.24 ± 0.54%ID/g at 20 min p.i.), but the activity remained fairly stable (4.76 ± 0.78%ID/g at 60 min and 3.90 ± 0.55%ID/g at 120 min). In tumor tissue, the initial uptake promptly reached (3.61 ± 0.22%ID/g at 20 min), and moderately declined over time (2.51 ± 0.99%ID/g at 60 min and 2.16 ± 0.24%ID/g at 120 min). ^{18}F-MISO showed a higher initial uptake in the liver, intestine and muscle compared to Al^{18}F-NOTA-NI. Normal organs showed slower depuration, and the specific activities in the liver and kidneys at 120 min p.i. were

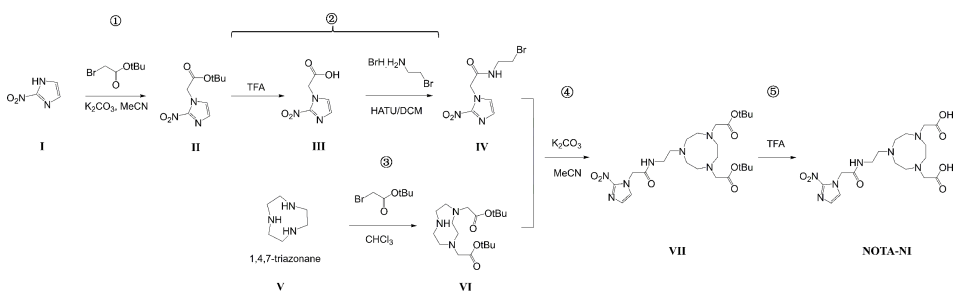

FIGURE 2 | Synthesis pathways of NOTA-NI, a 2-nitroimidazole derivative.

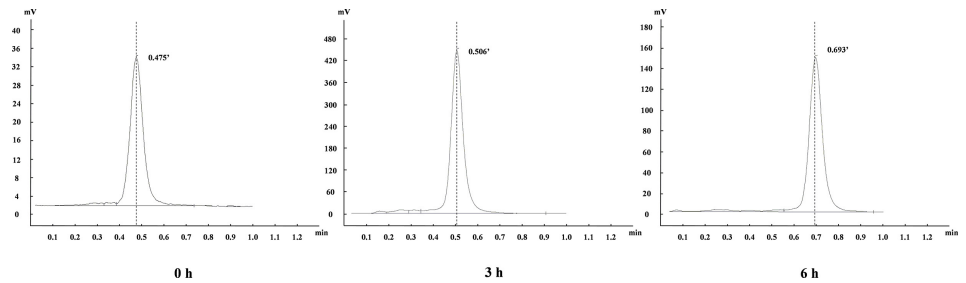

FIGURE 3 | Radiochemical purities determined by iTLC-SG at 0, 3 and 6 h.

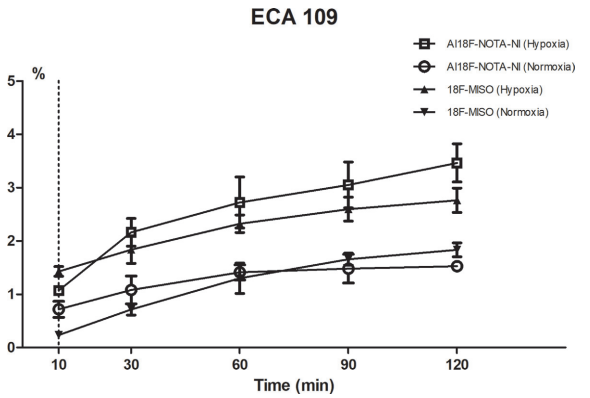

FIGURE 4 | Cell uptake of Al^{18}F-NOTA-NI under hypoxic and normoxic conditions (n = 4 per time point).

TABLE 1 | Hypoxia/normoxia uptake ratios of Al^{18}F-NOTA-NI and ^{18}F-MISO ($p < 0.05$).

Agent	Uptake Rate (mean ± SD, N = 3)
Al^{18}F-NOTA-NI	2.268 ± 0.353
^{18}F-MISO	1.533 ± 0.182

5.74 ± 0.33%ID/g and 2.57 ± 0.16%ID/g, respectively. According to the literature, ^{18}F-MISO is excreted through both the urinary and hepatobiliary tracts (15). Although tumor had a higher uptake of ^{18}F-MISO, more drug penetrated into surrounding soft tissues and blood at the same time point. T/B and T/M of Al^{18}F-NOTA-NI gradually increased, and were apparently higher at 120 min than those for ^{18}F-MISO (T/B: 2.32 ± 0.53 vs. 1.70 ± 0.39, $p = 0.253$; T/M: 2.67 ± 0.08 vs. 1.58 ± 0.24, $p = 0.004$). Statistically significant differences between T/M of the two agents could probably be attributed to the rapid clearance of the highly hydrophilic Al^{18}F-NOTA-NI.

MicroPET Imaging Study

MicroPET imaging studies were also conducted in ECA109 xenografted mice. Images were obtained from a two-hour dynamic scan and a series of static images were obtained at 30, 60 and 120 min after injection (**Figures 6A, B**). In the dynamic scan, the uptake of Al^{18}F-NOTA-NI in kidney was higher than that in liver and decayed with time, whereas radiotracer was accumulated in the bladder and intestine since 20 min p.i. The peak value of imaging ROI-derived SUV for tumor was 2.80 ± 0.24 at approximately 30 min, and the tumor region was visualized with good tumor-to-muscle contrast as early as 30 min p.i., corresponding to the findings of the biodistribution study. In static images, T/M SUV ratios were 4.09 ± 0.59, 4.37 ± 0.94 and 4.53 ± 0.20 at 30, 60, and 120 min, respectively. By pimonidazole labeling and immunofluorescence imaging, we detected hypoxic regions in xenograft tumors (**Figure 6C**).

DISCUSSION

Rasey et al. assessed pretreatment hypoxia in a variety of tumors using ^{18}F-MISO PET (17). In their study, hypoxia was heterogeneously distributed in 97% of the tumors studied, and tumor fractional hypoxic volume (FHV) (range 0 to 94.7%) did not correlate with the size, histology, or site. Hypoxic imaging is mainly used to detect tumor regions with hypoxia-related resistance to assess the prognosis. Qian et al (18). stated that the presence of hypoxia in imaging was associated with worse local recurrence, with a cumulative incidence of local recurrence at 12 months of 0% for patients without hypoxia versus 30% for patients with hypoxia (P <0.01). Since re-oxygenation strongly affects radiotherapy, the detection of hypoxia is needed to predict radiosensitivity, evaluate the therapeutic effect, and delineate the target volume. Generations of hypoxic tracers have been subjected to clinical tests and used in applications that lay the foundation for biological target volume (BTV) and biological intensity-modulated radiotherapy (BIMRT) (19–23). ^{18}F-MISO, a classical nitroimidazole agent, has the disadvantage of requiring a complex labeling method and the pharmacokinetics (tumor-nonspecific accumulation and washout) cause background impurities. To explore more efficient production processes and more sensitive hypoxic tracers, we designed and synthesized a novel nitroimidazole agent, Al^{18}F-NOTA-NI. In this compound, we added an amide bond at the linker between nitroimidazole and NOTA to produce a more hydrophilic agent. NOTA-NI was then labeled by ^{18}F with high efficiency in one

TABLE 2 | Organ uptake, tumor uptake, tumor/blood ratio and tumor/muscle ratio of Al18F-NOTA-NI and 18F-MISO.

Organ (Mean ± SD, %ID/g)	20 min			60 min			120 min		
	$Al^{18}F$-NOTA-NI	^{18}F-MISO	*p*-value	$Al^{18}F$-NOTA-NI	^{18}F-MISO	*p*-value	$Al^{18}F$-NOTA-NI	^{18}F-MISO	*p*-value
Liver	5.24 ± 0.54	9.16 ± 0.16	<0.001*	4.76 ± 0.78	6.08 ± 0.76	0.104	3.90 ± 0.55	5.74 ± 0.33	0.008*
Intestine	2.67 ± 0.37	2.67 ± 0.37	0.002*	1.91 ± 0.40	1.91 ± 0.40	0.114	2.21 ± 0.65	2.41 ± 0.10	0.648
Liver+ Intestine	7.91 ± 0.84	15.16 ± 0.57	<0.001*	6.67 ± 1.02	9.21 ± 0.74	0.025*	6.11 ± 0.98	8.14 ± 0.30	0.026*
Kidney	17.90 ± 0.81	7.49 ± 0.17	<0.001*	6.75 ± 1.31	3.29 ± 0.81	0.018*	4.50 ± 0.89	2.57 ± 0.16	0.060
Blood	7.38 ± 0.14	5.94 ± 1.24	0.180	1.53 ± 0.03	1.58 ± 0.31	0.811	1.01 ± 0.44	1.30 ± 0.21	0.361
Muscle	2.71 ± 0.18	6.03 ± 0.72	0.011*	1.13 ± 0.03	2.04 ± 0.37	0.013*	0.81 ± 0.11	1.40 ± 0.45	0.088
Tumor	3.61 ± 0.22	4.45 ± 0.56	0.072	2.51 ± 0.99	2.78 ± 0.56	0.705	2.16 ± 0.24	2.26 ± 0.94	0.862
Tumor/Blood Ratio	0.49 ± 0.03	0.76 ± 0.05	0.002*	1.63 ± 0.51	1.76 ± 0.12	0.743	2.32 ± 0.53	1.70 ± 0.39	0.253
Tumor/Muscle Ratio	1.33 ± 0.10	0.74 ± 0.01	0.013*	2.21 ± 0.70	1.37 ± 0.16	0.174	2.67 ± 0.08	1.58 ± 0.24	0.004*

*$p < 0.05$.

step at 100°C for 20 min. Our study showed that this tracer was stable at room temperature in PBS and at 37°C in human serum.

Esophageal cancer is an aggressive tumor with a poor prognosis and the 5-year survival rate for unresectable patients with concurrent chemoradiotherapy remains less than 20% (24). Overexpression of hypoxia-inducible-factor 1α (HIF-1α) strongly influence both tumor proliferation and lymph node metastasis in ESCC (25). So hypoxic detection was performed in esophageal carcinoma cell lines and xenografts. In vitro, we observed 2.27-fold greater contrast enhancement in hypoxic compared to normoxic esophageal squamous carcinoma cells ECA109, suggesting that this novel tracer is highly sensitive for detecting hypoxic tissue.

In contrast to the results in the *in vitro* uptake study, which depended on the oxygen partial pressure, in *in vivo* studies, the tracer's efficiency could be influenced by factors such as biodistribution and elimination. Hydrophilic agents have generally been demonstrated to exhibit reduced liver uptake and increased kidney excretion. The introduction of amide bonds would make the product more negatively charged and hence may facilitate rapid clearance through the kidneys (26). The biodistribution study showed that the kidneys had the highest uptake of $Al^{18}F$-NOTA-NI (17.90 ± 0.81%ID/g) 20 min p.i., while the liver had the highest concentration of ^{18}F-MISO (9.16 ± 0.81%ID/g). After the distribution of the drugs stabilized in each tissue, the tumor-to-kidney uptake ratios for $Al^{18}F$-NOTA-NI and ^{18}F-MISO were 0.48 ± 0.05 and 0.88 ± 0.37, respectively. The tumor to liver and intestines ratios were 0.36 ± 0.10 and 0.28 ± 0.12, respectively. ^{18}F-MISO was eliminated mostly through the entero-hepatic pathway, $Al^{18}F$-NOTA-NI was mainly washed out *via* the kidneys. Notably, the high concentration in the bladder and the low uptake in the intestine would obscure masses located in the abdominal area, while the tumor-to-nontumor (liver) ratios gradually increased. Due to its hydrophilicity, $Al^{18}F$-NOTA-NI hardly diffused into cells and showed lower uptake than ^{18}F-MISO in tumors, as well as in normal organs. The initial uptake values for the novel tracer in tumors and other normal tissues were 3.61 ± 0.22%ID/g and 48.00 ± 1.52%ID/g, while the values for ^{18}F-MISO were 4.45 ± 0.56%ID/g and 62.80 ± 2.17%ID/g. At two hours p.i., the uptake values for $Al^{18}F$-NOTA-NI in tumors and normal tissues decreased to 2.16 ± 0.24%ID/g and 15.84 ± 1.20%ID/g, and the values for ^{18}F-MISO fell to 2.26 ± 0.94%ID/g and 21.39 ± 0.71% ID/g, respectively. In this period of time, the uptake values in normal tissues for $Al^{18}F$-NOTA-NI and ^{18}F-MISO had statistically significant difference ($p = 0.002$), but there was no difference in tumor uptake values ($p > 0.05$). The novel tracer was eliminated more rapidly from surrounding tissues but was retained in hypoxic tumor cells, resulting in higher tumor-to-blood and tumor-to-muscle ratios. We analyzed the results by students t-test and demonstrated T/M ratio for $Al^{18}F$-NOTA-NI was statistically higher than that for ^{18}F-MISO, but the difference of T/B ratios had no statistical significance. Considering the small number of experimental mice, we will further expand the sample size to confirm the imaging characteristics of the new drug. Our results in PET imaging studies with ECA109 xenografted mice demonstrated that promising contrast between tumors and normal tissues appeared at 60 min after injection, and T/M SUV ratios continued to rise for two hours p.i. We have proved the presence of hypoxic regions in xenograft tumors by pimonidazole probe. Subsequently, we will further contrast the PET images with the immunofluorescence images on the same cross-section of the tumor, so as to estimate the correlation between the intratumoral uptake of the novel tracer and the hypoxia degree in a single xenograft tumor.

To improve the signal-to-background ratio of ^{18}F-MISO PET, alternative hypoxia tracers, such as ^{18}F-flortanidazole (^{18}F-HX4), have been proposed and subjected to preclinical and clinical trials. ^{18}F-HX4 is more water-soluble than ^{18}F-MISO (logP: -0.69 vs logP: -0.4) (27). In Carlin et al.'s comparative study in animal models, tumor uptake of ^{18}F-HX4 appeared to be broadly similar to that of ^{18}F-MISO, but with more prominent renal uptake and less liver accumulation at this time point (28). Wack et al (29). demonstrated that ^{18}F-HX4 showed a six-fold higher clearance than ^{18}F-MISO in clinical tests. Although the absolute tracer activity for ^{18}F-HX4 was lower at four hours p.i., ^{18}F-HX4 showed significantly higher median contrast (2.08, range 1.87-2.73) over all patients than ^{18}F-MISO (1.58, range 1.54-1.64). Another study in head and neck squamous cell carcinoma (HNSCC) patients showed that ^{18}F-HX4 had faster clearance and a shorter injection-acquisition time, to give a T/M ratio similar to that of traditional ^{18}F-MISO (1.5 h vs. 2 h p.i.) (30).

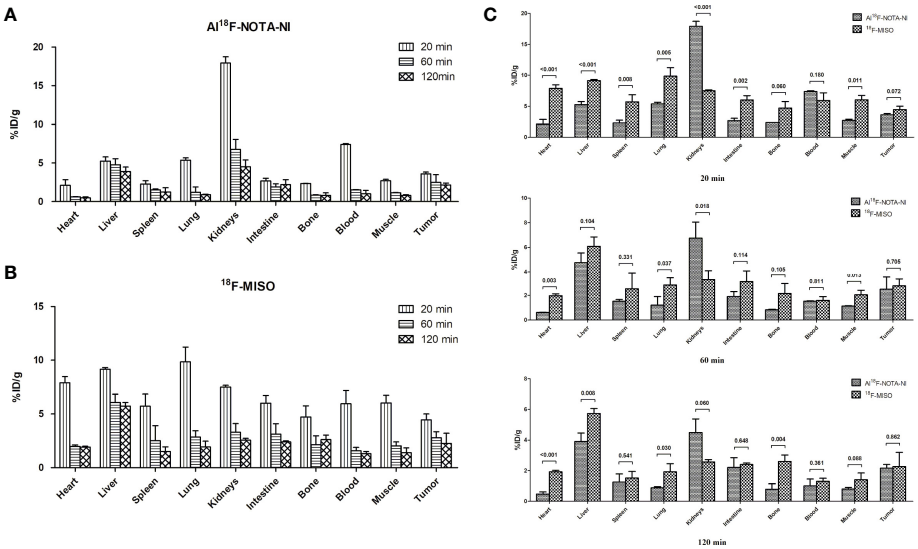

FIGURE 5 | **(A)** Percentages of injected dose per gram of tissue (%ID/g ± SD) in major organs of xenografted mice at 20, 60, and 120 min p.i. for Al^{18}F-NOTA-NI (n = 3 per time point). **(B)** Percentages of injected dose per gram of tissue (%ID/g ± SD) in major organs of xenografted mice at 20, 60, and 120 min p.i. for Al^{18}F-NOTA-NI (n = 3 per time point). **(C)** Uptake value (%ID/g ± SD) of Al^{18}F-NOTA-NI in comparison with that of ^{18}F-MISO in the same kind of organ at 20, 60, and 120 min p.i (n = 3 per time point).

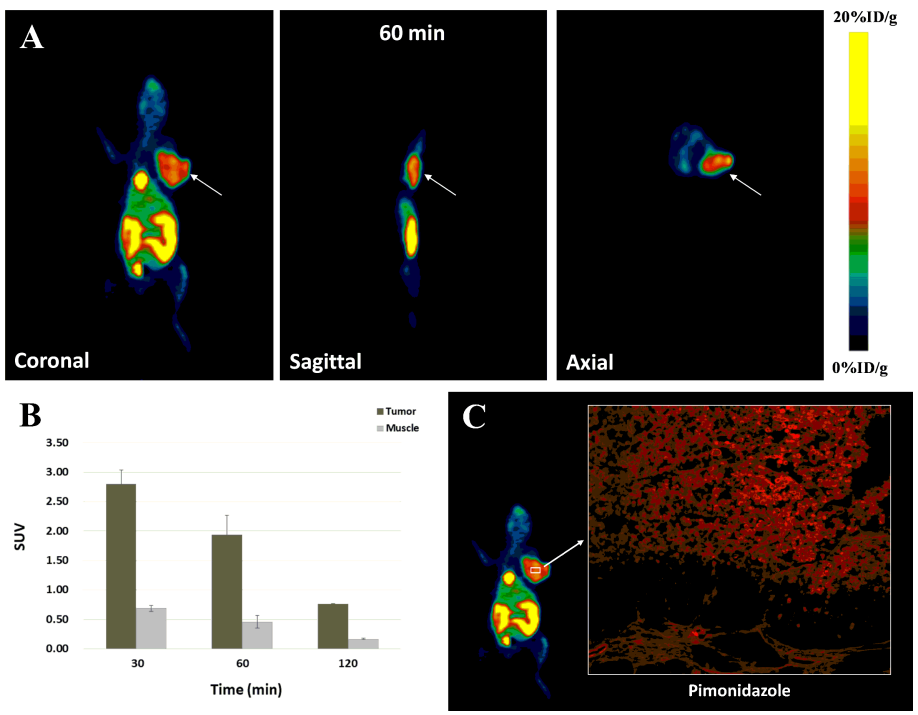

FIGURE 6 | **(A)** MicroPET images of Al^{18}F-NOTA-NI for tumor-bearing mice at 60 min after radiotracer administration (white arrows indicate tumors). **(B)** ROI-derived %ID/g for tumor and muscle, respectively (n = 4 per time point). **(C)** Immunofluorescence images of Pimonidazole in tissue sections of xenograft tumors (red).

The peculiarities of ^{18}F-HX4, as a hydrophilic drug, were in line with those of our tracer. We found that the contrast ratios for all tracers continued to increase over time, and an increase in hydrophilicity accelerated the appearance of obvious contrast between hypoxic tumors and nontarget tissues, due to the faster elimination of free tracer and the resulting decrease in background signal. However, we should not ignore the fact that nitroreductase, the target of nitroimidazole derivatives, is an intracellular enzyme. More hydrophilic tracers tend to have lower tumor uptake because there is insufficient agent to freely diffuse into cells. Therefore, we sought to weigh the pros and cons of reducing lipophilicity. The current experimental results showed that Al^{18}F-NOTA-NI had an ideal detection result in this aspect and significantly improved the contrast between tumor and background compared with ^{18}F-MISO. We can further compare it with ^{18}F-HX4 which is more hydrophilic.

A study indicated that fluorinated nitroimidazoles showed increased radiotracer uptake with not only pimonidazole but also CAIX staining, compared to the use of ^{64}Cu-ATSM to observe hypoxic regions with low staining (28). Dubois et al (31). showed that ^{18}F-HX4 imaging was highly correlated with the endogenous hypoxic marker CAIX so that it could reflect the tumor hypoxic state more accurately than ^{18}F-MISO. Further studies should perform immunofluorescence staining to evaluate the relationship between novel tracer distribution and the mechanism of hypoxia. Furthermore, additional preclinical research and clinical applications should be carried out to better understand individual variations in clearance and distribution.

CONCLUSIONS

We designed and synthesized an ^{18}F-radiolabeled 2-nitroimidazole derivative conjugated with the bifunctional chelating agent NOTA. We testified the radiochemical purity, stability, uptake by hypoxic cells, and target-to-background ratio in *in vitro* and *in vivo* experiments. This imaging agent was principally excreted *via* the kidneys and xenografted tumors were distinguished from para-carcinoma tissue. Our results revealed that this hydrophilic tracer was suitable for hypoxia imaging due to its outstanding pharmacokinetic properties, such as ideal infiltration into hypoxic tumors, fast clearance of free tracer and low uptake into normal tissues.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of Nanjing Medical University.

AUTHOR CONTRIBUTIONS

X-CS is the corresponding author that contributed to design and guide of this research. QZ is the corresponding author too. JL is the first author that contributed to synthesis the novel tracer and write this paper. CZ contributed to the experiments *in vitro* and *vivo*. XY contributed to 18F labeling. X-JY contributed to the experiments *in vitro* and *vivo*. All authors contributed to the article and approved the submitted version.

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