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Women in gastrointestinal cancers, volume II

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Edited by
Nadia M. Hamdy, Aditi Banerjee and Divya P. Kumar

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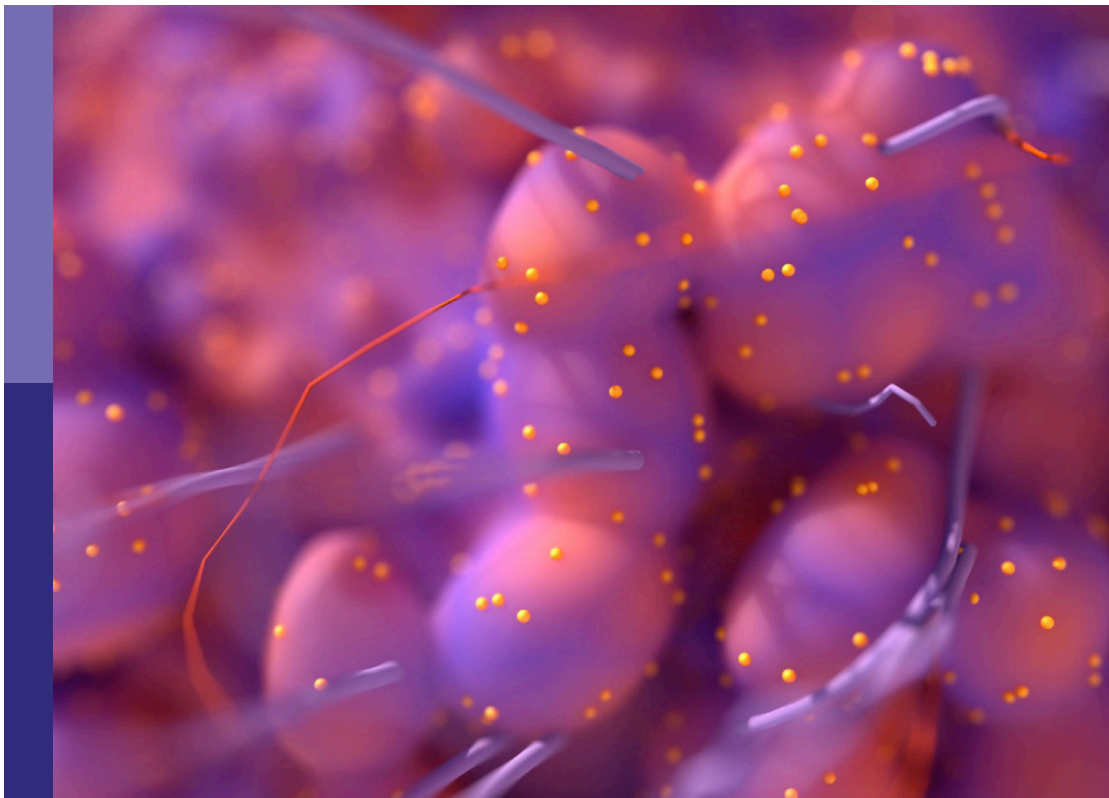

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Women in gastrointestinal cancers, volume II: 2022

Topic editors

Nadia M. Hamdy — Ain Shams University, Egypt

Aditi Banerjee — University of Maryland, United States

Divya P. Kumar — JSS Medical College & Hospital, JSS Academy of Higher Education and Research, India

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Yun Dai,
First Hospital, Peking University, China

*CORRESPONDENCE
Nadia M. Hamdy
nadia_hamdy@pharma.asu.edu.eg

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Editorial: Women in gastrointestinal cancers, volume II: 2022

Nadia M. Hamdy*, Lisardo Bosca[2], Sukh Mahendra Singh[3], Srinivasa Reddy Bonam[4], Igor Kiss[5], Divya P. Kumar[6] and Aditi Banerjee[7]

[1]Department of Biochemistry, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt, [2]Alberto Sols Biomedical Research Institute, Faculty of Medicine, Autonomous University of Madrid, Madrid, Spain, [3]School of Biotechnology, Banaras Hindu University, Varanasi, India, [4]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, United States, [5]Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute and Faculty of Medicine, Masaryk University, Brno, Czechia, [6]Department of Biochemistry, JSS Medical College, JSS Academy of Higher Education and Research, Mysore, India, [7]Department of Pediatrics, University of Maryland, School of Medicine, Baltimore, MD, United States

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women in science, personalized medicine, *in silico*/bioinformatics, CRC (colorectal cancer), gastrointestinal (GI) cancers

Editorial on the Research Topic
Women in gastrointestinal cancers, volume II: 2022

This editorial presents the inaugural Frontiers in Oncology (FIO) "*Women in Gastrointestinal Cancer, volume II: 2022*" series of article collections. The Research Topic collection highlights the diversity of research performed across the entire breadth of oncology research by women scientists pursuing STEM careers. Nine research articles, from the fourteen submitted to FIO within this section, have been published under this Research Topic by 92 authors with the aim of presenting advances in theory, experiment, and methodology and applications to compelling problems related to gastric cancers, such as gastrointestinal (GI) cancer, and hepato-pancreatic-biliary (HPB) cancers. One of the published articles is a brief research report article titled "the BET inhibitor JQ1 potentiates the anticlonogenic effect of radiation in pancreatic cancer cells". Another is a case report article titled "*a female with synchronous multiple primary malignant tumors in the esophagogastric junction, duodenum, and pancreas: Case report and review of the literature*". There is also a methodology clinical trial article titled "*implementing pharmacogenetic testing in gastrointestinal cancers (IMPACT-GI): Study Protocol for a Pragmatic Implementation Trial for Establishing DPYD and UGT1A1 screening to guide chemotherapy dosing*". Four out of the nine published articles are original research articles exploring the tumor–immune interactions in the tumor microenvironment (TME) and identifying new prognostic and/or therapeutic biomarkers to assist in novel decoding mechanism(s) of tumor immunotherapy, as well as being confined with personalized treatment based on geno/phenotypic evaluations. These four articles are, first, "*Evaluation of Galanin Expression in Colorectal Cancer (CRC): An Immunohistochemical (IHC) and*

Transcriptomic Study"; second, "*Swimming Impedes Intestinal Microbiota and Lipid Metabolites of Tumorigenesis in Colitis-Associated Cancer*"; third, "*The Novel LncRNA WASH5P Inhibits CRC Carcinogenesis via Targeting AKT Signaling Pathway*"; and fourth, "*Detection of Glycosylated Markers from Cancer Stem Cells With ColoSTEM Dx Kit for Earlier Prediction of Colon Cancer Aggressiveness*". Moreover, there are two review articles titled "*Prospective Medicinal Plants and Their Phytochemicals Shielding Autoimmune and Cancer Patients Against the SARS-CoV-2 Pandemic: A Special Focus on Matcha*" and, a systematic review, "*Interactions of CRC, Dietary Fats, and Polymorphisms of Arachidonate Lipoxygenase and Cyclooxygenase Genes*". The former review could be considered a study about the importance of herbal medicine (matcha) for protecting immunocompromised cancer patients against SARS-Cov-2. One experimental study used mice to test swimming as a physical activity that could influence metabolism and cancer susceptibility.

Most of the published articles performed bioinformatics and *in silico* analyses using several databases, either before the practical/experimental work or after for confirmation.

Cancer in general was mentioned in one case report paper, gastrointestinal cancers were addressed in three papers, and four papers presented CRC; however, pancreatic cancer was studied in only one article.

The emerging importance of *cancer-personalized treatment plans* is addressed in three research articles, in which the growing significance of cancer-tailored treatment strategies or personalized medicine is discussed.

It was claimed in a systematic review article by Gholamalizadeh et al. that dietary fat has an impact on the risk of CRC, which may be significantly influenced by the polymorphism of the genes arachidonate lipoxygenase (*ALOX*) and cyclooxygenase (*COX*). After further validation, it was suggested that dietary advice regarding fats for CRC prevention might be adopted based on the *ALOX* and *COX* personal genotypes.

Regarding the metabolism of fat and lipids, the role of physical activity was, interestingly, tested with the use of mice by Wang et al. by studying the protective effect of swimming against colitis-associated cancer (CAC). They claimed that swimming interferes with the connection between colonic lipid metabolites and prostaglandin E2 to its receptor signaling (PGE2/EP2 signaling). Swimming-induced genera given to the mice and probiotics increased the intestinal short-chain fatty acids (SCFAs) linked to the significant anti-inflammatory and anti-tumorigenic effects of swimming. Moreover, they were found to promote glycerophospholipid and choline metabolism in cancer cells. Therefore, the authors concluded that swimming is a potent preventive measure against CAC and implied that the differential lipid metabolites screened in the experiment are candidates for medicine with anti-inflammatory and tumorigenesis prevention properties. However, the results need further experimentation validation to identify/confirm their molecular basis.

Long non-coding RNAs (lncRNAs) and their differential expression levels have been implicated in the development and progression of CRC; thus, they are extensively studied nowadays. One article published by Wei et al. studied Wiskott–Aldrich Syndrome Homolog 5 Protein family (WASH5P). Compared to healthy controls, WASH5P expression was dramatically downregulated in CRC cell lines and tissues. The proliferation, invasion, and migration of CRC cells may be markedly reduced by the ectopic expression of WASH5P in these cells. When this happens, WASH5P overexpression can drastically reduce protein kinase B (AKT) activation by preventing AKT phosphorylation.

Talaat et al. performed an IHC and transcriptomic study of the neuropeptide human galanin to address its role in CRC. Galanin, which is widely distributed in the colon tissue and expressed in many cancers including CRC, was identified as a potential CRC-negative biomarker in this study. Galanin expression downregulation in the CRC was connected to cell cycle and cell division, autophagy, transcriptional regulation of TP53, immune system response, and advanced CRC staging.

Blondy et al. used the ColoSTEM Dx IHC kit developed by Carcidiag Biotechnologies to precisely detect glycosylated markers from cancer stem cells (CSCs) by identifying the glycan patterns that CRC stem cells (over)express. As a result, it provides a ground-breaking clinical tool for earlier tumor aggressiveness prediction, and it is of prognostic value for therapy response assessment in CRC patients.

Again, Varughese et al. reported the importance of pharmacogenetic (PGx) testing adoption in routine clinical care. Their current clinical trial (https://clinicaltrials.gov/ct2/show/NCT04736472) was conducted for "Implementing pharmacogenetic (PGx) testing in gastro-intestinal cancers IMPACT-GI Study protocol for a pragmatic implementation trial for establishing germline *DPYD* and *UGT1A1* variants". They established that when tested/screened individually, these variants are associated with reduced enzyme activity, and when being tested/screened, they will guide 5-fluorouracil, capecitabine, and irinotecan chemotherapy dosing to patients at high risk of severe chemotherapy-induced toxicity. Furthermore, the dihydropyrimidine dehydrogenase gene *(DPYD)* and UDP-glucuronosyltransferase 1A1 *(UGT1A1)* polymorphisms are associated with lower enzyme activity—dihydropyrimidine dehydrogenase (DPD) for breaking down 5FU and the UGT1A1 enzyme for inactivating the active metabolite of irinotecan.

Garcia et al.'s study addressed the use of the BET inhibitor JQ1 in pancreatic cancer cells with ionizing radiation (IR) as a combined potentiating treatment option before surgery. JQ1 decreases the expression of the DNA repair protein RAD51 in cancer cells by potentiating the anti-clonogenic effect of IR and increases cancer cell DNA damage. Therefore, Garcia et al. recommended that patients with borderline respectable pancreatic cancer would benefit from using BETi JQ1 + IR as a treatment option pre-surgery

Du et al. suggested, for patients with multiple primary cancers (MPCs), a personalized treatment plan set by a committed multidisciplinary healthcare team with an assessment of all options at various disease/treatment stages along the disease trajectory. This suggestion was made based on their case report and literature review of a female with synchronous multiple primary esophagogastric junction adenocarcinoma, duodenal

adenocarcinoma, and pancreatic ductal adenocarcinoma (PDAC), which had not been reported in the literature before.

Finally, one interesting review article by Kiriacos et al. addressed the impact of matcha as a prospective medicinal plant and novel potential protective and therapeutic phytochemical agent for cancer and immunocompromised patients during the SARS-CoV-2 pandemic. They claimed that matcha is a "tri-acting herbal tea having a potent antitumorigenic effect, immunomodulatory role, and proven anti-SARS-CoV-2 activity". The review mentioned the current status of patients with cancer and their autoimmune system after the emergence of SARS-CoV-2 variants. Moreover, they addressed the effects of all the available medicinal and edible herbs given to those patients, such as black and green tea ingredients and the different constituents of matcha in comparison to previously mentioned herbs.

Author contributions

NH and AB were the associate editors, and DK was the co-associate editor of the current Research Topic. NH wrote and revised the editorial paper text. LB, SS, SB, and IK acted as guest editors for one paper each in the FIO Research Topic: Women in GI tumors Vol II: 2022. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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REVIEW
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Prospective Medicinal Plants and Their Phytochemicals Shielding Autoimmune and Cancer Patients Against the SARS-CoV-2 Pandemic: A Special Focus on Matcha

Caroline Joseph Kiriacos[1], *Monika Rafik Khedr*[1], *Miray Tadros*[1] *and Rana A. Youness*[1,2*]

[1] Molecular Genetics Research Team (MGRT), Pharmaceutical Biology Department, Faculty of Pharmacy and Biotechnology, German University in Cairo, Cairo, Egypt, [2] Biology and Biochemistry Department, School of Life and Medical Sciences, University of Hertfordshire Hosted by Global Academic Foundation, Cairo, Egypt

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Edited by:
Nadia M. Hamdy,
Ain Shams University, Egypt

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Safaet Alam,
State University of Bangladesh, Bangladesh
Waseem El-Huneidi,
University of Sharjah, United Arab Emirates

***Correspondence:**
Rana A. Youness
r.youness@herts.ac.uk;
rana.youness21@gmail.com

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Background: Being "positive" has been one of the most frustrating words anyone could hear since the end of 2019. This word had been overused globally due to the high infectious nature of SARS-CoV-2. All citizens are at risk of being infected with SARS-CoV-2, but a red warning sign has been directed towards cancer and immune-compromised patients in particular. These groups of patients are not only more prone to catch the virus but also more predisposed to its deadly consequences, something that urged the research community to seek other effective and safe solutions that could be used as a protective measurement for cancer and autoimmune patients during the pandemic.

Aim: The authors aimed to turn the spotlight on specific herbal remedies that showed potential anticancer activity, immuno-modulatory roles, and promising anti-SARS-CoV-2 actions.

Methodology: To attain the purpose of the review, the research was conducted at the States National Library of Medicine (PubMed). To search databases, the descriptors used were as follows: "COVID-19"/"SARS-CoV-2", "Herbal Drugs", "Autoimmune diseases", "Rheumatoid Arthritis", "Asthma", "Multiple Sclerosis", "Systemic Lupus Erythematosus" "Nutraceuticals", "Matcha", "EGCG", "Quercetin", "Cancer", and key molecular pathways.

Results: This manuscript reviewed most of the herbal drugs that showed a triple action concerning anticancer, immunomodulation, and anti-SARS-CoV-2 activities. Special attention was directed towards "matcha" as a novel potential protective and therapeutic agent for cancer and immunocompromised patients during the SARS-CoV-2 pandemic.

Conclusion: This review sheds light on the pivotal role of "matcha" as a tri-acting herbal tea having a potent antitumorigenic effect, immunomodulatory role, and proven anti-SARS-CoV-2 activity, thus providing a powerful shield for high-risk patients such as cancer and autoimmune patients during the pandemic.

Keywords: SARS-CoV-2, herbal drugs, autoimmune diseases, nutraceuticals, cancer

INTRODUCTION

In October 2007, a warning letter was issued but no one responded (1). The warning letter was issued by Cheng and his colleagues mentioning that "Horseshoe bats resemble a large reservoir for SARS-CoV-like and the possibility of its reemergence with another novel virus should be taken into consideration because it is a time bomb" (1). The warning letter became a reality 12 years later in December 2019; the city of Wuhan in China experienced the emergence of a novel coronavirus that was initially called "Wuhan pneumonia" (2). It was further classified by the WHO on March 11, 2020 as the 5th documented pandemic since the 1918 Spanish flu pandemic (H1N1) (3).

SARS-CoV-2 has been the main cause of death in 2020 and 2021, accounting for more than 5 million deaths (4). Upon stratification of the mortality lists and the morbidity rates around the globe, several observations have been observed (5). Cancer and autoimmune patients such as those with asthma (6), rheumatoid arthritis (RA) (7), multiple sclerosis (MS) (8), and systemic lupus erythematosus (SLE) (7) were reported to be among high-risk patients during the pandemic (9).

In the case of cancer patients, their chemotherapy-induced immune-compromised status puts them at a higher risk to be easily infected by the virus, and at the same time, such patients should receive their treatment protocols to avoid complications from their oncological diseases (10, 11). Several reports from China (12–14), United States (15), and Italy (16–18) confirmed that cancer patients are at very high risk of developing severe complications upon SARS-CoV-2 infection. Among cancer patients, those with lung cancer are the least fortunate as it was reported that the highest incidence of comorbidity with SARS-CoV-2 was in lung cancer patients (19). Consequently, such patients experience severe symptoms of SARS-CoV-2 that may require intensive care admission and mechanical ventilation, or could result in loss of life (11). This issue encouraged oncological societies such as the European Association for Medical Oncology (ESMO) (20), the American Society of Clinical Oncology (ASCO), the National Comprehensive Cancer Network (NCCN), and many others to provide new guidelines for cancer patients' treatment protocols and diagnostic tests during the pandemic (20). The main ideology behind the new guidelines is to calculate the benefit: risk ratio and categorize cancer patients into high, medium, and low priority based on Ontario Heath Cancer Care as previously reviewed in (21).

The same goes for patients suffering from autoimmune disorders where their immune-compromised status puts them at a higher risk of infection by the virus and developing more severe symptoms (22). In addition, their treatment protocols are mainly dependent on immunomodulatory disease-modifying therapies (DMTs) including glucocorticoids and immunosuppressants that are mainly prescribed to mitigate the immune attacks towards their normal body organs (22). For instance, a study focused on MS patients highlights that younger MS patients with lower socioeconomic status are at a higher risk of exposure to an unfavorable course of SARS-CoV-2 infection (23). In the case of SLE patients, it was first predicted that hydroxyl-chloroquine in their treatment protocol might provide a type of protection from COVID-19 complications (24). Yet, preliminary results from the clinics showed total opposite morbidity and mortality rates (25, 26). Mathian's group reported that SLE patients also showed a high incidence of severe and even fatal cases of infection, confirming that, despite the co-treatment of SLE patients with antimalarial drugs, a high risk of unfavorable infection course has still been witnessed among SLE patients (25). Also, a more coherent study that included 417 SLE patients showed that the morbidity rates are moderately higher in the case of SLE patients (7).

Therefore, it is highly recommended that rheumatologists and oncologists encourage their patients to continue their ongoing treatment to avoid dangerous flare-ups of their autoimmune diseases or complications of their oncological diseases. It is imperative for those patients to have a nutritional plan that shields them from SARS-CoV-2 infection and at the same time improves their autoimmune status in the case of autoimmune patients and/or provide antitumor actions in the case of cancer patients.

In this review, we will show a glimpse of all the therapeutical trials that were carried out during the last couple of years to decrease the socioeconomic burden of such a pandemic. Yet, several failed attempts were witnessed starting from repurposing of conventional drugs, discovery of new medications that might take years of validation, to several vaccination approaches that go in parallel with the high viral mutational capacity (27, 28). However, less attention was given to the ideal remedy—"herbal drugs"—that might be the ultimate route to treat such deadly disease.

In this review, the authors will try to emphasize the significance of herbal drugs that should not be less than that of vaccines and antivirals during the pandemic. Herbal drugs have an edge regarding high-risk patients (cancer and autoimmune patients) in that they might play a dual/triple role in alleviating the primary disease and act as a protective shield during the pandemic.

Upon focusing on the herbal products with their immense roles starting from being antioxidants and holding anti-inflammatory and antiviral activities, we had a closer look at "matcha", which we expect to have a great impact in the upcoming years because of its potent immune-modulatory capabilities and its recent validated activity against SARS-CoV-2 (29, 30). Nonetheless, matcha was also reported to hold a lot of promise for cancer (31, 32) and autoimmune patients (33, 34). Yet, in this review, the authors shed light on the research gap concerning the molecular mechanism of actions underlying matcha as a potent immunomodulatory, anticancer, and antiviral activity.

METHODOLOGY

In this review, the authors screened literature covering the therapeutic effects of "matcha" as a SARS-CoV-2 antiviral herbal drug; this review also focused on the anticancer activity and immunomodulatory role of "matcha". To attain the purpose

of the review, research was conducted at the States National Library of Medicine (PubMed). For the search in databases, the descriptors used were "COVID-19"/"SARS-CoV-2", "Herbal Drugs", "Autoimmune diseases", "Rheumatoid Arthritis", "Asthma", "Multiple Sclerosis", "Systemic Lupus Erythematosus" "Nutraceuticals", "Matcha", "Green tea", "EGCG", "Quercetin", "Cancer", and key molecular pathways. Research papers, books, and published data were reviewed for their relevance to the aim of the review and summarized. Criteria for inclusion were complete, relevant publication, available online, in English, published between 1997 and 2022, and with detailed information about participants, methods, and analyses. Data collection was performed, and data abstracted were in the form of descriptive information, covering the type of samples used, techniques, and findings or effects reported. Bias was limited through the evaluation of the studies through their internal validity rather than the conclusion.

SARS-CoV-2 Structure and Life Cycle

SARS-CoV-2 has a spherical shape with a positive single-strand RNA composed of approximately 30,000 nucleotides and enclosed inside a capsid (35). The genome encodes four structural proteins and many non-structural proteins (nsp) as previously reviewed (36, 37). The structural proteins are Spike (S) protein, Envelope (E) protein, Membrane (M) protein, and Nucleocapsid (N) protein. Inside the capsid, there is a nuclear capsid or the N protein, which is bound to the positive single-stranded RNA and coating it as demonstrated in **Figure 1**. The SARS-CoV-2 life cycle is briefly described in **Figure 2**, since it has been extensively discussed and reviewed in previous reviews (35, 38).

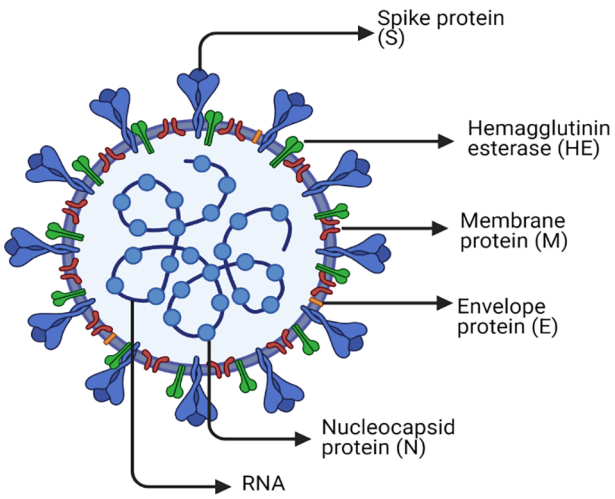

FIGURE 1 | SARS-CoV-2 structure. The figure represents a graphical representation of the viral structural proteins spike (S), envelope (E), and membrane (M), which are embedded in the lipid surface. The positive single-stranded RNA is bound to the nucleocapsid protein (N) in the core of the capsid. Each one of these proteins plays a crucial role in the replication life cycle of the virus. The spike protein (S) is the master that supports the attachment and entry of host cell *via* fusion. The nucleocapsid protein (N) is the one used in transcription, which is included in the replication cycle. The membrane protein (M) that is most abundant on the viral surface drives the viral assembly. Furthermore, the envelope protein (E) has an indispensable role in assembly, host cell membrane permeability, and interactions between the host and virus. Another surface protein is Hemagglutinin esterase dimer (HE) that is found to play a role in cell entry and its infection without having a role in the replication process itself. Finally, the lipid envelope encircles the approximately 30,000 nucleotides, which is the genome of the virus encoding its four structural and many nonstructural proteins (nsp).

Failed Trials to Handle the Pandemic

The current pandemic has urged the public health systems and pharmaceutical companies to develop new antiviral drugs and vaccines against SARS-CoV-2 after being the leading cause of death recently. In an attempt to find effective treatment for COVID-19 patients, enormous efforts were exerted in handling the pandemic. Several approaches were considered such as repurposing of FDA-approved drugs where the doctors were permitted to carry out such clinical trials using a combination of these drugs due to the urgent need to reduce cost, time, and risk of the drug development processes, but this was accompanied by several side effects and limitations as shown in **Table 1**. Thus, not all the repurposed drugs have been approved to be used in ameliorating this pandemic, and some of them were suspended by WHO such as chloroquine, hydroxychloroquine, remdesivir, and lopinavir/ritonavir (74). It is also important to note that all clinical trials highlighted in **Table 1** do not include any of the high-risk patients like cancer and autoimmune patients, who are the main concern of this review.

Throwing light on the currently available vaccines' effectiveness, it was reported that only 30.7% protection was acquired against the new variants of concern "delta" when compared to the "alpha" variant of the virus, which has provided 48.7% protection from a single dose of either BNT162b2 or ChAdOx1 nCoV-19 vaccines (75). However, two doses from these vaccines give a 93.7% protection against alpha and 88% protection against delta for BNT162b2, while for ChAdOx1 nCoV-19, it has an efficacy of 74.5% against alpha vs. 67% for delta (76). For the Pfizer/BioNTech vaccine efficiency, it has 88% protection against the alpha variant, and this percentage has significantly decreased against delta (76). Nonetheless, it was reported that certain mutations were identified in the most recent "omicron" variant that led to higher transmission ability, higher infectivity and binding affinity to ACE2 receptors, and increase in the failure of neutralizing antibodies and immune defense (77). Therefore, relying only on the significance of vaccinations to rescue us from such virulent variants is not a wise solution, especially since it has been well documented and experienced that the vaccines developed against the wild SARS-CoV-2 have lower efficiency rates against the mutated variants (78). Collectively, it has to be recognized that at this stage, vaccine development is important, but still the nourishment of our immune systems has a greater weight in fighting this ongoing pandemic.

After shedding light onto the evolution of new variants of SARS-CoV-2, it is essential to recall the long-lasting Influenza A virus as a live example, which can be compared in parallel with SARS-CoV-2 nowadays. The Influenza A virus causes one of the annual epidemics; even so, it continues to represent a significant threat to global public health due to its very high mutation rates and its ability to cross-transmit between species (79). The same

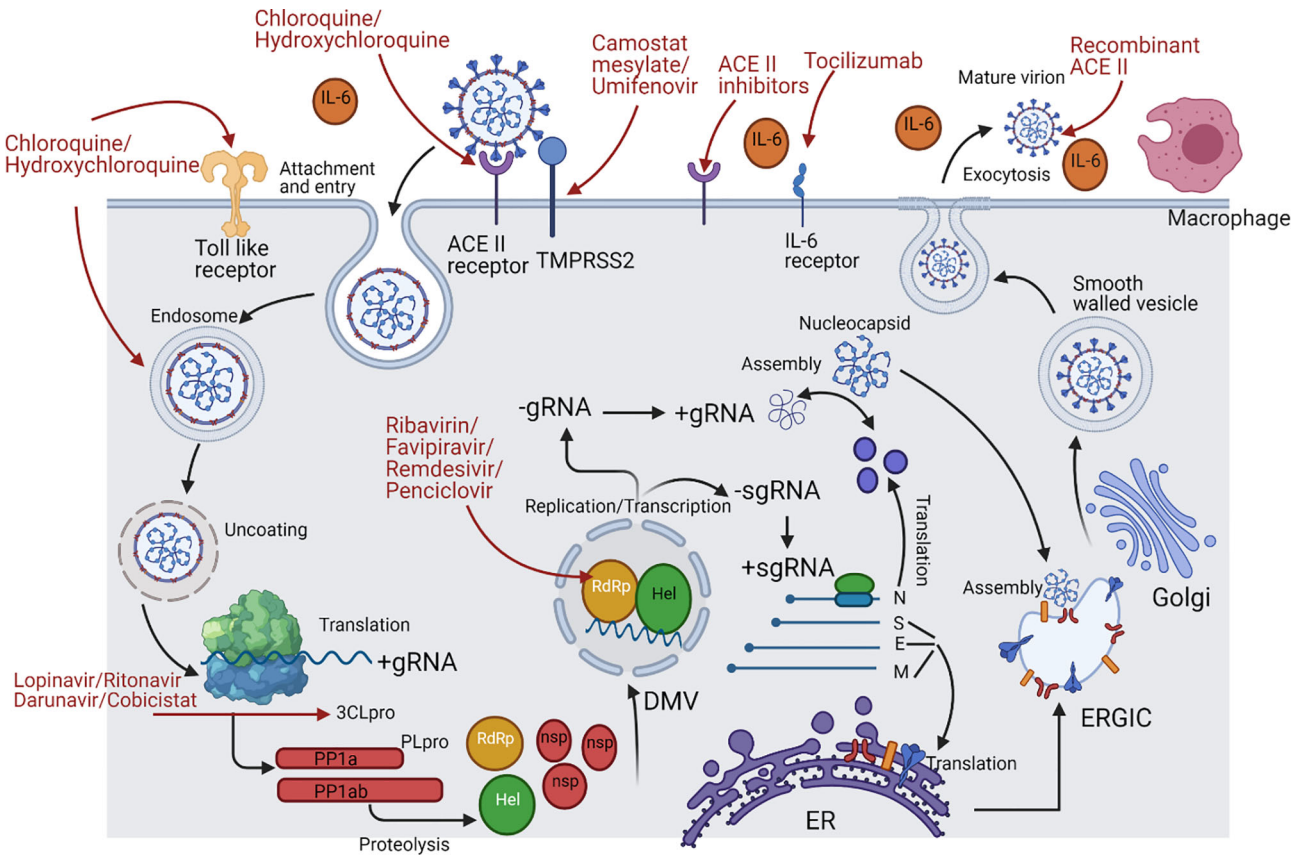

FIGURE 2 | SARS-CoV-2 life cycle and the repurposed drugs targeting specific stages throughout its life cycle. This figure represents a schematic description for the SARS-Cov-2 life cycle with the repurposed drugs targeting specific stages in it. ACE2 receptor on the lung cells is targeted by the RBD of the S1 region in the viral spike protein; however, this binding could be targeted by chloroquine and hydroxychloroquine, recombinant ACEII receptor, or ACEII inhibitors. After the attachment, transmembrane protease serine 2 (TMPRSS2) of the host cell makes a proteolytic cleavage between S1 and S2 subunits, thus separating RBD from the fusion domains, yet this step could be targeted by camostat mesylate and Umifenovir. Consequently, a major step is taken, which is the exposure of fusion peptide domain, enabling the virus to fuse with the cell and pave its way by endocytosis then enclosed in an acidified endosome. Proteasomes then act on the nucleocapsid protein (N), uncoating it and releasing the genetic material freely in the cytoplasm, but this can be inhibited by chloroquine and hydroxychloroquine due to rendering alkaline endosomal PH. Once the positive strand becomes free, translation of the open reading frame 1a/b and production of polyproteins pp1a and pp1ab takes place. The polyproteins undergo cleavage by the viral proteases Papain like protease (PLpro) and Chemotrypsin like protease (3C like protease or 3CLpro or Mpro). Lopinavir/Ritonavir and Darunavir/Cobicistat are the ones used to inhibit 3CLpro. On the other hand, the transcription process start since the Replication/Transcription complex (RTC) was translated, and at this point, there are many nucleoside analogue drugs that were repurposed for inhibiting RNA-dependent RNA polymerase (RdRp) such as Ribavirin, Favipiravir, Remdesivir, and Penciclovir. The RTC will supervise the formation of double membrane vesicle structures (DMV) in the cytoplasm to shield the transcription process. The positive strand is used as a template for making the negative strand, which is then transcribed to make more positive strands. Moreover, subgenomic mRNAs are produced by discontinuous transcription for the sake of being translated to form the 4 viral structural proteins. Once N protein is finished, it combines with a new positive strand for the nucleocapsid to be done. However, S, E, and M proteins proceed to the endoplasmic reticulum (ER) and then to the Golgi apparatus. Last but not least, both the nucleocapsid and structural proteins will be assembled at the ER-Golgi intermediate compartment (ERGIC) to the viral envelope followed by exocytosis of mature virions through smooth-walled vesicles. Many immune components can be released during the whole process such as IL-6, leading to a cytokine storm, so the monoclonal antibody Tocilizumab is used as well as the Toll-like receptor (TLR) inhibitors chloroquine and hydroxychloroquine.

scenario applies where Influenza A rapid evolution resulted in the loss of optimal efficacy for vaccines and antiviral drugs, to which the virus became resistant and thus complete eradication was not achieved (79). As a result, the scientific communities were compelled to use natural therapies and herbal products to boost the immune system as an alternative plan, which showed great success. Some of these herbal products include licorice roots, pomegranate, guava tea, vitamin C supplements, and zinc supplements (80).

Current Status of Cancer Patients and Autoimmune Patients After the Emergence of SARS-CoV-2 Variants of Concerns

Focusing on cancer patients and patients with autoimmune diseases, there are several studies that showed impaired antibody responses following dual COVID-19 vaccination in patients with chronic lymphocytic leukemia (81) and lung cancer (82). Furthermore, it was proved that humoral protection against the delta variant is markedly impaired among chronic

TABLE 1 | A list of currently available drugs for the treatment of SARS-CoV-2.

Drug	No. of Clinical Trial	Clinical Trial Status	No. of Participants	Participants' Average Age	Participants' Status	Intervention	Results	Side Effects	Limitations	Ref.
Ribavirin	NCT04551768	Completed	51	>18 years	Hospitalized	-50 mg/ml over 1 h, 2 times/day for 6 days -100 mg/ml over 30 min 2 times/day for 6 days	Pending	-Hemolytic anemia -Fatigue -Skin Rash -Leukopenia -Teratogenicity	-Excision ability of false nucleotides acquired to coronaviruses by nsp14	(39–42)
Favipiravir	NCT04694612	Completed (Published Results)	30	52.5	Hospitalized	Baloxavir/Marboxil + existing treatment: 80 mg/day on days 1 and 4 Favipavir + existing treatment: 1,600 or 2,200 mg then 600 mg 3 times/day Control: existing treatment	-No extra advantage on using Favipiravir -More vigorous clinical trials are needed to be approved for its international use	-Elevation of liver enzymes -Nausea -Vomiting	-Safety concerns about QT prolongation -Teratogenic potential is unclear	(39, 43–45)
Remdesivir	NCT04280705	Completed (Published Results)	1,062	≥18 years	Hospitalized	Remdesivir or Placebo, 200 mg IV on day 1 then 100 mg once/day for 10 days	No notable benefit specially for mild to moderate diseased patients at day 28	-Worsened respiratory problems -Nausea -Constipation -High alanine aminotransferase levels	-High mortality rate -Not sufficient as sole antiviral treatment -No improvement in mortality, time of virus clearance or time of clinical improvement	(46–48)
	NCT04871633	Completed	66	>12 years	Hospitalized	Remdesivir IV 200 mg followed by 100 mg/day for 5–10 days	Pending	N/A	N/A	
Camostat mesylate	NCT04321096	Recruiting	580	≥18 years	Hospitalized/Outpatients	Placebo: 2 pills 3 times/day for 5 days Camostat mesylate: 2 × 100 mg pills 3 times daily for 5 days	Pending	-Skin rash -Pruritus -Abdominal discomfort -Elevation of liver enzymes	-Early treatment initiation at the first phase of infection is needed	(49–51)
	NCT04608266	Recruiting	596	≥18 years	No initial hospitalization requirement	Placebo: 2 tablets every 8 h for 14 days Camostat mesylate: 2 × 100 mg tablets every 8 h for 14 days	Pending	N/A	N/A	
Darunavir	NCT04252274	Recruiting	30	Child, Adult, Older adult	N/A	Darunavir and cobicistat one tablet/day for 5 days + conventional treatments Conventional treatment only	Pending	-Increasing risk of cardiovascular diseases with increased exposure	-Well established pharmacological profile is needed	(49, 52, 53)
Lopinavir/Ritonavir	NCT04252885	Completed	86	18–80 years	N/A	-Lopinavir (200 mg) and ritonavir (50 mg) 2 tablets each, q12h, for 7-14 days +standard therapy	Pending	-Diarrhea -Nausea -Asthenia	-Further safety profile is needed -No significant improvement in	(54)

(Continued)

TABLE 1 | Continued

Drug	No. of Clinical Trial	Clinical Trial Status	No. of Participants	Participants' Average Age	Participants' Status	Intervention	Results	Side Effects	Limitations	Ref.
						-Arbidol (2 × 100 mg) tid, for 7-14 days +standard therapy -Standard treatment			mortality rate, viral load reduction or on clinical level	
Chloroquine/ Hydroxychloroquine	NCT04322123	Active; not recruiting	630	≥18 years	Hospitalized	Hydroxychloroquine 400 mg BID for 7 days -Hydroxychloroquine 400 mg BID + azithromycin 500 mg once a day -Standard treatment protocol	Pending	-Cardiac arrest -QT prolongation -High rate of hospital deaths	-Narrow therapeutic index -Cardiac toxicity -Safety and efficacy proofs are needed	(55–59)
Nitazoxanide	NCT04486313	Completed	1,092	12–120 years	N/A	Nitazoxanide, 2 × 300 mg tablets BID with food for 5 days -Placebo, 2 tablets BID with food for 5 days	Pending	-Abdominal cramps -Diarrhea	-Further evidence is required or hepatorenal -Cardiac toxicity -Teratogenic effects	(60)
Umifenovir	NCT04260594	Completed	236	18–65 years	N/A	-Arbidol 2 tablets, tid for 14–20 days -Ordinary treatment	Pending	-Nausea -Vomiting	-Limited safety and efficacy documents	(61)
ACE II inhibitors	NCT04364893	Recruiting	700	≥18 years	Hospitalized	- ACEI/ARBs discontinuation - ACEI/ARBs continuation	Pending	-Inflammatory lung diseases -Impaired lipid and glucose metabolism -Cardiac toxicity -Renal malfunction -Impaired immunity	-Negative impact on associated comorbidities as diabetes and hypertension	(62–64)
Recombinant ACE II	NCT04382950	Not yet recruiting	24	18–60 years	N/A	-rbACE2 IV 0.4 mg/kg BID for 7 days + Aerosolized 13 cis retinoic acid from 0.2 mg/kg/day to 4 mg/kg/day -Standard therapy	Pending	-Hypotension -Acute kidney injury	-More preclinical and clinical studies are still needed -Effective only at the early stage of infection	(65, 66)
Convalescent Plasma	NCT 04343261	Completed/ Has results	48	18–90 years	N/A	2 convalescent plasma infusions (2 × 200 ml) each for 1 h	-No significant change in number of days required to discharge, between testing positive and receiving plasma and same for those who died	N/A	-No improvement for critical cases -Finding suitable donors -Disease transmission risk	(67–70)
Tocilizumab	NCT04356937	Completed/ Has results	243	18–85 years	Hospitalized	-IV at dose 8 mg/kg + standard therapy - IV at dose 4 mg/kg + standard therapy	-Failure in death or intubation prevention in moderately ill hospitalized patients	-Septic shock -GIT perforations -Leukopenia -Lymphopenia	-High cost -Opportunistic infections risk -More studies are required	(39, 71–73)

TABLE 2 | Tri-acting (anticancer, immunomodulatory, and anti-SARS-CoV-2) natural products.

Natural Product	Active Constituent	Anti cancer Mechanism of Action	Immunomodulatory Mechanism of action	Anti-SARS-CoV-2 Mechanism of action	Ref.
Ginger	6-gingerol 6-shogaol 10-gingerol	-Induction of apoptosis by increasing caspase-3/7 in gastric cancer cells -Downregulation of cytosolic inhibitor of apoptosis (cIAP)-1 in gastric cancer cells -Inhibition of TRAIL-induced nuclear factor-kappaB (NF-κB) activation in gastric cancer cells	-Inhibiting the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages in multiple sclerosis -Inhibiting LPS-induced NO and production of pro-inflammatory cytokines by inhibiting the NF-kB activation in BV2 microglial cells in multiple sclerosis	-Inhibition of binding between S protein and ACE2 *in silico*	(91–95)
Turmeric	Curcumin	-Upregulation of miRNA-192-5p and suppression of PI3K/Akt signaling pathway in non-small cell lung cancer	-Reduced levels of pro-inflammatory cytokines (TNF-α and IL-1β) in the serum and synovial fluid in adjuvant-induced arthritis in rats -Regulates the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, leading to the suppression of various pro-inflammatory mediators, including MMP9 and MMP13 in arthritis -Inhibition of IL-12 signaling pathway in T cells in multiple sclerosis.	-Inhibition of Toll-like receptors, NF-κB, inflammatory cytokines and chemokines, and bradykinin, decreasing SARS-CoV-2 symptoms	(96–104)
Garlic	Diallyl disulfide (DADS) Alliin	-Proapoptotic effect by histone deacetylation, inhibition of ERK, activation of SAPK/JNK, and p38 pathways in MCF-7 breast cancer cells.	-Suppression of LPS inflammatory signals by generating an anti-inflammatory gene expression and preventing the increase in expression of pro-inflammatory cytokines IL-6 and MCP-1 in LPS induced inflammation in 3T3-L1 adipocytes.	-Attenuation of coronavirus infection by dual S-thioallylation of SARS-CoV-2 Mpro *in silico*	(105–113)
Flaxseed	Omega 3	Upregulation of BAX, downregulation of Bcl-2 and increase in DNA fragmentation in acute myeloid leukemia.	-Reducing the level of antibodies (anti-dsDNA), interleukins (IL-1α, IL-1β, and IL-2) and TNF-α in systemic lupus erythematous.	-Incorporation of Omega-3 in phospholipid bilayer of cell membranes leading to production of less pro-inflammatory mediators.	(114–116)
Citrus fruits	Hesperidin	-Induction of tumor suppressor miR-486-5p and repression of oncogenic long non-coding RNA H19 in breast cancer -Repression of metastatic mediator ICAM-1 in breast cancer	-Suppression of the levels of IL-4, IL-5, IL-13, and IgE levels in serum in mouse model for asthma -Increase the Treg cells production of interleukin IL-10, transforming growth factor (TGF-β), reduction in production of IL-17 and IL-6, decrease in the percentages of Th17 cells, % of Treg cells in the spleen and lymph nodes, reduces ROR-γt factor expression, but enhanced Foxp3 expression in mouse model for multiple sclerosis	-Binding to the TMPRSS2 and ACE2 and block the viral entry *in silico*	(117–120)
Black tea	Theaflavins Theaflavin-3, 3'-digallate (TFDG)	-Reduction in tumor-induced angiogenesis by downregulation of VEGF and HIF-1a in ovarian cancer cells	-Inhibiting the activation of NF-κB- and MAPK-signaling pathways in Rheumatoid arthritis.	-Blockage of viral RNA-dependent RNA-polymerase by *in silico* docking.	(121–125)
Green tea	EGCG Quercetin	-Decrease Bcl-2 expression, increase expression of caspase 3 and Bax in esophagus cancer -Induction of apoptosis and downregulation of PI3K, PKC, COX-2, and ROS. Increased expression of p53 and Bax in liver cancer.	-Reducing IgE and histamine levels, Decreasing FcεRI expression, regulating the balance of Th1/Th2/Th17/Treg cells and inhibiting related transcription factors in asthma.	-Inhibition of Mpro by *in silico* studies -Inhibition of 3CLpro and PLpro by *in silico* studies	(126–132)
***Tripterygium wilfordii* Hook. F.**	Extract Triptolide	-Accumulation has a small of p53 and apoptotic cell death in human prostatic epithelial cells - The sensitivity of gemcitabine-resistant cells to cisplatin treatment is enhanced by activation of mitochondria-initiated cell death pathway and suppression of HSP27 expression in pancreatic cells	NF-κB, NF-κB/TNF-α/vascular cell adhesion molecule-1, and TGF-β1/α-smooth muscle/vimentin signaling pathways induced by TNFs and TLR4 in rheumatoid arthritis -Downregulation of p38 MAPK and NF-κB signaling pathways in neuroinflammation	N/A	(133–136)
***Eucalyptus globulus* Labill.**	Extract 1,8-Cineol	-Suppresses the proliferation of human colon cancer cells by inducing apoptosis	-Reduces the expression of NF-κB target gene MUC2 in asthma	-Inhibition of Mpro *in silico.*	(137–139)

lymphocytic leukemia patients, indicating the urgent need for further optimization of immune protection in this patient cohort (81). Yet, not enough data were reported about the status humoral protection for patients with autoimmune diseases.

Herbal Drugs: From Passenger to Driver Seat During the Pandemic

Applying the concepts of ancient people about natural remedies in defending against colds and flu ensures that natural products were always side by side with any respiratory viral infection (83, 84). By now, most of the population had experienced the impaired protection of the currently available drugs against SARS-CoV-2 and vaccines due to the high rate of naturally occurring mutations. Consequently, a noteworthy concept is that we need an immune-modulatory and broad-spectrum antiviral agent with diverse mechanisms of action that can be readily used for the prevention of future pandemics. In this review, the authors will focus on candidates from herbal medicines exerting their immunomodulatory and antiviral effects especially for immune-compromised COVID-19 patients, and a special focus on the Japanese green tea "matcha" will be addressed. It is also worth mentioning that several reviews had shed light onto the potent role of natural compounds in the prevention of and/or as an adjunct treatment for COVID-19 (85–88). Yet, this review focuses on the tri-acting natural compounds that possess anticancer, immunomodulatory, and anti-SARS-CoV-2 activities, which were proposed as protective shields for cancer and autoimmune patients in particular during the pandemic.

Candidates from Herbal Medicine During the Pandemic

In this section, the authors will focus on candidates from the herbal medicine field that have been suggested to be used during the pandemic. During the last couple of years, a huge number of herbal medicines have been suggested as anti-SARS-CoV-2 agents, for example, purple coneflower, the bark of cinchona trees, Java turmeric, ashwagandha leaves, ginger, turmeric, garlic, flaxseed, tick berry leaves, oregano, elderberry, green tea, orange, and citrus peel as previously reviewed in (89, 90).

This review focuses on natural compounds that possess a triple action including anticancer, immunomodulatory, and anti-SARS-CoV-2 activities as listed below and as summarized in **Table 2**. The inclusion criteria include natural compounds that possess the 3 activities, with a known mechanism of action and molecular targets, and entered clinical trials in the case of anti-SARS-CoV-2 herbal drugs. A detailed list of natural compounds that entered clinical trials as anti-SARS-CoV-2 agents is shown in **Table 3**. The exclusion criteria used in this review include natural compounds that possess only one of the above-mentioned actions, and/or unknown mechanism of action.

Shufeng Jiedu

Shufeng Jiedu capsule (SFJDC) is an oral Chinese herbal medicine prepared from many different plants as rhizome and root of *Polygonum cuspidatum*, root of *Isatis indigotica* Fort, dried roots of *Phragmites communis*, and many others (53, 152). SFJDC was proven to have antibacterial, antiviral, anti-inflammatory, and antitumor effects (153). The capsule preparations are often used to cure Influenza, the thing that made these preparations to be suggested for investigating it against COVID-19 (53). Yet, it is worth mentioning that SFJDC is contraindicated in patients with known serious hypersensitivity to the product itself or any component of the dosage form.

Anticancer Activity of SFJDC

In a study held to discover the effects of combining SFJDC with doxorubicin to treat hepatocellular carcinoma cells, results showed higher incidence of apoptosis along with more inhibition in cancer migration and invasion, indicating that SFJDC could be a potential complementary anticancer medication (153).

Immunomodulatory Role of SFJDC

The anti-inflammatory action of SFJDC was studied using mouse models infected with HCoV-229E, and the study indicated the ability of SFJDC to decrease IL-6, IL-10, TNF-α, and IFN-γ in lungs. This has created the hypothesis about the ability of herbal medicines to attenuate the cytokine storm caused by COVID-19 (154). These effects could be explained by the following mechanisms in which SFJDC was found to be acting with them: the PI3K-Akt signaling pathway was attenuated and the NF-κB-mediated transcription of pro-inflammatory cytokines was inhibited as well (155, 156).

Anti-SARS-CoV-2 Activity of SFJDC

The main constituents of SFJDC are quercetin, wogonin, and polydatin, indicating their ability to bind to Mpro of SARS-CoV-2 by means of molecular docking studies (157). There are clinical data as well for the addition of SFJDC with the standard antiviral therapy, indicating the high probability of SFJDC to shorten the duration of COVID-19 symptoms in mild to moderate cases (157). Another study was conducted at Bozhou People's hospital where the effect of combining SFJDC with arbidol hydrochloride was studied in comparison to the arbidol hydrochloride alone (158). The results revealed clinical improvements in the combined group compared to the other one (152, 159).

Ginger

Zingiber officinale or ginger belongs to the family Zingiberaceae, it is an extremely beneficial herbal medicine used in many aspects. It originated in Southeast Asia but nowadays used worldwide as a food spice (160). Ginger rhizome is used for pain, nausea, and vomiting (161). A very wide range of active constituents are available and divided into two groups: volatile and non-volatile. The volatile group is definitely responsible for the odor and taste of ginger such as sesquiterpene and monoterpenoid hydrocarbons. However, gingerols, shogaols, parasols, and zingerone are the non-volatile constituents (162). Yet, it is worth mentioning that the usage of ginger might be accompanied by several side effects such as abdominal discomfort, diarrhea, heartburn, increased bleeding tendency, and mouth or throat irritation.

TABLE 3 | Herbal drugs in clinical trials against SARS-CoV-2.

Natural compound	Type	Dosage form	Clinical Trial No.	Results	Dose	Mechanism of action	Therapy type	Phase	No. of participants	Clinical trial state	Ref.
Echinacea purpurea	Nutraceuticals	Tablets (Echinaforce)	NCT05002179	Pending	Prevention: 800 mg 3 times/day Treatment: 800 mg 5 times/day	N/A	Primary therapy	Phase IV	122	Completed	(140)
Ashwagandha, Giloy, and Tulsi combination (Ayurveda Intervention)	Traditional medicine	Tablets	NCT04716647	Pending	Ashwagandha: Doses range from 250 mg to 5 g Giloy: Doses range from 500 mg to 1 g Tulsi: Doses range from 500 mg to 1 g	- Inhibitor of the main protease (Mpro or 3Clpro) - Inhibition of the TMPRSS2/ACE II complex	Primary therapy	N/A	28	Completed	(141, 142)
Turmeric	Nutraceuticals	Tablets (NASAFYTOL)	NCT04844658	Pending	1008 mg 8 times/day	- PLpro inhibitor	Supportive therapy	N/A	51	Completed	(89, 143)
Psidii Guava's	Herbal extract	Capsules	NCT04810728	Pending	2 caps 3 times/day	- Inhibitor of 3CLpro and PLpro (mainly quercetin)	Primary therapy	Phase III	90	Completed	(141)
Flaxseed	Nutraceuticals (omega3 fatty acid)	N/A	NCT04836052	Pending	2 mg 2 times/day	- Attenuate pro-inflammatory cytokines	Primary therapy	Phase III	372	Recruited	(144)
Hesperidin	Bioactive phyto-compound	Capsules	NCT04715932	Pending	500 mg 2 times/day	- Inhibitor of 3CLpro and PLpro	Primary treatment	Phase II	216	Completed	(145)
Ginger	Nutraceuticals	Tablets	IRCT20200506047323N1	Pending	1,000 mg 3 times/day	- Inhibitor of PLpro	Primary treatment	Phase III	86	Completed	(146, 147)
Green tea	Nutraceuticals	Capsules	IRCT20150711023153N3	Pending	450 mg 2 times/day	- Inhibitor of 3CLpro and PLpro -Inhibits complex formation with the virus	Supportive therapy	N/A	74	Completed	(30, 148)
EGCG	Nutraceuticals	Capsules	NCT04446065	Pending	250 mg	- Inhibitor of 3CLpro and PLpro -Inhibits complex formation with the virus	Primary treatment (prophylaxis)	Phase II	524	Not yet recruiting	(30, 148)
Colchicine	Bioactive metabolite	Tablets	NCT04363437	Pending	An initial dose of 1.2 mg followed by 0.6 mg after 2 h on day 1. After that, 0.6 mg of two doses up to the 14th day	- Disruption of microtubules and thus affect viral trafficking and the formation of double-membrane viral vesicles	Primary therapy	Phase II	70	Recruiting	(149)
Quercetin	Bioactive metabolite	Tablets	NCT04377789	Pending	500 mg of quercetin given daily to the prophylaxis group. The quercetin treatment group had confirmed cases of COVID-19 and they were provided with 1,000 mg quercetin daily.	- Inhibition of polymerases, proteases, and reverse transcriptase; suppressing DNA gyrase; and binding viral capsid proteins; thus, it possesses an effective antiviral activity	Primary therapy	N/A	447	Completed	(132)
Escin	Nutraceuticals	Tablets	NCT04322344	Pending	Oral administration of standard therapy Escin tablet for 12 days (40 mg thrice a day)	- Potent antiviral activity. Yet, the exact mechanism of action is still unknown.	Adjuvant therapy	Phase II/III	120	Recruiting	(150)

(Continued)

TABLE 3 | Continued

Natural compound	Type	Dosage form	Clinical Trial No.	Results	Dose	Mechanism of action	Therapy type	Phase	No. of participants	Clinical trial state	Ref.
Nicotine	Bioactive phytocompound	Patches	NCT04608201	Pending	As Nicotine patch 0.5 patch for day 1 and day 2 1 patch for day 3 and day 4 1.5 patches for day 5 and day 6 2 patches from day 7 to the day of discharge from hospital (Each patch contains 7 mg nicotine)	- Inhibits the penetration and spread of the virus - Prophylactic effect in COVID-19 infection	Primary therapy	Phase III	220	Recruiting	(151)

Anticancer Activity of Ginger

Ginger's active constituents 6-gingerol and 6-shogaol are the main anticancer agents. Ginger has a broad spectrum anticancer activity against an array of solid malignancies such as gastric, pancreatic, colorectal, and liver cancers as shown in **Table 2** and as previously reviewed in (91). The anticancer activity of ginger is accredited to its aptitude to repress several signaling pathways simultaneously such as the PI3K/AKT/mTOR pathway, the JAK/STAT pathway, the NF-κB pathway, COX-2 signaling, and caspase molecules (91).

Immunomodulatory Role of Ginger

Ginger is now considered a perfect choice for COVID-19 patients as it has analgesic, anti-inflammatory, antiviral, and immunomodulatory effects that can have a great role in the prevention of lung damage and respiratory disorders as listed in **Table 2**. Mechanistically, this analgesic effect is achieved by inhibiting prostaglandin (PG) production through cyclooxygenase (COX) and lipoxygenase (LOX) pathways. This is also achieved by its antioxidant activity where inhibition of the transcription factor Nf-κB occurs. It also acts as an agonist of vanilloid nociceptor, which represses the pain sensation (163). Considering the anti-inflammatory effect, several pathways are involved, but we will focus only on the effect of 6-gingerol, which inhibits the production of pro-inflammatory cytokines from LPS-stimulated macrophages as shown in **Table 2** (164). In the case of immune-compromised patients such as patients with rheumatoid arthritis, its manifestations are proved to be decreased by ginger as it increases the transcription factor forkhead box protein 3 (FoxP3) gene expression and decreases retinoic acid receptor-related orphan receptor γt (RORγt) and T-box expressed in T-cell (T-bet) gene expression (165).

Anti-SARS-CoV-2 Activity of Ginger

As illustrated earlier, one of the drug targeting mechanisms for SARS-CoV-2 is a papain-like protease (PL pro) that cleaves viral polyproteins that are very important for viral replication and survival. It was recently reported that ginger has the potential to act as a PL pro inhibitor for SARS-CoV-2, expressing its antiviral effect (89). Nonetheless, ginger has proven to relieve symptoms associated with COVID-19 infection such as chest pain. Ginger has proven to reduce chest pain and induce relaxation in airway smooth muscle, hindering airway resistance and inflammation as shown in **Table 3** (166).

Turmeric

Curcuma longa or turmeric is a widely known herbal medicine; its main active constituent is the polyphenolic compound curcumin. It belongs to the family Zingiberaceae and used as a food spice, same as ginger. In Asian countries, it is used as a supplement and medicine to treat many diseases such as diabetes mellitus, cardiovascular diseases, obesity, neurodegenerative diseases, inflammatory bowel disease, allergy or asthma, and psoriasis. As mentioned above, turmeric extract is known for its polyphenol curcumin, constituting up to 77%; it contains other active constituents such as demethoxy-curcumin and bis-demethoxy-curcumin (167, 168). Turmeric can be used as an

antiviral, antioxidant, anti-inflammatory, and anticancer agent. It is also important to note that turmeric does not usually cause severe side effects. Some users experience mild side effects such as abdominal discomfort, nausea, diarrhea, and dizziness.

Anticancer Activity of Turmeric

Turmeric is one of the well-investigated anticancer nutraceuticals. It was named the golden spice, whose use was passed on from the kitchen to the clinic (169). Curcumin shows an anti-neoplastic activity against solid malignancies such as breast, liver, colorectal, and prostate cancers, and several types of leukemias and lymphomas as shown in **Table 2** and previously reviewed in (170–172).

Immunomodulatory Role of Turmeric

Turmeric is ranked as one of the most common immunomodulatory herbal drugs as curcumin shows strong antioxidant and anti-inflammatory effects (173). Mechanistically, its anti-inflammatory effects are prominent through the inhibition of the pro-inflammatory molecules: toll-like receptor (TLR-4), phosphatidylinositol-3 kinase (PI3K), and nuclear factor-kappa B (NF-κB). Turmeric also has the potential to repress the production of an array of pro-inflammatory cytokines such as IL-6, tumor necrosis factor-alpha (TNF-α), and interleukin 1 beta (IL-1β) (174, 175).

Anti-SARS-CoV-2 Activity of Turmeric

Concerning the SARS-CoV-2 antiviral activity of turmeric, one of the proposed mechanisms of action is acting as a PL pro inhibitor, same as ginger (89). Yet, in the case of turmeric (curcumin), this is not the only known antiviral mechanism; it is also known to act as an ACE II inhibitor. As previously illustrated, the virus enters the host by its S protein binding to the ACE II receptor. ACE II expression is detected in nasal epithelial cells, alveolar epithelial type II cells (AEC type II) of lungs, and the luminal surface of intestinal epithelial cells. Consequently, it stops viral entry and invasion in these cells (176–178).

Garlic

Allium sativum or garlic is one of the world's oldest cultivated plants and has developed a well-established reputation across many cultures for embodying promising therapeutic benefits (179). More specifically, garlic is famed for its immunomodulatory role. Garlic contains a wide range of active constituents such as allicin, alliin, ajoenes, vinyldithiins, and diallyl sulfide. S-allyl-cysteine, S-ally-mercapto cysteine, and N-acetylcysteine resemble organosulfur examples, but concerning the flavonoidal constituents, quercetin is the main active constituent. The sulfur-containing phytochemicals are mainly responsible for its immunomodulatory, anti-inflammatory, anticancer, antitumor, antidiabetic, anti-atherosclerotic, and cardioprotective features (180, 181). Similar to other herbal drugs mentioned above, some mild side effects could accompany the usage of garlic such as unpleasant mouth or body odor, nausea, vomiting, and diarrhea.

Anticancer Activity of Garlic

The anticancer properties of garlic have been well-documented in several types of neoplastic conditions such as breast, nasopharyngeal, oral, esophageal, and gastric carcinomas, which were previously reviewed in (179). Digging deeper to understand the molecular mechanism of garlic as an anticancer agent, this could be directly related to the sulfur-containing active constituents as they provide a source of hydrogen sulfide (182). In particular, our research group has recently shown the vital role of hydrogen sulfide in cancer progression in different contexts (183, 184).

Immunomodulatory Role of Garlic

As previously mentioned, the immunomodulatory role of garlic extracts has a well-documented property that is mainly related to the sulfur-containing active constituents as well (180, 181). There are extensive mechanisms for the immunomodulatory and anti-inflammatory actions of garlic, specifically alliin, which effectively suppresses the expression of several proinflammatory cytokines such as interleukin 6 (IL-6) and mature plasma cell 1 (MCP-1) (110). In the case of asthmatic patients, garlic has also a proven anti-asthmatic property through repressing IL-4, IL-5, and IL-13 secretion (185). Moreover, the S-allyl cysteine constituent of garlic has also proven to ameliorate MS-related pathology and relieve the associated symptoms through altering tumor necrosis-α level in the MS-mouse model (186). However, concerning SLE patients, no information was reported concerning the impact of garlic on the pathogenesis of the disease or its associated symptoms. Collectively, garlic has been proven to have several features that could provide a protective shield for high-risk autoimmune patients in the current pandemic.

Anti-SARS-CoV-2 Activity of Garlic

Garlic has shown potential antiviral activity against a myriad array of viruses. Its antiviral activity against SARS-CoV-2 has been estimated. It was reported to act as a chymotrypsin-like protease ($3CL^{pro}$) inhibitor, resulting in hindering viral attachment to host cells. Such antiviral activity has been acknowledged to the alliin and quercetin constituents in the garlic (187). In the shadow of SARS-CoV-2-associated high risk of blood clots and increase in D-dimer levels that are directly proportional to mortality rate, it is important to decrease other risk factors for blood clots such as lipids, triglycerides, and cholesterol levels in high-risk patients in particular. In such context, black garlic extracts were proven to have an anti-atherosclerotic action, meaning to decrease the blood levels of total lipids, triglycerides, and cholesterol as they lower sterol regulatory element-binding protein 1 (SREBP-1C) mRNA expression causing downregulation of lipid and cholesterol metabolism (188).

Flaxseed

Linum usitatissimum or flaxseed has been known for its potential anticancer and anti-angiogenic properties against several solid and non-solid malignancies. Nonetheless, it has also been known for its promising immunomodulatory role and recent anti-SARS-CoV-2 activity. This has been associated with its high abundance of lignans and Omega 3. The most common lignin is secoisolariciresinol diglucoside (SDG).

Anticancer Activity of Flaxseed

Literature supports the anticancer activity of flaxseed oil and other isolated compounds from flaxseed both *in vitro* and *in vivo*

(189–191). Ezzat et al. have recently validated the anticancer activity of lignin-rich fraction from flaxseed against breast cancer cell lines and mice bearing tumors as well. It was reported that lignin-rich flaxseed fractions markedly repressed vascular endothelial growth factor (VEGF) and 1-α, metalloproteinases harnessing breast cancer metastasis *in vitro* and *in vivo* (191). Moreover, it was reported to activate the caspase-3-dependent apoptosis as a mechanism of its antiproliferative activity (190, 191).

Immunomodulatory Role of Flaxseed

For a very long time, PUFA has been known to treat metabolic, cardiac, inflammatory, and autoimmune diseases and reduce the risk of cancers (192). Generally, omega 3 PUFA has a great immunomodulatory effect in cases of acute pneumonia and acute respiratory distress syndrome (ARDS) by reducing reactive oxygen species and pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8 (193, 194).

Anti-SARS-CoV-2 Activity of Flaxseed

Since flaxseed's immunomodulatory role and its inhibitory impact on several cytokines that are reported to be dominant players in the cytokine storm manifested by SARS-CoV-2 patients were validated, the effect of flaxseed on SARS-CoV-2 patients was evaluated. It was found that omega 3 reduces lung inflammation caused by the SARS-CoV-2 infection by decreasing IL-6 production, extracellular signal-regulated kinases 1 and 2, COX-2 activation, and the nuclear translocation of NF-κB (144).

Citrus Fruits

Citrus fruits such as *Citrus sinensis* (sweet orange) are the most widely used functional food during the pandemic. This was definitely because of its highly relevant active constituents in combating SARS-CoV-2. Citrus fruits are rich in vitamin C, carotenoids, and flavanones (195). Nonetheless, even the hesperidin flavone is found in the peel and the white part (albedo) of citrus fruits (196). Hesperidin has manifold properties such as antiviral, antimicrobial, antioxidant, antitumor, antihypertensive, and immunostimulant activities (197).

Anticancer Activity of Citrus Fruits (Hesperidin)

Our research group has recently focused on the anticancer activity of hesperidin and its glycoside hesperetin, where we and others showed that hesperidin has potent anticancer properties against several hallmarks of breast cancer such as cellular viability, proliferation, colony-forming ability, migration, and invasion *in vitro* (198–200). Moreover, it was found to have a direct impact on the tumor microenvironment at the tumor-immune synapse through altering ICAM-1 and ULBP2 in MDA-MB-231 breast cancer cell lines.

Immunomodulatory Role of Citrus Fruits (Hesperidin)

Hesperidin has been recently reported to have a direct post immunomodulatory role on autoimmune patients. It was reported that hesperidin can reduce the neuroinflammation episodes experienced by MS victims as well as ameliorates the immunological outcome in an MS-mouse model (118). In a more comprehensive study, it was reported that hesperidin alleviates several neurological disorders including MS through its anti-inflammatory and potent antioxidant activities (201). It is also important to note that hesperidin was also found to have anti-arthritogenic effects in an experimental model of RA (202).

Anti-SARS-CoV-2 Activity of Citrus Fruits (Hesperidin)

More than one mechanism was proposed for the anti-SARS-CoV-2 activity of hesperidin; as explained before, SARS-CoV-2 is internalized by binding of the spike glycoprotein of the virus with ACE2 receptors. Hesperidin superimposes the ACE2-receptor-bidomain (RBD) complex, so it binds to the virus spike protein (197). Also, it was suggested that it binds "3Clpro" or "Mpro", preventing the processing of viral proteins pp1a and pp1ab into functional proteins in the host cells (145). Furthermore, it is considered a powerful antioxidant as it is powerful against superoxide and hydroxyl radicals that cause oxidative stress, and it can help control specific phases of the life cycle of SARS-CoV-2 and finally prevent cell death (197, 203–205). However, the main antioxidant effect of orange peel goes back to vitamin C content. It was suggested that increasing vitamin C daily intake during the COVID-19 pandemic is a useful protective measure as it stimulates antiviral immune responses and reduces the lungs' inflammatory status (144, 206, 207).

Echinacea purpurea

Echinacea purpurea or the purple coneflower is a well-known herb highly recommended for respiratory infectious diseases in Europe as it is already present in different forms such as extracts, tinctures, teas, and sprays, and at different dosages as well (208). The purple coneflower contains many bioactive compounds such as chicoric acid and caffeic acids, alkylamides, and polysaccharides (209). These active constituents were proven to have antiviral effects against enveloped viruses such as human coronavirus (209). Its supplements are widely recommended by naturopathic doctors for their immune support function (210). Moreover, it is well known for its various immunomodulatory, antioxidant, anti-inflammatory, and antibacterial properties (211, 212).

Anticancer Activity of Echinacea purpurea

The anticancer mechanism is still not clear, but a study showed that chicoric acid has the ability to induce apoptosis in colon cancer cells and to decrease the telomerase activity in HCT-116 cells (213). Another study done on human pancreatic cancer cells and colon cancer cells indicated the ability of the root extract to induce DNA fragmentation and increase the activity of caspase 3/7 in a dose- and time-dependent manner, thus inducing apoptosis (214).

Immunomodulatory Role of Echinacea purpurea

Extracts of *E. purpurea*, both aqueous and alcoholic, regulate the immune cells in both adaptive and innate systems (215–217). It works by improving $CD4^+$ and $CD8^+$ T lymphocyte and cytokine levels in blood. These cytokines include the three interleukins IL-6, IL-10, and IL-17 (218). To inhibit inflammation, it suppresses interleukins IL-2, IL-6, and tumor necrosis factor (TNF-α) (219).

Anti-SARS-CoV-2 Activity of Echinacea purpurea

E. purpurea seems to augment its antiviral response by influencing PRRs on the innate immune cells, pathogen-associated molecular pattern PAMPs on the virus (220). Such interaction triggers phagocytosis and initiation of other antiviral responses by the immune system (221). It was also noted that *E. purpurea* has an effective antiviral role against rhinoviruses (222), influenza virus (223), RSV (208), herpes virus (208), adenoviruses (208), and coronaviruses (224, 225).

Java Turmeric

Another potential candidate is Java turmeric, also known as *Curcuma zanthorrhiza*. It is a highly promising candidate as its major active constituent is xanthorrhizol (accounts for 44.5%) (226). Java turmeric is widely used in Southeast Asian countries and belongs to Zingiberaceae and *Curcuma* genus (90). This plant is usually used as an important food additive to enhance flavors (227), but as a treatment component, it is well approved for some diseases and can be used as supplements (226–229). This plant has a myriad of functions, namely, it has antimicrobial, antioxidant, antihyperglycemic, antihypertensive, antiplatelet, anticancer, and nephroprotective effects, and it can be used as a supplement in SLE (230–233). These characteristics make it a potential adjuvant therapy for COVID-19 patients and a preventive measure for high-risk patients, especially SLE patients.

Anticancer Activity of Java Turmeric (Xanthorrhizol)

The anticancer activity of Java turmeric can be due to the induction of the TP53-dependent mitochondrial pathway and thus induction of apoptosis (234–236). It can also induce caspase activation, which will lead to enzymatic proteolysis of DNA and cytoplasmic proteins leading to cell death (227). A study done on HCT166 colon cancer showed that xanthorrhizol leads to higher expression of NAG-1 and increases the activity of its promoter (237). NAG-1 (non-steroidal anti-inflammatory drug-activated gene 1) is a pro-apoptotic and is a member of (TGF-β) (237). Regulation of the MAPK pathway is another function for xanthorrhizol; it increases ROS levels intracellularly and enhances phosphorylation of p38 and JNK in SCC-15 oral squamous cell carcinoma (238).

Immunomodulatory Role of Java Turmeric (Xanthorrhizol)

It was proven that it inhibits the production of inflammatory cytokines from adipose tissue by downregulation of inflammatory cytokine genes and inhibits the expression of TNF-α as well (90). For SLE patients with hypovitamin D levels, a study showed that xanthorrhizol can lower the serum level of IL-6 and increase the serum level of TGF-β (229). Another study released the same results when done on hippocampal neurons and primary culture microglia, and this inhibition of inflammation could be due to inhibition of nitric oxide synthase (iNOS), and consequently, lower levels of nitric oxide (NO) are produced (235, 239, 240). Collectively, it may play an immunosuppressant role (90).

Anti-SARS-CoV-2 Activity of Java Turmeric (Xanthorrhizol)

Xanthorrhizol was shown to have a potent antiviral activity against SARS-CoV-2 variants such as GH clade strain and delta strain, so it can be a promising antiviral plant against COVID-19 (241).

Ashwagandha

Withania somnifera is widely known for its antiviral, immunomodulatory, anti-inflammatory, anti-stress, antihypertensive, and antidiabetic effects, and many clinical trials were made to study its safety profile in humans (which eventually confirmed its safe use in humans) (242, 243). Moreover, there are scientific proofs for the ability of *W. somnifera* to maintain immune homeostasis in states of infection and inflammation (244, 245). The main active constituent in this plant is called Withanolides, which is a group of C28 steroidal lactone triterpinoids, including withaferin A; withanolide A, B, and D; withanoside IV and V; withasomniferin A; withanone; sitoondoside IX and X; and 12-deoxywithastramonolide. Furthermore, there are other active constituents such as catechin, naringenin, and syringic acid p-coumarin. This combination of such significant components endows *W. somnifera* superior protective capability (245, 246).

Anticancer Activity of Ashwagandha

Withaferin A is considered to be the principal component in *W. somnifera*; it works by inhibition of β-tubulin and consequently stops the proliferation of cells (247); it also inhibits tumor proteasomal chymotrypsin (248). It was also proven that withaferin A inhibits the cancer chaperon Hsp90 as it stabilizes the signaling proteins (249). Moreover, Notch1, which mediates the survival of colon cancer cells, is inhibited as well by withaferin (250).

Collectively, withaferin A and withanone promote ROS signaling, so they induce cancer killing by oxidative stress along with other pathways (251, 252). Finally, a study was carried out in a mouse model that concluded that *W. somnifera* alcoholic extracts inhibit tumor proliferation and growth and increase life span (253).

Immunomodulatory Role of Ashwagandha

Withanolide A encourages B- and T-cell proliferation with improvements in TH1 response as well (254–258). In mice, *W. somnifera* extracts led to higher counts of leukocytes and platelets (259, 260), and in chicks, the count of $CD4^+$ and $CD8^+$ also increased when compared to normal levels (261, 262). *W. somnifera* extracts were found by a study to be an immunostimulant when administered with anupana as vehicle, and the results revealed the activation of T cells and NK cells after 4 days only with BID consumption (263).

Anti-SARS-CoV-2 Activity of Ashwagandha

W. somnifera can impede the viral replication cycle. Withanone destabilizes the complex between the ACE2 receptor (host) and spike protein (virus) (264), and in addition, withaferin A and withanone are responsible for blocking Mpro and TMPRSS2 enzymes, which could interfere with the entry of the virus (142, 264, 265). Withacoagin and withanolide B have the ability to block the spike protein and also the RdRp with a high affinity (266). It was reported that they prevent virus entry to the host through inhibition of the trans-membrane protease serine 2

(TMPRSS2)/ACE II complex, thus hindering SARS-CoV-2 entrance to host cells (142).

Green and Black Tea

Tea is from the plant *Camellia sinensis*, which is a highly consumed beverage worldwide, with approximately 2.5 million tons produced each year. The difference between green and black tea is in the manufacturing process as green tea, once harvested, is steamed to prevent fermentation, while black tea is left as it is, causing the dimerization of catechins to theaflavins (267, 268). Although the composition of tea can change according to the climate, leaves, season, etc., the main constituent in it is considered to be polyphenols (269). Tea is not just a normal beverage, as research has turned a spotlight on it to study its various effects whether *in vivo* or *in vitro* (269). The studies revealed that polyphenols present in the tea can have a role in several diseases including cancer, diabetes, and cardiovascular diseases (269).

Black Tea

The polyphenols present in black tea are mainly theaflavins and thearubigins (269). Derivatives of theaflavins are theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B), and theaflavin-3,3'-digallate (TF3) (270). Investigations of their biological properties found a myriad of benefits including antiviral, anti-inflammatory, antioxidant, antitumor, and antibacterial activities (271–273).

Anticancer Activity of Black Tea. Black tea shows a potential in the treatment of many types of cancer such as breast, prostate, lung, ovarian, cervical, and liver (274). *In vitro* studies for breast cancer showed 40% smaller tumor size for the intervention group when compared to controls (275). For prostatic cancer, significant inhibition for the androgen receptor promoter region along with inhibition of androgen receptor expression was noticed (276). Moreover, prostatic adenocarcinoma cell viability is inhibited in a dose-dependent fashion with TF1, TF2a, TF2b, and TF3 (277). A myriad of studies have proven the antiproliferative activity of theaflavins and the inhibition of survival and migration ability of cancer cells (274). There are studies that reveal the proapoptotic potential of theaflavins by observing higher levels of Bax (apoptotic protein) and lower levels of Bcl-2 (antiapoptotic protein) (274). Furthermore, P53 levels are increased by theaflavins and reduction in the levels of phosphorylated Akt, phosphorylated mTOR, and c-Myc occurs (274). Generally, theaflavins show a potential for cancer treatment and prevention (274).

Immunomodulatory Role of Black Tea. Theaflavins were proven to have the potential for inhibition of not only lipopolysaccharide (LPS)-induced intracellular adhesion molecule (ICAM)-1 but also the expression of the vascular cell adhesion molecule (VCAM)-1 by blocking pathways of NF-kB and c-Jun N-terminal kinase (JNK); this in turn will shut down the neutrophils since ICAM-1 and VCAM-1 are expressed on the endothelial cell surfaces (278–280). Theaflavins also have the capability to inhibit ROS and neutrophil elastase enzyme (the one that increases the permeability of alveolar epithelium) in a promising way (280–282).

Anti-SARS-CoV-2 Activity of Black Tea. The antiviral activity of black tea comes from TF1, TF2a, TF2b, and TF3, which were proven to have high affinity for 3CLpro and inhibit it (270). Theaflavins also showed a potential for inhibiting RNA-dependent RNA polymerase RdRp (283) and RBD in the spike at locations near the contact between ACE2 and spike protein (270). The roles of theaflavins go beyond treatment since TF3 was found to be able to bind to the ACE2 receptor, thus preventing spike RBD from attaching (284), leading to prophylaxis effects (285).

Green Tea

The main polyphenolics in green tea are quercetin and catechins, which include epigallocatechin-3-gallate (EGCG) (the most predominant one), epigallocatechin, epicatechin-3-gallate, epicatechin, gallocatechins, and gallocatechin gallate (269, 286). The main investigated biological effects were anti-inflammatory, antibacterial, antioxidant, antiproliferative, and antitumor (267), as shown in **Figure 3** and briefly described below. The main prominent effect for EGCG is being a potent antiviral more than the chemically synthesized drugs (270).

Regarding the scope of the review, their antiviral activity and their potential as anti-SARS-CoV-2 nutraceuticals will be the main focus especially since the anticancer activity and immunomodulatory role of EGCG and quercetin have been validated and previously reviewed (287, 288). This part will discuss some of the proposed mechanisms of EGCG (the main catechin) as an anticancer and immunomodulatory constituent of green tea as an introduction for its significance, then the spotlight on it, as well as on quercetin, will be turned on again in the matcha part.

Anticancer Activity of Green Tea. Apoptosis is the needed end result in the treatment of any cancer, so highlight was thrown on EGCG's ability to induce apoptosis. Studies showed its ability to induce apoptosis by generating ROS and activating caspase-3 and caspase-9. Consequently, this leads to cycle arrest at the G1 phase (289, 290). NF-kB, which has a major role in apoptosis inhibition in cancer (291), was inhibited by EGCG in breast cancer, lung cancer, and human non-squamous cell carcinoma (292, 293). Activator protein-1 (AP-1), which induces proliferation, is also downregulated by EGCG (294). Actually, EGCG was proven by several studies to inhibit VEGF production through inhibition of STAT-3 and NF-kB in breast and human non-squamous cell carcinoma (292). Another study indicated the efficacy of EGCG on the inhibition of the IGF/IGF-1R axis (295, 296). Even in epigenetics, EGCG inhibits the activation of DNA methyltransferase, leading to restoration of silenced tumor suppressor genes as an end result; these genes include retinoic acid receptor- β (RARβ), p16INK4a, and O^6-methylguanine-DNA methyltransferase (297).

Immunomodulatory Role of Green Tea. In an aim to investigate the effects of EGCG on cytokine level modulation, a study was done on activated human primary T cells to see the effect on atherogenesis (298). This study found that EGCG has successfully decreased the level of interleukins IL-2 and IL-4, INF-γ, and TNF-α. EGCG also decreased the level of phosphorylated c-Jun

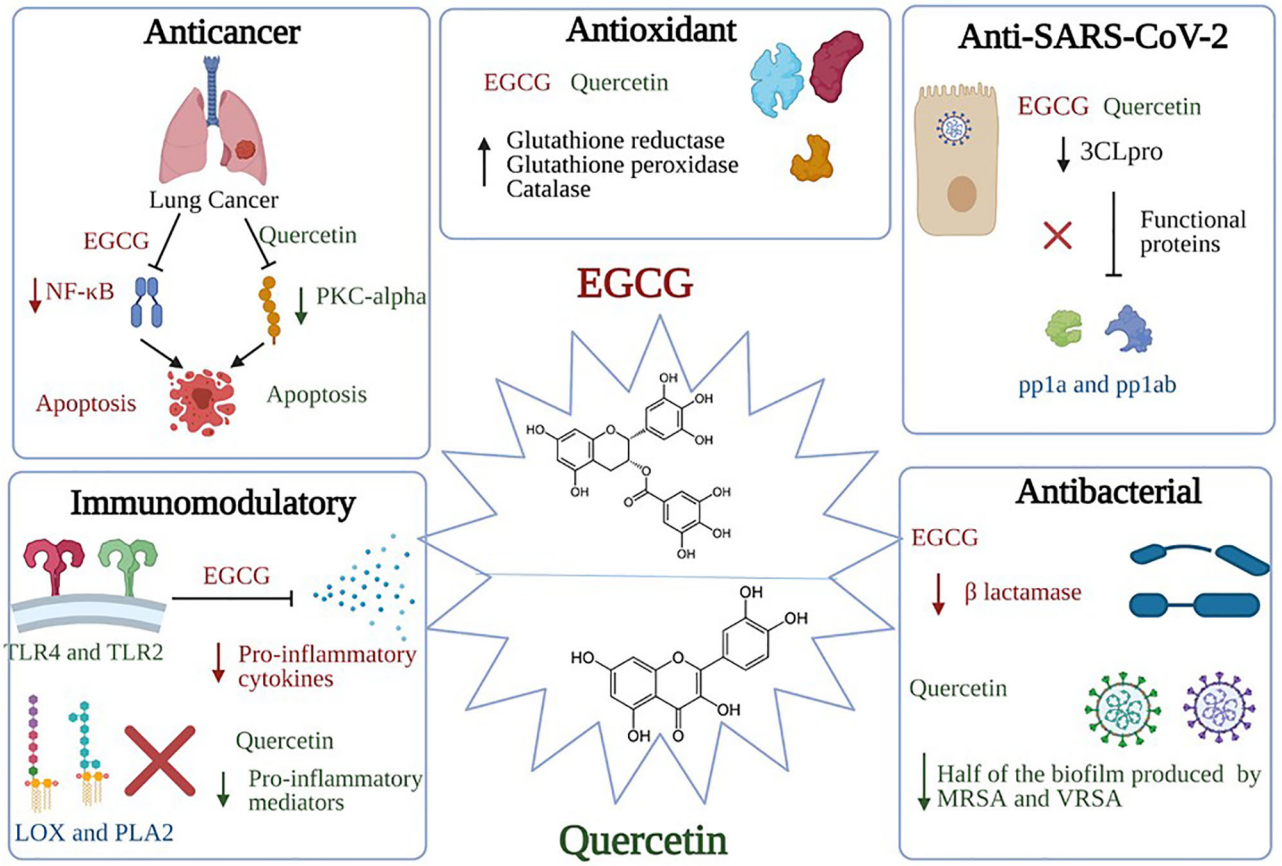

FIGURE 3 | Significant pharmacological activities of EGCG and quercetin. Green tea, with its main two active constituents EGCG and quercetin, contributes to a wide range of medicinal activities such as antioxidant, immunomodulatory, anticancer, antiviral, and antibacterial actions. EGCG anticancer activity is produced by suppressing the NF-κB signaling in A549 and H1299 cells; activation of apoptotic cascades are also initiated, resulting in a marked hindering of cellular proliferation. On the other hand, quercetin inhibits the protein kinase C (PKC-α), a survival signaling protein, repressing several cancer hallmarks. Concerning the immunomodulatory actions, EGCG induces TLR4 and TLR2 expression levels, thus depleting the mitogen-activated protein kinase (MAPK) pathway and repression of pro-inflammatory cytokines release. As for the quercetin, it inhibits pro-inflammatory mediators such as Lipoxygenase and Phospholipase A2. Besides, both constituents share the same antiviral mechanism against SARS-CoV-2, which is binding to "$3Cl^{pro}$" or "M^{pro}", preventing the processing of viral proteins pp1a and pp1ab into functional proteins in the host cells. Bona fida, the antioxidant effects of both EGCG and quercetin are mediated through scavenging and neutralizing the free radicals and boosting the enzymes that are responsible for detoxification such as glutathione reductase, glutathione peroxidase, and catalase. Finally, quercetin showed potential antibacterial activity through inhibiting half of the biofilm production by methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA). Similarly, EGCG showed antibacterial activities through inhibiting the B lactamase production and neutralizing the released toxins.

N-terminal (p-JNK) and extracellular signal-regulated kinase (p-ERK), and this could explain the mechanism used by EGCG to exert its anti-inflammatory effects (298). In addition, EGCG seems to have a role in symptoms reduction and pathology improvement in autoimmune diseases (299). Inhibition effects of EGCG on $CD4^+$ T-cell expansion in response to stimulation was observed (299). The differentiation of naïve $CD4^+$ T cells and that of Th1 and Th17 was also affected (299). This obstructed differentiation of Th1 and Th17 can be due to downregulation of transcription factors by EGCG, for instance, STAT1 and T-bet for Th1, while STAT3 and RORγt for Th17 (299). A study on multiple sclerosis in an animal model showed that EGCG weakened the disease severity in a dose-dependent manner and suppressed the proliferation of T cells along with reducing pro-inflammatory cytokine production (299). Besides, EGCG anti-inflammatory effects were proven as well in inflammatory arthritis disease (299).

EGCG has the ability to downregulate MAPK and NF-kb signaling pathways leading to the inhibition of pro-inflammatory cytokines as a result (300, 301). EGCG can weaken the transmigration of neutrophils through vascular endothelial cells (281) and decrease the neutrophil elastase enzyme, which increases the permeability of alveolar epithelium (302). As mentioned in black tea, EGCG in green tea can also inhibit LPS-induced ICAM-1 as well as the expression of the VCAM-1 by blocking pathways of NF-kB and c-Jun N-terminal kinase (JNK); this in turn will shut down the neutrophils since ICAM-1 and VCAM-1 are expressed on the endothelial cell surfaces (278–280). Last but not least, EGCG

can scavenge for ROS and neutrophil elastase enzyme (the one that increases the permeability of alveolar epithelium) in a promising way (280–282), making it a strong immunomodulatory agent that can help fight infections that consequently will have an impact on controlling COVID-19.

Anti-SARS-CoV-2 Activity of Green Tea. EGCG was shown to possess antiviral activity against many viruses such as porcine reproductive and respiratory syndrome virus (PRRSV), hepatitis C virus (HCV), ZIKA virus, chikungunya virus, influenza virus, and HIV-1 (303–307). Consequently, this inspired researchers to evaluate its antiviral potential against SARS-CoV-2. Initially, EGCG and quercetin were reported to be among the most effective inhibitors for 3CLpro as presented in **Figure 4** (148). EGCG was proven by molecular docking studies to be the most potent inhibitor for 3CLpro among all the nature-based phytochemicals (308). Then, it was reported that EGCG inhibits many structural proteins such as the HR2 domain, the post-fusion core of the S2 subunit, S protein, the RBD-ACE2 complex, and NSP15 endoribonuclease as shown in **Figure 4** (270). Also, another mechanism of action mediated by EGCG was the inhibition of the complex formation between glucose-regulated protein-78 (GRP-78) and the virus (309), as shown in **Figure 4**. GRP78 is a chaperone protein that is normally expressed in the lumen of the endoplasmic reticulum. Under cell stress conditions, overexpression of this protein occurs and is then translocated to the plasma membrane where SARS-CoV-2 interacts with it by the S protein, and subsequently, virus entry happens (310). Another molecular docking study was made on the binding affinity to the viral structural protein finding that EGCG has the highest affinity among the other substances that are included in the study. This study underlined a very important discovery: the affinity of EGCG to inhibition was higher than that of the well-known drugs used during the pandemic, remdesivir and chloroquine, suggesting a better antiviral activity for EGCG (270, 311).

Collectively, among all the natural active constituents isolated from phytochemical plants, EGCG and quercetin showed an exceptionally potent antiviral activity harnessing the SARS-CoV-2 life cycle through a myriad of mechanisms as summarized in **Figure 4**. Accordingly, our next step was to screen for herbal drugs that were reported to contain the highest phenolic contents of EGCG and quercetin. Undoubtedly, the choice was matcha, especially since it has been recently reported that several types of green tea could effectively block infection due to SARS-CoV-2 and its new variants by mainly abrogating the spike binding to the ACE2 receptor (29, 30).

Matcha: Filling the Gap During the Pandemic

Matcha powder is a herbal drug that was reported to contain at least three times higher EGCG content than green tea, providing

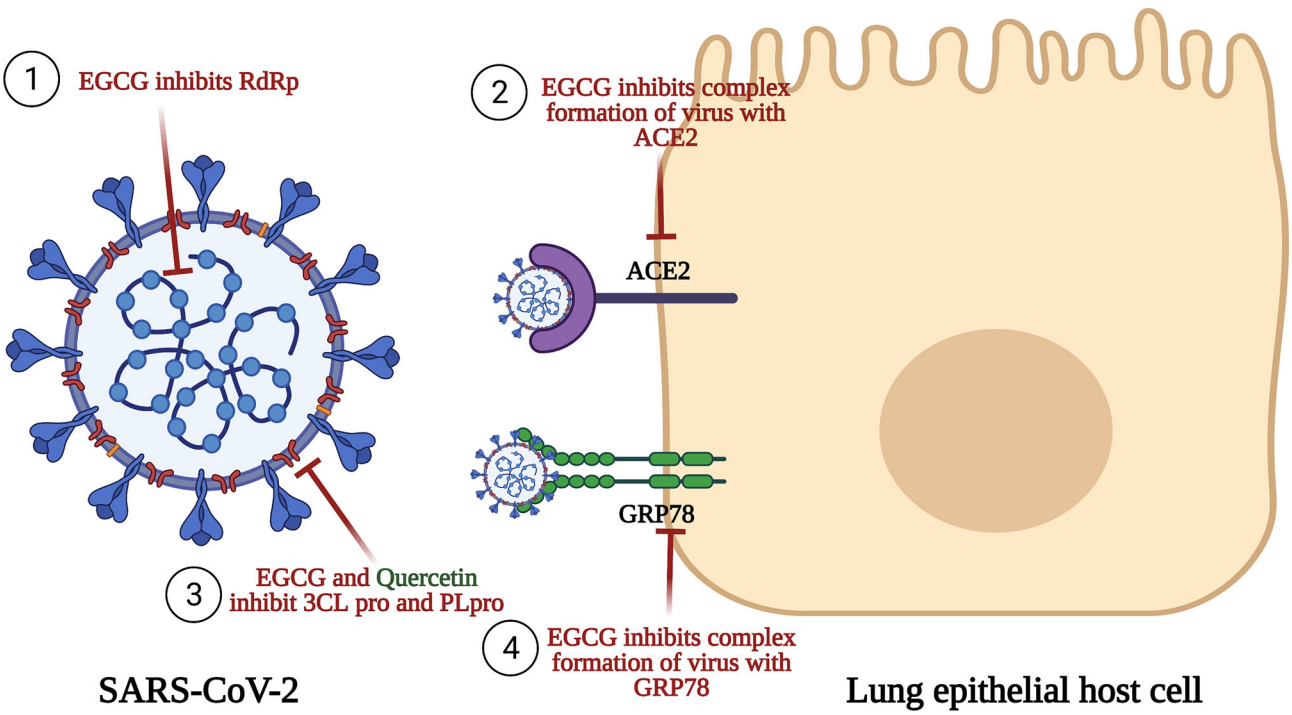

FIGURE 4 | Anti-SARS-CoV-2 activities of EGCG and quercetin. This figure illustrates the anti-SARS-CoV-2 for both EGCG and quercetin. EGCG inhibits RNA-dependent RNA polymerase (RdRp), an enzyme having an important role n replication and transcription of the virus. EGCG also inhibits the binding of the S1 region of the viral spike protein to the ACE2 receptor on the lung host cells. EGCG and quercetin prevent the processing of polyproteins pp1a and pp1ab by Papain-like protease (PL^{pro}) and Chemotrypsin-like protease (3C like protease or $3CL^{pro}$ or M^{pro}). Finally, EGCG inhibits complex formation of the virus with GRP78 receptor and thus inhibits viral entry.

an economic and beneficial beverage for SARS-CoV-2-infected patients and a preventive measure for high-risk patients such as cancer and autoimmune patients (312). Nowadays, matcha tea powder is widely known and used for its abundant health benefits and its exceptional quality. Matcha is the powdered form of green tea that originated in Japan (313). The high nutritional benefits of matcha come from the presence of many powerful active constituents as listed in **Table 4** below. The main forms of catechins and the most active ones found in higher amounts are (-) epigallocatechin 3-gallate (EGCG), caffeine, quercetin, phenolic acids, rutin, vitamin C, chlorophyll, and theanine (313). Catechins are present in four types: (-) epicatechin (EC), (-) epicatechin 3-gallate, (-) epigallocatechin (EGC), and (-) epigallocatechin 3-gallate (EGCG) (344, 345).

Matcha Main Constituents: EGCG

Catechins have an indisputable role as antioxidants by scavenging and neutralizing the free radicals and boosting the enzymes that are responsible for detoxification such as glutathione reductase, glutathione peroxidase, and catalase (346). It is also worth mentioning that the cellular redox homeostasis can be well maintained by the intake of catechins more often in the human diet (316). As previously mentioned, EGCG has a powerful antiviral effect. Compared to vitamin C, flavonoids, and glutathione, it has been proved that catechins have a higher antioxidant potential (316).

The Anti-Carcinogenic Activity of EGCG

This antioxidant effect contributes to one of the anti-carcinogenic mechanisms of EGCG, as it was previously mentioned that the catechins can quench the reactive oxygen species at any stage and consequently halt the malignant transformation process (347). Other studies have shown that the EGCG has several anticancer activities as shown in **Figure 5**. EGCG exhibits antitumorigenic properties in lung cancer. It suppresses the NF-κB signaling in A549 and H1299 cells; this leads to the inhibition of cell proliferation and induces apoptosis as shown in **Figure 5** (348). EGCG suppresses breast cancer progression through the tight binding of EGCG to signal transduction activator proteins of transcription 1 (STAT1) by its three hydroxyl groups of the B ring and one hydroxyl group of the D ring; this bond leads to the blockage of the phosphorylation of STAT1 by Janus Kinase 2 (JAK2) and inhibition of its carcinogenic effects since STAT1 in cancer cases can act as an oncogenic protein. It was worth noting that EGCG promotes Fas/CD95-mediated apoptosis in the neck and head squamous carcinoma by inhibiting JAK/STAT3 (317, 349).

Anti-Bacterial Activity of EGCG

As per the current treatment protocol for SARS-CoV-2, several antibiotics are prescribed especially in high-risk patients to avoid the complications of secondary bacterial infections, an act that would result in an antibiotic resistance catastrophe post-

TABLE 4 | Biological activities of matcha active constituents.

Active constituent	Biological activity	References
Chlorophyll	Anti-inflammatory Antioxidant	(314, 315)
Epigallocatechin 3-gallate (EGCG)	Decreases the ROS Increases enzymes for detoxification Anti-carcinogenic Anti-bacterial Antiviral Immunomodulatory	(300, 316–319)
Quercetin	Antioxidant Neuroprotective Decreases glucose absorption Increases insulin secretion and sensitivity Antiviral Immunomodulatory Anti-bacterial Anti-carcinogenic	(320–331)
Vitamin C	Strong exogenous antioxidant Enforces the immune system	(332)
Caffeine	Decreases the ROS Increases the antioxidant enzymes activity Increases glutathione levels Decreases pro-inflammatory cytokines	(333, 334)
Theanine	Gives distinctive and non-bitter taste	(335–337)
Phenolic Acids	Antioxidant Anti-inflammatory Hypoglycemic Neuroprotective Regulates carbohydrates/lipid metabolism	(333, 338–341)
Rutin	Antioxidant Anti-diabetic Anti-inflammatory	(332, 342, 343)

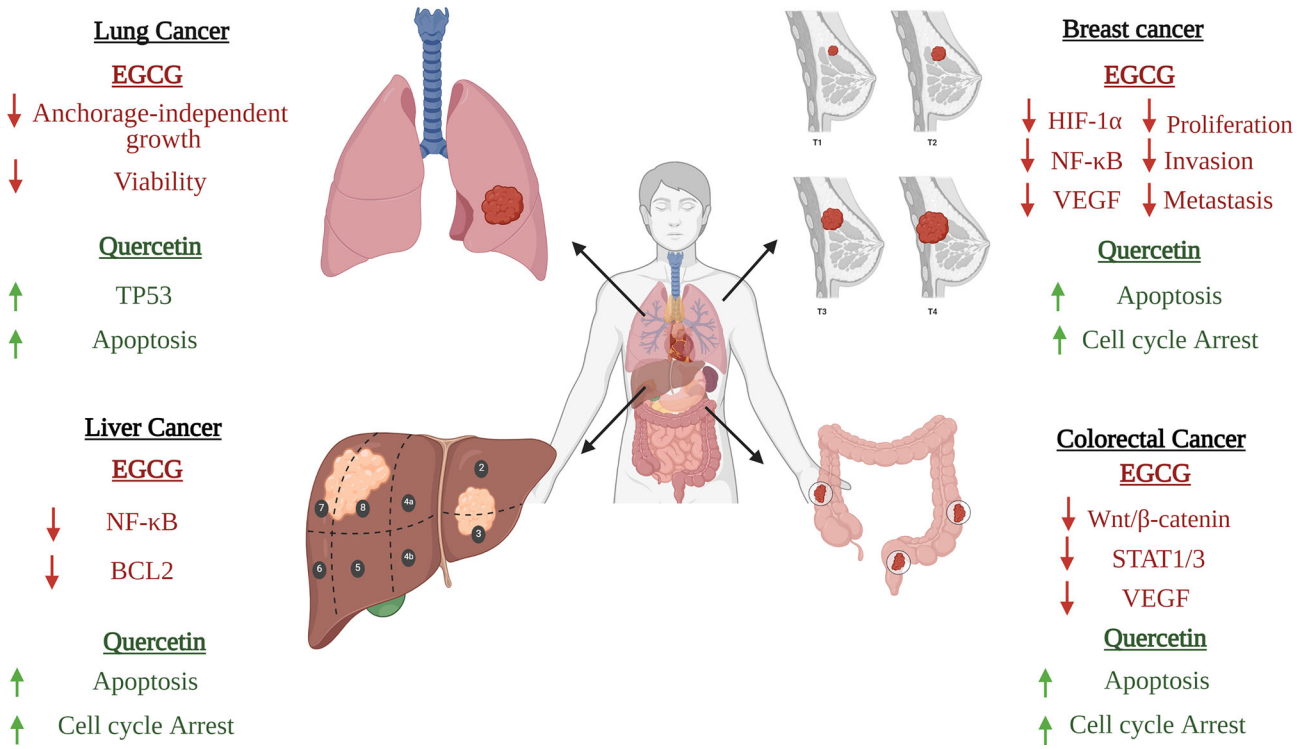

FIGURE 5 | Anticancer activities of EGCG and quercetin. Epigallocatechin-3-gallate (EGCG) and quercetin modulate several canonical oncological pathways such as Wnt/β-catenin and JAK/STAT pathways. Also, they modulate the expression of several anti-apoptotic proteins such as BCL2, tumor suppressor proteins such as TP53, and oncogenic drivers such as VEGF. Both active ingredients have proven to be effective in halting the malignant transformation process in several types of cancers such as breast, lung, liver, and colorectal cancers.

pandemic as described by the WHO in November 2020. As presented in **Figure 3**, EGCG has promising antibacterial activity while saving the world from the antibiotic resistance dilemma that would threaten our lives enormously. EGCG has shown bactericidal activity against staphylococci. Moreover, EGCG also shows an antibiofilm activity when co-administered with other antibiotics; it gives a powerful synergistic action. EGCG also can inhibit beta-lactamase production and neutralize the released toxins. The negatively charged property of EGCG makes it more effective against Gram-positive bacteria (318).

Immunomodulatory Role of EGCG

Nutrition immunity is a new concept that has been revolutionized during the pandemic (350). Massive attack on the respiratory epithelium (host cells for SARS-CoV-2) can lead to acute respiratory distress syndrome (ARDS), characterized by the uncontrolled release of pro-inflammatory cytokines leading to damaging the host cells, a vicious process termed as cytokine storm (350). It is important to note that the cytokine storm is also a common feature in the case of chemotherapy-treated cancer patients (351). Therefore, it is a crucially important measure to protect such high-risk patients during the pandemic. SARS-CoV-2-infected patients have a reduction in IFN-α and IFN-β levels, thus increasing the chance for the virus to invade and take over the immune system (319, 352). It is noteworthy that EGCG has a dominant immunomodulatory role by inducing TLR4 and TLR2 expression levels. Such induction occurs as a result of the repression of the mitogen-activated protein kinase (MAPK) and the pro-inflammatory cytokines as presented in **Figure 3** (300, 301). EGCG also modulates the immune system through inhibition of the RIG-I (acts as a RIG-I inhibitor), thus protecting the infected patient from the cytokine storm and its notorious consequences (353).

Matcha Main Constituent: Quercetin

As presented in **Table 4**, one of the main matcha constituents is quercetin. Quercetin possesses an array of pharmacological activities such as neuroprotection, antioxidant, and antineoplastic activities. It also has a vital role in diabetes mellitus patients, where it inhibits glucose absorption and thus it regulates carbohydrate metabolism, thus regulating the insulin secretion and sensitivity to tissues (320, 321, 354).

Anticancer Activity of Quercetin

Our research group has recently highlighted the potential anticancer activity of quercetin and its derivatives in liver and breast cancers (355, 356). Studies show that if quercetin was ingested on a daily basis, it was found to decrease the risk of cancer incidence (326). Quercetin was also reported to retain its antitumorigenic properties against several types of leukemias,

melanoma, lung, colorectal, and ovarian cancers (325). *In vivo* studies also supported the promising anticancer properties of quercetin in several animal models (324). Molecularly, quercetin inhibits protein kinase C signaling protein, resulting in the activation of apoptotic death signals and cell cycle arrest as shown in **Figure 5** (329). It also has powerful induction effects on TP53, Fas/FADD, caspases, and suppression of vital anti-apoptotic proteins (330, 331, 357). From an immune-oncological point of view, long-term intake of quercetin was proved to improve natural killer cells' cytotoxic activity, neutrophil chemotaxis, and lymphocyte proliferation (323, 358). In addition, quercetin induces T helper cells to produce TH1-derived interferon-gamma (IFN-γ) and downregulates TH2-derived IL-4 (327, 359). Altogether, it is quite evident that quercetin possesses potential intrinsic anticancer activity together with activating the innate and adaptive immune arms to halt oncological progression in several malignant contexts.

Antibacterial Activity of Quercetin

Similar to EGCG, quercetin has shown potent bactericidal properties against an array of bacteria, such as *Enterococcus faecalis* and *Listeria monocytogenes*. Both are resistant to several antibiotics and have a detrimental ability to produce biofilms on an artificial device such as stents. Quercetin was reported to effectively inhibit 95% of biofilm formation, also by stopping several glycolytic enzymes such as 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (GpmA) and ATP-dependent phosphofructokinase (PfkA) in *L. monocytogenes*. Quercetin also represses the secretion of the bacterial adhesion molecules that have a vital role in *L. monocytogenes* (foodborne illness bacteria) infection. Thus, the incorporation of quercetin as a food additive to minimize the adhesion, proliferation, and biofilm growth of the bacteria is a safe and economic idea (328). It is also important to note that quercetin was reported to inhibit half of the biofilm production by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), thus shedding light on its powerful antibacterial activity (328).

Immunomodulatory Role of Quercetin

Quercetin possesses an immunomodulatory role through repressing platelet aggregation, lipid peroxidation, inhibition of pro-inflammatory mediators such as lipoxygenase and phospholipase A2, and the expression levels of MHG class II and co-stimulatory molecules. Digging deeper, it was found that attenuation of several canonical and non-canonical immunomodulatory pathways such as arachidonic acid metabolism, the associated leukotriene/prostaglandins, and mTOR signaling pathways are the molecular mechanisms by which quercetin possesses its immunomodulatory role in several contexts (360, 361).

Matcha Other Constituents: Caffeine

Caffeine is one of the constituents of matcha; it has a strong antioxidant activity where it acts by neutralizing the ROS and it induces the antioxidant enzyme activities and also increases the glutathione levels, thus reducing oxidative stress. Besides the antioxidant effect, caffeine also has an anti-inflammatory activity where it reduces the secretion of pro-inflammatory cytokines. It was found that the caffeine content in matcha is greater than that in green tea, thus making matcha tea more effective (333, 334, 339).

Matcha Other Constituents: Phenolic Acids

Phenolic acids are found at their maximum levels in matcha tea. They are well known to have powerful antioxidant and anti-inflammatory effects as well as hypoglycemic and neuroprotective effects. Moreover, regulation of several metabolic disorders is controlled by some of the phenolic acids by regulating carbohydrates and lipid metabolisms (333, 338–341).

Matcha Other Constituents: Rutin

Rutin is a polyphenolic compound. Among all the kinds of tea available in the market, matcha contains very high amounts of rutin. It has several benefits such as antioxidant, antidiabetic, and anti-inflammatory effects (332, 342, 343, 362).

Matcha Other Constituents: Chlorophyll, Theanine, and Vitamin C

Chlorophyll is responsible for the bright green color of matcha. It was reported that it has powerful anti-inflammatory and antioxidant effects (314, 315). Nevertheless, the amino acid theanine provides the taste of matcha, which is distinctive and non-bitter. Also, it was found that the presence of caffeine with theanine improves efficiency rather than using them separately (335–337). Last but not least, the presence of vitamin C is also important and possesses several beneficial effects since it cannot be synthesized within the human body. It is considered a strong exogenous antioxidant, and it must be supplied *via* nutritional intake as it reinforces the immune system (363).

DISCUSSION

COVID-19 has made the world face a war with a different meaning this time—a war in which the entire world population are warriors, whose main slogan was "Stay Safe", a war whose weapons consist of open-ended practical trials that take place in research labs to find a solution to finally end the war. On one hand, the hospitals were at full capacity, and the demand for oxygen supplies was increasing. On the other hand, this virus impacted the entire globe with deleterious effects economically. The mess was escalating.

The trials for combating this virus were numerous, and carried out with different aims, whether for drug repurposing or trying to develop new antiviral agents. Vaccine development was also one of the main goals of researchers, yet many other researchers have shed light on the use of herbal medicine. *In silico*, *in vivo*, and *in vitro* studies were conducted all with only one aim, which is to find a solution to solve this mess. In fact, none of the studies could underestimate the significance of the others, and all can work in harmony with each other or help each other to reach the main curative goal at the end.

Herbal medicine was one of the major routes that were investigated throughout this pandemic by many researchers as plants have been proven to be a miracle drug throughout the generations for combating many diseases, which also gave many researchers hope for defeating COVID-19. Nature has never failed to protect us, that is why the first routine that was followed since the start of this pandemic is to eat fruits and vegetables because of their potential to strengthen our immune system and act as a preventive measure. For this reason, it was not surprising that many review articles, clinical trials, and molecular docking studies investigated the antiviral potential of many plants against SARS-CoV-2, and many of them showed a strong potential to improve the pandemic situation as previously reviewed in (364–366).

However, there was another dark side to the story, and this darkness relies mainly on the ones who were suffering every minute whether for fear of catching the virus because they know how weak their bodies are to defeat this enemy or for the difficulties they would face to follow their treatment plans in the hospitals or clinics during this pandemic. These sufferers are mainly the cancer patients and immune-compromised patients such as those with SLE, RA, and MS who have higher mortality rates and exacerbated conditions upon exposure to the virus (367) compared to other normal individuals. Although vaccines seemed to be a proper solution, there are still limitations that should be taken into consideration as regards the efficacy of the vaccine for this type of patients as well as the possible interactions between both the vaccine and their treatments or the disease condition (367). Moreover, these patients needed to be tracked routinely for any signs of unexpected adverse events or if they are on active cancer therapy, so the relation between the timing of the vaccine and its safety and efficacy with the treatments and immune deficiency should be evaluated (368). Because of this, it was logical to think of herbal medicines as an option for these people due to their potential to defend against a myriad of viruses and strengthen the immune system, and certain herbs could have a role in attacking cancer as well when compared to vaccines or synthetic drugs.

In this review, we focused on the significant role of herbal medicines in helping cancer and immune-compromised patients. A spotlight was thrown on many plants such as ginger, turmeric, garlic, flaxseed, citrus fruits, *Echinacea purpurea*, Java turmeric, ashwagandha, and black tea. All of the plants highlighted in this review have proven their efficacy as anticancer, immunomodulatory, and antiviral agents; many of them already show an anti-COVID-19 potential. The combination of these three actions suggests herbal medicines as a good option for these patients. Yet, it is worth mentioning that most of the herbal products' actions mentioned in this review are dose-dependent effects. For instance, it should be noted that garlic is an anticancer agent in several oncological contexts, but it is a source of organosulfur compounds, which are hydrogen sulfide donors (182). Hydrogen sulfide is a well-known biphasic gasotransmitter molecule that, at low concentrations, plays an oncogenic role while having an anticancer activity at higher concentration (183, 184).

One of the herbal plants that were discussed in this review was green tea, and while focusing on its constituents, which were mainly EGCG and quercetin, they were found to have very potent multiple mechanisms for defending against different cancer types, acting as immunomodulatory, anti-inflammatory, and antiviral agents specifically towards COVID-19. Matcha was able to obtain these protective properties in the highest possible amount.

Matcha is a Japanese green tea in which nowadays seems to be trendy in certain populations for its claimed ability to boost health and immunity, also it has been used recently in some of the cosmetic products for its ability to participate in a healthy skin conditions. Digging deeper in the Matcha constituents, we found that it contains EGCG and quercetin, the proven ones for their efficacy, in much concentrated amounts than in the normal green tea along with other constituents that were discussed as well in this review and as shown in **Figure 6**. This can explain the potential for that herbal tea in specific to be an indispensable way for cancer and immunocompromised individuals to protect themselves against COVID-19 along with alleviating their health states.

This review suggests matcha as one of the potential options that should be highlighted during this period as it offers a great amount of potential to battling the current pandemic.

FUTURE RECOMMENDATIONS

Since herbal medicines have always been able to tackle many health issues throughout the past pandemics and act as prophylaxis against a myriad of diseases, this encourages us to have a wider look in generalizing herbal medicines to the entire population. As reviewed, it has been documented that with the emergence of each new mutation, the efficacy of vaccines and drugs becomes negatively affected. Such information should encourage global health organizations to tackle this issue in a different way. More in-depth studies on herbal medicines need to be conducted using more clinical trials. *In vivo* and *in vitro* studies should be carried out for much more medicinal plants as well as *in silico* and molecular docking studies to further study and discover new effects for secondary metabolites. Such studies and clinical trials should also include the more susceptible populations such as cancer patients, immune disease patients, and children since they are not included in the current studies. Because of this, WHO will be encouraged to advise doctors about prescribing these herbal medicines along with other synthetic drugs if needed, and the media will also play a role as they will start to encourage people to use more herbal medicines and to make them aware of such medicines' benefits. Consequently, this can lead to the discovery of more new plants and the investigation of new research areas by pharmaceutical companies in order to meet the market need. This can be a plan for any upcoming SARS-CoV-2 outbreak or for any new pandemic for either normal or ill people, and this plan will most probably succeed. One of the herbal medicines that is highlighted by this review for future use is matcha.

Such a wide range of therapeutic potentials ofwfi 2 "matcha" constituents whether as an immunomodulatory agent or as an

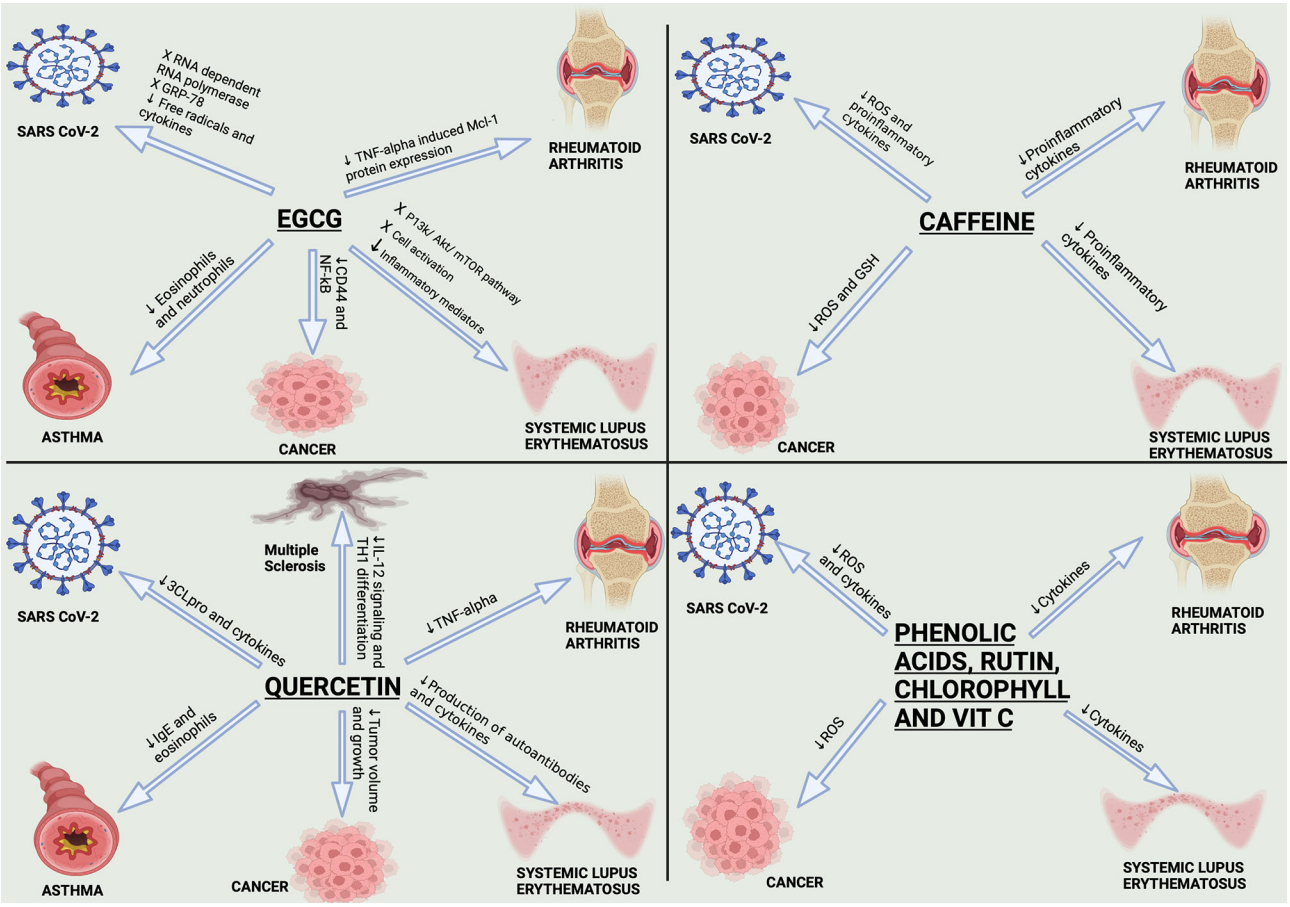

FIGURE 6 | Matcha beneficial effects in protecting cancer and autoimmune patients from SARS-CoV-2 infection. This figure highlights the dynamic constituents of matcha and its beneficial effects in preventing SARS-CoV-2 infection and also ameliorating SARS-CoV-2-positive patients. A special focus on cancer and autoimmune patients is presented. Patients who have caught SARS-CoV-2 were found to have a decrease in IFNs; thus, EGCG stimulates the expression of both TLR4 and TLR2, and this helps in reducing the pro-inflammatory cytokines and the cytokine storm. Since the viral nucleic acid activates the RIG-1 that increases IFN-1, EGCG showed to act as a RIG-1 inhibitor. In addition, EGCG has an antioxidant effect by neutralizing the free radicals and boosting the detoxification enzymes. Moreover, quercetin is a potent immunomodulatory in SARS-CoV-2, as it has many functions such as antiviral activity, platelet aggregation inhibition, and inhibition of proinflammatory mediators like Lipoxygenase (LOX) and Phospholipase A2. Rutin has an antioxidant and anti-inflammatory activity; thus, it is useful in the management of COVID-19. The chlorophyll content in matcha has proven to be beneficial due to its anti-inflammatory and antioxidant effect, which assists to overcome the cytokine storm. Besides, caffeine has a potential anti-inflammatory effect by decreasing proinflammatory cytokines, and its antioxidant effect is due to reduction of ROS and increase of glutathione. In addition, Vitamin C greatly stimulates antiviral immune responses and reduces the lungs' inflammatory state. It is essential to highlight that the intake of matcha tea not only will manage COVID-19 symptoms but also can prevent the virus itself from infecting humans.

anti-SARS-CoV-2 agent might be the reason why "matcha" would acquire a high market share in the upcoming years, especially since SARS-CoV-2 might behave like seasonal flu (after having more than eight waves to date). Nonetheless, we should not ignore the fact that more coronaviruses might appear at any time, since bats act as a reservoir or a storage tank for them. Collectively, this might highlight the potential of "matcha" to be the stone that could hit 3 birds (cancer, autoimmune disease, and SARS-CoV-2).

Although "matcha" seems to be very promising, there are a lot of challenges that may hinder its usage. For instance, "matcha" might not be accessible for many people due to its high price especially since we are focusing on cancer and autoimmune disease patients who already have very high expenses for their medications. Therefore, it is recommended to be produced in larger amounts for the sake of reducing its price, and this could be done by pharmaceutical companies. One of the major disadvantages that might discourage people to use "matcha" is its bad taste; however, this could be masked by the addition of flavors during the production phase, which again sheds light on the importance of pharmaceutical companies in "matcha" production. Therefore, this review elucidates the importance of having a cup of "matcha" to reinforce and strengthen the immune system in cancer and autoimmune disease patients who have a higher risk of catching SARS-CoV-2. Yet, this also can be generalized for everyone as it is powerful enough to prevent and protect them from catching the virus.

CONCLUSION

In conclusion, this review stresses the fact that the probability of the current pandemic to continue for a long time and the probability of developing future pandemics are extremely high, especially after the emergence of several VOCs. In this review, the authors highlight the great potential held by herbal medicine especially for high-risk patients such as cancer and autoimmune patients. Also, the authors shed light onto our new norm and how herbal products are considered risk-free solutions. In this review, it was set clear that after the SARS-CoV-2 pandemic experience, it should be noted that the development of new drugs and effective vaccines will not always be the easiest option. This review presents the current herbal medicines that could be used in preventing and fighting COVID-19, which happen to have three roles: as an immunomodulatory and anticancer agent, aside from displaying anti-SARS-CoV-2 activities. A special spotlight was turned on for the Japanese green tea "matcha". The authors elucidating the promising use of matcha as a prophylactic agent during the SARS-CoV-2 pandemic can have a significant impact on the socioeconomic and health status in general and on cancer and autoimmune patients in particular. This was mainly based on their major constituents: EGCG and quercetin and their well-reported anticancer activity, immunomodulatory effects, and their recent anti-SARS-CoV-2 activity. Yet, more detailed studies about the usage of "matcha" among cancer and autoimmune patients have to be conducted in the future.

AUTHOR CONTRIBUTIONS

CK, MK, and MT contributed to drafting the original draft of the manuscript and data collection (literature reviewing) and sketching the figures. The conception of the work, critical revision of the article, and data interpretation were performed by the principal investigator of the work, RY. The final version of the manuscript was approved and revised by all the authors.

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GLOSSARY

+gRNA	Positive single-stranded RNA
+sgRNA	Positive subgenomic RNA
2019-nCoV	2019 novel coronavirus
3C like protease	Chemotrypsin-like protease
3CLpro	Chemotrypsin-like protease
ACE II	Angiotensin-Converting Enzyme 2
Ad5	Adenovirus type 5
AKT	Protein kinase B
ALA	Alpha linolenic acid
ARDS	Acute respiratory distress syndrome
ASCO	American Society of Clinical Oncology
BAD	Bcl-2-associated agonist of cell death
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma extra large
CD 44	Cluster of differentiation 44
COVID-19	Coronavirus disease of 2019
CoVs	Coronaviruses
COX-2	Cyclooxygenase 2
CYP3A4	Cytochrome P450 3A4
DADS	Diallyl disulfide
DMTs	Disease-modifying therapies
DMV	Double-membrane vesicle
dsDNA	Double-stranded DNA
E	Envelope
EC	Epicatechin
EGC	Epigallocatechin
EGCG	Epigallocatechin 3-gallate
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum golgi intermediate compartment
ESMO	European Association for Medical Oncology
FADD	Fas-associated protein with death domain
Fas/CD95	Cluster of differentiation 95
FDA	Food and Drug Administration
FoxP3	Forkhead box protein 3
GIT	Gastrointestinal tract
GpmA	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase
-gRNA	Negative single-stranded RNA
GRP78	78-kDa glucose-regulated protein
GRP-78	Glucose-regulated protein 78
H1N1	Hemagglutinin type 1 and neuraminidase type 1
HCV	Hepatitis C virus
HE	Hemagglutinin esterase
HIV	Human immunodeficiency virus
ICAM-1	Intracellular adhesion molecule 1
ICTV	International Committee on Taxonomy of Viruses
IFN beta	Interferon beta
IFN gamma	Interferon gamma
IFN-1	Type I interferon
IgE	Immunoglobulin E
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase

(Continued)

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JAK2	Janus kinase 2
LNP	Liposomal nanoparticle
LOX	Lipoxygenase
LPS	Lipopolysaccharide
M	Membrane
MAPK	Mitogen-activated protein kinase
MCP 1	Monocyte chemoattractant protein 1
MCP-1	Mature plasma cell 1
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
miRNA	MicroRNA
MMP13	Matrix metallopeptidase 13
MMP9	Matrix metallopeptidase 9
Mpro	Main protease
mRNA vaccines	Messenger RNA vaccines
MRSA	Methicillin-resistant *S. aureus*
MS	Multiple sclerosis
mTOR	Mechanistic target of rapamycin
MUC 2	Mucin 2
N	Nucleocapsid
NCCN	National Comprehensive Cancer Network
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAID	National Institute of Allergy and Infectious Disease
NK cell	Natural killer cell
NO	Nitric oxide
NRVV	Non-replicating viral vector
Nsp	Nonstructural protein
Nsp1	Nonstructural protein 1
Nsp12	Nonstructural protein 12
Nsp13	Nonstructural protein 13
Nsp14	Nonstructural protein 14
NSP15	Nonstructural protein 15
Nsp2	Nonstructural protein 2
Nsp2-16	Nonstructural protein 2-16
Nsp3	Nonstructural protein 3
Nsp5-16	Nonstructural protein 5-16
Nsp7	Nonstructural protein 7
Nsp8	Nonstructural protein 8
P53	Tumor protein p53
PAF	Platelet-activating factor
PfkA	ATP-dependent phosphofructokinase
PG	Prostaglandin
PGE2	Prostaglandin E2
PH	Potential Hydrogen
PI3K	Phosphoinositide 3 kinase
PKC-alpha	Protein kinase C-alpha
PKC-delta	Protein kinase C-delta
PLpro	Papain like protease
PP	Polyprotein
PRRSV	Procaine reproductive and respiratory syndrome virus
PUFA	Polyunsaturated fatty acids
RA	Rheumatoid arthritis
RBD	Region binding domain
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene I
RORγt	Retinoic acid-related orphan receptor γt
ROS	Reactive oxygen species
RTC	Replication/Transcription Complex
S	Spike
saRNA	Self-amplifying messenger RNA
SARS-CoV-2	Severe Acute Respiratory Syndrome-Coronavirus-2
SDG	Secoisolariciresinol diglycoside
-sgRNA	Negative subgenomic RNA
SLE	Systemic lupus erythematosus
SOCS3	Suppressor of cytokine signaling 3
SREBP-1C	Sterol regulatory element-binding protein 1

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STAT	Signal Transducer and Activator of Transcription
STAT1	Signal Transducer and Activator of Transcription 1
STAT3	Signal Transducer and Activator of Transcription 3
T-bet	T-box protein expressed in T cells
TF1	Theaflavin
TF2A	Theaflavin-3-gallate
TF2B	Theaflavin-3'-gallate
TF3 or TFDG	Theaflavin-3,3'-digallate
TGF-β	Transforming growth factor beta
TH1	T helper 1 cell
TH17	T helper 17 cell
TH2	T helper 2 cell
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TMPRSS2	Transmembrane protease serine 2
TNF-α	Tumor necrosis factor alpha
ULBP2	UL16 binding protein 2
VAERD	Vaccine-associated enhanced respiratory disease
VEEV	Venezuelan equine encephalitis virus
VEGF	Vascular endothelial growth factor
VRSA	Vancomycin-resistant *S. aureus*

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CASE REPORT
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A Female With Synchronous Multiple Primary Malignant Tumors in the Esophagogastric Junction, Duodenum and Pancreas: Case Report and Review of the Literature

Yongxing Du[1†], Yunjie Duan[1†], Lipeng Zhang[1,2†], Zongting Gu[1], Xiaohao Zheng[1], Zongze Li[1] and Chengfeng Wang[1]*

[1] State Key Laboratory of Molecular Oncology and Department of Pancreatic and Gastric Surgery, National Cancer Center/ National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [2] Department of Burns and Plastic Surgery, The Fourth Medical Center of PLA General Hospital, Beijing, China

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***Correspondence:**
Chengfeng Wang
wangchengfeng62@163.com

[†]These authors have contributed equally to this work

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The incidence of multiple primary carcinomas (MPCs), which are defined as two or more malignancies detected in an individual person, is gradually increasing around the world. According to the timing of diagnosis for each constituent tumor, MPCs are classified into 2 categories: synchronous MPCs if constituent tumors emerge simultaneously or within 6 months or metachronous MPCs otherwise. In this report, we describe our recent observation and treatment of a female patient with synchronous primary esophagogastric junction adenocarcinoma, duodenal adenocarcinoma and pancreatic ductal adenocarcinoma (PDAC). To the best of our knowledge, this combination has not yet been reported in the literature. A crucial aspect is the decision regarding which tumor to treat initially and how to schedule further treatments according to individual tumor hazards. Our multidisciplinary team devised an individualized treatment regimen for this patient. The patient ultimately achieved an overall survival time of 18 months, which was much longer than the median survival time (6~11 months) of patients with locally advanced pancreatic cancer. Moreover, treating this rare combination raised a series of diagnostic, etiological and therapeutic questions, motivating us to carry out a critical review of the literature. In summary, an individualized treatment strategy with input from a dedicated multidisciplinary team and consideration of all options at different points along the disease trajectory is essential to optimize outcomes for patients with MPC.

Keywords: pancreas, multiple primary tumors, comprehensive treatment, literature review, prognostic analysis

INTRODUCTION

MPC, first reported by Billroth in 1879, refers to the simultaneous or subsequent occurrence of two or more cancers unrelated to each other in one patient, which may occur in different parts of the same organ or the same system or in different organs or systems (1). In recent research, MPC was defined by Moertel as two or more malignant tumors occurring within 6 months (2). The burden of MPCs is rising

as the aging population has increased over the last decades (2). Several studies have retrospectively investigated the incidence of MPCs. For example, the study of Alexia et al. showed that the incidence of MPCs in a cancer population varies between 2.4% and 8% within 20 years of follow-up (3). Moreover, another study from data of European cancer registries reported an overall incidence of multiple primary cancers of 6.3% (range, 0.4–12.9%) (4).

With the advancement of medical technology and improvements in comprehensive clinical diagnosis and treatment, previously difficult-to-find tumor lesions can now be detected easily, thereby increasing the chance of detecting multiple primary cancers. Nevertheless, owing to its low prevalence, many clinicians are not sufficiently aware of this disease and lack therapeutic experience. To date, the pathogenesis of this rare disease remains to be elucidated. MPC is clinically often confused with the recurrence or metastasis of malignant tumors which might greatly change the formula of patients. In view of above situation, we decided to report our recent observation and treatment of a female patient with synchronous primary esophagogastric junction adenocarcinoma, duodenal adenocarcinoma and PDAC. To the best of our knowledge, this combination has not yet been reported in the literature.

CASE REPORT

On January 23, 2019, a 77-year-old female patient was admitted to the Cancer Hospital, Chinese Academy of Medical Sciences, because of mild abdominal pain and intermittent fever (with a maximum temperature of 39°C) for more than 3 months, and a duodenal mass was detected 2 weeks prior. Moreover, she presented with transient jaundice and skin itching that lasted for approximately 5 days. She did not complain of any other discomfort and did not report any relevant family history, and she had not been treated at a nearby clinic or hospital. Because of the noninvasive, radiation-free, and convenient characteristics of abdominal ultrasound, the patient had a habit of regular medical examination with abdominal ultrasound every year. The patient's duodenal mass was diagnosed by ultrasound during her annual physical examination two weeks prior, then she came to our hospital for further tests and treatment.

Gastroscopy revealed superficial ulceration at the esophagogastric junction with irregular protrusion and a polypoidal protuberant mass in the duodenal papilla protruding into the lumen (**Figure 1**). Pancreatic magnetic resonance cholangiopancreatography (MRCP) reported soft tissue nodules at the end of the common bile duct approximately 2.6x1.7 cm in size that were prone to malignancy, with low biliary tract obstruction (**Figure 2A**). Another pancreatic somatic nodule was found in the body of the pancreas that was approximately 2.0x2.3 cm in size and poorly defined, so the possibility of malignancy could not be excluded (**Figure 2B**). The pathological results of endoscopic biopsy revealed adenocarcinoma in both lesions of the esophagogastric junction and duodenal papilla (**Figure 3A**). Hematologic examination revealed the following: ALT(88 U/L), ALP(280 u/L), DBIL(5.7 μmol/L), CA199(551 U/ml), and CA242 (107.190 U/ml).

The patient was 159 cm tall and weighed 70.0 kg, and her body mass index (BMI) was 27.7. She had a past history of hypertension, type-2 diabetes mellitus, and osteoarthritis. More than 20 years ago, she underwent thyroid surgery, and the pathology was benign. After the operation, the patient was treated with oral thyroid hormone drugs.

DIAGNOSTIC INVESTIGATION AND TREATMENT

Although the definition and criteria for the diagnosis of MPC have changed many times over the years, it is typically based on the

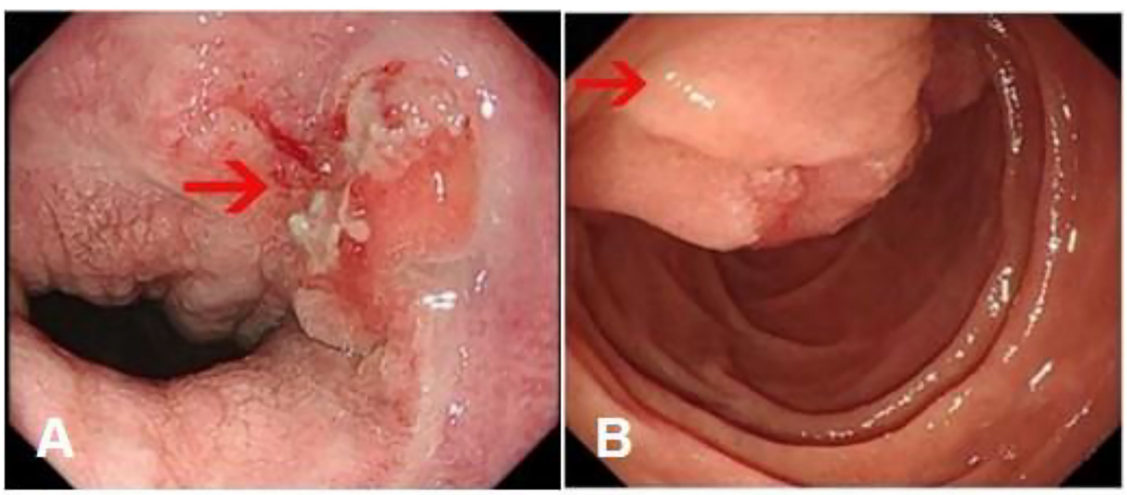

FIGURE 1 | Gastroscopy results. **(A)** Superficial ulceration at the esophagogastric junction with irregular protrusion. (Arrow: the location of the tumor) **(B)** A polypoidal protuberant mass in the duodenal papilla protruding into the lumen. (Arrow: the location of the tumor).

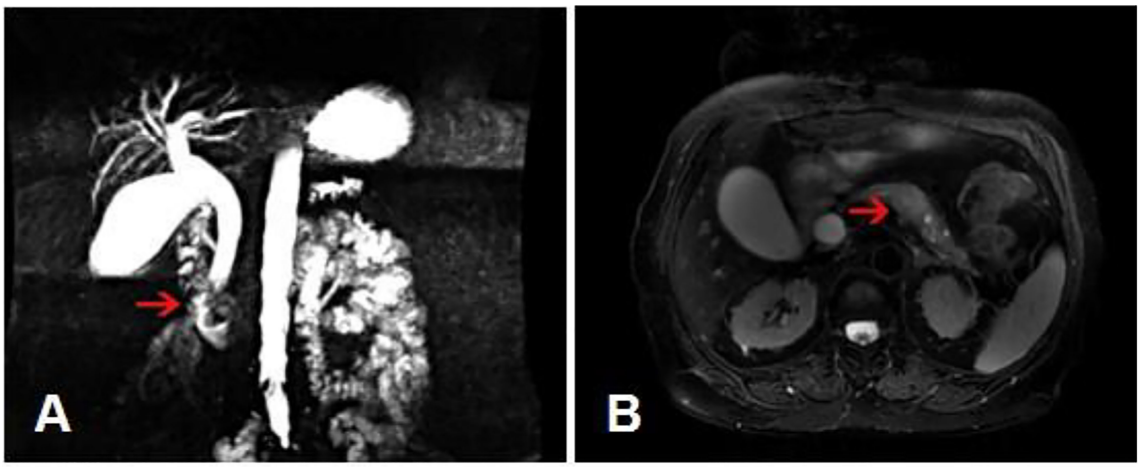

FIGURE 2 | MRCP results. **(A)** A soft tissue nodule at the end of the common bile duct with low biliary tract obstruction. (Arrow: the location of the tumor). **(B)** A poorly defined somatic nodule at the body of the pancreas. (Arrow: the location of the tumor).

criteria described by Warren and Gates (5): (a) each tumor must have a clear pathological diagnosis; (b) each tumor must have an independent initial site of disease; and (c) the lesions must be nonmetastatic tumors. We initially determined that this case fulfilled the diagnostic criteria for MPC by the above medical examinations, and then the case was discussed by a multidisciplinary team before treatment was initiated. Since the patient's family refused pancreatoduodenectomy due to the high

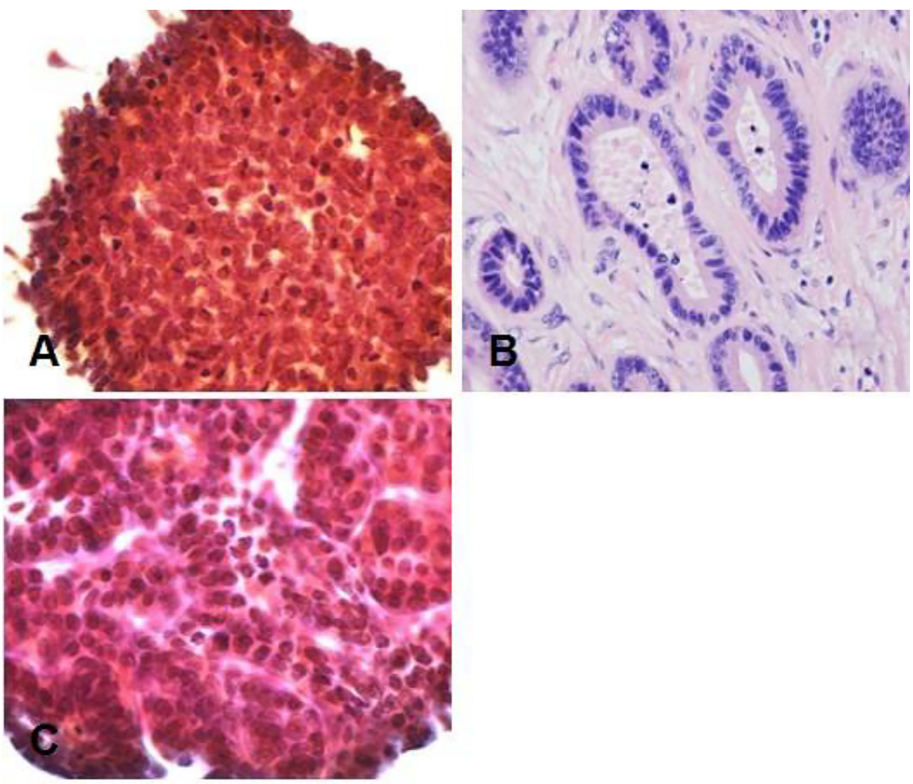

FIGURE 3 | Pathological results. **(A)** Duodenal adenocarcinoma. **(B)** Moderately differentiated adenocarcinoma (Lauren classification: intestinal type) at the esophagogastric junction. **(C)** Pancreatic adenocarcinoma at the pancreatic body. Hematoxylin–eosin (H&E) staining of biopsy samples (40×) magnification.

risk associated with the operation, our multidisciplinary team recommended that the tumors of the gastroesophageal junction be treated by endoscopic submucosal dissection because of the relatively early disease stage. For the pancreatic lesions and duodenal mass, the team recommended abdominal section exploration combined with intraoperative puncture biopsy of the pancreatic tumor, intraoperative radiotherapy, and gallbladder jejunostomy and gastrojejunostomy due to the high risk of radical surgery and to respect the wishes of the patient and her family.

The patient underwent two operations in our hospital. The first operation was endoscopic submucosal dissection (ESD) performed under general anesthesia on February 19, 2019. The final pathological result showed moderately differentiated adenocarcinoma (Lauren classification: intestinal type) (**Figure 3B**). It had invaded the submucosa at a depth of 1550 microns (1650 microns) and was staged as pT1b. Immunohistochemical results showed the following: AFP (-), CD10 (intramucosal +, submucosal region +, glandular surface +, partial intramucosal -), cdx-2 (2+), c-met (2+), EGFR(2+), GPC3(-), HER2(2+), MLH1(+), MSH2(+), MSH6(+), MUC2 (surface 1+, deep -), MUC5AC(1+), MUC6(1+), P53(90%+, missense mutated expression), SALL4(-), CD31, D2-40 (vascular staining), and Desmin (muscularis mucosa staining). Special staining results were observed for elastic fiber staining (showing veins). Gene detection did not show mutations in KRAS gene exons 2, 3 and 4, NRAS gene exons 2, 3 and 4, PIK3CA gene exons 9 and 20, or BRAF gene exons 11 and 15. On March 4, 2019, abdominal exploration was performed under general anesthesia. Intraoperative exploration revealed an unresectable tumor (the tumor was closely adhered to the mesenteric vasculature, and their separation was not possible) approximately 3x4 cm in size in the pancreatic body. The intraoperative pancreatic biopsy result was suggestive of adenocarcinoma (**Figure 3C**). Therefore, intraoperative radiotherapy with a dosage of 1500 cGy was delivered to the pancreatic mass. Gallbladder jejunostomy and gastrojejunostomy were also performed to relieve the obstruction of the biliary and gastrointestinal tracts. The final results of biopsy immunohistochemistry showed the following: braf-v600e (-), c-met (2+), HER2(1+), MLH1(+), MSH2(+), MSH6(+), PMS2(+), AE1/AE3(3+), CK18(3+), ChrA(-), Syno(-), and Ki-67 (30%+). In combination with the immunohistochemistry results, it was suggested that there was little infiltration of poorly differentiated adenocarcinoma into the fibrous adipose tissue with mucous secretion.

The patient recovered well, and she was discharged on the 14th postoperative day after the second operation. The first month after surgery, the patient had resumed her preoperative diet, her abdominal pain significantly improved, and no fever was reported. From June 10 to October 21, 2019, the patient underwent six cycles of chemotherapy (Abraxane combined with S-1) at the internal medicine department of our hospital. S-1, also known as Tegafur, Gimeracil and Oteracil Porassium Capsules, is a fluorouracil derivative oral anticancer agent that includes tegafur and the following two classes of modulators: gimeracil and oteracil. This medicine is mainly used for the treatment of unresectable locally advanced or metastatic gastric cancer and pancreatic cancer, etc. (6). After six courses of chemotherapy, her tumor markers were still not normalized (CEA, 15.25 ng/ml; CA199, 580.6 U/ml; CA242, >200 U/ml). However, no evidence of tumor progression or distant metastasis was found on multiple follow-up chest and abdominal CT and MRI scans during the entire period of chemotherapy. Four months later, the patient's level of CA199 increased to 20000 U/ml, and thoracic MRI revealed metastasis in the thoracic spine. Then, she received for further treatment in the form of proton radiation therapy at another hospital in February 2020. Radiotherapy achieved some encouraging treatment outcomes, and the patient's CA199 level decreased to 700 U/ml. Unfortunately, pulmonary metastasis was detected in June 2020, and the patient's level of CA199 had increased again. The patient ultimately died of cancer cachexia on August 27, 2020 (**Figure 4**).

DISCUSSION AND LITERATURE REVIEW

Liu reported that MPC most commonly occurs in the respiratory system and gastrointestinal tract, while intracranial origin is rare (1). Utada stated that esophageal cancer and oral/pharyngeal cancer are the most common types of MPC (5). Other studies have reported that the incidence of multiple primary cancers in the digestive system is 2% ~ 17%, and that of multiple primary cancer patients with pancreatic cancer is 5.2% ~ 23% (7–12). To gain further insights into the occurrence of pancreatic cancer with multiple primary cancers, we carried out a critical review of the literature. A summary of the previous studies describing MPC is shown in **Table 1**.

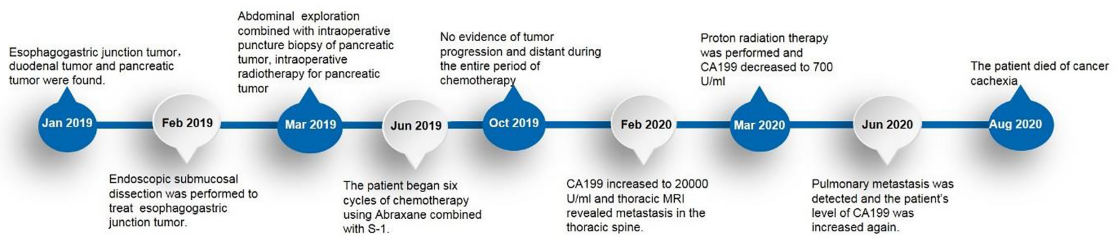

FIGURE 4 | Timeline of MPC progression in our female patient.

TABLE 1 | Summary of the previous studies describing multiple primary malignant neoplasms with pancreatic cancer.

Year	Author	1st Tumor	2nd Tumor	3rd Tumor	4th Tumor	Synchronous or Metachronous primary cancers	Case report or Retrospective cohort study	Number of patients	Outcomes	Genomic findings
1965	M Sasaki	Pancreatic carcinoma	Hepatocellular carcinoma			Synchronous	Case report	1	–	–
1966	P N Bodnar	Lung carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	–	–
1969	E Sasaki	Pancreatic carcinoma	Renal carcinoma			Metachronous	Case report	1	The patient died of cachexia.	–
1972	P Langeron	Cervical carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	–	–
1983	Y Kawaura	Pancreatic carcinoma	Appendiceal tumor			Synchronous	Case report	1	The patient died of cachexia.	–
1985	N Hori	Bladder carcinoma	Prostatic carcinoma	Pancreatic carcinoma		Synchronous	Case report	4	–	–
1986	M E O'Brien	Pancreatic carcinoma	Breast carcinoma			Synchronous	Case report	2	Patient 1 died of cardiac arrest. Patient 2 died of cachexia.	–
1987	Y Niv	Pancreatic carcinoma	Duodenal carcinoma			Synchronous	Case report	1	The patient died of liver failure.	–
1988	Niwa K	Pancreatic carcinoma	Ovarian carcinoma			Synchronous	Case report	1	–	–
1989	Yoshii K	Pancreatic carcinoma	Biliary tract carcinoma			Synchronous	Case report	1	The patient died of respiratory failure.	–
1990	A G Montag	Pancreatic adenocarcinoma	Pancreatic sarcoma			Synchronous	Case report	2	Patient 1 died of hypotensive shock. Patient 2 died of metastatic tumor.	–
1990	L C Childs	Biliary tract carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	The patient died of cachexia.	–
1992	N Ueda	Biliary tract carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	11 months without evidence of recurrence.	–
1994	Nishihara K	Pancreatic carcinoma	carcinoma of the papilla of Vater	Biliary tract carcinoma		Synchronous	Case report	1	The patient was still alive and well 4 years and 2 months after surgery.	The overexpression of p53 in the three tumors of the present case was found. / The DNA of the tumors in the present case were all aneuploid.
1994	P K Karak	Colonic carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	The patient died of metastatic tumor.	–
2000	Eriguchi N	Pancreatic carcinoma	Gastric carcinoma			Synchronous	Case report	3	The patient died of cachexia.	–
2000	Eriguchi N	Gastric carcinoma	Pancreatic carcinoma			Synchronous	Case report	2	The patient died of cachexia.	–
2000	Eriguchi N	Lung carcinoma	Pancreatic carcinoma			Synchronous	Case report	2	The patient died of cachexia.	–
2000	Eriguchi N	Colonic carcinoma	Pancreatic carcinoma			Synchronous	Case report	2	The patient died of cachexia.	–
2001	Joo YE	Pancreatic carcinoma	Colonic carcinoma			Synchronous	Case report	1	–	–

(Continued)

TABLE 1 | Continued

Year	Author	1st Tumor	2nd Tumor	3rd Tumor	4th Tumor	Synchronous or Metachronous primary cancers	Case report or Retrospective cohort study	Number of patients	Outcomes	Genomic findings
2003	Sato K	Gallbladder carcinoma	Biliary tract carcinoma	Pancreatic carcinoma		Synchronous	Case report	1	The patient died of cachexia.	The presence of p53-positive nuclei was recognized in the pancreatic cancer specimen.
2004	Brinster DR	Colonic carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	–	A germline mutation of the STK11/LKB1 tumor suppressor gene was recognized.
2004	Olgyai G	Renal carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	–	–
2006	I M'sakni	Pancreatic carcinoma	Gastrointestinal stromal tumor			Synchronous	Case report	2	The patients died of cachexia.	–
2008	Aurello P	Pancreatic adenocarcinoma	Pancreatic sarcoma			Synchronous	Case report	1	–	–
2010	Muroni M	Gastric carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	The patients died of metastatic tumor.	–
2008	Aurello P	Pancreatic adenocarcinoma	Pancreatic sarcoma			Synchronous	Case report	1	–	–
2010	Ozsoy O	Prostatic carcinoma	Pancreatic carcinoma			Synchronous	Retrospective cohort study	419	In patients with prostate cancer, abdominopelvic CT staging detects incidental second primary cancers with a greater frequency than that expected.	–
2011	Kenichiro Araki	Pancreatic carcinoma	Renal carcinoma			Synchronous	Case report	1	52 months without evidence of recurrence after the surgery.	–
2011	Maurea S	Pancreatic neuroendocrine carcinoma	Biliary tract carcinoma			Synchronous	Case report	1	–	–
2011	Power DG	Pancreatic adenocarcinoma	Pancreatic neuroendocrine carcinoma			Synchronous	Case report	2	Patient 1 is unknown. /Patient 2 succumbed to progressive disease 20 months after an initial diagnosis.	–
2011	Dasanu CA	Pancreatic carcinoma	Gastrointestinal stromal tumor			Synchronous	Case report	1	14 months without evidence of recurrence after the surgery.	–
2011	Gyorki DE	Esophageal adenocarcinom	Gastrointestinal stromal tumor			Synchronous	Case report	1	–	–
2013	Kim JS	Thyroid Carcinoma	Breast carcinoma	Pancreatic carcinoma	Gastric carcinoma	Metachronous	Case report	1	The patients died of cachexia.	–

(Continued)

TABLE 1 | Continued

Year	Author	1st Tumor	2nd Tumor	3rd Tumor	4th Tumor	Synchronous or Metachronous primary cancers	Case report or Retrospective cohort study	Number of patients	Outcomes	Genomic findings
2013	Kourie HR	Pancreatic carcinoma	Gastric carcinoma			Metachronous	Case report	2	–	The tumors of this syndrome demonstrate loss of protein expression of mismatch repair genes and are associated with microsatellite instability (MSI).
2014	Li Destri G	Colonic carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	The patients died of metastatic tumor.	–
2015	Ghothim M	Pancreatic carcinoma	Gastric carcinoma			Synchronous	Case report	1	The patient survived for 12 months.	–
2015	Ghothim M	Pancreatic carcinoma	Renal carcinoma			Synchronous	Case report	1	The patient survived for 19 months.	–
2016	Bansal A	Pancreatic carcinoma	Biliary tract carcinoma			Synchronous	Case report	1	–	–
2018	Vijayaraj P	Gallbladder carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	16 months without evidence of significant metastasis-related symptoms.	–
2019	Couch LL	Colonic carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	–	–
2020	Wang jun Zhang	Hepatocellular carcinoma	Pancreatic carcinoma			Synchronous	Case report	1	2 years without evidence of recurrence after the surgery.	–

Pancreatic Carcinoma and Hepatocellular Carcinoma

We retrieved two articles focusing on primary pancreatic carcinoma combined with hepatocellular carcinoma, and all cases were synchronous MPCs (13, 14) Sasaki and Zhang performed both hepatectomy and pancreaticoduodenectomy during the course of therapy. In the case report of Zhang, the patient received chemotherapy with gemcitabine combined with tegafur gimeracil oteracil potassium and anti-hepatitis C virus (HCV) therapy after the operation. Zhang investigated the possibility that HCV infection could increase the incidence of pancreatic cancers, but the biological mechanism underlying HCV-induced pancreatic cancers has not been fully elucidated. Finally, he highlighted the critical role of PET-CT as a tumor-related systemic examination method in the diagnosis and treatment of MPC.

Pancreatic Carcinoma and Biliary Carcinoma

Eight articles on synchronous pancreatic and biliary carcinomas were retrieved (15–22). Nishihara performed total pancreatectomy and extended cholecystectomy with regional lymph node dissection. The follow-up results showed that the patients survived for 3 years, and the recurrence-free survival time was 11 months without any adjuvant therapy after surgery. Sato adopted pancreaticoduodenectomy to treat MPC. Interestingly, the overexpression of p53 in the tumors observed in the present case was also reported by Nishihara, and he speculated that the oncogenic mechanisms of multiple synchronous cancers were caused by mutation of the p53 gene and abnormal DNA reparative mechanisms.

Pancreatic Carcinoma and Gastric Carcinoma

We retrieved seven articles on synchronous pancreatic and gastric carcinomas (23–29). The FOLFIRINOX chemotherapy regimen was adopted to treat synchronous pancreatic and gastric cancers in the study by Kouria. He planned to re-evaluate the surgical feasibility after two months of treatment. Meanwhile, Kourie emphasized that multiple tumors demonstrated loss of protein expression of mismatch repair genes and were associated with microsatellite instability (MSI). Therefore, MSI testing may be reasonable for patients with synchronous pancreatic and gastric cancers. For patients with

synchronous pancreatic carcinoma and gastrointestinal stromal tumor (GIST), pancreatectomy combined with postoperative concurrent chemoradiotherapy was performed in the study by Dasanu, and the patient was free of recurrence for 18 months following comprehensive treatment. Moreover, Dasanu suggested that screening for second cancers was warranted in patients with gastrointestinal stromal tumor (GIST) and early diagnosis of these cancerous lesions. A thorough search for second cancers, along with a multidisciplinary treatment approach, could further prolong patient survival and improve quality of life.

Pancreatic Carcinoma and Breast Carcinoma

Two articles on synchronous pancreatic and breast carcinomas were retrieved (27, 30). Among the cases previously reported, O'Brien performed modified radical mastectomy and palliative bypass procedures to treat breast carcinoma and pancreatic carcinoma, respectively. In his case report, he implied that high dietary intake of unsaturated fats was a carcinogenic factor for both carcinomas. If there is an association between breast and pancreatic adenocarcinomas, it may provide further evidence for the significance of dietary factors in the carcinogenesis of these two neoplasms in humans.

Pancreatic Carcinoma and Colon Carcinoma

A total of 6 articles on synchronous pancreatic and colon carcinomas were retrieved (23, 31–35). Among these reports, Karak administered a combined treatment of adjuvant chemotherapy consisting of 5-FU and leucovorin following colectomy combined with pancreaticoduodenectomy. Follow-up showed that the patients had a relatively good prognosis.

The associations between pancreatic carcinoma and other carcinomas of the lung, kidney, cervix, ovary, prostate, thyroid, etc. are weak and have been less frequently reported. However, many researchers have found that MPCs involve concurrent alterations in multiple gene pathways where both hypermethylation of tumor suppressor genes and hypomethylation of tumor-promoting genes occur; the main treatment approach is combination therapy, including surgery, radiotherapy and chemotherapy (30, 36–47).

Etiologies of MPCs

Some studies have speculated that the development of MPCs is associated with an unhealthy lifestyle, genetic susceptibility, side effects of chemotherapy and radiotherapy, weak immunity, etc. Moreover, as a tumor suppressor gene, inactivation of the TP53 gene plays a crucial role in the formation of tumors, and mutations in this gene exist in many solid tumors. Gerdes (11) reported that mutations of p16INK4a in the TP53 gene may be related to the occurrence of pancreatic cancer with multiple primary cancers. The patient in our case was overweight, with a BMI of 27.7, and high BMI is a well-known risk factor for the occurrence of malignant tumors, especially tumors of the digestive system (12). In addition, this patient had previously undergone partial thyroidectomy. Hormonal dysregulation may also be a risk factor for tumor development (12). Besides, another factor that could not be neglected is the weakened immunity associated with aging and increased tumor susceptibility (12).

Therapeutic Options for MPCs

Most MPCs are found in the same pipeline system. In the diagnosis and treatment of multiple primary cancers, surgical exploration or biopsy should be used as soon as possible to make a clear diagnosis. When primary cancer or metastatic cancer cannot be distinguished, as long as the tumor is limited or isolated and the patient's body condition allows, surgical resection should be performed to allow for the chance of cure. However, pancreatic cancer is an aggressive tumor with high mortality and poor prognosis. When pancreatic cancer patients have multiple primary cancers, priority should be given to a multidisciplinary combined treatment approach for pancreatic cancer. In this case, ESD was performed first to excise the esophagogastric junction lesions because this treatment was relatively easy to provide. Although we did not treat the tumor of the duodenal papilla or pancreatic tumor with surgery because pancreaticoduodenectomy could not be performed, intraoperative radiotherapy combined with postoperative adjuvant chemotherapy was used in a timely manner. Encouragingly, tumor progression or distant metastasis was not observed during the period of six cycles of chemotherapy, illustrating that intraoperative radiotherapy combined with postoperative adjuvant chemotherapy can effectively suppress advanced pancreatic cancer growth. Furthermore, this treatment result exemplifies the significant advantages of using multidisciplinary combined therapy for certain cancer patients.

The patient in our case received proton radiotherapy when she developed distant metastasis, and the level of CA199 presented a significant decrease (from 20000 U/ml to 700 U/ml). Proton radiotherapy is a type of external beam radiation therapy modality for the treatment of local disease that uses a proton beam to deliver a highly focused radiation dose to the tumor. In 1979, the University of Tsukuba began using proton cancer therapy with a booster accelerator for liver cancer, esophageal cancer, lung cancer, and brain tumors and achieved good curative effects (48). Proton radiotherapy has also gained popularity for the treatment of pancreatic cancer in recent years. Romaine, Michael and Dongha studied the efficacy of proton radiotherapy for the treatment of pancreatic cancer and found that patient survival was prolonged by this treatment (49–51). Here, our patient also exhibited a good treatment response. Although she ultimately passed away after an overall survival time of 18 months, this is much longer than the median survival time (6~11 months) of patients with locally advanced pancreatic cancer (52). The above evidence suggests that timely and effective multidisciplinary combined treatment for pancreatic cancer should be favored when pancreatic cancer patients are found to have multiple primary cancers. In a retrospective study of 147 cases of pancreatic resection for pancreatic cancer in Kumamoto University of Japan (53), it was

found that when treating multiple primary cancers in patients with pancreatic cancer, priority should be given to pancreatic cancer treatment, and the postoperative outcomes and survival conditions were similar to those of pure pancreatic cancer patients. This also supports the validity of giving clinical priority to pancreatic cancer treatment.

In conclusion, the treatment of malignant tumors needs to adhere to the concept of multidisciplinary comprehensive treatment, and individualized precision therapy is especially valuable for patients with multiple primary cancers. Our experience and further accumulation of these cases will provide guidance and a reference for clinicians.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YXD, YJD, and LZ wrote this paper. LZ and ZG collected relevant data of this patients. XZ and ZL followed up the patient. YXD, LZ, and CW managed the patient. CW and YXD revised this paper. All authors contributed to the article and approved the submitted version.

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Evaluation of Galanin Expression in Colorectal Cancer: An Immunohistochemical and Transcriptomic Study

*Iman M. Talaat[1,2,3], Nada M. Yakout[3], Ahmed S.A. Soliman[4], Thenmozhi Venkatachalam[2,5], Arya Vinod[2], Leen Eldohaji[2], Vidhya Nair[2], Amal Hareedy[6], Alaa Kandil[7], Wael M. Abdel-Rahman[2,8], Rifat Hamoudi[1,2,9] and Maha Saber-Ayad[1,2,10]**

[1] Department of Clinical Sciences, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates, [2] Sharjah Institute for Medical Research, University of Sharjah, Sharjah, United Arab Emirates, [3] Pathology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt, [4] Pathology Department, National Research Center, Cairo, Egypt, [5] Department of Physiology and Immunology, College of Medicine and Health Science, Khalifa University, Abu Dhabi, United Arab Emirates, [6] Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt, [7] Clinical Oncology and Nuclear Medicine Department, Faculty of Medicine, Alexandria University, Cairo, Egypt, [8] Department of Medical Laboratory Sciences, College of Health Sciences, University of Sharjah, Sharjah, United Arab Emirates, [9] Division of Surgery and Interventional Science, University College London, London, United Kingdom, [10] Pharmacology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

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Ain Shams University, Egypt

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Antonio Giovanni Solimando,
University of Bari Aldo Moro, Italy
Zhenying Guo,
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***Correspondence:**
Maha Saber-Ayad
msaber@sharjah.ac.ae

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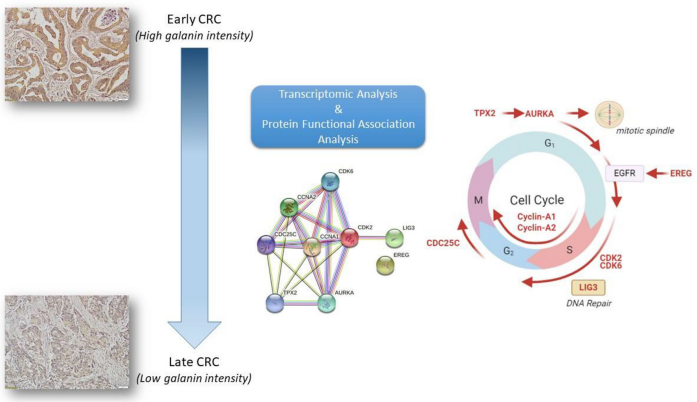
Colorectal cancer (CRC) represents around 10% of all cancers, with an increasing incidence in the younger age group. The gut is considered a unique organ with its distinctive neuronal supply. The neuropeptide, human galanin, is widely distributed in the colon and expressed in many cancers, including the CRC. The current study aimed to explore the role of galanin at different stages of CRC. Eighty-one CRC cases (TNM stages I – IV) were recruited, and formalin-fixed paraffin-embedded samples were analyzed for the expression of galanin and galanin receptor 1 (GALR1) by immunohistochemistry (IHC). Galanin intensity was significantly lower in stage IV (n= 6) in comparison to other stages (p= 0.037 using the Mann-Whitney U test). Whole transcriptomics analysis using NGS was performed for selected samples based on the galanin expression by IHC [early (n=5) with high galanin expression and late (n=6) with low galanin expression]. Five differentially regulated pathways (using Absolute GSEA) were identified as drivers for tumor progression and associated with higher galanin expression, namely, cell cycle, cell division, autophagy, transcriptional regulation of TP53, and immune system process. The top shared genes among the upregulated pathways are *AURKA, BIRC5, CCNA1, CCNA2, CDC25C, CDK2, CDK6, EREG, LIG3, PIN1, TGFB1, TPX2*. The results were validated using real-time PCR carried out on four cell lines [two primaries (HCT116 and HT29) and two metastatic (LoVo and SK-Co-1)]. The current study shows galanin as a potential negative biomarker. Galanin downregulation is correlated with advanced CRC staging and linked to cell cycle and division, autophagy, transcriptional regulation of TP53 and immune system response.

Keywords: galanin, immunohistochemistry, transcriptomic analysis, GALR1, colorectal cancer, bioinformatics, TIMER 2



GRAPHICAL ABSTRACT |

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide, representing 9.7% of all cancers (excluding non-melanoma skin cancer) (1). It is the second leading cause of cancer-related mortality in Europe and North America, with a global 900,000 deaths every year. The incidence has markedly accelerated in the Middle East and North Africa (8.8 in 1990 to 12.4 years in 2017) with 39.9%, most likely due to adopting the western lifestyle (2). CRC biomarkers have been extensively studied in Caucasians, but only a few studies have been conducted on the North African population.

Intriguingly, the gut is considered a unique organ with its distinguished hormonal and neuronal supply, highlighting the significance of studying the role of its neuropeptides. Human galanin is a 30-amino acid neuropeptide widely distributed in the central nervous system, heart and skin (3). The expression of galanin has been observed in many tumors. The level of expression was shown to correlate with differentiation or aggressiveness of the tumor (4). Galanin peptide is highly expressed in human pheochromocytoma, the first tumor in which galanin was identified (3, 5). It is also expressed in the pituitary adenoma (3, 6), gangliocytoma (7), neuroblastoma (8), and paraganglioma (9). Galanin was also detected in melanoma, small cell lung carcinoma, several breast cancers and head and neck squamous cell carcinoma and basal cell carcinoma (3, 4, 10). In 2007, Kim et al. showed that galanin is upregulated in colon cancer in Korean patients (11). Another study showed that high expression of galanin was associated with poor prognosis in stage II CRC in Japanese patients, as it was associated with higher invasiveness of cancerous cells. Galanin works in an autocrine and/or paracrine approach on G-protein coupled receptors. Galanin receptors are believed to be implicated in tumor progression, epithelial-mesenchymal transition (EMT) and chemotherapeutic sensitivity of colorectal cancer (12).

The aim of the current study was to quantify the expression of galanin and GALR1 in human tissue samples of Egyptian patients at different stages of CRC, and to correlate the results with various clinicopathological parameters. In addition, a subset of well-characterized samples from the early and late stages of CRC was subjected to transcriptomics analysis to explore the differentially expressed genes and pathways. Furthermore, the transcriptomics results were validated using primary and metastatic CRC cell lines.

PATIENTS AND METHODS

Patients and Sample Recruitment

The formalin-fixed paraffin-embedded (FFPE) tumor blocks of primary CRC were surgically managed in Alexandria & Cairo University Main Hospitals. Clinicopathological data of the patients along with the available follow-up data were retrieved from the medical records. The studied clinicopathological variables included age, gender, location, size, shape, histopathological variant, grade, depth of invasion (T), number of lymph node metastases (N), distant metastases (M), TNM staging, and state of the surgical margins. The present work was approved by the Research Ethics Committee (REC), Alexandria University and the University of Sharjah (Ref. No.: REC-17-11-23-01). The study was conducted according to the principles of the Declaration of Helsinki of 1975 (revised 1983). As this study was retrospectively conducted on FFPE blocks, the need for patients' consent was waived by the REC.

Histopathological Examination

The FFPE blocks of 81 cases diagnosed as primary CRC were cut into 4-μm thick sections and stained by Hematoxylin and Eosin, followed by microscopic examination. Two independent pathologists evaluated the histopathological parameters. The histologic type was assessed according to the 2019 WHO classification, and the staging was performed according to the TNM staging (8th edition).

Immunohistochemical Methods

In total, 81 samples were analyzed for the expression of galanin and galanin receptor 1 (GALR1) by immunohistochemistry (IHC). Manual immunostaining was performed on 4-µm-thick sections as described previously (13). In brief, FFPE sections were deparaffinized in xylene, rehydrated in graded alcohol, immersed in 0.01 M citrate buffer (pH 6.0), and heated in a domestic microwave oven at full power for 2x5 minutes and left in buffer to cool at room temperature. The sections were then incubated in 0.3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. Incubation with the primary antibodies [Anti-GAL (ab216399), and anti-GALR1 (ab150486); Abcam, Cambridge, UK] at a concentration of 1:200 diluted in 1% bovine serum albumin/tris-buffered saline was carried out overnight in a humid chamber at 4°C according to the manufacturer's instructions. The following day, the slides were washed and incubated first with biotinylated secondary antibody (SignalStain® Boost IHC Detection Reagent; Cell Signaling Technology) for 30 min at 20°C, then with avidin-biotin-peroxidase complex for 30 min at 20°C (Vectastain ABC kit; Abcam, Cambridge, UK). For visualization, the peroxidase/DAB DAKO Real ENVision detection system (DAKO, Glostrup, Denmark) was used, following the manufacturer's instructions. For each run of IHC staining, positive and negative control sections were included. Human skin sections (epidermis, sweat glands) were used as positive controls for GAL (14, 15). Moreover, galanin expression in Meissner's submucosal plexus in the colon and GALR-1 expression in immune cells in the colonic mucosa were considered internal positive controls (13).

Immunostaining Assessment

The immunoreactivity of galanin was assessed in the cancer cells based on the staining intensity (SI) and the percentage of positively stained cells (PP) to create the immunoreactive score (IRS) as follows: IRS=SI x PP, for each sample, as previously described (16). The intensity was scored as follows: 0: no staining, 1: weakly positive, 2: moderately positive and 3: strongly positive. The percentage of stained tumor cells was estimated by excluding the adjacent normal-appearing tissue, as well as necrotic or hemorrhagic foci if present and it was scored as follows: 0: 0%, 1: 0-9%, 2: 10-49% and 3: 50-100%. Accordingly, the IRS score ranged from 0 to 9, designated as a low expression for a score of 0 to 3, and a high expression for a score of 4 to 9 (5). A Semi-quantitative evaluation of the immunostained slides was performed blindly and independently by the pathologists, using an Olympus microscope (BX51; Olympus, Tokyo, Japan). All discordant cases were resolved within consensus meetings.

Whole Transcriptomics Analysis

Tissue curls were cut from FFPE blocks for representative samples from each category high and low galanin intensity as per IHC interpretation, and according to TNM staging [stage I (n=5), stage II (n=4) and stage III (n=2)]. The samples were classified into early (stage I) and late (stage II and III).

The tissue sections were subjected to RNA extraction using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Invitrogen) using the manufacturer's instruction. The extracted RNA was further purified and concentrated using Zymo RNA clean and concentrator kit (Zymo Research, USA). Thus, purified RNA was quantified using Qubit 3 fluorometer (Invitrogen) and ~50ng of RNA was sequenced using Ion Ampliseq transcriptome panel on S5 XL System (ThermoFisher) capturing more than 21,000 transcripts. In brief, ~30ng of Turbo DNase treated RNA was used for cDNA synthesis using SuperScript VILO cDNA Synthesis kit (Invitrogen) followed by amplification using Ion AmpliSeq gene expression core panel primers. The prepared library was purified using Agencourt AMPure XP Beads (Beckman Coulter) and the purified library was quantified using Ion Library TaqMan™ Quantitation Kit (Applied Biosystems). The libraries were further diluted to 100pM and pooled equally with four individual samples per pool. The pooled libraries were amplified using emulsion PCR on Ion OneTouch™ 2 instrument (OT2) and the enrichment was performed on Ion OneTouch™ ES following manufacturer's instruction. Thus, prepared template libraries were then sequenced with an Ion S5 XL Semiconductor sequencer using the Ion 540™ Chip.

Five early (stage I) cases of CRC showing low galanin intensity on IHC and six late cases (4 'stage II' and 2 'stage III') showing a high intensity of galanin were subjected to transcriptomics analysis.

Bioinformatics Analysis

RNA-seq data were analyzed using Ion Torrent Software Suite version 5.4 and the alignment was carried out using a modification of the Torrent Mapping Alignment Program (TMAP), optimizing it for aligning the raw sequencing reads against reference sequence derived from hg19 (GRCh37) assembly. The specificity and sensitivity were maintained by implementing a two-stage mapping approach by employing BWA-short, BWA-long, SSAHA (17), Super-maximal Exact Matching (18) and Smith-Waterman algorithm (19) for optimal mapping. Raw read counts of the targeted genes were performed using Sam tools (Sam tools view –c –F 4 –L bed_file bam_file) and the number of expressed transcripts was confirmed after Fragments Per Kilobase Million (FPKM) normalization. Differentially expressed gene (DEG) analysis was performed using an in-house script written in R programming language (version 3.6.3) with function calls to the DESeq2 package from Bioconductor libraries. Raw read counts from RNA Seq were subjected to quantile normalization and transcript counts ranked below 1 were excluded. Differentially expressed genes between the two sets of tissue samples [early-stage CRC with a high galanin intensity (n=5), and late stages with a low galanin intensity (n=6)] were assessed using a 2-tailed t-test. Differentially expressed genes with a p-value of <0.05 and a 5-fold difference were included for pathway analysis using Metascape.

Absolute GSEA was carried out as previously described (20). An R script to calculate the frequency of the gene recurrence across pathways was used to curate the shared genes among the top 5 differentially expressed pathways of interest. We further investigated the status of the resultant genes in COAD TCGA PanCancer datasets (n= 592). We used Gene Set Cancer Analysis (GSCALite), a web tool that provides a prediction of the gene activity in the cancer-associated pathway, among other functions

through the website: http://bioinfo.life.hust.edu.cn/web/GSCALite/, and http://bioinfo.life.hust.edu.cn/GSCA/#/expression (accessed on 14 April 2022) (21),. We studied the galanin expression in publicly available cancer data sets from CPTAC (n=197) and TCGA (n=194), to compare normal versus CRC specimens as well as the expression in different age groups, respectively. We used UALCAN, a web-based tool that uses OMICS data (http://ualcan.path.uab.edu accessed on 14 April 2022) (22).

Cell Lines

To validate the transcriptomics results, we used 4 human colorectal adenocarcinoma cell lines, namely; HCT116, HT29, SK-Co-1, and LoVo. HCT116 and HT29 are primary CRC cell lines, LoVo cells are derived from a metastatic lymph node, whereas Sk-Co-1 cells are derived from ascitic fluid from a patient with CRC. HCT116 and HT29 were maintained in RPMI. SK-CO-1 and LoVo were maintained in Minimum Essential Media, and Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham respectively. Both types of media were supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin). Cells were cultured in 75-cm^2 tissue culture flasks and incubated in a humidified incubator at 37°C in 5% CO_2 and 95% room air and sub-cultured every 3-4 days with trypsin.

Real-Time PCR

Total RNA from the cell lines was extracted using PureLink® RNA Mini Kit (ThermoFisher, USA) according to the manufacturer's instructions and quantified using Nanodrop (Invitrogen) A total of 500 ng RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit and Real-time PCR was then performed using 5X Firepol sybergreen master mix (Solis Biodyne, Estonia) on Quantstudio3 (Thermo, USA). Relative quantification was expressed as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct is the difference between the ΔCt values of the control and the starved cells and the samples analyzed in triplicates. The 18s rRNA gene was used as endogenous control and all the primers used in the RT–qPCR assays are listed in **Table 1**.

Statistical Analysis

Testing for normality was performed for continuous variables. When data were not normally distributed, the non-parametric Mann-Whitney U test was used to evaluate the significant differences between groups of samples, and to compare gene expression of different cell lines. Pearson Correlation was used to identify possible correlations of galanin intensity with various clinicopathological features; $p < 0.05$ denoted a significant difference. Adjusted $p < 0.8$ was used as a cutoff point for identifying the differentially expressed genes in the transcriptomics analysis. SPSS version 26 was used to perform statistical analysis (SPSS Inc, IBM, Chicago, Illinois).

RESULTS

Clinicopathological Data

In total, 81 CRC cases were recruited, with a mean age of 53.19 ± 13 years (range 22-86); 52 female patients and 29 male patients with a female: male ratio of 1.7:1. All specimens were diagnosed as adenocarcinoma (NOS) except for one case diagnosed as mucinous adenocarcinoma (not graded based on the degree of glandular differentiation) (23, 24). The clinicopathological data is shown in **Supplementary Table S1**. Staging of cancer showed significant negative correlation with the patients' age ($p = 0.006$, $r = -0.305$). Other parameters did not show significant correlations (**Supplementary Table S2**).

Galanin Intensity and TNM Staging

Immunohistochemical analysis of the 81 CRC samples revealed focal and diffuse galanin immunoreactivity in the majority of cases (**Figure 1**). Twenty-one cases revealed no expression, 28 showed mild, 22 were moderate, whereas only 10 cases revealed intense galanin expression. GALR1 expression generally showed the same trend in the CRC samples (**Supplementary Figure S1A** shows images of immunohistochemical staining of Galanin Receptor 1 (GALR1). **Supplementary Figure S1B** is a bar graph representing GALR1 intensity according to TNM

TABLE 1 | List of primers used in real-time PCR.

Gene	Primer (5'-3')	Primer (3'-5')	Amplicon size (bp)
AURKA	GCAACCAGTGTACCTCATCCTG	AAGTCTTCCAAAGCCCACTGCC	158
BIRC5	CCACTGAGAACGAGCCAGACTT	GTATTACAGGCGTAAGCCACCG	115
CCNA1	GCACACTCAAGTCAGACCTGCA	ATCACATCTGTGCCAAGACTGGA	118
CCNA2	CTCTACACAGTCACGGGACAAAG	CTGTGGTGCTTTGAGGTAGGTC	120
CDC25C	AGAAGCCCATCGTCCCTTTGGA	GCAGGATACTGGTTCAGAGACC	133
CDK2	ATGGATGCCTCTGCTCTCACTG	CCCGATGAGAATGGCAGAAAGC	97
CDK6	GGATAAAGTTCCAGAGCCTGGAG	GCGATGCACTACTCGGTGTGAA	106
EREG	CTCTACACAGTCACGGGACAAAG	CTGTGGTGCTTTGAGGTAGGTC	120
LIG3	GCTACTTCAGCCGCAGTCTCAA	GCAGTGGTTTGCCTGTCTTGTTG	147
PIN1	ACAGTTCAGCGACTGCAGCTCA	GCAGCGCAAACGAGGCGTCTT	101
TGFB1	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA	122
TPX2	TTCAAGGCTCGTCCAAACACCG	GCTCTCTTCTCAGTAGCCAGCT	131

Real-time PCR was carried out on Quant Studio 3 (Thermo Fisher) using HOT FIREPol® EvaGreen® qPCR Supermix. 18S was used as the housekeeping gene. The PCR reaction mixture and cycling conditions were performed according to manual instructions. All samples were amplified in triplicates. The average threshold cycle (Ct) values were obtained from each reaction and the gene expression was quantified using the 2(–ΔΔC(T)) relative method.

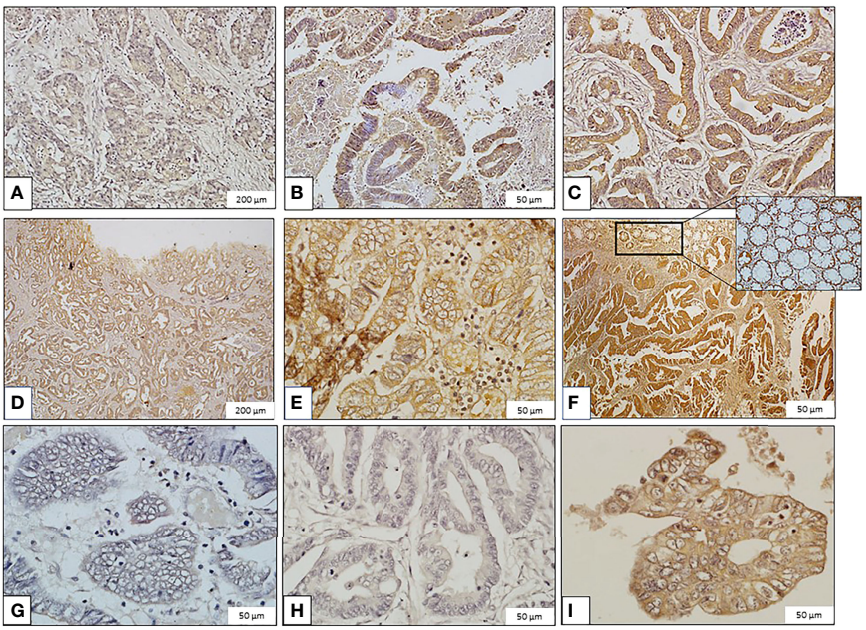


FIGURE 1 | Images of immunohistochemical staining of galanin in colorectal carcinoma (CRC) samples (Immunoperoxidase, scale bar: 200µm 'panels **A**, **D**' and 50µm 'panels **B**, **C**, **E**, **F**–**I**'). Galanin Expression: **(A)** Mild expression in grade II, stage I CRC. **(B)** Moderate expression in grade II, stage I CRC. **(C)** High expression in grade II, stage II CRC. **(D)** Mild expression in grade I, stage III CRC. **(E)** Moderate expression in grade I, stage III CRC. **(F)** High expression in grade I, stage II CRC. The inset shows galanin expression in normal colonic mucosa. **(G)** Minimal expression in grade III, stage IV CRC. **(H)** Mild expression in grade II, stage IV CRC. **(I)** Moderate expression in grade II, stage IV CRC.

staging). **Supplementary Table S3** shows the galanin intensity across the different TNM stages.

Galanin intensity showed a trend of negative correlation with TNM staging of the tumors (P-value =0.054, Pearson correlation of -0.215). The correlation with individual parameters revealed a negative correlation of galanin intensity with the "number of infiltrated lymph nodes" score (p-value <0.001, Pearson correlation of -0.384). N0: no lymph node metastasis, N1: 1-3 metastasis to regional lymph nodes, N2: metastasis to 4 or more regional lymph nodes, and with "distant organ metastasis" score (p-value of 0.039, Pearson correlation of -0.230), (0, 1 for absent or present distant metastasis, respectively). Galanin intensity was significantly lower in stage IV (n= 6) in comparison to other stages (p= 0.037, using the Mann-Whitney U test), **Figure 2**.

Transcriptomics Analysis

Whole transcriptomics analysis using NGS captured more than 21,000 transcripts, with 1337 differentially expressed genes. Gene Set Enrichment Analysis (GSEA) was performed as previously described (20). An R-script was used to calculate the frequency of the gene recurrence across cellular pathways. Differentially upregulated pathways in late CRC stages (TNM stage II and III with high galanin intensity) compared to early-stage (TNM stage I with low galanin intensity) are shown in **Figure 3**. The upregulated genes are members of five key pathways involved in CRC, namely cell cycle (170 genes), cell division (603 genes), autophagosome (40 genes), and transcriptional regulation by TP53 (362 genes) and immune system process (332 genes). The supplementary figures (**Supplementary Figures S2**–**S8**) show the normalized differential expression of different pathways.

The top shared genes among the upregulated pathways are *AURKA, BIRC5, CCNA1, CCNA2, CDC25C, CDK2, CDK6,*

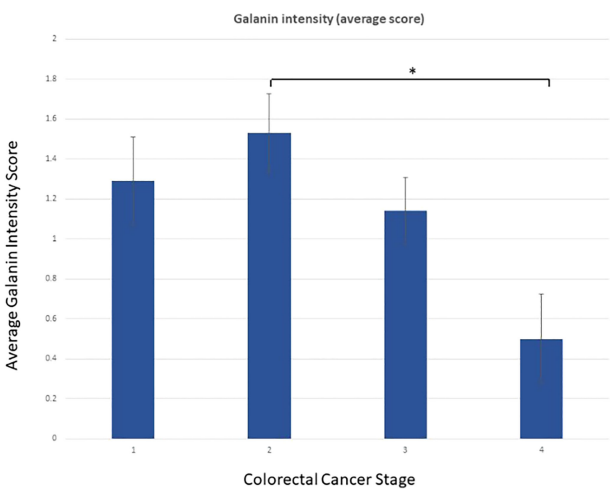


FIGURE 2 | Galanin intensity according to TNM staging. Galanin intensity is significantly lower in stage IV CRC compared to stage I-III (p= 0.037, using the non-parametric Mann-Whitney U test). * p-value <0.05.

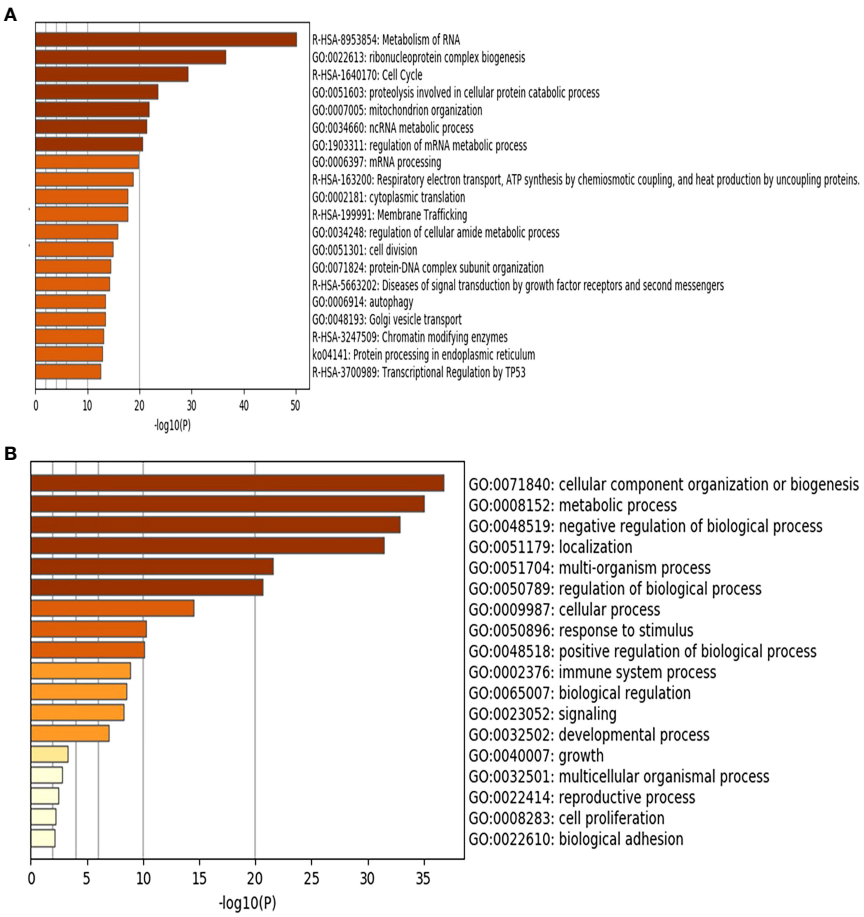


FIGURE 3 | Gene Set Enrichment Analysis (GSEA) of the differentially expressed genes showing the upregulated pathways in late stages of CRC (n=6) compared to early stages (n=5) using Metascape (http://metascape.org): a gene annotation and analysis online resource generating a graphical presentation. **(A)** Using Reactome and KEGG databases. **(B)** Using GO database.

EREG, LIG3, PIN1, TGFB1, TPX2 (**Table 2**). Functional protein analysis was performed to get more insight into the relationship among different coded proteins (**Figure 4**).

Validation of Transcriptomics Analysis

Galanin expression was significantly higher in two primary cell lines (HCT116 and HT29) compared to the two metastatic CRC cell lines (LoVo and SK-Co-1), **Figure 5**. We also examined the expressions of the top 12 DEGs shared among different pathways. Results were concordant with the transcriptomics analysis of patients' samples (**Figure 6**).

Bioinformatics Analysis

To further evaluate the effect of galanin expression in CRC, we performed bioinformatics analysis. We assessed the effect of shared genes on the activation or inhibition of cancer-associated pathways using Gene Set Cancer Analysis (GSCALite). **Figure 7** shows the percentage of colon adenocarcinoma cases in which shared gene expression activated (red) or inhibited (blue) specific pathways. Interestingly, there was a consistency in the expression of those genes in all studied CRC transcriptomes. **Supplementary Table S4** enlists the false discovery rate for each shared gene.

Then, Spearman's rank correlation coefficient was used to investigate the correlation between galanin expression and the immune-infiltrating-cell abundance in CRC using the TIMER 2 web tool. There was a negative correlation between the galanin expression and T cell CD8+, CD4+, neutrophil and natural killer (NK) cell numbers in the COAD tumor microenvironment (TME). However, there was a positive correlation between galanin expression and myeloid-derived suppressing cells (MDSC), denoting its association with a "non-inflamed" TME (cold TME), **Figure 8**.

Interestingly, galanin expression is significantly lower in CRC vs normal colonic mucosa (**Supplementary Figure S9**). The highest expression of galanin was found in the age group of 21-40 years compared to other age groups (**Supplementary Figure S10**).

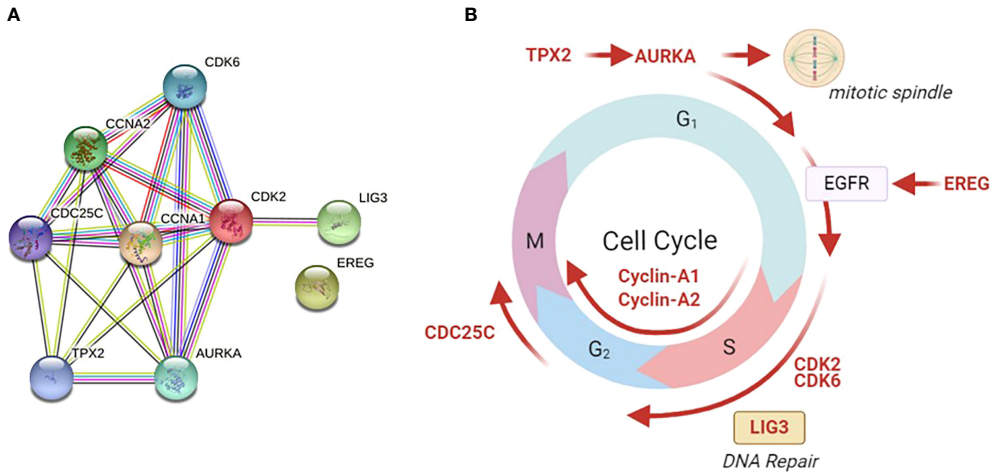


FIGURE 4 | Functional protein analysis of the most frequently shared genes among upregulated pathways in late versus early CRC stages. The analysis was performed using the STRING functional protein association network. **(A)** Functional protein analysis was performed for the most frequently shared kinases appearing twice in upregulated pathways in late versus early CRC stages (using STRING functional protein association network). TPX2 mediates AURKA localization to spindle microtubules. CDKs = cyclin-dependent kinases. AURKA = Aurora kinase A, EREG = Proepiregulin; Ligand of the EGF receptor/EGFR and ERBB4, LIG3 = DNA ligase 3, TGFβ = Transforming growth factor beta-1, TPX2 = Targeting protein for Xklp2. **(B)** The site of action of each gene is shown in different phases of the cell cycle. Galanin was not linked to any of those genes in the STRING functional protein association network.

DISCUSSION

The current study highlights the significance of galanin downregulation in CRC progression. In our study, galanin showed significantly lower expression in patients' samples of stage IV compared to earlier stages, evident by immunohistochemical staining. Although the galanin intensity is generally weakly correlated with TNM staging, galanin downregulation was associated with differential expression of key genes and driver pathways related to CRC progression, as revealed by our transcriptomics analysis. This was validated in the more controlled cell line experiments that showed a significant increase in the expression of galanin in the primary cell lines compared to the metastatic ones. In addition, the cell line experiments validated the results of differential expression of the transcriptomics analysis.

There is a complex interaction between the tumor and its microenvironment, leading eventually to further cancer cells' proliferation, invasion, and inhibition of apoptosis. Interestingly, it was reported that the number of galanin-containing neurons within myenteric plexuses located in the vicinity of the infiltrating cancer was higher in comparison to distal marginal regions (25). As a result of the invasion, nerve fibres of the enteric nervous system are destroyed, with subsequent atrophy of the myenteric plexus and the submucosal plexus. In the current study, in stage II CRC, galanin expression was at the highest in cancer cells, consistent with the previous findings of Nagayoshi et al. (12). With further progression, galanin expression declined, reaching its minimum in stage IV, where most neuronal plexuses are expected to be completely atrophied. However, this is controversial since galanin was reported in a previous study as a neuroprotective peptide that may inhibit the extrinsic pathway of apoptosis and, subsequently, promote cancer cell survival (26).

In the current study, transcriptomics analysis of representative samples of early-stage (TNM stage I) and late stages (stages II and III) identified five key pathways as drivers for tumor progression from early to late stages. Differentially upregulated pathways included cell cycle, cell division, autophagy, transcriptional regulation of *TP53* and immune system process. We identified twelve shared genes among different upregulated pathways and validated the results using Real-time PCR on cell lines.

Late stages of CRC in our study showed upregulation of the cell cycle and cell division pathways. One of the key characteristics of cancer is the uncontrolled proliferation due to derangement of cell cycle control through major checkpoints at G1–S transition and at G2–M transition (22, 23). Furthermore, autophagy plays an essential role in the progression of CRC under the increased mutational burden or through oncogenes or tumor suppressor genes. However, it is still doubtful if autophagy is an anti- or pro- cancerous (27). It was observed that expression of LC3B and SQSTM1 was associated with CRC's poor prognosis (28). In another study, although down-regulation of autophagy-related gene-5 (*ATG5*) was detected, its expression was associated with lymphovascular invasion (16). It was found that CRC with high microsatellite instability harbored at least one mutation in either *ATG12, ATG 9B, ATG 5, or ATG 2B* (29). In a study conducted by Lévy et al., depletion of ATG 7 in intestinal cells of mouse models was found to hinder cancerous growth (30). A gene signature related to autophagy was previously identified to group colon cancer patients into high or low risk in order to predict survival (31). Noteworthy, *TP53* mutations occur very frequently in CRC and are known to drive the progression from adenoma to adenocarcinoma (32). The

TABLE 2 | Most frequently shared genes among top differentially upregulated pathways in late CRC.

Gene	Annotation
AURKA	Aurora kinase A; Mitotic serine/threonine kinase that contributes to the regulation of cell cycle progression. It plays a critical role in various mitotic events including the establishment of mitotic spindle, centrosome duplication, centrosome separation as well as maturation, chromosomal alignment, spindle assembly checkpoint, and cytokinesis.
BIRC5	Baculoviral IAP repeat containing 5 (microtubule cytoskeleton organization). BRIC5 is also a member of the inhibitor of apoptosis gene family.
CCNA1	Cyclin-A1; is involved in the control of the cell cycle at the G1/S (start) and G2/M (mitosis) transitions. it belongs to the cyclin family.
CCNA2	Cyclin-A2; Cyclin which controls both the G1/S and the G2/M transition phases of the cell cycle. Functions through the formation of specific serine/threonine protein kinase holoenzyme complexes with the cyclin-dependent protein kinases CDK1 or CDK2.
CDC25C	M-phase inducer phosphatase 3; Functions as a dosage-dependent inducer in mitotic control. Tyrosine protein phosphatase required for progression of the cell cycle.
CDK2	Cyclin-dependent kinase 2; Serine/threonine-protein kinase involved in the control of the cell cycle; essential for meiosis, but dispensable for mitosis. It triggers duplication of centrosomes and DNA. Acts at the G1-S transition to promote the E2F transcriptional program and the initiation of DNA synthesis, and modulates G2 progression.
CDK6	Cyclin-dependent kinase 6; Serine/threonine-protein kinase involved in the control of the cell cycle and differentiation; promotes G1/S transition. Phosphorylates pRB/RB1 and NPM1.
EREG	Proepiregulin; Ligand of the EGF receptor/EGFR and ERBB4. Stimulates EGFR and ERBB4 tyrosine phosphorylation. Contributes to inflammation, wound healing, tissue repair, regulating angiogenesis.
LIG3	DNA ligase 3; Isoform 3 functions as heterodimer with DNA-repair protein XRCC1 in the nucleus and can correct defective DNA strand- break repair and sister chromatid exchange following treatment with ionizing radiation and alkylating agents.
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; Peptidyl-prolyl cis/trans isomerase (PPIase) that binds to and isomerizes specific phosphorylated Ser/Thr-Pro (pSer/Thr- Pro) motifs. - Regulates mitosis presumably by interacting with NIMA and attenuating its mitosis-promoting activity.
TGFB1	Transforming growth factor beta-1; Multifunctional protein that controls proliferation, differentiation and other functions in many cell types. It positively and negatively regulates many other growth factors.
TPX2	Targeting protein for Xklp2; Spindle assembly factor required for normal assembly of mitotic spindles. It mediates AURKA localization to spindle microtubules. Activates AURKA by promoting its autophosphorylation at 'Thr-288' and protects this residue against dephosphorylation.

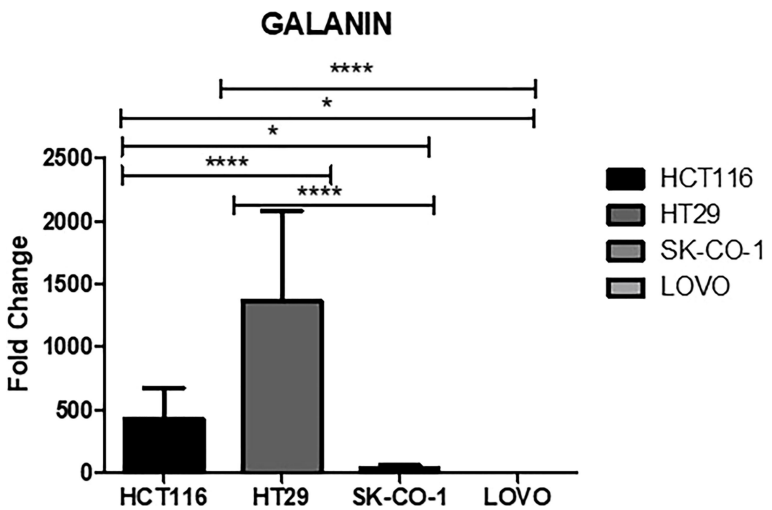


FIGURE 5 | Galanin expression in cell lines. Metastatic cell lines; LoVo and SK-Co-1 showed significantly lower expression of galanin compared to HCT116 and HT29 cell lines. The cells were starved overnight to ensure synchronization. Gene expression in each cell line was normalized to its corresponding non-starved cell line. * denotes p-value <0.05, **** p-value <000.1.

TP53 can harness autophagy by degrading autophagy protein LC3 in CRC cell lines. It was also found that loss of TP53 promoted the accumulation of LC3 initiating apoptosis (33).

The immune system process pathway is one of the differentially upregulated ones in late CRC samples in our study. Immune cells play a pivotal role in shaping the TME and determining the tumor progression (34), modulating inflammation (35), and metastasis (36). Thus, the TME, with its immune-cancer interaction, has a significant impact on the diagnosis and treatment of different malignancies, including CRC (37). The cellular, genetic, and molecular characteristics of the TME may affect the response to immune checkpoint inhibitors (38). In addition, chemotherapeutic agents used to treat CRC may also alter the expression of key molecules in the TME, e.g., capecitabine was recently reported to suppress the expression of Cytotoxic T lymphocyte antigen-4 (CTLA-4) in CRC, with potential enhancement of immunotherapy (39).

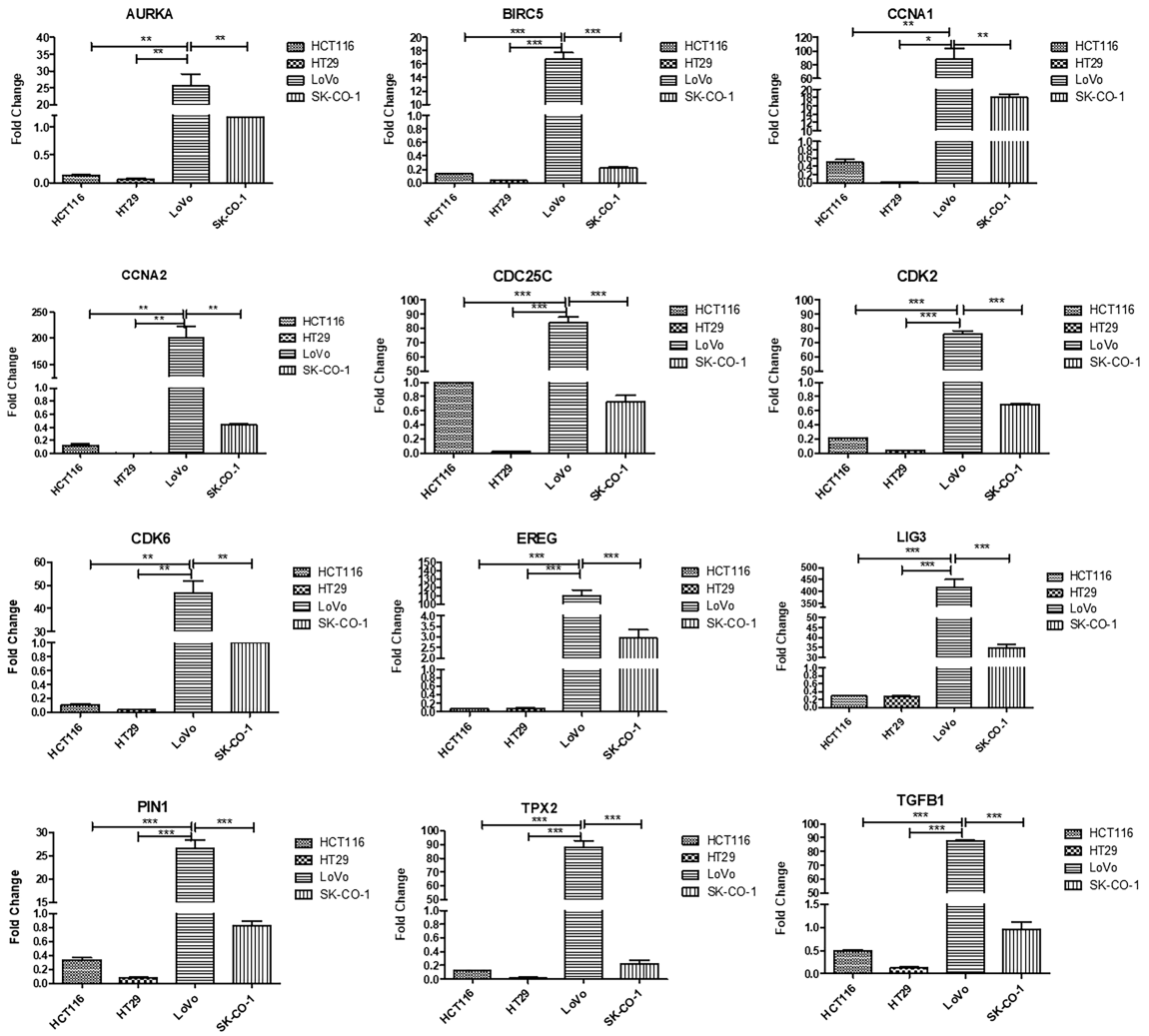


FIGURE 6 | Validation of top upregulated genes in metastatic CRC compared to earlier stages, using Real-time PCR for four cell lines; HCT116, HT29, LoVo and SK-Co-1. The cells were starved overnight to ensure synchronization. Gene expression in each cell line was normalized to its corresponding non-starved cell line. One way ANOVA was used to compare the expression among different cell lines. Metastatic cell lines; LoVo and SK-Co-1 showed significantly higher expression of *AURKA, BIRC5, CCNA1, CCNA2, CDK2, CDK6, EREG, LIG3, PIN1, TGFB1, TPX2*, compared to their expression in HCT116 and HT29. In contrast, CDC25C was also highly expressed in HCT116. * denotes p-value <0.05, ** denotes p-value <0.01, *** denotes p-value <0.001.

To further explore the relationship between galanin expression and the different immune cells in the TME, we conducted bioinformatics analysis. The analysis revealed a negative correlation of galanin expression with the number of several tumor-infiltrating immune cells, including CD8+ and CD4+ T-cells, neutrophils, and natural killer cells in the TME. In contrast, the galanin expression was positively correlated with the number of MDSC. Such findings denote the association of galanin expression with "cold" or "less inflamed tumors" with a poorer prognosis. They also represent a significant challenge of immunotherapy in different types of cancer, as there is a lack of adequate adaptive immune response (40).

Our results showed downregulation in the late stages of CRC of pathways involved in neuroactive ligand-receptor interaction compared to early stages. A neuroactive ligand-receptor interaction pathway was previously reported among the ones for which miRNAs were enriched in a study comparing responders and non-responders in terms of sensitivity to preoperative chemoradiotherapy for rectal cancer (41). Similarly, the neuroactive ligand-receptor interaction pathway was significantly different between colon tumor tissues and the adjacent noncancerous tissues using microarray data (42, 43).

We also examined the potential role of the top shared genes among the upregulated pathways, namely, *AURKA, BIRC5, CCNA1, CCNA2, CDC25C, CDK2, CDK6, EREG, LIG3, PIN1, TGFB1*, and *TPX2*. Collectively, the upregulation of those genes activates the cell cycle, EMT, PI3K/AKT and mTOR pathways.

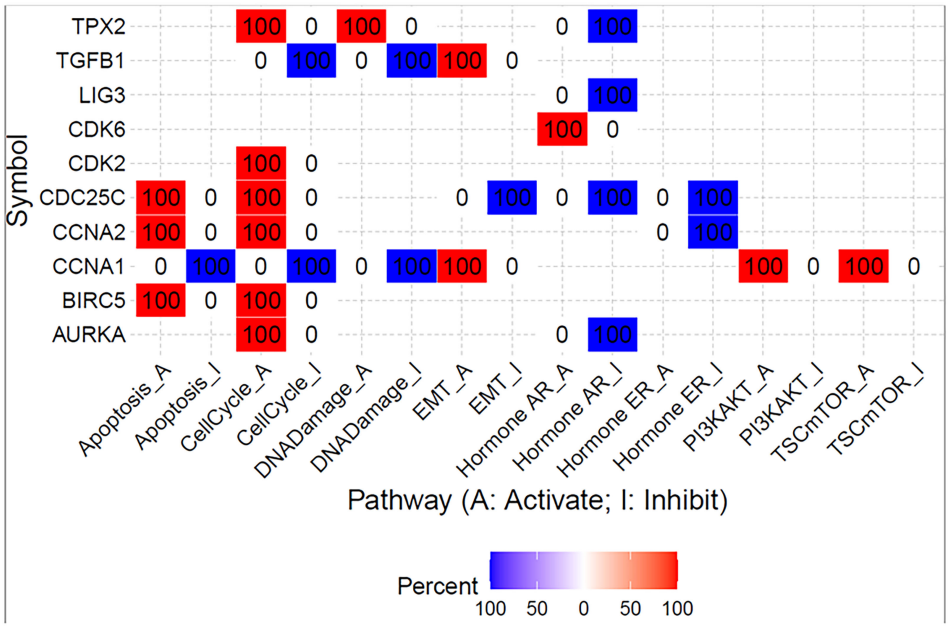


FIGURE 7 | Summary of the percentage of colon adenocarcinoma in which shared gene expression has a potential effect on pathway activity. The analysis was created using Gene Set Cancer Analysis (GSCALite) web tool and TGCA PanCancer COAD dataset.

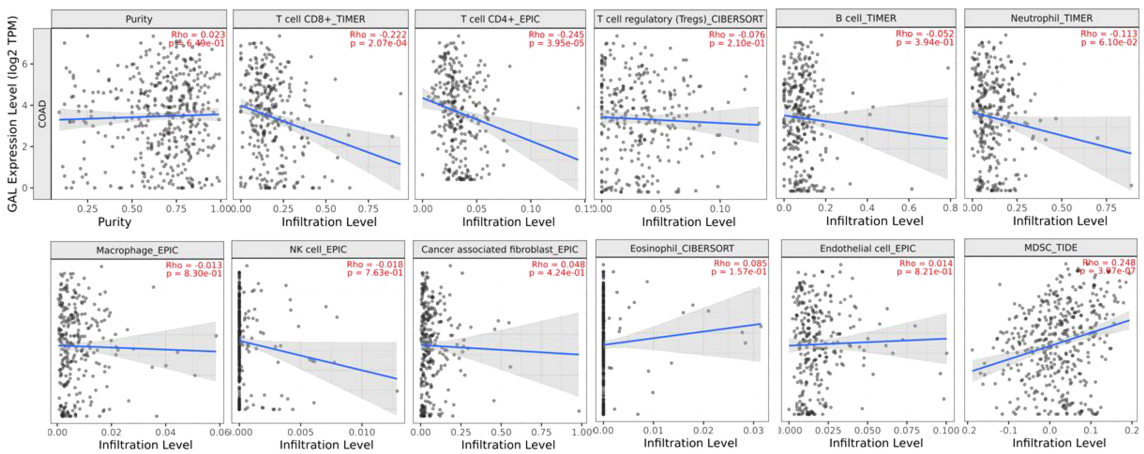


FIGURE 8 | Spearman's rank correlation coefficient of immune-infiltrating-cell abundance and GAL expression in COAD, using TIMER 2 web tool.

Our bioinformatics analysis revealed some interesting information about galanin; galanin expression is significantly lower in CRC vs normal colonic mucosa (consistent with our results). The highest expression occurs in CRC patients in the age group of 21-40 years. Though sporadic CRC is usually detected after the sixth decade of life, there has been an increase in CRC incidence in patients under the age of 40. Different molecular characteristics and a low suspicion of CRC in symptomatic young persons may underlie this incidence (44). Galanin expression may be linked to the early stage at which the disease is diagnosed at this young age group.

The current study has a few limitations, including the small patient cohort, with under-represented stages I and IV. In addition, we limited our transcriptomics analysis to the non-metastatic CRC patients' samples, considering the complexity and diversification of the biological programs underlying the metastatic CRC. Our current study mandates further biological validation to evaluate the different pathways detected by the

transcriptomics analysis in association with low galanin expression. However, the current study is the first to show the role of galanin in CRC progression at the molecular level in one of the Northern African populations. The transcriptomics analysis links galanin downregulation with key genes involved in key cancer-associated pathways.

CONCLUSIONS

There is a negative correlation of galanin intensity with CRC progression, being expressed most intensely in stage II and least in stage IV. Key driver pathways of tumor progression were revealed *via* transcriptomics analysis of late versus early-stage CRC tissue samples. The pathways included cell cycle and division, autophagy, transcriptional regulation of TP53 and immune system process.

The top shared genes among the upregulated pathways are *AURKA, BIRC5, CCNA1, CCNA2, CDC25C, CDK2, CDK6, EREG, LIG3, PIN1, TGFB1*, and *TPX2*. Interestingly, those genes are members of the cell cycle and cell division pathways. Moreover, BRIC5 is a member of the inhibitor of the apoptosis gene family. Galanin could represent a negative biomarker of CRC progression. Shared genes and pathways in late versus early stages of CRC provide insight into the progression of the disease and may serve as early biomarkers of metastatic disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number can be found at: https://doi.org/10.6084/m9.figshare.19895779.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee, University of Sharjah. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

IT: Conception, interpretation of data, preparation of the manuscript, supervision, MS-A: Conception, data analysis, preparation of the manuscript, supervision, NMY: data analysis, AS: data analysis, interpretation of data, TV: data analysis, Transcriptomics analysis and qPCR. AV: data acquisition, sample processing and preparation, AH: data acquisition, AK: clinical data acquisition, WA-R: interpretation of data, revising the manuscript, RH: conception, supervision, revising the manuscript. All manuscript authors agreed on the aforementioned contribution and have revised and approved the submitted version.

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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.2022.877147/full#supplementary-material

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BRIEF RESEARCH REPORT
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The BET Inhibitor JQ1 Potentiates the Anticlonogenic Effect of Radiation in Pancreatic Cancer Cells

*Patrick L. Garcia[1], Aubrey L. Miller[1], Ling Zeng[2,3], Robert C. A. M. van Waardenburg[1], Eddy S. Yang[1,2,4] and Karina J. Yoon[1]**

[1] Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, United States, [2] Department of Radiation Oncology, University of Alabama at Birmingham, Birmingham, AL, United States, [3] UAB Medicine Nursing, Oncology Services, UAB Hospital, Birmingham, AL, United States, [4] O'Neal Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL, United States

We reported previously that the BET inhibitor (BETi) JQ1 decreases levels of the DNA repair protein RAD51 and that this decrease is concomitant with increased levels of DNA damage. Based on these findings, we hypothesized that a BETi would augment DNA damage produced by radiation and function as a radiosensitizer. We used clonogenic assays to evaluate the effect of JQ1 ± ionizing radiation (IR) on three pancreatic cancer cell lines *in vitro*. We performed immunofluorescence assays to assess the impact of JQ1 ± IR on DNA damage as reflected by levels of the DNA damage marker γH2AX, and immunoblots to assess levels of the DNA repair protein RAD51. We also compared the effect of these agents on the clonogenic potential of transfectants that expressed contrasting levels of the principle molecular targets of JQ1 (BRD2, BRD4) to determine whether levels of these BET proteins affected sensitivity to JQ1 ± IR. The data show that JQ1 + IR decreased the clonogenic potential of pancreatic cancer cells more than either modality alone. This anticlonogenic effect was associated with increased DNA damage and decreased levels of RAD51. Further, lower levels of BRD2 or BRD4 increased sensitivity to JQ1 and JQ1 + IR, suggesting that pre-treatment levels of BRD2 or BRD4 may predict sensitivity to a BETi or to a BETi + IR. We suggest that a BETi + IR merits evaluation as therapy prior to surgery for pancreatic cancer patients with borderline resectable disease.

Keywords: pancreatic cancer, radiation, BET inhibitor, JQ1, DNA damage repair

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***Correspondence:**
Karina J. Yoon
kyoon@uab.edu

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INTRODUCTION

Pancreatic cancer (PC) is estimated to become the second leading cause of cancer related deaths in the United States in the next decade (1). The 5-year survival for patients with PC is ~10% (2). Surgical resection is the only potentially curative treatment, but only ~20% of patients are eligible for resection at diagnosis (3, 4). Criteria that determine eligibility for surgical resection have included absence of metastatic lesions and limited invasion into arteries of the mesenteric-portal axis (5). Several approaches to increase the number of eligible cases for resection have been evaluated for patients with pancreatic ductal adenocarcinoma (PDAC), the most common form of

PC. For example, combinations of a radiosensitizing agent and radiotherapy increases the number of patients with nonmetastatic disease that are eligible for resection, based on assessments that negative margin resection is possible post treatment (6–8). Two noteworthy studies evaluated the benefits of combining radiation with 5-fluorouracil (5-FU) or with FOLFIRINOX (5-FU, oxaliplatin, irinotecan, and leucovorin). The first, a prospective study, addressed the benefit of preoperative 5-FU + IR (50.4-56 gy) in borderline resectable PC. The study was conducted with 15 patients in 2001 by Mehta and colleagues (9). This approach converted 9/15 (60%) patients from surgically ineligible to eligible. The second, a retrospective study in 2014 by Christians, et al. analyzed the potential benefit for PC patients of FOLFIRINOX followed by gemcitabine or capecitabine and radiation (50.4 gy) (10). Twelve out of eighteen patients met the criteria for resection post therapy and had subsequent margin negative resections. Of these 12 patients, 7 were alive at termination of study 35 months post resection, and 5 of the 7 had no evidence of disease 18-35 months after diagnosis. Our study addresses whether the BETi JQ1, which we have shown to have anti-tumor efficacy as a single agent in preclinical models of PDAC, is also a radiosensitizer.

JQ1 inhibits the activity of BET bromodomain proteins (BRD2/3/4) by competitively inhibiting the association of BET proteins with acetylated lysine residues of binding partners (11). BRD2 and BRD4 are the main targets of BET inhibitors (12). The most well characterized effect of BET inhibitors is a decrease in binding of BET-dependent transcriptional complexes to promoter and enhancer regions of specific genes (13, 14). This inhibition, in turn, decreases expression of genes dependent on this mechanism of transcription. Recent literature suggests BET inhibitors may also decrease DNA repair mechanisms that depend on BRD4 activity (15–17). Consistent with this finding, we have shown that JQ1 increases levels of the DNA damage marker γH2AX and decreases expression of the NHEJ DNA repair protein Ku80 and the HR DNA repair protein RAD51 in models of PDAC of human origin (18).

Work presented here addresses whether JQ1 functions as a radiosensitizer in PC models *in vitro* and whether inhibition of BET protein activity or downregulation of BRD2 or BRD4 expression contributes to the potency of this combination. The work is based on the hypothesis that JQ1-mediated decreases in BET activity and consequent DNA repair deficiency sensitize PC cells to ionizing radiation. The long-range goal of the work is to determine if BET inhibition + IR comprises an effective pre-surgical treatment for patients that present with borderline resectable PDAC.

MATERIALS AND METHODS

Cell Culture and Chemicals

Panc1, BxPC3, and MiaPaCa2 PC cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 2 mM L-glutamine (Fisher Scientific). Each PC cell line was tested for mycoplasma using MycoAlert™ Plus Detection Kit (Lonza, Walkersville, MD, USA) and were negative. JQ1 (HY-13030, MedChem Express, Monmouth Junction, NJ, USA) stock solutions were prepared in DMSO, and diluted to the appropriate concentration in complete DMEM media. Vehicle control was DMSO (<0.01%).

Clonogenic Assay

PC cells were plated in 24-well plates (Panc1: 250 cells/well, BxPC3: 400 cells/well, and MiaPaCa2: 200 cells/well) and allowed to adhere for 24h. After being exposed to JQ1 for 72h or 120h and then irradiated (IR), cells were propagated in drug-free media for the equivalent of 5-6 cell doubling times (doubling times: Panc1, 48 hours; BxPC3, 52 hours; MiaPaCa2, 30 hours.). Cells were stained with crystal violet (0.025%) at 10, 14 and 7 days after exposure to drug. Quantification was performed by counting stained colonies of >50 cells, using an Olympus CK2 microscope (Olympus Scientific Solutions Americas Corp., Waltham, MA, USA). The plating efficiency (PE) was calculated as: PE = (number of colonies counted)/(number of cells plated). The clonogenic survival fraction (SF) for each treatment was calculated as: SF = [(# of colonies counted)/(# cells plated x PE) x 100] (15, 19–21). Data are reported as average survival fraction ± SEM.

Immunoblot

Cells were plated (3 x10^5 cells/well) in 6-well plates and allowed to adhere for 24h, exposed to JQ1 for 48h, and then irradiated (IR). Cells were harvested 1h or 24h post IR. Whole cell lysates were prepared in NP-40 containing protease inhibitors (Fisher Scientific). Primary antibodies used were: Cleaved-PARP (5625, Cell Signaling, Denvers, MA, 1:1,000), γH2AX (9718S, Cell Signaling, 1:1,000), RAD51 (ab88572, Abcam, Waltham, MA, 1:1,000), GAPDH (97166, Cell Signaling, 1:10,000), BRD2 (5848, Cell Signaling, 1:1,000) and BRD4 (13440, Cell Signaling, 1:1,000). Secondary antibodies used were: HRP goat anti-rabbit IgG (6721, Abcam, 1:50,000) and HRP anti-mouse IgG (7076, Cell Signaling, 1:5,000). Immunoblots were quantitated using ImageJ software. Data were normalized to respective loading controls and then to the DMSO control.

Immunofluorescence

BxPC3 cells were seeded (1 x 10^4 cells/well) on chamber slides (Lab-Tek®, Rochester, NY, USA) and incubated under standard conditions for 24h prior to drug or IR exposure. Cells were then exposed to JQ1 for 48h + 0, 4, or 8 gy IR and resuspended in drug-free medium for 1 or 24 hours, as indicated. At harvest, cells were washed with PBS, fixed with 10% neutral buffered formalin (NBF) (Fisher Scientific), and permeabilized with 0.1% Triton x-100 (Fisher Scientific). Blocking of non-specific binding was performed in 2% bovine serum albumin (BSA) (Fisher Scientific). γH2AX foci (05-636, Millipore Sigma, Burlington, MA, 1:500) were detected using AlexFluor 488 nm donkey anti-rabbit conjugated secondary antibody (1:500), and nuclei were stained with DAPI (1:20,000).

Images were taken using a Zeiss Observer Z.1 microscope and photomicrographs were processed with Zen 2011 Blue imaging software (Zeiss, Dublin, CA, USA).

Generation of Stable Transfectants

The protocol used to generate stable transfectants of Panc1 cells that express contrasting levels of BRD2 or BRD4 is published (18). Briefly, Panc1 cells were transfected using PEI (Polysciences Inc., Warrington, VA, USA) and Mission shRNA (Millipore Sigma) targeting BRD2 or BRD4. shGFP served as vector control (Addgene, Watertown, MA, USA). Selection of stable transfectants was carried out using 10 μg/ml puromycin (ENZO Life Sciences, Farmingdale, NY, USA).

Irradiation

Radiation experiments were carried out using a Kimtron IC-320 Series Biological Irradiator (Kimtron Medical Inc., Oxford, CT, USA). Cells were exposed to 4 or 8 gy IR using a 320 kV x-ray tube with a maximum output of 3200 watts, with parameters set on the SC-500 Series II controller (Kimtron Medical Inc.). In addition, the IR dose was also measured by a PTW Unidose E dosimeter (PTW, Freiburg, Germany).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 and 9.0 (San Diego, CA, USA). Quantitation of clonogenic assays, IF assays for BxPC3 cells and IB for Panc1, MiaPaCa2 cells was done using one-way ANOVA followed by Tukey multiple comparisons. $P<0.05$ was considered significant. All experiments were done a minimum of three times.

RESULTS

JQ1 + IR Reduced Clonogenic Potential More Than JQ1 or IR as a Single Modality

Clonogenic assays reflect cell reproductive capacity and are used as *in vitro* assessments of efficacy of IR and chemotherapeutic agents (20). We performed clonogenic assays using three PC cell lines to evaluate whether the combination of JQ1 + IR was more effective than either modality alone (**Figure 1**). The two concentrations of JQ1 used for each cell line reflect the approximate IC25 and IC50 for that cell line at two different exposure times. The two exposure times, 72h or 120h, were used to compare the impact of JQ1 at a higher dose for a shorter time with a lower dose for a longer time (**Figure 1A**). We used two doses of IR, 4 or 8 gy, to determine if increasing the dose provided added benefit when combined with a lower vs higher dose of JQ1. All concentrations used for JQ1 are achievable in plasma of murine models at nontoxic doses (22). Immediately after IR, cells were placed in drug-free media and propagated for an additional 5-6 cell doubling times (7-14 days, depending on the cell line).

Panc1 cells. Cells that were not exposed to JQ1 or IR (control) produced 40 ± 1 colonies. IR alone, 4 or 8 gy, reduced clonogenic survival fraction by 37% and 64%, respectively, compared to control (no JQ1 or IR). Exposure to JQ1 alone produced a dose-dependent reduction in colony number of ~32-56% compared to controls ($p<0.0001$) (**Figure 1B**). The combination of JQ1 + IR at all doses used was more effective than either modality alone. Notably, cells exposed to 8gy IR + 20 μM JQ1 for 72h or to 8 gy IR + 0.55 or 3 μM JQ1 for 120h completely suppressed colony formation. (**Figures 1B, C**). The data demonstrate that, in combination with each dose and time of JQ1 evaluated, 8 gy IR provided greater inhibition of clonogenic potential *in vitro* than 4 gy.

BxPC3 cells. Control BxPC3 cells produced 22 ± 1 colonies. 4 or 8 gy IR reduced colony number by 21% or 57%, respectively (**Figure 1D**). JQ1 alone (2 or 8 μM) decreased survival fraction by 39-82%, respectively. Similar to Panc1 cells, the combination of JQ1 + IR was more effective than either agent alone: BxPC3 survival fraction was completely inhibited when exposed to 4 gy IR + 8 μM JQ1 or 8 gy IR + 2 μM or 8 gy IR + 8 μM JQ1 at 72h (**Figure 1D**). A similar additive effect was seen in cells exposed to the lower doses of JQ1 for 120h (**Figure 1E**).

MiaPaca2 cells. Control MiaPaCa2 cells produced 24 ± 1 colonies. Radiation (4 or 8 gy) as a single treatment reduced survival fraction by 18%, or 56%, respectively (**Figure 1F**). JQ1 (80 or 250 nM) as a single modality decreased colony number by 41%, 50%, respectively. The combination decreased survival fraction by 58-99% compared to control (**Figure 1F**). As seen with Panc1 and BxPC3 cells, survival fraction was reduced to 99% following exposure to 8 gy IR + 250 nM JQ1 for 72h. Similar results were observed with cells exposed to JQ1 for 120h (**Figure 1G**).

Taken together, the data show that a higher dose of JQ1 x shorter exposure (72h) + IR and a lower dose of JQ1 x longer exposure (120h) + IR had similar impact on colony numbers. Further, concentrations of the BETi JQ1 achievable in murine plasma (up to 24 μM) at nontoxic doses can be effectively combined with IR to decrease the clonogenic potential of PC cells (22).

Immunofluorescence Data Demonstrate That JQ1 + IR Increased Levels of the DNA Damage Marker γH2AX and Decreased Levels of the DNA Repair Protein RAD51 in BxPC3 Cells

BET-associated transcription complexes regulate expression of many genes. We showed previously that JQ1 decreased expression of DNA repair proteins Ku80 and RAD51 in *in vivo* PDAC models (18). We also reported that JQ1 increased levels of DNA damage, as reflected by an increase in levels of the damage marker γH2AX (18, 23). These observations suggested that JQ1 interferes with DNA damage repair. We performed immunofluorescence (IF) and immunoblot (IB) assays to determine whether IR enhanced these two known effects of the BETi JQ1 in BxPC3 PC cells. Doses of JQ1, 2 or 8 μM represent approximate IC25 and IC50 concentrations for this cell line. The

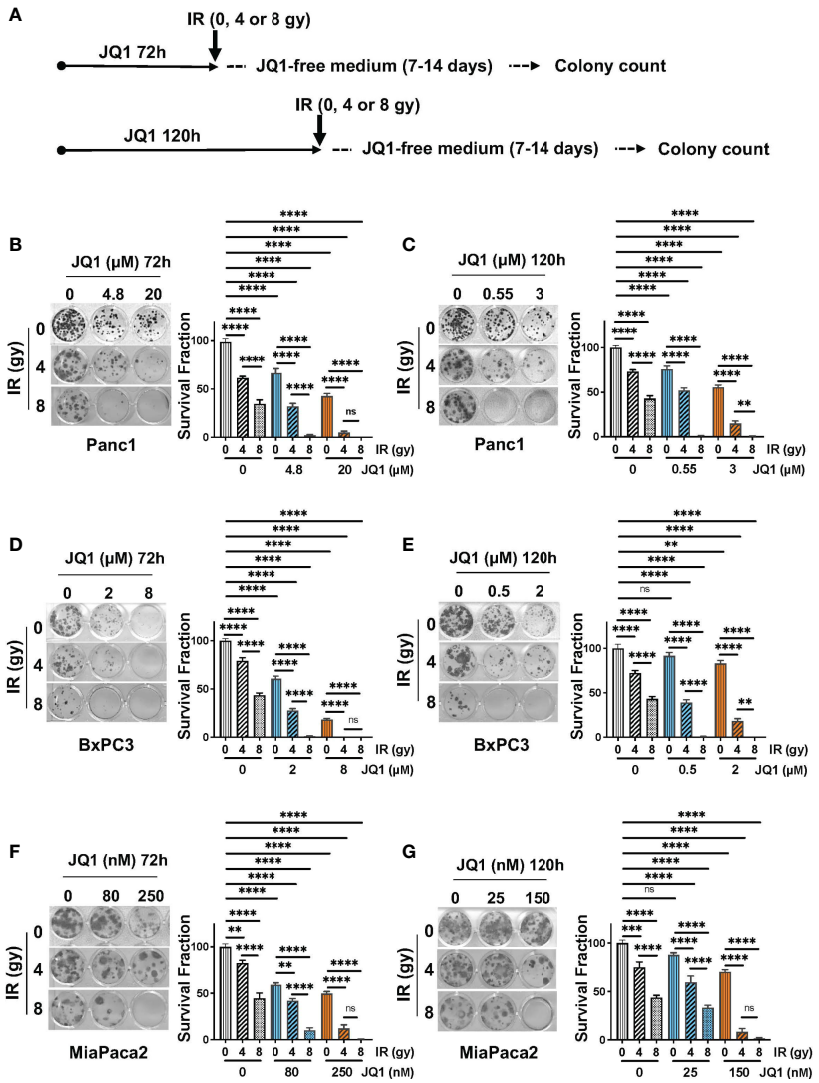

FIGURE 1 | The combination of JQ1 + IR reduces clonogenic potential more than JQ1 or IR as a single modality. **(A)** Schematic of the clonogenic assay protocol. Cells were exposed to JQ1 for 72h and 120h. **(B, C)** Images of representative plates showing colonies produced by Panc1 cells at **(B)** 72 hours or **(C)** 120 hours exposure to JQ1 ± IR. **(D, E)** Images of representative plates showing colonies (left panel) and survival fraction (right panel) of BxPC3 cells at **(D)** 72 hours or **(E)** 120 hours after treatment. **(F, G)** Images of representative plates showing colonies (left panel) and survival fraction (right panel) of MiaPaCa2 cells at **(F)** 72 hours or **(G)** 120 hours after treatment. Data are reported as average survival fraction ± SEM, as shown on bar graphs to the right of each plate image. One-way ANOVA followed by Tukey multi comparison analysis was performed as described in Materials and Methods. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. ns, not significant.

exposure time of 48h was based on laboratory experience indicating that increases in γH2AX are readily detectable 48h after exposure to JQ1.

Cells were exposed to JQ1 for 48h and irradiated. Analyses were performed at 1h and at 24h post IR (**Figure 2A**). We used IF to evaluate formation of γH2AX foci as a measure of DNA damage in BxPC3 cells exposed to JQ1 (2 or 8 μM) ± IR (4 or 8 gy) (**Figures 2B, C**). Cells with >5 foci were designated γH2AX positive. We used IB to assess the effect of JQ1 ± IR on expression of the DNA damage repair protein RAD51 (**Figures 2D, E**). At 1h post IR, the data show that: JQ1 increased γH2AX foci and decreased RAD51 levels compared to controls; that 4 gy IR augmented the effect of JQ1 on γH2AX; and that the effect of JQ1 + 4 gy IR was equivalent to that of JQ1 + 8 gy IR. In contrast, the effect of 4 vs 8 gy IR at 24h post irradiation (compare **Figures 2B, C**) at 2 or 8 μM JQ1, 8 gy IR increased γH2AX foci more than JQ1 + 4 gy IR ($p<0.01$). As anticipated, IR alone had little effect on the level of detectable RAD51 protein. The data suggest that JQ1 inhibits DNA repair and acts as a radiosensitizer and that the degree of sensitization is dose-dependent.

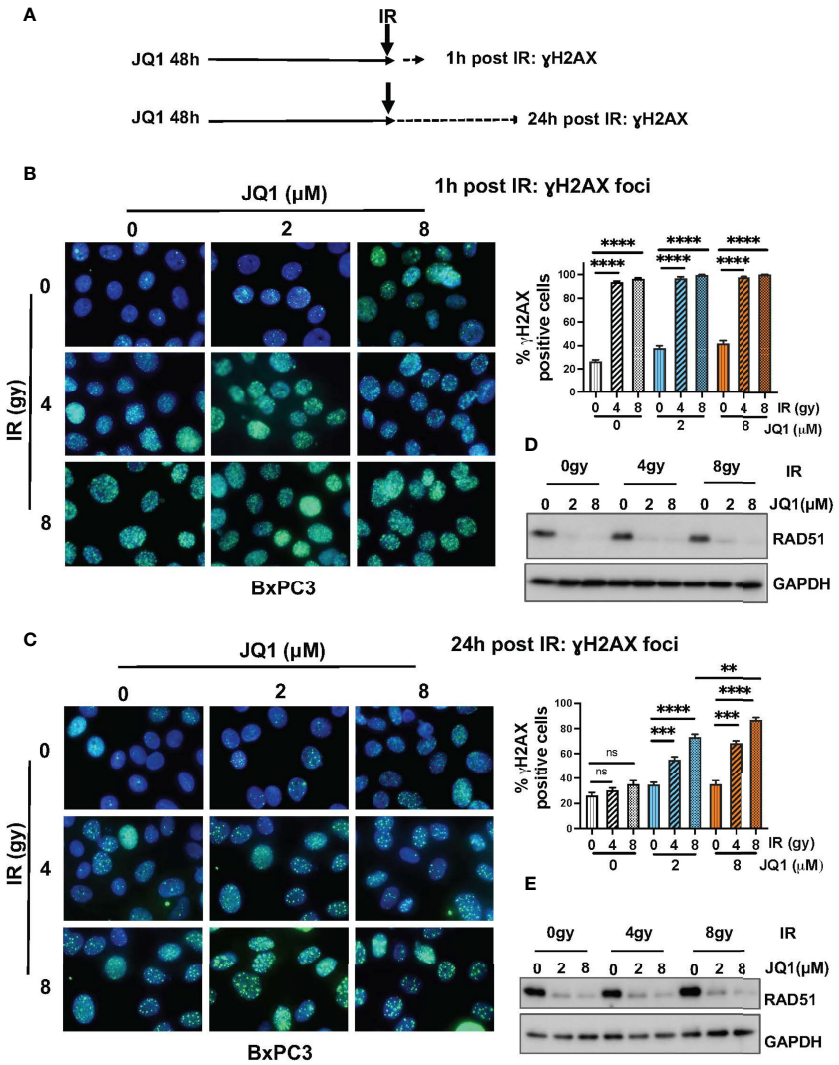

FIGURE 2 | Immunofluorescence and immunoblot data show that JQ1 + IR increases levels of the DNA damage marker γH2AX and decreases levels of the DNA repair protein RAD51 in BxPC3 cells. The combination is more effective than either single modality. **(A)** Schematic of exposure and harvest times of cells exposed to JQ1 and IR prior to evaluating levels of γH2AX and RAD51. **(B, C)** Images of representative immunofluorescence (IF) data at **(B)** 1 hour post IR or **(C)** 24h post IR. Quantitation of γH2AX foci is presented on bar graphs to the right of the IF images. **(D, E)** Representative immunoblots for RAD51 at **(D)** 1 hour and **(E)** 24 hours post IR. One-Way ANOVA followed by Tukey multi comparison analysis was performed as described in Materials and Methods. **p < 0.01, ***p < 0.001, ****p < 0.0001. ns, not significant.

Immunoblot Data Demonstrate That JQ1 + IR Increased Levels of the DNA Damage Marker γH2AX and Decreased Levels of the DNA Repair Protein RAD51 in Panc1 and MiaPaCa2 Pancreatic Cancer Cells, and That the Increase in γH2AX Was Concomitant With an Increase in the Apoptosis Marker Cleaved PARP

To confirm that data seen with BxPC3 cells was not unique to that PC cell line, we used IB to examine the effect of JQ1 ± IR on γH2AX and RAD51 levels in Panc1 and MiaPaCa2 cells (**Figure 3**). We also evaluated levels of cleaved PARP (Cl. PARP) to determine if this marker of apoptosis increased in parallel with an increase in γH2AX. We hypothesized that if the observed increase in DNA damage affected cell viability, Cl. PARP would increase simultaneously with γH2AX.

As was seen with BxPC3 cells, IR or JQ1 as single agents increased the level of γH2AX in Panc1 cells (**Figures 3A, B**) and JQ1 + IR increased the levels of γH2AX more than JQ1 or IR as a single modality. Also similar to data with BxPC3 cells, JQ1 decreased RAD51 levels ($p<0.0001$) at both time points post IR and IR had little if any effect on RAD51.

As with BxPC3 and Panc1 cells, in MiaPaCa2 cells JQ1 + IR increased γH2AX compared to JQ1 or IR alone ($p<0.0001$). Additionally, similar to Panc1 cells, JQ1 + IR increased levels of

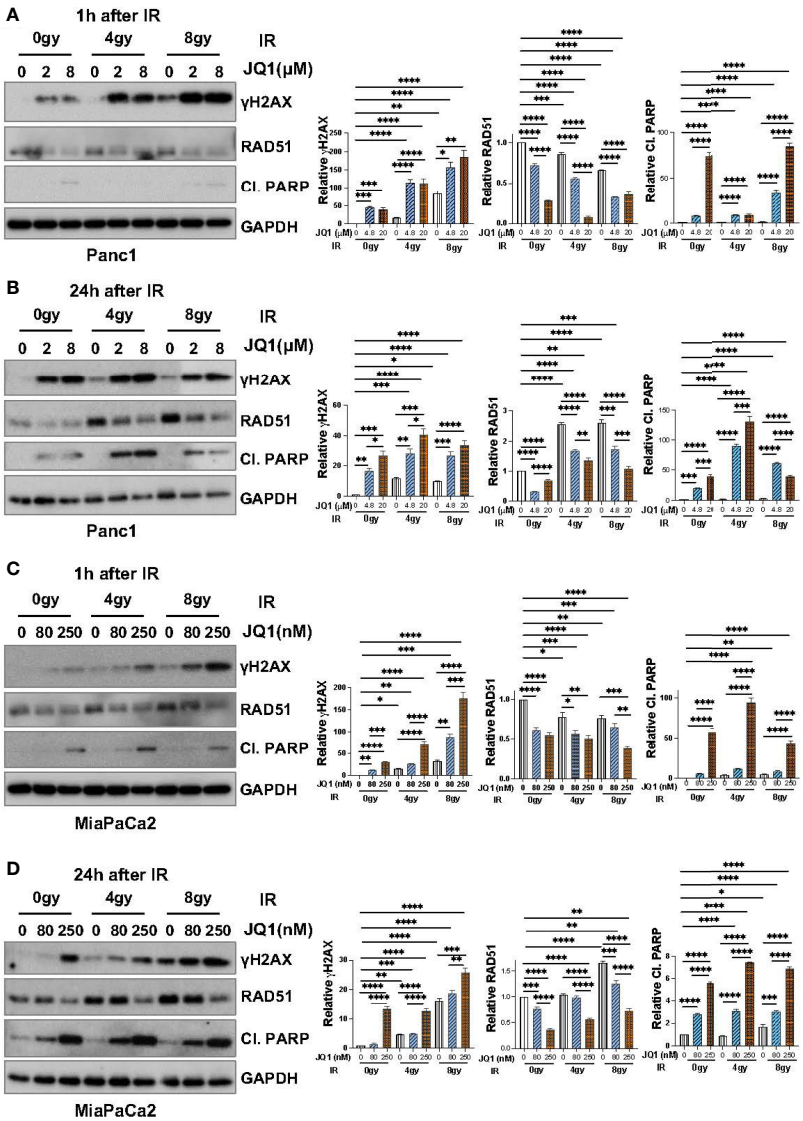

FIGURE 3 | Immunoblot data demonstrate that JQ1 + IR increases levels of the DNA damage marker γH2AX and decreases levels of the DNA repair protein RAD51 in Panc1 and MiaPaCa2 pancreatic cancer cells, and that the increase in γH2AX is concomitant with an increase in the apoptosis marker cleaved PARP (Cl. PARP). Images of representative immunoblots of Panc1 cells exposed to JQ1 ± IR, 1h after IR **(A)** or 24h after IR **(B)**, and of MiaPaCa2 cells 1h after IR **(C)** or 24h after IR **(D)**. Quantitation of γH2AX, RAD51 or Cl. PARP, was done using ImageJ software and analyzed using one-way ANOVA followed by Tukey multi comparison analysis. Quantitation is shown to the right of each immunoblot images as a bar graphs. *p < 005, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Cl. PARP at 24h, concomitant with the increase in γH2AX ($p<0.0001$) (**Figures 3C, D**). The data show that JQ1 and JQ1 + IR increase the levels of markers that reflect DNA damage and apoptosis. The data demonstrate simultaneous increases in DNA damage and apoptosis in cells exposed to JQ1 + IR greater than JQ1 or IR, and suggest that the BETi JQ1 functions as a radiosensitizer.

Downregulation of BRD2 and BRD4 Enhanced Sensitivity to JQ1 + IR

We next addressed a mechanistic aspect of JQ1 + IR on PC cells. BRD2 and BRD4 are the principal molecular targets of JQ1 (12). We considered the possibilities that lower levels of BRD2 or BRD4 would decrease JQ1 potency due to a relative lack of target protein(s) or, alternatively, lower levels of BRD2 or BRD4 would increase JQ1 potency because lower levels of drug might be needed to inhibit a greater percentage of BET protein activity. We transfected Panc1 cells with shBRD2 or shBRD4 to decrease expression of each protein by 96% or 99%, respectively, (**Figure 4A**) and evaluated the sensitivity of these and of shGFP control transfectants to JQ1 ± IR using clonogenic assays. As for experiments in **Figures 1B, C**, we used two concentrations of JQ1 and two exposure times: 4.8 or 20 μM for 72h (**Figures 4B, D, F**) and 0.55 and 3 μM for 120h (**Figures 4C, E, G**) ± 4 or 8 gy IR. Immediately after

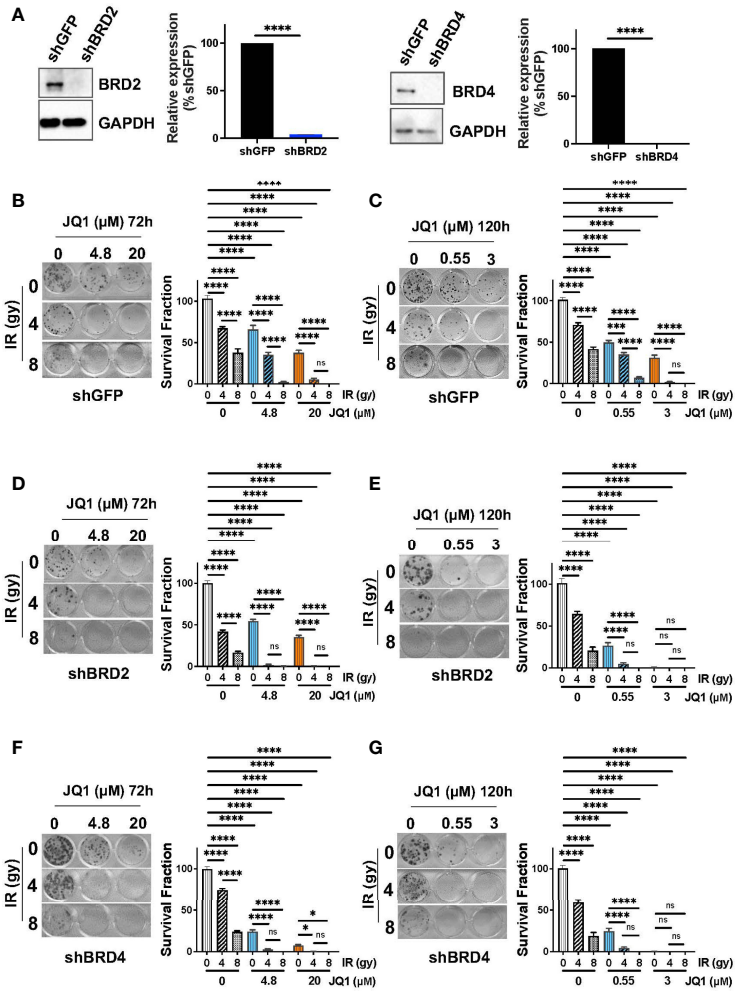

FIGURE 4 | Downregulation of BRD2 or BRD4 enhances sensitivity to JQ1 + IR. **(A)** Levels of BRD2 and BRD4 protein were lower in Panc1 cells transfected with shBRD2 or shBRD4 than in shGFP control transfectants. Quantitation of expression level was done as in Materials and Methods. ****p<0.0001. **(B, C)** Images of representative clonogenic assays showing colonies for shGFP transfectants exposed to JQ1 for **(B)** 72 hours or **(C)** 120 hours ± IR or for **(D, E)** shBRD2 transfectants or for **(F, G)** shBRD4 transfectants. Average survival fraction ± SEM is shown to the right of each image as a bar graph. Quantitation was done as in Materials and Methods. *p < 005, ***p < 0.001, ****p < 0.0001. ns, not significant.

irradiation, cells were placed in drug-free media and propagated for an additional 10 days.

In all three transfectants, JQ1 or IR as a single modality reduced colony number compared to controls (p<0.0001) and the combination reduced survival fraction more than either modality alone (p<0.0001). The data show that 4.8 or 20 μM JQ1 for 72h + 4 gy or 8 gy IR abrogated colonies in shBRD2 transfectants and reduced survival fraction by 97-100% in shBRD4 transfectants, compared to controls (**Figures 4B, D, F**). Also, 0.55 or 3 μM JQ1 x 120h abrogated colony formation in shBRD2 and shBRD4 transfectants (**Figures 4C, E, G**). PC cells with lower levels of BRD2 or BRD4 were more sensitive to JQ1 as a single agent and to JQ1 + IR. Notably, the combination abrogated clonogenic potential at concentrations of JQ1 that are achievable in murine plasma at nontoxic doses, in a dose- and time-dependent manner.

Taken together, our data suggest that the levels of BRD2 or BRD4 may represent a marker of sensitivity to BET inhibitors, and that a BET inhibitor can be effectively combined with IR to inhibit PC cell proliferation.

DISCUSSION

This study evaluated whether pharmacologic inhibition of BET bromodomain activity by the BETi JQ1 or downregulation of its molecular targets BRD2 or BRD4 potentiated the anti-clonogenic effect of IR *in vitro*. The data show that JQ1 + IR had greater effect than either treatment alone. The anticlonogenic effect of JQ1 + IR was associated with increases in DNA damage and decreases in expression of the DNA repair protein RAD51. Our data suggest that a combination of BET inhibitor and radiation

may be a useful strategy to reduce tumor proliferation and further as a neoadjuvant therapy to reduce tumor volume in patients with PC.

BET proteins contribute to multiple cellular processes in normal and tumor cells. The most well characterized function of BET proteins is to facilitate binding of BET-dependent transcription complexes to specific chromatin associated histones, to regulate transcription of target genes (24, 25). Preclinical studies demonstrate a potential utility BET inhibitors for treatment of a variety of solid malignancies including breast, lung, ovarian, and pancreatic cancers (26–30). Recent studies have sought to identify combination strategies that include BET inhibitors, to improve the efficacy of current standards of care. One such study by Karakashev et al. (2017) determined that the BETi JQ1 + the PARPi olaparib inhibited BRCA-proficient ovarian cancer cell proliferation *in vitro* and *in vivo* (16). The authors of that study conclude that the synergy observed with JQ1 + olaparib was associated with a JQ1-mediated decrease in expression of the G2 checkpoint kinase WEE1 and the DNA damage response protein TOPBP1. In 2019, our lab demonstrated the anti-tumor efficacy of this combination in pancreatic patient-derived xenograft (PDX) models (18). Mechanistic studies that were a component of that work showed that RNAi targeting either of the two molecular targets of JQ1, BRD2 or BRD4, decreased levels of the NHEJ repair protein Ku80 and the HR repair protein RAD51. Consistent with that finding, tumors of mice treated with JQ1 + olaparib had higher levels of DNA damage than tumors from mice treated with either drug as a single agent, as reflected by levels of the DNA damage marker γH2AX. The current study focused on RAD51 levels, since RAD51 is considered a representative marker for DNA repair proficiency and is frequently overexpressed in human PDAC tumors (31–34).

Recent studies indicate that this family of proteins also contributes directly to DNA damage repair. Li et al. (2018) demonstrated that the BET protein BRD4 binds to acetylated histones following IR-induced DNA damage and associates with the NHEJ DNA repair protein Ku80 to increase DNA repair in prostate cancer models (35). In that study, JQ1 alone did not increase levels of γH2AX. The latter observation contrasts with our observation in PC cells in that JQ1 as a single agent increases levels of γH2AX. A study by Cameros et al. (2020) showed that the BET inhibitor OTX015 + IR was more effective than OTX015 or IR alone, as reflected by increases in markers of apoptosis and of DNA damage *in vitro* in rhabomyosarcoma cells (36). In that study, increased DNA damage was associated with decreased levels of DNA damage repair proteins RAD51, ATM, and DNA-PK. Mechanistically, Wang et al. (2017) showed that JQ1 inhibits repair of DNA double-strand breaks induced by IR and promotes apoptosis in non-small cell lung cancer cell lines (37). Further, Yang et al. (2017) showed that JQ1 decreased HR DNA repair activity following 10 gy IR in 3 ovarian cancer cell lines (17). Similar to our study, these investigators observed an increase in γH2AX foci and decreased expression of the DSB repair protein RAD51. In that study, ChIP data showed that JQ1 inhibited binding of BRD2/3/4 to the RAD51 promoter, directly repressing expression of RAD51. Our study and published work using multiple types of tumor cell models indicate that JQ1 disrupts DNA damage repair (15–18, 38–40). Our data suggest that JQ1 may function as a radiosensitizer in pancreatic cancer, the first study to address this question. The data also suggest that a BET inhibitor + IR warrants further investigation to determine if this combination increases the number of PC patients eligible for resection.

Approximately 40% of patients diagnosed with PDAC have locally advanced disease at the time of diagnosis (41). For patients with borderline resectable tumors, neoadjuvant therapy of chemotherapy + IR has the potential to facilitate margin free resection and improve prognosis. Recently The National Cancer Research Institute Clinical and Translational Radiotherapy (CTRad) working group released consensus guidelines to encourage clinical trials conducted with novel compounds in conjunction with radiotherapy (42). Our study suggests that combining a BET inhibitor with IR may be a useful strategy to augment the anti-tumor efficacy of IR prior to resection in this patient population. Future work includes evaluation of BRD2 or BRD4 as a marker of sensitivity to JQ1 + IR and comparison of a BET inhibitor ± IR in low vs high level BRD2 or BRD4 expressing preclinical models of PC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conception and/or design: RvW, EY, KY. Acquisition of data: PG, AM, LZ. Data analysis and interpretation: PG, AM, EY, RvW, KY. Resources: EY, RvW, KY. Writing:-original draft preparation: PG, AM, KY. Writing-review and editing: PG, AM, LZ, EY, RvW, KY. Study supervision: EY, KY. All authors have read and agreed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Swimming Impedes Intestinal Microbiota and Lipid Metabolites of Tumorigenesis in Colitis-Associated Cancer

Weiyi Wang†, Ying Xu†, Xiaofeng Wang, Yimin Chu, Haiqin Zhang, Lu Zhou, Haijin Zhu, Ji Li, Rong Kuai, Fengli Zhou, Daming Yang and Haixia Peng**

Department of Endoscopy, Shanghai Tong Ren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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***Correspondence:**
Haixia Peng
PHX1101@shtrhospital.com
Daming Yang
ydm1100@shtrhospital.com

†These authors have contributed equally to this work and share first authorship

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Background: Accumulating data support that regular physical activity potentially inhibits chronic colitis, a risk factor for colitis-associated cancer (CAC). However, possible effects of physical activity on CAC and the underlying mechanisms remain poorly understood.

Methods: A pretreatment of swimming on azoxymethane/dextran sodium sulfate (AOM/DSS)-induced CAC mice was implemented to determine its protective effect. Inflammation and tumorigenesis were assessed using colorectums from C57BL/6 mice. In order to determine how swimming alters colonic lipid metabolism and gene expression, a comparative analysis was conducted. Meanwhile, alterations in intestinal microbiota and short-chain fatty acids (SCFAs) were detected and analyzed. Finally, an integration analysis of colonic lipid metabolism with gene expression and intestinal microbiota was performed respectively.

Result: Swimming pretreatment relieved bowel inflammation and minimized tumor formation. We demonstrated that prostaglandin E2 (PGE2)/PGE2 receptor 2 subtype (EP2) signaling as a potential regulatory target for swimming induces colonic lipid metabolites. Swimming-induced genera, Erysipelatoclostridium, Parabacteroides, Bacteroides, and Rikenellaceae_RC9_gut_group, induced intestinal SCFAs and affected the function of colonic lipid metabolites enriched in glycerophospholipid metabolism and choline metabolism in cancer.

Conclusion: According to our experiments, swimming pretreatment can protect mice from CAC by intervention in the possible link between colonic lipid metabolites and PGE2/EP2 signaling. Further, swimming-induced genera and probiotics promoted glycerophospholipid metabolism and choline metabolism in cancer, the major constituents of colonic lipid metabolites, and increased SCFAs, which were also important mechanisms for the anti-inflammatory and anti-tumorigenic effects of swimming.

Keywords: chronic inflammation, colonic lipid metabolites, colorectal cancer, intestinal microbiota, physical activity, swimming, tumorigenesis

INTRODUCTION

Colorectal cancer (CRC) ranks third among all cancers and as the second leading reason for cancer-related deaths globally (1). By applying early screening programs and developing new treatment regimens, colon cancer has witnessed a dramatic increase in 5-year survival rates. The 5-year survival rate of 91% is achieved for people with localized-stage CRC. However, only 15% of survival rate is achieved for people with metastatic-stage CRC (2). CRC has become a worldwide social and public health problem that imposes enormous humanitarian and financial costs on both the patients, the healthcare system, and society at large.

Healthy Lifestyle Score, comprising five high-potential alterable lifestyle factors (non-smoking, moderate alcohol consumption, a healthy diet, physical activity, and a healthy weight), was correlated with a lower risk of CRC, and the risk was to be further decreased as persistence in a healthy lifestyle score increased (3). A recent large-scale demographic-based study uncovered that a colonoscopy significantly reduces the absolute risk for CRC and that adherence to treatment can further reduce the genetically predetermined risk for CRC (4). Physical activity might protect against about 15% of CRC by its dose-dependent effect, making it an important part of a healthy lifestyle score, and its effect was independent of that of the weight control (5, 6). However, the molecular mechanism involved in the protective effects of physical activity are largely obscure yet.

From cellular changes to systemic metastatic spread, CRC has been undergoing a long development process. We therefore have more chances of discovering the risk factors of CRC and precancerous lesions early, intervening and treating them in time to reverse the development of the tumors. It is widely accepted that chronic inflammation has long been recognized as a crucial trigger of somatic tumorigenesis and progression (7–9). CRC can be categorized as two types: colitis-associated cancer (CAC) and sporadic CRC. Chronic relapsing inflammatory bowel disease (IBD) is an essential risk factor for initiating CAC, which is characterized as 2% of CAC incidence after 10 years and 18% after 30 years with a history of IBD (10, 11). There is opportunity for intraventive measures to halt or even reverse the development of the disease in this high-risk population, in light of the long process from IBD to CAC (12). In a rat model of chronic colitis evoked by the use of dextran sodium sulfate (DSS), Qin et al. found that physical activity treatment (i.e., swimming for 1 or 1.5 h per day, 5 days per week, for 7 weeks) in a dose-determined way prohibits colonic shortening, disruption of the colonic barrier, and splenomegaly. Swimming also alleviated dextran sodium sulfate (DSS)-induced chronic colitis by modulating inflammation, oxidative stress, and apoptosis (13). Additionally, these results may encourage further investigation into potential anti-inflammatory and even anticancer mechanisms associated with swimming.

This study used swimming to simulate the common whole-body aerobic activity people do to investigate whether physical activity prevents AOM/DSS-induced CAC, to research the underlying molecular mechanism, develop strategies for CRC prevention and treatment, improve life quality, and guide practical actions.

MATERIALS AND METHODS

Experimental Animals

Male C57BL/6 mice (age, 4 weeks) from the Animal Science Laboratory, School of Medicine, Shanghai Jiaotong University (Shanghai, China), were purchased. We had pathogen-free mice that are bred and raised in the Animal Care Facility of Tong Ren Hospital, Shanghai Jiaotong University School of Medicine, under laboratory conditions (23°C, 50% humidity, 12/12-h light/dark). Shanghai Tong Ren Hospital, Shanghai Jiao Tong University School of Medicine Ethics Committee authorized an experimental protocol.

Reagents

Azoxymethane (AOM), dextran sodium sulfate (DSS), and antibody against cyclooxygenase-2 were purchased from Sigma-Aldrich (St. Louis, MO, USA), MP Biomedicals (Santa Ana, CA, USA), and Servicebio Technology (Wuhan, China) respectively.

Experimental Procedure

The schedule of the experiment is shown in **Figure 1**. Thirty male C57BL/6 mice were assigned randomly to four groups: the Ctrl group (n = 5), Ctrl_swim group (n = 5), Model group (AOM/DSS only, n = 10), and Model_swim group (swimming while AOM/DSS, n = 10). Projections of AOM/DSS-induced CAC were carried out by our previous study protocol (14). On the first day, a single dose of AOM (10 mg/kg) was injected i.p. to the mice and thereafter 3 cycles of DSS treatment were applied. On each cycle of DSS, animals in the Model and Model_Swimming groups were treated by water with 2% DSS (w/v) continuously for 7 days, then sterile water for 2 weeks. The Ctr and Ctrl_Swim groups, which served as vehicle controls, received ordinary drinking water during the study period.

Mice in the Model_Swim and Ctrl_Swim groups performed swimming as previously described (15), with slight modifications. The mice were left to swim in a tank of 225-cm2 surface area, 15-cm depth, and 30–32°C water temperature. The animals swam initially for 20 min a day, with the swimming time increasing by 10 min per day, until reaching 90 min per day, up to the middle of the second week. After acclimatization, each day (10:00 am) the mice were trained for 2 months before the start of the experiment until the end of the experiment. The untrained mice were placed inside the same glass container containing water (kept up to 35°C) in a 2-cm depth.

Collection and Preparation of Samples

At the end of the experiment, euthanasia was performed on all animals, and laparotomy was performed immediately. From the distal cecum to the rectum, the colon was resected to remove adherent fatty tissue and dissected longitudinally, and collected feces were stored in -80°C; the colon was washed in ice-cold

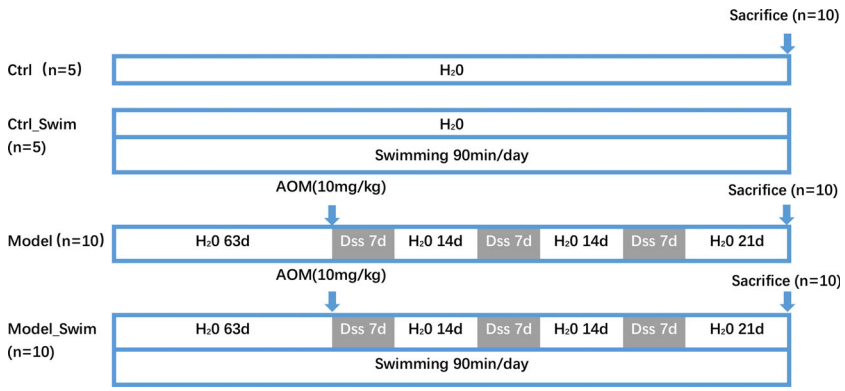

FIGURE 1 | Experimental schedule for AOM/DSS-induced CAC models in mice with swimming pretreatment. Ctrl, control; d, day.

saline for removing fecal debris, and photographs were taken. Histopathological, immunohistochemical, gene expression, and metabolic investigations were performed by utilizing the isolated colon. Feces were analyzed for 16S rRNA gene sequencing, metabonomic investigation, and SCFA detection.

Evaluation of Histopathology and Immunohistochemistry

Biopsied colon tissues were checked for tumorigenesis on a macroscopic scale. We fixed the tissues in formalin overnight and subsequently replaced the solution with 70% ethanol before paraffin embedding. H&E staining of paraffin sections was carried out following the criterion processes of histological evaluation (16).

RNA Isolation and Library Preparation

Total RNA was isolated from the colon tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Then libraries were then structured using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. OE Biotech Co., Ltd. (Shanghai, China), undertook the sequencing and analysis of the transcriptome.

Analysis of RNA Sequencing and Differentially Expressed Genes

Illumina HiSeq X Ten was utilized to sequence the library. Raw data (raw reads) of fastq format were firstly processed using Trimmomatic (17), and the low-quality reads were removed to obtain the clean reads. The clean reads were mapped to the Mus musculus genome (GRCm38.p6) with HISAT2 (18). Each gene's FPKM (19), as calculated by Cufflinks (20), and each gene's read counts were obtained from the HTSeq-count (21). Analysis of differential expression was conducted by the DESeq2 R package (22). Statistically significant differential expression was characterized as q value < 0.05, fold change > 2, or fold change < 0.5. On the basis of hypergeometric distribution, the DEG enrichment analysis was undertaken with R language for Gene Ontology (GO) as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) (23) pathways, respectively. RNA sequencing (RNA-seq) data were deposited in the Gene Expression Omnibus (GEO) of National Center for Biotechnology Information (NCBI) and will be accessible through GEO Series accession number GSE205173 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205173) after the indicated release date.

Analysis of the Metabolic Profile

Lumen Biotech (Shanghai, China) employed liquid chromatography–mass spectrometry (LC-MS) for evaluating the lipid metabolism in feces and colonic tissues. When extracting metabolites, the chemical properties of multitarget metabolites require consideration. Lipid metabolites were extracted from 20 mg of feces and 60 mg of tissue with storage at -20°C prior to LC-MS analysis.

A Dionex Ultimate 3000 RS UHPLC fitted with a Q Exactive Plus Quadrupole-Orbitrap Mass Spectrometer equipped with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA) was used for analyzing the metabolic profiles in ESI-positive and ESI-negative ion modes. Analysis in positive and negative modes was performed by means of an ACQUITY UPLC HSS T3 column (1.8 μ m, 2.1 × 100 mm). The binary gradient elution system comprised (A) water (with 0.1% formic acid, v/v) with (B) acetonitrile (with 0.1% formic acid, v/v); the following gradients were used to achieve the separation: 5% B, 0 min; 5% B, 2 min; 25% B, 4 min; 50% B, 8 min; 80% B, 10 min; 100% B, 14 min; 100% B, 15 min; 5% B, 15.1 min; and 5% B, 16 min. The flow rate was 0.35 ml/min while the column temperature was 45°C. In the course of the analysis, all samples were stored at 4°C. We set the injection volume to 1 μl, and the mass rises from m/z 100 to 1,000. The resolution of the full mass spectrometry scan at 70,000 and the HCD MS/MS scan at 17,500 was set. The collision energy was set in the ranges of 10, 20, and 40 eV. In accordance with the mass spectrometer, the spray voltage was 3,000 V (−) and 3,800 V (+); the flow rate of sheath gas was 35 arbitrary units; the flow rate of auxiliary gas was 8 arbitrary units; the temperature of the capillary was 320°C; the temperature of the Aux gas heater was 350°C; and the S-lens RF level was 50. Regular injections of

quality control (QC) (every 10 samples) were applied throughout the analytical process to demonstrate the reproducibility.

From Lumingbio, the target LC-MS database was used to identify lipid metabolites. Progenesis QI (Waters Corporation, Milford, USA) data processing software was applied to identify metabolites in this study, which was public database based, both http://www.hmdb.ca/ and http://www.lipidmaps.org/ in addition to self-built databases. To visualize metabolic alterations, we utilized principle component analysis (PCA) as well as (orthogonal) partial least square discriminant analysis (O) (PLS-DA). On the combination of a statistically significant variable influence on the projection (VIP) threshold and p values, when VIP > 1.0 and $p < 0.05$, the differential metabolites were selected.

Short Time sequence Expression Miner (STEM) was employed for identifying significantly different lipid metabolites ($p < 0.05$). Different-metabolite pathway enrichment was analyzed with the KEGG database. With these data, a gene–pathway–metabolite network was constructed by Cytoscape.

Short-Chain Fatty Acid Analysis

Short-chain fatty acids (SCFAs) of feces (20 mg) were extracted. Afterward, samples were placed at -20°C for at least 30 min, then a 0.22-μm organic-phase pinhole filter was applied through for subsequent ultra-performance LC (UPLC)-tandem MS (MS/MS) analysis by Luming Biotech Ltd. (Shanghai, China).

Sequencing Analysis of the 16S rRNA Gene

Fecal samples from experimental mice were extracted for DNA with a QIAamp Fast DNA Stool Mini Kit (Cat# 51604, QIAGEN, Venlo, Netherlands). Amplification of bacterial DNA was carried out by primers targeting the V3–V4 regions (5′-TACGGR AGGCAGCAG-3′, 5′-GGGTATCTAATCCT-3′). OE Biotech in Shanghai conducted DNA sequencing with a MiSeq PE300 platform from Illumina (CA, USA).

Trimmomatic software (17) is an application of preprocessing paired-end reads for detecting and cutting off obscure base(s) (N). In addition, it trimmed the quality score below 20 for low-quality sequences using the trimming method for sliding windows. Following trimming, FLASH software assembled the paired-end reads (24). The assembly parameters are as shown below: minimum overlap of 10 bp, maximum overlap of 200 bp, with a 20% maximum mismatch rate. We performed further denoising of the sequences in the following details: dropping reads with obscure, homologous sequences or less than 200 bp. Utilizing QIIME software (version 1.8.0), only 75% of the reads for bases above Q20 were retained (25). Followed by using VSEARCH, reads with chimeras were detected and removed (26). VSEARCH software was used to generate operational taxonomic units (OTUs) by clustering with a cutoff of 97% similarity. An RDP classifier was used to annotate the OTU against version 123 of the Silva database (70% confidence threshold) using QIIME software (27). In fecal samples, microbial diversity analysis was performed by the Chao1 index (28) and Shannon index (29). A binary Jaccard-based principal coordinate analysis (PCoA) was carried out by using the UniFrac distance matrix generated by QIIME software. Microbial multivariate statistical analysis used Kruskal–Wallis difference statistics. The bacterial species that differ among the four groups were identified by linear discriminant analysis and effect size measurements (LEfSe). OE Biotech Co., Ltd. (Shanghai, China), performed the sequencing and analysis of 16S rRNA. The 16S sequencing data were deposited in the NCBI BioProject under accession number PRJNA845074. The Sequence Read Archive (SRA) records will be accessible with the following link after the indicated release date: http://www.ncbi.nlm.nih.gov/bioproject/845074.

Statistics

By utilizing GraphPad Prism 9.0 software, Student's t tests were employed in analyzing gene expression, tumors number, tumors size on average, colon length, and bacterial diversity. Significant results were considered if 2-tailed p-values <0.05.

RESULTS

Swimming Pretreatment Attenuates AOM/DSS-Induced Tumor Development

The chemopreventive effect of swimming on CAC evaluated. The shortening of colonic length was significantly alleviated after swimming pretreatment, which indirectly suggested that swimming had chemopreventive effect on colitis (**Figure 2A**). We then focused on observing the neoplastic lesions on AOM/DSS induced CAC mice, and the macroscopic images of the colon in different groups of mice were shown in **Figure 2B**. Most tumors were located in the colorectum ranging from 1 mm to 5 mm, and a few were in the proximal colon. Swimming pretreatment significantly inhibited colitis-induced tumor's multiplicity and size, by a 60% tumorigenicity rate and an average of 2.7 gross tumors (mean diameter, 2.25 mm) each mouse of Swimming-pretreated mice vs a 100% tumorigenicity rate and 7 tumors (mean diameter, 2.45 mm) each mouse of the Model group (**Figures 2C–F**). In histological assessment, mice of the Model group displayed multiple adenomas and invasive adenocarcinomas, while mice of the Model_Swim group were characterized by disease mainly in the form of crypt dysplasia and adenomas (**Figure 2G**). In addition, swimming affected the expression of cyclooxygenase-2 (COX-2) in the colon. The expression of COX-2, one of inflammation-related enzyme and exercise-induced expression alteration biomarkers (9, 30), was upregulated in mucosal surfacing by AOM/DSS in Modle group (**Figure 2H**, Model), and was down in Swimming pretreatment group (**Figure 2H**, Model_Swim**).**

AOM/DSS and Swimming Pretreatment-Induced Changes in Transcription

We compared gene transcript expression between groups and selected the concatenation of differential genes, i.e. 1403 genes differentially expressed genes for subsequent analysis. Short Time-series Expression Miner (STEM) was applied for analysis

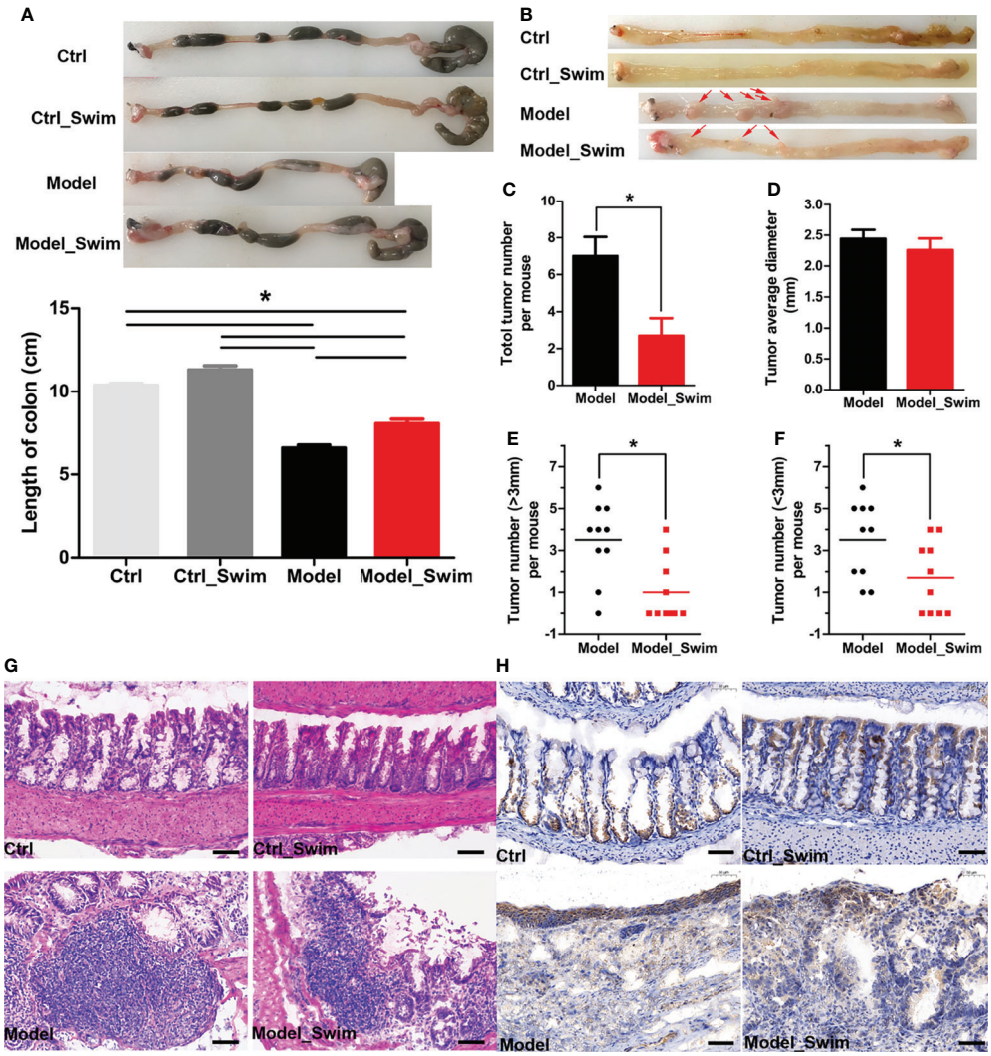

FIGURE 2 | Pretreatment with swimming alleviates colitis and tumorigenesis in AOM/DSS-induced CAC mouse models. **(A)** Representative images for both colon morphology and colonic length statistics. **(B)** Representative macrographic impressions. Remarkable red arrows represent tumors larger than 1 mm diameter. **(C–F)** Tumor morphology, tumor diameter and tumor distribution statistics. **(G)** The representative colonic H&E section. **(H)** Stained immunohistochemically by Cyclooxygenase-2 (COX-2). Results were presented by mean ± SEM. *P < 0.05, compared with Model group. Original magnification, ×200.

of 1403 differential genes (**Figure 3A**), and we considered that the differential expression of the genes, which were elevated or decreased in the Model group compared to the Ctrl and Ctrl_Swim groups and could be correspondingly decreased and increased in the Model_Swim group were influenced by swimming. In all, 1054 out of 1403 of the differentially expressed genes were significantly clustered in seven models, including profile 49 (**Figure 3B**), profile 29 (**Figure 3C**), profile 30 (**Figure 3D**), profile 28 (**Figure 3E**), profile 41 (**Figure 3F**), profile 22 (**Figure 3G**) and profile 42 (**Figure 3H**), only 279 genes of profile 30 (**Figure 3D**), profile 28 (**Figure 3E**) and profile 22 (**Figure 3G**) met our screening criteria and were screened (**Figure 3I**). KEGG enrichment analysis of the 279 genes showed many functions involving digestion and absorption, metabolism of nutrient elements, and about adhesion, migration, and cytokine interactions (**Figure 3J**), suggesting that swimming motivates body nutrient utilization and energy metabolism as well as acts as an inhibitor of colon cancer by affecting the gene expression of several aspects of inflammatory immune-related functions in AOM/DSS-induced CAC mice. We therefore selected these functionally enriched inflammatory immune-related genes which are directly related to tumorgenesis for analysis, a total of 48 differential genes were involved (**Figure 3K**). Among which we observed that only three genes expression, Fzd9, Cldn15 and Tfrc in the Model group were the lowest, while the other genes expression in each group were the opposite. Meanwhile, we observed that the AOM/DSS induced CAC mice upregulated Ptger2, Pla2g4f and Ptges expression, to increase synthesis of Prostaglandin E2 (PGE2) receptor 2 subtype (EP2), a G protein-coupled receptor, and that

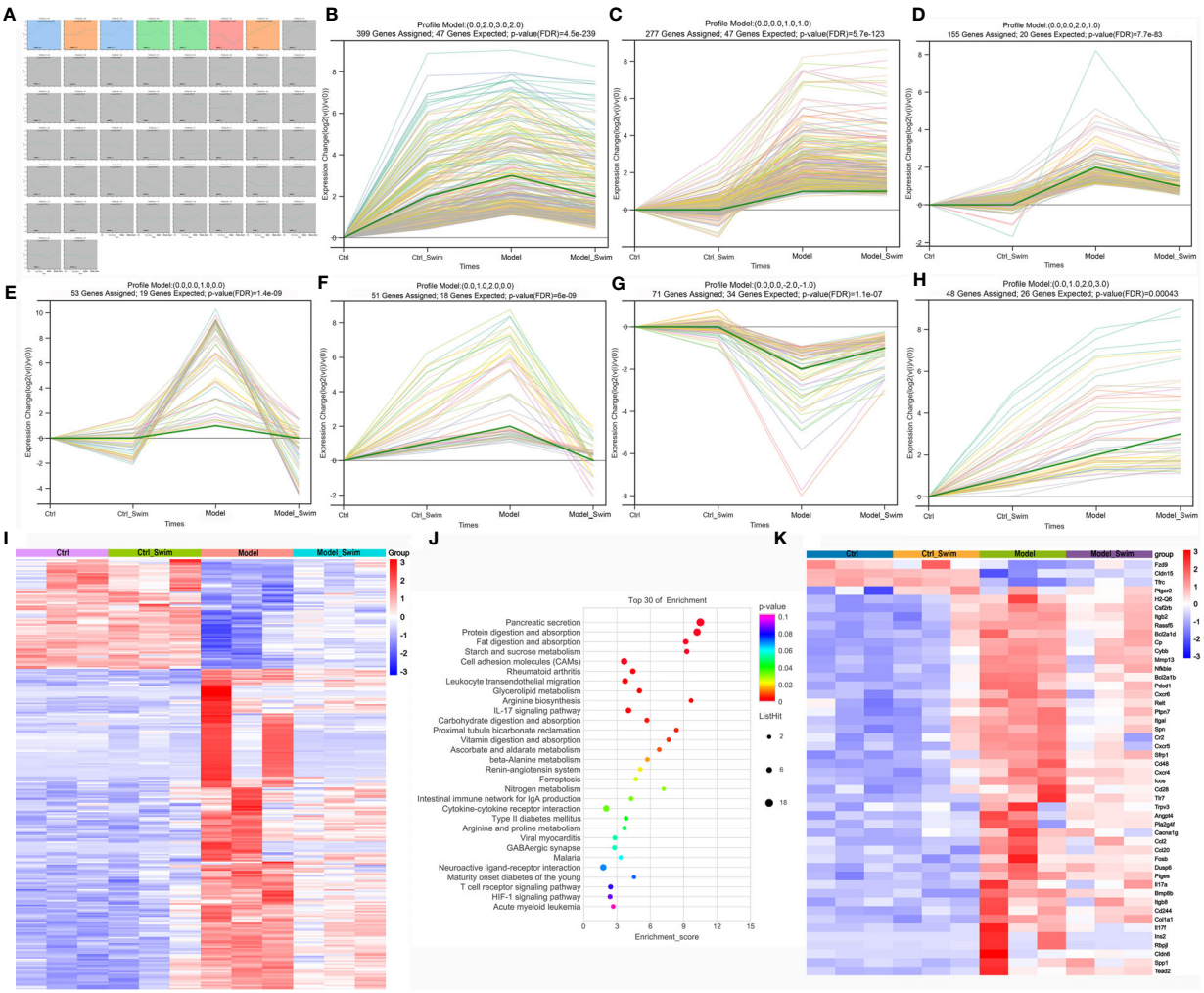

FIGURE 3 | Variations in transcription induced by AOM/DSS and swimming pretreatment. **(A–H)** Patterns of gene expression across the four groups inferred by Short time–series expression minor clustering analysis (STEM) analysis. **(I)** Heatmap of the differential genes caused by swimming in AOM/DSS-induced CAC Mice. **(J)** KEGG enrichment analysis of the differential genes caused by swimming in AOM/DSS-induced CAC Mice. **(K)** Heatmap of the screened 48 differential genes caused by swimming in AOM/DSS-induced CAC Mice.

PGE2, a downstream arachidonic acid metabolites of COX-2 and prostaglandin E synthase (PTGES), binds to EP2 to mediate multiple intracellular signaling pathways to promote colon cancer development (9, 31, 32). The rest of the genes were mainly related to Cell adhesion molecules (CAMs), Leukocyte transendothelial migration, IL-17 signaling pathway, Ferroptosis, Intestinal immune network for IgA production, Cytokine-cytokine receptor interaction, etc. Swimming may inhibit CAC processes by inhibiting PGE2/EP2 receptor-ligand binding and intracellular signaling pathways mentioned above.

Swimming Pretreatment Alters Composition of the Intestinal Microbiota Community

Overall, 16S rRNA sequencing produced 57,318 and 71,203 valid tags of average lengths 271.43 to 440.29 bp. In our result, we got 4666 operational taxonomic units (OTUs) that were part of 16 phyla, 24 classes, 73 orders, 119 families, and 246 genera. The species accumulation curve and the rarefaction curve(**Supplementary Figure. S1**) from a total of all samples reflected that the sequencing depth was adequate to characterize the bacterial richness and diversity of all samples. Following false discovery rate (FDR) correction (FDR < 0.05), 1606 OTUs, 0 phyla, 1classes, 12 orders, 23 families, and 55 genera were significantly different among the 4 groups.

At the phylum level, Bacteroidota increased after swimming (Ctrl_Swim:76.98%, Model_Swim:81.55% vs Ctrl: 71.77%, Model: 74.39%) and Firmicutes decreased (Ctrl_Swim:17.78%, Model_Swim:11.39% vs Ctrl:22.59%, Model:16.63%) (**Figure 4A**). At the genus level, the Model_Swim group had the lowest percentage of Muribaculaceae, Lachnospiraceae_NK4A136_group, Colidextribacter and the highest percentage of Alloprevotella,

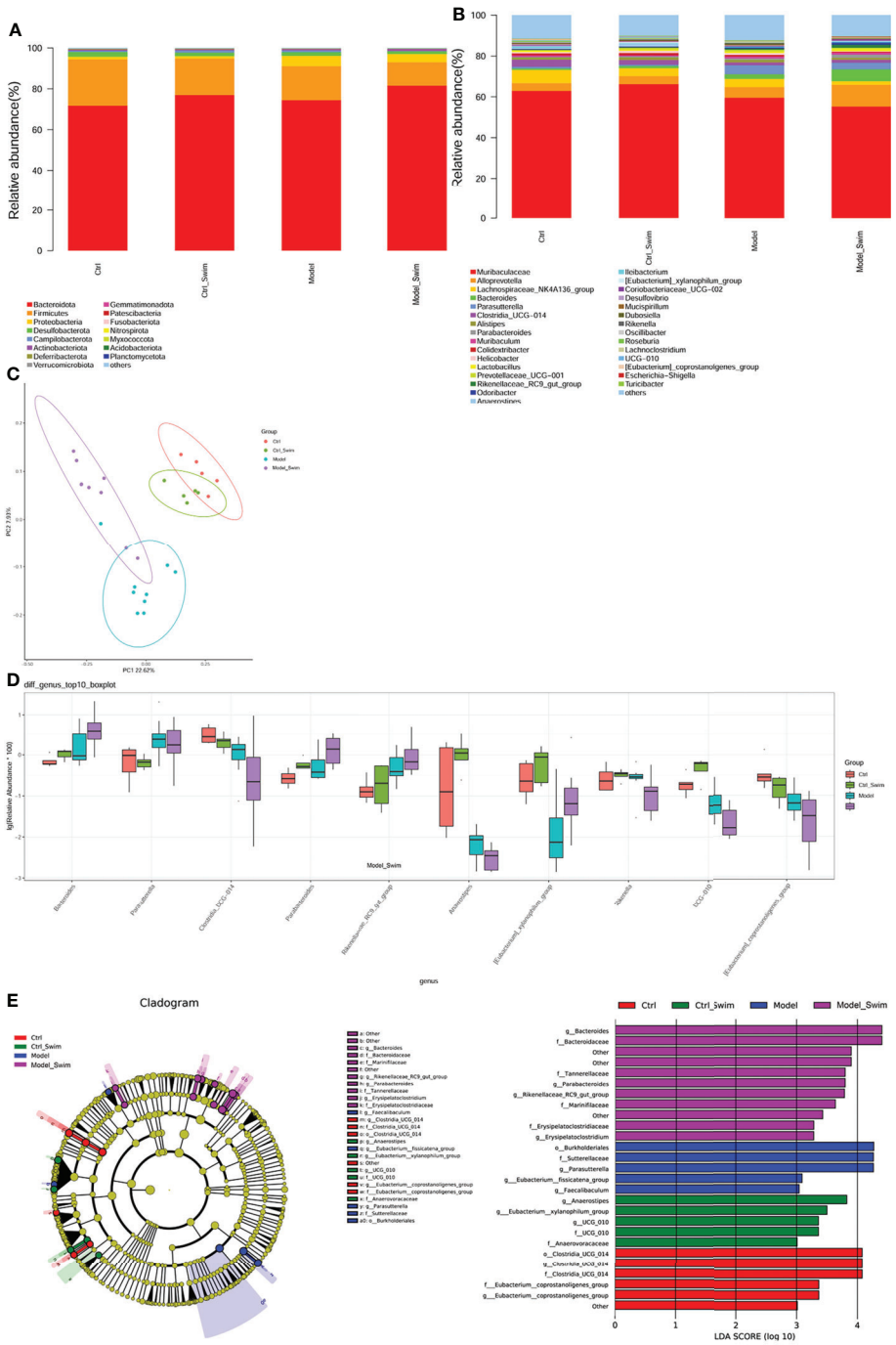

FIGURE 4 | Alteration of fecal microbial community composition caused by swimming pretreatment in AOM/DSS-induced CAC Mice. **(A)**, Relative abundance in each group for the major bacterial phylum. **(B)**, Relative abundance of the main bacterial genus in each group. **(C)**, Fecal microbial community diversity analyses. Binary jaccard-based PCoA plot base of the relative abundance of OTUs displaying bacterial structural clustering. **(D)**, The top 10 genera with significant differences for relative abundances among the Model, Model_Swim, Control and Control_Swim groups; Kruskal–Wallis, all $p < 0.05$. **(E)**, Analysis of the different species composition among the Model, Model_Swim, Control and Control_Swim groups by LEfSe.

Bacteroides, Parabacteroides among the four groups, and it was also observed that Lactobacillus, a related genus in our previous study (14), had the highest percentage among the four groups (**Figure 4B**).

Although Chao1 estimates and Shannon index did not differ significantly among the groups(**Supplementary Figure S2**), however, binary jaccard-based PCoA analysis combined with the results of adonis analysis (P=0.001), demonstrated significant differences in beta diversity among the groups (**Figure 4C**). In further multivariate statistical analysis, Kruskal Wallis difference statistics identified 55 differential genera, of which Bacteroides, Parabacteroides, Rikenellaceae_RC9_gut_group were significantly more abundant in the Model_Swim group than the other three groups, while Clostridia_UCG-014, Anaeroplasma, Rikenella, UCG-010, [Eubacterium]_coprostanoligenes_group were significantly less abundant in the Model_Swim group than the other three groups (**Figure 4D** and **Supplementary Table S1**).

A cladogram generated by Linear discriminant analysis coupled with effect size measurements (LEfSe) was used to identify the specific bacteria linked to swimmimg (**Figure 4E**). The result shown that Erysipelatoclostridium, Parabacteroides, Bacteroides, Rikenellaceae_RC9_gut_group were significantly dominant (LDA scores (log10) >3.0) in the Model_Swim group, while Faecalibaculum, Parasutterella, Eubacterium:fissicatena_group were the most abundant in the Model group (LDA scores (log10) >3.0). Together, these results suggest that changes in the intestinal microbiota composition were linked to swimmimg.

Lipid Metabolism Changes in Colonic Environment Induced by AOM/DSS and Swimming Pretreatment

The transcriptome sequencing and analysis showed that transcriptome functions were enriched to many digestion and absorption, nutrient metabolism, which hinted the effects of swimming on these aspects. As a natural lipids, the biosynthesis of PGE2 is inhibited by swimming, which attract us to investigate how swimming affects lipid metabolism in the colonic environment, which include colonic tissue and direct contacted feces, to inhibit PGE2 synthesis and its functional signaling pathway.

Firstly, we analyzed lipid metabolites in colonic tissue. We used PCA and (orthogonal) partial least squares discriminant analysis (OPLS-DA), and separate between Ctrl & Ctrl-Swim, Ctrl & Model, Ctrl-Swim & Swim, and Swim & Model, respectively. We separate between Ctrl and Ctrl_Swim (R2Y (cum) = 0.835 and Q2 (cum) = 0.598), Ctrl and Model (R2Y (cum) = 0.788 and Q2 (cum) = 0.642), Ctrl_Swim and Model_Swim (R2Y (cum) = 0.622 and Q2 (cum) = 0.82), and Model_Swim and Model (R2Y(cum) = 0.788 and Q2 (cum) = 0.642), respectively. Which demonstrated the presence of metabolic differences in these two groups. Excellent stability and reproducibility of the analytical platform were displayed by the permutation test (Ctrl & Ctrl_Swim, R2 = 0.965, Q2 = -0.137; Ctrl & Model, R2 = 0.959, Q2 = -0.16; Ctrl-Swim & Model_Swim, R2 = 0.974, Q2 = -0.151; Model_Swim & Model, R2 = 0.965, Q2 = 0.002), which can be employed in the follow-up metabolomics research (**Supplementary Figure S3**). Total of 685 metabolites were found. According to the STEM analysis (**Supplementary Figure S4**), 233 differential metabolites were found among the four groups, which decreased in the Model group but rebounded in the swimming groups(**Supplementary Figure S5**). The KEGG pathway enrichment analyses of 233 differential metabolites indicated that a total of 24 differential lipid metabolites with functional enrichment (**Figure 5A**) were related to Glycerophospholipid metabolism, Leishmaniasis, Choline metabolism in cancer, Retrograde endocannabinoid signaling, Autophagy – other, Systemic lupus erythematosus Neurotrophin signaling pathway, Kaposi sarcoma-associated herpesvirus infection, AGE-RAGE signaling pathway in diabetic complications, Autophagy – animal, Adipocytokine signaling pathway, Necroptosis, Amoebiasis, Sphingolipid signaling pathway, Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, Insulin resistance, Sphingolipid metabolism, Linoleic acid metabolism, alpha-Linolenic acid metabolism, Glycine, serine and threonine metabolism, and Arachidonic acid metabolism (**Figure 5B**).

Secondly, the fecal metabolome is considered as an intestinal microbiome functional readout. Fecal metabolic analysis as a new tool to explore the connection of microbiota composition with the host phenotype (33). As described in colonic lipid metabolism analysis, PCA, OPLS-DA, and permutation test were performed for quality control of fecal lipid metabolites analysis (**Supplementary Figure S6**). In total, 75 differential metabolites were observed in fece. On the base of a differentiated screening strategy for STEM analysis (**supplementary Figure S7**), 7 discriminating metabolites were found among the four groups, which were also significantly highly expressed in the swimming groups (**Figure 5C**). The KEGG pathway enrichment analyses revealed that these differential metabolites were involved in Glycerophospholipid metabolism and Choline metabolism in cancer (**Figure 5D**).

In addition, we performed SCFAs UPLC-MS/MS analysis on mouse feces and showed that SCFAs of the Model_Swim group was higher than that of the Model group, and the expression of Propionic Acid, Butyric Acid and Acetic Acid was higher in the Ctrl group and the Model_Swim groups, but lower in the Model group, especially Butyric Acid (P<0.05) (**Figure 5E**).

Integration Analysis Among Intestinal Microbiota Community, Colonic Lipid Metabolites, and Transcriptomics to Construct Anti-Inflammatory and Anti-Tumorigenic Transcriptional Networks

Since the development of CRC is influenced by the interaction of the tumor microenvironment (TME) and the intestinal microbiota (34). In the present experiments, the changes induced by swimming, both systemic within the organism and changes in the intestinal flora, and their effects on the TME are directly reflected in the alteration of colonic lipid metabolites. A Spearman correlation analysis was performed to analyze the covariation between 24 colonic differential lipid metabolites with functional enrichment and 48 differentially expressed genes we selected (**Figure 6A**). The results showed that colonic lipid metabolites: PS(18:0/22:6), PS(18:0,18:1), PS(18:0,18:1), PC(18:0/20:4), PC

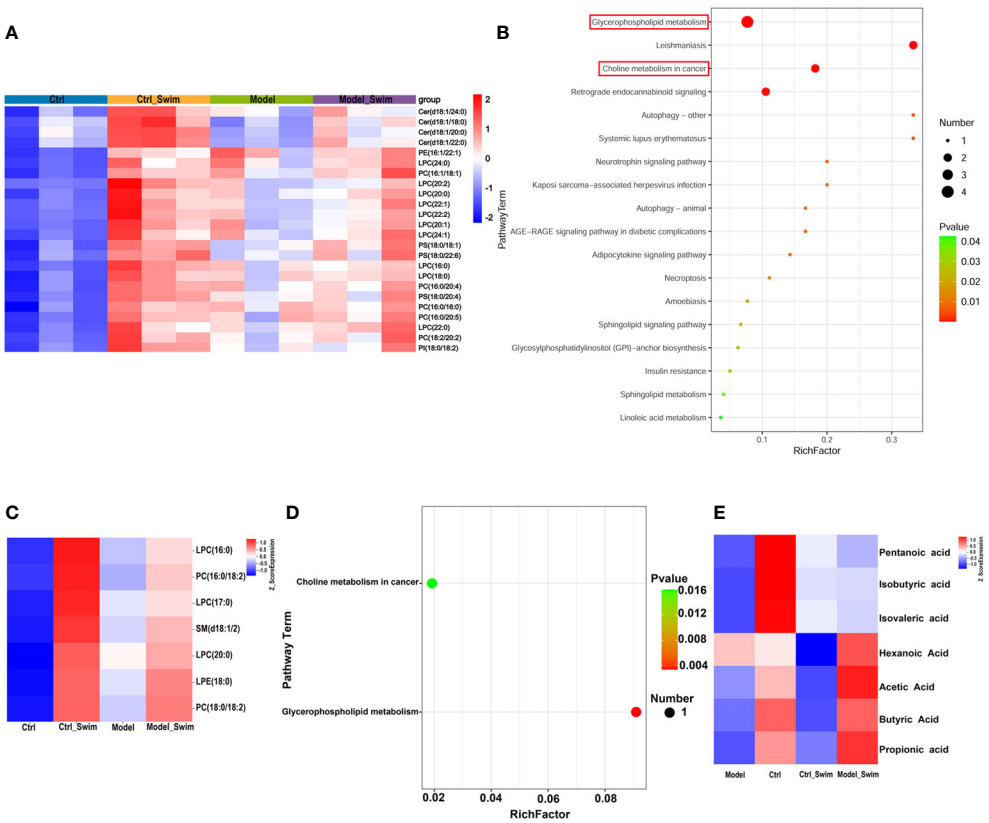

FIGURE 5 | Lipid metabolism changes in colonic environment induced by AOM/DSS and swimming pretreatment. **(A)** Heatmap of 24 differential colonic lipid metabolites with functional enrichment. **(B)** KEGG enrichment analysis of the differential colonic lipid metabolites caused by swimming in AOM/DSS-induced CAC Mice. **(C)** Heatmap of 7 differential fecal lipid metabolites caused by swimming in AOM/DSS-induced CAC Mice. **(D)** KEGG enrichment analysis of the differential fecal lipid metabolites caused by swimming in AOM/DSS-induced CAC Mice. **(E)** Heatmap of fecal SCFAs alteration by swimming in AOM/DSS-induced CAC Mice.

(18:2/20:2), LPC(22:1), LPC(20:2), Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/24:0) were closely related to Ptger2 expression, where PE(16:1/22:1) was closely associated with many gene expressions (e.g. Pla2g4f, Ptges). Cytoscape showed that the metabolites and gene functions were co-enriched for "arachidonic acid metabolism", suggesting that colonic lipid metabolites potentially inhibit inflammatory-cancer development by suppressing Ptger2, Pla2g4f and Ptges expression, PGE2/EP2 ligand/receptor binding and activation of intracellular related signaling (**Figure 6B**).

In addition, differential metabolites in fecal and colonic tissue were functionally co-enriched in Glycerophosphlipid metabolism and Choline metabolism in cancer, which means swimming induced dominant genera mainly regulate metabolites of these two functions to exert anti-inflammatory and anti-tumorigenic effects. The correlation analysis between the differential intestinal microbiota and colonic differential lipid metabolites showed that colonic differential metabolites were positively associated with Erysipelatoclostridium, Parabacteroides, Bacteroides, Rikenellaceae_RC9_gut_group (dominant genera in the Model_Swim group) (**Figures 6C**).

In short, colonic lipid metabolites may play an anti-inflammatory and anti-cancer role by inhibiting PGE2/PTGER2 ligand/receptor binding and activating intracellular related signaling, while the metabolites with a major function in Glycerophosphlipid metabolism and Choline metabolism in cancer are influenced by swimming-induced flora alterations.

DISCUSSION

Chronic inflammation is a recognized risk factor for CRC (9). The previous study showed that swimming attenuates DSS-induced chronic colonic inflammation by regulating inflammation, oxidative stress and apoptosis (13). Our research showed that swimming reduced neoplasia formation rate and tumor number in mice model of AOM/DSS-induced CAC. After being shown to have anti-inflammatory effects, swimming has again been shown to have anti-inflammatory and tumorigenesis prevention effects by animal models. While exhibited anti-inflammatory effects of swimming in the transcriptional dimension by modulating numerous inflammatory signaling pathways. Our experiments also showed that the expression of COX-2, PTGES, Group IV phospholipase A2 family (PLA2G4F) and EP2 were elevated in the model group. PLA2G4F, COX-2 and PTGES are important enzymes for the synthesis of its

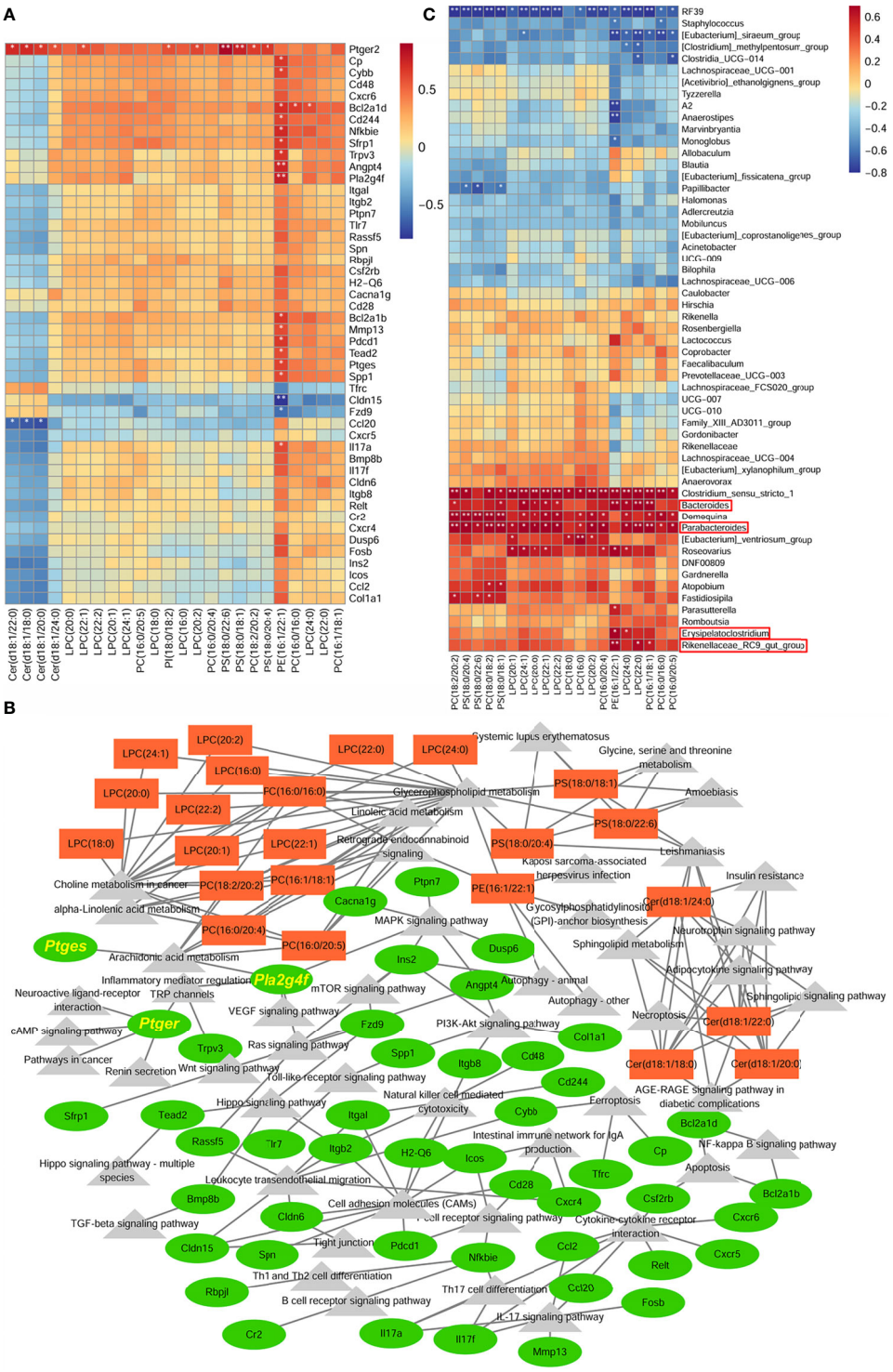

FIGURE 6 | Integration analysis between colonic lipid metabolites and transcriptomics to construct anti-inflammatory and anti-tumorigenic transcriptional network. **(A)** Heatmap of correlation between 24 colonic differential lipid metabolites with functional enrichment and 48 differentially expressed genes. **(B)** Correlation network analysis between differential genes and metabolites. **(C)** Heatmap of correlation between colonic differential lipid metabolites, functionally enriched in Glycerophosphlipid metabolism and Choline metabolism in cancer, and the differential fecal microbiota. *P < 0.05, **P < 0.01.

downstream bioactive lipid products, PGE2, which binds to EP2, and act as multiple signaling pathways of modulating various pathophysiological functions in the tumor microenvironment (TME), including chronic inflammation, invasion and metastasis, cell apoptosis angiogenesis, tumor immune evasion and tumor occurrence (31, 32). In contrast, the increasing of these PGE2/EP2 pathway markers were reduced in the swimming groups. In the latest decades, COX-2 and its prostaglandin products are attracting growing interest due to their important role in colon cancer as well as in other tumors progression. However, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) to inhibit COX-2 or specific COX-2 inhibitors has caused various side effects, including peptic ulcer and its complications and cardiovascular events (35, 36). These have limited the use of these drugs. The above animal experimental phenomena, consistent with preclinical studies (30), suggest that swimming may play an anti-inflammatory and tumorigenesis prevention role, and it is more acceptable to the general public and has no significant side effects risk.

Metabolic reprogramming is an important hallmark of cancer pathogenesis (37, 38). In our model, we observed that swimming reduces tumorigenicity in mice by modulating bioactive lipid products, Prostaglandin E2 (PGE2). It is suggested that lipid metabolites, as energy reserves and signaling transmitters, have a vital role in this process. The Swimming-induced changes of colonic lipid metabolites in CAC was mainly observed. We found that swimming induced the expression of 24 functionally enriched colonic lipid metabolites and regulated the expression of numerous genes closely associated with tumor development, and significant correlation between the metabolites and the genes were displayed by Correlation analysis and Cytoscape. These findings provide evidence for further investigation of the relevance of swimming-induced colonic lipid metabolites to tumors and their pathophysiological mechanisms.

With the researches on physical activity and diseases, we can easily relate to obesity, metabolic diseases prevention and control, for example, metabolic syndrome, type 2 diabetes, cardiovascular disease, etc. Researches had found that physical activity plays a role in preventing and reversing diseases by controlling inflammation, body weight and reducing insulin resistance (39, 40), however, the role in cancer prevention is not related to the body mass index (BMI) (41). Intensive studies of numerous physical activity intervention trials suggested a substantial association with physical activity and tumors development by exercise induced systemic alterations, such as endocrine system, immune response, metabolic changes (42–45). TME is a mixture of stromal and immune cells that determine cancer progression and influence response to tumor therapy, tumor-derived PGE2 undermines immunity against tumors via targeting natural killer (NK) cells, conventional type 1 dendritic cells (cDC1) (35). Besides, physical activity leads a rapid surge of catecholamines in the blood and an increasing influx of various immune cells into the tumor, e.g. NK cell, cDC1 (46). The altered lipid metabolite expression induced by swimming in our study was closely associated with inhibition of PGE2/EP2 signaling pathway, suggesting that these lipid metabolites exert anti-tumor effects by inhibiting immune escape caused by PGE2. Our results partially refine the mechanism of physical activity against cancer from a metabolic perspectives. Among these lipid metabolites, it is possible to screen for lipid metabolites with significant anti-inflammatory and tumoriogenesis prevention effects and potential to become pharmaceutical target for cancer treatment. As well as exploring the role of lipid metabolites as markers for clinical application in the diagnosis, staging and prognostic prediction of CRC.

There is a mutual and highly dynamic communication between tumor cells and TME that strongly influences all steps of tumorigenesis, moreover TME and tumors also have a systemic interaction with the whole organism, including the microbiota (47). The intestinal microbiota is a component of the formation and development of CRC along with its response on therapy. Several studies have attempted to reduce CRC risk by Dietary interventions, Weight reduction, Administration of probiotics (48). As one of the microbiota-derived metabolites, physical activity also increases fecal short-chain fatty acid (SCFAs, e.g. butyrate and propionate) which are produced from non-digestible carbohydrates in the gut, for instance, fiber and resistant starch, by the fermentation of intestinal microbiota (49–51). As an anti-inflammatory molecule, butyrate and propionate deregulate both pro-inflammatory cytokines, induce apoptosis in CRC cell lines, and modulate colonic regulatory T (Treg) cells to exert effective anti-inflammatory efficacy in animal models (52–57).. In our study, swimming changes the ratio of Firmicutes/Bacteroidetes, β-diversity, and LEfSe analysis showed significantly high abundance of 4 genera in Model_Swim group. Among the dominant genera, several species of Parabacteroides and Bacteroides supply of nutrients and vitamins both to the host and to other intestinal microbial inhabitants (58, 59). Which could partially explain why the content of several SCFAs in Model_Swim group higher than in Model group. These findings were also coherent to our previous preclinical research (14) suggesting that swimming exerts anti-inflammatory and tumorigenesis prevention role by altering intestinal microbiota and increasing SCFAs levels as oral supplementation. In addition, we identified seven additional lipid metabolites from feces that increased after swimming, which were functionally co-enriched with tissue-derived lipid metabolites in two functional signaling pathways, Glycerophosphlipid metabolism and Choline metabolism in cancer, suggesting that swimming-induced dominant genera were involved in anti-inflammatory and tumorigenesis prevention effects via these two functional pathways. Further correlation analysis of swimming-induced dominant genera with colonic lipid metabolites enriched in these two functions showed significant relevance further supporting this scenario.

Within contemporary exercise science, mice are widely employed to discover exercise intervention mechanisms for disease prevention. There are still limitations to the mouse model, however. First, mice do not mimic certain forms of movement in humans, but the swimming is the easiest kind of systemic aerobic exercise to simulate. Second, the CAC-induced

mouse model does not accurately capture the pathophysiological processes of CRC in human. Third, the functional database of lipid metabolites is not yet complete, and the functions of different lipid metabolites are not fully enriched, and only a small part of the anti-inflammatory and carcinogenesis prevention functional mechanisms of lipid metabolites can be explained by the existing database. Further research as well as optimization of the metabolite functional database would be necessary.

As a conclusion, we demonstrated that the swimming pretreatment relieved AOM/DSS-induced CAC, by exerting anti-inflammatory and tumorigenesis prevention functions. Positive impact of swimming were relevant to its capacity to regulate PGE2/EP2 signaling pathway by modifying colonic lipid metabolites. The swimming can increase SCFAs in the intestine by altering the intestinal microbiota, such as increasing abundance of probiotics, and the efficacy was exerted by swim-induced genera affecting the function of colonic lipid metabolites enriched in Glycerophosphlipid metabolism and Choline metabolism in cancer. These findings demonstrated swimming was a potent preventive measure against CAC, and implied that the differential lipid metabolites screened in the experiment were candidates of medicine with anti-inflammatory and tumorigenesis prevention properties. However, the exact mechanism of the beneficial effects attributed to the lipid metabolites still deserve to be elucidated. Therefore, the research should need further screening of highly efficacious anti-inflammatory and tumorigenesis prevention lipid metabolites and intensively study their molecular mechanisms, as well as screening of tumor markers for colon tumors.

DATA AVAILABILITY STATEMENT

RNA sequencing (RNA-seq) data were deposited in the Gene Expression Omnibus (GEO) of National Center for Biotechnology Information (NCBI) and will be accessible through GEO Series accession number GSE205173 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205173) after the indicated release date. The 16S sequencing data were deposited in the NCBI BioProject under accession number PRJNA845074. The Sequence Read Archive (SRA) records will be accessible with the following link after the indicated release date: http://www.ncbi.nlm.nih.gov/bioproject/845074.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethical Committee of Tongren Hospital, Shanghai Jiaotong University School of Medicine. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

WW designed the experiment. YC, HQZ, LZ, HJZ, JL, RK, and FZ performed the experiment. YX and XW processed the data. WW wrote the paper. DY and HP revised the paper. All authors approved the final version to be published and agree to be accountable for all aspects of the work.

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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.2022.929092/full#supplementary-material

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METHODS
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Implementing Pharmacogenetic Testing in Gastrointestinal Cancers (IMPACT-GI): Study Protocol for a Pragmatic Implementation Trial for Establishing *DPYD* and *UGT1A1* Screening to Guide Chemotherapy Dosing

*Lisa A. Varughese[1], Madhuri Bhupathiraju[1], Glenda Hoffecker[1], Shannon Terek[2], Margaret Harr[2], Hakon Hakonarson[2], Christine Cambareri[3], Jessica Marini[3], Jeffrey Landgraf[4], Jinbo Chen[5], Genevieve Kanter[6], Kelsey S. Lau-Min[7], Ryan C. Massa[7], Nevena Damjanov[7], Nandi J. Reddy[8], Randall A. Oyer[8], Ursina R. Teitelbaum[7] and Sony Tuteja[1]**

[1] Division of Translational Medicine and Human Genetics, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [2] Center for Applied Genomics, The Children's Hospital of Philadelphia, Philadelphia, PA, United States, [3] Department of Pharmacy, Hospital of the University of Pennsylvania, Philadelphia, PA, United States, [4] Information Services Applications, Penn Medicine, University of Pennsylvania, Philadelphia, PA, United States, [5] Department of Biostatistics, Epidemiology and Informatics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [6] Division of Medical Ethics and Health Policy, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [7] Division of Hematology/Oncology, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States, [8] Ann B. Barshinger Cancer Institute, Lancaster General Health, Penn Medicine, Lancaster, PA, United States

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Edited by:
Igor Kiss,
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Reviewed by:
Radim Nemecek,
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Andrea Botticelli,
Sapienza University of Rome, Italy

***Correspondence:**
Sony Tuteja
sonyt@pennmedicine.upenn.edu

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Background: Fluoropyrimidines (fluorouracil [5-FU], capecitabine) and irinotecan are commonly prescribed chemotherapy agents for gastrointestinal (GI) malignancies. Pharmacogenetic (PGx) testing for germline *DPYD* and *UGT1A1* variants associated with reduced enzyme activity holds the potential to identify patients at high risk for severe chemotherapy-induced toxicity. Slow adoption of PGx testing in routine clinical care is due to implementation barriers, including long test turnaround times, lack of integration in the electronic health record (EHR), and ambiguity in test cost coverage. We sought to establish PGx testing in our health system following the Exploration, Preparation, Implementation, Sustainment (EPIS) framework as a guide. Our implementation study aims to address barriers to PGx testing.

Methods: The Implementing Pharmacogenetic Testing in Gastrointestinal Cancers (IMPACT-GI) study is a non-randomized, pragmatic, open-label implementation study at three sites within a major academic health system. Eligible patients with a GI malignancy indicated for treatment with 5-FU, capecitabine, or irinotecan will undergo PGx testing prior to chemotherapy initiation. Specimens will be sent to an academic clinical laboratory

followed by return of results in the EHR with appropriate clinical decision support for the care team. We hypothesize that the availability of a rapid turnaround PGx test with specific dosing recommendations will increase PGx test utilization to guide pharmacotherapy decisions and improve patient safety outcomes. Primary implementation endpoints are feasibility, fidelity, and penetrance. Exploratory analyses for clinical effectiveness of genotyping will include assessing grade ≥3 treatment-related toxicity using available clinical data, patient-reported outcomes, and quality of life measures.

Conclusion: We describe the formative work conducted to prepare our health system for *DPYD* and *UGT1A1* testing. Our prospective implementation study will evaluate the clinical implementation of this testing program and create the infrastructure necessary to ensure sustainability of PGx testing in our health system. The results of this study may help other institutions interested in implementing PGx testing in oncology care.

Clinical Trial Registration: https://clinicaltrials.gov/ct2/show/NCT04736472, identifier [NCT04736472].

Keywords: pharmacogenetics, cancer, DPYD, UGT1A1, chemotherapy, toxicity, pragmatic trial, implementation science

INTRODUCTION

Clinical pharmacogenetics (PGx) is a promising tool that harnesses an individual's germline genetic information to optimize prescribing decisions and improve medication-related outcomes. Advances in genomic technologies and the increasing number of clinical practice guidelines have led to health system-wide precision medicine initiatives to support PGx programs (1–4). PGx testing can be leveraged in the oncology setting to guide chemotherapy dosing to minimize the severity of treatment-induced toxicity, thereby reducing the potential for costly emergency department visits and/or hospitalizations (5–7). Fluoropyrimidines (fluorouracil [5-FU] and its oral prodrug capecitabine) and irinotecan are well-known systemic chemotherapy agents used in a wide variety of tumors. Treatment-related toxicities manifesting as neutropenia, diarrhea, mucositis, and hand-foot syndrome are prevalent in 35-50% of patients receiving combination regimens (8). While interindividual differences in the severity of adverse events is partially due to clinical factors such as age, sex, organ dysfunction, and performance status, common genetic variation can further explain differences in chemotherapy response as it relates to its safety profile.

The *DPYD* gene encodes dihydropyrimidine dehydrogenase (DPD), the primary enzyme responsible for degrading more than 80% of an administered fluoropyrimidine dose. While fluoropyrimidines are generally well-tolerated, decreased DPD activity is associated with a greater than four-fold risk of severe or fatal toxicity from standard dosing (9). Further data suggests that carriers with variant alleles encoding for decreased DPD function have a 25.6-times increased risk of treatment-related death following standard dose fluoropyrimidine in solid tumors (10). Partial or complete DPD deficiency stems from approximately 40 different genetic aberrations, including exon skipping, deletions, frameshifts, missense mutations, and polymorphisms (11). The relationship between four *DPYD* variants (c.1905+1G>A [*2A], c.1679T>G [*13], c.2846A>T, and c.1129-5923 C>G/c.1236G>A [HapB3]) and fluoropyrimidine-induced toxicity have primarily been studied in populations of European ancestry, with a combined carrier frequency of approximately 2-8% (10, 12). Additional reports suggest the c.557A>G variant, evident in 3-5% in individuals of African ancestry, is also associated with reduced DPD activity and fluoropyrimidine toxicity (13, 14).

Results from prospective trials testing for common *DPYD* variants prior to treatment justify recommendations to perform initial dose reductions in variant carriers (5, 12). The Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines provide fluoropyrimidine dosing recommendations based on *DPYD* gene activity score, where a variant score of 1 corresponds to normal function, 0.5 as reduced function, and 0 as no function. Phenotypes, or metabolizer status, are assigned based on the sum of the two lowest variant activity scores. Individuals found to be intermediate metabolizers (Activity Score 1 or 1.5) are recommended to receive a 50% reduction in the starting dose. Poor metabolizers are recommended to avoid fluoropyrimidines (Activity Score 0) or a strongly reduced dose (<25% of the normal starting dose) due to the potential for life-threatening or fatal toxicity (Activity Score 0.5) (15).

Concurrent *UGT1A1* testing with *DPYD* screening can be considered to guide irinotecan dosing given its higher frequency of polymorphisms, regardless of if the agent is administered alongside a fluoropyrimidine or used in future lines of therapy. The UDP-glucuronosyltransferase 1A1 (UGT1A1) enzyme is encoded by *UGT1A1* and is responsible for inactivating SN-38 (the active metabolite of irinotecan) following glucuronidation. *UGT1A1* genotype assay results are reported by using the star (*) allele nomenclature or by the number of thymine-adenine (TA) repeats in the gene promoter region; wild-type contains six TA repeats [(TA)6TAA or 6/6]. Homozygosity or compound heterozygosity in the *28 [(TA)7TAA or 7/7] and *6 (c.211G>A) alleles are associated with reduced UGT1A1 activity, resulting in

increased exposure to SN-38 and a higher risk of severe neutropenia and diarrhea (16). The prevalence of these alleles in various geographic populations have led to revisions in the product labeling of irinotecan for poor metabolizers. The U.S. Food and Drug Administration recommends an initial reduction by one dose level in *28 homozygotes (*UGT1A1*28/*28) (17). The 2012 European Society for Medical Oncology (ESMO) consensus guidelines state testing for UGT1A1 polymorphisms should be considered when irinotecan is used at high doses (300–350 mg/m2) (18). However, doses in this range are rarely administered in the United States. More recent ESMO guidelines from 2016 continue to recognize that *6 and *28 polymorphisms are predictive biomarkers of irinotecan-related toxicity. This guideline further acknowledges that testing is not used in everyday practice (likely due to barriers in implementation), therefore phenotyping should be performed in patients with a suspicion of UGT1A1 deficiency as reflected by low conjugated bilirubin and in patients planning to receive doses of >180 mg/m2 (19). Other international groups acknowledge the increased toxicity risk in individuals with *UGT1A1*6/*6, *28/*28, and *6/*28 genotypes and recommend testing prior to treatment (20, 21). **Table 1** describes the function and prevalence of clinically actionable *DPYD* and *UGT1A1* alleles in different populations.

Preemptive testing in patients receiving fluoropyrimidine- and/or irinotecan-based regimens has shown to be feasible, safe, and cost-effective in both the academic medical center and community settings (5, 12, 22–25). While these data support clinical implementation, barriers to routine testing in the clinic often include access to timely results, lack of clinician experience in interpreting actionable findings, and test costs that may be incurred by patients. The average time frame for translating research findings into practice is seventeen years (26). Implementation science facilitates the timely integration of evidence-based practice into clinical care and expands the focus from the patient level to address provider, organization, and policy level barriers in healthcare delivery. Determinant frameworks such as the Consolidated Framework for Implementation Research (CFIR) and process frameworks such as Exploration, Preparation, Implementation, Sustainment (EPIS) provide foundational approaches to introducing and evaluating a new intervention, such as PGx testing, through systematic assessment of key constructs that influence implementation and effectiveness (27, 28). Both frameworks identify inner and outer contextual factors essential to implementation; EPIS uniquely highlights sustainability, a critical component for administrators and payers interested in longer-term fiscal considerations for PGx test coverage to adopt and maintain testing at the health system level. Successful clinical implementation of PGx testing holds the potential to identify at-risk patients, personalize chemotherapy dosing, and better manage toxicity. Therefore, we sought to address barriers to *DPYD/UGT1A1* testing identified in our institution using the EPIS framework through the Implementing Pharmacogenetic Testing in Gastrointestinal Cancers (IMPACT-GI) study to maintain PGx services as a new standard of care.

Guiding Implementation Science Framework

The design of this study was guided by the EPIS framework by Aarons and colleagues (**Figure 1**) (29). During the *Exploration* phase, we gauged interest in implementing *DPYD* testing from leadership within our healthcare system including the Director of the Penn Center for Precision Medicine, Director of the Cancer Center, the Chief Executive Officer, and the head of the gastrointestinal (GI) cancer service line. We evaluated various options for PGx testing such as establishing an institutional test, partnering with an academic clinical laboratory, or using a commercial laboratory. The *Preparation* phase involved an internal assessment of barriers and facilitators from the point of view of GI oncology providers curated during semi-structured qualitative interviews. We also performed a retrospective study to understand baseline rates of drug-related adverse events in patients receiving fluoropyridines and/or irinotecan. During the *Implementation* phase, we designed and refined strategies to address each of the barriers uncovered during the internal assessment; each of the strategies are described in detail in the next section. Once the study is complete, we will examine

TABLE 1 | Description and prevalence of actionable *DPYD* and *UGT1A1* pharmacogenetic variants tested in the IMPACT-GI study.

Gene	Variant Allele			Allele Frequency[15,31]				
	* Allele	c. Nomenclature	rsID	AA	CSA	EA	EU	LAT
DPYD	*2A	c.1905+1G>A	rs3918290	0.003	0.005	0.000	0.008	0.001
	*8	c.703C>T	rs1801266	NR	0.0002	0.000	0.0001	0.000
	*10	c.2983G>T	rs1801268	NR	NR	NR	NR	NR
	*12	c.1156G>T	rs78060119	NR	NR	NR	NR	NR
	*13	c.1679T>G	rs55886062	0.000	0.000	0.000	0.001	0.000
	HapB3	c.1236G>A	rs56038477	0.003	0.020	0.000	0.024	0.006
		c. 1129-5923C>G	rs75017182					
		c.483+18G>A	rs56276561					
		c.557A>G	rs115232898	0.012	NR	0.000	0.0001	0.001
		c.2846A>T	rs67376798	0.003	0.001	0.000	0.004	0.002
UGT1A1	*6	c.211G>A	rs4148323	0.004	0.045	0.146	0.008	0.012
	*28	c.-41_-40dupTA(TA7)	rs8175347	0.373	0.414	0.148	0.316	0.400

AA, African ancestry; CSA, Central/South Asian ancestry; EA, East Asian ancestry; EU, European ancestry; LAT, Latino ancestry; N/A, Not applicable; NR, not reported.
**Refers to the standardized "star" (*) allele nomenclature.*

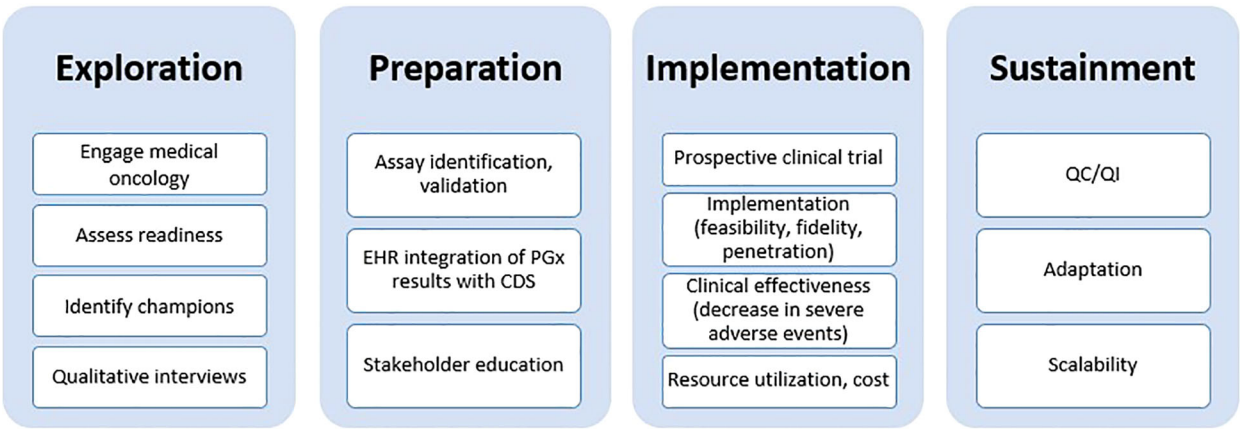

FIGURE 1 | Exploration, Preparation, Implementation, Sustainment (EPIS) framework used as a guide for implementing *DPYD/UGT1A1* pharmacogenetic testing. EHR, electronic health record; PGx, pharmacogenomics; CDS, clinical decision support; QC/QI, quality control/quality improvement.

Sustainment of PGx testing during the transition from research testing to clinical testing.

METHODS/DESIGN

Qualitative Interviews

We conducted a qualitative study with our GI oncologists and oncology pharmacists to elicit impressions about current dosing practices, attitudes toward using PGx results to tailor prescribing, and perspectives on an appropriate study design for a prospective PGx trial. An interview guide informed by CFIR constructs was created to facilitate semi-structured interviews. A brief survey was distributed during the interview to collect participant demographics and quantitatively assess level of comfort in interpreting PGx results. Barriers to testing highlighted by our clinicians included a limited evidence base and burdensome workflows related to testing (e.g., lengthy turnaround time, financial concerns, EHR integration); full results of this study are published in a separate manuscript (30). Our qualitative study allowed us to identify contextual factors deemed essential to test uptake within our institution and refine strategies to address barriers during the implementation study.

Retrospective Study

As part of our *Preparation* phase, we also conducted an institutional retrospective study to understand baseline drug-related adverse event rates in adult patients with a GI malignancy who initiated fluoropyridines and/or irinotecan over a six-month period in 2017 and 2018. We observed that approximately half of our cohort experienced at least one toxicity event (primarily related to the hematological or GI system), with 22% of patients requiring management in the emergency department or hospital. This cohort will serve as a control group for those receiving PGx testing in the prospective study. A subset of these participants have DNA available as part of the Penn Medicine Biobank and we are currently genotyping *DPYD* and *UGT1A1* variants.

Assay Validation

Our *Exploration* phase informed plans to partner with an academic clinical laboratory to offer germline testing. Prior to the availability of our PGx panel, genotyping was typically performed as a send-out laboratory test following chemotherapy-induced toxicity to confirm enzymatic deficiency. Results took approximately four weeks to return, rendering this prolonged timeline unfeasible and impractical to guide preemptive chemotherapy dosing. During *Preparation*, we collaborated with our academic clinical laboratory to develop and validate a custom panel of twelve *DPYD* variants and two *UGT1A1* variants: *2A, *5, *6, *8, *9A, *10, *12, *13, HapB3, c.557A>G (rs115232898), c.496A>G (rs2297595), and c.2846A>T (rs67376798) for *DPYD*; and *6, and *28 for *UGT1A1*. **Table 2** provides the corresponding activity scores for each *DPYD* variant listed. The Illumina™ Infinium Global Screening Array version 3 (GSAv3.0) is used to detect variants in the *DPYD* gene and the Applied Biosystems™ fragment analysis assay is used to assess for thymine-adenine (TA) tandem repeats in *UGT1A1*, followed by Sanger sequencing confirmation for both genes. CPIC tables describing *DPYD* and *UGT1A1* allele frequencies in major ethnic groups are reviewed for updates on a quarterly basis by the laboratory (15, 31). Genotyping costs are covered by the research study.

Integration of Pharmacogenetic Test Results Into the EHR

Prior to study initiation, most germline genetic data were reported in unstructured portable document formats (PDF) that fragmented workflows for personalized interpretation and application. The study team worked closely with Information Services (IS) within our institution and Epic Systems Corporation (Verona, Wisconsin, USA) to customize the Genomics Module and develop the PennChart Precision Medicine tab to serve as a centralized

TABLE 2 | *DPYD* allele function and activity score.

DPYD * Allele/rsID	Activity Score	Allele Function
*1	1	Normal
*2A	0	None
*5	1	Normal
*6	1	Normal
*8	0	None
*9A	1	Normal
*10	0	None
*12	0	None
*13	0	None
HapB3 (rs75017182, rs56038477, rs56276561)	0.5	Decreased
rs115232898	0.5	Decreased
rs67376798	0.5	Decreased
rs2297595	1	Normal

**Refers to the standardized "star" (*) allele nomenclature.*

location for pharmacogenomic information in the patient's medical record (32). *DPYD* and *UGT1A1* genotyping results are now stored in a discrete, computable format to enable electronic searching, clinical decision support (CDS), and secondary use for research and operations. PGx results are entered as diplotypes based on PharmVar star allele definitions (e.g., *UGT1A1* *1/*28) or CPIC activity score (e.g., *DPYD* Activity Score 1.5) and mapped to the corresponding phenotype (**Figure 2**). Integration of PGx results into the EHR serves as a key strategy to ensure sustainability of the testing long-term.

Epic's Genomic Indicators feature consists of tags added to a patient's chart indicating PGx phenotypes based on entered results. Displayed on the Snapshot tab, clinically actionable indicators drive automated CDS in the EHR to the care team (physicians, advanced practice providers, pharmacists, and nurses). Clinicians receive a best practice alert (BPA) notifying them of results at the genotype level within the Precision Medicine tab. For patients with actionable PGx results impacting their treatment regimen, an in-line warning and pop-up alert appear in their chart at the time of chemotherapy order entry and verification. These warnings succinctly summarize the drug-gene interaction and provide a guideline-concordant dosing recommendation (**Figure 3**). Results indicating high-risk genotypes immediately impacting patients are directly communicated to the ordering physician by study personnel and pharmacy staff to prevent delays in care. **Table 3** outlines the genotype-guided CDS on *DPYD* and *UGT1A1* results integrated in our EHR system.

Clinician Education

Our qualitative interviews highlighted the need for clinician education on PGx and the current evidence base for germline *DPYD* and *UGT1A1* screening. We used these findings to organize a Continuing Education program for oncology pharmacists and an in-service presentation for physicians one month prior to the initiation of the IMPACT-GI study. These educational initiatives focused on disseminating foundational PGx knowledge, from the role of actionable *DPYD* and *UGT1A1* variants in pharmacotherapy to current regulatory stances and evidence on the feasibility, safety, and cost utility of screening in the clinic. We also reviewed the test ordering and resulting processes in our EHR to maximize the learning experience for providers and promote its use in our oncology clinic. Ongoing PGx education is delivered through our CDS system and study newsletters. Our CDS tools provide

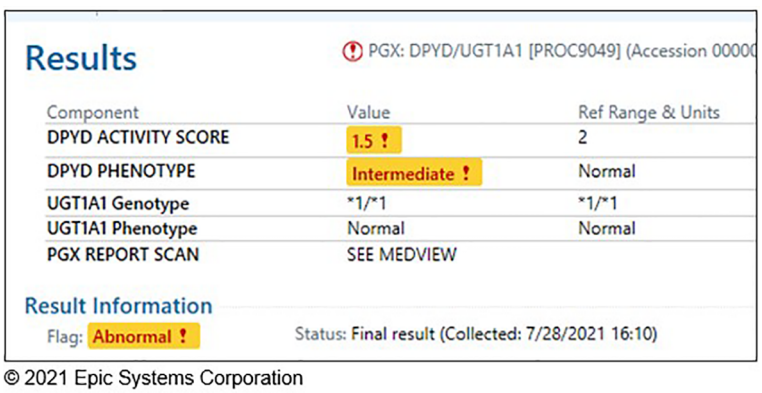

FIGURE 2 | Discrete *DPYD/UGT1A1* genotype results in a patient chart.

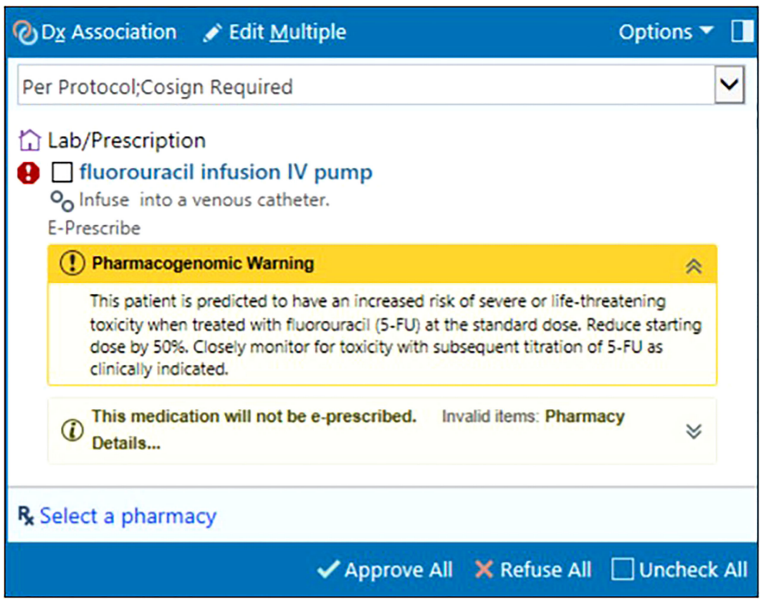

FIGURE 3 | In-line warning at fluorouracil order entry informing provider of actionable DPYD results, clinical implication, and dose recommendation. © 2021 Epic Systems Corporation

clinicians with digestible, patient-specific result interpretations and recommendations at the point of care. Study newsletters are distributed periodically to the GI oncology team and reviewed at standing clinical research meetings to maintain clinician engagement, obtain feedback on implementation processes, and report on enrollment trends with rates of test ordering and turnaround times.

Study Design of Implementation Trial

This is a pragmatic, non-randomized, open-label, multi-site trial performed within the University of Pennsylvania Health System (UPHS) (Philadelphia, Pennsylvania, USA). UPHS is an academic health system of six acute-care hospitals with approximately 3000 beds and over five million annual outpatient visits. Eligible participants are recruited at two oncology clinics in Philadelphia and an outpatient cancer center in Lancaster, Pennsylvania. **Figure 4** presents the study workflow.

Study Objectives

The primary objective of the IMPACT-GI study is to assess the feasibility of introducing *DPYD* and *UGT1A1* pharmacogenetic testing to guide initial fluoropyrimidine and irinotecan dosing in patients with GI malignancies. The secondary objectives are to evaluate the clinical effectiveness of the PGx testing service using clinical data, patient-reported outcome (PRO), and quality of life (QoL) measures. We hypothesize that the availability of a rapid turnaround PGx test with specific dosing recommendations will increase PGx test utilization to inform pharmacotherapy decisions and improve patient safety outcomes through our prospective clinical implementation study.

Study Outcomes

Primary endpoints for this implementation study are to determine (1): feasibility, defined by the proportion of PGx test results returned prior to the first dose of chemotherapy (2); fidelity, or the proportion of dose modifications made in agreement with genotype-guided dosing recommendations; and (3) penetrance, characterized by the rate of testing among eligible patients at our study sites.

Feasibility and fidelity data will be prospectively collected on a continual basis by examining the timestamp on test orders and pharmacy records. Penetrance will be determined by examining clinic schedules and study screening logs for eligible patients.

Exploratory outcomes for include (1): the proportion of patients experiencing ≥Grade 3 toxicity according to NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 (33) over the first six cycles of chemotherapy (or fewer if planned); 2) the relative dose intensity of fluoropyrimidine and irinotecan dosing over the first six cycles for comparison with the historical control cohort (3); PRO and QoL responses during the first six cycles of chemotherapy (34, 35); and (4) participant knowledge and attitudes towards PGx testing as determined by a patient survey. Additional exploratory endpoints include (1): the incidence of ≥Grade 3 toxicity by ancestry (2); minor allele frequencies for *DPYD* and *UGT1A1* reported by ancestry (3); survival analysis (progression-free survival, PFS and overall survival, OS) (4); medical service utilization and costs; and (5) assessment of exploratory biomarkers with fluoropyrimidine-associated toxicity.

The study team will review participants' medical records to collect demographic and clinical data at baseline, the first six cycles of chemotherapy (or fewer if planned), and survival data

TABLE 3 | Genotype-guided clinical decision support for *DPYD* and *UGT1A1* results.

Phenotype	Clinical Implication	Clinical Decision Support Alert Message	Reference
DPYD Normal Metabolizer (Activity Score 2)	Patient is predicted to have a normal risk of toxicity when treated with 5-FU or capecitabine.	None	15
DPYD Intermediate Metabolizer (Activity Score 1.5)	Patient is predicted to have an increased risk of severe toxicity when treated with 5-FU or capecitabine.	This patient is predicted to have an increased risk of severe or life-threatening toxicity when treated with fluorouracil or capecitabine at the standard dose. Reduce starting dose by 50%. Closely monitor for toxicity with subsequent titration of fluorouracil or capecitabine as clinically indicated.	15
DPYD Intermediate Metabolizer (Activity Score 1)	Patient is predicted to have an increased risk of severe toxicity when treated with 5-FU or capecitabine.	This patient is predicted to have an increased risk of severe or life-threatening toxicity when treated with fluorouracil or capecitabine at the standard dose. Reduce starting dose by 50%. Closely monitor for toxicity with subsequent titration of fluorouracil or capecitabine as clinically indicated.	15
DPYD Poor Metabolizer (Activity Score 0.5)	Patient is predicted to have an increased risk of severe toxicity when treated with 5-FU or capecitabine.	This patient is predicted to have an increased risk of severe or life-threatening toxicity when treated with fluorouracil or capecitabine at the standard dose. Avoid use of fluorouracil or capecitabine. If alternative agents are not considered a suitable option, administer fluorouracil or capecitabine at a strongly reduced dose (i.e. <25% of normal starting dose).	15
DPYD Poor metabolizer (Activity Score 0)	Patient is predicted to have an increased risk of severe toxicity when treated with 5-FU or capecitabine.	This patient is predicted to have an increased risk of life-threatening toxicity when treated with fluorouracil (5-FU) or capecitabine at the standard dose. Avoid use of 5-FU or capecitabine.	15
UGT1A1 Normal Metabolizer (*1/*1)	Patient is predicted to have a normal risk of toxicity when treated with irinotecan.	None	17, 20, 21
UGT1A1 Intermediate Metabolizer (*1/*28 or *1/*6)	Patient is predicted to have a normal risk of toxicity when treated with irinotecan.	None	17, 20, 21
UGT1A1 Poor Metabolizer (*28/*28, *6/*6, or *6/*28)	Patient is predicted to have an increased risk of severe toxicity when treated with irinotecan.	The patient is predicted to have an increased risk of severe toxicity when treated with irinotecan at the standard dose. Reduce starting dose by 30%. Closely monitor for toxicity with subsequent titration of irinotecan as clinically indicated.	17, 20, 21

**Refers to the standardized "star" (*) allele nomenclature.*

approximately six months from treatment initiation. PRO and QoL questionnaires will be distributed electronically *via* Research Electronic Data Capture (REDCap) (36) or by paper in clinic to better understand any treatment-related symptoms from the participant's perspective with each cycle. Information readily available in the EHR, such as clinical progress notes, telephone encounters, and patient portal messages, will be corroborated with available PRO and QoL responses to grade adverse events.

Study Participants

Patients aged 18 years or older with a pathologically confirmed GI malignancy for which treatment with a fluoropyrimidine and/or irinotecan is indicated and a life expectancy of at least six months are eligible. Initially inclusion was restricted to patients with an ECOG performance status of 0, 1, or 2, but the protocol was amended in December of 2021 to no longer restrict testing based on functional status. Participants must be able and willing to provide informed consent and undergo blood sampling for genotyping and comply with study procedures.

Exclusion criteria include (1): known *DPYD* and *UGT1A1* genotype status (2); unacceptable laboratory values, including (a) hepatic dysfunction, as defined by serum bilirubin ≥1.5 x upper limit of normal (ULN), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) ≥2.5 x ULN, or in case of liver metastases ALT and AST≥5 x ULN, (b) renal dysfunction as defined by serum creatinine ≥1.5 x ULN, or creatinine clearance <60 ml/min (by Cockcroft-Gault Equation), or (c) absolute neutrophil count of $<1.5 \times 10^9$/L or platelet count of $<100 \times 10^9$/L (3); women who are pregnant or breast feeding, or subjects who refuse to use reliable contraceptive methods throughout the study; and (4) treating physician does not want the subject to participate. Initially prior treatment with 5-fluorouracil or capecitabine was an exclusion, but this was later amended in December 2021 to enroll patients who had received these agents in the past.

The duration of patient recruitment is 18 months. The follow-up period for enrolled participants is six months from the first dose of chemotherapy. Participants are free to withdraw from participation in the study at any time without stating any reason nor affecting their medical care.

Sample Size

We plan to enroll 300 participants at the three sites. This number is a convenience sample based on known patient volume from institutional cancer registry data, estimating the number of patients that will be eligible for testing. The first 116 participants were enrolled prior to the study amendment.

Study Procedures

Recruitment

All recruitment is conducted at three Penn Medicine cancer clinics. Clinic schedules are screened by clinical research personnel for patients diagnosed with a GI tumor being evaluated for treatment. Prior to the scheduled initial office visit, the research coordinator confirms eligibility with the treating

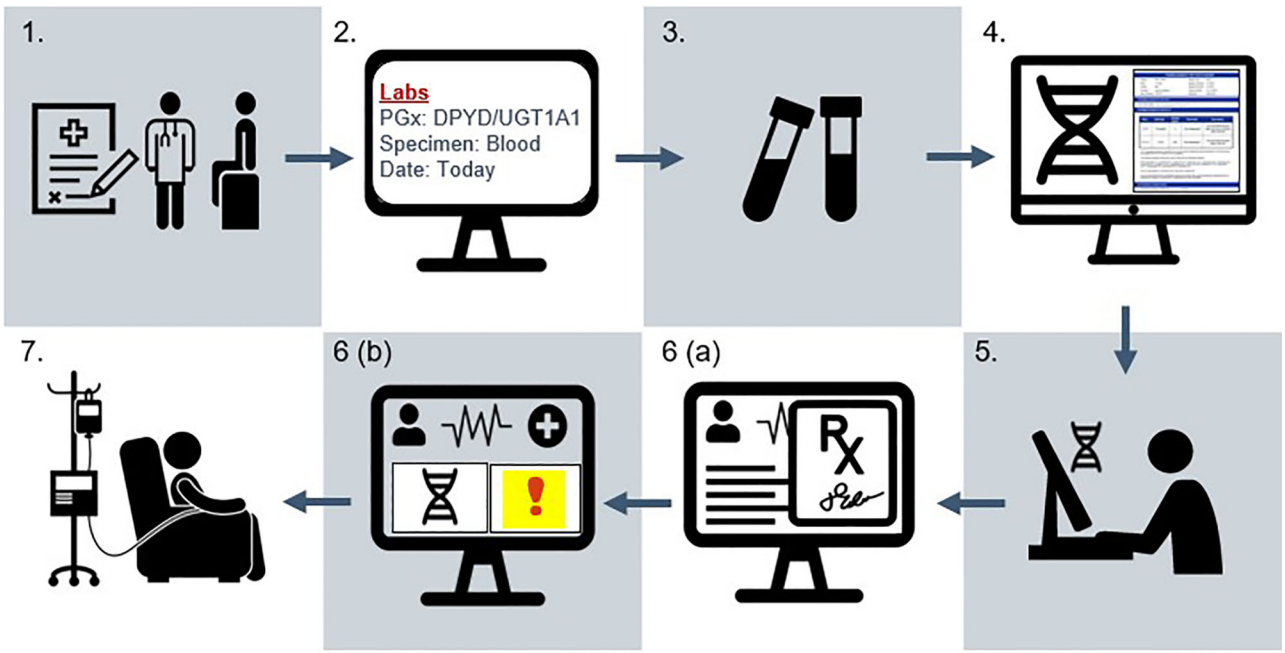

FIGURE 4 | Study schema for the IMPACT-GI study. (1) Patient provides informed consent at initial evaluation (baseline) visit in the gastrointestinal oncology clinic. (2) A laboratory order for pharmacogenetic testing is placed in the EHR. (3) A specimen is collected alongside routine laboratory collections by phlebotomy. (4) The specimen is sent to an external CLIA laboratory for genotyping and report generation. (5) Pharmacogenetic results are entered by the institutional lab into the precision medicine section of the EHR as discrete result components. (6a) When the care team signs and verifies chemotherapy orders, (6b) an alert indicating increased toxicity risk appears in the patient's chart for individuals with actionable results. Clinical decision support provides recommendations for dose adjustments. (7) Following order verification, chemotherapy is prepared and dispensed to the patient in the oncology infusion suite. Fidelity is demonstrated when the prescriber adjusts dosing according to the patient's genotype. Adverse event data is collected for the first six cycles of treatment. Patients complete a symptom questionnaire at each of these cycles and a one-time survey assessing attitudes towards pharmacogenetic testing after receiving at least two cycles. Tumor outcomes (progression-free survival, overall survival) are assessed at approximately six months following treatment initiation.

oncologist. The oncologist or research coordinator then discusses the study with the patient during this visit and obtains consent for study participation.

Following enrollment, an order is placed within the patient's medical record so that a blood sample for DNA genotyping is obtained alongside routine laboratory orders by clinic phlebotomists. High-throughput genotyping, interpretation, and report generation is carried out in a College of American Pathologists (CAP)-accredited and Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory at the Center for Applied Genomics at the Children's Hospital of Philadelphia (Philadelphia, PA, USA). The anticipated test turnaround time is ten business days.

Study Assessments

- Baseline (collected at time of enrollment):
  - o Demographics: age, gender, race/ethnicity, insurance status, work status, and contact information for electronic PRO, QoL, and survey responses
  - o Cancer history: GI tumor type, stage, history or planned surgical resection and/or radiation therapy, previous lines of therapy, and treatment intent with prescribed regimen
  - o Laboratory assessments: vital signs (height, weight, heart rate, blood pressure), routinely performed laboratory tests: complete metabolic panel (CMP), complete blood count (CBC), and Eastern Cooperative Oncology Group (ECOG) performance status
  - o Concomitant medications
- Ongoing (collected during chemotherapy cycles 1-6, or fewer if planned):
  - o Laboratory assessments: vital signs, CMP, CBC, and ECOG performance status
  - o Concomitant medications (changes from baseline or previous cycle)
  - o PRO and QoL responses (obtained by paper or electronically at each subsequent visit to reflect symptoms experienced in the previous cycle)
  - o Toxicity events and management (including treatment location as outpatient or emergency department/hospitalization, and changes to prescribed regimen)
- End of study (collected at six months from treatment initiation):
  - o Subject status (completion of planned toxicity assessments, study withdrawal, or death)

o Laboratory assessments: vital signs, CMP, CBC, and ECOG performance status

o Concomitant medications (changes from baseline or previous cycle)

o Oncologic outcomes: PFS and OS based on available clinical data

Patient Survey

Participant-reported knowledge and attitudes towards PGx testing will be captured by a REDCap survey instrument (**Supplementary Data Sheet 1**). To support standardization among participants enrolled at different time points, the survey is disseminated to genotyped participants who have received at least two cycles of treatment. A paper-based version is available for patients who wish to complete the survey in clinic or may lack internet access. Participants will be compensated with a $25 gift card for survey completion. Likert scale responses will be compared by sex, race/ethnicity, tumor type, and socioeconomic status using linear regression.

Statistical Plan

Primary implementation endpoints will be analyzed using descriptive statistics. The mean, standard deviation (SD), median, interquartile range, range, counts, and percentage will be used to describe and compare baseline characteristics between the prospectively genotyped group and the historical control cohort. Student's t-test or rank sum test will be employed for continuous variables and Fisher's exact test for categorical variables.

While our primary study outcomes are focused on evaluating implementation, our exploratory measures of clinical effectiveness will employ a multivariable regression model with adjustment for covariates (e.g. age, sex, tumor type, treatment regimen, ECOG performance status) to compare the proportion of ≥Grade 3 toxicities in variant carriers who received genotype-adjusted dosing in the prospective IMPACT-GI cohort to variant carriers who received standard chemotherapy dosing in the historical control group. Subgroup analyses will be performed by tumor type. PRO and QoL responses as assessed on a Likert scale will be reported as means (SD).

Concurrent Process Evaluation

To further delineate the barriers that arose during the implementation study, we will elicit feedback from central users involved in the implementation process. We will perform one-on-one semi-structured key informant interviews at the end of the study with the following individuals: oncology physicians, advanced practice providers, pharmacists, research and clinical support staff, Information Services staff, and laboratory staff. We will also examine perspectives from health system administrators and local payers. These efforts will also be essential in evaluating, refining, and sustaining future PGx efforts in our institution.

Present Status

The first patient was enrolled in March 2021. As of January 2022, 116 participants have been enrolled across the three sites.

TABLE 4 | Baseline characteristics of the first 116 participants.

	n=116
Age, years (mean + SD)	61 + 13.2
Sex, female, n (%)	57 (49)
Ancestry/Ethnicity, n (%)	
White	77 (66.4)
Black	25 (21.6)
Hispanic/Latino	7 (6.0)
East Asian	5 (4.3)
Other	2 (1.7)
Tumor type, n (%)	
Colorectal	51 (44)
Pancreas	33 (28.4)
Appendix	9 (7.7)
Gastric	5 (4.3)
Small intestine	5 (4.3)
Esophageal	4 (3.4)
Other	9 (7.7)
Treatment regimen, n (%)	
FOLFOX-based	47 (40.5)
Capecitabine-based	35 (30.2)
FOLFIRI-based	27 (23.3)
Other	7 (6)

Baseline characteristics of those patients are shown in **Table 4**. Recruitment of the last patient is expected in August 2022.

DISCUSSION

Precision medicine initiatives have accelerated the translation of genomics research into clinical practice and continue to gain traction in health systems. It is anticipated that clinical adoption of PGx testing will become more ubiquitous with growing stakeholder interest and increasing test coverage policies by major payers (37–39). Implementation science frameworks such as EPIS provide a roadmap for implementation and can facilitate the adoption of PGx testing into routine clinical care. We established implementation in one service line (GI oncology) to build necessary clinical operations to deliver PGx testing. Conducting qualitative interviews during the Exploration phase were vital in identifying barriers to implementation and at the same time provided an opportunity to engage and educate key personnel about the intervention (30).

While randomized controlled trials (RCTs) remain the gold standard for medical practice, alternative pragmatic methods should be considered for contributing to real-world PGx evidence and supporting its clinical use (40–42). Given that many actionable PGx markers occur at low frequencies in the population, it is not always feasible to conduct a RCT and demonstrate effectiveness with sufficient power. During our implementation planning process, many oncologists in our institution expressed ethical concerns with a randomized trial design, fearing that a *DPYD* or *UGT1A1* carrier may receive chemotherapy at standard dosing and thereby be exposed to an increased risk of toxicity. Bearing these contextual factors in mind, we pursued a non-randomized, open-label approach to our prospective study with the goal of establishing the PGx test as

part of routine care and removing barriers to its use. Additionally, well-powered effectiveness trials have already been performed showing that prospective *DPYD* testing reduces severe toxicity; our study design is similar to other implementation science trials with the primary goal of demonstrating feasibility and fidelity in individualizing chemotherapy dosing (12, 43). To our knowledge, this is the first study prospectively evaluating additional *DPYD* and *UGT1A1* variants such as *DPYD* c.557A>G and *UGT1A1* *6, which are observed more frequently in populations of non-European ancestry and reflect the diversity of patient populations receiving care in our health system. It should be further acknowledged that individual germline genetic variations play one role in the clinical outcomes of chemotherapy treatment, other factors such as gender, age, weight, lifestyle habits, performance status, organ dysfunction and concomitant medications must also be taken into consideration for determining treatment plans. An interdisciplinary clinical team that includes a pharmacist is crucial for evaluating drug-drug interactions and drug-drug-gene interactions from concomitant medications and PGx profiles for optimal dosing decisions, particularly in an aging cancer population where polypharmacy is highly prevalent (44, 45).

Following study conclusion, we intend to scale this testing to patients with other tumor types (e.g., breast, head and neck) considering fluoropyrimidine therapy, along with plans to offer testing of additional PGx variants to guide prescribing of supportive care medications administered during chemotherapy (e.g., anti-emetics, analgesics). Local laboratory partnerships, EHR infrastructure build, and new regional test coverage for PGx testing has laid the groundwork for future test panels in our health system. Implementation science framework will continue to shape implementation strategies across our multi-level health system to bridge gaps between the available evidence and delivery of care.

CONCLUSION

Fluoropyrimidines and irinotecan remain commonly prescribed chemotherapy agents for GI malignancies. Screening for germline *DPYD* and *UGT1A1* variants to tailor chemotherapy dosing to each patient's genetic profile can help identify those at highest risk for toxicity to improve patient outcomes while achieving favorable risk/benefit ratios of treatment tolerability and efficacy. This study leverages implementation science frameworks to evaluate the implementation of *DPYD* and *UGT1A1* testing, while developing infrastructure for genomic medicine in our cancer centers to ensure sustainability of PGx testing as standard of care.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by both the institutional review board (IRB) and the Abramson Cancer Center Clinical Trials Scientific Review and Monitoring Committee (CTSRMC) at the University of Pennsylvania as of 17 December 2020. It is registered at ClinicalTrials.gov (NCT04736472). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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.2022.859846/full#supplementary-material

Supplementary Data Sheet 1 | Perceptions of Pharmacogenetic Testing in Patients with GI Cancers (patient survey).

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ORIGINAL RESEARCH
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The Novel LncRNA WASH5P Inhibits Colorectal Cancer Carcinogenesis *via* Targeting AKT Signaling Pathway

Hongyun Wei, Tao Mao, Qian Zhang, Keyu Ren, Xingsi Qi, Yunmei Zhang, Bin Cao, Yanchun Jin, Zibin Tian and Linlin Ren *

Department of Gastroenterology, Affiliated Hospital of Qingdao University, Qingdao, China

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Lakshminarayan Reddy Teegala, University of Toledo Medical Center, United States

***Correspondence:**
Linlin Ren
renlinlin@qdu.edu.cn

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Emerging evidence has shown that long non-coding RNAs (lncRNAs) play an important role in colorectal cancer (CRC) carcinogenesis, so more specific mechanisms of key lncRNAs in CRC initiation and development are needed. Here, we evaluated the expression profiles of lncRNAs in CRC tissues and identified a novel lncRNA generated from the pseudogene Wiskott-Aldrich syndrome protein (WASP) family homolog 5, termed lncRNA WASH5P. However, the role and potential molecular mechanism of this novel lncRNA in diseases, including CRC carcinogenesis, is unknown. Our present study found that WASH5P was significantly downregulated in CRC cell lines and tissues compared with normal controls. The ectopic expression of WASH5P in CRC cells could significantly inhibit CRC cell proliferation, invasion, and migration. In addition, WASH5P could increase the expression of E-cadherin and decrease Vimentin expression. WASH5P-overexpressing CRC cells developed tumors more slowly in different mouse models. Meanwhile, the overexpression of WASH5P could significantly inhibit AKT activation *via* suppressing AKT phosphorylation. The treatment of PI3K/AKT (phosphatidlinositol 3-kinase /protein kinase B) signaling agonist 740Y-P rescued WASH5P-reduced AKT phosphorylation and abolished the inhibitory effects of WASH5P on cell viability, migration, and invasion. Moreover, 740Y-P restored the WASH5P-induced downregulation of p-AKT and vimentin and the upregulation of E-cadherin *via* Western blot. In summary, our findings suggested that the novel lncRNA WASH5P might be a potential candidate biomarker and therapeutic target that could inhibit CRC by repressing the AKT signaling pathway.

Keywords: LncRNA WASH5P, AKT signaling pathway, colorectal cancer, proliferation, cancer metastasis

INTRODUCTION

Although substantial progress has been made in cancer prevention, diagnosis, and treatment recently, all kinds of cancer are still the leading causes of death according to cancer statistics in 2021 (1). For colorectal cancer (CRC), it is still the fourth leading cause of cancer-related death worldwide (2), more than a hundred thousand new cases and fifty thousand deaths were estimated in 2021 (1). Genetic, environmental, epigenetic, and microbiological factors have been considered as the causing factors of CRC (3). However, molecular pathogenesis is still not fully clarified. Additionally, the current treatment strategies did not show a satisfying effect. Therefore, it is of critical importance to identify new effective biomarkers and elucidate the underlying mechanisms involved in CRC carcinogenesis.

As a class of RNA molecules, long non-coding RNAs (lncRNAs) with more than 200 nucleotides are located in the nucleus or cytoplasm (4), and play key roles in gene regulation in various human diseases, such as cancers (5, 6). Emerging evidences demonstrated the important role of lncRNAs in the initiation and maintenance of solid carcinomas (7–9). Recent studies have indicated that many lncRNAs, including MALAT1 (metastasis-associated lung adenocarcinoma transcript1), GAS5 (growth arrest-specific 5), and FLANC (flamingo non-coding RNA), were involved in the development of CRC (10–12). In addition, more and more studies revealed the relationship between lncRNAs and key signnaling pathways (13), including the AKT signaling pathway. Yao et al. showed that lncRNA MALAT1 could enhance the radioresistance of CRC *via* the AKT/YAP axis (14). In addition, lncRNA SPRY4-IT1 has functional interactions with PI3K/AKT signaling in the carcinogenesis of CRC (15). However, more specific mechanisms of key lncRNAs in CRC initiation and development need to be further teased out.

In the current study, we evaluated the expression profiles of lncRNAs in CRC tissues and identified a new lncRNA generated from the pseudogene Wiskott-Aldrich syndrome protein family homolog 5, termed lncRNA WASH5P. However, the expression level and potential function of WASH5P have not been reported until now. Here, we found that lncRNA WASH5P was significantly downregulated in both CRC tissues and cell lines. The biological roles of lncRNA WASH5P in CRC were genetically assessed in both *in vitro* and *in vivo* models. Further analysis revealed that lncRNA WASH5P inhibits CRC *via* suppressing the AKT pathway. In summary, we identified a novel lncRNA, WASH5P, which might be a potential candidate biomarker and therapeutic target in CRC *via* targeting the AKT signaling pathway.

MATERIALS AND METHODS

Chemical

740Y-P, an activator of the AKT/AKT signaling pathway, was obtained from Med Chem Express (Cat. No. HY-P0175). The agent was dissolved with DMSO (dimethyl sulphoxide) and used to activate the AKT/AKT pathway in CRC cells.

Bioinformatic Analysis

In the TCGA (The Cancer Genome Atlas) database, 30 paired normal and CRC samples from GSE74602 were included for further analysis. The related RNA-seq data were extracted. The expression of WASH5P was verified in TCGA and GTEx (the Genotype-Tissue Expression) databases. Statistical analysis was performed using R (v.3.5.1). Graphpad prism was used for plotting. Differential gene expression was considered significant if |logFC|>1 and adj.P.Val<0.05.

Cell lines and Cell Culture

Four human colorectal cancer cell lines SW480、HCT116、RKO、 and HT29 and one normal colon cell line (NCM460) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NCM460, SW480, and HT29 were maintained in an RPMI-1640 (Procell, PM150110 Wuhan, China) medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (NCM Biotech, Suzhou, China c125c5) at 37°C with 5% CO_2. HCT116 was maintained in a Dulbeco's modified Eagle (Procell, PM150210 Wuhan, China) medium and RKO in an MEM (Minimum Essential Medium) medium (Procell, PM150410 Wuhan, China). All cell lines were validated by short tandem repeat profiling.

Nuclear and Cytoplasmic RNA Fraction Isolations

Nuclear and cytoplasmic RNA was isolated with a Cytoplasmic and Nuclear RNA Purification Kit (Norgen Biotek Corp, Ontario, Canada) according to the manufacturer's instructions. The cells were added with lysis buffer J including β-mercaptoethanol and incubated on ice for 5 min. After centrifugation for 10 min with 14,000 g, the supernatant was collected as the cytoplasmic fraction and the precipitation as the nuclear fraction. Then, RNA was extracted.

RNA Extraction and Quantitative PCR

Total cellular RNA was isolated using a TRIzol reagent (Cat. No. 15596026, Thermo Fisher Scientific, Shanghai, China), dissolved in RNA-free H_2O and stored at −80°C. RNA (1 μg) was used to obtain high-quality (complementary deoxyribonucleic acid) cDNA using the reverse transcription kit (Genecopoeia, Guangzhou, China) according to the manufacturer's protocols. The PCR system was used to amplify all transcripts using the iQ SYBR Green Supermix' (1708882AP; Bio-Rad, Shanghai, China). The primer sequences were as follows: WASH5P (sense 5'- GCGGCTCGCAGGCAC-3', antisense 5'-GGATGAAGGGCACGGCA-3'). qPCR was designed with the following conditions: predenaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing and elongation at 60°C for 30 s. Relative transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated in triplicate.

Protein Extraction and Western Blot

The extracted proteins from tissues or cells were resolved by sodium dodecyl sulfate - polyacrylamide gel electrophoresis gradient gel (5% spacer gel and 10% or 15% separation gel). After electrophoresis, samples were transferred to a polyvinylidene fluoride membrane, blocked at room

temperature for 1 h with 5% milk, and incubated with a primary antibody at 4˚C overnight. Primary antibodies include a rabbit antibody against vimentin (1:2,000; Proteintech, 10366-1-AP), E-cadherin rabbit polyclonal antibody (1:2,000; Proteintech, 20874-1-AP), GAPDH rabbit polyclonal antibody (1:6,000; Proteintech, 10494-1-AP), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mouse monoclonal antibody (1:6,000; Proteintech, 60004-1-Ig), p-AKT antibody (1:1000; Y011054, abm, Richmond, BC, Canada), and AKT (1:1,000; Y409094, abm, Richmond, BC, Canada). The membranes were washed with a buffer three times, incubated with an HRP-conjugated anti-rabbit IgG (1:10,000; #14708, CST, USA) or anti-mouse IgG (H+L) (1:10000; CST, #14709, Shanghai, China) at room temperature for 2 h, and visualized with an ECL (Enhanced Chemiluminescence) kit (NCM Biotech, China).

Vector Construction and Cell Transduction

For the downregulation of WASH5P, the siRNA-1 (upstream: 5'-GATCCGCTTACAGACTCTCTGTTTAACTCGAGTTAAACAGAGAGTCTGTAAGCTTTTTG-3'; downstream: 5'-AATTCAAAAAGCTTACAGACTCTCTGTTTAACTCGAGTTAAACAGAGAGTCTGTAAGCG-3') and siRNA-2 (upstream: 5'-GATCCGCTTGGTGTTCGCAACTTTGACTCGAGTCAAAGTTGCGAACACCAAGCTTTTTG-3'; downstream: 5'-AATTCAAAAAGCTTGGTGTTCGCAACTTTGACTCGAGTCAAAGTTGCGAACACCAAGCG-3') sequence synthesized by Huada Technology (Shanghai, China) were cloned into the pLVX (pLVX-shRNA2-puro). In addition, a lentiviral plasmid containing the full length of WASH5P was constructed. The primer used for the overexpression of WASH5P is shown below: forward Primer 5'-GGATCTATTTCCGGTGAATTCGGAAGCGGCGGCGGGAGC-3', reverse primer: 5'-CGCTCTAGAACTAGTCTCGAGGGCAGGAGGAGGGTGTGG-3'.

WASH5P-overexpressing or WASH5P shRNA (short hairpin RNA) lentivirus was produced in HEK293T cells, respectively. HCT116, SW480, and RKO cells were then transfected with a different lentivirus in 6-well cell culture plates and selected with 5μg/ml puromycin. For rescue experiments, the CRC cells and WASH5P-overexpressed cells were pretreated with 740Y-P (25 μM) for 24 h at 37°C.

Cell Counting Kit-8 Analysis

Cell Counting Kit-8 (CCK-8) was used to detect cell viability. Cells were plated in 96-well plates at 5×10^3 cells/well. Approximately 10 μl of CCK-8 solution was added to each well, and the color intensity with an enzyme-mark analyzer was measured after 0h, 24h, 48h, and 72 h, respectively, at 450 nm.

Migration and Invasion Assay

The invasion assay was performed using 24-well Transwells (8-μm pore size; Millipore Bedford, MA, USA) that were coated with Matrigel (BD Biosciences, San Jose, CA, USA). Different cells were seeded in the serum-free medium into the upper chamber, whereas a medium supplemented with 20% FBS (fetal bovine serum) was applied to the lower chamber as a chemoattractant. After 48 h of incubation, the migrated cells at the bottom surface of the filter were fixed, stained, and counted.

The migration assay was performed using a 24-well transwell with no Matrigel. Treated cells were seeded in a serum-free medium into the upper chamber, whereas a medium supplemented with 20% fetal bovine serum was applied to the lower chamber as a chemoattractant. After 30 h of incubation, the migrated cells at the bottom surface of the filter were fixed, stained, and counted.

Animal Experiments

5–6-week-old male Balb/c nude mice, weighing 18–20g, were purchased from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China) and housed in an SPF animal facility. All the mice were randomly assigned to the normal control (HCT116 vector) and lncRNA-WASH5P overexpression (HCT116 + WASH5P OE) group (n=6). The animal experiment was approved by the Ethics committee for animal experiments of the Affiliated Hospital of Qingdao University (No.QYFY WZLL 26861). The mice were housed 6/cage, in a 12:12-h light:dark cycle environment, with *ad libitum* access to food and water. After adapting to the new environment for 2 weeks, the mice were implanted with unilateral axilla subcutaneous injection of 1×10^7 HCT116-vector cells or WASH5P-overexpressed HCT116 cells to induce primary tumors, respectively. The tumor length and width were measured every 5 days until the experiment was ended. After 4 weeks, the mice were sacrificed and the tumors were dissected and weighed.

In the metastatic model, all cells (1×10^6) were transplanted by tail vein injection. All mice were sacrificed after 45 days. Lung tissues were collected for further analysis.

Hematoxylin and Eosin Staining and Immunohistochemistry Analysis

Fresh tumor tissues from nude mice were fixed in formalin at room temperature for 24 h; then, the tissues were dehydrated with an ethanol and acetone series, embedded in paraffin, and sectioned onto glass slides at 4 μm. The slides were stained with hematoxylin and eosin (H&E) following deparaffinization. For IHC (immunohistochemistry), the sections were incubated at 65° C for 2 h and hydrated in xylene and graded ethanol. Then, the sections were immersed in a citrate buffer solution, heated in a microwave oven for 2.5 min, kept 5 min at room temperature, and heated in a microwave oven for 24 min. After washing with PBS (Phosphate Buffer Solution) three times, the slides were incubated with a blocking buffer for 10 min and then with a primary antibody overnight at 4°C. Primary antibodies include a rabbit antibody against vimentin (1:5,000; Proteintech, 10366-1-AP), the E-cadherin rabbit polyclonal antibody (1:1000; Proteintech, Wuhan, China, 20874-1-AP), p-AKT antibody (1:50; Y011054, abm, Richmond, BC, Canada), and AKT (1:200; Y409094, abm, Richmond, BC, Canada). After washing with Phosphate Buffer Solution, the sections were incubated with a secondary antibody, detected with DAB (diaminobenzidine),

and counterstained with hematoxylin. Positive staining was deemed as brown dots.

Statistical Analysis

R package clusterProfiler was used for GSEA (Gene Set Enrichment Analysis) analysis. The metrological data were expressed as means ± SD. Each experiment was performed at least 3 times. Significance was determined with the Student's t-test (when only two groups were compared) or one-way analysis of variance (ANOVA) followed by Dunnett's test (when more than two groups were compared). All statistical analyses were performed using SPSS (Statistical Product and Service Solutions) software (version 20.0), or GraphPad Prism 8.0 (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Long Non-Coding RNA WASH5P is Downregulated in Colorectal Cancer

To explore the key lncRNAs in CRC carcinogenesis, we analyzed the gene expression data in CRC (n=30) and paired adjacent tissues (n=30) in GSE74602 from the TCGA database. A total of 1,639 non-coding genes were differentially expressed, including 662 upregulated and 977 downregulated genes in CRC compared with the paired adjacent tissues (**Figure 1A**). The non-coding genes with the most significant differential expression in GSE74602 are listed in **Figure 1B**. Among these genes, lncRNA WASH5P was significantly downregulated in CRC tissues compared with adjacent tissues using the Wilcoxon rank-sum test in GSE74602 (logFC= -1.09, adj. P=0.0227) (**Figure 1C**). Summarized data from TCGA-COAD (The Cancer Genome Atlas-Colon Adenocarcinoma) and GTEx (the Genotype-Tissue Expression) validated that the expression level of WASH5P was significantly lower in cancer tissues than in normal tissues (P<0.001) (**Figure 1D**). Furthermore, Gene Set Enrichment Analysis analysis showed that lncRNA WASH5P was enriched in the hallmark PI3K-AKT pathway (**Figure 1E**).

To verify the expression level of lncRNA WASH5P, qPCR was performed in different CRC cell lines (SW480、HCT116、RKO、HT29) and one normal colon cell line (NCM460). The results showed that lncRNA WASH5P was significantly downregulated in CRC cell lines compared with normal colon cells ($p<0.05$) (**Figure 2A**). To figure out the location of lncRNA WASH5P in CRC cells, we analyzed the expression level of lncRNA WASH5P both in the nucleus and the cytoplasm and found that lncRNA WASH5P was mainly located in the cytoplasm (**Figure 2B**).

WASH5P Inhibits Colorectal Cancer Cell Proliferation, Invasion, and Migration

We then evaluated the role of lncRNA WASH5P in CRC cells. LncRNA WASH5P was successfully downregulated or upregulated in different CRC cell lines (**Figure 2C**). The overexpression of lncRNA WASH5P in HCT116 and SW480 could dramatically inhibit tumor cell viability (**Figures 2D, E**). The transwell assay revealed that lncRNA WASH5P upregulation could significantly inhibit tumor migration and invasion when compared with the control group (**Figures 2G–I**). Furthermore, the silence of lncRNA WASH5P could promote RKO CRC cell viability (**Figure 2F**), migration, and invasion (**Figures 2G–I**). In addition, the overexpression of WASH5P could inhibit the EMT process *via* targeting EMT markers, while the downregulation of WASH5P may promote EMT as shown in **Figure 2J**.

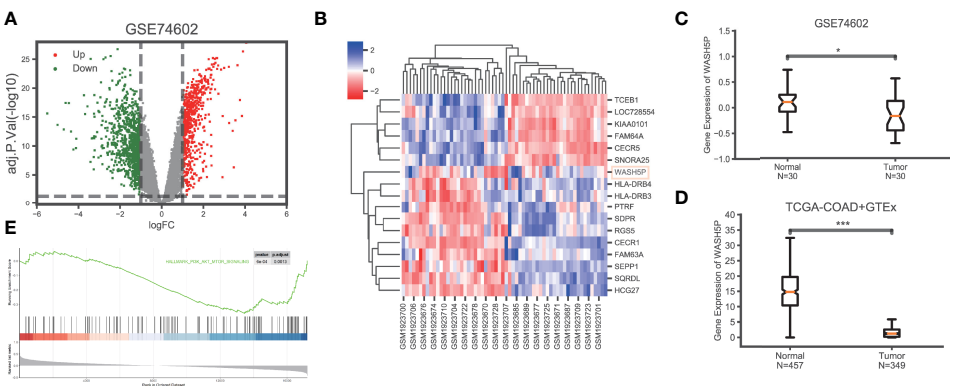

FIGURE 1 | LncRNA WASH5P is downregulated in CRC. **(A)** Identification of the differentiated expression of non-coding genes in colorectal cancer based on GSE74602 from the The Cancer Genome Atlas (TCGA) database: a total of 1,639 differentially expressed non-coding genes were screened, including 662 upregulated (the red dots) and 977 downregulated genes (the green dots). **(B)** Heat maps showing the most differentiated expression genes between colorectal cancer (n=30) and adjacent normal tissues (n=30) from the TCGA data set (GSE74602). The color of the square corresponds to the relative expression among cases. **(C)** The relative expression of lncRNA WASH5P in CRC tissues (n=30) and adjacent non-tumor colorectal tissues (n=30) was analyzed in data set GSE74602. **(D)** WASH5P expression levels in cancer tissues (n=349) and normal tissues (n=457) in the database from TCGA-COAD and GTEx.***. Correlation is significant at the 0.001 level. **(E)** GSEA analysis showed that lncRNA WASH5P was enriched in the hallmark PI3K-AKT pathway.

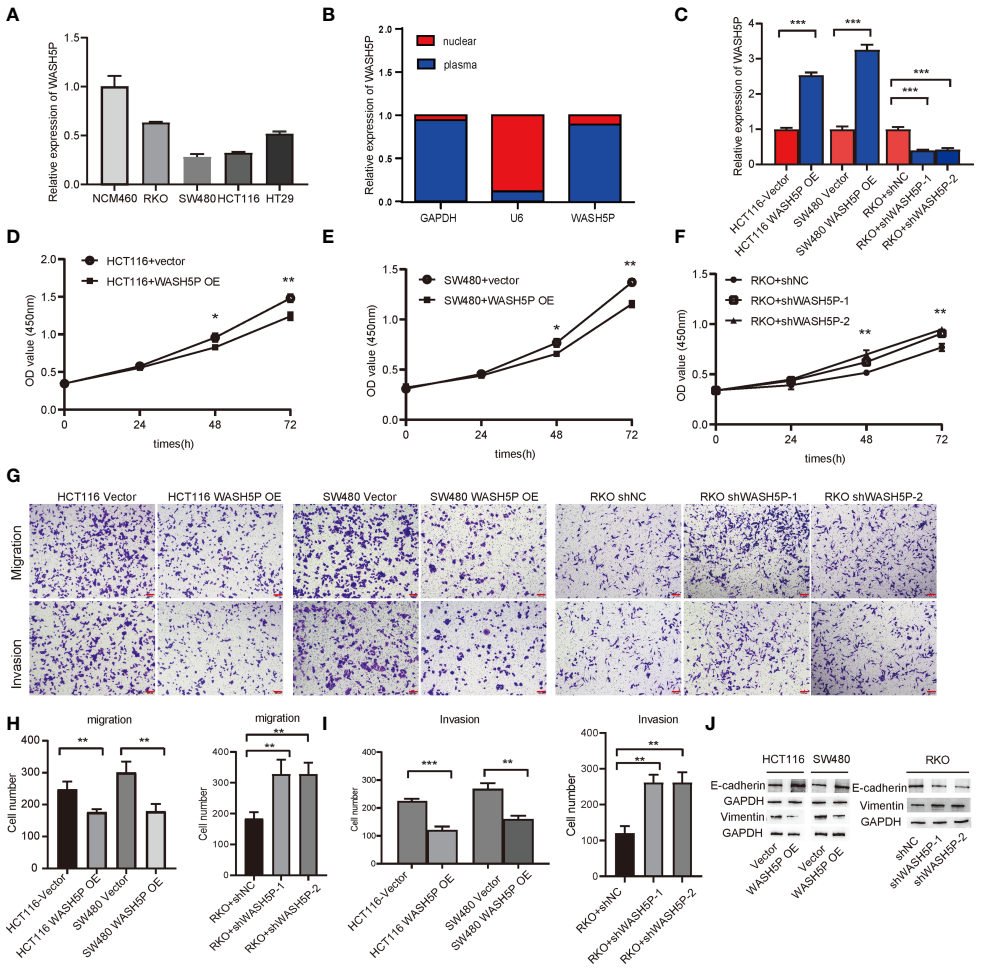

FIGURE 2 | LncRNA WASH5P inhibits the development of CRC *in vitro*. **(A)** LncRNA WASH5P expression was measured in the normal colon cell line NCM460 and four CRC cell lines by real-time PCR. **(B)** Real-time PCR was performed to examine lncRNA WASH5P levels in the cytoplasm and nucleus, respectively. **(C)** Knockdown and overexpression efficiency were confirmed by real-time PCR in different CRC cell lines. ***. Correlation is significant at the 0.001 level. **(D, E)** CCK-8 assay was performed in HCT116 and SW480 cells transfected with lncRNA WASH5P or a vector. Cell viability was measured from an absorbance reading at 450 nm. Data were expressed as relative viability (%) calculated: [A450(treated) – A450 (blank)]. *. Correlation is significant at the 0.05 level, **. Correlation is significant at the 0.01 level. **(F)** CCK-8 assay was performed in RKO cells transfected with shWASH5P (WASH5P shRNA) or shNC (control shRNA). **. Correlation is significant at the 0.01 level. **(G–I)** *In vitro* invasion and migration assays were performed using HCT116, SW480, and RKO cells. Cells were photographed under a light microscope and counted from five random microscopic fields (×200) per insert in triplicate. **. Correlation is significant at the 0.01 level, ***. Correlation is significant at the 0.001 level. Scale bars represent 100 μm. **(J)** Protein level of epithelial marker (E-cadherin) and mesenchymal marker (vimentin) were measured by Western blot in HCT116 and SW480 cell after WASH5P transfection, compared with vector; Immunoblots indicated the expression levels of epithelial (E-cadherin) and mesenchymal (vimentin) markers in RKO cells after being transfected with shWASH5P and shNC. GAPDH was used as a loading control.

WASH5P Plays an Essential Role in Inhibiting the Development of CRC Through the AKT Pathway

We have found that lncRNA WASH5P was enriched in the hallmark PI3K-AKT pathway in Gene Set Enrichment Analysis analysis (**Figure 1E**). PI3K-AKT is a classical signaling pathway that is involved in CRC tumorigenesis, so we further determined the relationship and mechanisms involved in WASH5P and CRC. Western blot and summarized data suggested that the overexpression of WASH5P in HCT116 and SW480 could significantly inhibit AKT pathway activation *via* suppressing AKT phosphorylation (**Figures 3A, B**). Therefore, we speculate that the AKT pathway might be a potential downstream regulator of WASH5P in CRC tumorigenesis. In order to confirm this, we then treated the WASH5P-overexpressed cells and the control group with 740Y-P, a specific activator of PI3K, to activate the PI3K/AKT pathway. Our data suggested that 740Y-P treatment could restore WASH5P-reduced CRC cell viability in WASH5P-overexpressed cells (**Figure 3C**). Moreover, 740Y-P abolished the inhibitory effects of WASH5P on cell migration and invasion in WASH5P-overexpressed cells *via* the cell migration assay and cell invasion assay (**Figures 3D–F**). In addition, 740Y-P could rescue the

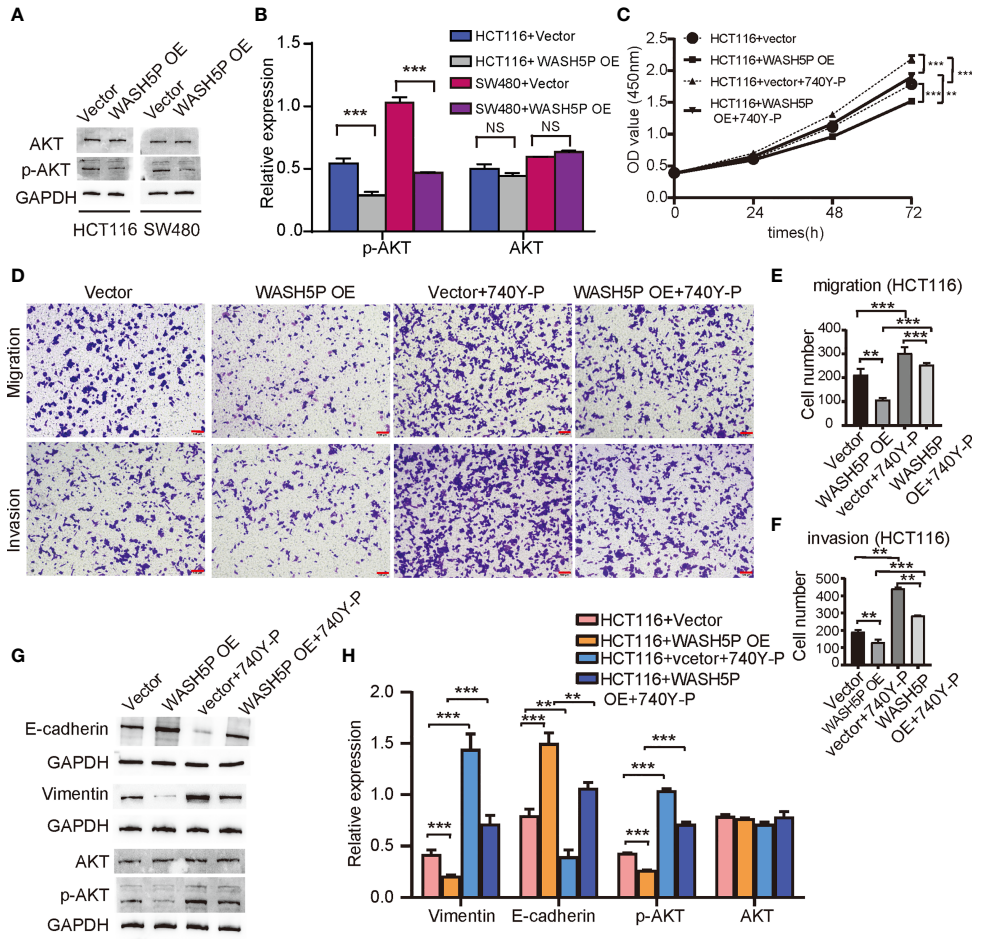

FIGURE 3 | LncRNA WASH5P plays an essential role in inhibiting the development of CRC through the AKT pathway. **(A, B)** Western blot analysis and summarized data showed that the expression of p-AKT was downregulated in HCT116 and SW480 cells transfected with lncRNA WASH5P compared with a vector. ***. Correlation is significant at the 0.001 level. **(C)** CCK-8 assay was performed in HCT116 cells transfected with lncRNA WASH5P or vector. 740Y-P was added to the medium and cultured for 24 h. Cell viability was measured from an absorbance reading at 450 nm. Data were expressed as relative viability (%) calculated: [A450(treated)-A450 (blank)]. **. Correlation is significant at the 0.01 level, ***. Correlation is significant at the 0.001 level. **(D–F)** Transwell Matrigel invasion assays and Migration assays were performed in HCT116 cells transfected with different viruses as indicated. Cells were observed under a light microscope and photographed. Cells were counted from five random microscopic fields (×200) per insert in triplicate. The migrated cell numbers were normalized to that of the control group. Data are shown as mean ± SD from three separate experiments. **. Correlation is significant at the 0.01 level, ***. Correlation is significant at the 0.001 level. Scale bars represent 100 μm. **(G, H)** Immunoblots and summarized data showed the expression of AKT, p-AKT, epithelial marker (E-cadherin), and mesenchymal marker (vimentin) in shWASH5P or shNC transfected HCT116 cells after 740Y-P treatment. GAPDH was used as a loading control. Data are shown as mean ± SD from three separate experiments. **. Correlation is significant at the 0.01 level, ***. Correlation is significant at the 0.001 level.

WASH5P-induced downregulation of p-AKT and vimentin and the upregulation of E-cadherin *via* Western blot (**Figures 3G, H**).

All these results demonstrated that WASH5P could inhibit the development of CRC *via* the AKT signal pathway.

WASH5P Significantly Inhibits CRC Carcinogenesis *In Vivo*

To further identify the involvement of WASH5P *in vivo*, we established the xenograft tumor model. WASH5P overexpression could inhibit tumor growth in the mouse model when compared with the control group (**Figures 4A–C**). Furthermore, we analyzed the expression of vimentin, E-cadherin, p-AKT, and AKT in the tumor mass and found that vimentin and p-AKT were decreased in the WASH5P-overexpressed group compared to normal control (**Figures 4D, E**). These data suggested that the overexpression of WASH5P inhibited CRC progression *via* regulating the AKT pathway *in vivo*.

In addition, we evaluated the effect of WASH5P on CRC in the metastatic mouse model. WASH5P overexpression could significantly inhibit CRC lung metastasis when compared with the control group *via* H&E staining (**Figures 4F–K**), indicating that WASH5P could inhibit CRC metastasis *in vivo*.

DISCUSSION

Emerging pieces of evidence showed that lncRNAs could function as important modulators in carcinogenesis, and

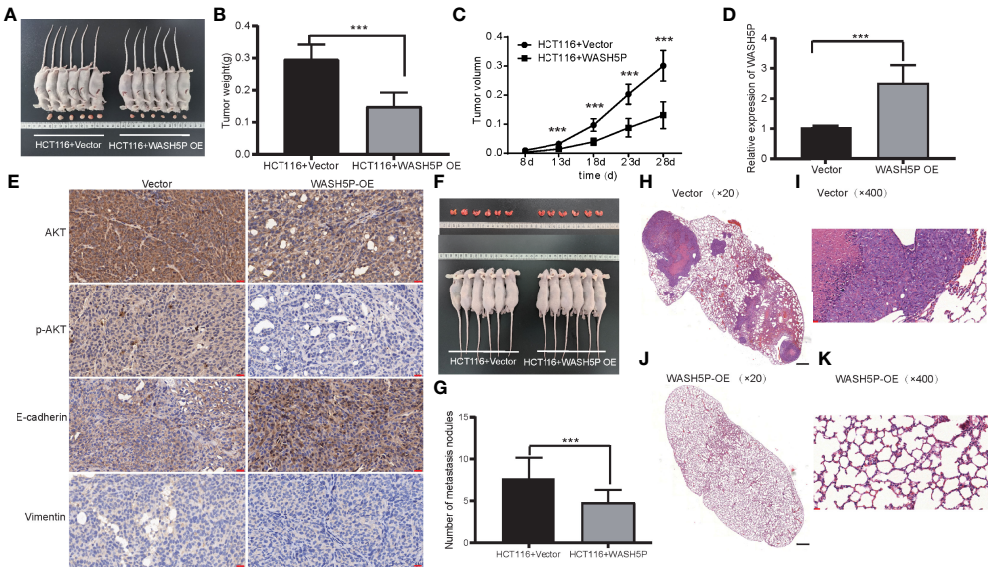

FIGURE 4 | LncRNA WASH5P significantly inhibits CRC *in vivo*. **(A)** Mice carrying xenograft tumors treated with lncRNA WASH5P (n=6, right panel) or vector (n=6, left panel) at the last time point. **(B)** The xenograft tumors were separated and weighted at the end of the experiments. ***. Correlation is significant at the 0.001 level. **(C)** Tumor volume was measured in lncRNA WASH5P or vector groups. Data represent the mean with a 95% confidence interval. ***. Correlation is significant at the 0.001 level. **(D)** Expression level of WASH5P was confirmed by real-time PCR in the xenograft tumors of the WASH5P-overexpressed group and vector group. ***. Correlation is significant at the 0.001 level. **(E)** Immunohistochemical analysis revealed the expression of AKT, pAKT, epithelial (E-cadherin), and mesenchymal (vimentin) markers in the tumors of different groups. Original magnification: ×200. Scale bars represent 20 μm. **(F)** Pulmonary images of mice in a metastatic mouse model treated with vector (n=6, left panel) or lncRNA WASH5P (n=6, right panel) at the last time point. **(G)** Summarized data of tumor lung nodules in nude mice at 45 days in lncRNA WASH5P or vector groups; n = 6. ***. Correlation is significant at the 0.001 level. **(H–K)** Representative H&E images of the lungs of nude mice metastasis at 45 days in two groups: vector group **(H, I)** and WASH5P-overexpressed group **(J, K)**. The black scale bars in H and J represent 500 μm. The red scale bars in I and K represent 20 μm.

emerge as oncogenes or tumor suppressors, *via* target genes or signaling pathways (9, 14, 16). Specific lncRNAs in clinical samples could serve as novel biomarkers or therapeutic targets and contribute to tumor diagnosis and better clinical outcomes (17–20).

To figure out the potential lncRNAs in CRC, we performed bioinformatics analysis based on the TCGA database, and the results showed that WASH5P was dramatically downregulated in CRC. Right now, no studies have been reported about the role of lncRNA WASH5P in any disease.

LncRNA WASH5P is one of the newest lncRNAs with a length of 400 bp and is mainly located in the cytoplasm. In our present study, we showed that lncRNA-WASH5P regulated the proliferation, migration, and invasion process of CRC. Firstly, WASH5P was downregulated in CRC tissues compared to adjacent controls. Secondly, WASH5P was decreased in four CRC cell lines. Thirdly, the silence of WASH5P could aggregate CRC cell proliferation, invasion, and migration *via* the AKT pathway. Additionally, the overexpression of WASH5P inhibits this phenomenon. Finally, the overexpression of WASH5P in HCT116 could inhibit CRC proliferation and invasion *in vivo*. All the above results indicated the therapeutic potential of WASH5P in CRC.

The pivotal role of the PI3K/AKT pathway in carcinogenesis has received much attention (21, 22). The PI3K/AKT pathway could participate in cell proliferation, differentiation, angiogenesis, and invasion in CRC (23, 24). More and more sheds of evidence have emphasized that lncRNAs could promote CRC carcinogenesis *via* activating the PI3K/AKT pathway (16, 25, 26). In the present study, Gene Set Enrichment Analysis analysis showed that lncRNA WASH5P was enriched in the hallmark PI3K-AKT pathway. The overexpression of WASH5P in CRC cells could significantly inhibit AKT pathway activation. The further functional experiment revealed that 740Y-P could restore the expression of p-AKT and promote CRC in WASH5P-overexpressed cells. Thus, our result provided evidence that WASH5P could inhibit CRC *via* targeting AKT signaling, indicating the possible mechanism of this potential biomarker.

Interestingly, emerging pieces of evidence showed that lncRNAs could be used as drugs to treat cancer due to their biological functions (18, 27). For example, lncRNA MEG3 was combined with exosome through engineering technology and used to treat osteosarcoma animal models (28). Furthermore, pieces of evidence were provided in clinical studies. A phase I/II clinical trial showed that BC-819, a plasmid with an lncRNA H19 promoter, showed 22% complete response rates and 44% partial response rates in treating bladder cancer (29). Antisense oligonucleotides (ASOs) containing lncRNAs were shown to be successful in treating neurodegenerative diseases both in preclinical studies and human clinical trials (30). In addition,

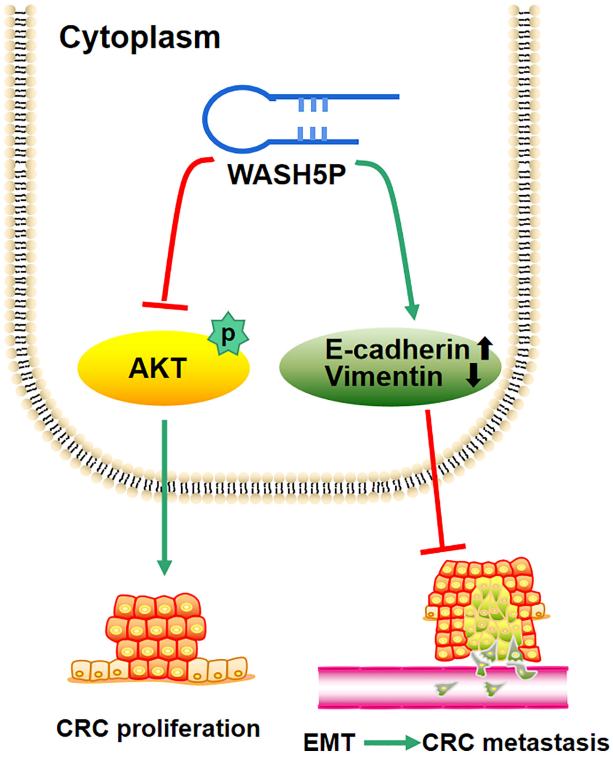

FIGURE 5 | Schematic model of the role of lncRNA WASH5P in colorectal cancer. lncRNA WASH5P could inhibit CRC *via* repressing the AKT signaling pathway and EMT.

the clinical trials of ASO- targeting lncRNAs have shown a promising therapeutic effect in advanced unresectable solid tumors (NCT02508441 (31)/NCT03985072 (32)).

In summary, the present study supplies the new evidence that the tumor suppressor WASH5P could inhibit CRC cell proliferation, invasion, and migration through the AKT signaling pathway both *in vitro* and *in vivo* (**Figure 5**). Therefore, the detection and targeting of WASH5P could monitor and suppress CRC development, providing a new potential biomarker and therapeutic target for CRC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics committee for animal experiments of the affiliated hospital of Qingdao university.

AUTHOR CONTRIBUTIONS

HW, ZT, and LR designed this research. HW, TM, and QZ performed the analyses and prepared the manuscript. HW, KR, YZ, and XQ performed literature search and the initial analyses. HW, BC, TM, YJ, and LR performed analyses and data interpretation. HW and LR edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Interactions of Colorectal Cancer, Dietary Fats, and Polymorphisms of Arachidonate Lipoxygenase and Cyclooxygenase Genes: A Literature Review

Maryam Gholamalizadeh[1], Nazanin Majidi[2], Shirin Tajaddod[2], Sepideh Abdollahi[3], Seyed Mohammad Poorhosseini[4], Mina Ahmadzadeh[5], Mohammad Naimi Joubani[6], Samaneh Mirzaei Dahka[7], Hanieh Shafaei[7], Mogge Hajiesmaeil[8], Atiyeh Alizadeh[9], Saeid Doaei[10] and Anahita Houshiar-Rad[11]**

[1] Cancer Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Department of Nutrition, Science and Research Branch, Islamic Azad University, Tehran, Iran, [3] Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran, [4] Department of Medical Genetics, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [5] Department of Clinical Nutrition and Dietetics, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [6] Research Center of Health and Enviroment, School of Health, Guilan University of Medical Sciences, Rasht, Iran, [7] Nursing and Midwifery School, Guilan University of Medical Sciences, Rasht, Iran, [8] Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Rome, Italy, [9] Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, [10] Reproductive Health Research Center, Department of Obstetrics and Gynecology, School of Medicine, Al-Zahra Hospital, Guilan University of Medical Sciences, Rasht, Iran, [11] Dept. of Nutrition Research, Faculty of Nutrition Sciences and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Objective: Genetics and dietary factors play important roles in the development of colorectal cancer (CRC). However, the underlying mechanisms of the interactions between CRC, gene polymorphisms, and dietary fat are unclear. This review study investigated the effects of polymorphisms of arachidonate lipoxygenase (*ALOX*) and cyclooxygenase (*COX*) genes in the association between CRC and dietary fat.

Methods: All the related papers published from 2000 to 2022 were collected from different databases such as PubMed, Science Direct, Scopus, and Cochran using related keywords such as colorectal cancer, *ALOX*, *COX*, polymorphism, and dietary fat. Non-English and unrelated documents were excluded.

Results: Some single-nucleotide polymorphisms (*SNPs*) in the *ALOX* and *COX* genes, such as rs2228065, rs6413416, and rs4986832 in the *ALOX* gene, and rs689465 in the *COX* gene may play significant roles in the association between the risk of CRC and dietary fats. SNPs of ALOX and COX genes may influence the effects of dietary fatty acids on the risk of CRC.

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***Correspondence:**
Saeid Doaei
Doaei@gums.ac.ir
Anahita Houshiar-Rad
sdoaei@sbmu.ac.ir

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Conclusion: Some polymorphisms of the *ALOX* and *COX* genes may have important roles in the effects of dietary fat on the risk of CRC. If future studies confirm these results, dietary recommendations for preventing colorectal cancer may be personalized based on the genotype of the *ALOX* and *COX* genes.

Keywords: colorectal cancer, polymorphism, dietary fat, lipoxygenase, cyclooxygenase

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death in women and the third in men worldwide (1) and cause about 0.9 million deaths worldwide in 2020 (2). It has been reported that CRC originates from a combination of genetic, environmental, and behavioral risk factors. Some behavioral factors are associated with dietary intake, including higher intake of calories, red meat, and fats (3, 4).

Recently, various types of fatty acids have been reported as effective dietary factors in CRC development. Some fatty acids, such as saturated fatty acids, may have an adverse effect, whereas other fatty acids, such as omega-3 fatty acids, may have a beneficial effect on CRC prevention (5–7). One main mechanism through which dietary polyunsaturated fatty acids (*PUFAs*) may affect colonic carcinogenesis is the formation of specific eicosanoids (oxygenated metabolites of *PUFAs*) such as prostaglandins (*PGs*), thromboxanes (*TXs*), leukotrienes (*LTs*), and lipoxins (*LXs*) (7). Two enzymatic pathways related to the synthesis of these eicosanoids are the arachidonic lipoxygenase (*ALOX*) pathways and prostaglandin-endoperoxide synthase (*PTGS*), which are also known as cyclooxygenase (*COX*) pathways (8). The function of ALOX enzymes, such as ALOX5, ALOX12, and ALOX15, eventually leads to LT and LX formation, and the COX enzymes, like COX1 and COX2, result in the production of PGs and TXs (9, 10). Evidence has shown that changes in the sequence of *COX* and *ALOX* genes as single-nucleotide polymorphisms (*SNPs*) can influence the risk of CRC (8).

In terms of *ALOX*, previous research has established that *ALOX*15 expression and concentration of eicosanoic metabolites are reduced in polyps and colorectal tumors in humans (11). In contrast, increased *ALOX*5 expression has been reported in colorectal cancer cells (12). Moreover, it has been reported that some mutations in *ALOX*12 are associated with tumorigenesis in epithelial cancers (13). Recent studies have identified that the expression level of the *COX2* gene and the levels of its metabolites, such as PGE_2, PGD_2, and $PGF2\alpha$, are significantly increased in the colon of obese mice. Also, it has been shown that the administration of *COX2* inhibitors can suppress inflammation, tumor growth, and tumor metastasis (14–16).

Notably, the effect of dietary fats on the risk of CRC may be influenced by gene polymorphisms (17–19). However, the interactions between CRC, dietary fat, and gene polymorphisms are still unknown. So, this review study investigated the effects of SNPs of the *ALOX* and *COX* genes on the association between dietary fats and CRC risk.

MATERIALS AND METHODS

Search Strategy

The literature search was performed using the PubMed, Science Direct, Scopus, and Cochran databases, and all related papers published from 2000 to 2022 were collected using the following keywords: "dietary fat or fatty acid or fat or lipid" and "*ALOX* or *COX* or prostaglandin-endoperoxide synthase or *PTGS* or cyclooxygenase or *COX* or arachidonic lipoxygenase or lipoxygenase" and "colorectal cancer or colon cancer or rectal cancer" and "polymorphism or genetic variation or genotype or *SNP*." All the collected papers and their references were reviewed.

Inclusion and Exclusion Criteria

All studies that examined the interaction of colorectal cancer with *ALOX* and *COX* genes, studies concerning the relationship between the *ALOX* and *COX* gene polymorphisms, and studies on the interactions between colorectal cancer, *ALOX* and *COX* genes, and dietary fat were included in this study. Unrelated and non-English papers, the review studies, studies on the relationship between *ALOX* and *COX* with other cancers, and animal studies were excluded.

Assessment of Methodological Rigor

In this review study, the quality of the collected studies was assessed by four researchers (MG, HS, SA, and SD). In the case of having opposing ideas, other researchers (MH and HS) would be involved in reaching an agreement. After collecting the papers, all unrelated studies were excluded from the review process according to their titles and abstracts. Then, the full texts of the relevant articles were studied precisely. The standard quality assessment method of the 'EPOC Risk of Bias Tool' was applied to assess the quality of the methodologies (20). The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (*PRISMA*) checklist (21) was used to extract the required data from the included studies. Finally, the data about the participants, intended comparisons, obtained results, and study planning (*PICOS*) were collected.

RESULTS

Description of the Identified Studies

The process of including the appropriate studies is presented in **Figure 1**. A total of 413 articles were collected in the primary search, of which 354 articles were excluded after the screening of their titles and abstracts. Also, 28 articles were excluded after

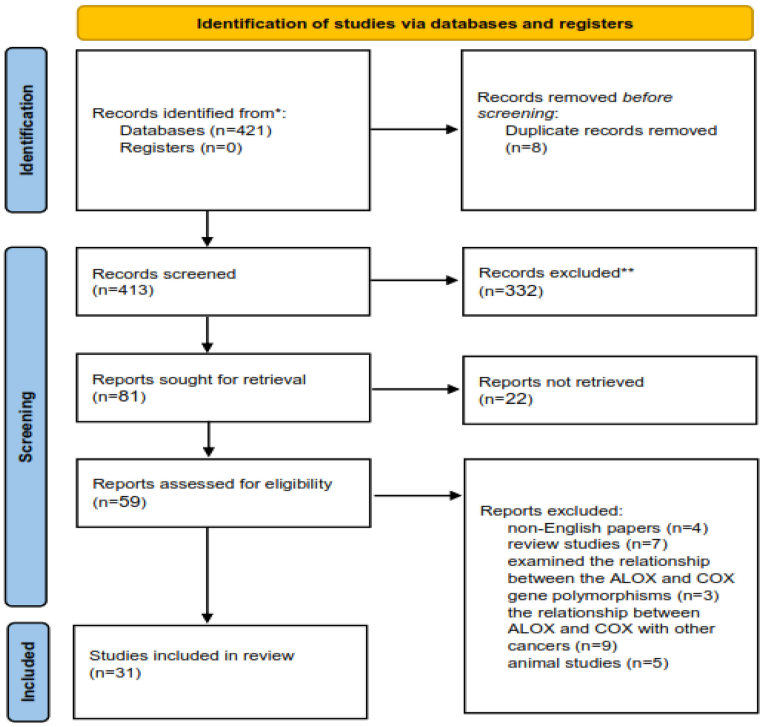

FIGURE 1 | The process of including the appropriate studies.

reading the full texts. Finally, 31 articles qualified to be included in the review process. All articles were published from 2000 to 2022 and were related to the interactions between *CRC, ALOX*, and *COX* genes and fat intake. The main characteristics of the studies are presented in **Tables 1**, **2**.

Arachidonate Lipoxygenase (*ALOX*) Gene Polymorphisms and Risk of CRC

The primary function of *ALOX* is to convert arachidonic acid (*AA*) into hydroperoxyeicosatetraenoic acid (*HPETE*) and eventually leukotrienes, a class of paracrine hormones involved

TABLE 1 | Summary of the studies related to *ALOX* gene polymorphisms and CRC risk.

Study	Ethnicity	Study design	Case/Control	Polymorphisms	Main finding
Goodman et al. (8)	African-Americans and Caucasians	Case–control study	468 cases and 304 controls	rs6413416, rs4986832 and rs2228065 in *ALOX*5, and rs1126667 in *ALOX*12	This study found that a haplotype including *ALOX*5 rs6413416 and rs4986832 was associated with decreased colorectal cancer risk in Caucasians.
Kleinstein et al. (22)	American	Case–control study	Colon cancer (1,424 cases/1,780 controls) rectal cancer (583 cases/775 controls), colorectal adenomas (485 cases/578 controls)	Four SNPs in FLAP (rs17239025), *ALOX* 12 (rs2073438), and *ALOX*15 (rs4796535 and rs2619112)	*ALOX12* (rs2073438) was associated with a lower risk of rectal cancer. *ALOX15* (rs4796535 and rs2619112) was associated with an increased risk of rectal cancer.
Tan et al. (23)	Chinese	Case–control study	1,000 cases and 1,300 controls	*ALOX12* (rs1126667)	*ALOX 12* rs1126667 was associated with a moderately increased risk of CRC.
Poole et al. (24)	Minnesota	Case–control study	517 adenomatous or 192 hyperplastic polyps versus 618 polyp-free controls	*ALOX*5 (rs4986832)	ALOX5 rs4986832 polymorphism did not have any association with the risk of colorectal polyps.
Ruan et al. (25)	China	Cross-sectional	438 tumor tissue samples and 41 adjacent tissue samples	*ALOX* gene family expression (*ALOX*E3, *ALOX*5, *ALOX*12, and *ALOX*12B)	The ALOX12 mRNA expression could be a diagnostic marker for colon adenocarcinoma and the expression of ALOXE3 combined with ALOX12 could have a prognostic value in colon adenocarcinoma.

ALOX, Arachidonic Acid Lipoxygenase; FLAP, Arachidonate 5-lipoxygenase-activating protein; CRC, Colorectal cancer.

TABLE 2 | Summary of the studies related to *COX* gene polymorphisms and CRC risk.

Study	Ethnicity	Study design	Case/Control	Polymorphisms	Main finding
Lin et al. (26)	African-American, Chinese (Hong Kong), Filipino, Hispanic, Indian (Asian), Japanese, Korean, Samoan, and Caucasian.	Case–control study	299 cases and 477 controls	V511A (rs5273) in PTGS2 (*COX*2)	The *COX*2 rs5273 polymorphism may reduce the risk of CRC in African-Americans
Cox et al. (27)	Chinese	Case–control study	292 cases and 272 controls	COX2 rs4648298, rs689469, rs689165, rs20417, rs20424, rs5277, rs20432, rs5275	COX2 rs4648298 and rs689469 polymorphisms had an association with an increased risk of CRC
Mosallaei et al. (28)	Isfahan, Iran	Case–control study	88 cases and 88 controls	COX2 rs4648298 polymorphism	There was a significant relationship between AA genotype and CRC risk reduction in the Iranian population (OR=0.14; 95% CI, 0.05-0.34; P <0.001).
Ulrich et al. (29)	American	Case–control study	680 cases and 584 controls	*COX*2 (rs20417)	The allele frequencies of *COX*2rs20417reduced the risk of CRConly among non-users of NSAIDs.
Hoff et al. (30)	Caucasian	Case–control study	326 cases and 369 controls	The *COX*2 rs20417 and rs689466	The -765GG genotype (rs20417) increased CRC risk, while GG/AC haplotype (rs20417) decreased CRC
Xing et al. (31)	Asian	Case–control study	137 cases and 199 controls	*COX*2 rs20417	COX2 rs20417 polymorphism appears to be related to an increased risk of CRC in the smoker.
Ueda et al. (32)	Winston-Salem and Charlotte, North Carolina	Case–control study	162 incident, sporadic colorectal adenoma cases and 211 controls	COX2 (765G>C, 8473T<C, 9850 A>G),COX1 (842 A<G)	COX2 8473T>C can reduce the CRC risk in individuals who consume NSAIDs drugs
Shomaf et al. (33)	Caucasian	Case–control study	239 cases and 115 controls	*COX*2 rs689466	COX2 rs689466 polymorphism may have a protective role against the risk of CRC.
Peters et al. (34)	Caucasian	Case–control study	85 cases and 218 controls	*COX*2 rs689466	There was overexpression of COX2 rs689466 GG genotype compared with AA genotype in patients with FAP.
Pereira et al. (35)	Caucasian	Case–control study	246 cases and 480 controls	*COX*2 rs689466	There was a nearly 6-fold increased CRC risk in smoker individuals with COX2 rs689466.

PTGS2, Prostaglandin-Endoperoxide Synthase 2; COX2, Cyclooxygenase; CRC, Colorectal cancer; NSAIDs, Non-steroidal anti-inflammatory drugs; FAP, Familial adenomatous polyposis.

in the inflammatory response. For example, *ALOX*12 converts AA into 12-hydroperoxyeicosatetraenoic acid (*12-HPETE*), which is involved in the expression of pro-inflammatory cytokine genes such as tumor necrosis factor-α (*TNF-α*) (36). The role of the *ALOX* gene in inflammatory diseases and colorectal neoplasia has been frequently reported. For example, the arachidonate-5 lipoxygenase (*ALOX*5) and 12-lipoxygenase (*ALOX*12) played pro-carcinogenic roles in colorectal cancer (22). Additionally, overexpression of *ALOX*5 with its related downstream metabolites has been reported in other cancers such as breast, esophageal, pancreatic, and prostate cancers by stimulation of cell proliferation, tumor angiogenesis, and survival (37). Moreover, it has been reported that *ALOX*15 is associated with an increased risk of colorectal cancer, particularly in people with higher inflammatory factors (38). In another study, Ruan et al. examined the diagnostic and prognostic values of the *ALOX* gene family mRNA expression in 438 colon adenocarcinoma tumor samples and 41 adjacent tissue samples of Chinese patients by bioinformatics analysis. They showed that the expression level of *ALOX*E3, *ALOX*5, *ALOX*12, and *ALOX*12B was upregulated in colorectal tumor samples. Finally, they reported that *ALOX*E3 and *ALOX*12 might serve as potential independent prognostic indicators of colon adenocarcinoma (25). Thus, *ALOX* pathways in the *AA* metabolism process can be considered crucial pathways in the development of CRC (**Figure 2**).

Notably, specific polymorphisms of the *ALOX* gene can affect the susceptibility to CRC. For instance, Goodman et al. assessed the effects of *ALOX*5 and *ALOX*12 gene polymorphisms on CRC in African-Americans and Caucasian patients. They found that the rs6413416 and rs4986832 polymorphisms of *ALOX*5 were associated with a decreased risk of CRC in Caucasians. They hypothesized that these polymorphisms could improve binding to the promoter region, leading to downregulation of *ALOX*5. In this way, they can lower the cancer risk by reducing enzymatic activity (8). However, the rs4986832 polymorphisms of *ALOX*5 had no association with the risk of colorectal polyps in Minnesota (24). Kleinstein et al. conducted a study on 2447 cases and 3133 controls regarding the effect of *ALOX* gene polymorphisms on the risk of CRC. The results showed that the rs2073438 polymorphism of *ALOX* 12 was related to a lower risk of rectal cancer (OR = 0.66, 95% CI: 0.42–1.04), while the rs4796535 and rs2619112 polymorphisms of *ALOX*15 were associated with an increased risk of rectal cancer (OR = 1.43,

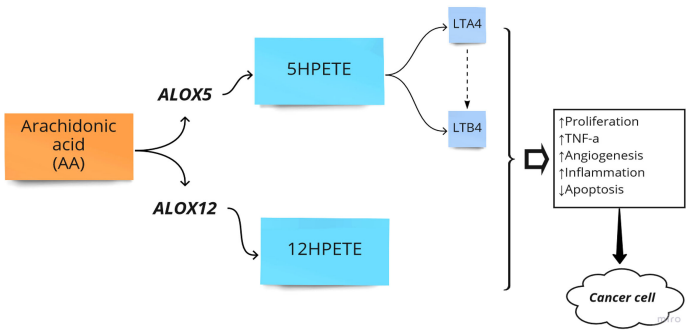

FIGURE 2 | Arachidonate lipoxygenase (ALOX) in the metabolism of Arachidonic Acid (AA). HPETE, hydroperoxyeicosatetraenoic acid; LT, Leukotriene; ↑, Increase; ↓, Decrease.

95% CI: 1.03–1.97 and OR = 1.13, 95% CI: 0.85–1.55, respectively) (22). Moreover, a positive association was found between the *ALOX*12 rs1126667 polymorphism and a moderately increased risk of CRC (OR = 1.38, 95% CI: 1.09–1.74) (23). However, the association between rs1126667 polymorphism of *ALOX*12 and the risk of CRC has been reported in African-Americans and Caucasian patients (8). This discrepancy can be due to differences in ethnic or statistical power. A summary of studies on the association between *ALOX* polymorphisms and CRC is provided in **Table 1**.

Cyclooxygenase (*COX*) Gene Polymorphism and Risk of CRC

Prostaglandin H synthase, also known as cyclooxygenase and prostaglandin-endoperoxide synthase (*PTGS*), catalyzes the first step in the biosynthesis of all prostaglandins and prostacyclins by converting arachidonic acid to prostaglandin H (39). Two forms of human *PTGS*, *PTGS1* and *PTGS2* (*COX1* and *COX2*), can be inhibited by non-steroidal anti-inflammatory drugs (*NSAIDs*). Also, the end products of *COX* are related to various biological pathways in stimulating tumor growth (26) (**Figure 3**).

Prostaglandins are upregulated in colorectal cancer, and it was reported that genetic polymorphisms in both *COX1* and *COX2* are associated with CRC (27, 37). *COX2* is involved in cell cycle control, and increased expression of *COX-2* in CRC patients compared to normal controls indicates its possible role in the progression of CRC (40). *COX2* influences cancer progression by increasing prostaglandin production, preventing tumor cell apoptosis, cell proliferation, and tumor angiogenesis (41). It was reported that aspirin plays a key role in preventing colon cancer by inhibiting *COX* (37). Ayiomamitis et al. examined the expression of *COX1*, *COX2*, prostaglandin-endoperoxide synthase 3 (*PTGES3*), and telomerase reverse transcriptase (*TERT*). They used bioinformatics analysis on the Cancer Genome Atlas Colon Adenocarcinoma (*TCGA-COAD*) and rectal adenocarcinoma (*READ*) datasets. The results showed an inverse relationship between *COX2* expression and telomerase activity in CRC. In the end, they identified differentially methylated patterns within the promoter regions of *COX2* and *TERT* (42). Joanna et al. observed *COX2* overexpression in the early stages of colorectal cancer and higher *COX2* gene expression in the advanced stages of the disease. The results also indicated that *COX2* expression level could affect carcinogenicity by modulating local inflammation (43). In addition, a case–control study on Iraqi patients reported that the expression level of *COX2* was upregulated at higher tumor grades (44). This result suggests that considering *COX2* as an early marker of progression or initiation of colorectal carcinoma should be investigated by further studies. Moreover, Labda et al. found that *COX2* expression was associated with tumor size and degree of differentiation in an observational study including 58 Indonesian CRC patients. However, there was no statistical correlation between *COX2* expression and tumor location (45). Jin et al. conducted a case-control study involving 213 Chinese patients with colorectal cancer and 200 controls and reported that the expression level of *IGF-IR* and *COX2* was directly related

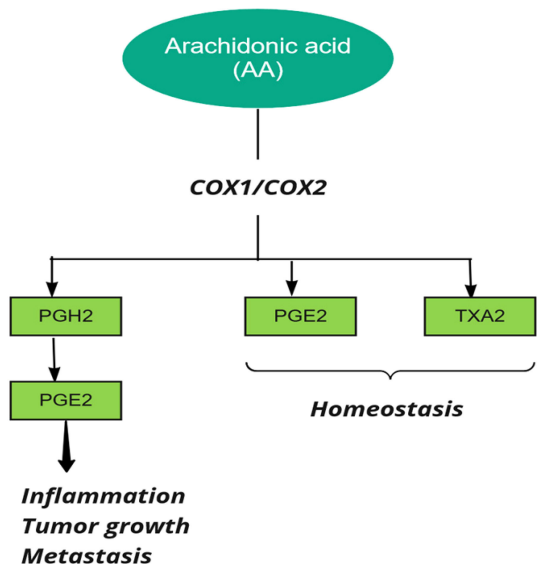

FIGURE 3 | Cyclooxygenase (COX) in metabolism of Arachidonic Acid (AA). PG, Prostaglandin, TX, Thromboxane.

to the degree of progression and lymphatic metastasis and inversely related to the mean survival rate in CRC patients (46). The results of a Chinese study also indicated that PGE_2 and *COX2* expression were significantly associated with tumor invasion, tumor differentiation, lymph node metastasis, and *TNM* stage and were inversely related to patient survival (47).

Polymorphisms of the *COX* gene can affect the risk of *CRC*. In this regard, Lin et al. found that the *COX2* rs5273 polymorphism, in about 5% of African Americans, was associated with a lowered risk of CRC (OR = 0.78, 95% CI: 0.49–1.23) (26). The *COX2* rs4648298 and rs689469 polymorphisms were reported to be associated with an increased risk of CRC. Analysis of haplotypes confirmed that people with these variants were at an increased risk of colorectal cancer (OR = 2.17, 95% CI: 0.97–4.84, P = 0.06) (27). In contrast with these results, Mosallaei et al. observed a significant relationship between *COX2* rs4648298 polymorphism (*AA* genotype) and a reduced risk of CRC in the Iranian population (OR = 0.14; 95% CI: 0.05–0.34; P <0.001). Interestingly, they found this significant association only in non-smokers (28). This finding suggests that environmental factors may influence the association between the *COX* gene polymorphism and CRC. Another study showed that the *GG* genotype of *COX2* rs20417 was associated with an increased risk of developing CRC in the Dutch population (OR, 1.45; 95% CI, 1.03–2.04) (30). Interestingly, Xing et al. observed the positive association between the *GG* genotype of the *COX2* rs20417 polymorphism and increased CRC risk in China, especially in smokers and in people with a high Body Mass Index (*BMI*) (OR: 1.107, 95% CI: 1.107–3.726; P = 0.022) (31). In this line, the Minnesota-based case-control study discovered that *COX2* gene expression or COX*2* enzyme activity is suppressed and the risk of colorectal polyps is reduced by NSAIDs in individuals with the GG genotype of *COX2* rs20417 (OR: 0.66; 95% CI: 0.48–0.92) (29). Ueda et al. investigated the association between the *COX2* position 765 G<C, 8473 T>C, and 9850 A>G and CRC risk and reported that among the studied polymorphisms, *COX2* 8473T>C may reduce the CRC risk in people who consume *NSAIDs* (OR: 1.57, 95% CI: 1.04–2.38) (32). Another previous study on 104 cases of adenomatous polyps and 115 matched control samples found that *COX2* rs689466 polymorphism may have a protective effect on the risk of development of CRC (33). In contrast, Peters et al. identified that overrepresentation of *COX2* was associated with a high risk for CRC development in patients with familial adenomatous polyposis (*FAP*) who had the rs689466 polymorphism *GG* genotype compared with *AA* genotype carriers (OR = 2.81; 95% CI = 1.00–7.91, P= 0.042) (34). In addition, Pereira et al. suggested that smoker people with *COX2* rs689466 polymorphism had a nearly 6-fold increased CRC risk compared with people without rs689466 risk allele (95% CI: 1.49–22.42, P = 0.011) (35). Some reasons for these conflicting results on the association between *CRC* and *COX* gene can be due to effects of different environmental factors such as lifestyle on this association. **Table 2** presents the summary of studies regarding *COX* polymorphisms and CRC risk.

Interaction Between CRC, *ALOX* and *COX* Polymorphisms, and Dietary Fatty Acids

COX enzymes (*COX1*, *COX2*) are important factors in the biosynthetic pathway of *PGs* from *AA*. *ALOX* enzymes (*ALOX5*, *ALOX12*, and *ALOX15*) convert *PUFA* to fatty acid hydroperoxides, which results in the production of *LTs*. Recent studies reported an association between the CRC risk with the amount of fatty acids intake and *COX* and *ALOX* polymorphisms (**Figure 4**). Habermann et al. identified an association between *COX1* rs10306110 polymorphism and low intake of docosahexaenoic acid (*DHA*), a fatty acid with anti-inflammatory properties, with an increased risk of colon cancer (OR = 1.6, 95% CI: 1.1–2.3, adjusted *P* = 0.06) (38).

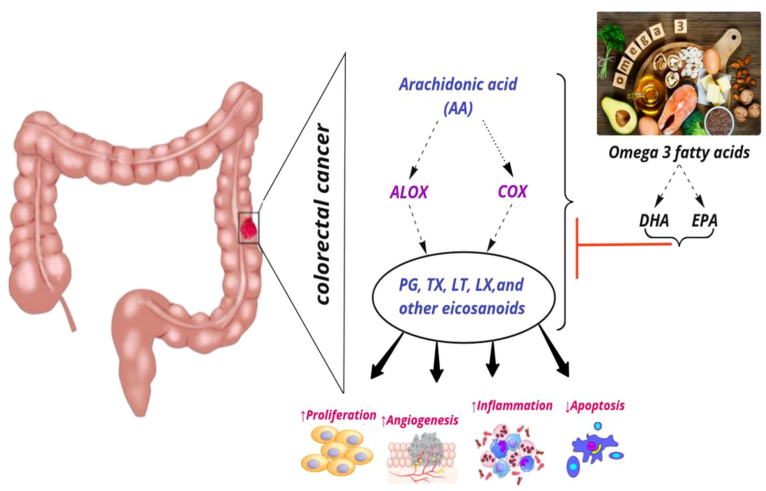

FIGURE 4 | Interaction among dietary fatty acids, ALOX (Arachidonate lipoxygenase) and COX (Cyclooxygenase) in metabolic pathway of AA (Arachidonic Acid), and CRC (colorectal cancer) risk. PG, Prostaglandin; TX, Thromboxane; LT, Leukotriene; LX, Lipoxin; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; ↑, increase; ↓, decrease.

Notably, supplementation with some fatty acids such as ω-3 fatty acids plays a protective role in colon cancer by attenuating the pro-inflammatory state and decreasing the production of PGE_2 (48). These results conform to studies that indicated that omega-3 long-chain polyunsaturated fatty acids (n−3 *LC-PUFA*) may lower cancer risk by suppressing oxidative stress, tumor apoptosis, and inflammatory pathways by modulation of *COX* activity and inhibition of arachidonic acid-derived eicosanoids (49–51). However, a case–control study on 310 patients with colorectal cancer and 1,177 controls provided epidemiological evidence for the possible link between *PGs* production from n−6 *PUFAs* through the enzymatic activity of *COX2* and increased risk of colon cancer. They reported an association between *COX2* rs20417 polymorphism and an increased risk of colon cancer in individuals with high n−6 *PUFA* intake (OR = 2.38, 95% CI = 1.23–4.59, P = 0.07). However, there was no association between this polymorphism and the risk of rectal cancer regardless of the dietary n−6 *PUFA* intake levels (52). These results emphasize the importance of lifestyle modification in the carriers of the high-risk allele of the *COX* gene. Moreover, Siezen et al. demonstrated that colorectal adenoma risk could be modified by the interaction between polymorphisms in *AA* pathway genes and fish consumption. They showed that the *COX2* rs5277 polymorphism in people with high fish consumption played a protective role against CRC compared with people with low fish intake (53). In another work, Siezen et al. confirmed the inverse association between high fish consumption and CRC risk. However, they could not find any significant interaction between CRC and *SNPs* in the genes involved in the *AA* pathway (54). Interestingly, another study indicated that the effects of n−3 *PUFA* intake and *NSAIDs* on CRC may differ in people with *COX1* polymorphisms. Among the wild-type homozygous individuals (*PP* genotype) with *COX1* rs3842787 polymorphism, high fish consumption and regular use of *NSAIDs* were associated with a decreased risk of CRC. In comparison, an inverse association was observed in individuals with at least one risk allele (PL, LL genotypes) in the *COX-1* rs3842787 polymorphism (55). Furthermore, dietary supplementation with n−3 *PUFA*, particularly *DHA* and *EPA*, was reported to have antineoplastic effects on CRC by modifying the epigenetic modification like *DNA* methylation (56). **Table 3** summarizes studies regarding the association between *ALOX*, *COX* polymorphism, dietary fatty acid, and CRC risk.

Regarding the interactions between *ALOX* and *COX* gene polymorphisms, Siezen et al. reported that these SNPs are associated with colorectal adenoma risk and that these associations are modified by fish consumption. No association was found between *SNP* rs5277 in the *COX2* gene and rs743646 in the *ALOX15* gene (53).

DISCUSSION

The results of this study indicated that some *SNPs* of the *ALOX* and *COX* genes can be associated with the interaction between dietary fats and the risk of CRC. The metabolizing effects of *ALOX* and *COX* enzymes on *AA* were reported to be associated with the production of carcinogenic factors in the colon (57). The association between *ALOX12* and colorectal neoplasia has been reported (22). However, Goodman et al. found that the *ALOX5* gene haplotype, including the rs6413416 and rs4986832 polymorphisms, was associated with a reduced risk of CRC in Caucasians. They assumed that these polymorphisms could augment the binding to the regulatory region of the promoter. Thus, attenuating the enzymatic activity could lead to a lower cancer risk. While this association was not observed in the African-American population, this inconsistency can be related to the existence of effective genetic or environmental factors in the African-American population (8).

Concerning the role of the *COX* enzyme in CRC risk, it has been reported that *COX2* is involved in the early stages of colon cancer development (42). Low *COX2* expression is observed in the early stages of colon cancer and *COX2* overexpression is more common in the advanced stages of the disease (43). *COX2* expression was significantly associated with CRC tumor invasion, tumor location, tumor size, degree of differentiation, and metastasis (45, 58). However, there was no significant relationship between *COX2* expression and the histological type of CRC (45). On the other hand, an inverse association was reported between CRC with *COX2* expression as well as methylation patterns within the promoter regions of *COX2* (42). Regarding the association between CRC and *COX*2 genotype, some studies found no association between rs20420 and rs5273 polymorphisms of the *COX2* gene and CRC risk (8, 59). In contrast, Lin and Schumaf reported the protective effect of *COX2* rs689466 and rs5273 polymorphisms against colorectal neoplasms (26, 33), and some other studies reported an increased risk of CRC carriers of some *COX2* polymorphisms such as rs689466 and rs20417 (31, 32, 35, 43). These conflicting results of the studies can be due to differences in ethnicity, environmental factors, and tumor type.

The role of polyunsaturated fatty acids (*PUFAs*) in the prevention of various types of malignancy, such as CRC, has been frequently reported (56). Recent studies found that dietary fatty acids may influence the association between CRC with ALOX and COX genes. For example, Habermann et al. indicated the effects of different fatty acid intake patterns on the association between colon cancer risk and *COX1* rs10306110 and *ALOX15* rs11568131 polymorphisms and also on the association between rectal cancer risk and *COX1* rs10306122 and *ALOX12* rs11571339 polymorphisms. They reported a possible increase in CRC risk among those with low intake of the marine sources of n−3 *PUFAs* such as *EPA* and *DHA* in people with a risk allele of *COX1* rs10306110 polymorphism (38). The evidence indicates that n−3 *LC-PUFA* may decrease cancer risk by suppressing oxidative stress, tumor apoptosis, and inflammatory pathways. They can decrease inflammation *via* the modulation of *COX* activity and inhibition of arachidonic acid-derived eicosanoids (49–51). It has also been reported that consuming higher n−3 fatty acids may reduce the production of pro-inflammatory eicosanoids, which may be involved in colon cancer (17). In this regard, Wilson et al. reported that ω-3 fatty acid

TABLE 3 | Summary of studies regarding interactions between *ALOX, COX* polymorphism, dietary fatty acid, and CRC risk.

Study	Ethnicity	Study design	Case/ Control	Polymorphisms	Main finding
Habermann et al. (38)	American	Case–control study	1,574 colon cancer and 791 rectal cancer and 2969 control	COX1 (rs10306110 and rs10306122), COX2 (rs4648276), ALOX15 (rs11568131)	There was a positive association between low intake of DHA and increased risk of colon cancer with COX1 rs10306110. There was a positive association between higher inflammatory score and increased risk of colon cancer with wild type ALOX15 rs11568131. There was an inverse association between low total fat intake and rectal cancer risk with COX1 rs10306122. There was an inverse association between low inflammatory score and rectal cancer risk with COX2 rs4648276.
Wilson et al. (48)	American	Cross-sectional study	90 participant	*PTGS*	Supplementation of some kind of fatty acids like ω-3 fatty acids can have a protective role in colon cancer by decreasing the production of PGE_2.
Koh et al. (52)	Asian	Nested Case–control study	310 colorectal cancer cases and 1177 controls	*COX2* rs20417	It was a statistically significant association between COX2 rs20417 polymorphism and CRC risk among high consumers of dietary n–6 PUFA.
Siezen et al. (53)	Netherlands	Case–control study	384 cases and 403 polyp-free controls	COX2 rs5277	COX2 rs5277 polymorphism in people with high consumption of fish had a protective role against CRC compared with people with low fish intake.
Siezen et al. (54)	Netherlands	Case–control study	508 cases and 772 controls	PTGS1 and PTGS2	Although there was a significant reduction in cancer risk for individuals with COX2 rs5277 in combination with high fish intake, no significant interaction was observed between the SNPs in genes involved in AA metabolism and fish intake.
Poole et al. (55)	Minneapolis	Case–control study	522 adenomas, 194 hyperplastic polyps and 626 polyp-free controls	COX -1 rs3842787	The results suggested that among individuals with the wild-type homozygous (PP) in COX1 rs3842787, increased fish consumption was associated with a slight reduction in the risk of adenoma, Whereas among people who had at least one different allele (LL, PL) in COX1 rs3842787, an inverse association was observed.
Sarabi et al. (56)	Shiraz, Iran	Cell culture	5 human CRC cell lines	Polyunsaturated fatty acids DNA methylation (DNMT)	PUFA significantly suppressed DNMT3a and DNMT3b expression in SW742 cells ($p < 0.05$) and PUFA treatment tends to coordinately suppress the expression of DNMTs in four CRC cells lines.

COX, Cyclooxygenase; ALOX, Arachidonic Acid Lipoxygenase; DHA, Docosahexaenoic acid; PTGS, Prostaglandin-Endoperoxide Synthase; CRC, Colorectal cancer; PUFA, Polyunsaturated fatty acids; DNMT, DNA methyltransferases.

supplementation upregulated *COX1* expression and reduced the pro-inflammatory state. Individuals with higher mRNA expression of *COX2* after ω-3 fatty acid supplementation had reduced colonic PGE_2 (48). The *ALOX* and *COX* gene expression level in CRC patients is supposed to be dependent on dietary fats. Koh et al. provided epidemiological evidence for a possible association between the production of prostaglandin n–6 *PUFAs* through *COX2* enzymatic activity and an increased risk of colon cancer. They also showed a significant association between the *COX2* rs20417 and colorectal cancer risk among people with a higher intake of n–6 *PUFA* (52). The ratio of omega-6 fatty acids (as precursors of inflammatory eicosanoids) to omega-3 fatty acids (as precursors of anti-inflammatory eicosanoids) may affect the extent to which the ALOX and COX genes affect colorectal cancer risk. These results highlight the importance of the intake of different types of dietary fats in carriers of the risk alleles of the ALOX and *COX* genes. However, few studies directly assess this interaction. Also, different factors, such as ethnic and racial differences, may influence the obtained results on the interactions of *CRC* risk with dietary fats and *ALOX* and *COX* genes. Further studies are needed to understand the interaction between dietary fat, genetics, and colorectal cancer. Moreover, other genes involved in enzymatic pathways for synthesizing eicosanoids from dietary fats and possible mechanisms for their relationship with CRC risk should be investigated. If the findings of this review study are confirmed in future longitudinal studies, it could be an important step in providing a specific diet to prevent colorectal cancer, especially in COX and ALOX risk allele carriers.

CONCLUSION

In conclusion, COX and ALOX genes may play a significant role in CRC risk. Additionally, dietary fats may play an essential role in the effects of the *ALOX* and *COX* genes on the risk of CRC. Generally, enhancing the knowledge of nutritional genomics can lead to finding new methods to prevent, treat, and manage CRC. The results of this review article emphasize that environmental factors, such as dietary fat intake, may influence the association between colorectal cancer and the genotype of an individual. If the results are confirmed in future longitudinal studies, the importance of personalized medicine and the recommendation

of personalized diets according to the genotype of individuals to prevent colorectal cancer will be further highlighted. Further longitudinal studies in this field of nutritional genomics can lead to the discovery of personalized dietary recommendations for CRC prevention.

AUTHOR CONTRIBUTIONS

MG, SMD, and AH designed the study, and were involved in the data collection, analysis, and drafting of the manuscript. NM, SA, SA, SP, MNJ, SD, HS, MH, MA, and AA were involved in the design of the study, analysis of the data, and critically reviewed the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Detection of Glycosylated Markers From Cancer Stem Cells With ColoSTEM Dx Kit for Earlier Prediction of Colon Cancer Aggressiveness

Sabrina Blondy[1†], Stéphanie Durand[2†], Aurélie Lacroix[2], Niki Christou[2,3], Charline Bouchaud[1], Maud Peyny[1], Serge Battu[2,4], Alain Chauvanel[2,5], Vincent Carré[1], Marie-Odile Jauberteau[2,6‡], Fabrice Lalloué[2*‡] and Muriel Mathonnet[2,3*‡]*

[1] Carcidiag Biotechnologies company, Guéret, France, [2] INSERM U1308 - CAPTuR "Control of cell activation, Tumor progression and Therapeutic resistance", Faculty of Medicine, University of Limoges, Limoges, France, [3] Department of Digestive Surgery, Dupuytren University Hospital, Limoges, France, [4] Laboratory of Analytical Chemistry, Faculty of Pharmacy, Limoges, France, [5] Department of Pathology, Dupuytren University Hospital, Limoges, France, [6] Department of Immunology, Dupuytren University Hospital, Limoges, France

Nowadays, colon cancer prognosis still difficult to predict, especially in the early stages. Recurrences remain elevated, even in the early stages after curative surgery. Carcidiag Biotechnologies has developed an immunohistochemistry (IHC) kit called ColoSTEM Dx, based on a MIX of biotinylated plant lectins that specifically detects colon cancer stem cells (CSCs) through glycan patterns that they specifically (over)express. A retrospective clinical study was carried out on tumor tissues from 208 non-chemotherapeutic-treated and 21 chemotherapeutic-treated patients with colon cancer, which were stained by IHC with the MIX. Clinical performances of the kit were determined, and prognostic and predictive values were evaluated. With 78.3% and 70.6% of diagnostic sensitivity and specificity respectively, our kit shows great clinical performances. Moreover, patient prognosis is significantly poorer when the MIX staining is "High" compared to "Low", especially at 5-years of overall survival and for early stages. The ColoSTEM Dx kit allows an earlier and a more precise determination of patients' outcome. Thus, it affords an innovating clinical tool for predicting tumor aggressiveness earlier and determining prognosis value regarding therapeutic response in colon cancer patients.

Keywords: colorectal carcinoma, early stage, cancer stem cells, glycosylated biomarkers, prognosis value, tumor aggressiveness

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Nobu OSHIMA, Kyoto University, Japan

***Correspondence:**
Fabrice Lalloué
fabrice.lalloue@unilim.fr
Stéphanie Durand
stephanie.durand@unilim.fr
Muriel Mathonnet
muriel.mathonnet@unilim.fr

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

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INTRODUCTION

Colon cancer represents the second leading cause of death from cancer (1–3). Diagnosis is usually based on the pathological staging classification (pTNM) (stages I to IV) (4). Surgical resection is the only curative method at present. Although the prognosis has improved in recent years, survival rates widely vary by stage, with 85% 5-years net survival for stage I and 50% for stage III (5). Indeed, nearly 10% of stage I, 30% of stage II and 55% of stage III will present a metachronous cancer or a

recurrence (locoregional or metastatic) within 5 years postoperatively (6). This high risk of relapse requires to further improve earlier detection of colon cancer and to achieve personalized evaluation of patients' outcome and prognosis. This approach implies a systematic and precise determination of disease aggressiveness in order to strengthen patient follow-up and management (7). Therefore, searching and using for molecular biomarkers pave the way to improve patients' prognosis and management. Since abnormal glycosylation is a common phenomenon that occurs in cancer cells (8), thus, glycan abnormalities profiles play important roles in cancer biology and therefore afford a potential tool for the characterization of tumor markers.

Glycosylation is one of the most important posttranslational modifications of lipids (glycolipids) and proteins (glycoproteins), by the highly coordinated action of glycosyltransferases and glycosidases. Glycoproteins and glycolipids regulate a diverse range of key biological and cellular functions, including differentiation, proliferation, growth, pluripotency *etc* … Alterations in glycosylation processes (*i.e.* aberrant glycosylation) are linked to colon cancer development, progression, metastases and therapeutic failures (9–11). Aberrant glycosylation constitutes a hallmark of Cancers and might even lead to the acquisition of a stemness phenotype (12–15). This mechanism is already known to be altered in proteins expressed on tumor differentiated cells but also in a specific cell subpopulation with acquired stemness properties, the cancer stem cells.

Cancer Stem Cells (CSCs) are highly tumorigenic cells, *i.e.*, they are able to give rise to complete tumor mass. In addition to their ability to regenerate tumor mass, the main CSCs properties are self-renewal and multipotent differentiation capacity. They also have a unique property of resistance to treatments. Given all these features, CSCs contribute to colon tumor initiation, progression (metastasis formation), aggressiveness and relapses (16–21) and represent new biomarkers for cancer prognosis due to their original stemness properties. Various CSCs markers were defined to identify and isolate colon CSCs. Most of them are (i) membrane receptors and surface molecules (such as CD44 and its splice variants (CD44v), CD133/1 (AC133 epitope) or epithelial cell adhesion molecule (EpCAM)), (ii) cytosolic enzyme, such as aldehyde dehydrogenase 1 (ALDH1) enzymatic activity, and transcription factors including OCT-4 (12, 16, 17, 19, 22, 23). However, these biomarkers currently failed to be used in clinic because of their concomitant expression in non-tumor stem cells (SCs) (12, 13). These data underline the importance of evidence of more specific colon CSCs biomarkers.

Some data reported a correlation between the alteration of glycosylation processes with the induction and/or regulation of CSCs phenotype and properties. Recent results evidenced that colon cancer stemness would be regulated by O-GlcNAcylation. Indeed, the inhibition of the O-GlcNAc transferase (OGT) and thus of the GlcNAc residue leads to an increase of colon cancer stemness characteristics and properties, concomitant with a more aggressive and malignant phenotype (24). Another study evidenced that overexpression of O-glycan truncated forms such as Tn antigen (Ag), is involved in the development and the induction of colon oncogenic features (tumorigenesis, cell growth, invasion, metastases and resistance to UV-induced apoptosis) (25). The expression of ß-1,4-N-acetylgalactosaminyltransferase 3 is upregulated in colonospheres and its knockdown decreases sphere formation and stemness marker expression (OCT-4 and NANOG) (26). Overexpression of α-2, 6-Sialyltransferase and α-N-acetylgalactosaminide α-2,6-sialyltransferase 1, are both correlated with (i) colon CSCs enrichment (increase of CD133 and ALDH1 expressions, as well as sphere forming ability), and (ii) acquired resistance to chemotherapy (irinotecan and 5-Fluorouracil) and EGFR-targeted therapy (gefitinib) (27–30). *FUT9* gene encoding the α-1,3 fucosyltransferase, plays a complex dual role in colon cancer development and malignancy. Alpha-1,3 fucosyltransferase knockdown strongly decreases sphere formation, growth of xenograft tumors and expression of OCT-4 and CD44, whereas it increases cell proliferation and migration. *FUT9* expression supports colon cancer aggressiveness. Its expression at early stages is required for CSCs expansion and colon cancer initiation. On the contrary, its downregulation at later stages promotes colon cancer progression (31). Most colon CSCs surface markers are glycoproteins. They differ from their normal counterpart by the expression of tumor specific glycans (15). Thus, CD44 splice variants carry oncofetal carbohydrate T and sialyl-Tn (sTn) Ag, correlating with the increased metastatic potential of colon cancer cells. The case of CD133 can also be mentioned since rather than the expression of total CD133 protein, it is the expression of a specific glycan epitope (AC133) that could constitute a "bona fide" CSCs marker. Altogether these data suggest that a better characterization of colon CSCs glycosylation profiles could pave the way to identify more efficient new CSCs biomarkers in order to improve specific detection within tumor and thus for targeting them.

Based on these knowledges and current clinical needs, Carcidiag Biotechnologies company has developed the ColoSTEM Dx kit, consisting of specific colon CSCs detection within heterogeneous tumor cell populations. There is currently no clinically standardized way (*i.e.*, efficient prognosis biomarkers) to provide a reliable and earlier prognosis for colon cancer patients. In this context, our kit provides innovating and reliable biomarkers, specific to colon CSCs, for a better and an earlier stratification of low- or high-risk patients to develop an aggressive disease and relapse. The ColoSTEM Dx kit represents a tool perfectly adapted to the personalized management of patients. More precisely, it is an innovative tool that uses a MIX of biotinylated plant lectins (UEA-1, Jacalin and ACA, mixed in a particular *ratio*) recognizing glycan patterns specifically (over)expressed by colon CSCs and tumor cells related to CSCs. These glycan patterns are not expressed neither by "normal" stem cells nor by differentiated cancer tumor cells. This colon CSCs specific MIX was evidenced by lectin-arrays and validated *in vitro* from research works carried out in collaboration with the University of Limoges, that have conducted to file two patents (national registration numbers WO2016FR53196 and WO2016FR53197).

Based on these results and in collaboration with Limoges University Hospital, we conducted a retrospective clinical study aiming at validating the ColoSTEM Dx kit for a routine clinical use by immunohistochemistry (IHC).

MATERIALS AND METHODS

Cell Culture

Colon adenocarcinoma cancer cell lines HT-29 were obtained from ATCC (HTB-38™; ATCC®, France). Cells were incubated at 37°C with 5% CO2 and 95% humidity and cultured in 1X McCoy's 5A modified medium (Gibco - ThermoFisher Scientific, France) supplemented with 10% fetal bovine serum (FBS; Gibco-ThermoFisher Scientific, France) and 1% penicillin/streptomycin (Gibco - ThermoFisher Scientific, France).

Indirect Magnetic Cell Sorting

MACS was realized from 10^7 cells using the CELLection™ Biotin Binder kit (Invitrogen - ThermoFisher Scientific, France) according to manufacturer's instructions, using 10µg of (i) the MIX (ColoSTEM Dx kit) or (ii) the AC133 biotinylated antibody (Ab) (Miltenyi Biotech, France), were used.

Evaluation of $EpCAM^{High}$ Immunostaining and $ALDH1^{bright}$ Activity by Flow Cytometry

$EpCAM^{high}$ cell percentages within the AC133 – and + sorted-cells, were analyzed by flow cytometry (FCM) from 5.10^4 cells. After saturation in 1% BSA in DPBS 1X calcium and magnesium free (10min, 4°C), cells were incubated for 45min at room temperature (RT) with an EpCAM mouse monoclonal Ab (clone VU1D9; Ozyme - Cell Signaling Technology, France) diluted at 1:150 in 1% BSA/DPBS. After a washing step in DPBS 1X (g x 300, 10min, 4°C), cells were incubated for 30min at RT in the dark with an Alexa-Fluor 633-conjugated goat anti-mouse secondary Ab (ThermoFisher Scientific, France) diluted at 1:1000 in DPBS 1X.

Enzymatic activity of ALDH1 in MIX+ and MIX- sorted-cells was analyzed from 10^5 cell/mL, using the ALDEFLUOR kit (Stem Cell Technologies, France) according to the manufacturer's recommendations.

Cells were extemporaneously stained with a DNA dye, i.e., 0.5µL propidium iodide (PI; λex=475-581nm/λem=583-697nm; BD Biosciences, France). $EpCAM^{high}$ immunostaining and ALDH1 enzymatic activity (ALDH1bright cells) were analyzed among live cells (PI-), with the BD AccuriC6 Plus FCM (BD Biosciences, France). Mouse IgG1 isotype control (R&D systems, France) and N, N-diethylaminobenzaldehyde (DEAB, ALDH1 specific inhibitor) were used to control for background fluorescence

Clonogenicity Assay

MIX- and MIX+ sorted-cells were seeded in ultra-low attachment 96-wells plates (Falcon Corning brand, France) in increasing cell densities, i.e., 600, 1250, 2500, 5000, 10000 cells. For each condition, cells were seeded in 3 wells in 200µL of defined medium composed of 1X Dulbecco's Modified Eagle's Medium (DMEM)/F12 (HAM 1:1) medium (Gibco - ThermoFisher Scientific, France) supplemented in 1X B27/Insulin (ThermoFisher Scientific, France), 10ng/mL Fibroblast Growth Factor (FGF; Peprotech, France) and 20ng/mL Epidermal Growth Factor (EGF; Peprotech, France). Twenty microliters of medium were added per well every week, for 4 weeks. The number of spheres formed per well and per condition was counted under optical microscope (Olympus CKX53; magnification, x100).

Patients and Samples

Ninety colon tumor tissues came from TMA (Tissue MicroArray) (HCol-Ade180Sur-08; AMSBIO, USA), which also includes the 90 corresponding healthy borders. Necrosed and absent tumor tissues (N=4) were excluded (N=86).

Forty-six colon tumor tissues were collected from patients having benefited from colon cancer resection at the Department of Digestive Surgery, General and Endocrine Surgery at Limoges University Hospital (France) and without any pre-operative chemotherapeutic treatment. Necrosed (N=4) were excluded (N=42).

Twenty-four tumor tissues were also collected from colon cancer patients with pre-operative chemotherapy at Limoges University Hospital (France). Necrosed (N=3) were excluded (N=21).

Supplementary colon tumor tissues were collected from two TMA constituted in a cohort of colon cancer patients with early stages (I and II) (without chemotherapeutic treatment) from the "Centre de Ressources Biologiques – Institut Régional du Cancer Montpellier (CRB-ICM, Montpellier, France, ICM-CORT-2016-26). Necrosed and absent or non-interpretable tumor tissues (N=15) were excluded (N=80). Both TMAs also include N=50 paired healthy samples.

Eighteen kidney tumors (clear cell carcinoma) in TMA were chosen as MIX negative control provided from AMSBIO society and are referenced as T8235714D-5 and T8235716D-5.

Clinicopathological data including pTNM stages, gender, age and survival status at 5- and 7-years were provided after baseline examinations and patients' diagnosis according to histological analyses of biopsies (American Joint Committee on Cancer staging manual) (32). Survival rates analysis of non-chemotherapeutic-treated and chemotherapeutic-treated patients from all stages (refer to "statistical analysis" described below) were realized respectively from N=128 and N=21 samples (**Tables S1** and **S2**). Survival rates analysis at 5 years of non-chemotherapeutic-treated patients from early stages (refer to "statistical analysis" described below) were realized from N=70 samples (N=29 from CRB-ICM, N=27 from Limoges Hospital and N=14 from the TMA (AMSBIO) (**Table S3**).

MIX and OCT-4 IHC Immunostaining

MIX staining was realized on N=208 and N=21 tumor tissues from respectively non-chemotherapeutic-treated and chemotherapeutic-treated patients with colon cancer (refer to "patients and samples" section; **Tables S1**, **S2** and **S3**). MIX/OCT-4 co-staining was performed on some tumor tissues from the non-chemotherapeutic-treated patients' cohort, i.e., N=42 tumor tissues from Limoges University Hospital (refer to "*Patients and Samples*" section; **Table S1**). MIX staining was also achieved on 18 kidney tumors samples in order to assess sensibility and specificity in comparison with MIX staining performed in colon cancer samples. Each staining was realized by IHC on paraffin-embedded histological sections (4µm in thickness), in three main steps using the Leica Bond Max

automatic staining platform (Leica Biosystems, France), according to the manufacturer's instructions: (i) Preparation and pretreatment of the tissues. Paraffin coating is removed using the Bond Dewax Solution (Leica Biosystems, France) and tissues are rehydrated under heat using the acidic buffer Bond Epitope Retrieval Solution 1, for 5min (pH 6; Leica Biosystems, France); (ii) Immunostaining. Activity of endogenous peroxidases and biotins was blocked using the Bond Intense R Detection kit (Leica Biosystems, France). Tissues were incubated for 20 min with either the MIX alone pre-diluted at 1:2 ratio in the diluent supplied in the ColoSTEM Dx kit, or with both MIX (1:2) and OCT-4 (OCT-4 polyclonal rabbit IgG Ab; ThermoFisher Scientific, France). MIX and/or OCT-4 staining was revealed using respectively the Bond Intense R Detection kit and the Bond Polymer Refine Red Detection kit (Leica Biosystems, France). Nucleus were counter-stained by incubation with hematoxylin (Leica Biosystems, France) for 8 min; (iii) Slides mounting. After dehydration by two successive baths of absolute ethanol (VWR, France) and toluene (ThermoFisher Scientific, France), for 5min each, tissue slides were mounted using the Leica CV Ultra (Leica Biosystems, France) and examined under the Leica photomicroscope DM4 B (Leica Biosystems, France; 200x magnification).

Scoring Method

MIX staining appears in brown at apical membrane and/or in cytoplasm. OCT-4 staining appears in red/pink within nucleus (in blue) and/or cytoplasm. Scoring method of both staining was adapted from a previous an well-known scoring method called "quickscore" (33–35). All tissues were stained either with the MIX alone, or with both the MIX and OCT-4. The total absence of staining (score 0) or the presence of stained cells constitutes the first element of analysis. The second element of analysis is related to the proportion of stained cells that is scored according to the followed gradation: 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100% of stained cells. The third element of analysis is related to the staining intensity, graduated as followed: 1 = Low, 2 = Medium and 3 = High staining intensity. Scores obtained from both gradations are then added together and the total obtained results into 6 intermediate scores, ranging from 2 to 7, which are finally grouped into 3 final scores (**Figure 1**). Final scores of 1 and 2 are considered as "Low staining" (MIX-Low and/or OCT-4-Low) and final score of 3 is considered as "High staining" (MIX-High and/or OCT-4-High).

Evaluation of Clinical Performances of the ColoSTEM Dx Kit

Clinical performances of the ColoSTEM Dx kit, were determined from N=166 tumor tissues (N=86 of the commercial TMA (AMSBIO) and N=80 of the cohort of CRB-ICM Montpellier) and N=136 non-tumor tissues (N=86 tumor borders of the commercial TMA (AMSBIO) and N=50 non-tumor samples of the cohort of CRB-ICM Montpellier). Diagnostic sensitivity is related to the percentages of tumor tissues stained with the MIX (true positives) relative to the unlabeled ones (false negatives), as

Scoring of staining intensity / Scoring of stained cells %	Low 1	Medium 2	High 3
1-25%: 1	2	3	4
26-50%: 2	3	4	5
51-75%: 3	4	5	6
76-100%: 4	5	6	7

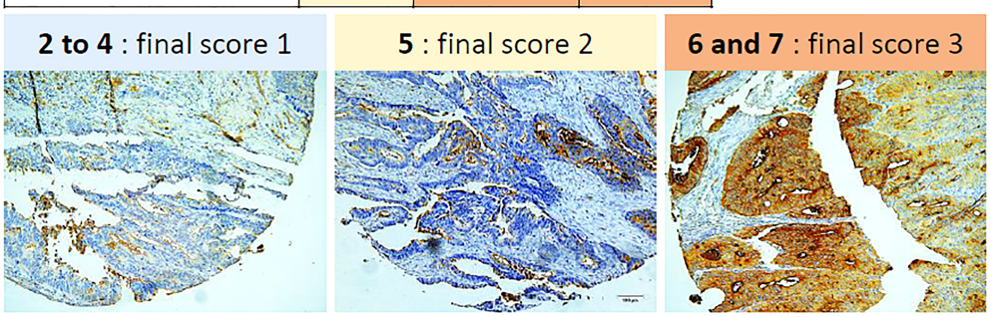


FIGURE 1 | Scoring method of IHC staining, according to percentages of stained cells and staining intensity. Percentages of stained cells are graduated into four scores (1 to 4) and staining intensity is graduated into three scores (1 to 3). The total some of both scoring results in intermediate scores ranging from 2 to 7. Intermediate scores ranging from 2 to 4 (in blue) result in a final score of 1. Intermediate score of 5 (in yellow) results in a final score of 2. Intermediate scores of 6 and 7 (in orange) result in a final score of 3. Final scores of 1 and 2 are considered as "Low staining" and final score of 3 is considered as "High staining". Representative illustrations of MIX staining (in brown), as observed by IHC (magnification, 200x), are depicted below table. *IHC: Immunohistochemistry*.

followed Sensitivity (%) = 100 x (True positives/(True positives + False negatives)). Diagnostic specificity is related to the percentages of tumor borders or non-tumor tissues unstained with the MIX (true negatives) relative to the labeled ones (false positives), as followed Specificity (%) = 100 x [True negatives/(True negatives + False positives)].

Statistical Analysis

Statistical analysis and graphics were performed with StatView 5.0 (USA), Prism 7 (GraphPad, USA) and R environment (version 4.0.3). Statistical analysis of *in vitro* clonogenicity assay was made with an ANOVA/ANCOVA test. Survival rates according to MIX/OCT-4 co-staining were analyzed from the non-treated patients from the Limoges Hospital cohort, at 5 years, *i.e.*, only patients whose survival is ≤ 60 months at the last visit time, were retained (N=42; refer to "patients and samples" and **Table S1**). Survival rates of all non-treated patients were analyzed according to their clinicopathological data and MIX staining at (i) 5 years and (ii) 7 years, i.e., patients whose survival is ≤ 84 months at the last visit time (respectively N=79 and N=128; refer to "patients and samples" and **Table S1**). Survival rates analysis at 5 years of non-treated patients from early stages were achieved by combining three cohorts composed of patients from Limoges' Hospital, CRB-ICM Montpellier as well as from a cohort provided by AMSBIO (N=70; refer to "patients and samples" and **Table S3**). Survival rates of treated patients were analyzed according to MIX staining, at 5 years (N=21; refer to "patients and samples" and **Table S2**). The prognostic value of each parameter for outcome was assessed using the Kaplan-Meier method and log-rank test (Mantel-Cox). For each variable, hazard ratio (HR) was estimated using a univariate Cox model and expressed with their 95% confidence interval (95% CI). Multivariate analysis was carried out using a Cox regression model on single features identified by the univariate Cox modeling. Survival analysis were performed in R using *survival* and *survminer* packages. The proportional hazards assumption for Cox regression model fit was verified using *cox.zph* function of survival package. A p-value below 0.05 was considered as significant.

RESULTS

The ColoSTEM Dx Kit Allows Efficient Isolation and Enrichment of a Cell Subpopulation With Stemness Properties

The ColoSTEM Dx kit originates from research works carried out by the EA3842 laboratory (Limoges' University; patent national registration number 1561763 – publication number 3044680). It aims at colon CSCs specific detection in both heterogeneous colon cancer cell populations and tumors. Indeed, it is based on the use a MIX of biotinylated plant lectins that recognize glycan patterns specifically (over) expressed by these cells, *i.e.*, not by differentiated cancer cells, within heterogeneous tumor colon tissues. ColoSTEM Dx kit proofs of concept, *i.e.*, MIX evidence by lectin-arrays and its validation in specific colon CSCs detection and enrichment from several colon adenocarcinoma cell lines (including HT-29), are reported in detail in the patent mentioned above.

Main and most relevant results have been recalled in **Figure S1**. Briefly, HT-29 cells were sorted by MACS, with either the MIX (MIX+ and MIX- sorted-cells) or an AC133 Ab (AC133+ and AC133- sorted-cells). Some of CSCs characteristics and properties were then evaluated: protein expression and enzymatic activity of stem cells (SCs) markers (EpCAM and ALDH1), and sphere forming ability. While there are as many $EpCAM^{High}$ cells in AC133+ sorted-cells as in AC133- (normalized to 1), there are 7.5-times more $EpCAM^{High}$ cells in MIX+ sorted-cells, compared to both MIX- (normalized to 1) and AC133+ cells (**Figure S1A**). Consistently, there are 4.7-times more $ALDH1^{bright}$ cells in MIX+ sorted-cells (74.73%) than in MIX- (15.6%) (**Figure S1B**). Finally, MIX+ sorted-cells have a significant capacity to form spheres compared to MIX- cells, even when seeded at low densities (**Figure S1C**).

The use of the ColoSTEM Dx kit is more efficient for detecting and enriching in specific colon CSCs than cell sorting using AC133. These results suggest that ColoSTEM Dx kit improves CSCs detection and cell sorting.

The ColoSTEM Dx Kit Improves Colon CSCs Detection and Allows a More Accurate Prognosis Than the Standard Stem Cell Marker OCT-4

MIX specificity in colon CSCs detection by IHC on tumor tissue, as well as its efficiency in patients' prognosis evaluation, were evaluated and compared to a standard SCs marker, OCT-4. MIX and OCT-4 staining were realized on N=42 tumor tissues from non-chemotherapeutic-treated patients (**Table S1** and **Figure S2**). Among stained samples, half of samples are MIX-Low or MIX-High (**Figure S2A**), while OCT-4-high staining is present in a broad panel of samples (almost 80%, **Figure S2B**), suggesting that OCT-4 is not able to discriminate CSCs from the heterogeneous cell subpopulations. In addition, when cells are double-stained with MIX and OCT-4, samples are mainly divided in MIX-Low/OCT-4-High or MIX-High/OCT-4-High (**Figure S2C**). Intensity of MIX staining is not linked to OCT-4 staining and is independent of clinicopathological characteristics of patients except for gender (**Table 1**). However, this association is not found later on larger patient cohorts (**Table 2**). Altogether, these results suggest that the ColoSTEM Dx kit is relevant for the discrimination of cancerous from healthy SCs. It also evidences a better specificity to detect colon CSCs, than OCT-4 whose staining within tumor colon epithelium does not seem to be restricted to CSCs, but to all SCs (healthy and cancerous) and progenitors.

Survival rates at 5 years were evaluated by Kaplan-Meier curves according to either OCT-4 staining (OCT-4-Low *versus* High; **Figure 2A**), MIX staining (MIX-Low *versus* High; **Figure 2B**) or both staining (OCT-4-High/MIX-Low *versus* OCT-4-High/MIX-High; **Figure 2E**). Univariate and multivariate Cox regression were performed to estimate prognosis values and risk scores associated to OCT-4 and MIX staining (**Figure 2C**). Representative pictures of MIX/OCT-4 co-staining, are depicted in **Figure 2D**.

Survival rates at 5-years are not significantly different regardless of OCT-4- staining (Low *vs* High; **Figure 2A**; p=0.91). On the

contrary, 5-years survival rates of the MIX-High subgroup are significantly poorer than of the MIX-Low (**Figure 2B**; p=0.017). No relevant difference in survival median having been noted between a MIX-Low and an OCT-4-Low staining (data not shown; p=0.28). Interestingly, even if results are not significant, it has been observed a lower survival median (decrease of 18 months) with a MIX-High compared to an OCT-4-High staining (data not shown; p=0.2). Cox univariate analysis indicated that only MIX staining is a predictive factor for OS, with a significantly increasing risk associated to a MIX-High staining (HR: 3.3, 95% CI 1.17 to 9.43, p=0.025; **Figure 2C**, left panel). Multivariate model confirms independence and relevance of MIX staining as prognostic factor (HR: 4.2, 95% CI 1.3 to 13.5, p=0.015; **Figure 2C**, right panel).

Finally, survival rates at 5 years were also evaluated according to MIX/OCT-4 co-staining on the same tissues (N=41), i.e., MIX-Low/OCT-4-Low, MIX-Low/OCT-4-High, MIX-High/OCT-4-Low, MIX-High/OCT-4-High. Due to not enough MIX-Low/OCT-4-Low and MIX-High/OCT-4-Low tumor tissues included (n=6 and 2, respectively; **Figure S2C**), Kaplan Meier curves were only depicted and analyzed for MIX-Low/OCT-4-High and MIX-High/OCT-4-High co-staining (**Figure 2E**). Interestingly, and consistently with previous observations, a MIX-High/OCT-4-High co-staining predicts significant poorer and worse prognosis than a MIX-Low/OCT-4-High co-staining, with strong survival median decrease (p=0.015; **Figure 2E**). High co-staining harbor a hazard ratio of 5.3 (95% CI 1.2 to 23.8, p=0.0298) in a univariate Cox regression model (not shown).

Contrary to OCT-4, MIX staining levels are closely associated with patient survival. Indeed, compared to OCT-4, MIX-High staining level improve significantly the detection and discrimination of colon CSCs. MIX-High staining might be a relevant CSCs biomarker for monitoring disease aggressiveness and could be useful to establish the prognosis upon treatment.

The ColoSTEM Dx Kit Allows Earlier Evaluation of Patients' Disease Aggressiveness and Prognosis, Regardless of Clinicopathological Data

In order to evaluate and confirm the prognosis value of the ColoSTEM Dx kit, all tumor tissues from non-treated patients (N=128, **Table S1**) were stained with the MIX. Survival rates were evaluated at 5- and 7-years of OS, according to clinicopathological data, *i.e.*, stages [early (I/II) and late (III/IV)], sex (men and women) and age (< and ≥ 60 years old) (**Table 2**).

Among the 79 tumor tissues included for survival rates analysis at 5-years, 6 were excluded due to a total absence of MIX staining. Fifty-six percent and 44% correspond respectively to early and late stages. Of the 41 early stages, 41% and 59% were respectively MIX-Low and MIX-High. Among the 32 late stages, 47% and 53% were respectively MIX-Low and MIX-High. Regarding OS at 5 years, we noted that MIX-staining is independent of tumor stage (**Table 2A**, p=0.809).

Survival rates at 7 years were then analyzed from 128 tumor samples. As previously described, we excluded tumor samples without MIX staining (n=13). Among the 115 retained tumors, 63% and 37% correspond respectively to early and late stages. Of the 73 early stages, 33% were MIX-Low whereas 67% were MIX-High. Regarding the 42 late stages, half of the population were MIX-Low or MIX-High. This result suggests that regardless of evaluated time point of OS, MIX staining is independent of tumor stages (**Table 2**).

Kaplan-Meier curves and Cox regression models were displayed and patients' survival rates were analyzed at 5- and 7-years, according to MIX staining levels and stages (**Figure 3** and **Table 3**). At 5-years, prognostic significance for OS of MIX scoring is clearly supported by survival curve (p=0.011; **Figure 3A**) and univariate Cox model (HR: 2.1 with 95% CI 1.17 to 3.75, p=0.013; **Table 3A**). On the contrary, no statistically relevant difference is shown between MIX-Low or -High at 7-years survival rates (**Figure 3B** and **Table 3B**).

Noted that sex and age have not a significant impact on survival rates, at both 5- (**Table 3A** and **Figure S3A**) and 7-years (**Table 3B** and **Figure S3B**). Kaplan-Meier curves performed on separately groups of patients (MIX-Low and -High), stratifying according to sex (male and female) or age (inferior or superior to 60 years old), failed to show any difference in survival rates according to low or high MIX staining (data not shown). In brief, no statistically relevant difference on survival rates between MIX-Low or -High staining was noted, regardless of age, even if a significant link was been previously identified by Chi-square test

TABLE 1 | Evaluation of the relationship between each clinicopathological characteristic (Sex, Age and pTNM staging) of non-chemotherapeutic-treated patients (n = 41, **Table S1**) and intensity of MIX staining (Low/High) by Chi-squared statistic.

		n	MIX staining		
			Low	High	P value
Sex	**Female**	18	12	6	**0.046**
	Male	23	7	16	
Age	**< 60yrs**	4	2	2	1
	≥ 60yrs	37	17	20	
Stage (UICC)	**Early (I/II)**	27	14	13	0.514
	Late (III/IV)	14	5	9	
OCT4 staining	**Low**	8	6	2	0.109
	High	33	13	20	

Association between scoring according to MIX and OCT-4 staining has been evaluated. pTNM, pathology Tumor-Node-Metastasis, UICC, Union for International Cancer Control; yrs, years. The p values less than or equal to 0.05 have been written in bold.

TABLE 2 | Relationship between intensity of MIX staining and clinicopathological characteristics of non-treated patients included at 5 years and 7 years of OS.

(A)

		n	MIX Staining		
			Low	High	P value
Sex	**Female**	35	18	17	0.239
	Male	38	14	24	
Age	**< 60yrs**	8	6	2	0.139
	≥ 60yrs	65	26	39	
Stage (UICC)	**Early (I/II)**	41	17	24	0.809
	Late (III/IV)	32	15	17	

(B)

		n	MIX Staining		
			Low	High	P value
Sex	**Female**	54	22	32	0.855
	Male	61	23	38	
Age	**< 60yrs**	16	11	5	**0.014**
	≥ 60yrs	99	34	65	
Stage (UICC)	**Early (I/II)**	73	24	49	0.087
	Late (III/IV)	42	21	21	

Numbers of tumor tissues from non-treated patients included at 5 years (A) and 7 years (B) of OS, for which there is a MIX-Low staining or a MIX-High staining, according to clinical and pathological data, i.e., gender, age and stage, were indicated. The association between MIX staining and clinicopathological characteristics of patients was evaluated by Chi-squared statistic. ***(A)*** *All tissues (n = 79) were stained with the MIX but 6 samples show absence of MIX staining (not reported in the present table). They are distributed as follows: gender: 3 Female/3 Male; age: 3 < 60 yrs/3 ≥ 60 yrs; stage: 3 early/3 late; vital status: 1 alive/5 dead. (B) All tissues (n = 128) were stained with the MIX but 13 samples show absence of MIX staining (not reported in the present table). They are distributed as follows: gender: 6 Female/7 Male; age: 4 < 60 yrs/9 ≥ 60 yrs; stage: 7 early/6 late; vital status: 7 alive/6 dead. OS, Overall Survival; UICC, Union for International Cancer Control; yrs, years.*

The p values less than or equal to 0.05 have been written in bold.

at 7 years patients' follow-up (p=0.014, **Table 2**). We conclude in the same way with regard to gender.

Since multivariate analysis revealed that late stages and a high-MIX score were independent prognosis factors at both 5-years and at 7-years of patients' follow-up (**Table 3**), we chose to combine these two parameters in order to assess their impact on survival rates. We confirmed high value of MIX score as risk factor for OS, regardless of pTNM staging (**Figure 4**).

Survival rates of MIX-high staining associated with late-stage patients are significantly poorer compared to MIX-low staining with a doubling HR, observed at 5-years and at 7-years of OS (**Figure 4**). The same tendency was observed for the early stage patients, even if only results acquired at 5-years of OS were significant (**Figure 4**).

Noteworthy that results are statistically more pronounced and relevant for 5-years follow-up: patients characterized by a MIX-Low staining have higher survival rates than MIX-High patients (**Figure 4A, C**). To accurately estimate the prognosis value of MIX staining according to given stages, survival analysis (Kaplan-Meier and univariate Cox regression) according to Low or High MIX subpopulations at early (I/II) or late (III/IV) stages, were performed (**Figure S4**). At 5 years of patients' follow-up, a MIX-High staining could be considered as a poor prognosis marker in both early (HR: 3.3 with 95% CI 1.2 to 9.1, p=0.021; **Figure S4A**) and late stages (HR: 2.2 with 95% CI 1 to 4.6, p=0.039; **Figure S4B**). At 7 years of patients' follow-up, MIX prognosis benefit is lost for early stages (**Figure S4C**) but is slightly maintained for late stages (HR: 1.95 with 95% CI 0.93 to 4.1, p=0.076; **Figure S4D**). Altogether, these results suggest that the MIX could be considered as an efficient prognosis marker to predict disease aggressiveness from early phase and within the 5 years post resection. Importantly, the significance of MIX prognosis value should be useful at early stage to adapt therapeutic strategy and improve patients' management.

Thus, the cohort of early stage subpopulation (initially made up of 16 stage 1 and 25 stages 2; **Table S1**) was implemented (**Table S3**) with a total of 41 patients of stage I and 29 patients of stage II, at 5 years of follow-up. Survival analysis performed on this cohort confirms the High-MIX staining as poor prognosis factor. Although a slightly difference is observed between MIX-high and -Low in early stage subpopulation (p=0.18), it can be noted that MIX-high has a moderate bad prognosis value in univariate Cox model (p=0.18, HR=1.764 and 95% CI 0.6514-4.779; data not shown). Thus, survival rates of I and II early stages were analyzed separately, by combining pTNM stages and MIX scoring (*i.e.*, Stage I/MIX-Low, Stage I/MIX-High, Stage II/MIX-Low, Stage II/MIX-High; **Figure S5**). Interestingly, survival rates of Stage II/MIX High patients are collapsed compared to Stage I/MIX High, suggesting that the relative risk is markedly increased when a High MIX staining is detected in stage II patients.

To resume, if we consider 7 years of OS, the ColoSTEM Dx kit does not allows prediction of disease evolution (i.e., patients' prognosis) regardless of their age or sex. However, concerning their stages, prognostic value of MIX staining appeared more reliable in the later stages of colon cancer

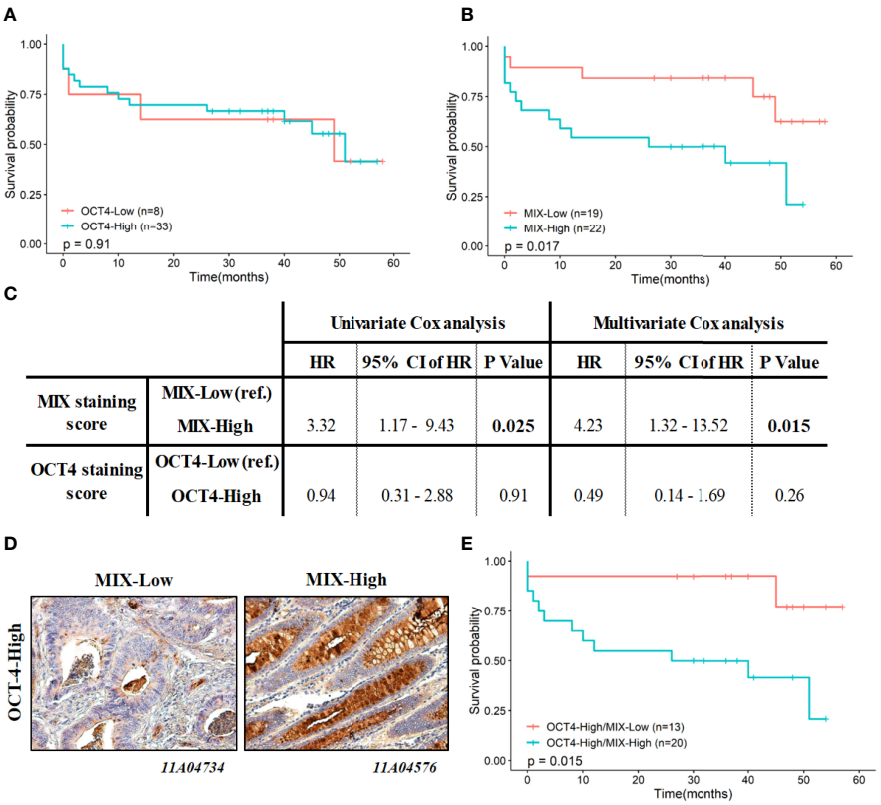


FIGURE 2 | Association between MIX and OCT-4 scoring with survival rates at 5 years (60 months). Kaplan-Meier curves at 5-years are depicted according to OCT-4-Low *versus* -High staining **(A)**, MIX-Low *versus* -High staining **(B)** and MIX-High/OCT-4-High *versus* MIX-Low/OCT-4-High co-staining **(E)**. P values indicated in each panel are related to the log-rank tests (Mantel-Cox) performed to survival curves comparison. **(C)** Prognostic value of MIX and OCT-4 scoring independently, was estimated using univariate and multivariate Cox regression models, and expressed with their HR and 95% CI. **(D)** Representative illustrations of MIX (brown) and OCT-4 (red/pink) staining, as observed by IHC, are depicted (magnification, 200x). *CI: Confidence Interval; HR: Hazard ratio; IHC: Immunohistochemistry.*

patients (**Figure S4C, D**). On the contrary, if we consider 5 years of overall survival, the ColoSTEM Dx kit markedly predicts disease aggressiveness and allows the stratification of patients with good or poor prognosis, with a high or low risk of relapse after curative surgery, especially from early stages. Altogether, these results evidence that specific glycan motif of colon CSCs detected by the ColoSTEM Dx kit, constitute independent prognosis factor from pTNM staging and other clinicopathological data. It allows to discriminate a better or worse prognosis, as well as to predict in a standardized way colon cancer aggressiveness within the first 5 years after curative surgery.

The ColoSTEM Dx Kit Displays Great Clinical Performances

Clinical performances of the ColoSTEM Dx kit, *i.e.*, diagnostic specificity and sensitivity, have been determined from N=166 tumor tissues (N=86 from AMSBIO TMA and N=80 from the CRB-ICM Montpellier cohort) and N=136 tumor edges (N=86 from AMSBIO TMA and N=50 from the CRB Montpellier cohort). Among the N=166 tumor tissues, N=36 depicted an absence of MIX staining (false negatives) and N=130 were stained (true positives). Among the N=136 non tumor tissues, N=96 depicted an absence of MIX staining (true negatives) and N=40 were stained (false positives). According to formula described in "*Materials and Methods*" section, diagnostic sensitivity and specificity reach respectively 78.3% and 70.6%. ROC curve built from results of MIX staining on non-tumor and tumor colon tissue permit to establish that AUC is acceptable (AUC = 0.7445; data not shown) and confirmed a good sensitivity and specificity of ColoSTEM Dx kit.

To evaluate the cellular type specificity of ColoSTEM Dx kit, we have performed MIX staining on 18 kidney tumors samples. Three kidney tumors were positive and 15 tumors were negative. Compared to colorectal tumors, where 130 were positive and 36 were negative after MIX staining, we can conclude that there is a positive association between MIX staining and colorectal tumors (confirmed by Fisher's exact test, p = 3.3x10-7).

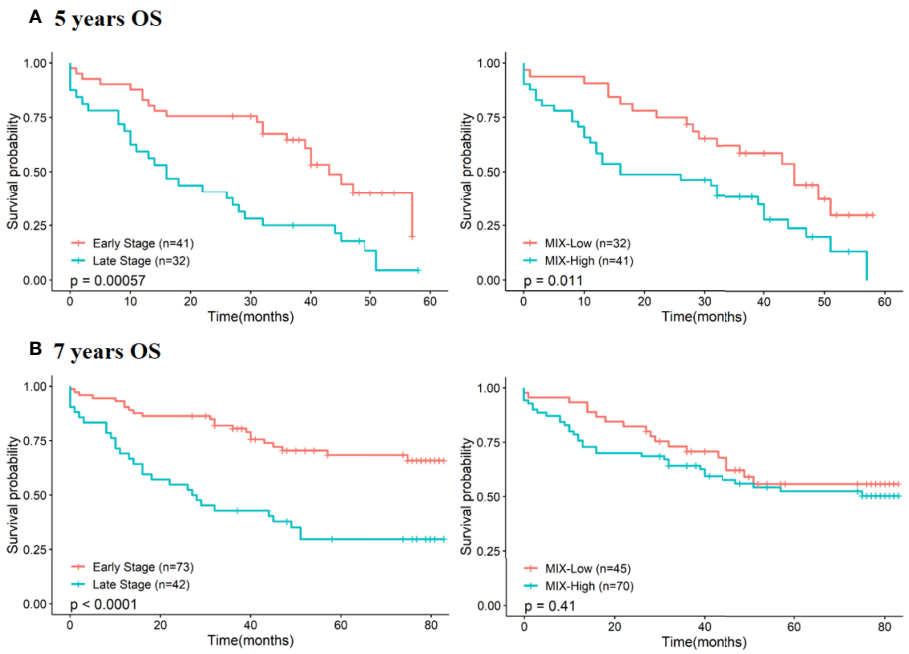


FIGURE 3 | Association between pTNM staging and MIX scoring with survival rates at 5- and 7 years (60 and 84 months respectively). Prognostic value of each feature (pTNM staging and MIX staining) was assessed using the Kaplan-Meier method and log-rank test by stratification of patients according to early and late stages or MIX-Low and High scoring at 5-years of OS **(A)** and at 7-years of OS **(B)**. *OS, Overall Survival; pTNM, pathology Tumor Node Metastasis.*

TABLE 3 | Prognostic values of clinicopathological features (gender, age, stage and MIX staining) at 5- and 7 years of patients' follow-ups (60 and 84 months respectively).

(A)

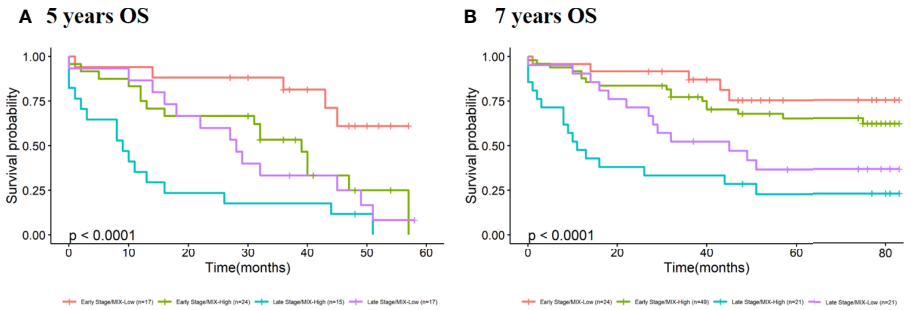
		Univariate Cox analysis			Multivariate Cox analysis		
		HR	95% CI of HR	P Value	HR	95% CI of HR	P Value
Sex	**Female (ref.)**						
	Male	1.38	0.78 - 2.44	0.269	/	/	/
Age	**< 60 yrs (ref.)**						
	> 60 yrs	0.78	0.33 - 1.85	0.573	/	/	/
Stage (UICC)	**Early (I/II) (ref.)**						
	Late (III/IV)	2.62	1.49 - 4.62	**0.001**	2.997	1.69 - 5.32	**0.0002**
MIX staining score	**MIX-Low (ref.)**						
	MIX-High	2.09	1.17 - 3.75	**0.013**	2.461	1.37 - 4.44	**0.003**

(B)

		Univariate Cox analysis			Multivariate Cox analysis		
		HR	95% CI of HR	P Value	HR	95% CI of HR	P Value
Sex	**Female (ref.)**						
	Male	1.07	0.62 - 1.85	0.818	/	/	/
Age	**< 60 yrs (ref.)**						
	> 60 yrs	1.30	0.56 - 3.06	0.542	/	/	/
Stage (UICC)	**Early (I/II) (ref.)**						
	Late (III/IV)	3.21	1.84 - 5.6	**3.99e-05**	3.77	2.12 - 6.71	**6.51e-06**
MIX staining score	**MIX-Low (ref.)**						
	MIX-High	1.27	0.72 - 2.26	0.411	1.82	1.00 - 3.30	**0.049**

*Resulting HR (with 95% CI), stratifying patients for 5-years of OS **(A)** and for 7-years of OS **(B)** according to clinicopathological features, were obtained by univariate Cox modeling (left panel). Multivariate analysis (right panel) was carried out using a Cox regression model using pTNM staging and MIX scoring. CI, Confidence Interval; HR, Hazard Ratio; OS, Overall Survival; pTNM, pathology Tumor Node Metastasis.*

The p values less than or equal to 0.05 have been written in bold.



C

	5-years of OS			7-years of OS		
	HR	95% CI of HR	P Value	HR	95% CI of HR	P Value
Early Stage + MIX-Low (ref.)						
Early Stage + MIX-High	3.136	1.15 - 8.59	**0.026**	1.67	0.61 - 4.52	0.317
Late Stage + MIX-Low	3.877	1.38 - 10.91	**0.010**	3.43	1.22 - 9.64	**0.019**
Late Stage + MIX-High	8.288	3.01 - 22.86	**4.38e-05**	6.54	2.39 - 17.90	**0.0003**

FIGURE 4 | Combination of pTNM staging and MIX scoring for survival analysis at 5- and 7 years (60 and 84 months respectively). Comparison of survival curves was performed using Kaplan-Meier method (with log-rank test) by stratification of patients according to early and late stages or Low- and High-MIX scoring, at 5- **(A)** and 7- **(B)** years. **(C)** Prognostic values of pTNM staging and MIX scoring for survival analysis at 5- and 7 years of patients' follow-ups were analyzed. Resulting HR (with 95% CI) for stratifying patients for 5- (left) and 7- (right) years of OS using stage and MIX scoring combination, were obtained by univariate Cox modeling. *CI, Confidence Interval; HR, Hazard Ratio; OS, Overall Survival; pTNM, pathology Tumor Node Metastasis*.

Specific Glycan Motifs of Colon CSCs Evidenced by the ColoSTEM Dx Kit Could Also Constitute Promising Predictive Biomarkers

In order to evaluate predictive values of the ColoSTEM Dx kit, 21 tumor tissues from chemotherapeutic-treated patients were stained with the MIX: 42.8% and 57.1% tissues were respectively MIX-Low and MIX-High (**Table S2** and **Figure 5A**). Kaplan Meier curves were achieved with treated patients' OS rates at 5-years according to MIX staining levels (Low *vs* High; **Figure 5B**). 5-years OS of the MIX-High subgroup is significantly poorer than the MIX-Low, with a strong decrease in survival median (p=0.016). Univariate Cox analysis revealed MIX score (p=0.03) and, to a lesser extent, age (p=0.066) and pTNM staging (p=012), as predictive factor for OS in treated patients (**Table 4**, left panel). Multivariate analysis confirm that MIX score is an independent prognostic factor (HR: 6.98 with 95%CI 1.1 to 44.03, p=0.0387, **Table 4** right panel).

These preliminary results evidence that the ColoSTEM Dx kit might also constitute a promising predictive tool, i.e., companion test, in order to (i) allow better prediction of therapeutic responses and relapses' risk and (ii) improve therapeutic management for each patient.

DISCUSSION

CSCs play a key role in colon cancer evolution and has major implications to cancer therapy. Currently, CSCs failed to be used as biomarkers in clinical routine although these cells could reflect tumor aggressiveness and might be of prime importance for

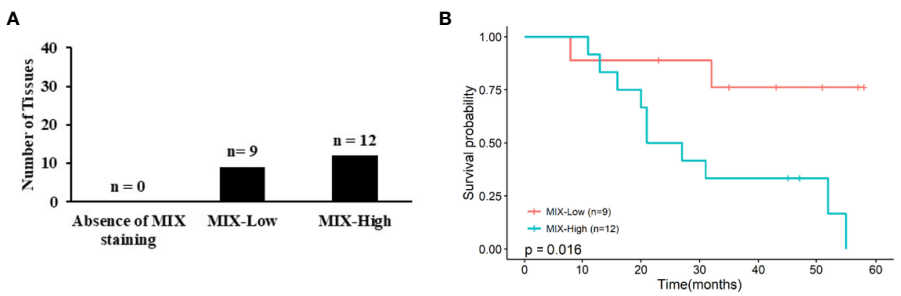


FIGURE 5 | Association between MIX scoring with chemotherapeutic-treated patients' survival rates at 5-years. **(A)** Graph represent numbers (N) of tumor samples from treated patients for which there is an absence of MIX, a MIX-Low or a MIX-High staining. All samples included (N=21) were stained with the MIX. **(B)** Corresponding Kaplan-Meier curves are displayed according to MIX staining (i.e., Mix-Low and -High). P values correspond to log-rank test.

TABLE 4 | Prognostic values of clinicopathological features (sex, age, stage and MIX staining) at 5 years of patients' follow-ups (60 months) of chemotherapeutic-treated patients' cohort.

		Univariate Cox analysis			Multivariate Cox analysis		
		HR	**95% CI of HR**	**P Value**	**HR**	**95% CI of HR**	**P Value**
Sex	**Female (ref.)**						
	Male	2.01	0.65 - 6.26	0.229	/	/	/
Age	**< 60 yrs (ref.)**						
	> 60 yrs	7.01	0.88 - 56.06	**0.067**	15.48	1.52 - 158.16	**0.021**
Stage (UICC)	**Early (I/II) (ref.)**						
	Late (III/IV)	2.91	0.76 - 11.19	0.12	4.23	0.94 - 18.98	0.0596
MIX staining score	**MIX-Low (ref.)**						
	MIX-High	5.42	1.17 - 25.07	**0.031**	6.98	1.11 - 44.03	**0.039**

Univariate and multivariate Cox regression were displayed for analysis of clinicopathological parameters and MIX scoring impact on prognostic value. CI, Confidence Interval; HR, Hazard Ratio; OS, Overall Survival; UICC, Union for International Cancer Control; yrs, years.
The p values less than or equal to 0.05 have been written in bold.

diagnosis/prognosis. Indeed, CSCs enable cancer therapeutic resistance to conventional treatments thereby conduce to therapeutic failure. Thus, the reliable detection of CSCs from patient samples might improve future patient management and survival. Nevertheless, no kits or devices developed for clinical or translational research are currently likely to specifically and efficiently detect CSCs within tissues. Furthermore, the clinical use and significance of CSCs biomarkers are still restricted due to the risk of confusing detection with biomarkers expressed by adult non-cancerous stem cells as well as differentiated cancer cells (10, 12, 19).

In this context, Carcidiag Biotechnologies decided to develop a new device for a specific detection of CSCs and tumor cells related to CSCs in FFPE tissues from patient solid biopsies, usable in a clinic-standardized way in order to improve patients' diagnosis and prognosis. ColoSTEM Dx kit, developed by Carcidiag Biotechnologies, is based on glycoproteins detection, known to be specifically (over)expressed at the surface of colon CSCs and tumor cells related to CSCs. More precisely, this diagnosis tool uses a MIX of biotinylated plant lectins that recognize glycan patterns specifically expressed or overexpressed by colon CSCs only, *i.e*, normal SCs or differentiated tumor cells are not detected by the lectin MIX. Strikingly, the MIX staining score in stained cells is higher suggesting a strong percentage of CSC. While the number of CSCs in colon cancer is generally reduced to a low percentage of total cells, this difference might be due to cell plasticity that contributes to increase the percentage of CSCs or tumor cells related to CSCs. Indeed, non-stem tumor cells can emphasize an oncogenic transformation enhancing their spontaneous conversion in cancer stem cell (CSCs)-like cells (36). This interconversion also occurs *in vivo*, CSCs-depleted fractions might give rise to tumors enriched in CSCs or cancer stem-like cells (37). A second mechanism combined to previous might explain the significant rise of MIX positive cells observed in score 3 (**Figure 1**) and thus the increase of CSCs number detected by ColoSTEM Dx kit. CSCs or tumor cells related to CSCs are likely to transfer their aggressiveness properties or stemness phenotypes to recipient non-CSCs *via* the dissemination of extracellular vesicles triggering their transformation in tumor cells with acquired stem-like properties (38, 39). Altogether, these data suggest the MIX staining might not detect only CSCs but also the colon cancer cells which gained some of "stemness" properties and are considered as cancer stem-like cells.

Here, it was evidenced that MIX-positive cells from HT-29 cell sorting show an enrichment of $EpCAM^{high}$ and $ALDH1^{high}$ cell subpopulations, consistent with a stem cell phenotype. Indeed, EpCAM expression and ALDH1 activity are both currently used to define CSCs populations in digestive cancers (40). In an experimental context, their expressions were associated with poor prognosis in both disease-free and overall survival for colorectal cancer. However, these markers are not adaptable to clinical routine use. In addition, MIX positive cells are highly able to form colonospheres, up to 8 times more than their negative counterpart, again reflecting the stem cell status of these cells. Based on this *in vitro* evaluation, the potential stem cell detection capacity of MIX was tested by IHC on 42 colorectal cancer tissue samples and the score obtained with the MIX staining (Low *vs* High) was compared to that of OCT-4 staining, a common SCs marker. While MIX-high staining seems restricted to a subset of tissues, OCT4-high staining is present in a broad panel of tissues. No significant association was been evidenced between intensity of staining with both markers. These results are consistent with previous work demonstrating that although OCT-4 is considered as a pluripotent SCs marker required to enhance the self-renewal ability, its expression was reported to be restricted in normal colon, polyp and colon cancer. Thus, OCT-4 analysis by IHC is poor of interest to characterize CSCs for diagnosis (41). Survival curves according low or high OCT-4 staining were homogeneous, while MIX-high staining reveals a significant decrease of OS, suggesting that this biomarker is clearly more relevant for monitoring patients and might be of prime interest for patient management. Consistently, it appeared that MIX-High staining is considered as an independent bad prognosis factor as validated by univariate

and multivariate Cox regression analysis (HR: 4.2). Poor prognosis value was confirmed by the decreased median survival of colon cancer patients characterized by an OCT4-High/MIX-High co-staining. As previously mentioned, we confirmed that OCT-4 staining alone (High *vs* Low) is unable to discriminate good or poor prognosis patients. These results suggest that MIX staining allows to recognize CSCs and tumor cells related to CSCs and could be useful for their detection in tumor samples and thus could predict the presence of CSCs and tumor cells related to CSCs as well as the associated risk of recurrence post-resection.

In this context, the prognosis significance of the ColoSTEM Dx kit has been assessed on several cohorts of non-chemotherapeutic-treated (total of N=208 tissues) and chemotherapeutic-treated (total of N=21 tissues) colon cancer patients, according to MIX scoring and clinicopathological data available (gender, age and stages). The MIX staining has revealed a significant prognosis value especially at early stages within the 5 years of patient follow-up, independently of pTNM staging. The prognosis value of MIX staining on OS at 5 years was confirmed but not demonstrated at 7 years suggesting that the MIX could be of prime importance at early stages of colon cancer and might be used to predict treatment outcome at this stage. Since the MIX staining constitute an independent poor prognosis factor, from pTNM staging and from other clinical parameters (age and gender), it could be crucial in the future management of patients. In a similar way, patients stratification using combination of MIX and pTNM stages allows improvement of the patient's classification. Indeed, this combined analysis highlights that MIX-High staining is a marker of poor prognosis and could be used as a predictive biomarker to improve management of patients. Indeed, regardless of stage, MIX-high is associated with a poor prognosis with a HR of 3 to 8 in early or late stage, respectively, at 5 years of follow-up. The same trend was observed at 7 years, without significance. Thus, patients who are still alive at 7 years in the OS cohort are probably very good prognosis colorectal cancer. The bad prognosis value of MIX staining has been confirmed in chemotherapeutic-treated patients' cohort, supporting that the MIX could also be likely to predict patients' treatment outcome at early stages and maybe in future to prevent tumor burden by early detection of recurrence.

Even if new tools such as Immunoscore and circulating tumor DNA aid to accurately characterize patients with minimal residual disease, they don't allow to identify the specific presence of CSCs or tumor cells related to CSCs within tumor. The presence of circulating cancer cells does not predict the presence of CSCs within the tumor mass; currently, no direct relationship between the presence of circulating cancer cells and the presence of CSCs was been found in the literature. On the contrary, the ColoSTEM Dx kit is efficient to detect CSCs and cancer stem-like cells even from early-stage tumor. It can therefore be complementary to current approaches. Nevertheless, further developments are required and will include validation in prospective multicenter interventional outcome studies in order to confirm on a wide cohort of chemotherapeutic-treated patients that MIX staining could have a predictive value in the early stages. This newly prognostic tool could be spread to any kind of solid cancer [39] and it appear very promising as there is currently no kit used in clinical routine for the detection of CSC and cancer stem-like cells to our knowledge.

In summary, it appears that ColoSTEM Dx kit demonstrated its significance to detect CSCs or tumor cells related to CSCs, more efficiently than OCT-4 and could be a new tool usefully in clinical management of colon cancer, due to their potential to predict tumor aggressiveness, even on colorectal cancer early stages.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Limoges University Hospital. Tumor samples were provided by Biological Resources Collection (BRC-Biolim-Cancer) from Limoges University Hospital certified NF S 96900 since 2014. Reporting number of collections held by the BRC-Biolim-Cancer: DC-2010-1074. Authorization number for the transfer of biological resources: AC-2013-1853.

PATENTS

The patent under the national registration number 1561763 (N° WO2016FR53196 and WO2016FR53197 publication number 3044680 and 3044681) results from a part of the work reported in this manuscript, i.e., experimental data depicted in the supplementary materials (**Figure S1**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Limoges University Hospital. Tumor samples were provided by Biological Resources Collection (BRC-Biolim-Cancer) from Limoges University Hospital certified NF S 96900 since 2014. Reporting number of collections held by the BRC-Biolim-Cancer: DC-2010-1074. Authorization number for the transfer of biological

resources: AC-2013-1853. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, SD, FL, M-OJ, VC and MM. Methodology, CB, MP, AL, SeB, SD, FL, M-OJ and MM. Validation, AL, SaB, SD, FL, M-OJ, VC and MM. Formal analysis, AL, CB, SaB, SD, FL. Investigation, FL, M-OJ and MM. Resources, AC, FL, M-OJ and MM. Data curation, AC and MM. Writing-original draft preparation, SaB, SD, NC, VC, FL and MM. Writing-review and editing, SaB, SD, VC, FL and MM. Visualization, SaB, SD, FL, M-OJ, VC and MM. Supervision, FL, M-OJ, VC and MM. Project administration, FL, M-OJ, VC and MM. Funding acquisition, FL, M-OJ, VC and MM. All authors have read and agreed to the published version of the 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.2022.918702/full#supplementary-material

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Conflict of Interest: Authors SB, CB, MP, and VC were employed by Carcidiag Biotechnologies company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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