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The impact of alkalizing the acidic tumor microenvironment to improve efficacy of cancer treatment

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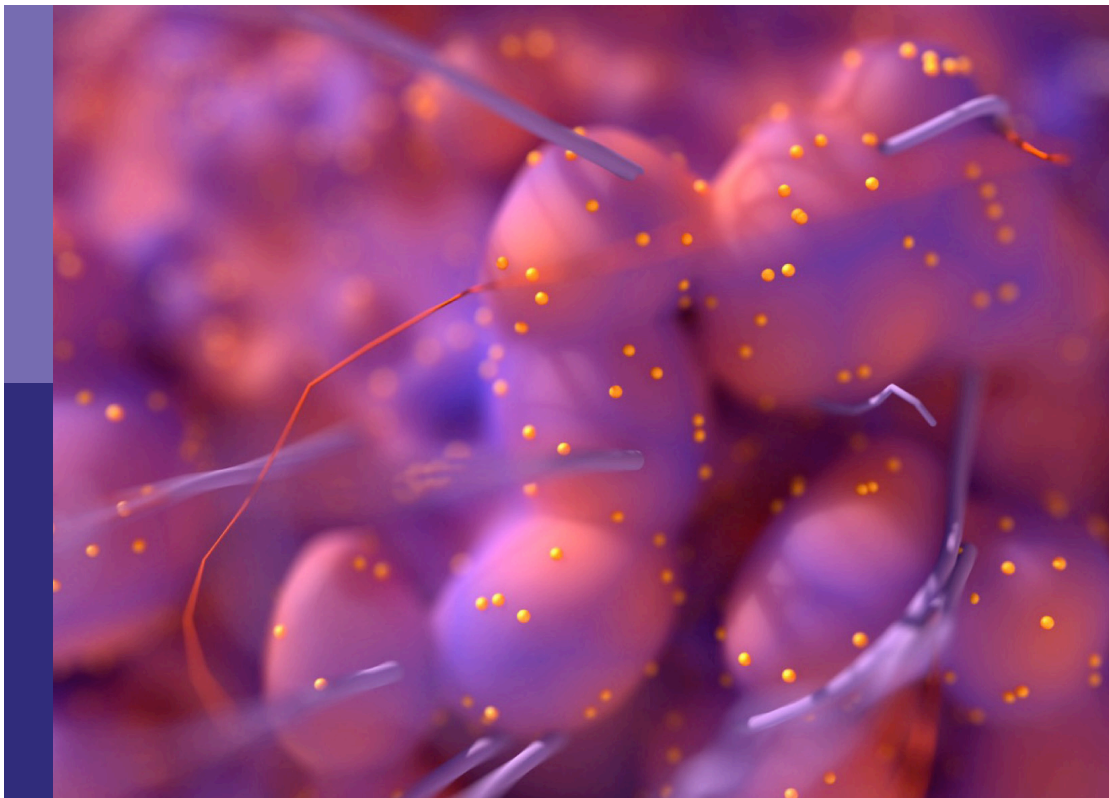

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The impact of alkalizing the acidic tumor microenvironment to improve efficacy of cancer treatment

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Editorial: The impact of alkalizing the acidic tumor microenvironment to improve efficacy of cancer treatment

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KEYWORDS

cancer, cancer metabolism, tumor microenvironment, alkalization therapy, multi-drug resistance, pH

Editorial on the Research Topic

The impact of alkalizing the acidic tumor microenvironment to improve efficacy of cancer treatment

In recent years, the acidic tumor microenvironment (TME) that is created by cancer-specific metabolism has attracted much attention in cancer therapy. In this Research Topic, we discuss the wide range of knowledge that has accumulated regarding cancer metabolism, focusing on the effects of acidity of the TME on cancer pathology. Points discussed include characteristics of the acidic TME (Bogdanov et al.), an overview of alkalization therapy (Hamaguchi et al., Wada et al.), a clinical trial of alkalizing agents on cancer patients (Gillies et al.), the association between acidic TME and glioblastoma (Seyfried et al.), the association between pH of the TME and the immunological state (Hosonuma and Yoshimura in press), role of the immunosuppressive TME in pancreatic cancer (Hashimoto et al.), *Drosophila* as an effective toolkit to investigate cancer metabolic abnormalities (Jiang et al.), acidic imaging positron emission tomography probes (^{89}Zr-labeled pH-low insertion peptides) (Bauer et al.), role of the proton-sensing G protein-coupled receptor GPR68 in breast cancer (Elemam et al.), the association between cancer and chronic heart failure focusing on mitochondrial abnormalities (Takada et al.), and the association between cancer metabolism and ascorbic acid (Maekawa et al.). To address cancer metabolism and target it as a treatment, it is necessary to recognize how cancer develops, and what characteristics of the metabolic process are involved. Here, we will outline the origins and metabolism of cancer, how to deal with it, and the importance of alkalization of the TME.

How does cancer develop? The most important point was reported by Otto Warburg in "On the origin of cancer cells" (1). Cancer cells develop when there is a lack of oxygen, but a supply of nutrition. Cancer cells are primarily glycolytic, as they perform fermentation, meaning that they are dependent on the glycolytic system rather than oxidative phosphorylation by cellular respiration. The essence of this is the presence of mitochondria in eukaryotic cells. Cancers comprise cells that have been forced to choose their own path of life without working, but not failing, mitochondria. Gilles R. and Gatenby R. et al. reported in detail that cancer cells are dependent on aerobic glycolysis for survival

as a result of Darwinian selection pressure (2). In addition, as Seyfried T. has stated, cancer can be considered as a metabolic disease (3). These points suggest that cancer cells try to survive on their own in an environment where there is a lack of oxygen but a supply of nutrients. In other words, cancer is comprised of cells that have lost their coordination with other cells in the body, and are living on their own.

How does cancer metabolism work? Cancer cells have a unique metabolism that differs from that of normal cells, and as enhanced glycolysis generates large amounts of acidic substances (protons) inside the cell, cancer cells expel protons to the outside of the cell by proton transporters, resulting in the inside of the cell being alkaline and the outside being acidic (4). The most important proton transporter that is involved in this phenomenon is sodium/proton (Na^+/H^+) exchanger isoform 1 (5). In the general biological environment, the extracellular pH of normal cells is maintained at pH 7.2 to 7.4, whereas the pH around cancer cells tends to be more acidic at pH 6.2 to 6.8 (6). This acidification of the TME has been reported to promote cancer progression. In this state, cancer cells become resistant to a variety of treatments, their proliferation is activated, and their metastatic potential is also increased (7, 8). In general, current cancer treatments do not target the pH balance of the TME that results from this cancer-specific metabolism. This means that adequate and satisfactory cancer treatment results have not yet been achieved.

What happens when the acidic TME is alkalized? Reversal of the pH gradient between the inside and the outside of cancer cells, i.e., extracellular acidification and intracellular alkalinization, attenuates the intracellular concentration of many anticancer drugs, and leads to resistance to anticancer drug treatments (5). For example, it has been reported that an increase in intracellular pH from 7.0 to 7.4, although in an experimental system, leads to a 2,000-fold increase in adriamycin resistance in human lung cancer cell lines (9). Conversely, lowering the intracellular pH (raising the extracellular pH) of cancer cells is expected to attenuate their resistance to various anticancer drugs, and to make anticancer drug therapy more effective. Furthermore, an acidic TME is known to decrease anticancer immune responses, and hence alkalinization of the acidic TME is expected to improve the function of immune cells, such as dendritic cells, natural killer cells, cytotoxic T cells, and macrophages (10, 11). In addition, this treatment method of lowering the intracellular pH (raising extracellular pH) may be sufficiently effective on its own (Wada et al.).

Clinical methods for alkalization of this acidic TME include alkalization therapy with alkalizing agents or proton pump inhibitors (Hamaguchi et al.). In addition, the influence of the daily diet should also be considered. Diets with alkalizing effects are rich in vegetables and fruits, which at the same time have anti-inflammatory and gut-regulating properties (12). Alkalization therapy is a treatment that acts on cancer metabolism, and can be used in combination with anticancer drugs, radiation therapy, and other therapies, and is also a safe treatment method. In the future, the combination of alkalization therapy and conventional therapy for the treatment of cancer needs to be further investigated in prospective clinical trials.

Author contributions

RH and HW performed the literature review and wrote the article. SU performed the literature review. All authors conceived and designed the study and gave final approval for publication.

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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.

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ORIGINAL RESEARCH
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Expression of GPR68, an Acid-Sensing Orphan G Protein-Coupled Receptor, in Breast Cancer

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Background: Breast cancer (BC) is the most diagnosed cancer and the leading cause of global cancer incidence in 2020. It is quite known that highly invasive cancers have disrupted metabolism that leads to the creation of an acidic tumor microenvironment. Among the proton-sensing G protein-coupled receptors is GPR68. In this study, we aimed to explore the expression pattern of GPR68 in tissues from BC patients as well as different BC cell lines. Methods: In-silico tools were used to assess the expression of GPR68 in BC patients. The expression pattern was validated in fresh and paraffin-embedded sections of BC patients using qPCR and immunohistochemistry (IHC), respectively. Also, in-silico tools investigated GPR68 expression in different BC cell lines. Validation of GPR68 expression was performed using qPCR and immunofluorescence techniques in four different BC cell lines (MCF-7, MDA-MB-231, BT-549 and SkBr3). Results: GPR68 expression was found to be significantly increased in BC patients using the in-silico tools and validation using qPCR and IHC. Upon classification according to the molecular subtypes, the luminal subtype showed the highest GPR68 expression followed by triple-negative and Her2-enriched cells. However, upon validation in the recruited cohort, the triple-negative molecular subtype of BC patients showed the highest GPR68 expression. Also, in-silico and validation data revealed that the triple-negative breast cancer cell line MDA-MB-231 showed the highest expression of GPR68. Conclusion: Therefore, this study highlights the potential utilization of GPR68 as a possible diagnostic and/or prognostic marker in BC.

Keywords: GPR68, acidosis, tumor microenvironment, breast cancer, triple-negative breast cancer

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INTRODUCTION

According to the World Health Organization (WHO), breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases. Furthermore, BC was reported to be the fifth leading cause of cancer-related mortality worldwide, with 685,000 deaths (1).

It is quite known that highly malignant and invasive cancers have disrupted metabolism and specifically an elevated glycolytic activity. This creates an acidic milieu, also known as the Warburg effect, which is an important hallmark of the tumor microenvironment (TME) (2). Such an environment regulates proliferation, apoptosis, and metastasis of cancer cells as well as modulate inflammation, anti-tumor immunity, and angiogenesis (3–5). Possible antagonizing approaches to this environment is the use of bicarbonate buffer that reduces growth and metastasis of cancers including melanoma, breast, prostate, pancreatic and lung cancers (6–8). Consequently, targeting tumor acidity may serve as a potential and promising therapeutic approach for cancers.

There are several acid-sensing cell surface receptors and ion channels that can sense acidity in the microenvironment; among them are proton sensing G protein-coupled receptors (GPCRs). GPCRs are considered the largest family of cell signaling receptors with over 800 GPCRs encoded in the human genome, representing approximately 3% of the human genome. They are seven-transmembrane spanning domain receptors that respond to numerous types of extracellular signals such as lipids, peptides, proteins, ions, and photons which regulate many physiological processes (9). Furthermore, GPCRs represent more than 30% of targets for FDA approved small molecules (10, 11). In tumors, GPCRs are known to regulate cellular processes that are critical for the initiation and progression of tumors, such as cell proliferation, inhibition of apoptosis, immune evasion, tumor invasion, angiogenesis, and metastasis (12, 13).

Among the members of the proton sensing GPCRs is GPR68, also known as ovarian cancer G protein-coupled receptor 1 (OGR1). It was first identified from the HEY human ovarian cancer cell line and is located on chromosome 14 band q31 (14q31) (14). So far, the only endogenous agonist of GPR68 is H^+ ions/acidic environment, where it is inactive at pH 7.8 and becomes activated at pH 6.8 (15). Being coupled with Gαq subunit, GPR68 activation triggers Ca^{2+} release from intracellular stores, stimulates protein kinase C (PKC) signaling and formation of inositol trisphosphate (IP3). Moreover, GPR68 activates the mitogen-activated protein kinase (MAPK) signaling pathways (16–19). Also, GPR68 acts as a double-edged sword, where it was found to be a tumor-suppressor in the prostate cancer (20), whereas other studies revealed that GPR68 has an oncogenic profile by promoting cancer outgrowth (21). In this study, we sought to investigate GPR68 expression in the breast tumor microenvironment that might aid in sensing acidosis and regulating BC progression.

SUBJECTS, MATERIALS AND METHODS

In-Silico Expression of GPR68 in Breast Cancer Patients and Cell Lines

In-silico tools TNM plot (https://www.tnmplot.com/) (22) and UALCAN TCGA data analysis (http://ualcan.path.uab.edu/index.html) (23) were used to assess the expression of GPR68/OGR1 in various cancers compared to normal tissues. Also, these tools were used to explore GPR68 expression in BC tissues compared to healthy ones. Moreover, the UALCAN tool was used to retrieve Kaplan-Meier plots in order to investigate if there is an association between GPR68 expression levels and the survival of BC patients. The UALCAN tool was also used to explore the association with the clinicopathological parameters of BC patients. On the other hand, GPR68 expression was explored in the different BC cell lines using the in-silico tool EMBL-EBI (https://www.ebi.ac.uk/gxa/home) by examining the data of RNA-seq in 934 human cancer cell lines from the cancer cell line encyclopedia.

Breast Cancer Tissues

The cohort included in this study was composed of a total of 98 female Egyptian BC patients who underwent conservative breast surgery/mastectomy in Alexandria University, Kasr El-Aini and the National Cancer Institute hospitals, Egypt. Pathologists confirmed the pathological diagnosis of all samples, and their clinicopathological parameters were summarized in **Table 1**. The mean age (±SD) of recruited patients was 47.18 (±11.40) years. Some of the adjacent normal counterparts of the cancerous tissues were resected (n=15), that were used in the comparison with the fresh BC samples (n=28). Also, other non-tumor fibrocystic breast tissues (n=20) were collected for histological comparison to the formalin-fixed paraffin-embedded (FFPE) BC tissues (n=70). All patients enrolled in this study agreed and signed informed consents. The study was approved by the research ethics committee of the University of Sharjah, UAE (REC-21-09-04-01). All experiments were performed in compliance with the ethical standards of the declaration of Helsinki.

Cell Culture of Breast Cancer Cell Lines

Four different cell lines were used in the study, hormonal luminal A cell line (ER^+, PR^+, $Her2^-$: MCF-7), triple-negative/basal-like cell lines (ER^-, PR^-, $Her2^-$: BT-549 and MDA-MB-231), and the $Her2^+$ SKBr3 (ER^-, PR^-, $Her2^+$). All four cell lines were obtained from ATCC, USA. MCF-7, BT-549, MDA-MB-231 cell lines were cultured in complete RPMI-1640 medium, while SkBr3 was cultured in complete DMEM media. All culture media were supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 71.5 µM 2-mercaptoethanol, and 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA).

RNA Extraction, Reverse Transcription, and qRT-PCR

Fresh breast tissues were snapped frozen in liquid nitrogen directly after collection and stored at -80°C. For RNA extraction from BC

TABLE 1 | Clinicopathological characteristics of the recruited cohort of breast cancer patients (n = 98).

Category	Frequency	Percent (%)
Age		
≤ 40	28	28.6
> 40	70	71.4
Tumor size		
T1	16	16.3
T2	50	51.0
T3	32	32.7
Histologic type		
Invasive ductal carcinoma	89	90.8
Invasive lobular carcinoma	5	5.1
Others	4	4.1
Histologic grade		
G1	8	8.2
G2	65	66.3
G3	24	24.5
G4	1	1.0
ER status		
Negative	47	48.0
Positive	51	52.0
PR status		
Negative	45	45.9
Positive	53	54.1
Her2 status		
Negative	78	79.6
Positive	20	20.4
Ki-67		
Low (<14)	26	26.5
High (≥14)	72	73.5
Molecular subtype		
Luminal A	33	33.7
Luminal B	24	24.5
Her2-enriched	9	9.2
Triple-negative	32	32.7
Nodal status		
N0	26	26.5
N1	32	32.7
N2	21	21.4
N3	19	19.4
Tumor stage		
Stage 1	9	9.2
Stage 2	36	36.7
Stage 3	53	54.1

tissues, Trizol RNA extraction method was applied. For cell lines, RNA was extracted using the RNeasy extraction kit (Qiagen, Germany). Complementary DNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermofisher Scientific, USA). GPR68/OGR1 expression was detected and quantified using the primers (Forward: GTTTGAAGGCGG CAGAAATG, Reverse: GTGGAATGAGGAGGCATGAA), HOT FIREPol EvaGreen qPCR Supermix (SolisBioDyne, Estonia) and Quantstudio 3 real time qPCR (Applied Biosystems, USA). Ribosomal 18S was used as housekeeping gene and relative quantification was calculated as $2^{-\Delta\Delta CT}$.

Immunohistochemical Staining and Scoring of GPR68 Expression

BC paraffin-embedded tissues were sectioned at 4 µm, after which they were stained with rabbit anti-human GPR68 antibody (Invitrogen, USA, Cat. no: 720277), at 1:200 dilution. The secondary conjugation and detection were done using UltraVision Quanto Detection System HRP and DAB Quanto (Thermofisher Scientific, USA). The images were captured with Olympus DP74 microscope digital camera attached to a BX43 microscope (Olympus Life Sciences, Tokyo, Japan). Immunoreactive score (IRS) was used to evaluate the expression status of GPR68 in the different samples according to the recommendations by Remmele and Stegner (24). IRS is usually generated by the multiplication of the staining intensity and the percentage of immuno-stained cells with a range from 0-12. Microscopic evaluation of the immunohistochemical stainings was performed by two independent investigators. Also, semiquantitative analysis of DAB staining of GPR68 was done using the immunohistochemistry (IHC) Toolbox plugin in Image J software (https://imagej.nih.gov/ij/index.html). Optical density (OD) was calculated as log (max intensity/mean intensity).

Immunofluorescence of GPR68 in Breast Cancer Cell Lines

The four different BC cell lines were seeded in 6 well plates, coated with cover slides. The cells were washed with PBS, fixed using 4% paraformaldehyde for 15 minutes and permeabilized using 0.1% Triton-X for 10 minutes. The cells were stained with the primary anti-human GPR68 antibody (Invitrogen, USA, Cat. no: 720277, 2 µg/ml), at 4°C and left overnight. Then, the secondary antibody AlexaFluor 488-conjugated goat anti-rabbit IgG (Invitrogen, USA, Cat. no: A-11008) was incubated for 45 minutes. After washing multiple times, coverslips were removed carefully and loaded on slides with DAPI nuclear stain (Invitrogen, USA). The images were captured with Olympus DP74 microscope digital camera attached to an BX43 inverted microscope (Olympus Life Sciences, Tokyo, Japan), at x400 and x1000. The blue color indicated the nucleus of the BC cell lines while the green color indicated the GPR68 expression.

Statistical Analysis

Statistical analysis was performed using SPSS 27 (IBM, Armonk, NY, USA) software package and GraphPad Prism 6 (San Diego, CA, USA). For SPSS analysis, descriptive univariate analyses were conducted using frequencies and percentages for categorical variables as well as means, medians, and standard deviations for scale variables. The Chi-square test was performed to assess the associations between categorical variables. The normality of continuous variables was tested visually using the Q-Q plots and statistically using the Kolmogorov-Smirnov test. Differences in the means of normally distributed continuous variables were analyzed using the independent t-test and ANOVA test, for two independent or multiple samples, respectively. Non-parametric tests, including Mann–Whitney or Kruskal–Wallis tests were used for skewed continuous outcomes. For GraphPad Prism analyses, normality tests were conducted, and the non-parametric Mann Whitney U-test was used to compare two groups. P-value <0.05 was considered statistically significant.

RESULTS

In-Silico Analysis of GPR68 Expression in Various Cancers

GPR68 expression was assessed across various cancer types using the online tools TNMplot (https://www.tnmplot.com) and UALCAN (http://ualcan.path.uab.edu/index.html). As shown in **Figure 1A**, the TNMplot tool explored the GPR68 expression in various cancer types, where BC was among the tumors with a significant differential expression. This expression pattern was also reported in the UALCAN tool data (**Figure 1B**). In particular, as shown in **Figure 1C**, BC tumor tissue showed higher GPR68 expression compared to adjacent normal tissues (p=1.15e-17). This was further validated by UALCAN tool where a similar expression pattern was observed in BC patients (n=1097) that showed significantly higher GPR68 compared to normal breast tissues (n=114) with a p-value of 1.63e-12 (**Figure 1D**).

Breast Cancer Patients' Survival Based on GPR68 Expression

It was crucial to explore whether GPR68 might have any effect on the prognosis and survival of BC patients. To investigate this issue, in-silico UALCAN tool was implemented. The results showed that GPR68 was not a potential prognostic factor in BC (p=0.85, **Figure 2A**). However, upon the classification of patients according to the molecular subtypes, luminal, Her2-enriched or triple-negative, GPR68 showed a significant effect on BC patients' survival (p=0.0064, **Figure 2B**).

Validation of GPR68 mRNA and Protein Expression in Breast Cancer Patients

The in-silico data was validated in BC patients' samples collected from different hospitals. As illustrated in **Figure 3A**, the mRNA of GPR68 expression was higher in BC patients compared to normal breast tissues (p<0.01). Further, the in-silico data revealed that GPR68 is differentially expressed in the various molecular subtypes of BC. High GPR68 expression was found to be in the luminal as well as the triple-negative molecular subtype, as shown in **Figure 3B**. To confirm these observations, quantitative real-time qPCR performed on our cohort showed that triple-negative and luminal B subtypes had high expression of GPR68 expression as compared to normal controls (**Figure 3C**, p<0.05 and p<0.0001, respectively).

In addition, GPR68 expression was validated in the recruited cohort and assessed by immunohistochemical staining of paraffin-embedded BC tissues. Different intensities were

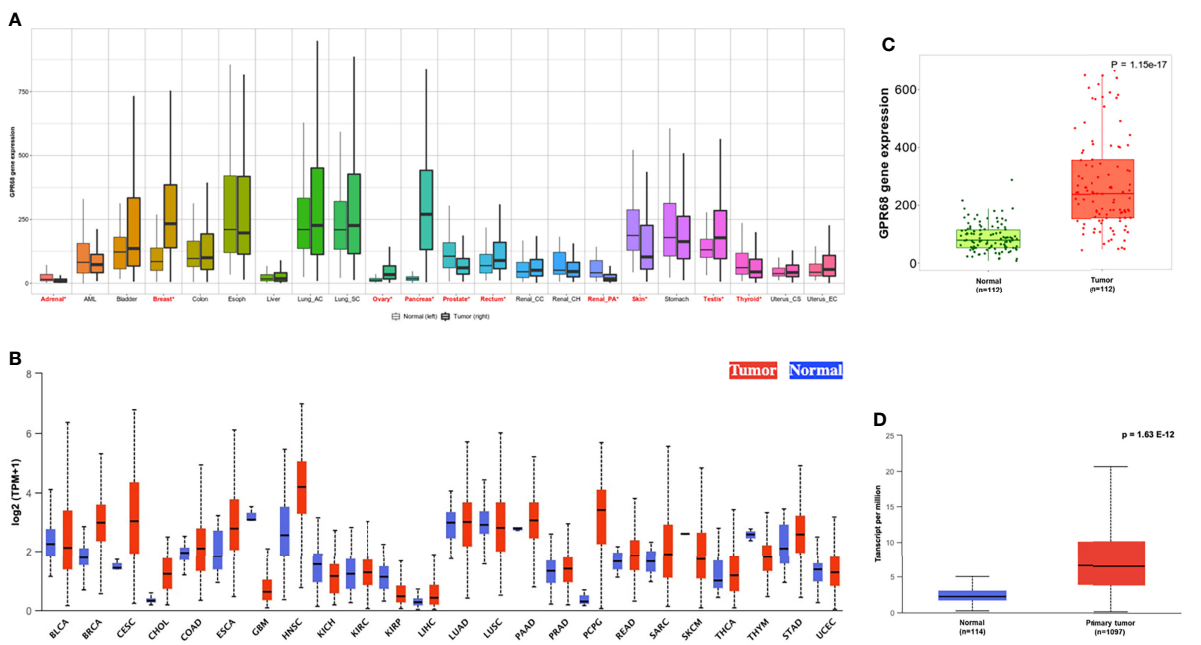

FIGURE 1 | In-silico expression of GPR68 across various cancer types. **(A)** TNMplot showing breast cancer to be among the cancers where GPR68 was upregulated in tumor tissues. The significant differences by the Mann-Whitney U test are marked with "red*". **(B)** UALCAN tool supporting GPR68 upregulation in breast cancer. **(C)** GPR68 was upregulated in the breast cancer tissues (n=112) compared to paired adjacent normal breast tissues using TNMplot data analysis. **(D)**UALCAN tool confirmed the upregulation pattern in 1097 breast cancer patients compared to 114 normal breast tissues. BLCA, Bladder urothelial carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and neck squamous cell carcinoma; KICH, Kidney chromophobe; KIRC, Kidney renal clear cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin cutaneous melanoma; STAD, Stomach adenocarcinoma; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC , Uterine corpus endometrial carcinoma.

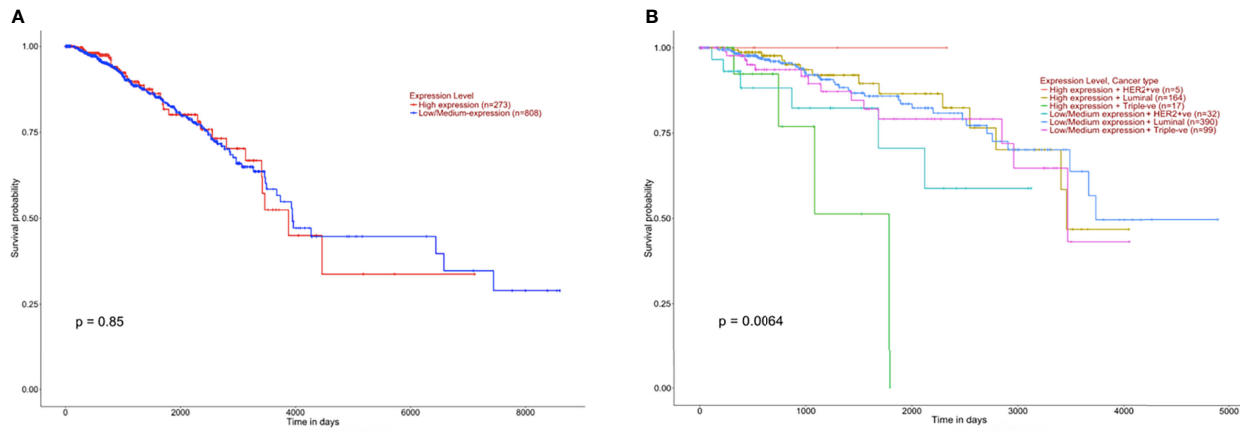

FIGURE 2 | Kaplan Meier survival graphs of breast cancer patients based on GPR68 expression using UALCAN in-silico tool. **(A)** Survival plot of breast cancer patients that are classified according to high and low GPR68 expression showing that it is not a prognostic factor. **(B)** Classification of breast cancer patients with low and high GPR68 expression as well as molecular subtypes (Her2-enriched, luminal and triple-negative) showed GPR68 to be a potential marker affecting the survival of breast cancer patients.

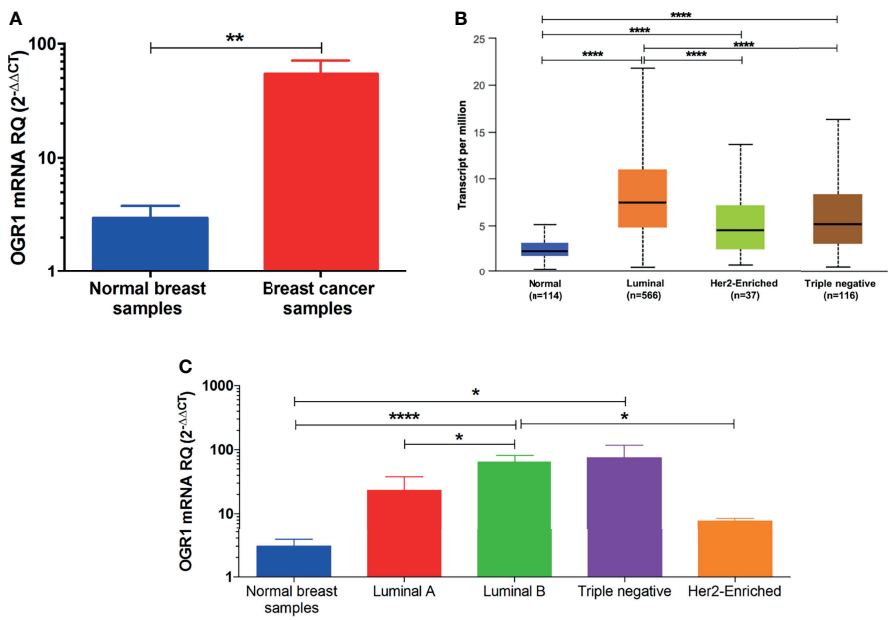

FIGURE 3 | Quantification of GPR68 mRNA expression in breast cancer patients using qRT-PCR. **(A)** GPR68 mRNA expression was upregulated in breast cancer tissue samples of the recruited cohort compared to their normal counterparts. **(B)** In-silico analysis of GPR68 among the various molecular subtypes of breast cancer. **(C)** Validation of GPR68 expression on the mRNA level in the luminal A, luminal B, Her2-enriched and triple-negative breast cancer subtypes. *p<0.05, **p<0.01, ****p<0.0001.

observed in BC tissues ranging from mild, moderate, to strong staining, with a cytoplasmic and/or membranous localization (**Figure 4A**). All BC sections were scored using the IRS system, that is usually generated by the multiplication of the staining intensity and the percentage of immuno-stained cells with a range from 0-12 (24).GPR68 was found to be higher in BC samples when compared to non-tumor breast tissues (**Figure 4B**). As shown in **Figure 4C**, upon the classification of BC patients, GPR68 expression in BC tissues showed a high expression in all the molecular subtypes. Such an expression pattern was further confirmed using the semi-quantification method via IHC Toolbox by Image J, where a higher expression of GPR68 was observed in BC tissues compared to non-tumor breast samples (**Figure 4D**). The expression across

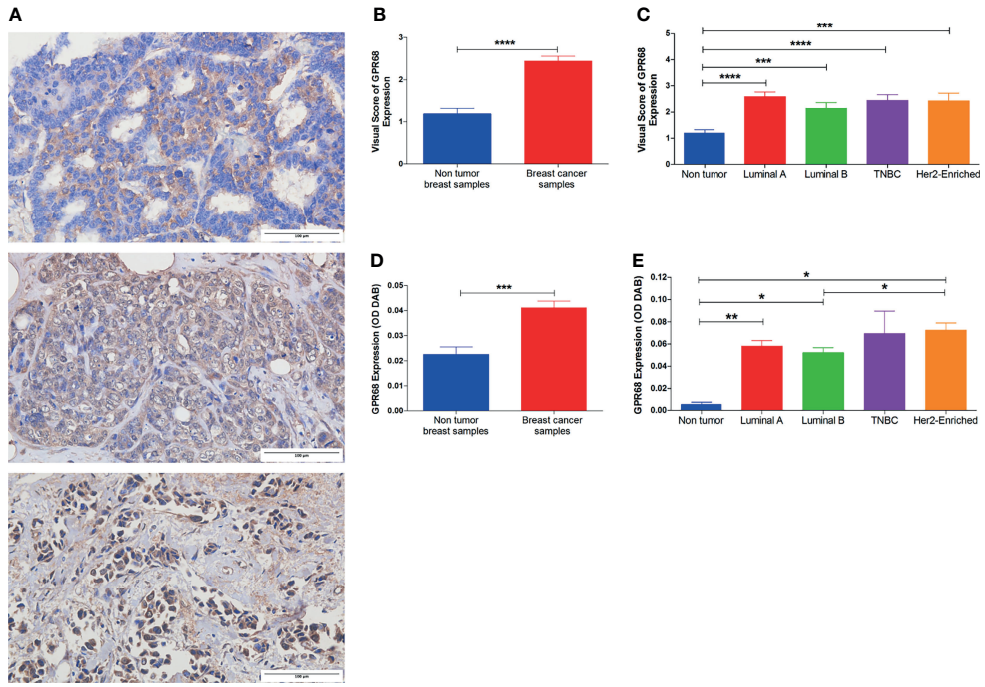

FIGURE 4 | Immunohistochemical assessment of GPR68 expression in paraffin-embedded breast cancer tissues. **(A)** Microscopic images showing various degrees of intensity: mild, moderate, and strong GPR68 expression. Images were captured at x400 magnification, with a scale bar representing 100 μm. Brown/DAB staining denotes GPR68 expression. **(B)** Immunoreactive scoring of GPR68 expression in immunohistochemical staining of BC tissues compared to non-tumor tissues. **(C)** GPR68 expression according to immunoreactive scores between the different molecular subtypes of breast cancer patients. **(D)** Semi-quantitative assessment of GPR68 expression in breast cancer patients compared to non-tumor breast tissues, by calculating the optical density of DAB substrate using IHC toolbox-Image J. **(E)** Semi-quantitative assessment of GPR68 expression across the different molecular subtypes of breast cancer patients. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

the different molecular subtypes was compared where the highest expression was observed in the triple-negative BC group, followed by the Her2-enriched and subsequently the luminal subgroup (**Figure 4E**). A negative control image as well as representative images of GPR68 expression in the different molecular subtypes of BC are illustrated in **Figure S1**.

Association of GPR68 Expression With the Clinicopathological Parameters of Breast Cancer Patients

It was important to assess the correlation between GPR68 expression and the clinicopathological parameters of BC patients, in the in-silico data and recruited cohort (**Table 2**). The in-silico data revealed GPR68 expression to be unaltered across the different age groups. This was similar to the association reported from our recruited cohort, where age was not found to affect GPR68 expression in BC patients. Upon investigating the association with the molecular subtypes of BC, the in-silico data showed that the luminal group had a higher expression as compared to the Her2-enriched and triple-negative patients ($p<0.0001$ for both). However, in our recruited cohort the semi-quantification of GPR68 expression was higher in non-hormonal BC patients (triple-negative and Her2-enriched) when compared to the hormonal luminal A and B ($p=0.046$). This was further supported by a higher GPR68 expression in the PR negative BC patients as compared to PR positive BC patients ($p<0.05$). Lastly, in-silico data reported a significant change according to nodal metastasis status, where BC patients with N1 and N2 profiles had higher GPR68 expression compared to those with N0 ($p<0.05$). However, this was not observed in the recruited cohort.

The observed discrepancy between the in-silico and validated GPR68 expression could be attributed to the different ethnicities between the BC patients. As mentioned earlier, our recruited cohort is comprised of Egyptian BC patients, i.e., African ethnicity, while the in-silico data was mainly composed of Caucasians, African Americans, and Asians. Intriguingly, the in-silico UALCAN tool showed that there was a significant increase in GPR68 expression in the Caucasian population in comparison to the African American population ($p<0.05$, **Figure 5**). This highlights the impact of race and ethnicity on GPR68 expression in BC.

Validation of GPR68 mRNA and Protein Expression in Breast Cancer Cell Lines

In order to assess the effect and mechanism of GPR68 in BC, four cell lines were selected as they showed various GPR68 expression

TABLE 2 | Association between GPR68 expression and the clinicopathological parameters of breast cancer patients, using data from the in-silico and the recruited cohort in the study.

	In-silico		Recruited Cohort		
	Categories	**p value**	**Categories**	**GPR68 expression**	**p value**
Age	21-40 yrs vs. 41-60 yrs	0.887	≤ 40	0.033492	0.782
	21-40 yrs vs. 61-80 yrs	0.832			
	21-40 yrs vs. 81-100 yrs	0.999			
	41-60 yrs vs. 61-80 yrs	0.897	> 40	0.044524	
	41-60 yrs vs. 81-100 yrs	0.91			
	61-80 yrs vs. 81-100 yrs	0.866			
Tumor Stage	Stage 1 vs. Stage 2	0.851	Early (1–2)	0.044313	0.308
	Stage 1 vs. Stage 3	0.24			
	Stage 1 vs. Stage 4	0.376			
	Stage 2 vs. Stage 3	0.105	Advanced (3–4)	0.040389	
	Stage 2 vs. Stage 4	0.381			
	Stage 3 vs. Stage 4	0.766			
Molecular Subtype	Luminal vs. Her2-enriched	**0.000002******	Non-hormonal:	0.048693	**0.046***
	Luminal vs. triple-negative	**0.000078******	Triple-negative & Her2-enriched		
	Her2-enriched vs. triple-negative	0.209	Hormonal:	0.027408	
			Luminal A & B		
Nodal Metastasis Status	N0 vs. N1	**0.011***	Negative	0.042336	0.529
	N0 vs. N2	**0.045***			
	N0 vs. N3	0.813			
	N1 vs. N2	0.764	Positive	0.038511	
	N1 vs. N3	0.241			
	N2 vs. N3	0.233			

*Bold text indicates significant findings. *p<0.05 and ****p<0.0001.*

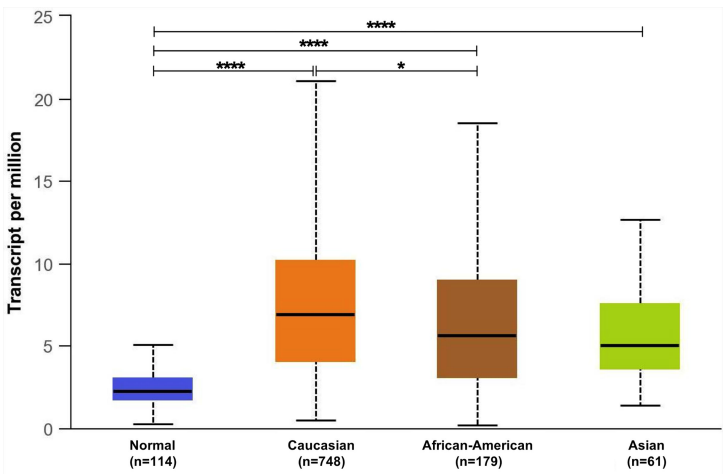

FIGURE 5 | In-silico analysis of GPR68 expression across the different ethnicities (Caucasians, African-American and Asians) of breast cancer patients compared to normal breast samples. *p<0.05 and ****p<0.0001.

according to the EBI tool using RNA-seq data of cancer cell line encyclopedia (**Figure 6A**). Upon validation of this data with qPCR, the triple-negative adenocarcinoma MDA-MB-231 showed the highest expression of GPR68 at the mRNA level, followed by the luminal A MCF-7 cell line, followed by $Her2^{+}$ SkBr3 and lastly the triple-negative invasive ductal carcinoma BT-549 (**Figure 6B**). This was further validated using immunofluorescence, where a cytoplasmic and membranous expression of GPR68 was observed (**Figure 6C**).

DISCUSSION

In this study, GPR68 expression in BC was explored using various approaches including in-silico analysis, fresh biopsies, FFPE tissues, and cell lines. A significant upregulation pattern was observed along with a differential expression in BC molecular subtypes, suggesting a potential role in BC pathogenesis that needs to be further studied.

The effects of acidosis on cancer cells have been previously investigated in different tumors (3). However, the exact

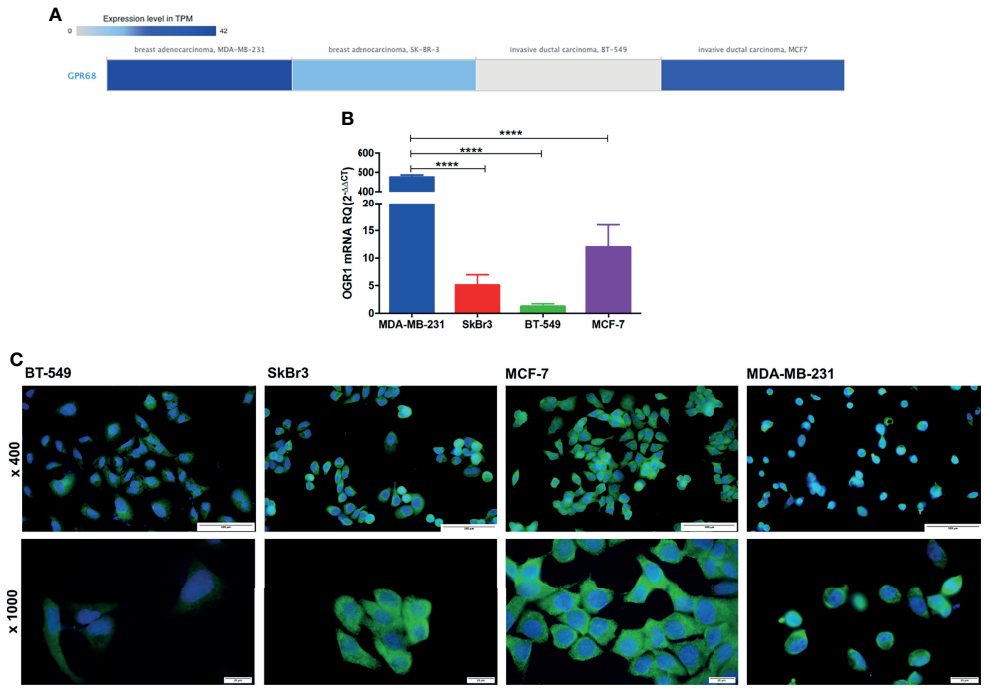

FIGURE 6 | GPR68 expression in breast cancer cell lines. **(A)** In-silico data showing GPR68 expression in 4 different breast cancer cell lines using the EMBL-EBI tool. The grey color represents the lowest expression while the darkest blue color represents the highest expression. **(B)** Validation of GPR68 mRNA expression using qPCR in breast cancer cell lines with MDA-MB-231 showing the highest expression. **(C)** Immunofluorescence of GPR68 in breast cancer cell lines, where the blue color indicates the nucleus, and the green fluorescence represents GPR68 expression. Microscopic images were captured at x400 and x1000 magnification, with a scale bar of 100 and 20 μm, respectively. ****p<0.0001.

mechanisms and receptors that might facilitate these effects still need to be further explored. Among the proton sensing GPCRs is OGR1/GPR68, which is considered a novel pH sensor that is activated by an acidic extracellular pH (15, 19). Such proton sensing GPCRs were reported to play a role in tumor development, metastasis, inflammation, and angiogenesis process (3). GPR68 expression has been investigated across cancer types including skin, head and neck squamous cancer as well as pancreatic ductal adenocarcinoma (25–28). Since BC is the most prevalent cancer globally, we aimed at investigating the expression pattern of GPR68 in order to understand its role in the BC microenvironment.

Our in-silico data revealed GPR68 expression to be highly upregulated in BC across the different tumor types. This goes in line with previous in-silico findings by Wiley et al. where the most prominent increased GPR68 expression was in pancreatic ductal adenocarcinoma, cervical squamous cell carcinoma, certain subtypes of breast adenocarcinoma and ovarian cancer (29). The in-silico data revealed a high transcript level of GPR68 BC patients as compared to normal breast samples. This was further confirmed at the mRNA and protein levels by qPCR and IHC, respectively in the recruited cohort. Furthermore, GPR68 didn't show any prognostic potential in BC patients unless they were classified according to their molecular subtypes, which is similar to the findings reported by Zhang et al. where the high GPR68 expression group did not have different survival rates (25). The in-silico data revealed that luminal subtypes have the highest GPR68 expression, followed by the triple-negative and Her2-enriched BC subtypes. Nevertheless, upon validation of GPR68 expression at the mRNA and protein levels, it was observed that the highest expression is in the triple-negative molecular subtype. Furthermore, there was a higher GPR68 expression in the PR negative BC patients when compared to the PR positive BC patients. Such discrepancy between the in-silico and validated GPR68 expression could be attributed to the different ethnicities between the BC patients, which was further supported by GPR68 expression across different ethnicities using the in-silico UALCAN tool. Such findings point out the effect of race and ethnicity on GPR68 expression, especially in BC.

Previous studies demonstrated the role of GPR68 in tumor development where GPR68 deficiency significantly reduced tumor allograft development in GPR68 knockout mouse model of prostate cancer cells (21). In addition, activation of GPR68 caused the stimulation and secretion of proinflammatory mediators such as IL-6 and IL-8 (CXCL8), which triggered tumor progression (30–32). The expression of GPR68 in BC cell lines was previously reported by Herzig et al., which showed a weak GPR68 expression in the BC cell lines MCF-7 and MDA-MB-231 (27, 33). Since the molecular subtype was found to affect GPR68 expression, it was essential to explore the baseline expression of GPR68 in four different BC cell lines. In-silico data, as well as the mRNA and protein expression of GPR68,

revealed a strong expression in the triple-negative adenocarcinoma MDA-MB-231 cell line, followed by the luminal A MCF-7 cell line, and $Her2^+$ SKBr3, with the lowest expression existing in the invasive ductal carcinoma triple-negative BT-549 cell line. Additionally, our data indicated a membranous and cytoplasmic GPR68 expression that could be possibly due to the internalization of GPR68 that might occur as a consequence of excessive activation, as previously reported (34). Such a process would need additional confirmation in future functional studies. Previous studies utilized MCF-7 cell lines to investigate the role of GPR68 in BC, where its overexpression inhibited cell migration by a Gα12/13-Rho-Ras-related C3 botulinum toxin substrate 1 (Rac1) pathway (33). Furthermore, overexpression of GPR68 increased the apoptosis of MCF-7 BC cells and inhibited cell growth, migration, and proliferation (33, 35). Our data revealed that MDA-MB-231 is a good candidate to investigate the function of GPR68 in BC.

In conclusion, this study is the first to report GPR68 expression in BC patients and its association with the clinicopathological parameters including molecular subtypes. Moreover, this study explores GPR68 expression across various cell lines showing MDA-MB-231 as a potential candidate for further studies to explore GPR68 in the BC microenvironment and allow researchers to understand its role in the pathogenesis of BC.

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 studies involving human participants were reviewed and approved by Research ethics committee of the University of Sharjah, UAE (REC-21-09-04-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, NE, IT, and AM. Methodology, NE and IS. Formal analysis, NE and AH. Investigation, NE and IT. Data curation, RY, NY, YE, and TM. Writing—original draft preparation, NE. Writing—review and editing, RY, AH, IT, and AM. Supervision, IT and AM. All authors have read and agreed to the final 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.2022.847543/full#supplementary-material

Supplementary Figure 1 | Representative images of immunohistochemical staining of breast cancer tissues. **(A)** A negative control for the immunohistochemical staining process. **(B)** Representative images of GPR68 expression in luminal A, luminal B, Her2-enriched and triple-negative breast cancer tissues. All images were captured at x200 magnification, with a scale bar of 200 μm.

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PET Imaging of Acidic Tumor Environment With ^{89}Zr-labeled pHLIP Probes

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Acidosis of the tumor microenvironment is a hallmark of tumor progression and has emerged as an essential biomarker for cancer diagnosis, prognosis, and evaluation of treatment response. A tool for quantitatively visualizing the acidic tumor environment could significantly advance our understanding of the behavior of aggressive tumors, improving patient management and outcomes. ^{89}Zr-labeled pH-low insertion peptides (pHLIP) are a class of radiopharmaceutical imaging probes for the *in vivo* analysis of acidic tumor microenvironments *via* positron emission tomography (PET). Their unique structure allows them to sense and target acidic cancer cells. In contrast to traditional molecular imaging agents, pHLIP's mechanism of action is pH-dependent and does not rely on the presence of tumor-specific molecular markers. In this study, one promising acidity-imaging PET probe ([^{89}Zr]Zr-DFO-Cys-Var3) was identified as a candidate for clinical translation.

Keywords: pH-low insertion peptides, membrane-insertion behavior, acidic tumor microenvironment, zirconium-89, PET imaging, human dosimetry estimates

INTRODUCTION

Cancer is a complex disease with potentially high heterogeneity between tumors and within an individual tumor and its metastases (1). Tumor growth and progression depend not only on tumor genotype but also on the metabolic status of cancer and the immune cells within the tumor microenvironment (TME). Cancer cells alter their metabolism (metabolic switch) to support their rapid proliferation and dissemination across the body, manifested in high rates of glucose consumption and an overexpression of surface carbonic anhydrases (e.g., CA IX), which catalyze the transformation of carbon dioxide and water into carbonic acid (2). As a result of anaerobic (3) and aerobic glycolysis (4) (Warburg Effect) and the overexpression of carbonic anhydrases, cancer cells contribute to the acidification of the TME. Also contributing to acidity are tumor-associated macrophages (TAMs). The progression of immune-excluded ("cold") tumors is associated with the presence of acidic metabolically active TAMs, which generate immuno-suppressive signals, enhance

angiogenesis, and promote metastases (5, 6). A better understanding of these processes could greatly impact patient outcomes.

The ability of the pHLIP family to sense and target acidic cancer cells and TAMs within the TME could be leveraged to investigate tumor biology and predict cancer treatment responses. As such, pHLIP technology is a highly active area of research (see reference for a comprehensive review) (7). Briefly, pHLIP is a water-soluble unstructured peptide at neutral and high pH values (state I, **Figure 1**). Being a moderately hydrophobic peptide, pHLIP exhibits a high affinity for the cell membrane (state II, **Figure 1**). Several carboxyl groups within the pHLIP sequence are protonated at a low cell surface pH, which triggers the peptide's folding and insertion across the cell membrane to form a stable transmembrane helix (state III, **Figure 1**). The dielectric environment at the membrane slightly increases the pKa values of the carboxyl groups. At low local pH values (6.0–6.5), found at the surface of metabolically active cells (8–10), the environment promotes the peptide's protonation. Variation and truncation of the original pHLIP sequences allowed us to identify pHLIP Var3 as the lead candidate for clinical translation because of its optimal pK values and improved insertion rates as well as suitable pharmacokinetic and pharmacodynamic properties (11, 12). A variety of imaging and therapeutic agents have been successfully delivered by pHLIP agents to tumors (in more than 20 different human and murine tumor models). The cargo (payload) is attached either to the N-terminus of the peptide — the end that remains in the extracellular space (**Figure 1**) — or to the membrane-inserting end (C-terminus) (13–29). The pHLIP's tumor uptake correlates with the tumor's extracellular pH (30–32) and can be enhanced by acidification using co-injection of glucose (33) and overexpression of CA IX (31). In addition to primary tumors, satellites near the primary tumor and micro-metastases in distant organs are targeted by pHLIP agents (33–36). It was also demonstrated that pHLIP conjugates target the acidic TAMs within the TME (28).

The acidic TME can be imaged with pHLIP-based PET-compatible radiotracers. Clinically, a pHLIP-based radiotracer could provide more information about the TME and tumor progression than a standard [^{18}F]FDG PET scan. Our groups have evaluated ^{18}F-labeled (37), ^{99m}Tc-labeled (32), ^{64}Cu- and [^{18}F]AlF-labeled (38), as well as $^{68/67}$Ga-labeled (39) pHLIP analogues in various tumor models. A phase I clinical trial on breast cancer with an investigational ^{18}F-labeled pHLIP probe (based on a D-amino acid sequence of Var3 pHLIP) was completed at Memorial Sloan Kettering Cancer Center (MSK) (NCT04054986). The phase I protocol was performed as a first-in-human PET/CT trial of five patients with metastatic breast cancer and demonstrated the safety and slow blood clearance (several hours) of the ^{18}F-labeled pHLIP conjugate. The short half-life and/or availability of the radionuclides mentioned above is a significant limiting factor for long-term circulating pHLIP compounds. In preclinical PET imaging, an optimal tumor contrast has been observed with pHLIP at or after 24 hours. For this reason, long-lived PET radionuclides, such as the widely available zirconium-89 (^{89}Zr), would likely maximize clinical diagnostic potential with pHLIP. Here, we investigated several ^{89}Zr-radiolabeled pHLIP imaging agents with the goal of introducing a novel PET pH-sensor with optimized pharmacokinetics and a high tumor uptake for a possible clinical translation.

MATERIALS AND METHODS

pHLIP Conjugates

The D-amino acid versions of the Var3 and WT pHLIPs were synthesized and conjugated with the chelators by the company CS Bio (Menlo Park, CA) with ≥ 95% purity. The HOPO chelator was provided by the laboratory of Dr. Lynn Francesconi (Chemistry Department at Hunter College, New York). The DFOsqa chelator was synthesized following a procedure described in the literature (40). ^{1}H- and, ^{13}C-NMR (nuclear

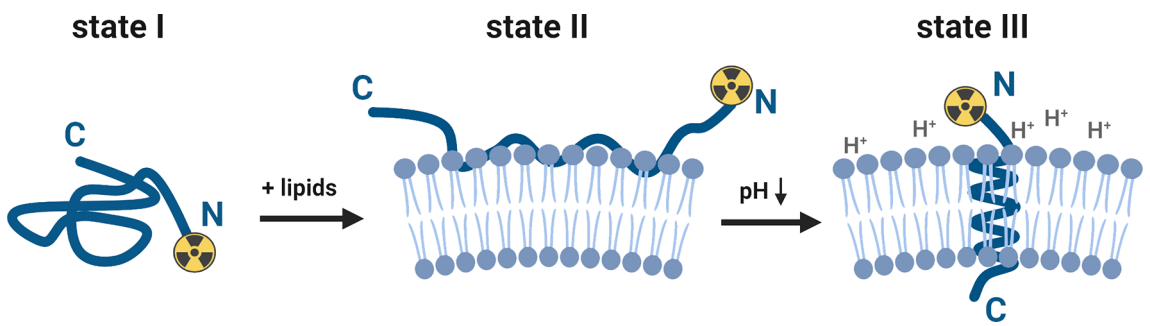

FIGURE 1 | Schematic presentation of the interaction of a pHLIP-cargo conjugate with the lipid bilayer of a membrane at neutral/high and low pH values (for purpose of this study, the cargo represents a [^{89}Zr]zirconium-chelate, attached to the N-terminus). The pHLIP sequence is an unstructured coil in the solution at neutral and high pH values (state I). The equilibrium shifts towards the membrane-bound state II when lipids (e.g., cell membranes) are added to the system. At a low surface pH (6.0–6.2), aspartic acid and glutamic acid residues are protonated and the overall hydrophobicity increases. This triggers the partitioning of the peptide into the lipid bilayer. The equilibrium at low pH values is shifted towards the membrane-inserted state III, which is accompanied by membrane-associated folding to form a transmembrane alpha-helix. This figure was created with BioRender.com.

magnetic resonance), and ESI MS (electrospray ionization mass spectrometry) analysis did match the literature records [SI (40)]. All other chemicals were purchased from commercial suppliers without further purification unless otherwise stated.

Radiochemistry

[^{89}Zr]Zr-oxalate in 1 M oxalic acid was received from 3D Imaging LLC (Little Rock, AR). All activities recorded in this study were determined by an Capintec® CRC-55tR dose calibrator. For the radiolabeling, the required activity (185 MBq/5 mCi) was transferred into a Protein LoBind® Eppendorf tube and adjusted with 1 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer [four-times the volume of (^{89}Zr)Zr-oxalate] to pH 7.0–7.4. To this, the pHLIP conjugate was added [60 µL of a 5×10^{-4} M pHLIP-solution in DMSO (dimethyl sulfoxide), equivalent to 30 nmol].

The mixture was incubated at 37°C for 30 minutes. The completion of the radiolabeling was checked by radio instant thin-layer chromatography (iTLC) using iTLC-SG chromatography paper (Agilent Technologies) and an aqueous EDTA (ethylenediaminetetraacetic acid) solution (50 mM, pH 4). The iTLCs were scanned on an radio-TLC imaging scanner (AR2000; Eckert & Ziegler Radiopharma GmbH). The radiochemical yield of all constructs exceeded 95%, and they were used without purification. The ^{89}Zr-radiolabeled pHLIP were prepared for intravenous injection by diluting the reaction mixture with sterile filtered phosphate-buffered saline (PBS) to the required volume (2.5 mL). Each mouse received 100 µL of PBS solution containing 1.2 nmol of pHLIP and 7.4 MBq/200 µCi of activity.

A potential degradation of the compounds was investigated by serum stability assay. For this, 100 µL of the prepared PBS solution (containing 1.2 nmol of pHLIP and 7.4 MBq/200 µCi) was added to 900 µL of human serum (heat inactivated from Millipore Sigma) and incubated for seven days at 37°C. The release of free $^{89}Zr^{4+}$ was measured by radio instant thin-layer chromatography (as described above) and the degradation of the whole construct was checked by radio high-performance liquid chromatography (HPLC). The analytical reverse-phase HPLC was performed on a Shimadzu system equipped with a Flow Count PIN diode radiodetector from BioScan, a DGU-20A degasser, two LC-20AB pumps, and an SPD-M20A photodiode array detector. A BetaBasic 18 column (150 Å, 5.0 µm, 4.6×150 mm, Thermo Scientific) was used with water (+ 0.1% trifluoroacetic acid) and acetonitrile (+ 0.1% trifluoroacetic acid) as solvents. The gradient started at 5% acetonitrile and increased to 95% over 15 minutes at a constant flowrate of 1 mL/min. The radiochemical purities of all ^{89}Zr-radiolabeled pHLIP conjugates were greater than 90% over the course of seven days.

Biophysical Studies

The interaction of the pHLIP agents with liposomes was investigated by recording the tryptophan fluorescence and circular dichroism (CD) spectra by using a PC1 spectrofluorometer (ISS, Inc) and an MOS-450 spectrometer (Biologic, Inc), respectively, with temperature control set to 25.0°C. Liposomes, consisting of large unilamellar vesicles, were prepared by extrusion. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) (Avanti Polar Lipids, Inc.) in chloroform was desolvated on a rotary evaporator and dried under vacuum for several hours. The phospholipid film was rehydrated in 10 mM phosphate buffer, pH 8.0, vortexed, and passed 21 times through the extruder (50 nm membrane).

Using an excitation wavelength of 295 nm, tryptophan fluorescence spectra were recorded from 310–400 nm. CD spectra were recorded from 190–260 nm with 1-nm steps. The concentration of the peptide and the POPC liposomes varied in different experiments: 5–15 µM of pHLIP agents and 1 mM of POPC liposomes.

The pH-dependent insertion of the peptides into the lipid bilayer of the POPC liposomes was studied by monitoring either the changes in tryptophan fluorescence spectra or changes in the molar ellipticity at 222 nm as a function of the pH value. After the addition of aliquots of HCl, the pH values of the solutions containing peptide and POPC liposomes were measured using an Orion PerHecT ROSS Combination pH Micro Electrode and an Orion Dual Star pH and ISE Benchtop Meter. Fluorescence spectra were analyzed using the Protein Fluorescence and Structural Tool Kit (PFAST) (41) to determine the positions of spectral maxima (λ_{max}). The λ_{max} and millidegree data were normalized to 0–1 and were plotted as a function of pH. The pH-dependence was fit with the Henderson-Hasselbach equation to determine the cooperativity (n) and the mid-point (pK) of transition:

$$\textbf{Normalized pH dependence} = \frac{1}{1 + 10^{n(pH-pK)}}$$

The tryptophan fluorescence kinetics were measured using an SFM-300 mixing system (Bio-Logic Science Instruments) in combination with the MOS-450 spectrometer with temperature control set to 25.0°C. All samples were degassed before the measurements to minimize air bubbles in the samples. The peptide and POPC samples were incubated overnight to reach equilibrium, to assure that most of the peptide is associated with the liposome lipid bilayers. To follow the peptide insertion, equal volumes of the peptide-POPC solution and of HCl were mixed to lower the pH from 8 to 4. To monitor fluorescence intensity changes during the peptide insertion into POPC liposomes induced by the pH drop, the tryptophan emission signal was observed through a cut off 320 nm filter at an excitation of 295 nm.

All data was fit to the appropriate equations by nonlinear least squares curve fitting procedures employing the Levenberg Marquardt algorithm using Origin 8.5.

Cell Preparation and Animal Models

The RM-1 and 4T1 cell lines were purchased from ATCC and cultured according to the recommended conditions at 5% CO_2 atmosphere and 37°C in DMEM (Dulbecco's Modified Eagle Medium) and RPMI 1640 medium, respectively, each containing 10% fetal bovine serum. The media were provided by the MSK Media Preparation Core. For the subcutaneous allografts, the cells were stripped in the absence of magnesium or calcium ions using a mixture of 0.25% trypsin and 0.53 mM EDTA in Hank's Balanced Salt Solution, concentrated in 1 mL of the corresponding medium, and a small aliquot was used to

determine the cell count (Beckman Coulter Vi-CELL XR). Another aliquot was diluted with the medium so that 50 µL contained 1×10^6 cells.

The imaging and biodistribution studies were performed with male (RM-1 model) and female (4T1 model) athymic nude mice and the kidney-blocking experiments were performed with female SCID mice received from Charles River Laboratories (Stone Ridge, NY). After arrival, the mice were kept in the MSK vivarium for one week before any experimental handling was performed. The animals were allowed free access to water and food, and all animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). For the subcutaneous allografts, a 1-to-1 ratio of Corning Matrigel Matrix and the cell solution was prepared and stored on ice for the injections. Each mouse received a subcutaneous injection of 100 µL (50 µL cell solution, viability > 90%, + 50 µL Matrigel) into the flank on the level of the right shoulder. The injections were performed under anesthesia (2% isoflurane, Baxter Healthcare, Deerfield, IL, USA). Palpable tumors of similar size (100–300 mm^3) developed 5–8 days after grafting.

Small Animal *in vivo* PET Imaging and Post-Mortem Biodistribution Studies

The mice were intravenously injected (tail vein) with the radiolabeled pHLIP conjugates (1.2 nmol, 7.4 MBq/200 µCi, 100 µL, in sterile filtered PBS). The activity of the syringe prior to injection and after injection was used to determine the percent of injectate administered.

PET images were obtained at the respective timepoints with the mice under anesthesia (2% isoflurane) on a microPET Focus 120 (Concorde Microsystems) or an Inveon PET-CT (Siemens) rodent scanner. All images were analyzed using the Medical Imaging Data Examiner Amide (version 0.9.0) or the Inveon reconstruction software suite. The counting rates in the reconstructed images were converted to percent of injected dose per weight (%ID/g) by applying a system-specific calibration factor. At the final timepoint, the remaining mice were euthanized (by CO_2 asphyxiation, followed by cervical dislocation) and their tumors were resected for histological analysis.

For the biodistribution studies the tumor-bearing mice were randomized before the injections. Only mice with suitable tumors (150–300 mm^3) were utilized. An exception was made for the 120-hour timepoint, for which only mice with smaller tumors (150 mm^3) were selected. The mice were grouped in cohorts of four. At the respective timepoint, the mice were euthanized (by CO_2 asphyxiation, followed by cervical dislocation) and their blood was collected by cardiac puncture as a terminal procedure, followed by quick removal of the tumors. After this, the other organs/samples were collected. All samples were weighed and the radioactivity for each one was counted for 1 minute using an automatic gamma counter (Wizard^2 2480 3″, PerkinElmer, Waltham, MA), resulting in less than 10% error due to counting statistics for each measurement. The exact activities of all samples of one mouse were determined by an Capintec® CRC-55tR dose calibrator. These decay-corrected measurements were used to determine a calibration factor and calibrate all counts to the corresponding activities. Activity concentrations were calculated as percentage injected dose per tissue weight (%ID/g wet tissue). All data was processed and visualized using Origin 8.5.

The animal studies, including the different radiotracers and animal models, are listed in **Table S1**.

Dosimetry

Briefly, human dosimetry estimates were extrapolated from the serial biodistribution and PET image data for [^{89}Zr]Zr-DFO-Cys-Var3 in female nude mice. The decay-corrected percentage of administered activity in human organ *i*, $\%ID_{i,human}$, was calculated using the following equation, which assumes interspecies equivalence of the organ-level mean standardized uptake values (42):

$$\%\mathbf{ID}_{\mathbf{i,human}} = \frac{\%\mathbf{ID}_{\mathbf{i,mouse}}}{\mathbf{m}_{\mathbf{i,mouse}}} \times \frac{\mathbf{m}_{\mathbf{mouse}}\mathbf{m}_{i,\mathbf{human}}}{\mathbf{m}_{\mathbf{human}}}$$

Here, $\%ID_{i,mouse}/m_{i,mouse}$ is the activity concentration in mouse organ *i* (see **Table S2**), m_{mouse} is the mouse total body mass (approx. 25 g); $m_{i,human}$ and m_{human} are the blood-inclusive source organ mass and total body mass, respectively, of the computational phantom used to represent the reference adult patient (43, 44). The activity concentration in the blood was assumed to be representative of the red bone marrow. For each organ, the $\%ID_{i,human}$ vs. time was modeled using mono- or bi-exponential functions, which were fit to the data using weighted least-squares regression using the Microsoft Excel SOLVERSTAT statistics package (45). The weight of each observation was taken as the inverse of the variance (computed from the SDs given in **Table S2**). The time-integrated activity coefficient (46) for human organ i,$\tilde{a}_i$ was computed from the analytical expressions for the integrals of the fit functions; the standard error in each $\tilde{a}_i$, $\sigma_{\tilde{a}_i}$ was computed *via* Gaussian error propagation of the standard errors in the fit parameters, taking the covariances into account. Finally, the absorbed dose and effective dose coefficients were computed with MIRDcalc software (47), using the $\tilde{a}_i$ and $\sigma_{\tilde{a}_i}$ as input. 3D-absorbed dose maps were generated using PARaDIM software (48). The results can be found in **Table S3**

Ex vivo Autoradiography, Staining, and Microscopy

The mice of the imaging groups were euthanized at the final timepoint (72 hours, by CO_2 asphyxiation, followed by cervical dislocation) and their tumors were resected, embedded in OCT compound (optimal cutting temperature compound) and frozen. The following day, a series of contiguous tumor sections of 10 µm thickness were cut and exposed to a phosphor-imaging plate (Fujifilm BAS-MS2325, Fuji Photo Film, Japan) for 24 hours at −20 °C and read using a Typhoon 8600 photographic film scanner (GE Healthcare). Digital images of radioactivity distribution at 50 µm resolution were obtained. The same sections were subsequently used for H&E (hematoxylin and eosin) and CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1, highlighting blood vessels) staining. The staining and scanning/digitalizing of all slices was performed by the MSK

Molecular Cytology Core Facility. Autoradiographic and histological images were registered using Adobe Photoshop CS3 software.

Cherenkov and Fluorescence Imaging

For the side-by-side imaging study, five male RM-1 tumor-bearing athymic nude mice were injected with a 100 µL PBS-solution containing [^{89}Zr]Zr-DFOsqa-Lys-Var3 (1.2 nmol of pHLIP and 7.4 MBq/200 µCi) and the fluorophore Alexa546-Cys-Var3 (5 nmol). Alexa546-Cys-Var3 was provided by the laboratory of Prof. Dr. Yana K. Reshetnyak. At 48 hours post-injection, the mice were sacrificed and the kidneys, livers and tumors were resected and imaged. Cerenkov luminescence imaging (recording time of 5 minutes) and fluorescence imaging (recording time of 1 minute) was performed with an IVIS Spectrum (PerkinElmer). Cerenkov luminescence was recorded in p s^{-1} cm^{-2} sr^{-1} and fluorescence in p s^{-1} cm^{-4} sr^{-1} μW^{-1}.

RESULTS

Preparing the pHLIP Conjugates

In this study, six ^{89}Zr-labeled pHLIP conjugates were investigated. The pHLIP sequence was originally based on the wild type pHLIP (WT), which was isolated from the C-helix of the bacteriorhodopsin protein, a proton pump found in archaea organisms (13, 49). Optimization of this sequence led to Var3 pHLIP compound, which is used in this study (12). At the N-terminus, the pHLIP sequences can be modified with either a lysine (Lys-Var3 or Lys-WT) or a cysteine (Cys-Var3) residue, which can be used to conjugate a chelator by harvesting the cysteine–maleimide or a lysine–isothiocyanate reaction, respectively. The N-terminus of the pHLIP sequence remains in the extracellular matrix, while the C-terminus crosses the cell membrane into the intracellular space as shown in **Figure 1**. Previous studies confirmed that the cargo can affect the pharmacokinetics of the pHLIP conjugates (34); therefore, the choice of the chelator can be a critical factor. We investigated six ^{89}Zr-labeled pHLIP conjugates (**Figure 2**) to identify the best candidate for a possible clinical translation. Five Var3 constructs were synthesized, using the zirconium-chelators DFO, DFO*, DFOsqa, and HOPO. The Zr^{4+}-ion forms a +1 charged chelate with DFO and DFOsqa, while DFO* and HOPO form a neutral-charge chelate. It was previously shown that the WT pHLIP exhibits lower tumor targeting than the Var3 pHLIP. The WT sequence was used in this study to demonstrate the benefits of the optimized Var3 pHLIP version.

The radiolabeling of all pHLIP with [^{89}Zr]zirconium oxalate conjugates resulted in a radiochemical yield > 95% (verified by

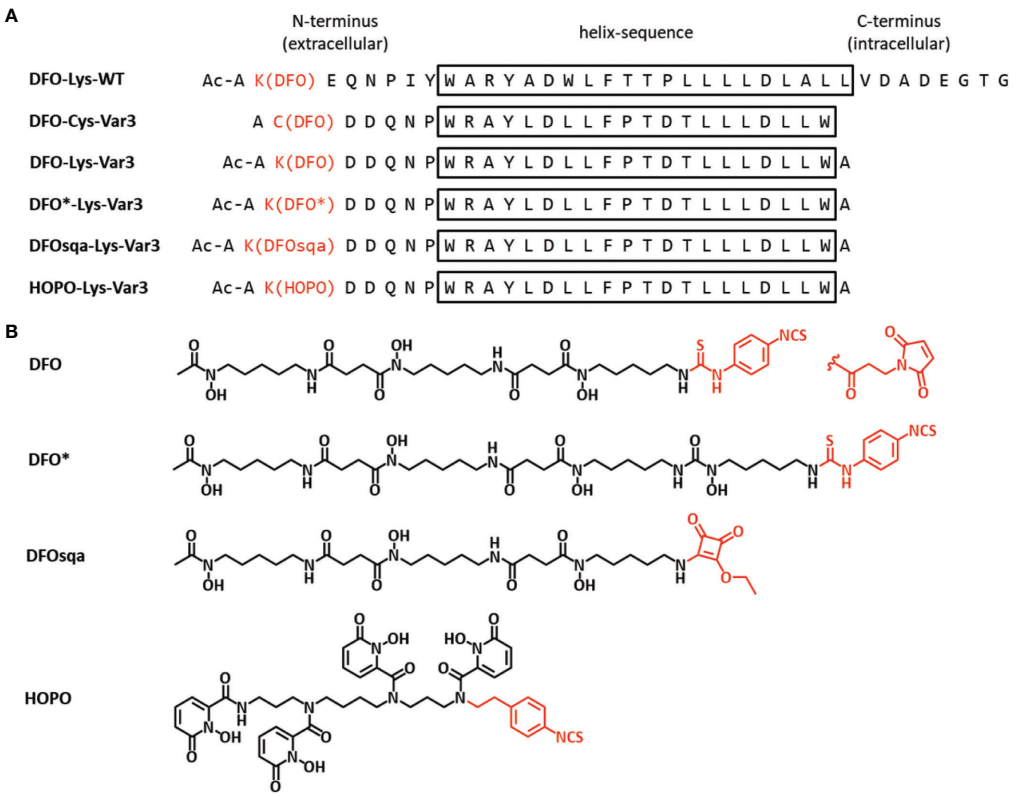

FIGURE 2 | **(A)** The pHLIP sequences (d-amino acids) of the six investigated constructs in single letter code and **(B)** the chemical structures of the four chelators (active conjugation side in red). The conjugation was either performed by reacting the Lys-pHLIP with the isothiocyanate version of the chelator (activated ethyl ester for DFOsqa) or reacting the Cys-pHLIP with DFO-maleimide.

iTLC and radio-HPLC) and no additional purification was necessary. The stability of the radiotracers was investigated in human serum at 37°C, indicating no release of unchelatssed $^{89}Zr^{4+}$-ions or breakdown of the pHLIP constructs over a period of 7 days (**Figure S1**).

Biophysical Characterization

A biophysical characterization of all pHLIP agents was performed to check on their membrane-insertion behavior. The pH-dependent bilayer interactions of the constructs were measured using POPC liposomes by changes in steady-state tryptophan fluorescence and CD. The tryptophan (Trp) fluorescence and CD was recorded for the pHLIPs in solution at a pH value of 8 (state I), and in presence of POPC liposomes at the pH of 8 (state II) and 4 (state III) (**Figures 1**, **S2, S3**, and **Table S4**). The fluorescence signal of the WT pHLIP cannot be directly compared with the emission of the Var3 constructs, since the Trp residues in the WT are located at the beginning and middle of the transmembrane helix, while the Trp residues in Var3 are located at the beginning and end of the transmembrane helix. Therefore, it is not surprising that the position of the fluorescence maximum of the DFO-Lys-WT in state III shows the lowest value (λ_{max} = 338.9 nm, **Table S4**). Among the five investigated Var3 agents, the spectral signals of the HOPO-Lys-Var3 were distinct, either because the HOPO-zirconium chelate absorbs light and/or its conjugation with pHLIP alters the pH-dependent behavior of the pHLIP. We also compared the spectral properties of the agents with and without chelated (natural) zirconium, and obtained very similar results (**Table S4**), indicating that the presence of the metal does not affect their spectral properties. Both DFO-Lys-WT and HOPO-Lys-Var3 were excluded from further biophysical studies. The pK value (midpoint of transition) and the cooperativity (*n*) of the peptides' insertion into the membrane was investigated for the four remaining Var3 agents. The low wavelength shifts of the Trp fluorescence (fluor. changes, **Figure 3A**) indicate the peptide's propagation into the hydrophobic environment of the lipid bilayer. The increase of their ellipticity (CD changes, **Figure 3B**) indicates a coil-helix transition. The normalized data fitted by the *Henderson-Hasselbalch* equation is shown in **Figure 3** and **Table S4**. All agents exhibit similar pK values with the highest cooperativity (*n* = 1.6) established for the DFO-Cys-Var3 coil-helix transition. The investigated agents demonstrated a fast insertion (msec) into the membrane (**Figure S4**) similar to the rate of the unmodified Var3 peptide (12). The influence of the metal-chelate complex on the peptide is minimal since it was attached to the membrane non-inserting end (N-terminus).

In vivo Imaging Studies in RM-1 and 4T1 Tumor Models

The murine cell lines RM-1 and 4T1, end-stage models for prostate and breast cancer, were respectively allografted subcutaneously into male and female athymic nude mice (1×10^6 cells/mouse). Both cell lines grow rapidly; within one week of allografting, an optimal tumor size (100–300 mm^3) was reached. Approximately 7.4 MBq/200 µCi (apparent molar activity of approx. 6.3 MBq/nmol) of the ^{89}Zr-labeled pHLIP conjugate was intravenously administered (0.1 mL) to the tumor-bearing mice. PET scans were performed at 4, 24, 48, and 72 hours post-injection. The PET scans obtained from the 4T1 model, injected with [^{89}Zr]Zr-DFOsqa-Lys-Var3, are displayed in **Figure 4A**. The variant for which serial biodistribution studies were performed, [^{89}Zr]Zr-DFO-Cys-Var3, was cleared from the blood with a (biological) half-life of (16.0 ± 0.4) hours. A good tumor-background ratio for all compounds was observed for the 48-hour timepoint. All radiotracers were additionally evaluated in the RM-1 model

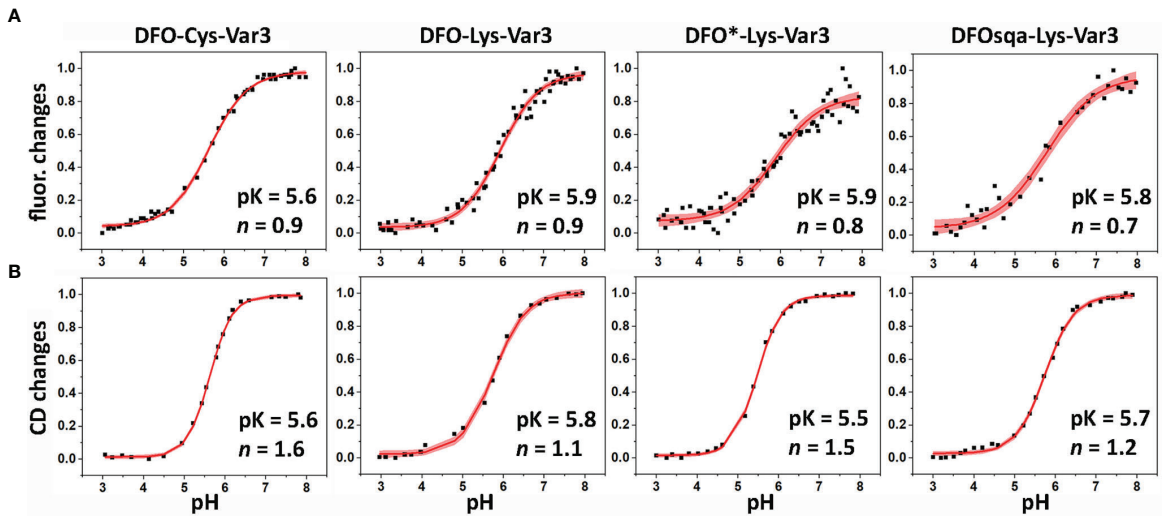

FIGURE 3 | The pH-dependent insertion of pHLIP agents into the lipid bilayers of POPC liposomes was studied by monitoring the changes in the position of the maxima of the tryptophan fluorescence spectra **(A)** and ellipticity of the CD signals measured at 222 nm **(B)** as function of the pH. The data was fitted using the Henderson-Hasselbalch equation. The fitting curves (red lines) and 95% confidence intervals (pink areas) are shown.

and PET images at multiple timepoints were recorded. For all compounds, the 48-hour timepoint showed the best tumor-to-background ratio (**Figure 4C**); the tumor-to-muscle uptake-ratios ranged from 11.6 ± 4.9 to 16.9 ± 6.8 for the Var3 conjugates and was 5.5 ± 1.5 for DFO-Lys-WT. A comparison of the PET images between the 4T1 and RM-1 tumor models revealed a similar biodistribution of the constructs with slight variations in the tumor- and kidney-uptake (**Figure S5**). The radiotracers' distributions are discussed in more detail in the "Biodistribution" section.

Histological Tumor Analysis

After the 72-hour timepoint, all mice used in the imaging study were sacrificed. The tumors were resected, frozen in OCT compound, and sectioned (10 µm slices) the following day, followed by the recording of autoradiographs. Additional slides were prepared for H&E and CD31 staining (murine blood vessels staining). The pHLIP agents were present in the entire tumor mass, and the highest activity areas overlapped with the tumor stroma, as displayed in **Figures 4B** and **S6**, indicating the acidic hotspots within the TME. This result is in line with previously reported findings (50) showing that pHLIP compounds target the tumor–stroma interface, which serves as an acidic dump for cancer cells to maintain an optimal intracellular pH. A similar tumor distribution was observed for all pHLIP constructs. Additionally, 10 µm kidney sections were prepared, revealing that within kidney the ^{89}Zr-labeled pHLIP compounds are present primarily in its cortex (**Figure S6**).

Biodistribution Studies

The *in vivo* PET study revealed optimal tumor uptake and tumor-to-background contrast at the 48-hour timepoint. For this reason, the 48-hour timepoint was chosen for a single timepoint biodistribution study to compare the six pHLIP constructs more thoroughly in the RM-1 tumor model. The biodistribution data of the organs with the highest uptake is shown in **Figure 5A**. Additional data can be found in **Table S5**. All Var3 agents demonstrate similar biodistribution. This differs from the biodistribution of the WT agent, which demonstrates the lowest tumor targeting and highest spleen, liver, and lungs uptake. All Var3 agents exhibit high tumor uptake with the highest value (12.4 ± 4.7) %ID/g observed for [^{89}Zr]Zr-DFOsqa-Lys-Var3. However, the high tumor uptake also correlates with a high kidney uptake, (82.5 ± 14.2) %ID/g for DFOsqa-Lys-Var3. The tumor-kidney-uptake correlation of all compounds is visualized in **Figure 5B**. It is reported that a significant pH gradient exists within the kidney parenchyma, related to the metabolic activity of the thick ascending limb of the loop of Henle, which might be of relevance for the acid-based homeostasis (51). This suggests that the radiotracers with a higher tumor uptake would also show an increased kidney accumulation.

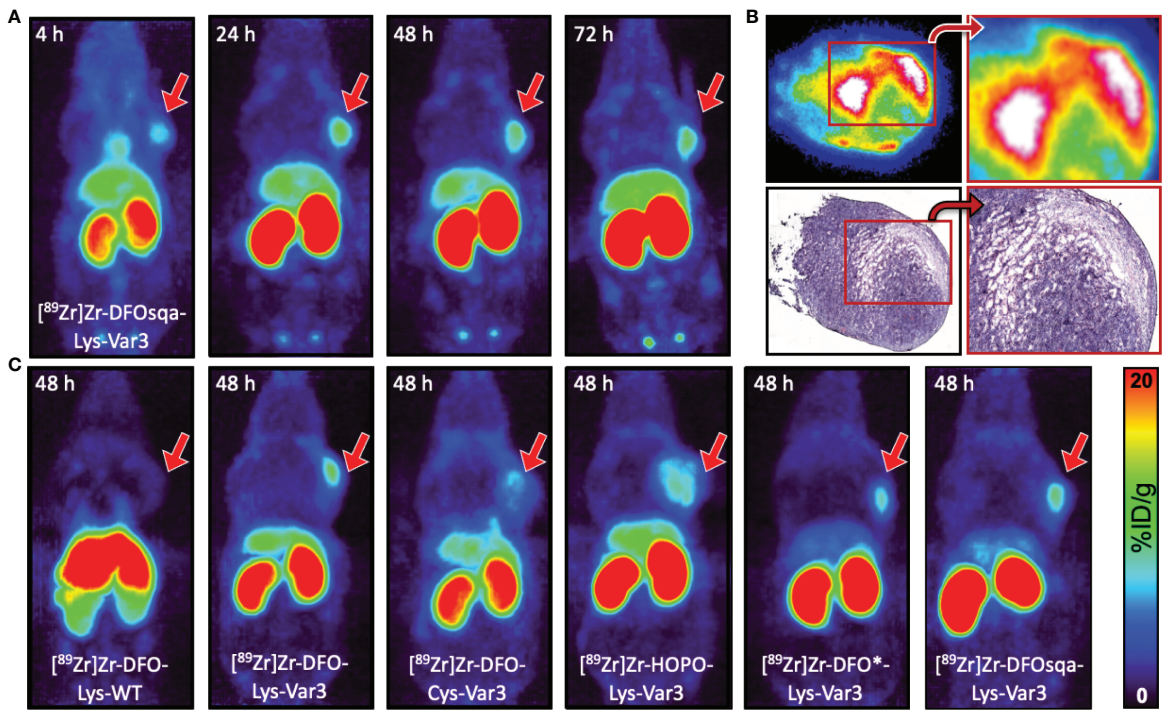

FIGURE 4 | **(A)** Coronal MIP PET images at 4, 24, 48, and 72 hours of female athymic nude mice bearing subcutaneous 4T1 tumor allografts on the right shoulder, administered with 7.4 MBq/200 µCi of [^{89}Zr]Zr-DFOsqa-Lys-Var3 (1.2 nmol pHLIP). **(B)** *Ex vivo* autoradiograph (top) and H&E staining (bottom) of 10 µm-tumor slices (excised at 72 hours post-injection), not scaled or calibrated. **(C)** Coronal MIP PET images at 48 hours of male athymic nude mice bearing subcutaneous RM-1 tumor allografts on the right shoulder, administered with 7.4 MBq/200 µCi and 1.2 nmol of the corresponding pHLIP conjugate. The scale bar applies to all PET images.

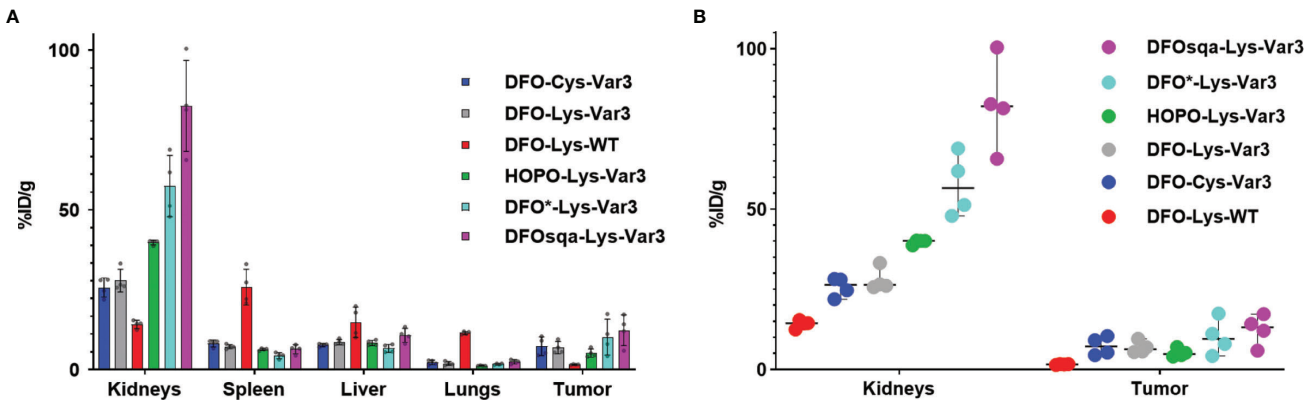

FIGURE 5 | **(A)** Biodistribution data of the six investigated pHLIP constructs at 48 hours post-injection evaluated in the RM-1 tumor model (n=4). **(B)** Direct comparison of the kidney and tumor uptake.

Cherenkov and Fluorescence Imaging

The distinct kidney uptake was observed for all ^{89}Zr-labeled Var3 conjugates. At the same time, according to the literature, fluorescently labeled pHLIP conjugates exhibit significantly less kidney uptake (34, 52) than radiometal-chelate-containing pHLIPs. Therefore, we carried out a side-by-side imaging study. Five male RM-1 tumor-bearing athymic nude mice were co-injected with [^{89}Zr]Zr-DFOsqa-Lys-Var3 and fluorophore Alexa546-Cys-Var3. The mice were sacrificed at 48 hours post-co-injection and the kidneys, livers, and tumors were resected and imaged. Cherenkov irradiation originating from the ^{89}Zr-decay and the Alexa546 fluorescence was recorded (**Figure 6**). Cherenkov imaging (expressed as radiance) and fluorescence imaging (expressed as radiant efficiency) are two different modalities. The organ uptake should be compared within the same modality. A slightly higher tumor-to-kidney ratio of Alexa546-Cys-Var3 was observed compared to the ratio for [^{89}Zr]Zr-DFOsqa-Lys-Var3 — indicating that the metal-chelator constructs exhibit higher kidney retention.

Blocking the Kidney Uptake

Additional experiments were performed to investigate the influence of various kidney-uptake blockers and diuretics. Healthy female SCID mice received a "blocking agent" 30 minutes prior to the injection of [^{89}Zr]Zr-DFOsqa-Lys-Var3 (7.4 MBq/200 µCi, 1.2 nmol). The goal was to achieve a significant kidney clearance (uptake < 80%ID/g), as determined by PET imaging. Five different drugs were investigated: amiloride (epithelial sodium channel inhibitor), 5-(N,N-dimethyl)amiloride (more potent amiloride analog), probenecid (inhibits kidney uptake of organic anions), chlorthalidone (inhibits sodium reabsorption), and acetazolamide (carbonic anhydrase inhibitor). However, none of these drugs led to a significant reduction of kidney uptake compared to the control mice, which did not receive any inhibitor. The experimental set-up can be found in **Table S6**.

Biodistribution and Dosimetry of the [^{89}Zr]Zr-DFO-Cys-Var3 Lead Candidate

Due to elevated uptake, the kidney was expected to be the organ limiting administered activity for clinical PET. Since none of the kidney blocking strategies were effective in reducing the kidney uptake of [^{89}Zr]Zr-DFOsqa-Lys-Var3, the [^{89}Zr]Zr-DFO-Cys-Var3 agent, which shows (7.3 ± 2.9) %ID/g tumor uptake and (25.7 ± 3.0) %ID/g kidney uptake, was selected as the lead compound. A multiple-timepoint biodistribution was performed (4, 24, 48, 72, and 120 hours) using the 4T1 tumor model and [^{89}Zr]Zr-DFO-Cys-Var3 (**Figure S7** and **Table S2**). Additionally, PET-CT images were recorded for each timepoint (**Figure S8**). The tumor- and kidney-uptake values [(9.7 ± 1.7) % ID/g and (47.6 ± 11.5) %ID/g, 48-hour timepoint] were significantly higher for this compound in the 4T1 model than in the RM-1 model [(7.3 ± 2.9) %ID/g and (25.7 ± 3.0) %ID/g, 48-hour timepoint].

Because the 4T1-tumors grew relatively quickly, smaller tumors (100 mm^3) had to be chosen for the 120-hour timepoint, most likely leading to a slightly lower (not significant) tumor- and kidney-uptake for this timepoint [(6.8 ± 0.7) %ID/g and (35.3 ± 5.9) %ID/g, 120-hour timepoint]. Overall, the highest tumor uptake was detected for the 48-hour timepoint and a small but not significant tumor- and organ-clearance was observed for the 72- and 120-hour timepoints. A progressive drop in radiotracer blood level was observed over this five-day interval. A trace amount of (0.2 ± 0.05) %ID/g was detected for the 120-hour timepoint.

Murine biodistribution data was extrapolated to reference human adults in order to obtain radiation dosimetry estimates for human i.v. administration of [^{89}Zr]Zr-DFO-Cys-Var3. The effective dose coefficient was 0.50 mSv/MBq. The organs with the highest absorbed dose coefficients were the kidneys [male, (1.83 ± 0.11) mGy/MBq; female, (2.18 ± 0.14) mGy/MBq], followed by the adrenals [male, (0.934 ± 0.047) mGy/MBq; female, (0.991 ± 0.038) mGy/MBq] A graphical summary of the dose coefficients is given in **Figure 7**; tabulated values can be found in **Table S3**.

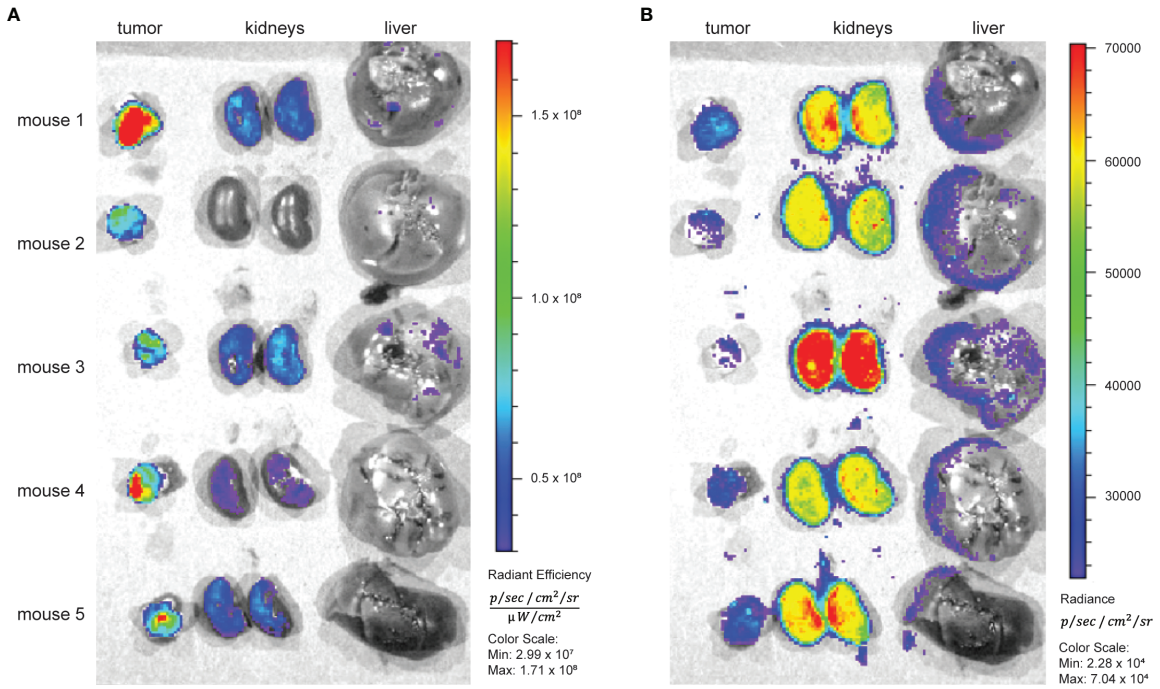

FIGURE 6 | Five RM-1 tumor bearing male nude mice received 7.4 MBq/200 μCi [^{89}Zr]Zr-DFOsqa-Lys-Var3 (1.2 nmol) and Alexa-546-Cys-Var3 (5 nmol) in a co-injection. At 48 hours the tumors kidneys and livers were resected, and fluorescence **(A)** and Cherenkov **(B)** images were recorded using the IVIS system.

DISCUSSION

Previously, ^{18}F-labeled (37), ^{99m}Tc-labeled (32), ^{64}Cu- and [^{18}F]AlF-labeled (38), as well as $^{68/67}$Ga-labeled (39) pHLIP analogs were investigated in various mouse models. However, the short half-life of the radionuclides or their availability is a limiting factor. The blood clearance of these radiolabeled pHLIP constructs was found to be several hours. For PET imaging, an optimal tumor-to-tissue ratio would be expected after 24 hours or even later. In **Figure 8**, the biodistribution data for [^{89}Zr]Zr-DFO-Cys-Var3 to [^{64}Cu]Cu-NO2A-cysVar3 and [^{18}F]AlF-NO2A-cysVar3, from a previously published study (38), are compared. The half-life of ^{18}F (110 minutes) does not allow for awaiting a suitable tumor-to-blood ratio. The four hour timepoint with a tumor-to-blood ratio of 0.51 ± 0.12 corresponds to two half-lives of [^{18}F]AlF-NO2A-cysVar3 (25% of activity remained). For [^{64}Cu]Cu-NO2A-cysVar3, a suitable tumor-to-blood ratio of 4.8 ± 1.3 was reported for the 48-hour timepoint. However, after 48 hours, only 6% of the short-life ^{64}Cu remains ($t_{1/2}$ = 12.7 hours) – challenging for imaging at late timepoints. For this reason, other radionuclides like the widely available ^{89}Zr are better suited. Zirconum-89 is a radiometal with a half-life of 3.3 days, which matches the goal of employing long-term imaging using the pHLIP conjugates. ^{89}Zr's relatively low positron energy (E_{Avg}=395 keV) allows for high-resolution PET imaging, comparable to fluorine-18 (53). As a further benefit, most medical centers are capable of producing ^{89}Zr using low-energy proton-accelerating cyclotrons (14.0–14.5 MeV) and the ^{89}Y(p, n)^{89}Zr production route (54). However, the release of the $^{89}Zr^{4+}$-ion from the pHLIP compounds should be avoided, since the free radiometal accumulates in the mineral bone, decreasing imaging sensitivity and elevating radiation absorbed doses to the red marrow and skeletal endosteum (55).

This study investigated the influence of different zirconium chelators on the pharmacokinetics of the pHLIP compound. For a higher *in vivo* stability of the peptide, the sequence was built with D-amino acids. Four different chelators were conjugated to the N-terminus of the Var3 and WT pHLIP sequence. The most prominent chelator for the Zr^{4+}-ion is DFO, which offers 6 donor atoms for the metal center. The DFO-Zr^{4+}-chelate carries a net +1 charge. Three DFO constructs were compared in this study: DFO-Lys-Var3, DFO-Cys-Var3, and DFO-Lys-WT. The cysteine variant used DFO-maleimide for the bifunctional chelator and DFO-NCS for the lysine variant. Despite it being the most commonly used zirconium chelator, reports indicate that DFO can lead to unwanted bone uptake of ^{89}Zr due to the chelate's instability (56). For this reason, we also investigated the chelators DFO* and HOPO, which both form a neutrally-charged chelate with the Zr^{4+}-ion. DFO* is structurally similar to DFO but offers an additional hyrdoxamate group, resulting in a total of 8 donor atoms and a more stable chelate (56). The HOPO chelator [3,4,3-(LI-1,2-HOPO)] offers the same amount of donor atoms and was reported to be an excellent alternative to DFO (57). Finally, we investigated DFOsqa, a deferoxamine conjugated to 3,4-diethoxy-3-cyclobutene-1,2-dione (squaric acid diethyl ester). It was reported that squaric acid conjugates enable a fast and simple conjugation to peptides under mild conditions and

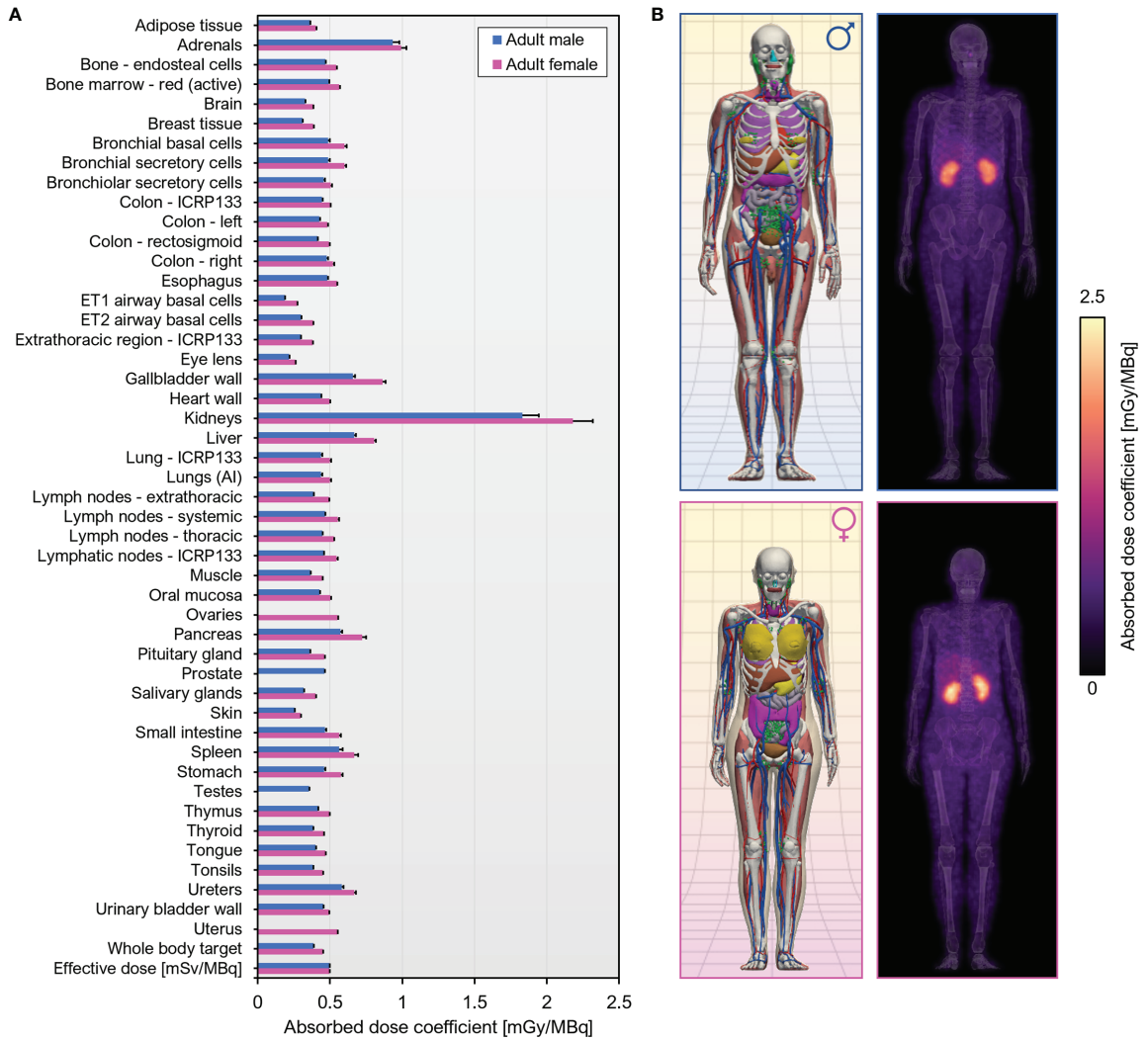

FIGURE 7 | Human dosimetry estimates for [^{89}Zr]Zr-DFO-Cys-Var3 projected from murine biodistribution. **(A)** Organ-level absorbed dose and effective dose coefficients computed for the ICRP 110 reference adult phantoms. Units are absorbed dose [mGy] per unit administered activity [MBq] unless otherwise specified. **(B)** Left, Computational phantoms used in dose calculations; Right, Maximum intensity projections of the 3D dose distribution.

that the squaric acid might even induce beneficial effects on the pharmacokinetic properties (58). Furthermore, the squaric acid is assumed to coordinate onto the zirconium resulting in a more stable chelate (58).

An efficient single method was developed to radiolabel all six conjugates under mild conditions and in only 30 minutes. The radiolabeling yield was greater than 95%, and no purification steps were necessary. All agents could be easily developed into a clinical kit-like product. The stability of the six [^{89}Zr]Zr-pHLIP conjugates was investigated in a serum stability assay and *in vivo* studies. All agents exhibited high stability: lack of degradation and no significant release of free [^{89}Zr]Zr^{4+}-ions. Biophysical studies confirmed that all agents preserved the ability to interact with the lipid bilayer, driven by a drop in pH. The membrane insertion and folding pK values (in the range of 5.5 to 5.9) and fast membrane-insertion kinetics indicated that agents could potentially sense a low pH (6.0–6.2) at the surface of activated cells within the TME.

The *in vivo* study performed on tumor models in mice, mimicking late-stage prostate and breast cancers, demonstrated a good targeting of the tumors by all Var3-based agents, as well as a high kidney uptake. Tumor targeting ranged from 7–12 % ID/g and kidney uptake increased from 25–83 %ID/g. In the side-by-side comparison study, tumor, kidney, and liver uptake were measured for co-injected radioactive and fluorescent pHLIPs. Fluorescent pHLIP showed significantly lower kidney uptake and slightly higher tumor targeting. Also, regulators of pH [acetazolamide; amiloride and 5-(N,N-dimethyl)amiloride], probenecid, an inhibitor the kidney uptake of organic anions; and chlorthalidone, an inhibitor of sodium reabsorption, failed to reduce the kidney uptake of [^{89}Zr]Zr-DFOsqa-Lys-Var3. The

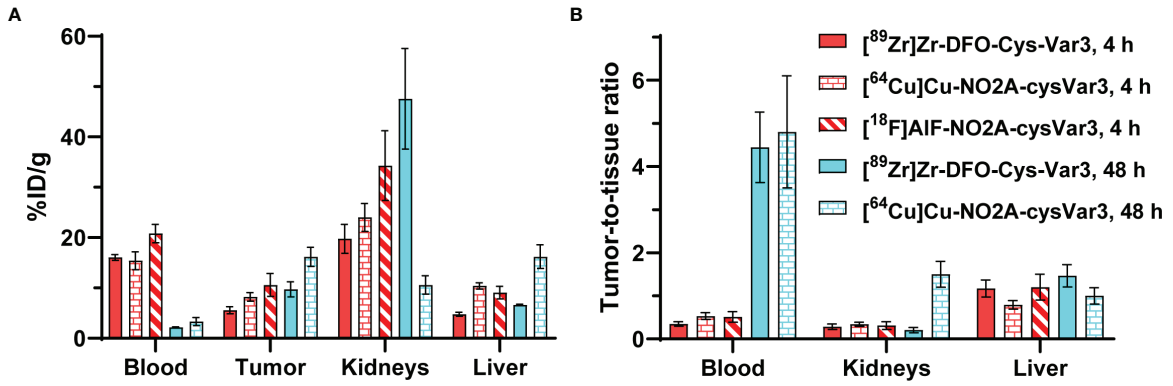

FIGURE 8 | **(A)** Comparison of the biodistribution in the 4T1 tumor model of [^{89}Zr]Zr-DFO-Cys-Var3 to [^{64}Cu]Cu-NO2A-cysVar3 and [^{18}F]AlF-NO2A-cysVar3 from a previously published study (38). **(A)** %ID/g of selected tissues at 4 and 48 hours post-injection of the radiotracers. **(B)** Selected tumor-to-tissue ratios at 4 and 48 hours post-injection of the radiotracers.

results clearly indicate that a high kidney retention is associated with the presence of a metal chelator rather than the pHLIP itself.

[^{89}Zr]Zr-DFOsqa-Lys-Var3 resulted in the highest tumor uptake amongst the investigated pHLIP compounds [(12.4 ± 4.7) %ID/g]. However, the kidney uptake was (82.5 ± 14.2) %ID/g, and the attempts to reduce this value by employing "kidney blocking agents" did not show the expected response. For this reason, we selected DFO-Cys-Var3 for a possible clinical translation, which exhibits high tumor targeting [(7.3 ± 2.9) %ID/g] and a moderate kidney uptake [(25.7 ± 3.0) %ID/g)]. The serial biodistribution study (4, 24, 48, 72, and 120 hours) confirmed the results of the imaging study using the 4T1 tumor model. Moderate blood clearance of [^{89}Zr]Zr-DFO-Cys-Var3 was observed. The blood-activity concentration at 24 hours was (5.8 ± 0.5) %ID/g; the biological blood clearance half-life was (16.0 ± 0.4) hours. The biological half-life for total body excretion was (415 ± 10) hours; minimal uptake was evident in the contents of the bladder or gastrointestinal tract on the PET images. Notably, in the PET images, kidney uptake was primarily localized to the cortex and minimal in the renal medulla or pelvis. An optimal tumor uptake was detected for the 48-hour timepoint (9.7 ± 1.7) %ID/g. Slight differences for the tumor- and kidney-uptakes were determined between the two tumor models and among different sizes of tumors. The differences can be related to the aggressiveness and acidity of the tumor. Human reference dosimetry estimates are required usage approval of [^{89}Zr]Zr-DFO-Cys-Var3 in investigational new drug studies and for documentation. In terms of the estimated human dosimetry, the effective dose coefficient was in line with full-length ^{89}Zr-labeled monoclonal antibodies (59). The major difference in dosimetry was that, for [^{89}Zr]Zr-DFO-Cys-Var3, the kidneys were the critical organ; however, no kidney toxicity is expected. Our preclinical biodistribution results, together with the human dosimetry estimates, suggest that [^{89}Zr]Zr-DFO-Cys-Var3 will be safe and effective at administered activities required to obtain diagnostic quality PET images in human patients.

CONCLUSION

Detailed investigation of the pharmacokinetics of the Var3 PET agents within the TME performed on tumor sections revealed that the agents stain the entire tumor mass and highlighted areas of acidification within the tumor–stroma interface. It was previously shown that targeting by fluorescent pHLIPs is not restricted to hypoxic areas. As sensors of the cell surface acidity, pHLIP constructs target the stroma (50), specifically metabolically active macrophages within the environment surrounding the cancer cells (28). In the era of immuno-oncology, this is an important finding toward improving therapy outcomes. An acidic TME created by both cancer cells and TAMs inhibits the presence of T-cells within the TME and suppresses the cytotoxic functions of T-cells (60). Acidity facilitates tumor growth, leads to drug resistance, and promotes immuno-suppression (60, 61). Thus, the development of novel imaging probes for tumor acidity has a high significance. Such probes would allow clinicians to predict and monitor the outcome of immunotherapies. pHLIP agents are very well suited for this task, but they exhibit slow blood clearance and tumor targeting, as established in preclinical and clinical studies. [^{89}Zr]Zr-DFO-Var3, containing the long-lived ^{89}Zr isotope, could be imaged for several days after administration, suggesting its potential for clinical translation for the identification of optimal imaging timepoints and, eventually, for supporting immuno-oncology therapeutics.

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 Institutional Animal Care & Use Committee (IACUC) at Memorial Sloan Kettering (MSK).

AUTHOR CONTRIBUTIONS

DB, YKR, and JSL conceived and designed this research. DB was involved in the chemistry, radiochemistry, and imaging. HV, AW, OAA, and YKR were involved in the biophysical characterization. DB and ZS were involved in the preparation of radiopharmaceuticals in this study. DB, ZS, and SK took part in the animal experiments. LMC calculated the dosimetry estimations. All authors contributed to the article 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.2022.882541/full#supplementary-material

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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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Meaning and Significance of "Alkalization Therapy for Cancer"

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Objectives of the Study: Our research aims to answer the following questions. Can cancer progression be stopped by changing the body condition of person with cancer? Can cancer be cured?If cancer progression can be stopped, what is the underlying mechanism?

Theoretical Rationale for Alkalization Therapy: Almost 70 years ago, Goldblatt H. & Cameron G. reported on the idea of alkalization therapy. Before that, Otto Warburg had been studying the metabolism of cancer and had discovered the essential nature of cancer. He published a review in Science in 1956 under the title "On the origin of cancer cells". From his phenomena described above, we established the theoretical rationale for alkalization therapy, based on the question of "How does cancer form and what is its nature"?

Limitations of Deductive Methods and Inductive Approaches: In this paper, we describe a method to reconstruct the limitations and weaknesses of modern cancer medicine as Science-based Medicine using an inductive method, and to present a new vision of cancer therapy. How should we treat cancer? (Case presentation): Using a specific clinical case, we present patients in whom were successfully treated with no or few anticancer drugs.

Summary: The biggest weakness of current cancer treatments is that they only treat the cancer and not the actual patient. The "alkalization therapy" that we advocate does not compete with any of the current standard treatments, but improves the effectiveness of standard treatments, reduces side effects, and lowers medical costs.

Keywords: cancer, metabolism, tumor microenvironment, alkalization therapy, urine pH, bicarbonate

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INTRODUCTION

Why did we decide to work in this kind of therapy? An experience was the beginning of this work. About 20 years ago (2001), when I was working at a university hospital, a patient with inoperable adenocarcinoma of the lung, clinical stage 3B, came to see me three years later. I had told him that his prognosis was probably about six months, but three years later he came to see me in good health.

When I asked him what he did to overcome the advanced cancer, he told me that he changed his diet. The diet was a low-calorie diet consisting mainly of vegetables and brown rice. I realized at the time, 'If you don't change the body of the cancer carrier, the cancer will not become suppressed. This can only be achieved through diet. It can only be reached by diet.

In the books I read later titled 'Radical Remission', the author, Kelly Turner, describes that "The first of the nine things that people who have had radical remission in common is changing their diet and drinking water." (1).

OBJECTIVES OF THE STUDY

Our aim was to consider the mechanisms by which cancer develops and to explain how these mechanisms can be applied clinically. Is it possible to stop the progression of cancer by changing the body of a person who has cancer? If the cancer stops growing, what is the mechanism by which this happens? Can it be cured? These are the questions that our research aims to answer.

THEORETICAL BASIS OF ALKALIZATION THERAPY

When we think about how it is that cancer can form in the human body, there must be some mechanism at work. An interesting experiment to consider this was reported 70 years ago. Fibroblasts of cardiac tissue are cultured under atmospheric conditions; they can be normally passaged. The fibroblast cultures were injected with nitrogen instead of air in a sealed container, and when about half of the cells had died, they were returned to their original atmospheric conditions. As a result, cancer cells developed in the nitrogen-injected culture, but no carcinogenesis was observed in the atmospheric conditions (2). It was during this period that Warburg studied the metabolism of cancer and discovered its essential nature. He published a review in Science in 1956 with the title 'On the origin of cancer cells' (3). In his review, Warburg described the work described above. The implication of this experiment is that the presence of nutrients but the absence of oxygen causes cancer. What happens when there is no oxygen? Mitochondria breakdown occurs in the cell. When the mitochondria break down, cytochrome C is released from mitochondria and the cell undergoes apoptosis and dies. Warburg et al. observed that in cells maintained under such anaerobic conditions, glycolysis was enhanced, and oxidative phosphorylation was reduced. This is what Warburg calls a cell with enhanced fermentation and reduced respiration. In other words, under anaerobic conditions, cells that avoid mitochondrial breakdown and choose to live by glycolysis are 'cancers'. This activated aerobic glycolysis, which is called the 'Warburg effect' is a hallmark of cancer metabolism and is known to be common to all cancers (4, 5).

It is a common understanding in biology that we, eukaryotic multicellular organisms, are made up of 'eukaryotic cells with the characteristics of archaea and the mitochondria formed by α-proteobacteria' (6, 7), and cancer cells are ancestral to archaea in terms of energy metabolism. In most cancer cells, mitochondria do not use oxygen, but do only substrate-levels phosphorylation dose produce minimal energy to maintain their own mitochondrial membrane potential. Cells that are forced to live on glycolysis in this way gradually develop abnormalities in gene expression. In most cases, this dysregulation occurs epigenetically in most cancers, but in rare cases, mitochondrial mutations have been reported to cause cancer recently.

More recently, Seyfried has proposed that cancer is not caused by a genetic abnormality but that it is a mitochondrial metabolic disease. He has experimentally shown that the cause of cancer is in the cytoplasm of the tumor, not in the nucleus. This is also the same proposal made by Warburg. The authors agree with this view and the importance of treating cancer as a metabolic disease (8, 9). Moreover, cancer patients with type II diabetes are known to have a poor prognosis (10), but we use metformin as needed to keep their hemoglobin A1c levels below 6, preferably around 5.8. When cancer cells start to live on glycolysis, their intracellular pH is always alkaline. In contrast, the intracellular pH of normal cells is almost neutral (11). Since cancer cells that live by glycolysis generate large amounts of H+ protons, they activate a mechanism to expel protons from the cell to keep the intracellular pH alkaline (12). As a result, the tumor microenvironment (TME) becomes acidic. Furthermore, extracellular acidic pH and intracellular alkaline pH of cancer cells is known to induce malignant behaviors, such as increased invasion and metastasis, multi drug resistance, and suppression of immune surveillance (13). One interesting example of this was reported in human lung cancer cell culture experiments, where 0.4 increase in intracellular pH was associated with a 2000-fold increase in the level of doxorubicin resistance in the tumor (14), proliferation and metastasis, expression of genetic abnormalities, growth factor activation, MDR and multidrug resistance, and vascular proliferation are activated.

It is well known that current cancer treatments often leave the TME acidic, resulting in poor therapeutic efficacy and severe side effects (4, 15). Therefore, our alkalization therapy aims to change the acidic TME to an alkaline. The actual methods are dietary interventions and the oral or intravenous administration of drugs that alkalize the body. As our bodies are made from the food that we eat, we believe that the act of alkalizing the body via food is a logical approach. A method to measure the pH of the TME has not yet been established to date, and hence we use urine pH as a surrogate indicator. The reason for this is that through our clinical practice, we have experienced that the urine of most patients who have achieved radical remission has an alkaline pH of 7.5 to 8. In the literature, it has been reported that Na+-H+ exchange 1 (NHE-1) becomes inactive when the extracellular environment is alkalized. NHE-1 has high ion transport activity under acidic conditions and its activity decreases with the shift to alkaline conditions, and that its activity decreases with the shift to alkalinity and completely disappears around pH 7.5 (16, 17). Thus, whether alkalization of TME really improves the therapeutic effect in clinical practice is an issue that should be examined in the future.

LIMITATIONS OF DEDUCTIVE METHOD AND INDUCTIVE APPROACHES: LEARNING FROM HEGEL'S "DIALECTIC"; PARADIGM SHIFT FROM EBM TO SBM

Georg Wilhelm Friedrich Hegel was a famous philosopher who advocated dialectics. Today's medicine is based on the concept of Evidence Based Medicine (EBM). This EBM is a "deductive method". What would you do if you were a cancer patient and you were told that stage 4 cancer cannot be cured, that there is only treatment to suppress it, or that treatment will not cure it? In deductive reasoning, we start from a given hypothesis and predict 'what we will see' if the hypothesis is true. In deductive reasoning, this prediction of 'what we will see' is always objective in the sense that it will be true if the hypothesis is true. The problem is that we cannot expand our knowledge beyond what is in the hypothesis. The biggest problem is that current medicine treats stage 4 cancer based on the assumption that it cannot be cured. To solve this problem, it is important to study people who have been cured of stage 4 cancer. In other words, it is important to "try to evaluate the significance of the results observed in a single experiment," i.e., to find "inductive" evidence. This combination of inductive and deductive perspectives provides a purely deductive method (called objective probability calculation) for drawing scientific conclusions from inductive ends (18).

From the point of view of Hegel's dialectic, the current EBM includes the antithesis that 'it is not possible to cure stage 4 cancer while receiving anticancer drugs. As a proposition to deny this, it is important to construct a hypothesis because 'there are people who have been cured of stage 4 cancer', and to perform inductively what those who have been cured have done in the treatment of stage 4 cancer. At present, it appears most appropriate to construct the hypothesis based on current molecular biological knowledge. Based on the results (Aufheben), the subsequent hypothesis needs to be tested, and new EBM needs to be constructed. Medical treatments based on the molecular biological findings necessary to construct this hypothesis is what is referred to as science-based medicine (SBM).

HOW DO WE DEAL WITH CANCER?

Alkalinizing the Acidic Tumor Microenvironment

So, what should we doctors do in actual clinical practice? As we have already mentioned, cancer is a cell that is based on a metabolic disorder, so the first thing to do is to stop this disorder. Cancer is said to undergo 'selfish' metabolic reprogramming, and it was mentioned above that the pH, which indicates the acid-alkaline level inside the cancer cell, must become 'alkaline'. To maintain the homeostasis of intracellular alkalization, ion transporters, such as NHE-1, vacuolar H+-ATPases, monocarboxylate transporters and carbonic anhydrases are expressed on the surface of cancer cells (12). The first thing to do is to stop these proton transporters.

The patient should first be advised to change his/her diet. It is important to measure the patient's urine daily with a litmus test paper. Under normal conditions, the urine pH is almost 5-5.5 acidic, but as the diet is changed to an alkalinizing diet (fruits and vegetables, no meat, no dairy products), the patient's urine pH will increase. The aim is to achieve a urine pH of 7.5-8 or higher, and if this is not achieved, alkalinizing agents may be given intravenously or orally. **Figure 1** is from our paper published in a journal and shows that in cases of small cell lung cancer, the urine pH was approximately 7.3 in the alkalization group and 6.4 in the control group, indicating an increase in urine pH in the alkalization group that showed efficacy increase in the alkalization group (19). Several other reports have also been made (20, 21).

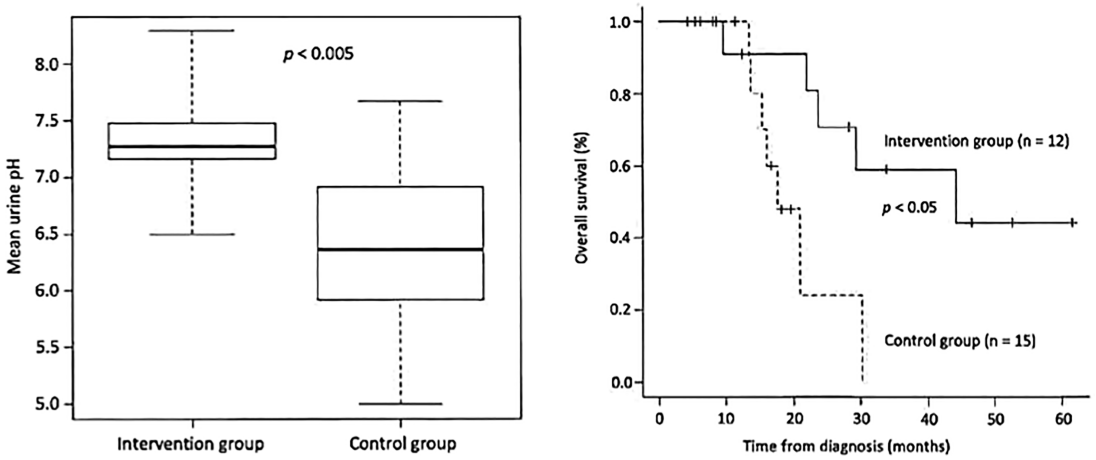

FIGURE 1 | (Left) The mean urine pH of the alkalization group of small cell lung cancer patients is shown to be higher than that of the control group. (Right) The overall survival of the alkalization group of small cell lung cancer patients is shown to be prolonged compared with that of the control group.

The next thing to do is to reduce inflammation. C-reactive protein (CRP) is used as an indicator. The patient should be taught to keep the CRP below 0.05. It is reasonable to assume that the elevated CRP in carcinoma carriers is due to acidification of the TME, where protons present in the TME attract primary immune cells such as neutrophils and macrophages (22, 23). A rapid reduction in CRP can be achieved by 'donating' electrons to the patient. A large intravenous dose of ascorbic acid (at least 4 grams) is very effective. Warming the patient's body and making him sweat are also helpful. Obesity also raises CRP, so patients should be advised to reduce their weight. At this time, it is important to improve the bacterial flora in the intestine. There is a growing consensus that abnormalities in the intestinal microflora are involved in carcinogenesis [Schwabe & Jobin (24) Zambirinis et al. (25) Johnson et. al. (26) Yu & Schwabe (27) Gopalakrishnan et al. (28) Routy et al., (29) Riquelme et al., (30)]. It has been reported that the human gut microbiota regulates many host processes, including metabolism, inflammation, immunity, and cellular responses, and that its composition is known to be altered in many diseases, including cancer (31). The gut microbiome may also affect the development of cancer. It has also been reported that the microbiome can worsen the prognosis of cancer, by producing carcinogenic toxins and metabolites. Therefore, to improve the gut microbiome, foods rich in 'water-soluble pectin' should be consumed.

The next step is to 'suppress the primary immunity and activate the secondary immunity' (32). Cancer is known to utilize inflammatory cytokines to grow. In the absence of cancer, this response is the first response in the "wound-healing process" and is called the primary (innate) immune response, but it is also known to support cancer growth (22). Normally, TMEs are acidic. Recently, it has been known that this acidification has a beneficial effect on cancer cell proliferation, migration, invasion, metastasis, therapeutic response and the function of stromal cells such as immune cells and vascular cells in cancer growth. It is believed that the activation of proton-sensitive GPCRs by acidosis causes the above conditions. Considering this aspect, it is safe to assume that elimination of protons presents in TME, i.e., alkalization of it, can inhibit cancer growth. Acidification of the TME is associated with the accumulation of myeloid-derived suppressor cells (MDSCs), which are a population of cells that proliferate during cancer, inflammation, and infection, and have a robust ability to suppress effector T-cell responses (33). It has been reported that about 80% of MDSCs eventually differentiate into neutrophils and the remaining 20% into macrophages in the TME, and that alkalization of the TME prevents these MDSCs from accumulation in the TME. When the TME becomes alkaline, the primary (innate) immune response is suppressed, but as a result the secondary (acquired) immune response becomes more active. The white blood cells that play a central role in this process are lymphocytes. There are several subsets of lymphocytes but identifying the specific subset does not appear to be important in making clinical decisions.

In our clinical experience, we found that patients who show improvement have a neutrophil/lymphocyte ratio of less than 2, or even less than 1.5, and a lymphocyte count of more than 1,500, or even more than 2,000, which we use to determine the clinical status of the patient. CRP is also useful in determining neutrophil activity, and a CRP of 0.05 or less is considered as low neutrophil activity. The goal is to achieve a urine pH of 7.5 to 8.0 or more. Once this is achieved, it is often our experience that anticancer drugs can be given at less than half the standard dose and still show satisfactory effects.

Causes of Drug Resistance, Invasion, and Metastasis of Cancer

It is well-known that cancer cells that survive anticancer drug treatments gradually become resistant to the drugs. This is closely associated with acidification of the TME around a cancer. Alkalization of the TME around malignant cells is known to attenuate the intracellular concentrations of anticancer drugs, such as vinblastine, adriamycin, cisplatin, paclitaxel, campthothecin, etc. (4), and hence tumors become resistant to anticancer drugs when the TME becomes acidic (34). As mentioned above, Keizer and Joenje H (14) demonstrated using human lung cancer cells that an increase in intracellular pH from 7 to 7.4 results in very high drug resistance, such as a 2,000-fold increase in adriamycin resistance. There is a strong positive correlation between the degree of MDR and the intracellular pH of cancer cells. This mechanism is likely to be associated with the high acidification of intracellular organelles (lysosomes, endosomes, Golgi network, etc.). The so-called protonation, ion capture effect, or the theory of 'Mathematical Modelling of Tumor Acidity Regulation of Intracellular pH' has been proposed to explain this hypothesis (35). In addition, with regard to metastasis, NHE-1 also regulates the formation of invadopodia (cellular structures that mediate the migration and invasion of tumor cells). Therefore, reducing NHE1 activity in cancer cells is linked to the inhibition of metastasis, and this can be achieved by 'alkalization' of the TME (11). In conclusion, tumors acquire resistance to anticancer drugs and the ability to metastasize because of the acidification of the microenvironment surrounding the tumor (TME), and therefore, alkalization of the TME is expected to substantially reduce cancer activity.

Purification of the Arterioles

Carcinogenesis occurs when, as Warburg states, 'nutrients are supplied but oxygen is lacking'. It is reasonable to assume that this is true in the human body because of the narrowing of the arterioles. When the narrowing of the arterioles occurs, it becomes difficult for blood cell components to flow, but liquid components can flow. The flow of fluid means that nourishment is being carried out, and the difficulty in the flow of blood cells means that without the flow of red blood cells, it is difficult for oxygen to be supplied. When white blood cells become blocked, a cytokine storm develops, leading to chronic inflammation (36). Most cancer patients consume large amounts of high-fat food. In addition to eating meat and drinking heavily, many male patients also smoke. Many female patients prefer sweet cakes with lots of cheese and cream. These dietary habits may be considered as a cause of narrowing of the arterioles.

In addition, soft stools and diarrhea are more common in male patients and constipation is more common in female patients. These are all associated with dysbiosis of the intestinal microflora. The gut microbiota as above also needs to be purified to become synbiotic (37). This study by Zitvogel showed that alterations in the human gut microbiota can lead to cancer. By avoiding high-fat diets and purifying the gut microbiota to a symbiotic state, it is possible to stop cancer in its tracks.

Anti-Tumor Effect of Intravenously Administered Ascorbic Acid

In our clinic we often use intravenous ascorbic acid in the treatment of cancer. There has been much debate as to whether ascorbic acid (vitamin C) is effective in the treatment of cancer, but it has recently been shown that the pharmacological effects of taking this substance orally and administering it intravenously are quite different. Although the function of vitamin C in vivo is not clearly and fully understood, it is understood that this substance acts in vivo as an electron donor (38). The antitumor effect of ascorbic acid can only be achieved by intravenous or intrathoracic or intraperitoneal administration. Oral administration does not have such an antitumor effect, which the authors intend to report in detail elsewhere (39–41).

DISCUSSION

The main problem with current cancer treatment is that it does not consider the condition of the cancer patient's own body. As stated earlier, "In conclusion, cancer is the result of cells being forced to choose glycolysis as a metabolic pathway to survive in the presence of nutrients but without oxygen. In the case of cancer, it is reasonable to assume that the first priority is to restore the body of the person with cancer to the state it was in when there was no cancer. In other words, it is important to 'make the cancer patient's body less susceptible to cancer' before treating it. All multicellular organisms are made up of eukaryotic (nucleated) cells. Eukaryotic cells emerged from prokaryotes 4 billion years ago through mitochondrial symbiosis, resulting in a drastic expansion of the number of genes expressed by 200,000 (6–7). This leap in genomic capacity, due to the power of mitochondria, was a prerequisite for the key evolutionary processes that led to the increasing complexity of eukaryotes (multicellular organisms) (6). Based on this complex network of genes and considering the results of the experiments of Goldblatt & Cameron (2), we can understand that hypoxia induces induction at the genetic level, the accumulation of which results in cancer.

Otto Warburg, studying cancer metabolism, states that 'the characteristics of cells which have survived hypoxia are altered, there is an increase in fermentation (glycolysis) and a decrease in respiration (oxidative phosphorylation), and these characteristics accumulate'. This first event in carcinogenesis has recently been reported to be the transmission of signals from the mitochondria to the nucleus, resulting in the condition described above. It is reasonable to assume that avoiding 'mitochondrial break-down' is a survival strategy for cells in oxygen-deprived conditions, as it does not lead to cell death. Mitochondrial retrograde signaling is a mitochondrial-to-nuclear signaling pathway that influences many cellular and biological activities during normal and pathophysiological conditions. In yeast, it is used as a sensor of mitochondrial abnormalities and initiates the readjustment of sugar and nitrogen metabolism, for which the RTG gene has been found to be responsible (42–45). However, although a similar response has been observed in human cells, the signaling players remain unclear.

Retrograde signaling has been suggested to be involved in tumor progression by inducing the invasion of non-neoplastic cells. It has been speculated that Nuclear Factor κ B (NFκB) may be the master regulator of this network in humans (46). In most tumors, the Warburg effect is a "selfish" metabolic reprogramming, with an initial overexpression of hypoxia-inducible factor-1 (HIF-1) (15). This suggests that the human retrograde signaling pathway is based on the activation of NFκB in the absence of oxygen, which somehow transmits a signal from the mitochondria to the nucleus to avoid mitochondrial disruption, the first genetic abnormality being the overexpression of hypoxia-inducible factor-1 (HIF-1). When carcinogenesis is triggered, the surrounding environment of the cancer cell becomes acidic and the cell adapts to this, leading to malignant transformation of the cell (47). When this occurs, the pH within the cancer cell becomes alkaline and the TME becomes acidic. The regulation of this intracellular alkalization and acidification of the extracellular microenvironment is crucial in the treatment of cancer.

There are many studies on proton pump inhibitors (PPIs) as a drug repositioning therapy targeting acidic TME (48–51). Several in vivo and in vitro studies have shown that combination of PPI and chemotherapies increases the chemotherapeutic responses (52–54). Furthermore, preclinical studies in human tumor cell lines have reported that administration of PPI alone, without chemotherapy, induced apoptosis of cancer cells and produced anticancer effects (55–57). Population-based studies have also suggested that PPI use may prevent the development of breast cancer (58–60). Although clinical studies are limited, three patients of advanced colorectal carcinoma were treated with high-dose PPI in combination with chemotherapy and reported favorable results (61). Furthermore, for patients with metastatic breast cancer, the combination of chemotherapy and PPI was reported to significantly prolong time to progression and overall survival compared to chemotherapy alone (62). On the other hand, several animal studies have also shown that systemic alkalization with buffer therapy using bicarbonate and/or alkalizing agents inhibits tumor progression (63–65). Moreover, although Amiloride and Cariporide are known as drugs that stop alkalization in cancer cells, they have not yet been applied clinically due to their adverse effects. There are many reported intervention methods to neutralize acidic TME, most of which are based on the concept of proton control by external intervention of drugs. 'Alkalization therapy' we advocate alkalizes TME using an alkalinizing diet and alkalizing agents to cleanse the body of cancer patients.

Alkalinizing diet and alkalinizing agents are used in the treatment. However, the measurement of the acid-alkaline level of the microenvironment surrounding the cancer is not available in current clinical practice (12, 66, 67). We found that in all cases where various cancer treatments were effective using our 'alkalization therapy', the urine pH became alkaline (19–21, 68, 69). Due to the impossibility of measuring the pH of the microenvironment surrounding the cancer, we use the patient's urine pH as a surrogate indicator to practice alkalization of it. This alkalization of the urine pH means a decrease in the number of acidic substances such as protons excreted in the urine and does not imply an extreme alkalization of the acid-alkaline balance of the body fluids.

When the TME is acidic, cancers are more resistant to all therapies, including anticancer drugs, radiotherapy, immune checkpoint inhibitors and molecular targeted therapy, and the side effects of these therapies are more severe (4, 47, 70). In the treatment of cancer, it is important to suppress primary immunity and activate secondary immunity. The primary immune system began over hundreds of millions of years ago when a series of specialized enzymes and proteins evolved to protect our primitive ancestors from attack by the outside world. This inflammatory immune response worked so well that its function has been conserved. Until now, cancer researchers have treated cancer with the belief that genes are the main underlying cause of cancer. However, in the last few decades we have come to understand that the inflammatory component of the primary (innate) immune system, which is normally part of the wound healing process, promotes carcinogenesis by aiding tumor development and growth. The reason why the inflammatory component of the primary (innate) immune system is activated in cancer development and growth is due to the acidification of the TME caused by the extracellular excretion of intracellular protons from glycolysis. It has been reported that cancer cells are more active in this acidic environment (22, 71). The tumor microenvironment is acidic. This acidification has a multifaceted effect on cancer cell proliferation, migration, invasion, metastasis, resistance to treatment and the function of stromal cells such as immune cells and vascular cells. However, the molecular mechanisms by which cancer cells and stromal cells sense and respond to acidic pH in the tumor microenvironment are not well understood.

The role of the family of pH-sensing G protein-coupled receptors (GPCRs) in tumor biology is increasingly being recognized as important to regulate cancer cell metastasis and proliferation, immune cell function, inflammation, and angiogenesis. Neutrophils sense protons of the TME via GPCRs expressed on their cell surface and aggregate in areas of high proton concentrations. Macrophages accumulate by a similar mechanism (72, 73). Acidification of TME is associated with pro-tumor polarization of tumor-associated macrophage phenotype (74), and it is reported that Inhibition of tumor acidosis through PPIs promote an antitumor phenotype of tumor-associated macrophages (75). MDSCs are a population of cells that proliferate during cancer, inflammation, and infection, and have the robust ability to suppress T-cell responses (33, 76). MDSCs accumulate at sites of activation of the innate immune system by the activity of neutrophils and macrophages, as described earlier, and NO and ROS are said to be involved in this mechanism. About 80% and 20% of MDSCs differentiate into neutrophils and macrophages, respectively, and if the acidic TME is left without intervention, inflammation in this environment will increase. It hence appears reasonable to assume that acidification of the TME is the most common cause of the resistance of cancers to treatment. Our strategy of alkalinizing the acidic TME is called 'alkalization therapy'.

The aims of this treatment are to achieve (1) a urine pH of 7.5 to 8.0 or more, (2) a CRP level of 0.05 or less, and (3) a neutrophil/lymphocyte ratio of 2.0 or less (1.5 if possible), and a lymphocyte count of 1,500 to 2,000 or more. The cause of the increased CRP is thought to be the presence of H+ and the development of arteriolitis owing to vascular narrowing in hyperlipidemia, obesity, diabetes, and heavy drinking. Alkalization of the body is very much influenced by the diet consumed and is determined using urine pH. In addition, the association between intestinal microflora and cancer will require further study in the future.

CONCLUSION

The treatments we advocate do not in conflict with conventional standard therapies but can be used in combination to increase their efficacy and reduce side effects. The human body is a dissipative structure, which means that it is an open system that is not in thermodynamic equilibrium. This means that it is possible to reduce the entropy of the body. It is our experience that alkalization of the TME enables various treatments to become more effective, and with the future collaboration of many researchers, we hope that it will soon become possible to treat intractable cancers using standard treatments combined with alkalization therapy (19, 21, 21, 68, 69, 77, 78).

PATIENTS DEMONSTRATING A SUCCESSFUL TREATMENT RESPONSE TO ALKALIZATION THERAPY

The cases shown here are patients whose treatment involved no anticancer drugs or, if used, exceedingly small amounts of oral medication. These case reports are comprehensively included in "Investigation of survival factors for cancer patients using data science methods" approved by Institutional Review Board of the Japan-Multinational Trial Organization. Written informed consent for publication of these case reports and accompanying images has been obtained from the patients.

(i) Male, 84 years old (at the time of first consultation), Unresectable renal pelvis cancer, cT3N2M0, Periaortic lymph node (LN) metastasis (+)

First visit to our hospital February 2021, January 2021: Visited T Hospital due to gross hematuria; CT scan showed venous invasion of right renal pelvis carcinoma and pararenal aortic LN metastasis. The same diagnosis was made at the University Hospital. He was told by the doctor that anticancer drug treatment would not be given because he was too old, and it would not be effective. An alkalizing diet and intravenous vitamin C showed a marked improvement in the renal pelvis cancer. No anticancer drugs were used.

After 4 months of alkalinizing diet + alkalinizing agents + intravenous vitamin C, the tumor in renal pelvis of the right kidney, which was found in January 2021, was significantly reduced in June 2021. The red circles indicate the sites of renal pelvis cancer (**Figure 2**). In February 2021, urine pH = 5.5, which increased to 8 in July (5 months after the visit) (**Figure 3**).

(ii) Female, 76 years old, Malignant lymphoma of stomach, 2015/12, First visit to Wada Clinic.

Her gastric malignant lymphoma disappeared two and a half years after her first visit to Wada Clinic, simply by changing her diet (**Figure 4**). All she did was to start an alkaline diet as alkalization therapy.

(iii) Female, 89 years old, malignant lymphoma of the right tonsil.

In February 2009, the patient presented with an enlarged right tonsil and swollen lymph nodes in the right mandible, which were biopsied and a diagnosis of malignant lymphoma was

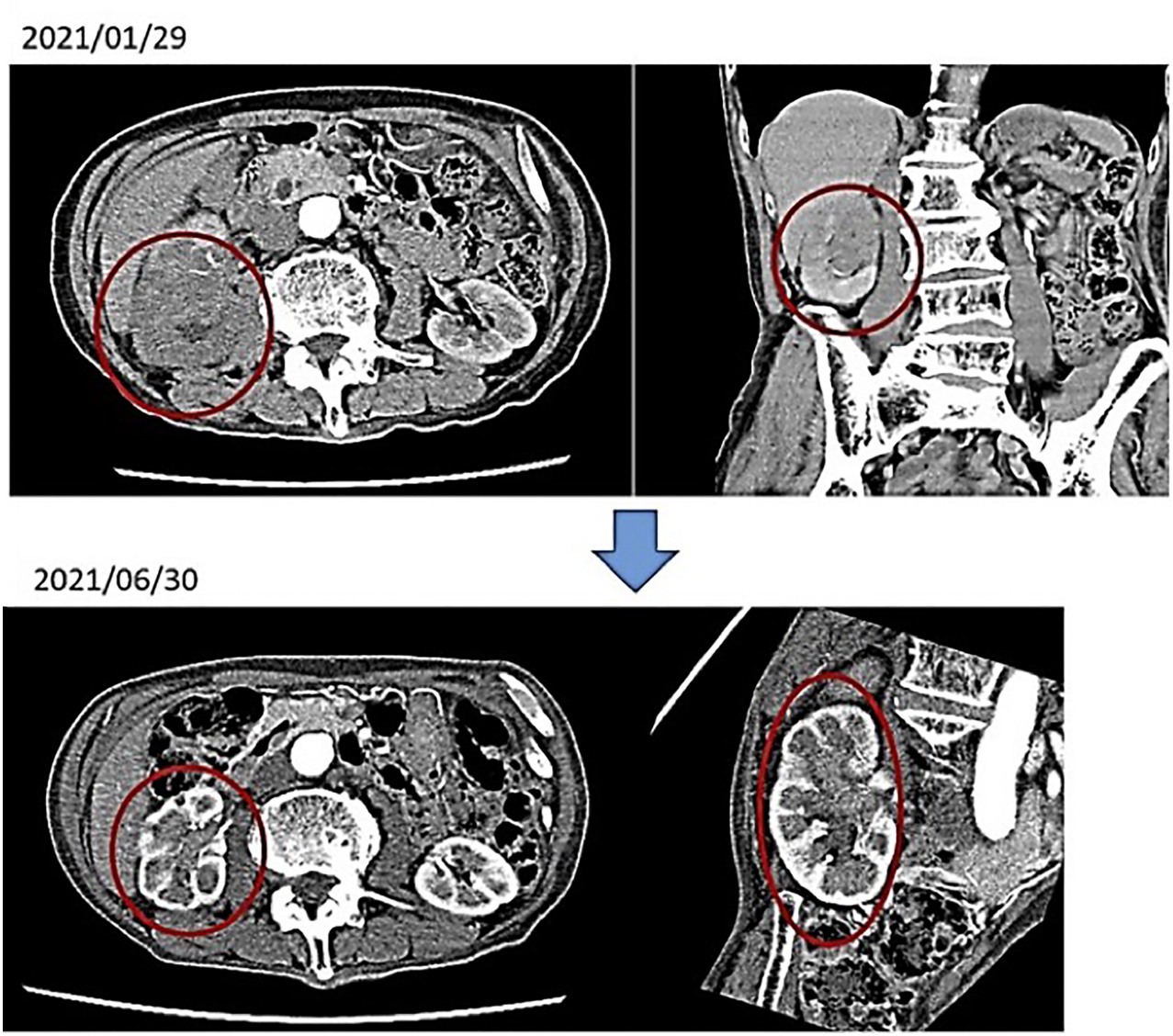

FIGURE 2 | A contrast enhanced computed tomography of abdomen are shown.

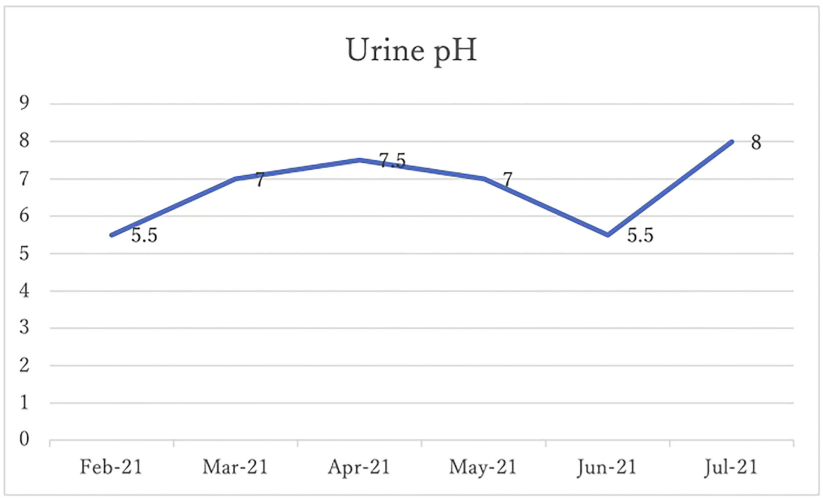

FIGURE 3 | His urine pH levels are shown.

made. The malignant lymphoma was diffuse large B-cell lymphoma, CD20 positive, CD79α positive and negative for epithelial marker A E1/AE3 negative.

The patient was advised to be hospitalized and receive anticancer therapy, and came to our clinic on April 2009 because of anxiety about side effects. At the time of the consultation, the patient had an ulcer on the right oral mucosa, which made opening the mouth a little difficult.

After the visit to our clinic, I recommended her to drink 2 bags of red bean cedar tea (Yunnan Yew tree) infused with 1 liter of tea per day to soak the affected area, and 3 bags of Misatol (plum extract supplement) in 3 portions to soak the affected area.

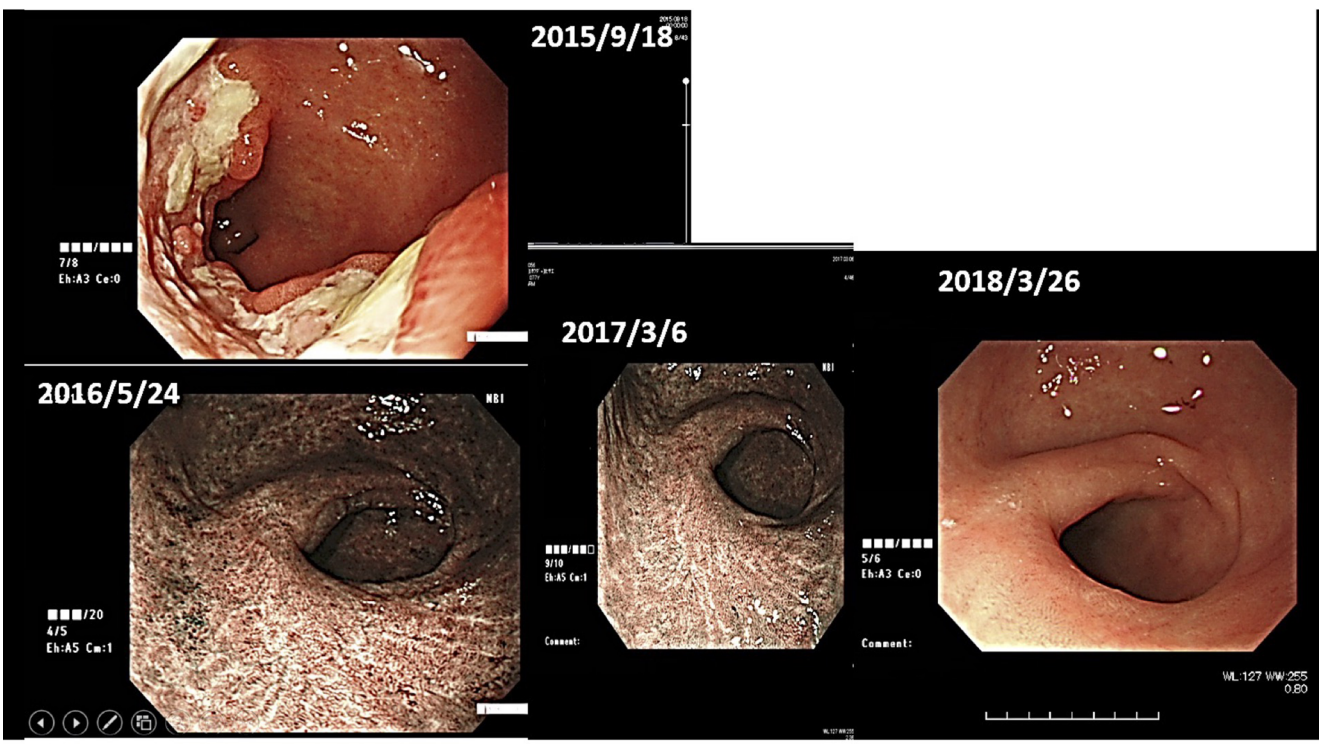

FIGURE 4 | Her images of upper gastrointestinal endoscopy are shown.

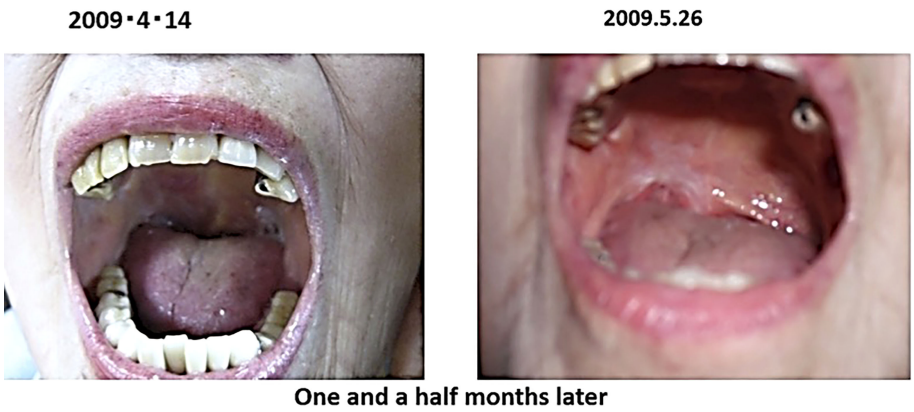

FIGURE 5 | Her photographs of right tonsil are shown.

The lymphoma disappeared after two months. The lymphoma almost disappeared after two months and has not recurred since (**Figure 5**). She lived her daily life in good health, but two years and eight months after the start of treatment, at the age of 91, she passed away as if asleep after breakfast one day.

(iv) Female, 15 years old, malignant lymphoma of tonsil.

The patient was first seen at our clinic in 2020/3. She was diagnosed as diffuse Large B Cell Lymphoma (DLBCL) of tonsillar origin at the Children's Hospital in 2019. PET-MRI showed no obvious accumulation except for enlarged right palatine tonsil. Prior to her illness, she was an imbalanced eater of foods. She was very fond of sweets. In the two months following the first visit, there was no increase in the lymphoma of the tonsils. However, in May 2020, the tumor became airway obstructed (**Figure 6**), causing breathing difficulties, and she underwent emergency surgery at the prefectural hospital. Since the resection, she has been taking apple pectin juice, citric acid preparations, red bean cedar tea (Yunnan Yew tree) and summer white chrysanthemum (feverfew) and has had no recurrence. She has not been treated with any anti-cancer drugs. Her IL-2R decreased after her visit to our clinic and has continued to decrease steadily after her surgery (**Figure 7**).

(v) Male, 64 years old. Gastric cancer (unresectable), multiple liver metastases, Adenocarcinoma(por), Group5. First visit to our clinic August 2020.

History of the disease: Introduced by Dr. B, Department of Surgery, Hospital A. On July, 2020, the patient was brought to the emergency room because of fatigue on standing up. When he came to the hospital, he was found to be anemic with Hb = 7.2 and was diagnosed with advanced gastric cancer on further examination. A contrast-enhanced CT scan revealed multiple liver metastases, and he and his wife decided not to take standard anti-cancer treatment and came to our clinic.

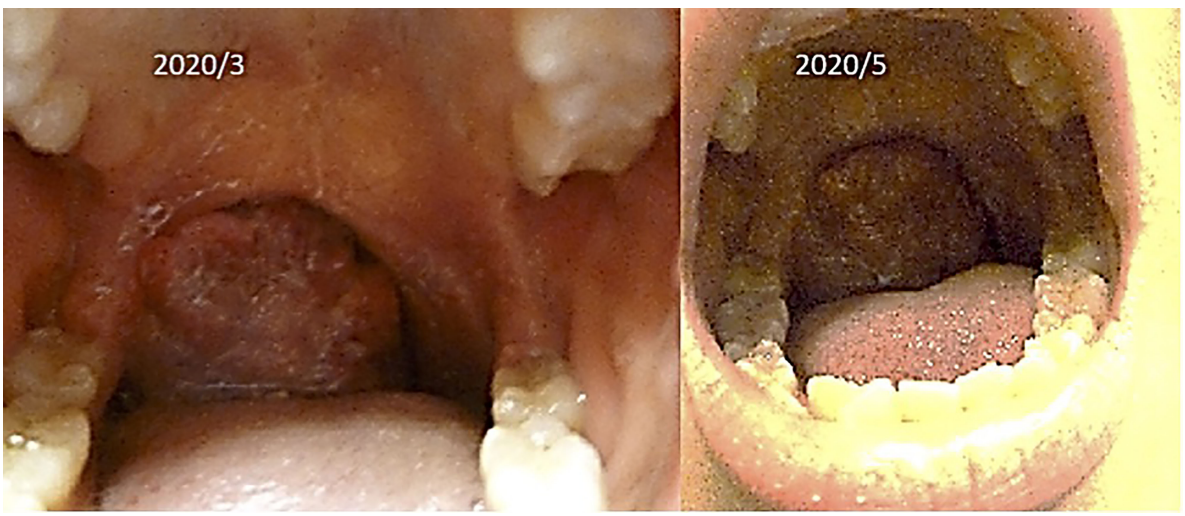

FIGURE 6 | Her tumor of tonsil is shown.

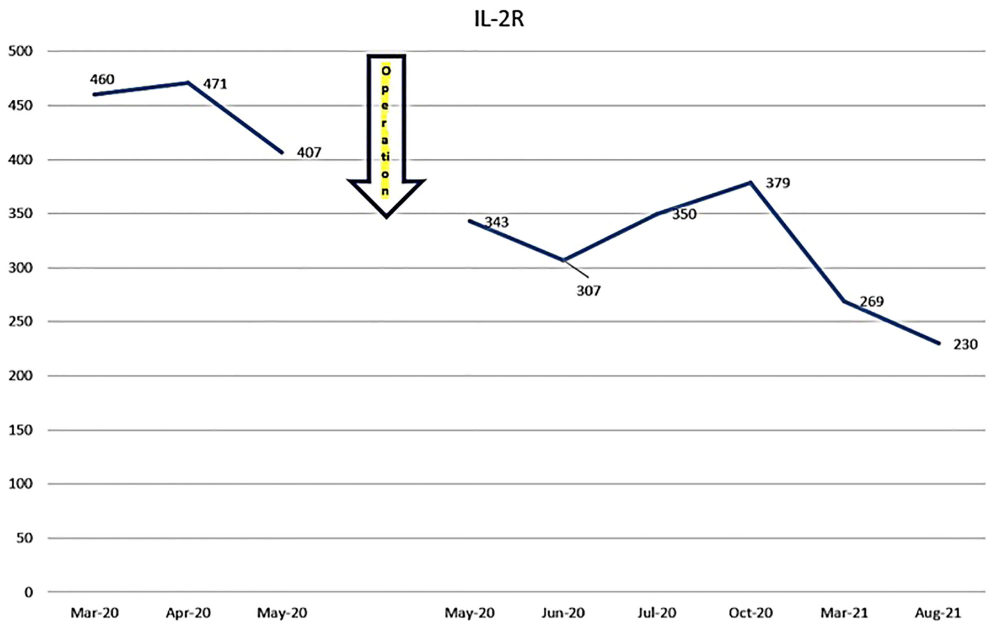

FIGURE 7 | Her IL-2R levels are shown.

(Diet before the onset of the disease) Dairy products: a lot. 350ml of milk, a pack of yoghurt, a lot of cheese, taste: strong.

(Course) Recently, he has been cooking by himself. He eats an alkalizing diet with lots of vegetables and fruit. He wants to reflect on his diet and way of life and challenge himself to live with cancer. He doesn't want to do standard therapy.

He has decided to do the following Things.

Start the Maruyama vaccine (immunostimulant) and receive lentinan (immunostimulants) intravenously once a week. Take one tablet of the oral anticancer drug TS-1 (5FU derivative) (20 mg) twice a week. Alkalization of the urine with citric acid preparations and sodium. The patient drinks 140 ml of apple pectin juice daily for three months and receives 25 g of Vit C intravenously. In order to increase the secondary immunity, mushrooms should be ground up and eaten in soups. Keep warm and exercise.

One year and three months later, gastric endoscopy showed that all cancers had disappeared (**Figure 8**), and PET scans also showed that all liver metastases had disappeared. (He has put this information on you-tube. The address is as follows: https://youtu.be/zHF_zUE9prI).

(vi) Male, 70 years old. Multiple gastric cancer, first visit in December 2012.

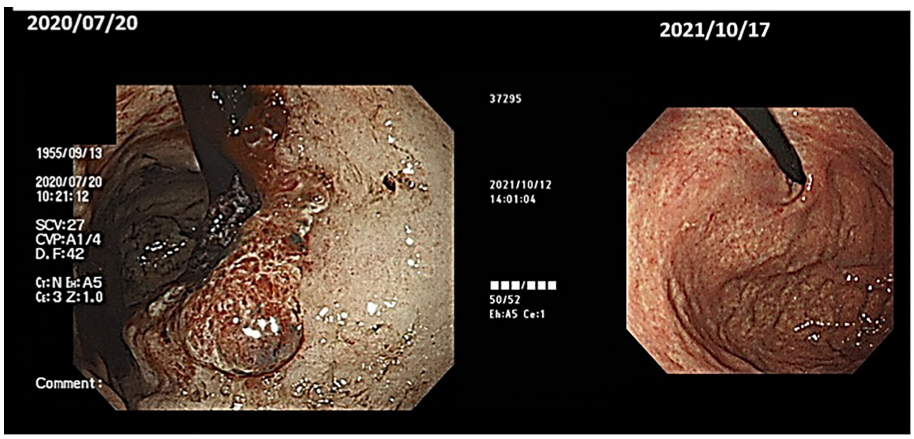

FIGURE 8 | An upper gastrointestinal endoscopy showed that tumors had disappeared.

At the beginning of October 2012, he had a check-up at a hospital in Kagawa. He went to a hospital in December. He was told that he would have to undergo an immediate resection, but when he asked to wait until next year, he was told that the polyps in the upper part of the stomach would be removed under endoscopy, and that the lower part of the stomach would be removed and a subtotal resection would be performed.He was positive for H. pylori and was put on medication.

He has been receiving intravenous lentinan (an immunostimulant) once a week since December 2012 and was started on TS-1 20 mg orally three times a week. After three years, his stomach cancer had disappeared after following a diet and lifestyle that was "light in taste, rich in fresh fruits and vegetables, dairy-free and meat-free" (**Figure 9**).

(vii) Male, 74 years old. First seen in our clinic on December 2014.

The patient was diagnosed with gastric cancer (early stage) after a thorough physical examination in 2012. He underwent gastrectomy (laparotomy) at a hospital in February 2012 and was told that there was no abnormality at the one-year check-up after the surgery. In November 2013, his physical examination revealed an abnormality in the remaining stomach, which was diagnosed as a recurrence of the remaining stomach, and he underwent a total gastrectomy in January 2014. In January 2014, he had a total gastrectomy. The pathological examination of the resected specimen showed that it was a scirrhous gastric cancer. He was told that the cancer was left at the anastomosis of the dissection. After his consultation, he was treated with intravenous lentinan (an immunostimulant) once a week and TS-1 capsules (an oral 5FU derivative, 20 mg) twice a month. Seven years later, in 2021, he is still alive and well.

(viii) Male, 68 years old (at time of first visit). Post-operative recurrence of gastric cancer; first visit to our hospital in May 2015.

In 2015, he went to the emergency room of his local doctor for hematemesis, was diagnosed as gastric hilar cancer and underwent emergency surgery.He underwent total gastrectomy and cholecystectomy and was diagnosed with anastomotic marginal gastric cancer remains. Postoperatively, tumor markers (CA72-4, CEA) were elevated, and recurrence was suspected.

In Wada clinic Lentinan (immunostimulant) was started intravenously and combined with TS-1 capsule (oral 5FU derivative, 20 mg) once a week. The patient is alive and well 6 years after surgery (**Figure 10**).

(ix) Female, 50 years old. Postoperative recurrence of breast cancer with multiple systemic metastases, including bone metastases. First visit to our clinic October 2019.

The resected tissue showed ER+, PgR+, HER2 1+ (negative), Ki67 26-29% (hormone receptor positive). In 2012, she underwent partial resection and lymphatic dissection for right breast cancer. In May 2020, a PET scan showed multiple metastases in the right axilla, vertebrae, and ribs, and she came to our clinic in October 2019. In terms of lifestyle, she loved sweets and enjoyed making pastries.

After her visit to our clinic, she has been on an 'alkalizing diet'. In Wada clinic, we recommended her to do the following.

She took 140 ml/day of apple pectin juice (apple shimmer) at home, followed an alkalizing diet and 'dairy products' were strictly forbidden (79).

At the Wada Clinic she was given 25g vitamin C intravenously once a week. Subcutaneous injections of a luteinizing hormone-releasing hormone derivative microcapsule sustained-release formulation and a human IgG2 monoclonal antibody formulation targeting RANKL were continued.

A PET/CT scan six months after the first visit showed that the tumors had disappeared. The patient was also put on sodium

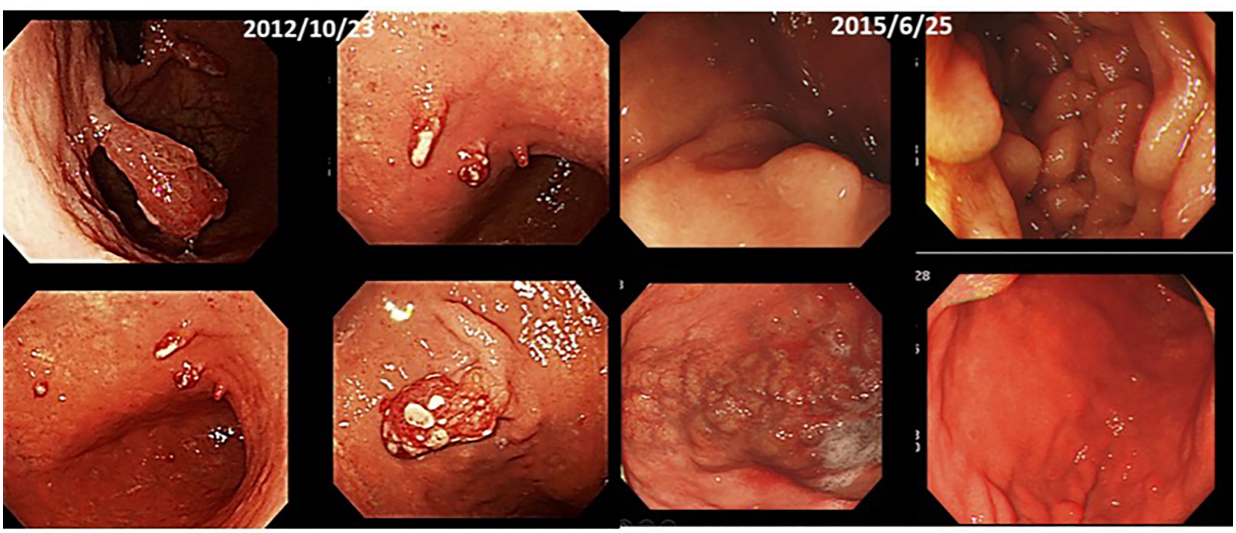

FIGURE 9 | An upper gastrointestinal endoscopy showed that all tumors had disappeared.

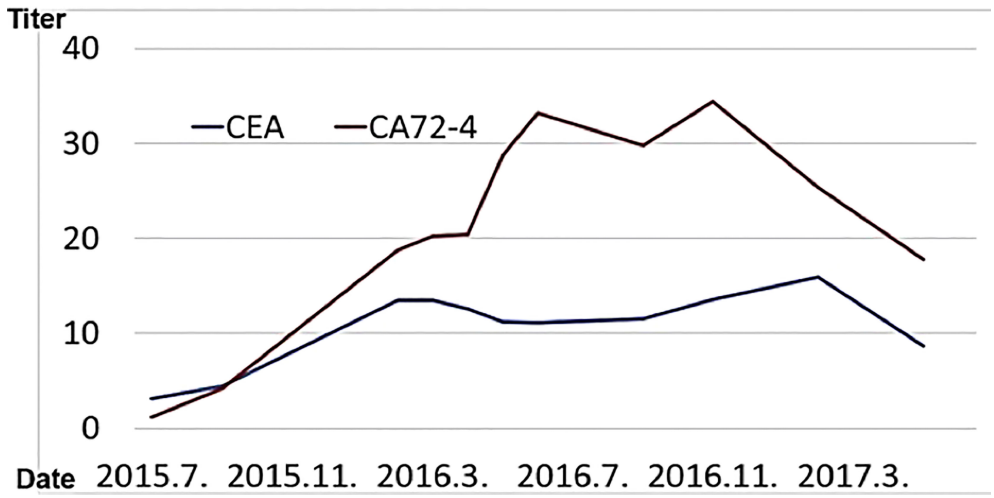

FIGURE 10 | His tumor marker (CEA, CA72-4) values are shown.

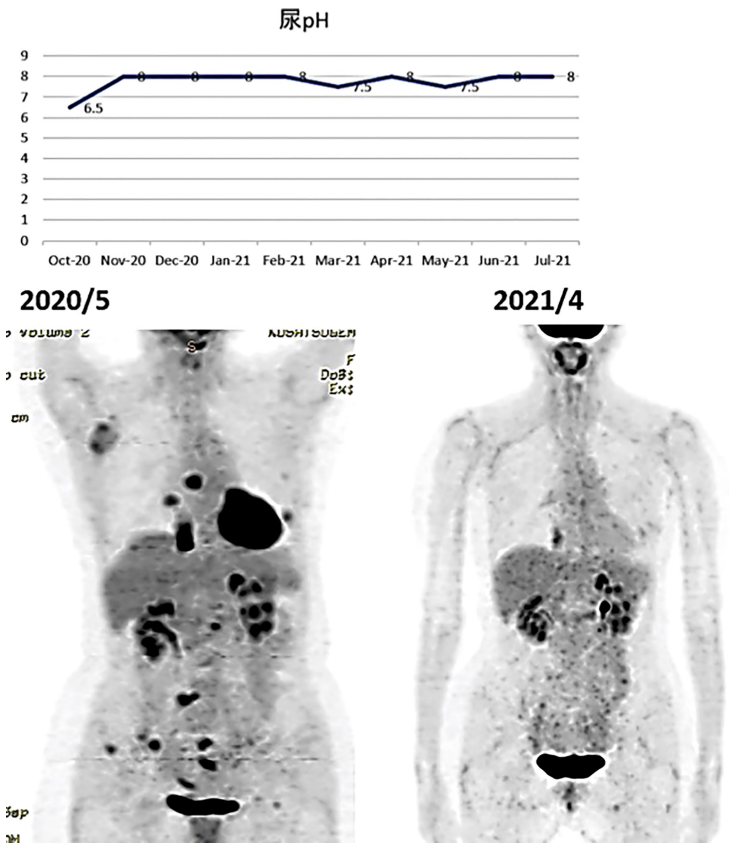

FIGURE 11 | (Upper) Her urine pH levels are shown. (Lower) A PET/CT scan showed that tumor had disappeared.

bicarbonate and her urine pH increased from 6.5 on visit to 8.0 one month later (**Figure 11**).

(x) Female, 45 years old (at first medical examination). First visit to Wada Clinic in January 2011.

Postoperative breast cancer, systemic metastasis including multiple lung and bone metastasis. Due to recurrent lung metastasis from breast cancer, she had breathing difficulties and started treatment at a cancer center, but the cancer did

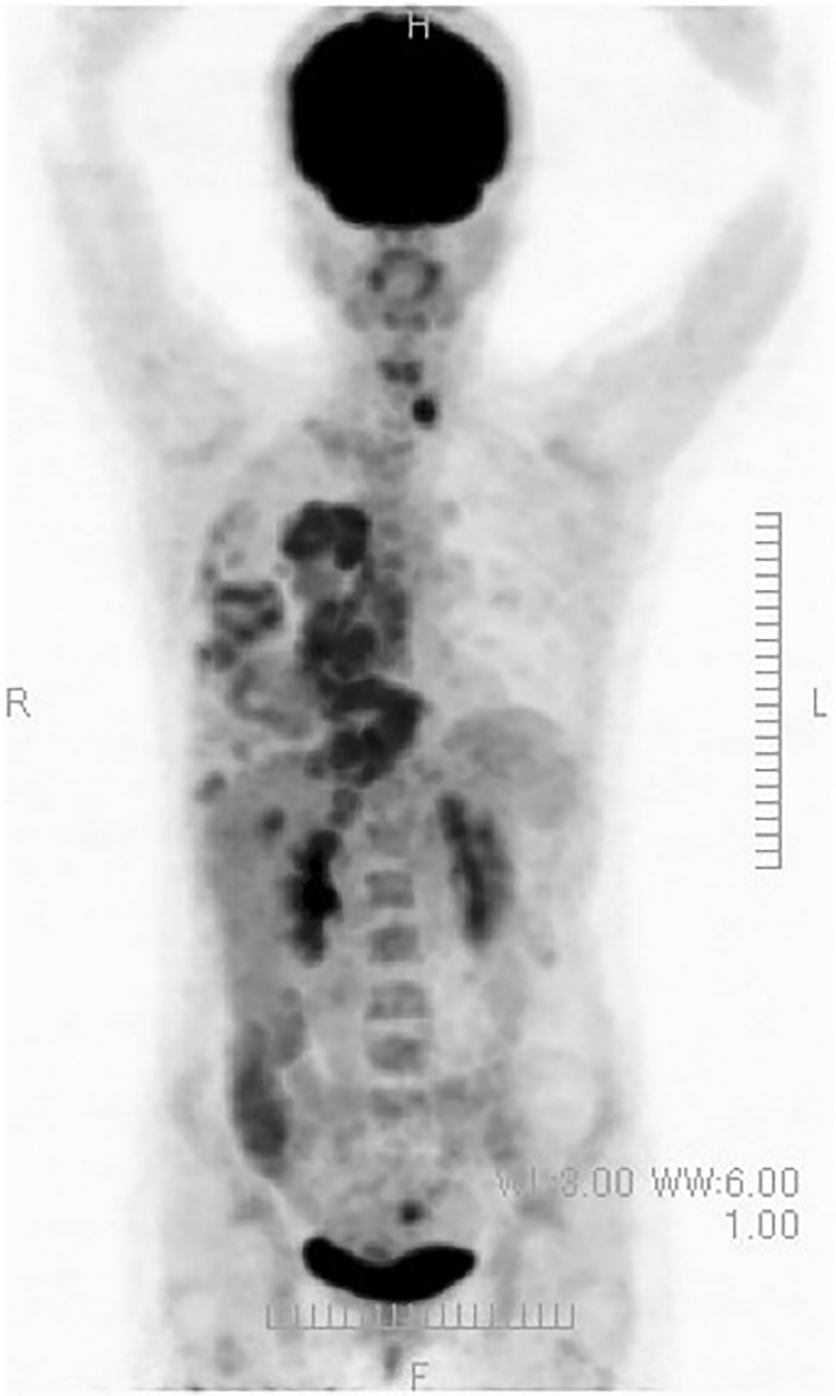

FIGURE 12 | A PET/CT scan shows abnormal accumulations on her chest.

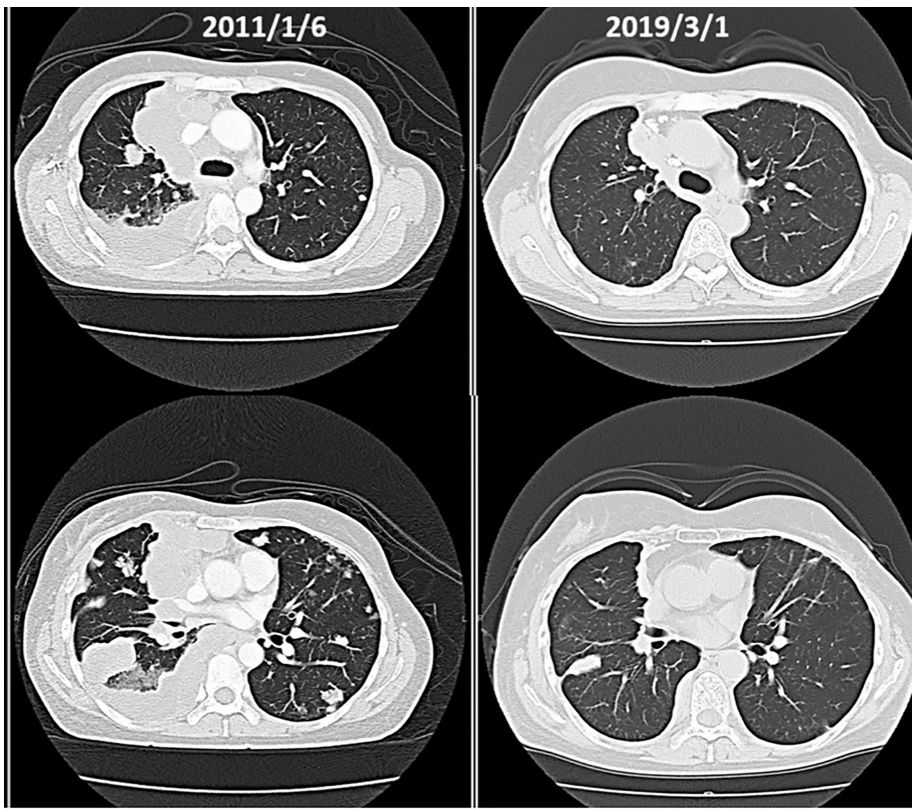

FIGURE 13 | CT scans reveals gradual shrinkage of the tumors.

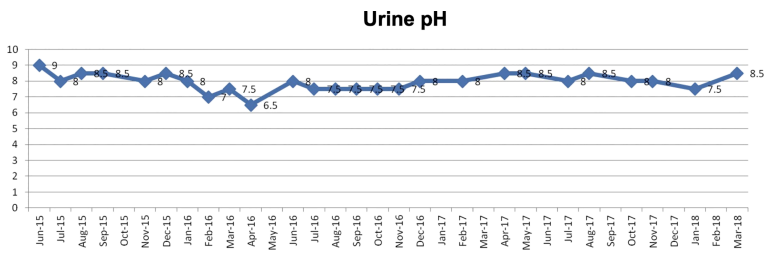

FIGURE 14 | Her urine pH levels are shown.

not shrink. After alkalizing diet, the cancer started to shrink (**Figure 12**, **13**). She has not received any anti-cancer drugs for more than 10 years. She drinks 2 liters of fruit and vegetable juice a day. In January 2022, when her urine is alkalized by diet alone (**Figure 14**), she is living her daily life in good health.

(xi) Female, 50 years old at the time of the first examination. Postoperative recurrence of breast cancer.

Breast cancer with axillary lymph nodes recurrence first visit of Wada Clinic on November 2016. She underwent breast cancer surgery in 2007 and had a recurrence in the right axillary lymph nodes (2014). We instructed her to eat an alkalizing diet, and had her take plume terpenes (Triterpenoids, Fatty acid synthase inhibitors), summer chrysanthemums (4) (Feverfew, NF kappa B inhibitors, parthenolide) and bicarbonate (3 g) (80–83). 85% regression was

observed in spring 2017, and 100% regression and tumor disappearance in spring 2018.

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 Japan-Multinational Trial Organization. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HW and RH performed the literature review and wrote the article. RN and HM performed the acquisition of data. All Authors conceived and designed the study and gave final approval for publication.

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Metabolic management of microenvironment acidity in glioblastoma

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Glioblastoma (GBM), similar to most cancers, is dependent on fermentation metabolism for the synthesis of biomass and energy (ATP) regardless of the cellular or genetic heterogeneity seen within the tumor. The transition from respiration to fermentation arises from the documented defects in the number, the structure, and the function of mitochondria and mitochondrial-associated membranes in GBM tissue. Glucose and glutamine are the major fermentable fuels that drive GBM growth. The major waste products of GBM cell fermentation (lactic acid, glutamic acid, and succinic acid) will acidify the microenvironment and are largely responsible for drug resistance, enhanced invasion, immunosuppression, and metastasis. Besides surgical debulking, therapies used for GBM management (radiation, chemotherapy, and steroids) enhance microenvironment acidification and, although often providing a time-limited disease control, will thus favor tumor recurrence and complications. The simultaneous restriction of glucose and glutamine, while elevating non-fermentable, anti-inflammatory ketone bodies, can help restore the pH balance of the microenvironment while, at the same time, providing a non-toxic therapeutic strategy for killing most of the neoplastic cells.

KEYWORDS

glutaminolysis, glycolysis, fermentation, succinate, lactate, glutamate, ketogenic diet, ketogenic metabolic therapy

Abbreviations: PPP, pentose phosphate pathway; OxPhos, oxidative phosphorylation; mSLP, mitochondrial substrate level phosphorylation; TMZ, temozolomide; D-β-OHB, D-β-hydroxybutyrate; KD, ketogenic diet; KMT, Ketogenic metabolic therapy; ROS, reactive oxygen species.

Introduction

Glioblastoma (GBM) has among the highest mortality rates for primary brain tumors and remains largely unmanageable. Despite the hype surrounding newer therapies, median life expectancy following GBM diagnosis is only about 11-15 months with some large patient data bases reporting few survivors beyond 30 months (1–7). The poor overall GBM patient survival is also astonishingly consistent across many surgical institutions Figure 1. Although remarkable advances in science and technology have occurred over the last 100 years in Western societies, no significant advances have been made over this same period in improving survival for GBM patients (2, 7, 8). This abysmal lack of therapeutic progress can be due in large part to the inability to recognize GBM as a metabolic disorder (7, 12). Acidification of the GBM microenvironment arises as a consequence of the fermentation metabolism within the neoplastic tumor cells and is largely responsible for therapy failure. This review provides the evidence supporting this statement.

Fermentation metabolism is responsible for GBM growth

GBM, like most major cancers, is dependent on fermentation metabolism for the synthesis of biomass and energy (ATP) regardless of the cellular or the genetic heterogeneity observed within the tumor (13, 14). A dependency on fermentation metabolism is the consequence of the well-documented abnormalities in the number, the structure, and the function of GBM mitochondria and mitochondrial associated membranes (MAM) and shown in Figure 2, and as described previously in detail (7, 14, 15, 17–25). In light of these structural and functional abnormalities, it would not be possible for GBM mitochondria to synthesize much if any ATP through OxPhos based on the foundational principle in evolutionary biology that structure determines function (14, 26, 27). The numerous reports suggesting that OxPhos is either normal or not seriously impaired in GBM cells is inconsistent with this foundational principle (28–40). It is important to recognize that oxygen consumption is not a reliable marker for OxPhos function in cancer cells (see below). It is unlikely that ATP synthesis through OxPhos could be normal in GBM cells that have documented abnormalities in mitochondria ultrastructure and function. Moreover, the large numbers of somatic mutations seen in GBM and in many other cancers, for that matter, arise as down-stream effects of OxPhos dysfunction with consequent ROS production (12). The somatic mutations in tumor cells will prevent adaptive versatility according to the evolutionary concepts of Darwin and Potts, thus locking in a dependency on fermentation metabolism for growth (41–44). It should be known, especially in the oncology field, that nothing in either general biology or in cancer biology can make sense except in the light of evolution (12, 45).

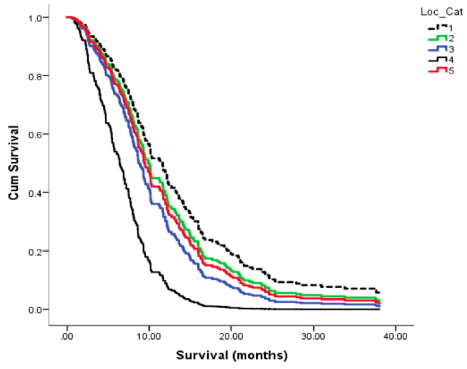

FIGURE 1
Kaplan-Meier plots for overall survival of GBM patients across five (1–5), Canadian surgical institutions. Each line represents patient survival for a particular institution as described (8). The GBM survival statistics recorded for these Canadian institutions are similar to those recorded in surgical institutions of other countries (2, 9, 10). These findings support the view of no major improvement of GBM patient survival in almost 100 years (8, 11). Image reproduced under a Creative Commons license from (8).

It is important to emphasize that a reduction in OxPhos of ~50% would dissipate the protonmotive force causing a reversal of the F_o-F_1 ATP synthase (7). The F_o-F_1 ATP synthase generally operates in forward mode (i.e., synthesizing ATP) only when the mitochondria are sufficiently polarized. The F_o-F_1 ATP synthase would be unable to generate ATP under a loss of electron transport chain operation on the order of 45-50% (7). This degree of loss would cause ATP hydrolysis, thus pumping protons out of the matrix. Reversal of the ATP synthase is what affords glutamine-driven mitochondrial substrate phosphorylation (mSLP) the critical role of providing ATP directly within the matrix when OxPhos becomes inhibited or impaired (7, 12). An inverse relationship between OxPhos efficiency and tumor aggression has been reported (46). A similar phenomenon has also been described with respect to the degree of fermentation and tumor growth, *i.e.*, the greater is the fermentation, the more aggressive is the cancer (14, 47–49). GBM cells, regardless of their cellular origin or genetic heterogeneity, are dependent on fermentation for survival due to abnormalities in mitochondrial structure and function.

A large part of the confusion on mitochondrial dysfunction in cancer comes from the incorrect assumption that oxygen consumption observed in cancer cells is linked to ATP synthesis through OxPhos (14, 28, 29, 40, 50–53). Many cancers, including GBM, can survive in hypoxia (0.1% oxygen) or in a solution of potassium cyanide, a Complex IV inhibitor, findings that would exclude normal OxPhos as a source of ATP synthesis (54–57). Cells with normal OxPhos function cannot survive for very long in cyanide or in hypoxia. While oxygen is necessary for cholesterol synthesis, GBM cells can obtain cholesterol from the

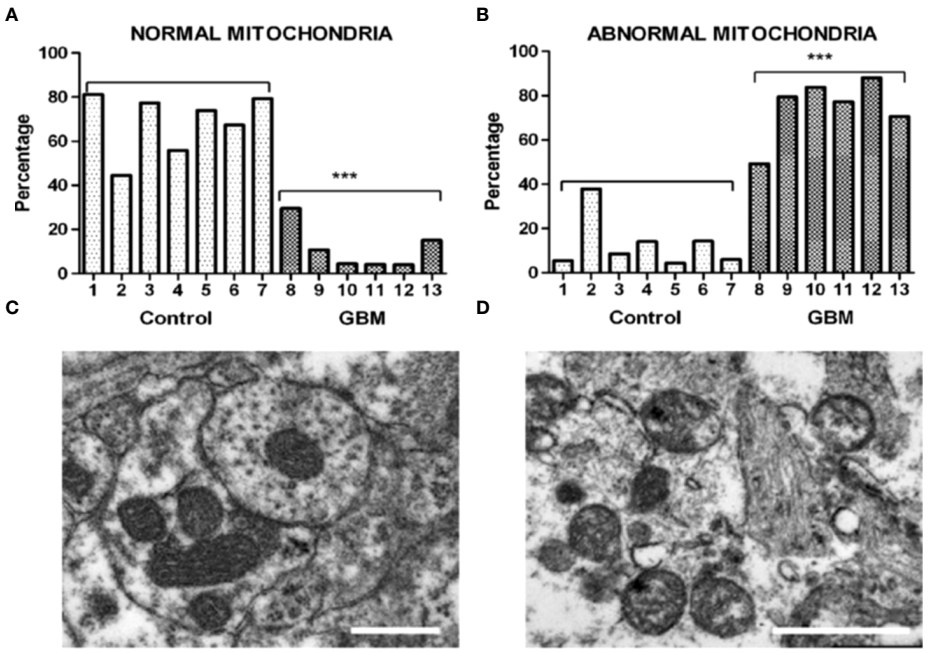

FIGURE 2
Morphological abnormalities seen in GBM mitochondria from the work of Deighton et al. (15). The morphology of 150 mitochondria was assessed in six GBM samples and in seven peri-tumoral control samples using Electron Microscopy (EM). **(A)** Percentage of normal mitochondria where cristae were visible throughout the mitochondria in peri-tumoral control and GBM samples (each bar represents one sample; *** p-value = 0.0001); **(B)** Percentage of abnormal mitochondria where cristae were sparse and abnormal in peri-tumoral control and GBM samples; *** p-value = 0.0001). **(C, D)** Representative EM images of normal and abnormal mitochondria, respectively. Cristolysis was significantly greater in mitochondria from GBM tissue than in mitochondria from normal surrounding brain tissue. The scale bars represent 0.5 um. The authors reported 117 mitochondrial proteins altered in GBM in association with ultrastructural mitochondrial abnormalities, similar to those described previously by Arismendi-Morillo et al. (16). ATP synthesis through OxPhos cannot be normal in tumor cells with these abnormalities. Image reproduced under a Creative Commons license from Deighton et al. (15).

microenvironment under hypoxic conditions (54, 58). Many normal cells and tumor cells will consume oxygen and ferment lactic acid when grown *in vitro*, but only tumor cells continue to ferment when grown *in vivo* (14, 47, 48).

The oxygen consumption in tumor cells is uncoupled and is used more for ROS production than for ATP synthesis through OxPhos (14, 23, 59–61). High-resolution oxygen consumption measurements and extracellular flux analysis, such as produced by Seahorse XF technology, cannot accurately measure OxPhos-driven ATP synthesis (53). Moreover, these measurements are highly variable in inter-laboratory settings (cell lines with exactly the same genetic background can display opposite metabolic profiles), are extrapolated using general, non-cancer specific ATP/O stoichiometries, and are limited by non-physiological and artefactual cell culture conditions (53, 62). It is not clear if most investigators using general purpose respirometry are aware of these facts.

Also contributing to misinformation on oxygen consumption and ATP synthesis is the failure to recognize glutamine-driven mSLP as a major source of energy for GBM cells (7, 14). Warburg was also unaware of this linkage, as he assumed that oxygen consumption was linked to OxPhos in his cancer cell preparations (7, 47, 48). Viewed collectively, these findings indicate that oxygen consumption alone cannot be used as a measure of OxPhos-derived ATP synthesis in most tumor cells including GBM.

Mitochondrial substrate level phosphorylation drives ATP synthesis and microenvironment acidification in GBM

Recent studies have described how mSLP at the succinyl CoA synthetase reaction in the glutaminolysis pathway can provision ATP synthesis in GBM (7, 14, 53, 63, 64). The glutamine nitrogen produced from glutaminolysis is essential for the synthesis of nucleotides and amino acids. The waste products of glutaminolysis (primarily glutamic acid and succinic acid) would also contribute to acidification of the GBM microenvironment (7,

14, 65, 66). The catabolism of glutamine towards succinate will generate CO_2 from the oxidative decarboxylation of the alpha-ketoglutarate dehydrogenation complex thus further acidifying the microenvironment. Additionally, succinate can stimulate NF-κB-driven inflammation and facilitate Hif-1α-driven glycolysis (7, 65, 67, 68). While the energetic competence of mitochondria in GBM and most other cancers is compromised in producing ATP through OxPhos, these mitochondria remain functional for other biosynthetic roles and in producing sufficient ATP through mSLP. Unlike OxPhos, however, that produces water and CO_2 as waste products, mSLP produces glutamic acid and succinate acid as waste products that contribute to microenvironment acidification.

Fermentation metabolites acidify the GBM microenvironment

The metabolic waste products of glucose and glutamine fermentation (lactic acid, glutamic acid, and succinic acid) will together acidify the GBM microenvironment. This acidification is ultimately responsible for drug resistance, enhanced invasion, immunosuppression, and metastasis (7, 14, 53, 69). Glucose carbons are essential for biomass synthesis through the glycolysis and the pentose phosphate pathways, with lactic acid and nucleic acid precursors produced as major end products (14, 70, 71). The pyruvate kinase M2 (PKM2) isoform, which is abundantly expressed in most malignant cancers, produces pyruvate-derived lactic acid with minimal ATP synthesis (14, 72–75). In other words, most of the glucose-derived lactic acid coming from the tumor cells is produced with little ATP synthesis through glycolysis. Some of the lactate acid produced in cancer cells can be returned to the tumor as glucose through the Cori cycle thus maintaining a constant supply of glucose to the tumor (76).

Calorie restriction, which lowers blood glucose and elevates blood D-β-OHB, reduces nuclear expression of phosphorylated NF-κB (p65), cytosolic expression of phosphorylated IκB, total IκB, and DNA promoter binding activity of activated NF-κB in the CT-2A astrocytoma (77). NF-κB is a major driver of inflammation in the GBM microenvironment. Figure 3A shows how the waste products of glucose and glutamine fermentation are largely responsible for the inflammation and acidification in the GBM microenvironment. Hence, therapies that can lower blood glucose while elevating D-β-OHB will mitigate microenvironment inflammation and acidification through multiple mechanisms.

Current therapies could enhance microenvironment acidity and recurrence of GBM

The current treatment for GBM management involves debulking surgery, radiotherapy, and temozolomide chemotherapy (TMZ) (1, 8, 9, 87). While the waste products of glucose and glutamine fermentation will contribute to microenvironment acidification and the rapid growth of untreated GBM, the current treatments used for GBM management could also accelerate these processes after a growth delay following surgical debulking (1, 88, 89). It is documented that radiotherapy produces significant necrosis and hypoxia in the tumor microenvironment (1, 90–92). Radiotherapy disrupts the tightly regulated glutamine-glutamate cycle in the neural parenchyma thus increasing the levels of glutamine and glutamic acid as described further in Figure 3A.

Glutamic acid is an excitotoxic amino acid that enhances GBM invasion (1, 80, 81, 86, 93–96). Radiotherapy also damages the brain microenvironment, which increases glucose and glutamine availability to the tumor cells thus driving tumor growth through hyperglycolysis, necrosis, and acidification. While chemo-radiotherapy might have a role in the treatment of low-grade non-neural tumors, these confounding variables are ultimately responsible for GBM therapy resistance (1, 90, 97–99).

Blood glucose is linked to rapid GBM growth

Linear regression analysis showed that blood glucose could predict the growth rate of the CT-2A malignant mouse astrocytoma, a stem-cell tumor (100, 101) (Figures 4A–C). Evidence also shows that survival is lower in GBM patients with higher blood glucose levels than in GBM patients with lower glucose levels (1, 103–111). Although the dexamethasone steroid is often prescribed along with standard treatments to reduce vasogenic edema, steroids will elevate blood glucose levels thus contributing indirectly to tumor growth (1, 112–114). Alternatives to dexamethasone for reducing vasogenic edema should receive consideration (115). Radiotherapy also increases blood glucose levels and facilitates hybridizations between tumor cells and macrophage/microglia thus producing highly invasive metastatic cells (1, 82, 108, 116–118). As glucose-derived lactic acid is the end product of glycolysis, GBM treatments that would elevate blood glucose levels will contribute to elevated lactic acid, microenvironment acidification, and tumor recurrence. Conversely, therapeutic strategies that would reduce glucose levels will lower lactic acid production, microenvironment acidification, and tumor recurrence (Figure 3B). It is clear from Figure 5 that calorie restriction, which lowers blood glucose while elevating ketone bodies, reduces microvessel density (angiogenesis) and increases tumor cell apoptosis in the CT-2A malignant astrocytoma. Hence, the dietary restriction of blood glucose can reduce microenvironment acidification through therapeutic effects on inflammation, angiogenesis and apoptosis.

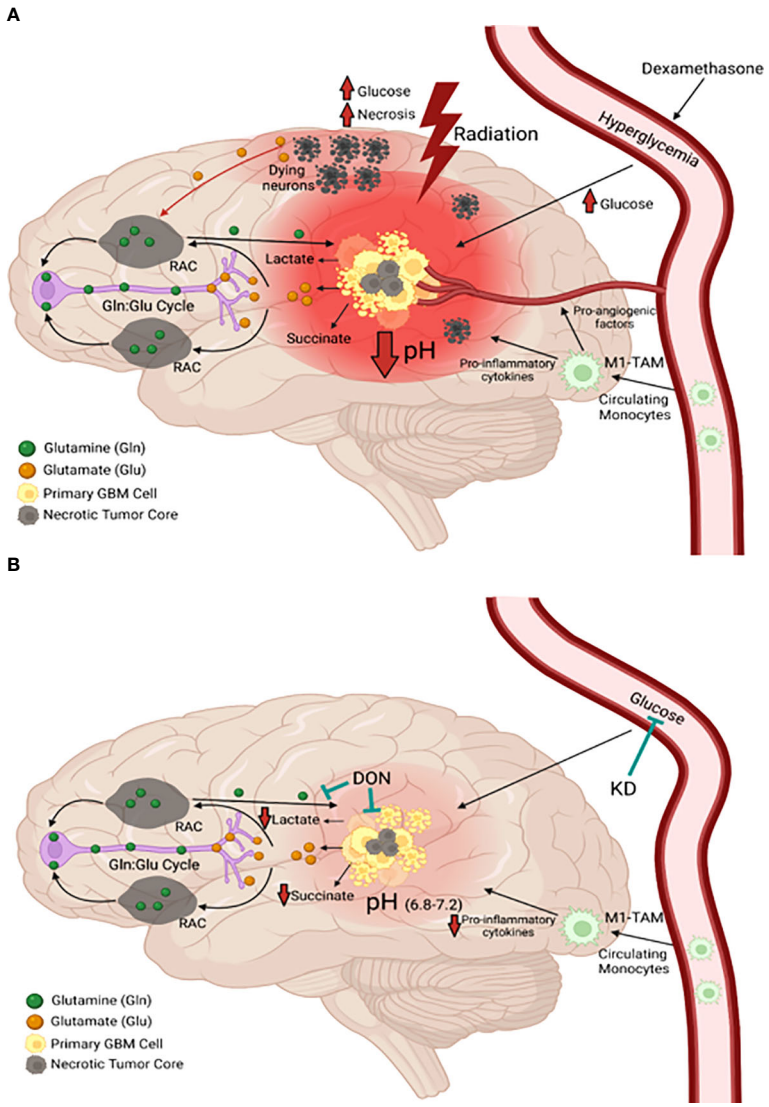

FIGURE 3
Origin and management of microenvironment acidity in GBM. **(A)** Glucose and glutamine are the primary energy metabolites necessary for driving rapid GBM growth. Glucose is the metabolic fuel necessary for nearly all brain functions under normal physiological conditions and is the major source of carbons for biomass synthesis through the glycolytic and pentose phosphate pathways in tumor cells. Tumor cells metabolize glutamine to glutamate, which is then metabolized to alpha-ketoglutarate. Significant energy is generated from the succinyl CoA ligase reaction (substrate level phosphorylation) in the glutaminolysis pathway using alpha-ketoglutarate-derived succinyl CoA as substrate (see Figure 7). In contrast to extracranial tissues, where glutamine is the most available amino acid, glutamine is tightly regulated in the brain through its involvement in the glutamate-glutamine cycle of neurotransmission (1, 78, 79). Glutamate is a major excitatory neurotransmitter that must be cleared rapidly following synaptic release in order to prevent excitotoxic damage to neurons (1, 79–81). Glial cells possess transporters for the clearance of extracellular glutamate, which is then metabolized to glutamine for delivery back to neurons. Neurons metabolize the glutamine to glutamate, which is then repackaged into synaptic vesicles for synaptic release (1). This cycle maintains low extracellular levels of both glutamate and glutamine in the normal neural parenchyma. Disruption of the cycle can provide neoplastic GBM cells access to glutamine. Besides serving as a metabolic fuel for the neoplastic tumor cells, glutamine is also an important fuel for cells of myeloid linage, which include macrophages, monocytes, microglia, and especially the invasive mesenchymal cells in GBM (1, 13, 82–84). In contrast to the proliferative GBM stem cells, the neoplastic GBM mesenchymal cells are thought to be derived from microglia or from microglia-stem cell fusion hybrids, which would have immuno-suppressive properties (82, 85). As long as GBM cells have access to glucose and glutamine, the tumor will grow and acidify the microenvironment making long-term management difficult. The current treatments for GBM (radiation and TMZ chemotherapy) will further increase glucose and glutamine availability, creating an unnecessary metabolic storm that will enhance microenvironment acidification and rapid tumor recurrence. The red hue is indicative of the inflammation and acidification of the tumor microenvironment (see text for further details). **(B)** The simultaneous restriction of glucose and glutamine, while elevating non-fermentable, anti-inflammatory ketone bodies, will reduce acidification, restore the pH balance of the microenvironment, and growth arrest or kill most of the neoplastic cells (11–13). RAC, reactive astrocytes; TAM, tumor-associated macrophages; Gln, glutamine; Glu, glutamate. These images were modified from that in (86).

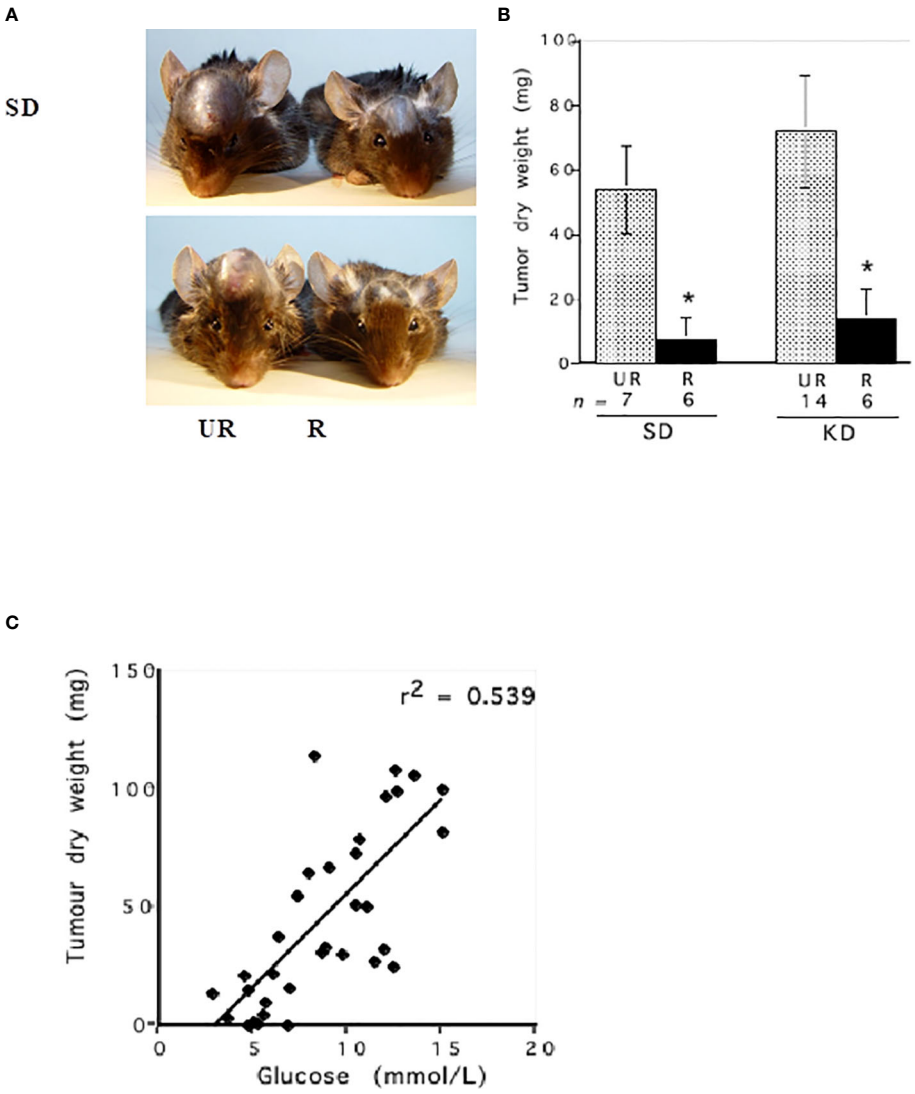

FIGURE 4
Influence of diet on the intracerebral growth of the CT-2A malignant astrocytoma. Dietary treatment was initiated 1 day after tumor implantation and was continued for 13 days. The visual representation **(A)** and quantitative assessment **(B)** of tumor growth in C57BL/6J mice receiving either the standard diet (SD) or ketogenic diet (KD) under either unrestricted (UR) or restricted (R) feeding. The asterisk indicates that the dry weights of the tumours in R groups were significantly lower than those in the UR groups at $P < 0.01$. **(C)** Linear regression analysis of plasma glucose and CT-2A-tumor growth in mice from both the SD and KD dietary groups combined (n = 34). These analyses included the values for plasma glucose and tumor growth of individual mice from both the UR and R-fed groups. The linear regression was highly significant at $P < 0.001$, indicating that blood glucose levels predict CT-2A tumor growth rate (100). The failure of the KD-UR to reduce blood glucose levels and tumor growth could be due to insulin insensitivity in this mouse strain (102). Images reproduced under a Creative Commons license.

Mesenchymal cells will contribute to GBM acidification

Accumulating evidence shows that the highly invasive mesenchymal cells seen in GBM are derived from neoplastic microglia or from microglia/macrophages that hybridize with non-invasive cancer stem cells, similar to that reported for other highly invasive metastatic cancers (82, 118, 120–124). Indeed, up to 60% of the cells in some GBM contain macrophage characteristics (125–128). We described how the neoplastic GBM cells with mesenchymal characteristics can be derived from transformed macrophages/microglia (13, 82, 129–131). As activated macrophages are immunosuppressive and acidify the microenvironment, it should be no surprise why immunotherapies have been largely ineffective in managing GBM (1, 132–134). The mesenchymal cells seen in GBM, whether part of the neoplastic cell population or part of the infiltrating cell population, will acidify the microenvironment

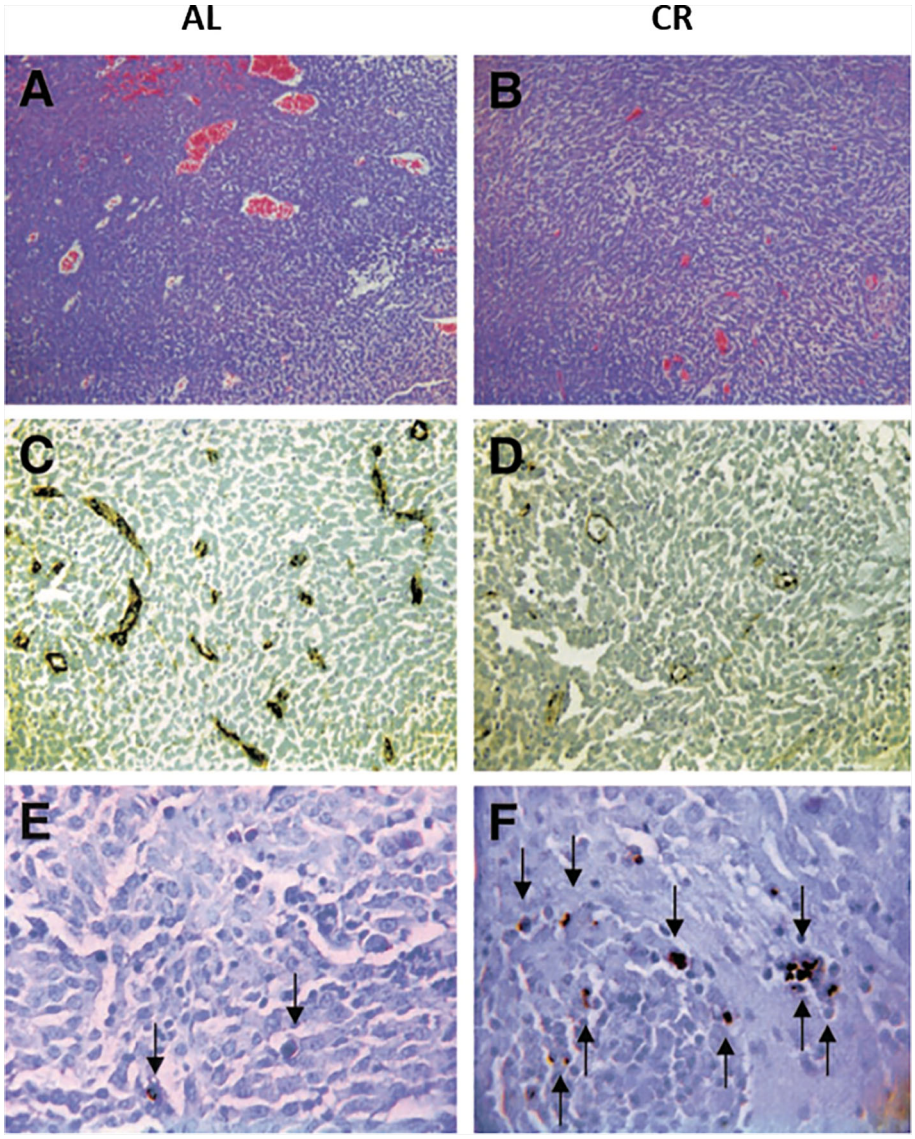

FIGURE 5
Influence of calorie restriction (CR) on microvessel density and apoptosis in the CT-2A malignant astrocytoma. CR was initiated 7 days before intracerebral tumor implantation and was continued for 11 days. H & E stained tumor sections in an *ad libitum* (AL) mouse **(A)** and in a CR mouse **(B)** (100X). Factor VIII immunostaining from the tumor grown in an AL mouse **(C)** and in a CR mouse **(D)** (200X). TUNEL positive apoptotic cells (arrows) from the tumor grown in an AL mouse **(E)** and in a CR mouse **(F)** (400X). Each stained section was representative of the entire tumor. All images were produced from digital photography. Image reproduced under a Creative Commons license from (119).

through a variety of inflammation-linked mechanisms (135, 136). Some researchers also consider tumor cell-derived lactate as a checkpoint due to its ability to block immunotherapies (69). As lactate is derived from glucose, glucose restriction should reduce this "so called" checkpoint inhibitor. Hence, the mesenchymal cell populations in GBM will not only contribute to microenvironment acidification, but will also contribute to their own survival using glutamine as a metabolic fuel (13, 137, 138).

Can metabolic therapy improve immunotherapy?

Immunotherapies have not yet been effective GBM management, but could be effective if there is evidence showing that they will not increase availability of glucose and glutamine in the tumor microenvironment, enhance inflammation, or cause hyper-progressive disease, as was documented in non-small cell lung cancer (139). Inflammatory oncotaxis, arising from surgical resection or

from biopsy of lower-grade brain tumors, could also contribute to the transformation to high-grade secondary GBM (140–143). As the neoplastic macrophage/mesenchymal cells seen in GBM are dependent to a large degree on glutamine (13, 144), glutamine restriction will be essential for targeting these cells as we recently demonstrated (13). Recent studies show that a ketogenic diet can enhance the efficacy of immunotherapy (145). Most importantly, the simultaneous restriction of glucose and glutamine could improve the therapeutic efficacy of immunotherapies.

GBM chemotherapy can contribute to microenvironment acidification

TMZ chemotherapy can contribute to microenvironment acidification through adverse effects on mitochondrial OxPhos function and increased production of GBM driver mutations (1, 9, 146, 147). In addition to increasing blood glucose levels, dexamethasone also increases glutamine levels through its induction of glutamine synthetase activity (7, 11, 86, 113, 148, 149). Bevacizumab (Avastin) is also widely prescribed to GBM patients to reduce angiogenesis (150–152). Bevacizumab, however, increases tumor necrosis while selecting for the most invasive and therapy-resistant tumor cells (153, 154). As both bevacizumab and TMZ damage mitochondria (155), these drugs will contribute further to tumor cell reliance on fermentation metabolism thus increasing microenvironment acidification (7, 11). Considered together, the current GBM chemotherapies inflict damage to the microenvironment and facilitate availability of glucose and glutamine to the neoplastic tumor cells, all of which will contribute to tumor recurrence, further acidification, and rapid progression (Figure 3A). It is not likely that overall patient survival could be improved when using therapies that increase distal tumor cell invasion and microenvironment acidification.

It should also be recognized that human cytomegalovirus (HCMV) infects many GBM that would further facilitate tumor cell use of glutamine and glucose (1, 156, 157). Recent studies show that vaccine-targeting of the HCMV pp65 protein could increase progression free and overall survival of some GBM patients (158). It would be interesting to determine if this therapeutic effect resulted in part from inhibition of the glycolysis or the glutaminolysis pathways in GBM cells (159, 160). Glucose and glutamine are required for synthesis of glutathione while glutamine is essential for the action of manganese superoxide dismutase (161–163). Consequently, the elevated use of glucose and glutamine, which increases anti-oxidant potential, will contribute to the resistance of GBM cells to chemotherapy and radiotherapy.

It is known that elevated aerobic fermentation (Warburg effect) also drives the multidrug resistant (MDR) phenotype, which protects GBM cells from toxic chemotherapy (1, 5, 41, 164). Hence, the treatment-linked increases in fermentable energy metabolites and disruption of the tumor microenvironment can explain in large part how overall survival remains so poor for most GBM patients treated with current standard of care (7, 97). The information presented in Figure 3A describes how current therapies can facilitate rapid GBM recurrence. It is our view that these therapies can account in large part the remarkable reproducibility of poor patient survival across multiple surgical institutions as seen in Figure 1. It is unlikely that GBM patient survival will improve significantly if therapies that increase microenvironment acidification and are inherently ineffective are continuously used (165).

Ketone bodies are non-fermentable and can reduce GBM acidification

As fermentation metabolism is ultimately responsible for rapid GBM growth and the acidification of the microenvironment, then therapies that target fermentation metabolism should reduce acidification and GBM growth. Metabolic therapy involves diet/drug combinations that target the availability of glucose and glutamine while also elevating non-fermentable, anti-inflammatory ketone bodies (13, 41, 166–170). Most importantly, GBM and other tumors cannot use ketone bodies for energy due to deficiencies in SCOT; the key mitochondrial enzyme needed for ketone body metabolism (171–173). No evidence has been presented, to our knowledge, showing that ketone bodies can replace glucose or glutamine in serum free media for the survival of any tumor cell. Ketogenic diets and water-only therapeutic fasting will lower circulating glucose levels while elevating circulating D-β-hydroxybutyrate (D-β-OHB) levels (41, 174–176). Water-only fasting in humans is comparable to a 40% calorie restriction in mice due to differences in basal metabolic rate that about six times faster in mice than in humans (177). Therapeutic strategies that lower blood glucose while elevating blood ketone bodies are anti-angiogenic, anti-edematous, anti-inflammatory, and pro-apoptotic. Evidence supporting this statement was described previously (13, 178). Diets that lower glucose and elevate D-β-OHB can also reduce circulating levels of insulin-like growth factor 1 (IGF-1), a known driver of tumor growth (Table 1). There is no known drug that can produce the broad range of therapeutic effects as can diets that reduce glucose while elevating D-β-OHB.

It is important to mention that blood glucose can be reduced to very low levels (less than 1.0 mM) in humans that are in therapeutic ketosis (6-8 mM, D-β-OHB) without producing hypoglycemic reactions (174, 179, 180). A whole-body transition from glucose-driven metabolism to D-β-OHB-driven metabolism will reduce circulating glucose levels thus reducing extracellular acidification from lactic acid production. At the same time, this transition will also produce metabolic stress on all neoplastic GBM cells that are dependent on glycolysis for growth (41, 172, 174, 181). Moreover, D-β-OHB metabolism enhances the $\Delta G'ATP$ hydrolysis in normal cells from -56 kJ/mole to -59 kJ/mole, thus providing normal cells with an energetic advantage over the fermentation-dependent tumor cells (41). We are not familiar with any therapies, besides ketogenic

TABLE 1 Influence of diet on plasma glucose, β-OHB, and IGF-I levels in mice bearing the CT-2A intracerebral brain tumour[a].

Diet[b]	Groups[c]	Glucose (mmol l^{-1})	β-OHB (mmol l^{-1})	IGF-I (ng ml l^{-1})
SD	UR	9.1 ± 0.9	0.6 ± 0.1	208 ± 25
	R	(7)[d]5.2 ± 1.1*	(7)1.4 ± 0.2*	(6)117 ± 36*
		(6)	(6)	(6)
KD	UR	11.4 ± 1.4	1.0 ± 0 .3	294 ± 30
		(14)	(14)	(5)
	R	5.7 ± 1.5*	1.3 ± 0.6	193 ± 57*
		(6)	(6)	(6)

[a]Values are expressed as means ± 95% confidence intervals. [b]Animals were fed either a standard chow diet (SD) or a ketogenic diet (KD). [c]UR (unrestricted feeding) and R (restricted to 60% of the SD-UR group. [d]Numbers in parentheses indicate the number of independent tumor-bearing mice examined in each group. The asterisks indicate that the values of the R groups differed from those of their respective UR groups at $P < 0.01$. The details of these experiments are as we described (100).

metabolic therapy (KMT), that can enhance the energetic advantage of normal cells over that of tumor cells (11, 41).

The energetic advantage of D–β-OHB metabolism in normal cells is seen predominantly with D-β-OHB, and is not seen with either the D/L- β-OHB racemic mixture or with fatty acids (174, 182, 183). On the other hand, racemic D/L-β-OHB tends to reduce blood glucose more through shifting redox state in the liver and can potentially increase ROS production in tumor cells through β-oxidation of the L-form (41). The L-β-OHB interconverts back to D-β-OHB (in tissues) through a racemase enzyme or gets converted to acetyl-CoA. The L-β-OHB also has greater potential as a signaling molecule since it remains in circulation longer and has similar effects at suppressing the NLRP3 inflammasome and epigenetic effects (184–186). Hence, D-β-OHB and D/L-β-OHB can stress tumor cell metabolism while enhancing the metabolism of normal cells through a variety of mechanisms.

The therapeutic effects seen with ketone bodies are generally best when blood glucose levels are low (generally below 3.6 mM), as little or no therapeutic benefit is seen in either preclinical GBM models or in human patients when glucose levels remain elevated (100, 171, 187). These therapeutic glucose levels could be difficult to achieve for many GBM patients, however, due to the glucose-elevating effects of the current standard treatments used to manage GBM. We also did not find any therapeutic benefit of sodium bicarbonate on the growth of the VM-M3 mouse glioblastoma suggesting that alkalinization using sodium bicarbonate was ineffective in managing this GBM model (L. Shelton, unpublished). It is the synergistic action of low blood glucose with elevated ketone bodies that provides the best therapeutic strategy for slowing growth and reducing microenvironment inflammation and acidification.

The simultaneous restriction of glutamine and glucose will reduce GBM growth and acidification

In addition to glucose, glutamine is the other major fuel that drives GBM growth especially the neoplastic mesenchymal cells (14, 137, 144). We showed that the glutamine-targeting analogue, 6-diazo-5-oxo-L-norleucine (DON), used with a calorie restricted ketogenic diet could significantly reduce growth and improve overall survival in preclinical models of GBM (Figure 6).

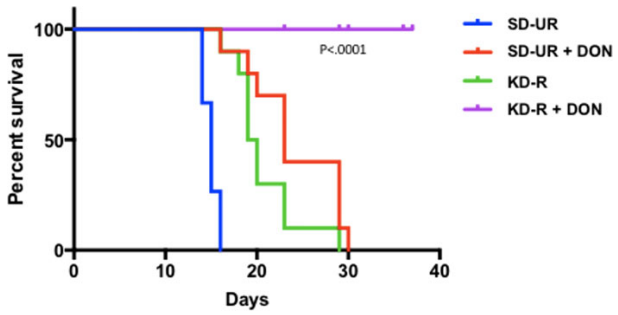

FIGURE 6
Influence of diet/drug therapy on overall survival of VM/Dk mice with the VM-M3 invasive GBM. A calorie restricted ketogenic diet (KD-R) was administered together with the glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine (DON) as we described (13). Overall survival was significantly longer in the tumor bearing mice receiving the diet/drug combination (KD-R + DON) than in the mice receiving the standard high-carbohydrate diet (SD-UR), the KD-R alone, or DON alone. It is important to mention that 2-3x more DON was delivered to the tumor of the mice fed the KD-R than to the mice fed the SD-UR indicating that the KD facilitates a non-toxic delivery of small drug molecules through the blood brain barrier (13, 188). Image reproduced under a Creative Commons license from (13).

Moreover, we found that ketogenic diets facilitated delivery of DON and other small molecules through the blood brain barrier (13, 188). This delivery may be due in part to the action of the content of caprylic acid in the diet (189). Hyperbaric oxygen therapy can also reduce angiogenesis and microenvironment inflammation especially in combination with therapeutic ketosis (41, 190–193). In addition to findings in preclinical models, we also described how the *IDH1* mutation could act as a therapeutic drug that simultaneous targets the glycolysis and glutaminolysis pathways to improve survival in a GBM patient (11) (Figure 7). The long term survival of this patient, now at eight years, was attributed to a combination of his younger age, his low-carbohydrate ketogenic diet, his acquired *IDH1* mutation, and finally to his avoidance of radiation, TMZ, and steroids (11). Ketogenic metabolic therapy involves the synergistic therapeutic action of the KD used with drugs and procedures that restrict availability of glucose and glutamine while providing normal cells with an energetic advantage over tumor cells that are limited to energy generation through fermentation (12, 41). More recent studies also support some of these observations in younger GBM patients (199). Persistent statements suggesting that tumor cells have a growth advantage over normal cells make no sense in the light of

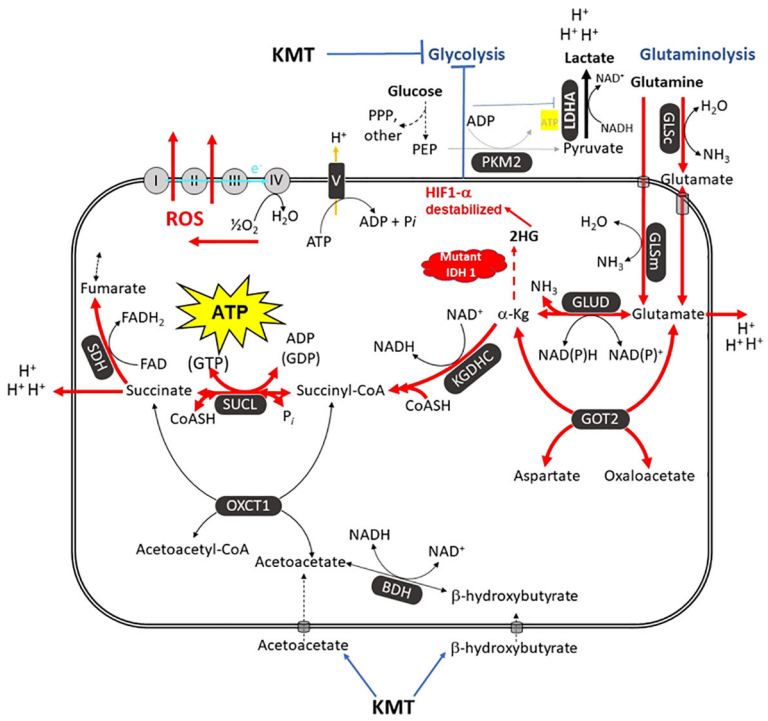

FIGURE 7
The metabolic pathways responsible for the acidification of the GBM microenvironment. GBM growth is dependent on glucose carbons for biomass synthesis through glycolysis and glutamine carbons for ATP synthesis through glutaminolysis. The glutamine nitrogen is necessary for protein and nucleic acid synthesis. The waste products of the glycolytic and the glutaminolysis pathways (lactic acid, glutamic acid, and succinic acid) will acidify the GBM microenvironment. The oxygen consumption is linked to ROS production, not to ATP synthesis. Excessive ROS produce somatic mutations and further increase inflammation and acidification of the microenvironment (7, 12, 14, 194). A calorie restricted KD will reduce glucose availability for glycolysis while also interfering with the glutaminolysis pathway (11). Glutamine-driven mSLP in the glutaminolysis pathway is a major source of ATP synthesis for GBM cells (7, 14). The glutaminolysis pathway (red) becomes dominant in tumor cells with inefficient OxPhos and that express the dimeric PKM2 isoform. PKM2 is expressed in GBM and produces less ATP through glycolysis than does the PKM1 isoform (73, 75, 195, 196). The elevation of ketone bodies (D-β-hydroxybutyrate and acetoacetate) through KD will indirectly reduce ATP synthesis through the succinate CoA ligase (SUCL) reaction by diverting CoA from succinate to acetoacetate. The *IDH1* mutation could reduce ATP synthesis through mSLP by increasing synthesis of 2-hydroxyglutarate that is derived from α-ketoglutarate and thus reduce the succinyl CoA substrate for the SUCL reaction (11, 14, 197). Besides its potential effect in reducing glutaminolysis, 2-hydroxyglutarate can also target multiple HIF1α-responsive genes and enzymes in the glycolysis pathway thus limiting synthesis of metabolites and one-carbon metabolism needed for rapid tumor growth (7, 14, 68, 198). The down regulation of Hif1-α-regulated lactate dehydrogenase A (LDHA), through the action of both the KD and the *IDH1* mutation, will reduce extracellular lactate levels thus further reducing microenvironment inflammation, acidification, and tumor cell invasion. Hence, the simultaneous inhibition of glycolysis and glutaminolysis through the synergistic effects KMT and the *IDH1* mutation will reduce the majority of signaling pathways necessary for rapid GBM growth and acidification of the microenvironment. BDH, β-hydroxybutyrate dehydrogenase; FAD, flavin adenine dinucleotide; GLSc, glutaminase cytosolic; GLSm, glutaminase mitochondrial; GLUD, glutamate dehydrogenase; GOT2, aspartate aminotransferase; KGDHC, α-ketoglutarate dehydrogenase complex; LDHA, lactate dehydrogenase A; NME, nucleoside diphosphate kinase; OXCT1, succinyl-CoA:3-ketoacid coenzyme A transferase 1; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PKM2, pyruvate kinase M2; SDH, succinate dehydrogenase; SUCL, succinate-CoA ligase. **KMT,** Ketogenic metabolic therapy. Reprinted with modifications from (14).

evolutionary theory (12). The metabolic pathways contributing to GBM microenvironment acidification and their management by KMT and the *IDH1* mutation are described in Figure 7.

Limitations

There are several limitations that currently prevent the application of metabolic therapy for reducing microenvironment acidification and the growth of GBM. First, the dosage, timing, and scheduling of the diet/drug combinations that can best target glucose and glutamine availability have yet to be optimized for most GBM patients (1, 11, 41, 87). Second, the findings that GBM is largely dependent on glucose and glutamine fermentation for growth due to OxPhos deficiency is inconsistent with the current dogmatic view that GBM and most other cancers are exceedingly complex genetic diseases requiring complicated Rube Goldberg-type solutions (12). Finally, the most important limitation for adapting metabolic therapy in the clinic is the absence of a business model that can generate sufficient replacement revenue using cost-effective, non-toxic metabolic therapies (200–203). We predict that major advances in overall GBM patient survival will be realized once GBM becomes recognized as a mitochondrial metabolic disease and when non-toxic metabolic therapies become the standard of care for management.

Conclusions

Microenvironment acidification is largely responsible for drug resistance, enhanced invasion, immunosuppression, and metastasis. The acidic waste products of glucose and glutamine fermentation metabolism (lactic acid, glutamic acid, and succinic acid), generated within the neoplastic tumor cells, are responsible for the acidification of the GBM microenvironment. Stated simply: The greater is the availability of fermentable fuels, the greater is the resistance to therapy. The cancer microenvironment will heal itself if the origin of the acidification can be removed. Therapeutic strategies that restrict the availability of fermentable fuels, while increasing levels of non-fermentable ketone bodies, will reduce acidification, eliminate the majority of neoplastic tumor cells, and thus improve GBM management.

Author contributions

All authors read and approved the information presented in the manuscript. ST wrote the manuscript. A-MG, ZG, LD, DT, EA, MJ, MP, TL, D'AD, KM, and CC made material contributions to content and revisions.

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Conflict of interest

Author KM was employed by the company BERG LLC. Author SL was employed by the company Matterworks. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as the potential conflict of interest.

Publisher's note

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Drosophila as a toolkit to tackle cancer and its metabolism

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Cancer is one of the most severe health problems worldwide accounting for the second leading cause of death. Studies have indicated that cancers utilize different metabolic systems as compared with normal cells to produce extra energy and substances required for their survival, which contributes to tumor formation and progression. Recently, the fruit fly *Drosophila* has been attracting significant attention as a whole-body model for elucidating the cancer mechanisms including metabolism. This tiny organism offers a valuable toolkit with various advantages such as high genetic conservation and similar drug response to mammals. In this review, we introduce flies modeling for cancer patient genotypes which have pinpointed novel therapeutic targets and drug candidates in the salivary gland, thyroid, colon, lung, and brain. Furthermore, we introduce fly models for metabolic diseases such as diabetes mellitus, obesity, and cachexia. Diabetes mellitus and obesity are widely acknowledged risk factors for cancer, while cachexia is a cancer-related metabolic condition. In addition, we specifically focus on two cancer metabolic alterations: the Warburg effect and redox metabolism. Indeed, flies proved useful to reveal the relationship between these metabolic changes and cancer. Such accumulating achievements indicate that *Drosophila* offers an efficient platform to clarify the mechanisms of cancer as a systemic disease.

KEYWORDS

cancer, *Drosophila*, genetics, metabolic reprogramming, drug discovery

Introduction

Cancer ranks the second leading cause of death worldwide, and its disease burden continues to increase yearly (1). This malignant disease involves genetic alterations which induce various cancer hallmarks such as sustaining cell proliferation and invasion to promote cellular transformation (2). To date, preclinical studies have typically used cultured cells and mouse models to elucidate the cancer mechanisms and to identify numerous therapeutic agents. However, developing novel therapeutics still faces many

challenges including low success rates in clinical trials, the high toxicity of therapeutic candidates and even approved drugs, and emerging resistance in patients (3). These challenges imply that it is inevitable to introduce additional approaches to complement the current efforts to clear the hurdles efficiently.

Here, we will introduce the fruit fly *Drosophila* as one of the ideal whole-body models to this end. *Drosophila* has a high rate of reproduction and low husbandry cost in laboratories. In addition, *Drosophila* is well-characterized for its genome with over 70% of disease-associated genes in humans (4, 5). Furthermore, flies show structural and physiological conservations in tissues/organs with mammals such as the brain, lung, heart, liver/adipose tissue, pancreatic islets, colon, and urinary system (Figure 1A). These similarities provide a powerful advantage in elucidating the mechanisms of tumorigenesis in specific organs. In light of modeling cancer genotype, flies offer a robust genetic toolkit to achieve precise genetic manipulation, which makes them a useful model organism in studies on cancer as a genetic disease. Indeed, there have been multiple fly models emerging, with single or multiple driver mutations to mimic cancer genotypes in patients (6). These models have allowed exploring the roles of such abnormalities in carcinogenesis and developing anti-cancer leads (Figure 1B) (6).

In addition to elucidating the mechanisms of cancer and to developing novel therapeutic strategies, flies also have contributed to delineating unique metabolic networks within cancer cells. Previous studies have demonstrated that tumor-associated metabolic reprogramming led by oncogenic mutations plays an important role in driving sustained cancer cell proliferation hence accelerating malignant progression (7). One of the first discoveries of such metabolic shift is aerobic glycolysis known as the Warburg effect, the vigorous glucose uptake to fuel glycolysis and secretion of lactate by cancer cells even in the presence of oxygen. Cancer cells intake extra glucose as compared with normal cells to produce extra energy and substances required for their survival (7, 8).

Meanwhile, the overproduction of reactive oxygen species (ROS) by dysfunctional mitochondria is another significant metabolic alteration in cancer cells attracting much attention these years. Excessive amounts of ROS cause cytotoxicity by inducing intracellular oxidative stress, which accumulates over time and ultimately leads to cell death (9). However, cancer cells

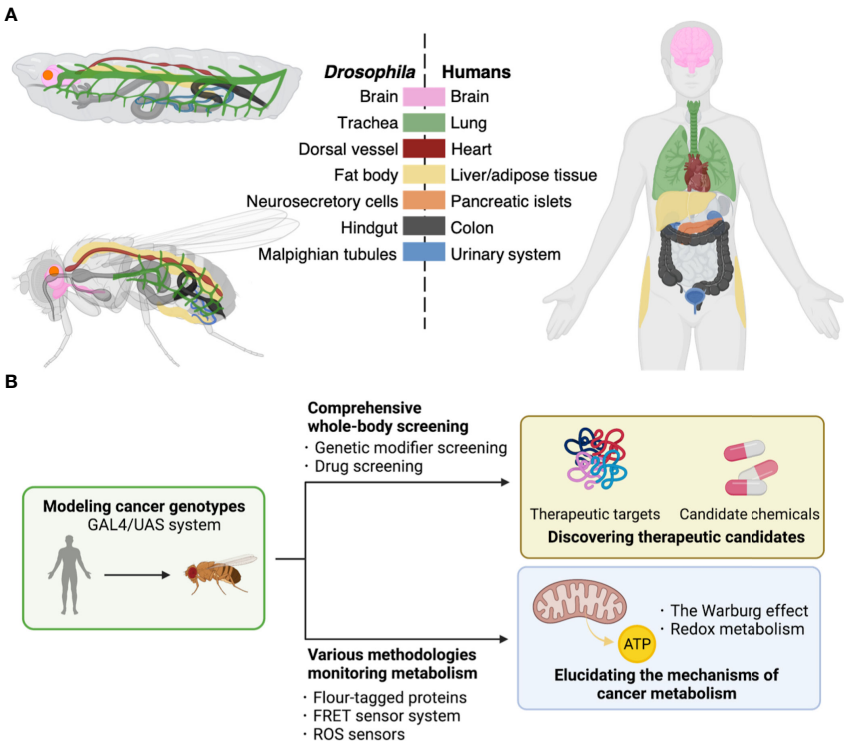

FIGURE 1
Drosophila platforms to study cancer and its metabolism. **(A)**, Corresponding tissues/organs regarding their structures and functions between *Drosophila* and humans. Each color indicates tissues/organs with similar functions among *Drosophila* larva (top upper left) and adult (bottom left), and human (right). Such similarities among metabolic pathways and physiological responses allow construction of fly models for human diseases of both cancers and metabolic disorders. **(B)**, The GAL4/UAS system enables induction of genes of interest in target fly tissues. These flies have allowed discovery of therapeutic targets including kinases and development of potent compounds for cancer treatment by comprehensive screenings (top). Furthermore, flies offer a useful toolkit including reporter lines to study cancer metabolism (bottom).

have flexible responses to produce reducing equivalents against intracellular oxidative stress and foster cancer cell proliferation at last (8, 10). Hence, therapeutic strategies targeting metabolic vulnerabilities of cancer show the potential to become effective treatments and to combat drug resistance of cancer cells (11, 12). However, our limited knowledge has yet unraveled the metabolic programming in cancers, which prevents us from going further in identifying novel therapeutic candidates.

In this review, we will first introduce fly models for various cancer genotypes (Section 2) and then introduce fly models for metabolic diseases including obesity, cachexia, and diabetes mellitus (Section 3). Lastly, we will put emphasis on fly studies that have provided novel insights into cancer metabolism (Section 4) (Figure 2).

Fly models for various cancer genotypes

In this section, we introduce cell type-specific fly models for cancer genotypes (Figure 2).

Adenoid cystic carcinoma

Adenoid cystic carcinoma (AdCC) is a rare gland tumor accounting for ~1% of all malignant tumors in the head and neck region and ~10% of all salivary gland cancers (13). AdCC generally grows slowly accompanied with perineural invasion, and more than 60% of the patients suffer local recurrence and/or distant metastasis (14). Such high recurrence and metastasis rates suggest that AdCC expands beyond surgical margins, causing hematogenous metastasis at early stages. The most common target organs of AdCC metastasis are lungs, bones, and livers (15).

Current treatment against AdCC includes radical surgical resection and adjuvant radiotherapy. Furthermore, clinicians often execute cytotoxic chemotherapy and targeted therapy against advanced AdCC. However, it is often difficult to confirm their therapeutic effect because AdCC responds to these treatments slowly. Even advanced AdCC sometimes becomes highly resistant to these treatments, which can lead to patients' death (13). These circumstances lead to the long-term poor prognosis of patients. In fact, the 5-year rate of overall survival (OS) is around 70% but the long-term OS rate drops significantly (10- and 15-year OS: 54% and 37%, respectively).

As per mutational landscape, recent progress in genomic sequencing revealed that over 50% of AdCC have the *MYB-NFIB* fusion gene (16). This abnormality leads to overexpression of *MYB*, hence its transcriptional target genes associated with apoptosis (*API5*, *BCL2*, *BIRC3*, *HSPA8*, *SET*), cell cycle (*CCNB1*, *CDC2*, *MAD1L1*), cell growth and angiogenesis (*MYC*, *KIT*, *VEGFA*, *FGF2*, *CD53*), and cell adhesion (*CD34*)

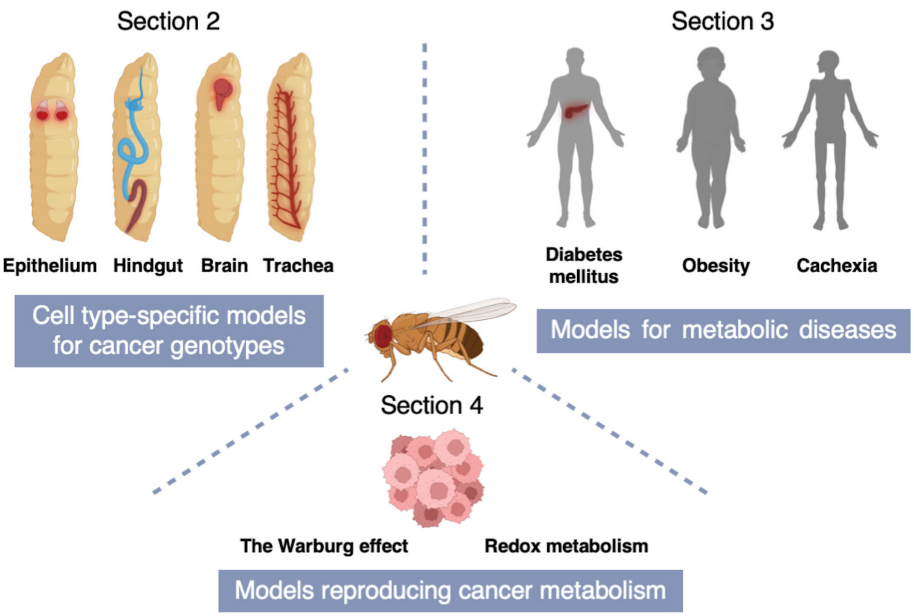

FIGURE 2
A schematic of the structure of this review. Section 2 describes cell type-specific models for cancer genotypes to mimic equivalent cancers in specific fly tissues [Adenoid cystic carcinoma (AdCC) and thyroid cancer (TC) in the fly wing disc epithelium; Colorectal cancer (CRC) in the fly hindgut; Non-small cell lung cancer (NSCLC) in the fly trachea; Glioblastoma (GBM) in the fly brain]. In Section 3, we introduce fly models for metabolic diseases including obesity, cachexia, and diabetes mellitus. Section 4 indicates fly models reproducing cancer metabolism which provide novel insights into the Warburg effect and redox metabolism.

(17). These findings suggest that *MYB* activation promotes AdCC development, and products of these genes offer novel therapeutic targets for this cancer.

Recently, several research platforms such as human AdCC organoids and mouse models carrying patient derived xenograft (PDX) of AdCC have emerged. These achievements significantly contributed to revealing fundamental aspects of AdCC such as its pathogenesis and novel therapeutic targets (18–20). However, a high difficulty in obtaining sufficient AdCC samples due to its low availability has made it extremely difficult to accelerate further analyses on AdCC using these mammalian models.

To overcome this issue, Bangi et al. took a *Drosophila*-based research approach for a patient with advanced AdCC (21). They developed a patient-specific transgenic fly strain as a 'personalized fly avatar' that modeled the patient's somatic mutations. To this end, they sequenced the patient's genome and selected six major alterations including *MYB-NFIB* fusion, *NOTCH1* overexpression, and missense mutations of *FAT1*, *3*, *4* and *ERCC2*. Next, they introduced into the fly avatar their fly orthologs. Regarding *MYB-NFIB*, they utilized truncated human *MYB* which mimicked an N-terminal product lacking the C-terminal regulatory domain (Table 1).

To test their effects on epithelial cells, the authors drove expression of these genes using the *patched* (*ptc*) gene enhancer/promoter in larval wing discs which proved useful to characterize cellular transformation (34). Upon induction of the transgenes, this fly avatar exhibited excessive proliferation of epithelial cells and decreased fly viability. They then conducted a large-scale screening of all FDA-approved drugs in this avatar by feeding them orally. Through this test they identified a three-drug cocktail as a therapeutic candidate to increase fly viability composed of vorinostat (the histone deacetylase inhibitor drug for cancer), pindolol (the non-selective beta blocker drug for high blood pressure), and tofacitinib (the JAK inhibitor drug for rheumatoid arthritis). Indeed, this cocktail led to disease stabilization and a partial metabolic response for 12 months in the patient. This study demonstrated that such a personalized approach using flies as a whole-animal platform can be useful in developing new treatments for AdCC.

Thyroid cancer

Incidence of thyroid cancer (TC) has been increasing worldwide. TC represents the most common endocrine malignancy, accounting for 3.4% of all cancers diagnosed annually in the U.S (35). In recent years, TC can be detected in earlier stages than before because screening tests for TC have been becoming available in the clinical practice. However, mortality rate of TC has been increasing slightly and makes it a significant unmet clinical need (36).

TC mainly originates from endoderm-derived follicular cells or neural crest-derived C cells in the thyroid, and the majority of TC can be divided into two subtypes depending on their origins: papillary TC (PTC) and medullary TC (MTC). PTC originating from the former cells is the main subtype of TC accounting for 84% of all TCs, while MTC is relatively rare accounting for 4% of all TCs (37).

In terms of genetic background, > 30% of PTC and > 50% of MTC harbor activation of RET, a receptor tyrosine kinase (RTK) (38). Specifically, PTC carries *RET* fusion genes upon a chromosomal translocation to produce constitutively active RET proteins (37). Especially, two *RET* fusions *CCDC6-RET* and *NCOA4-RET* account for more than 90% of all PTC fusion genes (37, 39). Regarding MTC, *RET* is also the most frequently mutated gene followed by *RAS* mutations and *RET* or *ALK* fusions (37). Particularly Met918 to Thr (M918T), the amino acid substitution in the RET kinase domain, is one of the most common mutations in MTC. This alteration leads to conformational changes in RET protein decreasing its autoinhibition mechanisms causing phosphorylation even in the absence of its ligands such as brain-derived neurotrophic factor (BDNF) (38, 40).

Despite these molecular findings, developing novel therapeutics for PTC and MTC has been problematic due to the lack of efficient experimental tools. Additionally, RET inhibition turned out harmful causing severe adverse effects (41). Also, RET inhibitors suppress other tyrosine kinases which are structurally similar to RET resulting in unexpected systematic reactions (38, 41).

To overcome these problems, groups including us have developed and utilized fly models for TC genotypes, discovering novel therapeutics efficiently. For example, Vidal et al. introduced an active form of *Drosophila Ret* (*dRet*^{M955T}, analogous to the human *RET*^{M918T}) to adult fly eyes to generate *Glass multimer reporter* (*GMR*)-*dRet*^{M955T} flies (Table 1) (22). This model displayed transformation of eye cells causing 'rough eye' phenotype due to cell proliferation. Using this model, they revealed that a tyrosine kinase inhibitor ZD6474 rescued this abnormality upon oral administration. Eventually, ZD6474 got approved in 2011 as the first targeted therapy vandetanib for MTC. This suggests that flies with cancer genotypes have potential to contribute to the development of therapeutics for human cancers.

Focusing on discovering compounds with higher anti-tumor effect than conventional kinase inhibitors, Dar et al. executed comprehensive chemical and genetic screenings in another fly model with MTC genotype (*ptc>dRet*^{M955T}; Table 1) (23). Kinase inhibitors such as RET inhibitors mentioned above typically interact with multiple targets beside their intended targets. This polypharmacological nature of a chemical affects various signaling pathways to modulate its efficacy and toxicity. Therefore, the authors attempted to optimize the polypharmacological profile of a kinase inhibitor. To this end, they developed an original chemical library targeting RET and other tyrosine kinases (91 in total) and found that one of the chemicals AD57 rescued

TABLE 1 *Drosophila* models of various cancer genotypes and drug development using these strains.

Patient			*Drosophila*			Ref.
Cancer type		**Genotype**	**Genotype**	**Phenotype**	**Therapeutic candidates**	
Adenoid cystic carcinoma (AdCC)		*MYB-NFIB*, *NOTCH1*^{act}, *FAT1/3*^{missense}, *FAT4*^{missense}, *ERCC2*^{missense}	*ptc>MYB-ΔC*, *N*, *Kug*^{RNAi}, *ft*^{RNAi}, *Xpd*^{RNAi}	Cell proliferation, cell migration	A personalized combination therapy with **vorinostat** (a histone deacetylase inhibitor drug for cancer), **pindolol** (a non-selective beta blocker drug for high blood pressure), and **tofacitinib** (a JAK inhibitor drug for rheumatoid arthritis)	(21)
Thyroid cancer (TC)	Medullary TC (MTC)	*RET*^{M918T}	*GMR-dRet*^{M955T}	'Rough eye' with partially fused and inconsistent patterns of ommatidia	**ZD6474**, approved as **vandetanib** by FDA as the first targeted therapy for MTC	(22)
			ptc>dRet^{M955T}	Cell proliferation, cell migration	**AD80**, which was modified structure of AD57 to reduce its toxicity	(23)
				Cell proliferation, cell migration	**APS6-45**, which was modified structure of sorafenib to reduce its toxicity	(6)
	Papillary TC (PTC)	*CCDC6-RET*, *NCOA4-RET*	*ptc>CCDC6-RET*, *ptc>NCOA4-RET*	Cell proliferation, cell migration	A combination of **sorafenib** plus a WEE1 inhibitor **AZD1775**	(24)
Colorectal cancer (CRC)		*KRAS*^{G12V}, *TP53*^{inact}, *PTEN*^{inact}, *APC*^{inact}, *SMAD4*^{inact}	*byn>ras*^{G12V}, *p53*^{RNAi}, *pten*^{RNAi}, *apc*^{RNAi}, *Med*^{RNAi}	Cell proliferation, EMT, cell migration	A combination of the proteasome inhibitor **bortezomib** and the PI3K pathway inhibitor **BEZ235**	(25)
		KRAS^{G13A}, *TP53*^{inact}, *APC*^{inact} *FBXW7*^{inact}, TGFBR2^{inact}, *SMARCA4*^{inact}, *FAT4*^{inact}, *MAPK14*^{inact}, *CDH1*^{inact}	*byn>ras*^{G12V}, *p53*^{RNAi}, *apc*^{RNAi}, *ago*^{RNAi}, *put*^{RNAi}, *brm*^{RNAi}, *ft*^{RNAi}, *p38*^{RNAi}, *shg*^{RNAi}	Hindgut expansion	A combination of the MEK inhibitor **trametinib** and a bisphosphonate **zoledronate**	(26)
		KRAS^{act}, *APC*^{inact}	*esg>ras*^{G12V}, *apc*$^{Q8/}$ *apc2*^{N175K}	Increased tumor burden	n/d	(27)
Non-small cell lung cancer (NSCLC)		*KRAS*^{G12V}, loss of *PTEN*	*btl>ras*^{G12V}, *pten*^{RNAi}	Cell proliferation, fly lethality	A combination of the MEK inhibitor **trametinib** and the HMG-CoA reductase inhibitor **fluvastatin**	(28)
		EGFR^{act}	*ppk>Egfr*^{A877T}	Tracheal epithelial cell malformation, larval death	A combination of the tyrosine kinases inhibitor **afatinib** and the JAK/STAT signaling inhibitor **bazedoxifene**	(29)
		EGFR^{act}, *KRAS*^{G12D}, *RAF*^{act}, *MAPK*^{act}, *PIK3CD*^{act}, *ALK*^{act}, *AKT*^{act}, *CTNNB1*^{act}	*ppk>Egfr*^{A887T}, *ras*^{G12V},*Raf*^{OE}, *Rolled*^{OE}, *Pi3K92E*^{OE}, *Alk*^{OE}, *Akt*^{OE},*Arm*^{OE}	Tracheal epithelial cell proliferation and thickening, larval death	The MEK inhibitor **trametinib**	(30)
Glioblastoma multiforme (GBM)		*EGFR*^{act}, *PIK3CA*^{act}	*repo>dEGFR*$^{\lambda}$, *dp110*^{CAAX}	Glial cell proliferation and invasion	A combination of the YAP/TAZ-TEAD transcriptional activation inhibitor **verteporfin** and the ACAT1 inhibitor **K-604**	(31–33)

ptc, *patched*; *GMR*, *glass multimer reporter*; *byn*, *brachyenteron*; *esg*, *escargot*; *btl*, *breathless*; *ppk*, *pickpocket*; *repo*, *reversed polarity*; EMT, epithelial-mesenchymal transition.

tumorigenic phenotypes in *ptc>dRet*^{M955T} flies efficiently. In this study, they also found in genetic screening that transformation in these flies was dependent largely on *Raf*, *Src*, and *S6K* (42). AD57 consistently inhibited these kinases, but it simultaneously inhibited *dTor* (a fly ortholog of human *mTOR*) which was an effector of Phosphoinositide 3-kinase (PI3K) and a suppressor of Raf (43). As a result of Raf deregulation hence dTor activation, AD57 caused high toxicity beside efficacy in flies. Accordingly,

they modified the chemical structure of AD57 to generate AD80 which did not inhibit dTor. As expected, AD80 rescued fly lethality and cellular transformation including malformations of wing veins and cuticles more efficiently than AD57 without showing obvious toxicity. Of note, AD80 suppressed TC xenograft in mice dramatically (23). Recently, AD80 demonstrated a 100- to 1000- fold higher anti-tumor effect on TC cell lines than other multikinase inhibitors approved for RET-dependent cancers including sunitinib, sorafenib, vandetanib, and cabozantinib (44). These studies prove that flies provide potential preferred and non-preferred targets of kinase inhibitors. Applying these achievements will reveal mechanisms of adverse effects of various anti-cancer drugs and eventually lead to establishment of potent anti-cancer drugs with reduced toxicity.

In order to develop therapeutic compounds that preserve their anti-tumor effect while reducing their toxicity in a more rational manner, we established a novel method for developing therapeutics by utilizing comprehensive chemical and genetic modifier screens in *ptc>dRet*^{M955T} flies (Table 1) (6). We first screened in this model all kinase inhibitor drugs approved by FDA for cancer therapy at the time of 2016, and confirmed that sorafenib showed the strongest but only marginal effects. In clinical practice sorafenib has given benefits to MTC patients, but severe adverse effects emerge such as skin damage, diarrhea, alopecia and even fatality in patients (45, 46).

We thus attempted to determine the cause of this toxicity through comprehensive chemical and genetic modifier screens of the kinome network in *ptc>dRet*^{M955T} flies. First, we developed a library of sorafenib analogs by their chemical synthesis and fed them orally to *ptc>dRet*^{M955T} flies, finding out several derivatives with improved efficacy measured by fly viability as a readout. Then, we executed genetic modifier screening (199 in total, covering more than 80% of all fly kinases) in the presence of sorafenib or such derivatives to elucidate the mechanisms of their efficacy and toxicity. Interestingly, inhibiting one of the sorafenib targets Lk6 [a fly ortholog of human Mitogen-activated protein (MAP) kinase-interacting serine/threonine-protein kinase (MKNK)] by removing one copy of *Lk6* gene in these flies (*ptc>dRet*^{M955T},*Lk6ptc>dRET*^{M955T}, *Lk6*$^{-/+}$) caused complete lethality in the presence of sorafenib. Control *Lk6*$^{-/+}$ flies presented almost 100% survival, therefore these results indicate that sorafenib has LK6 as an 'anti-target' whose inhibition accounts for its toxicity. These findings led us to derivatize sorafenib further to generate APS6-45 which *in silico* modeling predicted to have significantly reduced binding capacity with MKNK but not RET as compared with sorafenib. As expected, APS6-45 suppressed growth of human MTC cell line TT and its xenograft in mice without detectable toxicity, suggesting that APS6-45 offers a novel therapeutics for treating MTC. As such, this 'rational polypharmacology' integrating multiple fly screening platforms with computational chemistry with medicinal chemistry can accelerate development of novel high-efficacy and low-toxicity drugs.

As per PTC, a previous study generated fly models for PTC genotypes including *CCDC6-RET* and *NCOA4-RET* fusions, both of which are frequently observed in the patients (Table 1) (24). The authors employed these fly models to identify compounds for PTC treatment and to validate functional differences between two types of human *RET* fusions. These flies exhibited tumorigenic phenotypes in wing discs including cell migration and delamination. Of importance, flies with human *NCOA4-RET* fusion showed more severe phenotypes than those with human *CCDC6-RET*, consistent with outcome of PTC patients carrying distinct genetic abnormalities. In this paper, the authors revealed key roles of MAP kinase (MAPK) signaling pathway in PTC development. Notably, they also identified that these *RET* fusions activated distinct signaling pathways; *NCOA4-RET* but not *CCDC6-RET* activated Hippo and PI3K pathways. Furthermore, chemical screenings in these flies for FDA drugs and experimental small molecules (55 in total) revealed that these fusions conferred different drug sensitivity. Specifically, sorafenib and cabozantinib rescued lethality of *NCOA4-RET* flies, whereas gefitinib and vandetanib rescued that of *CCDC6-RET* flies. Therefore, they concluded that these two *RET* fusions activated different signaling pathways to promote transformation and determine distinct sensitivity to clinically relevant drugs. Their achievements indicate that fly platforms are useful not only for identifying therapeutic targets and chemicals against cancers but also for analyzing functions of human genes.

Collectively, transgenic flies successfully unveiled the fundamental effects of abnormalities in TC genome on cellular characteristics. Generating and testing more TC models will accelerate comprehensive determination of TC pathogenesis and novel therapeutics.

Colorectal cancer

Colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of cancer death globally accounting for 10% of total cancer cases and 935,000 deaths in 2020, respectively (47). Our previous work based on mammalian models has given us important insights into the CRC mechanisms. For example, we demonstrated that PGE2-EP2 and NOTCH-ABL-TRIO-RHO pathways promote CRC initiation and progression, respectively (48–53). Also, we identified the invasion/metastasis-suppressing *Aes* gene which inhibits NOTCH signaling (54–57).

Although plenty of previous studies including them have deepened our understanding of cancer signaling pathways, tackling CRC remains to be an important challenge. To solve this, *Drosophila* has proven a powerful whole-body model due to its significant similarities in both physiology and morphology of the digestive tract to mammalians (Figure 1A) (58). The fly gut has similar functions to its mammalian counterparts to digest

food, absorb nutrients, and execute the first-line defense against infection by innate immunity (59, 60). Based on their functions, the fly gut is divided into three parts: the foregut, midgut, and hindgut (61). Among them, the midgut is regarded as useful to study the contribution of signaling pathways and metabolism in CRC, because its architecture resembles digestive tracts of mammals (58, 62).

On the other hand, CRC onset has several related signaling pathways: *WNT*, *SMAD4*, *KRAS*, *PIK3CA*, and *TP53* (63). Inactivation of the *Adenomatous polyposis coli* (*APC*) gene, a tumor suppressor in the WNT signaling pathway, is the most common mutation in CRC occurring in 80-90% of patients (64). As the second most common mutation, approximately 50% of CRCs are homozygous for loss-of-function mutations in the *TP53* tumor suppressor gene, followed by gain-of-function mutations in the *KRAS* oncogene in around 40% of CRCs (65, 66). Their identifications led to genetic manipulation of two or more of them in combination *via* mouse genetics (67). Unfortunately, genetically engineered mouse models (GEMMs) with complex genotypes require enormous resources to generate and maintain. In this regard, *Drosophila* offers advantages because modeling multiple mutations is easy in flies. Hence, as we will state in this section, fly models for CRC genotypes contributed to unraveling the complexity of CRC regarding disease metabolism and drug response complementarily with mammalian models.

Modeling recurrent mutations in CRC, a group induced five cDNAs and knockdown siRNAs as transgenes in the fly hindgut, including *KRAS* (fly *ras*), *TP53* (*p53*), *PTEN* (*pten*), *SMAD4* (*Med*), and *APC* (*apc*) by using the *byn* (*brachyenteron*) enhancer/promoter (Table 1). These genetic modifications resulted in cellular transformation recapitulating hallmarks of human CRC including cell proliferation, disruption of the epithelial architecture, EMT, migration and dissemination to distant sites. By using these multigenic flies, the authors identified the mechanism of resistance against a PI3K/mTOR inhibitor BEZ235. They further discovered two-step therapy using bortezomib (the proteasome inhibitor) and BEZ235 to overcome this resistance in this model. This treatment was also effective in a CRC cell line DLD1 carrying a similar mutational signature to the multigenic flies, as well as in its xenografts in mice. This study provided important insights into the use of flies as a handy platform for rapid and large-scale functional exploration of human cancer genomes as well as drug discovery (25). Moreover, the authors published another milestone paper where they developed a personalized fly model of a patient with refractory metastatic CRC harboring *KRAS* mutation (Table 1) (26). In FDA drug screening, combination between trametinib [the drug targeting Mitogen-activated protein kinase kinase (MEK)] and zoledronate (a bisphosphonate) significantly suppressed anterior expansion of the hindgut. This treatment gave a significant response to the patient reducing the tumor volume by 45%. Notably, CRC remained stable for 11 months in the patient (26).

Following these studies, another group developed novel high-throughput assays for quantifying tumor burden (27). They reported two methods to evaluate proliferation of transformed cells. One method is to use a simple software they developed in ImageJ Fiji to automatically analyze the area that transformed cells occupy. Another is to use luciferase as a reporter to determine the number of transformed cells. By these two methods, they reported increased tumor burden in a fly model for CRC genotype carrying the *esg* (*Escargot*)-GAL4 driver to induce ras^{G12V} and $apc^{LOF\ (Loss\ of\ function)}$ transgenes specifically in intestinal stem cells (ISCs) (Table 1). Besides, another study designed an *in silico Drosophila* model for CRC genotype based on data of cell type-specific RNA-seq on FlyGut-seq database (68). They constructed a computational framework for the fly midgut, which successfully elucidated cell fate, validated drug cytotoxicity, and devised a personalized treatment candidate. To summarize, *Drosophila* is a useful preclinical whole-animal model due to its multiple applications in CRC studies.

Different from *byn* cells throughout the hindgut and a subset of posteriorly derived visceral muscles (26), another driver *esg*-GAL4 is frequently used in fly CRC studies. *esg*-GAL4 is active in progenitor cells in the posterior midgut, which are also known as ISCs (27). It has not been declared in papers that inducing transformation in fly ISCs mimics CRC tumorigenesis derived from mutated stem cells in patients. However, modeling CRC mutations in fly ISCs provided important clues of metabolic reprogramming in stem cells with tumorigenic potential (62, 69–71). Specifically, a study revealed that activation of *yorkie* (*yki*), a fly ortholog of the human oncogene *YAP1*, leads to proliferation of ISCs *via* upregulation of insulin/insulin-like growth factors (IGF) signaling and glycolysis (69). In addition, studies have shown that elevated lactate concentration caused by Ras/Raf activation in ISCs caused Warburg effect-like metabolic changes in transformed cells to induce proliferation of transformed cells (62, 70). Another study showed that tumor-like ISCs induced by Notch depletion proliferated and generated ROS, while ISCs with reduction of both Notch and β-integrin caused metastasis and ROS (71). Overall, these studies demonstrated that flies are practical in mechanistic analyses and drug discovery of CRC.

Lung cancer

Lung cancer is the top cause of global cancer mortality with a rising incidence (72). With a large number of diagnoses each year, reported 5-year survival remains ~15% for all patients and less than 4% for those with distant metastasis. Unfortunately, only minimal improvement has been made in these dismal statistics over the past decades (73). Because of such disappointing prognosis and significant systemic toxicities of even approved treatment, developing novel therapies has remained one of the major goals in lung cancer research.

To solve this issue, *Drosophila* has been used to model lung cancer genotypes to develop novel therapeutics recently (29), breaking the long-standing underestimation of the potential of *Drosophila* in lung cancer research (74). In fact, *Drosophila* is devoid of lungs. However, the respiratory systems in flies and mammals share lots of structural and physiologic similarities (Figure 1A) (75). For example, fly models of chronic lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are available in the field, demonstrating the feasibility of modeling lung diseases in flies by mimicking their genotypes in the respiratory system (76).

Non-small cell lung cancer (NSCLC) accounts for approximately 84% of all primary lung cancers (77). *KRAS* and *Epidermal growth factor receptor* (*EGFR*) are the two most common identifiable drivers, whose mutations cover 50%–60% of NSCLC cases (78, 79). Based on this information, several groups developed fly models of NSCLC genotypes and fly-based platforms for processing high-throughput chemical screening (28–30). For example, Levine and Cagan developed the first *Drosophila* lung cancer model by targeting ras^{G12V} alone or in combination with *PTEN* knockdown to the fly tracheal system using the *breathless* (*btl*)-GAL4 driver (Table 1). In this model, ras^{G12V} induced tracheal proliferation and fly lethality in the larval or pupal stage. Using this lethality as a readout, the authors screened the library of FDA-approved drugs and identified inhibitors of MEK and HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase as potential therapeutics (28).

While, other teams used the *pickpocket* (*ppk4*)-GAL4 driver to induce constitutive activation of *EGFR* in the fly trachea, causing malformation of tracheal epithelium and larval death. Among ~1,000 FDA-approved drugs, only tyrosine kinase inhibitors (TKI) afatinib, gefitinib, and ibrutinib rescued *EGFR*-induced larval lethality. By utilizing the fly-based whole-animal screening, they identified synergistic anti-tumor effects of a combination of afatinib and bazedoxifene, a novel GP130/STAT3 pathway inhibitor. These findings suggested therapeutic benefits by simultaneously blocking EGFR and JAK/STAT signaling in NSCLC (Table 1) (29).

Even after establishment of such fly-based high-throughput screening systems, it was still unclear if other driver mutations also caused transformation. To answer this question, the authors further developed modular *Drosophila* models for a larger number of human lung cancer oncogenes including *Egfr*, ras^{G12V}, *Raf*, *Rolled* (a fly ortholog of human *MAPK*), *Pi3K92E* (*PIK3CD*), *Alk*, *Akt*, and *Arm* (*CTNNB1*) (Table 1) (30). On the other hand, they established two complementary readouts which were simple, reliable, and adaptive to the needs of high-throughput screening. One of the readouts was rescue of fly lethality, and the other was reduction of a quantifiable tumor mass (30). This workflow demonstrated a possibility of *Drosophila* to provide various high-throughput screening measures and thus novel lung cancer treatments.

Glioblastoma

Gliomas, especially glioblastoma multiforme (GBM), is the most common primary malignant brain tumor in adults (80). The incidence of GBM is estimated at 3.2 per 100,000 population in the United States per year. The 5-year relative survival rate for this cancer is only 5%, making it one of the most deadly and recalcitrant tumors of all malignant solid tumors (81). In addition, even with surgical resection as the standard treatment, patients with GBM have a poor prognosis with a median survival of only 15 months (82). Considering such poor prognosis, developing effective therapies for GBM treatment is an urgent clinical need for improving clinical outcome.

According to genomic profiling including The Cancer Genome Atlas project, the most common genetic alteration for GBM is overexpression of *EGFR* (altered in 30-40% of total cases) and *PIK3CA* (8-10%) (83). Undoubtedly, identification of these key effectors involved in GBM are critical clues to develop novel measures for diagnosis and treatment (Table 1).

There are several studies demonstrating that the mechanisms of neural development are remarkably similar between flies and humans (Figure 1B) (84). Combined with the advantages of fly genetics, such similarities make flies an effective tool to model genotypes of gliomas to delineate their pathogenesis (85).

To investigate genetic basis and to determine novel therapeutic targets of GBM, Read et al. developed a fly model $repo{>}dEGFR^{\lambda}$,$dp110^{CAAX}$ based on *reversed polarity* (*repo*)-GAL4-driven co-overexpression of active forms of *Drosophila EGFR* ($dEGFR^{\lambda}$) and *PIK3CA* ($dp110^{CAAX}$) in glia. These alterations induced neoplasia causing proliferation and invasion of glial cells seen in human GBM (31). Thus, this fly model has been widely used in GBM studies and has brought novel insights into the molecular mechanisms of GBM (Table 1) (32, 86, 87).

Another group found that overgrowth and invasion of glial cells happen upon overexpression of other RTKs including *Pvr* [a fly ortholog of platelet-derived growth factor receptor (PDGFR)/vascular endothelial growth factor receptor (VEGFR)], *htl* [a fibroblast growth factor receptor (FGFR) ortholog], and *InR* (an insulin receptor ortholog) (86). Besides, they demonstrated that administration of the EGFR inhibitor drug gefitinib, the PI3K inhibitor wortmannin, and the Akt inhibitor triciribine can revert EGFR/PI3K-induced transformation (86).

After these studies, a kinome-wide genetic screening was conducted in $repo{>}dEGFR^{\lambda}$,$dp110^{CAAX}$ flies to discover effectors required for RTK- and PI3K-dependent neoplastic transformation. This test clarified that overexpression of right open reading frame (RIO) kinase driven by mTor-complex-2 (TORC2)-Akt signaling promoted cell proliferation and survival, which gives novel therapeutic opportunities for GBM (87). In the same model, *yki* was overexpressed in neoplastic glia, and its knockdown suppressed glial proliferation (32). This finding

raises an FDA-approved liposomal formulation of verteporfin as a novel therapeutic option for EGFR-driven GBMs as it suppresses transcriptional activity of YAP/TAZ which are mammalian orthologs of fly *yki* (Table 1) (32). However, it is possible that abnormalities in multiple RTKs limit efficacy of therapies targeting a single RTK. Therefore, combining several RTK inhibitors can offer more effective treatment for GBM than monotherapy (88).

Accumulating evidence indicates that metabolic reprogramming in the brain is a critical factor in the transition from non-neoplasm to neoplasm including GBM. Therefore, targeting essential metabolic pathways in glia may provide new therapeutic opportunities for GBM treatment (89). In *repo>dEGFR*$^{\lambda}$,*dp110*^{CAAX} flies, glia-specific knockdown of four genes essential for glial metabolism [*ALDOA* (*Aldolase* in flies), *ACAT1* (*CG8112*), *ELOVL6* (*Baldspot*), and *LOX* (*Lox*)] partly rescued glioma-induced phenotypes such as shorter lifespan and bigger tumor size. Of these four the authors especially focused on *ACAT1* which plays a role in regulating endoplasmic reticulum-cholesterol homeostasis and lipid metabolism. Then they found that silencing *ACAT1* maintained cholesterol homeostasis, and prevented brain hypertrophy and glioma trait-induced shortening of fly lifespan (Table 1) (33).

While, impaired insulin function leads to abnormal glucose metabolism and mitochondrial dysfunction (90). Based on the same fly model mentioned above, glioma-secreted *Imaginal morphogenesis protein-late 2* (*ImpL2*) was found to inhibit insulin pathway activity, which caused synaptic loss and consequently promoted neurodegeneration. Restoring insulin signaling in neurons by overexpressing *Rheb* (activation of insulin/TOR/S6K signaling pathway) partially rescued neurodegeneration and mortality of the model (91).

More than using flies to identify novel therapeutic targets, the utilization of the above-mentioned fly model verified that mitochondrial PTEN-induced kinase 1 (PINK1), a regulator of the Warburg effect, turned out to suppress GBM growth (92). *PINK1* overexpression attenuated GBM traits in both flies and orthotopic xenografts of human U87 cells in mice. In summary, these studies on flies have unraveled part of the mechanism by which significant alterations in metabolic pathways in cancer cells are associated with the onset and progression of GBM.

Fly models for metabolic diseases

Similarities among metabolic pathways and physiological responses between *Drosophila* and humans makes flies a useful whole-animal model for not only genetic diseases but also metabolic disorders (93, 94). In this section, we describe fly models to mimic metabolic disorders. In particular, we will focus on diabetes mellitus (DM), obesity, and cachexia introducing contributions of their fly models to understand these diseases (Figure 2).

Diabetes mellitus

Regarding sugar metabolism, different from mammals, flies take up simple sugars passively from the digestive tract into the fat body where they are converted to trehalose. Hence, trehalose is the primary circulating sugar in insects instead of glucose (95, 96). Despite this difference in substances, flies share functionally similar mechanisms with mammals to regulate sugar homeostasis through conserved pathways: *Drosophila* secretes an insulin equivalent insulin-like peptides (Ilps) and a glucagon analog adipokinetic hormone (AKH) to respond to high and low levels of circulating sugar, respectively (97–100). These facts generated an idea of modeling DM in flies.

Type 1 DM (T1DM) arises from the destruction of insulin-producing β-cells of the pancreas which results in decreased or complete loss of insulin. In flies, insulin-producing cells (IPCs) in the brain are equivalent to the β-pancreatic islet cells in mammals (Figure 1B). Upon ablation of IPCs, flies reproduced T1DM-like phenotypes such as growth defects and developmental delay (98, 99).

On the other hand, Type 2 DM (T2DM) is a disease of insulin resistance; namely hyperglycemia persists despite the presence of high levels of circulating insulin. Interestingly, feeding a high-sugar diet (HSD) caused insulin resistance in flies generating diabetic-like states such as hyperglycemia even with high levels of Ilps (101).

Epidemiological studies have shown an increased risk of several types of cancer in DM patients including pancreas, liver, breast, colorectal, urinary tract, and female reproductive organs (102). Hence, there have been studies to reveal the relationship between DM and cancer, and flies contributed to the discovery that HSD boosted tumor progression. One of the examples is that feeding flies with HSD promoted EGFR-driven epithelial neoplasia and metastasis through lactate dehydrogenase (LDH)-dependent aerobic glycolysis (103). Therefore, flies are practical toolkits to simultaneously reproduce tumorigenesis and systemic metabolic disorders to explore their mutual mechanisms.

Obesity

Regarding the cause of T2DM, obesity induced by caloric excess is a triggering factor for insulin resistance-associated diabetes. In fact, 55% of T2DM patients are obese (104). Consistently, a fly model for HSD-induced T2DM manifested also obesity as determined by accumulating fat within the body (101). In these flies, HSD also triggered alterations in insulin signaling, lipogenesis, and gluconeogenesis. Therefore, this model not only revealed pathological relationships between multiple metabolic disorders but also gave rise to novel therapeutic candidates such as Gomisin N, which relieves the endoplasmic reticulum stress to be a potential agent for preventing and treating obesity (105).

So far, several epidemiological studies provided strong evidence of an association between obesity and increased risk of various cancers (106). To understand the interplay between obesity and cancer, a study induced obesity by HSD in flies modeling activation of multiple oncogenes such as *Ras* and *Src* (107). In this study, the active form of fly *ras* and a null allele for fly *C-terminal Src kinase* (*Csk*) induced tumors. Intriguingly, HSD caused the $ras1^{G12V}$;$csk^{-/-}$ tumors to grow more aggressively than normal diet. Simultaneously, the authors demonstrated that Ras/Src-activated cells efficiently responded to nutritional signals of a SIK-Yki-Wg-InR signaling circuit and ensured tumor growth upon nutrient-rich conditions including obesity (107, 108). As such, studies with flies have significant potential in elucidating the mechanisms by which obesity influences development and progression of cancer.

Cachexia

Another cancer-related metabolic dysfunction cachexia is a multifactorial wasting syndrome that contributes to the clinical deterioration in patients with advanced cancer. Cachexia is characterized by weight loss, skeletal muscle wasting, and atrophy of the adipose tissue (109). Recently, some reports included *Drosophila* to recapitulate cachexia-like systemic wasting to obtain insights into the cachexia mechanisms (69, 110).

For example, a study developed $scrib^{-/-}$, ras^{G12V} tumors in flies. The authors found robust wasting of adipose and muscle tissues in flies developing tumors, which resembled cancer cachexia in patients (110). Another study established a model for systemic organ wasting in adult flies by overexpressing *yki* using *esg*-GAL4 driver active in ISCs (69). Both studies stated that insulin signaling was impaired in transformed cells demonstrating the central role of tumor-induced insulin resistance in cachexia.

Metabolic diseases and cancers

In addition to these models, flies turned out to be useful in modeling hepatic metabolic diseases, neurodegenerative diseases, and other types of metabolic dysfunctions (94, 111). These achievements come from high conservation of metabolic pathways between *Drosophila* and mammals. Therefore, it is possible to reproduce characteristic alterations in cancer metabolism in fly models of cancer genotypes to elucidate their mechanisms and their impact on each disease.

As one of such reactions in cancer, angiogenesis under hypoxic stress is an adaptive strategy in tumor progression to meet its metabolic needs (112). In analogy to human blood vessels, the fly tracheal system plays similar roles in transporting oxygen to internal organs (113). Previous studies showed that *Drosophila* transformed cells suffered from oxygen shortage similar to human cancers. Interestingly these cells released pro-tracheogenic factors, which led to identification of tracheogenesis as a novel tumor hallmark in flies (112). Therefore, *Drosophila* offers also a convenient whole-body organism to determine metabolic reprogramming in cancers.

Drosophila models for metabolic disorders are not only valuable for elucidating pathogenesis of these metabolic disorders but also able to contribute to cancer research. Several metabolic disorders such as obesity and DM have an important mutual influence on specific types of cancers (102, 114, 115). Intriguingly, up to 80% of pancreatic cancer patients are either hypoglycemic or diabetic in a presymptomatic phase. Therefore, new-onset diabetes is a potential clue to early diagnosis of pancreatic cancer (116). Indeed, metabolic disorders and cancer are too complex to recapitulate in mammalian models simultaneously. However, flies have provided a possibility to combine two models in one organism and have promoted understanding of the fundamental associations between metabolic diseases and cancers as discussed above. Moreover, flies contributed to elucidating the cachexia mechanisms such as cancer-host interactions. To summarize, fly studies provide us with simple and effective ways to explore critical insights not only of cancer development and progression but also of the connections between metabolic diseases and cancers.

Cancer metabolism revealed by fly studies

In this section, we present fly models for studying cancer metabolism (Figure 2).

The Warburg effect

Glucose metabolism is essential for cells to produce adenosine triphosphate (ATP) as an energy source to maintain their homeostasis and activity. In the process of glucose metabolism, normal cells break glucose into pyruvate in the cytosol by glycolysis, putting pyruvate into the tricarboxylic acid (TCA) cycle, also termed as Krebs cycle in mitochondria where pyruvate further gets metabolized (oxidized) into carbon dioxide and ATPs. It is well known that this glucose metabolism pathway is changeable depending on oxygen. In the presence of sufficient oxygen, most types of cells produce ATP through the TCA cycle and further steps of glucose metabolism including oxidative phosphorylation in mitochondria. Through this process, cells can generate 36 ATP molecules per one glucose molecule. Normally, cells obtain oxygen constantly from the blood circulation. On the other hand, cells under hypoxic conditions where oxygen is scarce largely count on glycolysis

yielding lactate and just two ATPs per one glucose molecule. Therefore, in light of ATP production, glycolysis is far from an efficient strategy compared with oxidation of pyruvate through the following process in mitochondria. Intriguingly, Warburg discovered that cancer cells tended to employ glycolysis to produce energy even in the presence of sufficient oxygen, publishing his findings as the 'Warburg effect' (117).

In the process of the Warburg effect, LDH plays an essential role in promoting glycolysis. Human LDH enzymes are encoded by four distinct genes (*LDHA*, *LDHB*, *LDHC*, and *LDHD*). Among them, LDHA primarily converts pyruvate to lactate. Meanwhile, *Drosophila* has one *LDH* gene *ImpL3*, whose product functions similarly to mammalian LDH. Therefore, it seems reasonable to study ImpL3 in flies to effectively understand essential functions of human LDH in tumorigenesis.

In human cells where glucose metabolism is active, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) generates nicotinamide adenine dinucleotide hydrogen (NADH) from nicotinamide adenine dinucleotide (NAD_+) in glycolysis. NADH is an essential molecule for the forthcoming process, oxidative phosphorylation, to generate additional ATPs. In this process, reducing the amount of NAD^+ results in decelerating the glycolytic process leading to growth restriction (118). In order to compensate for the shortage of NAD^+, cancer cells largely use NAD+ production by LDH which oxidizes NADH and produces NAD^+ in the process of converting pyruvate into lactate (119). Therefore, human LDH is responsible for maintaining the NAD^+/NADH redox balance in highly glycolytic cells such as cancer cells.

Intriguingly, flies have similar compensation mechanisms in glucose metabolism as in human cells. For instance, larvae lacking *ImpL3* are still able to produce lactate by accelerating glycerol-3-phosphate (G3P) production with increased activity of G3P dehydrogenase 1 (GPDH1), which allows normal larval development. Therefore, GPDH1 regulates the NAD^+/NADH redox balance and ATP level in larvae (120). Given the similar glycolytic processes and the conserved functions between ImpL3 and LDH, we speculate that flies give us clues to understand the fundamental mechanisms of the Warburg effect.

In recent years, several fly models for cancer genotypes have exhibited unique cell metabolism in transformed cells that are seen also in human cancer cells. Next, we introduce examples which give insights into the relationship between glucose metabolism and cancer (Table 2).

Firstly, fly models for cancer genotypes have shown to shift their glucose metabolism toward the Warburg effect. A study demonstrated that activation of PDGF/VEGF-receptor Pvr in imaginal discs induced epithelial tumors with upregulated ImpL3 and enhanced glycolysis (126). Indeed, *Pvr* activation induced glucose metabolic changes through stabilization of Hifα [a fly ortholog of human Hypoxia-inducible factor-1α (HIF-1α)], which transcriptionally upregulated glycolytic enzymes including ImpL3 inducing glycolysis. In this study, the authors employed a GFP-based enhancer trap reporter strain *ImpL3-GFP* to visualize *ImpL3* transcription, which enabled easy detection of endogenous *ImpL3* expression in fly tissues (Figure 3) (136). Additionally, the authors found that multiple oncogenic pathways inhibited activation of pyruvate dehydrogenase (PDH) which converts pyruvate into acetyl-CoA and is essential for driving the TCA cycle and oxidative phosphorylation.

Another paper also revealed elevated aerobic glycolysis in flies with misexpression of *Drosophila* Homeodomain-interacting protein kinase (Hipk). These *dpp>Hipk* flies displayed tumor formation, loss of epithelial integrity, and an invasion-like phenotype in their wing discs (132). The authors identified that *Hipk* triggered upregulation of *dMyc* in these transformed cells. It was already reported that induction of *dMyc* increased expression of glycolytic genes such as *ImpL3* hence upregulated glucose consumption as revealed in *Drosophila* S2 cells (137). As for *Hipk*-induced fly transformed cells, a study identified that *Hipk* triggers robust expression of glycolytic genes especially *ImpL3* and *Phosphofructokinase 2* (*Pfk2*, a fly ortholog of human *PFKFB*). Pfk2 catalyzes the synthesis of fructose-2,6-bisphosphate to stimulate further steps in glycolysis (122). Intriguingly, ectopic expression of *dMyc* was sufficient to increase *Pfk2* expression, leading to further *dMyc* accumulation in fly wing discs. These results suggest a positive feedback loop between *dMyc* and aerobic glycolysis (132). In this study, the authors monitored glucose metabolism in fly tissues with Förster resonance energy transfer (FRET)-based glucose sensor composed of a glucose-binding domain (GBD) combined with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) to determine the intracellular glucose level (Figure 3) (123, 138). Binding of GBD to glucose induces GBD's structural changes to increase the FRET efficiency (the ratio of YFP to CFP). These papers show that flies exhibit Warburg-like metabolic changes resulting from multiple molecular mechanisms, which has led us to better understand how the Warburg effect contributes to cancer cell metabolism. Moreover, fly platforms are able to provide novel insights of relations between signaling pathways involved in cancer and its metabolism.

Regarding Notch signaling pathway which is one of the most commonly mutated genes in cancer, previous papers demonstrated that *Notch*-induced fly models for cancer genotypes harbor accelerated glycolysis. One study established fly models with ectopic expression of *Drosophila* Notch intracellular domain (Nicd) in *ptc>Nicd* flies (139). In this paper, the authors performed a chromatin immunoprecipitation (ChIP) assay with antibody against Suppressor of Hairless [Su(H)], a key transcription factor in Notch signaling (140). This assay revealed that Notch signaling transcriptionally regulated several effectors in glycolysis including *ImpL3*. In addition, the aforementioned study by Slaninova et al. showed that knockdown of genes associated with glucose metabolism suppressed tissue overgrowth that Notch

TABLE 2 *Drosophila* models reproducing cancer metabolism.

Human Gene	*Drosophila* Genotype	GAL4 Driver	Metabolic Phenotype	Mode of action	Assessment methodology	Ref.
NOTCH^{act}	*Nicd*	*ptc* (wing disc)	Upregulation of glycolysis associated genes including *ImpL3*	Upregulation of N transcriptional activity	ChIP assay with α-Su(H) antibody, mRNA measurement by qPCR	(121)
PDGF/VEGF receptor^{act}	*Pvr*^{act}	*dpp* (wing disc)	Upregulation of ImpL3	Stabilization of Hifα	*LDH-GFP* reporter	(122)
HIPK^{act}	*Hipk*^{OE}	*dpp* (wing disc)	Upregulation of glycolysis associated genes including *ImpL3*	Upregulation of dMyc	mRNA measurement by qPCR, *LDH-GFP* reporter, FRET glucose sensor	(123)
COX7A^{inact}	*COX7a*^{RNAi}	*ey* (eye disc)	Upregulation of ImpL3, Increased glucose uptake and level of intracellular lactate	Inhibition of mitochondrial ETC	*LDH-GFP* reporter, FRET glucose/lactate sensor	(124)
DLG1^{inact}	*dlg* depletion	*en* (wing disc)	Elevated ROS	Loss of cell polarity	DHE, DCFH-DA	(125)
FLT1^{act}	*Pvr*^{act}	*dpp* (wing disc)	Elevated ROS	Glycolytic tumor	DHE, *GstD-GFP* reporter	(126)
KRAS^{G12V},*SCRIB*^{inact}	*ras*^{G12V},*scrib*$^{-/-}$	*en* (eye disc)	Elevated ROS	Loss of cell polarity	DHE, DCFH-DA	(127)
n/d	*brat*^{RNAi}	*da* (brain stem cells)	Elevated ROS	Brain stem cell tumor	ROS sensor CellRox	(128)
KRAS^{G12V},*SCRIB*^{inact}	*ras*^{G12V},*scrib*$^{-/-}$	*ey* (eye disc)	Elevated ROS/ Reduced ROS under *ras*^{G12V} regulation	Loss of cell polarity	MitoSOX	(129)
MYC^{act},*PI3K*^{act}	*dMyc*^{OE},*Pi3K92E*^{OE}	*hh* (wing disc)	Elevated ROS	Field cancerisation	DHE	(130)
n/d	*brat*^{RNAi}	*pnt*, *ase* (brain stem cells)	Elevated ROS	Brain stem cell tumor	ROS sensor CellRox	(131)
HIPK^{act}	*Hipk*^{OE}	*dpp* (wing disc)	Elevated ROS	Accumulated hyperpolarized mitochondria	DHE	(132)
KRAS^{G12V}	*ras*^{G12V}	*esg* (intestinal stem cells)	Elevated ROS/ Reduced ROS under *ras*^{G12V} regulation	Intestinal stem cell tumor	ROS sensor RoGFP2	(70)
BRAF^{act}	*Raf*^{act}	*esg* (intestinal stem cells)	Elevated ROS	Intestinal stem cell tumor	DHE	(133)
YAP1^{act}	*yki*^{OE}	*GMR*,*ey*,*dpp* (adult eye)	Elevated ROS	Cardiac dysfunction	DHE	(134)
NOTCH^{inact}, *ITGB1*^{inact}	*N*^{RNAi},*mys*^{RNAi}	*esg* (intestinal stem cells)	Elevated ROS	Intestinal stem cell tumor	DHE, MitoSOX, *GstD-GFP*	(21)
NOTCH^{act}	*N*^{OE}	*1407* (brain)	Elevated ROS	Brain tumor	DCFH-DA	(135)

ETC, Electron transport chain; Su(H), Suppressor of Hairless ISC, intestinal stem cell; BSC, brain stem cell; act, activation; inact, inactivation; OE, overexpression; n/d, not determined. *: Authors did not mention its ortholog in humans.

induced (139). These data suggest that a shift of normal glucose metabolism toward the Warburg effect is essential to promote tumorigenesis.

Another study also identified a relationship between Notch signaling pathway and the Warburg effect. The authors demonstrated how the Warburg effect causes cell proliferation using eye disc as a model tissue (141). They first performed genetic modifier screening with RNAi fly strains (109 genes in total) against *Notch*-induced fly tumor models with overexpression (OE) of *Drosophila* Notch ligand Delta (Dl) in eye discs [*eyeless* (*ey*)>*Dl*^{OE}]. This screening revealed that *COX7a* (a mitochondrial respiratory chain subunit Cytochrome C-oxidase subunit 7a) was a key enhancer of eye cell proliferation induced by Notch activation. Furthermore, they found that *COX7a* knockdown

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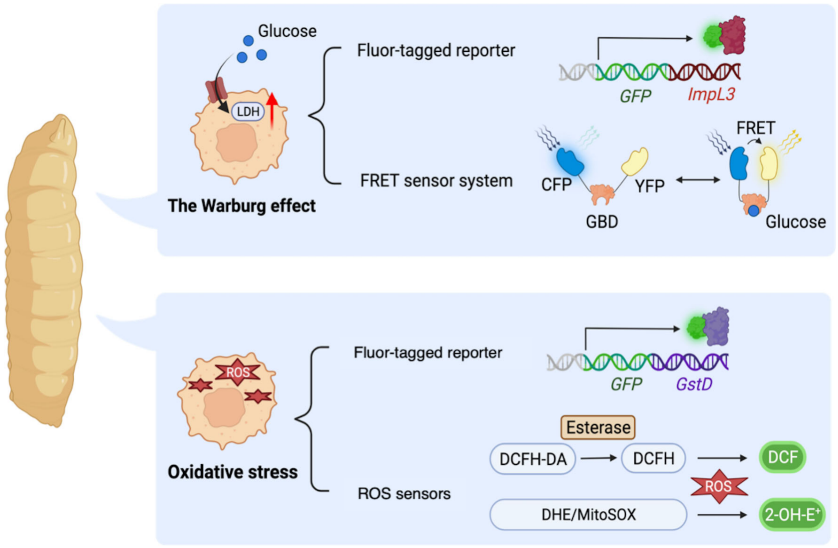

FIGURE 3
Drosophila methodologies to monitor metabolic alterations in a whole-body manner. *ImpL3-GFP*: a GFP-based enhancer trap reporter strain which enables easy detection of endogenous *ImpL3* expression in fly tissues. Förster resonance energy transfer (FRET)-based glucose sensor: a reporter strain carrying a glucose-binding domain (GBD), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP). FRET sensor determines the intracellular glucose level upon binding of glucose to GBD, which in turn changes the GBD's structure to increase the FRET efficiency (the ratio of YFP to CFP). *GstD-GFP*, a GFP-based endogenous *GstD* expression reporter strain to monitor oxidative stress response. DCFH-DA, 2′,7′-Dichlorofluorescin diacetate, a cell-permeable ester that can be hydrolyzed intracellularly by esterases to become DCFH which reacts with H_2O_2 and turns into highly fluorescent DCF. DHE, dihydroethidium which forms a highly fluorescent product 2-hydroxyethidium (2-OH-E^+). MitoSOX, a DHE derivative with an additional triphenylphosphonium group to target mitochondria monitoring ROS within mitochondria.

attenuated the mitochondrial electron transport chain (ETC), and this attenuation resulted in Warburg-like metabolic changes such as upregulation of *ImpL3* expression as well as the intracellular lactate level. Moreover, they identified that upregulation of *ImpL3* activity resulted in lactate accumulation, which reduced intracellular pH level and then contributed to proliferation of these transformed cells. Therefore, this paper demonstrated that the Warburg effect may not only be a feature of glucose metabolism specific to transformed cells but also be a key promoter of cell proliferation. These papers prove that utilizing fly genetics disclose associations between genes or signaling pathways and the Warburg effect, which has potential to understand the fundamental roles of glucose metabolism in cancer cells.

Other than focusing on upregulation of *ImpL3* under oncogenic stress, a previous study demonstrated that ImpL3 itself is attributed to promotion of tumor-like phenotype in flies (103). In this paper, they demonstrated that *ImpL3* cooperated with *EGFR* to induce neoplasia. Specifically, co-expression of *ImpL3* with *dEGFR* in *apterous* (*ap*)>*dEGFR*, *ImpL3* flies led to tumor-like phenotypes in wing discs accompanied with increased level of MMP1 and loss of cell polarity. Additionally, they used the same flies to show that HSD promoted *EGFR*-induced neoplasia in an *ImpL3*-dependent manner. Consistently, concomitant activation of *LDHA* and *EGFR* was associated with poor patient prognosis in breast cancer, sarcoma, and gliomas (103). Therefore, flies contribute to understanding the molecular basis of the Warburg effect as well as prognostic markers for cancer patients. It is interesting to speculate that these mechanisms provide links between cancers and high sugar conditions such as DM.

In summary, flies share regulators and processes in glucose metabolism with humans. Thus, flies are useful for elucidating the mechanisms of glucose metabolism and its relationship with tumorigenesis. What makes this possible includes various whole-body tools to monitor glucose metabolism such as *ImpL3-GFP* reporter and FRET systems.

Redox metabolism

Reactive oxygen species (ROS) is a group of highly reactive and heterogeneous molecules, including superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet$OH), which are reduced oxygen generated from electron-leakage in the electron transport chain (121, 142). In a normal

cell, ROS homeostasis is well sustained by the balance between ROS production and numerous detoxification processes regulated by antioxidant enzymes (124). On the other hand, the story is quite different in cancer cells: oxidative stress caused by excessive amounts of ROS can lead to oxidative modification-induced damage in intracellular macromolecules, which accumulates over time and ultimately causes cell death (9).

Along with aerobic glycolysis shown in section 4.1 (the Warburg effect), cancer cells also undergo reprogramming of mitochondrial metabolism, which causes the loss of redox homeostasis mainly by excessive production of ROS (Figure 2) (143). Indeed, a study reported that almost all cancer cells exhibited elevated levels of endogenous ROS (144). As such, oxidative stress is a result of metabolic reprogramming and is also known to be an important factor in tumor progression. In other words, ROS is a double-edged sword for tumor progression depending on its concentration. Namely, mild elevation of ROS makes it a second messenger necessary for many aspects of tumor development and progression (145). For example, low concentration of ROS stimulated proliferation of cultured human cells of various cancers such as breast and ovarian cancers by directly inhibiting GDP/GTP exchange within RAS hence activating RAS-ERK1/2 signaling through oxidative modification (145, 146). In contrast, high concentration of ROS is toxic to cancer cells by directly inducing cancer cell death through senescence, apoptosis, and ferroptosis (144, 145). Abundant ROS also inhibits cancer progression by sensitizing drug-resistant cancer cells (144). In addition, previous studies have revealed that exogenous H_2O_2 triggered cancer cell death with a high basal level of ROS in the pancreas and brain (147–149). In pancreatic cancer cells in particular, intracellular elevation of antioxidants derived from increased activity of antioxidant proteins is a prerequisite for the occurrence of tumor hallmarks including cell proliferation and metastasis (147). However, the complexity of ROS in cancers remains to be an important question to be addressed.

Therefore, researchers tried different ways to observe metabolic impacts of ROS. Increasing number of fly studies indicated that *Drosophila* is a well-suited model organism to study metabolic reprogramming in the redox process. This is because over 90% of ROS is derived from energy metabolism in mitochondria fly regulators of which are highly conserved with humans (150, 151). In fact, the utilization of flies as a whole-body organism to reveal the redox process in diseases including cancers has increased over the past few years. Previous studies of aging (152), obesity (93), diabetic retinopathy (153, 154), and neurodevelopmental diseases (155) have successfully modeled in flies the oxidative stress in human diseases.

On the other hand, emerging diversity of methodologies in flies to quantify the ROS amount also offers advantages to make *Drosophila* a suitable model for understanding redox metabolism. As mentioned above, metabolites and metabolic pathways in flies and mammals are highly conserved. Therefore, many established tools for studying metabolic changes in mammals can be directly applied to fly studies (156). So far, there exist a variety of tools established to directly measure intracellular ROS. For example, dihydroethidium (DHE) is one of the most frequently used dyes which fluoresces upon oxidation by superoxide (157). In addition, MitoSOX is used to distinguish the sources of ROS as a modified version of DHE with a mitochondrion-targeting group to observe ROS that mitochondria generate (156, 158). Another widely used fluorogenic probe for oxidative stress in mammals is 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) probe, which also proved efficient in flies (153, 155, 159, 160). Furthermore, as a useful genetic tool in flies, the reporter gene *GstD* can easily quantify intracellular ROS levels. The fly *GstD* is an oxidative stress response gene encoding for glutathione S-transferase (161). Since expression of *GstD* is positively correlated with intracellular oxidative stress, transgenic flies carrying *GstD-GFP* are developed to conveniently evaluate intracellular ROS levels in disease models (Figure 3) (71, 162).

For the past several years, an increasing number of fly studies unraveled ROS-related redox metabolic reprogramming in cancer cells with various genotypes. Indeed, these studies encompassed a wide range of signaling pathways (Table 2). For example, a group established a glycolytic tumor model in flies by activating the oncogenic *Pvr* (126). In this model, they found that excess ROS produced in transformed cells functioned as a feedback signal to consolidate glycolytic metabolic reprogramming. Moreover, *dMyc* induction in wing disc epithelium increased ROS substantially, which may transduce pre-cancerization effect by dMyc to adjacent tissues (130). In addition, *Hipk*-overexpression induced ROS in transformed cells by inhibiting mitochondrial energetics, which exacerbated tumors by potentiating JNK and its downstream MMP1 (132). Strikingly, almost all tumorigenic mutations tested thus far produced extra ROS in transformed cells regardless of tissue types in *Drosophila*. Exceptions include ras^{G12V} which suppressed ROS production (70, 129), and this outcome is in accordance with ROS detoxification *via* the RAS-RAF-NRF2 pathway (10).

Moreover, loss of cell polarity caused by elevated ROS is a well-studied phenotype related to redox metabolism (124). The discovery of important genes regulating cell polarity in *Drosophila* makes it possible to establish fly cancer models with loss of such regulators to unravel metabolic reprogramming in tumor cells that had lost their polarity (125, 127, 129, 163). For example, *discs-large* (*dlg)* and *scrib* are two important genes to maintain cell polarity in flies (164). Loss of *dlg* in epithelial cells of larval wing discs causes overgrowth due to loss of cell polarity. In addition, DHE staining of wing discs demonstrated higher superoxide levels in those transformed cells (125). Likewise, ras^{G12V},$scrib^{-/-}$ flies mimic loss of cell polarity in the context of cellular transformation (129) (127, 163). Though transformed cells carrying ras^{G12V} or $scrib^{-/-}$ alone did not show

intracellular oxidative stress, transformed cells with the concurrence of ras^{G12V} and $scrib^{-/-}$ produced ROS by structurally damaged mitochondria (129). Furthermore, another group investigated the role of ROS in signaling pathways of transformed cells and demonstrated that ras^{G12V}-activated caspases increased intra- and extra-cellular ROS rather than inducing apoptosis in transformed cells. These results indicated that ROS promoted a caspase-triggered amplification loop and promoted tumor progression (127).

Besides these ROS alterations under loss of cell polarity, elevated ROS also showed up in transformed cells in flies modeling brain cancer genotypes (128, 131, 135). A study established a tumor model *da>brat*^{RNAi} to show elevated levels of ROS and chromosomal instability (CIN) by depleting the *brain tumor* (*brat*) gene in the brain of third instar larvae using the *daughterless* (*da*) driver which is active in fly neurons. Moreover, extracellular antioxidants blocked overgrowth of *brat*-deleted tumors, showing the essential role of ROS elevation in CIN-dependent tumorigenesis. Hence, accumulated ROS can be a vulnerability for CIN-dependent tumors that can be targeted by metabolic intervention (128). However, whether accumulated ROS promotes tumor cell proliferation is currently inconclusive. In another study focusing on brain cancers, authors developed *brat*-deleted tumors by using *Pointed* (*pnt*) and *Asense* (*ase*) drivers targeting neuroblasts in larval brains of *pnt>brat*^{RNAi} and *ase>brat*^{RNAi} flies. Their results showed that scavenging ROS by antioxidant treatment did not affect the tumor progression, though the tumors contained significantly elevated ROS than normal larval brain. Instead of oxidative stress by ROS, reprogrammed redox homeostasis of NAD^+/NADH is primarily required for *brat*-deleted tumors to become immortalized (131). Therefore, the role of ROS elevation led by *brat*-deletion in fly neuroblasts still remains unraveled needing further investigation. On the other hand, a study induced *Notch* overexpression using the *1407*-GAL4 driver to develop proliferative transformed cells in the brain of *1407>N*^{OE} flies. These transformed cells exhibited elevated ROS production triggered by Notch-RET-signaling to contribute to Notch-induced neoplastic transformation (92, 135).

With the established ISC tumors that *esg*-GAL4 drives in flies, there have been multiple studies on the role of ROS under various physiological and pathological conditions (133). For example, a study identified an intrinsic homeostatic range of ROS in ISCs, indicating that the intracellular redox level is a critical determinant of cancer cell fate (71). In this study, tumor-like ISCs induced by depleting *Notch* in *esg>N*^{RNAi} flies and extracellular matrix (ECM)-deprived ISCs induced by depleting *β-integrin* (*mys*) in *esg>mys*^{RNAi} flies exhibited proliferative phenotype under a moderate increase of ROS. On the other hand, ISCs with both *N*- and *mys*-reduction (*esg>N*^{RNAi},*mys*^{RNAi}) displayed metastatic phenotypes accompanied by even higher ROS levels with cytotoxic oxidative stress (71). Additionally, another paper focused on tumor microenvironment regulating ROS. The authors utilized *esg>Raf*^{act} flies modeling benign gut tumors by targeting a constitutively active form of *Raf* to adult intestines (133). They confirmed that autophagy in cells around neoplasia was induced downstream of elevated ROS and activated JNK signaling in tumor cells. Transformed cells had significantly increased ROS, while ROS elevation was mild in their neighboring cells. Intriguingly, sparing expression of the antioxidase catalase gene in transformed cells efficiently blocked autophagy in surrounding cells and inhibited tumor proliferation (149).

Beside revealing the role of intracellular ROS in tumor cells, there are studies using *Drosophila* to identify the relationship between tumor-derived ROS and cardiac dysfunction. For example, fly models with *yki*-overexpression had a systemic increase in ROS, which resulted in compromised cardiac function (134).

To summarize, flies share conserved redox metabolism pathways with humans, and previous studies have provided novel insights into cancer redox metabolism using fly models of cancer genotypes. Emerging diversity of methodologies in flies to evaluate redox metabolism in transformed cells provides flies with potential in elucidating the mechanisms of cancer redox metabolism and its relationship with carcinogenesis.

Conclusion

In this review, we highlighted *Drosophila* studies on cancer demonstrating the cancer mechanisms and unique metabolism. Recently, we have access to flies produced to carry a variety of cancer driver mutations. These flies have surpassed the usage in studying cancer signaling pathways and contributed to drug discovery in a high-throughput manner. Furthermore, the broad application of flies in metabolic disease research has demonstrated that the high similarity between fly and human metabolism allows for the reproduction in flies of characteristic metabolic changes in human diseases to elucidate their mechanisms and their impact on concurring diseases. Based on this idea, many studies have come up with new insights into cancer metabolism by analyzing fly models for various cancer genotypes carrying markers. Therefore, we expect that *Drosophila* keeps playing a significant role in our future exploration of the nature of cancer as a systemic disease and in providing candidate targets for novel therapeutics against notorious cancers.

Author contributions

HJ, TK, HH: Writing—Original draft, Writing—Review and editing. MS, RY: Conceptualization, Writing—Review and editing, Project administration, Funding acquisition.

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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Tumor acidity: From hallmark of cancer to target of treatment

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Tumor acidity is one of the cancer hallmarks and is associated with metabolic reprogramming and the use of glycolysis, which results in a high intracellular lactic acid concentration. Cancer cells avoid acid stress major by the activation and expression of proton and lactate transporters and exchangers and have an inverted pH gradient (extracellular and intracellular pHs are acid and alkaline, respectively). The shift in the tumor acid–base balance promotes proliferation, apoptosis avoidance, invasiveness, metastatic potential, aggressiveness, immune evasion, and treatment resistance. For example, weak-base chemotherapeutic agents may have a substantially reduced cellular uptake capacity due to "ion trapping". Lactic acid negatively affects the functions of activated effector T cells, stimulates regulatory T cells, and promotes them to express programmed cell death receptor 1. On the other hand, the inversion of pH gradient could be a cancer weakness that will allow the development of new promising therapies, such as tumor-targeted pH-sensitive antibodies and pH-responsible nanoparticle conjugates with anticancer drugs. The regulation of tumor pH levels by pharmacological inhibition of pH-responsible proteins (monocarboxylate transporters, H^+-ATPase, etc.) and lactate dehydrogenase A is also a promising anticancer strategy. Another idea is the oral or parenteral use of buffer systems, such as sodium bicarbonate, to neutralize tumor acidity. Buffering therapy does not counteract standard treatment methods and can be used in combination to increase effectiveness. However, the mechanisms of the anticancer effect of buffering therapy are still unclear, and more research is needed. We have attempted to summarize the basic knowledge about tumor acidity.

KEYWORDS

cancer, metabolism, acidity, hallmark, treatment target

Introduction

Cancer cells have an inverted pH gradient: extracellular and intracellular pHs (pHe, pHi) are acid and alkaline, respectively (1). The acid shift in the tumor microenvironment (TME) is closely associated with hypoxia (2) but, more specifically, with highly activated glycolysis in tumor cells. Even in normoxia, about 80% of all

malignant tumors use aerobic glycolysis, described as the Warburg effect (3), which is an integral part of metabolic reprogramming and sustaining biosynthetic pathways in cancer cells (4).

According to the present knowledge, the shift in the tumor acid-base balance promotes proliferation, apoptosis avoidance, invasiveness, metastatic potential, aggressiveness, immune evasion, and treatment resistance (5–8). On the other hand, inversion of the pH gradient in tumors could be a weakness that will allow for the development of new promising therapies (Figure 1). It is possible to create acid stress inside cancer cells by inhibiting proton release systems or by using drugs that decrease mitochondrial activity to increase lactate production (5, 9, 10). The acidity of the TME could be used for the drug delivery of cytotoxic agents and/or carriers that are more active and/or change physicochemical properties under such conditions (11–13). It is very attractive to increase the pHe by a combination of an alkaline diet and bicarbonate therapy (14–16) or by direct local isolated perfusion of the tumor with bicarbonate solutions (17, 18).

Obviously, the altered acid-base state of the tumor affects every stage of cancer development, from dysplasia to metastatic disease (1, 2). In this mini-review, we have attempted to summarize the basic knowledge about tumor acidity from hallmark of cancer to target of treatment.

Tumor acidity as a hallmark of cancer

One of the causes of tumor heterogeneity is altered tumor vasculature, which leads to different perfusion of nutrients and oxygen and to the accumulation of acidic metabolites (19, 20). Due to the reprogramming of metabolism in such conditions and the use of glycolysis as a major source of ATP production, tumor cells have an acidic pHe (6.4-7.1) and an alkaline pHi (7.1-7.8). For normal tissues, the pHe is around 7.4, and the pHi is around 7.2 (2, 21). Large amounts of lactate produced during glycolysis result in a significant increase in the intracellular proton (H+) concentration. It should be noted that glutaminolysis is another way for ATP production and an additional source of lactate and H+ in cancer cells (21–24). In addition, glutamine uptake and metabolism in oxidative cancer cells can be promoted by lactate (25). However, even in the presence of oxygen, glucose is almost completely converted into lactate. At the same time, glutamine is not fully respired, but it is rather fermented into lactate or pyruvate. Increased glutamine flux can enhance aerobic glycolysis and make it optimal for tumor proliferation (22, 26).

As acid stress triggers apoptosis (27), cancer cells use several ways to evade it (28). Activation and expression of H+ (and lactate) transporters and exchangers are the main mechanisms of tumor cell adaptation to intracellular acidification and of the inverted pH gradient phenomenon (29–32). It should be noted that not only H+ ejection systems lead to an increase in the pHi, but also a reduction of CO_2 by decreased activity of the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS) (1, 10). Carbonic anhydrases (CAs) additionally support the pH regulation of cancer cells by catalyzing the reversible hydration of CO_2 to HCO_3– and H+ (32).

Acidosis of the TME is an essential stage associated with high rates of tumor cell proliferation (33). Numerous studies have shown a role for tumor acidity in acquiring aggressive cancer characteristics, so it is recognized as a hallmark of cancer (21, 31, 34–36). For example, melanoma cells exposed to acidosis are characterized by a high invasive potential, high resistance to apoptosis and drug therapy, fixed independent growth, and a phenotype of epithelial to mesenchymal transition (37). Under

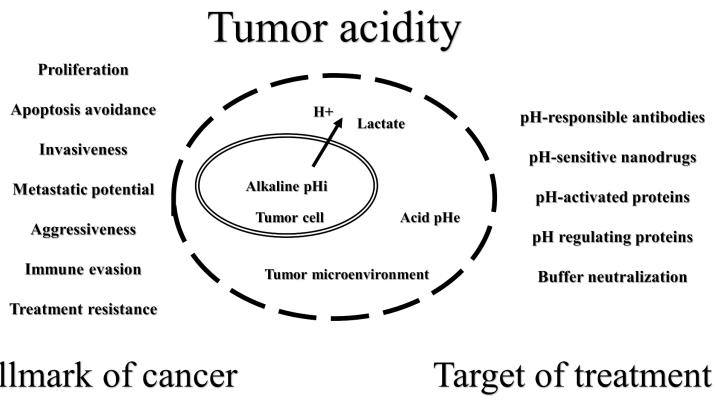

FIGURE 1
Schematic representation of tumor acidity properties.

growth factor limitations, alkaline pHi favors cancer cells survival (38). The acid adaptation of tumor cells leads to a gene expression response that correlates with human cancer tissue gene expression profiles and survival (39). Acidic TME improves the activity of regulatory T-cells and inhibits effector T-cells (40). In view of the foregoing, acidic TME could serve as an incubator that represses overabundant proliferation and cultures cells with a restricted growth rate but with strong proliferative potential (41). Clinicians should consider tumor acidity when diagnosing and determining optimal treatment, as it is also connected with poor cancer patients prognosis (39).

A wide range of non-invasive and minimally invasive imaging modalities have been studied preclinically for tumor pH monitoring, including magnetic resonance imaging (MRI) and spectroscopy, positron emission tomography, electron paramagnetic resonance, and optical and photoacoustic imaging (42). To date, among the methods used, MRI appears to be the most promising, particularly chemical exchange saturation transfer (CEST) MRI, which has good *in vivo* sensitivity for assessing tumor acidosis and changes in pH after therapeutic treatment, with a high spatial resolution to determine the heterogeneity of extracellular acidification. For example, CEST MRI has been used successfully to map tumor pH in a rabbit liver cancer model (43). In another study, tumor acidosis assessed by CEST MRI revealed the metastatic potential of breast cancer in mice (44). Translating the results of preclinical studies into clinical trials is only beginning to yield significant results. CEST MRI shows good results for measuring pH in ovarian cancer patients (45). In addition, CEST MRI has recently been shown to differentiate between benign and malignant liver tumors in patients (46). However, it is still difficult to routinely measure the pH of tumors in the clinic. In addition to direct measurements, tumor acidity can be assessed indirectly by determining the concentrations of bicarbonate (47) and lactate ions in the blood and using biopsy data (48). However, each clinical situation requires an individual approach.

Tumor acidity and tumor resistance

Cancer cell survival strategies in acidic TME promote resistance to radiation and chemotherapy. Radioresistance is closely related to hypoxia. Available clinical data show that the presence of large hypoxia areas in solid tumors is associated with a poor prognosis in cancer patients after radiotherapy (49). The cytotoxic effects of ionizing radiation are mainly due to damage to genomic DNA as a result of the indirect action of generated free radicals (50). Molecular oxygen must be present during irradiation, which is insufficient under hypoxic conditions. Hypoxia also prevents DNA repair and leads to the inhibition of the G1/S cell cycle checkpoint, an increase in DNA errors, and an increase in chromosomal instability. At the same time, the alkaline pHi of tumor cells prevents mitotic arrest initiated by activated checkpoints during DNA damage (21, 51). Thus, the inversion of the pH gradient of the tumor is a "partner" of hypoxia in creating conditions for radioresistance, and clinicians should consider acidic TME in the planning of radiation therapy.

Acidic TME itself can lead to chemoresistance due to ongoing physicochemical changes in the structure and charge of drugs. Weak-base chemotherapeutic agents, such as vincristine, mitoxantrone, doxorubicin, vinblastine, and paclitaxel, may have substantially reduced cellular uptake capacity due to neutralization or protonation ["ion trapping" (52)]. Therefore, the cytotoxic effects of these drugs may be reduced, resulting in a stable tumor phenotype. Interestingly, reversing the pH gradient may increase the intracellular concentrations of some weak-acid drugs, including cyclophosphamide and chlorambucil (53–56). Acidic TME induces p-glycoprotein (multiple drug resistance (MDR) protein) activity by promoting p38 mitogen-activated protein kinase (57–59). Tumor acidosis induces the expression of the transcription factor SOX2 by inhibiting vitamin D receptor-mediated transcription, which also results in drug resistance (60). Oxidation-induced lactic acidosis increases resistance to uprosertib, a serine/threonine protein kinase inhibitor, in colon cancer cells (61). To obtain the maximum effect of chemotherapy, the acidity of the TME must be considered.

Current knowledge strongly suggests that acidic TME inhibits the antitumor immune response, although the complication of experimentally measuring tumor acid-base status makes it difficult to obtain direct evidence (7, 62). For instance, a decrease in the pHe leads to a decrease in the activity and proliferation of T cells (63, 64). In an acidic environment, effector T cells require higher thresholds for full activation and co-stimulatory signals (e.g., CD28) and show increased negative regulatory signaling through upregulation of interferon gamma receptor 2 (IFN-γR2) and cytotoxic T cell-associated protein 4 (CTLA-4) (64). Acidic extracellular conditions reduce the expression of T-lymphocyte receptor components (65). Since the movement of lactate between the cytosol and the extracellular space depends on its concentration gradient, a high concentration of extracellular lactate in the TME prevents the export of lactate from T cells. This negatively affects the functions of activated T-lymphocytes dependent on glycolysis for ATP production (66). Notably, the functions of effector T-lymphocytes could be restored after normalization of pH (65–69), so the acidity does not have a cytotoxic effect. A significant effect of low acidity appears to be its negative effect on effector cytokines production by T cells, which is significantly reduced under acidic conditions (70–72). However, receptor interactions also play an important role. For example, in acidic TME, the V-domain Ig suppressor of T cell activation (VISTA), which is expressed by tumor-infiltrating myeloid suppressor cells, is activated and suppresses effector T cells (73). The inhibitory effect of acidic TME on dendritic cells is not related to the high

concentration of H+, which actually stimulates antigen presentation (74). This inhibition can be explained by the accumulation of lactate, which modulates the dendritic cell phenotype and causes increased production of anti-inflammatory (e.g., IL-10) and decreased production of pro-inflammatory (e.g., IL-12) cytokines (75, 76). An acidic pHe and a high concentration of lactate together lead to a decrease in the activity of natural killers, including the depletion of interferon gamma (IFN-γ) and their ability to infiltrate the tumor (71, 77, 78). At the same time, for example, an acidic environment stimulates regulatory T cells (Tregs) activity by involving lactic acid in metabolism (79). In addition, lactic acid promotes Tregs' expression of programmed cell death receptor 1 (PD-1) by absorption through monocarboxylate transporter 1 (MCT1). Thus, the PD-1 blockade activates PD-1-rich Tregs, resulting in treatment failure (80). Besides this, the acidity of the TME upregulates programmed death ligand 1 (PD-L1) in tumor cells (81).

It seems clear that the acidic conditions of the TME must be considered in monoclonal antibodies (mAbs) anticancer therapy. On the one hand, slightly acidic conditions are probably optimal for most mAbs (82), i.e., acidity in solid tumors may only slightly influence the deterioration of the therapeutic properties of mAbs. On the other hand, the possibility of the degradation of mAbs under such conditions cannot be excluded (7). For example, the rate of antibody Fc fragment oxidation and aggregation, which determines antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), has been shown to increase with decreasing pH (83, 84). Despite the fact that cancer immunotherapy uses immune checkpoint-blocking mAbs that are specifically modified to eliminate interactions with Fc receptors, fatal changes in other parts of mAbs that determine their activity are also possible at low pH values. For example, the chemical degradation of aspartic acid induced by acidic pH in the complementarity-determining region (CDR) of a monoclonal antibody against the epidermal growth factor receptor (EGFR) causes a loss of antibody-binding activity (85). The high structural and physicochemical affinity of mAbs to their targets is a condition for achieving a therapeutic effect. In particular, histidine residues in interacting sites can increase pH-mediated dissociation due to protonation under acidic conditions, favoring electrostatic repulsion between rigid domains in protein–protein interaction (86). The low pHe can also greatly affect the bioavailability of therapeutic mAbs. At the same time, the "useful" side of acidic TME is the possibility of creating therapeutic pH-selective mAbs (87, 88).

Tumor acidity as a target of treatment

Tumor-targeted pH-sensitive antibodies should be screened for low pH activity, and antibody engineering should not be limited to finding molecules with activity over a wide pH range (87). For example, despite the pH-independent affinity of CTLA-4 for ipilimumab, an analog was developed with up to a 50-fold affinity for CTLA-4 at pH 6.0 compared to pH 7.4 (89). A bispecific pH-responsive anticarcinoembryonic antigen-related cell adhesion molecule (CEACAM) 5 antibody that binds pH-independently to CEACAM6 was generated (88). Likewise, acidic TME allows for pH-activated molecular targets, such as VISTA. A combination of anti-VISTA mAb with anti-PD-L1 therapy demonstrated a significant survival benefit in tumor-bearing mice (90). Nanotechnologies also provide a good tool for creating pH-responsible anticancer drugs based on pH-responsible polymer nanomaterials, nanogels, etc. (91, 92). It has recently been summarized that several types of pH-sensitive nanoparticle conjugates with paclitaxel, doxorubicin, or others enhance drug delivery and potentiate anticancer effects in various experimental cancer cell lines (93).

Another approach to influencing tumor aggressiveness and/or therapeutic response is the regulation of tumor pH levels. First, since glycolysis is the main source of lactate and H+, would it be possible to reduce lactate production by limiting glucose? Also considering that hyperglycemia is known to be associated with reduced survival rates in some types of cancer (94–97), although this is still controversial, for example, in pancreatic (98–100) or colorectal cancers (101–103). Indeed, glucose restriction can reverse the Warburg effect and decrease lactate production *in vitro* (104). However, cancer cells can also use glycogenolysis, glycogen synthesis, and gluconeogenesis to compensate for glucose starvation (105–107). Many therapies targeting glucose metabolism (e.g., targeting glucose transporters, glycogen phosphorylase, glycogen synthase kinase 3β, hexokinase 2, glucose-6-phosphate isomerase, etc.) have been developed, but have not yet been successful in clinical trials (107). Furthermore, glycolysis is the main metabolic pathway of neutrophils, M1 macrophages, dendritic cells, naive T cells, effector T cells, etc. (108). For example, glucose-deficient TME limits the anaerobic glycolysis of tumor-infiltrating T cells and thus suppresses tumor-killing effects (109). Nutritional deficiencies in the TME, especially glucose, impair the metabolism of NK cells and their antitumor activity (110). It is important to note that human glucose levels may be reduced to very low levels without causing harm (111), and ketone bodies can be used for energy production with benefits for the organism (112, 113). For instance, a ketogenic diet improves the function of T cells (114, 115) and possibly creates an unfavorable metabolic environment for cancer cells (116, 117). However, ketone bodies utilization or formation may be a promoter for tumor cells proliferation and metastasis (118–121). Therefore, limiting glucose or its metabolism to reduce lactate production can have a completely ambiguous effect.

A more optimal way to reduce lactate production seems to be the inhibition of lactate dehydrogenase A (LDHA). This approach provides the simultaneous restriction of lactate synthesis from both glycolysis and glutaminolysis. Indeed, the

inhibition of LDHA *in vivo* redirects pyruvate to support OXPHOS (122, 123). To date, a large number of LDHA inhibitors have been studied preclinically, but unfortunately, the clinical utility of such inhibitors may be limited due to nonselective toxicity or complex interactions with other cellular components. Optimization of existing compounds and continued search and development of new LDHA inhibitors will be reasonable strategies to obtain direct antitumor effects or enhance, for example, immunotherapy results (48, 124, 125). For example, since the effect of immunotherapy can be prevented by lactate (79, 80) and high LDH levels before treatment are correlated with a poor response to immunotherapy (126, 127), inhibition of LDHA can improve the efficacy of anti-PD-1 therapy (128).

Alternate modality to regulate tumor acidity is the pharmacological inhibition of proteins responsible for regulating pHi or mitochondrial activity (5, 9, 10). For example, inhibition of mitochondrial pyruvate transporter (MPC) works to block lactate utilization while preventing oxidative glucose metabolism (129). Blocking the monocarboxylate transporter 1 (MCT1) (used to import lactate as an energy source in oxidative cancer cells) with the specific MCT1 inhibitor AZD3965 prevents lactate consumption, increases its concentration in the TME, and has an antiproliferative effect (130–132). Conversely, the inhibition of MCT4 (expressed to remove lactate in glycolytic tumor cells) causes intracellular lactate accumulation, a decrease in pHi, but also reduces tumor growth *in vitro* and *in vivo* (132, 133). The cooperative use of MCT1/MCT4 inhibitors or nonspecific MCT inhibitors has good therapeutic potential (125, 132, 134, 135). Also of great importance to decrease pHi values is the pharmacological inhibition of the proton pump H^+-ATPase (136), sodium-hydrogen antiporter 1 (NHE1) (137), and carbonic anhydrase IX (CAIX) (138). For example, according to the results of a phase III clinical trial (NCT01069081), intermittent use of a high dose of the proton pump inhibitor esomeprazole potentiates the effects of docetaxel and cisplatin chemotherapy in metastatic breast cancer without causing further toxicity (139). In a retrospective study, omeprazole was found to have a synergistic effect with chemoradiotherapy and to significantly reduce the risk of rectal cancer recurrence (140). Other ion exchangers and transporters are involved in tumor pH regulation, but their role in cancer progression remains unclear (2).

Another way to affect tumor acidity is the use of buffer systems, such as sodium bicarbonate. Preclinical and some clinical studies suggest that "direct" tumor deacidification may slow progression or improve therapeutic response (34). Oral administration of sodium bicarbonate can increase the efficacy of doxorubicin and mitoxantrone in model experiments (52, 55). Furthermore, peroral administration of sodium bicarbonate and other buffer solutions significantly reduced the invasion and metastasis of various experimental (including spontaneous) tumors in genetically modified animals but had no effect on the growth of primary tumors (141–146). Neutralization of tumor acidity improved the antitumor response to anti-CTLA-4 and PD-1 mAbs, as well as the adoptive transfer of T-lymphocytes in experiments using the B16 melanoma model and Panc02 pancreatic cancer in mice (69).

At the same time, the first three clinical trials of oral sodium bicarbonate (NCT01350583, NCT01198821, NCT01846429) to improve outcomes and reduce pain in pancreatic adenocarcinoma failed due to poor taste sensation and gastrointestinal disturbances, resulting in bad compliance (147). However, a recent clinical study successfully examined the effect of alkalinization therapy (an alkaline diet supplemented with oral sodium bicarbonate) in combination with chemotherapy on the survival of patients with advanced pancreatic cancer (UMIN 000035659). The median overall survival rate in patients whose urine pH became high (>7.0) after the start of therapy was significantly greater than in patients with low urine pH (≤7.0) (16.1 vs 4.7 months; $p<0.05$) (14). In another study (UMIN000043056), the combination of alkalinization therapy with intravenous vitamin C was also associated with favorable outcomes in patients with small cell lung cancer (SCLC) receiving chemotherapy. The median overall survival for the intervention group was 44.2 months vs. 17.7 months for the control group (15).

Parenteral administration of buffer systems to directly neutralize tumor acidity is also of great importance, but it must be done under the close supervision of medical personnel and can have some serious side effects (148). The use of nanoobjects to deliver buffers due to the enhanced permeability and retention effect (EPR) can overcome such limitations (149). For example, the administration of sodium bicarbonate-loaded liposomes in combination with subtherapeutic doses of doxorubicin in mice with triple-negative breast cancer resulted in a superior therapeutic response compared to drug administration alone (150). Performing an isolated infusion or perfusion of the tumor with buffer solutions is another option. In the ChiCTR-IOR-14005319 clinical study, the efficacies of transarterial chemoembolization (TACE) with or without local administration of 5% sodium bicarbonate solution in patients with large-focal hepatocellular carcinoma were compared. In the case of sodium bicarbonate, the objective response rate (ORR) was 100% vs. 44.4% in the case of conventional TACE in a nonrandomized cohort and 63.6% in a randomized study (151). In a preclinical study, it was found that intraperitoneal perfusion with 1% sodium bicarbonate solution significantly prolonged overall survival in mice with the ascitic form of Ehrlich's adenocarcinoma (median survival, 24 vs. 17 days; $p < 0.05$) when compared to 0.9% sodium chloride solution (18). In another study, perfusion was performed with a 4% sodium bicarbonate solution of rat limbs with a Pliss lymphosarcoma graft. The median survival in the sodium bicarbonate group was

17 days, while in the nonperfused group and in the isotonic saline group it was 13 days (17).

The mechanisms of the anticancer effects of alkalization (buffering) therapy remain unclear. While the improved chemotherapeutic effect can be explained by "ion trapping" (53, 54), the antitumor, antimetastatic, and immunotherapy-enhancing effects of buffered therapy may be much more complex and have been studied predominantly as a phenomenon until now. Buffering of the TME can reduce the optimal conditions for enzymes involved in tumor invasion, such as cathepsins and matrix metalloproteases (MMPs) (152). Neutralization of acidity in the TME can result in a reduction of PD-L1 expression, which is increased at low pH through proton-sensing G protein-coupled receptors (81). Neutralization of lactic acid with sodium bicarbonate reactivates metabolically altered (in an acid environment) T cells, enabling extracellular lactate as an additional source for their energy production (153). More research is needed on the mechanisms of the effectiveness of sodium bicarbonate and other buffer solutions in cancer patients. Alkalization (buffering) therapy does not conflict with standard treatment methods but can be used in combination to increase effectiveness (154).

Conclusion

Despite extensive studies on the acid-base status of malignant tumors over the past decades, the mechanisms of tumor adaptation to acidity, induction of invasion and metastasis, and the mechanisms leading to evasion of immune surveillance are still poorly understood. Further research in this direction is needed, including the development of approaches and drugs that directly or indirectly increase the pH of the TME for use in conjunction with chemotherapy, radiation therapy, and immunotherapy. However, it is clear that clinical options already exist to counteract tumor acidosis in patients. Additionally, the selectivity of acidosis in tumors versus healthy tissues holds promise for pH-activated or pH-targeted drugs, which are safer than traditional chemotherapy and are applicable to more cancers than many targeted drugs. Regardless of the complexity of the clinical assessment of the TME acidity, clinicians should consider acidosis in practice, and the continued development of methods for clinical assessment of tumor pH should allow for accurate diagnosis and selection of personalized treatment regimens.

Author contributions

AlB and VM contributed to the conception of the mini-review. AlB and AnB wrote the first draft of the manuscript. All authors contributed to the manuscript revision, read, 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.

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Clinical review of alkalization therapy in cancer treatment

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One of the most unique characteristics of cancer metabolism is activated aerobic glycolysis, which is called the "Warburg effect", and is a hallmark of cancer. An acidic tumor microenvironment (TME) resulting from activated anaerobic glycolysis is associated with cancer progression, multi-drug resistance, and immune escape. Several *in vitro* and *in vivo* studies reported that neutralization of the acidic TME by alkalizing agents, such as bicarbonate, resulted in the suppression of cancer progression and a potential benefit for anti-cancer drug responses. In clinical settings, alkalizing effects were achieved not only by alkalizing agents, but also by a following a particular diet. An epidemiological study demonstrated that more fruits and vegetables and less meat and dairy products are associated with an increase in urine pH, which may reflect the alkalizing effect on the body. However, it remains unclear whether alkaline dietary intervention improves the effects of cancer treatment. Moreover, there are few clinical reports to date regarding cancer treatments being performed on patients together with alkalization therapy. In this review, we investigated whether alkalization therapy, which includes an alkaline diet and/or alkalizing agents, improves cancer treatment.

KEYWORDS

cancer, cancer metabolism, tumor microenvironment, alkalization therapy, urine pH, chemotherapy

Introduction

There are numerous lines of evidence that pH gradient reversal, intracellular alkalization, and extracellular acidification are commonly seen in malignant tumors and are associated with the progression, metastasis, and multidrug resistance (MDR) of cancer cells (1–3). Activation of aerobic glycolysis, which is also known as the "Warburg effect", is a characteristic feature of cancer metabolism and a hallmark of cancer (4). Cancer cells require rapid adenosine triphosphate (ATP) generation to maintain their energy state, increase macromolecule biosynthesis, and maintain an appropriate cellular redox state for their survival and growth. Activated aerobic glycolysis produces reduced nicotinamide adenine dinucleotide phosphate, which is necessary to maintain redox

balance, and also acts as an antioxidant to protect against reactive oxygen species that are generated during rapid cancer growth (5). Therefore, aerobic glycolysis, which is a shift from ATP generation by oxidative phosphorylation to ATP generation by glycolysis, is observed even under normal oxygen concentrations (5–7). The constant increase in aerobic glycolysis is considered to be an adaptation to the hypoxia that occurs as precancerous lesions become increasingly distant from the blood supply (6). However, recent reports indicate that the glycolytic phenotype is an important component of the metabolic reprogramming of cancer cells that occurs early in carcinogenesis, i.e., before the development of tissue hypoxia (1, 5–7). Aerobic glycolysis can be caused by genetic instability, mutations, abnormal gene expression, or altered signaling pathways (1). Increased lactate production owing to increased glycolysis leads to acidosis of the extracellular tumor microenvironment (TME) (5, 6, 8). Moreover, the systemic extrusion of H^+ by different proton transporters, and the neutralization of protons in cancer cells by bicarbonate anions from the chloride bicarbonate exchanger are the main mechanism for reversing the pH gradient in cancer cells (5, 7, 9, 10). The extrusion of H^+ from cancer cells is positively regulated by several membrane-bound proton transporters, such as Na^+/H^+ exchanger 1 (NHE1), Na^+/K^+ ATPase pump, vacuolar H^+-ATPase (V-ATPase), H^+/Cl^- symporter, monocarboxylate transporter (MCT), and carbonic anhydrase (CA) (10).

Although emerging lines of evidence from both *in vivo* and *in vitro* studies suggest that the reversed pH gradient of cancer cells may be a promising new target of cancer treatment, the mainstream treatments for advanced cancer are chemo therapeutic drugs and molecularly targeted therapies, and there are few strategies aiming at the pH regulation of cancer cells in clinical settings. In this article, we aimed to summarize the association between the acidic TME and cancer treatments, and introduce several approaches of alkalizing the external TME and associated treatment strategies.

An acidic TME leads to resistance to cancer therapy

A direct cause and effect association among the degree of MDR, decrease in external tumor pH (pHe), and increase in internal tumor pH (pHi) has been reported, and the reversed pH gradient of cancer cells is known as a key factor in driving the progression of malignancy and resistance to conventional therapies (8, 11, 12). An *in vitro* study of human lung tumor cells demonstrated that a close to 2,000-fold increase in doxorubicin resistance was observed when the pHi increases from 7.0 to 7.4 (13). Furthermore, a decrease in pHe and increase in pHi mediated by proton-extruding mechanisms is responsible for not only the maintenance of MDR but also protection against the induction of apoptosis (14–16). P-glycoprotein, a drug efflux transporter, is regulated in a pH-dependent manner, and a decrease in pH of the TME has the potential to enhance its efflux function (17, 18). Moreover, the uptake of weakly basic chemotherapeutic drugs by tumors is highly affected by the pH of the TME and the ionization properties of the drug (19). That is, an acidic TME reduces the cellular uptake of weakly basic chemotherapeutic drugs, such as anthracyclines (doxorubicin, daunorubicin, mitoxantrone, etc.) because weakly basic chemotherapeutic drugs become trapped in extracellular compartments owing to being positively charged in acidic conditions (20–22). Characteristics of the TME, such as having an acidic pH, being hypoxic, and lacking nutrients, are associated with cancer stem cells that demonstrate self-renewal and multilineage potential, leading to heterogeneity within the tumor and contributing to treatment resistance and clinical relapse (23). It is also known that the acidic TME is associated with a decreased anti-cancer immune response. Lactic acid in the TME suppresses immune cells, such as dendritic cells, natural killer cells, cytotoxic T cells, and macrophages, resulting in the inhibition of antitumor immune responses, and cancer immune escape (24, 25). An *in vitro* study demonstrated that the acidic TME is associated with both the suppression of T-cell responses and a decrease in the secretion of IFN–γ and TNF–α, and the effects of anti-programmed cell death 1 therapy were reported to be enhanced by alkalization using bicarbonate in mouse models of melanoma (26).

In summary, reversal of the pH gradient of the TME of cancer cells leads to MDR and reduced cancer immunity, resulting in resistance to cancer therapy. Current cancer treatment strategies do not consider pH changes in cancer and its association with sensitivity to drug therapies, and treatment approaches aiming at pH regulation of the TME may hence be a future therapeutic strategy.

Approaches of alkalization of the acidic TME

There are two main therapeutic approaches that target the acidic pH of the TME. One is buffer therapy, in which alkalizing agents are administered to neutralize protons, and the other is the inhibition of proton efflux transporters expressed on the cancer cell membrane (Figure 1).

Alkalizing agents

Several studies have been reported on buffer therapies that neutralize the acidic TME of cancer cells. Alkalizing agents, such as bicarbonate, are commonly used in *in vitro* and *in vivo* studies. A

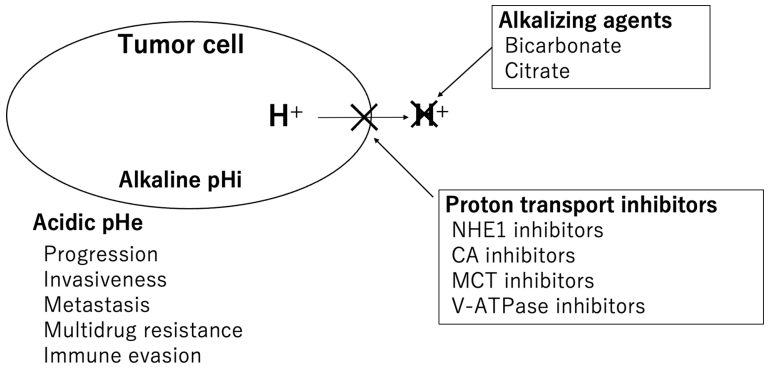

FIGURE 1
Therapeutic approaches targeting the acidic pH of the TME. Alkalizing agents and proton transporter inhibitors are shown.

mathematical simulation study showed that oral bicarbonate consumption as a systemic pH buffer increases the pH of the external TME and inhibits tumor invasion (27). In mouse models of metastatic breast cancer, it was reported that bicarbonate administration increased the pH of the TME, resulting in the suppression of metastasis and improvements of survival rates (28). It was also reported that alkalization of the acidic TME improves the anticancer immune response. As described above, the effects of anti-programmed cell death 1 therapy in mouse models of melanoma have been shown to be enhanced by alkalization through bicarbonate consumption (26). A prospective clinical trial in healthy volunteers was conducted for investigation of the safety of the long-term consumption of sodium bicarbonate for cancer care, and demonstrated that 90 days of sodium bicarbonate consumption (median 0.17 g/kg/day) was feasible and safe, and an increase in urine pH as a surrogate marker for buffering effect was observed following bicarbonate intake (29). It has also been reported that the oral administration of sodium potassium citrate as an alkalizing agent increases HCO_3^- concentrations in the blood and urine, leading to an increase in urine pH and neutralization of the acidic TME in a pancreatic cancer xenograft model, thereby enhancing the therapeutic effects of anticancer drugs (tegafur/gimeracil/oteracil) (30).

Proton transport inhibitors

NHE1 inhibitors

NHE1 is known to play not only an essential role in the survival of normal cells, but also a key role in cancer progression. In normal cells, NHE1 is quiescent in the steady-state resting intracellular pH, and is activated only upon cytosolic acidification. In cancer cells, NHE1 is activated even at resting pH, and the activation of NHE1 directly results in an increase in intracellular pH and a decrease in extracellular pH of cancer cells (7). NHE1 is a major plasma membrane pump that extrudes intracellular protons from cells, and is associated with tumor growth and progression (7). There are several NHE1 inhibitors, including derivatives of amiloride, such as 5-(N-ethyl-N-isopropyl) amiloride, 5-(N,N-dimethyl) amiloride, 5-(N,N-hexamethylene) amiloride (HMA), and cariporide (9). *In vitro* and *in vivo* studies using breast cancer cells have reported that cariporide improves doxorubicin sensitivity (31). It was reported that a patient with metastatic ovarian cancer who was treated with amiloride as a Na^+/H^+ exchanger inhibitor showed a favorable outcome (32). However, as NHE1 is widely present in many tissues and plays a fundamental role in important physiological processes, there is a potential risk of life-threatening side effects associated with NHE1 inhibitors. To take advantage of NHE1 inhibition in cancer therapy, it will be important to develop drugs that selectively target NHE1 in tumors (33).

CA inhibitors

CA acts as a catalyst to reversibly hydrate carbon dioxide to produce bicarbonate and protons, and the overexpression of CA isoforms IX and XII is involved in cancer progression and metastasis (34). These enzymes contribute to acidification of the extracellular pH of cancer cells (35). Inhibitors of CA IX and CA XII are considered as potential anticancer agents, and several clinical trials using these inhibitors have been conducted (34). A study using girentuximab, a chimeric antibody against CA IX, was reported and showed no significant effects on recurrence-free survival in clear cell renal cell carcinoma. However, subgroup analysis showed that patients with high CA IX expression have significantly longer recurrence-free survival than those with low CA IX expression (36).

MCT inhibitors

The activated glycolysis of cancer cells results in the overproduction of lactate, which is transported out across the

cancer cell membrane *via* the MCT (mainly MCT1) (9, 37). Expression of MCT1 and MCT4 has been reported to be a characteristic of cancer cells and to contribute to tumor invasiveness, and hence these MCTs are potential targets for cancer treatment (37). *In vivo* and *in vitro* studies on the effects of MCT1 inhibitors against diffuse large B-cell lymphoma and Burkitt lymphoma reported that the accumulation of intracellular lactate and cancer cell proliferation were reduced by these inhibitors (38).

V-ATPase inhibitors

V-ATPase is an ATP-dependent proton transporter that expels protons from cancer cells, and V-ATPase activation promotes the progression of cancer. The inhibition of V-ATPase was reported to reduce cancer cell growth and induce apoptosis in several *in vivo* and *in vitro* studies (39). Moreover, proton pump inhibitors (PPIs), which act as H^+/K^+-ATPases and are used for the treatment of gastric ulcers and gastroesophageal reflux, are also known to inhibit V-ATPase. *In vivo* and *in vitro* studies have shown that PPIs induce apoptotic cell death and lead to chemosensitization and reversal of chemoresistance *via* the inhibition of V-ATPase (40, 41). Population-based studies also reported that treatment with PPIs may prevent the progression of breast cancer (42, 43). Although clinical trials are limited, favorable results have been reported in three patients with advanced colorectal cancer treated with chemotherapy in combination with high-dose PPIs (44). In addition, in patients with metastatic breast cancer treated with a combination of chemotherapy and PPIs, significantly prolonged progression-free survival (PFS) and overall survival (OS) were observed compared with patients treated with chemotherapy alone (45).

Can diet affect the pH regulation of the TME?

It is known that diet is associated with cancer risk. The World Cancer Research Fund/American Institute for Cancer Research reported their recommendations associated with food intake to reduce cancer risk as follows: 'Eat a diet rich in wholegrains, vegetables, fruit and beans' and 'Limit consumption of red and processed meat' (46). Although the benefit of an alkaline diet on cancer risk still remains unclear, a case-control study reported that a diet with a high acid load may increase lung cancer risk (47). However, to our knowledge, there are no studies to date regarding the association between food intake and pH of the TME. On the other hand, the acid-base load on the body can be affected by food. In a study investigating the effects of food on urine pH, the acid and base precursors in food were quantified and the potential renal acid load was calculated to predict net renal acid excretion, and the potential renal acid load of meat was calculated as +9.5 mEq, whereas that of fruit was −3.1 mEq and vegetables was −2.8 mEq (48). An epidemiological study showed that an alkaline diet consisting of high fruit and vegetable and low meat intake had a significant association with an increase in urine pH (49). Therefore, the alkalizing effect of food results in an increase in urine pH; however, further studies are required to clarify the association between an alkaline diet and pH of the TME.

Clinical reports of alkalization therapy for cancer

Although pH regulation of the acidic TME is considered to be a potential target of cancer therapy, research on the effects of alkalizing agents and proton transport inhibitors on cancer are mainly limited to *in vivo* and *in vitro* studies, and there are few clinical reports regarding alkalization therapy for cancer treatment. In this section, we will introduce some retrospective studies of alkalization therapy for cancer conducted by our group.

First, we report on a retrospective study investigating the effects of an alkaline diet on advanced or recurrent non-small cell lung cancer patients with epidermal growth factor receptor (EGFR) mutations, who were treated with EGFR-tyrosine kinase inhibitor (TKI) (50). All patients in this study were given instructions to follow an alkaline diet as part of their routine clinical care. In this study, the mean urine pH (n = 11) was significantly increased after an alkaline diet, which was defined as that with a large amount of vegetables and fruits and minimal amount of meat and dairy products. Although the average dosage of EGFR-TKI administered to the patients was less than the standard dosage (56% ± 22% of the standard dosage), the median PFS was 19.5 (n = 11, range = 3.1–33.8) months. It is known that the median PFS reported in publications of a similar population treated with EGFR-TKI alone was 10.9–13.1 months (51, 52). This was a preliminary observational study that did not have a comparator group; however, the favorable results of these 11 cases might suggest the importance of the combination of alkalization and EGFR-TKI therapy.

Second, a retrospective study was conducted to investigate the effects of alkalization therapy performed concurrently with chemotherapy on recurrent or metastatic pancreatic cancer patients (53). A total of 28 patients with advanced pancreatic cancer who agreed to receive alkalization therapy, were treated with alkalization therapy, consisting of an alkaline diet with oral sodium bicarbonate (3.0–5.0 g/day). We found that alkalization therapy significantly increased the mean urine pH. A significantly prolonged median OS was observed in patients with a urine pH of higher than 7.0, compared with patients with a urine pH of 7.0 or lower (n = 28, 16.1 vs. 4.7 months; $p< 0.05$). Moreover, a retrospective case-control study was conducted to

investigate the effects of alkalization therapy on chemotherapy outcomes in recurrent or metastatic pancreatic cancer patients (54). Patients in the alkalization group (alkalization therapy plus chemotherapy, n = 36), which included patients from the above retrospective study, were compared with patients in the control group (chemotherapy only, n = 89). The median OS was significantly longer in the alkalization group than in the control group (15.4 vs. 10.8 months; $p< 0.005$) (Figure 2A). In addition, the median OS of patients with an increased urine pH (pH > 7.0) in the alkalization group (n = 13) was significantly longer than that of the control group (n = 89) (25.1 vs. 10.8 months; $p< 0.005$) (Figure 2B). These studies suggest that alkalization therapy may be associated with more favorable outcomes in advanced pancreatic cancer patients treated with chemotherapy. A prospective randomized study is required in the future to clarify the effects of alkalization therapy.

Third, we conducted a retrospective study investigating the effects of alkalization therapy combined with intravenous vitamin C treatment on small cell lung cancer patients treated with chemotherapy (55). Twelve patients who agreed to be assigned to the intervention group (alkalization therapy plus vitamin C treatment together with chemotherapy) were compared with 15 patients in the control group (chemotherapy only) who did not agree to receive interventional treatment. Similar to our previous studies, urine pH of the intervention group was significantly increased compared with that of the control group (Figure 3A). A prolonged median OS was observed in the intervention group compared with the control group (44.2 vs. 17.7 months; $p< 0.05$) (Figure 3B). Although this study was a retrospective study with a small number of patients, alkalization therapy may be associated with favorable outcomes in patients with small cell lung cancer receiving chemotherapy, and it is speculated that supplementary intravenous vitamin C may have also affected their treatment outcomes. However, the effect of intravenous vitamin C treatment in combination with alkalization therapy remains unclear, and further investigation is needed.

As described above, we summarized our clinical studies of alkalization therapy, consisting of an alkaline diet and alkalizing agents, such as bicarbonate. Alkalization therapy can be used in conjunction with any of the current standard chemotherapies, and may improve the outcomes of standard chemotherapies. However, these studies were not randomized, and were retrospective studies that analyzed a small number of patients from a single center, and hence the results should be interpreted with caution. Moreover, these clinical studies focused on patients with non-small cell lung cancer, pancreatic cancer, and small cell lung cancer, and did not investigate patients with other cancer types. In addition, our group has encountered patients with renal cancer, malignant lymphoma, gastric cancer, and breast cancer in whom alkalization therapy increased their urine pH, which may have been associated with their favorable outcomes. However, these are only case reports and require further investigation (56).

It was reported that intestinal alkalization by bicarbonate treatment showed a preventive effect for irinotecan-induced diarrhea in both *in vivo* and *in vitro* studies (57). In clinical studies investigating whether oral administration of bicarbonate (1.8–2.0 g/day) has preventive effects for irinotecan-induced diarrhea in patients with non-small cell lung cancer, small cell lung cancer, and colorectal cancer, no significant differences were observed in the effects of chemotherapy between the bicarbonate-treated and non-treated groups (58, 59). However, the effects of bicarbonate administration as alkalization therapy

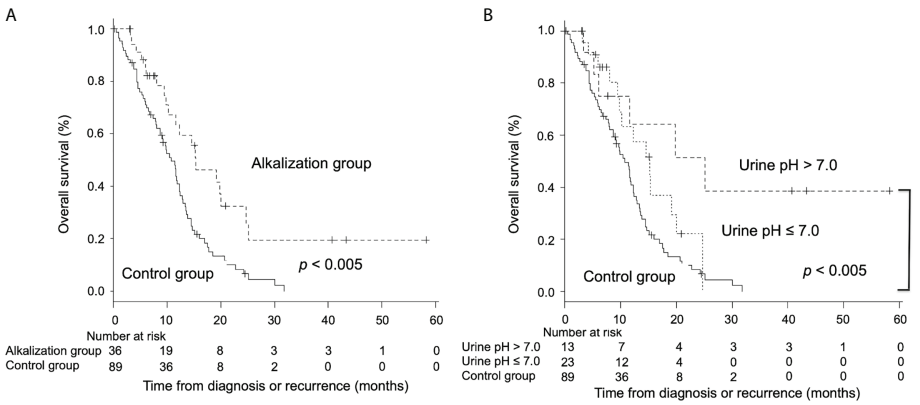

FIGURE 2
Overall survival between advanced pancreatic cancer patients who were treated with alkalization therapy plus chemotherapy and those who were treated with chemotherapy only. Kaplan–Meier curves of the OS of the alkalization group and the control group are shown. **(A)** The median OS of the alkalization group was significantly longer than that of the control group. **(B)** In patients with an increased urine pH (pH > 7.0), a more prolonged median OS was observed than in the control group. [Adapted from reference (54)].

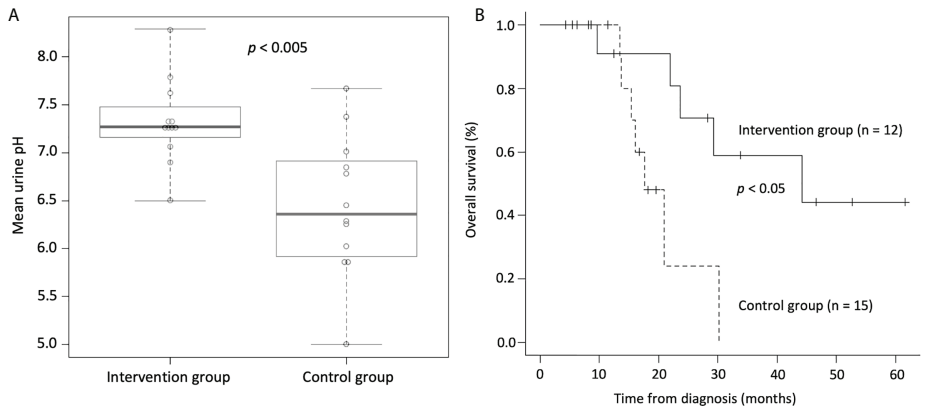

FIGURE 3
Urine pH and overall survival of small cell lung cancer patients who were treated with alkalization therapy plus vitamin C treatment together with chemotherapy and those who were treated with chemotherapy only. **(A)** Box-whisker plots of urine pH of the intervention group (alkalization therapy plus vitamin C treatment together with chemotherapy) and of the control group (chemotherapy only) are shown. Urine pH in the intervention group was significantly higher than that in the control group. The thick lines indicate the median values, the error bars indicate the maximum and minimum values, and the boxes indicate the values between the upper and the lower quartiles. **(B)** Kaplan–Meier curves of the OS of the intervention group and the control group are shown. The median OS of the intervention group was significantly longer than that of the control group. [Adapted from reference (55)].

requires further investigation, as the number of patients in these previous studies were also small, the amount of bicarbonate consumption was low, and urine pH was not measured. Thus, there are not enough clinical studies to date to validate the efficacy of alkalization therapy, and further studies focusing on the treatment of alkalizing agents or proton transport inhibitors are required to further clarify the effects of alkalization therapy.

Future directions of alkalization therapy

Alkalization therapy is a buffering therapy aimed at neutralizing the acidic TME. An animal study has shown that there is a correlation between changes in pH of the TME and changes in urine pH induced by alkalizing agents (30). Alkalization therapy tended to be more effective in patients with a higher urine pH in our clinical studies described above (50, 53–55), suggesting that urine pH may be an alternative indicator of the pH around cancer cells. It should be noted that these studies have not demonstrated the association between urine pH and tumor pHe/pHi ratio. Blood pH is tightly regulated, and the HCO_3^- buffer system plays an important role in maintaining blood pH homeostasis by balancing the composition of carbonic acid, HCO_3^- and carbon dioxide. In addition, renal filtration regulates the blood concentration of HCO_3^- through glomerular filtration and acid secretion (60). It is speculated that bicarbonate administration increases the blood HCO_3^- concentration, delivering excess HCO_3^- into the tumor, where HCO_3^- molecules traps H^+ ions in the TME and form carbonic acid, resulting in neutralization of the tumor pHe (28). However, further objective evaluation of the association between urine pH and pH of the TME is needed. One method of measuring pH in tumor tissue is ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS). It has been reported that measurement of pH by MRS is largely standardized, providing an accuracy of ± 0.1 pH units (61). Novel imaging probes have been developed to assess the acidic TME. ^{89}Zr-labeled pH-low insertion peptide is a radiopharmaceutical imaging probe for *in vivo* analysis to quantify the acidic TME using positron emission tomography, and has potential clinical applications (62). Acido-chemical exchange saturation transfer magnetic resonance imaging can measure the extracellular pH of the TME using the ratio of two pH-dependent signals, and may be useful in revealing the association between urine pH and pH of the TME (63, 64). It is also necessary to investigate how alkalizing therapy affects the expression of cancer-associated genes, and whether the response to alkalizing therapy differs depending on the gene expression status. In addition, as regulation of pH in the body is affected by daily diet and lifestyle, numerous factors are involved, and an exhaustive analysis using artificial intelligence may be useful in the future.

Conclusions

We here summarized the therapeutic approaches against cancer targeting pH regulation. Although alkalization therapy as a buffer therapy using alkalizing agents, and therapies inhibiting proton transporters expressed on cancer cells are potentially promising, their clinical applications remain still limited. Further clinical investigations are hence needed in the future.

Author contributions

RH performed the literature review and wrote the article. MI, RN, HM, and HW performed the literature review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Diverse antitumor effects of ascorbic acid on cancer cells and the tumor microenvironment

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Ascorbic acid has attracted substantial attention for its potential antitumor effects by acting as an antioxidant *in vivo* and as a cofactor in diverse enzymatic reactions. However, solid proof of its clinical efficacy against cancer and the mechanism behind its effect have not been established. Moreover, cancer forms cancer-specific microenvironments and interacts with various cells, such as cancer-associated fibroblasts (CAFs), to maintain cancer growth and progression; however, the effect of ascorbic acid on the cancer microenvironment is unclear. This review discusses the effects and mechanisms of ascorbic acid on cancer, including the role of ascorbic acid concentration. In addition, we present future perspectives on the effects of ascorbic acid on cancer cells and the CAF microenvironment. Ascorbic acid has a variety of effects, which contributes to the complexity of these effects. Oral administration of ascorbic acid results in low blood concentrations (<0.2 mM) and acts as a cofactor for antioxidant effects, collagen secretion, and HIFα degradation. In contrast, intravenous treatment achieves large blood concentrations (>1 mM) and has oxidative-promoting actions that exert anticancer effects *via* reactive oxygen species. Therefore, intravenous administration at high concentrations is required to achieve the desired effects on cancer cells during treatment. Partial data on the effect of ascorbic acid on fibroblasts indicate that it may also modulate collagen secretion in CAFs and impart tumor-suppressive effects. Thus, future studies should verify the effect of ascorbic acid on CAFs. The findings of this review can be used to guide further research and clinical trials.

KEYWORDS

ascorbic acid, antitumor effect, cancer, cancer-associated fibroblast, antioxidant, intravenous administration

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Introduction

Ascorbic acid, also known as vitamin C, is a low-molecular-weight compound with the chemical formula $C_6H_8O_6$ and a molecular weight of 176.12 g/mol. It is an essential water-soluble vitamin that cannot be synthesized in the human body (1). Instead, this vitamin must be acquired by consuming food. Inadequate provision of dietary vitamin C can lead to deficiencies such as scurvy (2–4). Ascorbic acid acts *in vivo* as an antioxidant and cofactor in various enzymatic reactions but has also attracted substantial attention for its potential antitumor effects (5, 6). However, the clinical efficacy of ascorbic acid as an anticancer treatment, and the mechanism behind its effects, have not yet been confirmed.

Cancer maintains its characteristic growth and progression by interacting with surrounding cells, forming a cancer microenvironment composed of various cells. Among these cells, cancer-associated fibroblasts (CAFs) play a significant role in cancer cell proliferation, invasion, and metastasis by providing growth factors and nutrients to cancer cells and reorganizing the extracellular matrix of the peri-cancer stroma (7–11). However, the effect of ascorbic acid on the cancer microenvironment is unclear. Moreover, the heterogeneity phenotype of fibroblasts in the peritumoral stroma of some carcinomas promotes tumor growth (12, 13). Therefore, elucidation of the heterogeneity of fibroblasts is urgently required for the effective destruction of cancer cells.

In this review, we discuss the differences between the antioxidant and oxidant-promoting effects of ascorbic acid, including the role of ascorbic acid concentration. Our current understanding of the concentration-dependent actions and processes of ascorbic acid is also explained. We then provide future perspectives on the antitumor effects of ascorbic acid on cancer cells and its effects on CAFs, which form a key cancer microenvironment.

Administration route and vascular concentration of ascorbic acid

Orally ingested ascorbic acid is absorbed by transporters of sodium-dependent vitamin C transporters (SVCTs) and glucose transporters (GLUTs) in the small intestine and excreted *via* the kidneys (14). *In vivo*, ascorbic acid exists as reduced ascorbic acid or oxidized ascorbic acid (dehydroascorbic acid (DHA)), which are respectively taken into cells through SVCTs and GLUTs (15–17). Rat experiments revealed variations between the oral and intravenous administration of ascorbic acid, whereby oral administration of 5 mg/g of body weight did not raise blood ascorbic acid concentrations, but intravenous administration of 5 mg/g boosted ascorbic acid concentrations to approximately 10 mM (18). However, since mice and rats can synthesize ascorbic acid in their bodies (<100 μM), it is necessary to be careful in applying the results of experiments with mice and rats to humans, whose systems cannot synthesize ascorbic acid. In human studies, oral administration of 400 mg or more of ascorbic acid maintained steady-state blood concentrations of 50–80 μM (19), with oral administration of 3 g of ascorbic acid every 4 h increasing the maximum blood concentration to approximately 220 μM. Conversely, intravenous administration of 50 g of ascorbic acid was predicted to increase the maximum blood concentration to approximately 13.4 mM (20). The half-life of ascorbic acid in the blood is 2.0 ± 0.6 h (21). Furthermore, in a report on patients with cancer, ascorbic acid concentrations in the blood reached 20.3–49.0 mM with intravenous administration of 60–70 g/m^2 or 1.5 g/kg of ascorbic acid (21–23). In other words, blood concentrations of ascorbic acid vary widely depending on the route of administration. Thus, the pharmacological effects of ascorbic acid resulting from the low concentrations achieved by oral administration (several hundred μM) may differ from those resulting from the high pharmacological concentrations achieved by intravenous administration (>1 mM). As such, the intended administration route of ascorbic acid must be considered. Adverse effects of ascorbic acid include effects on renal function and hemolysis caused by a deficiency of glucose-6-phosphate dehydrogenase (G6PD). Oral doses of more than 1000 mg per day increase renal excretion of urate and oxalate compared to lower doses, so caution should be exercised when administering high doses (19). G6PD is required for the proper function of glutathione peroxidase, especially in erythrocytes (24). However, many clinical trials in which high concentrations of intravenous ascorbic acid were administered as monotherapy or in combination with anticancer agents have shown no serious adverse effects (21, 25–27). Therefore, ascorbic acid is considered a drug with very low toxicity to the human body.

In vivo effects of ascorbic acid

Recent studies have demonstrated that ascorbic acid absorbed *in vivo* has both antioxidant and oxidant-promoting effects (28, 29). Ascorbic acid also exhibits various physiological effects by catalyzing Fe(II)- and 2-oxoglutarate-dependent dioxygenase reactions (14).

Ascorbic acid and reactive oxygen species

Ascorbic acid degrades reactive oxygen species (ROS) at average blood concentrations of 40–80 μM, reducing low-density lipoprotein oxidation associated with atherosclerosis and lipid oxidation of cell membranes (30–32). However, high pharmacological concentrations of ascorbic acid achieved *via*

intravenous administration produce H_2O_2 *in vivo* (18, 33, 34) and then hydroxyl radicals *via* the Fenton reaction (35). Intravascularly, ROS produced by high concentrations of ascorbic acid are degraded by catalase in serum, whereas extravascularly, ROS accumulate without degradation by ascorbic acid and act as a pro-oxidant. Thus, ascorbic acid is notable for its paradoxical activity, serving as an antioxidant at low doses and a pro-oxidant at high doses (28, 29). In addition, oral administration of ascorbic acid does not reach the same pharmacological concentrations as intravenous treatment (19, 20); therefore, intravenous administration of ascorbic acid is required for pro-oxidant activity to occur. In a rat study, intravenous administration of 0.5 mg/g of ascorbic acid increased the H_2O_2 concentration in the extracellular fluid from undetectable to 20 μM, and intraperitoneal injection of the same dose increased H_2O_2 concentration to approximately 5 μM. In contrast, no increase in H_2O_2 concentration in the extracellular fluid was detected after oral administration of ascorbic acid (18). In addition, in a mouse subcutaneous transplantation model, intraperitoneal administration of 4 mg/g of ascorbic acid increased the H_2O_2 concentration in the extracellular fluid around the tumor to approximately 150 μM (34, 35).

Ascorbic acid as a cofactor for dioxygenase

Members of the Fe(II) and 2-oxoglutarate-dependent dioxygenase families catalyze many oxidation reactions throughout biology. Ascorbic acid acts as a coenzyme that catalyzes the reactions that produce hydroxylation products using 2-oxoglutarate and oxygen as substrates (36). Particularly well-known are the reactions in collagen (37) and HIFα, which is a master regulator of the cellular hypoxia response pathway (38). The reaction in collagen is mediated by one of the proline hydroxylases, collagen prolyl-4-hydroxylase (C-P4H), which hydroxylates the procollagen proline (37). C-P4H has a high binding capacity to oxygen and is not affected by the oxygen concentration. Conversely, in the reactions in HIFα, ascorbic acid catalyzes two types of reactions: PHD1-3 in proline hydroxylase (38–40) and factor inhibiting HIF-1 (FIH-1) in asparagine hydroxylase (41–43). In the PHD reaction, ascorbic acid degrades HIFα *via* ubiquitination by pVHL proteins (44–46). In the reaction of FIH-1, it suppresses the interaction with CBP/p300, which is a transcriptional cofactor, and suppresses the transcriptional activity of HIFs (42). These reactions are dependent on the oxygen concentration; thus, ascorbic acid acts as an oxygen sensor in the cell because the reaction is reduced in a hypoxic environment and HIF is not degraded (36). The concentrations of ascorbic acid necessary to sustain enzymatic activity of PHDs and FIH-1 are 140-180 uM and 260 uM, respectively (37) and are well above the steady-state blood concentrations of 40–80 μM, suggesting that these reactions require sufficient blood and tissue concentrations of ascorbic acid (14, 47). Ascorbic acid also acts as a cofactor for ten-eleven translocations (TETs) of DNA hydroxylases; TETs are proteins that convert 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC) (48, 49). Ascorbic acid promotes DNA demethylation by accelerating the reaction of TETs (50).

Antitumor effect of ascorbic acid

Ascorbic acid exhibits antitumor effects in various carcinomas (5, 6, 51); however, clinical studies have not yet produced any significant evidence of these effects (52). Ascorbic acid exhibits antitumor effects through ROS-mediated mechanisms and as a cofactor. The mechanisms of ascorbic acid as a cofactor include effects on HIFα *via* PHDs and FIH-1 and epigenetic effects *via* DNA demethylases (6, 49). (Table 1) Ascorbic acid can also modulate metabolism and epigenetic gene expression in immune cells as well as cancer cells (64–67). Ascorbic acid is also known to inhibit EMT of tumor cells (58, 59). Here, we discuss the known antitumor effects of ascorbic acid.

ROS-mediated antitumor effects of ascorbic acid

The ROS-mediated mechanism is the most well-known mechanism of the antitumor effect of ascorbic acid in various carcinoma. Intravenous administration of high ascorbic acid concentrations acts as a pro-oxidant *in vivo*, producing ROS through the Fenton reaction (18). H_2O_2, a ROS formed outside of the cell, diffuses rapidly inside the cell (68) where it consumes antioxidants such as reduced glutathione and NADPH. In addition, in colorectal cancer with KRAS or BRAF mutations, lung cancer with KRAS mutations, and pancreatic cancer, GLUT1 expression is increased because of an accelerated glycolytic pathway, resulting in higher DHA absorption (69–72). ROS accumulation increases oxidative stress, such as DNA damage, and DNA damage increases PARP activity, thereby decreasing NAD^+ levels and limiting glycolytic system processes (73, 74). In addition, GAPDH, an enzyme of the glycolytic system, is inhibited in its enzymatic function by the reversible binding of oxidized glutathione to cystein152, which is reactive to oxidative stress (75). As a result, the glycolytic pathway produces less adenosine triphosphate (ATP), and cells suffer apoptosis. Indeed, in a report of metabolic changes induced by ascorbate in a colon cancer cell line with KRAS or BRAF mutations, metabolomic analysis using LC/MS/MS showed that upstream metabolites in the glycolytic reaction catalyzed by NAD^+ and GAPDH were accumulated, whereas downstream

TABLE 1 The types and effects of Fe (II) and 2-oxoglutaric acid-dependent dioxygenases in which ascorbic acid acts as a cofactor.

Effect	Collagen prolyl hydroxylases (C-P4H) Promotes collagen production by stabilizing the three-dimensional structure of procollagen through hydroxylation of its proline.	Proline hydroxylases Degrade HIFα *via* ubiquitination by pVHL proteins.	Factor inhibiting HIF-1 Inhibits the transcriptional abilities of HIF1α *via* the interaction with CBP/p300.	DNA/histone demethylases (TETs/JHDMs) Promote DNA demethylation and regulate epigenetic gene expression.
Ascorbic acid concentration in previous reports	100 μM (53–56)	25-1000 μM (36, 46, 57)		100-2000 μM (58–63)
Antitumor effects	Not clear.	Inhibit tumor cell proliferation by inhibiting angiogenesis and suppressing the promotion of glycolysis.		Reexpresses tumor suppressor genes and suppresses oncogenes. Prevent migration and metastasis by suppressing EMT of tumor cells.

C-P4H, collagen prolyl-4-hydroxylases; TETs, ten-eleven translocation enzymes; JHDMs, Jumonji-domain histone demethylases; HIFα, hypoxia inducible factor α; pVHL protein, the von Hippel-Lindau protein; CBP, CREB-binding protein; EMT, epithelial-mesenchymal transition.

metabolites were reduced (74). Because of redox imbalance, cancer cells are susceptible to ROS and the effects of ascorbic acid (76). In conclusion, the pro-oxidant effect of high doses of ascorbic acid induces cell death by generating ROS in cancer cells and limiting ATP generation through the glycolytic pathway.

Conversely, the balance of oxidative stress and antioxidant activity plays a crucial role in tumor development and progression. In melanoma, ROS are overproduced by mitochondria or NADPH oxidase, which promotes tumor development and progression through DNA damage-induced mutation of oncogenes and signal transduction *via* NF-κB (77, 78). In addition, melanoma acquires metastatic potential due to enhanced production of antioxidant enzymes such as catalase and tolerance to oxidative stress (78, 79). Ascorbic acid has a dual impact on melanoma, with high concentrations triggering cell death and low amounts promoting tumor growth (80).

Despite the above reported antitumor effects of ascorbic acid at high concentrations, the ROS-mediated antitumor effects of ascorbic acid remain insufficient for the following reasons. First, the Fenton reaction-mediated ROS-generating effect of ascorbic acid, which is recognized *in vitro*, may be inhibited at *in vivo* concentrations of $Fe2^+$ and $Fe3^+$ (81). Second, *in vivo*, iron ions are always chelated, so the Fenton reaction may not occur (30). Finally, the inhibitory effect of ascorbic acid on ATP synthesis, even in the presence of PARP inhibitors *in vitro*, may be exerted by ascorbic acid regardless of the reduction of NAD^+ levels by PARP (82). In conclusion, it is possible that *in vitro* results of the ROS-mediated antitumor effects of ascorbic acid are not compatible with its *in vivo* mode of action, suggesting that alternative anticancer mechanisms may be involved.

HIFα-mediated antitumor effects of ascorbic acid as a coenzyme

HIFα, which is expressed in many tumors such as melanoma, leukemia, and carcinomas, including colon, pancreatic, and lung cancer (83–87), is involved in angiogenesis and regulation of the glycolytic system, which are crucial processes for cancer growth and progression, suggesting that HIFα may be a novel cancer therapeutic strategy (88, 89). Ascorbic acid is a cofactor for Fe(II)- and 2-oxoglutarate-dependent dioxygenases and has various physiological effects, catalyzing the interaction of PHDs and FIH-1 and degrading the HIFα activity (44–46). Ascorbic acid concentrations in human tumor samples were negatively connected with HIF1α expression in colon cancer, with higher ascorbic acid concentrations associated with prolonged recurrence-free survival (83). In human endometrial tumors, patients with higher ascorbic acid levels in tumors had lower protein expression of HIF1α, VEGF, and GLUT1 and lower malignancy (90). In human pancreatic cancer cell lines, *in vitro*, low ascorbic acid concentrations (25 μM) reduced HIF1α expression and suppressed tumor growth under hypoxic conditions (57). In a model of subcutaneous lung tumor transplantation in rats, intraperitoneal injection of ascorbic acid (1 g/kg) suppressed HIF1α expression in tumors and decreased tumor growth and vascular density (91). In a mouse model of human B cell lymphoma implanted subcutaneously, oral treatment of ascorbic acid (5 g/L) reduced HIF1α expression and prevented tumor development (92).

Thus, the activity of ascorbic acid as a coenzyme may suppress HIFα expression and activity in tumor cells and may inhibit tumor cell proliferation by inhibiting angiogenesis.

Ascorbic acid regulates epigenomic modifications

Ascorbic acid catalyzes the reaction of DNA hydroxylase TETs and Jumonji C domain-containing histone demethylases (JHMDs), thereby having epigenetic antitumor effects (6, 49, 93). TET is a member of the same family of iron- and 2-oxoglutarate-dependent dioxygenases as PHDs, which convert 5-methylcytosine (5mC) into 5-hydroxymethylcytosine, promoting histone demethylation and contributing to oncogene suppression and the re-expression of tumor suppressor genes (94). In hematologic tumors such as acute myeloid leukemia and myelodysplastic syndromes, loss-of-function mutations in TET2 are known to occur frequently, resulting in decreased and hypermethylated 5hmC. In these hematologic tumors, administration of several hundred μM ascorbic acid has a gene reprogramming effect that restores TET function and increases 5hmC levels, suppressing cell proliferation and promoting myeloid progenitor cell differentiation (60, 94). In malignant melanoma, 5hmC is known to decrease as the disease progresses, and administration of 100 μM ascorbic acid restores 5hmC *via* TET, induces apoptosis in tumor cells, and shows antitumor effects (61, 62). For colon cancer, administration of 1 mM ascorbic acid has also been reported to increase 5hmC *via* TET *in vitro*, showing antitumor effects when combined with an inhibitor of isocitrate dehydrogenase (IDH) mutations (63). JHDMs are histone demethylases that use Fe2 + and α-ketoglutarate as cofactors to demethylate histones and regulate gene expression (95). Isocitrate dehydrogenases (IDH) mutations reduce α-ketoglutarate, a substrate for TETs and JHDMs, and promote DNA methylation in cells with IDH mutations, regulating gene expression that leads to carcinogenesis such as glioma (96). Ascorbic acid is necessary for the proper activity of JHDMs and may correct gene expression that promotes oncogenesis by promoting histone demethylation (93, 97). Essentially, ascorbic acid has antitumor effects by improving the hypermethylation state observed in tumors *via* TETs and JHDMs, and by reprogramming gene expression.

Ascorbic acid downregulates EMT

Ascorbic acid regulates the epithelial-mesenchymal transition (EMT), which is important for metastatic tumor potential. *In vitro*, ascorbic acid suppressed EMT in human pancreatic cancer cells by decreasing Snail and increasing E-cadherin at concentrations of 1-1.5 mM (98). Ascorbic acid, in conjunction with 5-azacytidine (5-AZA), a potent DNA methyltransferase inhibitor, regulated EMT inhibition and cell cycle progression in human HCC cells *in vitro* by suppressing Snail expression *via* TET (58). Interestingly, ascorbic acid produced two distinct reactions in human breast cancer *in vitro*. A low dose (100 μM) of ascorbic acid decreased E-cadherin and increased the mesenchymal marker vimentin, while a high dose (2 mM) of ascorbic acid conversely increased E-cadherin and decreased vimentin, reversing TGF-β 1-induced EMT and, as a result, suppressing the formation of lung metastases *in vivo* (59). Ascorbic acid at concentrations of 1 mM or higher is thought to suppress EMT in tumors, possibly by inhibiting the effect of TGF-β1 or by regulating Snail expression by TETs.

Effects of ascorbic acid on fibroblasts

Ascorbic acid is known to enhance collagen synthesis (99, 100) and wound healing (101), reduce UV-induced damage (102, 103), and exhibit anti-inflammatory effects (104, 105); however, these effects are primarily skin-confined. Recently, the CAF cancer microenvironment has attracted considerable attention (10, 11), although few studies have described the effects of ascorbic acid on CAFs. Here, we describe the effects of ascorbic acid on fibroblasts.

Ascorbic acid and dermal fibroblasts

Ascorbic acid acts as a cofactor for C-P4Hs when taken up by human dermal fibroblasts and promotes collagen production by stabilizing the three-dimensional structure of procollagen through hydroxylation of its proline (4, 106, 107). *In vitro*, a low concentration of ascorbic acid (100 μM) in human skin fibroblasts increases the expression of type1,3,4 collagen and SVCT2 at the mRNA level (53–55) as well as increasing proliferation (56), suggesting a direct effect on fibroblasts. In human clinical data, oral ascorbic acid intake with exercise stimulation doubled the amino-terminal propeptide of collagen I in the blood, indicating enhanced collagen production (108). In addition, ascorbic acid concentrations as low as 0.17 mM in human skin fibroblasts increase the contractile phenotype of myofibroblasts in the presence of TGF-β1 through enhancement of the expression of TGFb1-responsive genes, but do not increase such a phenotype in the absence of TGF-β1 (109). Ascorbic acid promotes collagen production and proliferation of skin fibroblasts as a coenzyme. Moreover, in these studies, ascorbic acid increases in collagen synthesis and secretion occurred at concentrations as low as several hundred μM.

Ascorbic acid and other fibroblast reports

According to a study on tumor stroma, intraperitoneal administration of ascorbic acid at a high dose of 4 g/kg in an orthotopically transplanted mouse model of human pancreatic cancer resulted in tumor reduction, reduced metastasis, and enhanced tumor stroma due to increased collagen secretion (98). In the 4T1 breast cancer orthotopic model utilizing ascorbic acid-deficient (gulonolactone oxidase knockout mouse) mice, oral administration of ascorbic acid increased type 1 collagen to form a capsule around the tumor, and tumor boundaries were more clearly defined than in the control group (110). Thus, ascorbic acid may increase collagen production in the tumor stroma at both high and low doses. However, it is unknown whether this effect is on tumor cells or CAFs, and further research is needed to determine whether ascorbic acid activates CAFs in the tumor microenvironment and increases collagen production. In contrast, hepatic stellate cells, which are responsible for liver fibrosis, were inhibited *in vitro* by low doses of ascorbic acid (50-200 M), which decreased intracellular TGF-1 in rat cell lines (111). In a report examining the development of pulmonary fibrosis by paraquat treatment, intraperitoneal administration of 150 mg/kg of ascorbic acid inhibited pulmonary fibrosis in a mouse model by inhibiting inflammatory cell infiltration into the bronchoalveolar lavage fluid, suppressing apoptosis by increasing antioxidant activity in the lung, and inhibiting TGF-β in the lung (112). As a result, ascorbic acid may inhibit fibrosis by inhibiting inflammatory cell infiltration and reduction of TGF-β in tissues. In our study, we also found that *in vitro*, human pancreatic-derived fibroblasts, whose proliferation is promoted when co-cultured with cancer cells, receive high doses (>1 mM) of ascorbic acid for growth inhibition. (Figure 1) In conclusion, the effects of low and high doses of ascorbic acid on CAFs, such as enhanced collagen production and inhibition of fibrosis development, differ from organ to organ or disease model to disease model and remain unclear.

Clinical trials on ascorbic acid

In the 1970s, clinical trials involving ascorbic acid revealed that a small sample of patients treated with intravenous and oral ascorbic acid lived longer than a control group (113, 114). At that time, the mechanism of the antitumor effect of ascorbic acid efficacy remained unclear, and subsequent randomized, double-blind, placebo-controlled trials with oral ascorbic acid failed to demonstrate a survival benefit (115, 116). Therefore, the antitumor effect of ascorbic acid was viewed unfavorably. Multiple mechanisms of ascorbic acid's antitumor effect were subsequently proven *in vitro*, along with differences in ascorbic acid blood levels between oral and intravenous administration methods. Furthermore, the fact that blood levels of ascorbic acid

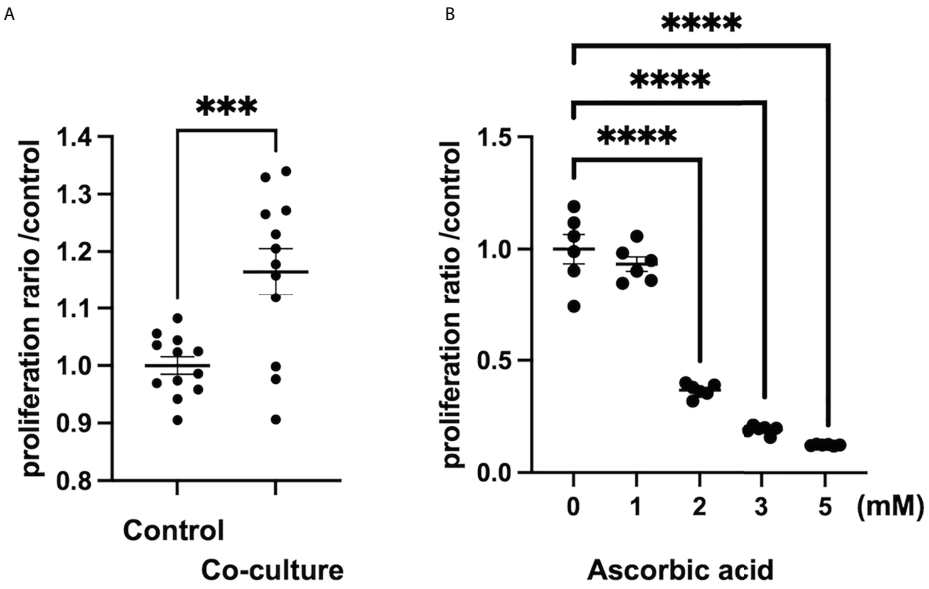

FIGURE 1
Ascorbic acid reduces the proliferation of human-derived pancreatic fibroblasts (hPFs). **(A)** Proliferation of hPFs is increased in a co-culture with a pancreatic cancer cell line (MIAPaCa2). ***P < 0.001 versus control, means ± SEM, n = 12. **(B)** Proliferation of hPFs is dose-dependently reduced by high-dose ascorbic acid treatment. ****P < 0.0001 versus ascorbic acid 0 mM, means ± SEM, n = 6. Statistical analysis was performed by GraphPad Prism 9 and significance was determined by Student's t-test.

were decreased in cancer patients (117, 118) and that the adverse effects associated with ascorbic acid administration were extremely low, led to the expectation that ascorbic acid could be used for therapeutic applications. (Table 2) There were a few scattered case reports showing tumor shrinkage with ascorbic acid treatment (131, 153–158), and there were also reports of antitumor effects in a small number of studies (25, 124, 159, 160). Ascorbic acid in combination with chemotherapeutic agents has also been researched, and some reports of reduced side effects and improved quality of life have been observed (21, 161, 162). In contrast, there have been no large-scale clinical trials that have demonstrated an additional antitumor effect by ascorbic acid (6, 76, 131, 163), and several ongoing clinical trials of ascorbic acid alone or in combination with chemotherapeutic agents for advanced colon cancer, pancreatic cancer, lung cancer, and other malignancies are expected to provide results in the near future (140, 148, 149, 151) (Table 2).

Discussion

Ascorbic acid is a medicine that has been widely investigated and used for a long time; however, its beneficial effects against cancer have not yet been proven by clinical trials. The contrasting *in vivo* effects of ascorbic acid may explain this. That is, the oxidative-promoting impact at high concentrations is detrimental to cancer cells, whereas the antioxidant effect at low concentrations may promote cancer (164). Because of this paradoxical effect, the administration route of ascorbic acid should be carefully considered. In addition, future research should explain the different activities of multiple dioxylases as cofactors, such as HIFα degradation, immune cell modulation, and epigenetic regulation of gene expression, in relation to the cancer microenvironment (Figure 2).

Additionally, research on the effects of ascorbic acid on CAFs implies the existence of novel therapeutic possibilities. Since the diversity of gene expression in fibroblasts *in vivo* differs among organs and pathological conditions (165), the effects of ascorbic acid on CAFs are also expected to vary among organs and pathological conditions. One of the potential effects of ascorbic acid may be the inhibition of tumor-promoting CAFs. Tumor-promoting CAFs support cancer growth by supplying cancer cells with nutrients and growth factors (7–11). Moreover, tumor-promoting CAFs control ECM secretion and protease secretion, remodel the ECM, and generate invasive routes necessary for solid tumor invasion (166, 167). Furthermore, in tumors with a high stromal component, such as pancreatic and breast cancer, the stromal fluid pressure in the tumor area increases, reducing drug delivery and indicating resistance to treatment (168, 169). Tumor-promoting CAFs promote cancer through cross-talk functions with cancer cells, ECM remodeling functions, and physical drug barrier functions. Ascorbic acid has an inhibitory effect on fibroblasts through a reduction in TGF at low doses and an inhibitory effect on cell proliferation *via* a

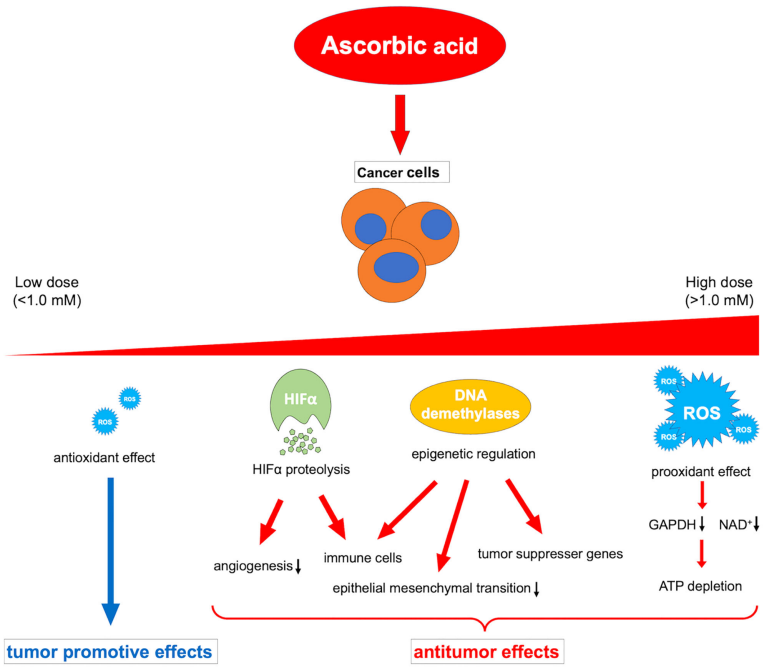

FIGURE 2
Overview of the various dose-dependent effects of ascorbic acid on cancer. ROS, reactive oxygen species; HIFα, hypoxia-inducible factor-alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide.

TABLE 2 Clinical trials on ClinicalTrials. gov.

Study[a]	Cancer types	Phase of study	Design of study	Therapy	Number of patients (with/ without ascorbic acid) or estimated enrollment	Result or primary outcome measures (if trials are not reported)
Published clinical trials						
NCT00954525 (25, 119)	pancreatic cancer	Phase I	single group assignment	Ascorbic acid (IV 50-100 g, 3 infusions per week), gemcitabine and erlotinib for 8 weeks per cycle	9	Seven patients were SD and 2 patients were PD. Time to progression was 89 days (standard deviation 77 days) and overall survival was 182 days (standard deviation 155 days)
NCT00006021 (120, 121)	multiple myeloma	Phase I/ II	single group assignment	Ascorbic acid (IV 1 g, 5 infusions per week) and arsenic trioxide for 5 weeks per 7 week	6	Two patients were PR, and 4 patients were SD.
NCT00317811 (122, 123)	multiple myeloma	Phase II	single group assignment	Ascorbic acid (oral 1g, days 1-4 every 2 weeks), bortezomib and melphalan	31	Five patients were CR, 3 patients were VCPR, 6 patients were PR, 9 patients were MR, 6 patients were SD, and 2 patients were PD.
NCT01049880 (124, 125)	pancreatic cancer	Phase I	single group assignment	Ascorbic acid (IV 50-125 g, 2 infusions per week) and gemcitabine	9	Time to progression and overall survival were 26 ± 7 weeks and 13 ± 2 months. (Means ± SEM)
NCT01050621 (26, 126)	all cancer	Phase I/ II	single group assignment	Ascorbic acid (IV 1.5 g/kg, 2 or 3 infusions per week) and chemotherapy	14	Three patients had unusually favorable experiences that were deemed highly unlikely to result from chemotherapy alone.
NCT01080352 (127, 128)	prostate cancer	Phase II	single group assignment	Ascorbic acid (IV week 1, 5 g; week 2, 30 g; and weeks 3–12, 60 g, once a week)	23	This treatment was not found to be effective.
NCT01364805 (98, 129)	pancreatic cancer	Phase I/ IIa	single group assignment	Ascorbic acid (IV 75-100 g, 3 infusions per week) and gemcitabine	14	Median progression-free survival and median overall survival were 3 months and 15.1 months.
NCT00228319 (130, 131)	ovarian cancer	Phase I/ IIa	parallel assignment, randomized	Arm 1: carboplatin and paclitaxel chemotherapy and ascorbic acid (IV 75-100 g, 2 infusion per week) for 6 months/Arm 2: carboplatin and paclitaxel chemotherapy	25 (13/12)	There were no statistically significant difference in overall survival and the median time for disease progression/relapse.
NCT02655913 (132, 133)	non-small-cell lung cancer	Phase I/ II	parallel assignment, randomized	Arm 1: administration of ascorbic acid (IV 1 g/kg, 3 infusions per week) for total 25 times, modulated electrohyperthermia, and supportive care/Arm 2: supportive care	97 (49/48)	Progression-free survival (3 months *vs.* 1.85 months, $P < 0.05$) and overall survival (9.4 months *vs.* 5.6 months, $P < 0.05$) were significantly prolonged by combination therapy compared to BSC alone.
NCT01905150 (134, 135)	pancreatic cancer	Phase II	parallel assignment, randomized	Arm 1: G-FLIP/G-FLIP-MD and ascorbic acid (IV 75-100 g, 2 infusions per week)/Arm 2: G-FLIP/ G-FLIP-MD	26 (we could confirm only abstract, and it did not describe details)	Ascorbic acid may avoid standard 20-40% rates of severe toxicities.
Ongoing or unpublished clinical trials						
NCT01754987 (136)	hepatocellular carcinoma	Phase I/ II	parallel assignment, non-randomized	Arm 1: ascorbic acid (IV 100 g, 3 infusions per week) for 16 weeks and sorafenib/Arm 2: sorafenib only	5 (5/0)	Number of participants that experience serious adverse events. (Time Frame: 16 weeks +/- 2 weeks)
NCT03410030 (137)	pancreatic cancer	Phase Ib/II	single group assignment	Ascorbic acid (IV ≥20 mM), nab-paclitaxel, cisplatin, and gemcitabine	36	Disease control rate (CR+PR+SD x18 weeks) (Time Frame: approximately 63 days)

(Continued)

TABLE 2 Continued

Study[a]	Cancer types	Phase of study	Design of study	Therapy	Number of patients (with/without ascorbic acid) or estimated enrollment	Result or primary outcome measures (if trials are not reported)
Published clinical trials						
NCT03964688 (138)	multiple myeloma and lymphoma	Phase II	parallel assignment, randomized	Arm 1: ascorbic acid (IV during hospitalization, after oral, total 6 weeks.)/Arm 2: placebo	47	Immune recovery (Time Frame: day 14-28)
NCT02905578 (139)	pancreatic cancer	Phase II	parallel assignment, randomized	Arm 1: ascorbic acid (IV 75 g, 3 infusions per week), gemcitabine, and nab-paclitaxel/Arm 2: gemcitabine and nab-paclitaxel	65	Overall survival (Time Frame: Every 2 months for up to 20 years post-treatment)
NCT03146962 (140)	colorectal, lung, and pancreatic cancer	Phase II	single group assignment	Cohort A: ascorbic acid (IV 1.25 g/kg, 4 infusions per week) for 2-4 consecutive weeks/Cohort B: ascorbic acid (IV 1.25 g/kg, 4 infusions per week) up to 6 months/Cohort C: ascorbic acid (IV 1.25 g/kg, 4 infusions per week) for 1-3 weeks and Yttrium-90 radioembolization of hepatic metastases	78	Change in antitumor activity measured by pathologic response based on tumor regression grading in cohort A patients. (Time Frame: cohort A - 8 weeks) Three-month disease control rate will be evaluated using RECIST v 1.1 in cohort B patients. (Time Frame: Cohort B - 3 months) Maximal tolerated dose of high dose vitamin C in combination with Y90 radioembolization (Time Frame: Cohort C - 16 weeks)
NCT03418038 (141)	high grade B-cell lymphoma with MYC and BCL2 or BCL6 rearrangements, recurrent diffuse large B-cell lymphoma, recurrent Hodgkin lymphoma, recurrent lymphoma, refractory diffuse large B-cell lymphoma, and refractory lymphoma	Phase II	parallel assignment, randomized	Arm A: ascorbic acid (IV) on days 1, 3, 5, 8, 10, 12, 15, 17, and 19, and combination chemotherapy./Arm B: placebo (normal saline) (IV) on days 1, 3, 5, 8, 10, 12, 15, 17, and 19, and combination chemotherapy./Arm C: ascorbic acid (IV) on days 1, 3, 5, 8, 10, 12, 15, 17, and 19, and another combination chemotherapy from Arm A and B.	147	Overall response rate (Arms A and B) (Time Frame: Up to 2 years) Overall response rate (Arm C) (Time Frame: Up to 2 years)
NCT03433781 (142)	myelodysplastic syndromes	Phase Ib/IIa	single group assignment	Ascorbic acid (continuous intravenous infusion/24 hours 50 g, 5 infusions every 4 week)	18	Measure of serum bioavailability of ascorbic acid in Myelodysplastic syndrome patients with ten-eleven translocation 2 mutations (Time Frame: 6 Months)
NCT03508726 (143)	soft tissue sarcoma	Phase Ib/II	single group assignment	Ascorbic acid (IV 62.5 or 75 g, 3 infusions per week)	25	Tumor response as assessed by pCR rate (Time Frame: Start of treatment up to 6 weeks after the last ascorbate infusion)
NCT03682029 (144)	myelodysplastic syndromes, chronic myelomonocytic leukemia-1, and cytopenia	–	parallel assignment, randomized	Arm 1: ascorbic acid (oral 1000 mg, daily) for 12 months/Arm 2: placebo	100	Median change from baseline in variant allele frequency at 12 months (Time Frame: At baseline and at 12 months)
NCT03799094 (145)	non-small-cell lung cancer	Phase I/II	parallel assignment, randomized	Arm 1: ascorbic acid (IV 30 g, once a week) and daily taking tyrosine kinase inhibitor/Arm 2: daily taking tyrosine kinase inhibitor	150	Progression free survival (Time Frame: From the start date of treatment until the date of first documented progression or death, assessed up to 2 years)
NCT03999723 (146)	myelodysplastic syndromes, acute myeloid leukemia, and chronic myelomonocytic leukemia	Phase II	parallel assignment, randomized	Arm 1: ascorbic acid (oral 1000 mg, daily) and azathioprine/Arm 2: placebo and azathioprine	196	Event-free survival (Time Frame: 0-54 months)

(Continued)

TABLE 2 Continued

Study[a]	Cancer types	Phase of study	Design of study	Therapy	Number of patients (with/ without ascorbic acid) or estimated enrollment	Result or primary outcome measures (if trials are not reported)
Published clinical trials						
NCT04033107 (147)	hepatocellular cancer, pancreatic cancer, gastric cancer, and colorectal cancer	Phase II	single group assignment	Ascorbic acid (IV 1.5 g/kg, D1-3, every 2 weeks) and metformin	30	Progression-free survival (Time Frame: up to 12 weeks)
NCT04046094 (148)	bladder cancer	Phase I/ II	single group assignment	Ascorbic acid (IV 25 g, 2 infusions per week) for 4 weeks	21	Post treatment pathological staging (Time Frame: 10 weeks)
NCT04516681 (149)	colorectal cancer	Phase III	parallel assignment, randomized	Arm 1: ascorbic acid (IV 1.5g/kg/ day, D1-3, every 2 weeks) and FOLFOXIRI+/- bevacizumab/Arm 2: FOLFOXIRI+/- bevacizumab	400	Objective response rate (Time Frame: up to 5 years)
NCT04634227 (150)	sarcoma, soft tissue sarcoma, unresectable soft tissue sarcoma, metastatic bone tumor, and bone sarcoma	Early Phase I	single group assignment	Ascorbic acid (IV 20-30 mM) on days 1, 2, 8, 9, 15 and 16 of a 28-day cycle, and gemcitabine	20	Determine the 12 weeks progression free survival at 12 weeks post treatment initiation (Time Frame: 12 weeks post-treatment)
NCT04801511 (151)	rectal cancer	Phase II	single group assignment	Ascorbic acid (IV 24 g, 25 times) with preoperative concurrent intensity-modulated radiation therapy and mFOLFOX6 chemotherapy, and then preoperative mFOLFOX6 chemotherapy	60	pCR rate (Time Frame: 2 year From the first subject underwent surgery to the last subject underwent surgery.)
NCT02516670 (152)	prostate cancer	Phase II	parallel assignment, randomized	Arm 1: ascorbic acid (IV 25 g, 2 infusions per week) for 3 weeks and docetaxel/Arm 2: placebo and docetaxel	50	Terminated (insufficient clinical response per DSMB)

[a]This table describes clinical trials since 2000.
IV, intravenous injection; CR, complete response; VGPR, very good partial response; PR, partial response; SD, stable disease; PD, progressive; pCR, pathologic complete response; DSMB, Data and Safety Monitoring Board.

prooxidant effect at higher doses, suggesting that it may have an inhibitory effect on tumor-promoting CAFs.

Conversely, collagen is known to form a barrier that physically obstructs cell migration without protease degradation (170, 171). In a mouse model lacking -SMA-positive fibroblasts, the tumor suppressive effects of CAFs have been demonstrated to induce an undifferentiated tumor phenotype and dramatically reduce survival (172). The increase in cancer stroma, tumor shrinkage, and metastasis inhibition effects of ascorbic acid may be attributed to the activation of tumor suppressive fibroblasts and the formation of collagen barriers that inhibit tumor progression.

Ascorbic acid may also affect CAFs *via* suppression of HIF1α. Tumor-induced ROS-mediated "pseudo-hypoxia" in CAFs leads to the accumulation of HIF1α and enhanced aerobic glycolysis (173, 174). Furthermore, high expression of HIF1α in CAFs induces protein expression in myofibroblasts in CAFs, and inhibition or knockout of HIF1α improves their phenotype (175). Stimulation by TGF-β or PDGF also suppresses IDH3 expression and decreases 2-oxoglutarate in fibroblasts, resulting in HIF1α accumulation and regulating fibroblast differentiation into CAFs (176). Ascorbic acid may inhibit the accumulation of HIF1α by promoting the reaction of 2-oxoglutarate-dependent dioxygenases such as PHDs and FIH-1, thereby suppressing fibroblast differentiation into CAFs. The JAK1/STAT3 pathway is also an important pathway that maintains actomyosin contractility and the CAF phenotype (177), and methylation of the promoter of protein tyrosine phosphatase non-receptor type 6 (SHP-1), which negatively regulates the JAK/STAT pathway, allowing for sustained signaling (167). For this epigenetic reorganization, DNA demethylase-mediated effects such as TETs of ascorbic acid may be exerted. However, CAFs have an enhanced glycolytic system due to chronic hypoxia in the tumor microenvironment and subsequent epigenetic reorganization by demethylation of HIF1α and promoters of enzymes of the glycolytic system (178),

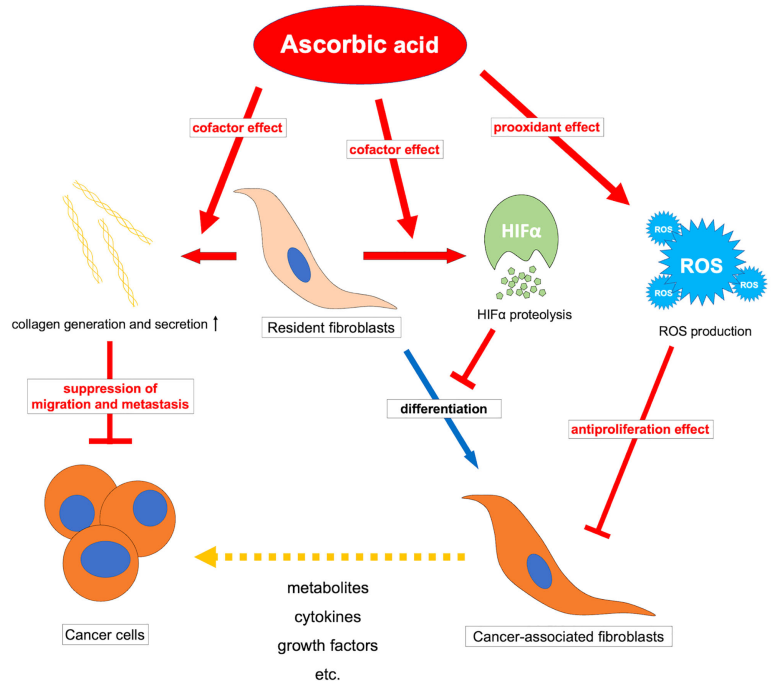

FIGURE 3
Overview of the antitumor effects of ascorbic acid on cancer-associated fibroblasts. ROS, reactive oxygen species; HIFα, hypoxia-inducible factor-alpha.

and there may be unexpected epigenetic effects of ascorbic acid that should be clarified in the future. Ascorbic acid may have a tumor suppressive effect by affecting CAFs and reprogramming them into normal fibroblasts. (Figure 3) It is possible that the antitumor effect of ascorbic acid can be improved by examining the method of administration and adapting it to the expression status of HIF in tumors and CAFs. Furthermore, elucidating the effects of ascorbic acid targeting not only tumor cells but also tumor microenvironments such as CAFs may help to reveal further antitumor effects of ascorbic acid.

Author contributions

TMa and TMi drafted the manuscript, which was subsequently critically revised by MT and SU. 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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Cross-disease communication between cancer and heart failure provides a rational approach to prevention and treatment of both diseases

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Accumulating clinical data have demonstrated a clear positive association between cancer and cardiac disorders, particularly chronic heart failure (CHF). These two diseases can be mutual drivers of each other, and hence frequently co-occur in patients. The immune system is the core mechanism that eliminates transformed cells from our bodies. However, immune cells often play distinct or even conflicting roles in cancer and CHF. Moreover, CHF alters the properties of immune cells, particularly those of regulatory T cells. Our previous study showed that the oxidative phosphorylation capacity of peripheral blood mononuclear cells is impaired in CHF, leading to the increased production of reactive oxygen species. Therefore, the co-occurrence of cancer and CHF becomes a serious problem, affecting the treatment of both diseases, and consequently negatively affecting patient survival rates. To date, few methods have been identified that effectively treat both diseases at the same time. Mitochondria activity may change in immune cells during their activation and exhaustion, and in CHF. Mitochondria activity is also largely affected in myocardia in CHF. We here focus on the mitochondrial abnormalities of immune cells in cancer and CHF, and discuss possible ways to treat cancer and CHF at the same time by targeting mitochondrial abnormalities. Many cancer cells are inevitably produced daily in our bodies, mostly owing to enzymatic nucleotide errors of DNA replication and repair. Therefore, the possibility of ways to prevent cancer by preventing the onset of heart failure will also be discussed.

KEYWORDS

immune-checkpoint inhibition, mitochondrial oxidative phosphorylation, disease prevention, myokine, PBMC, exercise, diet, reactive oxygen species

Introduction

Cancer and cardiac disorders, including chronic heart failure (CHF), represent two major causes of morbidity and mortality in developed countries (1, 2). Epidemiological studies have shown that the risk of developing cancer in patients with CHF is approx. four times greater than in those without CHF (3–6). Conversely, cancer patients can be at increased risk of cardiac disease due to deterioration of their lifestyle behaviors (*e.g.*, inactivity and an unbalanced diet) (7), and also due to treatment toxicity, as many anticancer drugs are known to cause cardiotoxic side effects (8–11). Therefore, cancer and cardiovascular diseases can be mutual disease drivers, and hence co-occur frequently in patients (Figure 1). Moreover, immune cells, particularly regulatory T (Treg) cells, play distinct or even conflicting roles in cancer and CHF (12, 13). Hence, the co-occurrence of cancer and cardiovascular disease is a serious problem, affecting the treatment of both diseases, and consequently negatively affecting survival rates (14, 15). To date, however, treatments exist only for each disease. Mitochondria are central to ATP production by oxidative phosphorylation (OXPHOS) and to metabolism. To address above problems, we here focus on the mitochondrial abnormalities of immune cells during CHF and cancer, and discuss possible methods to treat cancer and CHF at the same time by targeting these mitochondrial abnormalities; and, moreover, discuss possible ways to prevent cancer by preventing the onset of CHF.

Immune system mediates the crosstalk between cancer and CHF

T-cell dysfunction, particularly of tumor-infiltrating lymphocytes (TILs), is highly detrimental to antitumor immunity and immunotherapy (16). Recently, Koelwyn et al. reported that the adjusted relative risk of death from breast cancer is increased by approx. 60% in the presence of a cardiovascular event (17). They also demonstrated by using mouse models that myocardial infarction (MI), which leads to HF, accelerates breast cancer development (17). Molecularly, it was shown that MI epigenetically reprogrammed $Ly6C^{hi}$ monocytes, which are macrophage precursors in the bone marrow reservoir, to an immunosuppressive state, and increased their circulation and infiltration into tumors, whereas their depletion abrogated tumor growth (17). Moreover, tumors of MI mice had fewer T lymphocytes than control mice, in which T_{reg} cells are predominant. These changes that occur in MI mice may be beneficial to the heart, but they all promote tumor growth and survival (17). Therefore, certain populations of immune cells clearly play a central role in cross-disease communication between cancer and CHF.

CHF affects mitochondrial OXPHOS of immune cells

Mitochondrial OXPHOS plays a central role in lymphocyte activity (18). Mitochondria are also fundamental to the development and fate determination of peripheral lymphocytes (19, 20). Suppressed glycolysis and OXPHOS were shown to be early drivers of $CD8^+$ T-cell exhaustion (21). Moreover, TILs are constantly exposed to tumor antigens, and may also experience metabolic stress, which is thought to occur frequently in the tumor microenvironment. A recent report demonstrated that continuous antigen stimulation together with hypoxia impairs the mitochondrial functions of T cells, and hence promotes terminal T-cell exhaustion (22). Molecularly, it was shown that continuous antigen stimulation upregulates B lymphocyte–induced maturation protein 1, and represses peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1), resulting in the suppression of mitochondrial biogenesis and T-cell functions (23).

Our group has found that mitochondrial respiratory capacity of peripheral blood mononuclear cells (PBMCs), which are predominantly lymphocytes, declines with the progression of CHF, with class III (i.e., moderate to severe CHF) patients by New York Heart Association (NYHA) criteria having 10-24% lower mitochondrial respiratory capacity than NYHA class I/II (i.e., mild CHF) patients, in which mitochondrial ROS production of PBMCs was increased by 13-24% in patients with NYHA class III compared to those with NYHA class I/II (24). Such changes were observed even in the early stages of HF, and were closely associated with the severity of CHF. We moreover found that the capacity of complex II, but not complex I, of the mitochondrial OXPHOS of PBMCs was specifically decreased in CHF (24). It has been reported in monkeys that there is a close association among the mitochondrial OXPHOS activities of circulating monocytes, cardiac cells, and skeletal muscle cells (25).Therefore, ROS levels in PBMCs can be a marker indicating the onset and the severity of HF. As PBMCs mostly consist of unprimed lymphocytes, it awaits to be clarified whether activated lymphocytes are also affected in CHF patients.

Activating mitochondria of immune cells improves tumor immune therapies

Mitochondrial function of $CD8^+$ T cells in lung cancer patients can be a marker for determining the efficacy of anti-PD-1 immune checkpoint inhibition therapy (26). Scharping et al. have shown that restoration of mitochondrial activity and T-cell function by reversing the loss of PGC-1α in tumor-specific T cells resulted in increased antitumor immune responses (23). Yu et al. demonstrated that administering nicotinamide riboside (NR), a

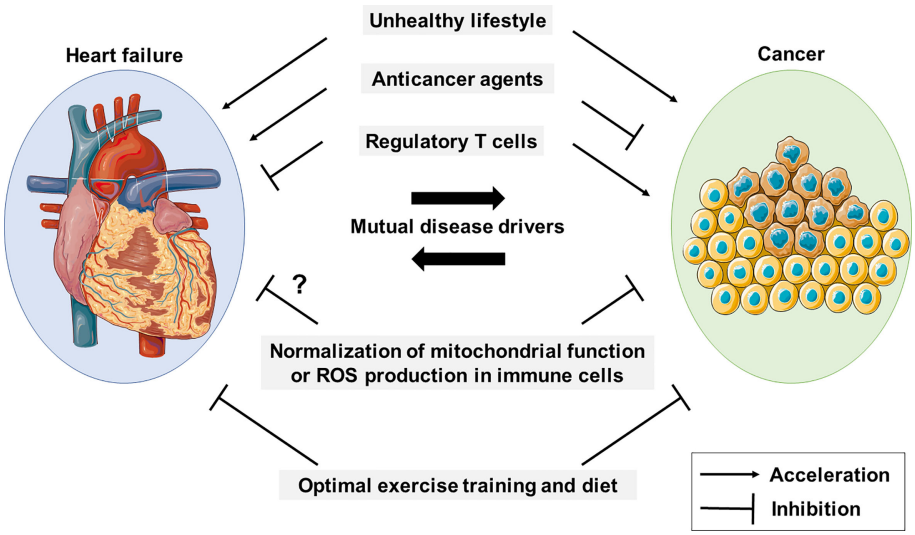

FIGURE 1
Cancer and HF mutually promote each other. An unhealthy lifestyle contributes to the development of cancer and CHF, and they are mutual disease drivers. Anticancer drugs and regulatory T cells appear to have conflicting roles in cancer and HF. Mitochondrial function and reactive oxygen species (ROS) production in immune cells are potential therapeutic targets in both diseases.

precursor of nicotinamide adenine dinucleotide, may be able to restore mitochondrial activity, prevent T-cell exhaustion, and sustain the antitumor responses of T cells in tumor-bearing mice (27). NR supplementation was moreover found to facilitate antitumor immune activity, when used in conjunction with the anti-PD-1 antibody (27). Vardhana et al. demonstrated that N-acetylcysteine (NAC), which is known to increase glutathione synthesis and neutralize ROS, reverses the metabolic defects of exhausted T cells, and promotes their antitumor immune activity, to act synergistically with anti-PD-L1 immunotherapy in lymphoma and melanoma (28). Therefore, activating immune cell mitochondria may improve the efficacy of immune checkpoint inhibition-based tumor immunotherapy. However, it should be noted that the administration of molecules such as NR or NAC may also activate cancer cells to more malignant states, and it is hence unclear whether they will be effective in the treatment of patients. It is also well documented that the reinvigoration of T cells, once they are deeply exhausted, might be very difficult (29). Another way to improve the efficacy of cancer immunotherapy would be to enhance the new production of T cells, and diversify the T-cell receptor repertoire, as has been demonstrated with radiation (30), but this might also be difficult in patients with CHF because of their poor health condition.

Future perspectives

When cancer and CHF coexist, the treatment of either disease alone is inadequate. Normalization of mitochondrial activity and the function of immune cells, which are frequently impaired in CHF, is a rational strategy to improve cancer therapeutics. For example, identification of a molecular basis for the downregulation of mitochondrial respiratory capacity in the PBMCs of CHF patients, which we have shown previously (24), and if such a mechanism occurs specifically in PBMCs but not in tumor cells, improving mitochondrial respiratory capacity in PBMCs may be promising for the treatment of cancer in patients who also have CHF. Such a strategy targeting immune cells' mitochondria may also enhance tumor growth suppression in cancer treatment by immune checkpoint inhibitors, although cardiac assessment with a careful follow-up is necessary because immune checkpoint inhibitors are known to have a cardiotoxicity with low incidence rate (<1%) with single use of them (31). Furthermore, activation of immune cells is beneficial for chemotherapy (32), and thus, mitochondria-targeted treatment strategy may help chemotherapy improve outcomes of cancer patients with or without CHF, although robust clinical evidence is still lacking.

Lifestyle habits, such as a proper diet and daily exercise are important preventive measures of cancer and CHF. Regarding the molecular bases, skeletal muscles secrete various myokines, which have positive effects on mitochondria in different organs and tissues, and may also promote immunity (33–35). Proper exercise by patients can also suppress tumor growth and promote anti-tumor immunity, and may improve the therapeutic effects of immune-checkpoint inhibitors, whereas the types of myokines and immune cells therein involved have been shown to differ depending on the types of cancer (36–38). On the other hand, muscle dysfunction occurs not only in CHF

(35, 39), but is also a widespread phenomenon of cancer patients regardless of cancer type or stage (40). Therefore, the identification of the singular point (41) before which exercise can be effective in the treatment of cancer and CHF, along with identification of effective exercise regimens and the related drugs, will be the major challenge of medicine in the future.

Author contributions

ST, SK, HH, TY, and HS wrote the manuscript. 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.

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Orchestration of mesenchymal plasticity and immune evasiveness *via* rewiring of the metabolic program in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is the most fatal cancer in humans, due to its difficulty of early detection and its high metastatic ability. The occurrence of epithelial to mesenchymal transition in preinvasive pancreatic lesions has been implicated in the early dissemination, drug resistance, and cancer stemness of PDAC. PDAC cells also have a reprogrammed metabolism, regulated by driver mutation-mediated pathways, a desmoplastic tumor microenvironment (TME), and interactions with stromal cells, including pancreatic stellate cells, fibroblasts, endothelial cells, and immune cells. Such metabolic reprogramming and its functional metabolites lead to enhanced mesenchymal plasticity, and creates an acidic and immunosuppressive TME, resulting in the augmentation of protumor immunity *via* cancer-associated inflammation. In this review, we summarize our recent understanding of how PDAC cells acquire and augment mesenchymal features *via* metabolic and immunological changes during tumor progression, and how mesenchymal malignancies induce metabolic network rewiring and facilitate an immune evasive TME. In addition, we also present our recent findings on the interesting relevance of the small G protein ADP-ribosylation factor 6-based signaling pathway driven by *KRAS*/*TP53* mutations, inflammatory amplification signals mediated by the proinflammatory cytokine interleukin 6 and RNA-binding protein ARID5A on PDAC metabolic reprogramming and immune evasion, and finally discuss potential therapeutic strategies for the quasi-mesenchymal subtype of PDAC.

KEYWORDS

EMT, inflammation, metabolic reprogramming, immune evasion, ARF6, ARID5A, IL-6

Introduction

Pancreatic ductal adenocarcinoma (PDAC) originates from epithelial cells of the exocrine pancreas, which is composed of secretory acinar cells and ductal cells (1). PDAC patients often have an unfavorable prognosis, and the 5-year overall survival rate has been reported to be only 11% in the United States (2). Only 20% of PDACs are confined to pancreatic tissue at diagnosis, approximately 30% have metastasized to regional lymph nodes, and more than 50% have disseminated to other tissues, primarily the liver and lungs (3).

Four major driver mutations have been identified in PDAC, including *KRAS*, *TP53*, *CDKN2A*, and *SMAD4/DPC4* mutations (4–6). Constitutive active mutations of *KRAS* occur in more than 90% of patients, often demonstrate oncogenic activity, and have been shown to be involved in the initiating event of PDAC tumorigenesis (6–9). In addition, oncogenic *KRAS* has been shown to promote tumor signaling through metabolic reprogramming (10) and stromal interactions (11) to facilitate tumor growth. Mutations in *TP53* also often result in oncogenic activity, and are present in up to 70% of PDACs, typically occurring at late stages of PDAC carcinogenesis, and are frequently associated with invasive and metastatic phenotypes (6, 12). Furthermore, *TP53* mutations play an important role in inducing platelet-derived growth factor (PDGF) receptor B expression, which associated with reduced disease-free survival in PDAC patients (13).

Because of the lack of effective diagnostic biomarkers for PDAC and the absence of early symptoms, the diagnosis of PDAC is often made at advanced, terminal stages. Current treatment options include surgery, if possible, or chemotherapy (gemcitabine, FOLFIRINOX [fluorouracil, leucovorin, irinotecan, and oxaliplatin], etc.), and radiation therapy, all with limited efficiency and achieving only slightly prolonged survival (14, 15). Immune checkpoint-based immunotherapies have been incorporated, albeit to a limited extent, into treatment modalities for some other cancers, but clinical trials targeting checkpoint molecules, such as CTLA4, PD-1/PD-L1, or their other cognate ligands have been unsuccessful for the treatment of PDAC. So far, there have been no successful clinical trials against PDAC, even those targeting multiple immune checkpoints (16–18).

PDAC cells also demonstrate a poor nutritional status, high levels of oxidative stress, inflammatory stress, extracellular acidosis, hypoxia, and decreased angiogenesis (15, 19, 20). Consistently, these are strong selection pressures that enable only cells that have adapted their metabolism to these hostile conditions to survive and proliferate. Notably, accumulating lines of evidence suggest that these adaptations also make PDAC cells more invasive, metastatic, stem cell-like, and resistant to therapeutic treatments (21). Consistently, several genome-wide gene expression profiling and genomic sequencing approaches to elucidate the molecular landscape of PDAC have demonstrated that the so-called basal-like (also known as quasi-mesenchymal-like or squamous) subtype is associated with a less favorable prognosis than other subtypes (22–25). Importantly, PDAC metabolite profiling and transcriptional analysis confirmed that the quasi-mesenchymal-like subtype is associated with the glycolytic subtype (26–28). This reorganization of pancreatic cancer cell metabolism opens the way for new therapeutic opportunities (20). However, the substantial heterogeneity in gene expression and metabolic characteristics, the plasticity of pancreatic cancer cells, and the pathological changes associated with their linked physicochemical and biological changes in the tumor microenvironment (TME) make PDAC a challenging disease to cure (26, 27, 29).

In this review, we summarize recent studies on how gene expression changes *via* intrinsic genetic mutations and epigenetic alterations involved in the acquisition of mesenchymal traits in PDAC cells, particularly post-transcriptional dysregulation of expression, are linked to metabolic reorganization associated with immunosuppressive TME formation during the development and malignant progression of PDAC.

Recently, PDAC has been hypothesized to be associated with two morphologically distinct precursors, i.e., pancreatic intraepithelial neoplasia (PanIN) and intrapapillary mucinous neoplasia (IPMN). PanIN can progress to invasive carcinoma in a stepwise and linear manner, which is an established mechanism of PDAC progression (30). Multiple studies have reported the sequential accumulation of PDAC driver gene mutations in PanIN, with *KRAS* mutations being the earliest known genetic alterations, being present in more than 90% of all PanINs regardless of cancer grade (31). On the other hand, the inactivation of *CDKN2A* is rare in low-grade PanIN, but has been reported to occur in more than 70% of high-grade PanIN (32). Mutations in *TP53* and *SMAD4* occur during the late stages of PanIN progression, and are almost exclusively found in high-grade PanIN and invasive PDAC. In contrast, IPMN is driven by four driver gene mutations of pancreatic tumorigenesis similar to PanIN, including early mutations in *KRAS* and late mutations in *CDKN2A*, *TP53*, and *SMAD4* (33). However, there are also two frequently altered driver genes specific to the IPMN pathway. Mutations in the oncogenic hotspot of *GNAS* are known to occur early in IPMN tumorigenesis (33–35). In addition, although inactivating mutations in *Ring finger protein 43* (*RNF43*), which encodes a ubiquitin ligase involved in WNT signaling (often with loss of heterozygosity) are also common in IPMNs (36), the precise timing of the occurrence of *RNF43* mutations in IPMN tumorigenesis has not yet been clarified to date.

In addition, we present our recent findings on the intriguing relevance of the small G protein ADP-ribosylation factor 6 (ARF6)-based signaling pathway driven by *KRAS/TP53* mutations, as well as the inflammation amplifying signals

mediated by the inflammatory cytokine interleukin 6 (IL-6) and the RNA-binding protein AT-rich interactive domain 5a (Arid5a) on PDAC metabolic reprogramming and immune evasion. We will present our recent findings on the relevance of these pathways, and finally discuss potential therapeutic strategies for the quasi-mesenchymal subtype of PDAC.

Plasticity of adult pancreatic tissues

The pancreas is an important organ responsible for metabolic control in the body, and is composed of two morphologically and functionally distinct components. The exocrine pancreas, accounting for more than 95% of total organ mass, is composed of acinar cells, which produce digestive enzymes, and ductal cells, which deliver these enzymes to the intestine. On the other hand, the endocrine islets of Langerhans consist of five different cell groups (α, β, δ, PP, and ε cells) that secrete various hormones, such as insulin and glucagon, and play crucial roles in the regulation of glucose metabolism. The exocrine and endocrine pancreas are associated with different diseases. Pancreatitis and pancreatic cancer, mostly PDAC, arise from the exocrine pancreas, whereas rare pancreatic neuroendocrine tumors arise from the endocrine islets, and diabetes is also a result of endocrine islet dysfunction (37). The mammalian pancreas has the capacity for regeneration after injury even in adults, with the acinar compartment having the highest plasticity in humans. Through epigenetic transcriptional regulation, acinar cells can dedifferentiate into an embryonic progenitor-like phenotype, and commit to either insulin$^+$ β-cells (38) or ductal cells (known as acinar to ductal metaplasia [ADM]) (39, 40). ADM transdifferentiation occurs in chronic pancreatitis *via* nuclear factor-κB (NF-κB) activation, and is associated with pancreatic intraepithelial neoplasia, which is a necessary step for the generation of neoplastic precursor lesions called PanINs (Pancreatic intraepithelial neoplasia) (41–43). Thus, it has been speculated that the acinar cells of the exocrine pancreas maintain plasticity to adapt to changes in the external environment, and that their dysregulation leads to pancreatitis and pancreatic cancer.

Heterogeneity of PDAC

To date, gene expression studies of PDAC have included comprehensive analyses focusing on subtyping of primary tumors obtained by surgical resection. Representative reports include the three-group classification by Collisson et al. (classical, quasi-mesenchymal, exocrine-like) (22), the two-group classification by Moffitt et al. (basal-like, classical) (23), and the four-group classification by Bailey et al. (squamous, immunogenic, pancreatic progenitor, and aberrantly differentiated endocrine exocrine) (24). Each of these classifications has been able to predict the prognosis of patients with resected PDAC on multivariate analysis. Notably, in about half of PDAC tumors, increased expression levels of hypoxia-associated genes were observed by RNA sequencing (RNAseq), and were substantially associated with basal-like subtypes, although there was no redundancy in the identified gene sets (44). Regarding morphology, PDACs are classified as having more or less than 40% glandular histogenesis, and are strongly associated with classical or basal subtypes, respectively (45). The squamous morphology found in more than 30% of invasive tumors has also been associated with basal-like tumors by several groups (16, 45). However, the mechanism by which PDAC diverges into various subtypes in the process of tumor evolution remains unclear. Recently, it has been reported that re-categorization of PDAC subtypes in a combined cohort of primary and metastatic tumors using single-cell RNAseq (scRNAseq) can lead to the extension of the two groups of basal-like and classical into five groups: "basal-like A", "basal-like B", "classic A", "classic B", and "hybrid" (46). These data sets, combined with cohort of patients with PDAC, enable the broad categorization of basal-like A and basal-like B into two disease subtypes, localized and metastatic disease, respectively. Thus, it is suggested that PDAC proceeds as a mixture of both expressed phenotypes, and that the behavior of the dominant phenotype and subtype is due to plasticity in both (46). The driver mutations for the classical and basal-like subtypes were shown to be biallelic loss of *SMAD4* with *GATA6* amplification, and biallelic loss of *TP53* and/or *CDKN2A* with mutant *KRAS* allele amplification, respectively, but none of the features were completely exclusive (45, 46). Therefore, whereas scRNAseq analysis of precancerous lesions to determine whether these expression phenotypes are established in PanIN has not been performed to date, the early acquisition of asymmetric driver gene mutations is itself dynamic, presumably dictating PDAC behavior, suggesting that both clonality and plasticity of PDAC cells are responsible for the histological and biological heterogeneity.

Current diagnosis and treatment methods of pancreatic tumors

Symptoms of PDAC and its diagnosis

Symptoms of PDAC are often vague and nonspecific, and hence it is sometimes referred to as the 'silent killer'; in fact, 30% to 35% of patients are diagnosed with locally advanced stages and 50% to 55% with metastatic stages of disease. Biomarkers for the early detection of PDAC have not yet been identified. The most common site of this tumor is the head of the pancreas, which causes biliary obstruction, resulting in dark urine, jaundice, appetite loss, fatigue, weight loss, and exocrine pancreatic insufficiency (47).

As early symptoms of PDAC are less frequent than those of any other cancer, and a method for its early diagnosis has not been established, multidisciplinary examinations are required to detect the pancreatic tumor. The pancreas is a digestive organ that also acts as an endocrine system, and hence has abundant blood vessels. This feature makes PDAC easy to metastasize and difficult to resect. There are four clinical stages in PDAC; 1) I–II resectable (5-year survival rate, 35%–45%), 2) II–III borderline resectable (10%–15%), 3) II–III locally advanced (10%–15%), and 4) metastatic (< 5%). Pancreas computed tomography (CT) angiography with chest and pelvis CT can be used for assessment of the vascular anatomy of the pancreas. The degree of contact between the tumor and local blood vessels is classified into three levels; uninvolved, abutted, or encased. The difference between abutment and encasement is the degree of circumferential tumor-vessel involvement; existence of the tumor more than 180 degrees around the vessel implies encasement. Magnetic resonance imaging and cholangiopancreatography are also helpful to assess the possibility of metastasis in indeterminate liver lesions, and are also useful for the identification of cancers that are poorly characterized on CT imaging (47).

Conventional treatments and ongoing notable clinical trials

Patients with nonresectable tumors are treated by chemotherapy according to their cancer stage and Eastern Cooperative Oncology Group (ECOG) performance status (48). Combinations of cytotoxic chemotherapies were developed in the previous decade and are still the basis of current treatments for metastatic pancreatic cancer (Figure 1) (49). Two multidrug regimens are now offered; FOLFIRINOX, and gemcitabine combined with nanoparticle albumin-bound paclitaxel (nab-paclitaxel). Gemcitabine alone is offered to patients with ECOG performance status 2 (within the five ECOG criteria, the groups in which patients are capable of self-care but are unable to carry out any work activities; i.e., patients are up and about > 50% of their waking hours, Figure 1). To classify patients eligible for either FOLFIRINOX or gemcitabine plus nab-paclitaxel as first-line drugs, Knox et al. demonstrated that a low level of GATA6, which is a characteristic of basal-like tumors, is a useful biomarker for selecting gemcitabine plus nab-paclitaxel in first-line therapy (50). The PASS-01 study analyzing the usefulness of GATA6 as a surrogate marker is now ongoing (NCT04469556).

Recent scientific advances have made incremental progress for the treatment of specific subgroups of pancreatic tumors. The American Society of Clinical Oncology guidelines updated in 2020 state three recommendations for pancreatic cancer; [1] early testing of both germline and tumor cells for microsatellite instability/mismatch repair deficiency, *BRCA* mutations, and *NTRK* gene fusions, [2] larotrectinib or entrectinib after first-line therapy for patients with tumors harboring *NTRK* fusions, and [3] continued treatment, including chemotherapy or olaparib, for patients with a germline *BRCA1* or *BRCA2* mutation who have received first-line platinum-based chemotherapy (51).

Although oncogenic *KRAS* mutations are observed in almost 90% of PDACs, a lack of drug-accessible pockets in the KRAS protein has hindered the development of their inhibitors for many years. However, X-ray crystallography identified a cryptic pocket of $KRAS^{G12C}$ potentially useful for drug development (52,

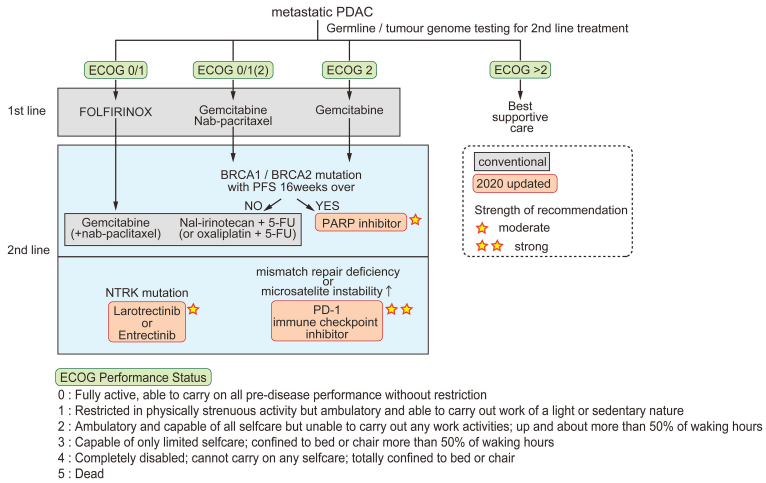

FIGURE 1
Conventional and updated guidelines for metastatic PDAC. Patients are classified by ECOG performance status for first-line chemotherapy. For second-line therapy, three options are recommended in the 2020 updated guidelines. The strength of recommendation is indicated by the number of stars.

53). A phase 1/2 clinical trial for the clinical-grade $KRAS^{G12C}$ inhibitor AMG-510 (sotorasib) is currently ongoing (NCT03600883). Other drugs targeting mutant KRAS proteins are also being developed (54).

Clinical trials of immune checkpoint inhibitors (ICIs) against PDAC were started with great expectations, but let researchers down because of their limited efficacy compared with their efficacy against other solid tumors, including melanoma and lung cancer (16, 17). These disappointing results were attributed to the unique characteristics of PDAC, which are explained in the following sections. Given these facts, ICI treatment in combination with other types of agents to increase treatment efficacy have been widely considered for the treatment of PDAC (54).

The concept of targeting cancer metabolism has existed for almost a century, since Otto Warburg's observation of aerobic glycolysis in cancer cells and Sidney Farber's paper describing anti-folate-induced remission of childhood acute lymphocytic leukemia (55, 56). Their concepts were eclipsed for some time during which knowledge on oncogenes accumulated and molecular-targeting therapies showed substantial effects on patient survival. However, recent technological innovations leading to various omics analyses have clarified the connection between tumor-associated genes and metabolism (57). Mitochondria, which play various roles in cancer metabolism and malignancy, are typical targets of metabolic agents (58). The lipoate analogue CPI-613, which inhibits pyruvate dehydrogenase and α-ketoglutarate dehydrogenase and therefore disrupts mitochondrial function (59), is being evaluated in a phase III trial of metastatic PDAC (NCT03504423) (60). In this trial, both groups (with or without CPI-613) are treated with FOLFIRINOX, because it has been reported that CPI-613 enhances FOLFIRINOX cytotoxicity in some PDAC cell lines (14). Another treatment target of PDAC is autophagy, which is activated in PDAC (61). A clinical trial of combinatorial treatment of hydroxychloroquine, an inhibitor of lysosomal scavenging, a MEK inhibitor, and ICIs for PDAC patients is now ongoing (NCT04214418). As discussed here, the paradigm of targeting not only tumor cells but also the TME, including immune cells, could bring a bright future to PDAC therapy.

Acquisition of mesenchymal plasticity of PDAC cells, and clinical implications of EMT in PDAC

TME and mesenchymal plasticity of PDAC

A variety of stimuli, including mechanical stress, low pH, hypoxia, innate and adaptive immune responses, changes in the extracellular matrix (ECM), and treatment with antitumor drugs can activate epithelial-mesenchymal transition (EMT) in cancer cells (62). It has been shown in real clinical settings that EMT plays a role in pancreatic cancer cell dissemination to distant organs in the precancerous stage prior to and/or in parallel with primary tumor formation in PDAC (63). The fact that almost all patients who undergo complete surgical resection and are free of metastases at that time eventually die within 5 years is consistent with the early-seeding model (64–66), suggesting important roles for EMT in PDAC progression and its contribution to the poor outcome.

PDAC has been well documented to be a desmoplastic stroma consisting of a dense ECM infiltrated with heterogeneous cell populations, including immune cells, endothelial cells, and cancer-associated fibroblasts (CAFs) (67). The high density of the stroma limits oxygen supply to and diffusion in the TME, leading to the creation of a hypoxic environment. Desmoplasia is observed in the bulk of the ECM, and contains collagen, fibronectin, laminin, and hyaluronic acid. These ECM components are primarily produced by CAFs. CAFs are also involved in producing various cytokines, such as transforming growth factor β (TGF-β), IL-1, IL-6, and tumor necrosis factor, and facilitate EMT signaling pathways (68).

PDACs are characterized by hypovascular tumors in a hypoxic microenvironment, in which high interstitial fluid pressure occurs owing to desmoplasia (69). However, microvessel density (MVD) has been shown to vary considerably among PDAC tumors with its decline being associated with poor survival in inverse correlation with stromal surface area (70). The hypoxic microenvironment has broad effects on the biological behavior and malignant phenotype of PDAC, including pathological angiogenesis and metabolic reprogramming, synergistically contributing to PDAC development and therapeutic resistance. Hypoxia-inducible factors (HIFs) are essential for hypoxia-induced angiogenesis in PDAC through transcriptional activation of various angiogenic factors, such as vascular endothelial growth factor (VEGF). It has been shown that under hypoxic conditions, NF-κB activates the transcription of HIF-1α and its target gene *VEGF-A*, resulting in the increased secretion of VEGF, and enhanced angiogenesis in hypoxic pancreatic cancer cells (71). Phosphorylated signal transducer and activator of transcription 3 (STAT3) is also a hypoxia-responsive nuclear transcription factor that has been shown to act synergistically with HIF-1α to regulate angiogenesis under hypoxia in pancreatic cancer cells (72). Indeed, increased production of VEGF has been demonstrated in human PDAC cell lines and resected PDAC tumor tissues (73), showing that VEGF is produced under the control of activated HIF-1α and STAT3 under conditions of oxygen deprivation (74, 75). VEGF produced by human PDAC cell lines has functional activity to promote endothelial cell growth *in vitro*, and in large tumors in immunocompromised mouse xenograft models (76). In addition, the anti-VEGF

strategy was shown to markedly reduce the growth of human PDAC cell lines orthotopically implanted into mice with a decrease in tumor MVD (77, 78). Despite these preclinical data suggesting that angiogenesis is important in PDAC, the use of anti-angiogenic agents has not been clinically successful for treating PDAC. Chronic treatment with VEGF antibodies was found to induce hypoxia and lead to increased collagen deposition, epithelial plasticity, and metastatic burden (79). These results may underly the lack of success of angiogenesis inhibitors in clinical trials of PDAC.

We previously showed that ARF6 is activated by VEGF in endothelial cells and is required for VEGF-induced tubular formation and migration. Furthermore, we have shown that ARF6 signaling is involved in choroidal neovascularization, which is a major cause of vision loss in patients with age-associated macular degeneration. We also found that ARF6 signaling is involved in VE-cadherin recycling, and may be involved in the sprouting process of angiogenesis associated with VE-cadherin-based cell-cell junctions as well as cell migration/tubular network formation activities (80). In addition, we found that high expression of the Arf6 effector AMAP1 is associated with the fibrosis of pancreatic cancer (81).

Treatment strategies for PDAC targeting angiogenesis have been pointed out as a way to normalize the tumor vasculature, such as strategies that prune immature and inefficient blood vessels, eliminate unproductive vasculature, and enable the reliable delivery of intravenous cancer drugs (82, 83). The inhibition of ARF6 signaling, which is important for pathological angiogenesis and fibrosis, may contribute to therapeutic strategies for PDAC.

Recent analyses have redefined the view that cellular senescence is the onset of the tissue remodeling that operates during normal embryonic development and tissue damage. To this end, senescent cells cease their own proliferation and recruit phagocytotic immune cells to promote tissue regeneration (84). On the other hand, it is well known that senescence is associated with cancer; in PDAC, senescence appears to produce tumor suppressive effects at the earliest stages. However, some lines of evidence indicate that senescent cells in the TME can produce a senescence-associated secretory phenotype (SASP), mediated by NF-κB and CCAAT/enhancer-binding protein-β, including the secretion of proinflammatory cytokines (IL-6 and IL-8), chemokines (monocyte chemoattractant proteins [MCPs], macrophage inflammatory proteins [MIPs], TGFβ, and granulocyte–macrophage colony-stimulating factor [GM-CSF]), and proteases (84), and play protumorigenic roles during tumor progression (85). SASPs have been shown to induce cell plasticity by stimulating cancer cell proliferation, motility, and invasion, and by generating an inflammatory TME (86). Thus, in the PDAC microenvironment, SASP may be involved in promoting EMT.

Role of EMT in PDAC metastasis

An important aspect of the EMT program in cancer biology may be its involvement in not only facilitating cellular motility and invasiveness, but also in orchestrating the cancer stem cell state (CSCs) *via* epithelial-mesenchymal plasticity (87–89). Mechanistically, intrinsic oncogenic mutations, epigenetic gene expression conversion, and extrinsic inflammatory signals may enable highly epithelial and highly mesenchymal non-CSCs to reversibly transition to an intermediate quasi-mesenchymal state; in the case of epithelial cells, the transition is accompanied by EMT, whereas in the case of highly mesenchymal cells, it is induced by mesenchymal-epithelial transition. Presumably, similar responses might occur in normal epithelial tissue when stem cells are lost. Thus, in the invasion-metastatic cascade, the EMT program is thought to enable the seeding of cells from the primary tumor into the parenchymal layer of distant tissues, and subsequently confers stemness, giving the disseminated tumor cells the ability to form metastatic colonies (87–89).

Although it is clear that EMT is involved in tumor metastasis, the exact function of EMT in cancer is still being debated. Indeed, some studies on the effects of the EMT-transcription factors (TFs) SNAIL and TWIST in pancreatic cancer have questioned the role of EMT in metastasis. A study using PDAC model KPC (*Pdx1-cre; LSL-Kras^{G12D};Tp53$^{R172H/+}$*) mice, in which *TWIST* and *SNAIL* were independently conditionally knocked out, resulting in *Pdx1-cre; LSL-Kras^{G12D};Tp53$^{R172H/+}$;Twist1$^{flox/flox}$* and *Pdx1-cre; LSL-Kras^{G12D};P53$^{R172H/+}$;Snai1$^{flox/flox}$* mice, respectively, found that although EMT was suppressed, the deficiency of *SNAIL* or *TWIST* did not affect tumor progression, regional invasion, or dissemination. Thus, it has been argued that EMT is not required for invasive and metastatic activities of cancers. On the other hand, mice bearing abrogation of EMT-transcription factor (EMT-TF) have been shown to be correlated with chemosensitivity to gemcitabine, indicating EMT induces chemotherapy resistance in pancreatic cancer (90). Similar results have been reported in breast cancer models (91). However, other groups have shown using the same KPC mouse PDAC model that ZEB1 conditional knockout mice (*Pdx1-cre; LSL-Kras^{G12D};Tp53$^{R172H/+}$;Zeb1$^{flox/flox}$*) have significantly reduced PanIN and PDAC formation, and invasion and metastasis, thus clearly demonstrating a crucial role for the EMT-TF ZEB1 in the PDAC progression (92). Taken together, these studies indicate a trend toward the differential functions of EMT-TF; SNAIL and TWIST do not appear to be necessary, whereas ZEB1 conversely appears to be an important factor that is not compensated by other EMT-TFs.

Metabolic characteristics of PDAC

Glucose metabolism

Glucose is the principal carbon and energy source for the growth and maintenance of mammalian cells. Glucose catabolism occurs by two metabolic pathways; glycolysis and the tricarboxylic acid (TCA) cycle. These pathways not only fuel adenosine triphosphate (ATP) production, but also produce carbon intermediates that support macromolecular biosynthesis. One contribution of oncogenic *KRAS* mutations to the oncogenesis and progression of pancreatic cancer is oncogenic *KRAS* mutation-driven metabolic rewiring. Transcriptome and metabolomic analyses indicated that the activity of oncogenic *KRAS* mutations promoted the upregulation of key metabolic enzymes involved in glucose metabolism, including glycolysis, hexosamine biosynthesis leading to the synthesis of uridine diphosphate N-acetyl-glucosamine, which is a significant substrate for protein glycosylation, and the pentose phosphate pathway producing NADPH and ribose 5-phosphate, which are essential for nucleic acid synthesis (10). This analysis also indicated that oncogenic *KRAS* mutations enhance glucose consumption in PDAC through the increase in transcription of the glucose transporter 1 (GLUT1, also known as solute carrier family 2 member 1 [SLC2A1]) the enzymes hexokinase 1 and hexokinase 2 (HK1 and HK2), and lactate dehydrogenase A (LDHA) (Figure 2). Thus, *KRAS* contributes to the unregulated growth of pancreatic cancer cells, and directly targeting metabolic pathways as a therapeutic target is a major challenge (93).

We previously showed that mutant *KRAS*, which is a major driver gene in PDAC cells, acts in a eukaryotic translation initiation factor 4A (eIF4A)-dependent manner to promote the translation of ARF6 mRNA, which is a member of the ARF family of GTPases with a quadruplex structure in the 5′-untranslated region, and upregulates ARF6 protein expression (94). Recently, it was also reported that silencing of ARF6 inhibits the Warburg effect, which is associated with aerobic glycolytic processes, in *KRAS*-mutated PDAC cells (95). The oncogene *c-Myc* is a transcription factor that regulates aerobic glycolysis through the upregulation of many key glycolytic genes, such as *GLUT1*, *HK2*, and *LDHA* (96, 97), and is

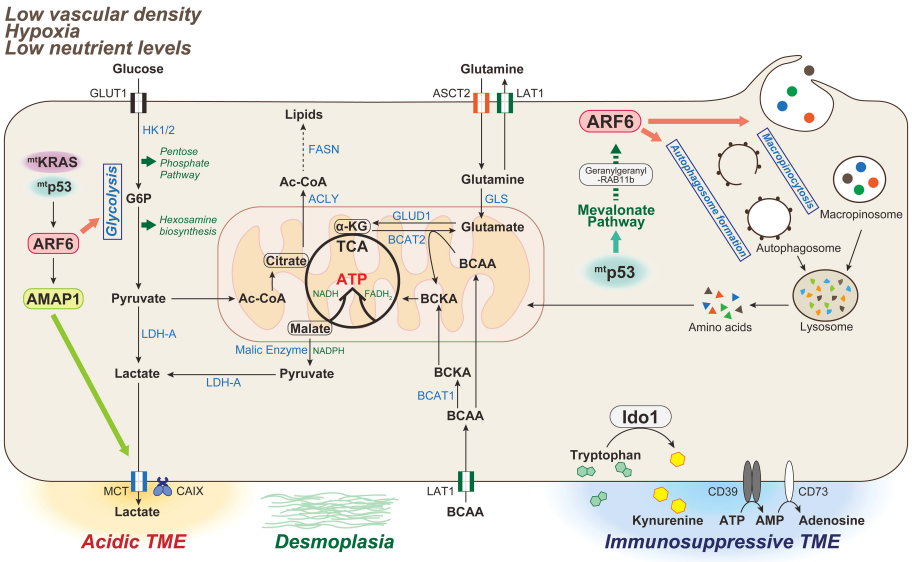

FIGURE 2
Metabolic characteristics of PDAC associated with the ARF6-based pathway. The tumor microenvironment (TME) in PDAC is characterized by low vascular density, resulting in severe hypoxia and low nutrient levels. PDAC is also characterized by a dense desmoplastic stroma. In mammals, glucose and glutamine are two of the most abundant nutrients that support cell survival and growth. Oncogenic *KRAS* mutations induce metabolic reprogramming by triggering the uptake of glucose, leading to increased glycolytic flux, carbon donation to the pentose phosphate pathway and hexosamine biosynthetic pathway, and lactate production driving acidic TME. Glutamine is also used as an energy substrate in the TCA cycle, and maintains the intracellular redox state of PDAC cells in an oncogenic *KRAS*-driven manner. Double mutations of *KRAS*/*p53* cooperatively promote the expression and activation of the ARF6-AMAP1 pathway, and ARF6 is involved in maintaining the Warburg effect to meet the abnormal nutritional and energy demands of PDAC cells, as well as those required for autophogosome and macropinosome formation. Mutant p53 promotes ARF6 activation *via* the enhanced expression of mevalonate pathway enzymes, and also intracellular trafficking of ARF6 mediated by geranylgeranylation of RAB11b. TCA, tricarboxylic acid; HK-1/2, hexokinase-1/2; G6P, glucose 6-phosphate; LDH-A, lactate dehydrogenase-A; MCT, monocarboxylate transporter; CA, carbonic anhydrase; Ac-CoA, Acetyl-CoA; ACLY, ATP-citrate lyase; FASN, fatty acid synthase; GLS, glutaminase; GLUD, glutamine dehydrogenase; BCAA, branched-chain amino acid; BCAT, branched-chain amino acid transaminase 1; IDO, indoleamine 2,3-dioxygenase.

associated with the transcriptional activation of ARF6. ARF6 has also been shown to be associated with the regulation of the expression of *GLUT1, LDHA*, and *HK2*, as well as *c-Myc* (95). Thus, it is possible that ARF6 is involved in the regulation of aerobic glycolysis *via* the regulation of *c-Myc* in PDACs. Interestingly, in several cancers, including PDAC, the upregulation of GLUT1 in cancer cells correlates with the low infiltration rate of cytotoxic $CD8^+$ T cells (98–100). This suggests that tumor cells successfully compete for glucose, suppressing antitumor immunity while simultaneously maintaining high metabolic and proliferative rates (101, 102). Importantly, this also indicates that antitumor immune cells are unable to obtain sufficient energy, thus impairing their function.

As solid tumors progress, large areas of the tumor often become deprived of oxygen, which interferes with ability of the immune system to combat the tumor (103). PDAC is characterized by a very hypoxic TME, and it has been noted that the high malignancy and poor curative efficacy of PDAC are mostly due to the hypoxic TME (103, 104). PDAC also shows increased accumulation of stromal tissue, i.e., desmoplasia, which may collapse blood vessels, and subsequently impede perfusion and promote maintenance of the hypoxic TME. Hypoxia and desmoplasia induce the expression of HIF-1α and its stabilization (105). HIF-1α is a key regulator of cellular responses to changes in oxygen concentration, and supports tumor cell adaptation to hypoxia in an oxygen-deprived TME.

Under hypoxic conditions (usually below 3% to 5% O_2), the HIF-1α subunit stabilizes and forms a dimer with the β-subunit aryl hydrocarbon receptor nuclear translocator (ARNT), which translocates to the nucleus to promote O_2-regulated gene expression. HIF-1α is considered to play a crucial role in of metabolic reprogramming (106). Several studies have confirmed that HIF-1α meets the metabolic needs of pancreatic cancer cells by increasing the expression of glycolysis-associated enzymes and the production of lactate (107–110). Indeed, the stabilization of HIF-1α has been reported to induce GLUT1 expression in a HIF-1α -dependent manner, increasing cellular glucose uptake and supporting aerobic glycolysis in cancer cells (111, 112). HIF-1α is also known to enhance the expression of LDHA (113, 114) and monocarboxylate transporter 4 (MCT4; encoded by *SLC16A3*) (115). LDHA reduces the dependence on oxygen-dependent mitochondrial oxidative phosphorylation (OXPHOS) by converting pyruvate to lactate, and the cell preferentially uses oxygen-independent glycolytic pathways to maintain sufficient ATP production to meet bioenergetic requirements, whereas MCT4 removes lactate from the cell by transporting it out of cells. Thus, HIF-1α drives the conversion from oxidative to glycolytic metabolism during hypoxia, which is not only beneficial for bioenergetic homeostasis, but may also promote tumor survival and growth.

Interestingly, it has been shown that the stabilization of HIF-1α by di-methyl-oxaloylglycine treatment markedly increases the level of ARF6 mRNA (116), and ARF6 activity is significantly promoted under hypoxia (117). As mentioned previously, ARF6 is also associated with the enhanced expression of genes involved in glycolytic metabolism in malignant pancreatic cancer with *KRAS* mutations, so hypoxia may potently promote glycolytic metabolism through the induction of HIF-1α and ARF6, thereby regulating the adaptive responses to a hypoxic environment.

In addition to glucose deprivation *via* tumor cells in the TME, higher rates of aerobic glycolysis in tumor cells may promote the production of lactic acid, which in turn increases the acidity of the TME. Excess lactate produced in tumor cells can also suppress $CD8^+$ T and NK cell activation, and enhance the function of immunosuppressive cells, such as myeloid subsets, and M2-polarized macrophages to an immunosuppressive phenotype (118, 119). This makes it difficult for immune cells to survive, but tumor cells can often adapt, survive, and multiply despite these harsh conditions. Tumor cells can respond to extracellular acidic pH conditions and regulate cellular acid homeostasis by altering the expression of proteins associated with pH regulation, such as monocarboxylate transporters and carbonic anhydrase (CA) (120). In *in vitro* models of melanoma, exposure to lactic acidosis has been shown to induce the EMT phenotype (121). In pancreatic cancer cells, lactate enhances the expression of IL-8 and contributes to EMT and metastasis (122–124), and tumor cells can use lactate as an alternative energy fuel to promote their proliferation (125). Indeed, high levels of lactate in PDAC have been shown to correlate with poor patient prognosis (126). Therefore, it is strongly suggested that the acidic environment in tumor tissue is involved in the acquisition of mesenchymal traits, and the augmentation of an immunosuppressive PDAC TME.

Lipid metabolism

Lipids are major components of biological molecules, and play important roles in various processes. Lipids are composed of thousands of different molecules, including phospholipids, sphingolipids, fatty acids, cholesterol, cholesteryl esters, and triglycerides. Such lipids are implicated in a variety of cellular processes, and are important components of biological membranes (127–132). Lipid uptake, accumulation, and lipogenesis are increased in various cancers, including pancreatic cancer, and provide energy for rapid tumor growth. In the early step of *de novo* lipid synthesis, ATP-citrate lyase (ACLY) catalyzes the conversion of citrate to acetyl-CoA, which is then converted to malonyl-CoA by acetyl-CoA carboxylase. The acyl groups of malonyl-CoA and acetyl-CoA bind to the acyl-carrier protein domain of fatty acid synthase (FASN) in an NADPH-dependent way to produce long-chain saturated fatty acid (133) (Figure 2).

Expression levels of lipogenic enzymes, including ACLY, are known to be often upregulated in PDAC (134, 135). Inhibition of ACLY activity suppresses PDAC cell growth in xenograft tumor models (136). Furthermore, PDAC patients, highly expressing

FASN, have been shown to have shorter overall survival than those expressing low levels of FASN (137). Overexpression of the *FASN* gene may be correlated with resistance to radiotherapy and gemcitabine in pancreatic cancer patients (138), and inhibition of FASN results in high cytotoxicity of this drug. As higher lipogenic activity has been shown in PDAC cells compared with normal cells, genetic and pharmacological inhibition of FASN and other lipogenic enzymes appears to be a promising therapeutic strategy.

The mevalonate pathway (MVP) is essential for cellular lipid metabolism, including cholesterol biosynthesis and the post-translational prenylation of proteins (139). The rate-limiting enzyme in this pathway, 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) reductase, has been considered as a prominent target for MVP inhibition, and is increased in a KRAS-driven PDAC mouse model (125, 140). Statins, which are reductase inhibitors, are used for the treatment of hypercholesterolemia (141). The anticancer effects of statins have also been analyzed *in vitro* in various cancer cell lines. Several studies have reported that simvastatin inhibits cancer cell proliferation by promoting apoptosis and reducing cell cycle progression *via* several kinds of signaling pathways, including mitochondrial apoptotic signaling pathways and the Rho signaling pathway involved in cell cycle arrest (142, 143). In addition, lipophilic statins (lovastatin, simvastatin, etc.) have been shown to be potent vaccine adjuvants *via* modulation of post-translational protein prenylation. Mechanistically, statins inhibit geranylgeranylation of the small GTPase Rab5, such as in antigen-presenting cells, causing inhibition of endosome maturation, sustained antigen retention, reinforced antigen presentation, and activation of T cells (144). Therefore, the MVP pathway is a potential target for cancer immunotherapy.

We have previously shown characteristic features that predict responders of MVP-based cancer treatment. We found that the Arf-GTPase ARF6, and its downstream effector AMAP1 (also called ASAP1/DDEF1), are often overexpressed in various types of cancer, including PDAC, and closely associated with poor patient survival (145–149). Interestingly, we found that the MVP is crucial for ARF6 activation in breast cancer cells. In this process, the MVP is essential for geranylgeranylation of RAB11b, which promotes intracellular trafficking of ARF6 to the plasma membrane where it is activated by RTKs. Furthermore, consistent with reports that gain-of-function mutants of p53 activate the MVP, it is clear that mutant p53 is essential for ARF6 activation (148, 150). Our *in vitro* experiments showed that the presence of statins improved the sensitivities of breast cancer cells to various drugs. In contrast, inhibition of MVP is ineffective when cancer cells do not overexpress components of the ARF6-based pathway. We have also shown that statins inhibit not only ARF6 activity and invasive potential but also recycling of the immune checkpoint molecule PD-L1 to the plasma membrane in pancreatic cancer cells (94). The chemopreventive effects of statins have been shown in pancreatic cancer cell lines (151–153) and pancreatic cancer model mice (154). Thus, the MVP may be crucial for promoting cancer cell invasion, metastasis, drug resistance, and PD-L1 recycling through the overexpressed ARF6 pathway activated by RTKs.

Glutamine metabolism

Glutamine addiction is common in various cancers, including PDAC (155–160). Glutamine may be a mitochondrial substrate for synthesis of macromolecules in cancer cells by supplying carbon to fuel the TCA cycle, and is a major nitrogen donor for the production of nucleotides and nonessential amino acids (155). In mitochondria, glutamine has essential roles in the synthesis of energy in the form of ATP through the TCA cycle and the OXPHOS process. Mitochondrial metabolism has been demonstrated to be important for tumor growth in several types of cancer, including PDAC (161, 162). Glutamine is the most abundant nonessential amino acid in the blood and plays various roles in cell metabolism (158, 163). Glutamine is first catalyzed to glutamate by the enzyme glutaminase. Glutamate is then converted to α-ketoglutarate through a deamination reaction catalyzed by glutamate dehydrogenase in the mitochondria. Subsequently, α-ketoglutarate enters the TCA cycle to supply metabolic intermediates, such as citrate and malate, producing NADH and FADH2 to generate ATP. Malate is converted to pyruvate leading to NADPH production, and then pyruvate is in turn transformed to lactate. Glutamine can also produce substantial amounts of the cofactor NADPH by glutaminolysis, in which malate is converted to pyruvate by malic enzyme. Glutamine-derived α-ketoglutarate is reductively carboxylated by mitochondrial isocitrate dehydrogenase 2 (IDH2) to isocitrate, which can then be isomerized to citrate. Citrate produced in the mitochondrial matrix is transported to the cytoplasm and then converted to isocitrate by aconitase in a reversible reaction. Cytosolic isocitrate is metabolized to α-ketoglutarate through cytosolic isoform of IDH1, which can also produce NADPH, which may be used for lipid synthesis. PDAC cells maintain cellular redox homeostasis, which is necessary for cell growth, by metabolizing glutamine in response to NADPH (157).

Circulating glutamine can be taken up *via* transporters, such as alanine-serine-cysteine transporter 2 (ASCT2, also known as SLC1A5), and can be exported or imported *via* large neutral amino acid transporter 1 (LAT1, also known as SLC7A5), in exchange for branched-chain amino acids (BCAAs; leucine, isoleucine, and valine). BCAAs are broken down by branched-chain amino acid transaminase 1 (BCAT1) on the cytosolic side and BCAT2 on the mitochondrial side to produce branched-chain α-keto acid and glutamate (Figure 2). Early-stage pancreatic cancer driven by mutant *KRAS* has been shown to

increase plasma BCAA levels (164). BCAT2, but not BCAT1, has been shown to be highly expressed in PanIN and PDAC ductal cells. Thus, it has been noted that the BCAA-BCAT2 axis driven by KRAS is important for PDAC development (165). In addition, some amino acid transporters (ASCT2 and LAT1) are overexpressed in pancreatic cancer (166), and associated with poor prognosis. PDAC cells are known to be highly dependent upon glutamine for tumor growth (157, 167). However, whereas the treatment of BPTES, a glutaminase inhibitor to target the glutamine metabolism, significantly inhibited PDAC proliferation, it did not affect PDAC cell death. Glutamine deprivation has been reported to activate macropinocytosis-associated autophagy and maintain proper intracellular glutamine levels by regulating glutamine metabolism. Furthermore, both glutamine deprivation and autophagy inhibition have been shown to robustly activate apoptotic cell death (168). Glutamine plays various roles in PDAC metabolic processes, suggesting that therapeutic strategies targeting the acquisition and utilization of this amino acid may be promising. However, glutamine deprivation was shown to promote the EMT signature *in vitro* and *in vivo* through an increase in the EMT master regulator Slug *via* ERK signaling and ATF4 activation (169). Thus, evaluating the effects of the simultaneous inhibition of distinct aspects of glutamine metabolism, such as the induction of autophagy and EMT on PDAC growth and metastasis may lead to new therapeutic approaches.

Recently, comprehensive analysis of metabolic enzymes by large-scale targeted proteomics demonstrated an enhanced metabolic system in malignant cancers to utilize glutamine-derived nitrogen for DNA synthesis (a shift in glutamine nitrogen metabolism) (170). In malignant cancer cells, the expression level of the metabolic enzyme phosphoribosyl pyrophosphate amidotransferase (PPAT), which transfers the nitrogen from glutamine to nucleic acid precursors, was markedly increased, whereas the metabolic enzyme responsible for glutaminolysis, namely, glutaminase (GLS) was decreased, indicating a shift toward nucleotide biosynthesis. In addition, meta-analyses of human cancers have shown that PPAT is most strongly associated with malignancy among the metabolic enzymes, particularly prominent in neuroendocrine cancers, including small cell lung cancer (SCLC) (170). Interestingly, the hazard ratio for PPAT is high in pancreatic cancer, whereas GLS expression does not significantly correlate with cancer prognosis. In PDAC mouse models, GLS inhibition does not demonstrate any anti-tumor effects *in vivo*, indicating an adaptive metabolic network that sustains proliferation (171). In cancers in which glutamine supply from the circulation is limited, such as PDAC, glutamine synthesis mediated by glutamate ammonia ligase, an enzyme involved in *de novo* glutamine synthesis, and the associated nitrogen assimilation and transfer to nitrogen-containing macromolecules, such as nucleotides, has been shown to be important (172). Thus, shifts in glutamine nitrogen metabolism that promote nucleotide biosynthesis *via* the increased expression of PPAT while suppressing the GLS response, as demonstrated in SCLC, are important in cancer malignancy, and may be a potential therapeutic target for pancreatic cancer in a glutamine-limited environment.

Autophagy/micropinocytosis

PDACs also rely upon metabolic pathways, such as autophagy and macropinocytosis, to survive and maintain metabolic homeostasis in harsh environments, such as those with low nutrient levels, hypoxia, desmoplasia, and high interstitial pressure. Autophagy is an indispensable intracellular pathway that provides intracellular energy by degrading unnecessary organelles and macromolecules in response to stimuli, such as starvation and accumulation of unfolded proteins (173). The molecular mechanism of autophagy is strictly regulated by more than 30 autophagy-related (ATG) proteins that are responsible for the dynamic autophagy pathways, and can be divided into the following series of steps: phagophore (isolation membrane) growth, closed double-membrane vesicle (autophagosome) formation, autophagosome-lysosome fusion, degradation within the lysosome, and recycling of the degradation products.

One of the characteristic features of PDAC is known to be increased autophagy. This is because owing to the tumor microenvironment of PDAC, in which the low vascular density results in severe hypoxia and limited nutrient utilization (61, 174), PDAC cells must rewire their metabolism to sustain proliferation. Indeed, the inhibition of autophagy by the genetic or pharmacological inhibitor chloroquine (an inhibitor of lysosomal acidification) resulted in mitochondrial metabolic abnormalities leading to decreased OXPHOS, reduced proliferation *in vitro*, and inhibited tumor growth *in vivo* (61). Furthermore, the significance of autophagy in PDAC tumorigenesis was confirmed by crossing a conditional knockout mouse of the autophagy essential gene *Atg5* with a PDAC mouse model (175, 176). This autophagy inhibition in mouse studies may exert anti-tumor effects by cooperating with the TME (177). Indeed, the crosstalk between stromal cells and tumor cells in PDAC is important, indicating that autophagy is required for stromal cells to secrete alanine, which is then taken up by tumor cells to support their growth (178). In a study using a PDAC mouse model expressing a tetracycline-inducible dominant-negative ATG4B protein which can reversibly and acutely inhibit autophagy in fully formed tumors, the inhibition of autophagy was shown to suppress tumor growth *via* intrinsic as well as extrinsic factors in tumor cells (61). This study also showed that the effect of inhibiting autophagy in the tumor itself on tumor regression was partially mediated by macrophages, indicating that induction of the immune system *via* autophagy

inhibition is also important for the anti-tumor effects. This may mean that there is autophagy-dependent metabolic crosstalk between tumor cells and the stroma, and hence autophagy is necessary to support the metabolism, tumorigenesis, and survival under harsh conditions of tumors.

PDAC does not respond well to ICIs, such as anti-PD1 and anti-CTL4A antibodies, and typically has a highly immunosuppressive TME that is characterized by marked infiltration of myeloid-derived suppressor cells (MDSCs) and lack of active cytotoxic $CD8^+$ T cells (179–182). Resistance to ICI therapy is known to be associated with major histocompatibility complex class I (MHC-I), which is essential for endogenous antigen presentation by cancer cells (183–185). PDAC cells have been shown to have reduced expression of MHC-I molecules on the cell surface, and instead localize predominantly to autophagosomes and lysosomes (186, 187). Indeed, it has been demonstrated in human and mouse PDAC that MHC-I is degraded by an autophagy-dependent mechanism to induce immune evasion (188). In addition, autophagy inhibition increased the surface levels of MHC-I, leading to the promotion of antigen presentation, enhanced anti-tumor activity of T-cell responses, and suppression of tumor growth in orthotopically transplanted syngeneic mice. Systemic autophagy inhibition by chloroquine, as well as the tumor-specific inhibition of autophagy, in combination with ICIs, showed synergistic effects. These findings provide a molecular mechanism by which autophagy promotes immune evasion, and provide a rationale for further research toward the development of new therapies targeting the autophagy-lysosome system in PDAC.

When glucose is deprived in PDAC cells, large amounts of reactive oxygen species are produced to activate autophagy, and provide the nutrients necessary for growth (189). On the other hand, glutamine starvation increases the degree of macropinocytosis in PDAC cells, and hence glutamine is important for regulating the degree of macropinocytosis in PDAC cells (190). Macropinocytosis is a process involving membrane ruffles, which are used to internalize extracellular materials, such as soluble molecules, nutrients, and antigens. After the nonspecific uptake of extracellular fluids by endocytic processes, the formation of vesicular structures, named macropinosomes, which contain the internalized proteins fuse with lysosomes, resulting in proteolytic degradation. The free amino acids produced by this process support the metabolic requirements of tumor cells (191). Thus, macropinocytosis is a nonselective endocytotic program capable of taking up content from extracellular fluid in a nutrient recycling and scavenging pathway that has been recognized as a key mechanism supporting pancreatic cancer growth (192).

PDAC cells expressing oncogenic *KRAS* mutation exhibited high enhancements of basal macropinocytosis consuming extracellular proteins for rapid tumor proliferation, which is closely linked to autophagy (174, 193–198). It has been shown that autophagy is required for the micropinocytosis-mediated degradation of extracellular proteins, and autophagy plays an important role in the breakdown of macromolecules internalized by macropinocytosis, to provide amino acids, particularly glutamine, in PDAC cells (168). The dynamic balance between glutamine metabolism and macropinocytosis-associated autophagy may ensure PDAC cell growth. Although these studies suggest that macropinocytosis is a potential therapeutic target for PDAC, understanding how macropinocytosis and autophagy cooperate is crucial for establishing treatments for PDAC.

ARF6 has been shown to regulate autophagy and colocalize with proteins mediating the initiation of autophagosome formation, i.e., the formation of pre-autophagosomal structures and phagophores (199, 200). Mechanistically, activation of the lipid-modifying enzyme PIP5K by ARF6 may contribute to autophagy, as PIP2 produced by PIP5K affects membrane trafficking for phagosome formation, by regulating plasma membrane endocytosis. Interestingly, ARF6 has been shown to be required for macropinocytosis in HT180 cells, a human fibrosarcoma cell line (201). In PDAC expressing high levels of ARF6, ARF6 may be a potential target for autophagy and micropinocytosis, and combination therapy, such as ICIs, may lead to a new treatment for PDAC. We also demonstrated that combination therapy with the eIF4A inhibitor silvestrol, which inhibits ARF6 protein production, and anti-PD-1 antibodies improves the efficacy of anti-PD-1 therapy in PDAC (202). However, it remains unclear whether ARF6 inhibition actually affects therapeutic efficacy by inhibiting autophagy and macropinocytosis.

Other types of metabolism

Amino acid availability in the TME, particularly arginine and tryptophan, is an important determinant of antitumor immunity. Increased arginine levels play an important role in T-cell activation by inducing metabolic changes, including a shift from glycolysis to OXPHOS, and the promotion of memory T-cell differentiation (203). Indoleamine 2,3-dioxygenase (IDO), which catalyzes the conversion of tryptophan to kynurenine, is often overexpressed in PDAC (204). Tryptophan depletion and kynurenine production in TME promote the establishment of a suppressive immune environment, and attenuate anti-tumor T-cell responses (205).

Extracellular ATP levels may be rapidly and robustly increased by hypoxia (206, 207). ATP, which has immunostimulatory properties on its own, may be ultimately converted to the nucleoside adenosine through stepwise process. Canonically, ATP is first catalyzed to AMP *via* the ectonucleotidase CD39. AMP is then dephosphorylated by CD73 and degraded into adenosine. Adenosine can then act on purinergic receptors, such as A1, A2a, A2b, and A3 (208), and regulates various aspects of physiology and

pathophysiology (209, 210). A2a receptors and A2b receptors are primarily responsible for the downstream signaling of immunosuppression associated with intracellular cAMP accumulation (211). In PDAC, high expression of CD73 was demonstrated to be associated with an immunosuppressive TME and poor survival, as well as decreased $CD4^+$, $CD8^+$, and $CD21^+$ TILs (212). Therefore, CD73 may also play a significant role in regulation of the immunosuppressive microenvironment of PDACs and promote their tumor progression.

Immunosuppressive TME in PDACs

The emergence of cancer immunotherapy, particularly ICIs, has offered hope to many patients with tumors that are not curable by conventional therapies. However, PDAC is known to be less sensitive to ICIs than other solid tumors, such as melanoma and lung adenocarcinoma. On the other hand, in PDAC patients, neoantigen quality has been shown to be associated with overall survival, suggesting that PDAC is associated with acquired immunity (213). In particular, the preclinical success of ICI therapy in PDAC patients with microsatellite instability (MSI high) and mismatch repair defects, as well as the therapeutic potential of autologous T-cell-based therapy in PDAC patients, holds promise for adaptive immune-based treatment strategies for PDAC (214, 215). At present, there is an ongoing study testing the effects of ICIs in patients with MSI-high PDACs (NCT02628067), which may provide insights into the subset of patients who respond to immunotherapy and the underlying mechanisms related to efficacy and resistance for ICIs. Overall, clinical results have been disappointing, but in some cases, correlative immunophenotypic studies have demonstrated that these therapies elicit adaptive T-cell responses. This suggests that immunosurveillance is operating in PDAC, however, a rational approach to countering its highly heterogenous and plastic immune evasiveness is needed.

TME of PDACs

Pancreatic cancer is known to have an immunologically cold microenvironment. Overall, immunosuppressive TME in PDAC is often associated with the presence of a tumor-promoting immune cell population (216). Analysis of PDAC mouse models has shown that the expression of oncogenic KRAS itself leads to robust inflammation, and initiates the cycle of inflammation associated with carcinogenesis (11, 179, 217, 218). Furthermore, whereas the expression of KRAS mutant during embryogenesis is sufficient to promote the onset of PDAC proliferation, chronic inflammation is required for malignant transformation in adult PDAC mouse models, indicating that oncogenic mutations alone cannot induce PDAC malignancy (97, 219, 220). Therefore, the inflammatory environment and oncogenic mutations work in concert to promote tumor progression. Thus, inflammation caused by cytokines and chemokines released from PDAC cells that have acquired mesenchymal traits is often associated with the infiltration of innate immune cells that facilitate an immunologically tolerant environment rather than an antitumor immune response (221). A low level of T-cell infiltration correlates with mortality in PDAC (222). Biochemical (production of chemokines and other factors in TME) and physical (deposition of the ECM) barriers in the stroma surrounding the TME inhibit T-cell infiltration (Figure 3).

Fibrosis

Although there are multiple factors that cause ICI treatment resistance, one of the main possible contributors is a dense fibrous stroma (desmoplasia) occupying 80% to 90% of the tumor mass in PDAC (223, 224). Desmoplasia is caused by the proliferation of α-smooth muscle actin-positive fibroblasts or activated pancreatic stellate cells, and work as a physical barrier against drug and immune cells. The trigger that causes these cells to proliferate is still unknown, but the communication among tumor cells and these cells have been identified. Two main components constitute desmoplasia: cells including fibroblasts and infiltrating immune cells, and noncellular proteins, such as collagen types I, III, and IV, fibronectin, and hyaluronan. A comprehensive review of the pancreatic cancer stroma has been published recently (15).

Heterogeneous fibroblasts

Fibroblasts exist in every solid organ, to maintain their morphology and function by depositing ECM proteins and secreting soluble factors (225). For instance, TGF-β secreted from fibroblasts is used by epithelial cells to cure skin injuries. Histological similarities, such as mesenchymal morphology, are maintained among fibroblasts in various organs, but their genomic landscapes differ depending on the organ in which they are located (226). Many studies have demonstrated that some fibroblasts contribute to tumor initiation, progression, and metastasis, and they are known as CAFs (227). Pancreatic cancer has a dense fibrotic architecture, and therefore, it will be useful to clarify the biology of CAFs in PDAC. Recent studies have demonstrated that the functional roles of CAFs in PDAC TME are more complicated than their expected simple tumor-promoting role (15).

Three-dimensional *in vitro* coculture of pancreatic stellate cells (PSCs) and KPC mouse-derived PDAC organoids induced two kinds of CAFs (228). Cocultured directly, PSCs turned into myofibroblastic CAFs (myCAFs) with highly upregulated α-SMA expression and myofibroblastic gene profiles (228). Although CAFs are thought to literally be 'associated' with tumors, myCAFs have anti-tumor activity (229), which

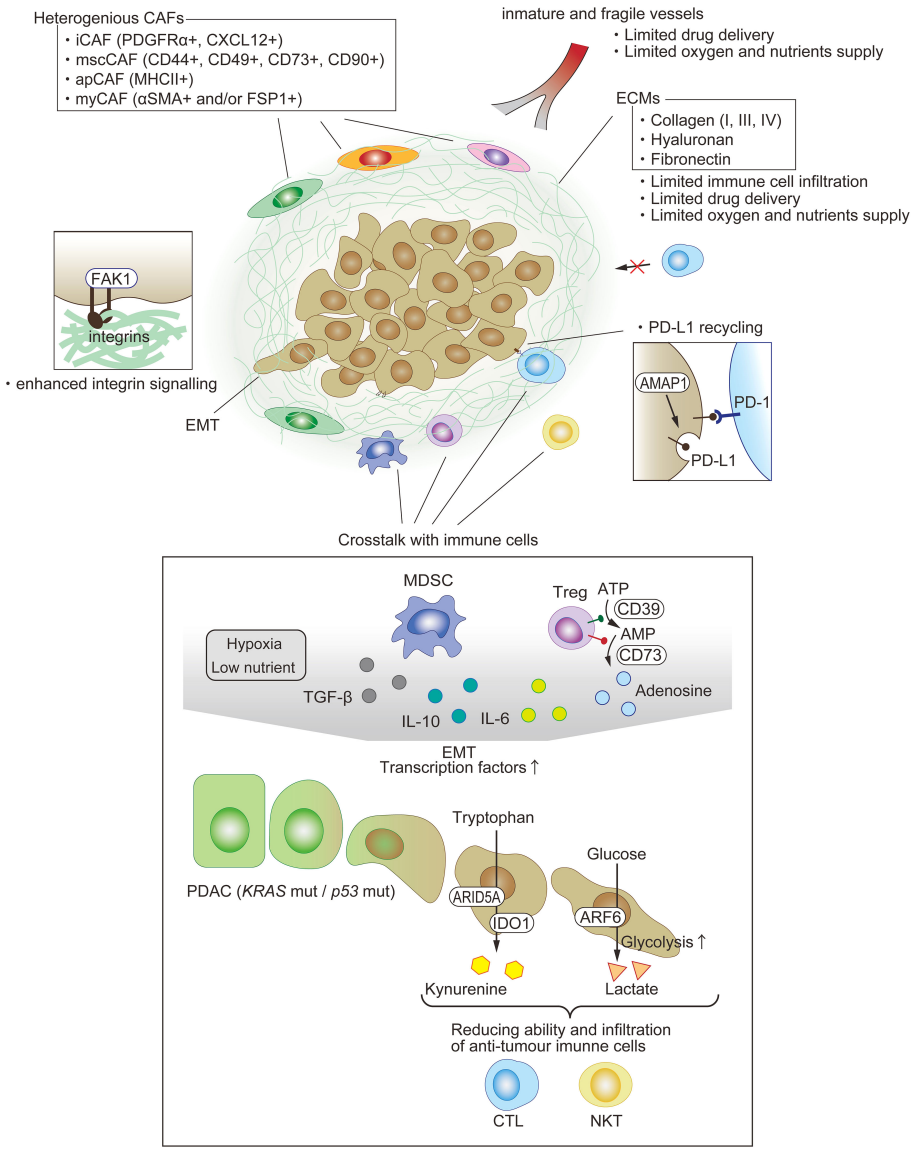

FIGURE 3
Immunosuppressive TME in PDAC. One of the features of PDAC is dense fibrosis, which limits immune-cell filtration, drug delivery, and oxygen supply. A variety of cells exist and compose the immunosuppressive milieu. Each type of fibroblast appears to play a key pro-tumor or anti-tumor role. The EMT of PDAC is among the factors enhancing anti-tumor immunity through not just cell intrinsic functions, such as PD-L1 recycling, but also crosstalk with other immune cells.

requires further investigation for elucidation of the mechanism. On the other hand, indirect coculture transforms PSCs into inflammatory CAFs that display inflammatory cytokines, such as IL-6. It is thought that CAF-derived IL-6 contributes to immune evasion (228).

scRNAseq by several investigators supported the existence of these two populations, and moreover, identified two other groups, namely, mesenchymal stem cell CAFs (mscCAFs) and antigen-presenting CAFs (apCAFs) (230, 231). MscCAFs are characterized by the expression of previously identified mesenchymal stem cell markers (CD44, CD49a, CD73, and CD90), and originate from bone marrow (232). They preferentially express GM-CSF, thus promoting macrophage polarization towards an immunosuppressive phenotype that results in the inhibition of CTL activity (179, 233, 234). Coexpression of MHC class II, including CD74 and

podoplanin, a pan-CAF marker, is a signature of apCAFs (230). It has recently been reported that apCAFs are derived from mesothelial cells and can induce the transformation of naïve $CD4^+$ T cells into regulatory T cells by their direct ligation with a specific antigen (235). This group also demonstrated that targeting mesothelin expressed in the mesothelium may be an effective treatment owing to the inhibition of apCAF formation and regulatory T (Treg) cell induction (235).

T cells

T-cell exclusion in tumors is primarily mediated by CAFs that express fibroblast activation protein, and secrete the chemokine CXCL12 (236). Additionally, activation of integrin-binding protein and non-receptor tyrosine kinase focal adhesion kinase (FAK) is associated with increased collagen I deposition and immunosuppression (237, 238). Highly phosphorylated FAK levels in pancreatic cancer patients were associated with decreased tumor-infiltrating $CD8^+$ T cells and reduced survivability (239). In addition, the expression of FAK in patients with PDAC is associated with decreased tumor cellularity and survival (239).

Acquiring a terminally differentiated T-cell state can diversely impact disease outcome, either countering tumor proliferation through antigen-limited tumor-killing immune responses, or promoting cancer progression by actively inducing immunosuppression (216, 240, 241). In particular, $CD8^+$ cytotoxic T lymphocytes and polarized $CD4^+$ T cells known as T helper type 1 (Th1) cells exert protective effects against tumors in PDAC mouse models, and have been shown to be associated with prolonged survival of human PDAC patients (242). Conversely, $CD8^+$ T-cell deficiency, low amounts of neoantigens, and $CD4^+$ Th2 and Treg cells are associated with tumor-permissive anergy (242–245). Cytokines produced by Th2, particularly IL-4 and IL-13, can not only reduce anti-tumor immune responses, but also can directly accelerate tumor growth induced by KRAS transformed cells (246). PDAC tumors are also accompanied with abundant lymphocyte infiltrates that are typically associated with the gastrointestinal mucosa (247). Th17 cells comprise approximately 5% of the $CD4^+$ T cells in PDACs. The role of Th17 cells in the TME is also context-dependent. In PDAC, IL-17 secretion from γδ T cells and Th17 cells may enhance antitumor immune responses (248). However, early stages in PDAC carcinogenesis, IL-17 has a direct mitogenic effect on *KRAS* mutation-induced PanIN cells expressing IL-17R (218). Whereas the effects of distinct T-cell subsets depend on the underlying immune context of the tumor due to various physiological conditions and environments, and may be altered during the tumor progression of PDAC, the regulation of differentiation and function of T cells in PDAC TMEs play crucial roles in tumor immunity.

B cells and myeloid cells

To date, it has become clear that distinct cell populations derived from lymphocyte and myeloid cells can act in a pro- or anti-tumor manner, depending on the situation. B-cell subsets have become apparent as key immunomodulators in PDAC (249–252). Furthermore, suppressive myeloid cell programming is a major cause of tolerogenic T-cell programming in PDAC. Macrophages are thought to serve a major function in the induction of immunosuppression in PDAC; $IL\text{-}10^+Arg1^+MHCII^{lo}$ tumor-associated macrophages (TAMs) are predominant in the PDAC TME, and are effective in promoting Th2 cell differentiation, but ineffective in inducing $CD8^+$ T-cell immunity (253–257). Similarly, immature MDSCs, collectively referred to as bone marrow-derived $Gr1^{hi}CD11b^+$ granulocyte lineage MDSCs, are characterized by a short half-life and strong suppressive effects in the TME (258). Although endogenous normal dendritic cells (DC) in the TME can produce anti-tumor T cells, the number of DCs in PDAC is low and probably insufficient to sustain robust adaptive immune responses. Furthermore, tumor-derived colony-stimulating factor 3 is found to inhibit the development process of DC in the bone marrow (259). Certain DC subsets have been understood as activators of immune evasiveness in PDAC. $CD11b^+CD103^-$ DCs with high expression of IL-23 and TGF-β are predominant in PDAC, drive the differentiation of $FoxP3^-$ tumor-promoting type I^+ T cells, and promote metastatic spread (260, 261). Moreover, it has been shown that Treg cells directly interact with tumor-associated DCs and suppress anti-tumor immunity by downregulation of costimulatory ligands expression that are important for activation of $CD8^+$ T-cell (262).

Stimuli that recruit myeloid cells to the TME in PDAC are only partially understood. In mouse models, tumor-derived factors have been presented to accumulate MDSCs in the PDAC TME. In the same way, CCL2 produced by tumor cells and CSF1 produced by tumor-associated fibroblasts contribute to the generation of M2-like macrophages (263, 264), and CXCL1 production by tumor cells has been linked to increased myeloid cell populations and decreased tumor infiltration of cytotoxic $CD8^+$ T cells (265). In particular, focusing on the CSF1-CSF1R, CCL2-CCR2, and CXC chemokine-CXCR2 axes to target in the PDAC TME may contribute to pancreatic cancer progression.

Microbiota

The normal pancreas has long been believed to be a sterile, protected site from bacteria. However, recent studies have shown that the pancreas contains bacteria that invade through the Vater's vastus. Interestingly, it has been reported that in the inflammatory environment of PDAC, the bacterial content of pancreatic tumor tissue increases by approximately 1,000-fold compared with normal

tissue (266–268). Furthermore, the bacterial species found in the tumorigenic pancreas are different from those in the gut, and low microbial diversity in the tumor results in a low survival rate of patients with PDAC, whereas high tumor microbiome diversity is associated with long-term survival (269). Mechanistically, the primary PDAC microbiome has a potent immunosuppressive effect on the inflammatory TME, driving the protumor inflammatory responses of PDACs *via* the activation of Toll-like receptors on bone marrow-monocyte cells, and inducing the expansion of MDSCs and anti-inflammatory M2-polarized macrophages. These innate immune cells with tolerogenic functions enhance the differentiation of immunosuppressive $CD4^+$ T-cell populations and inhibit the expansion of cytotoxic $CD8^+$ T cell populations (268). Consistently, microbial ablation in mice resulted in increased infiltration of Th1-polarized $CD4^+$ and $CD8^+$ T cells, decreased accumulation of MDSCs, and a TAM reprogrammed to a tumor-protective M1-like phenotype (268, 270). Potentially, targeting the microbiome by oral antibiotics might reverse myeloid cell-mediated adaptive immunosuppression and promote the efficacy of ICI therapy in PDAC.

Novel mechanisms bridging mesenchymal malignancy and immune evasiveness *via* rewiring of the metabolic program of PDAC

Immune evasion is an essential characteristic of cancer. Every day, the adult body produces mutant cells owing to genetic mutations *via* various intrinsic and extrinsic factors, and most mutant cells are detected and eliminated by the immune surveillance system. However, in rare cases in which mutant cells acquire traits that enable them to evade the immune surveillance system, the cells evade attack by immune cells and proliferate, eventually manifesting as cancer.

As mentioned above, the major immunosuppressive factors in the TME of PDAC include hypoxia, a low-nutrient environment, expression of immune checkpoint molecules, accumulation of immunosuppressive cell populations, such as Tregs and MDSCs, production of immunosuppressive cytokines, such as TGF-β and IL-10, immunosuppressive metabolic enzymes, such as Ido, arginase, and CD39/CD73, and metabolites, such as lactate and kynurenine. In addition, cancer-associated inflammation induced by IL-6, IL-1, IL-17, IL-22, and IL-23 is not only a driver of carcinogenesis, but is also associated with tumor progression by inducing EMT, whereby epithelial cells acquire malignant mesenchymal properties, such as detachment from other cells, invasion into adjacent tissues, and accelerated metastatic spread to other distant organs (21, 271–275). Thus, it is easy to speculate that the factors involved in the suppression of the immune environment of the TME are diverse and complex in their mechanisms of action, as they are produced not only by cancer cells, but also by the various stroma cells, including several kinds of immune cells and heterogenous fibroblasts in the TME.

On the other hand, international cancer-related consortiums, such as The Cancer Genome Atlas (TCGA) have promoted comprehensive genome-wide gene expression analyses of various cancers, but these efforts have not led to the development of effective diagnosis and treatment methods, particularly in the case of PDAC. There may be various reasons, such as the fact that the collected tissue sections are bulk preparations containing not only cancer cells but also stromal cells. Recently, the transcriptome and proteome have been compared worldwide, and it has been shown that there is a very poor correlation between the mRNA and protein levels of most genes (276–280). This strongly suggests that post-transcriptional mechanisms play an important role in the regulation of gene expression. Here, we present our studies from two different aspects on the molecular mechanisms linking the acquisition of mesenchymal plasticity and immune evasion in PDAC, with a focus on post-transcriptional mechanisms.

Functional roles of ARF6-AMAP1 axis as a mesenchymal executioner in PDAC

ARFs, a family within the Ras superfamily of small GTPases, are evolutionally the most ancient of the small GTPases. The ARFs are conserved throughout eukaryotes, including in species that branched off early, such as *Giardia lamblia*, in which no members of the Ras family nor heterotrimeric G-proteins are found (281, 282). *Giardia lamblia* is an anaerobic eukaryote parasite of the gut, which is evolutionally inferred to be an amitochondrial-type eukaryote that developed before the creation of mitochondria (283). This implies that eukaryotic cell features, such as nuclei and flagella, predate mitochondrial endosymbiosis, suggesting that ARF family molecules have been deeply involved in the maintenance of life homeostasis under anaerobic conditions during the evolution of eukaryotes. The human ARF family consists of six isoforms, ARF1–6, which are classified into three classes based on sequence homology, as follows: class I (ARF1–3), class II (ARF4–5), and class III (ARF6) (284). Class I and class II Arfs primarily regulate vesicular transport between the Golgi and endoplasmic reticulum (284, 285). Although ARF6, the only class III member, has virtually identical effector-interacting domains as the other ARFs, it is the most divergent of the ARF proteins, and predominantly localizes to the plasma membrane and recycling endosomal compartments, and functions in intracellular events associated with membrane dynamics, including recycling of plasma membrane components (including both endocytosis and recycling-back to the plasma membrane), as well as in actin-cytoskeletal rearrangement at the cell periphery (286–288).

We identified the ARFGAP protein AMAPs as molecules that are induced during macrophage differentiation, bind to the

integrin-associated protein, paxillin, and are involved in its intracellular dynamics. Furthermore, we found that AMAPs are ARF6-specific ARFGAP proteins that are commonly involved in enhancement of the cell motility of macrophages and epithelial cells (289–291). In addition, we identified a novel mechanism of action in which AMAP functions as an effector of activated ARF6 through steady-state binding to GTP-bound ARF6 *via* its ARFGAP domain in the presence of Mg^{2+} (290, 291). Consistently, Wittinghofer and colleagues demonstrated that Ca^{2+} spikes stimulate the ARF6-specific GAP activity of AMAPs, but not other members of the ArfGAP family (292).

Subsequently, we identified GEP100 as a guanine-nucleotide exchange factor that activates Arf6 in the acquisition of invasive and metastatic traits of breast cancer cells upon activation of the epidermal growth factor (EGF)receptor pathway (145). We also identified the mechanism of action by which GEP100 activates Arf6 by binding directly to the phosphotyrosine moiety of the activated EGF receptor *via* the Pleckstrin-homology domain. Furthermore, we found that the simultaneous expression of Arf6 and GEP100 in MCF7 human epithelial-like breast cancer cells induced EGF-stimulation-dependent EMT-like changes. Subsequently, pathological analysis demonstrated that GEP100 expression is present in approximately 80% of invasive breast cancers (145). Our present study suggests that Arf6-based signaling pathways play an important role in the acquisition of invasive and metastatic traits *via* EMT induction in cancer cells. In this pathway, AMAP1 binds to different proteins, such as cortactin, and protein kinase D2 to promote cortical actin remodeling and integrin recycling (293, 294). AMAP1 also binds to EPB41L5 (148, 149), which shows increased expression during TGF-β-induced EMT (295). Furthermore, we demonstrated that the EMT-TF ZEB1 is involved in *EPB41L5* gene expression, and that high expression levels of ZEB1 and EPB41L5 in cancer cells are associated with p53 mutations. This study demonstrated that the ARF6 pathway is a signaling pathway responsible for advanced cancer-specific mesenchymal traits associated with mutant p53 (296).

A series of our studies have identified that high protein levels of ARF6, AMAP1, and EPB41L5 were associated with invasiveness of several kinds of solid tumors, including breast cancer, clear cell renal cell carcinoma, lung adenocarcinoma, and PDAC and importantly that these expression levels were statistically correlates with poor prognosis (94, 145, 146, 149).

Notably, *ARF6* and *AMAP1* mRNAs are both rich in G/C content in their 5′-untranslated regions (UTRs) (74% and 88%, respectively) (297). Moreover, *ARF6* mRNA contains a G-quadruplex structure at the 5′-UTR (94), indicating that efficient translation is dependent upon the RNA helicase eIF4A, a member of Cap-dependent translation initiation factors (298, 299). On the other hand, the 5′-UTR of *AMAP1* mRNA contains a 5′-terminal oligopyrimidine-like sequence, indicating the mTOR complex 1 kinase-dependent translation control (300, 301). We found that the eIF4A inhibitor silvestrol suppresses protein levels of ARF6 in *KRAS* mutant cells, but only moderately in KRAS intact cells (202), and the mTOR inhibitors rapamycin and Torin1 suppress AMAP1 expression in *KRAS* mutant cells (94). Mechanistically, oncogenic *KRAS* mutations are the major cause of the aberrant overexpression of ARF6 and AMAP1, in which KRAS signaling enhances eIF4A-dependent *ARF6* mRNA translation and eIF4E-dependent *AMAP1* mRNA translation. In addition, gain of function mutations of *TP53* promoted the activation of ARF6 by PDGF *via* MVP-mediated geranylgeranyl lipid modification of Rab11b in PDAC cells (94, 148). Moreover, we revealed that the ARF6-AMAP1 pathway is closely associated with immune evasion in a KPC mouse model. Thus, the cooperation between eIF4A/4E-dependent mRNA translation and MVP has been identified as a link in which representative pancreatic driver mutations empower an ARF6-based pathway, activated by external ligands, to promote tumor cell motility, PD-L1 dynamics, and immune evasion. A recent clinical study by another group confirmed the importance of ARF6 in this context (302). We hence propose that targeting eIF4A, or eIF4E, as well as mutant KRAS, provides novel methods to improve the efficacy of anti-PD-1 therapy, in which ARF6 and AMAP1 overexpression may act as biomarkers to identify patients with drug-resistant disease in PDAC. Additionally, the ARF6-AMAP1 pathway was also found to be involved in acidosis and fibrosis of the TME, both of which are well known to be barriers against immune attack to cancer cells (81, 303), indicating that the ARF6-AMAP1 pathway may also be a valuable target in modifying the TME from pro-tumor, which makes PDAC resistant to treatment, towards an anti-tumor state. Taken together, given the importance of the ARF6-AMAP1 pathway in the pathophysiology of PDAC, its clinical application as a therapeutic target may broaden options for the treatment of PDAC (Figures 2, 3).

Arid5a acts as a dual regulator in malignant PDAC to generate an immunosuppressive TME

Arid5a was identified as an RNA-binding protein that binds directly to the 3′-UTR of *Il6* and stabilizes *Il6* mRNA (304). Recent studies have demonstrated that Arid5a plays an important role in innate and adaptive immune responses (305). In macrophages and embryonic fibroblasts, stimulation by LPS, IL-1, and IL-6 induces Arid5a expression (304, 305). Importantly, in untreated rheumatoid arthritis (RA) patients, expression of Arid5a in $CD4^+$ T cells is increased, whereas treatment with the anti-IL-6 receptor antibody tocilizumab is associated with decreased Arid5a expression (306), indicating that the IL-6-ARID5a axis may be involved in RA pathogenesis. Consistently, Arid5a has been shown to be involved

in several immune-associated pathologies. For example, Arid5a deficiency reduces IL-6 production under LPS-induced endotoxemia. Furthermore, in an experimental autoimmune encephalomyelitis (EAE) model, Arid5a deficiency significantly suppresses Th17 cell differentiation and lowers IL-6 serum levels, resulting in the reduced development of EAE (304). In addition, Arid5a regulates the stability of mRNAs for other genes involved in immune regulation, such as *Stat3*, *Tbx21*, *Ox40*, and *Il17* (307–310). In addition, IL-6 increases its own mRNA stability by increasing Arid5a levels *via* a positive feedback loop (311). Consistently, Arid5a-deficient mice show impaired LPS-stimulated *Il6* and *Ifnγ* expression, and are resistant to lethal endotoxic shock (304, 308). Thus, Arid5a-mediated upregulation of these factors may be involved in the enhancement of Th1 and Th17 cell polarity and function in acute inflammatory responses and autoimmune diseases.

Several cytokines have been shown to be actively involved in metabolic reprogramming in physiological and pathological conditions (312). For example, during cancer cachexia, the overproduction of cytokines significantly increases energy expenditure and leads to weight loss (313). In particular, circulating levels of IL-6 have been shown to positively correlate with cachexia in cancer patients, and importantly, IL-6 levels were found to negatively associate with their survival (314–317). Furthermore, treatment with the humanized anti-IL-6 receptor antibody tocilizumab increased body weight and serum levels of triglycerides and cholesterol in human cancer patients (318). *Il6*-deficient mice have been shown to develop adult-onset obesity with impaired glucose and lipid metabolism (319). The overexpression of IL-6 in high-fat diet-induced obese mice reduced their body weight and improved their obesity-induced fatty liver and insulin resistance (320). Consistently, Arid5a$^{-/-}$ mice showed reduced IL-6 production; mice with long-term loss of Arid5a developed adult-onset severe obesity. In contrast, mice with forced expression of Arid5a are highly resistant to high-fat diet-induced obesity (321). These results suggest that Arid5a is involved in IL-6-mediated metabolic regulation.

Recently, we showed that Arid5a mRNA and protein expression levels were significantly increased in mesenchymal tumor subtypes of PDAC and colorectal cancer (CRC), such as quasi-mesenchymal and consensus molecular subtype 4 subtypes, respectively. In addition, Arid5a expression was enhanced in *in vitro* EMT models, induced by IL-6 and TGF-β stimulation (322) (Figure 4). Furthermore, Arid5a enables mesenchymal tumor models of PDAC and CRC to facilitate immune evasiveness *via* promoting tumor infiltration of immunosuppressive granulocytic MDSCs (gMDSCs; also known as polymorphonuclear MDSCs (323)) and Tregs (324), and suppressing the recruitment and activation of anti-tumor effector T cells (322). Interestingly, Arid5a acts as a dual regulator leading to the formation of immunosuppressive TMEs in malignant tumors, triggering the metabolic reprograming and recruitment of suppressive immune cells. First, Arid5a induces the inhibitory effect of Ido1 on effector CD4$^+$/CD8$^+$ T cells *via* the post-transcriptional stabilization of Ido1 mRNA by binding to its 3′-UTR, and a reduction in intratumoral tryptophan concentration (325, 326). Additionally, Ido1 expression in tumor tissues promotes Treg differentiation/activation by generating kynurenine through tryptophan catabolism, and ultimately activating aryl hydrocarbon receptors (AhR) (327, 328), and AhR activation extensively mobilizes gMDSCs (329). Second, Arid5a upregulates chemokine Ccl2 expression in the TME *via* post-transcriptional stabilization of its mRNA, and then Ccl2 leads to enhancement of the infiltration of immunosuppressor cells, such as Tregs and gMDSCs (330–333), to the TME (322).

Therefore, these findings provide insights into the molecular basis of the acquisition of mesenchymal plasticity and immune evasiveness by PDAC and CRC *via* augmentation of the RNA-binding protein Arid5a, and indicate that Arid5a is a promising target for tumor immunotherapy, in addition to inflammatory diseases (Figure 4).

Conclusion and perspectives

In tumorigenesis, metabolic changes and chronic inflammation associated with genetic mutations in normal cells enable transformed cells to escape the homeostatic defense mechanisms of tissues, and to reprogram their intrinsic signaling mechanisms, as well as reprogram populations of stromal cells within the TME and the metabolic balance of the entire organism. In this process, tumor cell populations that adapt to the abnormal microenvironment form diverse, hierarchically organized colonies, and eventually acquire mesenchymal plasticity that promotes their dissemination, reduces the immune system's ability to counter tumor growth, and finally directly causes death of the organism. Elucidating the metabolic adaptations that tumors rely on to promote these changes and maintain growth in a metabolically unfavorable environment, as well as the molecular mechanisms that trigger the acquisition of mesenchymal plasticity and immune evasion capacity, will help towards developing new diagnostic and therapeutic approaches and dietary combinations for the treatment of PDAC.

Increased levels of IL-6 in the serum have been associated with poor overall survival prognosis in patients with high-grade pancreatic cancer (334), and the increased activity of IL-6/STAT3-mediated signaling has been reported to be associated with poor prognosis in post-resection PDAC patients (335). IL-6 also activates STAT3 and induces the mesenchymal phenotype in human pancreatic cancer cells *via* the induction of SNAI1 (336). In chronic pancreatitis, IL-6/STAT3-mediated ADM transdifferentiation occurs and is associated with PanIN, which is a necessary step for the generation of tumorigenic precursor lesions (220, 337). For example, in the KRAS-induced PDAC mouse model (220, 337), pancreatic epithelial cells with

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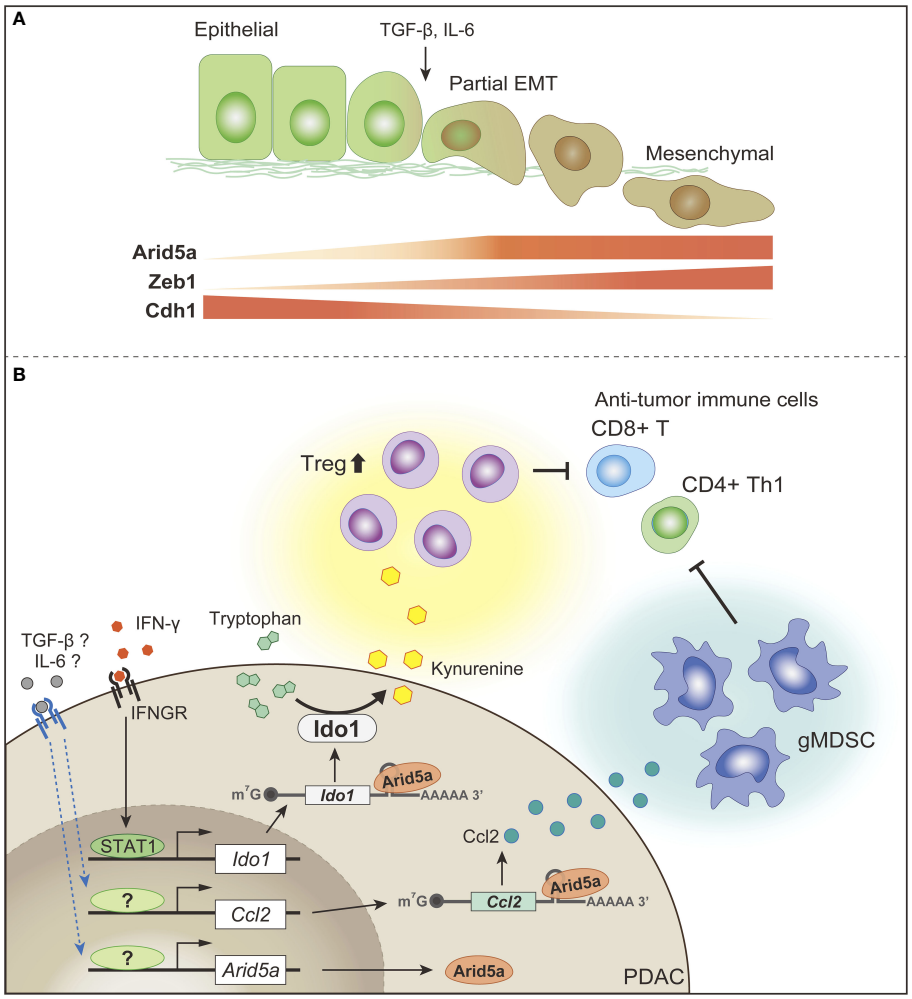

FIGURE 4
Involvement of Arid5a in the acquisition of mesenchymal plasticity and immune evasiveness in PDAC **(A)** Arid5a expression is associated with acquisition of the mesenchymal phenotypes of PDAC and CRC. Especially, cells showing partial EMT and mesenchymal-like cell lines show much higher expression levels of ARID5A than epithelial-like cell lines. During TGF-β- or IL-6-induced EMT, Arid5a level is augmented in cells that have acquired mesenchymal phenotypes. **(B)** Arid5a acts as a dual regulator in malignant tumors, such as the mesenchymal subtypes of PDAC, to promote an immunosuppressive TME; Arid5a upregulates Ido1 expression *via* post-transcriptional stabilization of its mRNA and then enhances the suppressive effects of Ido1 on anti-tumor immune cells, such as $CD8^+$ T cells and $CD4^+$ Th1 cells *via* a reduction in intratumoral tryptophan concentration, and a promotion of the differentiation and activation of Treg cells. Additionally, Arid5a post-transcriptionally induces the expression of the chemokine Ccl2 in the TME, which recruits immunosuppressive cells, such as Treg cells and gMDSCs, to the TME.

constitutively active KRAS mutations ($KRAS^{G12D}$) have been reported to cause inflammatory activation by recruiting immune cells. In particular, myeloid cells have been reported to promote the production of IL-6 and soluble IL6R (sIL6R), activate STAT3 *via* IL-6 trans-signaling, and furthermore, the complex of IL-6 and sIL6R binds to gp130-expressing cells (220). Aberrant STAT3 activation owing to the homozygous loss of *SOCS3* in the pancreas results in the accelerated progression of PanIN and the development of PDAC (220). It has also been shown that KRAS activation increases the levels of cytokines, such as IL-6 and IL-11, in epithelial cells, followed by STAT3 activation in an autocrine manner, and that STAT3-triggered matrix metallopeptidase 7 is required for tumor progression but not tumor development, and may be regulated by other STAT3 targets (337). As already mentioned, the TME of PDAC is severely hypoxic, and nutrient availability is limited by a low vascular density, so PDAC cells show increased autophagy that rewires their metabolism to enable survival in a harsh environment, and to maintain metabolic homeostasis. In a mouse model of PDAC caused by *KRAS* mutations, IL-6-induced STAT3 activation was shown to be involved in the increase in autophagy. As a mechanism, receptors for advanced glycation products have been reported to promote the IL-6-driven activation of STAT3 signaling in mitochondria, bridging

autophagy and the IL-6/STAT3 signaling pathway (338). Furthermore, IL-6 signaling has been implicated in the pathogenesis of cachexia in PDAC patients, by inducing a metabolic rewiring (339). Thus, it is clear that the activation of IL-6/STAT3 signaling is involved in the development of PDAC from the PanIN stage, continuing to malignant transformation.

Since its approval in 2009, tocilizumab has been shown to inhibit IL-6/STAT3 signaling in patients with autoimmune diseases, such as rheumatoid arthritis caused by the overexpression of IL-6, acute inflammatory diseases caused by chimeric antigen receptor T-cell therapy, and cytokine storms associated with SARS-CoV-2 infection. On the other hand, in clinical practice, few effective therapeutics have been developed as cancer treatments targeting IL-6/STAT3 signaling (340–343). As mentioned above, cancer is caused by a complex interplay of diverse cell populations, which leads to malignant transformation. Therefore, analysis of the expression and function of molecules associated with IL-6/STAT3 activation may enable the assessment of the local malignant potential and steady state of cancer, but may not be sufficient to predict the stage and detailed course of cancer. Furthermore, it has become clear that not only IL-6/STAT3 signaling, but also various groups of molecules are involved in cancer development. The mode of interaction between these molecules also requires further study.

In the future, it will be essential to introduce spatiotemporal gene expression analysis technology that analyzes multiple cell populations, improve detection technology to analyze the associations among aging, inflammation, and metabolism, and develop artificial intelligence technology to analyze cancer development and progression, and mathematical analysis technology to integrate these technologies. To this end, it is also indispensable to enhance the convergence of life science, physical science, engineering, and computational science to create the next generation of cancer diagnostics and therapeutics.

Author contributions

AH, HH, and ShH conceived and designed the manuscript. All authors wrote the manuscript, and approved the final manuscript.

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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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Back to basic: Trials and tribulations of alkalizing agents in cancer

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"Dysregulated" metabolism is a characteristic of the cancer cell phenotype. This includes persistent use of glycolytic metabolism in normoxic environments (Warburg effect) leading to increased acid production and accumulation of protons in the interstitial space. Although often thought to be disordered, altered cancer metabolism is the outcome of intense Darwinian selection and, thus, must have evolved to maximize cancer cell fitness. In an evolutionary context, cancer-induced acidification of the microenvironment represents a niche construction strategy to promote proliferation. Ecological advantages conferred on the cancer population included remodeling of the extracellular matrix to promote local invasion, suppression of potential competitive proliferation of fibroblasts, and suppression of host immune response. Preclinical data demonstrates that increasing the serum buffering capacity (through, for example, oral sodium bicarbonate and TRIS) can neutralize the acidic tumor microenvironment with inhibition local invasion and proliferation which can be synergistic with the effects of chemotherapy and immunotherapy agents. Here, we describe the proton dynamics in cancer and their influence on tumor progression and metastasis. Additionally, we will discuss targeting the tumor acidosis with alkalizing agents including our bicarbonate clinical trial results.

Clinical Trial Registration: clinicaltrials.gov, identifier NCT01350583, NCT01198821 and NCT01846429.

KEYWORDS

cancer, buffer therapy, clinical trial, alkalizing, acidosis

Introduction

Due to a mis-match between glucose fermentation and perfusion the extracellular pH (pHe) of the tumor microenvironment is profoundly acidic (1). This acidity, as first described by the Warburg, occurs even in the presence of oxygen (2). The acid accumulated extracellularly is removed *via* different proton transporting systems,

including sodium hydrogen exchanger, monocarboxylate transporters (3). This proton dynamics results in a decrease in pH and increase in an intracellular pH (pHi). The extracellular pH of a solid tumor can reach 6.5 (4). These conditions are highly toxic to normal mammalian cells and, thus, the cancer phenotype must evolve adaptive strategies to survive and proliferate in acidic conditions.

While the Warburg effect (i.e., "aerobic metabolism" - maintaining inefficient glycolytic metabolism even in the presence of oxygen) is often described as "dysregulated", this view is inconsistent with the evolutionary model of cancer. That is, because cancer cells are subject to constant Darwinian selection, it's metabolism must, in fact, represent an optimal phenotypic state that maximizes fitness. We have proposed that excess acid generation production represents a niche construction strategy that confers a competitive advantage on cancer cells by killing or suppressing the growth of potential normal cell competitors such as fibroblasts, causing breakdown of ECM to promote invasion, and blunting the immune response (5–7).

Functionally, cells can be classified as oxidative or fermentative in the tumor microenvironments. Oxidative cells will convert the lactic acid to pyruvate, that enter the TCA cycle and oxidized yielding ATP and CO_2. Carbonic anhydrase 9 or 12 (CA9, CA12) on the outside o the cell will hydrate this CO_2 producing $HCO3^+$ and H^+. In fermentative cells, Glucose will enter the cell *via* glucose transports 1 or 3 (GLUT-1, GLUT-3), and enter glycolysis after phosphorylated to Glucose 6-phosphate (G6P). The proton produced by this oxidative step is transported by sodium/hydrogen exchanger, NHE1, as well as CA9. The lactic acid produced *via* glycolysis is transported *via* monocarboxylate 1 and 4 transporters (MCT1/4) (8).

The extracellular acidity can negatively affect the normal tissue. Specifically, remodel extracellular matrix and allow tumor cells to invade and metastasize to surrounding and distal organs (9). It has been shown by us and others that acidity can also suppress immunity. *In vitro* and *in vivo* studies demonstrated that acidosis inhibits CD8 T cell function (10–12), and promote the pro -inflammatory macrophages phenotype (M2) (13).

Beyond invasion and metastases, tumor derived acid pH is also a known mediator of cancer-associated pain. In recent years, it is becoming apparent that metastasis-associated bone pain involves the reduction of peri-tumoral pH and activation of nociceptors, including acid-sensing ion channels, ASICs (14, 15). The two major nociceptor ASIC are the transient receptor potential vanilloid subtype 1, TRPV1, a.k.a. the capsaicin receptor (16, 17) and ASIC-3 (18). The expression of these transporters is decreased with bis-phosphonates, which have led some to speculate that the acid is derived from tumor-associated osteoclasts (19, 20). Osteoclasts exacerbate the tumor-derived acidity at the bone interface through their own export of protons *via* a Vacuolar type H-ATPase (21).

Although treatments to reduce intratumor acidity are often describe as "alkalizing agents," this represents a misnomer. Typically, such agents are actually physiologic buffers which promote a pH near 7.4. Thus, they will tend to alkalinize the acidic tumor pH in the sense that the typically increase it toward the physiologic range. However, in normal tissue and physiological pH, such buffers will have no effect on the acid concentrations (22). Multiple studies have demonstrated that tumor acidity can be significantly improved with oral buffers, and this can reverse some of the consequences of acidity, including invasion to the surrounding tissue, distal organ metastasis, and modulation of immune function. For instance, we have proved that chronic treatment of animals with 200 mM sodium bicornate ad lib significantly decreased invasion and metastasis in spontaneous and experimental metastasis cancer models (23–26), and enhanced the effect of immune checkpoint inhibitors as well as adoptive T cell transfer (12). In our previously published work, we showed that Lysin has a pH dependent effect on prostate tumors metastasis. Lysin with a pH (8.0) below pka value has no effect on metastasis while Lysine with pH (10.4), a higher pka value, significantly decreased metastasis, clearly suggesting buffering is mediating the antimetastatic effect (27). In another study in transgenic prostate model (TRAMP), treating the mice before development of the tumor (~ 4 weeks after weaning) prevented the development of the interepithelial neoplasia (PIN), furthermore doubling the concentration of bicarbonate treatment after the development of the PIN lesions(~10 weeks after weaning) prevented tumor cells metastasis (28). While the mechanisms under the buffer therapy is still not completely revealed; we observe by histopathology that the buffer treated tumors are less invasive and more benign (29).

Targeting tumor acidity with buffer therapy is the most direct approach, particularly by oral sodium bicarbonate $NaHCO_3$ or THAM. We have shown that mice can tolerate orally up to 200mM sodium bicarbonate with no changes to the systemic pH. Tumor volume at the time of the bicarbonate treatment influence the outcome. We observed no effect of sodium bicarbonate on large primary tumors compare to the small tumors (19, 22).

The anti-tumor effect of alkalinizing agents may be systemic and at the level of tumors. In fact, a milestone paper by our group (28) showed that the oral administration of sodium bicarbonate 100% prevented the development of prostate cancer in TRAMP mice, denoting that a daily alkalinization with either sodium bicarbonate or other buffers may well prevent cancer. Still, this probably occurs through a primary effect on the gastric pH since our stomach is not simply a digestive bug but rather an exocrine gland that produces H+ for the whole body, actively participating in the pH balance of our organism.

Recently, Helix BioPharma developed a target for tumor acidosis, L-DOS47, which can serve as an alternative to sodium bicarbonate. L-DOSE47 is a urase base extracted from Jack Bean that targets Carcinoembryonic antigen-related cell adhesion molecule 6(CECAM-6 antigen) overexpressed by several

cancer types, such as lung, colon, and pancreatic (65). They urease enzyme will convert the urea present around the tumors to ammonia($2NH_4$+) and bicarbonate (1 HCO_3 -) alkalizing the tumor microenvironment. L-DOS47 is now in phase I/II clinical trial (NCT02309892) in lung cancer (66).

Clinical trials using proton pump inhibitors remain the only evidence-based support for an anti-acidic approach against cancer that was reported; this includes a clinical study in patients with osteosarcoma (30); a case-control study in patients with metastatic breast cancer, in which an arm has been successfully treated with PPI alone (31); a pilot study in patients with GI cancers (32). Moreover, two clinical studies have been performed in domestic animals with malignant tumors, one combining standard chemotherapy with lansoprazole (33) and the other combining metronomic chemotherapy with lansoprazole and alkalinized water (34). These clinical results supported the evidence-based use of proton pump inhibitors as a new therapeutic approach, at least in combination with chemotherapy (35). However, three preclinical papers have shown that PPI alone had a potent anti-cancer effect in the absence of chemotherapy or other anti-tumor therapies in three different human tumors (36–38); suggesting that high dosage PPI should be considered in the future anti-cancer therapies. A clinical result partly supported this pre-clinical evidence in the above-quoted paper, a study performed on triple-negative breast cancer patients. In fact, at the end of the study, one arm of patients was treated with PPI alone compared to those left untreated, and the results showed a significant increase in OS in PPI-treated patients (31).

Moreover, three retrospective metanalysis have proposed PPI as an effective combined therapy with standard chemotherapy (39) and preventive treatment for breast cancer (40, 41). In a way, at least three reviews have proposed repositioning PPI for cancer therapy (42–44). Papers showed that alkalinization by oral administration of either a potent buffer (45) or alkalinized water (46), respectively, controlled the growth of a very aggressive melanoma and prevented the development of prostate cancer in TRAMP mice. Moreover, the control of melanoma growth was consistent with an increase in tumor pH and the treated mice's urines, suggesting that a buffering approach exerted its role in inducing both tumor and systemic alkalinization.

Preclinical studies performed by us, and others suggested that oral sodium bicarbonate can be translated to clinic. Three clinical trials were conducted, phase I/IIa clinical trials to test the tolerability of oral sodium bicarbonate. We calculated the amount of sodium bicarbonate needed by comparing the amount of sodium bicarbonate that mice consumed which is around 4.2 mL per day (25). This was equivalent to 2.8 g/kg/d. By inter-species allometric scaling, human dose will be around 16.3 g/d for a 70kg human (47). In a clinical trial study for children with Sickle Cell Anemia, oral administration of 21 gm/day was safe and complication free (48). Side effects of overdose of sodium bicarbonate can include metabolic alkalosis, hypokalemia, hypernatremia, and metabolic disorders such as hypoxia, however, side effects for our dose proposed is not anticipated (49). Gastrointestinal irritability and discomfort, as well as poor taste were that main causes for the low compliance in all our three trials.

Materials and methods

Protocols

Clinical trial NCT01350583, NCT01198821 and NCT01846429 protocols are publicly available at clinicaltrials.gov.

Results

Trial 1: Pilot Study. The first of these trials was conducted under IND106881 for powdered $NaHCO_3$ for use in pain management. NCT01350583 was a palliative trial opened on 08/08/2010 entitled "A Pilot Study of Oral Bicarbonate as Adjuvant for Pain Reduction in Patients with Tumor Related Pain". The rationale for this study was the prior observation that the major nociceptive (pain-sensing) receptor in cancer pain was TRPV, which has been shown to be an acid receptor (50). Target accrual was 28 patients for the 3 + 3 dose escalation study design. This trial accrued two female patients and was closed on 04/03/2012. Patient 1 completed her dose schedule of (0.5 g/kg/d) over 4 weeks. Patient 2 left voluntarily, withdrawing after 3 weeks. One grade 1-2 limb edema was reported and one each grade 1 nausea and vomitus were also reported. Both patients died 10 and 14 months after going off study.

Trial 2: GemTABS. The second trial was for pancreatic cancer (NCT01198821) patients being treated with gemcitabine under IND108551, entitled "A Phase I Study of Oral Sodium Bicarbonate in Patients with Unresectable Pancreatic Carcinoma Treated with Gemcitabine (Gem-TABS)" with a 27-month projected accrual of 35 patients. Gemcitabine has complex ionization behavior with an acid pKa of 11.65 (neutral below this value) and a base pKa of 4.47 (neutral above this value) indicating that its ionization state would not be altered between a native tumor pH of 6.5 or the bicarbonate treated pH of 7.0. Projected dose levels 1-4 of $NaHCO_3$ were 0.3, 0.5, 0.7, 1.0 g/kg/d; with same patient dose escalation allowed after 2 weeks at dose if the patient experienced no treatment-related adverse events. The trial was opened 08/27/2010 and closed on

TABLE 1 GemTABS adverse events.

	Grade 1	Grade 2	Grade 3	Grade 4
diarrhea	1		1	
vomiting	1	1		
edema		2		

06/22/2011. A total of eight (8) patients were accrued to this trial. Treatment-related adverse events included diarrhea, limb edema and vomitus (Table 1). Half of the patients reported grade 1-3 fatigue, which was not ascribed as treatment related. One patient acquired a non-treatment related biliary tract infection, leading to hospitalization. The first three patients completed the level 1 dose with no grade 3 AEs. Escalation to dose level 2 was performed on two patients, who voluntarily withdrew from the trial after 11 and 26 days. As a consequence, subsequent patients were accrued at dose level 1. Overall Survival (OS) ranged from 44-718 days after initiation of trial, with a median OS of 220 days after consent (Table 2). A median OS of 170-177 days was reported in the gemcitabine monotherapy arms in the registration (51) and follow-up (52) trials. The difference between OS in the treated and bicarbonate groups was not significant.

These trials demonstrate multiple limitations of powdered $NaHCO_3$. First, virtually every patient complained about the taste, which led to poor compliance. Second, there was an issue of dosing as, upon questioning, patients experiencing GI issues were likely taking too large of doses of $NaHCO_3$, which is an emetic, at a single setting. The trial was designed so that doses were split between 3-4 equal doses throughout the day, which may have led to GI issues, such as vomitus and diarrhea.

Trial 3: PainCAPS. To mitigate these problems, $NaHCO_3$ was re-formulated as 940 mg capsules under IND118182. NCT01846429 was designed as a phase I/II palliative trial entitled "A Phase I/II Study of Oral Bicarbonate as Adjuvant for Pain Reduction in Patients with Tumor Related Pain". The phase I component included 3 patients per cohort (12 total) with escalating dose levels of 10, 20, 30 and 40 capsules taken throughout the day. Dose level 2 included a lead-in period of 3 weeks at dose level 1 prior to escalating to dose level 2. Each dose level was designed to last 3 weeks, after which patients were allowed to choose to leave the trial, stay on trial at the same dose, or escalate dose. The trial was opened on 09/10/2013 and terminated on 10/12/2015 with a final accrual of 9 evaluable subjects. 100% of the patients reported grade 1-2 GI disorders including vomitus. One had grade-2 limb edema, and one patient experienced grade 3 hypokalemia and was removed from study (Table 3). All nine evaluable patients stayed on study for the planned 3-weeks, and eight of these opted to continue at the same dose or escalate to dose level 2 following the initial lead in (Table 4). Patients were asked to maintain a pain diary, and tumor-related pain levels were recorded weekly on a scale of 1 (no pain) to 10 (excruciating). Across all participants, there was a significant (by Wilcoxon signed-rank test; trial NCT01846429) reduction in perceived pain from the baseline 5.25 ± 1.16 to 3.62 ± 1.88 ($P = 0.010$) within the first 3 weeks, and this level of significance was no different for patients who stayed on therapy for >6 weeks: 3.86 ± 1.54 ($p = 0.013$). On an individual patient basis, 4 of 9 patients had a reduction in pain score after 3 weeks that was greater than 1 S.D. from a baseline established over the first 3 measurements in the first 2 weeks.

TABLE 2 GemTABS accrual.

pt #	gender	final dose	days on Tx	OS (days)
1	f	1	126	239
2	m	1	56	197
3	m	1	63	88
4	f	2	11	319
5	m	2	26	201
6	f	1	28	320
7	f	1	82	718
8	m	1	15	44

TABLE 3 PainCAPS adverse events.

	Grade 1	Grade 2	Grade 3	Grade 4
hypokalemia			1	
GI/vomitus	23	9		
edema				

TABLE 4 PainCAPS accrual.

pt #	gender	final dose	days on Tx	OS (days)
1	f	1	36	NA
2	m	1	28	116
3	m	1	32	42
4	f	2	7	NA
5	f	2	15	NA
6	f	2	34	34
7	f	2	32	218
8	m	2	35	132
9	m	2	41	NA

Discussion

We could not increase the dose of any of the oral sodium bicarbonate trials, because of taste, GI, and edema. Hence, we presume that sodium bicarbonate buffer monotherapy is not clinically feasible but there is some data to suggest the strategy of reducing intratumoral acidosis may have a favorable effect on cancer related pain. We suggest coupling the sodium carbonate therapy with other treatments to enhance the efficacy, this includes chemotherapy, and immunotherapy. As we mentioned previously, adding buffer therapy to immune blockade in mice increased response rates up to 3-fold (12).

A potential alternative strategy might reduce the requirement for supplement $NaHCO_3$ therapy be following an "alkaline diet", as discussed in (27, 53). One of the counteracts to the buffer therapy benefit is western diets since it is typically acidic. Hence,

modifying the diet to include high protein content can add to the buffering benefit of the sodium bicarbonate. As published previously, this diet should contain low sulfur concentration because its oxidation will result in increased acidity and thus inhibit the net buffering effect. Essential amino acids have to be added to diet since it cannot by synthetized by the body (54).

The potential renal acid load (PRAL) is an effective way to measure the amount of acid produced by different types of food. Measuring the urine pH that correspond to each food dose level, coupled with the protein to potassium ratio (protein/K+) (55). This food buffering mechanism completely differ from the sodium bicarbonate buffering. The bicarbonate buffering creates "*compensated metabolic alkalosis*", where kidneys secrete hydrogen ions because of the increased bold bicarbonate levels (56), which then lead to the increase of pHe in tumor microenvironment (57).

We have shown in multiple pre-clinical systems that oral buffers (*e.g.*, bicarbonate, imidazoles, Tris, lysine) explicitly increase tumor pH without changing systemic pH balance. These rarely affect growth of large primary or metastatic tumors but do inhibit small cancers thus preventing carcinogenesis or spontaneous metastases. To translate these studies into the clinic we started phase I/II clinical trials of buffer as a single agent in pain management trials (NCT01350583/01846429) and pancreatic cancer patients (NCT01198821). However, these trials failed to accrue due to poor compliance because of unpleasant taste and/or GI disturbances. However, data from one trial did show a decrease in tumor-related pain suggesting clinical efficacy may be significant if alternative treatment strategies can be devises. Thus, we suggest investigating pharmacological alternatives to achieve the same result (*i.e.*, reducing tumor acidity). As one possible solution to this issue, we have developed a point-based plan to achieve the same buffering with a combination of diet, supplements and buffers (Urbase®), that will be added to a trial with support from Anti-cancer Foundation (Brussels).

Data availability statement

The datasets [NCT01350583/ 01846429] and pancreatic cancer patients [NCT01198821] for this study can be found in publicly the [clinicaltrials.gov].

Ethics statement

The studies involving human participants were reviewed and approved by Moffitt Cancer Center under IND106881, the clinical trial NCT01350583. Under IND108551, the clinical trial NCT01198821. Under IND118182 the clinical trial NCT01846429. The patients/participants provided their written informed consent to participate in this study.

Author contributions

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.

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Association between pH regulation of the tumor microenvironment and immunological state

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The tumor microenvironment (TME) is characterized by interactions among various cells, including tumor cells, immune cells, stromal cells, and blood vessels mediated by factors such as cytokines and metabolites. The development of cancer immunotherapy in recent years has facilitated a more comprehensive understanding of the TME. The TME changes with cancer type and host immune status, as well as with therapeutic intervention. However, studies on pH regulation of the TME have been mostly based on lactate, a metabolite of tumor cells. Notably, the Warburg effect results in the increased production of secreted lactate, thereby acidifying the extracellular microenvironment and affecting the surrounding cells. Lactate inhibits the activation and proliferation of CD8+ T cells, M1 macrophages, natural killer (NK) cells, and dendritic cells, contributing to tumor cell immune escape. It is also involved in angiogenesis and tissue remodeling, as well as promotes tumor growth and invasion. In this review, we have discussed the lactate-based pH regulation in tumor cells in the TME and its effects on the other constituent cells.

KEYWORDS

pH, Warburg effect, lactate, immune escape, tumor microenvironment

Adjustment of acid-base balance

In humans, the extracellular fluid has a slightly alkaline pH of 7.40 ± 0.02. This corresponds to an H+ ion concentration of 40 ± 2 nEq/l and is regulated by the excretion of volatile and non-volatile acids produced in the body. Volatile acids, such as H_2CO_3, are produced as CO_2 from carbohydrates and fats, and approximately 15,000 mEq/day is discharged from the lungs. Conversely, non-volatile acids include approximately 100 mEq/day of amino acid metabolites and 30 mEq/day of phosphoric acid, a metabolite of nucleic acids and ATP. Approximately 70 mEq/day (approximately 1 mEq/kg body weight) of

non-volatile acids are neutralized by the kidneys using approximately 60 mEq/day of bases derived from the diet. The non-volatile acids produced are promptly scavenged by buffering substances, thereby minimizing pH alterations. The bicarbonate buffering system accounts for 60% of the extracellular fluid, the bone buffering system, and Hb buffering system outside the cells, whereas the HPO_4^{2-} and protein buffering systems are responsible for buffering inside the cells. However, in pathological conditions such as ischemia, inflammation, and systemic respiratory failure, these buffer systems are dysregulated, thereby leading to local and occasionally systemic acidemia (1). The gastrointestinal tract controls the physiological pH by regulating the local neutral range. Appropriate pH adjustment for local enzyme activation regulates digestive function, especially because the different parts of the digestive tract have different pH ranges (stomach pH 1.5–2, duodenal pH 3–5, small intestinal pH 6, and large intestinal pH 7) (2). Furthermore, an appropriate pH promotes diversity in the gut microbiota, which produces metabolites that skillfully regulate host immunity (3).

Osteoclasts promote an acidic microenvironment during bone resorption. Osteoclasts adhere to the bone at the sealing zone, which consists of polymerized actin; the demineralization of the bone is promoted by releasing acid from cells through proton pumps (4). These instances demonstrate the mechanisms by which living organisms maintain their functions by adequately adjusting local acid-base balance under physiological conditions.

Acidification mechanisms in the tumor microenvironment

The extracellular pH, which is maintained at pH 7.4 in normal tissues, decreases to approximately pH 6.8 in tumors. Decreased extracellular pH in tumors has been reported in epithelial tumors such as lung cancer, breast cancer, and melanoma, as well as in non-epithelial tumors such as sarcoma (5–10). However, tumor tissue is not uniformly acidic, and the pH varies from near neutral to strongly acidic (11–14). This acidic TME is primarily attributed to hypoxia and increased lactate levels owing to increased glycolysis in cancer cells. The TME has a lactate concentration of 10–30 mM, whereas that under physiological conditions is approximately 1.5–3.0 mM (15).

Hypoxia is presumed to be caused by tumor vascular abnormalities. Endothelial cell adhesion is looser in tumor blood vessels than in normal blood vessels, resulting in increased vascular permeability. Furthermore, the thickness of type IV collagen, which constitutes the vascular basement membrane, is varied and irregular in tumors depending on the site of the blood vessel; therefore, tumor blood vessels have varying diameters and random vasculature; thus, tumor arteries, veins, and capillaries lack a hierarchical structure compared with that of normal blood vessels (16). Consequently, cancer tissues have low blood flow despite the abundance of blood vessels, thereby creating a hypoxic microenvironment within the tumor tissue. Increasing dysfunctional tumor blood vessels does not improve the hypoxic microenvironment but promotes tumor growth (17). The interaction between blood vessels and cells within the tumor further promotes angiogenesis and tumor growth, thereby inducing a hypoxic environment (18, 19).

In the hypoxic environment, increased stability of hypoxia-inducible factor 1 (HIF1) results in increased glycolysis and a subsequent decrease in extracellular pH (20). HIF1 stabilization promotes glucose uptake and metabolism by enhancing the expression of glucose transporter type 1 (GLUT1). This metabolic process produces ATP, which increases the levels of lactate and protons (H+), consequently resulting in decreased intracellular pH. To maintain a constant intracellular pH, membrane proteins such as the Na+/H+ exchanger isoform 1 (NHE1) and ATPase and monocarboxylate transporters 1, 4 (MCT1, 4) excrete lactate and protons outside the cell, resulting in a decrease in external pH (21, 22). Notably, the TME lactate is presumed to increase tumor angiogenesis by promoting CXCL8 production from vascular endothelial cells, thereby exacerbating hypoxic conditions and reducing the extracellular pH (23).

Adaptation of tumor cells to the acidic tumor microenvironment

In 1924, Otto H. Warburg proposed a phenomenon known as the Warburg effect, in which cancer cells exhibit increased lactate production in an aerobic environment; this opposes the Pasteur effect, which reports the suppression of lactate production by oxygen. However, the Warburg effect does not indicate suppression of aerobic respiration, and mitochondrial aerobic respiration in cancer is enhanced compared with that in normal tissue (24). Furthermore, intracellular acidification inhibits enzymes, such as phosphofructokinase-1, involved in glycolysis; however, decreasing the TME pH does not necessarily promote glycolytic metabolism. Nonetheless, oxidative phosphorylation results in the production of 36 ATP molecules per glucose molecule, whereas glycolysis results in the inefficient production of 2 ATP molecules. Therefore, the preference for inefficient glycolytic metabolism in cancer cells has been actively investigated. The Warburg effect is a bona fide phenomenon observed *in vitro* and *in vivo* in animal models and patients with cancer (25). Furthermore, H+ accumulation occurs in the non-hypoxic regions of the tumors, suggesting that cancer cells purposefully select aerobic glycolysis depending on the time and environment (11, 12, 26). Aerobic glycolysis utilizes glycolytic intermediates for the *de novo* synthesis of nucleotides, lipids, and amino acids required for cell proliferation and, together with TCA cycle metabolites, supports tumor growth (24, 27–29). Thus, tumor cells increase lactate production and induce a decrease in extracellular pH, whereas intracellular pH remains unaltered or is slightly higher than that of normal cells.

Tumor cell pH is determined by anion exchangers (SLC4A1, SLC4A2, and SLC4A3), proton transporter vacuolar ATPase (V-ATPase), mono-carboxylate transporters (MCT1, MCT2, MCT3, and MCT4), chloride/bicarbonate exchanger (SLC4A8), and the

Na+/H+ exchanger 1 (SLC9A1), NHE1, Na+/K+ ATPase pump, H+/Cl− symporter, and carbonic anhydrase (CA) (22, 30). Furthermore, the transitional utilization of lactic acid has been reported. Metastatic breast cancer cells found in bone produce lactate, suggesting that they promote osteoclast differentiation and metastatic niche formation (31). Furthermore, in glioma cells, lactate stimulates transforming growth factor-β2 (TGF-β) expression, a key regulator of cancer cell migration, invasion, epithelial-to-mesenchymal transition, and metastatic niche formation (32). Furthermore, as elucidated later in the text, an acidic environment inhibits the action of anti-tumor immune cells, including T lymphocytes, natural killer cells, and M1 macrophages. Conversely, it activates immunosuppressive cells such as regulatory T cells and M2 macrophages. Glycolytic selection in the aerobic environment of tumor cells is not necessarily favorable for cancer cell growth *per se*. By creating an acidic environment, acid-induced immunosuppression is relatively beneficial and may form a favorable tumor microenvironment for cancer.

Adaptation of T and NK cells to the acidic tumor microenvironment

Effector T cells (CTL), which play a crucial role in anti-tumor immune responses, differentiate and proliferate from naive CD8+ T cells *via* stimulation from IL-2 produced by CD4+ T cells presented with cancer antigens by dendritic cells. Activated CTLs kill cancer cells by producing IFN-γ and perforin. In contrast, regulatory T cells (Treg), which are immunosuppressive, play a critical role in immune tolerance and avoid immune responses against self while suppressing anti-tumor immune responses by CTLs. In humans, Tregs are mainly released from the thymus to the periphery as naïve Tregs and transform into effector Tregs upon antigen stimulation. Effector Tregs suppress the maturation of antigen-presenting cells, consume IL-2, and produce inhibitory cytokines (such as TGF-β and IL-10), thereby suppressing the activation of cytotoxic T lymphocytes (CTLs) and CD4+ helper T cells. Kumagai et al. reported that PD-1 inhibitor treatment benefited patients with lung and gastric cancers and high and low PD-1 expression on effector T cells and Tregs, respectively (33).

Extracellular acidosis suppresses T cell-mediated immunity, and neutralization of tumor acidity reportedly improves antitumor responses to immunotherapy. Lowering the pH of the TME likely induces anergy in human and mouse tumor-specific CD8+ T cells through mTORC1 inhibition, thereby reducing cytolytic activity and cytokine production (34).

Several studies have reported on the effect of lactate on T cells, which is the primary cause of TME pH reduction. Many effector T cells are inactivated by glucose depletion and elevated lactate levels triggered by tumor cells, as their proliferation and cytokine production are highly dependent on glycolysis (35). Inhibition of glycolysis in CD4+ helper T cells and CTLs also reduces cell motility associated with decreased responsiveness to chemokines (36). Furthermore, the high lactate concentration in the TME inhibits lactate efflux from T cells, thereby reducing cytokine production and cytotoxic activity (37, 38). In contrast, in Treg cells, the master transcription factor forkhead box P3 (FOXP3) makes energy production less reliant on glycolysis and more on oxidative phosphorylation, which improves survival and maintains immune suppressive function in low-glucose and high-lactate environments (39, 40). Thus, TME lactate elevation reduces effector T cell function and attenuates anti-tumor immunity without affecting Treg cell function. Furthermore, lactate in the TME reduces the release of soluble granule contents such as perforin and granzyme from NK cells, decreases the production of cytokines such as IFN-γand TNF-αand indirectly suppresses NK cell function by increasing MDSCs (41–43). Moreover, the effects of the acidic TME on NK cells are reversible: oral administration of bicarbonate to a lymphoma mouse model and raising the TME pH to the physiological pH of 7.2–7.5 increased the production of IFN-γ by NK cells and suppressed tumor growth (44).

Adaptation of macrophages to the acidic tumor microenvironment

Macrophages are divided into M1 and M2 phenotypes. M1 macrophages are responsible for innate immune responses through the secretion of inflammatory cytokines, phagocytosis of foreign substances, and the presentation of antigens. They are involved in Th1-type responses. Th1 cytokines such as IFNγ and IL-12 and foreign antigens such as lipopolysaccharide (LPS) induce differentiation to the M1 phenotype. Conversely, M2 macrophages are induced by Th2 cytokines such as IL-4, IL-10, and IL-13 and play pivotal roles in immunosuppression, tissue remodeling, and angiogenesis. TAMs often exhibit M2-like traits in many malignant tumors and act as tumor promoters (45). IL-10 and TGF-β secreted by TAMs suppress Th1, as well as induce regulatory T cells, thereby suppressing T cell immune responses (46).

An acidic TME favors polarization to M2 macrophages *in vitro* and *in vivo* and additionally increases angiogenic vascular endothelial growth factor (VEGF) production (47, 48). The lactate-induced M2 macrophage polarization reportedly involves the ERK-STAT3 signaling pathway (49), HIF1α stabilization (50), and G protein-coupled receptor 132 (GPR132) activation. Furthermore, Zhang et al. reported that post-translational modification of histone proteins by lactyl groups derived from lactate induces M2 polarization (51). Furthermore, lactate inhibits monocyte differentiation into dendritic cells, and high lactate levels in the TME may interfere with dendritic cell formation and accumulation (52).

Adaptation of myeloid-derived suppressor cells to the acidic tumor microenvironment

Myeloid-derived suppressor cells (MDSCs) are classified into granulocytic/polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) according to their origin. A hallmark of MDSCs is their ability to inhibit immune responses, including those mediated by T, B, and NK cells. M-MDSCs and PMN-MDSCs share features that facilitate suppression of immune

responses, including the activation of STAT3 expression, induction of ER stress, expression of arginase 1, and expression of S100A8/A9. Furthermore, PMN-MDSCs preferentially use reactive oxygen species (ROS), peroxynitrite, arginase 1, and prostaglandin E2 (PGE2) to mediate immunosuppression, whereas M-MDSCs mediate nitric oxide (NO), induce immunosuppression through immunosuppressive cytokines such as IL-10 and TGFβ and immunomodulatory molecules such as PDL1 (53).

MDSCs reportedly upregulate PD-L1 expression and PD-1-mediated suppression of T cells through the lactate-induced HIF1α pathway in TMEs (54). Furthermore, MDSCs may promote the formation of tumor blood vessels by enhancing the production of angiogenic factors such as VEGF through the lactate-induced HIF1α pathway in TMEs, further contributing to the hypoxic conditions (43). VISTA, an immune checkpoint molecule expressed in MDSCs, is directly induced by acidification, resulting in immunosuppression (55).

Adaptation of cancer-associated fibroblasts to the acidic tumor microenvironment

In some cancers, such as breast and pancreatic cancers, cancer-associated fibroblasts (CAFs) are the most prominent stromal cell type, and their presence is associated with a poor prognosis. They have various origins, including resident tissue fibroblasts educated by primary cells, mesenchymal cells recruited from the bone marrow to the TME, and adipocyte-derived progenitor cells. The functions of CAFs in the TME are also diverse and participate in promoting tumor progression, including direct cancer cell proliferation, immunosuppression, angiogenesis, and promotion of extracellular matrix (ECM) remodeling. These functions are mediated by complex reciprocal signaling interactions with cancer cells, the ECM, and infiltrating immune cells (56).

CAF directly interacts with prostate cancer cells to promote lactate production through the expression of the glucose transporter GLUT1 and to induce TME acidification by releasing lactate *via* monocarboxylic acid transporter-4 (MCT4). Simultaneously, it induces Th1 cell suppression and Treg-induced immunosuppression (57, 58). Thus, CAF promotes metabolic-based tumor growth with TME acidification by interacting with tumor cells.

Approaches of alkalization of the acidic TME

As described above, TME acidification by cancer cells is considered to be one of the immune escape mechanisms and causes poor clinical outcomes. Therefore, in addition to alkalizing agents such as bicarbonate, inhibitors against membrane-bound proton transporters, such as NHE1, Na+/K+ ATPase pump, V-ATPase, H+/Cl− symporter, MCT, and CA have been attempted to be developed as alkalizing therapy for TME (59, 60). In clinical practice, there is a report that the prognosis was improved by alkalizing therapy, using urinary pH as an indicator of alkalinization (61–63). In addition, CAIX inhibitors, which are intensively researched (64), have been reported to enhance ICI antitumor effects in preclinical models, and clinical applications of combined immunotherapy and alkalizing therapy are expected in the future.

Conclusion

Tumors exploit the local acidification using lactate to interact with the cells that constitute the TME and facilitate immune escape, which involves the suppression of immune cells with anti-tumor activity, activation of immunosuppressive cells, and promotion of the malignant transformation of CAF-forming stroma and proliferation of tumor blood vessels. The development of therapeutics that inhibit pH-responsive proteins, such as MCT, and the administration of buffers to adjust the pH level of the TME may be further explored as potential therapeutic alternatives.

Author contributions

MH and KY wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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.

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