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Advances of novel approaches to enhance therapeutic efficacy and safety in human solid cold tumor

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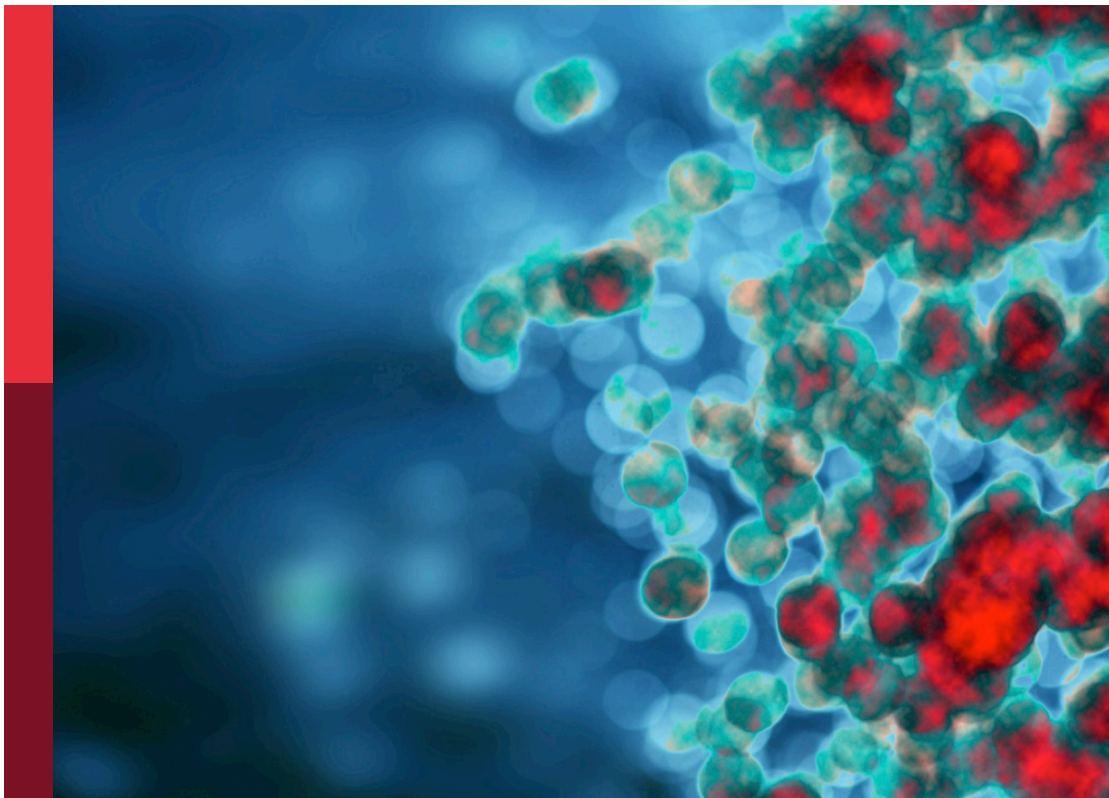

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Advances of novel approaches to enhance therapeutic efficacy and safety in human solid cold tumor

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Editorial: Advances of novel approaches to enhance therapeutic efficacy and safety in human solid cold tumor

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Editorial on the Research Topic
Advances of novel approaches to enhance therapeutic efficacy and safety in human solid cold tumor

Tumor immunotherapy has garnered significant attention since its introduction in clinical practice. Unlike traditional treatments such as surgery, radiotherapy, and chemotherapy, immunotherapy offers the benefits of high specificity and minimal side effects. By stimulating the body to generate a targeted immune response against tumors, immunotherapy works to inhibit and destroy cancer cells. Immune checkpoint inhibitors, particularly CTLA-4 and PD-1/PD-L1 blockers, are crucial components of immunotherapy, as they help overcome the inhibitory signals that tumors send to immune cells, allowing for a more effective anti-tumor immune response (1, 2). Despite the promising potential of ICI in cancer therapy, a significant number of cancer patients exhibit resistance to this treatment. Additionally, the occurrence of immune-related adverse events (irAE) is not uncommon during ICI therapy, with severe cases potentially resulting in irreversible organ damage and life-threatening complications (3).

The effectiveness of immune checkpoint inhibitors (ICI) in treating cancer is influenced by various factors, including immune checkpoints, antigen presentation, the tumor microenvironment (TME), and inflammatory signals. “Cold tumors” like pancreatic cancer, colorectal cancer, and small cell lung cancer have low immunoreactivity, lack of effector T cells, high levels of immunosuppressive regulatory T cells and M2-polarized macrophages, limited antigen availability, and low T cell infiltration, making them less responsive to ICI treatment. Transforming “cold” tumors into “hot” tumors through strategies that enhance immunoreactivity could significantly improve the efficacy of immunotherapy (4). In this Research Topic, we focus on showcasing innovative approaches to enhance the effectiveness and safety of immunotherapy, discovering fresh biomarkers for “cold tumors”, enhancing results with cancer immunotherapy, and investigating methods to reshape the TME for promoting T-cell infiltration and immune reactions.

PD-1/PD-L1 inhibitors have shown remarkable therapeutic potential in clinical practice. Ni et al. found that, compared with EP/EC plus PD-L1 inhibitors, IP/IC

combined with camrelizumab (a humanized high-affinity IgG4-κ anti-PD-1 monoclonal antibody) combined with apatinib has better efficacy and controllable safety in the treatment of untreated extensive-stage small-cell lung cancer (ES-SCLC). It was also found that the occurrence of irAE was associated with longer progression-free survival (PFS), which may be a potential prognostic factor for patients treated with ICIs. Sheng et al. used next-generation sequencing (NGS) for tumor gene expression profiling and PD-L1 immunohistochemical expression to achieve a precise diagnosis in a patient with primary cancer of unknown (CUP) who achieved complete remission after six cycles of nivolumab plus carboplatin and albumin-bound nanoparticle paclitaxel. In addition, Chen et al. innovated an attempt to use immunotherapy combined with etoposide-platinum (EP) regimen in the treatment of tracheal small cell carcinoma. The patient received six cycles of PD-L1 inhibitor (adebrelimab) combined with EP treatment, which significantly relieved symptoms and completely disappeared the tracheal mass. Wang et al. also tested that the addition of adebrelimab to etoposide-platinum-based chemotherapy may result in longer survival than durvalumab and atezolizumab in patients with ES-SCLC. Zhu et al. found that the strategic introduction of TACE and microwave ablation during PD-1 monoclonal therapy in patients with pancreatic cancer enhanced the release of tumor antigens, thereby amplifying the immune response. It not only shows the advantages of PD-1/PD-L1 inhibitors, but also implies the prospect of combination therapy in tumor immunotherapy. In addition to combination therapy, some emerging therapies, such as DNA/RNA vaccines, are also emerging. Liu et al. demonstrated that intramuscular injection of OVA-encoding plasmid DNA (pDNA) in combination with pDNA encoding α-PD-1 could enhance tumor therapy through *in situ* gene delivery and enhancement of a potent muscle-specific promoter. Similarly, in refractory brain tumors, antigen-specific personalized mRNA vaccines have shown effective anti-tumor responses in clinical brain tumor models, providing new ideas for therapeutic approaches targeting classical immunotherapy resistance and some "cold tumor" types (5). Muraro et al. also found that targeting BRAF mutations in cutaneous melanoma (CM), using an immune weapon as antibody-dependent cytotoxicity (ADCC) of anti-receptor tyrosine kinase (RTKs) antibodies, it effectively and specifically killed EGFR-expressing BRAFi resistant CM cells both *in vitro* and *in vivo* in a humanized mouse model.

Recent research has identified new biomarkers for "cold tumors" beyond the traditional PD-1/PD-L1 and CTLA-4 pathways. These biomarkers include surface proteins and non-coding RNA/DNA, offering new avenues for treating "cold tumors". Lodewijk et al. have demonstrated that post-translational modifications on tumor cells can create tumor-associated antigens (TAAs), which are specific targets that can provoke anti-tumor immune responses. They have highlighted three TAAs: CD44v6, truncated carbohydrates like Tn and STn, and altered ganglioside expression such as GD2 and O-GD2. These TAAs show promise for developing antibodies, antibody-drug conjugates, nanodrugs, vaccines, and CAR T cell therapy. Additionally, researchers have observed that the escape of the prostate-specific antigen STEAP1 can lead to drug resistance in metastatic prostate cancer. Combining tumor-targeted interleukin-12 therapy with STEAP1-directed CAR T cell therapy has shown potential in overcoming STEAP1 antigen escape and enhancing the anti-tumor effects in prostate cancer (6). In addition to targeting surface proteins, certain researchers have employed an exosome-mediated CRISPR/Cas9 delivery system to target the YTHDF1 gene *in vivo*. This approach can effectively inhibit the translation of lysosomal genes, thereby restricting the lysosomal proteolysis of major histocompatibility complex class I (MHC-I) and antigens. Ultimately, this method helps to restore tumor immune surveillance (7). Likewise, certain researchers have employed mRNA lipid nanoparticles encoding just the N terminus of gasdermin to stimulate pyroptosis, resulting in a robust anti-tumor immune response and rendering "cold tumors" susceptible to checkpoint immunotherapy (8). By increasing the amount of cytoplasmic double-stranded DNA to activate the classical cGAS-STING-pTBK1/pIRF3 axis, IR and ATRi can enhance CD8+ T cell infiltration and improve the efficacy of anti-PD-L1 therapy in a mouse CRC model (9).

Remodeling the TME is a viable option for enhancing the immune reactivity of "cold tumors". In a study by Hu et al., targeting neutrophils and combining various doses of radiation effectively inhibited tumor growth, disrupted the tumor cell cycle, and facilitated the release of neoantigens from tumor cells to enhance the immune response and prolong the cytotoxic effects of functional T cells. Additionally, some researchers have demonstrated that using nanomedicines to target different inhibitory immune cells can help minimize the side effects of drugs (10). By utilizing single-cell RNA sequencing (scRNA-seq), researchers can conduct a detailed analysis of the specific immune cell populations, their phenotypes, and other microenvironmental elements in the tumor microenvironment (TME) of different cancer types. This approach offers a more accurate method for tailoring immunotherapy treatments using immune checkpoint inhibitors (ICIs) (11). In a similar vein, Hapuarachi et al. propose that exercise training could potentially convert a "cold tumor" to a "hot tumor," resulting in alterations to the tumor microenvironment (TME) and systemic immune system that could improve the efficacy of immunotherapy. Moreover, the administration of platinum drugs may trigger the release of cytokines and provoke varied responses within the TME, ultimately causing type I hypersensitivity that poses a challenge to patient treatment. To address this issue, Li and Yin suggest a 5-step platinum desensitization protocol to reduce the risk of allergic reactions.

This Research Topic explores novel approaches to enhance the effectiveness and safety of immunotherapy for "cold tumors" as well as underscores the dynamic field of tumor immunotherapy, highlighting the shift towards personalized and more targeted approaches to overcome the challenges associated with "cold tumors" and improve patient outcomes. These strategies include the identification of new biomarkers for "cold tumors", targeting immune cells to modify the tumor microenvironment, and utilizing combination therapy to enhance the outcomes of cancer immunotherapy. These approaches offer a fresh perspective on the application of immunotherapy in treating "cold tumors". While the potential for these strategies is promising, there are significant challenges in translating them into clinical practice. The discovery of new targets is eagerly anticipated, as they may offer additional avenues for improving tumor

immunotherapy and advancing cancer prevention, diagnosis, and treatment.

Author contributions

SG: Supervision, Writing – original draft, Writing – review & editing. XX: Writing – original draft. YC: Writing – review & editing. YL: Writing – review & editing. LL: Conceptualization, Writing – review & editing.

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Conflict of interest

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Functional plasticity of neutrophils after low- or high-dose irradiation in cancer treatment – A mini review

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Over the last several decades, radiotherapy has been considered the primary treatment option for a broad range of cancer types, aimed at prolonging patients' survival and slowing down tumor regression. However, therapeutic outcomes of radiotherapy remain limited, and patients suffer from relapse shortly after radiation. Neutrophils can initiate an immune response to infection by releasing cytokines and chemokines to actively combat pathogens. In tumor immune microenvironment, tumor-derived signals reprogram neutrophils and induce their heterogeneity and functional versatility to promote or inhibit tumor growth. In this review, we present an overview of the typical phenotypes of neutrophils that emerge after exposure to low- and high-dose radiation. These phenotypes hold potential for developing synergistic therapeutic strategies to inhibit immunosuppressive activity and improve the antitumor effects of neutrophils to render radiation therapy as a more effective strategy for cancer patients, through tumor microenvironment modulation.

KEYWORDS

neutrophils, low-dose radiotherapy, high-dose radiotherapy, tumor-associated neutrophils, cancer, tumor

1 Introduction

Radiotherapy, a therapy that has been applied worldwide for several decades to reduce tumor size and relieve pain from metastatic cancer patients. Two-thirds of cancer patients benefit from radiation which delivers high-energy X-rays or particles to destroy tumors (1). There is ample evidence that high-dose radiotherapy is associated with prolonged overall survival, as demonstrated by a broad spectrum of cancer patients' clinical therapeutic results and preclinical experiments. Patients who received low-dose irradiation following high-dose

irradiation at local tumor triggered a systematic antitumor response rate of metastatic burden, as observed in preclinical studies (2, 3). So far, many studies demonstrated that high-dose irradiation triggers system toxicity and boosts antitumor immunity in cancer patients; low-dose irradiation, however, is under more investigation especially its influence on profoundly reprogramming the tumor immune microenvironment (TIME) and cytotoxic T cell recruitment which can effectively reverse cold tumors and priming the antitumor immunity with less toxicity (4, 5).

Neutrophils, a type of polymorphonuclear (PMN) cells, have short lifespan and play an indispensable role in immune defense by their tumoricidal activity, granules release and etc. that are necessary for tumor suppression (6). Neutrophils regulate the microenvironment through pro- and anti-inflammatory cytokines expression; in turn, cytokines in the microenvironment mediate the function of neutrophils (7). Various subtypes of tumor-associated neutrophils (TANs) either promote or inhibit tumorigenesis, metastasis, and recurrence (8, 9).

TANs are distinguished from the potent immune-suppressive polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) not by cell surface markers but only by functional characteristics (9). A recent study by Condamine et al. found that the lipid metabolism-related protein lectin-type oxidized LDL receptor-1 can distinguish PMN-MDSCs from neutrophils, yielding a potential target in medical oncology (10). However, in some cancer types, neutrophils have disparate roles in either supporting or suppressing the tumor cells proliferation, depending on the cancer types (11).

The contribution of neutrophils after low or high radiotherapy doses to patients' metastasis-free survival (MFS) and local tumor control is still a matter of controversy. Elevated numbers of peripheral neutrophils result in radioresistance by activating the mitogen-activated protein kinase (MAPK) pathway (12, 13). Neutrophil depletion through antibodies or pharmacological approaches before radiotherapy boost cancer immune response of cancer patients (12). However, compelling evidence also indicates that fractionated radiation doses initiate neutrophil recruitment and promote antitumor immunity (14). These antitumor effects of neutrophils are amplified by concurrent administration of granulocyte colony-stimulating factor (G-CSF) and radiotherapy through PI3K/Akt/Snail signaling pathway activation (15, 16). Therefore, the distinction between "bad" and "good" neutrophils after low- or high-dose irradiation may pave the way to enhance "good" neutrophils and inhibit the "bad" neutrophils as a potential target to increase radiation efficacy (17, 18).

In this review, we will emphasize several phenotypes of neutrophils after fractionated low- or high-dose radiation, respectively and provide the potential target to enhance the combination therapy.

2 The functional versatility of neutrophils in response to conventional high- or low-dose irradiation therapies

In the early stages of human lung tumors, TANs compose up to 25% of cells from isolated tumor samples, indicating neutrophils' indispensable role in tumor development and further progression (19). At the same time, crosstalk between T cells and neutrophils in this period encourages cytotoxic signaling expression on the surface of neutrophils, which fosters T cell proliferation (9, 19). Neutrophils also demonstrate functional diversity in the late stage of BALB/C mice bearing 4T1 mammary carcinoma. High-density neutrophils (HDN) from tumor-bearing mice prompt antitumor activity while low-density neutrophils (LDN) induce immunosuppressive properties (20, 21).

Neutrophils are divided into three major subtypes: pre-neutrophils, immature and mature neutrophils from previous reports, and these subtypes have various functional capacities (22). Neutrophil to lymphocyte ratio (NLR), an indicator and prognostic factor of progression-free and overall survival, a higher ratio is proportional to a lower immune response and more frequent relapsing rate in metastatic Non-small-cell lung carcinoma (NSCLC) (23). NLR provides an extensive range of cancer types with prognostic biomarkers after high- and low-dose irradiation treatment (23–25). In cervical cancer autochthonous mouse models of soft tissue sarcoma, elevated neutrophil numbers suppress the immune response and promote resistance to radiotherapy after the tumor receives a total 20 Gy *in situ* radiation (12). The accumulation of monocytic (M-)MDSCs in the tumor sites of MC38 tumor-bearing mice after 20 Gy irradiation stimulation results in radioresistance through STING pathway activation (26).

The effects of repeated radiation doses on positively or negatively regulating the immune can be influenced by the neutrophils that are infiltrated into tumors. Despite the immunosuppressive properties of neutrophils, neutrophils also participate in the first line of innate and adaptive immune response in tumor models after radiotherapy (14, 15, 27). Fractionated radiation of 8Gy leads to a remarkable increase of phosphorylated histone H2AX (γH2AX) which considerably induces CXCL1, CXCL2, and CCL5 inflammatory chemokine secretion and improves radiosensitivity. Neutrophils recruited by hypofractionated radiation facilitate mesenchymal-epithelial transition (MET) *via* the ROS-mediated PI3K signaling pathway and impel tumor elimination. A combination of high- or low-dose radiotherapy and granulocyte colony-stimulating factor (G-CSF) enhances radiosensitivity and antitumor activity with anticancer functional neutrophils (14, 15, 28, 29).

3 Neutrophils are reprogrammed in the tumor microenvironment after either low- or high-dose radiotherapy

Neutrophils are a critical component of the innate immune system, the pro- or anti-inflammatory functions of neutrophils depends on cytokines stimulation in the TIME (30–32). MDSCs, for example, is a type of the immunosuppressive neutrophils that involve in tumor progression in numerous cancer types after pathogen activation (33). Antitumor neutrophils can be regulated by ICAM-1 and TNFα, leading to reduced neutrophil extracellular

traps (NETs) and improved radiotherapy response, resulting in prolonged survival in cancer patients (8, 9, 34, 35). With a better understanding of the crosstalk between neutrophils and the TIME, patients could benefit from improved diagnosis, more effective immune protection strategies, and targeted therapies.

4 Antitumor effects of neutrophils after high-dose or low-dose irradiation

4.1 Type 1 tumor-associated neutrophils

Similar to tumor-associated macrophages (TAMs), neutrophils undergo a reprogramming process under the stimulation of chemokines, tumor necrosis factors, colony-stimulating factors (CSFs), and interferons (IFN), which results in non-identical polarized phenotypes. Some are antitumor (N1 TANs), while others promote tumor progression and metastasis and pro-tumor TANs (36). However, there is currently no definitive method for identifying N1 TANs and N2 TANs (pro-tumor TANs) based on specific functional characteristics (37).

IFN-α and IFN-β, also referred as type 1 IFN, are considered as potential anticancer agents to maintain immune system surveillance capabilities. IFN- β can polarize neutrophils into an antitumor N1 phenotype (38). The presence of IFN-β in the early stage of tumor development restricts angiogenesis and backing neutrophils to antitumor N1 phenotype. This conversion leads to an increase in cytotoxic T cells and suppression of tumor cell proliferation, leading to improved outcome of cancer patients (36, 38). Antitumor N1 TANs have been shown, through immunohistochemical analysis in various cancer types, to promote leukocyte recruitment by producing cytotoxic reactive oxygen species (ROS), tumor growth inhibitor matrix metalloproteinase (MMP)-8, Fas-ligand for antibody-dependent cell-mediated cytotoxicity (ADCC), and multiple cytokines. These mechanisms result in similar antitumor effects regardless of whether high or low doses of radiation are used (36, 39, 40).

Several syngeneic mouse tumor models were utilized to gain deeper insight into the early immunological effects of different doses of radiation on neutrophils in TIME. In EG7-bearing C57BL/6 mice, which is a radiosensitive syngeneic graft tumor model, showed remarkable impact on tumor viability with 1.3Gy radiation and enhanced the antitumor effects through $CD11b^{+}Gr\text{-}1\ high^{+}$ neutrophils infiltration. However, 4T1-bearing Balb/c mice required a high dose (15 Gy) to reach similar anti-neoplastic influence through heightened production of ROS neutrophils (15).

4.2 Antitumor neutrophil extracellular traps

Neutrophils play vital roles in protecting the host against infections through NETs. They immobilize and eliminate invading pathogens by activating the downstream ROS pathway and inducing chromatin decondensation promoters such as protein-arginine deiminase type 4 (PAD4), myeloperoxidase (MPO), and neutrophil elastase (NE) (41, 42). Although the mechanisms by which NETs are involved in the TIME and their potential anti- or pro-neoplastic activation have been explored for decades, it remains unclear how NETs regulate different types of tumor progression and elicit radioresistance or radiosensitivity after different doses radiation. One proposed mechanism by which NETs may promote tumor growth is the induction of chronic inflammation in the TIME. Moreover, the DNA in the NETs can act as a danger signal to trigger the activation of innate immune cells and further exacerbating inflammation in the TIME. However, reports also mentioned the anti-tumor effects of NETs. For example, NETs can trap circulating tumor cells and prevent tumor dissemination, which can reduce the tumor metastasis (11, 39, 42).

Histology analysis of adenocarcinoma patients demonstrates that the concentration of NETs reaches a peak in tumor sites in response to tumor cells, and neutrophil infiltration decreases gradually from tumor tissues to distal sites. *In vitro* evidence confirms that cultured Caco-2 colorectal adenocarcinoma cells and acute myeloid leukemia (AML) undergo apoptosis processes when encountering NETs (43). Toll-like receptors (TLRs) stimulate the formation of NETs (44). Furthermore, specific TLRs selectively produce corresponding NETs structurally and functionally distinct, which offers a promising foundation for further application in disease intervention (45). Co-culture with endothelial cells promote NETs formation, causing damage to tumor-dependent blood vessels and slowing tumor growth; this antitumor effect could be abrogated *via* NADPH oxidase inhibition (46). Histones, a component in NETs, can trigger host cell cytotoxicity, suggesting that NETs could be a promising target to increase tumor-killing efficacy (47).

5 Pro-tumor effects of neutrophils after high-dose and low-dose irradiation

Despite the extensive literature on the antitumor functions of TANs mentioned above in modified tumor cell lines or after receiving specific therapies, there are a substantial number of studies suggest their pro-tumor roles (48, 49). Increasing reports support the contribution of neutrophils in tumor progression through tumor angiogenesis, chemokine and cytokine release in TIME, which induce the pro-cancer role of neutrophils and resistance to radiotherapy (12, 26, 50). Pro-tumor TANs are associated with tumor metastasis, tumor cell proliferation, and a high frequency proportion of relapse. Neutrophil depletion through antibodies or genetic methods increases radiotherapy sensitivity (12). Neutrophils support the extravasation of disseminated carcinoma cells and inhibit intraluminal cell clearance mediated through NK cells; furthermore, such pro-cancer neutrophils show an extended life span (51–53).

5.1 Myeloid-derived suppressor cells

A subtype of immature and mature neutrophils, identified as inhibitory immune cells partly through promoting the proliferation of regulatory T cells (Tregs) and macrophage differentiation at pre-metastatic niches, are known as MDSCs (31, 54). Prostaglandin E2 (PGE-2), an essential cell growth and regulatory factor whose receptor is expressed on the surface of MDSCs, encourages differentiation of $Gr1^{+}CD11b^{+}$ MDSCs. PGE-2 inhibits the capacity of Th1, CTL, and NK cell and enhances Th2, Treg, tumor-infiltrating T helper type 17 (Th17), and tumor-infiltrating helper T cells inhibitory properties. PGE-2-deficient BALB/c mice display lower MDSCs expression and delayed tumor growth compared with wild-type mice when incubated with 4T1 tumor cells (55, 56). Activation of CXCL12/CXCR4 pathway leads to PEG-2-dependent accumulation of MDSCs, which in turn migrating *via* COX2 and promote tumor progression in ovarian cancer (57). Previous findings demonstrated that specific COX2 inhibitors, nonsteroidal anti-inflammatory drugs and other agents could be further developed as potential anticancer production by reducing PGE-2 synthesis or increasing neutrophil superoxide anion production to delay tumor progression and reduce metastasis (58, 59).

Radiation induces the infiltration of MDSCs into different types of tumors, with either low- or high- dose radiation (54, 60). The development of bone marrow-derived myeloid cells, including TAM and MDSCs, is dependent on CSF1/CSF1 receptor (CSF1R) (33, 61, 62). In prostate cancer, both multiple fractions of 3 Gy or a single 30Gy *in situ* radiation induce a systematic increase of MDSCs and CSF1/CSF1R in various organs through tumor-infiltrating myeloid cells (TIM) employment. Selective blockade of CSF1R suppresses MDSCs infiltration and facilitates MDSCs loss results in improved therapeutic outcomes (50, 63). In a report on MC 38 and CT26 tumor bearing mice, it reports that hypofractionated irradiation (>= 20Gy) suppresses the accumulation and infiltration of MDSCs. However, lower doses of irradiation tended to facilitate the recruitment of MDSCs into tumor (64, 65).

The influence of radiation on MDSCs infiltration and therapeutic outcomes are different between types of cancers. For example, a single dose of 25Gy radiation can recruit $CD11b^{+}Gr\text{-}1^{+}$ MDSCs to infiltrate tumors and affects last longer than 14 days in TRAMP-C1 intramuscular tumor model (66). However, there are significant differences between tumors and radiation doses. In high grade gliomas (HGG), a single dose of 4Gy radiation downregulates M2 TAMs and M-MDSCs and encourages T cell proliferation (67). Similar findings were shown in intracranial CT2A subcutaneous mouse model, where nanoparticles-based fractionated 2Gy repolarizes M2 pro-tumor phenotype to M1 antitumor phenotype and boosts ROS generation (66). High-dose radiation, which is over 45 Gy, can have various effects on MDSCs population in head and neck cancer patients (68).

Previous reports also suggest that MDSCs are responsible for abscopal effects inhibition. A melanoma patient who received radiotherapy showed decreased MDSCs concomitant with abscopal effects, suggesting a manageable way to investigate the relationship between radiation-induced MDSCs and tumor abscopal effects (69).

The depletion of MDSCs, specifically targeting the crosstalk between MDSCs and NK cells, has been shown to mediate immunosuppressive influence and inhibit tumor growth after no more than 0.2Gy LDRT (70). Moreover, TGF-β secretion by MDSCs and N2-type TANs activation damage NK cells after receiving low radiation (63, 70) (Figure 1). The literature mentioned above reveals that the MDSCs which receives lose-dose and high-dose irradiation therapy establish various, and this yields a potential target to increase MDSC-based antitumor immunity in combination with different doses radiation with specific timing.

5.2 NETs formation

Neutrophils can prevent fungal and bacterial cells from invading through degranulation and nuclear chromatin expulsion and forming the NETs (42). However, in the context of radiation therapy, formation of NETs after radiation therapy can facilitate tumor progression, and its inhibition post-radiation improves the therapeutic outcome and overcomes radioresistance in syngeneic bladder cancer mode in a TLR-4 dependent manner (71). Tissue damage caused by radiation before tumor formation promotes Notch pathway activation, which leads to neutrophils' recruitment to tumor sites and subsequent cancer metastasis (72).

Extracellular DNA accumulation is a marker of NET formation, and DNA components of NETs (DNA-NETs) promote cancer metastasis. DNase, which destroys DNA scaffolds through CCDC225, has been shown to abrogate NET-mediated metastasis (73, 74). Preclinical models have shown that NETs inhibition through PAD4 inhibitors (citrullination) increases sensitivity to anti-PD-1 and anti-CTLA 4 and achieve significant therapeutic efficacy (73).

Mitochondrial biogenesis and tumor cell proliferation have been found to be correlated with NETs formation and PAD4 expression in human cell lines (75–77). High dose of radiation (10 Gy) induces NETs formation in tumors, which capture tumor cells and shield them from detection by CTLs to facilitate tumor cell metastasis to distal sites (50). PAD4-KO tumors have the properties of elevated apoptosis, mitochondrial membrane potential and less ATP production, indicating the potential target for clinical appilicability (50, 75). However, a study also shows low-dose radiation (2Gy) has no influence on NETs formation in bladder cancer model (50). The involvement of NETs in radiation resistance demands further exploration.

5.3 N2 TANs

Substantial evidence suggests that functional disparity by factors in the TIME is the basis of the heterogeneity of neutrophils. For example, tumor releases IL-8, IL-10, PGE-2, and TGF-β to interstitially induce tumor progression N2 TANs and

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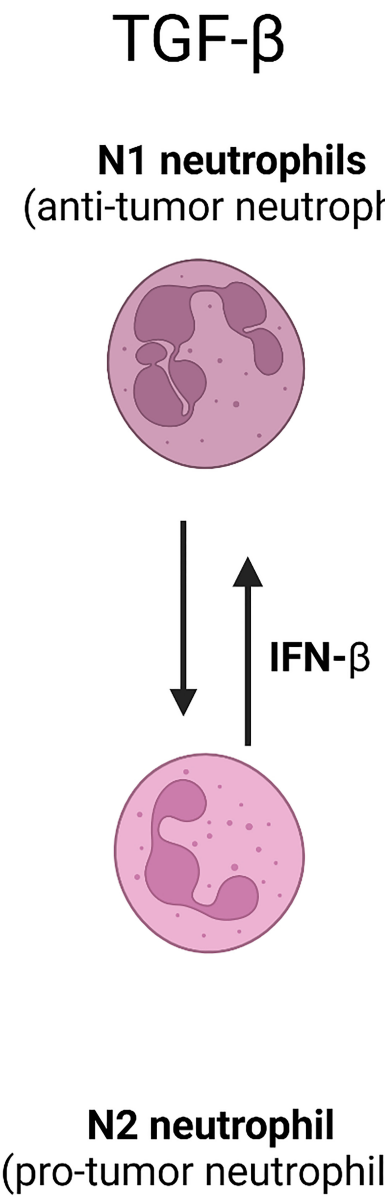

FIGURE 1
The involvement of TGF-β in neutrophil polarization.

promote tumor growth and metastasis (12, 78). In addition, VEGF and TNF production from N2 TANs promote tumor vascularization and Matrix Metalloproteinases (MMP)-9 secretion, which participates in the tumor extracellular matrix reconstruction and contributes to subsequent tumor metastasis (9, 79, 80).

The tumor microenvironment and the patient's overall TIME promote the conversion of neutrophils to the N2 TANs phenotype, as previously described that the absence of IFN-β stimulation leads to N2 TANs to promote tumor growth (36). Moreover, neovascularization was accompanied by increased invading N2 TANs that expressed more VEGF and CXCR4 (19, 81).

In contrast, differentiated N2 TAN secretes copious hydrolases, cytokines and chemokines to reinforce the immunosuppressive state of the TIME and promote tumor proliferation and migration. Tumor cells, pro-neoplastic neutrophils and even tumor-derived fibroblasts secrete MMP-9 to mediate the degradation of the tissue basement membrane type IV collagen to promote tumor growth and stimulate VEGF to promote vascularization (82, 83) (Figure 2).

N2-type neutrophils in tumor tissues stimulate Th17 proliferation and differentiation through CCL-20, IL-23 secretion and inhibition of TNF-α production, which in turn strengthens the immunosuppressive effects of Th17 and inhibit antitumor effects of $CD4^+$ Th1 to negatively regulate functional homeostasis to tumor onset (14, 84–86). In the preclinical transgenic lung tumor mouse model, abnormal neutrophil accumulation correlated with an N2-like $SiglecF^{pos}$ and Ly-6G downregulation. Furthermore, incomplete neutrophil deletion mediated with Ly-6G along with the radiotherapy retards tumor growth and triggers durable tumor regression (82).

6 Conclusion and perspectives

Radiation therapy induces a systematic immune response in cancer patients and improves survival. However, the cytotoxic effects are not persistent and patients develop radioresistance after several months. Therefore, more concurrent administration strategies are needed to refine radiotherapy.

Factors determine the function and polarization of neutrophils in the TIME; thus how the signalings and chemokines are involved in inducing the chemotaxis has been explored for several decades but still needs more elucidation. Neutrophil-targeted therapies offer a promising therapeutic route to strengthen therapeutic effects in a wide range of cancers from preclinical to clinical cancer patients. In multiple cancer patients' histology samples, NLR is regarded as a reliable and accessible biomarker for predicting prognosis as cancer progresses. Low- or high radiotherapy slows tumor growth, interrupts the tumor cell cycle and releases neo-antigen from tumor cells to recruit immune cells and reinforce anti-neoplastic neutrophils and or dampen pro-tumor neutrophils to accelerate tumor shrinkage or undermine radioresistance, the mechanisms are still unclear. Chemotherapies or ICI and different doses of synergistic radiotherapy strategies render cancer patients responsive to radiation therapy to amplify immune responses and extend the cytotoxic effects of functional T cells. Cancer patients are benefited from combinational therapy with tumor shrinkage and extended survival. However, owing to the TIME and tumor gene mutation burden (TMB) in multiple cancer types, further research is necessary to better illustrate the interactions between neutrophils and high- and low-dose radiation therapy.

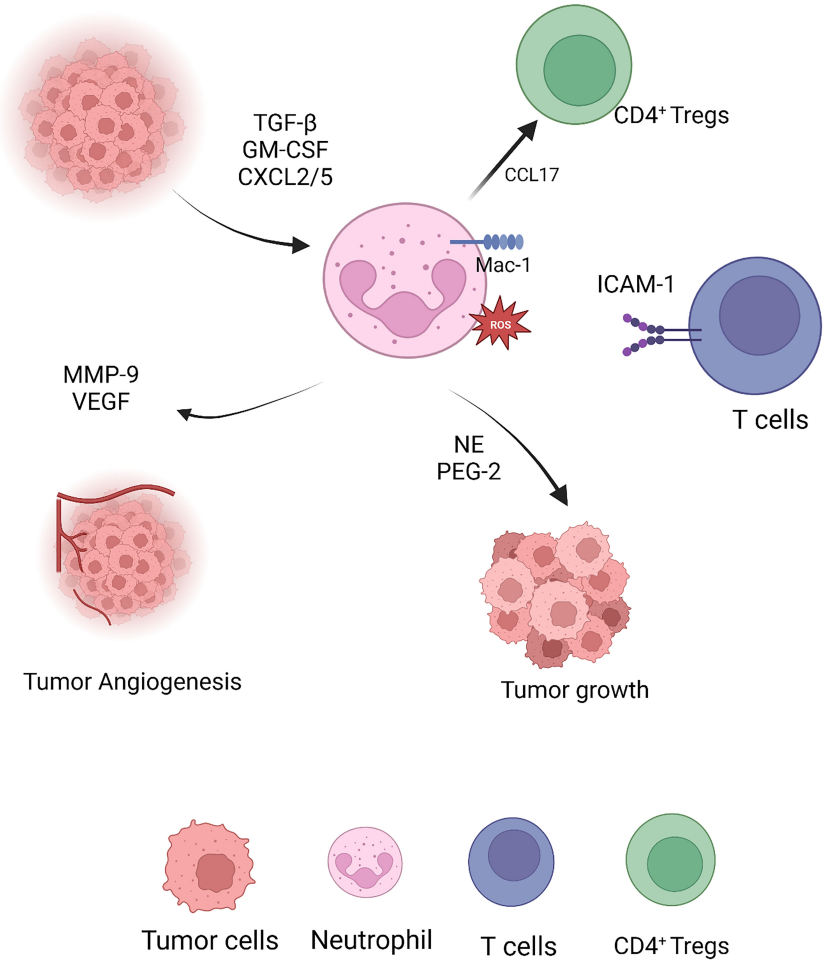

FIGURE 2
Pro-tumor capacities of neutrophils.

In addition, limited documents elucidate the appropriate application time and doses of radiation when combined with neutrophil-target therapies across various cancer classifications, which highlights the necessity for expanding our understanding of the precise underlying mechanisms of how tumor-induced cytokines and chemokines modify neutrophils to inhibit immunosuppressive neutrophils and extend immunotoxic neutrophils.

Author contributions

Conceptualization, JH and MW. Writing—review and editing JH, MW, MP, YW and YZ. All 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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Camrelizumab plus platinum-irinotecan followed by maintenance camrelizumab plus apatinib in untreated extensive-stage small-cell lung cancer: a nonrandomized clinical trial

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Background: Programmed cell death-ligand 1 (PD-L1) inhibitors plus chemotherapy have made substantial progress in extensive-stage small-cell lung cancer (ES-SCLC), but the survival benefit is still limited. This study aimed to evaluate the preliminary efficacy and safety of camrelizumab plus platinum-irinotecan (IP/IC) followed by maintenance camrelizumab plus apatinib in patients with untreated ES-SCLC.

Methods: In this non-randomized clinical trial (NCT04453930), eligible patients with untreated ES-SCLC received 4-6 cycles of camrelizumab plus IP/IC, followed by maintenance with camrelizumab plus apatinib until disease progression or unmanageable toxicity. The primary endpoint was progression-free survival (PFS). Patients who received PD-L1 inhibitors (atezolizumab or durvalumab) plus platinum-etoposide (EP/EC) were selected as the historical control.

Results: Nineteen patients received IP/IC plus camrelizumab and 34 patients received EP/EC plus PD-L1 inhibitor. At a median follow-up time of 12.1 months, the median PFS was 10.25 months (95% CI: 9.40-NA) in the IP/IC plus camrelizumab group and 7.10 months (95% CI 5.79-8.40) in the EP/EC plus PD-L1 inhibitor group, respectively (HR=0.58, 95% CI 0.42-0.81). The objective response rate of IP/IC plus camrelizumab and EP/EC plus PD-L1 inhibitor was 89.6% and 82.4%, respectively. The most common treatment-related adverse events in the IP/IC plus camrelizumab group was neutropenia, followed by reactive cutaneous capillary endothelial proliferation (RCCEP) and diarrhea. The occurrence of immune-related adverse event was found to be associated with a prolonged PFS (HR=4.64, 95% CI 1.92-11.18).

Conclusions: IP/IC plus camrelizumab followed by maintenance camrelizumab plus apatinib showed preliminary efficacy and acceptable safety profile in patients with untreated ES-SCLC.

KEYWORDS

small cell lung carcinoma (SCLC), platinum, irinotecan, camrelizumab, immune checkpoint inhibitors

Introduction

Approximately 15% of lung cancer cases are small-cell lung cancer (SCLC), which has a poor prognosis (1). Platinum-based chemotherapy has been the standard first-line treatment for both limited-stage (LS) and extensive-stage (ES) disease since the 1980s, with an objective response rate (ORR) of about 65% and a one-year survival rate of 30%-40% for ES-SCLC (2, 3).

Since the advent of immune checkpoint inhibitors (ICIs), treatment for many solid tumors has been subverted. Furthermore, SCLC has a high mutation rate, making ICIs an attractive therapeutic option (4). Two phase 3 trials evaluated the addition of programmed cell death-ligand 1 (PD-L1) inhibitor atezolizumab (IMpower133) (5, 6) or durvalumab (CASPIAN) (7, 8) to first-line chemotherapy (etoposide and cisplatin [EP]) followed by maintenance PD-L1 inhibitor in patients with ES-SCLC, and showed a significant improvement in overall survival (OS). Based on the above two studies, PD-L1 inhibitor combined with chemotherapy followed by maintenance PD-L1 inhibitor has been the standard care for untreated ES-SCLC now. However, compared with the unprecedented success of PD-L1 combined with EP in the first-line treatment of ES-SCLC patients, programmed cell death 1 (PD-1) inhibitors showed unsatisfactory efficacy outcomes. KEYNOTE-604 study reported an improvement in progression-free survival (PFS) but not in OS with first-line pembrolizumab combined with EP in patients with ES-SCLC (9). Other therapeutic regimens with PD-1 inhibitors therefore remain to be explored.

Camrelizumab is a humanized high-affinity IgG4-kappa anti-PD-1 monoclonal antibody, which has been approved in combination with chemotherapy (carboplatin plus paclitaxel or pemetrexed) for the first-line treatment of advanced non-small-cell lung cancer (NSCLC) (10, 11). However, the efficacy and safety of camrelizumab plus chemotherapy for ES-SCLC patients remains unknown. In patients with ES-SCLC, two of the most commonly used first-line chemotherapy regimens are platinum (cisplatin or carboplatin) combined with etoposide (EP/EC) or irinotecan (IP/IC). A phase III trial conducted in Japan demonstrated a survival benefit of IP regimen over EP regimen for previously untreated ES-SCLC (12). Moreover, the PASSION study found that the ORR of apatinib (an antiangiogenic agent) combined with camrelizumab was 34.0%, with a median PFS of 3.6 months and a median OS of 8.4 months in patients with ES-SCLC who failed platinum-based chemotherapy (13). Thus, based on the potential therapeutic effect of IP in Asian patients and the promising efficacy of camrelizumab plus apatinib as second-line regimen for ES-SCLC, this non-randomized trial was conducted to explore the preliminary efficacy and safety of camrelizumab plus platinum-irinotecan followed by maintenance camrelizumab plus apatinib in patients with untreated ES-SCLC, with comparison of our historical cohort who received PD-L1 inhibitors plus platinum-etoposide.

Methods

Study design and patients

This non-randomized clinical trial was approved by the ethics committee of Peking Union Medical College Hospital, and was registered with ClinicalTrials.gov (NCT04453930). All patients signed the informed consent before any procedure. The key inclusion criteria were patients aged 18 to 75 years old; pathologically or cytologically confirmed ES-SCLC; previously untreated (including radiotherapy, chemotherapy, vascular endothelial growth factor receptor [VEGFR] inhibitors and ICIs); with an Eastern Cooperative Oncology Group performance status (ECOG PS) score of 0-1. Patients with active brain metastasis or meningeal metastasis were excluded. Detailed inclusion and exclusion criteria were presented in the Supplementary Materials.

Procedure

Eligible patients were administrated camrelizumab (200 mg, day 1), irinotecan (65 mg/m^2, days 1 and 8) plus platinum (cisplatin: 30 mg/m^2, days 1 and 8; or carboplatin: area under curve [AUC]=4~5, day 1) every 3 weeks for 4-6 cycles as induction therapy. After induction therapy, patients were assessed for efficacy per Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1). If progressive disease (PD) was not occurred, patients then received maintenance therapy with camrelizumab (200 mg, day 1, every 3 weeks) and oral apatinib (250 mg, once daily) until disease progression or unacceptable toxicity. Prophylactic brain irradiation was allowed after the induction therapy.

Efficacy was assessed per RECIST 1.1 criteria after the first cycle, and every two cycles thereafter in both induction treatment phase and maintenance treatment phase. For patients without PD but who

discontinued the treatment, efficacy was assessed every eight weeks until PD or death. Follow-up for survival was conducted every eight weeks until death or loss of follow-up. Adverse events (AEs) were recorded and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE, version 5.0).

Endpoints

The primary endpoint was PFS, which defined as the time from enrollment to PD per RECIST 1.1 criteria or death from any cause, whichever occurred first. The secondary endpoints included OS (defined as the time from enrollment to death from any cause), disease control rate (DCR, defined as the percentage of patients with a complete response [CR], partial response [PR] or stable disease [SD]), ORR (defined as the percentage of patients with a CR or PR), and duration of response (DoR, defined as the time from the first document CR or PR to PD or death from any cause). The safety profiles included treatment-related AEs (TRAEs) and immune-related AEs (irAEs).

Historical cohort

Using the electronic medical record system of Peking Union Medical College Hospital, ES-SCLC patients who received ICIs combined with chemotherapy as the first-line treatment were retrospectively reviewed. Among them, patients who received EP/EC combined with PD-L1 blockades (atezolizumab or durvalumab) were selected as the historical control. Information of baseline characteristics, treatment pattern, efficacy and safety profiles of patients were reviewed.

Statistical analysis

All statistical analyses were conducted using SPSS version 23.0 and RStudio 1.2.5001. Continuous variables were expressed as median and range, and classification variables were expressed as frequency and percentage. Kaplan-Meier method was used to evaluate PFS and DoR, and Cox proportional hazards model was used to estimate hazard ratio (HR) and 95% confidence interval (CI). PFS was analyzed in different subgroups according to age (≤65 years, >65 years), sex (male, female), metastatic sites (with or without brain metastasis, with or without liver metastasis), modified neutrophil to lymphocyte ratio (dNLR, ≤3, >3), serum sodium (<135, ≥135), serum lactate dehydrogenase (LDH, ≤260, >260), the absolute value of CD4+ cells (≤600/μl, >600/μl), the absolute value of CD8+ cells (≤450/μl, >450/μl), CD4+/CD8+ (1-2, <1 or >2) and the occurrence of irAEs (with or without irAEs).The HR and 95%CI of subgroups were calculated using the non-stratified Cox risk ratio model, and the association between different subgroups and median PFS was calculated. $P < 0.05$ was considered statistically significant.

Results

Patient baseline

From March 2020 to December 2021, 19 ES-SCLC patients were enrolled in this study and received study medications. Of them, the median age was 63 (range 51-70) years old, and 17 (89.5%) patients were male. Most patients (89.5%) were stage IV. Two patients had liver metastasis and one patient experienced brain metastasis (Table 1).

In addition, a total of 54 ES-SCLC patients received ICIs combined with chemotherapy as the first-line treatment were retrospective reviewed form January 2013 to December 2021, of which 34 patients treated with EP/EC combined with a PD-L1 inhibitor (atezolizumab or durvalumab) were selected as the historical control group. The median age of these patients was 64 (range 42-77) years old and 27 (79.4%) patients were male. Two patients had an ECOG PS score of 2 or above. The majority (97.1%) were stage IV. Six (17.6%) and three (8.8%) patients had liver metastasis and brain metastasis, respectively. There were no significant differences in the baseline characteristics of patients between two groups (Table 1; Figure S1).

Efficacy profiles

At the time of data cut-off (March 16, 2022), the median follow-up time was 12.1 months. Nine (47.4%) patients experienced PD and four (21.1%) died in the IP/IC plus camrelizumab group, with a median PFS of 10.25 (95% CI 9.40-NA) months. In the EP/EC plus PD-L1 group, 18 (52.9%) patients developed PD and six (17.6%) patients died, with a median PFS of 7.10 (95% CI 5.79-8.40) months. IP/IC plus camrelizumab showed better PFS than EP/EC plus PD-L1 as first-line treatment in patients with ES-SCLC (HR=0.58, 95% CI 0.42-0.81; P=0.0013) (Figure 1). The median OS was not achieved in both groups.

In the IP/IC plus camrelizumab group, 17 (89.5%) patients had PR, and two (10.5%) had SD, with a median DoR of 8.74 months. In the EP/EC plus PD-L1 inhibitor group, 28 (82.4%) patients had PR, four (11.8%) had SD, and two (5.9%) had PD. The median DoR was 4.4 months. The DCR and ORR were 100% and 89.6% in the IP/IC plus camrelizumab group, as well as 94.1% and 82.4% in the EP/EC plus PD-L1 inhibitor group, respectively (Table 2).

As of the last follow-up, 18 (53.9%) patients with EP/EC plus PD-L1 inhibitor and nine (47.4%) patients with IP/IC plus camrelizumab developed PD. Post-progression treatment was at the discretion of the investigator depending on the patient's condition. Of these patients with PD in the EP/EC plus PD-L1 inhibitor group, eight (23.5%) patients subsequently started receiving PD-L1 inhibitor plus other chemotherapy regimens, one (2.9%) patient received topotecan, one (2.9%) patient received albumin paclitaxel monotherapy, and eight (53.9%) patients started receiving IP/IC plus PD-1 inhibitor. In the IP/IC plus camrelizumab group, four (10.5%) patients started treatment with EP/EC plus PD-L1 inhibitor (atezolizumab/durvalumab) following progression, four (10.5%) patients began EP/EC treatment, and one patient (5.3%) received anlotinib monotherapy.

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TABLE 1 Baseline characteristics of patients.

Characteristics	IP/IC+camrelizumab (N=19)	EP/EC+PD-L1 (N=34)	P value
Age, median (range), years	63 (51–70)	64 (42-77)	0.392
Sex (male)	17 (89.5%)	27 (79.4%)	0.324
Smoking status			0.090
Never smoked	4 (21.2%)	14 (41.2%)	
Smoking index ≥400*	11 (52.6%)	17 (50%)	
ECOG PS score			0.725
≤1	19 (100%)	32 (94.1%)	
≥2	0	2 (5.9%)	
TNM staging			0.074
Stage IIIC	2 (10.5%)	3 (8.8%)	
Stage IV	17 (89.5%)	31 (91.2%)	
Liver metastasis	2 (10.5%)	6 (17.6%)	0.497
Brain metastasis	1 (5.3%)	3 (8.8%)	0.125
Bone metastasis	5 (26.3%)	14 (41.2%)	0.288
Adrenal metastases	2 (10.5%)	4 (11.8%)	0.894
Subcutaneous muscular metastasis	0	1 (2.9%)	0.460
Paraneoplastic syndrome	1 (5.3%)	5 (14.7%)	0.307
dNLR			0.285
≤3	13 (68.4%)	28 (82.4%)	
>3	6 (31.6%)	6 (17.6%)	
Serum sodium (Na)			0.093
≥135	18 (94.7%)	26 (76.5%)	
<135	1 (5.3%)	8 (23.5%)	
Serum LDH			0.613
≤260U/L	12 (63.2%)	19 (55.9%)	
>260U/L	7 (36.8%)	15 (44.1%)	
Absolute value of CD4+ T cells			0.789
≥600/μl	13 (68.4%)	22 (64.7%)	
<600/μl	6 (31.6%)	12 (35.3%)	
Absolute value of CD8+ T cells			0.955
≤450/μl	13 (68.4%)	23 (67.6%)	
>450/μl	6 (31.6%)	11 (32.4%)	
CD4+/CD8+			0.527
1.0~2.0	10 (52.6%)	21 (61.8%)	
<1 or >2	9 (47.4%)	13 (38.2%)	
Serum ProGRP			0.641
≤3 ULN	5 (26.3%)	7 (20.6%)	
>3 ULN	14 (73.7%)	27 (79.4%)	

(Continued)

TABLE 1 Continued

Characteristics	IP/IC+camrelizumab (N=19)	EP/EC+PD-L1 (N=34)	P value
Serum NSE			0.327
≤3 ULN	9 (47.4%)	21 (61.8%)	
>3 ULN	10 (52.6%)	13 (38.2%)	
Number of ICI cycles, median (range)	7 (3-26)	6 (2-17)	0.051
Number of chemotherapy cycles, median (range)	6 (3-6)	5 (2-6)	0.060

EP/EC, (etoposide plus cisplatin, or etoposide plus carboplatin); IP/IC, (irinotecan plus cisplatin, or irinotecan plus carboplatin); ECOG PS, Eastern Cooperative Oncology Group performance status; dNLR, modified neutrophil to lymphocyte ratio; LDH, lactate dehydrogenase; ProGRP, pro-gastrin releasing peptide; NSE, neuron-specific enolase; ULN, upper limit of normal; ICI, immune checkpoint inhibitor.
*The smoking index was calculated as cigarettes per day x duration of smoking (years).

Safety profiles

In the IP/IC plus camrelizumab group, the median cycle number was six for chemotherapy and seven for camrelizumab, respectively; while in the EP/EC plus PD-L1 inhibitor group, the median cycle number was five for chemotherapy and six for immunotherapy. The most common TRAE in the IP/IC plus camrelizumab group was neutropenia, followed by reactive cutaneous capillary endothelial proliferation (RCCEP) and diarrhea (Table 3). The irAEs in the IP/IC plus camrelizumab group included RCCEP (52.6%), abnormal liver function (21.1%), rash (10.5%), hypothyroidism (5.3%), oculomotor paralysis (5.3%) and kidney injury (5.3%) (Table 4).

Subgroup analysis

The IP/IC plus camrelizumab showed better PFS than EP/EC plus PD-L1 inhibitor among all subgroups, but the difference was not statistically significant (Figure S2). For all enrolled patients, 19 patients experienced irAEs. The median PFS in patients with irAEs was 14.5 months (95% CI 7.94-NA), which was significantly longer than that in patients without irAEs (14.5 months vs. 6.3 months, HR=4.64, 95% CI 1.92-11.18) (Figure 2).

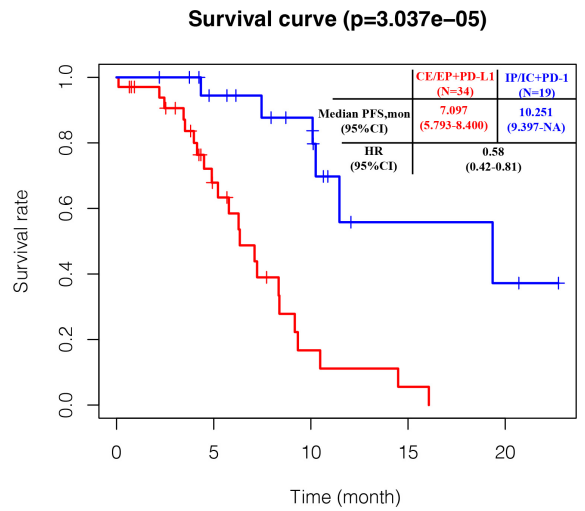

FIGURE 1
Kaplan-Meier curves of progression-free survival.

Discussion

In the past 30 years, platinum-based chemotherapy has been used as a first-line treatment for ES-SCLC patients (14, 15). Because SCLC has high tumor mutation burden, ICIs offers a new way to treat this disease and prolong patient survival. There is no doubt that PD-L1 inhibitor in combination with chemotherapy has become the standard first-line treatment for ES-SCLC patients. However, the survival benefit of ICI treatment on SCLC patients is less than that on NSCLC patients, and the OS of SCLC is still limited (4, 16). Therefore, exploring new combined treatment options is urgently needed. To our knowledge, this is the first study to report the efficacy and safety of IP/IC plus camrelizumab followed by maintenance camrelizumab plus apatinib as the first-line treatment in ES-SCLC. Compare with PD-L1 inhibitor plus EP/EC (the historical control), IP/IC plus camrelizumab had the comparable ORR (89.6% vs 82.4%), but achieved a better median PFS (10.25 months vs. 7.10 months, HR=0.58, 95% CI 0.42-0.81). The OS data was immature in both groups.

PD-1 inhibitors are able to block both PD-1/PD-L1 pathways as well as PD-1/PD-L2 pathways, whereas PD-L1 inhibitors only inhibit PD-1/PD-L1 (17). Therefore, when PD-L1 inhibitors are used, the tumor can escape through the PD-1/PD-L2 axis. Based on a meta-analysis, NSCLC patients treated with PD-1 inhibitor plus chemotherapy had a lower death risk compared to those treated with PD-L1 inhibitor plus chemotherapy (RR=0.66, 95% CI 0.48-0.90) (18). However, a different scenario was shown in untreated ES-SCLC. In the first-line treatment of ES-SCLC, IMpower133 and CASPAIN studies have demonstrated the survival benefit of PD-L1 inhibitor atezolizumab or durvalumab plus chemotherapy, while KEYNOTE-604 study of PD-1 inhibitor pembrolizumab plus chemotherapy only showed a PFS benefit but failed to show an OS benefit (5–8, 19). Nevertheless, was too early to draw a conclusion that that ES-SCLC would not benefit from the first-line therapy with PD-1 inhibitor (20). In our study, a superior PFS

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TABLE 2 Treatment efficacy.

Variables	IP/IC+camrelizumab (N=19)	EP/EC+PD-L1 (N=34)
PR	17 (89.5%)	28 (82.4%)
SD (≥6w)	2 (10.5%)	4 (11.8%)
PD	0	2 (5.9%)
DoR, months (95% CI)	8.74 (0.72-19.09)	4.4 (0.23-14.62)
Still response at the last follow-up	10 (52.6%)	14 (41.2%)
DCR	19 (100%)	32 (94.1%)
ORR	17 (89.6%)	28 (82.4%)
Median PFS, months (95% CI)	10.25 (9.40-NA)	7.10 (5.79-8.40)

EP/EC, (etoposide plus cisplatin, or etoposide plus carboplatin); IP/IC, (irinotecan plus cisplatin, or irinotecan plus carboplatin); PR, partial response; DoR, duration of response; SD, stable disease; PD, progression disease; DCR, disease control rate; ORR, objective response rate; PFS, progression-free survival.

was also observed in the IP/IC plus camrelizumab group compared with in the EP/EC plus PD-L1 inhibitor group, whereas OS data were not yet mature at the time of data cutoff. Considering that the chemotherapy regime and maintenance therapy were distinct from the above-mentioned studies, mature OS data from our study are worthy of anticipation.

Chemotherapy and radiotherapy have the ability to increase the immunogenicity of tumor cells, increase major histocompatibility class I (MHC-I) molecule expression, activate immune effector factors such as nature killer cells, and promote immune response by targeting tumor immunosuppressive cells (21–23). In addition, radiotherapy and chemotherapy can reduce the immunosuppressive properties of tumor

TABLE 3 Treatment-related adverse events.

TRAE	IP/IC+camrelizumab (N=19)		EP/EC+PD-L1 (N=34)	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Neutropenia	10 (52.6%)	7 (36.8%)	19 (55.9%)	9 (26.5%)
RCCEP	10 (52.6%)	0	0	0
Diarrhea	7 (36.8%)	1 (5.3%)	0	0
Nausea and vomiting	7 (36.8%)	0	11 (32.4%)	1 (2.9%)
Platelet count decreased	5 (26.3%)	2 (10.5%)	6 (17.6%)	1 (2.9%)
Hemoglobin decreased	5 (26.3%)	1 (5.3%)	10 (29.4%)	1 (2.9%)
Weakness	5 (26.3%)	0	9 (26.5%)	0
Abnormal liver function	3 (15.8%)	1 (5.3%)	0	3 (8.8%)
Alopecia	3 (15.8%)	0	3 (8.8%)	0
Rash	1 (5.3%)	1 (5.3%)	0	0
Hypothyroidism	1 (5.3%)	0	3 (8.8%)	0
Kidney injury	1 (5.3%)	0	0	0
Constipation	0	0	4 (11.8%)	0
Pneumonia	0	0	2 (5.9%)	0
Thrush	0	0	1 (2.9%)	0
Fever	0	0	1 (2.9%)	0
Myelitis	0	0	0	1 (2.9%)
Oculomotor paralysis	0	1 (5.3%)	0	0
Creatine kinase increased	0	0	1 (2.9%)	0

EP/EC, (etoposide plus cisplatin, or etoposide plus carboplatin); IP/IC, (irinotecan plus cisplatin, or irinotecan plus carboplatin); TRAE, treatment-related adverse event; RCCEP, reactive cutaneous capillary endothelial proliferation.

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TABLE 4 Immune-related adverse events.

irAE	IP/IC+camrelizumab (N=19)		EP/EC+PD-L1 (N=34)	
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
RCCEP	10 (52.6%)	0	0	0
Abnormal liver function	3 (15.8%)	1 (5.3%)	1 (2.9%)	2 (5.9%)
Rash	1 (5.3%)	1 (5.3%)	0	0
Oculomotor paralysis	0	1 (5.3%)	0	0
Kidney injury	1 (5.3%)	0	0	0
Hypothyroidism	1 (5.3%)	0	3 (8.8%)	0
Pneumonia	0	0	2 (5.9%)	0
Myelitis	0	0	0	1 (2.9%)
Creatine kinase increased	0	0	1 (2.9%)	0

EP/EC, (etoposide plus cisplatin, or etoposide plus carboplatin); IP/IC, (irinotecan plus cisplatin, or irinotecan plus carboplatin); irAE, immune-related adverse event; RCCEP, reactive cutaneous capillary endothelial proliferation.

cells, thereby stimulating T cell activation and resulting in tumor shrinkage (24). SCLC is a disease that is highly sensitive to chemotherapy and radiotherapy. The preferred chemotherapy regimen for initial treatment of patients with ES-SCLC is EP/EC, followed by IP/IC; whereas two meta-analyses of randomized controlled trials reported a favorable OS and less hematological toxicities in irinotecan/platinum regimens compared with etoposide/platinum regimens in this population (25, 26). Therefore, in contrast to the EP/EC regimen used as a combination with immunotherapy in the IMpower133 and CASPIAN studies (5, 8), we chose IP/IC regimen when combined with camrelizumab in this study.

Maintenance PD-L1 inhibitor was administrated in IMpower133 and CASPIAN studies and pembrolizumab plus chemotherapy was continued until disease progression in KEYNOTE-604 study, whereas in our study camrelizumab combined with apatinib was used for maintenance therapy. Preclinical data have shown that apatinib could relieve hypoxia, enhance CD8+ T cell infiltration, decrease the recruitment of tumor-associated macrophages in tumors and the amount of TGF-β in both tumors and serum, and generate synergistic antitumor effects with PD-L1 blockade in lung cancer (27). Moreover, the PASSION study evaluated the efficacy of camrelizumab combined with apatinib in the treatment of recurrent ES-SCLC, and showed promising efficacy and manageable safety profile (13). These suggested this combination may have promising therapeutic effects on untreated ESCLC;

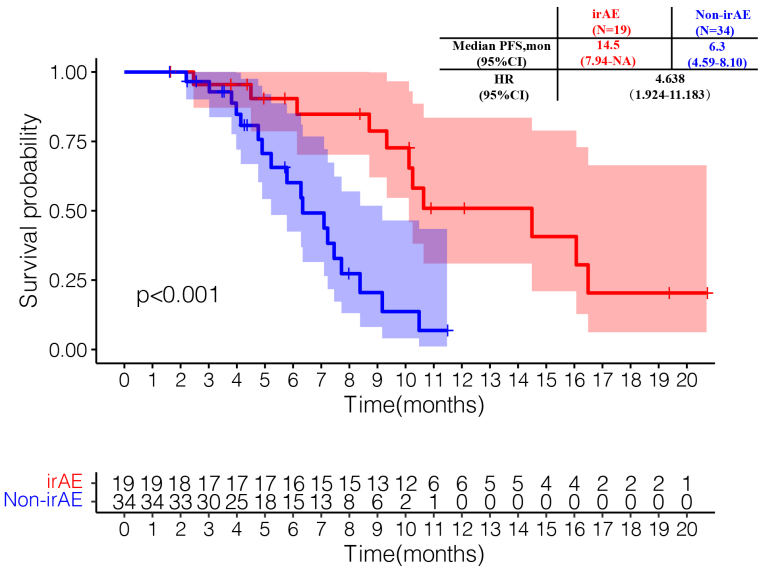

FIGURE 2
Kaplan-Meier curves of progression-free survival by immune-related adverse events (irAEs) in the irAE-evaluable population.

however, further studies are needed to elucidate its exact molecular mechanism.

The median number of chemotherapy cycles in our study was six for IP/IC and five for EP/EC, compared to the standard four cycles. Although the latest NCCN guideline for SCLC also recommends 4 cycles of treatment for untreated ES-disease, patients may receive up to 6 cycles based on response and tolerability after 4 cycles (28). Additionally, similar overall safety profiles and grade 3-4 TRAEs were observed in two groups. The most common grade 3 or higher TRAE in both groups was neutropenia, which was reversible by symptomatic treatment (such as with granulocyte-colony stimulating factor), and its incidence rate was higher with IP/IC plus camrelizumab than with EP/EC plus PD-L1 inhibitor. Neutropenia is often induced by chemotherapy. Previous retrospective study reported that the development of severe neutropenia with IP regimen might be associated with improved prognosis as opposed to EP therapy in patients with ES-SCLC (29). In addition, RCCEP and diarrhea were the two most common TRAEs of any grade in the IP/IC plus camrelizumab group, whereas none occurred in the EP/EC plus PD-L1 inhibitor group. Diarrhea was generally reversible with dose modification or antidiarrhea therapy. Given that the vast majority of diarrhea were grade 1-2, whether prophylactic loperamide is necessary deserves further exploration. RCCEP was observed in more than half of the IP/IC combined with camrelizumab group, which may be related to the activation of vascular endothelial cell proliferation by camrelizumab through regulating vascular receptor VEGFR2, leading to vascular proliferation (30, 31). No new safety signals were observed in this study.

Notably, the subgroup analysis showed that the occurrence of irAEs was associated with a longer median PFS, which was consistent with the results of SCLC with larger samples (32), NSCLC (33), malignant melanoma (34), and metastatic renal clear cell carcinoma (35). In 2020, a multicenter retrospective study showed that the occurrence of irAEs was an independent protective factor for ES-SCLC patients treated with ICIs (PFS: HR=0.44, 95% CI 0.29-0.66; OS: HR=0.47, 95%CI 0.32-0.71) (32). Besides, it was demonstrated that the development of irAEs was associated with survival outcome in patients with advanced or recurrent NSCLC treated with nivolumab (median PFS: 9.2 months vs. 4.4 months, P=0.04; median OS: NA vs. 11.1 months, P=0.01) (33). A retrospective study of 195 NSCLC patients treated with nivolumab found that the incidence of irAEs was 43.6%, and that the ORR (43.5% vs 10%, P<0.001) and median PFS (5.7 months vs 2.0 months, P<0.001) of patients with irAEs were significantly improved (36).

This study has some limitations. First, the control group was retrospectively reviewed with selective bias, so the results of this study cannot fully reflect the efficacy and safety of the EP/EC plus PD-L1 inhibitor in patients with ES-SCLC. Second, since this study was a single-center analysis, the results might be influenced by the level of diagnosis and treatment at our center. Third, the sample size was small, and finally only 53 patients with ES-SCLC were analyzed, resulting in insufficient statistical power of our study. Last, the data of OS was immature, and the patients' survival situation should be continuously tracked. Further large-scale randomized controlled trials are warranted to confirm our results.

Conclusion

In conclusion, our study demonstrated the preliminary efficacy and manageable safety of IP/IC plus camrelizumab followed by maintenance camrelizumab plus apatinib in untreated ES-SCLC, compared with EP/EC plus PD-L1 inhibitor. The occurrence of irAEs was associated with a longer PFS, which may be a potential prognostic factor for patients treated with ICIs.

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 Peking Union Medical College Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LZ and XZ contributed to the conception of the study. JN, XS and HW performed the experiment. JN contributed to analysis and manuscript preparation. JN performed the data analyses and 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1168879/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Baseline characteristics of patients by treatment pattern.

SUPPLEMENTARY FIGURE 2
Forest plot of subgroup analysis of progression-free survival.

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Enhancedanti-tumor efficacy through a combination of intramuscularly expressed DNA vaccine and plasmid-encoded PD-1 antibody

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Immune check inhibitors (ICIs) have moderate response rates (~20%–30%) in some malignancies clinically, and, when used in combination with other immunotherapeutic strategies such as DNA tumor vaccines, there is evidence to suggest that they could optimize the efficacy of cancer treatment. In this study, we validated that intramuscular injection of plasmid DNA (pDNA) encoding OVA combined with pDNA encoding α-PD-1 (abbreviated as α-PD-1 in the following treatment groups) may enhance therapeutic efficacy by means of *in situ* gene delivery and enhanced muscle-specific potent promoter. Mice treated with pDNA-OVA or pDNA-α-PD-1 alone showed weak tumor inhibition in the MC38-OVA-bearing model. In comparison, the combined treatment of pDNA-OVA and pDNA-α-PD-1 resulted in superior tumor growth inhibition and a significantly improved survival rate of over 60% on day 45. In the B16-F10-OVA metastasis model, the addition of the DNA vaccine enhanced resistance to tumor metastasis and increased the populations of $CD8^+$ T cells in blood and spleen. In conclusion, the current research shows that a combination of pDNA-encoded PD-1 antibody and DNA vaccine expressed *in vivo* is an efficient, safe, and economical strategy for tumor therapy.

KEYWORDS

DNA vaccine, immune checkpoint inhibitors, intramuscular gene delivery, pDNA, L/E/G

Introduction

An effective anti-tumor treatment requires not only the activation of immunity but also the inhibition of some inhibitory targets. Immunotherapy has attracted interest as a new method for cancer treatment. Blocking immune checkpoint molecules, including the CTLA4, PD-1, and PD-L1 molecules, was proposed as a highly effective anti-tumor treatment for most types of cancer (1, 2), with a 20%–50% success rate in different

clinical trials (3). However, immune checkpoint inhibitor (ICI) therapy is still ineffective in most cancers, with the majority of cancer patients receiving little benefit from it. Nevertheless, its combination with other treatment methods is considered to be good treatment protocol and could have a tangible curative effect (4, 5).

In the growing field of immunotherapy, because of ability to promote extensive immune activation, DNA vaccines have good prospects as a treatment method for the prevention and cure of many cancers (6). Nevertheless, DNA vaccines have not received enough attention in clinical applications because of insufficient immune response as far. However, the application of new technologies has increased the immunogenicity of DNA vaccines; for instance, the optimization of plasmids and the utilization of gene delivery and genetic adjuvants has been improved through *in vivo* electrotransfer (7). The combined use of immune checkpoint inhibitors and DNA vaccines has a solid theoretical foundation. The transfection of plasmid DNA (pDNA) *in vivo* enables specific tissues to successfully produce desired proteins; pDNA can then either encode antigens or therapeutic proteins and is therefore a promising prospect in the treatment of various diseases. Recently, a number of studies have used the *in vivo* transfection of plasmids to produce therapeutic antibodies for tumor therapy (8–10).

To enhance this gene delivery efficiency, using an effective transport technique is of the utmost importance, as the phospholipid bilayer renders it impossible for any polar molecule, including DNA, to shuttle freely through the cell membrane. Numerous strategies have been explored to enhance the efficiency of penetrating cell membranes. In recent decades, researchers have made many efforts to boost the immunogenicity of DNA vaccines and gene delivery efficiency in bids to increase their clinical application potential. These strategies include the use of electroporation (EP) (7), which aims to increase the permeability of cell membranes and thus allow polar molecules to pass through.

Skeletal muscle is extensively distributed throughout the human body, and is both large in surface are and thick with blood vessels. To ensure that genes can be expressed at high levels in skeletal muscle without affecting the normal function of different tissues, we screened and developed muscle-specific promoters named efficient muscle-specific (EMS) promoters. By way of EMS promoters, the pDNA carrying the target gene is transferred to patien's skeletal muscle cells. The skeletal muscle cells function as “factories” that produce therapeutic proteins/polypeptides that are then secreted from cells, identifying their targets, and transported to them through blood vessels (11). A new finding related to the use of electroporation-mediated DNA shuttling is that several neo-antigens have been shown to effectively induce the anti-tumor function of $CD8^+$ T cells in animals (12).

Here, we outline a method that utilizes DNA vectors as vaccine and immune checkpoint blockade (ICB) protein-encoding gene carriers. The pluronic L64 (L)/electroporation (E)/epigallocatechin gallate (G) (EGCG) (referred to as L/E/G) system was utilized to promote gene delivery efficiency. The aggregate of plasmid DNA encoding ovalbumin (OVA) (referred to as pOVA) vaccine with pVAX-α-PD-1 caused enormous tumor regression, which is often attributed to the elevated cytokine stage and proportion of $CD8^+$ T and $CD4^+$ T cells in tumors. We clearly demonstrate that this aggregate routine, via the L/E/G system, can prevent lung metastasis in B16F10-ovalbumin (OVA)-bearing mice models. In summary, we show that OVA vaccines with a modified delivery route can increase the efficacy of ICI, and that the plasmid encoding α-PD-1 provides a safer and more economical treatment choice for major cancer patients.

Materials and methods

Plasmids

The published complementary DNA (cDNA) sequences were used to construct the mouse PD-1 antibody (EP 1445 264 A1), and the PD-1 antibody sequence was cloned onto the pVAX1 vector. The OVA gene was cloned on the pcDNA3.1(+) and efficient muscle-specific plasmid (pEMS) to construct the DNA vaccine. The pEMS is a reconstructed plasmid backbone, replacing the cytomegalovirus (CMV) promoter of pcDNA3.1(+) with EMS (Patent CN 113106094 B). In accordance with the manufacturer's instructions, all plasmids were obtained using an Endofree Plasmid Extraction Kit (Cwbio, Jiangsu, China) and dissolved in phosphate-buffered saline (PBS). The purities and concentrations of plasmids were determined using agarose gel electrophoresis and spectrophotometry.

Cells

HEK293T, MC38, and B16F10 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, ThermoFisher, USA) containing 10% fetal bovine serum (FBS) (Sigma Aldrich, Taufkirchen, Germany) and 1% penicillin/streptomycin (Sigma Aldrich, Taufkirchen, Germany). MC38 cell line (MC38-OVA) and B16F10 cell line (B16F10-OVA) stably expressing ovalbumin were established by *Lentivirus* transfection. MC38-OVA and B16F10-OVA cells were cultured in the above-mentioned DMEM media and supplemented with 2 μg/mL puromycin at 37°C at a concentration of 5% CO_2 to sustain the expression of OVA. All cell lines were routinely tested and confirmed as being free of *Mycoplasma* contamination.

Mice

C57BL/6J 6- to 8-week-old male mice were purchased from Sipeifu Biotechnology Company (Beijing, China). The mice were fed in a pathogen free environment, and kept in a room with a light/dark cycle of 12 hours. All animal experiments were approved by the Biomedical Research Ethics Committee of West China Hospital in Sichuan Universit in accordance with the guidelines set out by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). MC38-OVA cells (5×10^5) resuspended in PBS were subcutaneously injected into C57BL/6 mice. In this experiment, the date of tumor inoculation was set as

day 0. The tumor volume was measured once every 3 days with an electronic digital caliper, which calculated the volume as follows: (width^2 × length)/2. Results were displayed with a Kaplan–Meier survival curve and tumor growth curve. The mice to which MC38-OVA tumors were attached were euthanized on the 14th day after tumor injection; after their death, tumor, blood, and spleen samples were collected for further analysis. The mice were euthanized when close to death or when their attached tumors were larger than 3,000 mm^3 in size. To establish the lung metastasis model, B16F10-OVA (1×10^6) cells resuspended in PBS were inoculated on C57BL/6 mice via the caudal vein. The remaining mice were euthanized on day 29, and the organs needed for the experiment were collected.

L/E/G system

Intramuscular gene delivery was administrated using the previously established L/E/G system (13). Briefly, L/E/G refers to a combined pluronic L64/electroporation/EGCG system. Following this system, pDNA was dissolved in EGCG (E4143, Sigma-Aldrich, Germany) working solution and incubated for 30 minutes at room temperature. Subsequently, the EGCG/pDNA complex was mixed with 0.4% (W/V) L64 (435449, Sigma-Aldrich, German) until L64 reached a concentration of 0.1%. Finally, the EGCG/pDNA/L64 complex was injected into the right anterior tibial muscle of the mice; 1 hour later, the mice were stimulated with an electrical pulse to promote gene transfer.

Immunoblotting analysis

Proteins in the supernatant that was collected from the transfected HEK293T cells were separated by 10% SDS-PAGE gel using 100 V for 2 hours and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skimmed milk (RM00014, ABclonal, China) for 1 hour at room temperature and then incubated with HRP goat anti-mouse lgG (H+L) secondary antibodies (31430, ThermoFisher, USA), which can bind the Fc region of α-PD-1 expressed protein in mice, at a 1:5,000 dilution overnight at 4°C. The next day, the membranes were washed three times with tris-buffered saline with 0.1% Tween® 20 detergent (TBST) and, subsequently, the protein bands were detected using enhanced chemiluminescence (ECL) with a ChemiDoc XRS machine and an image analyzer (Bio-Rad, USA).

Immunohistochemical analysis

Immunostaining of lymphocytes in excised tumor tissues was carried out in accordance with the method described in a previous study (14). The paraffin sections were probed with primary anti-CD4 antibody (14–0042–82, ThermoFisher, USA) and anti-CD8 antibodies (14–0081–82, ThermoFisher, USA) mAb.

Side effects study

Blood was collected and left to coagulate at room temperature for 30 minutes. The clotted blood was then centrifuged at 3,500 rpm for 15 minutes at room temperature. After the serum was collected and stored at –80°C, the frozen serum samples were shipped to Li lai Co. Ltd. (Chengdu, China) for aminotransferase (AST) and alanine aminotransferase (ALT) analysis. The weights of spleens, lungs, and tumors were measured on day 29.

Enzyme-linked immunospot

The IFN-γ Single-Color enzyme-linked immunospot (ELISpot) assay was carried out on the mice samples in accordance with the manufacturer's instructions (2210002, Dakewe Biotech, China). The procedure carried out was as follows: 1×10^5 fresh splenocytes were diluted in 100 μL of CTL-Test medium (Dakewe Biotech, China) and incubated overnight at 37°C on an anti-IFNγ-coated 96-well plate. For stimulation, 10 ng/μL of $TRP2_{180–188}$ peptide (SVYDFFVWL) (GenScript, Nanjing, China) was added to the plate and cultured for 2 days. A cell stimulation cocktail (CT317-PR2, Dakewe Biotech, China) was used as a positive control and PBS and a P815 irrelevant peptide (LPYLGWLVF) were used as negative controls. Finally, the spots were counted using an ELISpot reader system (Xingjianya, China).

ELISA

For the quantitative determination of cytokine levels in serum, the blood of mice was collected from the orbital veins, then serum was separated at room temperature and stored at -80°C. The concentrations of interleukin-2 (IL-2) (pg/mL) (RK00007, ABclonal, China), IL-4 (pg/mL) (RK00036, ABclonal, China), and IL-10 (pg/mL) (RK00016, ABclonal, China) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits.

Binding ELISA was conducted to verify the affinity of antibody in the supernatant from pVAX-α-PD-1-transfected cells. The procedure carried out was as follows: 96-well plates were pre-coated with 10 μg/mL of PD-1 protein (Sigma) overnight at 4°C. They were subsequently washed with 0.1% Tween 20/PBS and then blocked with 5% bovine serum albumin (BSA) in a humid chamber for 1 hour at room temperature. After blocking, plates were washed three times and incubated at room temperature with supernatant from cells for 1 hour. Next, plates were washed and incubated with HRP goat anti-mouse lgG (H+L) secondary antibody at a dilution of 1:10,000 for 1 hour. The optical density was measured at 450 nm with an automatic ELISA reader (Synergy H1 Microplate Reader, BioTek, USA).

Flow cytometry analysis

Peripheral blood and spleens were collected 14 days after tumor implantation. Spleens were pressed into single-cell through a 70 μm cell strainer (251200, Sorfa, China), followed by removal of the red blood cells with RBC lysis buffer (00430054, ThermoFisher, USA). Cells were incubated for 10 minutes on ice with anti-mouse CD16/32 antibody (14–0161–82, Thermo Fisher, USA) for Fc blocking. For $CD4^+$ and $CD8^+$ T-cell detection, cells were stained with eBioscience™ Fixable Viability Dye eFluor™ 450 (65–0863–14, Thermo Fisher, USA), anti-CD45 (63–0451–82, Super Bright™ 600 rat anti-mouse CD45, Thermo Fisher, USA), anti-CD3 (11–0031–82, FITC rat anti-mouse CD3, Thermo Fisher, USA), anti-CD4 (17–0042–82, APC rat anti-Mouse CD4, Thermo Fisher, USA), and anti-CD8 (45–0081–80, PerCP-Cyanine5.5 rat anti-mouse, Thermo Fisher, USA) antibodies for 30 minutes at 4°C. Blood samples were stained directly, then mixed with 1 mL of RBC lysis buffer and incubated at 37°C for 5 minutes so that red blood cells could be lysed. Sample data were acquired with LSRFortessa (BD bioscience, NJ, USA) and analyzed with FlowJo software (FlowJo, Ashland, OR, USA).

Statistical analysis

Statistical analyses were carried out using GraphPad Prism software for Windows. For all experiments, statistical significance was determined using one-way analysis of variance (ANOVA) or a Student's *t*-test. The survival rate of mice was calculated using the log-rank test. In all cases, differences between groups were considered significant when the *p*-value was < 0.05 ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$); in some cases there was no significant difference (NS).

Results

pVAX-α-PD-1-Fc can express antibodies with mouse PD-binding activity *in vitro*

A DNA molecule capable of directing *in vivo* antibody production was designed by creating a cassette consisting of the single-chain variable fragment of anti-mouse PD-1(J43 clone) and the mouse Fc region with a D265A point mutation which leads to the total loss of cytolytic function and cloned onto pVAX plasmid (Figures 1A, B). To ensure that the plasmid production of PD-1 antibody, human 293T cells were transfected with pVAX and pVAX-α-PD-1-Fc. In the culture media of cells transfected with pVAX-α-PD-1-Fc, western blot assay under non-reducing conditions detected secretion of 120-KDa protein (Figure 1C). The binding affinity of α-PD-1 to the corresponding mouse antigen PD-1 was detected using an ELISA assay. The ELISA assay showed that α-PD-1 from cell culture media bound to PD-1 protein in a dose-dependent manner and that it had a significantly

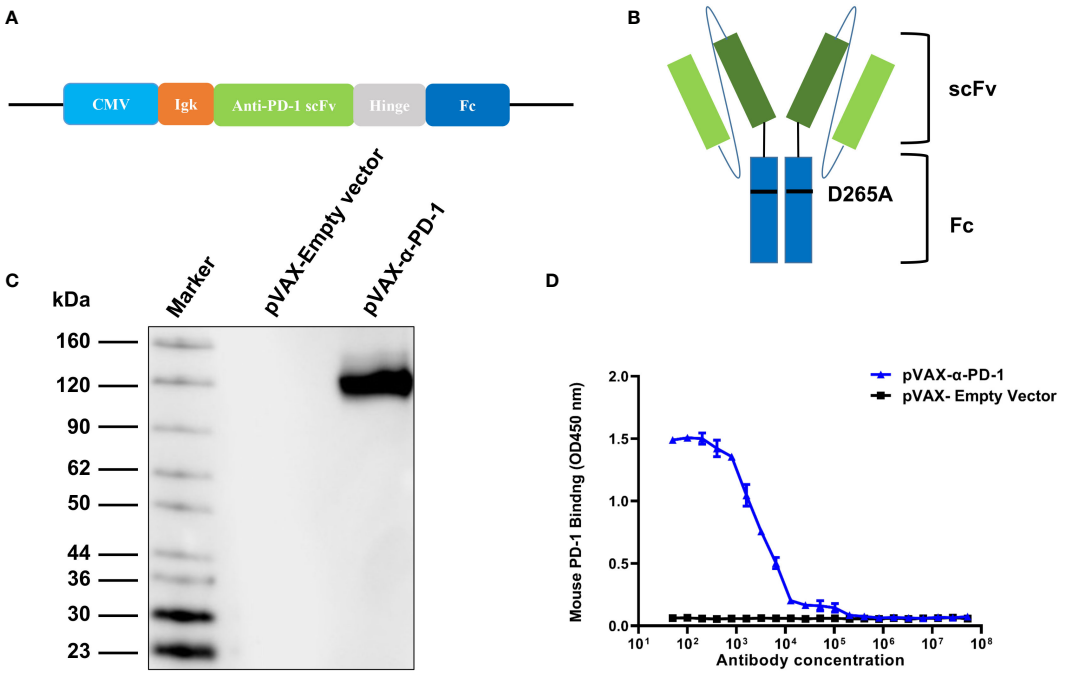

FIGURE 1
Expression and binding of PD-1 antibody. **(A)** Schematic of DNA construct encoding PD-1 antibody. The α- PD-1-Fc gene was cloned onto the pVAX vector and driven by the CMV promoter. **(B)** Structure of PD-1 antibody. α-PD-1-Fc contained scFv of mouse PD-1 antibody and the Fc region of mouse IgG1 with a D265A mutation. **(C)** Western blot analysis of mouse IgG from supernatants of pVAX-α-PD-1-Fc transfected 293T under non-reduced conditions. The pVAX-empty vector served as a control. Numbers indicate molecular weight. **(D)** The binding ELISA of α-PD-1-Fc from cell culture supernatants with mouse PD-1 antibodies. Results are representative means ± SDs of three independent experiments.

higher binding capacity than the control group (Figure 1D). The results of the assay also demonstrated that pVAX-α-PD-1 was capable of encoding functional mouse PD-1 antibodies.

pEMS-OVA vaccine combined with PD-1 antibody expressed by plasmid delivery intramuscularly *in vivo* increased the anti-tumor activity in MC38-OVA model

We examined the tumor inhibition effect induced by the pCMV/EMS-OVA DNA vaccine combined with pVAX-α-PD-1 on the tumor progression in MC38-OVA-bearing mice. The mice were divided into three groups according to treatment type, i.e., the pCMV-empty vector, pCMV-OVA, and pEMS-OVA groups. These groups were evenly divided into two groups after tumor formation, and they were treated with pVAX-Ctrl or pVAX-α-PD-1, respectively. DNA vaccine injections, tumor inoculation, and pVAX-α-PD-1 injections were administered on different days (Figure 2A), and we examined the effects of these different vaccination pathways on tumor sizes. The pCMV-OVA vaccination showed little tumor inhibition effect, and the pEMS-OVA vaccination caused the greatest delay in tumor growth (Figure 2B). All mice that received the pVAX-α-PD-1 treatment displayed slower tumor growth than those administered with DNA-only vaccine treatment (Figures 2C–E). In particular, mice that were in the pEMS-OVA combined with pVAX-α-PD-1 treatment group showed significant tumor regression (Figure 2F). For safety reasons, we monitored the mice for changes in body weight after tumor inoculation. All mice, irrespective of group, displayed stable weight gain (Figure 2G), and this preliminarily finding indicated that these treatments were safe enough. However, mouse survival rate varied significantly across the groups, and all mice in the pCMV-empty vector combined with pVAX-Ctrl treatment group (EV) died within 23 days of tumor inoculation. The pEMS-OVA vaccine combined with pVAX-α-PD-1 treatment group (pEMS-OVA + p-α-PD-1) showed superior results, with about 60% of the mice surviving for more than 45 days. By contrast, approximately 40% of the mice in the pCMV OVA vaccine combined with pVAX-α-PD-1 treatment group (pCMV-OVA + p-α-PD-1) survived for more than 45 days and approximately 20% of the mice in the pVAX-α-PD-1 single treatment group (EV + p-α-PD-1) survived for more than 45 days (Figure 2H). The results above demonstrate that the pEMS-OVA vaccine combined with pVAX-α-PD-1 treatment provided the greatest therapeutic benefit.

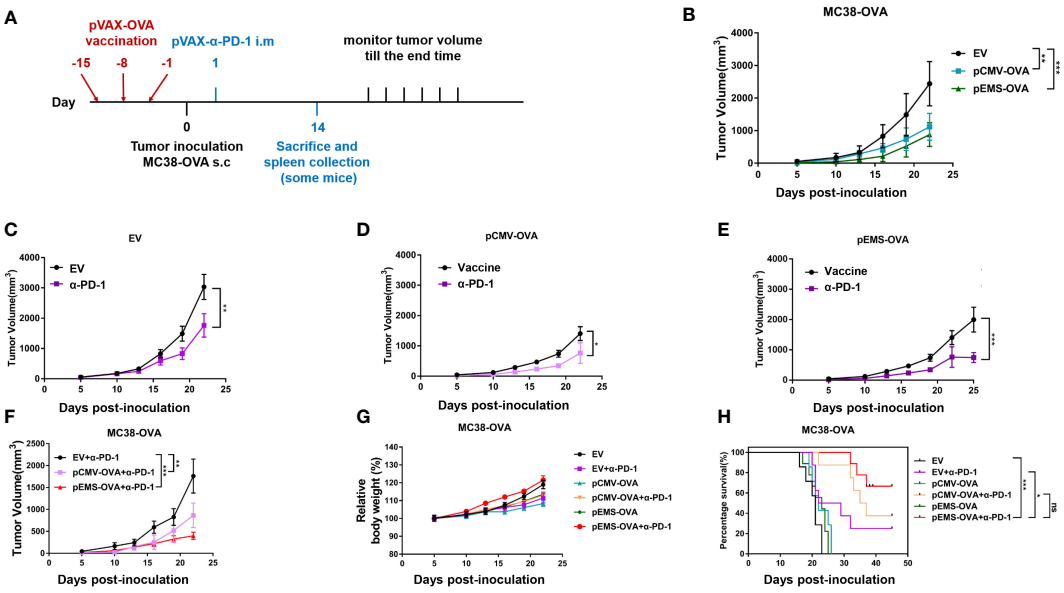

FIGURE 2
Anti-tumor activity of DNA vaccination and intramuscular gene transfer of PD-1 antibody combination in MC38-OVA model. **(A)** Schematic protocol of the tumor inoculation, DNA vaccination and pVAX-α-PD-1-Fc injection, and tumor measurement in MC38-OVA model. The DNA vaccines (pCMV-OVA or pEMS-OVA) were pre-intramuscularly electroporated 15 days, 8 days, and 1 day before 2×10^5 MC38-OVA tumor cells were subcutaneously injected into the right flanks of C57BL/6 mice. pVAX-α-PD-1-Fc were intramuscularly electroporated on day 1. Thereafter, the tumor volume was monitored every 3 days until mice were sacrificed. **(B)** Tumor growth curves of three different vaccine groups. The tumor growth curves of the empty vector **(C)**, pCMV-OVA **(D)**, and pEMS-OVA **(E)** DNA vaccination combined with pVAX-α-PD-1-Fc groups. **(F)** Tumor growth curves of three kinds in the pVAX-α-PD-1-Fc group. Relative body weight change **(G)** and percentage of survival curve **(H)** of mice receiving six different treatments. The error bars represent the mean ± SEM; $n = 6$ per group. Statistical analysis was carried out using one-way ANOVA with Dunnett's multiple comparisons test or a Student's *t*-test. The log-rank (Mantel-Cox) test was used to calculate the significance of survival (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). ANOVA, analysis of variance.

DNA vaccination in combination with PD-1 antibody expressed by intramuscular plasmid transfer enhanced the immune response of MC38-OVA-bearing mice

To study the activation levels of positive response in tumor microenvironment (TME) brought about by a combination of vaccines and pVAX-α-PD-1 therapy, the concentrations of IL-2, IL-4, and IL-10 cytokines in the serum were determined. The concentrations of IL-2, IL-4, and IL-10 were at very low levels in the vaccine-only treatment groups (i.e., the pCMV-empty vector, pCMV-OVA and pEMS-OVA groups). However, the concentration of IL-2 cytokines was slightly increased in the pCMV-empty combined with pVAX-α-PD-1 treatment group. Meanwhile, the concentrations of IL-2, IL-4, and IL-10 cytokines were significantly increased in the serum after the DNA vaccine combined with pVAX-α-PD-1 treatment, indicating that responses that were not triggered by DNA vaccine had been elicited (Figure 3A). We further evaluated the capacity of the DNA vaccine combined pVAX-α-PD-1 treatment to elicit a particular IFN-γ response by ELISpot. Without the vaccine, very few spots were detected, but the splenocytes from the DNA vaccine-treated mice showed a significant increase in the number of spots. In addition, the stimulation of immune cells with the DNA vaccine combined with pVAX–α-PD-1 treatment resulted in a significantly increased number of spots being observed (Figures 3B, C). To investigate whether the EMS promoter is more beneficial for immune activation after intramuscular injection of plasmids. In the next experiments, the mice were limited to three main combination groups that revealed significant efficacy after treatment. Fifteen days after tumor induction, MC38-OVA-bearing mice were euthanized and tumors were excised. The levels of $CD8^+$ T and $CD4^+$ T cells were higher in the tumor tissues of the therapeutic groups than those of the empty vector group (Figures 3D, E).

These results showed that the pEMS-OVA vaccine combined with pVAX-α-PD-1 treatment activated more antigen-specific lymphocyte cells than the pVAX-α-PD-1 treatment alone. In

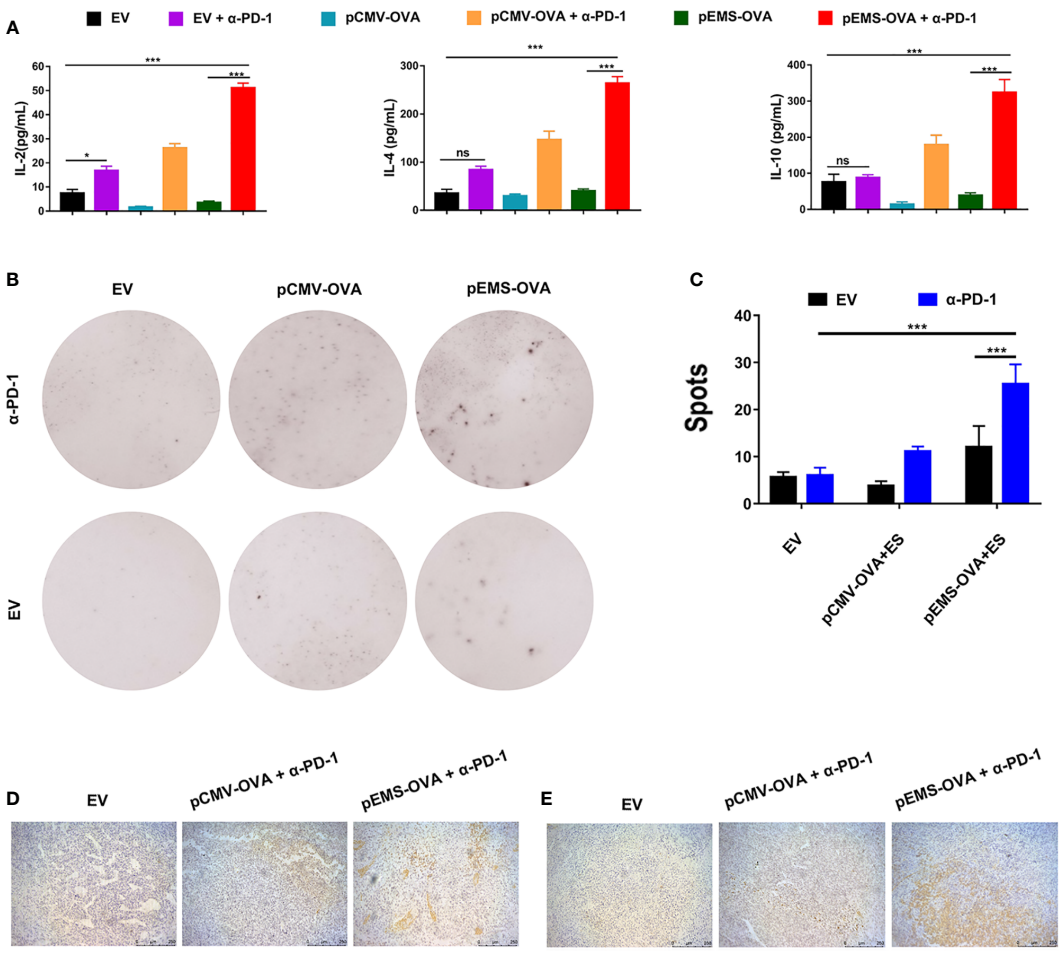

FIGURE 3
Immune response effect of DNA vaccination and intramuscular gene transfer of PD-1 antibody combination for MC38-OVA-bearing mice. **(A)** Levels of IL-2 (left), IL-4 (middle), and IL-10 (right) inflammatory mediators in the serum of mice receiving six different treatments were detected by ELISA on day 29. **(B)** Representative IFN-γ ELISPOT wells for splenocytes isolated from various six treatment groups. **(C)** Quantification of IFN-γ positive spots in the six treatment groups. Immunohistochemical analysis of $CD4^+$ **(D)** and $CD8^+$ **(E)** T-cell infiltration in MC38 tumors from mice treated with three kinds of vaccine combined pVAX-α-PD-1-Fc groups. Scale bars: 250 μm. Results are representative of three independent experiments and are expressed as the mean ± SD; $n = 5$ per group. One-way ANOVA was used to calculate the p-value, $*p < 0.05$, $***p < 0.001$. ANOVA, analysis of variance; ELISPOT, enzyme-linked immunospot.

addition, increased PD-1 expression in cytotoxic T lymphocyte (CTL) indicated T-cell exhaustion and the loss of effector function (15, 16), and led to tumor growth. These results indicate that the combined pEMS-OVA vaccine and pVAX-α-PD-1 treatment leads to the increased recruitment of CTL into tumors, and thus has a significant anti-tumor effect.

DNA vaccinations potentiated the inhibition of melanoma pulmonary metastases by intramuscular pDNA-based PD-antibody

Because cancer cells can spread from primitive tumors to different parts of the body even in the early stages, the inhibition of metastasis is an essential attribute of cancer vaccines. We tried to determine if pOVA vaccines (i.e., pCMV-OVA and pEMS-OVA) could enhance the inhibitory effect of intramuscular pDNA-based PD-1 antibody using a poorly immunogenic B16-F10 lung metastases model. We used the same treatment protocol as for the MC38-OVA model (Figure 4A). The weakest inhibitory effect on lung metastasis was observed in the empty vector treatment group. We also discovered the importance of the L/E/G system to vaccination, as we observed that, as in the empty vector group, there was weak suppression of lung metastasis in the DNA vaccination without L/E/G (pCMV-OVA-None + p-α-PD-1) treatment group. The pCMV-OVA combined with pVAX-α-PD-1 (pCMV-OVA + p-α-PD-1) therapy strongly suppressed the lung metastasis of tumors, which was consistent with findings in previous studies (17–19). The effect of α-PD-1 therapy is mainly dependent on innate immunity and the infiltration of lymphocytes in tumors (20, 21). We observed that pEMS-OVA combined with pVAX-α-PD-1 (pEMS-OVA + p-α-PD-1) therapy further prevents the formation of pulmonary nodules (Figures 4B, C).

In excised lungs, a trend for increased lung weight was observed in the DNA vaccination without L/E/G (pCMV-OVA-None + p-α-PD-1) treatment group, although there was no statistical difference in comparison to the other groups (Figure 4D). Similarly, the hemoxylin and eosin (H&E) staining of lung tissues identified intensive metastases in control mice and mice in the DNA vaccination without L/E/G treatment group, whereas there were notably small numbers of nodules detected on the lung sections of mice in the pCMV-OVA combined with pVAX-α-PD-1 and the pEMS-OVA combined with pVAX-α-PD-1 treatment groups. Remarkably, the combination of pVAX-α-PD-1 and pEMS-OVA vaccine therapy brought about almost normal lung histopathology (Figure 5), and this efficacy may be attributed to increased $CD8^+$ T-cell ratios in the blood and spleen (Figures 6B, D). There was no demonstrable difference, however, in the proportion of $CD4^+$ T cells, both in the blood and spleen, across the treatment groups (Figures 6A, C). However, in the pEMS-OVA combined with pVAX-α-PD-1 treatment group, the proportion of $CD8^+$ T cells was increased by up to 45%, and the proportion in the spleen was increased by up to 27%. The proportion of $CD8^+$ T cells in the spleen and peripheral blood were also significantly elevated (Figures 6B, D).

Finally, we evaluated the safety of the treatments. The results showed that there was no increase in lung water content or splenomegaly after any treatment (Figures 4D, 6E). Moreover, mouse body weight increased slightly during the treatment course

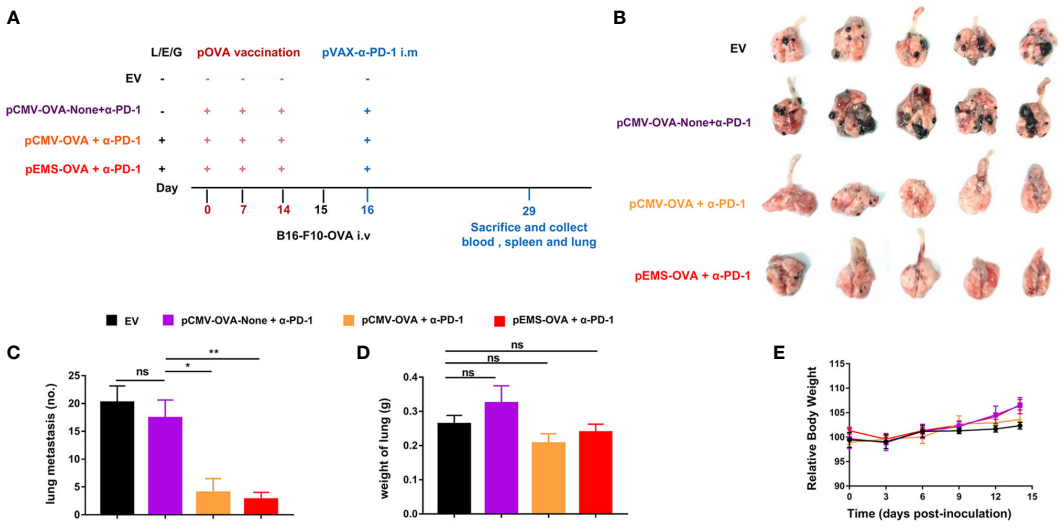

FIGURE 4
Lung metastasis inhibition of combination DNA vaccination and intramuscular gene transfer of PD-1 antibody in B16F10-OVA model. **(A)** Schematic protocol of the B16F10-OVA inoculation and combination therapy of DNA vaccination with pVAX-α-PD-1-Fc. C57BL/6 mice were divided into four groups: the combination of pOVA vaccine with α-PD-1 antibody (pCMV-OVA + α-PD-1 and pEMS-OVA + α-PD-1), the combination of vaccine without L/E/G and α-PD-1 (pCMV-OVA-none + α-PD-1) and no vaccine (empty vector). *N* = 5 per group. **(B)** Images of lung metastasis taken 2 weeks after B16F10-OVA inoculation. Number of metastases nodules on the surface of lung **(C)** and the wet weight of lungs **(D)** of the four groups. **(E)** Relative body weight change of mice receiving four different treatments. The data **(C–E)** represent the means ± SDs and were subjected to a one-way ANOVA; "NS" indicates no significant difference. $*p < 0.05$; $**p < 0.01$. ANOVA, analysis of variance; pluronic L64 (L)/electroporation **(E)**/epigallocatechin gallate **(G)** (EGCG) (referred to as L/E/G).

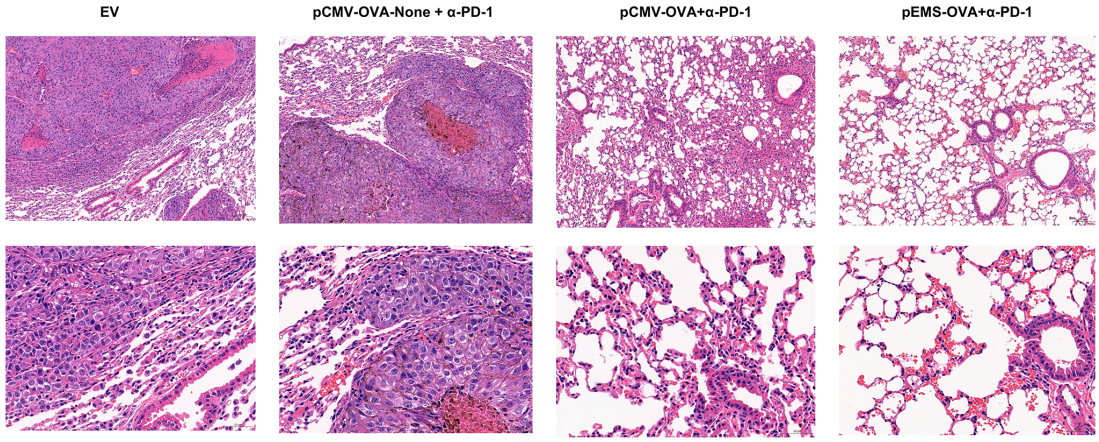

FIGURE 5
H&E staining of lungs collected from the treated B16F10-OVA-bearing mice. Representative H&E staining images of lungs isolated from B16F10-OVA-bearing mice following treatment with a combination of different DNA vaccination and intramuscular gene transfer of PD-1 antibody. H& E staining, hematoxylin and eosin staining.

(Figure 4E). No significant difference was detected in ALT levels between the four groups (Figure 6F). However, AST levels were increased in the serum of the pCMV-OVA-none combined with pVAX-α-PD-1 treatment group when compared with the empty vector group, and this may be related to the malignant metastasis activity of the tumors. However, in the sera of the two vaccines with combined PD-1 antibody treatment groups, we observed decreased AST levels when compared with the empty vector group, but these differences were not significant (Figure 6G).

Overall, our data indicate that the pEMS-OVA combined with pVAX-α-PD-1 treatment had no apparent serious side effects and had enhanced anti-tumor effects. Potential mechanisms for

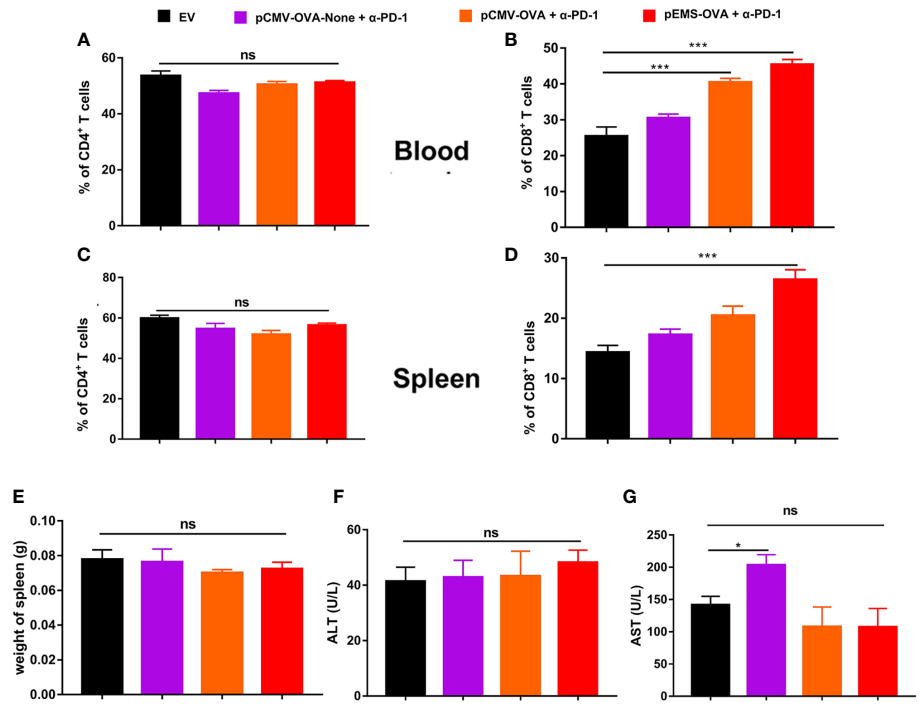

FIGURE 6
Immune cell populations and biochemical analysis of combination DNA vaccination and intramuscular gene transfer of PD-1 antibody in B16F10-OVA model. All mice were sacrificed at day 29. Immune cell populations analysis of spleen and blood from mice receiving four different treatments. **(A)** Percentage of $CD4^+$ T cells in the blood. **(B)** Percentage of $CD8^+$ T cells in the blood. **(C)** Percentage of $CD4^+$ T cells in the spleen. **(D)** Percentage of $CD8^+$ T cells in the spleen. **(E)** Spleen weight. **(F)** ALT level in the serum of mice. **(G)** AST level. Data are shown as means ± SDs. Statistical analyses were performed using one-way ANOVA with Dunnett's multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ANOVA, analysis of variance; AST, aminotransferase; ALT, alanine aminotransferase. .

synergistic effects of DNA vaccine may be attributed to the vaccine's ability to boost $CD8^+$ T cells, the exhaustion of which may have been prevented by the inhibition activity of PD-1 antibodies, expressed *in vivo*.

Discussion

Immune checkpoint inhibitors, notably antibodies targeting PD-1 and CTLA4, have revolutionized the treatment strategies for many cancers, especially advanced melanoma, for which, currently, nearly 50% of tumors in patients can either regress or be controlled for long periods, compared to the historical figure of less than 10% (22). But achieving this ideal response with this treatment strategy is still difficult for many tumors. Recently, antibodies targeting PD-1, including pembrolizumab and nivolumab, have been approved as a second-line treatment plan for HCC patients. However, their objective response rates (ORR), being 20% and 16.9% for nivolumab and pembrolizumab, respectively, are not satisfactory (23, 24). This indicates that there are numerous patients with limited response to immunomonotherapy, and this has promoted the exploration of new tumor treatment strategies that may improve the effectiveness of immunotherapy for these unresponsive tumors.

Although satisfactory results have been obtained in preclinical models, the low immunogenicity of DNA vaccines and the adverse reactions of many patients to the blocking of immune checkpoints still limit their application in clinical environments. Previous studies have demonstrated that the intramuscular delivery of DNA is a promising therapeutic method. Because of its large therapeutic area, its accessibility, and the presence of many antigen-presenting cells within it, skeletal muscle was selected as an appropriate target, especially for the delivery of DNA plasmids encoding a variety of tumor antigens or immune checkpoint inhibitory antibodies. To make gene transfer by this means efficient, a variety of measures have been exploited to increase transfection efficiency (25).

Gene electrotransfer is a mature non-viral delivery strategy that has been used to deliver naked DNA or RNA to various types of tissue. As a commonly used delivery system, lipid nanoparticles (LNPs) provide support for the delivery and site-specific release of mRNA and DNA (26). An mRNA vaccine prepared with LNPs as carriers can therefore better resist the degradation of nuclease, making intravenous injection and other delivery routes possible. However, the clinical application potential of cationic liposomes is limited by their toxicity, non-specific immunogenicity, circulatory instability, and rapid clearance *in vivo* (26, 27). If LNPs are to be used more widely in clinical settings, more in-depth research is needed to solve the problems of biological distribution, insufficient immune cell recruitment, and clearance *in vivo* in target tissues (28, 29). mRNA is rapidly translated in the cytoplasm. The expression of therapeutic antibodies based on delivered RNA is more transient compared with that based on DNA or viral vector-mediated gene transfer (30). Although viral vectors can promote high levels of expression in the long term, it is difficult to repeatedly administer them and they may produce genetic markers in patients, and more attention ought be directed toward potential concerns with the safety of their use (31).

The L/E/G system has not been previously applied in the field of vaccine preparation. Under normal conditions, its components cause an immune reaction at the injection site and recruit cells such as macrophages, so it may be particularly suited to the production of vaccines. In this study, we used the L/E/G system to improve the transfer efficiency of plasmids. It can help plasmids to enter the nucleus efficiently. Traditional DNA vaccines must first be successfully transcribed in the nucleus before entering the cytoplasm to start the expression of antigen proteins. The nucleus's multi-layer barrier makes it difficult for DNA to enter the reaction site and therefore makes immune activation difficult. In this study, the L/E/G system could disturb the cell membrane and help plasmids to enter the muscle cells. In addition, it could deform pDNA so that it had a suitable architecture with an appropriate surface charge, prevent the degradation of nucleic acids, and improve delivery efficiency, thereby increasing the expression levels of exogenous genes in skeletal muscle cells (13). Using EMS promoter, OVA can be efficiently expressed in muscle, which is conducive to enhancing immune activation, thereby overcoming the obstacle of immune activation. Compared with the pathological alteration caused by the accumulation of lipids in liver of LNPs, L/E/G/does not cause pathological changes to main organs. Although the L/E/G system used here cannot overcome the relevant shortcomings of LNP, we still showed that it represents an available, economical, and efficient alternative to existing methods of gene delivery.

In this study, we explored the use DNA vaccine or plasmid encoded antibodies and the combined treatment of α-PD-1 with DNA vaccines through the L/E/G system, and found that the pEMS-OVA DNA vaccine can not only can prevent tumor attacks from occuring in the body, but also cooperate with pVAX-α-PD-1 plasmid to suppress tumors.

Based on studies related to neoantigen vaccination and immunomodulatory therapy, this study selected MC38 to construct a colon tumor model. It has been established that MC38 cells trigger spontaneous $CD8^+$ T cell-induced immune responses in tumor-bearing mice. However, because of their immunosuppressive microenvironment, these immune cells are inactive and cannot eradicate tumor cells completely (32). In the early stages of tumor progression, the treatment of tumor-bearing animals with immunomodulatory antibodies targeting PD-L1 or PD-1 triggers effector T-cell responses, which can induce tumor cell death (33). In this study, we revealed that pOVA vaccines, in combination with α-PD-1 expressed by pVAX-α-PD-1, can trigger tumor death in a $CD8^+$ T cell-dependent manner, and that treatment with α-PD-1 antibody alone moderately inhibited tumor growth.

In this study we also used a murine colon cancer model to determine what factors affected immune checkpoint inhibitor blockade, and, even though the vaccine vectors used were different, we observed that tumors were smaller in mice treated with pVAX-α-PD-1 than in those in the pVAX-empty vector group 15 days after tumor inoculation, which means that pVAX-α-PD-1 monotherapy can suppress tumor growth. In addition, tumor growth and the survival rate were substantially improved by pVAX-α-PD-1 treatment in the following experiment.

Furthermore, when we examined different methods of immunization, we discovered that the pEMS-OVA expressed vaccine was associated with the highest tumor-suppressing efficiency. Our most important finding, however, was that the pEMS-OVA combined with pVAX-α-PD-1 treatment ameliorated the survival rate and reduced tumor growth. This synergistic effect originated from the ability of DNA vaccines and ICBs to stimulate T cells to fight specific cancer antigens, thereby increasing T-cell activity and tumor infiltration. Therefore, ICB creates a good microenvironment for the activity of cancer DNA vaccines. The pVAX-α-PD-1 treatment was found to significantly increase the quantity of IFN-γ-secreting $CD8^+$ T cells. When used in combination, pOVA and pVAX-α-PD-1 not only increased levels of IL-2, IL-4, IL-10, and IFN-γ production but also demonstrably increased those of $CD8^+$ and $CD4^+$ T cells. IL-2 can not only adjust tolerance of the dysregulated immune system through regulatory T cells (Tregs) but also promote immune response through activating effector T cells and memory T cells (34). In early studies, IL-2 was co-administered with autologous lymphocytes for cancer treatment or for the amplification of patients' natural killer (NK) cell and effector T-cell populations (35, 36). The combination of immunostimulatory plasmid DNA encoding IL-2 and siRNA delivered by TT-LDCP NPs targeting PD-L1 in the treatment of hepatocellular carcinoma (HCC) has also been shown to significantly increase tumor infiltration and $CD8^+$ T-cell activation (37). The significant increase in the levels of IL-2 in the pEMS-OVA+α-PD-1 group may also indicate the up-regulated infiltration of $CD8^+$ T cells. IL-4 is a Th2 cytokine that has a wide range of biological effects, especially with regard to immune responses (38). In the treatment of B-ALL with CD19/CD22/CD3 trispecific antibody, IL-4 was up-regulated in the treatment group (39). Another study showed that Man-CTS-TCL NPs increased the concentration of IL-4 concentration compared with the control group (40). After OVA stimulation, IL-4 levels in the spleen cells of mice increased significantly (41). Earlier studies have mainly focused on the immunosuppressive role of IL-10, but recent studies have shown that IL-10 can also reprogram the metabolic profile of T cells and restore the function of depleted T cells to produce a response (42). The tumor-triggered amplification of Tregs is an obvious obstacle to immunotherapy. Strikingly, however, AV-IL-27 treatment has been shown to induce the rapid elimination of Tregs in lymphoid organs and peripheral blood. AAV-IL-27 therapy also leads to tumor regression without significant adverse events, partly because of its induction of IL-10 (43). Further research has shown that IL-10 promotes the preservation of the effector function of antitumor $CD8^+$ T cells (44). Interestingly, PD-1 blockade results in the compensatory release of IL-10 through tumor-infiltrating myeloid dendritic cells (45). Increased levels of IL-2/IL-4/IL-10 cytokine secretion after PD-1 antibody expressed by pVAX-α-PD-1 treatment were also observed in our study. The increased levels of IL-10 secretion in combination treatment groups may function through the the mechanism of compensatory IL-10 secretion by dendritic cells after PD-1 blockade. The production of cytokines is only one of many factors that affects tumor volume, so it is difficult to ensure that changes in tumor volumes are completely attributable to these observed trends. Immune activation starts to occur in the early phases of tumor development, especially when therapy combining DNA vaccines and ICBs is used.

In B16F10-OVA lung metastasis mice models, the combined use of ICBs and pOVA vaccines allowed us to reduce the doses of antibodies and induced a potentially more powerful immune response when compared with single therapy. The early stages of tumor development, that is up to 14 days after tumor injection, have been proven to be key to metastasis, and we therefore analyzed their role in the TME. The efficacy that suppresion of pulmonary metastases was benefit by an increase in the proportion of CD8. Consistent with these blood results, the combined use of ICBs and pOVA DNA vaccines can increase the percentage of $CD8^+$ T cells in the spleen. In addition, pVAX-α-PD-1 without L/E/G treatment failed to inhibit lung metastases, indicating that the L/E/G system established in our previous study is sufficient to promote target gene expression and subsequent effects (13).

It is now clear that if a balance can be established between immune activation and immunosuppression, it is possible to achieve an effective anti-tumor response (46, 47) Therefore, the disadvantages of DNA vaccine or ICBs injection alone can be overcome by combining multiple treatment methods (48). In the case of cell vaccines, numerous in-depth studies have been conducted on the combined use of ICB and cancer vaccines (49, 50). Cell vaccine studies using triple therapy found that it is significantly superior to any dual or single therapies (49, 51). Strong anti-tumor effects were observed after treatment with a combination of cell-based vaccines and inhibitors. These can be attributed to antigen-specific effects, the increased proliferation of $CD4^+$ T and $CD8^+$ T cells, the release of antigen-specific cytokines, and the up-regulation of pivotal signaling molecules crucial to T-cell function (49). In contrast, few preclinical studies have tested the efficacy of DNA vaccines combined with immune checkpoint inhibitors (52). Published studies have explored the combination of single or dual CTLA4/PD-1 inhibitors and MYB oncoprotein targeting DNA vaccines (53), SSX2 cancer antigen (54), PSMA prostate-specific antigen (55), and TRP-2 and gp100 melanoma-specific antigens (56). The common characteristic of these studies is that combination therapy had better anti-tumor effects than single therapy. Some previous studies have demonstrated the feasibility of combined treatment of vaccines with ICI (57–60). As we know, the ICBs used in the above studies are antibodies (52, 61, 62), the costs of which are high, and the families of most cancer patients therefore cannot consider them as a treatment option. pVAX-α-PD-1 in this study utilized muscle cells as the production base of therapeutic antibodies. Currently, our research does not allow for the regulated expression of genes *in vivo*, and exploring gene switch platforms that control functional gene expression *in vivo* to enable self-regulatory therapy related to disease progression may provide a more valuable complement to this strategy (63). In addition, we can adjust the dosage of antibodies by changing the number of plasmids injected. Whether or not the effectiveness of this combined strategy is equally applicable to other tumor vaccines requires further study. The success of this strategy in preclinical research could make it possible for cancer patients to use cheap and affordable antibody drugs.

Our study reports on a specific combined treatment method. Driven by the muscle-specific promoter EMS, therapeutic proteins were specifically expressed in skeletal muscle to improve the effect of the vaccine. These observations show that the PD-1 antibody produced by intramuscular plasmid injection can synergize with DNA vaccines, most probably because of the highly increased effector function by expanding the frequencies of tumor-specific T cells. This combined strategy could represent a simple, convenient, economical, and effective means of oncology treatment.

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 animal study was reviewed and approved by the West China Hospital of Sichuan University Biomedical Research Ethics Committee.

Author contributions

XL and YY performed the experiments, analyzed data, and wrote the manuscript. GW and ML were involved in study conception and design. XZ was involved in data analysis and contributed intellectually to the research. All authors contributed to manuscript editing. 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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Immunochemotherapy achieved a complete response for metastatic adenocarcinoma of unknown primary based on gene expression profiling: a case report and review of the literature

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Background: Cancer of unknown primary (CUP) is a malignant and aggressive tumor whose primary origin is still unknown despite thorough evaluation. CUP can be life-threatening with a median overall survival of less than 1 year based on empirical chemotherapy. Gene detection technology advances the driver gene detection of malignant tumors and the appropriate precise therapy. Immunotherapy has ushered in a new era in cancer therapy, changing the way advanced tumors, including CUP, are treated. Combined with comprehensive clinical and pathological investigations, molecular analysis of the original tissue and detection of potential driver mutations may provide therapeutic recommendations for CUP.

Case presentation: A 52-year-old female was admitted to hospital for dull abdominal pain, with peripancreatic lesions below the caudate lobe of the liver and posterior peritoneal lymph nodes enlargement. Conventional biopsy under endoscopic ultrasonography and laparoscopic biopsy both revealed poorly differentiated adenocarcinoma based on immunohistochemical series. To help identify tumor origin and molecular characteristics, 90-gene expression assay, tumor gene expression profiling with Next-generation sequencing (NGS) method and Immunohistochemical expression of PD-L1 were employed. Although no gastroesophageal lesions discovered by gastroenteroscopy, the 90-gene expression assay yielded a similarity score and prompted the most likely primary site was gastric/esophagus cancer. NGS revealed high TMB (19.3mutations/Mb) but no druggable driver genes identified. The Dako PD-L1 22C3 assay IHC assay for PD-L1 expression revealed a tumor proportion score (TPS) of 35%. Given the presence of negative predictive biomarkers for immunotherapy, including adenomatous polyposis coli (APC) c.646C>T mutation at exon 7 and Janus kinase 1(JAK1), the patient received immunochemotherapy instead of immunotherapy alone. She was successfully treated with nivolumab plus carboplatin and albumin-bound nanoparticle

paclitaxel for six cycles and nivolumab maintenance, which achieved a complete response (CR) maintained for 2 years without severe adverse events.

Conclusions: This case highlights the value of multidisciplinary diagnosis and individual precision treatment in CUP. Further investigation is needed as an individualized treatment approach combining immunotherapy and chemotherapy based on tumor molecular characteristics and immunotherapy predictors is expected to improve the outcome of CUP therapy.

KEYWORDS

cancer of unknown primary, immunochemotherapy, case report, gene expression profiling, metastatic adenocarcinoma

Introduction

Cancer of unknown primary (CUP) is a malignant tumor whose primary lesion remains unknown despite extensive examination. It has been histologically confirmed as metastatic, accounts for 2-5% of all diagnosed cancers worldwide and is characterized by early and aggressive metastases (1).

CUP can be life-threatening and has an extremely poor prognosis, with a median overall survival of less than 1 year (2). CUP was roughly divided into a good prognosis group and a poor prognosis group, of which the poor prognosis group accounted for about 80% of the total and the poor prognosis group was dominated by empirical paclitaxel and platinum-based chemotherapy (3).

Due to the heterogeneous presentation of CUP, it is difficult to adequately answer important questions involving immunohistochemical testing, biological characteristics, tissue-of-origin molecular profiling, and novel therapies through traditional prospective randomized trials. However, in combination with comprehensive clinical and pathological investigations, molecular analysis of the original tissue and detection of potential driver mutations may provide therapeutic recommendations for CUP (4). It is important to formulate the best individualized treatment strategy combining clinical and molecular biological characteristics to improve the treatment outcome of CUP patients.

Here we describe a case of unfavorable CUP with multimodality diagnostic procedures and successfully treated with nivolumab plus carboplatin and albumin-bound nanoparticle paclitaxel for six cycles and nivolumab maintenance. The patient has achieved complete response (CR) which maintained for 2 years.

Case presentation

In January 2021, a 52-year-old female was admitted to the Medical Oncology Department at Sir Run Run Shaw Hospital for dull abdominal pain. The patient was previously healthy, denying hypertension, diabetes and other diseases. Physical examination found obvious pain under xiphoid process, no tenderness or rebound pain in other parts. CT enhancement of the abdomen showed peripancreatic lesions below the caudate lobe of the liver. The enlarged lymph nodes in the posterior peritoneum were suspected of malignancy. Peripheral blood tumor markers indicated normal AFP, CA199, CEA, and CA125 levels. There was only a slight increase in CA72-4 at initial diagnosis, and the baseline result was 13.96 U/ml (reference, <6.90 U/ml). Gastroenteroscopy did not reveal gastrointestinal space occupying lesions. Further endoscopic ultrasound was performed to puncture the hypoechoic mass in the hepatopancreatic space and the pathology indicated poorly differentiated carcinoma (Figure 1D). Immunohistochemical results on hepatopancreatic lesions by endoscopic ultrasound biopsy were CK-pan (+), CK7 (partial+), CK20 (-), P63 (-), P40 (-), CgA (-) and Syn (-).

To help identify the primary lesion, the patient underwent a positron emission tomography-CT (PET-CT) examination (Figure 1). The results showed that there were soft tissue mass shadows in the hepatic hilar region and pancreas head, with a maximum cross section of 35 mm x 34 mm, accompanied by increased FDG metabolism and SUVmax of 17.5. The local boundary with the pancreas neck is not clear. It also found multiple retroperitoneal lymph node enlargements with increased FDG metabolism, with SUVmax of 21.4, and the larger one with a short diameter of about 18 mm. These changes were considered to be associated with malignancy, pancreatic origin, or at least involvement.

As the immunohistochemistry (IHC) results on hepatopancreatic lesions by endoscopic ultrasound biopsy were unable to determine the origin of this CUP case, this patient underwent MDT consultation and was recommended for minimally invasive laparoscopic surgical exploration and biopsy. General anesthesia with endotracheal intubation was performed in the supine position. After disinfection, an inverted L-shaped incision was made in the upper right abdomen, and the abdominal adhesion was separated layer by layer. There was slight adhesion in the abdominal cavity. The right paracolic sulci was opened, the right retroperitoneal space and hepatoduodenal ligament

Abbreviations: VAF, variant alle frequency; NGS, Next Generation Sequencing; GEP, gene expression profiling; TMB, tumor mutation burden; IHC, immunohistochemistry; APC, adenomatous polyposis coli; JAK1, janus kinase 1.

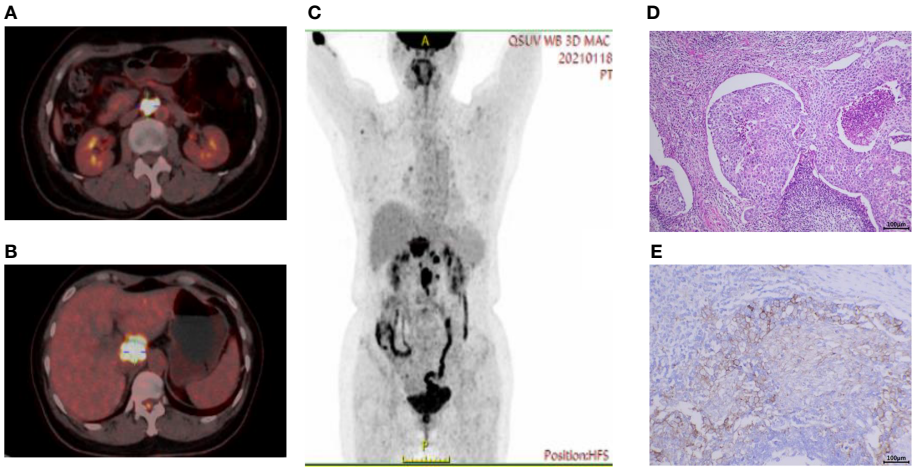


FIGURE 1
Baseline PET-CT imaging and pathologic results. **(A)** High uptake of fluorodeoxyglucose (FDG) in enlarged retroperitoneal lymph nodes and **(B)** peripancreatic lesions below the caudate lobe of the liver. **(C)** Coronal view showed multiple hypermetabolic lesions in the abdominal cavity. **(D)** Hematoxylin and eosin staining on hepatopancreatic lesions under endoscopic ultrasound showed poorly differentiated carcinoma. **(E)** The Dako PD-L1 22C3 assay immunohistochemistry assay for PD-L1 expression revealed that the tumor proportion score (TPS) was 35%. Scale bar = 100μm.

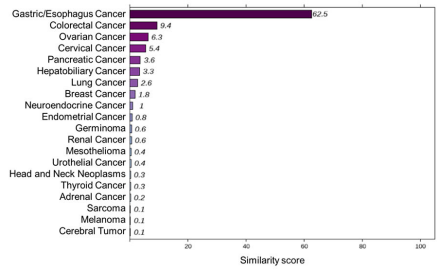
were separated, and masses in the portal vena cava space and beside the left renal vein, approximately 4cm x 4cm and 3cm x 2cm in size, were exposed. They were hard in texture and the lesion boundaries were unclear. No obvious abnormality was found in other organs of abdominal cavity. Pathological immunohistochemical series results based on surgical biopsies were positive for CK-pan, CK7, SALL4 and Villin. In addition, CK20, CK5/6, CgA, Syn, CD56, PAX-8, Calretinin, TTF-1, GATA-3, Oct-4 and AFP were negative. As summarized in Figure 2A, the final pathological diagnosis was a metastatic poorly differentiated carcinoma, combined with an immunohistochemical predisposition to digestive system origin.

Total RNA was isolated from FFPE tissue sections using an FFPE Total RNA Isolation Kit (Canhelp Genomics, Hangzhou, China). The 90-gene expression assay yielded a similarity score for each of the 20 tumor types based on formalin-fixed, paraffin-embedded cancer tissues. The top three predictions were gastric/esophagus cancer (62.5), colorectal cancer (9.4), and ovarian cancer (6.3), which indicated the most likely site was gastric/esophagus cancer (62.5, Figure 2B). The Dako PD-L1 22C3 assay immunohistochemistry assay for PD-L1 expression revealed a tumor proportion score of 35% (Figure 1E). NGS revealed MSS and high tumor mutation burden (TMB) as 19.3mutations/Mb.

A

IHC results on hepatopancreatic lesions by endoscopic ultrasound biopsy		IHC results on retroperitoneal mass by surgical biopsy		PD-L1 IHC (22C3 pharm Dx assay)
Positive	Negative	Positive	Negative	
CK-pan + CK7 partial+	CK20 P63 P40 Syn CgA	CK7+ SALL+ Villin +	CK20 PAX-8 Calretinin TTF-1 GATA-3 Oct-4 AFP	TPS =35% CPS=36

B



C

Gene	Exon	Nucleotide variation	Amino acid variation	VAF (%)
ABL1	exon11	c.2458C>T	p.R820W	8.12
ABL1	exon7	c.1100G>A	p.R367Q	7.40
ALK	exon15	c.2623G>A	p.G875R	7.61
APC	exon7	c.646C>T	p.R216*	4.10
ASXL1	exon12	c.1934del	p.G645Vfs*58	6.15
ATM	exon51	c.7543A>G	p.K2515E	5.56
BRCA1	exon17	c.5096G>A	p.R1699Q	8.83
BRD4	exon9	c.1687del	p.S563Afs*21	8.2
BRIP1	exon 7	c.632del	p.P211Lfs*63	12.45
CDKN2B	exon2	c.313C>T	p.R105W	6.24
EPHA5	exon 3	c.877G>A	p.A293T	3.92
ERF	exon 4	c.640C>A	p.L214M	6.27
FGFR3	exon 16	c.2066C>T	p.T689M	7.68
IL7R	exon 8	c.1322T>C	p.L441P	4.48
JAK1	exon 5	c.425del	p.K142Rfs*26	7.44
KDR	exon 16	c.2359C>T	p.R787W	8.69
KMT2C	exon 38	c.8390del	p.K2797Rfs*26	10.12
MSH6	exon 5	c.3209del	p.G1070Vfs*9	6.97
NOTCH2	exon 25	c.4055G>A	p.R1352K	2.81
PALB2	exon 4	c.839del	p.N280Tfs*8	7.52
PREX2	exon 10	c.1206del	p.K402Nfs*2	5.76
RNF43	exon 9	c.1975_1976del	p.G659Sfs*87	10.22
SLX4	exon 15	c.5497C>T	p.R1833W	7.01
SNCAIP	exon 6	c.1076G>T	p.R359M	2.44
STAT5A	exon 10	c.1102del	p.Q368Rfs*2	2.49
TP53	exon 7	c.733G>A	p.G245S	7.00
TP53BP1	exon 12	c.2145del	p.E716Nfs*12	9.32

FIGURE 2
Summary of histopathological and molecular pathological information. **(A)** Pathological and immunohistochemical (IHC) results. **(B)** 90-gene tumor tissue traceability expression assay results. The maximum similarity score for tumor tissue traceability was 100. **(C)** Gene expression profile of tissue specimens analyzed by NGS. VAF, variant alle frequency. NGS, Next Generation Sequencing.

Besides, hot spot exon regions and some intron regions of more than 600 genes related to tumorigenesis and cancer development were sequenced by Illumina high-throughput sequencing platform. No druggable driver genes were found. The summary results are listed in Figure 2C.

To understand the pathways and biological functions involved, we further analyzed gene ontology (GO) and KEGG pathways. Finally, we identified the top 9 enriched GO terms as shown in Supplementary Figure S1. These findings indicated that gene expression profiling (GEP) results from this CUP may involve cell cycle regulation, protein tyrosine kinase activity, and homologous recombination.

Given the presence of negative predictive biomarkers for immunotherapy, including adenomatous polyposis coli (APC) c.646C>T mutation at exon 7 and janus kinase 1 (JAK1) c.425del mutation at exon5, the patient is at risk of disease hyper-progression if given immunotherapy alone (Figure 2C). Therefore, she received nivolumab 200mg intravenously combined with albumin-bound nanoparticle paclitaxel 400mg intravenously and carboplatin 550mg intravenously, every 21 days, followed by regular nivolumab 200mg intravenously every 21 days for 2 years. Clinical and laboratory evaluations were required weekly during first treatment cycle then within 3days of each subsequent cycle. After six cycles of combination therapy, CT imaging indicated complete remission (Figure 3). Baseline elevated CA72-4 returned to normal after four cycles of treatment. With the addition of early supportive treatment, the patient's chemotherapy-related nausea and vomiting and myelosuppression were satisfactorily controlled. Only mild nausea, fatigue, and grade 1 anemia occurred during combination therapy. The patients were satisfied with the therapeutic effect and showed good compliance. There were no significant adverse events during nivolumab maintenance, and regular follow-up CT scans showed that baseline lesions maintained complete remission within 2 years, without any previously unappreciated gastroesophageal lesions found.

Discussion

Here we describe a case of unfavorable CUP with multimodality diagnostic procedures including tumor imaging and extensive review of pathology. With the help of tissue-origin-sequencing and gene expression profiling, the patient received the tailored combination strategy. After six cycles of nivolumab plus carboplatin and albumin-bound nanoparticle paclitaxel, she achieved complete response (CR) which maintained for 2 years during nivolumab maintenance.

One widely accepted definition of CUP is defined by the National Comprehensive Cancer Network (NCCN) as histologically proven metastatic malignant tumors whose primary site cannot be identified during pretreatment evaluation. Median survival was less than 1 year when empirical chemotherapy was used as the primary treatment for CUP. For CUP with poor prognosis, both the National Comprehensive Cancer Network (NCCN) and ESMO guidelines recommend platinum, taxanes, or gemcitabine-based empiric chemotherapy and participation in clinical trials. Objective response rate (ORR) of different chemotherapy regimens ranged from 10% to 50%, and median overall survival (OS) ranged from 4 to 13 months (5–7).

The development of molecular tumor profiling in CUP patients over the past decade has helped predict the location of tumor origin. Researchers have discovered genes and specific expression patterns related to the origin of tumor tissue (8, 9). Several novel assays of gene expression have been shown to be effective in predicting the primary source of a wide range of tumor types, supporting its

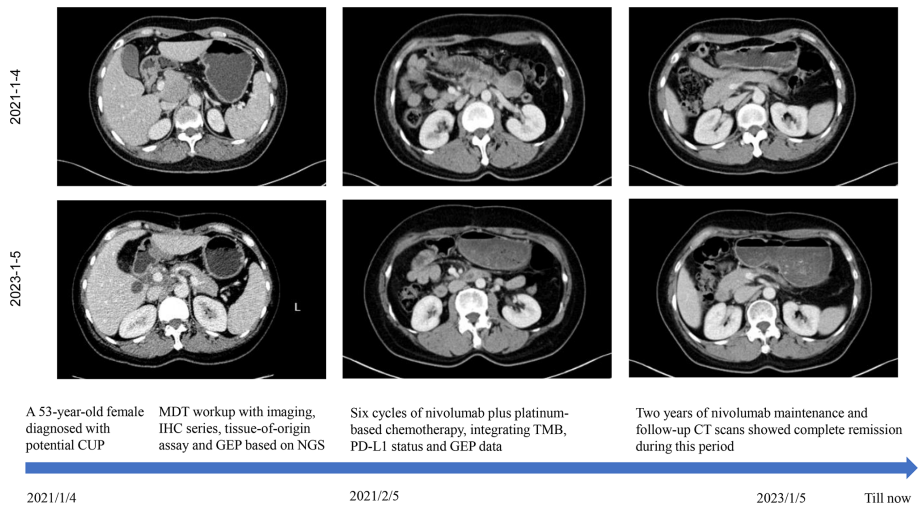


FIGURE 3
The patient's clinical course schedule. Computed tomography scans of abdominal lesions at baseline (upper line) and after treatment (middle line) with nivolumab plus carboplatin and albumin-bound nanoparticle paclitaxel for six cycles and nivolumab maintenance. This case introduced an MDT workup with imaging, IHC series, tissue-of-origin assay, and NGS-based GEP. Immunochemotherapy incorporating TMB, PD-L1 status and GEP data benefited this patient with long-lasting response.

diagnostic utility for molecular classification in difficult-to-diagnose metastatic cancer (10, 11). Using gene expression profiling, the "90-gene expression assay," a real-time PCR assay, was recently developed to identify 21 common cancer types. It calculates the test sample similarity score for each cancer type, reflecting how closely the test sample's gene expression pattern is similar to the global gene expression pattern of a known cancer. The tumor type with the highest similarity score was considered the predicted tumor type. In a retrospective cohort of 609 clinical samples, the gene expression assay was 90.4% accurate for primary tumors and 89.2% accurate for metastatic tumors (10). In addition, the gene expression assay was able to provide insightful predictions of primary tumors in 82.3% of patients in a real-world cohort of 141 CUP patients (116/141) (10). These data support the diagnostic utility of the 90-gene expression assay for molecular classification in difficult-to-diagnose metastatic cancer.

Compared with immunohistochemistry, the detection method based on gene expression profile improves the sensitivity and specificity of tumor origin tissue, but its guiding value for treatment is still controversial (12, 13). A meta-analysis included 5 original studies with a total of 1114 patients, of whom 454 received site-specific therapy and 660 received empiric therapy. Site-specific therapy was not significantly associated with improvements in PFS or OS compared to empirical therapy. Subgroup analyses, however, found that site-specific therapy significantly increased OS performance in the high-precision predictive analysis subgroup (HR 0.46, 95% CI 0.26-0.81, P = 0.008). Site-specific therapy also improved survival for patients with more responsive tumor types compared to patients with less responsive tumor types (14).

The development of gene detection technology promotes the discovery of the driving genes of malignant tumors and the corresponding precise therapy. Patients with CUP may potentially benefit from comprehensive genomic profiling-informed treatment. A multi-omics study of 97 CUP patients revealed frequent actionable genomic alterations with high levels of evidence among CUP patients, and better clinical outcomes in patients with high versus low degrees of precision matching. However, the molecular landscape of each patient was unique (15). Although it has been reported that 30% of CUPs have clearly targetable therapeutic genetic alterations (16), no pharmacologically tractable molecular alteration was found by NGS on tissue samples in our case.

Immunotherapy, represented by immune checkpoint inhibitors, has ushered in a new era of cancer treatment, changing the paradigm of treatment for many patients with advanced tumors. The approval of immunocheckpoint inhibitors has improved the prognosis of patients with CUP. Programmed cell death-1 (PD-1) inhibitor pembrolizumab is the world's first approved anti-tumor drug for use regardless of tissue origin. In 2020, the FDA accelerated the approval of pembrolizumab for the treatment of advanced solid tumor patients who have progressed after previous treatment and no alternative treatment plan available, and whose tumor mutation load is more than 10 mutations/million bases. It is worth mentioning that the efficacy of single immunotherapy agents for CUP is limited. A phase II clinical study of pembrolizumab in advanced uncommon tumors demonstrated preliminary antitumor activity, in which 22 CUP patients who received at least one course of pembrolizumab had a progression-free survival rate of 33% and an ORR of 23% at 27 weeks (17). In another Phase II study in CUP, the effect of pembrolizumab was further investigated (18). Poorly differentiated carcinoma was present in 14 (56%) of the 25 eligible and evaluable patients (29 enrolled). Prior to enrolment, patients had already received a median of two lines of treatment. With acceptable safety profiles, the ORR of pembrolizumab was 20.0% (95% CI: 6.8–40.7), median PFS and OS were 4.1 (95% CI: 3.1–5.1) and 11.3 (95% CI: 5.5–17.1), respectively (18). In addition, Nivolumab's efficacy in treating subtypes of CUP with poor prognosis was evaluated in the NivoCUP study, a multi-center Phase II study, which found that the ORR of CUP patients who had previously received chemotherapy was 22.2%, and the median OS was 15.9 months (19). In light of these results, nivolumab was also approved as the first immunological agent for the treatment of CUP in Japan. Moreover, nivolumab was more effective in the subgroup with high expression of programmed cell death ligand 1 (PD-L1), high tumor mutation load, and MSI-H (19). This suggests that it may be possible to further improve the efficacy of immunotherapy in CUP patients by identifying biomarkers that benefit from immunotherapy. Using a multiplex testing approach, 28% of CUP carried one or more predictive biomarkers (MSI-H, PD-L1 and/or TML-H) to the immune checkpoint blockade (20). The CUP patient we report is an microsatellite (MS) stable type, but the TMB result was 19.3 mutations/Mb with moderately positive for PD-L1 (22C3, TPS=35% and CPS=36), indicating a possible benefit from immunotherapy. When practitioners considered systemic treatment options, the presence of negative predictive biomarkers (JAK1 loss of function mutations for our patient) to immune checkpoint inhibitors also needs to be take into account to avoid tumor hyper-progression.

The strategy of nivolumab combined with chemotherapy in the neoadjuvant phase of NSCLC, advanced NSCLC or advanced gastroesophageal malignancy has been fully demonstrated to be effective and well-tolerated (21–24). Moreover, pembrolizumab combined with platinum-based chemotherapy has also achieved good efficacy in a previous reported CUP case (25). Since PD-L1 TPS was less than 1%, that patient received six cycles of pembrolizumab plus platinum and pemetrexed. However, tumor cells in that case were positive for thyroid transcription factor-1 (TTF-1) and cytokeratin 7, indicating the possibility of NSCLC origin.

Targeted therapy and immunotherapy guided by molecular biological detection are the main directions of future research (26). It is hoped that treatment based on molecular biological characteristics can surpass platinum-based empirical chemotherapy and improve the prognosis for CUP (16). To date, this case is the known one with the longest follow-up time and the most comprehensive diagnosis and treatment of CUP. Our case highlights the importance of the multidisciplinary diagnosis and

individual precision treatment based on integrated tissue-origin-sequencing with molecular profiling information. It should be noted that this is a case study and further research and clinical trials are needed to confirm these findings.

Last but not least, the experience in this case may have implications for reassessing the diagnosis and treatment of CUP. Current guidelines for systematic treatment of CUP include conventional chemotherapy regimen, directed molecular targeted therapy based on driver genes, and immunotherapy recommendations for patients with MSI-H or dMMR, high expression of TMB or PD-L1 (27). However, existing treatment guidelines for systematic treatment of CUP may overlook the strategic value of immunotherapy-based combination therapy. Although molecular profiling of tumor tissue and NGS based on solid tumor biopsy or liquid biopsy as an alternative have been recommended for therapeutic decision-making, predictors of immunotherapy efficacy found by tests, especially negative predictors, should also be fully considered in CUP treatment decisions. Immunochemotherapy, antiangiogenic therapy, and dual immunotherapy are worthy of future exploration in CUP. In the future, the optimal individualized treatment strategies and the design of clinical trials combined with molecular biological characteristics are expected to prolong the survival of CUP patients.

Conclusion

Our case suggests that the individualized treatment plan combined with immunotherapy and chemotherapy based on tumor molecular characteristics and immunotherapy predictors is expected to improve the outcome of CUP treatment and deserves further study.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board (IRB) of Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JS: Conceptualization, Methodology and Writing- Original draft preparation. WH: Supervision, Writing- Reviewing and Editing. HP: Writing- Reviewing and Editing. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1181444/full#supplementary-material

SUPPLEMENTARY FIGURE 1
GO enrichment plot and KEGG pathway analysis based on gene expression data. The count indicates the number of genes related to the enriched GO pathway. The color of the bar denotes the -log10 (p.adjust). The top 3 terms of enriched biological process (BP) were DNA recombination, negative regulation of cell cycle phase transition, and negative regulation of cell cycle. The following cellular composition (CC) terms significantly correlated with this GEP were DNA repair complex, condensed chromosome, chromosomal region. Furthermore, molecular function (MF) enrichment analysis showed that gene expression profiling (GEP) was significantly correlated with protein tyrosine kinase activity, protein serine/threonine/tyrosine kinase activity, transmembrane receptor protein tyrosine kinase activity. Interestingly, KEGG pathway analysis demonstrated pathways related to homologous recombination, Fanconi anemia pathway, and microRNAs in cancer.

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The efficacy of adebrelimab compared with durvalumab and atezolizumab in untreated extensive-stage small-cell lung cancer: a survival analysis of reconstructed patient-level data

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Background: Adebrelimab showed excellent efficacy in the first-line treatment for extensive-stage small-cell lung cancer (ES-SCLC). However, whether adebrelimab is superior to durvalumab and atezolizumab remains unclear. Therefore, we, in this study, aimed to compare the survival data of adebrelimab (CAPSTONE-1 trial) with durvalumab (CASPIAN trial) and atezolizumab (IMpower133 trial) in the first-line setting of ES-SCLC patients.

Methods: Online databases, including PubMed, Embase, Web of Science, and Cochrane CENTRAL, were systematically searched on December 2, 2022. The *metaSurvival* and *IPDfromKM* methods were used to analyze the summary survival curves and the reconstructed patient-level data. The main endpoints were median overall survival (OS) and progression-free survival (PFS).

Results: In this analysis, survival data in the CASPIAN, IMpower133, and CAPSTONE-1 trials were collected from five published studies. The pooled median OS and PFS were 14.0 months (95% CI 11.2-16.6) and 5.6 months (95% CI 4.7-6.7) when ES-SCLC patients received chemotherapy (etoposide and cisplatin/carboplatin) and anti-PD-L1 therapy. Based on the reconstructed patient-level data, adebrelimab significantly prolonged survival outcomes against atezolizumab (OS: Hazard ratio [HR]0.76, 95% CI 0.60-0.95; PFS: HR 0.67, 95% CI 0.54-0.83) and durvalumab (OS: HR 0.75, 95% CI 0.60-0.92).

Conclusion: For previously untreated ES-SCLC patients, longer survival time might be benefited from adding adebrelimab to etoposide-platinum chemotherapy. In future studies, further real-world evidence or head-to-head clinical trials are warranted to confirm the differences between the PD-L1 inhibitors.

KEYWORDS

PD-L1 inhibitor, survival, first-line, extensive-stage, small-cell lung cancer

Introduction

The prognosis of extensive-stage small-cell lung cancer (ES-SCLC) remains poor (1). In the era of chemotherapy, etoposide plus platinum was the standard of care. However, the median overall survival (OS) of ES-SCLC might be hard to exceed one year. Additionally, patients, who received first-line chemotherapy, suffered disease progression within approximately six months.

With the exploration and development of immunotherapy, over one year of OS in ES-SCLC patients is coming true (2). Adding anti-PD-L1 therapy to chemotherapy has revolutionized the first-line treatment framework for ES-SCLC. In the CASPIAN trial, the addition of durvalumab to chemotherapy significantly improved the OS and progression-free survival (PFS) compared with placebo plus chemotherapy (3, 4). Subsequently, the IMpower133 trial demonstrated the clinical benefits of atezolizumab in patients with ES-SCLC (5, 6). Based on these two trials, anti-PD-L1 therapy plus etoposide-platinum chemotherapy has become the optimal first-line treatment option for ES-SCLC (2).

The pace of progress continues. ES-SCLC patients in the CAPSTONE-1 trial treated with adebrelimab and chemotherapy achieved a 15.3 months OS with an acceptable safety profile. Nevertheless, the median PFS in all these trials remained within six months (7). Based on these trials, physicians and patients could be confused about which PD-L1 inhibitor might be more suitable for ES-SCLC patients (3, 5, 7).

At the current stage, physicians and patients are eager to ask about the overall benefits in the era of anti-PD-L1 plus chemotherapy and the optimal option of PD-L1 inhibitors because no direct head-to-head trials have been performed across these treatments (adebrelimab vs. durvalumab vs. atezolizumab). In this study, we reconstructed the patient-level survival data in the CASPIAN, IMpower133, and CAPSTONE-1 trials using a new indirect comparison method, the *IPDfromKM* method (8, 9). Through this survival analysis, we intended to provide valuable survival information and inform readers more about this new area of original research.

Methods

Literature search

A systematic literature search was carried out to identify the randomized controlled trials eligible for the survival analysis. This search was performed in PubMed, Web of Science, Embase, and Cochrane CENTRAL on December 2, 2022. Search terms included "small-cell lung cancer OR SCLC", "extensive-stage OR extensive-disease", "immunotherapy OR PD-L1" and "first-line OR previously untreated OR treatment-naïve". Relevant records in the bibliography were searched for more eligible trials.

Inclusion and exclusion criteria

This analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guideline (10). The inclusion criteria were: (1) treatment-naïve ES-SCLC patients; (2) patients in the treatment arm received anti-PD-L1 therapy plus etoposide-platinum chemotherapy; (3) placebo-controlled randomized clinical trial; (4) primary or update time-to-event data reported as a Kaplan-Meier curve. Meeting abstracts and *post-hoc* subgroup analyses were excluded due to immature data and unbalanced backgrounds.

Data extraction

Detailed therapeutic regimens, doses, and cycles were collected. Kaplan-Meier curves were captured from enrolled trials. Scanlt software (version 2.0.8.0) was used for digitizing the Kaplan-Meier curves.

Statistical analysis

The *metaSurvival* package of R software (version 4.2.2) was applied to generate the synthesized OS and PFS curves, indicating the overall efficacy of chemotherapy plus anti-PD-L1 therapy in patients with ES-SCLC (11). With the comparisons between PD-L1 inhibitors, *IPDfromKM* method was used to reconstruct the individual patient data (8). Hazard ratio (HR) and 95% CI were carried out by Cox statistics.

Results

Through searching the online databases, we identified 373 records. After the systematic screening, three placebo-controlled randomized clinical trials reported in five studies (CASPIAN, IMpower133, and CAPSTON-1) were eligible for further survival analysis (Figure 1) (3–7).

For enrolled ES-SCLC patients, treatment included induction and maintenance phases (Figure 2). During the induction phase, patients in the CASPIAN and IMpower133 trials were treated with up to four cycles of durvalumab/atezolizumab and etoposide-platinum chemotherapy. In the CAPSTONE-1 trial, the cycles ranged from four to six. Another difference existed in the maintenance phase. Atezolizumab and adebrelimab were administrated every three weeks, while durvalumab was prescribed every four weeks.

Although the ES-SCLC patients were treated with chemotherapy and anti-PD-L1 therapy, the survival outcomes differed between the trials. The median OS was 12.9 months (95% CI 11.3-14.7) in the durvalumab group, 12.3 months (95% CI 10.8-15.8) in the atezolizumab group, and 15.3 months (95% CI 13.2-17.5) in the adebrelimab group; The median PFS was 5.1 months (95% CI 4.7-6.2) in the durvalumab group, 5.2 months (95% CI 4.4-5.6) in the atezolizumab group, and 5.8 months (95% CI 5.6-6.9) in the adebrelimab group. Subsequently, we synthesized the survival data from these three trials to better understand the overall efficacy of combining chemotherapy with immunotherapy in the first-line treatment of ES-SCLC patients. The pooled median OS and PFS

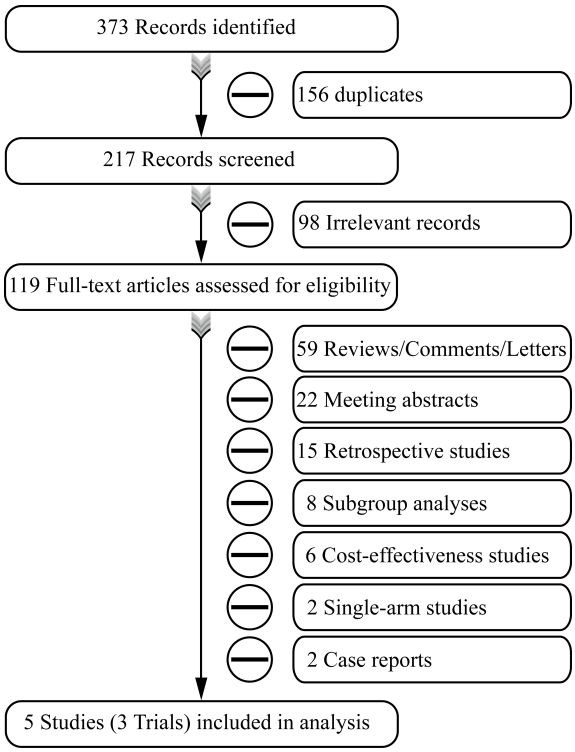

FIGURE 1
Flowchart of the selection process.

were, respectively, 14.0 months (95% CI 11.2-16.6) and 5.6 months (95% CI 4.7-6.7) (Figure 3).

Comparisons between PD-L1 inhibitors were conducted based on reconstructed patient-level data. In terms of OS, adebrelimab significantly decreased the risk of death versus durvalumab (HR 0.75, 95% CI 0.60-0.92) and atezolizumab (HR 0.76, 95% CI 0.60-0.95) (Figures 4A, B). Durvalumab and atezolizumab showed a comparable OS in treating ES-SCLC patients (HR 0.98, 95% CI

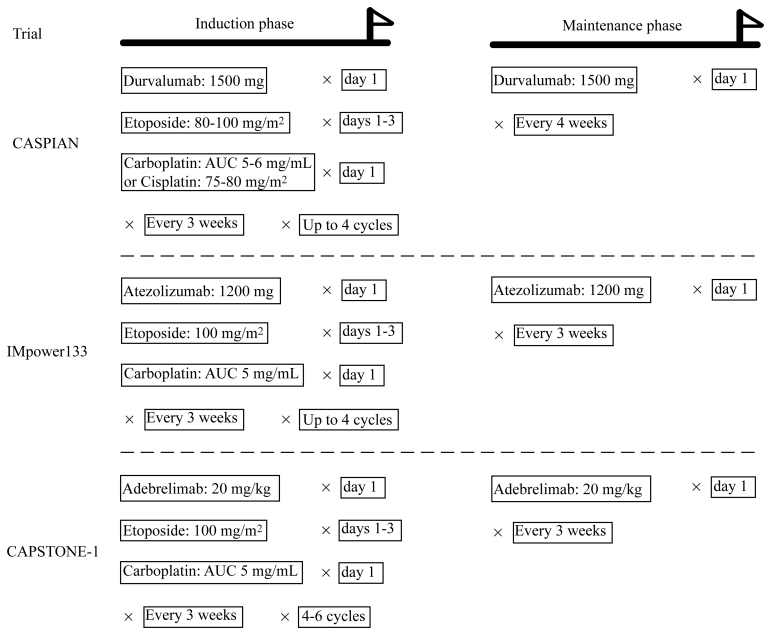

FIGURE 2
Therapeutic strategies in the enrolled trials.

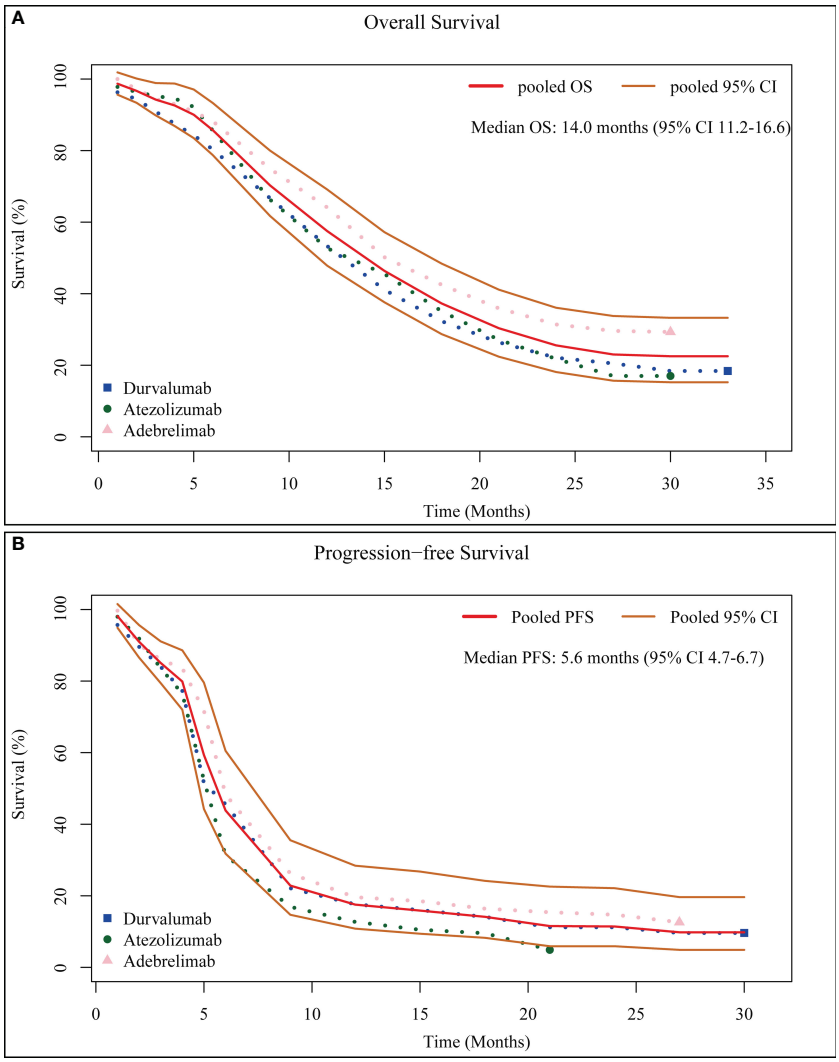

FIGURE 3
Pooled Kaplan-Meier OS **(A)** and PFS **(B)** curves.

0.79-1.21) (Figure 4C). Adebrelimab had higher 1-year and 2-year OS rates (64%, 95% CI 58-70; 31%, 95% CI 25-39) compared with durvalumab (53%, 95% CI 47-59; 22%, 95% CI 17-28) and atezolizumab (53%, 95% CI 46-60; 22%, 95% CI 16-29).

Regarding the reconstructed PFS, adebrelimab (HR 0.83, 95% CI 0.68-1.00) and atezolizumab (HR 0.82, 95% CI 0.67-1.01) showed similar risks of disease progression or death against durvalumab (Figures 5A, C). Nevertheless, adebrelimab achieved a lower risk of disease progression or death than atezolizumab (HR 0.67, 95% CI 0.54-0.83) (Figure 5B). Additionally, patients in the adebrelimab group showed the highest PFS rates at 6 months (50%, 95% CI 44-57) and 12 months (20%, 95% CI 15-26), followed by durvalumab (46%, 95% CI 40-52; 18%, 95% 14-23) and atezolizumab (32%, 95% CI 26-39; 13%, 95% CI 9-19).

Discussion

In this original patient-level survival analysis, we synthesized the OS and PFS in ES-SCLC patients treated with anti-PD-L1 therapy plus chemotherapy, with a median OS of 14.0 months (95% CI 11.2-16.6) and a median PFS of 5.6 months (95% CI 4.7-6.7). In addition, patients in the adebrelimab group showed the lowest risks of death or disease progression and highest survival rates compared with durvalumab and atezolizumab. However, it appears to be a debate on whether adebrelimab is superior to durvalumab and atezolizumab.

For patients in the placebo group of the CAPSTONE-1 trial, the median OS was 12.8 months, much longer than the reported data in the CASPIAN (10.5 months) and IMpower133 (10.3 months) trials. Except for the comparable rates of liver metastasis, brain metastasis, and stage IV disease in these three trials, the most apparent difference was that patients in the CAPSTONE-1 received antiangiogenic drugs (24% in the adebrelimab groups and 30% in the placebo group). Nevertheless, the authors did not make any more explanations or discussions on the administration of antiangiogenic therapy. The prolonged OS in the CAPSTONE-1 trial might be partly attributed to antiangiogenic agents (12–14).

Considering OS data could be impacted by subsequent second or later-line treatments, we suggest that median PFS and PFS rate

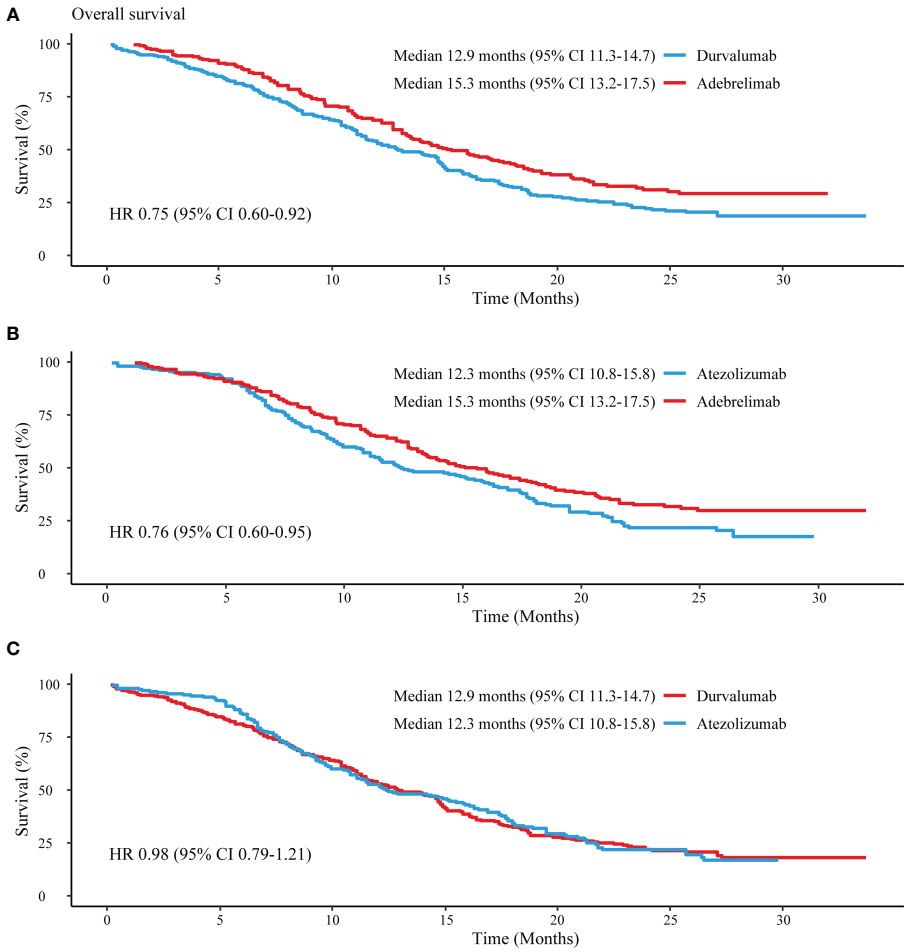

FIGURE 4
Reconstructed median OS comparisons between PD-L1 inhibitors. **(A)** Adebrelimab vs. Durvalumab; **(B)** Adebrelimab vs. Atezolizumab; **(C)** Atezolizumab vs. Durvaluamb.

might be more suitable to be applied to assess the efficacy of PD-L1 as the first-line treatment for ES-SCLC (15). Another reason was that PFS data could directly reflect the improvement achieved by adding anti-PD-L1 therapy to chemotherapy. In the CASPIAN trial, the median PFS was 5.1 months in the durvalumab group versus 5.4 months in the placebo group, and the rates of PFS at 12 months were 17.9% in the durvalumab group and 5.3% in the placebo group; In the IMpower133 trial, the median PFS was 5.2 months in the atezolizumab group versus 4.3 months in the placebo group, and the rates of PFS at 12 months were 12.6% in the atezolizumab group and 5.4% in the placebo group; In the CAPSTONE-1 trial, the median PFS was 5.8 months in the adebrelimab group versus 5.6 months in the placebo group, and the rates of PFS at 12 months were 19.7% in the adebrelimab group and 5.9% in the placebo group. According to these results, the atezolizumab group achieved a wider gap (0.9 months) than the durvalumab (-0.3 months) and adebrelimab (0.2 months) groups. Although median PFS failed to be vastly elevated (less than one month), the PFS rates at 12 months were improved. Therefore, PFS data can be considered as primary endpoints in studies investigating the first-line treatment of ES-SCLC.

Regarding the subgroup analyses, more details deserve our attention. Less than 65 years old patients might benefit from adebrelimab, while patients ≥ 65 could choose durvalumab and atezolizumab. In addition, patients with brain or liver metastasis might be hard to profit from adding anti-PD-L1 therapy to chemotherapy owing to the short lifetime. However, we should read the results of subgroup analyses rigorously because the well-balanced backgrounds of patients were lacking.

Physicians and patients may not be satisfied with the current anti-PD-L1 therapy and chemotherapy. There is a long way to prolong the PFS and OS. During the maintenance phase, the addition of radiotherapy to anti-PD-L1 therapy could effectively treat the primary lung or distant metastatic lesions (16, 17). Moreover, antiangiogenic agents have been reported to enhance the effects of immunotherapy in ES-SCLC patients (12, 18).

This survival analysis had both advantages and limitations. Data in the placebo groups were not taken into the analysis, which might increase the reliability of our findings. A Bayesian or frequency network analysis could solve the problem, but it is not feasible at the current stage due to the limited enrolled trials. However, readers can intuitively understand the survival differences between the PD-

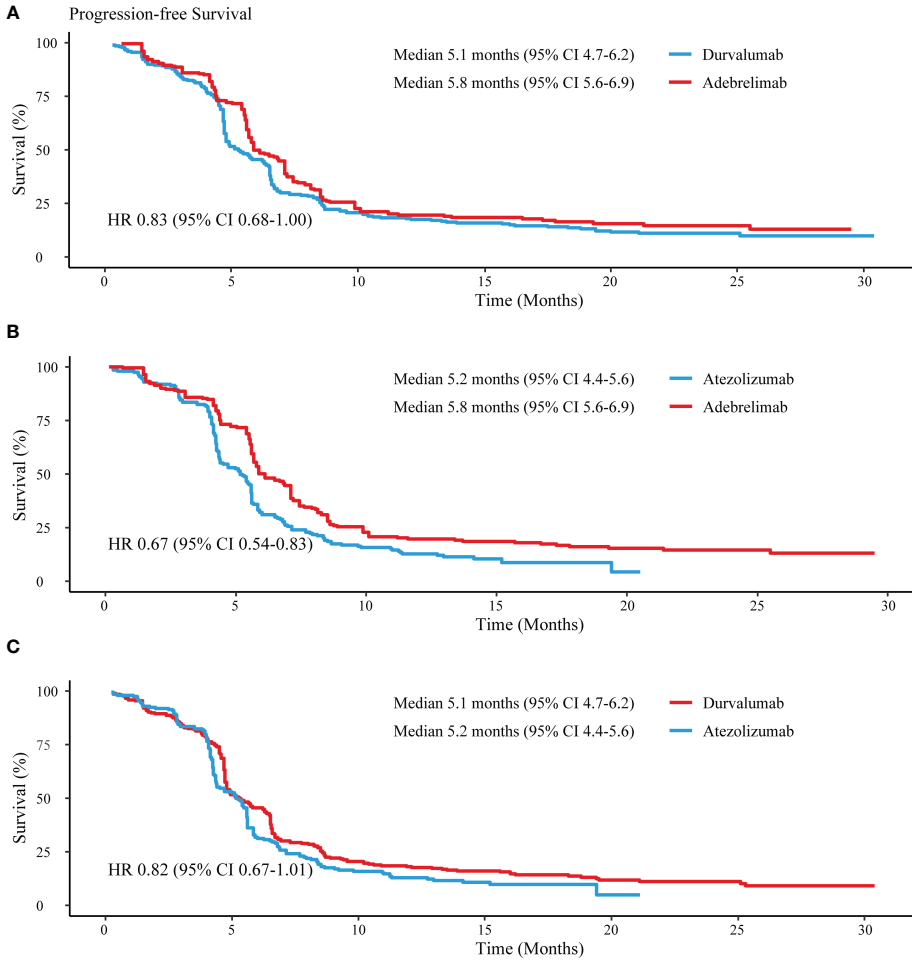

FIGURE 5
Reconstructed median PFS comparisons between PD-L1 inhibitors. **(A)** Adebrelimab vs. Durvalumab; **(B)** Adebrelimab vs. Atezolizumab; **(C)** Atezolizumab vs. Durvaluamb.

L1 inhibitors from our reconstructed survival curves. Combining *IPDfromKM* method with Bayesian network analysis is warranted to balance the inhomogeneity among trials. Additionally, we did not intend to further analyze the immune-related adverse events, mainly because PD-L1-related toxicities could be well controlled.

Finally, anti-PD-L1 therapy has improved the survival time of ES-SCLC patients. Although our results showed that patients in the adebrelimab group achieved the most prolonged survival benefits, durvalumab and atezolizumab have their strong points. We hope future studies can provide more real-world comparisons and effective therapeutic strategies.

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.

Author contributions

Study design: B-CW. data extraction: B-CW and CF. data analysis: B-CW and CF. Manuscript writing and edition: all authors. 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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CD44v6, STn & O-GD2: promising tumor associated antigens paving the way for new targeted cancer therapies

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Targeted therapies are the state of the art in oncology today, and every year new Tumor-associated antigens (TAAs) are developed for preclinical research and clinical trials, but few of them really change the therapeutic scenario. Difficulties, either to find antigens that are solely expressed in tumors or the generation of good binders to these antigens, represent a major bottleneck. Specialized cellular mechanisms, such as differential splicing and glycosylation processes, are a good source of neo-antigen expression. Changes in these processes generate surface proteins that, instead of showing decreased or increased antigen expression driven by enhanced mRNA processing, are aberrant in nature and therefore more specific targets to elicit a precise anti-tumor therapy. Here, we present promising TAAs demonstrated to be potential targets for cancer monitoring, targeted therapy and the generation of new immunotherapy tools, such as recombinant antibodies and chimeric antigen receptor (CAR) T cell (CAR-T) or Chimeric Antigen Receptor-Engineered Natural Killer (CAR-NK) for specific tumor killing, in a wide variety of tumor types. Specifically, this review is a detailed update on TAAs CD44v6, STn and O-GD2, describing their origin as well as their current and potential use as disease biomarker and therapeutic target in a diversity of tumor types.

KEYWORDS

CD44v6, STn, O-GD2, Cancer, Targeted Therapy, O-glycans, Tn, GD2

1 Introduction

Immunotherapy, cell therapies, and vaccines are areas of active research and development aimed at harnessing the body's immune system to fight diseases including cancer and infectious diseases. Antigens play a crucial role in these therapies as they elicit an immune response which leads to the activation and targeting of immune cells against specific disease-associated targets.

Tumor-associated antigens (TAAs) are structures expressed by tumor cells that are recognized by the immune system as foreign (1). These antigens can be used as targets for cancer immunotherapies such as adoptive cell transfer (ACT) or cancer vaccines (2, 3). With the growth of technological development and new research techniques every year new TAAs are being developed in order to reach preclinical research and clinical trials, but few of them actually change the therapeutic landscape. Difficulties either to find antigens that are solely expressed in tumors or the generation of good antibodies or specific molecules that recognize these TAAs represent a major bottleneck.

Basic research in specialized cellular machineries such as post-translational modifications (PTMs) and differential splicing is a good source of neo-antigen expression (4–6). Post-translational modifications (PTMs), including phosphorylation, methylation, glycosylation, sialylation and others, introduce structural chemical changes to existing proteins. Even though these proteins are self-proteins, the attachment of an additional glycan or sialyl-group transforms them into foreign antigens. PTMs occurring on tumor cells can therefore give rise to TAAs. Regarding differential splicing, the generation of TAAs can arise from an alternative splicing process during gene expression by which particular exons of a gene are either included or excluded from the final mRNA (7).

Changes in these processes generate surface proteins that, instead of increasing or decreasing protein antigens, as happens with EGFR or other surface proteins driven by increased mRNA processing, produce surface molecules that are aberrant in nature and therefore more specific as targets to elicit a specific anti-tumor therapy. Overall, ongoing research and advancements in understanding the immune system, genomics, and protein engineering are continually expanding the range of potential antigens that can be targeted for immunotherapy, cell therapies, and vaccine development.

It is worth noting that the development and approval of new antigens for immunotherapy, cell therapies, and vaccines involve a complex and rigorous process of preclinical and clinical trials to assess safety and efficacy. Regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) play a critical role in evaluating and approving these therapies and vaccines (8).

In this review, we introduce a selection of promising TAAs that have shown potential as targets for cancer monitoring, targeted therapy, and the development of new immunotherapy tools. These tools include recombinant antibodies and CAR-T or CAR-NK cells, which can be utilized for precise tumor eradication in various types of tumors. This comprehensive review focuses on the latest information about three specific TAAs: CD44v6, STn, and O-GD2 the first is a variant generated from differential splicing of the well-known CD44 surface marker and the other two are very good examples of cancer associated antigens generated by aberrant glycosylation patterns such as sialylation and O-glycosylation. The review covers their origins, as well as their current and potential applications as disease biomarkers and therapeutic targets across a diverse range of tumor types.

2 CD44 alternative splicing as a source of TAAs

The role of the CD44 adhesion protein family in neoplastic transformation and invasive potential of carcinomas has been widely considered (9). CD44 represents a complex transmembrane glycoprotein encoded by a single gene on the short arm of chromosome 11 (Figure 1, upper). In humans, the CD44 encoding gene consists of 20 exons that give rise to a wide variety of multifunctional glycoproteins due to alternative splicing processes. The standard form, known as CD44s glycopeptide, represents the smallest and most abundant CD44 structure, comprised of exons 1-5 and 16-20. The constant region of the variable isoforms (CD44v) is represented by the same exons, while alternative splicing of the remaining exons (exons 6-15 or CD44v1-10) gives rise to the different CD44v isoforms. It is of note that isoform CD44v1 has not been detected in humans due to the presence of a stop codon in exon 6 (also known as exon v1) (10). The different isoforms of CD44 generated by alternative splicing can act as TAAs and have been implicated in tumor progression and immune evasion. Some CD44 isoforms, such as CD44v6 and CD44v9, have been found to be frequently expressed in many types of cancer, including pancreatic (11, 12), colon (13, 14), and prostate cancer (15, 16). These isoforms are associated with increased invasiveness, metastasis, and resistance to apoptosis.

2.1 CD44v6

Alterations of CD44 glycoprotein expression have been shown to play an important role in the progression of various malignancies (17). Specifically, overexpression of the CD44v6 variant has been found in the majority of squamous cell carcinomas and a variety of adenocarcinomas, but has not frequently been observed in non-epithelial tumors (18). In this way, CD44v6 has been described to negatively impact the prognosis of patients with multiple myeloma (19), colorectal cancer (CRC) (20, 21), osteosarcoma (22), esophageal carcinoma (22), gastric cancer (23) as well as head and neck squamous cell carcinoma (HNSCC) (24, 25) patients, among others. Moreover, altered CD44v6 expression has been associated with tumor development, migration, invasion and metastatic potential in a broad variety of tumor types, such as HNSCC (24), oral cancer (26), laryngeal carcinoma (27), esophageal squamous cell carcinoma, gastric cancer (28–30), pancreatic cancer (11, 31), liver cancer (32, 33), CRC (34), lung cancer (35, 36), breast carcinoma (37) and gynecologic malignancies (38) such as ovarian (39, 40) and prostate cancer (41), among others (Figure 1).

Circulating tumor cells (CTCs) highly expressing CD44v6 were found in blood samples from metastatic CRC patients (34, 42). Nicolazzo et al. (2020) (43) described treatment failure in those patients with CD44v6-positive CTCs undergoing first-line chemotherapy and proposed that the presence of those cells might be a valuable predictive biomarker of therapy resistance. Indeed, many

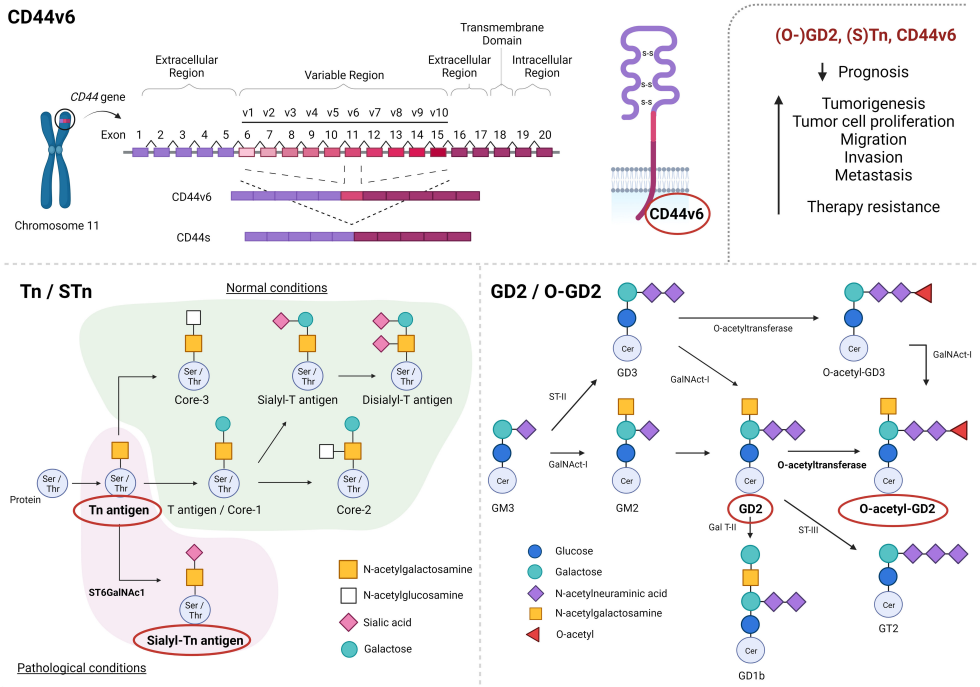

FIGURE 1
Schematic representation of CD44-alternative splicing, giving rise to CD44 standard form (CD44s) and CD44 isoforms (CD44v1-10), as well as alternative glycosylation processes, generating O-glycans sialyl-Tn (STn) and O-acetyl-GD2 (O-GD2). CD44v6, (S)Tn and (O-)GD2 have been described to negatively impact prognosis as well as to promote tumorigenesis, tumor progression and therapy resistance. Created with BioRender.com.

studies have associated CD44v6 expression with chemoresistance. For example, Gaggianesi et al. (2022) (44) demonstrated that tumor microenvironmental cytokines promote CD44v6 expression in CRC stem cells, conferring resistance to standard anti-tumor therapeutic options, whereas Wang et al. (2019) (45) found that the downregulation of CD44v6 augments chemosensitivity of CRC cells *in vitro*. CD44v6 has been described to enhance CRC therapy resistance by a variety of mechanisms, including activation of the PI3K/Akt/mTOR pathway leading to increased multidrug resistance, up-regulation of the MAPK/ERK pathway stimulating autophagosome formation, and blocking of the Fas-FasL interaction preventing cell apoptosis. Pereira et al. (2020) (46) found similar results, showing that CD44v6+ gastric cancer cells were more resistant to chemotherapy treatment compared to the CD44v6 cells. Even though the exact mechanism in gastric cancer remains unknown, they proposed that therapy resistance might be the result of concomitant CD44v6 expression and either STAT3 or P38 activation, depending on the cellular context. Wang et al. (2021) (45) proposed the involvement of CD44v6 expression in cisplatin resistance in ovarian cancer cells due to the interaction of CD44v6 with P-glycoprotein (P-gp) and, therefore, the acquisition of multidrug resistance. Sagawa et al. (2016) (47) found a correlation between CD44v6 expression and chemoradiotherapy resistance in nasopharyngeal carcinoma, and prostate cancer chemosensitivity was associated with CD44v6 by Ni et al. (2020) (41). Both studies indicated the activation of the PI3K/Akt/mTOR pathway as the major player in the acquisition of therapy resistance. Specifically, in the case of prostate cancer, the interaction between CD44v6 and hyaluronic acid, a component of the extracellular matrix of epithelial and connective tissues, could augment multidrug resistance through up-regulation of the PI3K/Akt/mTOR pathway.

Even though some studies hypothesized the potential role of the majority of CD44 isoforms, such as CD44v3-5 and CD44v8-10, in tumorigenesis and cancer progression, none has been as extensively described as CD44v6 (48). The extensive description of all these different isoforms and their potential implication in tumor development and/or disease progression goes beyond the scope of this review. Beyond alternative splicing process giving rise to different CD44 isoforms, extensive posttranslational modifications, such as N- and O-glycosylation processes as well as the addition of glycosaminoglycans add another layer of complexity to the CD44 transmembrane glycoprotein structure and function (49).

3 STn and O-GD2, the result of alternative glycosylation processes

Altered glycosylation processes are known to be widely involved in tumorigenesis and cancer progression (50). In early cancer stages, the normal synthesis of glycans present in normal epithelial tissues is often impaired due to altered expression of glycosyltransferases, which causes the biosynthesis of truncated carbohydrate structures (such as Tn and STn) which can be observed in a variety of tumor types (49). In the same way, altered ganglioside expression (like GD2 and O-GD2 expression) due to modified glycosyl- and sialyltransferase expression has been found in different cancers such as melanomas and neuroblastomas (51). The role of Tn/STn

and GD2/O-GD2 expression in tumorigenesis and cancer progression will be discussed below (Figure 1, lower).

3.1 Tn & STn

Truncated O-glycan Tn (GalNAcα1-Ser/Thr) and its sialylated variant sialyl-Tn (STn) (Neu5Acα2,6GalNAcα1-Ser/Thr) represent two modifications observed in a variety of proteins which give rise to two tumor-associated antigens that are not expressed in normal cells. Even though O-glycan Tn formation does occur in normal tissue, the Tn glycopeptide represents an immature structure which is normally elongated into other structures by additional glycosyltransferases. Furthermore, altered expression of ST6GalNAc1 glycosyltransferase modifies the Tn glycopeptide by the addition of a sialyl-acid, resulting in STn glycopeptide formation (Figure 1). Therefore, the presence of both O-glycan Tn and STn are described to be restricted to pathological conditions, including tumorigenesis (49).

The structural simplicity and biological complexity of the Tn antigen have been extensively reviewed by Ju et al. (2011) (52). Even though the exact role of the Tn antigen in tumor development and progression is only starting to be unraveled, the presence of the Tn antigen on tumor cells was observed as early as 1969 (53). The first correlation between the Tn antigen and cancer was made by Springer et al., who described expression of the Tn antigen in 90% of breast carcinomas. Subsequently, several studies have reported altered levels of the Tn antigen in many tumor types, such as lung, gastric, CRC, bladder, cervical, prostate and ovarian tumors (54–56). Additionally, altered expression of the Tn antigen has been associated with tumor progression, migratory capacity and metastasis as well as poor prognosis in a wide variety of cancer types, including lung cancer (57), pancreatic cancer (58), CRC (59, 60), breast carcinomas and cervical cancer (59, 61).

Similar to the Tn antigen, the presence of the STn antigen has been reported in several tumor types, such as lung cancer, gastric cancer, pancreatic cancer, CRC, breast carcinomas, cervical cancer, prostate and ovarian cancer (62–64). Nevertheless, STn expression in tumors has been described to be rather heterogeneous, with STn-positive cells ranging from 5% to 100% independent of tumor type origin (64). A growing body of evidence also suggests that the role of STn in tumorigenesis might be cancer type-specific and/or organ-specific as STn can be carried by different glycoproteins. Increased expression of STn was associated with cell proliferation and metastasis in gastric, breast and pancreatic cancer (65). In prostate cancer, however, Munkley et al. (2015) (66) described that STn expression is likely to promote cancer cell dissemination and invasion, and this expression is up-regulated in primary prostate carcinoma. Concordantly, Davidson et al. (2000) (67, 68) reported higher levels of STn-positive ovarian cancer cells at the invasive site of primary tumors than in metastatic lesions, and Ferreira et al. (2013) (69) reported increased migratory and invasive capacity of bladder cancer cells upon STn expression. The current hypothesis behind these observations suggests that the STn antigen inhibits tumor formation by reduction of cell-cell and cell-matrix interactions, which facilitates tumor cell spreading. Even though the STn antigen does not seem to provide cell adhesive properties needed for the formation of metastatic lesions, these specific adhesive characteristics are essential for extravasation and invasive capacity. Accordingly, a transient role for STn in cancer progression has been proposed.

Recently, STn expression has also been associated with tumor-microenvironment interactions. Interestingly, a protective role for glycan STn with regard to tumor cell recognition and degradation has been described (70). Either by receptor masking or the inhibition of cytolytic activity, STn has been reported to play an essential role in immunosuppression (71, 72). In bladder cancer, STn expression has been suggested to induce a tolerogenic phenotype in innate and adaptive immune cells. However, extensive studies in a variety of cancer types will be needed to further unravel the role of STn in cancer progression, invasion and metastasis (73).

The STn antigen can be detected in serum when considerable tumor size is reached, either due to O-glycoprotein secretion from tumors or by its expression on CTCs. Therefore, the presence of the STn antigen in serum is thought to be correlated with advanced cancer and, thus, poor prognosis. Indeed, Carvalho et al. (2020) (74) recently reported an association between STn expression and advanced bladder cancer stage and grade. However, the correlation between the STn antigen and prognosis seems to be rather ambiguous and cancer type-specific (64). STn expression was associated with poor prognosis in ovarian cancer (75, 76), but has not been associated with overall survival in either lung (77) or cervical cancer (68). Additionally, contradictory findings have been published for several tumor types, such as breast cancer, esophageal cancer, gastric cancer and CRC, reflecting the potential cancer subtype-specific role of the STn antigen in tumorigenesis and cancer progression (64).

3.1.1 Interaction between CD44v & STn

As mentioned above, STn can be carried by different glycoproteins and glycosylation processes add another layer of complexity to the CD44 glycoprotein. With these observations in mind, CD44 has indeed been reported as carrier protein for STn in gastric and colon cancer. Campos et al. (2015) (78) described the presence of glycan STn on CD44 glycoprotein which leads to altered CD44 molecular features such as molecular weight and antibody recognition in gastric cancer. Mereiter et al. (2019) (79) also reported activation of the receptor tyrosine kinase RON due to increased colocalization of CD44v6 with this receptor in the presence of STn, leading to enhanced hyaluronan binding capacity. Additionally, improved CD44v9 detection by the expression of immature O-glycan structures, such as STn, has recently been proposed by Moreira et al. (2020) (80). Finally, Singh et al. (2001) (81) showed the presence of the STn antigen on CD44 splice variants in CRC, further emphasizing the abovementioned interaction between STn and CD44. Taken together, it might be clear that not only STn or CD44 (v) expression, but also their combination might be very useful with regard to biomarker detection as well as therapeutic targeting.

3.2 GD2 & O-GD2

Even though the exact biological mechanisms remain to be elucidated, altered activity of glycosyl- and sialyltransferases seems to be mainly responsible for the modification of ganglioside expression in tumorigenesis (51). For example, increased N-acetylgalactosaminyltransferase I (GM2/GD2 synthase) expression has been found to provoke enhanced GD2 ganglioside levels in melanoma and neuroblastomas (82). Additionally, even though high expression levels of GD2 have been associated with reduced apoptosis as well as enhanced tumor cell proliferation, adhesion, angiogenesis, migration and invasion capacity in a variety of tumor types such as breast cancer (83, 84), bladder cancer (85), lung cancer (86), osteosarcoma (87), Ewing sarcoma (88), retinoblastoma (89) and brain tumors (90), some expression of this ganglioside has been also observed in some normal tissue in healthy adults, like the central nervous system and peripheral nerves (90), Even so, this ganglioside has been used as a biomarker of cancer in serum samples (91) associated with advanced disease and poor prognosis in neuroblastoma by (92), and (85) in high-grade bladder cancer compared to low-grade disease (84). Also, a correlation between GD2 expression and malignant phenotypes of lung cancer has been described by Yoshida et al. (2002) (86) and Esaki et al. (2020) (93).

Even though GD2 expression might be a valuable prognostic marker and biomarker, its expression on healthy cells complicates its potential as therapeutic target (detailed below). Therefore, interest has currently focused on O-acetyl-GD2 (O-GD2), which is formed by the addition of an O-acetyl ester to the GD2 backbone by 9 (7)-O-Acetyl transferase (Figure 1, lower right). Fleurence et al. (2017) (51) and Cavdarli et al. (2019) (91) have extensively modeled the complex biosynthetic processes giving rise to the different members of the O-acetylated ganglioside family. Interestingly, O-GD2 has been found to be coexpressed with GD2 on tumor cells. Indeed, the presence of O-GD2 in GD2 positive tumors, such as lung carcinoma, melanoma, osteocarcoma, brain tumors and neuroblastoma, has been confirmed by various studies (94, 95), whereas no expression of O-GD2 was observed in either peripheral nerves or a large variety of other healthy tissues (96).

Even though these findings strongly support O-GD2's potential as valuable biomarker and therapeutic target, its regulatory mechanisms of expression as well as its role in tumorigenesis remain largely unknown due to the complexity of studying this antigen within the extensive biosynthesis network of the whole ganglioside family. Fleurence et al. (2017) (51) suggested that O-GD2 expression in a cell type depends on the balance and activity of at least 4 different enzymes involved in the biosynthetic model of the ganglioside family.

4 CD44v6, STn and O-GD2 as therapeutic targets: past, present and future

4.1 CD44v6

Due to its expression pattern, CD44v6 has been considered an attractive target for antibody-based cancer therapy (17). Thus far, a variety of recombinant antibodies and antibody-drug conjugates have been evaluated in different phase I clinical trials, mainly for their use in HNSCC treatment (96–104). In addition, CD44v6-directed CAR-T cell therapies have shown promising results in preclinical studies, which has led to a phase I/IIa trial in AML and MM patients to study efficacy, safety and feasibility of CD44v6-directed CAR-T cell therapy (105). Even though CAR-T cell therapy for hematological cancers has been very successful, its use for the treatment of solid cancers remains very challenging. Therefore, a variety of studies has evaluated the potential of CD44v6-directed CAR-T cell therapy in solid CD44v6-expressing tumors (105–108). Recently, CD44v6-directed CAR-cytokine induced killer cells (CIK) have demonstrated anti-tumor activity in preclinical studies among different cancer types such as high grade soft tissue sarcomas where the anti-sarcoma activity of CD44v6-CAR-CIK bipotential killers was confirmed in a STS xenograft model in which killing activity was significantly higher compared with unmodified CIK, especially at low effector/target (E/T) ratios: 98% vs 82% (E/T = 10:1) and 68% vs 26% (1:4), ($p<0.0001$) (109) (Figure 2). An extensive overview on the development and potential of the abovementioned immunotherapy strategies can be found below.

4.1.1 Anti-CD44v6 antibody & antibody-drug conjugates

The first studies into the potential of anti-CD44v6 antibody-based immunotherapy were performed almost 30 years ago, when anti-CD44v6 antibodies VFF18 and U36 were characterized as candidates for immunotherapy in squamous cell carcinoma (97–99).

Heider et al. (1996) (97) showed essential therapeutic features for radiolabeled the anti-CD44v6 VFF18 antibody in an *in vivo* model of human epidermoid squamous cell carcinoma. Additionally, a first clinical study was performed by Bree et al. (1995) (98), who demonstrated high and selective tumor uptake for the radiolabeled 99mTc-labeled anti-CD44v6 U36 antibody in HNSCC patients. In agreement with the previous finding, Van Hal et al. (1996) (99) demonstrated that the CD44v6 antigen targeted by anti-U36 is an appropriate target for the treatment of head-and-neck carcinoma by the use of radiolabeled anti-CD44v6 U36 antibody. The biodistribution studies *in vitro*, after which a first phase I clinical trial with 186RE-labeled chimeric antibody U36 among patients with HNSCC showed excellent tumor targeting (100). Even though stable disease as well as reduction in tumor size were observed and the antibody was found to be well-tolerated, myelotoxicity represented a dose-limiting factor. Preclinical characterization of two other radiolabeled anti-CD44v6 antibodies, 111In- and 177Lu-labeled U36 antibodies, using HNSCC *in vitro* and *in vivo* models has also been described as showing a tumor uptake of 72 h.p.i which makes it interesting for therapies (110, 111) cancer detection tool (Table 1).

Another phase I clinical study reported high tumor uptake and well-tolerated administration of the murine 99mTc-labeled anti-CD44v6 antibody BIWA1 (initially called VFF18) in patients with HNSCC (101) showing a tumor intake with a mean value of 14.2+/-8.4% of the injected dose/kg tumor tissue and a mean tumor:

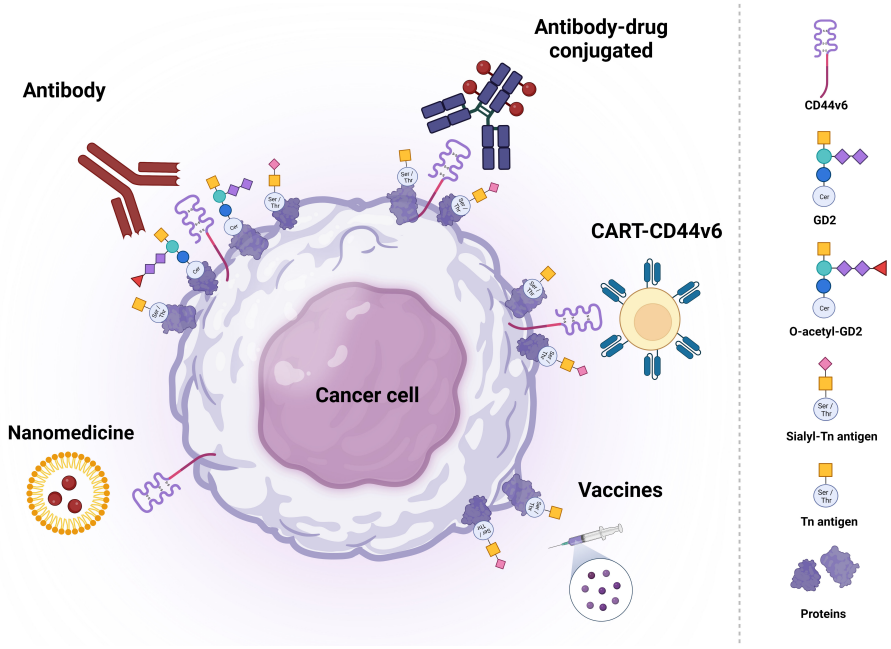

FIGURE 2
Schematic representation of potential therapeutic strategies based on CD44v6, (S)Tn and (O-)GD2 targeting. Each type of therapy is grouped with different antigens. Created with BioRender.com.

blood ratio of 2.0+/-1.4 at 40 h after injection with a mean biological half-life in blood (34.5+/-6.1 h). With the aim of clinical application, a humanized 99mTc-labeled anti-CD44v6 antibody BIWA4 antibody, known as bivatuzumab, was then developed, and a phase I clinical trial with HNSCC patients showed selective and high tumor uptake by radioimmunoscintigraphy showing targeting of primary tumors and lymph node metastases in 8 of 10 and 1 of 5 patients, respectively. The highest tumor uptake and tumor to nontumor ratios was observed for the 50-mg dose group with a tumor intake of 26.2 +/- 3.1% of bivatuzumab, without evidence of safety concerns (102). At the same time, phase I clinical studies were performed with HNSCC patients and patients with recurrent or metastatic HNSCC receiving humanized 186Re-labeled bivatuzumab, which showed anti-tumor activity and high tumor to non-tumor targeting ratio (103, 104). Several parallel phase I clinical trials in patients with HNSSC, esophageal carcinoma and metastatic breast cancer were also started with antibody-drug conjugate Bivatuzumab mertansine (105, 109, 114, 115), but the clinical development of this drug was discontinued after a lethal outcome due to toxic epidermal necrolysis in one of the trials. Nevertheless, in 2012, Gurtner et al. (127) reported improved local tumor control at acceptable toxicity levels in an *in vivo* model using lower doses of Bivatuzumab mertansine (the maytansine derivative DM1 as cytotoxic agent) in combination with fractioned irradiation (Table 1).

4.1.2 CD44v6-based CARs cell therapy

Other therapeutic strategies based on anti-CD44v6 antibody constructions, which have recently generated interest, include the use of chimeric antigen receptor (CAR) T cell approaches. In 1996, Hekele et al. (110) reported tumor growth suppression in a rat pancreatic adenocarcinoma *in vivo* model when receiving genetically manipulated cytotoxic T lymphocytes (CTLs), expressing the scFv of rat-specific anti-CD44v6 antibody 1.1ASML. The same 1.1ASML antibody was used for the construction of a bispecific F(ab9)2 antibody conjugate (BAC) which recognizes both the CD44v6 tumor cell moiety and the complement receptor CR3 on macrophages in order to redirect and induce efficient macrophage-mediated tumor cytotoxicity. However, *in vitro* and *in vivo* models of the abovementioned pancreatic carcinoma showed lower anti-tumor activity for the BAC compared to the naked anti-CD44v6 1.1ASML antibody (111). So far, promising results regarding CAR-T cell mediated anti-tumor activity have been described for several cancer types such as leukemia (19), multiple myeloma (19, 116), HNSCC (117), lung adenocarcinoma (107) and ovarian cancer (107). In *in vivo* models of acute myeloid leukemia (AML) and multiple myeloma (MM), CD44v6 CAR-T cells were found to provide significant anti-tumor activity without affecting either CD44v6-expressing keratinocytes or hematopoietic stem cells (19). A phase I/IIa clinical trial has subsequently been performed to study safety, efficacy and feasibility of CD44v6 CAR-T cell immunotherapy in AML and MM patients mediating a potent antitumor effects against primary AML and MM while sparing normal hematopoietic stem cells and CD44v6-expressing keratinocytes (105) mediating a potent antitumor effects against primary AML and MM while sparing normal hematopoietic stem cells and CD44v6-expressing keratinocytes (105) mediating a potent antitumor effects against primary AML and MM while sparing normal hematopoietic stem

TABLE 1 Overview of the CD44v6 directed therapies described in this study.

Target	Type of therapy	Therapeutic construct	Example	Reference
CD44v6	Anti-CD44v6 antibody & antibody-drug conjugates	Radiolabeled murine/chimeric anti-CD44v6	^{99m}Tc-U36	(98)
			^{99m}Tc-VFF18 (BIWA1)	(101)
			^{186}Re-U36	(100)
			^{111}In-U36	(112)
			^{177}Lu-U36	(113)
		Radiolabeled humanized anti-CD44v6	^{99m}Tc-BIWA4 (bivatuzumab)	(101)
			^{186}Re-bivatuzumab	(103, 104)
		Anti-CD44v6 drug conjugate	Bivatuzumab-mertansine	(105, 109, 114, 115)
	CD44v6-CAR-based cell therapy	Anti-CD44v6 CAR-T	scFv 1.1ASML	(19, 107, 116, 117)
		Anti-CD44v6 CAR-CIK		(118, 119)
	CD44v6-based nanomedicine	Anti-CD44v6 conjugated nanoparticle drug delivery	Arsenic trioxide	(120)
			Gemcitabine + siRNA	(121, 122)
			Gemcitabine + miRNA21	(122)
			Bevazucimab	(123)
			MG2477	(124)
		Multi-walled carbon nanotubes (MWNTS)	CXCR4 + gemcitabine	(125)
			CXCR4 + oxaliplatin	(125)
		Polymeric micelles		(126)

This table includes therapeutic target, type of therapy, therapeutic construct and antigenic determinant.

cells and CD44v6-expressing keratinocytes (105) mediating a potent antitumor effects against primary AML and MM while sparing normal hematopoietic stem cells and CD44v6-expressing keratinocytes (105). Additionally, Porcellini et al. (2020) (107) showed CD44v6 CAR-T cell infiltration and proliferation at the tumor site and tumor growth inhibition, leading to enhanced overall survival in xenograft mouse models of ovarian and lung adenocarcinoma with more than 30% of CD44v6-CAR T treated mice still alive after 2 months of observation (106, 117). T cells expressing CD44v6 CAR have been shown to be effective against lung and ovarian adenocarcinomas in mice (107), urothelial carcinoma (106) and pancreatic adenocarcinoma (108). Recently, studies have also focused on optimization of the CAR structure in order to improve CD44v6 CAR-T cell functionality (105) and generating not just CAR-T but CAR-NK cells, which in comparison to CAR-T are proposed to show less cytokine release syndrome associated problems (105) and generating not just CAR-T but CAR-NK cells, which in comparison to CAR-T are proposed to show less cytokine release syndrome associated problems (105) and generating not just CAR-T but CAR-NK cells, which in comparison to CAR-T are proposed to show less cytokine release syndrome associated problemsRaferty et al. (108) demonstrated the efficacy of CD44v6-CAR-NKs in triple negative breast cancer model demonstrating cytotoxic function in both 2D and 3D models of triple-negative breast cancer (108). Nearby to CAR-T and CAR-NK cells, CD44v6-directed CAR-cytokine induced killer cells (CIK) have demonstrated anti-tumor activity in *in vitro* and *in vivo* models of high-grade soft tissue sarcomas such as undifferentiated pleomorphic sarcoma, liposarcoma, fibrosarcoma, leiomyosarcoma and gastrointestinal stromal tumor, among others (118). Moreover, a recent preclinical study proposed the use of CAR-CIK in leukemia patients due to intrinsic anti-tumor activity as well as enhanced safety (119).

4.1.3 CD44v6-based nanomedicine

Finally, CD44v6-targeting nanomedicine has been explored as a promising tool for cancer therapy. Qian et al. (2013) described the use of anti-CD44v6 conjugated nanoparticles carrying the anti-cancer agent Arsenic trioxide in a pancreatic cancer mouse model and its successful accumulation at the tumor site, followed by tumor growth control (120). The same animal model was used for the evaluation of nanoparticle-mediated co-delivery of a chemotherapeutic agent and target genes, either Gemcitabine and small-interfering RNA or Gemcitabine and microRNA-21 (121, 122). Both strategies confirmed CD44v6-directed tumor cell targeting and they were found to provide efficient inhibitory effects on tumor growth and metastasis. Kennedy et al. (2018) (128) reported the specificity and stability of anti-CD44v6 Fab-conjugated poly(lactic-co-glycolic acid (PLGA) nanoparticles *in vivo* in a gastric cancer model, emphasizing its potential as a

carrier for cancer therapy in solid tumors, and Baião et al. (2020) (123) described the application of anti-CD44v6 PLGA-PEGylated nanoparticles for bevacizumab delivery in a colorectal cancer model demonstrating intracellular levels of bevacizumab significantly higher in cells incubated with v6 Fab-PLGA-PEG NPs and these nanoparticles resulted in a significant decrease in the intracellular VEGF compared to untargeted nanoparticles and free bevacizumab. In addition, the use of anti-CD44v6 conjugated PEGylated, organic-modified silica (ORMOSIL) nanoparticles carrying the anti-cancer agent 3N-cyclopropylmethyl-7-phenyl-pyrrolo-quinolinone (MG2477) has been shown to provide significantly increased cytotoxic activity towards CD44v6-expressing cells demonstrating that nanoparticles conjugated with a smaller amount of targeting agent being more effective than the ones conjugated with a larger amount of antibody, an effect that will probably be dependent of the affinity of the antigen-antibody interaction (123) demonstrating that nanoparticles conjugated with a smaller amount of targeting agent being more effective than the ones conjugated with a larger amount of antibody, an effect that will probably be dependent of the affinity of the antigen-antibody interaction (123) demonstrating that nanoparticles conjugated with a smaller amount of targeting agent being more effective than the ones conjugated with a larger amount of antibody, an effect that will probably be dependent of the affinity of the antigen-antibody interaction (123) demonstrating that nanoparticles conjugated with a smaller amount of targeting agent being more effective than the ones conjugated with a larger amount of antibody, an effect that will probably be dependent of the affinity of the antigen-antibody interaction (123). Finally, multi-walled carbon nanotubes (MWNTS) and polymeric micelles have been considered as vectors for drug delivery in cancer therapy. Recently, Yin and Qian (2021) (125) showed enhanced anti-tumor activity after treatment with anti-CD44v6 MWNTS, carrying CXCR4 and either gemcitabine or oxaliplatin, in *in vitro* and *in vivo* models of ovarian cancer, whereas Andrade et al. (2021) (126) described the efficacy of anti-CD44v6 polymeric micelles as an anticancer agent carrier (Table 1).

Although the above experimental evidences require further proof, the targeted nanoparticle development present clinical potential and provide a launching point for future improvements and therapeutic and/or diagnostic opportunities.

4.2 Tn & STn

Based on the restricted expression of O-glycans Tn and STn among human carcinomas, these antigens represent an excellent feature for targeted therapy. As for CD44v6, several recombinant antibodies and antibody-drug conjugates have been evaluated in a variety of phase I/II clinical studies for their anti-tumor efficacy in different tumor types (129–133). Additionally, STn-based CAR-T cell therapy has shown promising results in preclinical studies and, subsequently, reached phase I clinical trials for the treatment of solid tumors (134–136). Nevertheless, the best characterized STn-targeting therapy remains a STn-based vaccine, which has been extensively evaluated in phase III clinical trials (51) (Figure 2). An extensive overview on the development and potential of the abovementioned immunotherapy strategies can be found below.

4.2.1 Anti-Tn/STn antibody & antibody-drug conjugates

Many Tn-recognizing antibodies have been generated over the last 35 years. The Tn antigen seems to represent an easy antigenic determinant due to its simple chemical structure (GalNAcα1-Ser/Thr). However, even though Trabbic et al. (2018) (137) published the recognition of a single Tn residue by the anti-Tn Kt-IgM-8 antibody, a single Tn determinant has often been found not be enough for anti-Tn antibody function (138). Accordingly, either the presence of a peptide backbone or multiple consecutive Tn residues has been found to be essential for a wide variety of anti-Tn antibodies. Reis et al. (1998) (89) reported that the PMH1 antibody requires an additional MUC2 apomucin peptide chain, whereas the Tn-MUC1 complex was found to represent the antigenic determinant for several anti-Tn antibodies, including SM3 (139), PankoMab (140) and 5E5 (141). Furthermore, multiple anti-Tn antibodies, such as MLS128 (142), 83D4 (143), KM3413 (144) and Ca3638 (145), have been described to require at least two consecutive Tn residues. Finally, the importance of the Tn backbone composition has been described for some antibodies. For example, Mazal et al. (2013) (146) demonstrated specific recognition of a tri-Serine Tn backbone by the 15G9 antibody (Table 2).

Some of the abovementioned mouse anti-Tn antibodies have been converted into chimeric or humanized antibodies for therapeutic use in the clinic. The mouse-human chimeric antibody, cKM3413, was found to induce ADCC and direct killing activity, which was accompanied by increased survival *in vivo* (144). Additionally, the humanized 5E5 antibodies CIM301-1 and CIM301-8 were reported to enhance NK cell activation and cytotoxicity *in vitro* (147), and humanized PankoMab-GEX was described to be safe, well-tolerated and exhibit promising anti-tumor activity in advanced disease in ovarian cancers, in a phase I clinical trial (129, 130). In 60 evaluable patients with ovarian cancer the clinical benefit included one complete response in a patient treated 483 d and confirmed disease stabilization in 19 patients lasting a median (range) of 23 (10–102) weeks (126). Additionally, the combination of PankoMab-GEX with an anti-EGFR antibody was evaluated in a phase I clinical study and showed anti-tumor activity in lung cancer and CRC patients (131). From this clinical trial were 2 and 4 RECIST partial responses in the first and second part of the study, all in CRC patients. There were 2 responses in each subgroup and the duration of best response was 7.2 months. The PFS for NSCLC was 5.3 months and 2 heavily pretreated patients achieved a prolonged control of disease of 10.6 and 9.4 months. Sedlik et al. (147) has used another mouse-human chimeric antibody, ChiTn, derived from the mouse anti-83D4 antibody, as an ADC for cancer treatment. The ChiTn antibody selectively accumulated in the solid tumor, but not in healthy tissue. When conjugated to saporin (SAP) or to auristatin F, the Chi-Tn ADC exhibited effective cytotoxicity to Tn-positive tumor cells *in vitro* and conjugated to MMAF also induced a delay of tumor growth *in*

TABLE 2 Overview of the (S)Tn directed therapies described in this study.

Target	Type of therapy	Therapeutic construct	Antigenic determinant	Example	Reference
Tn	Anti-Tn antibody & antibody-drug conjugates	Anti-Tn antibody	Tn single residue	Kt-IgM-8	(137)
			Tn-MUC2 complex	PMH1	(89)
			Tn-MUC1 complex	SM3	(139)
				PankoMab	(140)
				5E5	(141)
			Consecutive Tn residues	MLS128	(142)
				83D4	(143)
				KM3413	(144)
				Ca3638	(145)
			Tri-Serine Tn	15G9	(146)
		Humanized anti-Tn antibody	Consecutive Tn residues	cKM3413	(144)
			Tn-MUC1 complex	CIM301-1	(147)
				CIM301-8	(147)
				PankoMab-GEX	(129, 130)
			Consecutive Tn residues	ChiTn	(148)
		Anti-Tn drug conjugate	Consecutive Tn residues	ChiTn - drug conjugate	(148)
		Humanized anti-Tn antibody combination therapy	Tn-MUC1 complex	PankoMab-GEX + anti-EGFR	(131)
	Tn-based vaccines	Anti-Tn vaccine	Tn single residue	MAG-Tn-TT	(149)
	Tn-based CAR-T cell therapy	Anti-Tn CAR-T	Tn-MUC1 complex	Anti-Tn 5E5 CAR-T	(150)
				Anti-Tn SM3 CAR-T	(135, 136)
				Anti-TnMUC1 CAR-T	(151)
STn	Anti-STn antibody & antibody-drug conjugates	Radiolabeled murine anti-STn		^{177}Lu-CC49	(152)
				^{131}I-CC49	(153)
		Radiolabeled murine anti-STn combination therapy		^{131}I-CC49 + IFN	(153)
		Humanized anti-STn antibody		HuCC49ΔCH2	(154)
				^{225}Ac-DOTaylated-huCC49	(155)
		Anti-STn drug conjugate		CC49-Br-MMAE	(156)
				SF3-MMAE	(157)
	STn-based vaccins	Anti-STn vaccin		Theratope	(158–162)
	STn-based CAR-T cell therapy	Anti-STn CAR-T		Anti-STn CC49 CAR-T	(134, 163, 164)
		Dual-specific CAR-T		Dual STn- and CD30-specific CAR-T	(165)
				Dual STn- and CD47-specific CAR-T	(166)

This table includes therapeutic target, type of therapy, therapeutic construct and antigenic determinant.

vivo, validating for the first time the use of an anti-Tn antibody as an effective ADC (148).

The anti-STn antibody CC49 has shown promising. Radiolabeled 177Lu-CC49 was initially demonstrated to provide significant anti-tumor activity *in vivo* (152), which led to its evaluation in phase I and phase I/II radioimmunotherapy clinical trials (132, 133). Here, the 177Lu-CC49 antibody was shown to be well-tolerated and anti-tumor effects were confirmed in patients with chemoresistant ovarian cancer. A differently radiolabeled CC49 antibody, 131I-CC49, was tested in patients with hormone-resistant metastatic prostate cancer. Phase II clinical studies demonstrated enhanced tumor uptake and anti-tumor activity for this antibody in the presence of interferon (IFN) which promoted increased tumor antigen expression and localization compared to the use of the 131I-CC49 antibody alone (153). However, the generation of human anti-murine antibody responses and bone marrow suppression represented two major adverse side-effects, which limited the therapeutic use of these radiolabeled anti-CC49 antibodies. A solution was reported by Rogers et al. (2005) (154) who presented the development of a humanized CC49 antibody, huCC49ΔCH2, and demonstrated less bone marrow toxicity as well as prolonged median survival *in vivo*, probably due to the acceptance of higher radiation doses without limiting side effects and off-side toxicity. In 2021, Minnix et al. (155) described the use of another humanized CC49 antibody construction, 225Ac-labeled DOTaylated-huCC49, in an *in vivo* murine model of ovarian cancer, which showed reduced tumor growth and increased survival without considerable off-side toxicity. Minnix et al. (2020) (156) also proposed an alternative therapeutic strategy using antibody-drug conjugates. The same abovementioned murine model was used to evaluate treatment with the anti-STn antibody-drug conjugate, CC49-Br-monomethyl auristatin E (MMAE, developed from CC49 antibody and monomethyl auristatin E). Reduced tumor growth as well as increased survival were found in mice receiving CC49-Br-MMAE treatment. Finally, another anti-STn antibody-drug conjugate, SF3-MMAE, was reported to be well tolerated and inhibit tumor growth in murine breast and colon cancer models (157) (Table 2).

4.2.2 Tn/STn-based vaccines

Another therapeutic strategy is the use of Tn- and STn-based vaccines. In a phase I clinical trial among breast cancer patients with high-risk of relapse, Rosenbaum et al. (2020) (149) evaluated the efficiency of the multiple antigenic glycopeptide-Tn-tetanus toxoid-derived TT830-844 (MAG-Tn-TT) vaccine. This study revealed high levels of Tn-specific antibodies which were found to induce complement dependent cytotoxicity (CDC) and subsequent tumor cell death in all vaccinated patients. It is of note that carbohydrates can induce immune tolerance towards the tumor, which complicates the clinical success of glycan-based cancer vaccines. The development of efficient Tn-based vaccines, such as the MAG-Tn-TT vaccine, might therefore be a complicated process rather than standard therapeutic strategy (167–170).

The same holds for the STn antigen. The generation of an efficient STn-based anti-tumor immunotherapy is challenging due to its poor immunogenicity (71, 171). However, surprisingly, the best characterized STn-targeting therapy is a STn-based vaccine called Theratope. As delayed tumor growth by efficient antibody responses was observed in *in vivo* models receiving Theratope (158, 159), anti-STn immune responses and increased survival were initially also observed in phase I and II clinical studies with Theratope-treated pancreatic, colorectal, breast and ovarian cancer patients (160–162). Nevertheless, phase III clinical trials with metastatic breast cancer patients did not show this overall beneficial anti-tumor activity of Theratope, either alone or in combination treatment with endocrine therapy (172). An extensive review on Theratope´s composition and all clinical studies performed with this STn-based vaccine was published by Julien et al. (2012) (51).

4.2.3 Tn/STn-based CAR-T cell therapy

The construction of anti-STn CAR-T cells and their anti-tumor activity *in vitro* was first described by Hombach et al. (163) in 1997, after which therapeutic benefit of anti-STn CC49-based CAR-T cells was proposed based on *in vivo* models of colon and endometrial carcinoma by McGuinness et al. (164) in 1999. Based on these preliminary findings, several anti-STn CAR-T cell engineering strategies have been evaluated. For example, dual-specific CAR-T cells targeting a second additional antigen, such as CD30 or CD47, have been developed to potentiate the anti-tumor activity of anti-STn CAR-T cells (165, 166). Indeed, Shu et al. (2021) (166) reported delayed tumor growth in low STn expressing tumors when treated with the dual STn- and CD47-specific CAR-T cells, but not after therapy with the single anti-STn CAR-T cells in an *in vivo* ovarian cancer model. It is of note that single anti-STn CAR-T cell therapy was found to be sufficient to obtain the same result in high STn expressing tumors. Finally, the first phase I clinical trials with anti-STn CC49-based CAR-T cells as immunotherapy for solid tumors were published by Hege et al. (134) in 2017. Even though these anti-STn engineered T cells were found to be relatively safe, no considerable clinical efficacy was observed in metastatic CRC patients. Here, rapid clearance of those anti-STn CAR-T cells was proposed as one of the limitations to be overcome for significant therapeutic benefit (Table 2).

CAR-T cell strategies targeting Tn or STn have been extensively studied as well. Posey et al. (2016) (150) used the scFv of the abovementioned anti-Tn 5E5 antibody for the development of anti-Tn CAR-T cells, which subsequently showed anti-tumor activity in murine models of pancreatic cancer and leukemia. Anti-Tn CAR-T cells with alternative Tn recognition domains have also been developed and showed therapeutic benefit in *in vivo* models of head and neck cancer (173) and breast and gastric carcinoma (174). In this last case, as an alternative to the use of αβ T cells a subtype of γδ T cells were used, and comparison of both CAR-Ts demonstrated a better cytotoxic effect with the γδ CAR-T cells both *in vitro* and *in vivo*. Additionally, anti-Tn SM3 antibody specificity has been used for the generation of multiple CAR-T constructs, which have been tested in a variety of clinical studies (135, 136). Significant clinical efficacy without evidence of adverse side-effects and safety concerns has been observed in phase I trials including patients with

metastatic seminal vesicle cancer (135) and lung cancer (136). Finally, therapeutic efficacy as well as tolerability and safety of anti-TnMUC1 CAR-T cells are being evaluated in a phase I clinical trial among patients with TnMUC1+ multiple myeloma and solid tumors such as lung cancer, breast cancer, pancreatic cancer and ovarian cancer, by Gutierrez et al. (2021) (151). Preliminary efficacy assessed by RECIST v1.1 at Day +28 demonstrate stable disease in all patients that followed lymph depletion chemotherapy.

4.3 GD2 & O-GD2

GD2 might be the most relevant glycan in the clinic. Two different antibodies, namely anti-GD2 antibodies 3F8 and 14.18, have been the basis for the generation of clinically successful anti-GD2 therapy (Figure 2).

After a variety of phase I clinical studies indicating clinical safety and effectiveness without significant toxicity, either after single or repeated ch14.18 antibody administration (175, 176), a phase II clinical trial showed that the use of ch14.18 combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL2) compared to the ch14.18 alone in metastatic neuroblastoma patients was associated with a significantly improved outcome as compared with standard therapy in patients with high-risk neuroblastoma (177). This observation was emphasized by Simon et al. (2004) (178), who demonstrated that the administration of ch14.18 alone did not improve progression-free survival in stage 4 neuroblastoma patients. In 2010, Yu et al. (179) reported a significant increase in event-free survival and overall survival for high-risk neuroblastoma patients treated with a combination of anti-GD2 mAb ch14.18 with IL2 and GM-CSF compared to patients who received standard treatment in a phase III clinical study. Based on these results, Dinutuximab (anti-GD2 ch14.18 antibody) in combination with IL2 and GM-CSF was approved by the United States Food and Drug Administration (FDA) in 2015 for the treatment of high-risk neuroblastoma patients (180). The mechanism of action behind Dinutuximab is based on GD2 binding and subsequent induction of antibody-dependent cell-mediated cytotoxicity (ADCC) as well as CDC, after which recruitment of granulocytes and NK cells finally provoke tumor cell death. In 2017, Dinutuximab β (ch14.18/CHO) was approved by the European Commission for the treatment of high-risk neuroblastoma patients in Europe, whereas Naxitamab (a humanized mAb Hu3F8) in combination with GM-CSF was approved by the FDA in 2020 for the treatment of relapsed high-risk neuroblastoma patients or those who show refractory disease in the bone or bone marrow (181). Currently, several clinical trials are ongoing which will evaluate the potential of Dinutuximab and other hu14.18- and hu3F8-based anti-GD2 antibodies as therapeutic strategies for neuroblastoma and other cancer types including lung cancer, melanoma and osteosarcoma (138, 182). Additionally, alternative therapeutic constructs of anti-GD2 antibodies are being extensively studied, such as radiolabeled antibodies, antibodies modified for drug delivery, GD2-based vaccines, GD2-specific chimeric antigen receptor (CAR) T cells and bispecific antibodies, among others (138, 182). Accordingly, the number of clinical trials evaluating anti-GD2 antibodies for cancer immunotherapy is rapidly growing. An extensive review on approved anti-GD2 antibody-based cancer treatments as well as the many ongoing clinical trials was recently published by Nazha et al. (2020) (182) and Berois et al. (2022) (138).

However, a major bottleneck to the clinical application of anti-GD2-based immunotherapy is the presence of significant adverse side-effects such as neuropathic pain, due to GD2 expression among peripheral nerves (96). In addition to further studies into strategies that circumvent or diminish those significant side effects, alternative approaches for anti-GD2 cancer immunotherapy are being evaluated. Accordingly, the O-GD2 antigen has shown great potential as therapeutic target for cancer immunotherapy due to its absence on healthy tissues, supposing safer therapeutic options and improved treatment tolerance (96). Decades ago Cerato et al. (1997) (183) developed a mouse 8B6-antibody specific for O-GD2 (Table 3). Over time, this antibody has been proven to be as efficient as anti-GD2 antibodies regarding the induction of ADCC and CDC (95, 96, 184). Additionally, Cochonneau et al. (2013) (185) proposed a role for the anti-O-GD2 8B6 antibody in tumor cell death by the induction of cell cycle arrest and apoptosis. Even though the exact mechanism behind this observation remains to be elucidated, the apoptosis inducing activity of this O-GD2 antibody might clinically be of major importance, especially in the treatment of tumors that are able to protect themselves from immunological cytotoxicity. For clinical purposes, an alternative mouse/human chimeric version of the 8B6 antibody (c.8B6), maintaining antigen binding affinity and specificity characteristics, was developed by Terme et al. (2014) (184) (Table 3). The c.8B6 O-GD2 antibody was found to be as effective as the ch14.18 GD2 antibody, but did not provoke any significant adverse side effects in an *in vivo* model, emphasizing the potentially major clinical benefit of O-GD2-based therapeutic approaches. Nevertheless, whereas many clinical trials with anti-GD2-based antibody therapies are ongoing, clinical trials with an anti-O-GD2 c.8B6 antibody are still awaited.

5 Concluding remarks & future perspectives

Taken together, the reviewed antigens represent potential biomarkers and attractive therapeutic targets for personalized medicine in cancer treatment.

The three mentioned TAAs could serve as biomarkers in liquid biopsy and provide valuable information about the presence, progression, and characteristics of tumors. Liquid biopsy involves the analysis of various biomarkers, including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and proteins, obtained from a patient's blood or other bodily fluids (Lodewijk et al., 2018). TAAs can be detected and measured in these samples and offer insights into the underlying cancer biology. Even though some studies have reported the presence of CD44v6, STn and GD2 antigens in serum, no extensive evaluations of their potential as valuable diagnostic, prognostic and/or predictive biomarkers in liquid biopsy have been

TABLE 3 Overview of the (O-)GD2-directed therapies described in this study.

Target	Type of therapy	Therapeutic construct	Example	Reference
GD2	Anti-GD2 antibody	Anti-GD2 combination therapy	Dinutuximab (anti-GD2 ch14.18) + IL2 + GM-CSF	(179)
			Naxitamab (anti-GD2 hu3F8) + GM-CSF	(181)
		Anti-GD2 antibody	Dinutuximab β	(180)
		Anti-GD2 radiolabeled antibodies	Dinutuximab and other hu14.18- and hu3F8-based anti-GD2 antibody constructs	(138, 182)
		Anti-GD2 antibodies for drug delivery		
		GD2-based vaccins		
		GD2-specific CAR-T cells		
		Bispecific antibodies		
O-GD2	Anti-O-GD2 antibody	Murine anti-O-GD2 antibody	Anti-O-GD2 8B6 antibody	(183)
		Chimeric anti-O-GD2 antibody	Anti-O-GD2 c.8B6 antibody	(184)

This table includes therapeutic target, type of therapy, therapeutic construct and antigenic determinant.

described. It is of note that the presence of promising biomarker and therapeutic target O-GD2 in serum has not been reported. However, this might be explained by both the recent interest and limited approaches to study the regulatory mechanisms of O-GD2 expression, and further studies into the expression of this antigen are needed to examine the presence of this glycan in serum. Importantly, CD44v6 expression has only been detected on CTCs, whereas STn glycans have been found to be present either on CTCs or as secreted products from tumors. Contrarily, GD2 expression has not been observed on CTCs and is found in the serum as secreted products either from the tumor or exosomes. This observation emphasizes the importance of evaluation of different components of liquid biopsies in order to characterize the presence of newly described antigens in liquid biopsies. Moreover, liquid biopsies not only include serum, but various biological fluids, such as pleural liquid, cerebrospinal fluid, saliva and urine. Therefore, dependent on the cancer type, it might be important to examine CD44v6, STn and O-GD2 expression in other fluids additional to serum. For example, as expression of all those antigens has been observed in lung cancer, the evaluation of pleural liquid might be of great interest. It's important to note that the choice of TAAs as biomarkers in liquid biopsy depends on the specific type of cancer and the unique genetic alterations associated with it. Different cancers have distinct TAAs, and ongoing research continues to identify and validate novel TAAs for liquid biopsy applications. The utilization of TAAs as biomarkers in liquid biopsy holds promise for non-invasive cancer detection, monitoring, and personalized treatment decisions.

In conclusion, the identification and targeting of tumor-associated antigens have opened up exciting new avenues in cancer treatment. Immunotherapies based on TAAs, such as immune checkpoint inhibitors, cancer vaccines, and CAR-T-cell therapy, have shown great promise in improving patient outcomes. However, it is important to note that the field of TAA-based therapies is still evolving, and further research is needed to optimize treatment strategies, overcome resistance mechanisms, and broaden the application to a wider range of cancer types. Nonetheless, the progress made so far suggests the tremendous potential for the future of cancer treatment.

Author contributions

CR: Conceptualization, Supervision, Writing – review & editing, Data curation, Validation, Visualization, Funding acquisition, Investigation, Project administration. IL: Conceptualization, Data curation, Investigation, Resources, Writing – original draft. MD: Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Validation, Visualization, Writing – original draft. JP: Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Validation, Visualization, Writing – review & editing.

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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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Case Report: Overcoming challenges in pancreatic cancer with liver metastases: a personalized therapeutic odyssey of TACE, ablation, and immunotherapy

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Pancreatic cancer represents a malignant neoplasm originating from pancreatic cells. The optimal approach to cancer treatment remains uncertain, lacking a definitive consensus. Here, we present a compelling case of a 49-year-old female with pancreatic head cancer with liver metastases, as identified by CT and confirmed by biopsy. PET-CT indicated widespread metastatic involvement. TACE therapy with gemcitabine and cisplatin was initiated, yielding a stable disease response. The patient's high PD-L1 expression prompted TACE-PD-1 monoclonal antibody combination therapy. Subsequent treatments, including ablation, sustained PD-1 immunotherapy, and consolidation TACE, culminated in a complete response, as evidenced by imaging and tumor marker dynamics. Our case underscores the potential of multifaceted strategies in managing aggressive pancreatic cancer.

KEYWORDS

pancreatic cancer, immunotherapy therapy, transarterial chemoembolization (TACE), PD-L1, PD-1, microwave ablation

Introduction

Pancreatic cancer (PC) is a formidable malignancy with a mere 11% 5-year survival rate (1). While strides in immunotherapy and targeted interventions have propelled favorable outcomes across various malignancies (2, 3), these strides have yet to encompass PC. Surgical intervention emerges as the primary treatment avenue for localized early-stage pancreatic cancer, whereas chemotherapy prevails as the frontline regimen for advanced and metastatic cases (4–6). Although surgical intervention thrives in managing early-stage cases, most patients present with advanced stages, often accompanied by liver metastases, rendering them ineligible for surgical intervention. Thus, the quest for pioneering and

efficacious therapeutic avenues paramount significance in elevating survival rates and enhancing the quality of life for pancreatic cancer patients.

Transarterial chemoembolization (TACE), a minimally invasive interventional radiology technique orchestrating the targeted delivery of chemotherapy and embolic agents via the arterial blood supply, has been integrated with systemic chemotherapy in hepatocellular carcinoma management, yielding significant survival extensions (6, 7). Though the application of TACE in addressing pancreatic malignancies encounters hurdles due to the anatomical intricacies and distinctive biological behavior of pancreatic cancer (8), the potential efficacy of TACE alongside systemic chemotherapy shows a promising therapeutic course for those individuals with concurrent hepatic metastases. TACE delivers concentrated chemotherapy agents directly to intrahepatic metastases, facilitating localized control of tumor proliferation. Furthermore, embolic agents exert a pivotal role in interrupting tumor blood supply, ameliorating the tumor microenvironment, and potentiating the cytotoxic impact of chemotherapeutic agents. Cooperatively, the adjunctive use of systemic therapy (including chemotherapy, targeted modalities, immunotherapy, etc.) functions systemically to inhibit tumor propagation and progression. Microwave ablation harnesses microwave energy to heat tumor cells rapidly, expediting the generation of high temperatures and making it particularly suited for more extensive and challenging-to-access tumor lesions (9). Its application in liver cancer therapy is attracting increasing interest (10). Moreover, within the realm of clinical practice involving pancreatic cancer liver metastases, microwave ablation emerges as a promising innovative approach that augments the overall therapeutic efficacy by diminishing tumor foci (11). Concurrently, despite PD-1 monoclonal antibody therapy not yet attaining the status of a definitive standard for pancreatic cancer treatment, its potential therapeutic gains in patients exhibiting elevated PD-L1 expression warrant careful consideration (12).

Consequently, combining diverse treatment modalities such as TACE, microwave ablation, and immunotherapy unfolds expansive vistas for creating custom therapeutic regimens for individual patients. This comprehensive and multimodal treatment strategy holds the potential to yield marked clinical advancements and survival benefits for individuals navigating the complexities of pancreatic cancer accompanied by liver metastasis. Here, we present a compelling case study of a PC diagnosis achieved through CT imaging and liver biopsy. Our patient underwent a comprehensive therapeutic approach, synergizing TACE, microwave ablation, and immunotherapy, yielding satisfying therapeutic outcomes with commendable tolerability. The amalgamation of TACE, microwave ablation, and immunotherapy effectively enhances the therapeutic impact in managing PC, surpassing the confines of singular modalities.

Case presentation

A 49-year-old female was found to have a pancreatic head mass and multiple low-density lesions in the liver on computed tomography (CT) in October 2020, consistent with pancreatic head cancer with liver metastases. A liver biopsy was conducted, and subsequent cytological analysis confirmed the presence of cancer. Positron emission tomography-computed tomography (PET-CT) showed a pancreatic mass with high 18F-fluorodeoxyglucose (FDG) uptake, suggesting metastasis, liver metastasis, portal vein tumor thrombus, and metastasis to peripancreatic and retroperitoneal lymph nodes. Histopathological examination of the liver biopsy showed poorly differentiated carcinoma with an adenocarcinoma-like tendency. Based on the clinical presentation, pancreatic cancer with metastasis cannot be excluded. Notably, there exists an absence of prior tobacco and alcohol consumption within the patient's history, as well as an absence of previous exposure to pharmaceutical treatments. There is no reported history of malignant tumors within the patient's familial lineage.

Subsequently, the patient initiated a treatment plan involving TACE (transarterial chemoembolization) therapy, which coincided with gemcitabine, cisplatin, and pirarubicin (THP). These sessions occurred on October 28, November 24, and December 18, 2020. The procedure encompassed the administration of gemcitabine at a dose of 0.8mg and cisplatin at 30mg into the celiac trunk and superior mesenteric artery, correspondingly. Additionally, a dosage of THP60mg and super liquefied lipiodol at 2ml was introduced into the liver tumor's feeding artery via the superior mesenteric artery route.

On December 18, a CT scan revealed infiltration of the portal vein and superior mesenteric vein by the pancreatic neck mass, with suspected involvement of celiac trunk branches. Multiple metastatic lymph nodes were also observed in the peripancreatic region, retroperitoneum, and porta hepatis. The liver exhibited various metastatic nodules, some showing changes after treatment. Relative to the CT scan findings from October 14, 2020, the tumor size was not significantly reduced and didn't escalate in its dimensions; hence, the patient's response to the three times TACE interventions was evaluated as stable disease (SD).

Given the positive PD-L1 test results from the patient's tumor biopsy sample (Figure 1), we adjusted the patient's treatment strategy to TACE combine with a PD-1 monoclonal antibody therapy. Briefly, starting from December 19, 2020, the initiation of PD-1 inhibitor therapy has been established on a regimen of administration once every three weeks, devoid of interruptions. TACE interventions were conducted on January 13, February 4, and March 11, 2021. The procedure involved injecting 1.6g of gemcitabine into the celiac trunk and superior mesenteric artery. Additionally, 200mg of albumin-bound paclitaxel (Abraxane) was administered into the hepatic tumor-feeding artery, while THP 60mg, along with 2 ml of highly liquid lipiodol, was introduced into the superior mesenteric artery's feeding artery. In the follow-up CT on February 4, 2021, several smaller nodular masses were observed above the pancreas's neck, the liver's hilar region, and the retroperitoneum compared to previous scans. While multiple nodules were still present in the liver, some exhibited slight changes following treatment, although not significantly different from before (Figure 2). As a result, the collective impact of the three interventions, along with PD-1 immunotherapy, was classified as a partial response (PR).

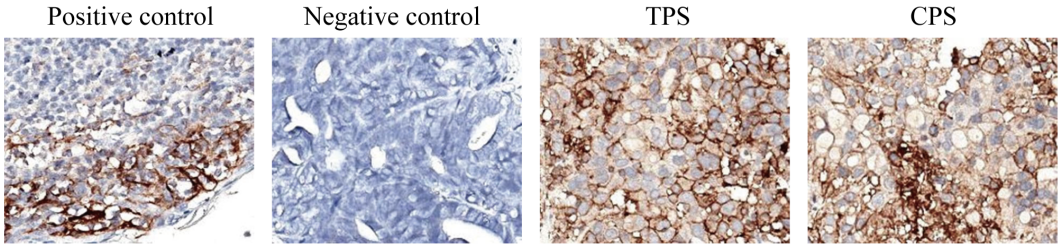

FIGURE 1
The PD-L1 test results from the patient's tumor biopsy sample. The TPS is over 95%, and the CPS is over 90. (TPS, tumor proportion score, the ratio of PD-L1 stained tumor cells to total tumor cells x 100%; CPS, combined positive score; the ratio of PD-L1 stained cells to total tumor cells, including tumor cells, lymphocytes, and macrophages x 100. The negative expression of PD-L1 is TPS < 1%; the low expression is TPS > 1 and < 49%; and the high expression is TPS ≥ 50%).

As the reduced size of the liver metastatic lesions, in alignment with microwave ablation treatment protocols, microwave ablation of the liver metastasis was executed successfully on April 15, 2021, at our institution. The patient exhibited favorable postoperative recuperation. A subsequent magnetic resonance imaging (MRI) scan on May 20, 2021, revealed multiple nodules above the pancreas's neck, within the hilar region of the liver and retroperitoneum, consistent with previous observations; Multiple liver masses with potential for post-treatment changes; Multiple small cysts in the liver may also be seen, with some edges slightly blurry. The strategy entails sustaining PD-1 immunotherapy to consolidate the therapeutic approach further. The administration regimen for PD-1 treatment involves a frequency of once every three weeks, with a dosage of 200 mg per session by intravenous infusion for one hour per session. Meanwhile, patients underwent TACE treatment on May 21, July 20, September 22, and November 24, 2021. Upon MRI evaluation on May 20, 2021, the tumor lacked developmental progression, primarily characterized by a perfusion-oriented nature, negating the necessity for embolization. Hence, the regiment deducted the THP 60mg and 2ml of highly liquid lipiodol, only injecting 1.6mg of gemcitabine into the celiac trunk and superior mesenteric artery and infusing 200mg of Abraxane into the abdominal trunk and superior mesenteric artery. Following these four consolidation TACE sessions, the patient's therapeutic response consistently maintained a PR. Then, continue to PD-1 monoclonal antibody treatment consistent with the previous regimen. 2022.01.27 follow-up PET-CT showed no obvious space-occupying lesions, no increased FDG metabolism, two slightly low-density foci in the liver, and no increase in FDG metabolism. Peripancreatic and retroperitoneal metastatic lymph nodes, as well as the portal vein tumor thrombus, disappeared. These findings collectively indicated that the patients' treatment had achieved complete response (CR). After that, the patients were treated with PD-1 monoclonal antibody consistent with the

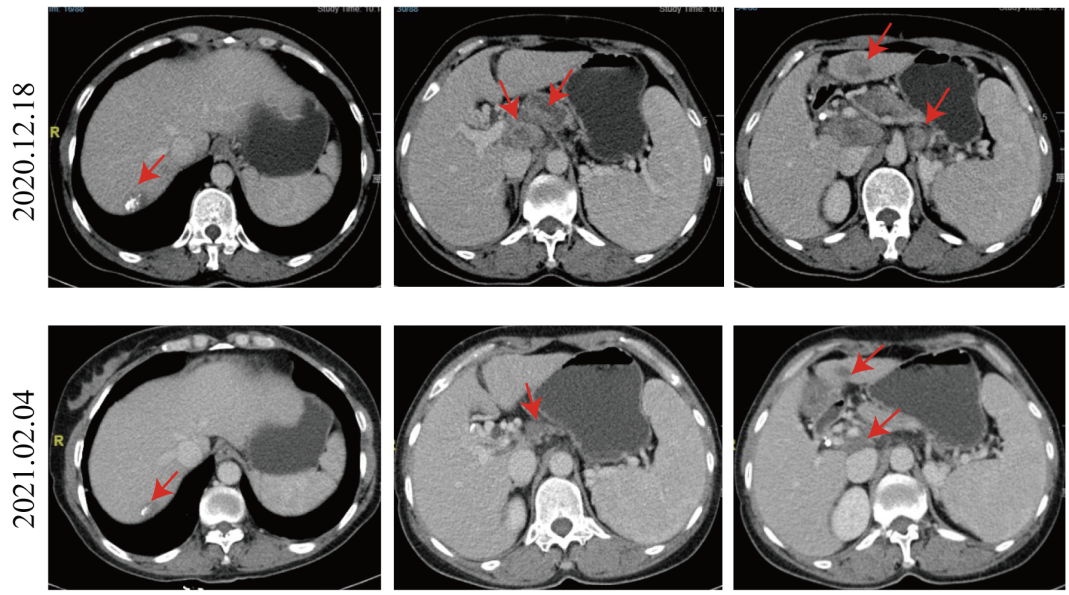

FIGURE 2
Comparison of CT images taken on December 18, 2020, and February 4, 2021. Compared to the CT image of 2020.12.18, the nodular masses of the pancreas's neck, the liver's hilar region, and the retroperitoneum were smaller. Multiple nodules were still present in the liver, and some exhibited slight changes following treatment, not significantly different from before.

previous regimen, and PET-CT was examined again on 2023.01.03. The results were the same as those of 2022.01.27, indicating that the patients maintained the state of CR after PD-1 monotherapy (Figure 3). Furthermore, we supervised alterations in blood tumor markers, including CA125, CA199, and CA50, from the start of treatment (Figure 4), which exhibited a transient elevation during the initial treatment phase and a subsequent decline below baseline levels. CA199, the most prevalent and well-studied pancreatic cancer marker, measures tumor burden but not metastatic potential (13). CA 50, a tumor marker for pancreatic cancer, is typically used with CA 199 to assess the prognosis (14). CA125 is a tumor marker for ovarian cancer and the most significant predictor of pancreatic cancer with metastases in recent years (15, 16). Our clinical experience and prior studies show that monitoring CA199, CA125, and CA50 as a group can better assess the prognosis of pancreatic with metastases (15, 17). This observation underscores the effectiveness of TACE coupled with systemic therapy in this patient.

Discussion

This case report encapsulates a compelling therapeutic exploration and intervention journey in a 49-year-old female patient diagnosed with pancreatic head cancer and concurrent liver metastases. The timeline of the patient's treatment is described in Figure 5. The complex coordination of precise therapeutic interventions, informed by advancing diagnostic knowledge, has significant ramifications for managing advanced cancerous conditions. The initial diagnostic process, driven by computed tomography (CT) and cytological investigation, revealed an intricate scenario marked by a tumor in the pancreas

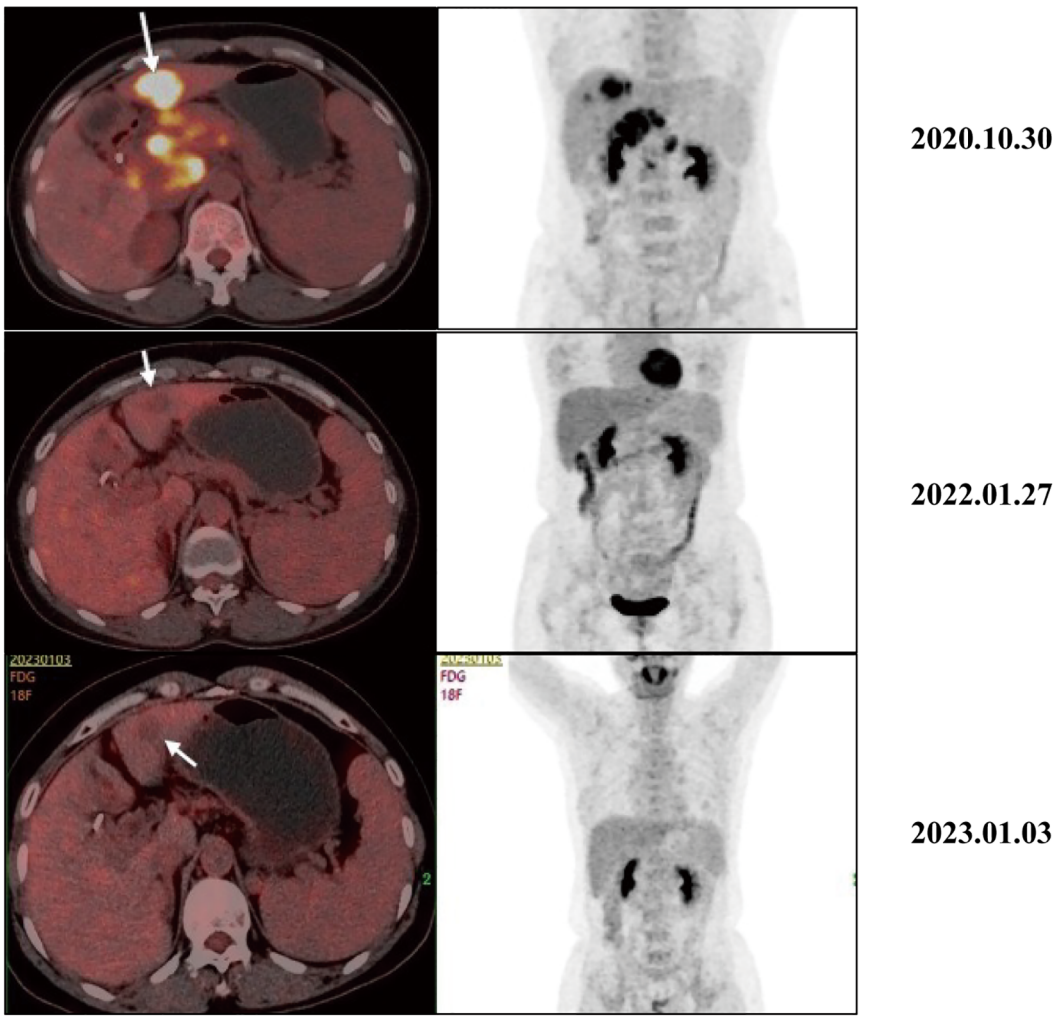

FIGURE 3
Typical PET-CT images at different time points before and after receiving treatment. The PET-CT of 2020.10.30 showed a mass with high FDG uptake, suggesting metastasis, liver metastasis, portal vein tumor thrombus, and metastasis to peripancreatic and retroperitoneal lymph nodes. After receiving treatment, intrahepatic lesions initially showed a significant decrease in size and FDG metabolism. In the 2022.01.27 and 2023.01.03 images, no increase in FDG metabolism was observed, and the primary peripancreatic and retroperitoneal metastatic lymph nodes and portal vein cancer thrombi have subsided.

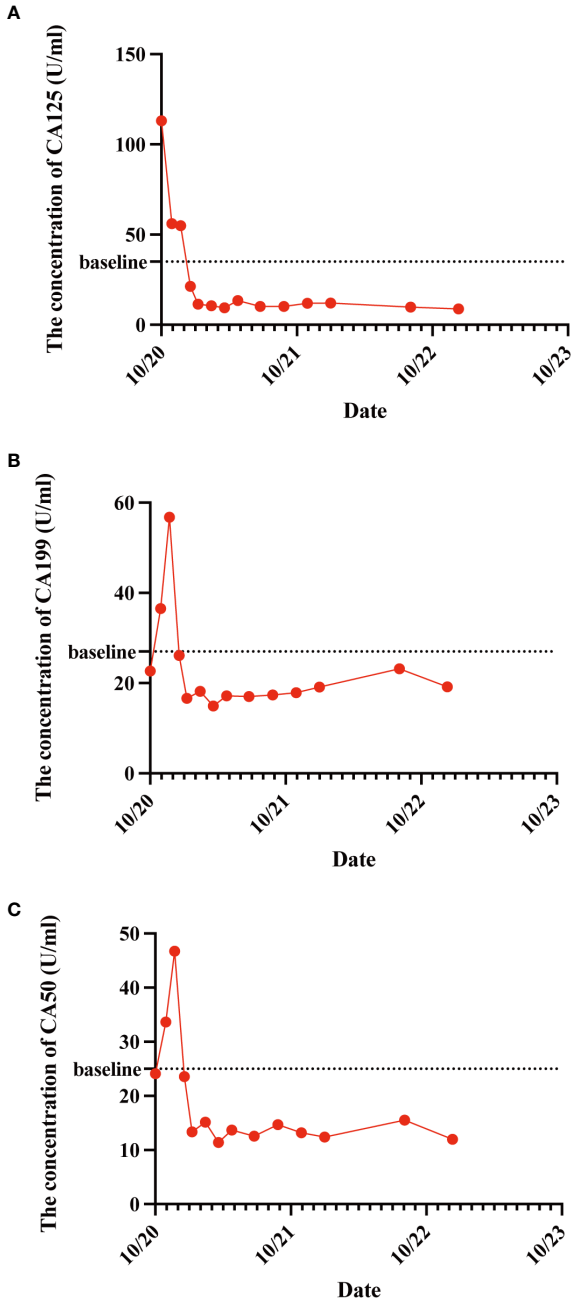

FIGURE 4
The variations in patient CA125 **(A)**, CA199 **(B)**, and CA50 **(C)** concentration levels before and after treatment administration. These tumor markers exhibited a transient elevation during the initial treatment phase and a subsequent decline below baseline levels.

head and liver lesions that suggest metastasis. Significantly, the subsequent PET-CT scan revealed a range of metastatic spread extending from the pancreatic mass to the surrounding peripancreatic and retroperitoneal lymph nodes and a tumor thrombus in the portal vein. The histological examination of the liver biopsy provided further insight into the malignancy, revealing a poorly differentiated carcinoma with adenocarcinoma-like features, thereby reinforcing the ominous nature of the disease.

The treatment trajectory commenced with TACE interventions strategically synchronized with gemcitabine and cisplatin administration. These sessions yielded an SD status and paved the path for further refinements in the therapeutic strategy. The revelation of positive PD-L1 evaluation results from the patient's tumor biopsy sample prompted a paradigm shift. A harmonized approach was adopted, combining TACE PD-1 monoclonal antibody therapy. The ensuing PD-1 immunotherapy regimen, marked by consistent administration, reflected the commitment to harnessing the potential of immunomodulation. Microwave ablation's judicious application further accentuated the comprehensive approach. Its success in diminishing liver metastatic lesions demonstrated the merit of multidisciplinary interventions. Subsequent assessments, compared with earlier

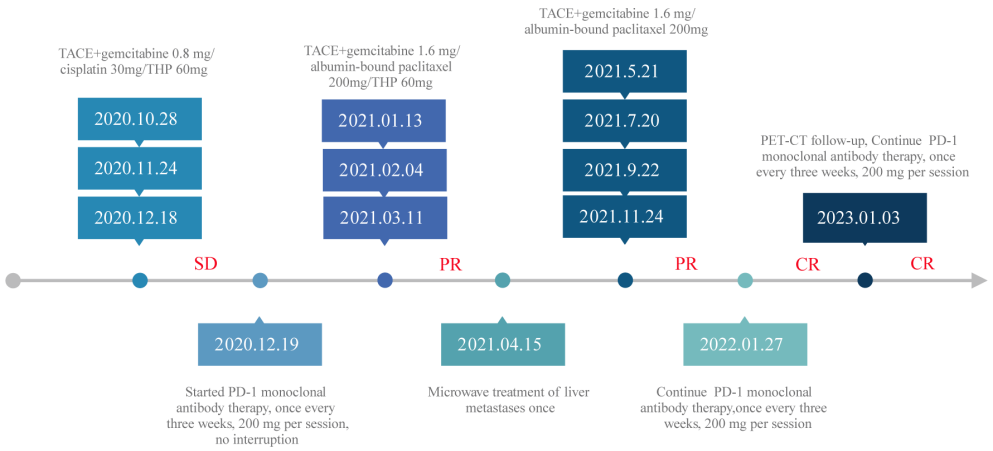

FIGURE 5
The timeline of the patient's treatment.

imaging, underscored the effectiveness of this strategy, revealing a transition to PR and emphasizing the cumulative effect of the therapeutic synergism. Continued vigilance and adaptability remained the cornerstone of this intricate endeavor. The strategic sequencing of subsequent TACE sessions fortified the therapeutic gains, culminating in a sustained PR. Notably, with the continuous treatment of PD-1 monoclonal antibody, the patient's trajectory culminated in CR status, substantiated by the vanishing of metastatic foci, nodules, and tumor thrombus, as evidenced in imaging studies by follow-up PET-CT scans and blood tumor markers. The fluctuation of blood tumor markers, CA125, CA199, and CA50, presented a dynamic narrative, mirroring the treatment trajectory. The transient elevation during initial treatment, followed by a decline, underscored the intricate interplay between therapies and disease dynamics. This is a testament to the clinical relevance of tumor marker surveillance, reflective of therapeutic efficacy.

However, it is imperative to acknowledge that our case report has limitations. The study's reliance on a solitary case report curtails how much our findings can be extrapolated to a broader populace. Furthermore, including a solitary patient in the study might not comprehensively encapsulate the diverse spectrum of individuals grappling with the intricacies of pancreatic cancer and liver metastases. Nevertheless, it is essential to underscore that our case represents an incipient stride towards tailoring treatment strategies in accordance with individual patient profiles. This pivotal endeavor serves as a bedrock for the precision-based management of malignancies. In the trajectory ahead, we strongly advocate for the inclusion of a broader and more appropriate cohort of patients to conduct methodologically robust clinical trials. This prospective undertaking holds promise in unearthing novel avenues for treating advanced pancreatic cancer, enriching the therapeutic landscape.

In conclusion, a significant factor contributing to CR in this case was our keen understanding of the patient's disease trajectory, which enabled us to tailor treatment plans accordingly. Initially, a combination of TACE and chemotherapy was swiftly employed to stabilize the patient's condition. After positive outcomes from the patient's PD-L1 testing and considering their overall health, an opportunity for immunotherapy emerged. Promptly, PD-1 monoclonal antibody treatment was administered. Concurrently, we maintained vigilant surveillance of treatment response and strategically introduced TACE and microwave ablation during immunotherapy to enhance the release of tumor antigens, thereby amplifying the immune response. Consequently, this success can be attributed to the meticulous fusion of TACE, PD-1 monoclonal antibody therapy, and microwave ablation, harmonized with the patient's individual medical profile. It is imperative to note that this innovative approach is an initial endeavor, and its replicability in yielding such favorable outcomes for other patients warrants further in-depth investigation through additional case studies.

Conclusion

In summary, this case report underscores the remarkable efficacy of our combined therapeutic approach, showcasing the transformative potential of precision medicine in achieving and maintaining complete remission in advanced metastatic pancreatic cancer. Integrating TACE and PD-1 immunotherapy and ablative interventions, guided by comprehensive imaging and tumor marker monitoring, offers a promising paradigm for personalized treatment strategies in similar clinical scenarios. Our findings emphasize the importance of multidisciplinary collaboration and vigilant follow-up in optimizing patient outcomes. Further research and validation are warranted to solidify this innovative approach as a cornerstone in managing aggressive malignancies.

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 humans were approved by Fudan University Shanghai Cancer Center Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

ZM: Validation, Writing – review & editing, Funding acquisition, Resources, Supervision, Visualization. YZ: Conceptualization, Project administration, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. ZN: Methodology, Resources, Supervision, Validation, Writing – review & editing.

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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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Exercise to transform tumours from cold to hot and improve immunotherapy responsiveness

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Exercise provides significant health benefits to patients diagnosed with cancer including improved survival outcomes, quality of life and reduced cancer recurrence. Across multiple murine cancer models, aerobic exercise and resistance training has exhibited anti-tumour properties illustrated by inhibited tumour growth, reduced metastatic potential and modulation of the tumour microenvironment to allow the recognition and destruction of cancer cells. Clinical studies have demonstrated the rapid mobilisation and circulatory release of mature lymphoid populations, myokines and cytokines that occurs with exercise along with tumour vasculature normalisation. Tumour microenvironments enriched with immune cells with anti-cancer potential, such as CD8+ T cells, are termed 'hot', whilst those favouring an immunosuppressive environment and lacking in effector immune cells are classed as 'cold'. Pre-clinical evidence suggests exercise training has the potential to reprogramme cold tumours to become hot, although this requires validation in clinical studies. This hot environment could potentiate immunotherapy responsiveness, improving survival outcomes of patients undergoing cancer immunotherapy and allow those with typically cold tumours to benefit from immunotherapy. This review discusses the complex interactions between exercise and cancer, including exercise-induced alterations within the tumour microenvironment and systemic immunity. The potential role exercise may play in improving cancer immunotherapy responsiveness is explored. This review also highlights the need for translational studies exploring the role of exercise in patients with cancer with the potential to widen the spectrum of tumours that derive significant benefit from immunotherapy.

KEYWORDS

exercise, tumour, immunity, microenvironment, immunotherapy, hot, cold

1 Introduction

1.1 The immune climate of the tumour microenvironment: cold vs hot

Immunotherapies, such as immune checkpoint inhibitors (ICIs), have significantly extended survival outcomes across multiple different cancers. Immune system evasion is a key tactic utilised by cancer cells to allow their growth, migration and invasion. ICIs facilitate cancer cell recognition and immune cell destruction by inhibiting pathways they use to evade the immune system. Although ICIs have demonstrated significant benefits in cancer care, they are limited by a spectrum of potentially serious side-effects, including fatigue and immune-mediated toxicities such as hepatitis, colitis and pneumonitis (1). Fewer than 40% of patients derive significant benefit from ICIs which highlights the need to better understand the mechanisms for resistance to immunotherapy and how to overcome this to improve responsiveness (1, 2). ICI response correlates with the degree of immune cell infiltration within the tumour microenvironment (TME), which varies significantly between tumours on the cold to hot spectrum.

When a TME lacks effector immune cells such as CD8+ T cells and Natural Killer (NK) cells, as in the cases of colorectal and pancreatic cancer, this is considered a 'cold tumour' (3). Myeloid derived suppressor cells (MDSCs) and tumour associated macrophages (TAMs) can be found in abundance within cold tumours creating an immunosuppressive environment and consequently, tumours are able to propagate without significant resistance from the host immune system (3–5). This less favourable immune profile correlates to a limited, if any, response to ICIs (4, 5).

In comparison, 'hot' tumours, such as melanoma, are highly immunogenic with a rich CD8+ T cell population within their TME (5). Therefore, hot tumours hold the potential for immune-mediated recognition and destruction of tumour cells, which cold tumours inherently lack. The abundance and potential anti-cancer activity of these immune components can vary between different hot tumours, partly explaining the varying responses to ICIs amongst hot tumours. Dual ICIs with nivolumab and ipilumumab have some success in the treatment of advanced mesothelioma, which could be classed as a hot tumour due to the presence of immunogenic cells within the tumour microenvironment, although only 40% respond with a median overall survival of 18.1 months (6). This is a relatively modest benefit when compared to ICI response to a classically hot tumour such as melanoma, where the overall survival exceeds 60 months with dual ICIs (7). The TME in mesothelioma is complex with NK cell, CD4+ and CD8+ T cell infiltration supporting its classification as a hot tumour, however functional aberrations prevent their full utility (8). This coupled with high concentrations of pro-tumour TAMs suppress the degree of responsiveness seen with ICIs (8). This implies that tumours lie on a spectrum from cold to hot with some tumours exhibiting both cold and hot characteristics.

There is a growing body of evidence that exercise training can modulate local tumour and systemic immunity in patients with cancer, skewing the immune profile to favour anti-tumour activity. Exercise training could be a simple, safe, cost-effective method to help shift tumours towards the hot end of the spectrum and to support ICIs' mechanism of action. This would lead to improved responses and survival outcomes in patients with hot tumours undergoing immunotherapy, as well as expanding the range of cancers that can obtain benefit from immunotherapy, such as pancreatic cancer.

Furthermore, exercise has known health benefits including enhancing quality of life and ameliorating adverse effects associated with cancer and its treatment (2, 3). The evidence for the effect exercise exerts on different tumour types within the cold-hot spectrum via different mechanisms is described in detail below.

2 Exercise and cancer

The definition of physical activity is "*any bodily movement produced by skeletal muscles or that requires contraction of your muscles and energy expenditure*" (9). In order to be classed as exercise, activity would need to consist of organised, repeated movements which when done regularly, can have a positive impact on cardiovascular and respiratory function, physical fitness and general overall health (9).

Exercise has been shown to have health benefits in the general population, both physically by improving cardiovascular, respiratory and musculoskeletal functioning and mentally (9). The United Kingdom National Health Service (NHS) and the American College of Sports Medicine (ACSM) guidelines recommend regular aerobic exercise (150-300 minutes of moderate intensity or 75-150 minutes of high intensity per week) and resistance training twice weekly (10, 11). These recommendations are provided for the general population, however there is currently no tailored exercise advice to patients with advanced cancer. Given the wide range of functional abilities and background health conditions, a 'one size fits all' exercise regime would not be appropriate.

Evidence shows that the added benefits in patients with cancer include improving overall survival, reducing cancer-related and treatment induced fatigue along with reducing cancer recurrence (4, 12). It would be prudent to define the exercise event to provide a reproducible, standardised intervention in cancer care (4). Acute exercise includes independent exercise activities compared to regularly repeated exercise lasting months with exercise training or more than a year with chronic exercise (4). Studies mainly correlate exercise training at moderate intensity aerobic exercise with the physical and mental health benefits seen in cancer patients (13, 14). Furthermore, the FITT criteria (Frequency, Intensity, Time and Type) would provide information on dosing required to exhibit intended benefits and does require further research in order to cater to the advanced cancer population (4).

2.1 Cold tumours

Colorectal cancer is classically a cold tumour when it possesses microsatellite stability rendering ICIs ineffective (15). There is strong evidence that exercise has a protective role against

colorectal cancer with an estimated relative risk reduction in colorectal cancer development of 12 to 28% (16). One of the proposed mechanisms behind this is the exercise-induced reduction in adipose tissue and the metabolic benefits including optimising insulin sensitivity, although studies suggest other pathways contribute to the reduced cancer risk (16). Although the indirect mechanisms described above are well documented, the direct anti-cancer processes induced by exercise require further exploration.

Zylstra et al. reviewed the effect of incorporating combined moderate intensity supervised and home-based aerobic exercise activities into the pre-operative treatment pathway, consisting of multi-agent neoadjuvant chemotherapy, for potentially resectable oesophageal cancer, which is a cold tumour (13). This single centre controlled study showed that the exercise cohort had a significantly enhanced tumour response to treatment compared to controls (13). This was demonstrated by a higher rate of Mandard Tumour Regression Grades (MTRG) of 1-2 (13). MTRG is a scoring system from 1-5, where a score of 1 indicates no evidence of active cancer cells (complete response) and a score of 5 suggests no evidence of cancer regression (no response) within the pathology specimen. There are multiple limitations to this study including non-randomisation due to geographical issues in delivering supervised exercise activities, small sample size and the modification in clinical guidelines during the study with regards to optimum chemotherapy regimen (13). Given the relatively poor prognosis of oesophageal cancer and limited treatment options, a robust randomised controlled trial is required to support exercise as an effective anti-cancer intervention and determine the effect on more relevant clinical endpoints such as overall survival.

2.2 Hot tumours

Exercise significantly inhibits tumour development and growth in preclinical models of hot tumours including transplanted Lewis lung cancer, diethylnitrosamine induced liver cancer and melanoma (GrM1) mouse models (17). Pedersen et al. (12) demonstrated anti-cancer benefits in female mice that performed exercise training over four weeks. This involved wheel running at an average of 4.1km a day for each mouse, before both subcutaneous implantation and intravenous administration of B16F10 melanoma cells (12). A statistically significant 61% reduction in tumour size and lower incidence of metastatic lung disease in the exercised mice were seen (12). This demonstrates exercise plays a dual role in primary tumour inhibition and prevention of metastases.

Only female mice were inoculated with this model introducing an inherent gender bias. Although the positive effect of wheel running on cancer regression was also demonstrated in five different murine models including diethylnitrosamine induced liver cancer in male Naval Medical Research Institute (NMRI) mice (12). To further explore the mechanism behind the inhibited tumour growth in the B16F10 melanoma model, microarray analysis confirmed increase in immune cell activity with enhanced gene activation, cytokine expression and immune cells both of pro-inflammatory and anti-inflammatory nature (12). Chronic inflammation correlating with increased expression of pro-inflammatory immune components can be associated with the carcinogenic process. Microarray analysis specifically noted increased interleukin (IL)-1α and inducible nitric oxide synthase (iNOS levels) (12), both involved in signalling pathways that promote tumour growth and a raised IL-1α level was shown to be a poor prognostic factor in gastric cancer and squamous cell carcinoma affecting the head and neck (18, 19). Despite the upregulation of these pathways, an overall reduction in tumour growth and incidence suggests exercise induces a series of complex immune processes, which favour anti-tumour activity.

3 Exercise and immunity

Evidence is increasing in support of the role of exercise in modulating the immune system and TME through multiple mechanisms in cancer patients (4). 'Exercise-induced leucocytosis' refers to the immediate increase in circulatory leukocytes after a single exercise activity (20). Murine models have demonstrated that exercise up-regulates immune pathways in tumours including natural killer (NK) cells, B cells, T cells and dendritic cells (17). NK cell circulatory release appears to be the most sensitive and have an immediate response to acute exercise driven by catecholamine release (17). NKG2D and NKp46 are NK cell activating receptors that are upregulated within the TME with exercise (21). Within the hour after exercise cessation, T cells continue to produce cytokines and *in vitro* studies have shown NK cells are more efficiently cytotoxic against myeloma and lymphoma cell lines (4, 22). Improved outcomes can be seen across many different cancers with an NK cell rich TME likely due to their cytotoxic function (21). Pederson et al. were able to show in the B16F10 melanoma murine model, lower tumour burden was associated with raised NK cell tumour infiltration (12). They were able to demonstrate tumour specificity of NK cell concentration as opposed to lymphoid organ NK cell accumulation seen in exercised control mice (12). This highlights that exercise-induced immune responses can be directed to local tumour immunity rather than a non-specific generalised mechanism and help to create a hot TME.

Despite this well evidenced exercise driven leucocytosis, the converse depletion of NK cells and CD8 T cells below baseline levels is observed after three hours of exercise completion (23). However, this lymphopaenia may reflect their redistribution from circulation to peripheral tissue supporting a continued enhancement of immune function (23).

A high abundance of NK cells within the systemic circulation and intratumourally has been correlated with a favourable prognosis and specifically, a reduced metastatic potential in a range of hot to cold tumours including renal cell carcinoma (hot) and gastric cancer (cold) (24). Davis et al. correlated improved survival outcomes in patients with pancreatic cancer with higher circulatory NK cell count (25). Metastatic disease in all solid malignancies is reliant on the ability of cancer cells to evade regulated immune-mediated destruction during separation from the primary tumour, manipulation and restructuring of the extracellular matrix and movement through the circulatory

system (24). CD8+ T cells, effector cells of adaptive immunity, rely on presentation of tumour antigen on MHC class I molecules, thus NK cells are able to act synergistically to eliminate cancer cells that lack MHC class I expression (26). Utilising this exercise-induced burst of NK cells may be of particular benefit to in reducing the metastatic potential of tumours.

Importantly, many studies showing correlation between exercise and increasing immune cell tumour infiltration in murine models have been undertaken in hot tumours with TMEs rich in tumour inhibiting T cells and cytokines such as interferon gamma (IFN-γ) (5) and therefore, inherently immunogenic. Multiple diverse mechanisms can result in systemic immune dysfunction and cold tumours such as pancreatic and prostate cancer, have been shown to have low immune cell populations and reduced immune activity within their TMEs as demonstrated in Figure 1 (4, 5).

Cancer cells are able to survive and proliferate by hiding from the immune system and preventing destruction by a number of different mechanisms (21). One of these mechanisms seen in pancreatic ductal adenocarcinoma (PDA), the most common subtype of pancreatic cancer, is reconfiguring immune cell production with consequent proliferation of myeloid cells, with a preference towards an immature subset and a reduction in lymphoid cells that exhibit anti-cancer activity, subsequently promoting an immunosuppressive environment (27). The paucity of anti-tumour immune cell concentrations in PDA and consequent lack of responsiveness to immunotherapies, highlight the specific need to optimise local tumour and systemic immunity in cold tumours.

Schwappacher et al. identified statistically significant changes in PANC-1 (primary human pancreatic cancer cell) viability following incubation with serum taken from pancreatic cancer patients at baseline, 6 and 12 weeks into a whole body electromyostimulation (WB-EMS) programme; a method used to simulate the effect of resistance training (28). Supporting this, Kurz et al. demonstrated exercise induced benefits within different PDA mouse models showing tumour mass was decreased by 20-30% in exercised mice with their methodology accounting for potential confounding factors of reduced body and muscle mass (27). The reasoning behind this was explored using single cell RNA sequencing, demonstrating that exercise caused the shift towards mature lymphoid populations, such as cytotoxic CD8 T cells whilst reducing MDSCs within the pancreatic TME (27), thereby reducing the immune evading ability of the tumour. Exercise reduced CXCR2 expression, which is a myeloid cell receptor involved in signalling pathways that enhance MDSC populations (27).

Ex vivo studies demonstrated higher Ki-67 levels, indicating proliferative activity, in CD8 T cells when cultured with MDSC isolated from tumours from exercised mice compared to controls indicating increased T cell activity and a reduction in the MDSC immunosuppressive effect (27). To the best of our knowledge, this currently is the only mouse study demonstrating the direct anti-cancer activity of exercise on PDA and its ability to turn this cold tumour hot. Given the poor prognosis, survival outcomes and high morbidity and mortality, this warrants further evaluation including in human studies.

A favourable immune environment has also been demonstrated in human studies with significantly raised CD3+ and CD8+ T cells and lowered Tumour Necrosis Factor alpha (TNFα) levels seen in the exercise cohort of oesophageal cancer patients in the study by Zylstra et al. (13). These studies have demonstrated the promising abilities of exercise to allow effector T cell infiltration and MDSC depletion within cold TMEs creating hot tumours.

4 Exercise and myokines

Myokines, including IL-6, IL-7 and I-15, released on muscle contraction play a role in immune system mediation along with many other physiological processes and therefore play a critical role in exerting the systemic effects of exercise (4, 29). As discussed before, many components of the immune system can exhibit opposing mechanisms of action. IL-6 is involved in both pro-inflammatory and anti-inflammatory pathways, and it is the mechanism of release along with local and systemic conditions and duration of exposure which skew the direction of immune activity with exercise-induced short-term rises in IL-6 demonstrating more of an anti-inflammatory effect (4). One *in vitro* study using LoVo cell line (colorectal cancer cell line KRAS mutant TP53 wildtype) incubated with human serum taken pre and post exercise, showed a 4.2% reduction in cell proliferation following exercise and demonstrated a 24.6% rise in serum IL-6 post exercise (16). The study demonstrated, using recombinant IL-6 on LoVo cells, that increasing IL-6 led to a proportionate decrease in LoVo cell proliferation and γ-H2AX expression which relates to DNA damage (16). This study supports exercise-induced IL-6 as having potential anti-tumour properties although the complex dynamics and interactions of exercise-induced IL-6 *in vivo* would need to be examined and compared to the pro-inflammatory carcinogenic and immunosuppressive actions associated with long-term exposure to IL-6, which has been documented in murine models as enhancing CCR5 expression and subsequent promotion of MDSC activity (30).

IL-7 and IL-15 play an important role in modulating T cell levels and activity and are released with muscle contraction during exercise (4). IL-7 is involved in early signalling pathways stimulating naïve T cell proliferation and along with IL-15, promotes effective memory T cell concentrations after exposure to antigen (4). Kurz et al. (27) demonstrated the key anti-cancer role exercise-induced IL-15 plays by blocking IL-15 downstream signalling in PDA models *in vivo* and negating the beneficial effects. Therefore, the release of IL-7 and IL-15 during exercise may help shape the immune landscape and turn cold tumours hot.

There have been studies identifying different, novel myokines which exhibit anti-tumour properties, such as the secreted protein acidic and rich in cysteine (SPARC) myokine, oncostatin M (OSM) and irisin (31–33). SPARC levels rise instantaneously with acute exercise with a gradual decline over six hours following exercise cessation (31). Kim et al. demonstrated a significant rise in myokines oncostatin M and SPARC in patients with metastatic castrate resistant prostate cancer (mCRPC) who underwent a 6 month supervised exercise regimen combining resistance training

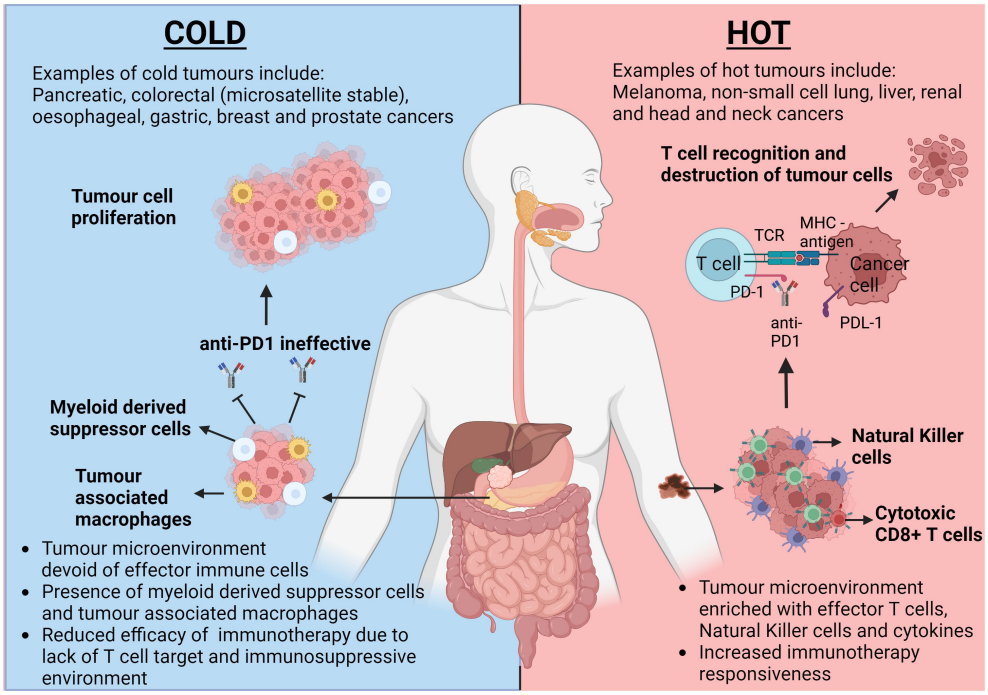

FIGURE 1
The opposing responses to immunotherapy dependant on the tumour microenvironment climate. This figure highlights the differences in tumour microenvironments (TMEs) between a classically hot tumour such as melanoma and a classically cold tumour such as pancreatic cancer. Intratumoral T cells in hot tumours provide a target for immunotherapies, such as anti-PD1, to allow inhibition of PD-1/PDL-1 interaction and allow subsequent cancer cell lysis. Due to the inherent lack of T cells within cold tumours, tumour cell proliferation remains uninhibited. TCR, T cell Receptor; MHC, Major Histocompatibility Complex; PD-1, Programmed Cell Death Protein 1; PDL-1, Programmed Cell Death Ligand 1. Created with BioRender.com

with aerobic exercise (32). Colon-26 cancer cell proliferation was suppressed when mouse recombinant SPARC was added (31) and there was reduced cell growth *in vitro* when DU145 prostate cancer cells were cultured with their myokine enriched serum compared to the control arm (32). However, the suppression plateaued at SPARC concentrations higher than 2 μg/ml and it is therefore difficult to conclude that raised SPARC levels above baseline in humans would have a similar effect.

Limitations include small sample size and hence an inadequately powered study and the lack of specificity correlating the rise in myokines and their subsequent signalling pathways to tumour inhibition, highlighting the need for further review of the potential cytotoxic mechanisms in prostate cancer with exercise induced myokines (32).

Irisin is another myokine which exhibits significant metabolic effects leading to increased energy expenditure with an immediate increase in circulatory levels demonstrated following both aerobic exercise and resistance training (33, 34). Gannon et al. showed in an aggressive epithelial breast cancer cell line, a significantly reduced cell number and viability and increased apoptotic signalling with enhanced caspase-3/7 activity with human recombinant non-modified irisin (33). Liu et al. demonstrated irisin's inhibitory effects on cancer cell growth in pancreatic cancer cell lines (35). This provides convincing *in vitro* evidence behind irisin's anti-cancer potential although *in vivo* studies would further strengthen the correlation between exercise-induced irisin and tumour inhibition. Schwappacher et al. provide evidence supporting anti-migratory properties of WB-EMS against pancreatic cancer cells implicating myokines, such as IL-10 and CCL4, as promoters of caspases3/7 apoptotic signalling (28). By inhibiting cancer cell migration, required for metastatic disease formation, this further supports the role resistance training-induced myokines play on reducing the metastatic potential of tumours. *In vivo* studies would also allow exploration of how exercise-induced myokines modulate cold TME and prevent metastasis formation. Figure 2 summarises the effect exercise has on the proliferation and circulatory release of immune cells and myokines leading to the reprogramming of cold tumours to become hot and eventual cancer cell death.

5 Exercise and the tumour vasculature

Due to the dysregulated and exponential proliferation of cancer cells, tumour angiogenesis results in an inefficient and flawed vessel structure perpetuating an oxygen-depleted environment and tumour expansion (36). Vascular endothelial growth factor-α (VEGF-α) is associated with angiogenesis and has been shown to be significantly raised in the immunotherapy groups with the combined exercise and nivolumab model in Martin-Ruiz's study having the highest VEGF-α tumoural levels as well as the highest

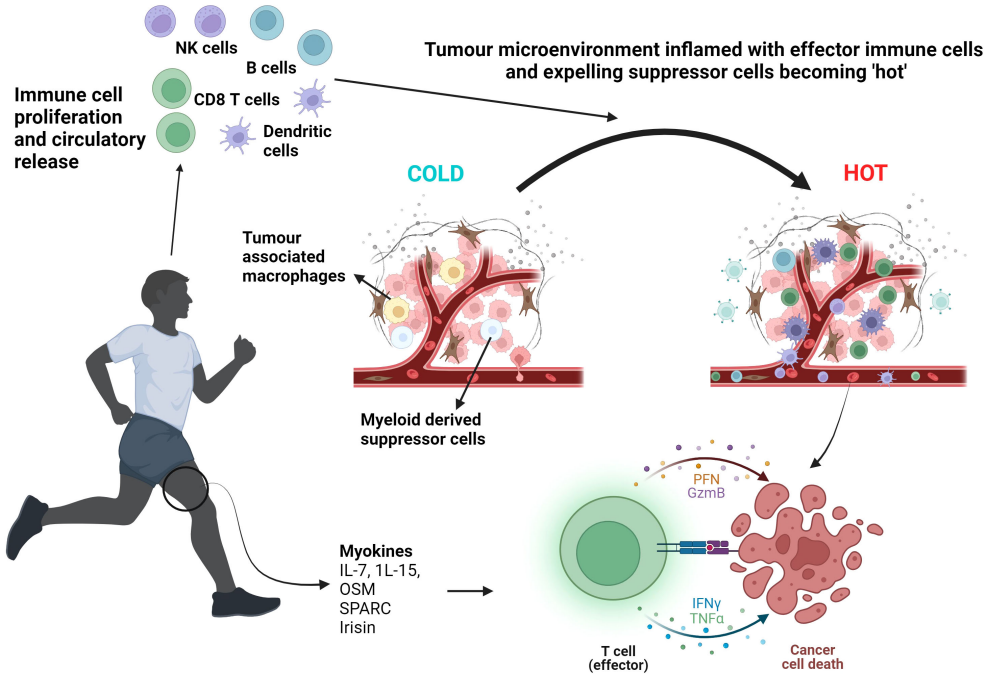

FIGURE 2
Exercise induced myokine and immune cell proliferation and release leading to T cell activation and cancer cell death. Aerobic exercise and resistance training mobilise myokines such as IL-7. IL-15, OSM, SPARC and irisin alongside inducing a circulatory leucocytosis, including NK cells and T cells. The TME becomes 'inflamed' with effector immune cells. Effector T cell function is enhanced resulting in tumour lysis. NK cells, Natural Killer cells; OSM, Oncostatin M; SPARC, Secreted Protein Acidic and Rich in Cysteine; PFN, Perforin; GzmB, Granzyme B; IFNγ, Interferon gamma; TNFα, Tumour Necrosis Factor alpha. Created with BioRender.com

necrotic index (37). However, there is conflicting evidence with regards to VEGF-α expression and exercise with both an up-regulation and down-regulation reported in the literature (37).

Within the TME, hypoxia fuels a signalling pathway resulting in raised hypoxia-inducible factor-1 (HIF-1) (38). Jones et al. noted a positive correlation between exercise and HIF-1α leading to enhanced VEGF expression within the TME along with ANGPT2, a gene marker of angiogenesis (38). In this study, C57BL/6 mice orthotopically implanted with transgenic adenocarcinoma of mouse prostate (TRAMP) C-1 cells undertook aerobic exercise using a voluntary wheel running method in a study exploring the association between exercise and tumour hypoxia and vessel structure (38).

They demonstrated using dynamic magnetic resonance (MR) scans, a significant increase in blood flow through tumours in exercised mice indicating vasculature normalisation (36, 38). The authors hypothesise that exercise training may be linked to an alteration in the mechanism of action of HIF-1 combined with cytokine release to support tumour blood flow, illustrated in Figure 3 (38). This suggests tumour vasculature normalisation occurs secondary to the complex, synergistic interactions between exercise and the TME rather than a single pathway. This further supports the theory that exercise leads to enhanced VEGF-α expression and so optimising intratumoural drug delivery (37).

Bedoya et al. demonstrated that a home-based combined aerobic exercise and resistance training regimen prior to surgery for PDA exhibited a favourable change in the tumour vasculature with increased microvessel density and patency (14). Although exercise was performed concurrently with either chemotherapy, combination of chemotherapy and radiotherapy or during the recovery period between pre-operative treatment and surgery, the effect of exercise on treatment responses and clinical outcomes was not explored, subsequently missing a potential translational endpoint (14, 39).

Exercise leads to increased cardiac output and a redistribution of blood flow away from constricted vessels within the splanchnic organs allowing supply to the contracting skeletal muscles (36). Betof et al. demonstrated, using the 4T1 breast cancer murine model, a significant reduction in EF5 (marker of hypoxia) within the tumours of exercised mice compared to sedentary mice (40). One theory behind the proposed benefit of exercise on tumour hypoxia includes the inherent nature of tumour vasculature to ignore vasoconstrictive instructions and consequently take advantage of the raised cardiac output and redirected blood flow (36).

Gomes-Santos et al. identified a simultaneous reduction in tumour hypoxia and an increased CD8+ T cell intratumoural influx with exercise training in mice implanted with breast cancer (41). Furthermore, Hatfield et al. were able to show that increasing the proportion of inspired oxygen in mice reduced tumour hypoxia and allowed a comparable increase in breast tumour infiltrating CD8+ T cells (42). These studies suggest that reduced tumour

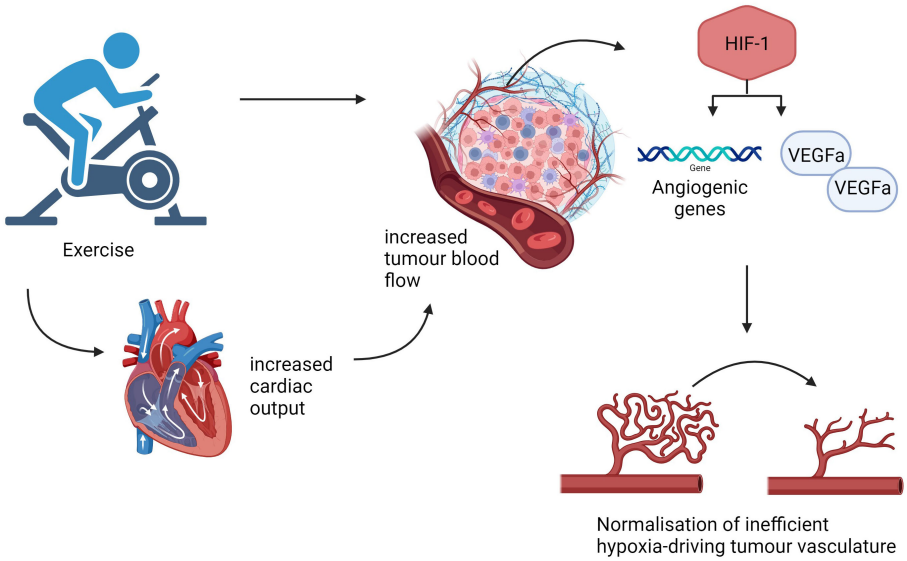

FIGURE 3
Exercise induced normalisation of tumour vasculature. An outline of some pathways that aerobic exercise may utilise to normalise tumour vessels, increase tumour blood flow and reduce hypoxia. Created with BioRender.com

hypoxia could be a contributing factor to this CD8+ T cell tumour infiltration, providing a favourable hot TME for their stimulation and enhanced function (36).

Therefore, exercise-induced tumour vasculature normalisation of cold tumours could lead to improved oxygenation and CD8+ T cell infiltration indicating a different mechanism of reprogramming cold tumours to become hot. By modulating PD-L1 expression and CD8+ T cell infiltration, exercise-induced tumour oxygenation could potentially improve the efficacy of ICIs working within the PD-1/PD-L1 pathway (36).

6 Exercise and immunotherapy

Pre-clinical studies suggest exercise can modulate systemic and local tumour immunity by complex pathways upregulating immune components with anti-tumour activity, turning cold tumours hot and therefore, could play an adjunctive role to immunotherapy in patients with cancer.

Immunotherapy has revolutionised treatments across many different cancers and has shown to be particularly effective in hot tumours (5). ICIs, including anti- Programmed Cell Death Protein 1 (anti-PD1) and anti- cytotoxic T lymphocyte antigen 4 (anti-CTLA-4), are commonly used as standard of care in both curative and palliative settings. However, ICIs are not effective across all cancers, particularly cold tumours, highlighting the need for further research to reprogramme tumours to become more responsive to ICIs.

Martin-Ruiz et al. reviewed the effect of exercise (following inoculation with cancer cells) and nivolumab (anti-PD1) in NOD-SCID γ mice transplanted with human poorly-differentiated squamous non-small cell lung cancer (NSCLC), a hot tumour (37). A significantly raised necrotic index was observed in exercised mice with nivolumab compared with the non-exercise and isotype control group with the latter showing the greatest proliferating cell rate with Ki67 immunostaining (37). The necrotic index was not significantly different between the exercised and non-exercised groups receiving nivolumab and this may be due to the limited sample size (37). This study included combined aerobic and resistance training within the intervention, however we would be unable to draw any conclusions with regards to anti-cancer activity associated with resistance training alone. Proposed mechanisms of the anti-proliferative effects include promoting AMP-activated protein kinase (AMPK) signalling with subsequent suppression of the mammalian target of rapamycin (mTOR) oncogenic pathway (37, 43). A statistically significant reduction in key components of mTOR activation was observed in a study where human exercised serum was applied to A549 NSCLC cells (37, 43). Although no difference was seen in tumour growth or volume in mice receiving nivolumab irrespective of exercise, a reduced tumour growth rate and volume was found in the exercised mice in comparison to non-exercised mice, both groups having received isotype control (37). The immune deficient NOD-SCID γ mice would be unlikely to mount a sufficient response with anti-PD1 due to the inherent absence of T cells, providing difficulty in successfully evaluating the effect of exercise on cancer immunotherapy responses (44).

Buss et al. (44) examined EO771 'cold' breast tumour and B16-F10 'hot' melanoma mouse models and the interactions between post-implant exercise and anti-PD1 on local and systemic immunity, TME and growth. They observed a negative effect of exercise in these aggressive tumour models with a skew towards an immunosuppressive TME and exercise reduced CD8 T cell infiltration in the EO771 model and no effect on tumour growth seen when combined with anti-PD1 (44). Conversely, Wennerberg et al. demonstrated significant tumour growth inhibition with exercise during anti-PD-1 and radiotherapy treatment in a breast

cancer murine model compared to sedentary mice receiving the same regimen (4, 45). They showed that exercise induced a local and systemic preference for anti-tumour immune cells and suppression of MDSC promoting the conversion of the breast cancer cold TME to a hot TME (4). Validity of the results and anti-PD1 used in Buss et al.'s study could be questioned as anti-PD1 alone exerted no tumour inhibitory effects and TME modulation also occurred with the isotype control (44). In clinical practice, pseudo-progression describes the inflammatory process with ICIs causing a paradoxical increase in assumed tumour volume, masking tumour response and may also provide a barrier to detecting the true anti-cancer effect of ICIs in murine models.

Further murine breast cancer models have also shown that utilising exercise alongside ICIs enhanced tumour suppression (41). In clinical practice, we utilise the combination of ICIs and chemotherapy for advanced triple negative breast cancer, however ICIs have shown little benefit outside of this sub-group, although multiple other treatments are available. Further studies into the role of ICI and exercise in breast cancer including hormone-positive and HER2-positive subgroups are required to support the clinical utility of exercise across all breast cancer sub-types.

Kurz et al. demonstrated that exercise optimised local immunity when combined with anti-PD1 by raising CD3 T cell and cytotoxic CD8+ T cell infiltrations and subsequently led to significant tumour size reduction compared to anti PD-1 alone in their PDA murine model (orthotopic transfer of KPC cells to wild type C57BL/6 mice) (27). The results are particularly promising due to the limited effective treatment for pancreatic cancer and the lack of responsiveness of cold tumours to immunotherapy, providing evidence that exercise could possibly expand the spectrum of tumours that could benefit from ICIs.

7 Discussion - incorporating exercise into clinical practice

7.1 Exercise is feasible in the advanced cancer population

This review has discussed the different mechanisms by which exercise training can reprogramme the cold TME with effector T cell infiltration, MDSC depletion, anti-tumour myokine secretion and tumour vasculature normalisation creating a hot TME.

Mouse models of both cold and hot tumours have showed additional benefits of combing immunotherapy with exercise training. Further exploration of this potentially advantageous combination is required with human studies. There is a clear unmet need in patients with advanced cancer to improve overall response to immunotherapy and survival outcomes. Within the advanced cancer population, reduced functional reserve, exercise tolerance and respiratory function may provide significant obstacles to implementing exercise training into their cancer management. It is unlikely there will be a generalised regime applicable to all patients with cancer. Therefore, there should be a role for exercise specialists, with expertise in working with patients with cancer, to be involved in the delivery of exercise interventions. Exercise protocols will need to be tailored to the individual patient's needs and abilities, whilst ensuring intensity levels are reached to provide a sufficient immune response. This may not be possible in some cancer patients with frailty, cancer cachexia and disease burden potentially limiting their exercise abilities. Possible strategies to overcome these challenges could include utilising chair-based exercise activities, using a graded exercise intensity to build exercise tolerance and collaboration with nutritionists to minimise weight loss and maintain muscle mass.

However, the cancer population eligible for immunotherapy are inherently required to possess an Eastern Cooperative Oncology Group (ECOG) Performance Status of 0-1, indicating a good baseline level of activity and functioning (41). This further supports the feasibility of tailored exercise in these cohorts.

Mikkelsen et al. showed that strength training in combination with a home-based walking programme over 12 weeks was feasible in an elderly population affected by either advanced pancreatic cancer, biliary tract cancers or non-small cell lung cancer (46). They demonstrated a 69% adherence rate and 94% completion rate of attended sessions occurring alongside first line palliative oncological treatment (46). The acceptability of this exercise intervention by an older age population and the inclusion of hard-to-treat advanced cold tumours is promising for the future incorporation of exercise into clinical practice in these populations.

A combination of cycling and resistance training was incorporated into a rehabilitation programme for patients with advanced lung cancers including mesothelioma (47). Only one third of eligible patients managed to be recruited with a 56.6% completion rate of the 8-week programme (47). This highlights the need to review reasons behind reduced participant acceptability of exercise training, cater interventions, and acknowledge limitations within the advanced cancer population.

7.2 Moderate intensity aerobic exercise and resistance training promote anti-cancer activity

Importantly, to incorporate exercise as an evidence-based standard intervention in cancer management, the FITT principles allow prescription of a measured dose of exercise, although there will still undoubtedly be challenges in determining the exact 'dose' of exercise required to exhibit treatment effect. Tables 1A–C outline pre-clinical and clinical studies researching the relationship between exercise and cancer in cold and hot tumours. The *in vitro* studies required pre- and post- exercise conditioned serum of healthy participants or patients with cancer depending on the study (16, 22, 31, 32). The exercise regimes utilised varied from moderate –intensity to high-intensity interval training and largely centred on cycling. The window of opportunity studies (13, 14) reviewing exercise as a prehabilitation measure in oesophageal and pancreatic cancer patients, respectively, used a combination of aerobic exercise and resistance training at moderate intensity, with Bedoya et al. incorporating a walking activity over 2-6 months (14) and Zylstra et al. not specifying the type of aerobic

exercise but occurring alongside neoadjuvant chemotherapy lasting 8-9 weeks (13).

Animal studies in non-cancer models have shown that exercise training at moderate intensity, with a maximal oxygen uptake of around 70%, was able to promote cytotoxic immune function and support a Th1 cytokine profile, playing a protective role against infection (48). As immunity against infection overlaps with anti-tumour immunity, this further supports the use of moderate intensity exercise in cancer care.

Conversely, the opposite may be seen with chronic exercise at high intensity, for example in those at professional standards of exercise (48). After showing an initial increase within 30 minutes of high intensity endurance exercise, CD8+ T cells dropped along with Th1 cytokines IL2 and IFNγ (49). Previously, a 3-hour post exercise

TABLE 1A Pre-clinical studies with cold tumours.

Study authors	Cancer type (s)	Cell line/ animal model/ human	Exercise intervention	Study findings
In vitro				
Orange et al. (16)	Colon	LoVo	Males with risk factors for colon cancer (n=16). Moderately intense cycling (5 minute sessions performed 6 times with 2.5 minute breaks)	Serum of exercised individuals significantly decreased LoVo cell proliferation and γ-H2AX expression compared to serum collected prior to exercise. Exercise led to increased IL-6 levels.
Aoi et al. (31)	Colon	C2C12 myocytes and colon-26	Healthy males 30 minutes uninterrupted cycling at 70% VO2 max Group 1: (n=10) One session Group 2: (n=9). Three weekly sessions for four weeks	Exercise increased SPARC levels in mice and humans following a single exercise session. SPARC reduced colon-26 cell proliferation.
Kim et al. (32)	Metastatic castrate-resistant prostate cancer (mCRPC)	DU145	mCRPC patients. Exercise group (n=13) 6 months of combination aerobic exercise (high-intensity, interval training) and resistance training. Control group (n=12) self-directed exercise	Supervised exercise training increased myokines oncostatin M (OSM) and SPARC compared to control. DU145 proliferation reduced with 6-month exercise-conditioned serum 12 to 61 hours after incubation but not at 72 hours.
Mouse studies				
Kurz et al. (27)	Pancreatic Ductal adenocarcinoma (PDA)	KPC cells in C57BL/6 mice	Running on a treadmill. 30 minutes, 5 times a week (mild intensity 15cm/s)	Exercise decreased tumour mass by 20-30%. Exercise increased intra-tumoural CD8+ T cells and reduced MDSCs with upregulation of 1L-15 signalling pathway.
Buss et al. (44)	Breast	Female C57BL/6 with orthotopic EO771 cells	Wheel running	Exercise did not affect NK cell levels. Exercise reduced CD8+ T cell proportions. Exercise combined with anti-PD1 had no significant effect on immune populations.
Wennerberg et al. (45)	Breast	4T1 in BALB/ c mice	30 minutes 5 times a week. Running on a treadmill at 18m/min compared to control.	Exercise reduced tumour volume and MDSCs in the spleen. Exercise provided additional benefit in tumour inhibition when combined with radiotherapy and anti-PD1 compared to the latter two without exercise, as well as increased intratumoural NK cell activity.

TABLE 1B Clinical studies with cold tumours.

Study authors	Cancer type (s)	Cell line/ animal model/ human	Exercise intervention	Study findings
Human studies				
Zylstra et al. (13)	Oesophageal cancer	Oesophageal cancer patients for possible curative surgery	Combined moderately intense aerobic exercise/weight training (n=21) compared to controls (n=19)	Enhanced pathological response and increased CD3 and CD8 T cells following pre-operative chemotherapy seen in the exercise group compared to control group.
Bedoya et al. (14)	PDA	Pancreatic cancer patients for possible curative surgery	70 patients undergoing neoadjuvant chemotherapy or combination chemotherapy/radiotherapy prior to surgery. Combination moderate level unsupervised aerobic exercise (1 hour per week of walking) and weight training (1 hour per week).	33/70 patients had their cancer surgery, although only 23 patients' pathology specimens were able to be included. Compared to non-exercised controls, exercise increased microvessel density and patency in the tumours. Exercise did not influence the amount of tumour regression.

TABLE 1C Hot tumours.

Study authors	Cancer type (s)	Cell line/animal model/human	Exercise intervention	Study findings
Mouse studies				
Pedersen et al. (12)	Melanoma, liver and lung	B16F10 in female mice, Diethylnitrosamine (DEN) in Naval Medical Research Institute NMRI male mice, Lewis Lung Carcinoma (LLC) in female mice	Wheel running. Average daily running distance per mouse: 4.1km (female) 6.8km (male)	All models: Tumour size, growth and metastatic disease reduced with exercise. B16 and LLC – increased pro- and anti-inflammatory immune components including IL-6 and NK cells.
Martin-Ruiz et al. (37)	Non-small cell lung cancer (NCSLC)	NOD-SCID gamma mice – patient derived xenograft model	40-60 minutes 5 times a week for 8 weeks. Combination treadmill running (performed at 40-80% maximal velocity) and resistance training.	Exercise led to reduced tumour growth and volume and in combination with nivolumab (anti-PD1), significantly increased the necrotic index comparative to double control, although necrotic index, cell proliferation and tumour growth were no different in the nivolumab groups irrespective of exercise.
Buss et al. (44)	Melanoma	Female C57BL/6 with subcutaneous B16F10 cells	Wheel running	Exercise did not affect NK cell levels. Exercise reduced CD8+ T cell proportions. Exercise combined with anti-PD1 had no significant effect on immune populations.

lymphopaenia had been addressed as potential redistribution from blood to tissue, however, a higher predisposition to developing infection has been noted (23, 49).

High intensity interval training was directly compared to moderate intensity continuous exercise over a 12-week programme in participants with significant risk factors for breast cancer in a randomised controlled trial (50). This study demonstrated a moderate intensity aerobic exercise regime over 12 weeks was superior to high intensity in increasing CD8 effector memory T cell proliferation and promoting improved immune responses by reducing senescence across different T cell subpopulations (50). Graff et al. were able to show that resistance training of the major muscle groups within a sedentary older age population exhibited a greater degree of leucocytosis, including cytotoxic CD8 T cells, than aerobic exercise (51). Pre-clinical and clinical evidence suggests the optimal exercise intervention to promote anti-cancer immunity would be moderate intensity, aerobic exercise training over a duration of 8-12 weeks with the inclusion of resistance training.

In the previously mentioned exercise studies, small sample sizes and significant heterogeneity in the study populations, exercise interventions and immune outcome measures limit our ability to draw firm conclusions. Furthermore, there may be selection bias of more physically active patients, who would be more likely to accept recruitment and contamination bias, due to the inability to prevent self-directed exercise in control groups. These would provide significant obstacles to defining the optimal exercise dose in patients with cancer.

Further clarification with larger studies focusing on a standardised effective exercise dose in a single disease site is required to allow implementation of exercise as a prescribed intervention in clinical practice.

Incorporating moderate intensity aerobic exercise for patients with cancer undergoing immunotherapy is likely to be more feasible and effective in promoting a favourable immune response. However, the translatability of these animal studies to patients with cancer should be considered given the differences in which an exercise intervention can be performed between humans and mice. Currently, there is limited evidence of the effectiveness of different exercise intensities within the advanced cancer population and is an area that warrants further exploration.

7.3 Future research

Despite the growing pre-clinical evidence of exercise-induced benefits on immunity and immunotherapy response in mouse models, there may be limitations in its translation to the human population. Notable differences in anatomy, physiology and immunology between mice and humans may limit extrapolating these beneficial effects of exercise interventions to humans. Differences in leucocyte subpopulations and Th1/Th2 profiles are present between mice strains as well as mice and humans (52). Therefore, importantly the utility of exercise as an effective intervention and an enhancer of immunotherapy responsiveness should be explored in ongoing human studies. ERICA is a prospective clinical trial looking into the practicalities and acceptability of undertaking exercise immediately prior to infusion of combination immunotherapy and chemotherapy in a French cohort of patients with metastatic NSCLC (53). Exploring the association between high intensity aerobic exercise and NK cell release in NSCLC, HI AIM is a randomised controlled trial that hypothesises augmented immunotherapy responses induced by exercise as a consequence of intratumoural and systemic immune cell influx (54). Importantly, these studies are investigating the clinical application of exercise in metastatic cancer populations, an area in oncology with vast room for improvement. NSCLC is typically a hot tumour and although there is scope for improving immunotherapy responses, to enhance the breadth of cancers that respond to immunotherapy, further research is required into cold tumours where immunotherapy is not effective.

Mechanisms by which drugs and oncolytic viruses can reprogramme cold tumours to become hot are being reviewed however, there is a research gap in demonstrating the ability of exercise to transform cold tumours to hot in patients with cancer. Future research could include ex-vivo work assessing immune

responses when cold tumours are excised from patients and cultured with exercise-conditioned serum from participants undertaking moderate intensity aerobic exercise and resistance training.

Due to lack of efficacy, immunotherapy alone cannot be used as a comparator to an exercise – immunotherapy combination in cold tumours. Therefore, a clinical study assessing the latter combination in malignancies such as advanced pancreatic cancer, could be assessed as a maintenance strategy in patients that have completed first line palliative systemic anti-cancer treatment and have either responded or have stable disease. If the patient remains well and deemed appropriate by the clinical team, they normally will undergo surveillance with regular Computed Tomography (CT) scans to assess for disease progression. Figure 4 illustrates a potential multi-arm study design to explore exercise as an adjunct to immunotherapy in advanced pancreatic cancer in the maintenance setting following first line palliative treatment. Subsequently, if the combination of exercise and immunotherapy is deemed to be effective in the maintenance palliative setting, it can be explored at other stages and clinicians would be able to prescribe a dose of exercise alongside immunotherapy to improve treatment responses and overall survival.

8 Conclusion

There is an increasing volume of evidence supporting various exercise-induced direct cytotoxic mechanisms in different cancer settings along with a crucial role exercise may play in local tumour and systemic immunity via anti-tumour immune cell infiltrations, favourable cytokine and myokine profiles and tumour vasculature normalisation (9, 21, 30). In pre-clinical models, these different exercise-mediated changes appear to act synergistically to remodel the immunosuppressive cold TME to become hot.

Immunotherapy has improved survival outcomes for many patients with cancer although immunotherapy-resistance and severe immune-mediated toxicities limit its universal utility. A significant barrier to immunotherapy effectiveness lies within the rigid, immunosuppressive and hostile TMEs of cold tumours such as pancreatic cancer (5, 27). Evidence suggests that exercise training, including aerobic exercise and resistance training, may have a role in shifting tumours towards the hot end of the cold-hot TME spectrum. There is a need to validate these findings in humans.

Due to the complex relationship between different forms of exercise and immune components as well as the diverse nature of different cancers, defining an optimum dose of exercise to enhance immunity remains problematic, although studies suggest a favourable immune profile with moderate intensity aerobic exercise and resistance training. In practice, it is likely that exercise activities would need to be tailored to the individual, taking into account their personal limitations and their cancer phenotype. Large, clinical randomised controlled trials are needed to assess the utility of specific exercise interventions as adjuncts to immunotherapy across different cancer settings. Following this, if implemented into clinical practice, exercise training could potentially be a promising, safe and

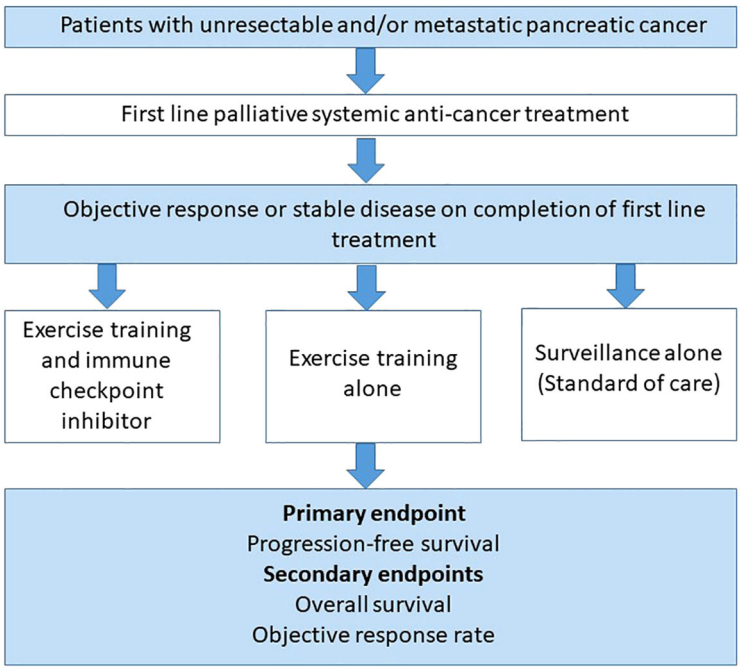

FIGURE 4
A multi-arm study design to explore the effectiveness of combining exercise training with immunotherapy as a maintenance strategy in previously treated advanced pancreatic cancer patients. A proposed randomised controlled trial design involving patients who have had an objective response or stable disease on completion of first line palliative systemic treatment for advanced pancreatic cancer. Patients would be randomised to either a combination of exercise training alongside a single agent immune checkpoint inhibitor or exercise training with standard of care or standard of care alone. All participants in each group will have regular Computed Tomography (CT) imaging every 3 months as standard of care. The primary endpoint would be progression-free survival (PFS), with overall survival (OS) and objective response rate (ORR) as secondary endpoints.

cost-effective measure to overcome some of the obstacles to optimising immunotherapy responsiveness.

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Platinum desensitization therapy and its impact on the prognosis of ovary high-grade serous adenocarcinoma: a real world-data

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Background: To examine the value of five-step platinum desensitization therapy in epithelial ovarian cancer

Methods: A retrospective study was conducted on the high-grade serous adenocarcinoma of the ovary (HGSAO) patients who developed a platinum allergy during treatment and received desensitization therapy between January, 2016 and December, 2020. The logistic-regression was adopted to analyze the relationship between platinum desensitization therapy and prognosis in HGSAO patients.

Results: 92 HGSAO patients were included in the study. Among these, 35 patients (38.0%) experienced mild allergic reactions, 51 (55.4%) experienced moderate allergic reactions, and 6 (6.5%) experienced severe allergic reactions. The desensitization therapy was successful in 86 patients (93.5%). Six patients had desensitization failure, of which five experienced severe allergic reactions during desensitization. The logistic-regression analysis revealed no significant correlation between platinum desensitization therapy and progression-free survival (PFS) or overall survival (OS) of patients ($P < 0.05$). However, the subgroup analysis demonstrated that the success or failure of platinum desensitization therapy significantly impacted the OS of patients who were platinum-sensitive recurrence. The patients who had successful desensitization therapy had a superior OS.

Conclusion: Five-step platinum desensitization therapy has potential application value in patients who were platinum-sensitive recurrence after first-line treatment but may bear the risk of severe allergic reactions.

KEYWORDS

high-grade serous adenocarcinoma of the ovary, platinum allergy, platinum desensitization therapy, platinum-sensitive recurrence, prognosis

Highlights

- Platinum desensitization therapy has potential value for platinum-sensitive recurrence ovarian cancer.
- Platinum desensitization therapy may bear the risk of severe allergic reactions.
- Five-step platinum desensitization therapy is safe.

1 Introduction

Platinum-based chemotherapy is the first-line chemotherapy regimen for the initial treatment of epithelial ovarian cancer (1). The incidence of allergic reactions caused by these agents has increased and attracted attention with the widespread clinical application of platinum-based chemotherapy agents. Studies showed that the risk of allergic reactions increased significantly with the increase in drug treatment courses, and most of the reactions occurred in patients who had received more than five or six courses of treatment (2). Markman et al. (3) showed that the incidence of carboplatin allergy in the first five courses was less than 1% in patients who received carboplatin chemotherapy for the first time, but increased to 6.5% in the sixth course. It was as high as 27% in patients receiving more than seven courses. The risk of allergic reactions increased significantly in recurrent patients or patients who received eight or more courses of treatment, with an incidence rate of up to 44% (4–7).

Platinum desensitization therapy has become an essential clinical technique for reintroducing platinum-based chemotherapy and is widely used in clinical practice (8–11). Rapid drug desensitization has become the standard treatment for patients with platinum drug allergies since the early, 2000s (12–14). Eroglu et al. (14) reported a 6-h, 12-step desensitization protocol for carboplatin allergy, including pretreatment with leukotriene receptor antagonists, antihistamines, and corticosteroids, as well as extended infusion time. In their study, 186 eligible patients were included, with 155 (83%) receiving platinum-based treatment and 104 (56%) completing three or more cycles of therapy during the desensitization period. Overall, the patients completed 694 cycles. Further, 79 (42%) patients experienced breakthrough hypersensitivity reactions during desensitization, with 4 patients requiring epinephrine and 84 (45%) discontinued desensitizing agents due to disease progression. Paksoy et al. (15) demonstrated that the 6-h, 12-step rapid drug desensitization protocol was safe and effective, with a total survival time of 42.2 months (range: 25.3–59.1 months) after the first desensitization treatment (0S2). The 1-, 2-, and 5-year survival rates were 92.6%, 75.6%, and 47.2%, respectively. The objective response rate was 78.5%. However, various complex clinical confounding factors may affect the long-term prognosis of patients, making it unclear whether desensitization treatment can significantly improve the progression-free survival (PFS) or overall survival (OS) of patients with ovarian cancer. Multivariate analysis can further eliminate the influence of other confounding factors, thereby determining the correlation between predictor variables and response variables. In addition, it is easier to find problems by comparing the results of Univariate and Multivariate. If the results of Multivariate analysis and Univariate analysis are consistent, the conclusion is more stable and easier to explain. Therefore, this study aimed to use multifactorial analysis to investigate the impact of platinum desensitization outcomes on the prognosis of patients with ovarian cancer, providing a basis for better-serving patients with platinum desensitization therapy.

2 Materials and methods

2.1 Research participants

This study was focused on patients with high-grade serous adenocarcinoma of the ovary (HGSAO) who developed a platinum allergy during cancer treatment and received concurrent desensitization therapy from January, 2016 to December, 2020. The inclusion criteria were as follows: patients aged 18 years or older, diagnosed with HGSAO by two independent pathologists based on the pathological slides, having platinum allergy during initial treatment or at recurrence, and receiving concurrent platinum desensitization therapy. Platinum agents included cisplatin, carboplatin, oxaliplatin, and nedaplatin. The exclusion criteria were as follows: patients who developed a platinum allergy during treatment but did not receive platinum desensitization therapy or those without HGSAO.

The scholars collected the clinical information of the patients through the hospital's electronic health record system, including age, pathological type, the International Federation of Gynecology and Obstetrics (FIGO) stage, neoadjuvant chemotherapy, number of chemotherapy cycles, surgical outcomes (R0, R1, or R2), maintenance therapy, allergy medication (cisplatin, carboplatin, oxaliplatin, or nedaplatin), desensitization medication (cisplatin, carboplatin, oxaliplatin, or nedaplatin), desensitization outcomes (success or failure), severity of allergy (mild, moderate, or severe), and prognostic outcomes (OS and PFS1: first recurrence; PFS2: first recurrence after desensitization).

2.2 Treatment

All patients received initial surgical treatment (comprehensive surgical staging) or interval cytoreductive surgery (after neoadjuvant chemotherapy), followed by platinum-based chemotherapy. All patients voluntarily underwent genetic testing and maintenance therapy and were followed up regularly in outpatient clinics. Platinum-sensitive recurrent patients received platinum-based chemotherapy. Interval cytoreductive surgery was performed before chemotherapy in patients with a chance of achieving R0 after surgery. Personalized treatment was adopted in platinum-resistant patients based on the National Comprehensive Cancer Network (NCCN) guidelines.

The platinum desensitization protocol is shown in Figure 1. The platinum desensitization protocol used in this study consisted of five-step. The first step involved administering dexamethasone 10 mg orally, once every 12 h, for a total of two doses. The second step involved administering diphenhydramine 50 mg via intramuscular injection. The third step to the fifth step involved preparing three different concentrations of platinum solution. The third step involved 1/100 of the total dose of platinum, and diluted with 250 mL of 5% glucose solution. The infusion process for the first concentration of platinum was as follows: 2 mL/h (for 15 min), 5 mL/h (for 15 min), 10 mL/h (for 15 min), and 20 mL/h (for 15 min). The fourth step involved 1/10 of the total dose of platinum, and diluted with 250 mL of 5% glucose solution. For the second concentration of platinum, it was 5 mL/h (for 15 min), 10 mL/h (for 15 min), 20 mL/h (for 15 min), and 40 mL/h (for 15 min). The fifth step involved the remaining dose of the total dose of platinum, and diluted with 250 mL of 5% glucose solution. For the third concentration of platinum, it was 10 mL/h (for 15 min), 20 mL/h (for 15 min), 40 mL/h (for 15 min), and 75 mL/h (until the end). Desensitization treatment was immediately stopped after the occurrence of allergic reaction symptoms.

The evaluation of patients who experience allergic reactions includes categorization of reactions as mild (cutaneous symptoms), moderate (cutaneous, respiratory, and gastrointestinal involvement), and severe (changes in vital signs, syncope, seizures, and cardiac or respiratory arrest). Successful desensitization is defined as symptom-free during the desensitization. If any degree of allergic reaction occurs during the desensitization process, it is considered a desensitization failure.

We have emergency response plans for severe allergic reactions. If a severe allergic reaction occurs when a patient undergoes chemotherapy or desensitization treatment, we will immediately initiate an emergency process. This process consists of multiple members, including oncologists, internists, anesthesiologists, emergency doctors, nurses, workers, etc. When an allergic reaction occurs, treatment must be prompt and timely, every second counts. Stop the medication immediately, lie down, inhale oxygen, and keep warm. Closely observe and record blood pressure, pulse, respiration, urine output, etc. Immediately subcutaneously inject 0.5 to 1 ml of 0.1% epinephrine hydrochloride. If the symptoms are not relieved, 0.1% epinephrine hydrochloride 0.5 ml or 0.1% epinephrine hydrochloride 0.5ml + 50% GS 40 ml intravenously can be injected subcutaneously every 10 to 30 minutes until out of the dangerous period. At the same time, dexamethasone 20mg + 50% GS 40ml intravenously is given, followed by dexamethasone 20mg + 5% GS 500ml intravenous infusion, or hydrocortisone 200 ~ 300mg + 5% ~ 10% glucose intravenous infusion. Antihistamine Drugs such as promethazine hydrochloride 25 to 50 mg or 10% calcium gluconate 10 to 20 ml are diluted and then injected intravenously or diphenhydramine 40 mg is injected intramuscularly. After the above treatment, if the condition does not improve and the blood pressure does not rise, it is necessary to establish 1 to 2 infusion channels in time to supplement the blood volume and give rescue drugs intravenously in a timely manner. If the blood pressure still does not rise, consider vasopressors, such as dopamine, alamin, norepinephrine, etc. When shock is accompanied by tracheospasm, immediately intravenously inject aminophylline 0.25, dexamethasone 10 mg, and 50% GS 20 ml, followed by intravenous infusion of 10% GS 500 ml, aminophylline 0.5mg, and dexamethasone 10 mg. In case of cardiac arrest, 1 ml of 0.1% epinephrine is injected intracardially, and external or intrathoracic cardiac massage is performed.

2.3 Statistical analysis

Statistical Analysis System (SAS) 9.2 was used for the statistical analysis of the data. The continuous data were presented as mean ± standard deviation, while the count data were presented as percentages. A *P* value of <0.05 indicated a statistically significant

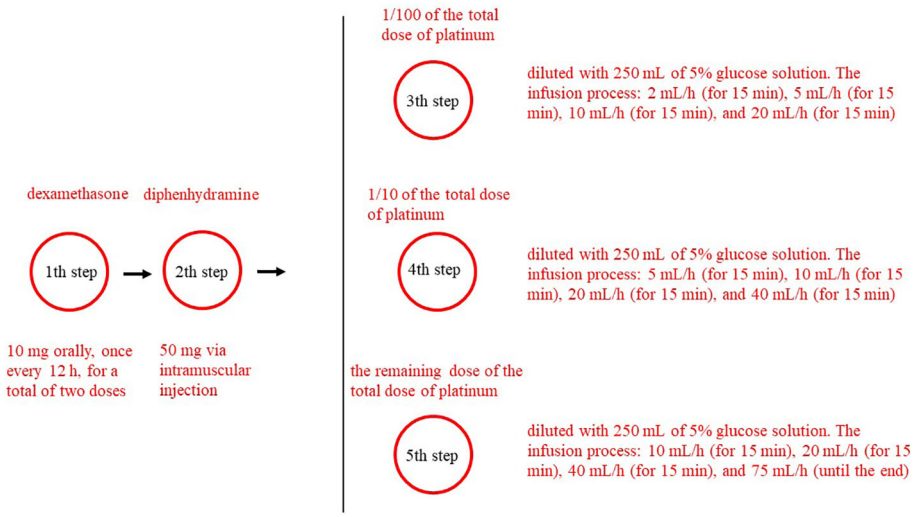

FIGURE 1
The platinum desensitization protocol.

difference. The hazard ratio (HR) was used to evaluate the impact of variables on the outcome event (death or recurrence), where an HR point estimate greater than 1 indicated an increased risk of the outcome event with the increase in the variable value, and vice versa. The single-factor statistical modeling was used for preliminary evaluation and screening of variables, and multiple-factor statistical modeling was performed by incorporating variables with clinical significance.

Single-factor statistical modeling is used for preliminary evaluation and screening of variables, and multi-factor statistical modeling is implemented by incorporating clinically significant variables based on clinical considerations. $P<0.05$ indicates that the difference is statistically significant, but it is not an absolute standard. Failure to meet statistical significance does not mean that it is without clinical significance. After all, the overall sample size is limited, and statistical power does not necessarily support the conclusion of statistical significance. Tables 1, 2 are examples. The first recurrence will be modeled by considering factors in turn. FIGO stage, neoadjuvant chemotherapy, number of chemotherapy, and preliminary assessment of allergy drug categories may have a certain impact on recurrence. Comprehensive clinical considerations, these factors were incorporated into a multi-factor model for unified modeling, and it was found that neoadjuvant chemotherapy, the more severe the allergy, the number of chemotherapy, and allergy drugs have statistically significant effects on the duration of recurrence. At the same time, surgery also has a significant impact on the duration of recurrence. There is likely to be an impact. Other factors showed no statistically significant effects, possibly due to insufficient statistical power due to insufficient samples.

3 Results

3.1 Basic characteristics of the study participants

The basic characteristics of the study participants are shown in Tables 3, 4, and Figures 2, 3. A total of, 1592 patients with HGSAO were treated over 5 years, with 127 (7.98%) experiencing platinum-based agent allergies. Among the 92 patients with HGSAO who met the inclusion criteria, 35 (38.0%) experienced mild allergic reactions, 51 (55.4%) experienced moderate reactions, and 6 (6.5%) experienced severe reactions. A total of 552 desensitization treatments were administered to the 92 patients, with 6 cases of failures. The success rates for desensitization treatment were 93.5% (86/92) and 98.9% (546/552). Of the 92 allergic patients, 19 (20.7%) were allergic to cisplatin, 55 (59.8%) to carboplatin, 12 (13.0%) to oxaliplatin, and 6 (6.5%) to nedaplatin. The desensitization agents used in the 92 desensitization-treated patients were cisplatin in 1 case (1.1%), carboplatin in 65 cases (70.7%), oxaliplatin in 15 cases (16.3%), and nedaplatin in 11 cases (11.9%).

Six cases of desensitization failure, one experienced mild allergic reactions, five experienced severe allergic reactions during desensitization and required rescue treatment, such as adrenaline, to alleviate symptoms. Desensitization failure in 6 patients occurred after multiple desensitizations.

The patients were followed up for an average of 48.9 months during the study. Further, 78 patients (84.8%) had experienced recurrence by the end of the follow-up period, while 4 patients (4.4%) had experienced disease progression during initial treatment. Among the 78 patients who experienced recurrence, 68 were platinum-sensitive and 10 were platinum-resistant. Moreover, 34 patients (37.0%) had deceased by the end of the follow-up period and all 34 patients died of ovarian cancer. A total of 36 patients (39.1%) did not experience recurrence, 23 patients (25.0%) experienced recurrence after stopping the therapy for more than 6 months, 4 patients (4.4%) experienced recurrence within 6 months of stopping the therapy, and 29 patients (31.5%) experienced disease progression during the period from desensitization treatment to the end of follow-up.

3.2 Effects of desensitization therapy on PFS or OS

The risk factors for PFS or OS included the FIGO stage, neoadjuvant chemotherapy, severity of the allergy, number of chemotherapy cycles, desensitization outcome, surgical outcome, maintenance therapy, and allergy agent. The univariate analysis showed that the desensitization outcome did not affect the initial PFS outcome (PFS 1), the PFS outcome (PFS 2) after desensitization treatment and OS in patients with ovarian cancer ($P > 0.05$). The results of the multivariate analysis were consistent with those of the univariate analysis. The details are presented in Table 2.

TABLE 1 Cross-tabulation of recurrence before and after desensitization treatment.

		Relapse distribution after desensitization therapy (%)				
		unrecurrence	>6 months	<6 months	progression	total
Recurrence at the end of follow-up	unrecurrence	9 (90.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)	10 (10.9%)
	>6 months	24 (35.3%)	22 (32.3%)	1 (1.5%)	21 (30.9%)	68 (73.9%)
	<6 months	3 (30.0%)	1 (10.0%)	3 (30.0%)	3 (30.0%)	10 (10.9%)
	progression	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (100%)	4 (4.4%)
	total	36 (39.1%)	23 (25.0%)	4 (4.4%)	29 (31.5%)	92 (100%)

TABLE 2 Multivariate analysis of influence on Patient's prognosis.

variable types		DF	PE	STD ERR	X^2	P	HR	HR 95%CI	
OS	FIGO	1	0.32577	0.35054	0.8636	0.3527	1.385	0.697	2.753
	neoadjuvant chemotherapy	1	0.78097	0.36020	4.7010	0.0301	2.184	1.078	4.424
	allergic	1	0.25763	0.34749	0.5497	0.4584	1.294	0.655	2.557
	Surgical outcome	1	0.11328	0.22536	0.2527	0.6152	1.120	0.720	1.742
	Maintenance treatment	1	-0.66046	0.45183	2.1367	0.1438	0.517	0.213	1.252
PFS1	FIGO	1	-0.12379	0.23182	0.2852	0.5933	0.884	0.561	1.392
	neoadjuvant chemotherapy	1	0.92902	0.28269	10.8000	0.0010	2.532	1.455	4.407
	allergic	1	0.54727	0.21326	6.5856	0.0103	1.729	1.138	2.625
	Chemotherapy cycles	1	0.80586	0.20833	14.9632	0.0001	2.239	1.488	3.368
	Surgical outcome	1	0.31210	0.17067	3.3443	0.0674	1.366	0.978	1.909
	Maintenance treatment	1	-0.34536	0.22409	2.3751	0.1233	0.708	0.456	1.098
	Allergy drug	1	0.35025	0.15831	4.8950	0.0269	1.419	1.041	1.936
PFS2	FIGO	1	0.65887	0.39556	2.7745	0.0958	1.933	0.89	4.196
	neoadjuvant chemotherapy	1	-0.40879	0.52347	0.6098	0.4349	0.664	0.238	1.854
	allergic	1	-0.14548	0.41474	0.1230	0.7258	0.865	0.384	1.949
	Chemotherapy cycles	1	-0.43906	0.34058	1.6619	0.1974	0.645	0.331	1.257
	desensitization outcome	1	0.95401	1.25883	0.5743	0.4485	2.596	0.220	30.608
	Surgical outcome	1	0.26510	0.28226	0.8821	0.3476	1.304	0.750	2.267
	Maintenance treatment	1	1.06397	0.36182	8.6469	0.0033	2.898	1.426	5.889
	Desensitization drugs	1	0.11367	0.29666	0.1468	0.7016	1.120	0.626	2.004

FIGO, The International Federation of Gynecology and Obstetrics; PFS, progression-free survival; OS, overall survival; DF, degree of freedom; PE, Parameter Estimation; STD ERR, standard Error; HR, Hazard Ratio; CI, confidence interval.

3.3 Effects of desensitization therapy on the prognosis of patients with different types of recurrence

The risk factors for the prognosis of patients with different types of recurrence included the FIGO stage, neoadjuvant chemotherapy, severity of the allergy, number of chemotherapy cycles, desensitization outcome, surgical outcome, maintenance treatment, and allergy agent. The univariate and multivariate analyses revealed that the desensitization outcomes did not affect the OS outcomes in platinum-resistant recurrent patients after initial treatment or patients with progressive ovarian cancer ($P > 0.05$). However, it significantly affected the OS outcomes of platinum-sensitive recurrent patients, with successful desensitization leading to better OS outcomes ($P < 0.05$). The details are presented in Table 5.

4 Discussion

4.1 Principal finding

Platinum desensitization therapy has potential application value in patients who were platinum-sensitive recurrence after first-line treatment but may bear the risk of severe allergic reactions.

4.2 Results in the context of what is known

Platinum agents can cause type I reactions, cytokine release reactions, and mixed reactions. Evidence supporting an immunoglobulin E (IgE)-mediated hypersensitivity reaction (HSR) mechanism of platinum agents was first reported in refinery workers exposed to platinum salts, followed by the detection of carboplatin-specific IgE (12). Most reactions occurred immediately, during, or within hours after infusion. The clinical features of platinum-based agent allergies were diverse and varied in severity, with most presenting as mild-to-moderate reactions such as rash, itching, chest tightness, and palpitations, and a few presenting as severe reactions such as difficulty breathing or even anaphylactic shock. No studies to date reported deaths due to platinum agent allergies.

Rapid drug desensitization (RDD) is a treatment modality that renders mast cells less responsive, thereby protecting patients from allergic reactions. In IgE-sensitized patients, desensitization inhibits the mechanisms of mast cell activation (12). The NCCN guidelines recommend desensitization as an option for patients who have experienced drug reactions (1). Platinum desensitization therapy has also been successful in many cases. Confino-Cohen et al. (16) reported a safe and effective desensitization therapy for patients with tumors

TABLE 3 The basic characteristics of patients.

variable types	n (%)
FIGO stage	
FIGO I stage	5 (5.4%)
FIGO II stage	10 (10.9%)
FIGO III stage	66 (71.7%)
FIGO IV stage	11 (12.0%)
neoadjuvant chemotherapy	
Y	55 (59.8%)
N	37 (40.2%)
Maintenance treatment	
no	76 (82.6%)
before desensitization	11 (12.0%)
after desensitization	5 (5.4%)
chemotherapy cycles	
Low-5	16 (17.4%)
5-10	26 (28.3%)
10-High	49 (53.3%)
Surgical outcome	
R0	60 (65.2%)
R1	17 (18.5%)
R2	15 (16.3%)

R0: No cancer remain; R1: All large lesions have been removed, and there are cancer on the resection margin under the microscope; R2: Tumor remains visible to the eyes; FIGO: The International Federation of Gynecology and Obstetrics.

TABLE 4 Basic characteristics related to desensitization.

Variable types	n (%)
Allergy medicine	
Cisplatin	19 (20.7%)
Carboplatin	55 (59.8%)
Oxaliplatin	12 (13.0%)
Nedaplatin	6 (6.5%)
Desensitization drugs	
Cisplatin	1 (1.1%)
Carboplatin	65 (70.7%)
Oxaliplatin	15 (16.3%)
Nedaplatin	11 (11.9%)
Desensitization outcome	
Desensitization successful	86 (93.5%)
Desensitization failed	6 (6.5%)

who were allergic to carboplatin. Among 228 patients with ovarian cancer or primary peritoneal cancer and 26 patients with endometrial serous papillary carcinoma who received carboplatin monotherapy or carboplatin-based combination chemotherapy, patients who developed immediate hypersensitivity reactions to carboplatin were administered a carboplatin skin test. Further, the patients who had a positive skin test were given carboplatin desensitization therapy for 6 h in each subsequent treatment. The desensitization therapy involved preparing four concentrations of carboplatin solution, with the first three containing 1/1000, 1/100, and 1/10 total dose of carboplatin, respectively, diluted in 150 mL of 5% glucose, and the fourth containing the remaining carboplatin. The infusion started with the 1/1000 carboplatin solution, and the infusion time for each concentration was more than 90 min. If the previous concentration was infused successfully, the next higher concentration was infused immediately. Twenty-three patients were allergic to carboplatin and had positive skin tests. Twenty patients received desensitization therapy while continuing carboplatin chemotherapy. Only one patient experienced mild skin rash in the first desensitization therapy and discontinued the treatment, whereas the other 19 patients had no adverse reactions and tolerated 80 courses of desensitization chemotherapy. Lee et al. (17) reported a 6-h, 12-step desensitization protocol for carboplatin allergy. The desensitization protocol involved preparing three concentrations of carboplatin solution, with the first two containing 1/100 and 1/10 total dose of carboplatin, respectively, diluted in 250 mL of 5% glucose solution, and the third containing the remaining carboplatin. The first concentration of carboplatin was used in steps 1–4, the second concentration in steps 5–8, and the third in steps 9–12. In the first 11 steps, the carboplatin infusion speed was adjusted every 15 min (doubled) in each step. The infusion started with 1/100 carboplatin, and the infusion time for each concentration was more than 90 min. Ten patients who received carboplatin monotherapy or carboplatin-based combination chemotherapy were treated, including eight patients with ovarian cancer, 1 with primary peritoneal cancer, and 1 with endometrial cancer. All 10 patients successfully completed 35 desensitization courses against carboplatin, with 31 courses showing no reactions. Four patients developed symptoms during the first (n = 3) or third (n = 1) desensitization courses, but repeat injections were tolerated without further reactions. The desensitization protocols for two patients who had developed cutaneous reactions were adjusted, while no adjustments were made for one patient who had developed a mild rash. All three patients could tolerate subsequent desensitization therapy without adverse reactions. The fourth patient had a reaction during desensitization therapy and could not receive further carboplatin treatment due to disease progression. Of the five patients who underwent carboplatin skin testing, four had positive blisters and erythema. One patient had a negative skin test for carboplatin after desensitization therapy. Kokabu et al. (18) reported successful nedaplatin desensitization, suggesting that the nedaplatin desensitization regimen could be a new alternative for hypersensitivity reactions to platinum-based agents. In this study, a 5-step platinum desensitization protocol was used for 92 patients, and 552 desensitization treatments were performed for platinum agents including cisplatin, carboplatin, oxaliplatin, and nedaplatin. Only six treatments failed, resulting in a high success rate of 98.9%. The results

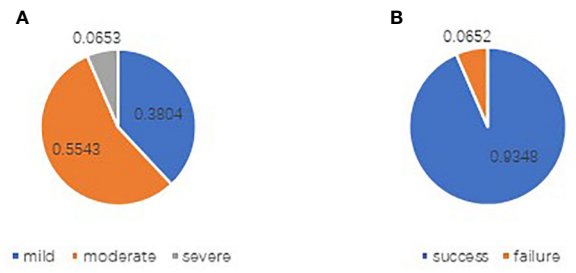

FIGURE 2
Distribution of allergy levels in patients with platinum allergy **(A)** and outcomes of desensitization treatment **(B)**.

indicated that platinum desensitization therapy was effective, and all platinum agents could be desensitized using the five-step desensitization protocol. However, among the six cases of desensitization failure, five experienced severe allergic reactions during desensitization and required rescue treatment, such as adrenaline, to alleviate symptoms. We have emergency response plans for anaphylactic shock. After rescue and treatment, all patients who failed desensitization were significantly relieved. Although the success rate of desensitization was high, the potential risk of severe allergic reactions in patients who failed desensitization might be high too.

Platinum-based chemotherapy is effective in ovarian cancer treatment, with an initial response rate as high as 60%–80% (19). Whether platinum desensitization treatment is associated with the prognosis of patients with ovarian cancer and whether it is worth bearing the potential risk of fatal allergic reactions during desensitization treatment after an allergic reaction to platinum chemotherapy agents remain unclear due to the lack of clinical data. This study retrospectively analyzed the impact of platinum desensitization on the prognosis of patients with ovarian cancer, taking into account confounding factors such as patient's FIGO stage, neoadjuvant chemotherapy, surgical outcome, maintenance therapy, and severity of allergic reactions. The present study revealed that desensitization treatment improved the prognosis of platinum-sensitive recurrent patients, but did not improve the prognosis of platinum-resistant recurrent patients or patients with disease progression during initial treatment. The desensitization treatment may provide survival benefits to platinum-resistant recurrent patients who experience a platinum allergy reaction, but the potential risk of severe allergic reactions caused by desensitization treatment needs to be considered.

4.3 Clinical implications and research implications

Various complex clinical confounding factors may affect the long-term prognosis of patients, making it unclear whether desensitization treatment can significantly improve the progression-free survival (PFS) or overall survival (OS) of patients with ovarian cancer. Therefore, this study aimed to use multifactorial analysis to investigate the impact of platinum desensitization outcomes on the prognosis of patients with ovarian cancer, providing a basis for better-serving patients with platinum desensitization therapy. The success rate of desensitization in 6 patients with severe allergic reactions was 100%. Was there a relationship between the degree of allergy reaction and the successful rate of desensitization? Because the sample size was too small, we were unable to answer this question. A large sample study is needed. We are planning a multicenter, large-sample, real-world study.

4.4 Strengths and limitations

This study was a single-center retrospective clinical study with small sample size and a long treatment time span. The influence of patient's genetic status and other unpredictable confounding factors was not investigated. Therefore, the results of this study should be evaluated with caution. Notably, this study was novel in exploring the application value of platinum desensitization in ovarian cancer through multivariate analysis and analyzing various outcomes of

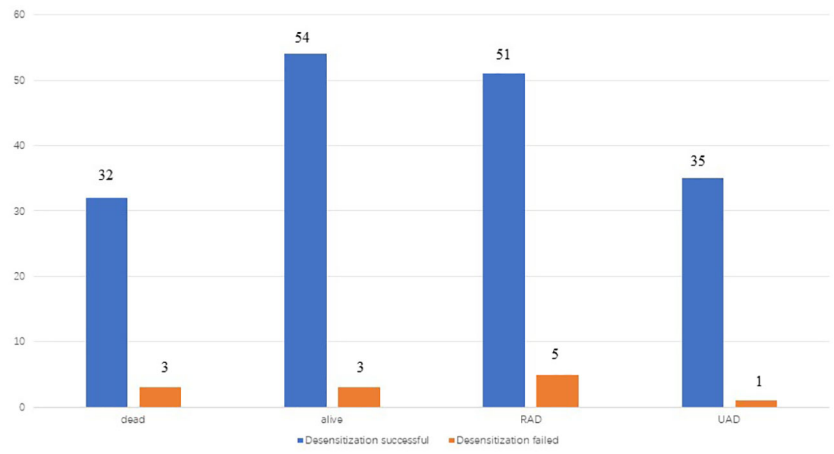

FIGURE 3
recurrence and death of patients in two groups(desensitization successful and desensitization failed) by the end of follow-up.

TABLE 5 Subgroup analysis: Multivariate analysis of influence on Patient's prognosis (platinum sensitive relapse).

variable types		DF	PE	STD ERR	X^2	P	HR	HR 95%CI	
PFS2	FIGO	1	0.14757	0.49896	0.0875	0.7674	1.159	0.436	3.082
	neoadjuvant chemotherapy	1	0.01693	0.62015	0.0007	0.9782	1.017	0.302	3.429
	allergic	1	-0.65006	0.50724	1.6424	0.2000	0.522	0.193	1.411
	chemotherapy cycles	1	-0.86049	0.41662	4.2659	0.0389	0.423	0.187	0.957
	desensitization outcome	1	0.52742	1.25318	0.1771	0.6739	1.695	0.145	19.759
	Surgical outcome	1	0.24655	0.29059	0.7199	0.3962	1.280	0.724	2.262
	Maintenance treatment	1	1.08510	0.36610	8.7849	0.0030	2.960	1.444	6.066
	Desensitization drugs	1	0.36415	0.37561	0.9399	0.3323	1.439	0.689	3.005
OS	FIGO	1	-0.0299	0.51491	0.0034	0.9537	0.971	0.354	2.663
	neoadjuvant chemotherapy	1	1.72302	0.54462	10.009	0.0016	5.601	1.926	16.288
	allergic	1	0.27486	0.42365	0.4209	0.5165	1.316	0.574	3.020
	chemotherapy cycles	1	0.23943	0.35939	0.4438	0.5053	1.271	0.628	2.570
	desensitization outcome	1	-1.7540	0.72829	5.8003	0.0160	0.173	0.042	0.721
	Surgical outcome	1	-0.0777	0.30764	0.0638	0.8005	0.925	0.506	1.691
	Maintenance treatment	1	-0.7743	0.51750	2.2389	0.1346	0.461	0.167	1.271
	Desensitization drugs	1	-0.3111	0.41561	0.5604	0.4541	0.733	0.324	1.654

FIGO, The International Federation of Gynecology and Obstetrics; PFS, progression-free survival; OS, overall survival; DF, degree of freedom; PE, Parameter Estimation; STD ERR, standard Error; HR, Hazard Ratio; CI, confidence interval.

platinum allergy and desensitization at the same time, demonstrating significant clinical guidance value.

5 Conclusions

In summary, the five-step platinum desensitization protocol was found to be safe and effective. The platinum-sensitive recurrent patients may benefit from platinum desensitization treatment, but the potential risk of serious allergic reactions should be considered. Further, large-sample prospective high-quality clinical studies are needed to confirm these findings.

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 humans were approved by Medical Ethics Committee of West China Second University Hospital, Sichuan University. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because exempt from signing informed consent by Medical Ethics Committee.

Author contributions

KL: Conceptualization, Formal Analysis, Methodology, Software, Writing – original draft, Writing – review & editing. RY: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing.

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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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Case report: A novel perspective on the treatment of primary tracheal small cell carcinoma: a patient's experience with immuno-combined EP therapy and literature review

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Tracheal small cell carcinoma (SCC) is a rare malignancy, for which the optimal treatment strategy has yet to be determined. Currently, treatment largely aligns with the therapeutic guidelines established for small cell lung cancer, although numerous unresolved issues remain. This paper details a case study of a patient with Stage IIIB primary tracheal SCC, who was treated with an immune-combined etoposide-platinum(EP) regimen. This treatment offers valuable insights into innovative approaches for managing such malignancies. Furthermore, the study includes a comprehensive literature review to better contextualize the findings. The patient, admitted on May 2, 2023, had been experiencing persistent symptoms of airway discomfort for 15 days. A bronchoscopy performed on May 4 revealed tracheal SCC, classified as T4N2M0, IIIB. Following the CAPSTONE-1 study's methodology, the patient underwent six cycles of PD-L1(adebrelimab) combined with EP therapy, leading to significant relief of symptoms and the eventual disappearance of the tracheal mass.

KEYWORDS

tracheal small cell carcinoma, immune-combined therapy, etoposide-platinum regimen, adebrelimab, optimal treatment strategy

Introduction

Primary tracheal cancer, a relatively uncommon malignant respiratory tumor, represents approximately 0.2% of such neoplasms (1). Its pathological subtypes include squamous cell carcinoma, adenoid cystic carcinoma, among others, with rarer forms like adenosquamous carcinoma, mucinous epidermoid carcinoma, undifferentiated glandular carcinoma, melanoma, and chondrosarcoma (2). In the early stages of the disease,

symptoms often lack specificity (3), and some individuals may exhibit manifestations like cough, sputum production, chest tightness, and shortness of breath, often leading to misdiagnosis as other respiratory conditions like chronic obstructive pulmonary disease or bronchitis (4). Diagnosis is definitively established through imaging and histological examinations. As the tumor grows, obstruction of the tracheal lumen may occur, resulting in symptoms of respiratory distress. Involvement of blood vessels may lead to hemoptysis, and when lymph nodes are affected, symptoms such as lymphadenopathy and tenderness may manifest (2). Among these, SCC of the airway is particularly rare, with only a limited number of cases described. The clinical characteristics of tracheal SCC closely resemble those of squamous cell carcinoma of the airway, encompassing factors such as patient age, gender, ethnicity, disease severity, lymph node involvement, and treatment modalities, including surgery and radiation. Notably, patients with tracheal SCC are more likely to receive chemotherapy (5). Some scholars have posited that treatment regimens effective for small cell lung cancer might also be applicable to tracheal SCC (6). However, the optimal therapeutic approach for tracheal SCC continues to be a topic for further investigation. Addressing the need for a more comprehensive understanding of tracheal SCC, this paper reports on a case of airway SCC where significant relief was achieved through the adoption of immunotherapy combined with EP treatment, informed by the CAPSTONE-1 study methodology. methodology, this report includes a thorough review of relevant literature.

Case presentation

On May 2, 2023, an 83-year-old male was admitted, reporting a 15-day history of recurrent cough, sputum production, chest tightness, and wheezing. The patient described an exacerbation of these symptoms, characterized by white, viscous sputum, and noted that the chest tightness and wheezing intensified with physical activity. Despite self-medicating (details unspecified), the symptoms showed no significant improvement. Consequently, the patient sought medical care at our outpatient clinic. A pulmonary CT scan conducted on the same day revealed a lesion at the tracheal prominence indicative of malignant tumor (MT), enlarged mediastinal lymph nodes suggestive of lymphatic metastasis, a small nodule in the right lower lobe, and scattered inflammatory lesions in both lungs. The scan also showed signs of bronchiectasis and pulmonary emphysema (Figure 1A). The patient's medical history was notably unremarkable for major illnesses or exposures, and there was no history of substance abuse or familial diseases. Comprehensive examinations conducted on May 3, 2023, including blood tests and scans, yielded normal results, apart from elevated tumor markers (CEA, NSE, and ProGRP).

On May 5, 2023, a bronchoscopy identified an extrinsic compressive growth causing tracheal stenosis, with mucosal swelling and an irregular surface, approximately 13 cm below the glottis. The narrowed tracheal lumen measured about 10x5 mm, where a mucosal biopsy was obtained (Figure 2A). Histopathological examination and immunohistochemistry combined with diagnosis of tracheal SCC (Figure 3), with the tumor tissue expressing CD56+, TTF-1+, NapsinA-, P40-P63-, and LCA-Ki-67 approximately 90% positive. The patient, classified as stage IIIB (T4N2M0) with an ECOG performance status of 1, tolerating the treatment well and exhibiting no significant adverse reactions. Follow-up pulmonary CT on June 28 (Figure 1B), August 22 (Figure 1C), and November 1, 2023 (Figure 1D), demonstrated a marked reduction in the tracheal prominence An electron bronchoscopy on November 2, 2023, revealed slight scarring and some point-like projections at the lower end of the tracheal prominence, without tracheal obstruction (Figure 2B).

Additionally, from the initial admission until November 5, 2023, the patient was subjected to regular monitoring encompassing blood routine, liver and kidney function, myocardial enzyme spectrum, and thyroid function tests. During this monitoring period, mild fluctuations were observed in thyroid hormone levels, which ranged between 40-46 mg/L and a slight leukopenia, varying between 1-2 degrees, were noted. Other parameters, including liver and kidney function tests and myocardial enzyme spectrum, consistently indicated no significant abnormalities. Importantly, throughout this period, the patient did not develop any immune-related dermatomyositis.

Discussion

Primary tracheal tumors are considered rare, with primary SCC of the trachea being notably elusive, and its optimal treatment strategy remains insufficiently explored. Presently, clinical approaches often draw upon the treatment protocols established for small cell lung cancer. Nonetheless, significant inconsistencies are observed in the staging of tracheal malignant tumors compared to lung malignancies, along with variations in diagnostic and therapeutic procedures. To address this, a distinct staging and diagnostic-treatment workflow has been developed, employing the TNM staging system for classification (Table 1). This system stratifies cases into four stages based on diverse TNM manifestations (Table 2). Despite the availability of this independent staging protocol, ta gap persists due to the lack of large-scale clinical research focused on optimal treatment strategies. As a result, treatment procedures are predominantly based on clinical staging (Table 3) (7, 8). The primary treatment approach for these cases typically involves a combination of radiation therapy and chemotherapy; however, the efficacy of various regimen remains to be definitively established. Furthermore, the management of tracheal SCC is characterized by its diversity, potentially influencing the overall treatment effectiveness. Building upon the foundation of TNM staging, this study entails a retrospective analysis of global tracheal SCC cases reported since 2004. The subsequent table (Table 4) delineates the current challenges in treatment (4, 6, 9, 10):

In the four cases under consideration, the predominant symptoms included respiratory distress and hoarseness, with some patients also presenting with cough and swallowing difficulties, indicating possible esophageal involvement. Of these

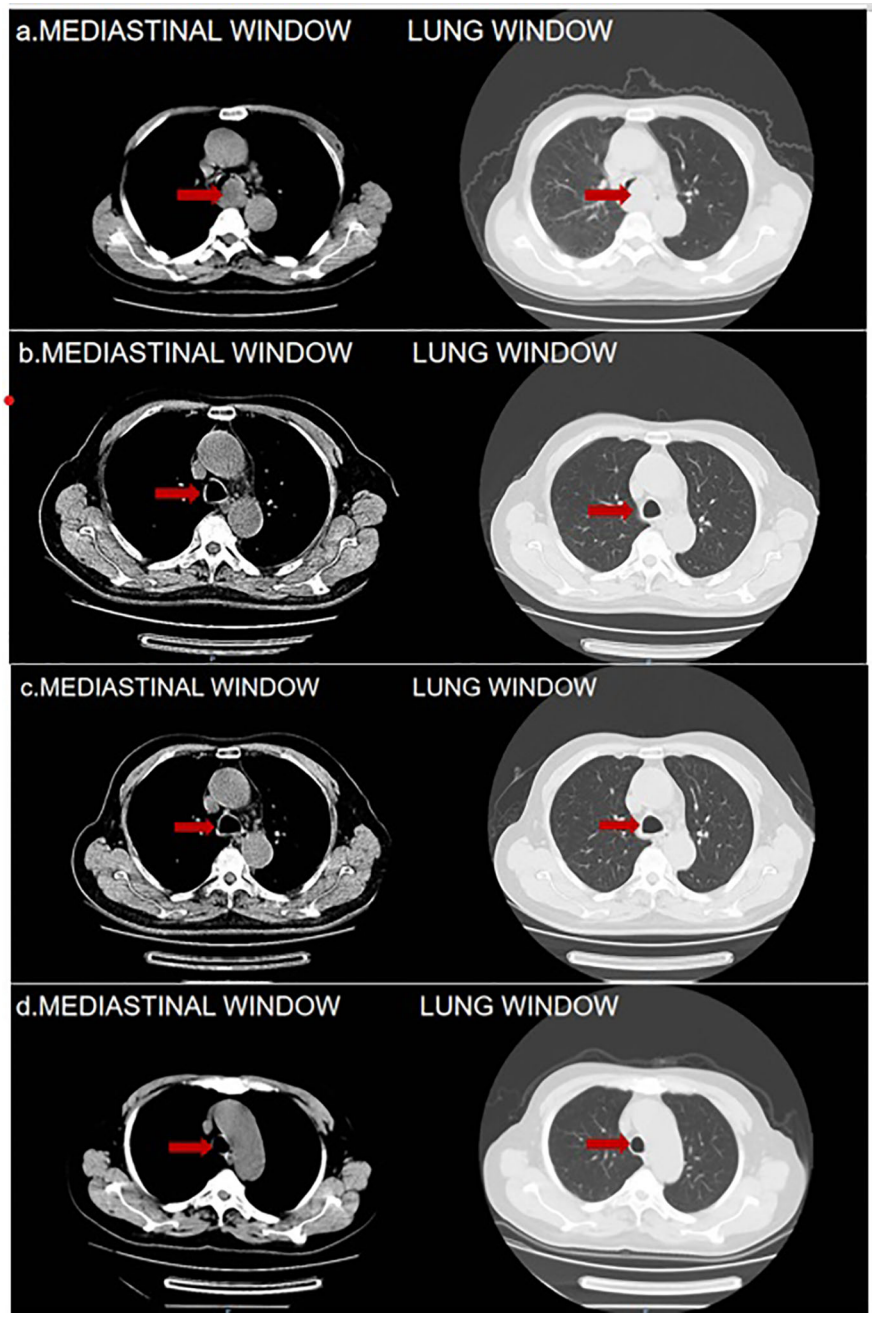

FIGURE 1
(A) 2023-5-2 Pulmonary CT Findings: Tracheal Prominence Occupancy. **(B)** 2023-6-28Pulmonary CT Findings: Significant Reduction in the Mass Below the Tracheal Prominence. **(C, D)** 2023-8-22 and 2023-11-1Pulmonary CT Findings: Absence of Mass Below the Tracheal Prominence in Both. The red arrows are used to indicate the location of tumor.

cases, three were identified as primary, while one was classified as secondary. All cases were treated with a combination of chemotherapy and radiotherapy. The maximum recorded survival period was 5 years, though the total survival durationfor some cases has not been comprehensively documented. Notably, tracheal SCC is characterized by a wide age range at onset, prolonged overall case duration, scarce reported instances, extremely low incidence rates, and no apparent gender predilection. These cases highlight that the optimal treatment modalities for tracheal SCC necessitate further in-depth investigation. Typically, tracheal SCC presents as an intrabronchial mass, and currently, there are no well-established treatment guidelines. The primary therapeutic strategies are derived from historical treatment experiences and include surgical intervention alone, combined surgery and chemotherapy, or chemotherapy alone (6). Over the past few decades, EP chemotherapy has remained the standard frontline treatment for extensive-stage small cell lung cancer (ES-SCLC), with no significantly superior alternatives yet identified. This regimen typically involves cisplatin and etoposide, administered with or without concurrent radiotherapy. Despite the initial sensitivity of

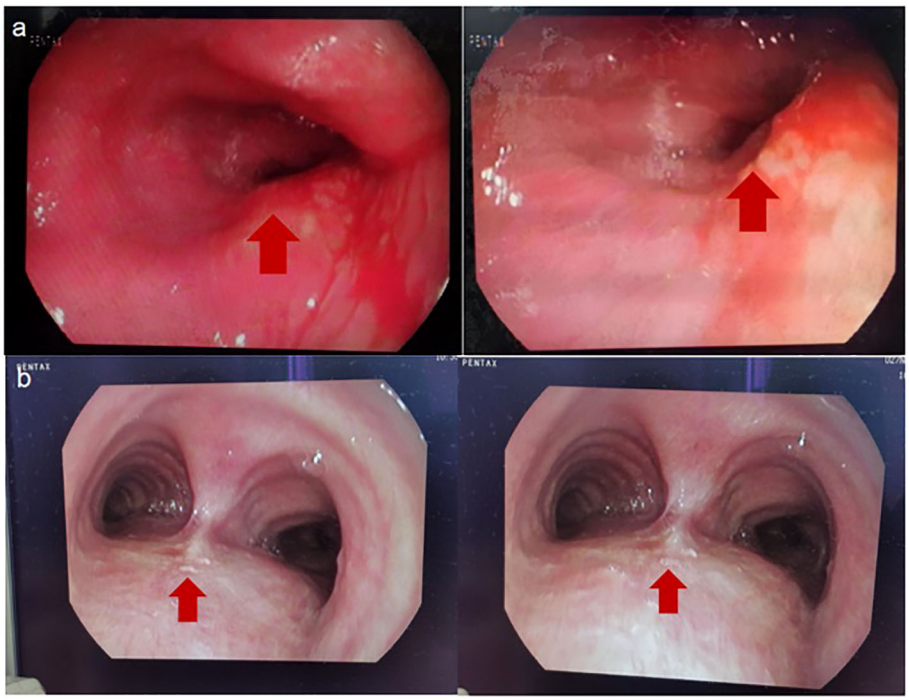

FIGURE 2
(A) 2023-5-5 Fibrobronchoscopy Reveals: Compression of the Tracheal Glottis, Narrowing of the Lumen, and Mucosal Swelling and Congestion. **(B)** 2023-11-2 Fibrobronchoscopy Reveals: The Tracheal Eminence Mass Has Disappeared, Localized with Slight Nodular Protrusions. The red arrows are used to indicate the location of tumor.

ES-SCLC to EP chemotherapy, resistance development is nearly inevitable, leading to tumor recurrence within six months and an objective response rate of approximately 50-60%. Notably, over the past two decades, no significant breakthrough in medical interventions have been achieved, and patient outcomes have not demonstrated marked improvement (11). In contrast, monotherapy with chemotherapy tends to yields less favorable results, and the addition of radiotherapy can introduce unpredictable risks (12). The optimal therapeutic approach and overall efficacy for tracheal SCC remain elusive, presenting significant challenges in its clinical management. Consequently, there is a pressing need for further research and comprehensive clinical studies to establish a more effective andpersonalize treatment strategy for this rare malignancy.

Prior to the introduction of immune checkpoint inhibitors (ICIs), this innovative class of drugs has revolutionized immunotherapeutic options for patients with ES-SCLC, significantly improving survival rates. The combination of ICI with the EP treatment regimen has become a cornerstone in the therapeutic strategy for patients with ES-SCLC (11, 13). Notably, the IMpower 133 regimen, which incorporates the PD-L1 antibody atezolizumab into platinum-based chemotherapy, has demonstrated enhanced overall survival (OS) compared to chemotherapy alone. In a similar vein, the CASPIAN regimen, which integrates the PD-L1 antibody durvalumab with chemotherapy, has also yielded improvements in overall survival (OS) (14). Consequently, adebrelimab is being considered as a

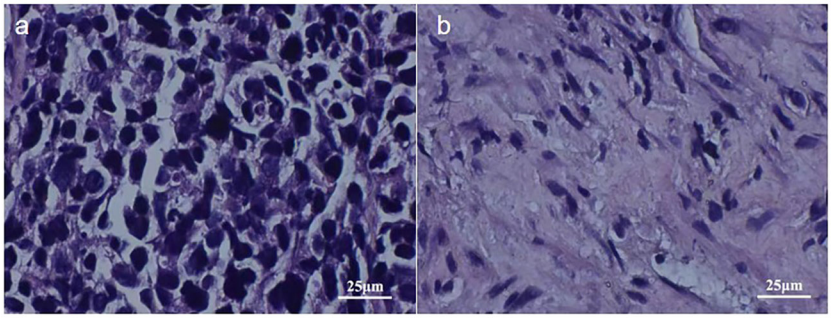

FIGURE 3
2023-5-5 Pathological Images: Image **(A)** Displays Tumor Tissue Cells Under 400x Magnification, Image **(B)** Presents Cells of Para-cancerous Tissue Under 400x Magnification.

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TABLE 1 Paolo Macchiarini TNM staging of tracheal malignant tumors.

Tumor (T) Staging	Tx	Primary tumor cannot be evaluated
	Tis	Tumor in situ, no invasion
	T1a	<3cm, confined to mucosa
	T1b	≥3cm, confined to mucosa
	T2	Tumor invades cartilage or outer membrane; tumor involvement of the membrane is at least classified as T2, regardless of the depth of invasion
	T3	Tumor invades the trachea or larynx
	T4a	Tumor invades the carina or main bronchus
	T4b	Tumor invades adjacent structures
Lymph Node (N) Staging	Nx	Regional lymph nodes cannot be assessed
	N1	Positive regional lymph nodes (N1a <3cm; N1b ≥3cm)
	Upper 1/3	Superior mediastinal lymph nodes; upper tracheal lymph nodes; prevascular and retrotracheal nodes
	Middle 1/3	Upper tracheal lymph nodes; prevascular and retrotracheal nodes; lower tracheal lymph nodes; paratracheal nodes (aortopulmonary window)
	Lower 1/3	Upper tracheal lymph nodes; prevascular and retrotracheal nodes; subaortic nodes (aortopulmonary window)
	N1A	1-3 nodes in upper one-third
	N1B	>3 nodes in upper one-third
	N2	Positive regional lymph nodes
	Upper 1/3	Lower tracheal lymph nodes; subaortic nodes (aortopulmonary window)
	Middle 1/3	Superior mediastinal lymph nodes; subaortic nodes (aortopulmonary window)
	Lower 1/3	Upper tracheal lymph nodes; ligamentum arteriosum
Metastasis (M) Staging	Mx	Distant metastasis cannot be evaluated
	M0	No distant metastasis
	M1	Distant metastasis beyond N1 and N2 regions
	M2	Distant metastasis (e.g., in the lungs)

potent therapeutic agent for tracheal SCC. Adebrelimab is a recombinant fully humanized IgG4 monoclonal antibody, exhibits high affinity and specificity for PD-L1. It was officially approved for cancer therapy by the China Drug Evaluation Center (CDE) in 2022 (15). The results of the multicenter phase III clinical trial of adebrelimab, employing the CAPSTONE-1 study design, have now solidified its role as a first-line treatment regimen for ES-SCLC (16). Specifically, this study enrolled a total of 462 patients with ES-SCLC, dividing them equally into two groups: the adebrelimab group, comprising 230 patients (50%) received adebrelimab in combination with chemotherapy, while the placebo group, also consisting of 232 patients (50%), received a placebo alongside chemotherapy. Patients in the adebrelimab group were treated with 4-6 cycles of carboplatin (area under the curve 5 mg/mL per minute, day 1) and etoposide (body surface area 100 mg/m^2, days 1-3), augmented with adebrelimab (20 mg/kg, day 1). In contrast, the placebo group underwent 4-6 cycles of the same chemotherapy regimen, but with a placebo, over a treatment cycle of 21 days. Notably, the median overall survival in the adebrelimab group exhibited significant improvement compared to the placebo

TABLE 2 Paolo Macchiarini TNM staging of tracheal malignant tumors.

Staging	T	N	M
0	Tis	N0	M0
Ia	T1a	N0	M0
Ib	T1b-2	N0	M0
IIa	T1b-2	N1	M0
IIb	T1b-2	N2	M0
IIIa	T3	N0	M0
IIIb	T3	N1-2	M0
IVa	All	N1-2	M1
IVb	All	N1-2	M2

TABLE 3 Diagnostic and therapeutic workflow for malignant tracheal tumors.

Staging	Substage	Objective	Management Strategy
0/I	0/I	Treatment or Observation	Surgical and other local curative treatments or observation
II	IIa/IIb	Cure	Surgical and other local curative treatments, with some adjuvant therapy
IIIa	IIIa/IIIb	Potential cure possibility	Surgical and other local curative treatments for some; majority may require combined systemic therapy and multidisciplinary care
IIIb	IIIb	Potential cure possibility	Multidisciplinary care, surgical and other local curative treatments, and systemic therapy for the majority
IVa	IVa	Potential cure possibility	Multidisciplinary care, systemic therapy for the majority, and some surgical and other local curative treatments
IVb	IVb	Potential cure possibility	Mainly systemic therapy with multidisciplinary care, symptomatic supportive treatment, and palliative care

group. The most common grade 3 or 4 treatment-related adverse events were decreased neutrophil count, white blood cell count, platelet count, and hemoglobin level. The incidence of severe treatment-related adverse events was notably lower in the adebrelimab group. Importantly, there were two cases of treatment-related deaths reported in both the adebrelimab and placebo group among the four cases documented (17). Consequently, the addition of adebrelimab is observed to significantly enhance overall survival rate in patients with ES-SCLC, while maintaining an acceptable safety profile. These findings advocate for the implementation of this combination therapy as a novel first-line treatment option for patients with ES-SCLC.

This article presents a case of primary tracheal SCC in an 83-year-old male patient. The lung CT scan showed bronchiectasis and pulmonary emphysema, along with a lesion occupying the tracheal carina. Bronchoscopy revealed a neoplasm obstructing the lower part of the glottis, situated approximately 13 cm below the vocal cords. The histopathological analysis of the bronchial biopsy specimen, supplemented with immunohistochemical studies, confirmed the diagnosis of trachealSCC. This diagnosis was established based on the clinical data, morphological characteristics, and immunohistochemistry findings, indicating primary tracheal SCC without metastatic involvement. Tracheal SCC typically manifests as an intrabronchial mass. In this case, involving an 83-year-old patient with compromised baseline health and diagnosed with primary tracheal SCC(not secondary to other malignancies), we adhered to the treatment protocol from the CAPSTONE-1 study. Given the proven efficacy of immunotherapy combined with chemotherapy in treating ES-SCLC, we opted for a similar therapeutic regimen. Due to the patient's advanced age and potential for chemotherapy resistance, a reduced dose of carboplatin was administered, alongside etoposide at 0.1g on days 1-5 and carboplatin 200mg on day 5, combined with adebrelimab 1.2g. Remarkably, after just two cycles of chemotherapy and immunotherapy, there was a significant reduction in the tracheal mass below the carina, and following six cycles, the mass completely disappeared without any severe adverse reactions. This combined approach of chemotherapy and immunotherapy demonstrated superior efficacy compared to chemotherapy plus radiotherapy,

TABLE 4 Reported cases of tracheal small cell carcinoma in the last 20 years.

Case No.	1	2	3	4
Gender	Male	Female	Male	Male
Age (years)	28	25	73	77
Discovery Year	2005	2007	2016	2023
Symptoms	Cough, Hoarseness, Swallowing Difficulty	Dyspnea, Hemoptysis	Dyspnea, Hoarseness	Dyspnea
Treatment Regimen	Cisplatin and etoposide for 4 cycles; 1.5 Gy irradiation for 30 sessions	Cisplatin for 6 cycles; Conformal radiation therapy with 6 MV photons, total dose of 5600 cGy in 28 fractions over 38 days	6 cycles (etoposide 100 mg/m^2, cisplatin 80 mg/m^2, every 3 weeks) + 10 sessions of radiation therapy (3000 cGy)	Cycle 1: carboplatin (AUC = 5mg/mL/min) and etoposide (80 mg/m^2). Cycle 2 onwards: cisplatin and etoposide (60 mg/m^2); Cycles 3-4 with added AHF (45 Gray/30 fractions). After complete remission, whole-brain irradiation (25 Gray/10 fractions)
Primary	Yes	No	Yes	Yes
Overall Survival Time(Follow-up time)	21 months	In the referenced article, the patient's survival time is not mentioned	5 years	In the referenced article, the patient's survival time is not mentioned

offering the patient a less distressing treatment course, maintaining overall well-being, and suggesting a novel therapeutic strategy for similar cases.

Bronchial carcinomas are relatively rare with small cell bronchial carcinoma being even more uncommon. Currently, the optimal treatment approach for such rare diseases remains unclear, The standard regimen typically involves platinum-based chemotherapy, commonly combining cisplatin and etoposide. This particular case of SCC offers a new perspective on potential treatment strategies. Taking into account the patient's physical condition, the integration of immunotherapy with EP regimen can effectively enhance survival rates for patients with tracheal SCC, thereby presenting a novel therapeutic avenue.

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

We asked the ethics committee and they said this type doesn't require approval. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from a by- product of routine care or industry. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YC: Writing – original draft. HZ: Writing – review & editing. DW: Resources, Writing – review & editing. YY: Resources, Writing – review & editing. JG: Resources, Writing – review & editing.

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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

Supplementary material

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Antibody dependent cellular cytotoxicity-inducing anti-EGFR antibodies as effective therapeutic option for cutaneous melanoma resistant to BRAF inhibitors

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Introduction: About 50% of cutaneous melanoma (CM) patients present activating BRAF mutations that can be effectively targeted by BRAF inhibitors (BRAFi). However, 20% of CM patients exhibit intrinsic drug resistance to BRAFi, while most of the others develop adaptive resistance over time. The mechanisms involved in BRAFi resistance are disparate and globally seem to rewire the cellular signaling profile by up-regulating different receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR). RTKs inhibitors have not clearly demonstrated anti-tumor activity in BRAFi resistant models. To overcome this issue, we wondered whether the shared up-regulated RTK phenotype associated with BRAFi resistance could be exploited by using immune weapons as the

antibody-dependent cell cytotoxicity (ADCC)-mediated effect of anti-RTKs antibodies, and kill tumor cells independently from the mechanistic roots.

Methods and results: By using an *in vitro* model of BRAFi resistance, we detected increased membrane expression of EGFR, both at mRNA and protein level in 4 out of 9 BRAFi-resistant (VR) CM cultures as compared to their parental sensitive cells. Increased EGFR phosphorylation and AKT activation were observed in the VR CM cultures. EGFR signaling appeared dispensable for maintaining resistance, since small molecule-, antibody- and CRISPR-targeting of EGFR did not restore sensitivity of VR cells to BRAFi. Importantly, immune-targeting of EGFR by the anti-EGFR antibody cetuximab efficiently and specifically killed EGFR-expressing VR CM cells, both *in vitro* and in humanized mouse models *in vivo*, triggering ADCC by healthy donors' and patients' peripheral blood cells.

Conclusion: Our data demonstrate the efficacy of immune targeting of RTKs expressed by CM relapsing on BRAFi, providing the proof-of-concept supporting the assessment of anti-RTK antibodies in combination therapies in this setting. This strategy might be expected to concomitantly trigger the crosstalk of adaptive immune response leading to a complementing T cell immune rejection of tumors.

KEYWORDS

cutaneous melanoma, BRAF, drug resistance, receptor tyrosine kinases, antibody dependent cell cytotoxicity

1 Introduction

Cutaneous melanoma (CM) is a very aggressive malignancy that originates from melanocytes, and shows a continuously increasing incidence in industrialized countries, with a more frequent diagnosis in young and middle-aged adults (1). Early tumor recognition and subsequent surgical treatment are usually curative for CM. However, diagnosis of CM may be difficult and it may be clinically misdiagnosed in a significant number of cases (2). CM escaping the clinical recognition frequently present in an advanced stage, are essentially unresponsive to conventional therapies, and show a poor prognosis (3, 4). Efforts in defining the biology of this malignancy have identified activating BRAF mutations in about 50% of CM patients. The constitutive activation of MAPK signaling caused by mutant BRAF appears a major driver of CM proliferation, survival, and progression. Accordingly, small molecule inhibitors of BRAF (BRAFi) demonstrated important clinical activities in BRAF-mutant CM, with remarkable response rates, and a significantly improved progression-free and overall survival in the advanced disease (5–9). However, the clinical effectiveness of these targeted therapeutics is greatly impaired by the almost invariable onset of an early drug resistance, which leads to tumor progression within about 7 months from the start of treatment in 50% of patients (8, 10, 11). Besides, about 20% of CM patients show intrinsic resistance to BRAFi and do not respond to treatment (12). The underlying mechanisms of resistance so far described are various and heterogeneous (7, 13–23).

Though the underlying mechanisms are disparate, a unifying feature of CM resistance to BRAFi appears the rewiring cellular signaling profiles (18, 20, 21, 24–28), which is frequently associated with a *de novo*, up-regulated or "positively selected" expression of different receptor tyrosine kinases (RTKs), including AXL, EGFR, IGF-1R, PDGFRα, and PDGFRβ (18–20, 24, 29–32). In this context, whether the expression of specific RTK is essential for the maintenance of the resistant phenotype is not fully defined. Indeed, literature data are discrepant, including reports showing cooperation between small molecule RTK inhibitors (RTKi) (e.g., the EGFR inhibitor gefitinib, the AXL inhibitor R428) and BRAFi [e.g. PLX4032 (vemurafenib), PLX4720] in reducing the *in vitro* and *in vivo* growth of BRAFi-resistant CM cells (19, 29, 30, 33–35), together with studies reporting negligible activity of RTKi on sensitivity to BRAFi after resistance is acquired (20, 24, 36). In addition, a recent study by Molnar et al. indicated that BRAFi-resistant CM cells with higher EGFR expression were more resistant to the treatment with erlotinib respect to those that expressed low levels (37). Besides, EGFR inhibitors simultaneously administered with other agents (38) or inhibitors of common RTK downstream pathways, such as those targeting SRC (e.g. Dasatinib) or PI3K (e.g.

GDC0941), were shown to be more effective in re-sensitizing to BRAFi (7, 20, 29, 36, 39), suggesting that the signaling alterations responsible for the resistant phenotype are broad, and thus, likely not strictly dependent on the activity of a single RTK. In line with these notions, recent single cell CM sequencing approaches are suggesting the co-existence in the same tumor of very different populations that are resistant to targeted therapies still being characterized by importantly dissimilar cellular programs, while pre-existing rare populations of CM cells marked by surface EGFR expression have been proposed as possible seeds for relapsing tumors (18).

These complex redundant signaling networks that are emerging as the main drivers of BRAFi resistance appear difficult to target by mechanistic approaches, and likely amenable to further resistance by signaling rewiring. Nevertheless, the shared phenotype of BRAFi resistant cells may itself represent a therapeutic target that could be actionable by drugs already available in the clinic. Among these, monoclonal antibodies (mAbs) directed to RTK appear particularly suited and appealing as compared to small molecule RTKi, since mAbs are able to act also via immune-mediated mechanisms. Indeed, when antibodies bind antigens exposed on cells, their Fc region can ligate and crosslink the Fcγ receptor (FcγR) expressed on immune effector cells, mainly Natural Killer (NK) cells, but also myeloid-derived effectors (40). Upon FcγR engagement, effector cells are activated and release cytokines as well as cytotoxic granules that ultimately lead to target cell killing in a process referred to as Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) (40). In this process, antibodies of the IgG1 isotype (e.g., the anti-CD20 rituximab, the anti-HER2 trastuzumab, the anti-EGFR cetuximab) are particularly effective (41).

From a therapeutic point of view, the immune-mediated activities of anti-RTK mAbs were shown to significantly contribute to their clinical activity. This is well established for trastuzumab, both in pre-clinical and clinical settings (42–44), and recent data support a role of ADCC also in delivering part of the clinical activity of cetuximab (45). Indeed, several immunogenetic studies associated high-affinity FcγR genotypes to improved therapeutic efficacy of cetuximab in colorectal cancer patients (46–49), and the ability of patients' NK cells to mount *ex vivo* an effective cetuximab-triggered ADCC associated with an improved clinical benefit of colorectal and head and neck squamous cell carcinoma patients (49, 50). Notably, ADCC activity could allow therapeutic targeting of RTK-expressing cells independently of the effects on signaling, contributing to trigger a broader anti-tumor immune response as well (44, 51, 52).

Based on the above notions, we sought to comprehensively investigate the feasibility of targeting RTKs, in particular EGFR, as a strategy to treat CM patients relapsing on BRAFi. A panel of 9 BRAF mutant metastatic CM cell cultures, which were made resistant to the PLX4032 BRAFi, was used as a model to evaluate: i) the expression of RTK and their ligands in CM cells acquiring BRAFi resistance; ii) the effect of EGFR targeting by small molecule inhibitors, mAbs, and genomic editing approaches on BRAFi sensitivity; and iii) the anti-tumor efficacy of EGFR targeting mAbs through ADCC.

Our data demonstrate that, in our model, signaling through EGFR-up-regulation/*de novo* expression is not required for the maintenance of BRAFi resistance, and, as such, inhibition of signaling *via* single RTK may fail in restoring sensitivity to BRAFi. Nevertheless, EGFR targeting through immune-mediated mechanisms is effective in killing tumor cells by ADCC, both *in vitro* and *in vivo*. These data support the potential clinical activity of anti-RTK mAbs in BRAFi-resistant CM and provide the grounds for their clinical evaluation in combination therapies for the treatment of CM patients relapsing on BRAFi.

2 Results

2.1 Expression of RTKs and their ligands in CM cell cultures with acquired resistance to BRAFi

In an attempt to generate an *in vitro* model of resistance to BRAFi, resistant cell cultures (vemurafenib resistant, VR) were obtained from 9 BRAF-mutant metastatic CM cell lines through sequential adaptation to escalating concentrations of the BRAFi PLX4032. Parental cell lines (P) carried BRAF V600E or BRAF V600K activating BRAF mutations and were all wild-type for NRAS (Supplementary Table 1). Dose-response curves confirmed an extreme resistance of VR cells to PLX 4032 (IC_{50} ranging from 16.7 μM of Mel 262 to 29.6 μM of Mel 599) as compared to their highly sensitive P counterparts (IC_{50} ranging from 0.06 μM of Mel 593 to 0.44 μM of Mel 336) (Supplementary Figure 1). RNA sequencing was then performed on three cell lines (Mel 599, Mel 611 and Mel 767) in order to define changes in RTKs expression profile in VR cells (Supplementary Table 2). As shown in Figure 1, different RTKs and several ligands were *de novo* expressed/up-regulated in VR cell lines (Figures 1A, B). In addition, an augmented expression of a number of transcription factors that have been described as positive determinants of BRAFi resistance was also observed (Figure 1C). Among them, a consistent increase in androgen receptor (AR) expression was found in VR cells. Interestingly, AR has been recently described as a positive determinant of BRAFi resistance and EGFR expression (53). Hence, all VR cells were quantitatively evaluated for AR and EGFR transcripts. An up-regulation of AR expression was found in all but one VR cell lines (Figure 1D, Supplementary Table 3), whereas a significant increase in EGFR mRNA expression levels was detected in 4 (Mel 593, Mel 599, Mel 611 and Mel 767) out of the 9 VR cell lines investigated, as compared to their P isogenic counterpart (Figure 1E, Supplementary Table 3), thus suggesting that AR might be partially responsible for EGFR overexpression in our *in vitro* model. EGFR transcript up-regulation was paralleled by that of its NRG1 ligand (Figures 1F, H, Supplementary Table 3), while no consistent co-expression was found with the other ligands tested (i.e. EGF) (Figures 1G, Supplementary Table 3). In line with molecular data, flow cytometry analyses confirmed a *de novo* cell-surface expression of EGFR in VR Mel 593, Mel 611 and Mel 767 cells as compared to their respective P cells, while a constitutive

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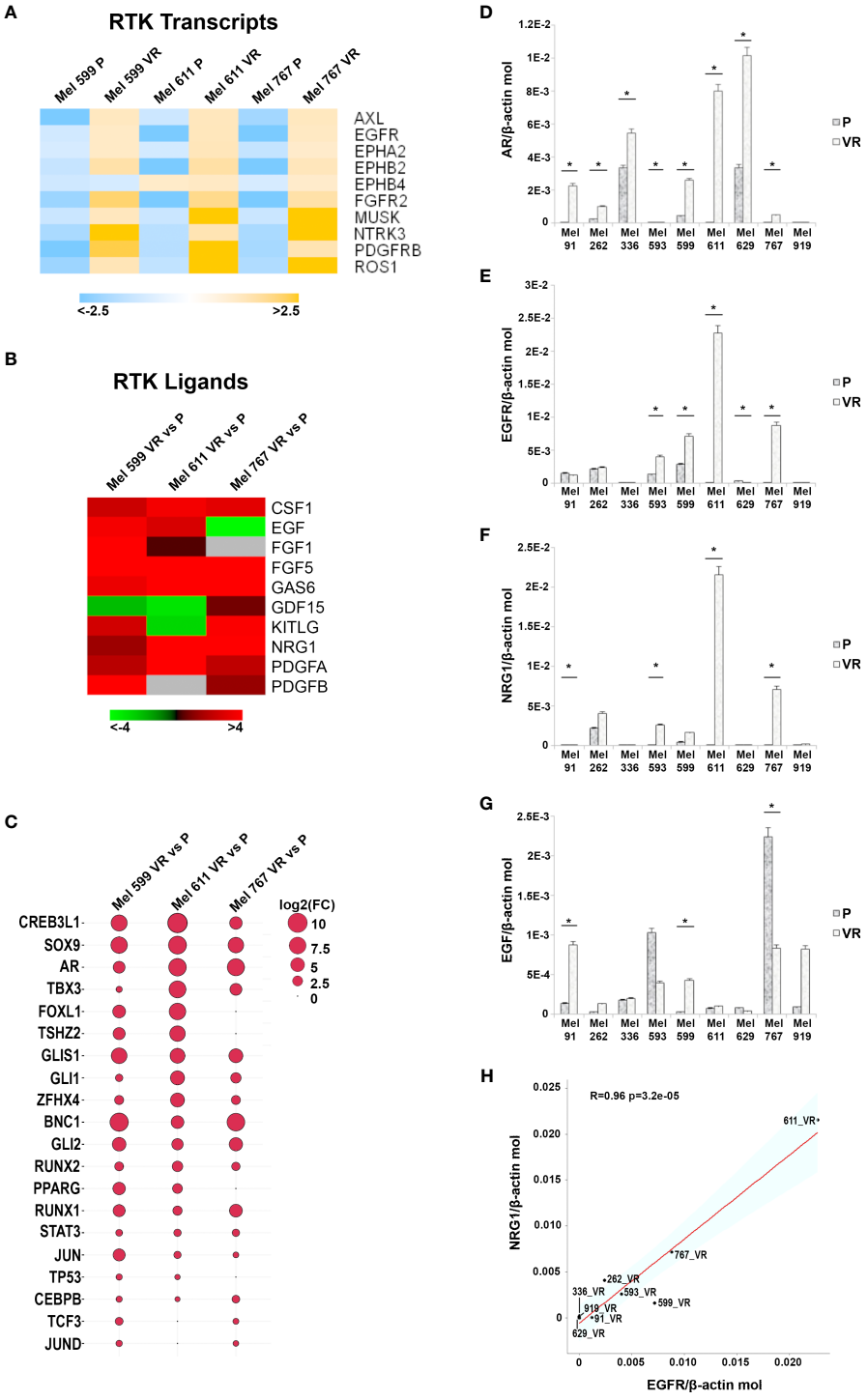

FIGURE 1
Transcripts analysis in P and VR CM cells. **(A)** Heatmap summarizing expression data for the differentially expression RTK transcripts. Data are shown as normalized expression values in log2 scale and centered on the median value. **(B)** Heatmap showing the Fold-Change values of RTK ligands. **(C)** Ballon-plot representing the Fold-Change values of the putative transcription factors regulating the RTK transcripts. The dimension of the ballon is correlated to the Fold-Change value. **(D–G)** Total RNA was extracted from P and VR cell cultures, retro-transcribed and subjected to SYBR Green quantitative real-time PCR analysis using assays for transcripts encoding AR, EGFR and its ligands NRG1 and EGF, as well as the housekeeping gene β-actin. Level of gene expression is reported as number of molecules of the target gene normalized to the number of β-actin molecules. Data are presented as mean + standard deviation of values obtained from at least 3 independent experiments; * $p \leq 0.05$. **(H)** Plot showing the Pearson correlation between NRG1 and EGFR mRNA values. The Student's t-test was used to compute the p-value.

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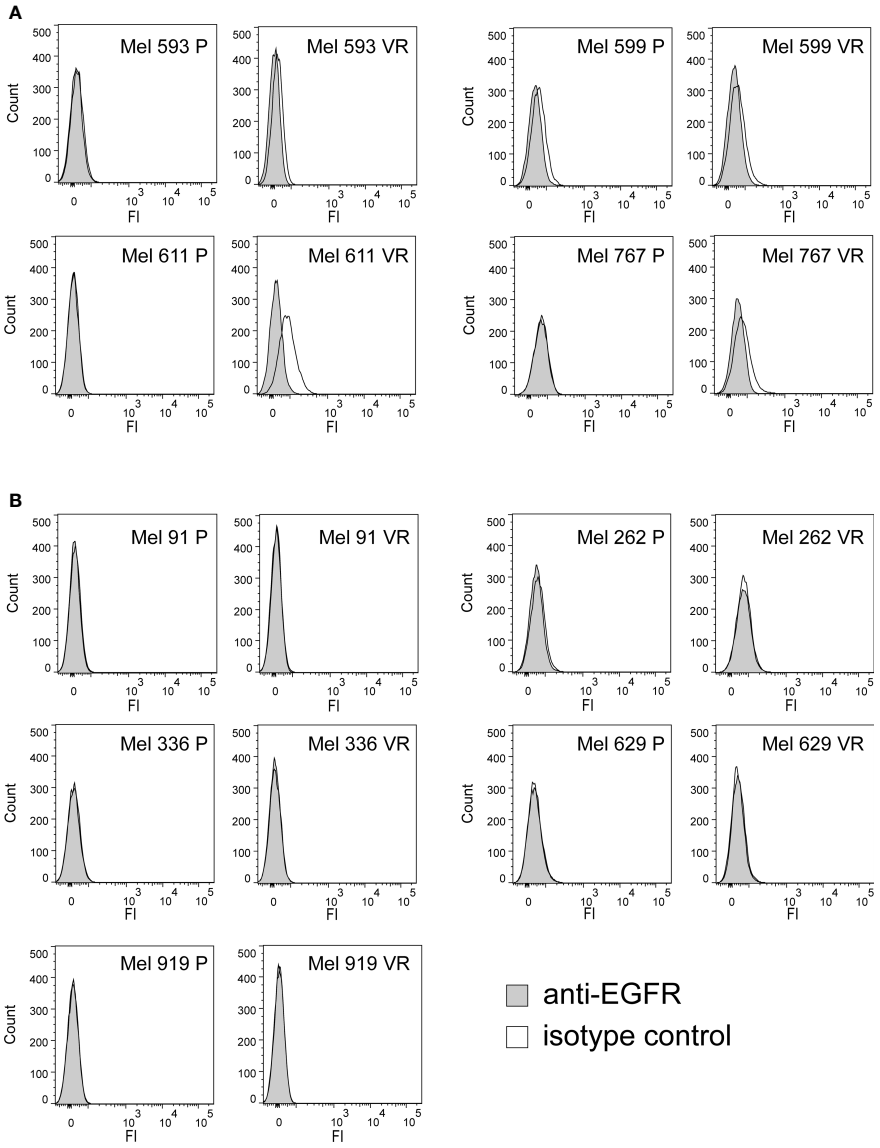

FIGURE 2
Cell surface expression of EGFR on P and VR CM cells. The cell-surface expression of EGFR was evaluated on P and VR isogenic cell cultures by flow cytometry. Filled gray histograms refer to isotype labeled cells; black empty histograms refer to cells labeled with anti-EGFR antibody. **(A)** Pairs of isogenic cultures where VR cells show surface EGFR expression, either acquired following gaining of resistance or constitutively present also on P cells. **(B)** Pairs of isogenic cultures where VR cells have no surface EGFR expression. Y axis, counts; X axis, fluorescence intensity.

EGFR surface expression was found in both P and VR Mel 599 cells (Figure 2). The increased EGFR expression in VR cells lines observed by flow cytometry was then confirmed by the results of western blotting analyses (Figure 3). The above information is of particular value, suggesting that EGFR expressed on the cell membrane following establishment of BRAFi resistance could be targeted by EGFR-specific antibodies. Notably, the analysis of TCGA data confirmed a significantly higher expression of transcripts of several RTKs and RTK ligands, including EGFR and NRG1, as well as transcription factors positively associated to BRAFi resistance in CM samples predicted to be intrinsically resistant to BRAFi (Supplementary Figure 2) based on a previously described scoring (33).

2.2 Signaling correlates of *de novo* EGFR expression in CM cells with acquired resistance to PLX 4032

Although the molecular mechanisms responsible for the resistance have not been investigated in this study, to evaluate whether the *de novo* expression of EGFR in VR cells was associated with modifications in cell signaling that could contribute to the maintenance of the PLX4032-resistant phenotype, Western blot analyses were carried out on P and matched VR cells (Figure 3, Supplementary Figure 3). Constitutive EGFR phosphorylation was heterogeneous, being evident in Mel 767 VR, Mel 599 VR and Mel 593 VR, while very limited in Mel 611 VR cells. EGFR

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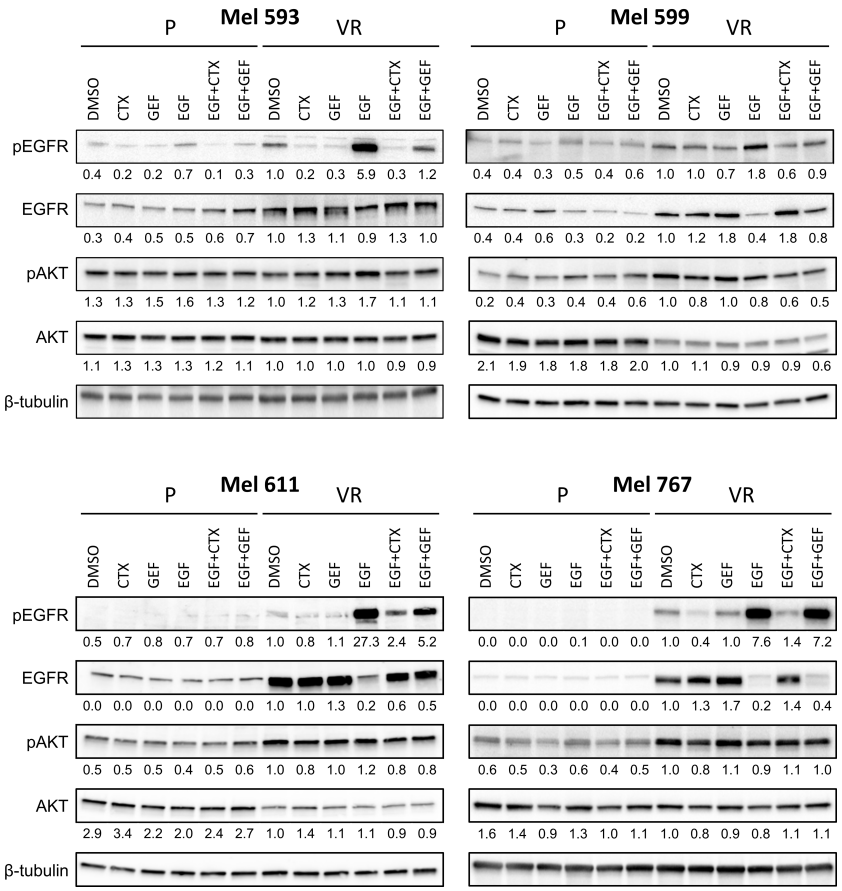

FIGURE 3
EGFR signaling in P and VR cell cultures. P and VR Mel 593, Mel 599, Mel 611, and Mel 767 CM cells were seeded in 6 well plates and treated for 24 h with either DMSO, 20 µg/ml cetuximab (CTX) or 2.5 µM gefitinib (GEF), with or without the addition of 20 ng/ml EGF. VR cells were maintained without PLX4032 for the duration of the assay. Immunoblotting was performed on cell lysates to evaluate EGFR signaling by EGFR and AKT phosphorylation (pEGFR, pAKT). Total EGFR and AKT (EGFR, AKT) served as reference, β-tubulin served as loading control. Quantifications are reported under the panels, and represent the densitometry values of the protein normalized to those of β-tubulin and referred to VR DMSO set to 1. Representative blots of at least 3 independent experiments.

phosphorylation following addition of exogenous EGF was triggered in all VR cells examined. Notably, exogenous EGF slightly upregulated EGFR phosphorylation also in Mel 599 P cells, suggesting that the EGFR constitutively expressed by these cells is functional. EGF stimulation triggered downregulation of EGFR, in line with literature data reporting degradation of activated EGFR (54–61). Independent of the degree of constitutive EGFR phosphorylation, activation of the AKT pathway emerged as a common feature of VR cells, as demonstrated by the increased phosphorylation of AKT1 found in 3 out of 4 VR cell cultures as compared to their P isogenic cells. Intriguingly, exogenous EGF did not further increase AKT1 phosphorylation (Figure 3), suggesting that the constitutively hyper activated AKT pathway in VR cells is not amenable to additional significant activation by EGFR stimulation (Figure 3, Supplementary Figure 3), similarly to what reported in the literature in other settings (62–64). Accordingly, significant enrichment in PI3K/AKT and EGFR signaling pathways was observed both in VR cell lines and in CM samples predicted to be intrinsically resistant to BRAFi (Supplementary Figure 2).

2.3 Activity of small molecule or antibody EGFR inhibitors on sensitivity to BRAFi

On the grounds of the above information, and of literature data involving EGFR signaling in resistance to BRAFi, we evaluated whether VR cells became "addicted" to EGFR oncogenic activity. To this end, VR cells were treated with either the small molecule EGFR inhibitor gefitinib or the anti-EGFR antibody cetuximab, which are able to inhibit EGFR phosphorylation through different mechanisms (65, 66). The extent of EGFR signaling inhibition induced by these drugs correlated with the level of constitutive EGFR activation, with cetuximab being effective in reducing the constitutive EGFR phosphorylation in Mel 767 VR cells, while having limited effect on Mel 593, Mel 599 and Mel 611 VR cultures. On the other hand, both gefitinib and cetuximab were able to counteract EGFR phosphorylation triggered by exogenous EGF, as clearly observed in Mel 599 and Mel 611 VR, and to a lesser extent in Mel 593, with cetuximab delivering the strongest activity (Figure 3, Supplementary Figure 3). Intriguingly, neither gefitinib

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nor cetuximab reduced AKT1 phosphorylation, suggesting a role for different or additional pathways in sustaining this activation rather than being completely or mainly relying on EGFR activation. In line with this observation, and with the hypothesis that AKT pathway activation could contribute to the acquired resistance to BRAFi, we observed that the addition of either gefitinib or cetuximab was unable to restore the sensitivity of VR cells to PLX-4032, as evaluated by dose-response curves measuring cell viability of P and VR cells cultured in the presence of escalating concentrations of PLX-4032 (Figures 4A, B). Similar results were found when sensitivity to PLX-4032 was evaluated in clonogenic assays in the presence or absence of gefitinib or cetuximab (Supplementary Figure 4). To evaluate whether EGFR might have a role in sustaining PLX-4032 resistance in our model, EGFR expression in Mel 611 VR was knocked down by using the CRISPR/CAS9 technology (Figures 4C, D), and the resulting cells were evaluated for sensitivity to PLX-4032. As shown in Figure 4E, EGFR-negative Mel 611 VR cells retained a complete resistance to PLX-4032, showing a dose-response curve superimposable to that of the un-edited Mel 611 VR cells, thus confirming that EGFR expression can be dispensable for maintaining the BRAFi resistant phenotype.

2.4 Targeting EGFR-positive BRAFi-resistant CM cells by ADCC-mediating anti-EGFR antibodies

Since inhibition of EGFR-driven signaling did not appear mandatory for maintaining BRAFi resistance in our model, we hypothesized that the frequent EGFR expression on CM cells acquiring BRAFi-resistance may still represent a valuable

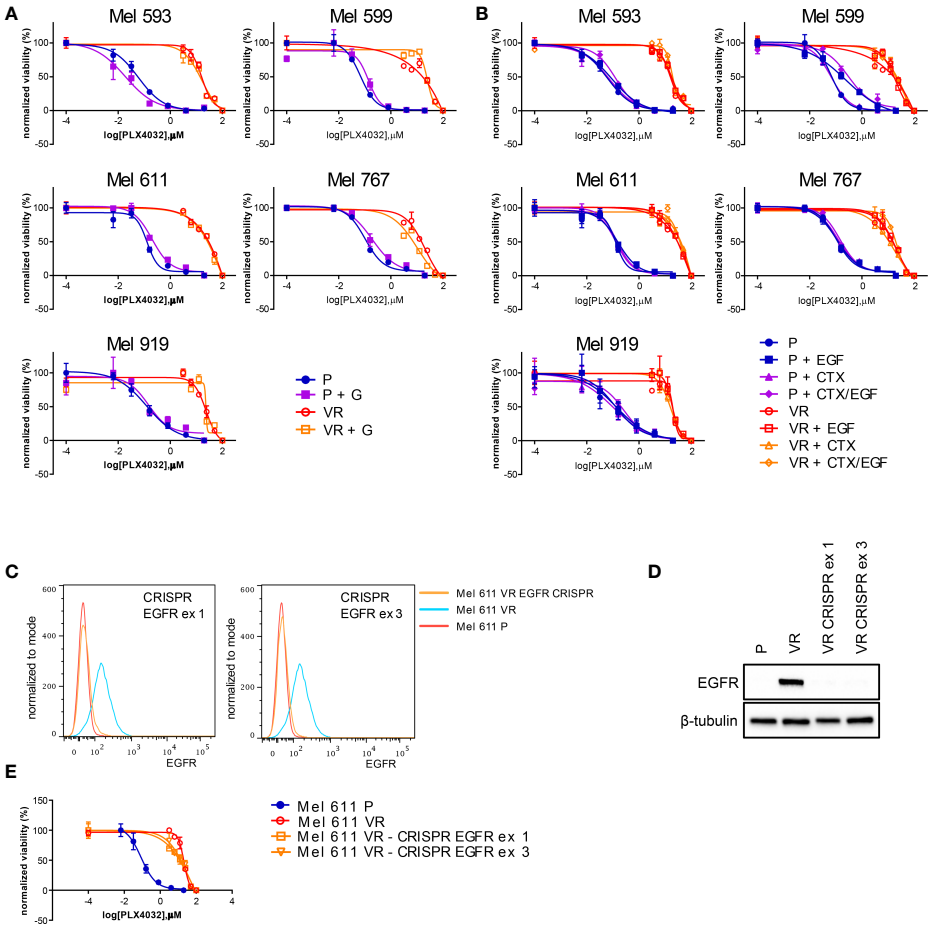

FIGURE 4
PLX4032 sensitivity of CM cells following EGFR targeting by small molecules, antibodies or genetic editing. P or VR CM cells were seeded in 96 well plates and treated for 72h with scalar doses of PLX4032, with or without the addition of 2.5 µM of the EGFR small molecule inhibitor gefitinib (GEF) **(A)**, or 20 µg/ml of the anti-EGFR mAb cetuximab (CTX), in the presence or absence of 20 ng/ml EGF **(B)**. **(C, D)** the expression of EGFR protein in Mel 611 VR cells was knocked down by CRISPR-CAS9 targeting EGFR at either exon 1 or exon 3. EGFR-negative Mel 611 VR cells were viably sorted by FACS and used for further assays. **(C)** Flow cytometry analysis confirming absence of cell surface EGFR expression on Mel 611 VR CRISPR-edited (orange line) at EGFR exon 1 (left panel) or exon 3 (right panel). **(D)** Western blot analysis confirming absence of EGFR protein in Mel 611 VR CRISPR-edited. **(E)** Dose-response curves to PLX4032 of Mel 611 P, VR and VR CRISPR-edited at EGFR exon 1 (Mel 611 VR - CRISPR EGFR ex1) or exon 3 (Mel 611 VR - CRISPR EGFR ex3). Cells were seeded in 96 well plates and treated for 72h with scalar doses of PLX4032. For all dose-response experiments, cell viability was evaluated using standard 4h MTT assays. Dose-response curves report means and standard deviations of three replicates vs. logarithmic scale of PLX4032 concentration. Normalized viability (%) is relative to vehicle-treated cells.

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therapeutic target. Along this line, the ability of antibodies to mediate ADCC is a particularly attractive feature that led us to investigate whether the anti-EGFR antibody cetuximab could target EGFR-expressing VR cells through immune-mediated mechanisms. The ability of cetuximab to kill CM cells by triggering ADCC was first investigated using peripheral blood mononuclear cells (PBMCs) from healthy donors. As shown in Figure 5A, cetuximab significantly induced ($p<0.05$) killing of EGFR-expressing VR cells by ADCC at all effector:target ratios examined. Cell lysis ranged from 44% to 48% in Mel 611 VR, and from 22% to 29% in Mel 767 VR, at effector: target ratios of 20:1 to 80:1, respectively. The extent of cell lysis correlated with the level of cell surface expression of EGFR (Figures 2A, B), being higher in Mel 611 VR as compared to Mel 767 VR, and to Mel 599 VR (Supplementary Figure 5). Triggering of ADCC by cetuximab was specifically dependent on EGFR expression on VR cells. Indeed, specific lysis due to ADCC was not observed towards EGFR-negative Mel 611 P and Mel 767 P cell cultures, nor towards EGFR-negative P or VR Mel 919 cells. The specificity of these observations was further confirmed by the inability of the anti-CD20 antibody rituximab to mediate ADCC against any of the CM cell cultures investigated, ruling out potential non-specific activities of the added antibodies (Figure 5A).

It is well known that CM can promote a vast remodeling of host's immune system, whose activity is frequently skewed towards an immunosuppressive and tumor-tolerating state within tumor microenvironment. To provide supportive evidence that the above reported observations could be potentially effective also *in vivo* in CM patients, we evaluated whether, in the presence of cetuximab, also PBMCs obtained from allogeneic metastatic CM patients were able to effectively kill *ex vivo* EGFR-positive VR CM cells through ADCC. As shown in Figure 5B, cetuximab significantly ($p<0.05$) mediated ADCC of Mel 611 VR by PBMCs from 3 metastatic CM patients at effector: target ratios of 40:1 to 80:1, with a maximum lysis of 30%, confirming that PBMCs from CM patients retained the ability to deliver cetuximab-triggered ADCC.

The ability of cetuximab to trigger ADCC towards EGFR-positive VR CM cells was further detailed by using an independent test, which evaluates the capability of antibody-cell combinations to activate the signaling cascade required for NK cell activation and target cell killing (Figure 5C). As expected, cetuximab was able to induce a significant ($p<0.001$) activation of "artificial" NK cells only when incubated with EGFR-positive Mel 611 VR, Mel 767 VR and Mel 599 VR cells, but not with EGFR-negative Mel 611 P, Mel 767 P or Mel 911 P or VR cells. No activation of the effector cells was observed following the addition of

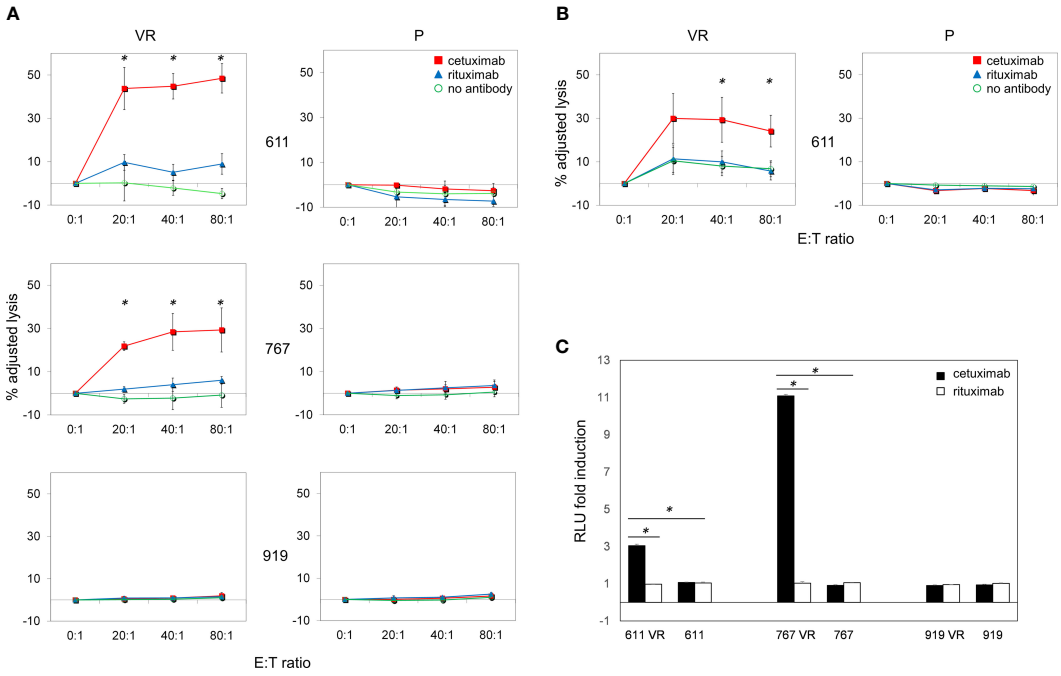

FIGURE 5
Cetuximab-mediated ADCC on P and VR CM cells. Antibody-Dependent Cell Cytotoxicity (ADCC) mediated by cetuximab in CM cell lines. **(A)** Representative graphs of ADCC mediated by cetuximab (red square) in P and VR CM cell lines. Data are shown as mean and standard deviation of 3 independent experiments performed with PBMCs obtained from 3 different healthy donors at 4 different effector:target ratios (80:1, 40:1, 20:1, 0:1), co-cultured overnight with target cells at 37°C and 5% of CO_2. ADCC efficiency is expressed as adjusted lysis, calculated as 100-adjusted survival, i.e. 100*(survival with effectors/survival without effectors). The specific lysis values measured in the presence of rituximab (blue triangle) and in the absence of antibodies (green circle) are considered as negative controls. The X axis reported the different effector:target (E:T) ratios used. **(B)** ADCC efficiency mediated by PBMCs obtained from 3 different CM patients. **(C)** ADCC Reporter Bioassay response to cetuximab (black histograms) and rituximab (white histograms) obtained using the FcγRIIIa/NFAT-RE-luciferase expressing Jurkat cell line against P and VR CM cell lines. Results are expressed as fold induction of luminescence (Relative Light Unit, RLU) measured respectively in the presence or absence of antibodies. *$p\leq0.05$.

the anti-CD20 antibody rituximab, used as negative control (Figure 5C, Supplementary Figure 5).

2.5 Therapeutic efficacy of cetuximab treatment of BRAFi resistant melanoma cells in humanized mice

Preliminary experiments allowed the identification of Mel 767 P and VR as a suitable model for vemurafenib resistant CM in mice based on their ability to form tumors *in vivo*, although the Mel 767 VR cells showed a slower growth rate, as previously shown for BRAFi-resistant cell lines (20). To test the ADCC-mediated therapeutic effect of cetuximab *in vivo*, we have set up a humanized mouse system based on the engraftment of human donor PBMCs in NSG-SGM3 mice. This mouse strain was chosen due to the expression of human stem cell factor, human Granulocyte/Macrophage-colony stimulating factor 2 and interleukin 3 as transgenes, which allow superior engraftment of human myeloid, B cells and T cells (67, 68). To confirm that the Mel 767 VR tumors retained the same EGFR up-regulation observed in the *in vitro* cultured cell line, Mel 767 P and VR tumors grown in NSG-SGM3 mice were harvested at ethical endpoint and investigated for EGFR expression via immunohistochemistry. As shown in Figures 6A, B, Mel 767 VR tumors retained an up-regulated EGFR phenotype with a mean tumor H-score of 189.9/

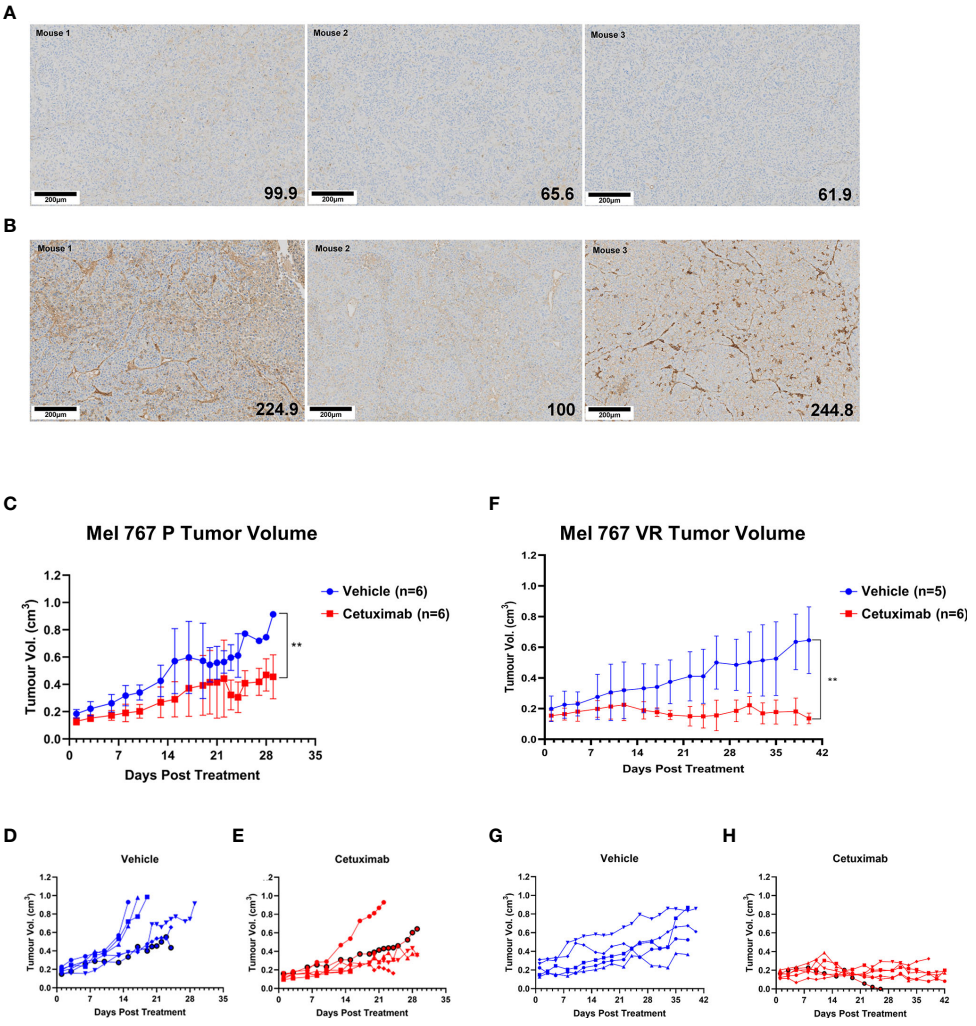

FIGURE 6
Antitumor activity of cetuximab in humanized mice. **(A, B)** EGFR IHC analysis of Mel 767 xenograft tumors. Tumor tissues harvested at ethical endpoints from NSG-SGM3 mice bearing Mel 767 P **(A)** and Mel 767 VR **(B)** were fixed, sectioned and stained for EGFR (31G7). Bottom right number indicating EGFR H-score. Tissue sections were analyzed and scored using QuPath digital pathology program. Scale bar represents 100 µm. **(C)** Mel 767 P *in vivo* xenograft tumor growth curves in humanized mice with (red) or without (blue) bi-weekly cetuximab therapy. Data are shown as mean Mel 767 P tumor volume of each treatment arm over time (days post treatment), n=6 per treatment arm. Error bars represent SD. Kruskal-Wallis test, ** p=0.0048. **(D)** Individual tumor growth curves of humanized mice bearing Mel 767 P xenograft (vehicle control). **(E)** Individual tumor growth curves of humanized mice bearing Mel 767 P xenografts treated with cetuximab. **(F)** Mel 767 VR *in vivo* xenograft tumor growth curves in humanized mice with (red) or without (blue) bi-weekly cetuximab therapy. All mice were treated with PLX4032 every two days. Data are shown as mean Mel 767 VR tumor volume of each treatment arm over time (days post treatment), n=5-6 per treatment arm. Error bars represent SD. Kruskal-Wallis test, ** p=0.0015. **(G)** Individual tumor growth curves of humanized mice bearing Mel 767 VR xenograft (vehicle control). **(H)** Individual tumor growth curves of humanized mice bearing Mel 767 VR xenografts treated with cetuximab.

300 respect to Mel 767 P tumors with a mean tumor H-score of 75.8/300. It is noted that Mel 767 parental cells were not completely EGFR-negative when grown *in vivo* as compared with the same cells grown *in vitro* and investigated for EGFR expression by Western blotting.

We first investigated the efficacy of cetuximab in NSG-SGM3 mice humanized with freshly isolated human PBMCs (10 x 10^6 cells/mice), supplemented with IL-15/IL-15RαFc engrafted with EGFR-low, vemurafenib sensitive MEL767 P cells. Based on earlier pilot tumor grow curve studies, mice were injected with 2.5 x 10^6 Mel 767 P cells with Matrigel (1:1 ratio). Once Mel 767 P tumors were established and grown to a palpable size of ~0.2cm^3, mice were bi-weekly i.v. injected with cetuximab (400 µg/mouse) for 3 weeks or treated with vehicle (saline) (n=6/group). As shown in Figures 6C–E, tumor growth in mice treated with cetuximab was slightly, although significantly ($p = 0.0048$), inhibited compared to the control group, with only 1/6 treated mouse undergoing faster tumor progression.

To investigate the *in vivo* efficacy of cetuximab against VR cells, NSG-SGM3 mice were injected with 3.5 x 10^6 Mel 767 VR cells with Matrigel (1:1 ratio) and treated with 10 mg/kg vemurafenib every 2 days to ensure that these tumors maintained also *in vivo* their vemurafenib-resistant phenotype along with EGFR up-regulation. As for the experiment with vemurafenib sensitive cells, NSG-SGM3 mice were humanized with freshly isolated human PBMCs (10 x 10^6/mice), supplemented with IL-15/IL-15RαFc when Mel 767 VR tumors were established and grown to a palpable size of ~0.2cm^3 and treated with cetuximab or vehicle (n=5-6/group). As shown in Figures 6F–H, cetuximab induced a strong and significant inhibition of Mel 767 VR tumors ($p < 0.008$) whose growth was stabilized in all cases except for one mouse which had tumor clearance but had to be culled early due to early onset of graft vs. host disease (GvHD). By contrast, all animals treated with the vehicle (5/5) (Figures 6F, G) developed tumors showing sustained growth, consistently with their VR phenotype. These findings indicate that cetuximab may be an effective therapeutic option to control BRAFi-resistant CM.

3 Discussion

In the present study, we provide evidence indicating that targeting a shared phenotype imposed by BRAFi resistance constitutes an effective modality to kill BRAFi-resistant cells both *in vitro* and *in vivo*, independently of the underlying resistance mechanism. In particular, we confirmed that gain of EGFR expression is a common phenotypic trait of CM cells acquiring resistance to BRAFi, and demonstrated that the anti-EGFR, ADCC-mediating, mAb cetuximab can effectively kill EGFR-positive BRAFi resistant CM cells through an immune-mediated mechanism.

Among the BRAFi-resistant cell lines generated in our study, 3 out of 9 (33%) showed *de novo* cell surface expression of EGFR, while 1 proved EGFR-positive both in the VR and in the respective P BRAFi-sensitive cultures. Even though the number of cell lines used in this study is limited, the prevalence of *de novo* EGFR expression in VR cultures is in line with those reported for tissues from a study by Sun C et al. (20). Subsequently, Ji Z et al. reported that 4/5 CM tumor pairs from CM patients treated with BRAF ± MEK inhibitors showed increased EGFR expression in the post-relapse samples compared to pre-treatment samples (16). Similarly, Wang J. et al. compared EGFR expression in autologous pairs of CM specimens obtained from 12 different patients before and after treatment with BRAFi, and EGFR expression post-treatment was significantly higher in almost all BRAFi resistant recurrent tumors (30). In a more recent study, EGFR mRNA levels could be measured in 3/5 CM patients who relapsed after 3-15 months from the end of the treatment with vemurafenib (69). Taken together, these data suggest that therapeutic approaches based on anti-EGFR ADCC-mediating mAbs could be applicable in at least 30% of BRAFi relapsing patients. Besides, immunohistochemistry performed on tumor samples from 19 patients showed EGFR tumor positivity in pre-therapy lesions in 16% of patients (Supplementary Table 4), suggesting its potential targeting independently from BRAFi-treatment or in CM that are intrinsically resistant to BRAFi and that are marked by EGFR upregulation (36). The potential clinical utility of immune targeting EGFR-positive CM cells even before or concomitantly to targeted therapy could find additional rationale from a recent paper showing the presence of rare cell populations variants, marked by surface EGFR-expression, that are poised to resistance to targeted therapies and which could contribute to tumor outgrowth upon BRAF/MEK inhibition (70). Unfortunately, post-BRAFi lesions were not sufficiently represented in the patient cohort that we had available to allow us to reliably assess the prevalence of EGFR expression on tumor tissues following gain of BRAFi-resistance.

ADCC can be mediated by both NK cells, mainly activated by IgG1 isotype antibodies, and myeloid-derived effectors, triggered by both IgG1 and IgG2 antibodies (71). In our model, anti-EGFR mAb-triggered ADCC of VR cells was essentially delivered by NK cells since targeting EGFR by the IgG2 isotype mAb panitumumab did not result in a significant killing of EGFR-positive VR cells (data not shown), suggesting a negligible, if any, contribution of myeloid-derived effectors in mAb mediated killing in this setting. In line with this finding, IgG1 class anti-EGFR mAbs (e.g., cetuximab, nimtuzumab, and necitumumab) appear preferable for combined therapies targeting EGFR-positive BRAFi-relapsing CM. A further improvement in this respect could be achieved by using anti-EGFR antibodies optimized to deliver enhanced ADCC activity, which demonstrated a superior anti-tumor activity as compared to cetuximab in mouse models (72).

As shown by Ji Z et al., CM lineage reprogramming contributes to BRAFi resistance through induction of autocrine EGFR ligand expression that triggers EGFR signaling (16). Engagement of this autocrine loops is potentially shared by other RTKs expressed following establishment of BRAFi-resistance, as demonstrated by the coordinate *de novo* expression of AXL and PDGFRβ (Supplementary Figure 6, Supplementary Table 5) and their ligands (i.e. GAS6 and PDGF) in our VR cell lines (Figures 1A, B). Accordingly, we confirmed that the EGFR-ligand NRG1 was consistently co-expressed with EGFR in VR cell cultures (Figure 1H). Since cetuximab binds EGFR at the ligand binding

site, autocrine ligand production could be able to interfere with the binding/activity of the mAb. Though we did not specifically address this issue from a mechanistic point of view, our data suggest that autocrine EGFR ligand expression did not significantly prevent the binding of cetuximab, nor the killing of VR CM cells by ADCC, supporting the efficacy of targeting RTKs through immune-mediated mechanisms even in the presence of autocrine ligand production.

From a perspective therapeutic point of view, a major advantage of using anti-EGFR (or more in general anti-RTK) ADCC-mediating mAbs is their ability to kill RTK-expressing VR CM cells independently of the requirement of downstream signaling for sustaining resistance to BRAFi, as it has already been demonstrated in other contexts (51, 73). Indeed, in our model, we found that cetuximab is at least as effective as gefitinib in down-modulating EGFR phosphorylation in VR cells, either it being constitutive or following ectopic EGF, in a cell context-dependent manner. Nevertheless, we failed to demonstrate a significant impact on BRAFi sensitivity of EGFR inhibition through either small molecule inhibitors or cetuximab. In addition, complete knock-down of EGFR protein expression in Mel 611 VR cells by genomic editing approaches confirmed that, at least in this specific cell line, EGFR expression is unnecessary for maintaining BRAFi-resistance. These observations are in line with reports suggesting that BRAFi resistance may result from a deep reprogramming of cellular signaling triggered by a switch to a different cellular state. In this context, the alternative cellular state was consistently marked by specific RTK profiles, but resistance was independent of the activity of distinct RTKs (20, 24, 36). Accordingly, inhibition of single RTK (e.g., EGFR, AXL) did not result in markedly restored sensitivity to BRAFi, as it did, in contrast, the targeting of common downstream signaling nodes/mediators (e.g. AKT, SRC) or multiple RTK targeting (20, 24, 29, 36, 74, 75).

Our data do not completely exclude that single RTK could play a prominent role in maintaining BRAFi resistance (19, 29, 30, 33, 38), nonetheless they clearly indicate that EGFR expression is dispensable for maintaining resistance to PLX4032 in specific CM cells. In these experimental conditions, the ability of cetuximab to mediate ADCC effectively killed EGFR-positive VR cells, suggesting that the use of ADCC-mediating anti-RTK mAbs could prove therapeutically valid, thanks to their immune-mediated mechanisms of action, independently of their effect on signaling. These therapeutic properties could further benefit from the ability of cetuximab-activated NK cells to induce an adaptive T-cell response against tumor antigens expressed by EGFR-positive cancer cells (52), which could boost the adaptive immune response to EGFR-expressing VR cells.

Our observations are likely not limited to EGFR but could prove valid also for other RTKs that are *de novo* expressed on VR cells. Among these, AXL is particularly appealing since it is included in the RTK signatures of BRAFi resistant CM (Figure 1A), and it has been proposed to play a potentially important role in acquiring resistance to BRAFi and in increasing the invasive potential of CM cells (24, 33, 76–78). Noteworthy, a recent single cell sequencing study revealed that a population resistant to targeted-therapy labeled as "invasive" and expressing high levels of AXL became frequently expanded in CM patient derived xenografts progressing on combined BRAF/MEK/RXR inhibition (18). We confirmed AXL to be *de novo* expressed on the cell surface of 2 out of the 9 VR CM cultures analyzed (Supplementary Figure 7). As seen with EGFR, AXL expression appeared to be dispensable for maintaining resistance to BRAFi, since its genetic knock down did not restore CM sensitivity to vemurafenib (Supplementary Figure 8). Independently on its role on BRAFi-resistance, AXL expression on VR cells makes it an attractive target for the ADCC-mediating anti-AXL mAbs that are currently being developed (79, 80). However, whether the expression of AXL on both tumor-associated and normal endothelial cells (Supplementary Table 4) could lead to systemic toxicities of anti-AXL mAbs has to be considered and explored.

In consideration of their ability to favor the cross-talk between innate and adaptive immune responses, an intriguing therapeutic option for the clinical use of ADCC mediating anti-RTK antibodies in BRAFi-relapsing CM is the combination with currently used immune checkpoint inhibitors (ICIs) that demonstrated to provide long term clinical control of the disease in this malignancy (81–83). Moreover, CM cells acquiring BRAFi resistance through activation of alternative signaling pathways driven by *de novo* expressed RTK (s) were shown to up-regulate the cell surface expression of PD-L1 molecule. This molecular event could further contribute to the progression of BRAFi resistant CM by favoring their immune escape mediated by triggering of the inhibitory PD1 immune checkpoint in tumor infiltrating effector T cells (84–86). Of interest, RNA sequencing analysis revealed that the expression of PDCD1LG2, also known as PD-L2, was significantly increased following acquisition of BRAFi resistance (Supplementary Figure 9). PD-L2 has been described as a second ligand for PD-1 in addition to PD-L1 (87), thus representing a potential target for cancer immunotherapy with PD-1 inhibitors. Hence, in this context, targeting of EGFR-positive BRAFi-resistant CM cells by combined treatment with cetuximab and ICIs might appear an extremely attractive option that would take advantage of the concomitant action on tumor and immune cells.

Our results obtained in humanized mice are consistent with the observation that cetuximab may exert some degree of therapeutic efficacy *in vivo* against BRAFi-responsive melanoma cells favored by a low basal level of EGFR expression. More important in a clinical perspective is our finding that cetuximab strongly inhibited the growth of a VR melanoma cell line in humanized mice undergoing concomitant vemurafenib treatment. Considering the emerging relevance of humanized mice as fundamental preclinical platforms for drug testing and therapeutic screening, as recently stated by the FDA (88), these findings provide the proof-of-principle to activate clinical trials aiming at assessing the therapeutic efficacy of cetuximab to improve the control of BRAFi in CM. In this context, it will be noteworthy to first evaluate the potential side effects of cetuximab therapy on EGFR-expressing normal epithelial cells. Several studies reported frequent dermatological toxicities of EGFR-targeting agents due to the key function of EGFR in skin biology (89). Acneiform eruptions were reported in patients affected by lung, colorectal, or head and neck cancers and treated with EGFR-inhibitors. However, severe skin

toxicities could be well prevented by oral antibiotics administration (90). Interestingly, cetuximab treatment in locally advanced cutaneous squamous cell cancer was associated with only mild acneiform skin rash, thus suggesting the potential safety profile of this drug in cutaneous malignancies (91).

In conclusion, our study, conducted on a wide panel of P and VR pairs of CM cells, confirmed a frequent *de novo* expression of RTKs in CM cells acquiring BRAFi resistance, and demonstrated that their targeting by ADCC-triggering mAbs can efficiently and reliably kill RTK(s)-expressing VR CM cells and mediate therapeutic effects *in vivo*. These data provide the grounds for the evaluation of the therapeutic activity of anti-RTK mAbs in CM relapsing on BRAFi, where combinations with immune checkpoint inhibitors appear a particularly suited setting.

4 Materials and methods

4.1 Cells cultures and reagents

Cell cultures were established from metastatic lesions surgically removed from cutaneous CM patients that did not underwent prior BRAFi therapy who were referred to the Centro di Riferimento Oncologico, IRCCS-National Cancer Institute, Aviano, Italy, as previously described (92). VR cell cultures were generated from BRAF V600 mutant, PLX4032-sensitive, CM cells by sequential culturing in the presence of doubling concentrations of PLX4032 (Selleck Chemicals), starting from 0.25 μM until they were able to grow in 8 μM PLX4032. Cell cultures were grown in RPMI 1640 Medium, supplemented with 2mM L-glutamine (Sigma-Aldrich) and 10% heat-inactivated fetal calf serum (FCS, Lonza). VR cell cultures were propagated under the same culture conditions, with the addition of 8 μM PLX4032. The identity of paired P and VR cells was confirmed by short tandem repeat profiling using the Power Plex 1.2 kit (Promega). Mutation status in BRAF and NRAS genes was determined as previously described (93, 94). PBMCs from healthy donors and CM patients were prepared as previously described (95). The study was approved by the Internal Review Board of the Centro di Riferimento Oncologico, IRCCS-National Cancer Institute, Aviano, Italy (IRB number 07-2017). Stock solutions of gefitinib and PLX-4032 (SeleckBio) were prepared in cell-culture grade DMSO (Sigma) as per manufacturer's indications. Cetuximab (MERK) and EGF (Life Technologies) stocks were diluted in RPMI medium.

4.2 RNA sequencing

Libraries preparation was performed as described in Montico et al. (96). RNA purity and integrity were assessed with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a 4200 TapeStation instrument (Agilent Technologies, Santa Clara, CA, USA), respectively. For RNA purity, an A260/280 ratio of ˜2.0 and an A260/230 ratio of 2.0-2.2 were considered acceptable; for RNA integrity, an RNA Integrity Number (RIN) of 9.0-10.0 has been obtained for all samples, indicating the absence of degradation and high integrity of RNA samples. For a precise estimation of the RNA concentration, a Qubit 2.0 fluorometer assay (Thermo Fisher Scientific, Waltham, MA, USA) has been employed. For RNA sequencing, 1 μg of high-quality total RNA was used for library preparation with a TruSeq Stranded Total RNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced (paired-end, 2 x 75 cycles) on the NextSeq 500 platform (Illumina, San Diego, CA, USA). For each experimental condition, three biological replicates were considered. RNA sequencing data analysis was performed as described in Casarotto et al. (97). In detail, the raw sequence files generated (.fastq files) underwent quality control analysis using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adapter sequences were removed using Trimmomatic version 0.38 (98). Filtered reads were aligned on human genome (assembly hg38) considering genes present in GenCode Release 37 (GRCh38.p13) using STAR v2.7.9a (99) with standard parameters. Quantification of expressed genes was performed using featureCounts (100) and differentially expressed genes were identified using DESeq2 (101). A given RNA was considered expressed when detected by at least ≥ 10 raw reads. Differential expression was reported as |fold-change| (FC) ≥1.5 along with associated adjusted p-value ≤ 0.05 computed according to Benjamini-Hochberg. Functional analysis on differentially expressed genes was performed using Ingenuity Pathway Analysis (IPA, Qiagen). Only Canonical Pathways and Molecular Function with a p-value < 0.05 were considered for further analysis. Prediction of Transcriptional regulators of the differentially expressed genes was performed using ChEA3 (102). The tables with the normalized values of expressed transcripts are available in BioStudies repository (https://www.ebi.ac.uk/biostudies) with the accession number S-BSST1225. The raw data of the RNA sequencing datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

4.3 Quantitative RT-PCR analysis

Real-time quantitative PCR analyses were performed as described (103) using Power SYBR Green Master Mix (Life technologies). Primers sets used are listed in Supplementary Table 6. The absolute copy number of cDNA of target genes and of the reference gene β-actin were measured in each sample from standard curves. The number of target gene cDNA molecules in each sample was normalized to the number of cDNA molecules of β-actin. The Student's t-test for two tailed distributions and paired data was used to compare normalized gene expression between VR and P CM cell lines. Differences were considered statistically significant when p≤ 0.05.

4.4 Dose-response curves

CM cells were seeded in flat bottom 96-well plates at a density of 2500 or 5000 cells/well, depending on their growth rate. After 24h, scalar doses of PLX-4032, or an equal volume of DMSO, used as negative control, were added into triplicate wells. When

combination experiments were performed, fixed concentrations of gefitinib (2.5 μM), cetuximab (20 μg/ml) and/or EGF (20 ng/ml) were added to both DMSO-treated and PLX-4032-treated wells. Cell viability was evaluated 72h after the addition of PLX-4032 by a standard MTT assay (Life Technologies). Generation of sigmoidal dose-response curves using a four-parameter nonlinear regression model, and calculation of PLX-4032 IC_{50} values were achieved by the Prism 6.0 software (GraphPad Software).

4.5 Flow cytometry analysis

Flow cytometry analyses were performed essentially as previously described (104). EGFR was detected by the phycoerythrin-conjugated anti-EGFR clone EGFR.1 mouse IgG2b mAb (Becton Dickinson); a phycoerythrin-conjugated mouse IgG2b isotypic antibody (Becton Dickinson) served as negative control. Antibodies were used following the manufacturer's instructions. Data acquisition was performed with a FACSCanto II flow cytometer (Becton Dickinson) and analyzed with Diva 5.0 (Becton Dickinson) and FlowJo software (Tree Star, Inc).

4.6 Western blot analysis

CM cells were plated in 6-well plates. Twenty-four hours after plating, cells were added with DMSO or 20 μg/ml cetuximab, 2.5 μM gefitinib, with or without 20 ng EGF, and were analyzed following 24h incubation. Whole cell lysate preparation and western blotting were performed as previously described (104). Ten μg cell lysate were separated on Criterion TGX 4-15% gradient gels (BioRad) and blotted onto nitrocellulose membranes. Immunoblotting was performed using the following antibodies, under the manufacturer's instructions: anti-phospho-Akt (Ser473) (#9271), -total-AKT (#9271), -phospho-EGFR (Tyr1068) (#3777), and -total-EGFR (#2232) antibodies from Cell Signaling Technology; anti-β-tubulin (H-235) (sc-9104), used as a loading control, from Santa Cruz Biotechnology. Revelation was performed using the Clarity Western ECL Substrate (BioRad) or the SuperSignal Femto reagent (Pierce) through Chemidoc XRS+ instrument (Biorad). Image analysis was performed with the Image Lab v6.1 (Biorad) software. The Student's t-test for two tailed distributions was used to compare data. Differences were considered statistically significant when $p \leq 0.05$.

4.7 CRISPR/Cas9 genomic editing

Guide sequences targeting EGFR were either previously described [TGCAAATAAAACCGGACTGA, exon 3 (105)] or designed (TCCTCCAGAGCCCGACTCGC, exon 1) using the CRISPR design software available at http://crispr.mit.edu. Complementary oligonucleotides containing cloning overhangs were synthesized at Sigma, annealed, and the obtained double stranded oligonucleotide was cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid, kind gift from Feng Zhang (Addgene plasmid # 48138), as per inventor's protocol (106). Plasmids were then transfected into CM cells using Lipofectamine 2000 reagent (Life Technologies) following the manufacturer's instructions. EGFR-negative VR CM cells were sorted 1 week after transfection using FacsARIA III (Beckton Dickinson).

4.8 *In vitro* assays to quantify ADCC

The ability of cetuximab to mediate ADCC in EGFR-expressing CM cell lines was evaluated through 2 different *in vitro* assays: a flow cytometry-based assay [modified from Hermans et al. (107)] and the ADCC Reporter Bioassay (Promega). In the flow cytometry approach, P and VR CM cell lines were labeled with 2 different concentrations (0.3125μM and 3.75μM respectively) of Carboxyfluorescein succinimidyl ester (Vybrant® CFDA SE cell Tracer, CFSE; Molecular Probes, Life Technologies) for 10 minutes in 500 μl of PBS; FCS (Lonza) was added for 20 minutes to quench the reaction, and cells were washed 4 times. VR and P CFSE-labeled cells were then counted and incubated together at a concentration of $2x10^5$/ml in Hank's Balance Salt Solution (Sigma) added with 10% FCS (Lonza). So treated target cells were then co-cultured overnight together with PBMCs obtained from healthy donors or CM patients, at different effector:target ratios (80:1; 40:1; 20:1; 0:1), in the presence/absence of 20 μg/ml of cetuximab, using 96-well ultra-low attachment microplates (Corning) at 37°C and 5% of CO_2. Rituximab (20 μg/ml)-labeled cells were used as negative control. Samples were collected and acquired with a FC500 flow cytometer (Beckman Coulter) and data were analyzed with the FlowJo software (Treestar). Specific lysis was calculated using Sytox® Orange Dead Cell Stain (Life Technologies) as cell death marker. ADCC efficiency was expressed as adjusted lysis, calculated as 100-adjusted survival, i.e. 100*(survival with effectors/survival without effectors). The Student's t-test for two tailed distributions and paired data was used to compare ADCC efficiency in the presence or absence of antibodies, or mediated by cetuximab versus rituximab. Differences were considered statistically significant when $p \leq 0.05$. The ADCC Reporter Bioassay (108) was performed under manufacturer's instructions. Briefly, P and VR CM cell lines were incubated at a 1:1 ratio with FcγRIIIa/NFAT-RE-luciferase expressing Jurkat cell line for 6 hours at 37°C and 5% of CO_2 in the presence/absence of cetuximab or rituximab (10 μg/ml). Luciferase activity was quantified using the Bio-GloTM Reagent and luminescence was measured using Tecan Infinite 200 Pro (Tecan Group Ltd). Data were expressed as luminescence (Relative Light Unit, RLU) fold of induction = RLU induced (cetuximab or rituximab)/RLU no antibody control. The Student's t-test for two tailed distributions and paired data was used to compare RLU fold induction in the presence of cetuximab or rituximab, or induced by cetuximab/rituximab in VR or P CM cell lines. Differences were considered statistically significant when $p \leq 0.05$.

4.9 *In vivo* efficacy studies

NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG) 1Eav/MloySzJ (NSG-SGM3) mice between 6-8 weeks of age of

mixed gender were obtained from Prof. Kristen Radford's research group (Mater Medical Research) originally sourced from JAX laboratories (Maine, USA). Animal experiments were approved by the University of Queensland Animal Ethics Committee (AEC 060/19). For tumor cell engraftment, Mel 767 P and VR cells were resuspended in 1:1 PBS : Matrigel (corning) suspension in a cell density of 2.5 x 10^7 and 3.5 x 10^7 cells/mL respectively and kept on ice till injection. Mice were anesthetized with 2-3% isoflurane and 100 µL of cell suspensions (Mel 767 P: 2.5 x 10^6 cells/mice, Mel 767 VR 3.5 x 10^6 cells/mice) were injected subcutaneously into the right or left flank. Mice bearing Mel 767 VR tumor cells were treated with 10 mg/kg vemurafenib (PLX4032) (MedChemExpress) intraperitonially (i.p) every 2 days. When tumors reach 0.2-0.3 cm^3 in size, tumor bearing NSG-SGM3 mice were humanized with freshly isolated human PBMC from healthy donors (human ethics HREC/2018/QMS/44046). 10 x 10^6 human PBMCs in 100 µL volume of PBS was injected via tail vein intravenous (i.v) injection. After human PBMC injection, mice were monitored and scored for signs of GvHD. Submandibular blood collection of humanized animals for immune analysis was performed two weeks post human PBMC injection to confirm successful engraftment of human immune cells. For maintenance of NK cells post PBMC engraftment, mice were injected i.p with 2.5 µg of recombinant human IL-15 (Peprotech) complexed with recombinant Human IL-15Rα Fc Chimera (R&D systems) (huIL-15/IL-15RαFc) once weekly. Cetuximab treatment commenced immediately a day after humanization and 400 µg/mice of cetuximab were injected into mice bi-weekly via tail vein i.v injection for 3 weeks. In vehicle control group, mice received saline injection. Mice were scored, weighed and tumors were measured via caliper measurement every 2 days.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/biostudies/studies/S-BSST1225?key=1e247f11-3430-47c2-bc66-ad69b16dc5d0.

Ethics statement

The studies involving humans were approved by Internal Review Board of the Centro di Riferimento Oncologico, IRCCS-National Cancer Institute, Aviano, Italy (IRB number 07-2017). The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from metastatic lesions surgically removed from cutaneous CM patients that did not underwent prior therapy with BRAF inhibitors who were referred to the National Cancer Institute of Aviano (Italy), as described in "Altomonte M et al. Differential Expression of Cell Adhesion Molecules Cd54/Cd11a and Cd58/Cd2 by Human Melanoma Cells and Functional Role in Their Interaction with Cytotoxic Cells. Cancer Res (1993) 53(14):3343-8". Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. The animal study was approved by Animal procedures were approved by the University of Queensland Animal Ethics Committee (approval number: UQDI/491/15/NHMRC/ARC). The study was conducted in accordance with the local legislation and institutional requirements. 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

EM: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. BM: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. BL: Data curation, Investigation, Writing – review & editing. FC: Investigation, Writing – review & editing. GG: Data curation, Investigation, Software, Writing – review & editing. AS: Writing – review & editing, Data curation, Investigation. RG: Writing – review & editing, Investigation. AR: Writing – review & editing, Investigation. EC: Writing – review & editing, Investigation. VC: Writing – review & editing, Data curation. AA: Writing – review & editing, Data curation. MD: Writing – review & editing, Data curation, Investigation. RM: Writing – review & editing, Data curation, Investigation. MM: Writing – review & editing, Data curation, Investigation. AW: Writing – review & editing, Data curation, Funding acquisition. MP: Writing – review & editing, Data curation, Funding acquisition. FS: Data curation, Funding acquisition, Investigation, Writing – review & editing. RD: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – review & editing. EF: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. LS: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1336566/full#supplementary-material

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