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Research, clinical trials, drug resistance and safety for cancer immunotherapy in head and neck cancers and brain tumors

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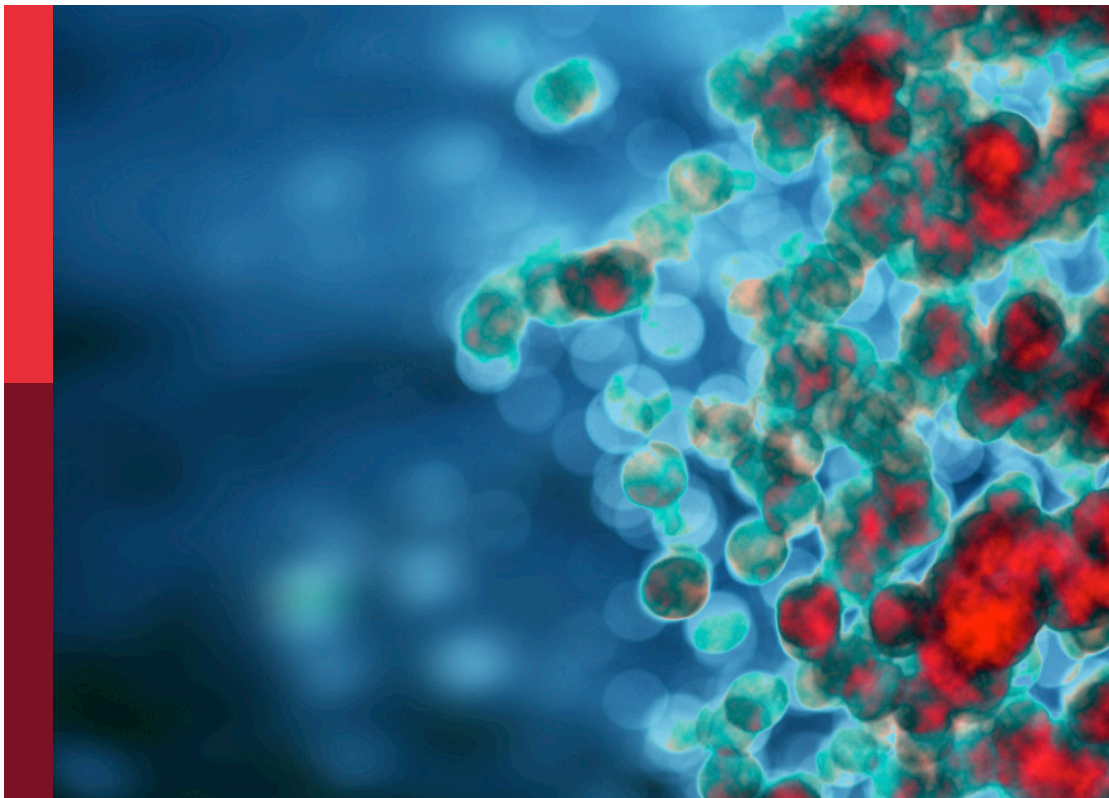

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Research, clinical trials, drug resistance and safety for cancer immunotherapy in head and neck cancers and brain tumors

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Acircadian rhythm-related gene signature for predicting survival and drug response in HNSC

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Head and neck squamous cell carcinoma (HNSC) represents one of the most common malignant carcinomas worldwide. Because the 5-year survival rate of patients with HNSC is poor, it is necessary to develop an effective signature for predicting the risk of HNSC. To identify a circadian rhythm (CR)-related predictive signature, we analyzed the RNA-seq data of patients with HNSC from The Cancer Genome Atlas and Gene Expression Omnibus cohorts. Nine CR-related genes (*PER2*, *PER3*, *GHRL*, *CSF2*, *HDAC3*, *KLF10*, *PRKAA2*, *PTGDS*, and *RORB*) were identified to develop a CR-related signature. The area under the curve values for 5-year overall survival were 0.681, 0.700, and 0.729 in the training set, validation set, and an external independent test set (GSE41613), respectively. The Kaplan–Meier curve analysis showed that the high-risk group had a reduced relapse-free survival compared with the low-risk group in the training set, validation set, and test set ($P < 0.05$). Finally, we observed that the CR-related gene signature was associated with the tumor immune microenvironment, somatic nucleotide variation, and drug response in HNSC. In conclusion, we developed a circadian rhythm-related gene signature for predicting overall survival in HNSC.

KEYWORDS

circadian rhythm, prognosis, head and neck squamous cell carcinoma, drug response, gene signature

Introduction

Head and neck squamous cell carcinoma (HNSC) represents one of the most common malignant carcinomas worldwide (1) and is characterized by heterogeneity and aggressiveness. Despite substantial efforts invested into the therapeutic development of HNSC, the 5-year survival rate of patients with HNSC remains poor (2). Therefore, it is clinically necessary to identify a comparatively reliable and applicable prognostic

signature for HNSC to guide clinical decision-making. Several gene signatures have been developed to predict the prognosis of HNSC (3–5). However, due to the heterogeneity of HNSC, the predictive ability of these indicators is not satisfactory. Therefore, identifying a novel biomarker for predicting overall survival for HNSC is urgent.

Circadian rhythms are 24-h oscillations that affect multiple biological functions in humans (6). Circadian rhythm disorders are linked to aggressive tumor behaviors and unwanted clinical outcomes. Circadian-related genes have been implicated in the pathogenesis of colorectal cancer (6), prostate cancer (7), and bladder cancer (8). Moreover, emerging evidence suggests the involvement of circadian rhythm in the tumor immune microenvironment (9–11). Because the tumor immune microenvironment has an important influence on the effect of tumor immunotherapy, the circadian rhythm may affect the sensitivity of immunotherapy by affecting the immune microenvironment.

While circadian rhythm has recently become a hot topic in the cancer research field, the specific mechanisms of its role in humans are still not fully understood. Specifically, the impacts of circadian rhythm disruption on the prognosis of HNSC and the immunotherapeutic effect remain unclear. Considering the involvement of circadian rhythm disturbance in aggressive tumor behaviors and unwanted clinical outcomes in several types of cancer, we hypothesized that a circadian rhythm (CR)-related gene signature may be used to predict prognosis and immunotherapeutic effects in HNSC patients.

Human papillomavirus (HPV) has emerged as a reliable predictor for the progression of HNSC (12). In addition, HPV infection has been directly linked to a higher morbidity of oropharyngeal cancer in men under 50 who do not smoke or drink (13). HPV infection affects the mutational landscape and correlates with an improved prognosis (14). Therefore, a hypothesis has been proposed that HPV infection may be involved in gene expression regulation (14). In the present study, we investigated whether circadian rhythm disruption is related to HPV infection.

To establish a CR-related predictive signature for HNSC patients, we investigated bulk RNA sequencing (RNA-seq) profiles from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) to provide an applicable gene signature for predicting the prognosis of HNSC patients.

Materials and methods

Data acquisition

Gene expression profiling and clinical information for 493 HNSC patients from TCGA were obtained from UCSC Xena on July 1, 2022 (https://xena.ucsc.edu/). Notably, there are cancerous samples and paracancerous normal samples of the HNSC patients from TCGA. In the present study, to extract potentially qualified genes to establish a CR-related gene signature for predicting survival in HNSC, we only included the cancerous samples of HNSC patients, and thus the gene expression profiles used for the downstream analysis were all from cancerous samples. Among 493 HNSC patients, 112 HNSC patients had a clear HPV status, including 34 HPV+ and 78 HPV- patients with HNSC. The microarray RNA-seq data and survival information of 76 HNSC patients were obtained from the GSE41613 dataset in GEO. The microarray expression data and the corresponding disease-free survival (DFS) data of 109 HNSC patients were obtained from the GSE27020 cohort in GEO. The GSE41613 dataset was based on the GPL570 platform (Affymetrix Human Genome U133 Plus 2.0 Array), and the GS27020 dataset was based on the GPL96 platform (Affymetrix Human Genome U133A Array). RNA-seq data from TCGA and GSE27020 were normalized in the form of transcripts per million values and then log2 (x + 1)-transformed. The somatic nucleotide variation (SNV) data of HNSC patients were obtained from TCGA database. As discussed previously, the SNV data were also from the cancerous samples of HNCS patients from TCGA. Detailed information for the datasets is shown in Table 1. There were 84 circadian rhythm-related genes, which come from the molecular signature database (15), used to select qualified candidate genes in the present study (Supplementary Table S1).

Estimation of enrichment scores for individual patients

To quantify the expression levels of the CR gene set in individual patients, we estimated the enrichment score (ES) of the CR-related gene set for individual HNSC patients using single-sample gene set enrichment analysis (ssGSEA) (16).

TABLE 1 Sample information in the datasets.

Database	Normalization method	Sample number with survival information	Survival time type	HPV status
Training set	TPM	345	OS	23 HPV+, 57 HPV-
Validation set	TPM	148	OS	11 HPV+, 21 HPV-
GSE41613	gcRMA algorithm	76	OS	76 HPV -
GSE27020	TPM	109	DFS	Unknown

TPM, transcripts per million; RMA, robust multiarray analysis; OS, overall survival; DFS, disease-free survival.

ssGSEA is a mathematical methodology to estimate the relative expression levels of a given gene set using RNA-seq data. The parameters used in this study were as follows: min.sz = 1, max.sz = Inf, and tau = 0.25, where min.sz represents the minimum size of the resulting gene sets, max.zs represents the maximum size of the resulting gene sets, and tau represents the exponent defining the weight of the tail in the random walk performed by ssGSEA.

Construction of the gene signature

A total of 493 HNSC patients from TCGA were randomly divided into the training set (n = 345) and validation set (n = 148). We assigned a number to each of the 493 patients, ranging from 1 to 493. Then, we randomly selected 70% of the patients as the training set based on a random sampling method that can be performed using the 'sample' function in R. Then, the rest of the patients were considered as the validation set. The CR-associated genes were first screened for eligible genes to establish the predictive signature using univariate Cox regression and then further analyzed using least absolute shrinkage and selection operator (LASSO) regression. The eligible genes in LASSO were utilized to construct a gene signature based on the expression levels of the eligible genes and their corresponding coefficients in LASSO using the following formula: *PER2* × (-0.2371) + *PER3* × (-0.0807) + *GHRL* × (-0.2003) + *CSF2* × (0.0277) + *HDAC3* × (0.5065) + *KLF10* × (0.1196) + *PRKAA2* × (0.2072) + *PTGDS* × (-0.0698) + *RORB* × (-0.1509).

Assessment of the predictive performance of the gene signature

The predictive ability of the gene signature was assessed by two analyses, namely, the receiver operating characteristic (ROC) curve and Kaplan–Meier curve, based on the risk score calculated using the abovementioned formula. The area under the curve (AUC) and log-rank test were performed in the training set, the validation set, and an independent test set. Notably, the published signatures that were used to compare with our gene signature have their own formulas for estimating the risk score, which are available in the corresponding published papers. Thus, we calculated the risk score based on the gene mRNA expression levels and the coefficients that were generated in each study.

Functional enrichment analysis

Functional enrichment analysis was performed using the "clusterProfiler" package in R (version: 3.18.1) (17), which can determine whether canonical biological processes and signaling pathways are significantly enriched in a given patient cohort based on gene expression profiles. Information on canonical biological functions and signaling pathways is available in the GO.db and KEGG.db Bioconductor annotation data.

Statistics

Statistical analysis was performed using R software (Version 4.0.1). The optimal cutoff value was estimated using the 'surv_cutpoint' function in "survival" package in R. Independent-sample t-tests or Wilcoxon signed rank tests were utilized according to the homogeneity of variance and normal distribution of data. Spearman's correlation coefficient was utilized to investigate the relationship between two continuous variables. Statistical significance was considered when the P-value was less than 0.05.

Results

Construction of a circadian rhythm-related gene signature

CR has been reported to play a role in cancer; however, it remains unclear whether it has an effect on HNSC. To investigate its association with HNSC, we compared the expression levels of circadian rhythm genes between HNSC and normal tissues using RNA-seq data from HNSC patients from TCGA cohort. The expression levels of the circadian rhythm signaling pathway were quantified as an ES using the ssGSEA algorithm based on the RNA-seq data of 493 HNSC samples from TCGA cohort. The findings showed that the circadian rhythm gene set was significantly reduced in HNSC samples compared with normal samples adjacent to the cancer (Figure 1A). Moreover, we divided the HNSC patients into low-ES and high-ES groups according to the optimal cutoff value of ES and performed a survival analysis. High-ES patients had an improved overall survival (OS) compared with low-ES patients (Figure 1B). Moreover, circadian rhythm levels accurately discriminated tumor patients from normal patients with an area under the curve of 0.770 (Figure 1C). These findings suggested that circadian rhythm is correlated with HNSC.

Based on the relationship between CR and HNSC, we next investigated if CR can predict the prognosis of HNSC patients by developing a CR-related gene signature to predict the survival of HNSC patients. To identify eligible CR-related genes in HNSC, we first performed a univariate Cox regression analysis for 84 CR-related genes, and we obtained nine genes (*PER2*, *PER3*, *GHRL*, *CSF2*, *HDAC3*, *KLF10*, *PRKAA2*, *PTGDS*, and *RORB*; $P < 0.05$; Figure 1D). The nine genes were further filtered using LASSO regression analysis to eliminate multicollinearity, which indicated that all nine genes (*PER2*, *PER3*, *GHRL*, *CSF2*, *HDAC3*, *KLF10*, *PRKAA2*, *PTGDS*, and *RORB*) could be used for the establishment

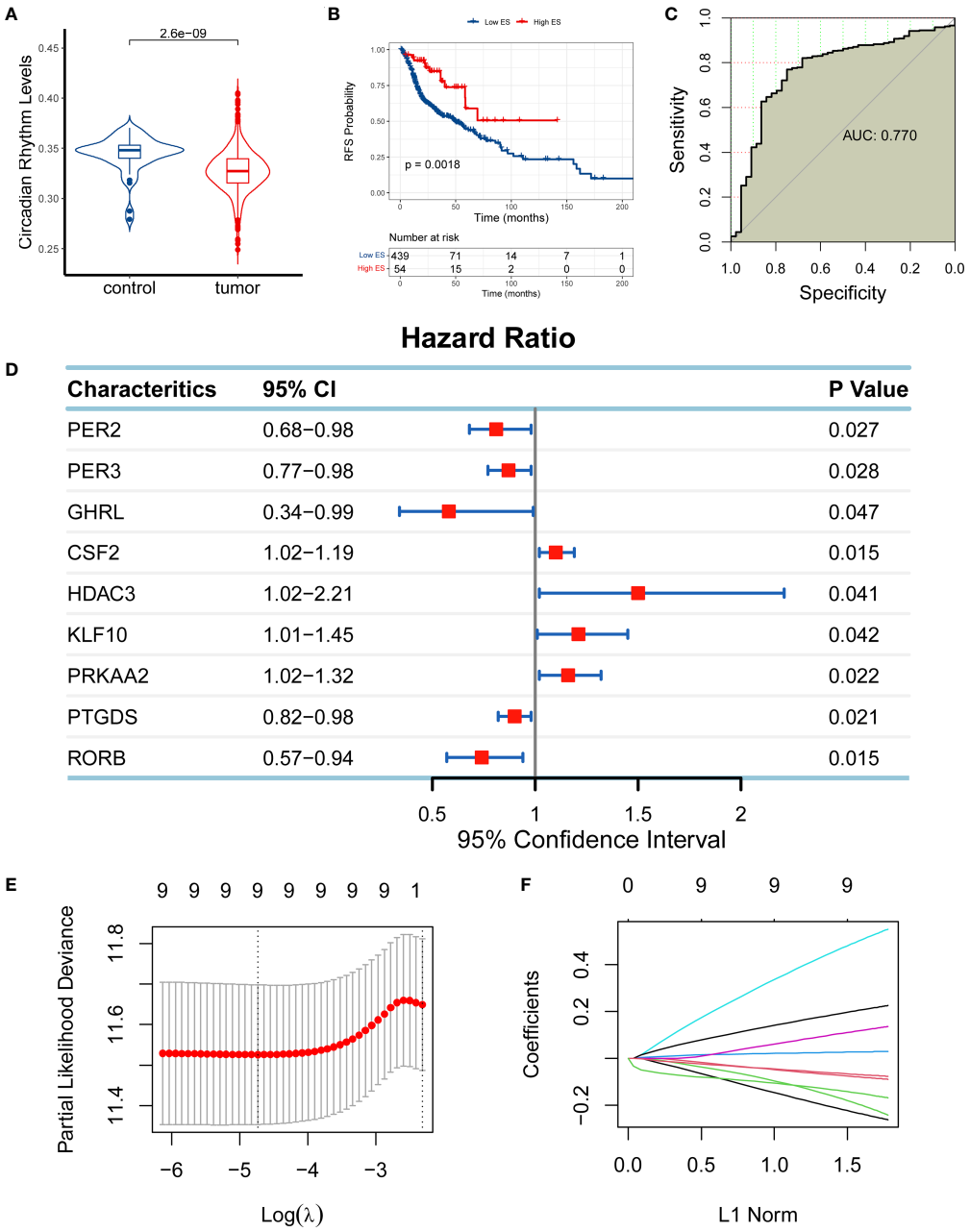

FIGURE 1
Establishment of a circadian rhythm (CR)-related gene signature in head and neck squamous cell carcinoma (HNSC). **(A)** The enrichment score (ES) of the CR-related gene set was significantly reduced in HNSC samples compared with normal samples adjacent to the cancer. **(B)** High-ES patients had an improved overall survival (OS) compared with their counterparts. **(C)** The CR-related gene set was positively enriched in normal tissue compared with tumor tissue. **(D)** Nine CR-related genes qualified for univariate Cox regression analysis (*PER2*, *PER3*, *GHRL*, *CSF2*, *HDAC3*, *KLF10*, *PRKAA2*, *PTGDS*, and *RORB*; $P < 0.05$). **(E, F)** Nine qualified genes were further validated using least absolute shrinkage and selection operator regression analysis to eliminate multicollinearity for the establishment of a CR-related gene signature for predicting OS in HNSC patients.

of a CR-related gene signature for predicting OS in HNSC patients (Figures 1E, F). The CR-related gene signature was quantified based on the mRNA expression levels and the corresponding coefficients of seven CR-related genes using the following formula: *PER2* × (-0.2371) + *PER3* × (-0.0807) + *GHRL* × (-0.2003) + *CSF2* × (0.0277) + *HDAC3* × (0.5065) + *KLF10* × (0.1196) + *PRKAA2* × (0.2072) + *PTGDS* × (-0.0698) + *RORB* × (-0.1509). The coefficients represented the influence of genes on OS, in which positive coefficients represented a risk factor for OS and negative coefficients represented a protective factor for OS.

Assessment of predicting the performance of the CR-related gene signature

The predictive capability of the CR-related gene signature was assessed using ROC curves in the training set (n = 345), the validation set (n = 148), and the external test set (GSE41613; n = 76). The AUC values for predicting 5-year overall survival were 0.681, 0.700, and 0.729 in the training set, validation set, and test set, respectively (Figures 2A–C), suggesting its ability to predict OS. We then quantified the CR-related gene signature using the abovementioned formula and divided the patients into low- and high-risk groups according to the median risk score. The principal component analysis showed that the low-risk patients were distinct from the high-risk patients in Dim 1, suggesting a discriminative ability of the CR-related gene signature (Figures 2D–F). Consistently, the survival analysis also revealed that the low-risk group had an improved OS compared with the high-risk group in the training set, validation set, and external test set (log-rank test, P < 0.001; Figures 2G–I). These results indicated that the mRNA levels of the critical genes could reflect prognosis, which is consistent with the previous study that the pivotal genes with the highest survival scores could be used as predictive and prognostic biomarkers (18). Collectively, these findings confirmed that the CR-related gene signature has a predictive capability for OS in HNSC.

We also compared the performance of the CR-related gene signature to other published signatures using ROC curves and Kaplan–Meier curves in the validation set, training set, and external test set. The CR-related gene signature exhibited the highest AUC value among the tested signatures (Supplementary Figures S1A, B).

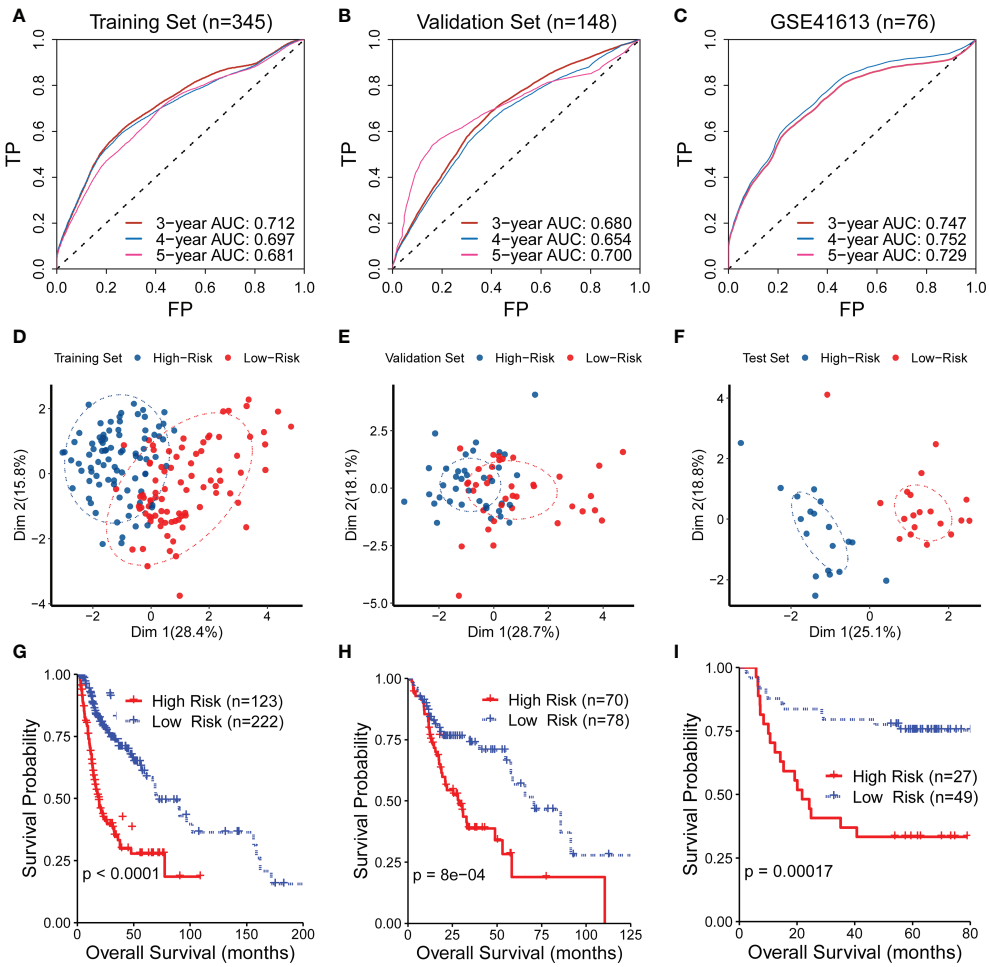

FIGURE 2
Evaluation of the performance of the circadian rhythm-related gene signature. **(A–C)** The area under the receiver operating characteristic curve (AUC) values for predicting the 5-year overall survival (OS) were 0.681, 0.700, and 0.729 in the training set, validation set, and test set, respectively. **(D–F)** The principal component analysis showed that the low-risk patients were distinct from the high-risk patients in Dim 1 in the training set, validation set, and test set. **(G–I)** The survival analysis also revealed that the low-risk group had an improved OS compared with the high-risk group in the training set, validation set, and external test set.

The other three published signatures showed a discriminating ability in the Kaplan–Meier curve with a significantly improved survival in the low-risk group compared with the high-risk group (Supplementary Figures S1C, E). We next investigated the statistical significance of the performance improvement for the CR-based signature compared with the other reported gene signatures. To this end, we compared the performance of these gene signatures 100 times using 80% of HNSC patients randomly sampled from the test set, and we compared the 100 AUC values of each signature to determine whether there was a significant difference. The results demonstrated that there was a significant distinction as shown in Supplementary Figure S1F.

Comparison of the predictive capability of the CR-related gene signature with other indicators

To further evaluate the predictive capability of the CR-related gene, we compared the predictive capability of the CR-related gene signature with other indicators of OS, including clinical characteristics and three other reported gene signatures. The results showed that the CR-related gene signature showed an improved predictive performance compared with other clinical indicators (clinical T staging, clinical N staging, clinical M staging, and clinical stage) with a maximum AUC value of 0.683 (Figure 3A). Actually, the relationship of the signature with TNM staging had been verified in an earlier study where many kinases have expressions that correlate with T, N, and M staging (18). Moreover, the CR-related gene signature (Signature 1) also showed an improved predictive performance compared with the other three reported gene signatures, namely, an immune-related gene signature (5) (Signature 2), an eight-gene signature (4) (Signature 3), and an autophagy-related gene signature (3) (Signature 4), with AUC values of 0.700 *vs.* 0.479, 0.631, and 0.528, respectively (Figure 3B). Collectively, these findings indicated that the CR-related gene signature has a better predictive ability than the other gene signatures.

Furthermore, we assessed the ability of the CR-related gene signature to predict the progression-free interval (PFI), disease-free interval (DFI), and disease-specific survival (DSS) in TCGA cohort. Surprisingly, the low-risk group had a significantly improved PFI, DFI, and DSS compared with the high-risk group (log-rank test, $P < 0.001$; Figures 3C–E). Moreover, the low-risk group had a significantly better DFS compared with the high-risk group in another independent cohort (GSE27020; log-rank test,

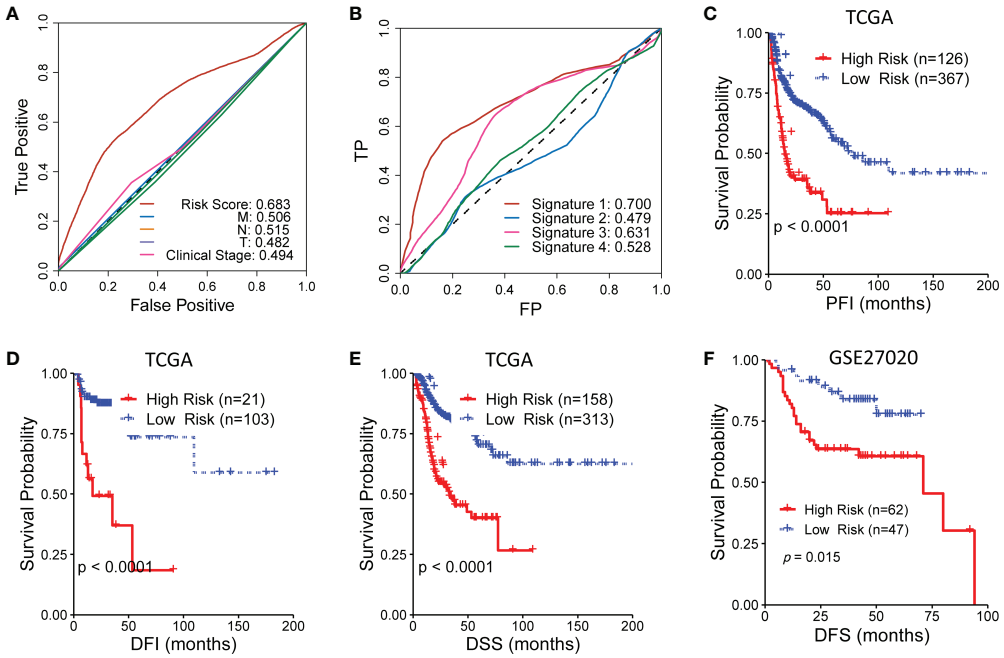

FIGURE 3
Comparison of the circadian rhythm (CR)-related gene signature with other indicators for overall survival in head and neck squamous cell carcinoma. **(A)** The CR-related gene signature showed an improved predictive performance compared with clinical T staging, clinical N staging, clinical M staging, and clinical stage. **(B)** The CR-related gene signature (Signature 1) also showed an improved predictive performance compared with the three reported gene signatures, namely, an immune-related gene signature (5) (Signature 2), an eight-gene signature (4) (Signature 3), and an autophagy-related gene signature (3) (Signature 4). **(C–E)** The low-risk group had a significantly improved progression-free interval, disease-free interval, and disease-specific survival compared with the high-risk group in The Cancer Genome Atlas. **(F)** The low-risk group had a significantly improved disease-free survival compared with the high-risk group in GSE27020.

P = 0.015; Figure 3F). These results further supported the acceptable prognostic ability of the CR-related gene signature in HNSC.

Clinical significance of the CR-related gene signature

To further investigate the clinical significance of the CR-related gene signature, we analyzed the relationship between the CR-related gene signature and clinical characteristics. We found that the older patients had a higher risk score than the younger patients (cutoff value of 60 years old; $P < 0.05$; Figure 4A), whereas the risk score was not related to clinical stage, TNM staging, and smoking exposure (Figures 4B–F). Based on the median value of 61 years old, the cutoff value was 60 years old; to facilitate practical clinical application, we artificially positioned the threshold to 60 years old. We next explored the relationship of the risk score with survival status and the expression levels of the nine genes. The findings showed that higher risk scores were associated with higher mortality and higher expression levels of CSF2, KLF10, PRKAA2, and HDAC3 in the training set (Figure 4G) and validation set (Figure 4H).

Furthermore, we analyzed the relationship between CR-related gene expression and age. Except for *PER3*, no other genes were

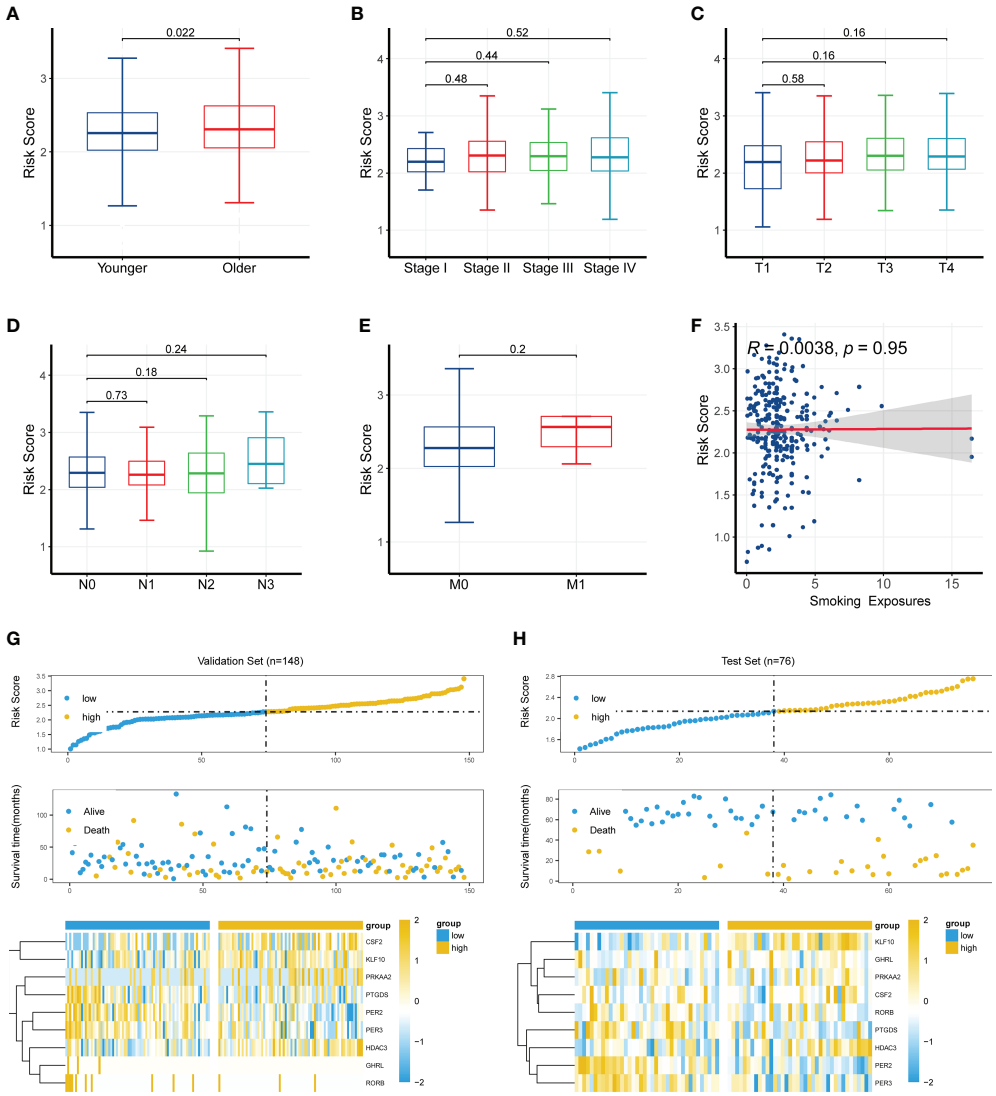

FIGURE 4
Clinical significance of the circadian rhythm-related gene signature. **(A)** Older patients had a higher risk score than younger patients (cutoff value of 60 years old; $P < 0.05$). **(B–F)** The risk score was not related to clinical stage, TNM staging, or smoking exposure. **(G)** A higher risk score was associated with a higher mortality and a higher expression level of CSF2, KLF10, PRKAA2, and HDAC3 in the training set. **(H)** A higher risk score was associated with a higher mortality and a higher expression level of CSF2, KLF10, PRKAA2, and HDAC3 in the validation set.

significantly changed between the younger and older groups (Supplementary Figures S2A–I). However, different proportions of HPV+ status were present in different age groups, which may have influenced the risk score and performance of the CR-related gene signature (Supplementary Figures S2J–L).

Functional enrichment analysis

To investigate the biological functions associated with the CR-related gene signature, we performed functional enrichment analysis for genes that were correlated with the CR-related gene signature. We calculated the correlation coefficients between the gene signature and all genes, which included 405 qualified genes ($P < 0.01$, $R > 0.3$) (19). The analysis using the "clusterProfiler" package in R indicated that these genes were mainly enriched in ribosome biogenesis, focal adhesion, and protein localization (Figures 5A–D; Supplementary Table S2). The gene set enrichment analysis showed that some enriched biological functions were associated with immune functions, including complement and coagulation cascades as well as the Fc epsilon RI signaling pathway and the T cell receptor signaling pathway (Figures 5E, F).

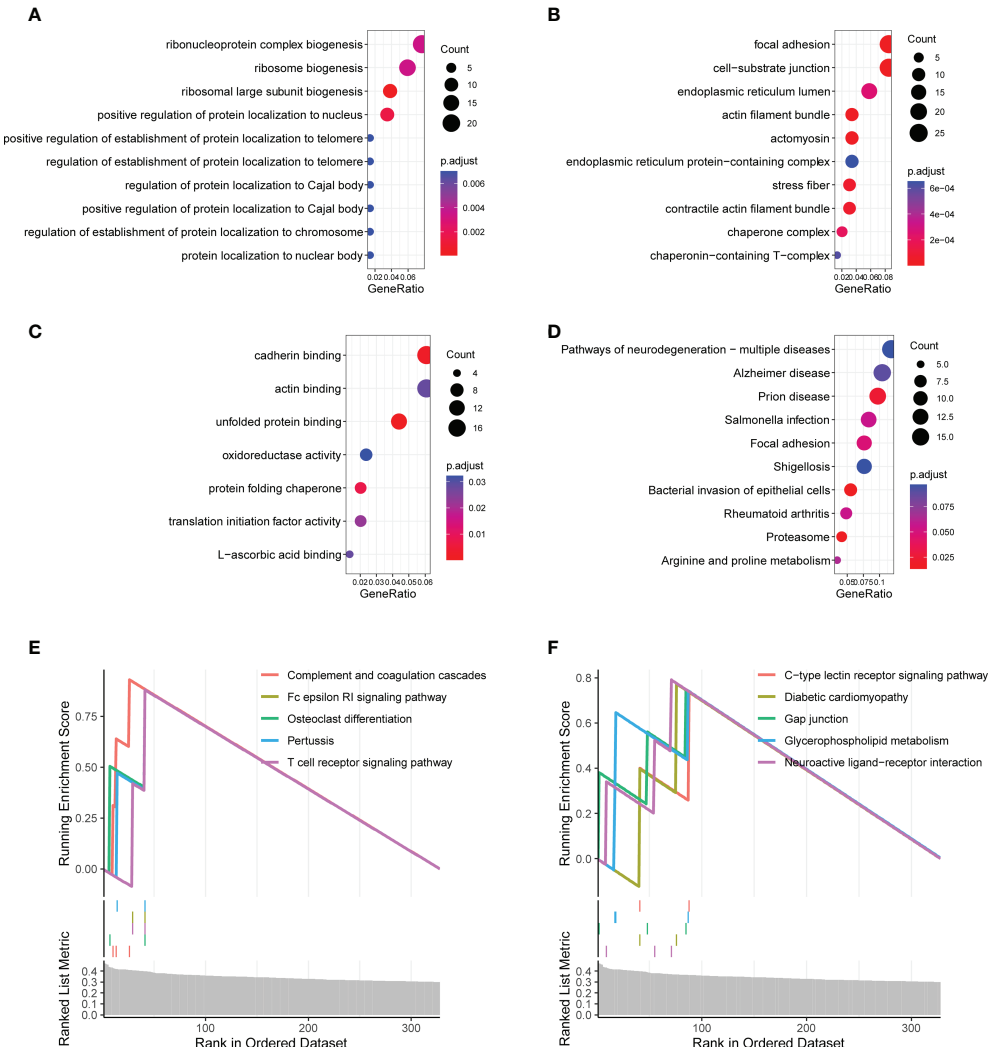

FIGURE 5
Functional enrichment analysis of the circadian rhythm-related gene signature. **(A)** Enriched biological processes included ribonucleoprotein complex biogenesis, ribosome biogenesis, and ribosomal large subunit biogenesis. **(B)** Enriched cell components included focal adhesion, cell–substrate junction, endoplasmic reticulum lumen, and actin filament bundle. **(C)** Enriched molecular functions included cadherin binding, actin binding, and unfolded protein binding. **(D)** Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways included focal adhesion, proteasome, arginine, and proline metabolism. **(E, F)** The gene set enrichment analysis showed the top 10 KEGG signaling pathways, including complement and coagulation cascades as well as the Fc epsilon RI signaling pathway and T cell receptor signaling pathway.

Association of the CR-related gene signature with the tumor immune microenvironment

Considering that the functional enrichment analysis implicated immune function in HNSC, we analyzed the changes in the abundance of immune cells between the high- and low-risk groups using bulk RNA-seq data. The abundance of immune cells was estimated using the CIBERSORT algorithm. The results showed that B cell plasma, $CD8^+$ T cells, memory resting $CD4^+$ T cells, T cell follicular helper cells, regulatory T cells, activated NK cells, monocytes, and M1 macrophages were significantly upregulated in the low-risk group ($P < 0.05$; Figure 6A), whereas resting NK cells, M0 macrophages, and resting mast cells were significantly upregulated in the high-risk group ($P < 0.05$; Figure 6A). The heat map analysis also indicated a distinct expression of these cells between groups (Figure 6B). The correlation analysis showed that activated NK cells were significantly positively correlated with CD8 T cells, T cell follicular helper cells, and M1 macrophages (Figure 6C). Consistently, we found that the risk score was negatively correlated with $CD4^+$ T cells, $CD8^+$ T cells, gamma delta T cells, and activated NK cells (Figure 6D; $P < 0.05$).

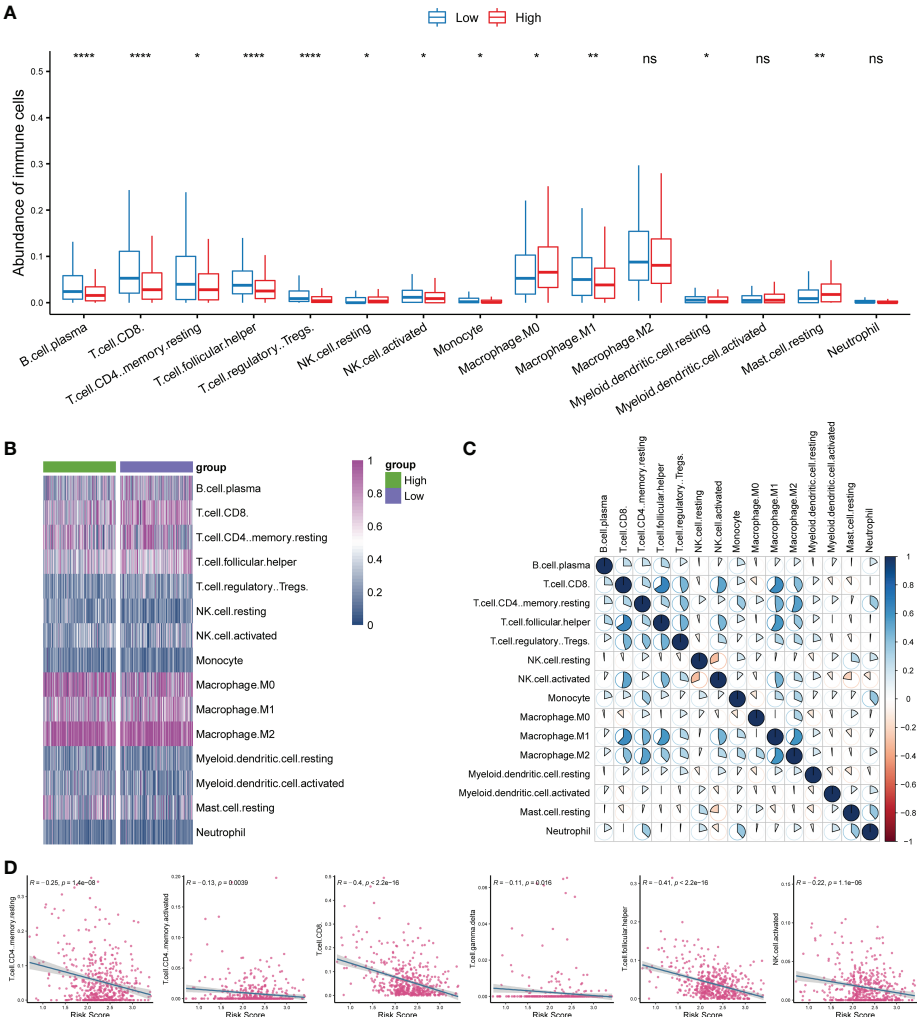

FIGURE 6
Investigation of the correlation of the immune microenvironment with circadian rhythm. **(A)** Comparison of the abundance of immune cells between the high- and low-risk groups. **(B)** The heat map analysis indicated a distinct expression of tumor-infiltrating immune cells between groups. **(C)** Activated NK cells were significantly positively correlated with CD8+ T cells, T cell follicular helper cells, and M1 macrophages. **(D)** The risk score was negatively correlated with $CD4^+$ T cells, $CD8^+$ T cells, gamma delta T cells, and activated NK cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Profiling of somatic nucleotide variation in HNSC patients between the low- and high-risk groups

To investigate the association of the CR-related gene signature with the mutation levels in HNSC patients, we profiled the mutation landscape of the low-risk and high-risk groups by analyzing the somatic nucleotide variation data of HNSC patients from TCGA cohort using the "maftool" package in R. To better compare the potential distinction of the mutation landscape between different risk score groups, we defined the patients with the top 25% risk score as the high-risk group, and we defined the patients with the bottom 25% risk score as the low-risk group. The waterfall plot demonstrated that the high-risk group had a higher nucleotide variation rate than the low-risk group (96.75% *vs.* 84.30%, Figures 7A, B). Consistent with the findings of the waterfall plot, the bar plot showed that the risk score was significantly increased in the high-mutation group compared with the low-mutation group (Figure 7C), and the box plot also demonstrated that the risk score was significantly increased in the high-mutation group compared with the low-mutation group (Figure 7D).

Effects of the CR-related gene signature on drug response

Because the gene signature was associated with the tumor immune microenvironment and somatic nucleotide variation in HNSC, we next investigated its relationship with drug response. We compared the risk scores between responders and nonresponders in TCGA and found that nonresponders had a significantly higher risk score than responders (Figure 8A; $P = 0.0014$). We then compared the mRNA levels of multiple immune checkpoint genes between the high- and low-risk groups. Consistently, we found that the mRNA levels of *TNFRSF9*, *LAG3*, *CD40LG*, *IDO1*, *CTLA4*, *TIGIT*, and *PDCD1* were all significantly upregulated in the low-risk group compared with the high-risk group (Figure 8B), further verifying the ability of the CR-related risk score to predict drug response. In addition, we compared the expression levels of more immune checkpoint molecules between the low- and high-risk groups. We retrieved 59 immune checkpoint genes from previous literature (20–23), and we found that 42 of these were differentially expressed between the low- and high-risk groups (Supplementary Table S3).

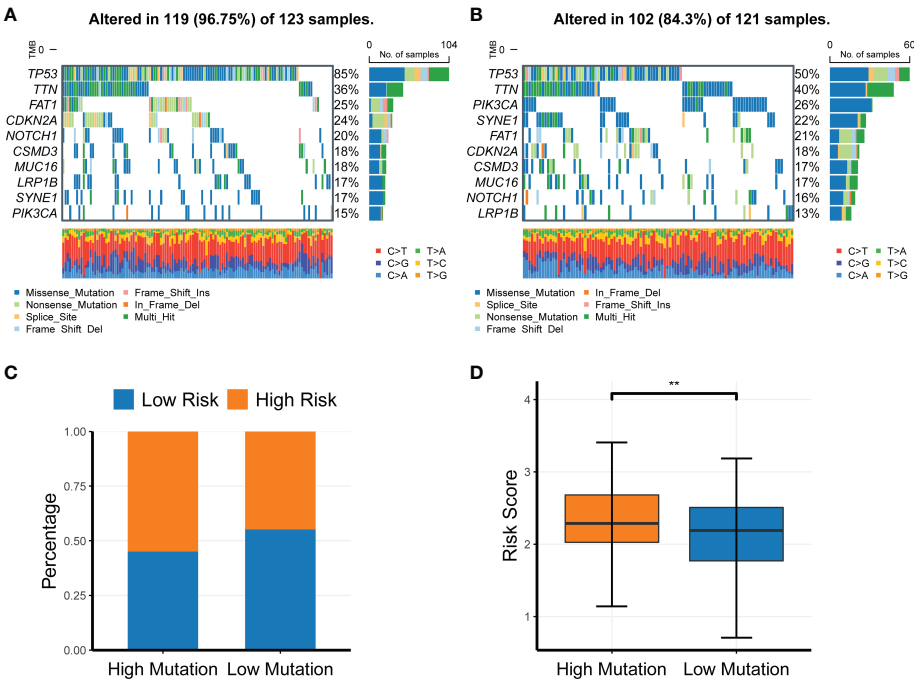

FIGURE 7
Profiling of somatic nucleotide variation for head and neck squamous cell carcinoma patients of different risk groups. **(A, B)** Waterfall plot of the nucleotide variation rate in the high-risk group and the low-risk group. **(C, D)** Bar plots and box plots of risk scores in the high-mutation group and low-mutation group. $^{**}P < 0.01$.

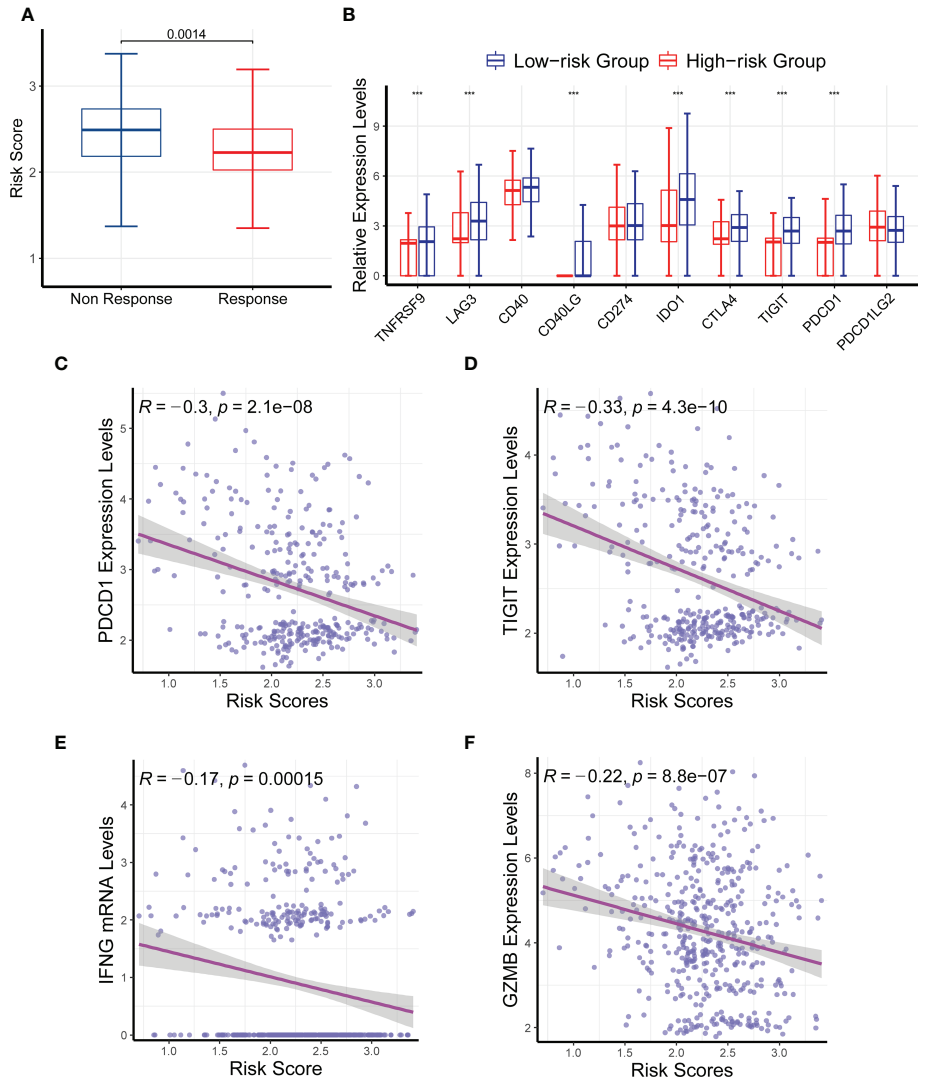

FIGURE 8
Effects of the circadian rhythm-related gene signature on drug response. **(A)** The nonresponders had a significantly higher risk score than the responders in The Cancer Genome Atlas. **(B)** The mRNA levels of *TNFRSF9*, *LAG3*, *CD40LG*, *IDO1*, *CTLA4*, *TIGIT*, and *PDCD1* were significantly upregulated in the low-risk group compared with the high-risk group. **(C–F)** The risk score was significantly correlated with the expression levels of *PDCD1*, *TIGIT*, *INFG*, and *GZMB*. ***$P < 0.001$.

We also analyzed the correlation between the risk score and several immune checkpoint genes, and we found that the risk score was significantly correlated with the expression levels of *PDCD1*, *TIGIT*, *INFG*, and *GZMB* (Figures 8C–F; $P < 0.05$). Collectively, these findings further confirmed the ability of the CR-related gene signature to predict drug response to immune checkpoint inhibitors in HNSC.

Discussion

We developed and validated a nine-gene CR-related signature for predicting prognosis in patients with HNSC, which may serve as a precision medicine tool. Several immune-related biological processes were enriched in HNSC, including complement and coagulation cascades as well as the Fc epsilon RI signaling pathway and the T cell receptor signaling pathway. Moreover, we observed that the CR-related gene signature was associated with somatic nucleotide variation and drug response in HNSC. Overall, these findings provide a tool for predicting prognosis and drug response in patients with HNSC, which will help to develop precision medicine.

One of the main contributions of this study is the establishment of a CR-related prognostic signature for predicting the survival of patients with HNSC. The performance of the signature was verified in an external dataset (GSE41613), which

indicated that the gene signature is highly reliable and widely applicable. The predictive power of this gene signature is superior to common clinical characteristics and several reported gene signatures for predicting the 5-year overall survival in HNSC patients. Surprisingly, the maximum AUC value of the CR-based model was not consistent between comparisons, which may be due to missing clinical data points or inconsistent baselines across populations. Specifically, we investigated the optimal performing test set and observed that it consisted of all HPV-negative patients. A subsequent analysis demonstrated that the CR-related gene signature performed better in the HPV-negative cohort. In addition, we observed that most clinical features of the CR-related gene signature were not different, and only a few clinical features were significantly different, which may be due to an unbalanced sample size between primary and metastatic patients (491 *vs.* 2) or some commonly used clinical features, such as N staging, may not be an ideal predictor for risk. Further investigation of this aspect is warranted.

Another important finding was that several important biological processes were identified to be involved in CR and high risk as follows: AM immune function, complement and coagulation cascades, Fc epsilon RI signaling pathway, and T cell receptor signaling pathway. Consistent with our findings, complement and coagulation cascades have been reported to be involved in HNSC (24). We also showed that the T cell receptor signaling pathway was enriched in the high-risk group. Similarly, a pancancer analysis revealed that disrupted circadian rhythm is associated with T cell exhaustion (25), and the response of T cells to antigens has a circadian variation (26, 27). Here we found that the CR-related gene signature was associated with these immune-related signaling pathways, suggesting the role of circadian rhythm disruption in the tumor immune microenvironment.

The nine genes comprising the CR-related gene signature may be potential biomarkers for HNSC. The dysfunction of *PER2* and *PER3* has been related to cancer development and progression (28–30) as well as poor prognosis in HNSC (31). In addition, high levels of *KLF10* are associated with a favorable prognosis in patients with HNSC (32). Colony-stimulating factor 2 (*CSF2*) plays an important role in macrophage polarization (33) and may be associated with poor prognosis in breast cancer and colorectal cancer (34, 35). However, further investigations on the role of *GHRL* and *RORB* in HNSC are warranted.

The present study has important implications for the treatment and prognosis of HNSC. First, our study provided a novel prognostic signature that may aid clinical treatment strategies for HNSC. Second, we revealed several critical oncogenes and pathways that may serve as promising therapeutic targets for the treatment of HNSC. Nevertheless, further *in vitro* and *in vivo* investigations are warranted to study the role of these pivotal genes in HNSC and their precise mechanisms of action.

The present study had several limitations that warrant further research. First, the key genes and signaling pathways in this study were identified using bioinformatics analysis, and further *in vitro* and *in vivo* studies are required to explore their physiological mechanisms of action. In addition, the risk score was identified to predict the drug response of HNSC, warranting further clinical investigation.

In conclusion, we successfully constructed and validated a novel CR-related signature that predicts the prognosis of patients with HNSC, thereby providing a rationale for the further investigation of HNSC.

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

CZ and DD collected the data. MY, CZ, and DD analyzed the data and wrote the manuscript. HW, JD and SS contributed to manuscript revision. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1029676/full#supplementary-material

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Incorporation of a TGF-β2-inhibiting oligodeoxynucleotide molecular adjuvant into a tumor cell lysate vaccine to enhance antiglioma immunity in mice

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Introduction: Transforming growth factor β2 (TGF-β2), also known as glioma-derived T-cell suppressor factor, is associated with the impairment of tumor immune surveillance. Therefore, blocking TGF-β2 signaling probably be a feasible strategy to develop a novel type of adjuvant for glioma vaccines to enhance antitumor immunity.

Methods: A TGF-β2 inhibitory oligodeoxynucleotide, TIO3, was designed with sequences complementary to the 3' untranslated region of TGF-β2 mRNA. The expression of TGF-β2 and MHC-I was detected by qPCR, western and flow cytometry in vitro. All the percentage and activation of immune cells were detected by flow cytometry. Subsequently, TIO3 was formulated with Glioma cell lysate (TCL) and investigated for its antitumor effects in GL261 murine glioma prophylactic and therapeutic models.

Results: TIO3 could efficiently downregulate the expression of TGF-β2 while increase the MHC-I's expression in GL261 and U251 glioma cells in vitro. Meanwhile, TIO3 was detected in mice CD4+ T, CD8+ T, B and Ly6G+ cells from lymph nodes after 24 hours incubation. Moreover, TCL+TIO3 vaccination significantly prolonged the survival of primary glioma-bearing mice and protected these mice from glioma re-challenge in vivo. Mechanistically, TCL+TIO3 formulation strongly evoke the antitumor immune responses. 1) TCL+TIO3 significantly increased the composition of CD4+ and CD8+ T cells from draining lymph nodes while promoted their IFN-γ production and reduced the expression of TGF-β2 and PD1. 2) TCL+TIO3 activated the NK cells with the elevation of CD69 or NKG2D expression and PD1 reduction. 3) TCL+TIO3 increased the glioma-specific lysis CTLs from spleen. 4) TCL+TIO3 downregulated PD-L1 expression in glioma tissues and in Ly6G+ cells among glioma-infiltrating immune cells.

Conclusion: TIO3 is a promising adjuvant for enhancing TCL-based vaccines to produce a more vigorous and long-lasting antitumor response by interfering with TGF-β2 expression.

KEYWORDS

adjuvant, tumor vaccines, TGF-β2, inhibitory oligodeoxynucleotide, glioma

1 Introduction

Glioma is a frequent type of brain tumor that is characterized by a poor prognosis and high mortality. Currently, the most common therapeutic treatments include surgery, radiation, and chemotherapy; however, the median survival of patients with glioma is just 15 months, emphasizing the need for novel therapeutic approaches (1). With the development of cancer treatments, tumor immunotherapy has become an innovative maintenance approach for the treatment of glioma. Immunotherapy can effectively promote immune effector cells to infiltrate the brain and specifically destroy residual tumor cells with no collateral damage in critical neural tissues, as confirmed in many preclinical studies (2).

Tumor vaccines are a promising form of immunotherapy that can activate the immune system to combat tumor growth. Over the last two decades, tumor vaccines have attracted increasing attention for their application in tumor prevention and treatments. Remarkably, Provenge, as the first tumor vaccine for the treatment of advanced prostate cancer, was approved by the U.S. Food and Drug Administration (FDA) in April 2010 (3). Moreover, numerous strategies have been developed to generate prophylactic or immunotherapeutic tumor vaccines with encouraging antitumor immune responses that have been undergoing clinical trials in cancer patients (4). Tumor vaccines based on single or combined tumor-associated antigens (TAAs) are a common formulation and preparation strategy (5). However, these vaccines are restricted to the subset of patients whose tumors express the known TAAs, and increasing immune responses may ultimately have limited efficacy due to tumor heterogeneity and loss of antigen expression over time (6). Compared with single tumor antigen strategies, tumor cell lysate (TCL) as the source of various antigens offers the potential advantage of inducing a broad T-cell response against multiple known and unknown TAAs expressed by the specific tumor, which facilitates a reduction in tumor escape (7). In a clinical research trial, dendritic cells (DCs) loaded with glioma TCL displayed a more potent antitumor effect than glioma-associated antigen-loaded DCs (8). Although glioma lysates offer a large variety of antigenic peptides, the immunogenicity of each antigen can vary widely depending on how efficiently it is bound to major histocompatibility complex (MHC) molecules and presented by DCs, resulting in suboptimal or inconsistent immune responses against tumors (9). Therefore, glioma TCL needs to be prepared into a formulation with effective adjuvants to enhance the immune responses against glioma.

Accumulated evidence has revealed that immune-stimulating cytokines or immunosuppressive cytokine inhibitors are considered potent adjuvants in tumor cell vaccines (10). Transforming growth factor-beta 2 (TGF-β2) is a cytokine that plays a very important role in tumor initiation, progression, and many other important processes in malignancy. Generally, TGF-β2 has been identified to negatively modulate the expression of MHC molecules to downregulate both innate and adaptive immune responses. Several studies found that TGF-β2 can inhibit the expression of MHC I, MHC II and CD80, CD86 and CD40 molecules on macrophages and dendritic cells and partially block the rat astrocyte autoantigen presentation and upregulation of MHC I and MHC II induced by interferon-γ (IFN-γ) (11, 12). Additionally, TGF-β2 can induce the differentiation of naive $CD4^+$ T cells into conventional $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) by binding to TβRIII (13). Therefore, inhibition of TGF-β2 may promote antigen-presenting cells (APCs) to express MHC molecules/costimulatory molecules and reduce the generation of Tregs to enhance immune responses.

TGF-β2 inhibitors have been used in cancer therapy studies. Short hairpin RNAs (shRNAs) and antisense transgenes have been used extensively in mediating the knockdown of TGF-β2 expression in cells. An oncolytic adenovirus expressing a TGF-β2 shRNA and granulocyte-macrophage colony stimulating factor (GM-CSF) can significantly enhance the anti-melanoma efficacy of melanoma antigen Melan-A (MART1) in mice (14). Similarly, the coexpression of GM-CSF and TGF-β2 antisense transgenes in an irradiated autologous whole-cell vaccine was used in a phase I/II clinical trial for treating patients with advanced prostate cancer, colon carcinoma, gastric cancer and leiomyosarcoma (15). Moreover, a therapeutic vaccine known as Belagenpumatucel-L, which is comprised of four TGF-β2 interfering gene-modified, irradiated, allogeneic non-small cell lung cancer (NSCLC) cell lines, has also been evaluated in a phase III clinical trial (16). Together, these data implied that TGF-β2 inhibitors could facilitate the stimulation of immune responses against TAAs and break immune tolerance, eliciting more vigorous antitumor responses. As a promising TGF-β2 inhibitor, specific oligodeoxynucleotides (ODNs) targeting TGF-β2 have also been developed to directly treat some types of cancer, such as high recurrence of malignant glioma, pancreatic cancer, melanoma, and triple-negative breast cancer (17–20). However, it is still uncertain whether TGF-β2 antisense oligodeoxynucleotides (ASOs) can act as effective tumor vaccine adjuvants, and the specific mechanism by which TGF-β2 ASOs enhance antitumor immune responses is still elusive due to limited research findings.

In a previous study, we designed a TGF-β2 inhibitory oligodeoxynucleotide (TIO3) with sequences complementary to the 3' UTRs of TGF-β2 mRNA and demonstrated that TIO3 could enhance multiple microbial vaccines to induce strong and persistent antibody responses by suppressing TGF-β2 expression in immune cells (12). In the current study, TIO3 was used as an adjuvant in a formulation with the glioma TCL, which was used to investigate the antitumor effects of TGF-β2 inhibition in mouse GL261 glioma models. Our results indicated that TIO3-TCL regulates the activation of potent glioma-specific cytotoxic T lymphocytes and prolongs the survival of mice with *in situ* glioma. Furthermore, TIO3 activated T and NK cells in the lymph nodes and inhibited PD1 and PD-L1 expression in mice bearing GL261 glioma. These data support the impact of TIO3 on antitumor immunity, allowing the advancement of TIO3 as a promising adjuvant for glioma vaccines.

2 Materials and methods

2.1 Oligodeoxynucleotides

Two ODNs fully phosphonothioate-modified were used in our study: TIO3, 5'-TTACCACTAGAGCACCACA-3' (19 nt); control ODN (cODN), 5'-ACTTACTCGAGAACCCCAA-3' (19 nt). All ODNs, including 3' Alexa Fluor 488-conjugated TIO3 and 3' Cy3-conjugated TIO3, were synthesized by Sangon Biotech (Shanghai, China).

2.2 Cell lines

GL261 cells (a murine glioma cell line) and U251 cells (a human glioblastoma cell line) were kindly provided by the Transfusion Research Institute of the Academy of Military Sciences, Beijing, China. Cells were maintained in RPMI-1640 or DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, TBD, Tianjin, China), 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified 5% CO_2 incubator at 37°C. In this study, GL261 cells were used for *in vitro* cell culture experiments and *in vivo* tumor challenge experiments. For *in vivo* tumor challenge experiments, GL261 cells were digested with trypsin-EDTA (0.25%), harvested, centrifuged for 5 min at 300 g, resuspended and then adjusted to a concentration of 5×10^6 cells/mL in serum-free RPMI-1640 for inoculating the mice with 2×10^4 cells through an intracranial injection.

2.3 Mice

Six-week-old female C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Experimental procedures and manipulation involving mice were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Scientific Investigation Board of Science & Technology of Jilin Province and the ethics committee of the College of Basic Medical Sciences of Jilin University (number 2019-01).

2.4 Preparation of GL261 tumor cell lysate

The tumor cell lysates were prepared as described previously (21). Briefly, GL261 cells were cultured and harvested *in vitro*, then 10^6 cells were intraperitoneally inoculated into the mice. Glioma mass was formed in about 20 days, then the mice was euthanized to isolate glioma tissue for preparing glioma cell lysate. To acquire high-quality glioma cell lysates, we first minced the glioma tissues with scissors, resuspended in 0.85% pathogen-free saline buffer and then disrupted in a glass homogenizer followed by filtration with a 40 μm nylon cell strainer. Cells were adjusted with the concentration at 10^7 cells/mL in saline and then disrupted by high pressure homogenizer at 800 Bar 5 cycles. After centrifugation at 10,000 g for 10 mins, the cell supernatant was separated and passed through a 0.2 μm filter. At last, the tumor cell lysate was conducted freeze-dried for storage at -80 °C (21). TCLs were detected under a microscope (Olympus Corporation, Tokyo, Japan) using trypan blue staining (Sigma-Aldrich, St. Louis, MO, USA) (22).

2.5 RNA isolation and reverse transcription

GL261 and U251 cells were prepared for isolating total RNA with TRIzol™ Reagent (Invitrogen, Carlsbad, CA). A UV2800 ultraviolet spectrophotometer was used to analyze the RNA concentration and purity. M-MLV reverse transcriptase (part: 28025021; Invitrogen, UK) was used for reverse transcription reactions. Briefly, the RT reaction mixture, in a volume of 20 μL, contained $MgCl_2$ (3 mM), dNTP mix (0.5 μM), 1 μl M-MLV, 1 μg of total RNA, oligo(dT)15 primer (0.5 μg) and reaction buffer. The RT reaction was incubated at 42°C for 1 h, and the RT enzyme was inactivated by incubation at 70° C for 15 min.

2.6 Real-time quantitative polymerase chain reaction

To quantify the mRNA expression of TGF-β2 and MHC-I, we used primers synthesized by Sangon Biotech (Shanghai, China), which are listed in Table 1, to amplify the target gene. Quantitative real-time polymerase chain reaction (RT–PCR) was performed using two-step SYBR green RT–PCR assays (Transgene Biotech, G31227) in a Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (12). The procedure for qRT–PCR was one cycle at 95°C (30 s) followed by 40 cycles at 95°C (5 s) and 64°C (31 s) (12). *GAPDH*, the most commonly used gene for normalization of qPCR data in glioma research, was used (23). Relative mRNA expression was calculated after normalizing cycle thresholds against *GAPDH* and is presented as the fold change value ($2^{\Delta\Delta}$comparative threshold) relative to the control.

2.7 Flow cytometry assay

All anti-mouse or anti-human monoclonal antibodies (mAbs) in this study were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

To analyze the surface TGF-β2 (sTGF-β2) and MHC-I expression on GL261 cells, GL261 cells were cultured with PMA (100 μg/mL), PMA+TIO3 (10 μg/mL), or PMA+cODN (10 μg/mL) for 24 h and then stained with an anti-MHC-I mAb (PE-conjugated, 566776) or goat-anti-mouse TGF-β2 antibody (AB-112N, R&D system, USA) for approximately 40 minutes at 4°C in the dark. After being washed twice with FACS buffer (PBS, containing 2 mmol/L EDTA and 20

TABLE 1 Primers used in real time-PCR.

Primer name	Oligonucleotides sequences (5′-3′)
mTGF-β2-F	TCGACATGGATCAGTTTATGCG
mTGF-β2-R	CCCTGGTACTGTTGTAGATGGA
mMHC-I-F	TACCTGAAGAACGGGAACGC
mMHC-I-R	CCATTCAACTGCCAGGTCAG
mGAPDH-F	ATCACCATCTTCCAGGAGCGA
mGAPDH-R	TCTCGTGGTTCACACCCATCA
hTGF-β2-F	CAGCACACTCGATATGGACCA
hTGF-β2-R	CCTCGGGCTCAGGATAGTCT
hMHC-I-F	CAGATACCTGAAGAACGGGAAC
hMHC-I-R	GCACCTCAGGGTGACTTTAT
hGAPDH-F	GGAGCGAGATCCCTCCAAAAT
hGAPDH-R	GGCTGTTGTCATACTTCTCATGG

mL/L FBS), the cells were incubated with the Alexa Fluor®488-conjugated donkey-anti-goat IgG secondary antibody (ab150129) diluted with PBS at 1:1000 for 40 min and then analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA).

To analyze the effect of TIO3 on the expression of TGF-β2 and IFN-γ in $CD4^+$ T cells and $CD8^+$ T cells, the mice were intramuscularly immunized with TCL, TCL+TIO3, TCL+cODN or PBS at the cervical lymph node area on Day 0 and Day 10. On Day 14, the lymphocytes from drainage lymph nodes were isolated. The cells were first stained with PE-labeled CD4 mAb and goat-anti-mouse TGF-β2 antibody or with PE-labeled CD8 mAb and goat-anti-mouse TGF-β2 antibody for 40 minutes on ice in the dark. After being washed twice, the cells were incubated with Alexa Fluor®488-conjugated donkey-anti-goat IgG secondary antibody diluted with PBS at 1:1000 for 40 min. For intracellular staining, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% saponin before staining with APC-labeled IFN-γ mAb (554413), followed by flow cytometry analysis. Similarly, to investigate the effect of TIO3 on the expression of CD69, NKG2D and PD1 in NK cells and $CD4^+$ and $CD8^+$ T cells, lymphocytes isolated from immunized mice were first stained with APC-conjugated anti-NK1.1 mAb (561117), PE-conjugated anti-CD4 mAb (553048), PE-conjugated anti-CD8 mAb (553033), PE-conjugated anti-CD314 mAb (NKG2D, 558403), FITC-labeled CD279 (PD1) and/or FITC-conjugated anti-CD69 mAb (553236) for 40 minutes on ice in the dark and then analyzed by BD FACS Calibur with Cell Quest software (BD Bioscience, San Jose, CA).

To explore the effect of TIO3 on glioma-specific T cells in tumor-bearing mice, C57BL/6 mice were intramuscularly immunized with 100 μL of TCL (100 μg TCL in PBS), TCL + TIO3 (100 μg TCL plus 10 μg TIO3 in PBS), TCL+ cODN (100 μg TCL plus 10 μg control ODN in PBS), or PBS on Day 0 and Day 10. On Day 14, the mice were challenged with 2×10^4 GL261 cells intracranially (i.c.). On Day 28, draining lymph nodes were isolated and minced into a single-cell suspension, and the lymphocytes were stained with PE-labeled anti-CD4 (553048) or PE-labeled anti-CD8 mAb (553033) and FITC-labeled CD69 mAb (553236) at 4°C in the dark for 30 min. After being washed twice, the lymphocytes were analyzed by flow cytometry.

2.8 Cytotoxicity assay

The mice were intramuscularly injected with TCL, TCL+TIO3, TCL+cODN or PBS at the cervical lymph node area on Day 0 and Day 10. On Day 14, the spleen was isolated, and then the splenocytes were used to perform the cytotoxicity assay. Splenocytes were cocultured with GL261 (1×10^4 per well per 100 μL) cells at a ratio of 100:1, 50:1 or 25:1 for 8 h at 37 °C in a 5% CO_2 humidified atmosphere. A methylthiazolyldiphenyl-tetrazolium bromide assay was conducted to determine the CTL activity.

2.9 Fluorescence staining and confocal microscopy

To observe whether TIO3 could enter T cells, lymphocytes isolated from naive mice were seeded on poly-L-lysine-coated 24-well plates at a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS. The cells were cultured with Cy3-labeled-TIO3 (10 μg/ml) in a 5% CO_2 incubator for 24 h at 37°C. After washing twice, the cells were fixed with 4% paraformaldehyde for 10 min at 37°C. The cells were washed twice, permeabilized with 0.1% Triton X-100 at 4°C for 10 min, and then blocked with 5% BSA at 37°C for 1 h. The cells were incubated with PE-conjugated CD4 or CD8 mAb at a dilution of 1:50 for 30 min at 4°C. Unbound antibody was washed and removed with PBS. The stained cells were visualized under a laser scanning confocal microscope (Olympus FV1000) and analyzed by AndorIQ2 software.

2.10 Effect of TIO3 as a prophylactic and therapeutic glioma vaccine adjuvant in an *in vivo* study

To assess the effect of TIO3 as a prophylactic glioma vaccine adjuvant in brain glioma in situ, 32 female C57 BL/6 mice (n=8 each group) were intramuscularly (i.m.) injected with TCL (100 μg/ml), TCL (100 μg/ml) + TIO3 (10 μg/ml), TCL (100 μg/ml) + cODN (10 μg/ml), or PBS in the neck draining lymph node of the mice on Day 0 and Day 10. On Day 14, each mouse was anesthetized and intracranially (i.c.) injected with 2×10^4 GL261 cells at 2 mm to the right of the bregma and 3 mm deep using a stereotaxic instrument (Kopf Instruments) (21). Any animals showing clinical symptoms or abnormal neurological signs 1-3 days after surgery were excluded from the experiment. Subsequently, the survival period of the mice after tumor inoculation was recorded.

To explore the effect of TIO3 as a therapeutic glioma vaccine adjuvant in brain glioma in situ, 44 female C57BL/6 mice were split into four groups (n=11), i.c. injected with 2×10^4 GL261 cells on Day 0 and then i.m. injected with TCL, TCL+TIO3, TCL+cODN or PBS on Days 1, 8, 15 and 22. The mice were monitored daily. In each group, 3 mice were euthanized on Day 18, and the brain tumor tissues were separated and fixed in formalin solution for 24 hours, embedded in paraffin, sectioned (4 μm), and stained with hematoxylin-eosin (H&E) for tumor histopathological analysis under a microscope.

2.11 Isolation of tumor-infiltrating immune cells

To isolate the infiltrating immune cells in brain glioma tissues, brain tissue pieces were mechanically processed using collagenase. To digest with collagenase, we first transferred glioma tissue pieces (1 g) to a P60 dish and then incubated them with collagenase IA (5 ml, 100 U/ml in RPMI) at 37°C for 30 minutes. A 5 ml pipette was used to resuspend the sample, which was then transferred into a 15 ml tube and incubated for another 30 minutes at 37°C (24). This step was repeated twice. Thereafter, we filtered the cell suspension by passing it through a 40 μm cell strainer. After centrifugation at 300 g for 10 min at 4 °C, the pellet was resuspended in 4 mL of 30% Percoll (GE) and overlaid on the top of a gradient containing 3.5 mL of 37% and 3.5 mL of 70% Percoll solution (21). Percoll was diluted with Hanks' balanced salt solution (HBSS) (Bio-Whittaker). The gradient was centrifuged at 500 × g for 20 min at 4 °C; cells were collected from the 37% to 70%

interface (approximately 5 mL) and then washed once with HBSS containing 10% fetal bovine serum (FBS). After being washed twice with PBS buffer (containing 20 mL/L FBS), the cells were stained with PE-conjugated CD274 (PD-L1, MIH5) or APC-conjugated Ly6G (560599) mAb for 30 min on ice in the dark. After washing twice, the cells were counted and processed for viability staining and flow cytometric analysis.

2.12 Western blotting

To explore the effect of TIO3 on TGF-β2 and PD-L1 expression in glioma tissues, we first i.m. injected the mice with TCL, TCL+TIO3, TCL+cODN, or PBS on Days 0 and Day 10 and then challenged the mice with GL261 cells intracranially on Day 14. On Day 28, the glioma was isolated from the mice and used to assess TGF-β2 and PD-L1 expression by western blotting. The glioma cells were cut into small pieces by scissors first and then digested with collagenase for 1 h. After centrifugation, the cells were lysed with ice-cold radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing the protease inhibitor PMSF at 4°C for 30 min. Then, the cell lysates were centrifuged at 14,500 × g for 10 min at 4°C. The cell supernatant was collected and quantified with a BCA protein assay kit (Wanleibio, Shenyang, China) followed by separation on 12% SDS –PAGE. After running the gel at 120 V for 1.5 h, we transferred the protein to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore USA) at 120 V for 1 h at 4°C. Then, the membranes were blocked with TBST containing 5% nonfat dried milk, shaken at room temperature (22-25°C) for 2 h, and probed with an anti-TGF-β2 antibody (AB-112-NA, R&D Systems, USA), anti-PD-L1 antibody (PA5-20343, Invitrogen) or anti-GAPDH antibody (60004-1-Ig, Proteintech) overnight at 4°C. After being washed with TBST [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.5% Tween 20] twice for 10 min intervals, the membranes were incubated with the secondary antibody HRP-conjugated anti-goat IgG (HAF017, R&D Systems, USA) at 25°C for 1 h. After washing three times, substrate was added to the membrane, and the target protein bands were visualized by Hyperfilm ECL (Amersham Biosciences). The intensity of the immunoreactive bands was determined by a densitometric analysis program (Image Gauge V3.12; Fuji Photo Film, Tokyo, Japan).

2.13 Statistical analysis

A Student's 2-tailed unpaired t test was used to determine the statistical significance of differences between two groups. For multiple sets of data analysis, 1- or 2-way ANOVA was used, followed by Scheffé's *post hoc* test. The Kaplan–Meier method was used to evaluate the survival curve of mice, and the log-rank test was used for comparison. SPSS 19.0 computer software was used for statistical analysis. The data come from four or more independent experimental groups. Data are expressed as the mean ± SEM. Differences in tumor size among the various groups were determined by repeated-measures analysis of variance (ANOVA).

3 Results

3.1 Regulatory effect of TIO3 on the expression of TGF-β2 and MHC-I in glioma cells

Advanced glioma overproduces TGF-β2, whose autocrine and paracrine actions promote tumor growth, invasion, and metastasis. In this study, we used different types of antigens to induce tumor cells to secrete TGF-β2 and observed the inhibitory effect of TIO3. As expected, TIO3, a specific TGF-β2 inhibitory oligodeoxynucleotide designed in our lab (12), significantly suppressed TGF-β2 mRNA and surface-bound TGF-β2 (sTGF-β2) expression in both GL261 (murine) and U251 (human) glioma cell lines ($p<0.05$) (Figures 1A, B, E-F). Moreover, the results also showed that TIO3 significantly upregulated the mRNA and protein expression of MHC I molecules on the surface of GL261 and U251 cells ($p<0.05$) (Figures 1 C, D, G, H), which may result in an enhancement of the antitumor immune responses.

3.2 Cellular uptake of TIO3 into murine immune cells

To observe whether TIO3 could be taken up into T cells and neutrophils, murine drainage lymph node (DLN) cells were isolated and incubated with Cy3- or Alexa-488-labeled TIO3 for 24 h and then stained with FITC-labeled mAbs against CD4, CD8, or V450-labeled mAbs against Ly6G, followed by counterstaining with DAPI, a blue-fluorescent DNA dye for staining the nucleus. Confocal microscopy revealed that both TIO3 and cODN could enter $CD4^+$ T cells and $CD8^+$ T cells, but TIO3 had a higher efficacy than cODN in entering these cells (Figures 2A, B). Similarly, TIO3 was also taken up by $Ly6G^+$ immune cells (Figure 2C), which may result in the inhibition of neutrophil-dependent production of TGF-β2. In addition, TIO3 was confirmed to be ingested by B cells in our previous study (Figure S1), facilitating the activation of B cells (12).

3.3 Prophylactic protective effect and long-term immune memory of TIO3+TCL in a murine glioma *in situ* model.

To explore whether TIO3 could be formulated as an adjuvant with glioma TCL to induce antitumor immunity, mice were immunized twice with PBS, TCL, TCL+TIO3 or TCL+cODN (TCL plus control ODN) on Day 0 and Day 10. On Day 14, the mice were intracranially inoculated with GL261 cells. We found that the tumor-bearing mice treated with TCL or PBS exhibited symptoms, including weight loss, hunched back, irregular breathing, prostration, paresis, and convulsions. More excitingly, none of the eight mice in the TCL +TIO3 group died within 180 days (Figures 3A, B). The results showed that TIO3 exhibited a potent adjuvant effect and facilitates TCL to induce antitumor immunity against glioma in a prophylactic mouse model.

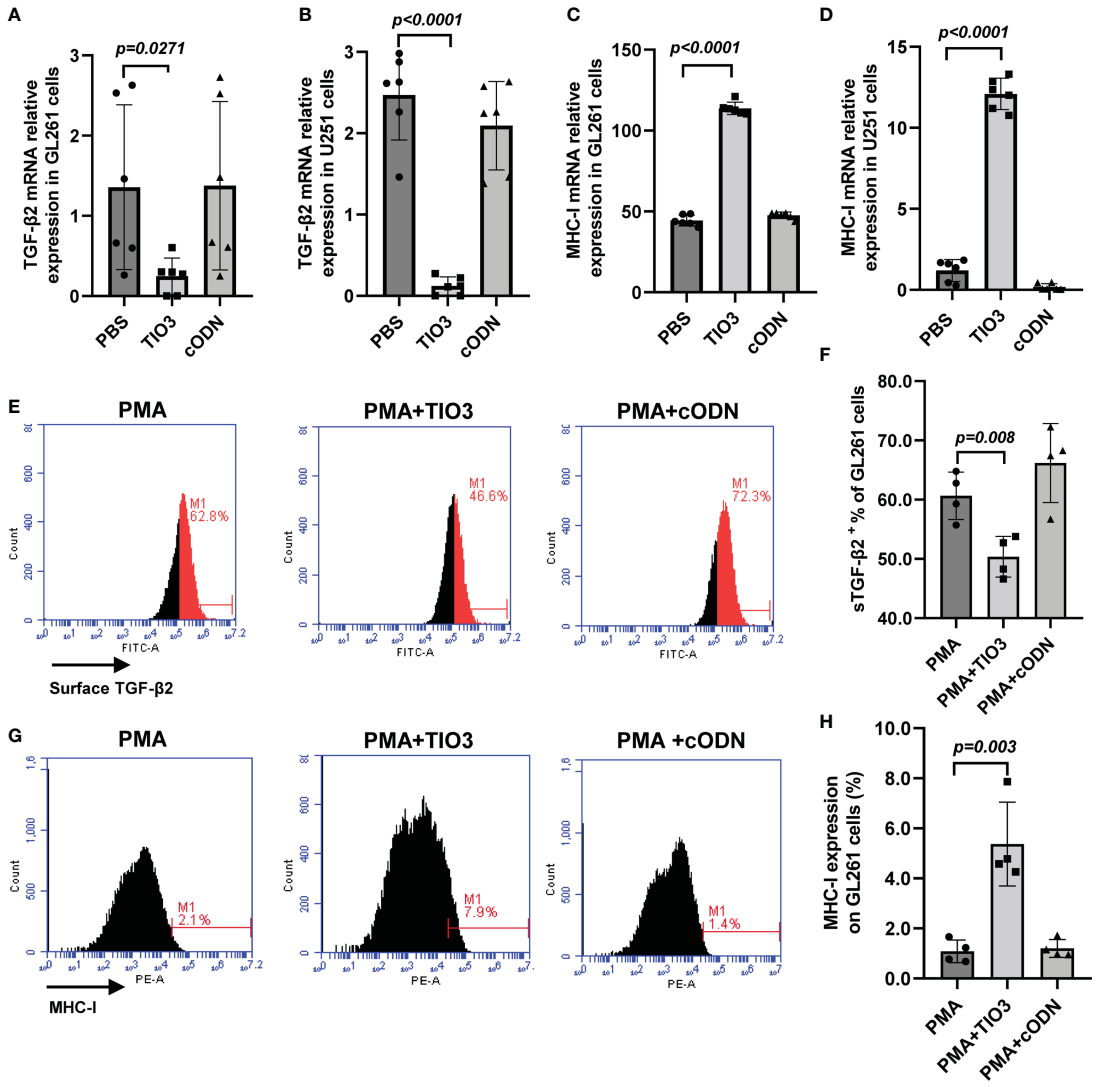

FIGURE 1
Effect of TIO3 on the expression of TGF-β2 and MHC-I in cultured GL261 cells or U-251 cells. GL261 or U-251 cells were cultured with TCL, TCL+TIO3 (10 μg/mL) or TCL+cODN (control ODN, 10 μg/mL) for 24 h, respectively, and the mRNA expression of TGF-β2 **(A, B)** and MHC I **(C, D)** were measured by qRT-PCR. In addition, GL261 cells were cultured with PMA (100 μg/mL), PMA+TIO3 or PMA+cODN for 48 h, and the protein expression of TGF-β2 **(E, F)** and MHC I **(G, H)** were detected by flow cytometry.

To observe the immune memory against glioma in TCL+TIO3-immunized mice, the eight surviving mice in the TCL+TIO3 group were rechallenged with intracranial transplantation of GL261 cells on Day 168 (Figure 3A). Surprisingly, 50% of the mice survived 110 days after glioma rechallenge, reflecting long-lasting immunity against tumors (Figure 3C). The data implied that TIO3 could assist TCL in generating tumor-specific immunologic memory.

3.4 Effect of TIO3 as a glioma vaccine adjuvant on the activation of $CD4^+$ and $CD8^+$ T cells

To explore whether TIO3 as an adjuvant coupled with the TCL vaccine (TCL+TIO3) could induce the generation of effective T cells in mice, C57BL/6 mice were intramuscularly injected with TCL, TCL +TIO3 or TCL+cODN or PBS on Day 0 and Day 10. One day after the second immunization, the lymphocytes were isolated, and the percentage and activation of T cells were immediately determined by flow cytometry (Figure 4A). The results showed that TIO3 significantly facilitated the ability of TCL to increase the percentages of $CD4^+$ and $CD8^+$ T cells in lymphocytes (Figures 4B, C). T-cell activation is vital to antitumor immune responses, and therefore the expression of inhibition and activation markers on T cells was observed in this study. As an inhibitory signal of antitumor immunity, the expression of sTGF-β2 on $CD4^+$ and $CD8^+$ T cells in the TCL+TIO3 group was significantly decreased compared with that in the TCL group (Figures 4D, E). IFN-γ secreted by activated T cells can kill tumor cells. As expected, the IFN-γ secretion frequencies of $CD4^+$ and $CD8^+$ T cells in the TCL+TIO3 group were higher than those in the other groups (Figures 4F, G). These results indicated that TIO3 could facilitate TCL's induction of the activation of T cells.

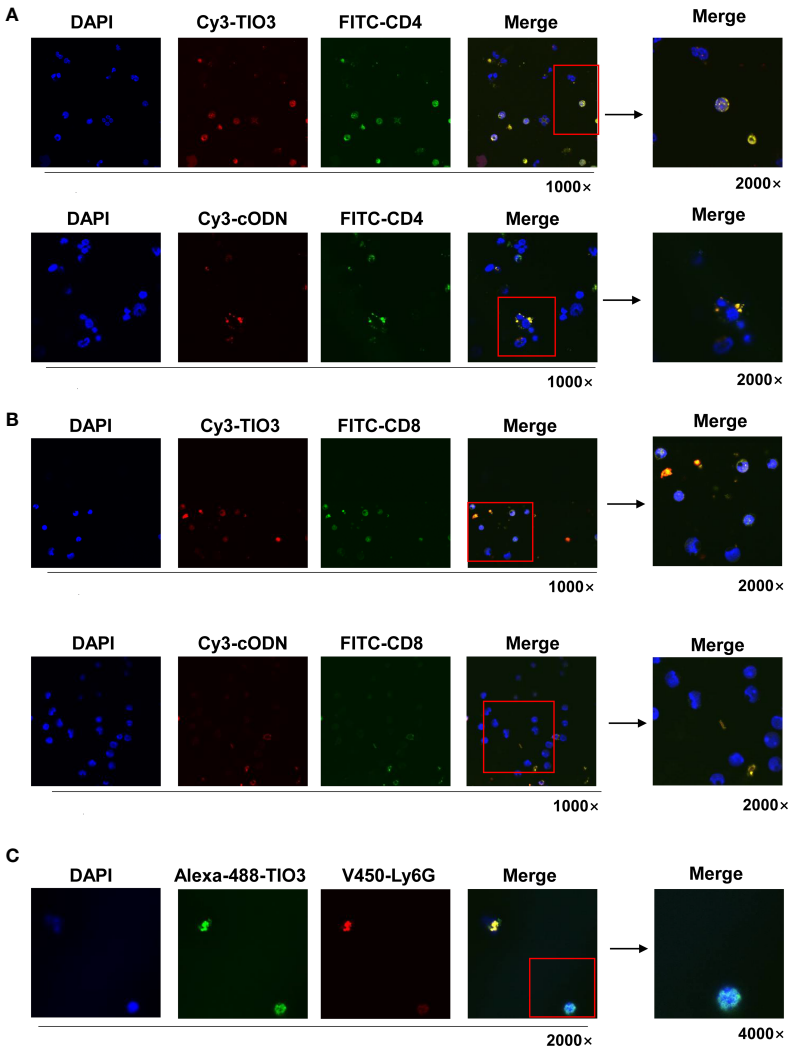

FIGURE 2
The TIO3 distribution in CD4$^+$ T and CD8$^+$ T cells. The lymph node (LN) cells from naive mice (n = 4) were incubated with red-fluorescent Cy3-TIO3 or Cy3-cODN or with green-fluorescent Alexa-488-TIO3 for 24 h, then stained with green-fluorescent (FITC-labeled) mAbs against CD4 **(A)** or CD8 **(B)** or red-fluorescent (V450-labeled) mAb against ly6G **(C)**, respectively, followed by counterstaining with DAPI, a blue fluorescent DNA dye for staining the nucleus. The resultant cells were observed under the confocal microscope. (Magnification, × 1000, × 2000, × 4000).

3.5 Effect of TCL+TIO3 on the activation of cytotoxic T lymphocytes and NK cells in glioma-bearing mice

To further explore whether TCL+TIO3 also activated specific cytotoxic T lymphocytes (CTLs) in glioma-bearing mice, mice were intramuscularly injected with TCL, TCL+TIO3, TCL+cODN or PBS in the neck on Days 0 and Days 10, and then the mice were i.c. injected with 2×10^4 GL261 cells in the caudate nucleus. On Day 28, the mice were sacrificed to isolate lymphocytes and analyze T-cell activation, and TGF-β2 expression in glioma tissue was also observed (Figure 5A). Consistent with the TGF-β2 expression pattern in lymphocytes, western blotting analysis demonstrated that TCL +TIO3 also significantly downregulated TGF-β2 expression in glioma tissue (Figure 5B). Similarly, our results showed that TCL +TIO3 obviously increased the percentage of CD4$^+$ and CD8$^+$ T cells in the lymphocytes of glioma-bearing mice (Figures 5C, D) compared with the TCL and PBS groups (p *<0.001*). To verify the effect on cytotoxic T cells induced by TCL+TIO3, splenocytes from each group were isolated and cocultured with GL261 cells for 8 h at effector/target ratios of 100:1, 50:1 and 25:1. The results clearly revealed that TCL +TIO3 induced a stronger cytotoxicity against GL261 tumor cells than TCL and PBS *in vitro* (p *<0.001*) (Figure 5F), suggesting that TCL+TIO3 could induce significant specific CTLs in glioma-bearing mice. Moreover, we also detected the IFN-γ production of lymphocytes isolated from the mice immunized with TCL+TIO3. The results showed that TCL+TIO3 induced more IFN-γ production than TCL or PBS (Figure S2). The IFN-γ production represents that specific CTLs and non-specific NK cell activity.

In addition to T cells, NK cells also play a major role in antitumor immune responses. Thus, the ratio and activation of NK cells in lymphocytes from the immunized mice were also determined by flow cytometry analysis. The results revealed that the ratio of NK cells in the spleen was significantly upregulated by TCL+TIO3 compared with PBS or TCL (p*=0.0004*) (Figure 5E). NKG2D and CD69 are activating immune markers expressed by NK cells (25). Our results

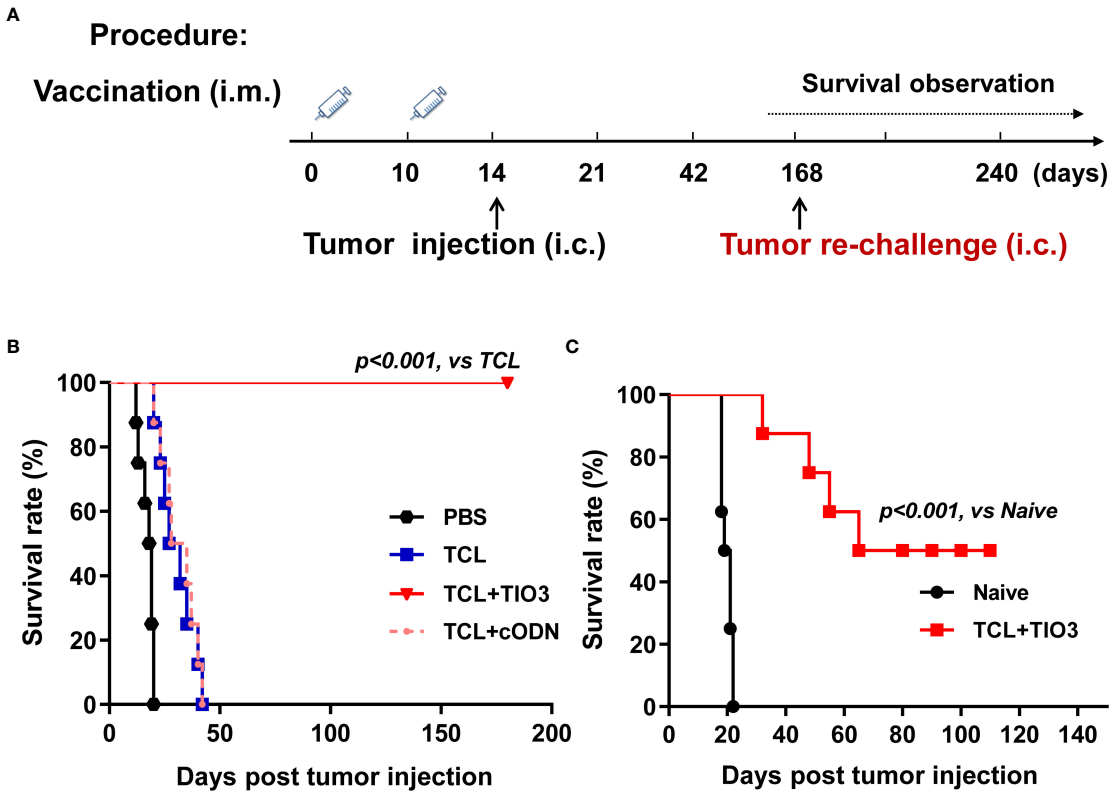

FIGURE 3
Prophylactic protective effect and anti-tumor immune memory of TIO3 as vaccine adjuvant on the *in-situ* glioma model in mice. **(A)** The experimental procedure: C57BL/6 mice (n=8) were intramuscularly (i.m.) immunized with PBS, TCL, TCL+TIO3 or TCL+cODN twice on day 0 and day 10, and then were challenged intracranially (i.c.) inoculated with 2×10^4 GL261 cells on day 14. Then, the survived mice were re-challenged with 2×10^4 GL261 cells on day 168, naive mice (n=8) were intracranially inoculated with 2×10^4 GL261 cells; **(B)** The survival rate of GL261-bearing mice in different groups after the first tumor inoculation. **(C)** The survival of the mice after tumor re-challenge were observed for another 110 days.

showed that TCL+TIO3 induced higher ratios of $CD69^+$ or $NKG2D^+$ NK cells in lymphocytes than TCL ($p<0.01$) (Figures 5G, H), indicating the activation of NK cells.

3.6 Inhibitory effects of TCL+TIO3 on PD1 or PD-L1 expression in immune cells in immunized mice

PD1 is an inhibitory receptor that is expressed by immune effector cells, including T cells, B cells and NK cells. To explore whether TCL +TIO3 could affect PD1 expression in immune cells, C57BL/6 mice were immunized with PBS, TCL, TCL+TIO3 or TCL+cODN on Day 0 and Day 10, and 24 h after the second immunization, splenocytes isolated from the mice were assessed for the expression of PD1 on $CD4^+$ T, $CD8^+$ T and NK cells by flow cytometry analysis (Figure 6A). Our results showed that TCL+TIO3 significantly inhibited PD1 expression in the $CD4^+$ T, $CD8^+$ T and NK cells compared with TCL alone (Figures 6B-D).

Since the high expression of PD-L1 in tumor tissues or neutrophils enables the suppression of T-cell function (24, 26), we determined whether TIO3 could impact the expression of PD-L1 in tumor tissues and tumor-infiltrating neutrophils. Mice were immunized with TCL, TCL+TIO3, TCL+cODN or PBS twice and then challenged with GL261 cells. On Day 14 after glioma inoculation, glioma tissues and tumor-infiltrating neutrophils were isolated to assess PD-L1 expression by western blotting and flow cytometry, respectively (Figure 6E). The results demonstrated that TIO3 significantly decreased PD-L1 expression and the percentage of $Ly6G^+$ $PD\text{-}L1^+$ neutrophils in glioma tissues (Figures 6F-I). These data indicated that TIO3 was capable of downregulating PD1-PDL1 inhibitory signaling in multiple immune cells, resulting in more potent activation of antitumor immunity.

3.7 Protective effect of TCL+TIO3 against murine glioma on *in situ* therapeutic model mice

To explore whether the glioma vaccine TCL+TIO3 could prolong the survival of mice with glioma in an *in situ* therapeutic model, mice were injected with GL261 cells on Day 0 and then immunized four times with TCL, TCL+TIO3, TCL+cODN or PBS on Days 1, 8, 15 and 22. Interestingly, the survival results showed that TCL+TIO3 significantly prolonged the survival of mice bearing glioma, and no mice died within 40 days (Figures 7A, B), while all mice in the other groups died within 23 days. These data indicated that TCL+TIO3 also induced a significant protective effect against murine glioma in an *in situ* therapeutic model.

Furthermore, brain tissues were taken for histopathological examination on the 18th day post-injection. Although glioma cells

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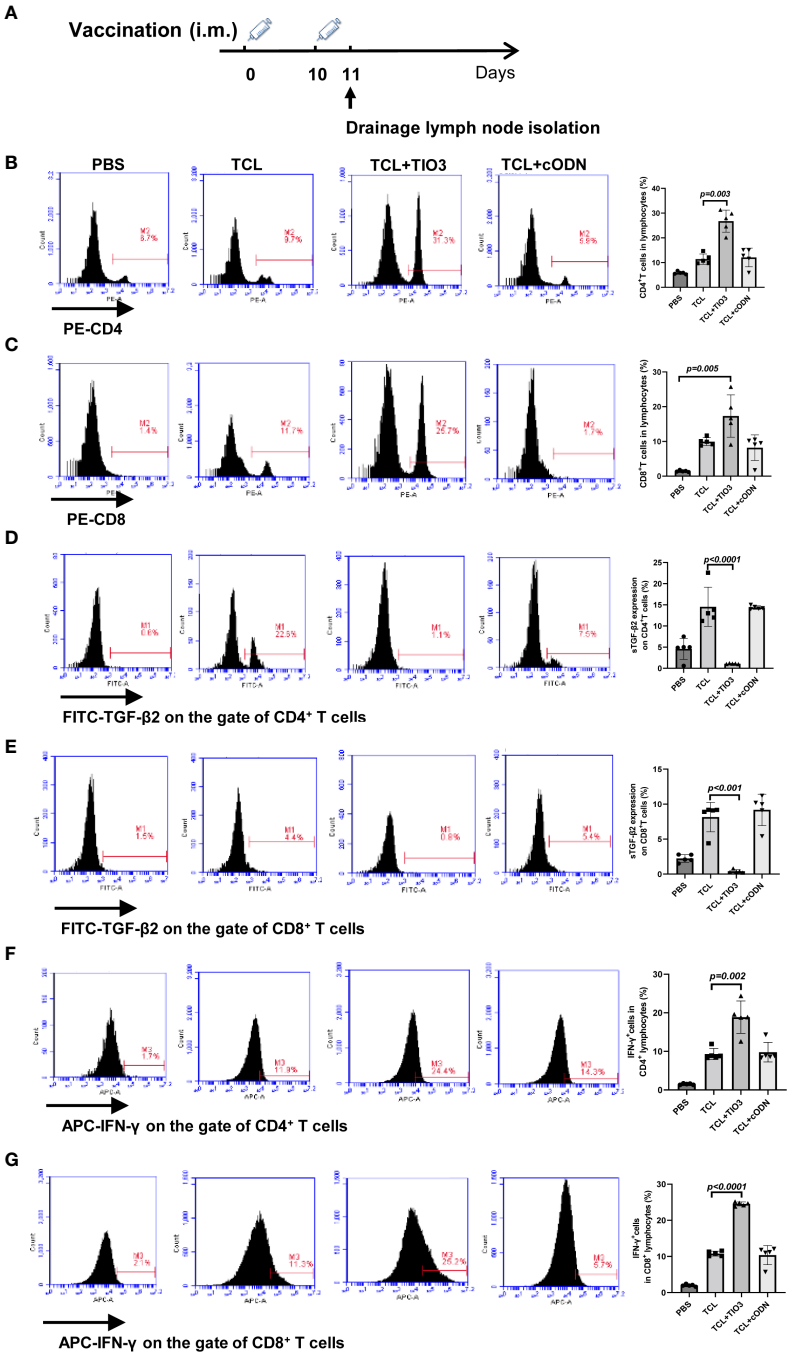

FIGURE 4
Effect of TIO3 as glioma vaccine adjuvant on the activation of T lymphocyte cells. C57BL/6 mice (n=6) were intramuscularly injected with PBS, TCL, TCL +TIO3, or TCL+cODN on day 0 and day 10, and then the lymphocytes from mouse drainage lymph nodes were isolated and analyzed by FACS. **(A)** Experimental procedure. **(B)** the percentage of $CD4^+$ T cells; **(C)** the percentage of $CD8^+$ T cells; **(D-G)** the expression percentage of sTGF-β2 and IFN-γ in $CD4^+$ T and $CD8^+$ T cells.

could be found in the mice of each group, the amount of tumor cells within the isolated tissue area of TCL+TIO3 mice was less than that of the PBS, TCL and TCL+cODN groups. The microscopy results demonstrated that the glioma tissue in those groups showed enlarged intercellular spaces, irregular arrangement, and uneven arrangement of intercellular substances. Moreover, in the TCL+TIO3 group, increased infiltration of immune cells appeared in the brain tumor tissue, which may be associated with the enhancement of anti-glioma immunity (Figure 7C). The above results suggested that TCL+TIO3 also induced massive immune cell infiltration into brain tissues to enhance antitumor immunity, which facilitated limiting the growth of tumors and prolonging the survival of glioma-bearing mice.

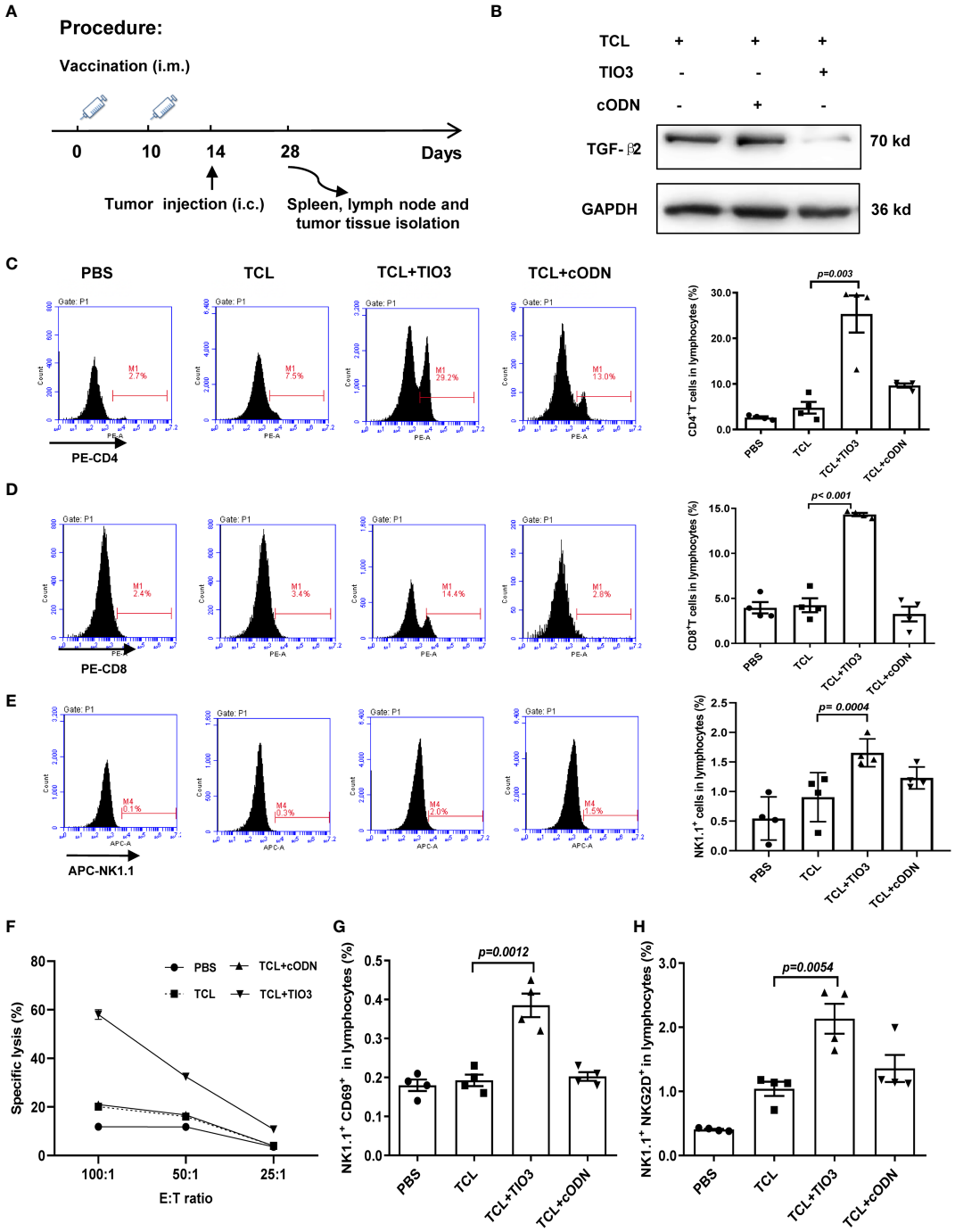

FIGURE 5
Effect of TCL+TIO3 on the activation of cytotoxic T lymphocytes and NK cell in glioma-bearing mice. The mice (n=4) were intramuscularly immunized with PBS, TCL, TCL+TIO3 and TCL + cODN in the neck on day 0 and 10, and then and then challenged i.c. with 2×10^4 GL261 cells on day 14. 4 mice were sacrificed on day 28. **(A)** Experimental procedure. **(B)** TGF-β2 expression in glioma tissue isolated from the mice on day 28 by western blotting. **(C-E)** Percentages of $CD4^+$ T, $CD8^+$ T and NK cells in lymph nodes. **(F)** Glioma-specific CTL detection. Splenocytes were co-cultured with GL261 cells for 8 h at effector/target ratios of 100:1, 50:1 and 25:1, and methylthiazolyldiphenyl-tetrazolium bromide assays was performed for the cytotoxicity. **(G-H)** The expression of CD69 and NKG2D on NK cells in lymphocytes. TIO3 plus TCL *versus* TCL.

4 Discussion

TGF-β2 inhibitors, including monoclonal antibodies, small molecules, and antisense oligonucleotides, have been used alone to treat tumors. However, little is known about their use as tumor vaccine adjuvants. In our previous study, a self-designed TGF-β2 inhibitory oligodeoxynucleotide, TIO3, was used as an adjuvant that was formulated with multiple microbial vaccines to induce strong and persistent antibody responses (12). Therefore, it is worthwhile to investigate whether TIO3 can also be used as a tumor vaccine adjuvant. In this study, we found that TIO3 is capable of being used as an effective vaccine adjuvant coupled with glioma TCL (TCL +TIO3) to induce potent antitumor immune responses and prolong the survival of glioma-bearing mice in prophylactic and therapeutic *in*

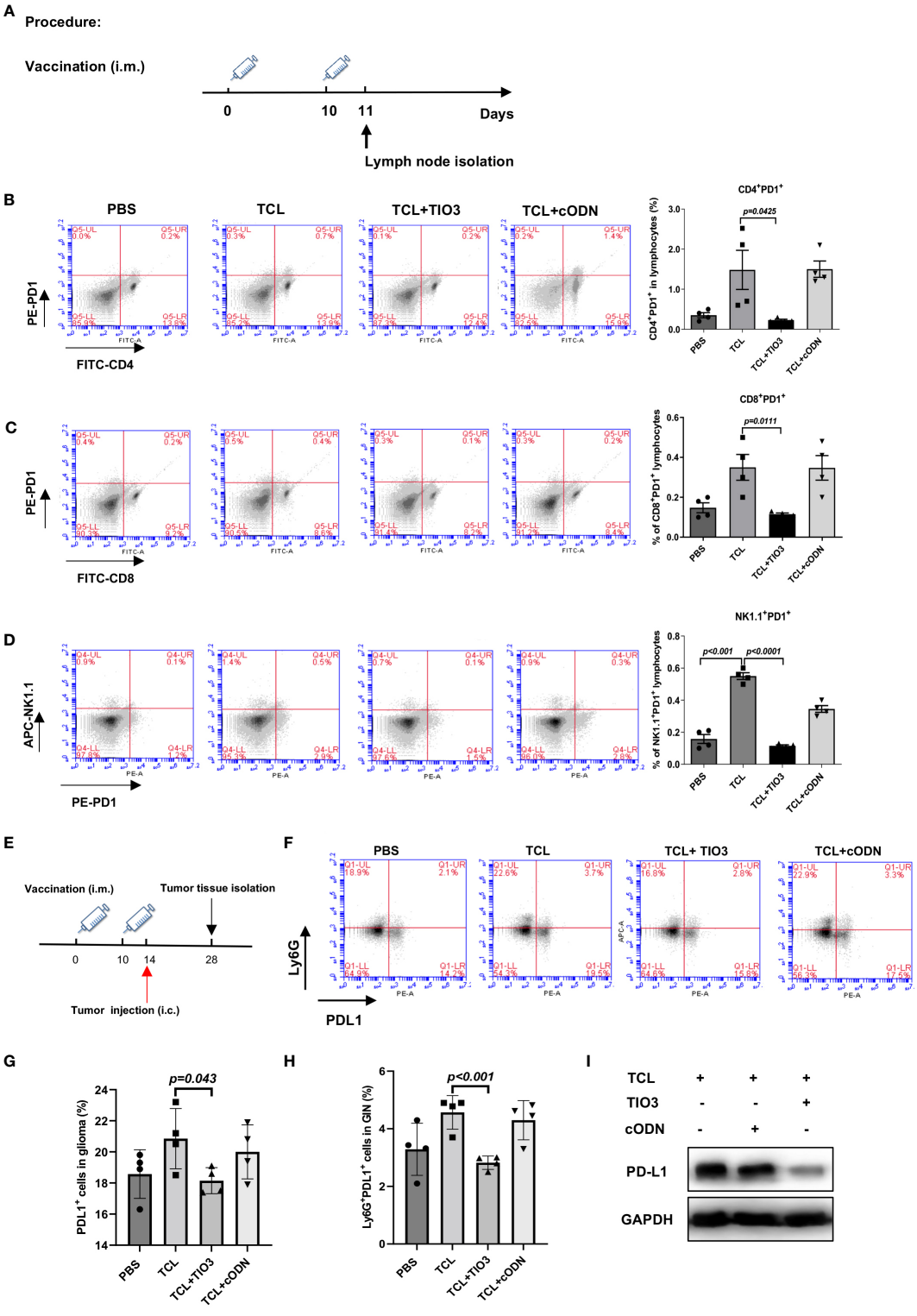

FIGURE 6
Inhibitory effects of TIO3 as glioma vaccine adjuvant on the expression of PD1 and PD-L1 on immune cells and tumor tissues in mice. **(A)** C57BL/6 mice (n = 4) were intramuscularly injected in the neck with PBS, TCL, TCL+TIO3 or TCL+cODN on day 0 and day 10, and 24h after the second immunization, the lymphocytes isolated from the mice were detected for the expression of PD1 on $CD4^+$ T cells **(B)**, $CD8^+$ T cells **(C)**, NK cells **(D)**. **(E)** The mice (n=4) were intramuscularly immunized with PBS, TCL, TCL+TIO3 and TCL + cODN in the neck on day 0 and 10, and then and then challenged i.c. with 2×10^4 GL261 cells on day 14, 4 mice were sacrificed on day 28. **(F-H)** Percentage of $PDL1^+$ cells in glioma tissue and glioma infiltrating neutrophils ($Ly6G^+$) (GIN). **(I)** PD-L1 expression in glioma tissue isolated from the mice on day 28 by western blotting.

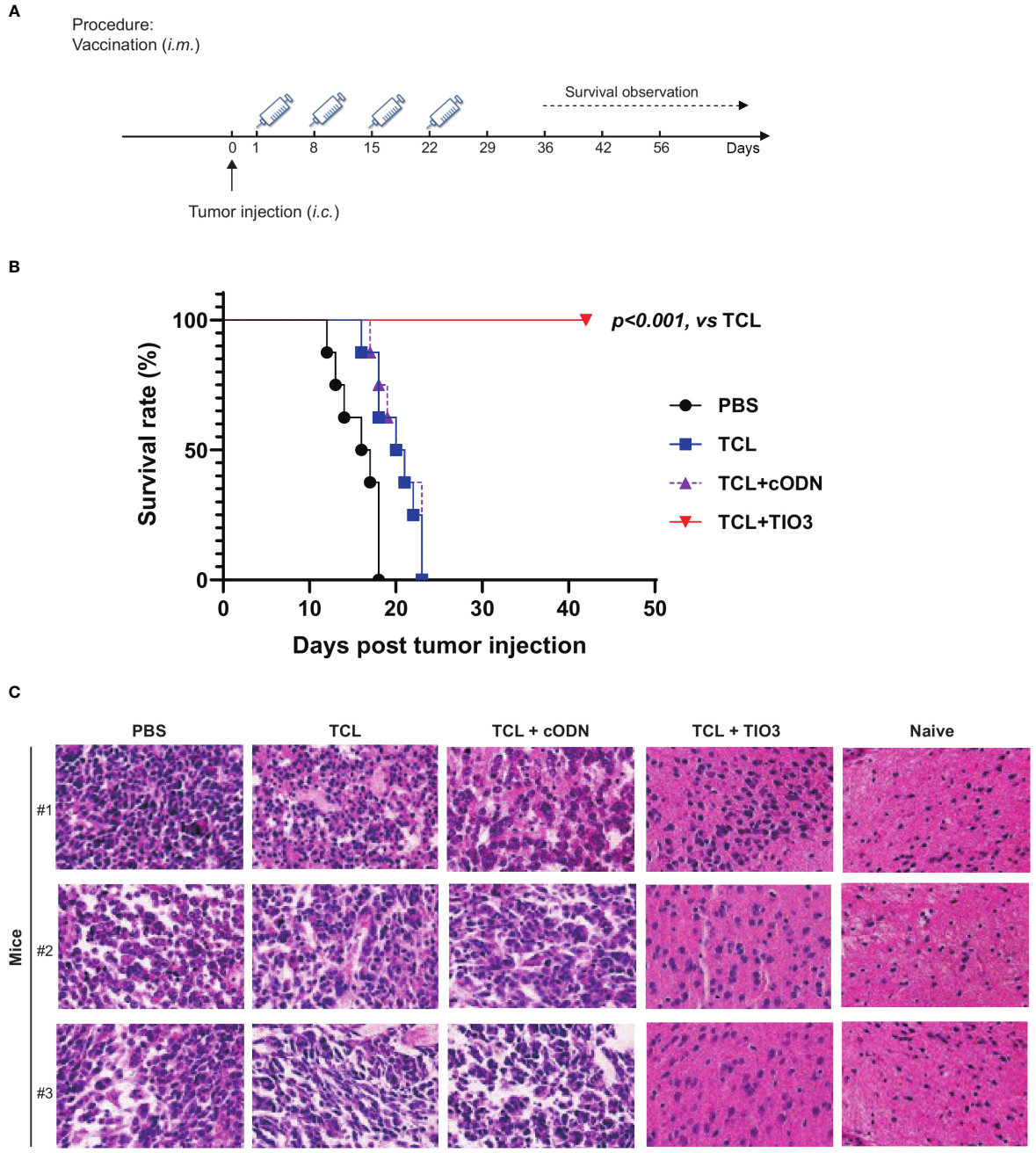

FIGURE 7
Therapeutic protective effect of TIO3 as vaccine adjuvant on the *in-situ* glioma model in mice. TIO3 as a glioma vaccine molecular adjuvant combined with GL261 TCL to treat GL261-bearing mice. **(A)** The experimental procedure: C57BL/6 mice (n=8) were intracranial (i.c.) inoculated with 2×10^4 GL261 cells in right caudate nucleus of the brains on day 0, and then were i.m. immunized in the neck with PBS, TCL, TCL+TIO3 or TCL+cODN on day 1, 8, 15 and day 22. **(B)** The survival of GL261-bearing mice. **(C)** Brain tissues were taken for histopathological examination with HE staining analysis on the 18th day. The three mice received PBS died within 18 days after the GL261 cell inoculation, and their brain samples were analyzed immediately after death. (Magnification, ×400).

situ models. In particular, TCL+TIO3 not only induced the activation of $CD4^+$ T, $CD8^+$ T and NK cells but also inhibited PD1 and PD-L1 expression in multiple immune cells and glioma tissues by suppressing the expression of TGF-β2.

TIO3 could significantly inhibit the expression of TGF-β2 in various cells. For example, TIO3 formulated in microbial vaccines dramatically reduced surface-bound TGF-β2 expression on $CD19^+$ B cells in splenocytes, resulting in an elevation of IgG levels (12). In this study, we demonstrated that TIO3 also broadly inhibits sTGF-β2 expression in tumor cell lines and glioma tissues, as well as in antitumor effector $CD4^+$ and $CD8^+$ T cells. TIO3 has also been confirmed capable of entering lung tumor tissues and tumor cells *in vitro* and *in vivo* (27). Our previous study revealed that the percentages of $Cy3^+$ cells reached 100% of the cultured LLC cells at 24 h after Cy3-TIO3 addition, and 50% of tumor tissue cells from tumor-bearing mice at 24 h post i.p. injection of Cy3-TIO3. Thus, we

think the actual targets of TIO3 are T cells and tumor cells in tumor immunotherapy. As reported, TGF-β2 can negatively regulate the expression of HLA-DR antigen on the surface of human malignant glioma cells. Moreover, TGF-β2 is sufficient to downregulate the surface expression of MHC molecules and may enhance the ability of tumor cells to evade immune responses (28). Thus, TGF-β2 has also been named glioblastoma-derived T-cell suppressor factor, a central molecule maintaining the malignant phenotype of glioblastoma (29). As expected, TIO3 significantly upregulated the mRNA and protein expression of MHC I molecules in GL261 and U251 cells. The upregulation of MHC-I molecules that present tumor antigens could promote the lysis of tumor cells by cytotoxic T lymphocytes. In addition, TIO3 also upregulated the expression of CD40, CD80, CD86, and MHC II molecules on $CD11c^+$ dendritic cells (12), facilitating antigen presentation to induce cytotoxic effects against microbes and tumors.

TIO3 is a short, single-stranded ODN, usually, ODNs enter cells *via* endocytosis (30). Also, it could be a ligand for TLR9. So, we determined whether TIO3 could activate TLR9 signals. In our previous study (27), there were no changes of TLR9 expression between the TIO3 and PBS groups. Moreover, the TIO3 didn't affect the mRNA expression of the expression of downstream molecules of TLR9 signals, such as the IRF5, TNF-α an IFN-α. Thus, we think TIO3 doesn't bind to TLR9 and stimulate innate immune response. Noticeably, the TIO3 was specially designed for the treatment of gliomas, and it was confirmed that it specifically inhibited the mRNA expression of TGF-β2 but not the expression of TGF-β1 and TGF-β3 (12). This is because the TIO3 complementary sequence is 100% identical to the original mRNA sequence of TGF-β2, while the mRNA sequence of TGF-β1 and TGF-β3 just have 63% and 57.89% similarity, respectively. TGF-β2 triggers signaling in cells *via* binding to a TGF-β receptor complex composed of two type I TGF-β receptors and two type II TGF-β receptors. Then, these receptors recruit and phosphorylate receptor regulated Smad2, Smad3 and other Smads to forms a heterodimeric complex. This complex then translocates into the cell nucleus where it binds with nuclear co-factors to regulate the transcription of various target genes involved the cancer progression [7]. Our result showed that TIO3 reduced the phosphorylation level of Smad3 in GL261 cells, compared to the PBS and cODN (Figure S3). This result is consistent with the results of TGF-β2 inhibition and tumor recession.

T cells and NK cells are both cytotoxic effector cells of the immune system against tumors. We found that TCL+TIO3 induced the activation of $CD8^+$ T cells, which resulted in the secretion of IFN-γ, which can kill tumor cells directly. In addition to the cytokine effect, $CD8^+$ T cells can release cytotoxic granules and activate the Fas/FasL pathway, resulting in tumor cell apoptosis (31). Moreover, the cytotoxic effect confirmed that TCL+TIO3 induced significant specific CTLs in glioma-bearing mice. Indeed, specific $CD4^+$ T-cell responses are also closely related to the tumor-killing effect (31). In particular, most tumor neoantigens are recognized by $CD4^+$ T cells and not $CD8^+$ T cells (32). The reason may be that MHC class II molecules are not constitutively expressed in most tumor cells, and therefore it is necessary for DCs to present tumor neoantigens and activate tumor-specific $CD4^+$ T cells. Thus, tumor vaccines prepared with new epitopes of $CD4^+$ T cells can effectively control the development of advanced tumors. TCL contains many neoantigens, and immunization of mice with new epitopes restricted by immunodominant MHC-II molecules can trigger strong tumor-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses (33, 34). Our data also confirmed that TIO3 exerts an antiglioma effect by promoting the activation of $CD4^+$ T cells. In addition, NK cells play an important role in innate defenses against glioma growth. NK cells not only kill tumor cells but also induce tumor cells to change the expression of HLA-I, PD-L1, or NKG2D-L and modulate their susceptibility to the immune response (35). In this study, TCL+TIO3 induced an increased population of NK cells and higher ratios of $CD69^+$ or $NKG2D^+$ NK cells in splenocytes compared with those of the TCL group, resulting in stronger cytotoxic activities against glioma.

In this study, a massive number of glioma-specific lymphocytes were induced in the periphery by TCL+TIO3. Generally, lymphocytes are recruited from the blood circulation to the tumor site and infiltrate the tumor mass, such as melanoma and colorectal and ovarian carcinoma. Tumor-infiltrating lymphocytes (TILs) are considered to be important antitumor effector cells, and the accumulation of activated lymphocytes at tumor sites is considered a good indicator of the regulation of tumor development and growth. As reported, TILs are mainly composed of T cells, B cells, and NK cells (36). However, it is difficult for lymphocytes to infiltrate glioma tissues through the blood–brain barrier (BBB). The presence of the BBB strictly restricts the movement of immune cells and soluble mediators from the periphery to the central nervous system. However, TILs have been detected in glioma patients (37). Glioma is a very aggressive tumor that is characterized by extensive proliferation and migration. Uncontrolled and fast growth of glioma can lead to the disruption of chimeric and fragile vessels, resulting in damage to the BBB. Therefore, lymphocytes can enter the brain through the damaged BBB to contain and kill glioma cells. Additionally, lymphocytes can enter the perivascular or mesenteric space from the endothelial cell wall of the blood–brain barrier *via* multiple adhesion molecules and signaling molecules and then cross the boundary membrane of glial cells and enter the central nervous system (38). Our histopathological results showed increased TILs in brain glioma tissues in the TCL+TIO3 group, which may be associated with the enhancement of antiglioma immunity. In line with these results, glioma patients after surgery were administered an autologous glioma TCL extracranially, which induced the massive infiltration of T lymphocytes into the brain tissues from the periphery, facilitating the clearance of the remaining glioma cells and preventing the recurrence of gliomas (34).

In addition to the activation of antitumor effector cells, TIO3 also reduced the expression of PD1 on $CD4^+$ T cells, $CD8^+$ T cells and NK cells and inhibited PD-L1 expression in glioma tissues and tumor-infiltrating neutrophils. Immune checkpoint signals such as PD1 and PD-L1 are key modulators of the antitumor T-cell immune response. In a tumor state, the interaction of PD-L1 on tumor cells with PD1 on T cells can inhibit the cytotoxic ability of T cells against tumor cells (39, and PD-L1 expression on neutrophils also enables the suppression of T-cell function. Moreover, TGF-β promotes PD1-PDL1 signaling in tumor immune escape. TGF-β can upregulate the expression of PD1 and CTLA-4 on T lymphocytes and attenuate the

cytotoxicity of T lymphocytes toward tumor cells *in vitro* and *in vivo* (40). Additionally, TGF-β present in the tumor microenvironment orchestrates tumor cell expression of the PD-L1 molecule, and therefore the PD-L1 level is highly correlated with the tumor TGF-β level (41). Thus, immunotherapy with inhibitors of PD1 or PD-L1 appears to prevent the tumor from evading the immune system in this way (39). Similarly, switching to a new strategy that inhibits TGF-β2 expression could reduce PD1 and PD-L1 production. As shown by the results of this study, TIO3 had a synergistic effect on the inhibition of PD1 expression in immune cells and PD-L1 expression in glioma cells by interfering with the expression of TGF-β2, resulting in more potent activation of antitumor immunity.

Overall, TIO3 as a vaccine adjuvant activated antitumor effector T cells and NK cells by inhibiting the immunosuppressive cytokine TGF-β2 and PD1 and PD-L1 expression on immune cells and tumor cells. Importantly, TIO3 formulated with TCL effectively prolonged the survival of glioma-bearing mice in both *in situ* prophylactic and therapeutic models. Interestingly, we found that TIO3 also had a monotherapeutic effect on the xenograft and *in situ* therapeutic mouse models of glioma (Figures S4, S5), but the survival dates of mice were shorter than those with TCL+TIO3 immunization. This result suggests that TIO3 formulated with glioma cell lysate as a new type of glioma vaccine is a more promising immunotherapy strategy against glioma. Particularly, the preventive strategy of TCL+TIO3 in tumor immunotherapy induced a strong tumor-specific immunologic memory. In fact, the preventive tumor immunotherapy at early stage of tumor development or for preventing tumor recurrence could induce the formation of a tumor microenvironment (TME), which is conducive to the DC activation as well as the recruitment and activation of the effector immune cells, particularly $CD8^+$ T cells and NK cells (27). Therefore, it has great potential application value for immunizing patients with TIO3 formulated with autologous TCL derived from excised glioma tissues, with the expectation of preventing the recurrence of glioma after surgery.

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 ethics committee of the College of Basic Medical Sciences of Jilin University.

Author contributions

MY and YLY contributed to the conception and design of the study and wrote the manuscript. LT, XS, LY performed experiments, analyzed data, and performed the statistical analysis. ZW, YPY, LW, XZ and PZ contributed to manuscript revision, read and approved the submitted version. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1013342/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The TIO3 distribution in B cells. The lymphocytes and spleen cells from naive mice (n = 4) were incubated with green-fluorescent Alexa-488-TIO3 or Alexa-488-cODN for 24 h, then stained with red-fluorescent (PE-labeled) mAbs against CD19, respectively, followed by counterstaining with DAPI, a blue fluorescent DNA dye for staining the nucleus. The resultant cells were observed under the confocal microscope (Scale bar represents 50 μm).

SUPPLEMENTARY FIGURE 2
The IFN-γ production of the cultured lymphocyte from the mice immunized with TCL+TIO3. The lymphocytes were isolated from draining lymph nodes of mice in PBS, TCL, TCL+TIO3 or TCL+cODN groups, and co-cultured with GL261 cells *in vitro*. 48h later, we collected the supernatant from the medium and detected the expression of IFN-γ by ELISA.

SUPPLEMENTARY FIGURE 3
Western blot analysis of the expression of p-Smad3 in each group. GL261 cells was cultured with PBS, TIO3 (10 μg/mL) or cODN (control ODN) for 24 h, respectively, and the phosphorylation of SMAD3 protein was measured by western blot.

SUPPLEMENTARY FIGURE 4
Protective effect of TIO3 as a therapeutic agent on the xenograft therapeutic mouse model. We subcutaneously inoculated mice with 5×10^5 GL261 cells, then PBS or TCL+TIO3 were intraperitoneally immunized four times with TIO3 or PBS on Days 1, 8, 15 and 22. Tumor growth **(A)** and survival **(B)** of the mice were monitored throughout the procedure.

SUPPLEMENTARY FIGURE 5

Protective effect of TIO3 as a therapeutic agent on the *in situ* therapeutic mouse model. **(A)** The experimental procedure: C57BL/6 mice (n =6) were intracranially (i.c.) inoculated with $2x10^4$ GL261 cells on day 0, and then were intraperitoneal injection (i.p.) with TIO3 or PBS on day 1, day 3, day 5 and day 7. **(B)** The survival rate of GL261-bearing mice was recorded.

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Neoadjuvant immune checkpoint inhibition in the management of glioblastoma: Exploring a new frontier

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Brain tumors are one of the leading causes of cancer related death in both the adult and pediatric patient population. Gliomas represent a cohort of brain tumors derived from glial cell lineages which include astrocytomas, oligodendrogliomas and glioblastomas (GBMs). These tumors are known to grow aggressively and have a high lethality with GBM being the most aggressive tumor in this group. Currently, few treatment options exist for GBM outside of surgical resection, radiation therapy and chemotherapy. While these measures have been shown to marginally improve patient survival, patients, especially those diagnosed with GBM, often experience a recurrence of their disease. Following disease recurrence, treatment options become more limited as additional surgical resections can pose life threatening risk to the patient, patients may be ineligible for additional radiation, and the recurrent tumor may be resistant to chemotherapy. Immune checkpoint inhibitors (ICIs) have revolutionized the field of cancer immunotherapy as many patients with cancers residing outside the central nervous system (CNS) have experienced a survival benefit from this treatment modality. It has often been observed that this survival benefit is increased following neoadjuvant administration of immune checkpoint inhibitors as tumor antigen is still present in the patient which enables a more robust anti-tumor immune response. Interestingly, results for ICI-based studies for patients with GBM have been largely disappointing which is a stark contrast from the success this treatment modality has had in non-central nervous system cancers. In this review, we will discuss the various benefits of neoadjuvant immune checkpoint inhibition such as how this approach reduces tumor burden and allows for a greater induction of an anti-tumor immune response. Additionally, we will discuss several non-CNS cancers where neoadjuvant immune checkpoint inhibition has been successful and discuss why we believe this approach may provide a survival benefit for GBM patients. We hope this manuscript will foster future studies aimed at exploring whether this approach may be beneficial for patients diagnosed with GBM.

KEYWORDS

glioblastoma, GBM, immunotherapy, ICI, neoadjuvant, brain

Introduction

Brain tumors are now the leading cause of cancer related death in males aged 39 years and below, and females aged 19 years and below (1). Gliomas are one of the drivers of brain tumor mortality as these tumors are known to grow aggressively and respond poorly to chemoradiation therapy (2). Gliomas arise from various glial cell types, whose normal function is to support neurons within the brain and include tumors defined as astrocytomas, oligodendrogliomas, and glioblastomas (GBMs) (2, 3). Oligodendrogliomas are characterized by 1p/19q chromosomal codeletion which is accompanied by an IDH mutation (4). The median survival time for those diagnosed with an oligodendroglioma is 10-12 years, with 5-year progression-free (PFS) and overall survival (OS) rates of 51-83% (5–8). IDH mutant astrocytomas, often do not boast as good of a prognosis as these tumors are known to grow more aggressively as compared to oligodendrogliomas (4). GBM which is classified as an IDH wildtype tumor is the most common glioma and associated with a dismal prognosis (4). The five-year survival for patients diagnosed with GBM aged 20-44, 45-54 and 55-64 is 22%, 9%, and 6% respectively (9). The current standard of care for patients diagnosed with GBM includes surgical resection, chemotherapy, and radiation therapy (10). However, these treatments often induce a host of negative side effects for patients and only marginally improve a patient's OS.

Multiple factors contribute to GBM having such a high lethality. The first is that GBMs typically grow in a diffuse manner and infiltrate the surrounding brain (11). This makes it nearly impossible for surgeons to completely resect the tumor as tumor cells reside beyond the main tumor bulk mass. These infiltrative tumor cells are difficult to visualize during surgery and are also difficult to be imaged by MRI. The inability to achieve a full resection of the tumor often leads to tumor recurrence within months after initial surgery and the ultimate demise of GBM patients (11).

A second contributor to GBM progression is the immunosuppressive nature of the tumor microenvironment (TME) (12) (13). Specifically, GBM overexpresses immune inhibitory proteins such as ICAM-1 which interacts with LFA-1 and enables myeloid derived suppressor cell (MDSC) accumulation within the TME (14). MDSCs suppress anti-tumor T-cells through the expression of anti-inflammatory molecules such as TGF-beta and arginase (15). GBMs also express Fas ligand, CD70, as well as PD-L1 (16). These molecules result in either T-cell death or T-cell inhibition. It has also been well documented that there are a limited number of T-cells that are present within the TME. T-cells that are present within the TME are typically classified as exhausted and may even function as immune suppressors (17). This immunosuppression is only amplified in GBM patients as most patients receive dexamethasone to control cerebral edema (18). Dexamethasone has been shown to upregulate immunosuppressive checkpoint molecules such as CTLA4 which inhibits T-cell anti-tumor activity (18). Additionally, dexamethasone has been shown to lead to an overall reduction in T-cell proliferation (18).

Intertumoral heterogeneity is an additional contributor to GBM progression as some studies have shown that 50% of recurrent GBM samples share only half the genetic mutations that were housed in the original tumor (19). Studies by Soeda and colleagues were some of the first to show that GBM subclones, derived from a single patient, had distinct cell populations and the sensitivity of each subclone to an inhibitor of epidermal growth factor receptor were dissimilar (20, 21). Later studies by Dirkse and colleagues expanded upon this work, by revealing that phenotypic heterogeneity that is observed in GBM, is derived from cancer stem cells undergoing reversible state transitions that are instructed by the tumor microenvironment (22). Additionally, it has been observed that the anatomical location of GBM within different sites of the brain, impacts the mutational landscape of the tumor (23).

A final contributor to GBM progression is the blood brain barrier (BBB) (24). The BBB is a complex of tight junctions joining endothelial cells which prevent most substances from passing into the cranial vault (25). This blocking of substances also includes the blocking of most antineoplastic therapies due to their large size. Therapies that can make it past the BBB are often not targeted directly to the tumor which leads to the host of treatment-associated negative side effects observed in patients (26).

Immunotherapy has shown tremendous promise for treating cancers residing outside the CNS. However, in brain tumors, immunotherapy trials have had underwhelming results (Table 1). Clinical trials such as the CheckMate 143 study were aimed at evaluating the effectiveness of using an ICI (nivolumab) compared to an anti-angiogenic agent (bevacizumab) in patients diagnosed with recurrent GBM (27). The findings for this trial were a disappointment for the field as patients who received nivolumab did not experience a survival benefit compared to bevacizumab-treated (control) patients (27). While the findings from this trial were quite disappointing, subgroup analysis indicated that corticosteroid use at baseline appeared to be unfavorably associated with outcomes in patients that received nivolumab (27). Corticosteroids have previously been shown to negatively impact T-cell function (12, 18, 28). Additionally, it was reported that patients bearing tumors with MGMT promoter methylation had a longer median overall survival compared to patients with unmethylated tumors (27). An additional study that is critical to highlight is a phase 3 study by Lim and colleagues which found that nivolumab in addition to chemoradiation therapy did not improve survival for patients with newly diagnosed GBM with methylated or indeterminate MGMT promoter as compared to patients receiving placebo and chemoradiation (29). The findings from these glioma-targeted ICI-based studies as well as others clearly highlight the need for the field to reconsider whether adjuvant immune checkpoint inhibition is beneficial for patients with GBM.

Trials evaluating vaccines targeted to GBM have failed as well (10, 30–33). Often, what many of these trials have in common is that these

Abbreviations: GBM, Glioblastoma; TME, Tumor Microenvironment; MDSC, Myeloid Derived Suppressor Cell; BBB, Blood Brain Barrier; ICI, Immune Checkpoint Inhibitor; CNS, Central Nervous System; OS, Overall Survival; PFS, Progression Free Survival; DDFS, Distant Disease-Free Survival; LND, Lymph Node Dissection; OSS, Overall Response Rate; RFS, Relapse-Free Survival; DMFS, Distant Metastasis-Free Survival; trAE, Treatment Related Adverse Event; MBM, Melanoma Brain Metastases; NSCLC, Non-Small Cell Lung Cancer; MPR, Major Pathological Response; TNBC, Triple Negative Breast Cancer; pCR, Pathological Complete Response; TLS, Tertiary Lymphoid Structures; TIL, Tumor Infiltrating Lymphocyte; RECIST, Response Evaluation in Solid Tumors22. ECOG, Eastern Cooperative Oncology Group23.

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TABLE 1 Major immune checkpoint inhibitor studies targeting gliomas.

Trial Number	Phase Number	Glioma Type	Treatment	Progression-Free Survival	Overall Survival	Trial Status
NCT04396860	Phase III	Glioblastoma	Arm 1: Radiation therapy, Temozolomide Arm 2: Radiation therapy, Ipilimumab, Nivolumab	Not reported due to termination	Not reported due to termination	Suspended
NCT02017717	Phase III	Glioblastoma	Arm 1: Nivolumab Arm 2: Bevacizumab	Arm 1: 5.8% at 18 months Arm 2: 8.9% at 18 months	Arm 1: 21.7% at 18 months Arm 2: 21.6% at 18 months	Completed
NCT02667587	Phase III	Glioblastoma	Nivolumab, Temozolomide, and Radiotherapy	10.64 months	28.91 months	Active, not recruiting
NCT02617589	Phase III	Glioblastoma	Arm 1: Nivolumab, Radiation Therapy Arm 2: Temozolomide, Radiation Therapy	Arm 1: 6.01 months Arm 2: 6.21 months	Arm 1: 13.40 months Arm 2: 14.88 months	Completed
NCT04817254	Phase II	Glioblastoma	Arm 1: Nivolumab, Ipilimumab 1mg/kg, Temozolomide Arm 2: Nivolumab, Ipilimumab 3mg/kg, Temozolomide	Not reported at time of review	Not reported at time of review	Recruiting
NCT04479241	Phase II	Glioblastoma	Lerapolturev, Pembrolizumab	Not reported at time of review	Not reported at time of review	Active, not recruiting
NCT03047473	Phase II	Glioblastoma	Avelumab, with Temozolomide and Radiotherapy	Median: 9.7 months	Median: 15.3 months	Completed
NCT04396860	Phase II	Glioblastoma	Arm 1: Radiation Therapy, Temozolomide Arm 2: Radiation Therapy, Ipilimumab, Nivolumab	Not reported due to termination	Not reported due to termination	Suspended
NCT02798406	Phase II	Glioblastoma	DNX-2401, Pembrolizumab	Not reported at time of review	Not reported at time of review	Completed
NCT03673787	Phase II	Glioblastoma	Ipatasertib, Atezolizumab	Not reported at time of review	Not reported at time of review	Recruiting
NCT05074992	Phase II	Glioblastoma	Ipilimumab, Surgery, Chemoradiotherapy	Not reported at time of review	Not reported at time of review	Recruiting
NCT03430791	Phase II	Glioblastoma	Arm 1: Nivolumab Monotherapy Arm 2: Nivolumab, Ipilimumab	Results submitted, awaiting quality control review	Results submitted, awaiting quality control review	Terminated
NCT02550249	Phase II	Glioblastoma	Nivolumab	Not reported at time of review	Not reported at time of review	Completed
NCT03367715	Phase II	Glioblastoma	Nivolumab, Ipilimumab, Short-course radiation therapy	5.92 months	80% at 1 year Median: 16.85 months	Completed
NCT03890952	Phase II	Glioblastoma	Arm A: Nivolumab and Bevacizumab, Salvage surgery Arm B: Nivolumab and Bevacizumab, No salvage surgery	Not reported at time of review	Not reported at time of review	Active, not recruiting
NCT04195139	Phase II	Glioblastoma	Arm 1: Radiotherapy, Nivolumab, Temozolomide Arm 2: Radiotherapy, Temozolomide	Not reported at time of review	Not reported at time of review	Active, not recruiting
NCT04145115	Phase II	Glioblastoma	Ipilimumab, Nivolumab	Not reported at time of review	Not reported at time of review	Recruiting
NCT03743662	Phase II	Glioblastoma	Arm 1: Re-irradiation, Bevacizumab, Nivolumab, No surgery Arm 2: Re-irradiation, Bevacizumab, Nivolumab, Re-resection surgery	Not reported at time of review	Not reported at time of review	Active, not recruiting
NCT03452579	Phase II	Glioblastoma	Arm 1: Nivolumab, Bevacizumab 10mg/kg Arm 2: Nivolumab, Bevacizumab 3mg/kg	Not reported at time of review	Not reported at time of review	Active, not recruiting
NCT04704154	Phase II	Glioblastoma	Regorafenib, Nivolumab	Not reported at time of review	Not reported at time of review	Active, not recruiting

(Continued)

TABLE 1 Continued

Trial Number	Phase Number	Glioma Type	Treatment	Progression-Free Survival	Overall Survival	Trial Status
NCT03718767	Phase II	IDH-mutant Astrocytoma	Nivolumab	Not reported at time of review	Not reported at time of review	Recruiting
NCT02794883	Phase II	Glioblastoma	Arm 1: Tremelimumab Arm 2: Durvalumab Arm 3: Tremelimumab, Durvalumab	Arm 1: 2.746 months Arm 2: 4.356 months Arm 3: 4.913 months	Arm 1: 7.246 months Arm 2: 11.71 months Arm 3: 7.703 months	Completed

experimental therapies are administered to patients following surgical resection, rather than before. This approach is problematic as surgical resection removes the bulk of the antigen present within the patient which decreases the number of antigenic targets for the immune system. A study by Cloughesy and colleagues reinforces this hypothesis as the research team found that neoadjuvant ICI with anti-PD1 promoted a survival benefit in patients diagnosed with GBM (34).

There are numerous advantages to administering neoadjuvant immunotherapy to patients diagnosed with solid tumors. The first advantage is that this approach allows for a decrease in the size of the tumor allowing for an increased likelihood of achieving a full surgical resection (35). A study by Xu and colleagues found that neoadjuvant immune checkpoint inhibition in patients with squamous cell lung cancer aided in facilitating surgical resection of the disease due to a decrease in tumor size (35). An additional advantage of using a neoadjuvant approach is that one can fully assess whether a patient has the capacity to respond to immunotherapy. Following surgical resection, patients are often immunosuppressed due to receiving agents aimed at reducing cerebral edema (dexamethasone) in addition to receiving cytotoxic chemotherapy and radiation therapy (12, 18). Additionally, the antigen that was present has now been removed due to surgical resection. This combination of factors makes patients poor candidates to receive immunotherapy and limits a clinician's ability to properly assess whether the patient can respond to immunotherapy. Trials such as NCT04817254 are aimed at designing a novel approach that can assess whether patients with GBM or Gliosarcoma can respond to ICIs.

In this review we will highlight clinical trials and laboratory studies where neoadjuvant ICIs were administered to combat solid tumors residing outside the CNS. In addition to evaluating whether this approach provided an OS and/or PFS benefit, we will also discuss the specific ways this approach enhanced immunological response. We will compare these observations to what has been observed in brain tumor immunotherapy studies as we hope to inspire future studies that evaluate whether neoadjuvant immunotherapy for GBM is an efficacious approach.

Melanoma

Historically, chemotherapy and interleukin-2 have been the standard of care for advanced melanoma despite their inability to demonstrate a meaningful survival advantage (36). Even with advances in adjuvant immune and targeted therapies, the risk of relapse for higher risk melanomas (stage IIC and IIIB-D) remains high with 10-year OS rates of 24% to 77% (37). The application of neoadjuvant immunotherapy could be viewed as a major disruptor to the current standard of care, with a promise for greater longevity for patients with advanced melanoma. In the last decade, randomized controlled trials using anti-BRAF/MEK targeted therapies such as dabrafenib/trametinib (DAB + TRAM) or vemurafenib/cometinib, paired with ipilimumab or nivolumab, have demonstrated a dramatic improvement in PFS and OS for unresectable melanoma (38–40). As it stands, there are 52 active, planned, or ongoing interventional trials evaluating neoadjuvant approaches in high-risk melanoma that are registered on clinicaltrials.gov (41). This section will focus on several clinical trials where immunotherapies were administered in a neoadjuvant setting with significant pathological response rates.

In a study of neoadjuvant versus adjuvant ICI in the treatment of clinical stage III melanoma, Song and colleagues reported a neoadjuvant associated 3-year improvement in distant disease-free survival (DDFS) (42). Even after adjusting for ICI agents received, neoadjuvant sequencing remained associated with improved 3-year DDFS as compared to adjuvant therapy. A pathological response was evaluated in 39 patients who received neoadjuvant treatment, with 59% of patients receiving a pathologic partial response and 13% receiving a pathologic complete response. The study enrolled 59 patients, with 18 (31%) receiving adjuvant therapy and 41 (69%) receiving neoadjuvant therapy. Adjuvant therapy was defined as ICI administration after lymph node dissection (LND), while neoadjuvant was defined as administration of one to two cycles of ICI prior to LND, followed by continuation of therapy after surgery. ICI regimens included ipilimumab 3 or 10mg/kg every 3 weeks, pembrolizumab 200mg every 3 weeks, nivolumab 240mg every 2 weeks or 480mg every 4 weeks, or combination ipilimumab 3mg/kg and nivolumab 1mg/kg every 3 weeks in the induction phase followed by nivolumab 240 or 480mg in the maintenance phase.

A phase two study that enrolled patients with high-risk resectable melanoma, evaluated the efficacy and safety of neoadjuvant nivolumab versus combined ipilimumab with nivolumab (43). Despite the trial's emphasis on monotherapy *vs.* combined neoadjuvant therapy, its findings support the overall efficacy of neoadjuvant immunotherapy administration. The RECIST overall response rates (ORR) was 25% with nivolumab monotherapy and 73% with combined ipilimumab and nivolumab therapy. Additionally, pathologic complete response rates were 25% and 45%, respectively. Although not statistically significant, combination therapy treatment was associated with improved PFS, relapse-free survival (RFS), distant metastasis-free survival (DMFS), and OS over treatment with nivolumab alone. Additionally, improved RFS, DMFS and OS were observed in pathologic complete response patients who received neoadjuvant therapy versus those who did not. However, these results did not reach statistical significance, likely due to small

sample size. Consequentially, toxicity rates differed significantly in patients who received neoadjuvant treatment, with reports of grade 3 treatment related adverse events (trAEs) in 8% and 73% of monotherapy and combination therapy patients respectively. A total of 23 patients were enrolled in the trial, with 12 patients randomized to nivolumab therapy monotherapy and 11 patients to combined therapy with ipilimumab and nivolumab. Monotherapy patients received up to four doses of nivolumab 3mgkg^{-1} on weeks 1, 3, 5 and 7, while combined therapy patients received up to three doses of nivolumab 1mgkg^{-1} and ipilimumab 3mgkg^{-1} on weeks 1, 4 and 7.

A phase Ib clinical trial evaluated the feasibility of combined neoadjuvant ipilimumab and nivolumab treatment as compared to an adjuvant regimen for the treatment of melanoma. The study enrolled 20 patients (10: adjuvant; 10: neoadjuvant) with palpable stage III melanoma who were randomized to receive either four courses of combined adjuvant ipilimumab 3mgkg^{-1} and nivolumab 1 mgkg^{-1} every 3 weeks starting at week 6 post-surgery, or two courses of the same regimen, but with neoadjuvant administration every 3 weeks prior to surgery. The study concluded that neoadjuvant-treated patients demonstrated more expanded tumor-resident T-cell clones compared to the adjuvant cohort (44). Additionally, in the neoadjuvant group, 9 of 10 patients were found to have pathological response, 78% of which achieved profound pathological response.

Melanoma brain metastasis

Melanoma has the highest propensity for the development of brain metastasis among all solid tumors, with studies revealing that up to 44% of all patients with stage IV melanoma develop brain metastases, and 75% of patients have CNS involvement identified at autopsy (45). Until recently, many trials testing the efficacy of newer immunotherapy treatments have excluded patients with melanoma brain metastases (MBM).

A phase 2 clinical trial assessing the efficacy of ipilimumab in patients with melanoma and brain metastases reported activity in some patients, more specifically when metastases were small and asymptomatic (46). Patients older than 16 years with histologically confirmed metastatic melanoma were eligible to be enrolled in this study if they had at least one measurable index brain metastasis of 0.5-3cm in diameter, or two measurable lesions larger than 0.3cm in diameter (46). In the first stage of this study, patients were enrolled into cohort A if they were asymptomatic, to assess the effect of ipilimumab monotherapy on brain and extracranial metastases (46). Patients enrolled in this study received four doses of 10mg/kg of ipilimumab, one every four weeks, and should these patients be clinically stable at week 24, they would then be eligible to receive 10mg/kg of ipilimumab every 12 weeks (46). The primary endpoint for this study was the proportion of patients with complete response, partial response, or stable disease after 12 weeks which was assessed using modified WHO criteria (46). Following the reaching of efficacy parameters, the study proceeded into stage two where patient enrollment into cohort A continued, and patients with symptomatic metastasis controlled with corticosteroids began being enrolled into cohort B (46). The study reported intracranial and extracranial disease control rates of 24% and 27% respectively, in neurologically asymptomatic patients (46). In symptomatic patients, intracranial and extracranial disease control rates were 10% and 5% respectively (46).

Another phase 2 trial with pembrolizumab administration also showed activity in brain metastases in patients with melanoma or NSCLC (47). Patients were enrolled in this study if they were 18 years or older, were diagnosed with melanoma or NSCLC with untreated brain metastases, and had at least one untreated brain metastasis between 5 and 20mm in diameter without associated neurologic symptoms or a need for corticosteroids (47). A total of 18 patients with melanoma and 18 patients with NSCLC were enrolled in this study and patients were given 10mg/kg of pembrolizumab every 2 weeks until progression (47). The primary endpoint for this study was brain metastasis response assessed in all treated patients (47). Brain metastases were assessed by a neuroradiologist with unidimensional evaluation using Response Evaluation in Solid Tumors (RECIST) criteria (version 1.1) (47). Findings from this study were encouraging as a brain metastasis response rate of 22% and 33% was observed in melanoma and NSCLC patients respectively (47).

Finally, a combined nivolumab and ipilimumab phase 2 study also showed greater efficacy in patients with asymptomatic melanoma with brain metastases than prior monotherapy studies (48). In this study, patients aged 18 years or older with measurable melanoma brain metastases that were 0.5-3.0cm in diameter were enrolled into either cohort A if they were asymptomatic, had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, no neurologic symptoms or baseline corticosteroid use, or cohort B if they were symptomatic, had an ECOG performance status of 0-2 with stable neurological symptoms (48). Patients in cohort B could be receiving low-dose dexamethasone (48). Patients in both cohorts received nivolumab 1mg/kg plus ipilimumab 3mg/kg every three weeks for four doses, followed by nivolumab 3mg/kg every 2 weeks for up to 2 years until either disease progression or unacceptable toxicity (48). The primary endpoint for this study was intracranial clinical benefit rate (complete responses, partial responses, or stable disease lasting 6 months or more) assessed in all patients that were treated (48). Response was determined *via* radiographic assessment that was performed every 6 weeks for the first year of study enrollment, and then every 12 weeks thereafter, until documented disease progression, using RECIST (version 1.1) criteria (48). The study reported an intracranial clinical benefit of 57.4% and 16.7% for neurologically asymptomatic and symptomatic patients respectively (48).

Non-small cell lung cancer

Many clinical trials have indicated that neoadjuvant immune checkpoint inhibition for patients with NSCLC could be a promising treatment modality. Few trials exist that have evaluated the efficacy of adjuvant ICIs for patients with NSCLC, let alone trials that have compared neoadjuvant ICI administration to adjuvant ICI administration. Given these findings, this section will focus on clinical trials where ICIs were administered in a neoadjuvant setting.

In a phase 2 clinical trial that evaluated the efficacy of administering neoadjuvant atezolizumab to patients with resectable NSCLC, 21% of the patient population achieved a major pathological

response and 7% of patients achieved a complete pathological response (49–51). These findings were correlated with a major pathological response rate of 33% in 50% of patients expressing the highest PD-L1 protein level (49–51). The 50% of patients expressing the lowest PD-L1 protein levels had a major pathological response rate of 11% (49–51). NCT02259621 is another study that evaluated the efficacy of administering neoadjuvant checkpoint inhibition to patients with stage IB-IIIA NSCLC. The trial enrolled 15 patients who received 3 mg/kg of nivolumab and 1 mg/kg of ipilimumab intravenously 6 weeks prior to surgical resection (52). 3 mg/kg of nivolumab was then given again 4 and 2 weeks preoperatively. Due to toxicity the study was terminated early (52). However, of the six patients who underwent surgical resection three patients were alive with no recurrence of disease, two patients experienced a recurrence, and one patient died postoperatively of acute respiratory distress syndrome (52).

The NADIM trial enrolled patients with resectable stage IIIA NSCLC. These patients underwent three cycles of neoadjuvant nivolumab and chemotherapy prior to surgery, and one year of adjuvant nivolumab monotherapy following surgery. Following tumor resection, it was observed that 85.4% of patients survived without any recurrence of their disease (53). Additionally, down-staging occurred in approximately 90% of the cases within this study (53). Interestingly, the team notes that there were no significant associations identified between any clinical or molecular parameters analyzed at the time of diagnosis and a patient's pathological response. However, the research team did observe that a PD-L1 tumor proportion score of 25% or more was associated with major or complete pathological response (53). However, this metric was insufficiently sensitive as 58% of patients with a PD-L1 tumor proportion score of less than 25% also demonstrated major or complete pathological responses (53). This trial enrolled a limited number of patients which may have impacted the ability to identify significant associations between clinical or molecular parameters identified at the time of diagnosis and pathological response.

Recently, a phase 2 study called the NEOSTAR trial was completed. The trial enrolled patients with surgically resectable NSCLC. This study sought to evaluate whether there was a survival benefit associated with administering neoadjuvant nivolumab and ipilimumab as opposed to administering nivolumab alone. This trial enrolled 44 eligible patients with 23 patients being assigned to the nivolumab monotherapy arm and 21 patients being assigned to the nivolumab plus ipilimumab cohort (54). Patients received doses of either nivolumab alone (3 mg/kg) or nivolumab and ipilimumab (3 mg/kg and 1 mg/kg respectively) at days 1, 15 and 29 (54). This regimen was followed by surgical resection which was planned for at least 21 days after and within 42 days of receiving the first dose of nivolumab. The research team found that 38% of patients in the nivolumab plus ipilimumab cohort achieved a MPR of 38% as compared to a 22% MPR in the nivolumab monotherapy cohort (54). In patients that underwent surgical resection, the MPR for the nivolumab plus ipilimumab cohort was 50% as compared to 24% for the nivolumab monotherapy cohort (54). Patients in the nivolumab plus ipilimumab cohort had higher complete pathological response rates (38% as compared to 10%), less viable tumor, and a greater number of effector, tissue resident memory, and effector memory T-cells (54).

A final study that is critical to highlight is a phase 3 trial led by Forde and colleagues which found that neoadjuvant nivolumab plus chemotherapy had promising findings in patients with NSCLC. In this study patients with stage IB to IIIA resectable NSCLC were randomly assigned to receive nivolumab (360mg) plus platinum-based chemotherapy or platinum-based chemotherapy alone every three weeks for three cycles (55). Surgery was planned to take place within 6 weeks following the completion of neoadjuvant treatment (55). Following surgery, patients in either the neoadjuvant nivolumab plus chemotherapy group, or the neoadjuvant chemotherapy alone group could receive up to four cycles of adjuvant chemotherapy, radiotherapy, or both (55). There were two primary endpoints for this trial, the first being event-free survival (55). The second primary endpoint was pathological complete response (55). In this trial, 352 patients received treatment, with 176 patients receiving neoadjuvant nivolumab plus chemotherapy, and 176 patients receiving chemotherapy alone (55). Findings from this trial were encouraging as the median event-free survival was 31.6 months for patients in the nivolumab plus chemotherapy group as opposed to a median event-free survival of 20.8 months for patients in the chemotherapy alone group (55). The percentage of patients with a pathological complete response was 24% for patients in the nivolumab plus chemotherapy group, and 2.2% for patients in the chemotherapy alone group (55). These findings highlighted that neoadjuvant nivolumab plus chemotherapy resulted in significantly longer event-free survival and a higher percentage of patients with a pathological complete response.

Pembrolizumab is an additional ICI that has been approved for the treatment of advanced NSCLC however there are few trials that have evaluated its usefulness in a neoadjuvant setting. In the NEOMUN trial, patients with stage IIA-IIIA NSCLC will receive two cycles of pembrolizumab as a neoadjuvant immunotherapy and clinical and pathological tumor response will be assessed (56).

Breast cancer

Breast cancer is the most common cause of cancer in women worldwide. The development of new immunotherapies to treat cancer, such as ICIs, have shown success in treating cancers such as malignant melanomas, non-small cell lung cancer, colon, and rectal cancer. While these types of treatments have shown efficacy in other types of cancer, breast cancer pathogenicity is somewhat unique in that it's considered to be immunologically "cold," thus immunotherapeutic approaches to treating breast cancer still have many unresolved issues (57). Immunotherapy, when provided in the neoadjuvant setting, is expected to be used as a new treatment modality when treating breast cancer, especially for phenotypes which have high immunogenicity, such as triple negative breast cancer (TNBC). Here, we will review clinical trials where neoadjuvant immunotherapeutic treatments were administered to patients with breast cancer.

A randomized phase 2 I-SPY2 trial examined the efficacy of neoadjuvant treatment that included pembrolizumab, on participants with early-stage, high-risk, ERBB2 (formerly HER2)-negative breast cancer. In the neoadjuvant setting, drug efficacy can be evaluated using pathological complete response (pCR) as a survival

endpoint, which is defined as the absence of invasive tumor in breast and regional lymph nodes at the time of surgery (58). Based on accumulated results, pCR after neoadjuvant treatment correlates significantly with the PFS and OS rate (59). Out of the 250 women included in the final analysis, 181 were randomized to receive standard NACT therapy (control arm), which included paclitaxel for 12 weeks, in addition to 4 cycles of doxorubicin plus cyclophosphamide every 2 to 3 weeks (AC). 69 participants in the intervention group received the standard NACT therapy in addition to neoadjuvant pembrolizumab every 3 to 4 cycles concurrently with paclitaxel. 40 participants were hormone receptor (HR) positive and 29 were triple-negative. Estimated pCR rates showed an increase in pCR for all cohorts of pembrolizumab *vs.* control, (44% *vs.* 13%) in ERBB2-negative, (30% *vs.* 13%) in HR-positive/ERBB2-negative, and (60% *vs.* 22%) in triple-negative. Adverse reactions to the intervention included thyroid abnormalities (13%) and adrenal insufficiency (8.7%). As stated, pCR significantly correlates with survival rate and this study showed that participants with pCR following pembrolizumab plus chemotherapy had high 3-year event-free survival rates (93% at 2.8 years' median follow-up). Findings from this study showed that the addition of pembrolizumab to standard neoadjuvant chemotherapy more than doubled pCR compared to chemotherapy alone for hormone receptor-positive/ERBB2-negative and triple-negative breast cancer (60).

A phase 3 KEYNOTE-522 clinical trial similarly evaluated the benefit of adding neoadjuvant pembrolizumab to neoadjuvant chemotherapy for participants diagnosed with early TNBC. Participants diagnosed with untreated stage 2 or stage 3 TNBC were randomly assigned to receive either 4 cycles of neoadjuvant chemotherapy (paclitaxel and carboplatin) plus placebo every three weeks (control), or 4 cycles of pembrolizumab in addition to paclitaxel and carboplatin every 3 weeks. Both groups also received doxorubicin-cyclophosphamide or epirubicin-cyclophosphamide. Following surgery, participants then received 9 cycles of adjuvant pembrolizumab or chemotherapy every 3 weeks. After subsequent analysis, it was reported that out of the 602 participants who were randomized in the study, the pCR rates for the pembrolizumab-chemotherapy group and the chemotherapy-placebo groups were 64.8% and 51.2%, respectively. The estimated treatment difference was calculated to be 13.6%. After follow-up, 7.4% of participants in the pembrolizumab-chemotherapy group and 11.8% of participants in the chemotherapy-placebo group were found to have either disease progression post-surgery, distal or local recurrence of disease, a second primary tumor, or died from any cause. Findings from this study demonstrated that among participants diagnosed with early, untreatable stage 2/3 TNBC, the pCR was significantly higher among participants in the cohort who received neoadjuvant pembrolizumab plus neoadjuvant chemotherapy compared to those who received neoadjuvant chemotherapy plus neoadjuvant placebo (61).

A randomized, double-blind phase 3 clinical trial, IMpassion031, compared the efficacy and safety of the drug atezolizumab *vs.* placebo in combination with nab-paclitaxel followed by doxorubicin plus cyclophosphamide for patients with TNBC. Participants of the study had to be 18 years or older and diagnosed with previously untreated stage 2 or stage 3 TNBC. Participants received either neoadjuvant IV atezolizumab plus chemotherapy every 2 weeks or received neoadjuvant placebo plus chemotherapy. The chemotherapy regimen consisted of nab-paclitaxel every week for 12 weeks, followed by doxorubicin and cyclophosphamide every 2 weeks for 8 consecutive weeks then followed by surgery. Out of the 333 eligible participants of the study, 165 were randomly assigned to receive atezolizumab plus chemotherapy and 168 were assigned to receive placebo plus chemotherapy. The median follow-up for the atezolizumab plus chemotherapy group was 20.6 months and 19.8 months for the group who received placebo plus chemotherapy. A pCR rate of 58% was reported in 95 patients who received the atezolizumab plus chemotherapy and a pCR rate of 41% was reported in 69 participants who received the placebo plus chemotherapy. The rate difference between the two groups was reported as 17%. For the population of participants that were PD-L1 positive, pCR was reported as 69% in 53 out of the 77 participants that received the atezolizumab plus chemotherapy. For the PD-L1 positive participants who received placebo plus chemotherapy, 37 out of the 75 participants reported a pCR rate of 49%. The rate difference between the two groups was reported as 20%. Findings from this study demonstrated that in patients with previously untreated, diagnosed early-stage TNBC, neoadjuvant treatment with atezolizumab in combination with chemotherapy significantly improved pCR rates compared to patients who received placebo in combination with chemotherapy (62).

Colon and rectal cancer

Numerous strategies have been utilized to activate cancer immunity in colorectal cancer (CRC) with major modalities of immunotherapy including monoclonal antibodies, ICIs, cancer vaccines, adoptive cell therapies, and bispecific T-cell engagers (63). Implementation of immunotherapy has long been a desired goal for treating CRC due to its tailorability and promising potential for inducing longer-term forms of immune surveillance, theoretically decreasing risks of future recurrence of disease (63). Even though much of the evidence supporting the use of ICIs is most abundant in cases of metastatic treatment-refractory cases of gastric cancer and hepatocellular carcinoma (64); these findings have highlighted the potential of using ICIs for the treatment of CRC. Previous studies found that response rates were independent of biologic marker status, such as microsatellite instability-high (MSI-H)/deficient MisMatch Repair (dMMR) or programmed cell death-ligand 1 (PD-L1) expression (63). Despite initial studies of ICIs demonstrating limited activity in unselected CRC patients, this section will focus on several clinical trials where ICIs were administered in a neoadjuvant setting allowing for significant pathological response rates.

In a phase three clinical trial that evaluated efficacy of administering neoadjuvant pembrolizumab to patients with MSI-H advanced CRC (65), patients experienced a median PFS of 16.5 months compared to 8.2 months when chemotherapy alone was administered. Correlative to these findings was an increase in overall response (complete or partial response as evaluated with Response Evaluation Criteria in Solid Tumors), with 43.8% of patients in the pembrolizumab group and 33.1% in the chemotherapy group achieving an overall response. Of the patients with an overall response, 83% in the pembrolizumab group, compared with 35% in

the chemotherapy group, had continual responses at 24 months, changing the standard of care for metastatic CRC with dMMR (66). The trial enrolled 307 patients who received 200 mg of pembrolizumab every three weeks or chemotherapy (5-fluorouracil-based therapy with or without bevacizumab or cetuximab) every two weeks. The study allowed patients in the chemotherapy cohort to subsequently convert to pembrolizumab therapy with any sign of disease progression.

A phase two study that enrolled patients with metastatic MSI-H/deficient MisMatch Repair (dMMR) CRC, evaluated the two-year long-term efficacy and safety of neoadjuvant nivolumab plus low-dose ipilimumab (67). Patients were treated with nivolumab every two weeks plus low dose ipilimumab every six weeks until disease progression. An objective response rate and disease control rate of 69% and 84%, respectively, were observed, with a complete response rate of 13%. The team noted that even though a median duration of response was not reached, 74% of responders had ongoing responses at data cutoff. Interestingly, a *post hoc* analysis of 14 patients who had discontinued treatment was also performed and ten remained progression-free. Additionally, at the end of the 24-month period, the team noted a median PFS and OS of 74% and 79% respectively. A consequential finding of this study was that regardless of baseline demographic and tumor characteristics, including *BRAF* or *KRAS* mutation status, clinical benefit was still observed (67).

Another study that is important to highlight is a phase two study that investigated the activity of a neoadjuvant nivolumab/ipilimumab regimen in both dMMR and proficient MisMatch Repair (pMMR) early-stage (Stage I-III) resectable colon adenocarcinomas (68). Patients received combination treatment of ipilimumab and nivolumab on day one ($1mgkg^{-1}$ and $3mgkg^{-1}$, respectively) as well as a dose of nivolumab ($3mgkg^{-1}$) on day fifteen. Following radical tumor resection at four weeks post initial treatment, it was observed that all (100%) patients with dMMR tumors showed pathological response *via* tumor regression. 95% of those patients had a major pathological response (MPR, <10% residual viable tumor). Additionally, 27% of patients with pMMR tumors showed a pathological response, with 50% of those showing complete pathological response. Changes in the microenvironments were also noted for dMMR tumors, with a significant increase in CD8+ and CD3+ T-cell infiltrates, as well as IFN-γ scores compared to pretreated biopsies (68). At the time of resection, these tumors also noted a significant increase in tertiary lymphoid structures (TLS), which have been found to harbor most PD-1+ tumor infiltrating lymphocytes (TILs) in lung cancer (69). Forty patients were enrolled in this trial with 21 dMMR and 20pMMR tumors (one patient had both pMMR and dMMR colon cancer).

Finally, results from the NICHE-2 study, a study treating patients with deficient mismatch repair (dMMR) colon cancer with neoadjuvant nivolumab and ipilimub has shown promising findings. Specifically, in this study, patients with non-metastatic dMMR colon cancer were treated with one dose of ipilimumab (1mg/kg) and two doses of nivolumab (3mg/kg) followed by surgery (70). The two primary endpoints for this study were safety, and 3-year disease-free survival (DFS) (70). A total of 112 patients were treated in this study with radiographic assessment performed at baseline revealing 89% of tumors to be stage III, 77% being high-risk stage III and 64% being considered T4 tumors (70). A major pathological response was observed in 95% of patients, and complete response was observed in 67% of patients (70). The findings from this trial were some of the first to clearly indicate that there is a strong potential for neoadjuvant immunotherapy to become the standard of care for patients with dMMR colon cancer (70).

Brain tumors

There have been numerous studies where patients with brain tumors have undergone adjuvant immune checkpoint inhibition. Many of these trials have failed which have led many to believe that ICIs may not be a useful treatment for brain tumors, especially GBM (27, 71–73). While there are numerous contributors that may have led to the failure of these trials some of the most likely reasons include, patients receiving corticosteroids prior to study enrollment or while on the study, and tumor resection prior to treatment initiation.

One of the first studies to explore the effectiveness of administering neoadjuvant immunotherapy to patients with brain tumors was a study by Cloughesy and colleagues which evaluated whether patients with recurrent GBM experience a survival benefit when receiving neoadjuvant anti-PD1 prior to undergoing surgical resection (34). Patients with recurrent GBM receiving neoadjuvant anti-PD1 were compared to those with recurrent GBM receiving adjuvant anti-PD1 following surgical resection. There were 35 total patients enrolled in this study with 16 patients being assigned to the neoadjuvant cohort and 19 patients being assigned to the adjuvant cohort (34). Following patient enrollment and cohort assignment, those patients assigned to the neoadjuvant cohort received 200 mg intravenous infusions of pembrolizumab 14 ± 5 days prior to surgical resection. Following resection, patients in both cohorts received 200 mg intravenous infusions of pembrolizumab every three weeks until tumor progression or until the occurrence of an adverse event requiring treatment discontinuation.

The research team found that patients receiving neoadjuvant anti-PD1 had a median OS of 13.7 months and those receiving adjuvant anti-PD1 had a median OS of just 7.5 months. Additionally, PFS was enhanced for those receiving neoadjuvant therapy as these patients had a median PFS of 3.3 months as compared to those receiving adjuvant therapy who had a median PFS of 2.4 months (34). Outside of a survival benefit, it was also noted that patients receiving neoadjuvant therapy experienced an increase in T-cell and interferon-gamma related gene expression within their tumors as well as a downregulation of cell cycle-related gene expression (34). This was not observed in patients receiving adjuvant therapy. It was also observed that those undergoing neoadjuvant treatment had an enhanced clonal expression of T-cells as well as a decreased expression of PD-1 on T-cells within the peripheral blood (34). This study was one of the first of its kind and highlighted the promise of not only using immunotherapy to treat GBM, but also the potential of administering this therapy neoadjuvantly.

Since the study by Cloughesy and colleagues there have been few studies that have explored the benefit of administering neoadjuvant

immunotherapy to patients with GBM. A study by Prins and colleagues found that neoadjuvant anti-PD1 induces T-cell and cDC1 activation in patients with recurrent GBM however, this treatment modality failed to overcome immunosuppressive tumor associated macrophages that have a large presence in the TME of patients with GBM (74). A clinical trial by a group of collaborators at Duke University (NCT04434560) explored the efficacy of administering neoadjuvant ICIs to patients with brain metastases however this trial was terminated due to poor enrollment.

Few other studies have been performed to date that explore the efficacy of administering neoadjuvant immunotherapy to patients with GBM. Additionally, studies that have been performed have only evaluated whether there is a survival benefit for patients with *recurrent* GBM. This is problematic as patients with recurrent GBM are typically immunosuppressed as they have undergone treatment with corticosteroids, cytotoxic chemotherapy as well as radiation therapy. More studies are needed that explore whether there is an even larger benefit to treating patients with their initial GBM with neoadjuvant immunotherapy as these patients have not undergone the immunosuppressive milieu of treatments that most GBM patients undergo.

While some success has been observed in studies targeting ICIs to brain metastases, few ICI studies have demonstrated success when adjuvant ICIs were targeted to GBM. One of the reasons for this lack of success could be differences in the tumor microenvironment between brain metastases and GBM. While there is a paucity of literature that clearly outlines differences in the tumor microenvironment of brain metastases compared to that of GBM, some studies have shown that both microenvironments are characterized by high numbers of myeloid cells that are associated with an immunosuppressive phenotype (75, 76). However, a key difference in the microenvironments of brain metastases and GBM may involve spatial heterogeneity, as a recent study by Schaettler and colleagues found that GBMs display more spatial heterogeneity at the genomic and neoantigen levels as compared to brain metastases (77). Additionally, the spatial diversity that was observed in this study was recapitulated in T-cell clone distribution as some GBMs possessed highly expanded yet spatially restricted clonotypes as compared to less spatially restricted clonotypes for brain metastases (77). These findings clearly indicate that far more research is needed to fully understand differences in the tumor microenvironments of GBMs and brain metastases as these differences may highlight areas of vulnerability that can be exploited when targeting neoadjuvant immunotherapies to GBM.

Conclusion

In this review we have highlighted several clinical studies that have demonstrated the effectiveness of administering neoadjuvant ICIs to patients diagnosed with tumors of the skin, lung, breast, as well as the colon and rectum (see Table 2). We selected these tumors due to their comparability to GBM, as these tumors are often classified as "immunologically cold", grow aggressively, and are often accompanied by a poor patient prognosis. In most of the studies highlighted throughout this review neoadjuvant immune checkpoint inhibition had a profound impact on OS as well as PFS, as this treatment modality allowed for an increased patient immune response. Beyond the malignancies treated with neoadjuvant immune checkpoint inhibition that we have highlighted in this manuscript, promising results have also been observed in patients diagnosed with squamous-cell carcinoma (78). In a phase 2 trial led by Gross and colleagues, patients with stage II, III, or IV cutaneous squamous-cell carcinoma received cemiplimab at a dose of 350mg every 3 weeks for up to four doses prior to undergoing surgery with curative intent (78). A pathological complete response was observed in 51% of patients that received neoadjuvant cemiplimab, and an objective response on imaging was observed in 68% of patients (78).

While results have been encouraging for treating cancers outside the brain with neoadjuvant ICIs, very few studies have evaluated the effectiveness of administering neoadjuvant ICIs to patients with GBM. We believe this is an area in need of further investigation as currently, GBM patients receive ICIs following surgery when the antigen has been removed from the cranial compartment. This antigen removal can be a barrier to the induction of a robust anti-tumor immune response due to decreased antigen load.

Additionally, GBM patients are often immunosuppressed and are therefore not best positioned to fully respond to ICIs (79, 80). Studies by Fecci and colleagues observed that patients with GBM have T-cell sequestration in the bone marrow due to the loss of the S1P1 receptor,

TABLE 2 Neoadjuvant immune checkpoint inhibitor studies targeting non-CNS and CNS cancers.

Trial Number	Phase	Cancer Type	Neoadjuvant Treatment	Adjuvant Treatment	Progression-free Survival	Overall Survival	Trial Status
NCT01274338	Phase III	Melanoma Stage III	Arm 1A: Ipilimumab	Arm 2A: Ipilimumab	Arm 1A-C: 62% at 36 months	Not reported at time of review	Recruiting
			Arm 1B: Ipilimumab + Nivolumab	Arm 2B: Ipilimumab + Nivolumab	Arm 2A-C: 31% at 36 months		
			Arm 1C: Pembrolizumab or Nivolumab	Arm 2C: Pembrolizumab or Nivolumab			

(Continued)

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TABLE 2 Continued

Trial Number	Phase	Cancer Type	Neoadjuvant Treatment	Adjuvant Treatment	Progression-free Survival	Overall Survival	Trial Status
NCT02519322	Phase II	Melanoma Stage IIIB-IV	Arm 1: Nivolumab	All patients undergoing surgery were offered 13 doses of Nivolumab post-surgery	Arm 1 patients: 58% at 22.6 months	Arm 1 patients: 76% at 22.6 months	Active, not recruiting
			Arm 2: Nivolumab + Ipilimumab		Arm 2 patients: 82% at 17.2 months	Arm 2 patients: 100% at 24 months	
			Both arms completed cycles prior to surgery				
NCT00623766	Phase II	Melanoma with Brain Metastases	N/A	Arm 1: Ipilimumab	Arm 1: 1.4 months	Arm 1: 26% at 36 months	Completed
				Arm 2: Ipilimumab + corticosteroid	Arm 2: 1.2 months	Arm 2: 10% at 36 months	
NCT02437279	Phase I	Melanoma Stage III	Arm 1: Nivolumab + Ipilimumab	Arm 2: Nivolumab + Ipilimumab after Complete Lymph Node Dissection	Arm 1: 80% at 25.6 months	Arm 1: 90% at 25.6 months	Active, not recruiting
			Complete Regional Lymph Node Dissection at Week 6		Arm 2: 60% at 25.6 months	Arm 2: 60% at 25.6 months	
NCT02927301	Phase II	Non-Small Cell Lung Cancer Stage IB-IIIB	Atezolizumab	N/A	Not a reportable outcome	Not a reportable outcome	Active, not recruiting
			Resection at day 40 +/- 10 days				
NCT02259621	Phase II	Non-Small Cell Lung Cancer Stage IB-IIIA	Nivolumab + Ipilimumab	All patients offered standard postoperative adjuvant chemotherapy+/- radiation	33% at 25 months	Not a reportable outcome	Recruiting
NCT03081689	Phase II	Non-Small Cell Lung Cancer Stage IIIA	Nivolumab + Paclitaxel + Carboplatin	Adjuvant treatment commenced 3-8 weeks post-surgery	77.1% at 24 months	89.9% at 24 months	Recruiting
			Surgical resection planned 42-49 days after 1st day of third treatment cycle				
NCT03158129	Phase II	Non-Small Cell Lung Cancer Stage I-IIIA	Arm 1: Nivolumab	Standard adjuvant chemotherapy and/or postoperative radiation allowed at the discretion of treating physician	Median PFS was not reached	Median OS was not reached	Recruiting
			Arm 2: Nivolumab + Ipilimumab				
			Surgical resection at least 3 weeks and within 6 weeks post last dose of nivolumab				
NCT03197467	Phase II	Non-small Cell Lung Cancer Stage II/IIIA	Pembrolizumab	Adjuvant chemotherapy will be recommended according to the current national and international guidelines	Not reported at time of review	Not reported at time of review	Active, not recruiting
			Surgical resection Day 50-60				
NCT03036488	Phase III	Triple Negative Breast Cancer	Arm 1: Chemotherapy + Paclitaxel + Carboplatin + Doxorubicin or Epirubicin-Cyclophosphamide	Adjuvant pembrolizumab or chemotherapy was given every 3 weeks for up to 9 cycles	Arm 1: 85.3% at 18 months	Not reported at time of review	Active, not recruiting
			Arm 2: Pembrolizumab + Paclitaxel + Carboplatin + Doxorubicin or Epirubicin-Cyclophosphamide		Arm2: 91.3% at 18 months		
			Surgical resection 3-6 weeks after last cycle				
NCT03197935	Phase III	Triple Negative Breast Cancer	Arm 1: Nab-paclitaxel + Doxorubicin + Cyclophosphamide	Arm 1: Nab-paclitaxel + Doxorubicin + Cyclophosphamide	Not reported at time of review	Not reported at time of review	Active, not recruiting
				Arm 2: Atezolizumab			

(Continued)

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TABLE 2 Continued

Trial Number	Phase	Cancer Type	Neoadjuvant Treatment	Adjuvant Treatment	Progression-free Survival	Overall Survival	Trial Status
			Arm 2: Atezolizumab + Nab-paclitaxel + Doxorubicin + Cyclophosphamide				
			Surgical resection followed				
NCT01042379	Phase II	*ERBB2*-Negative Breast Cancer	Arm 1: Paclitaxel + Doxorubicin + IV Cyclophosphamide	Adjuvant treatment was not mandated by this trial	Not reported at time of review	Not reported at time of review	Recruiting
			Arm 2: Paclitaxel + Doxorubicin + IV Cyclophosphamide + Pembrolizumab				
			Definitive surgery followed				
NCT02563002	Phase III	Stage IV Colorectal Carcinoma	Arm 1A: Oxaliplatin + Leucovorin + 5-Fluoropyrimidine	N/A	Arm 1A-F: 18.6% at 24 months	Arm1A-F: 52% at 32.4 months	Active, not recruiting
			Arm 1B: Oxaliplatin + Leucovorin + 5-Fluoropyrimidine + Bevacizumab		Arm 2: 48.3% at 24 months	Arm 2: 63% at 32.4 months	
			Arm 1C: Oxaliplatin + Leucovorin + 5-Fluoropyrimidine + Cetuximab				
			Arm 1D: Irinotecan + Leucovorin + 5-Fluoropyrimidine				
			Arm 1E: Irinotecan + Leucovorin + 5-Fluoropyrimidine + Bevacizumab				
			Arm 1F: Irinotecan + Leucovorin + 5-Fluoropyrimidine + Cetuximab				
			Arm 2: Pembrolizumab				
NCT04008030	Phase III	Metastatic Colorectal Cancer	Nivolumab + Ipilimumab	N/A	Median PFS was not reached (74% at 24 months)	Median OS was not reached (79% at 24 months)	Recruiting
NCT03026140	Phase II	Early-Stage Colon Cancer	Arm 1: Nivolumab	N/A	Not reported at time of review	Not reported at time of review	Recruiting
			Arm 2: Nivolumab + Ipilimumab				
			Arm 3: Nivolumab + Ipilimumab + Celecoxib				
			Surgery resection at 6 weeks				
NCT04201873	Phase I	Glioblastoma	Arm 1: Pembrolizumab	Arm 2: Pembrolizumab	Arm 1: 99.5 days	Arm 1: 417 days	Recruiting
					Arm 2: 72.5 days	Arm 2: 228.5 days	
NCT04434560	Phase II	Brain Metastases	Pembrolizumab & Ipilimumab	Standard postoperative adjuvant treatment	Not reported due to termination	Not reported due to termination	Terminated
			Surgical resection 7 days post treatment				

TABLE 3 Summary of the potential benefits of neoadjuvant administration of ICIs.

Benefits of Neoadjuvant Immunotherapy	Citation
Reduces tumor burden prior to surgery, allowing for more optimal resection	(82)
Allows for the ability to fully assess pathologic response to immunotherapy	(83)
Allows for the utilization of treatment response as surrogate outcome markers for relapse-free and overall survival	(82)
Allows for a greater chance of immune to tumor microenvironment interaction, resulting from preservation of the lymphatic system	(84)
Induces a stronger immune response (tumor specific $CD8^+$ T-cells) against a higher tumor antigen load	(85)
More cost effective than adjuvant treatment as a result of fewer treatments needed	(84)
Can attenuate the immunosuppressive effects of surgery, such as the systemic release of glucocorticoids, which suppress T-cell proliferation and induce apoptosis of naïve T-cells	(86)

while Giles and colleagues describe how dexamethasone (a corticosteroid commonly given to GBM patients to control cerebral edema) induces immunosuppression (18, 81). These are just some of the many ways that GBM patients become immunosuppressed throughout their tumor course which ultimately contribute to treatment failure (10, 12, 28). There may be major benefits to neoadjuvant immune checkpoint inhibition as this is when patients may be best positioned to respond to treatment as they are earlier in their treatment course and have not undergone surgical resection as surgery is known to create an anti-inflammatory tumor microenvironment. Given the large number of ICI clinical studies that have failed in neuro-oncology, phase 0 studies may be beneficial in evaluating whether neoadjuvant ICI is beneficial for patients with GBM as these studies are often first in human, enroll a small number of patients (lowering study-associated expenses), and can help investigators determine (*via* blood and/or tissue analysis) whether neoadjuvant ICI better primes the immune system as compared to adjuvant ICI.

In Table 3 we highlight several other benefits for the neoadjuvant administration of immune checkpoint inhibitors such as a reduction in tumor burden prior to surgery, the assessment of a pathologic immune response to immunotherapy, in addition to other benefits. We believe that the time is now for the field of neuro-oncology to begin evaluating whether there is increased benefit to administering ICIs prior to surgery as this may be the time the patient is most likely to respond to treatment and experience a survival benefit.

Author contributions

SF, CD, JB, and AD performed a review of the literature and wrote the manuscript. XZ and CH provided their expertise on the clinical aspects of the manuscript. GK conceptualized and supervised the writing of the manuscript. 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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The short-term efficacy and safety of induction chemotherapy combined with PD-1 inhibitor or anti-EGFR in locoregionally advanced nasopharyngeal carcinoma

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Purpose: This study aimed to investigate the short-term efficacy and safety of induction chemotherapy (IC) combined with PD-1 inhibitor or anti-EGFR in the treatment of locoregionally advanced nasopharyngeal carcinoma (LA-NPC).

Methods and materials: We retrospectively reviewed the clinical data of 206 patients with LA-NPC, including IC combined with anti-PD-1 (57 patients), IC combined with anti-EGFR (28 patients), and IC alone (121 patients). The short-term efficacy was assessed at the end of IC and one month after overall treatment. According to the RECIST v1.1, the short-term efficacy of cervical lymph nodes and primary nasopharynx foci was divided into complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD). The overall response (ORR) was defined as the sum of CR and PR. Acute toxicities were graded according to the CTCAE v5.0. One-way analysis of variance (ANOVA) was used to compare differences in the numerical variables among groups. Fisher Freeman-Halton test or Pearson Chi-square test was used to compare classified variables.

Results: The ORR rates of primary nasopharynx foci in IC, anti-EGFR, and anti-PD-1 group were 68.60%, 67.9%, and 94.7%, respectively, and the corresponding rates of ORR in cervical lymph nodes were 78.5%, 71.4%, and 93.0%, respectively. There was a statistical difference in the ORR between the three groups. Further analysis showed that after IC or overall treatment, the CR rate of primary nasopharynx foci in the anti-PD-1 group was significantly higher than the other two groups. The most common adverse effects were hematotoxicity, gastrointestinal toxicity, and transaminase elevation. However, there were no

statistical differences in the frequency of any common adverse effects between the three groups.

Conclusions: The addition of anti-PD-1 based on IC significantly improved the short-term efficacy of LA-NPC and toxicities were tolerable.

KEYWORDS

nasopharyngeal carcinoma, locoregionally advanced, induction chemotherapy, short-term efficacy, PD-1

Introduction

Nasopharyngeal carcinoma (NPC) is an aggressive epithelial malignancy that arises from the epithelium of the nasopharynx (1). The incidence of NPC is low worldwide, and its distribution has a unique ethnic and geographic distribution pattern, with the highest incidence in southern China and Southeast Asia, especially in the Chinese province of Guangdong (1, 2). Histopathologically, almost all NPC is poorly differentiated or undifferentiated squamous carcinoma, characterized by a high malignant degree, rapid growth, vague symptoms, early cervical lymph node metastasis, and distant metastases (3). Therefore, the majority of NPC patients often appeared with the advanced locoregionally disease at first diagnosis (1–4).

Due to its radiosensitivity, radiotherapy or a combination of radiation and chemotherapy is the mainstay treatment for NPC. For locoregionally advanced nasopharyngeal carcinoma (LA-NPC), the main reasons for the treatment failure of radical radiotherapy (RT) or concurrent chemoradiation (CCRT) are distant metastasis and local recurrence (5, 6). Induction chemotherapy (IC) can decrease the tumor burden, narrow lesions and eliminate distant micro-metastasis over a short period of time, and quickly relieve the symptoms and signs of the compression effect of the tumor on surrounding tissues. Therefore, oncologists have done lots of research on whether the addition of IC to CCRT can further improve the prognosis of patients with LA-NPC. Due to differences in the population studied, sample size, and chemotherapy regimen, previous clinical studies showed that the addition of IC to CCRT has improved the disease-free survival (DFS) but did not significantly improve overall survival (OS) in LA-NPC patients (7–9).

Recently, the final results of several randomized studies on IC have been published, which have demonstrated the prognostic value of IC combined with CCRT in high-risk LA-NPC patients. IC added to CCRT significantly improved DFS and OS, compared with CCRT alone, among patients with LA-NPC (10–12). Following the LA-NPC diagnosis, the standard treatment option is IC, followed by CCRT. The common chemotherapy protocols mainly consist of TPF (docetaxel, cisplatin, and fluorouracil), TPC (aclitaxel, cisplatin, and capecitabine), TP (docetaxel, cisplatin), PF (cisplatin, fluorouracil), GP (gemcitabine, cisplatin) and so on (10–14). These chemotherapy regimens showed similar clinical efficacy and favorable safety profiles in their respective clinical studies.

In addition, NPC is regarded as a highly immune inflammatory tumor because of its unique immune environment, and EGFR is usually highly expressed. Anti-EGFR therapy (Nimotuzumab) plus CCRT in the treatment of LA-NPC (15, 16) and anti-PD-1/PD-L1 immunotherapy combined with chemotherapy (17, 18) in the treatment of refractory for recurrent or metastatic nasopharyngeal carcinoma (RM-NPC) have made significant progress. Currently, clinical investigations on the addition of anti-EGFR or anti-PD-1/PD-L1 immunotherapy to IC are ongoing. Therefore, we conducted this retrospective study to evaluate the short-term efficacy and safety of anti-EGFR or anti-PD-1 immunotherapy with IC for LA-NCP.

Materials and methods

Patients

In this study, we retrospectively analyzed the clinical data of NPC patients who received IC at our institution between February 2019 to February 2022. The inclusion criteria were as follows: (1) Age ≥18 years old; (2) Pathologically diagnosed as NPC; (3) LA-NPC including stage III/IVa in accordance with the Eighth Edition of the AJCC staging system, but clinical stage T3/T4N0 was excluded; (4) All patients had a measurable primary lesion and at least one measurable metastatic lymph node according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The short-term efficacy evaluation is evaluated by experienced radiation therapists and re-examined by senior physicians; (5) Patients were treated with definitive IC followed by definitive CCRT or RT; (6) Before receiving IC, the patient's renal function, hepatic function, and hematological parameters were normal. The

Abbreviations: Nasopharyngeal carcinoma, NPC; radiotherapy, RT; induction chemotherapy, IC; concurrent chemoradiation, CCRT; disease-free survival (DFS); overall survival, OS; locoregionally advanced nasopharyngeal carcinoma, LA-NPC; refractory for recurrent or metastatic nasopharyngeal carcinoma, RM-NPC; complete remission, CR; partial remission, PR; stable disease, SD; progressive disease, PD; the overall response, ORR; the disease control rate, DCR; nasopharyngeal carcinoma, NPC; EBV, Epstein–Barr virus.

exclusion criteria included the following: (1) With incomplete clinical data;(2) Have undergone surgery before receiving induction chemotherapy;(3) Patients with apparent immune system diseases, concomitant inflammatory diseases, or blood system diseases; (4) History of severe cardiovascular or pulmonary disease;(5) Patients with a second primary tumor. Written informed consent from the participants was exempted owing to its retrospective design. We have de-identified all patient details such that the identity of any person may not be ascertained in any way.

Induction chemotherapy

In this study, all patients received two to three cycles of platinum-based IC, including modified TPF (nab-paclitaxel, raltitrexed, and cisplatin), GP (gemcitabine plus cisplatin), and TP (docetaxel/nab-paclitaxel plus cisplatin) regimens. The modified TPF was administered as 260 mg/m^2 nab-paclitaxel intravenously on day 1, 80 mg/m^2 cisplatin intravenously on day 1, and 3 mg/m^2 raltitrexed intravenously on day 1; 2 to 3 cycles were administered at intervals of 3 weeks. GP regimen was administered as gemcitabine (1000 mg/m^2 on days 1 and 8) and cisplatin (80 mg/m^2 on day 1) intravenously every 3-week cycle for 2 to 3 cycles. TP consisted of nab-paclitaxel (260 mg/m^2, d1, intravenous infusion) plus cisplatin (80 mg/m^2, d1, intravenous infusion) administered every 3 weeks for 2 to 3 cycles. Whether patients received IC combination of PD-1 inhibitors or anti-EGFR depends on the joint decision of the patient, the family, and the attending physician. If the patient received IC combined with anti-PD-1 immunotherapy or anti-EGFR targeted therapy, 200 mg camrelizumab, 240 mg toripalimab, or 200 mg nimotuzumab were given intravenously on the first day of each cycle IC.

Radiotherapy

All patients received RT or platinum-based CCRT (cisplatin 100mg/m^2 or lobaplatin 30 mg/m^2 every three weeks) after the completion of IC, and approximately 50% of these patients continued to receive anti-EGFR (200 mg nimotuzumab, 5-6 cycles) or anti-PD-1 immunotherapy (240 mg toripalimab, 2-3 cycles) during the radiotherapy period. Radiation therapy was delivered with the volumetric modulated arc therapy technique (VMAT). Gross tumor volume (GTV) consisted of the primary tumor and enlarged lymph nodes (GTVnx, the sum of the primary nasopharyngeal site and enlarged retropharyngeal nodes; GTVnd, the clinically involved cervical lymph nodes). High-risk clinical target volume (CTV1) was defined as the area where the metastatic positive lymph nodes are located and the lymphatic drainage area at the next station. The low-risk lymphatic drainage area (CTV2) is defined as the cervical lymphatic drainage area that needs prophylactic irradiation, except CTV1. For the planning target volumes of GTVnx, GTVnd, CTV1, and CTV2, total radiation doses of 69.96 Gy, 69.96 Gy, 60.06 Gy, and 54.45 Gy, respectively, were administered in 33 fractions delivered five times per week, starting on the first day of the first CCRT cycle.

Therapeutic efficacy and toxicity assessment

The short-term efficacy was assessed at the end of IC and one month after the end of overall treatment by physical examination, nasopharyngeal fiberscope examination, and magnetic resonance imaging (MRI) of the nasopharynx/neck. According to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1), the short-term efficacy of cervical lymph nodes and primary nasopharynx foci was divided into complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD). The overall response (ORR) was defined as the sum of CR and PR, and the disease control rate (DCR) was the sum of CR, PR, and SD.

Other evaluations included routine hematological, biochemistry tests, and physical examinations. Acute hematological and nonhematological toxicities during IC and CCRT/RT were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) v5.0.

Statistical analysis

Data analysis was performed using SPSS (IBM SPSS 23.0, SPSS Inc). Descriptive statistics were generated for relevant clinical characteristics. Fisher Freeman-Halton test or Pearson Chi-square test was used for the comparison of classified variables. One-way analysis of variance (ANOVA) was used to compare differences in the numerical variables among groups. A $p<0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 206 patients with LA-NPC were included in this study, including 121 (58.7%) patients with IC alone, 57 (27.7%) patients with IC combined with anti-PD-1, and 28 (13.6%) patients with IC combined with anti-EGFR. According to the eighth edition of the AJCC staging system, 66 (32.0%) patients were in stage III and 140 (68.0%) in stage IVA. There was no significant difference in age, gender, T stage, N stage, TNM stage, and pathological characteristics among the IC group, anti-PD-1 group, and anti-EGFR group, but there were differences in specific IC regimens and treatment protocols after IC. Overall, the specific IC regimen was mainly GP (66, 32.1%) alone and GP combined with anti-PD-1 (47, 22.8%), while after IC (84, 41.3%) patients received CCRT and CCRT combined with anti-EGFR (81, 39.3%). The basic clinical characteristics of the LA-NPC patients and the specific regimens are available in Table 1.

Short-term efficacy after IC for primary nasopharynx lesions

At the end of induction chemotherapy and one month after the end of overall treatment, the efficacy of primary nasopharynx

TABLE 1 Basic clinical characteristics of the patients and the specific regimens.

Characteristic	IC	anti-PD-1	anti-EGFR	χ2/F	*p*-value
No. of the patients (n, %)	121 (58.7)	57 (27.7)	28 (13.6)	/	/
Age, years (Median, Range)	46 (18–77)	45 (24–73)	51.5 (20–76)	1.14	0.321
Gender (n, %)				0.398	0.820
Male	90 (74.4)	40 (70.2)	21 (75.0)		
Female	31 (25.6)	17 (29.8)	7 (25.0)		
Clinical stage[&] (n, %)					
III	44 (36.4)	16 (28.1)	6 (21.4)	2.899	0.235
IVA	77 (63.6)	41 (71.9)	22 (78.6)		
T stage[&] (n, %)				8.205	0.223
T1	15 (12.4)	10 (17.5)	3 (10.7)		
T2	12 (9.9)	8 (14.0)	0 (0.0)		
T3	53 (43.8)	26 (45.6)	12 (42.9)		
T4	41 (33.9)	13 (22.8)	13 (46.4)		
N stage[&] (n, %)				5.772	0.217
N1	21 (17.4)	6 (10.5)	5 (17.9)		
N2	56 (46.3)	20 (35.1)	10 (35.7)		
N3	44 (36.4)	31 (54.4)	13 (46.4)		
Histology (nonkeratinizing) (n, %)					
differentiated	17 (14.0)	7 (12.3)	4 (14.3)	0.116	0.943
undifferentiated	104 (86.0)	50 (87.7)	24 (85.7)		
IC regimens (n, %)					
GP	66 (54.5)	47 (82.5)	2 (7.1)	49.395	**<0.001***
TP	23 (19.0)	10 (17.5)	14 (50.0)		
TPF	32 (26.4)	0 (0.0)	12 (42.9)		
IC followed by RT regimens (n, %)				54.412	**<0.001***
CCRT	67 (55.4)	15 (26.3)	3 (10.7)		
CCRT+anti-EGFR	43 (35.5)	20 (35.1)	18 (64.3)		
CCRT+anti-PD-1	1 (0.8)	14 (24.6)	1 (3.6)		
RT	5 (4.1)	3 (5.3)	0 (0.0)		
RT+anti-EGFR	5 (4.1)	5 (8.8)	6 (21.4)		

SD, standard deviation; RT, radiation therapy; IC, induction chemotherapy; GP, gemcitabine plus cisplatin; TP, docetaxel/nab-paclitaxel plus cisplatin; TPF, nab-paclitaxel, raltitrexed and cisplatin; RT, radical radiotherapy; CCRT, concurrent chemoradiation; [&], clinical staging according to the Eighth Edition of the AJCC staging system. Bold indicates the significant values (*p < 0.05).

lesions and cervical lymph node metastasis was evaluated according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1).

After induction chemotherapy, the clinical efficacy of primary nasopharynx lesions was evaluated: the response in the IC alone group was CR in 0.8%, PR in 67.8%, and SD in 31.4%; for anti-PD-1 group CR in 14%, PR in 80.7% and SD in 5.3%; for anti-EGFR group CR in 0%, PR in 67.9% and SD in 31.2%; while no patient showed disease progression in the primary site.

The ORR rate of IC, anti-EGFR and anti-PD-1 groups were 68.60% (83/121), 67.9%(19/28), and 94.7% (54/57), respectively. The ORR was statistically different between the three groups (χ

2 = 17.30 p < 0.001). There was no significant difference between IC and anti-EGFR group (χ 2 = 0.006, p=0.94), but there was significant difference between IC and anti-PD-1 group (χ 2 = 14.936, p < 0.001), and between anti-PD-1 and anti-EGFR group (Fisher Freeman-Halton test: p = 0.002). Further information is given in Table 2.

Short-term efficacy after IC for cervical lymph nodes

The short-term efficacy of cervical lymph nodes was evaluated: the response in the IC alone group was CR at 22.3%, PR at 56.2%, and SD at 21.5%; for the anti-PD-1 group CR at 36.8%, PR at 56.1%, and SD in 7.0%; for anti-EGFR group CR in 28.6%, PR in 42.9% and SD in 8.0%. No patients progressed by the end of treatment.

The ORR rate of IC, anti-EGFR, and anti-PD-1 was 78.5% (95/121), 93.0% (53/57), and 71.4% (20/28), respectively. The ORR was statistically different between the three groups (χ 2 = 7.60, p = 0.021). Regarding the ORR of cervical lymph nodes, significant differences were observed between anti-PD-1 and IC group χ 2 = 5.789, p = 0.016), and between anti-PD-1 and anti-EGFR group (Fisher Freeman-Halton test: p = 0.016); however, no significant difference was observed between IC and anti-EGFR group (χ 2 = 0.648, p = 0.421) Table 2.

Subgroup analysis of short-term efficacy in GP and GP + anti-PD-1

In this retrospective study, GP was the main chemotherapy regimen, so we compared the short-term efficacy between the GP and the GP + anti-PD-1 group by case sample size. The ORR rate of primary nasopharynx lesions in GP group and GP + anti-PD-1 group was 72.7% vs 95.7%, which was statistically significant (χ 2 = 9.984, p = 0.002); while the ORR rate in cervical lymph nodes was 86.4% vs 91.5%, which had no statistical difference (χ 2 = 0.708, p = 0.400) Table 3.

Short-term efficacy of the different IC regimens

We made a subgroup analysis of the short-term efficacy of 121 patients treated with induction chemotherapy alone. The ORR rates of GP, TP, and TPF to the primary nasopharynx lesions were 72.7%, 56.5%, and 68.8%, respectively. The three groups had no statistical difference (χ 2 = 2.080, p = 0.353). At the same time, there was no statistical difference between GP and TP (χ 2 = 2.077, p = 0.150), GP and TPF (χ 2 = 3.017, p = 0.082), TP and TPF (χ 2 = 0.278, p = 0.598).

The ORR rates of GP, TP, and TPF to the cervical lymph nodes were 86.4%, 65.2%, and 71.9%, respectively. There was no statistical difference among the three groups (χ 2 = 5.657, p = 0.059). At the same time, there was no statistical difference between GP and TP (Fisher Freeman-Halton test, p = 0.035 > 0.017), GP and TPF (χ 2 = 3.017, p = 0.082), TP and TPF (χ 2 = 0.278, p = 0.598) Table 4.

Short-term efficacy after overall treatment

After overall treatment, the ORR rate of nasopharyngeal lesions and cervical lymph nodes in all patients was close to 100%, only three patients who only received induction chemotherapy before radiotherapy were evaluated as SD (2 nasopharyngeal lesions and one cervical lymph node).

Further analysis found that after overall treatment, the CR rate of nasopharyngeal lesions in the anti-PD-1 group was significantly higher than the IC or anti-EGFR group (84.2% vs. 52.9% vs. 53.6%, χ 2 = 18.240, p <0.001). There was no significant difference between IC and anti-EGFR group (Fisher Freeman-Halton test, χ 2 = 0.228, p=1.0), but there was significant difference between IC and anti-PD-1 group (Fisher Freeman-Halton test, χ 2 = 16.817, p < 0.001), and between anti-PD-1 and anti-EGFR group (χ 2 = 9.188, p = 0.002). The short-term efficacy of cervical lymph nodes did not differ significantly between the three groups (Fisher Freeman-Halton test, χ 2 = 5.358, p <0.217) Table 5.

TABLE 2 Short-term efficacy after induction chemotherapy.

Lesion site	Group	No.	CR	PR	SD	PD	ORR	χ2 test	
		(n)	(n, %)	(n, %)	(n, %)	(n, %)	(n, %)	χ2	p
Nasopharynx								15.49	<0.001
	anti-EGFR	28	0 (0%)	19 (67.9%)	9 (31.2%)	0 (0%)	19 (67.9%)		
	anti-PD-1	57	8 (14%)	46 (80.7%)	3 (5.3%)	0 (0%)	54 (94.7%)		
	IC	121	1(0.8%)	82 (67.8%)	38 (31.4%)	0 (0%)	83 (68.6%)*&		
Lymph node								7.60	0.021
	anti-EGFR	28	8(28.6%)	12 (42.9%)	8 (28.6%)	0 (0%)	20 (71.4%)		
	anti-PD-1	57	21(36.8%)	32 (56.1%)	4 (7.0%)	0 (0%)	53 (93.0%)		
	IC	121	27(22.3%)	68 (56.2%)	26(21.5%)	0 (0%)	95 (78.5%)*&		

IC, induction chemotherapy; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; ORR, the sum of CR and PR; Note: CR + PR were considered effective; SD + PD were considered invalid. Bold indicates the significant values (p < 0.05). *compared with the anti-EGFR group, p < 0.017; & compared with the anti-PD-1 group, p <0.017.

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TABLE 3 Comparison of short-term efficacy between GP and GP + anti-PD-1 group.

Lesion site	Group	No.	CR	PR	SD	PD	ORR	χ2 test	
		(n)	(n, %)	(n, %)	(n, %)	(n, %)	(n, %)	χ2	*P*
Nasopharynx								9.984	0.002
	GP+ anti-PD-1	47	8 (17.0%)	37 (78.7%)	2 (4.3%)	0 (0%)	45 (95.7%)		
	GP	66	1 (1.5%)	47 (71.2%)	18 (27.3%)	0 (0%)	48 (72.7%)		
Lymph node								0.708	0.400
	GP+ anti-PD-1	47	15 (31.9%)	28 (59.6%)	4 (8.5%)	0 (0%)	43 (91.5%)		
	GP	66	18 (27.3%)	39 (59.1%)	9 (13.6%)	0 (0%)	57 (86.4%)		

IC, induction chemotherapy; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; ORR, the sum of CR and PR; Note: CR + PR were considered effective; SD + PD were considered invalid; GP, gemcitabine plus cisplatin; Bold indicates the significant values ($p < 0.05$).

Toxicity

Acute toxicity during induction chemotherapy was assessed between the three groups, and no severe adverse reactions occurred, as shown in Table 6. The most common adverse effects were hematotoxicity, gastrointestinal toxicity, and the elevation of transaminase. However, there were no statistical differences in the frequency of any common adverse effects between the three groups (p>0.05).

Discussion

Currently, the multimodal treatment approach, combining radiotherapy and chemotherapy, is the primary treatment strategy in locoregionally advanced nasopharyngeal carcinoma (LA-NPC); up to 10–20% of LA-NPC patients still develop local or metastatic relapse and die from this disease (19, 20). Induction chemotherapy (IC) can decrease the tumor burden, narrow lesions, eliminate distant micro-metastasis over a short period, and quickly relieve the symptoms and signs of the compression effect of the tumor on surrounding tissues. Therefore, oncologists have done lots of research on whether adding IC to concurrent chemoradiation (CCRT) can further improve the prognosis of patients with LA-NPC. The final overall survival (OS) analysis of a multicenter, randomized phase III trial showed that gemcitabine and cisplatin induction chemotherapy before concurrent chemoradiotherapy (CCRT) significantly improved OS in patients with LA-NPC without increasing the risk of late toxicities due to cancer therapies (12). Furthermore, It is particularly noteworthy that the depth of the tumor response to IC for LA-NCP significantly and positively correlates with OS, the 5-year OS of patients with complete response (CR), partial response (PR), and stable/progressive disease (SD/PD) was 100%, 88.4%, and 61.5%, respectively ($p<0.05$). Therefore, we conducted this retrospective study to evaluate the short-term efficacy and safety of anti-EGFR targeted therapy or anti-PD-1 immunotherapy with IC for LA-NCP. In this study, we found that the addition of anti-PD-1 immunotherapy in induction chemotherapy increased the short-term efficacy (anti-PD-1 vs IC vs anti-EGFR, the ORR of primary nasopharynx lesions/cervical lymph nodes, 94.7%/93.0% vs 68.6%/78.5% vs 67.9%/71.4%, p <0.001/p=0.021), especially significantly

TABLE 4 Subgroup analysis by the specific chemotherapy regimens.

Lesion site	IC Group	No.	CR	PR	SD	PD	ORR	χ2 test	
		(n)	(n, %)	(n, %)	(n, %)	(n, %)	(n, %)	χ2	*p*
Nasopharynx								2.080	0.353
	GP	66	1 (1.5%)	47 (71.2%)	18 (27.3%)	0 (0%)	48 (72.7%)		
	TP	23	0 (0.0%)	13 (56.5%)	10 (43.5%)	0 (0%)	13 (56.5%)		
	TPF	32	0 (0.0%)	22 (68.8%)	10 (31.3%)	0 (0%)	22 (68.8%)*&		
Lymph node								5.657	0.059
	GP	66	18 (27.3%)	39 (59.1%)	9 (13.6%)	0 (0%)	57 (86.4%)		
	TP	23	2 (8.7%)	13 (56.5%)	8 (34.8%)	0 (0%)	15 (65.2%)		
	TPF	32	7 (21.9%)	16 (50.0%)	9 (28.1%)	0 (0%)	23 (71.9%)*&		

IC, induction chemotherapy; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; ORR, the sum of CR and PR; Note: CR + PR were considered effective; SD + PD were considered invalid; GP, gemcitabine plus cisplatin; TP, docetaxel/nab-paclitaxel plus cisplatin; TPF, nab-paclitaxel, raltitrexed and cisplatin; Bold indicates the significant values ($p < 0.05$). *compared with the TP group, $p < 0.017$; & compared with the TPF group, p<0.017.

TABLE 5 Short-term efficacy after overall treatment.

Lesion site	Group	No.	CR	PR	SD	PD	F# test	
		(n)	(n, %)	(n, %)	(n, %)	(n, %)	χ2	*p*
Nasopharynx							18.240	**<0.001**
	anti-EGFR	28	15 (53.6%)	13 (46.4%)	0 (0%)	0 (0%)		
	anti-PD-1	57	48 (84.2%)	46 (15.8%)	0 (0%)	0 (0%)		
	IC	121	64 (52.9%)	55 (45.5%)	2 (1.7%)	0 (0%)*&		
Lymph node							5.358	0.217
	anti-EGFR	28	20(71.4%)	8 (28.6%)	0 (0%)	0 (0%)		
	anti-PD-1	57	50(87.7%)	7 (12.3%)	0 (0%)	0 (0%)		
	IC	121	92(76.0%)	28 (23.1%)	1 (0.8%)	0 (0%)		

IC, induction chemotherapy; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; F#, Fisher Freeman-Halton test. Bold indicates the significant values ($p < 0.05$). *compared with the anti-EGFR group, $p < 0.017$; & compared with the anti-PD-1 group, $p<0.017$.

increased the CR rate of primary nasopharynx lesions, and did not increase the treatment-related side effects.

Epstein-Barr virus (EBV) infection has been consistently identified as an essential risk factor for the progression of nasopharyngeal carcinoma (NPC) (21). Chronic EBV infection, numerous lymphocyte infiltrates, high PD-L1 expression, and several key immune molecules involved in T cell activation can be frequently observed in EBV-induced NPC tumor tissues (22). In addition to having very potent antigens, NPC has an elevated level of interferon response and is associated with a higher proportion of antigen-presenting cells (23). Based on the particular immune landscape of NPC, NPC is considered a highly immuno-

TABLE 6 Major adverse events during induction chemotherapy.

Adverse event	IC	anti-PD-1	anti-EGFR	χ2/F*	
				χ2	*p*
Leukopenia				5.044	0.283
G0 + 1	71 (58.7%)	26 (45.6%)	18 (64.3%)		
G2	35 (28.9%)	19 (33.3%)	8 (28.6%)		
G3 + 4	15 (12.4%)	12 (21.1%)	2 (7.1%)		
Neutropenia				4.280	0.369
G0 + 1	62 (51.2%)	25 (43.9%)	16 (57.1%)		
G2	34 (28.1%)	13 (22.8%)	7 (25.0%)		
G3 + 4	25 (20.7%)	19 (33.3%)	5 (17.9%)		
Hemoglobin				8.467	0.051
G0 + 1	107 (88.4%)	43 (75.4%)	27 (96.4%)		
G2	11 (9.1%)	13 (22.8%)	1 (3.6%)		
G3 + 4	3 (2.5%)	1 (1.8%)	0 (0%)		
Thrombocytopenia				4.342	0.325
G0 + 1	108 (89.3%)	48 (84.2%)	28 (100%)		
G2	7 (5.8%)	4 (7.0%)	0 (0%)		
G3 + 4	6 (5.0%)	5 (8.8%)	0 (0%)		
ALT/AST elevated				3.522	0.172
G0 + 1	117 (96.7%)	52 (91.2%)	25 (89.3%)		
G2 + 3	4 (3.3%)	5 (8.8%)	3 (10.7%)		

(Continued)

TABLE 6 Continued

Adverse event	IC	anti-PD-1	anti-EGFR	χ2/F*	
				χ2	*p*
Nausea				0.200	0.905
G0 + 1	106 (89.1%)	52 (91.2%)	25 (89.3%)		
G2	13 (10.9%)	5 (8.8%)	3 (10.7%)		
Vomiting				3.911	0.102
G0 + 1	114 (94.2%)	57 (100%)	28 (100%)		
G2	7 (5.8%)	0 (0%)	0 (0%)		

IC, induction chemotherapy; ALT, alanine aminotransferase; AST, aspartate transaminase; χ2/F*, Chi-square test or Fisher Freeman-Halton test.

inflammatory disease, which makes patients more suitable for immunotherapy.

In recent years, the blockade of inhibitory immune checkpoints programmed death-1/programmed death ligand-1 (PD-1/PD-L1) has made a breakthrough treatment advance. The efficacy and safety of many PD-1/PD-L1 inhibitors have previously been demonstrated in patients with refractory for recurrent or metastatic nasopharyngeal carcinoma (RM-NPC). One of the studies was on the efficacy and safety of camrelizumab in RM-NPC. The results of the phase I trials showed that 34% (95% CI 24-44) of evaluable patients had an overall response with a median follow-up of 9.9 months (24). The combination of camrelizumab and GP regimen showed promising anti-tumor activity and manageable safety. Subsequently, randomized phase III trials explored the addition of camrelizumab to the GP regimen as the first-line treatment for RM-NPC. 263 eligible patients were randomly assigned to the camrelizumab or the placebo group. Results of the evaluation showed that the progression-free survival (PFS) was significantly prolonged in the carellizumab group (9.7months vs. 6.9months, *p*=0·0002), and the most common grade 3 or above toxicities were hematotoxicity in both groups (18). Another multicenter randomized phase III trial included 289 patients with RM-NPC, mainly evaluating the efficacy and safety of the GP regimen combined with toripalimab or placebo in the first-line treatment. The prespecified interim PFS analysis showed that the PFS in the toripalimab group significantly improved compared to the placebo group (11.7 versus 8.0 months, HR = 0.52 (95%CI: 0.36-0.74), p = 0.0003). The grade 3 or higher adverse events were similar between the two groups (89.0 versus 89.5%). Based on these two studies, toripalimab and camrelizumab were approved for the treatment paradigms of RM-NPC in China.

Based on the preclinical evidence and promising results of anti-PD-1/PDL-1 immune checkpoint inhibitors in RM-NPC, the addition of immunotherapy in the current combined treatment modalities for LA-NPC is a hot clinical research topic, and many prospective phase II or III trials are ongoing (25). Ongoing studies mainly focus on immunotherapy's role in locally recurrent NPC. For LA-NPC at initial diagnosis, the phase III clinical trials on adding camrelizumab, toripalimab, or sintilimab to IC and CCRT are currently in the participant recruitment phase (NCT04453826, NCT03700476, NCT04557020, NCT04557020) (25).

Since August 2020, our hospital has been trying to add camrelizumab or toripalimab to IC for newly diagnosed LA-NPC, so we conducted this retrospective analysis. Our retrospective data showed that the addition of anti-PD-1 immunotherapy to IC has a remarkable short-term efficacy, which is significantly better than that of patients with simple IC or IC combined with targeted therapy (anti-EGFR), and the frequency of any of the common adverse effects between the three groups are similar (p >0.05). For primary nasopharynx lesions, the ORR rate of IC, anti-EGFR and anti-PD-1 group were 68.60% (83/121), 67.9%(19/28)and 94.7% (54/57), respectively (χ 2 = 17.30 p < 0.001). The short-term efficacy of cervical lymph nodes also was evaluated: the ORR rate of IC, anti-EGFR and anti-PD-1 group were 78.5% (95/121), 93.0%(53/57)and 71.4% (20/28), respectively (χ 2 = 7.60, p = 0.021). After overall treatment, the ORR rate of nasopharyngeal lesions and cervical lymph nodes in all patients was close to 100%. Only three patients who received IC before radiotherapy were evaluated as SD (2 nasopharyngeal lesions and one cervical lymph node). Further analysis found that the CR rate of nasopharyngeal lesions in the anti-PD-1 group was significantly higher than the IC or anti-EGFR group (84.2% vs. 52.9% vs. 53.6%, χ 2 = 18.240, p <0.001). However, because the specific induction chemotherapy regimen was not uniform in our retrospective study, we conducted a subgroup analysis of the GP regimen combined with anti-PD-1 immunotherapy and GP regimen according to the sample size of patients. However, because the specific induction chemotherapy regimen was not uniform in our retrospective study, we conducted a subgroup analysis of the GP regimen combined with anti-PD-1 immunotherapy and GP regimen according to the sample size of patients. Subgroup analysis revealed that the ORR rate of primary nasopharynx lesions in the GP group and GP + anti-PD-1 group was 72.7% vs. 95.7%, which was statistically significant (χ 2 = 9.984, p = 0.002). In particular, the CR rate of primary nasopharynx lesions in the GP + anti-PD-1 group was significantly higher than in the GP group, which were 17.0% and 1.5%, respectively.

In addition, previous studies have demonstrated that epidermal growth factor receptor (EGFR) is closely related to the malignant transformation of squamous cells and the proliferation of tumor cells. Molecular-targeted therapies using EGFR as the target is widely used in the clinical treatment of head and neck tumors. It is known that the levels of EGFR are overexpressed in more than

70% of patients with NPC and are closely associated with poor prognosis (26, 27). Nimotuzumab is the first monoclonal antibody authorized in China for EGFR-targeted therapy. The combination of CCRT with nimotuzumab has shown an excellent efficacy and safety profile in the treatment of NPC. A retrospective study of 730 patients with stage III-IVb NPC showed that the 5-year OS rate in the CCRT plus nimotuzumab group was significantly higher than that in the CCRT group (88.91% versus 78.30%, p = 0.006). The preliminary results of a recent phase III clinical study came from the 2022 ASCO annual meeting, which included 482 patients with locally advanced NPC. The results showed that the OS in the nimotuzumab combined with the CCRT group were significantly higher than those in the control group (76.9% versus 64.3%, log-rank = 4.125, p = 0.042), and the adverse effects were similar in both groups (15). However, there is currently no high-quality study on adding nimotuzumab to induction chemotherapy in the treatment of LA-NPC. Our study showed that adding nimotuzumab to IC did not significantly increase the ORR rate of patients. This might be attributed to the short follow-up period and the insufficient number of cases. Furthermore, the clinical practice of LA-NPC patients involves the application of various IC regimens, such as TPF, TP, PF, and GP, all of these chemotherapy regimens showed similar clinical efficacy and favorable safety profiles in their respective clinical studies (10–14). We made a subgroup analysis of the short-term efficacy of 121 patients treated with induction chemotherapy alone. There was no significant difference in ORR rates of cervical lymph nodes and primary nasopharynx lesions between the GP, TP, and TPF groups.

Nevertheless, we acknowledge that several limitations exist concerning this study. First, this is a retrospective study with a small number of patients, especially only 28 patients who received IC combined with targeted therapy, which may have resulted in potential selection biases. Furthermore, the IC regimens in this study were not unified, although our subgroup analysis showed no significant among-protocol difference in short-term efficacy. Finally, due to the short follow-up time in this study, only the short-term efficacy was assessed, but no long-term efficacy indicators such as OS or DFS were measured. Despite the above limitations, this study emphasizes that combining anti-PD-1 immunotherapy and chemotherapeutic drugs in IC regimens can significantly enhance the short-term efficacy of patients with LA-NPC.

Conclusions

In conclusion, our results showed that the addition of anti-PD-1 immunotherapy on the basis of induction chemotherapy significantly improved the short-term efficacy of locoregionally advanced nasopharyngeal carcinoma and toxicities were tolerable. However, its long-term efficacy should be confirmed by further follow-up and phase III randomized clinical trials.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the institutional ethics committees of Cancer Hospital, Chinese Academy of Medical Sciences. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JJ and YZ were responsible for the primary concept and the design of the study. XX and PC performed the data capture and analysis. XX drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Cell-based and cell-free immunotherapies for glioblastoma: current status and future directions

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Glioblastoma (GBM) is among the most fatal and recurring malignant solid tumors. It arises from the GBM stem cell population. Conventional neurosurgical resection, temozolomide (TMZ)-dependent chemotherapy and radiotherapy have rendered the prognosis of patients unsatisfactory. Radiotherapy and chemotherapy can frequently induce non-specific damage to healthy brain and other tissues, which can be extremely hazardous. There is therefore a pressing need for a more effective treatment strategy for GBM to complement or replace existing treatment options. Cell-based and cell-free immunotherapies are currently being investigated to develop new treatment modalities against cancer. These treatments have the potential to be both selective and successful in minimizing off-target collateral harm in the normal brain. In this review, several aspects of cell-based and cell-free immunotherapies related to GBM will be discussed.

KEYWORDS

temozolomide, cell-based immunotherapies, cell-free immunotherapies, treatment strategy, glioblastoma

1 Introduction

As the most common type of primary intracranial tumor, gliomas develop from a variety of neuroglial cells in the brain. According to the 2016 World Health Organization (WHO) histopathological and clinical criteria, gliomas are classified as grades I-IV (1). The use of Roman numerals in the intra-tumor grading system raises the risk of confusion between 'II' and 'III' or 'III' and 'IV', and uncorrected typographical errors may compromise treatment outcomes (2). The WHO Central Nervous System (CNS) 5 of 2021 recommends grading using Arabic numerals, where WHO grade 1 gliomas are usually considered benign, curable by complete surgical excision and rarely evolve into more

advanced lesions (3). In contrast, WHO grade 2 or 3 gliomas (mesenchymal astrocytomas and mesenchymal/malignant gliomas) are aggressive, progress to more advanced lesions and have a poorer prognosis (1, 2, 4). WHO grade 4 tumors are highly malignant and present with a poor prognosis (4).

Glioblastoma (GBM) has been described as a grade 4 tumor by the WHO and is among the most fatal and recurring malignant solid tumors to date (5) accounting for 57% of all gliomas and 48% of primary CNS malignancies (6). The median survival of GBM patients is 14.6 months. GBM is presumably caused by Glioblastoma stem cells (GSC), which have rapid self-renewal and a high rate of appreciation, and decreasing GSC is useful in limiting the progression of GBM (7, 8). Since 2005, the Food and Drug Administration (FDA) has authorized only two medications and one device for the treatment of GBM, namely temozolomide (TMZ) (9), bevacizumab (BVZ) (10) and Therapeutic Tumor Fields (TTFields) (11). The prognosis of GBM patients is still unsatisfactory despite decades of efforts and advances in surgery, radiotherapy and chemotherapy. The reason for this result is directly linked to the tumor immune microenvironment of GBM. Due to the existence of the blood-brain barrier, there are very few immune cells from the blood circulation in the brain parenchymal under physiological circumstances (12). When a tumor forms, multiple types of immune cells can move to the tumor region, either to exhibit antitumor effects or to be affected by tumor cells to create an immunosuppressive phenotype while cause suppressive functions (13, 14). At this period, inflammatory variables rule the suppressive immune microenvironment of GBM, which promotes tumor development. This discouraging clinical outcome has made GBM an urgent topic for cancer research. Here, we will discuss the progress made by immunotherapy in the treatment of GBM in recent years.

As early as the mid-nineteenth century, it was proposed that cancer treatment could be achieved by modulating the body's immune system to combat cancer (15). Its distinct scientific and clinical benefits have given rise to the idea of immuno-oncology, which is to enhance the immune response to tumor cells through the adaptive or innate immune system, eliminating them while reducing collateral damage (16). The evaluation of the therapeutic effect of glioma has always been a difficult clinical problem. RANO/iRANO proposed by Harvard Medical School has been recognized by the neurooncology community as a therapeutic response evaluation standard for high-grade glioma, and has also become a common evaluation standard for high-grade glioma clinical trials (17). Both criteria have significant characteristics. The limitation of RANO criteria is that if patients receive immunotherapy, their immune response is different due to different constitutions. However, iRANO standard does not require a large number of case verification, but is constantly discussed and verified by experts in clinical practice. This is why immuno-oncology medications, including cellular treatments, oncolytic virus immunotherapy, and immunological checkpoint blockade therapy are being researched intensively. In this paper, a wide range of possibilities for a new generation of cell-based and cell-free immunotherapies is demonstrated, such that the recent history of GBM immunotherapy can be summarized (Figure 1).

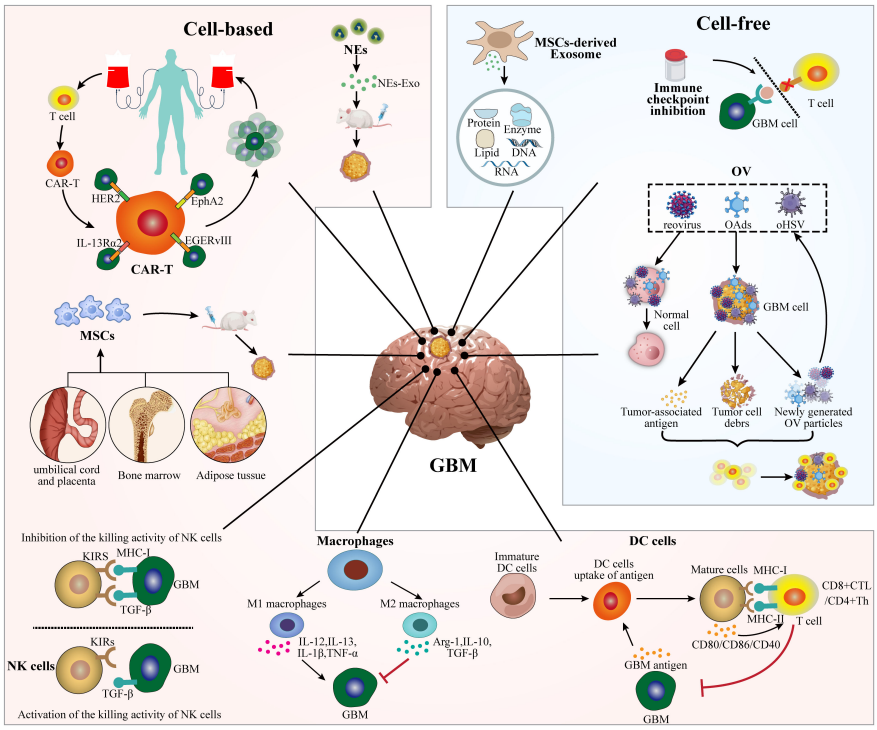

FIGURE 1
Schematic representation of cell-based and cell-free immunotherapies for GBM.

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2 Cell-based Immunotherapies for GBM

2.1 CAR-T cell therapy

Chimeric antigen receptor (CAR) T cell therapy holds one of the most promise as an anti-cancer therapeutic technology. CAR are synthetic molecules formed by four regions, the antigen recognition structural domain (variable region of monoclonal antibodies with a single chain variable fragment scFv), the extracellular hinge region, the transmembrane structural domain (consisting of a hydrophobic α-helix across the cell membrane) and the intracellular T cell signaling structural domain (zeta ζ signaling chain) (18) intended to guide T cells to particular antigens. First generation CAR is a fusion protein consisting of an extracellular antigen-binding domain, generally in the form of a single-chain variable fragment of an antibody, attached to an intracellular signaling domain, most often the CD3ζ chain of the T cell receptor (TCR) (18). In second-generation CAR, the activity of CAR-T cells is enhanced through the addition of co-stimulatory structural domains fused to CD3ζ, such as CD28 or CD137 (also referred to as 4-1BB), and the involvement of these intracellular signaling domains improves anti-apoptosis, cytokine secretion, T cell proliferation and *in vivo* persistence (19). Third generation CARs that incorporate multiple co-stimulatory structural domains (e.g. CD28-41BB, CD28-OX40), have also been developed (19). Fourth generation CAR, also known as TRUCK or armored CAR, have been further augmented with factors that enhance anti-tumor activity, persistence and T cell expansion. These potentially include cytokines such as IL-2, IL-5, IL-12, enzymes that degrade the extracellular matrix of solid tumors and co-stimulatory ligands (20). The fifth generation CAR is based on the second generation CAR with the addition of co-stimulatory structures and domains that activate other signaling pathways and is still in the development stage (Figure 2).

CAR-T cells were first used to treat hematological malignancies and have shown remarkable efficacy (21, 22). For example, the FDA has authorized two medicines for the treatment of hematological malignancies. The first one is Tisagenlecleucel for treating B-cell acute lymphoblastic leukemia (21) and the other is Axicabtagene Ciloleucel for treating large B-cell lymphoma. These CAR-T cells target CD19 on B cells to induce effective tumor cell death (22). Given the extraordinary success in hematological malignancies, CAR-T therapy in solid tumors has also been a rapidly developing research hotspot in recent years. These include interleukin 13 receptor alpha 2 (L13Rα2), epidermal growth factor receptor variant III (EGFRvIII), type A liver ligand protein receptor 2 (EphA2) and human epidermal growth factor receptor 2 (HER2), all of which have been tested as targets for several clinical CAR-T cell therapies (Table 1). However, identifying good antigens in solid tumors is always a challenge, and such antigens have to be highly expressed on most cancer cells but virtually absent in normal tissue (23). Under such conditions, CAR-T cells can't be efficiently transported to the center of solid tumor masses, and the adverse tumor microenvironment (TME) inhibits T cell activity (24).

2.1.1 IL-13Rα2

IL-13Rα2 as a target for CAR-T in the treatment of GBM. It's a membrane receptor with a high affinity for the anti-inflammatory cytokine interleukin 13 and it has been discovered to be overexpressed in a variety of solid tumors, most notably GBM, and has been related to poor prognosis (25). IL-13Rα2 is overexpressed in 76% of GBM, but not in the normal brain tissue, which makes it a highly selective target for immunotherapy (26).

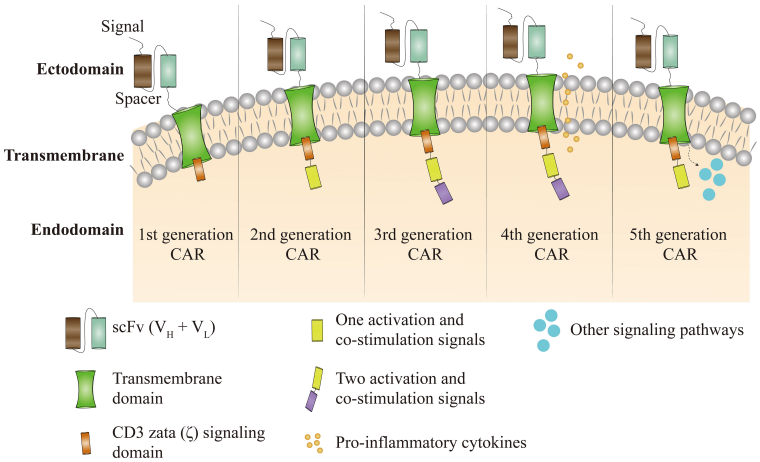

FIGURE 2
Figure 2 CAR-T cell therapy has been through five technical generations. The first generation of CAR depended only on CD3ζ to activate T cells. Clinical effectiveness is limited by a lack of intracellular co-stimulatory signaling, which prevents persistent T-cell proliferation and long-term anti-tumour effects. To the first generation CAR, the second generation CAR incorporates activation and co-stimulatory signals such as CD28 or CD137. Third generation CAR supplement first generation CAR with two co-stimulatory and activation signals, such as CD28, CD137, CD134, and OX40. Based on the third generation of CAR, the fourth generation of CARs incorporates pro-inflammatory cytokines like as IL-12 and co-stimulatory ligands with the goal of overriding tumor immune microenvironment suppression. The fifth generation of CAR is still under development and is based on the second generation of CAR with the inclusion of co-stimulatory structural domains that activate additional signaling pathways.

TABLE 1 CAR-T cell based clinical studies in GBM patients that have been completed or are ongoing.

Molecular target	Clinical trial NCT number and title	Study phase	Interventions	Study Results	Sponsor/Collaborators
IL13Rα2	NCT04003649 Evaluate IL13Rα2-Targeted Chimeric Antigen Receptor (CAR) T Cells Combined with Checkpoint Inhibition for Patients with Resectable Recurrent Glioblastoma	I	Biological: IL13Ralpha2-specific Hinge-optimized 4-1BB-co-stimulatory CAR/ Truncated CD19-expressing Autologous TN/MEM Cells Biological: Ipilimumab Biological: Nivolumab	Recruiting	City of Hope Medical Center (National Cancer Institute)
	NCT04661384 Evaluate IL13Rα2-Targeted Chimeric Antigen Receptor (CAR) T Cells for Adult Patients with Leptomeningeal Glioblastoma, Ependymoma or Medulloblastoma	I	Biological: IL13Ralpha2-specific Hinge-optimized 41BB-co-stimulatory CAR Truncated CD19-expressing Autologous T-Lymphocytes	Recruiting	City of Hope Medical Center (National Cancer Institute)
	NCT02208362 Cellular ImmunoTx Using Memory Enriched T Cells Lentivirally Transduced to Express an IL13Rα2-Specific, Hinge-Optimized, 41BB-Costimulatory Chimeric Receptor and a Truncated CD19 for Pts with Rec/Ref MaligGlioma	I	Biological: IL13Ralpha2-specific Hinge-optimized 4-1BB-co-stimulatory CAR/ Truncated CD19-expressing Autologous TN/MEM Cells Biological: IL13Ralpha2-specific Hinge-optimized 41BB-co-stimulatory CAR Truncated CD19-expressing Autologous T-Lymphocytes	After treatment with IL13Rα2-targeted CAR T cells, GBM regression was observed, and this clinical response persisted for 7.5 months (doi: 10.1056/NEJMoa1610497)	City of Hope Medical Center (National Cancer Institute; Food and Drug Administration)
EGFRvIII	NCT02209376 Autologous T Cells Redirected to EGFRVIII-With a Chimeric Antigen Receptor in Patients With EGFRVIII+ Glioblastoma	I	Biological: CART-EGFRvIII T cells	After 18 months of follow-up, only one of ten treated GBM patients exhibited residual stable disease (doi: 10.1126/scitranslmed.aaa0984)	University of Pennsylvania (University of California, San Francisco)
	NCT03726515 EGFRvIII-Directed CAR T Cells Combined With PD-1 Inhibition in Patients with Newly Diagnosed, MGMT-Unmethylated Glioblastoma	I	Biological: CART-EGFRvIII T cells Biological: Pembrolizumab	Completed	University of Pennsylvania
	NCT01454596 CAR T Cell Receptor Immunotherapy Targeting EGFRvIII for Patients with Malignant Gliomas Expressing EGFRvIII	I/II	Biological: CART-EGFRvIII T cells transduced PBL Drug: Aldesleukin Drug: Fludarabine Drug: Cyclophosphamide	Median overall survival was 6.9 months. Two patients survived over one year, and a third patient was alive at 59 months. (doi: 10.1097/CJI.0000000000000260)	National Cancer Institute
	NCT04197934 WSD0922-FU for the Treatment of Glioblastoma, Anaplastic Astrocytoma, or Non-small Cell Lung Cancer with Central Nervous System Metastases	I	Drug: EGFR/EGFRvIII Inhibitor WSD0922-FU Procedure: Therapeutic Conventional Surgery	Recruiting	Mayo Clinic (National Cancer Institute; Wayshine Biopharm, Inc.)
HER2	NCT01109095 CMV-specific Cytotoxic T Lymphocytes Expressing CAR Targeting HER2 in Patients with GBM (HERT-GBM)	I	Biological: HER.CAR CMV-specific CTLs	clinical benefit for 8 of 17 patients (doi: 10.1001/jamaoncol.2017.0184)	Baylor College of Medicine (The Methodist Hospital Research Institute; Center for Cell and Gene Therapy)
	NCT03389230 Memory-Enriched T Cells in Treating Patients with Recurrent or Refractory Grade III-IV Glioma	I	HER2(EQ)BBζ/CD19t+ T cells	Recruiting	City of Hope Medical Center (National Cancer Institute)

In preclinical studies, Christine E. Brown et al. assessed the potential of immunotherapy targeting IL13Rα2 to eliminate GSCs and heavily differentiated populations. This research looked at GSCs as a possible treatment resistance barrier in tumor cells (27). Despite preclinical studies showing that CAR-T cells can produce effective anti-glioma *in situ* mouse models, this approach has not yet been validated in patients. In 2015, they published the first promising human clinical study of intracranially administered IL13Rα2-specific CAR-T cells for GBM, which set the stage for the future application of improved peripatetic CAR-T cells therapy (28). This

was followed by a case study they published the following year, with the administration of CAR-modified T cells targeting the tumor-associated antigen IL13Rα2 to a patient with recurrent multifocal GBM (29). Regression of all intracranial and spinal tumors was observed subsequent to CAR-T cell therapy, and there was a corresponding increase in cytokine and immune cell levels in the cerebrospinal fluid. Such clinical responses persisted for 7.5 months after the initiation of CAR T-cell therapy (ClinicalTrials.gov, NCT02208362) (29). However, these studies also highlight a few obstacles to achieving more sustained clinical outcomes. First, tumor heterogeneity may promote relapse through the supply of a subsequently scalable pool of target-deficient tumor cells. To address this issue, there is a need to find CAR-T cell approaches that target multiple antigens. Secondly, an absence of persistence of therapeutic CAR-T cells may be another major factor. To address these limitations, Christine E. Brown et al. (30) describe the optimization of IL13Rα-specific CAR-T cells that contain a 4-1BB (CD137) co-stimulatory structural domain (IL13BBζ) to enhance the anti-tumor potency of the IgG4 Fc spacer (L235E, N297Q) and mutation reduction with Fc γ receptor binding. Enhanced anti-tumor activity and T cell persistence in patients with IL13BB-CAR-T cells as compared to first-generation IL13-CAR CD8+ indicates the biological activity of T cells. Given the widespread use of corticosteroids in the clinical care of GBM, they evaluated their effects and found that modest dosages of dexamethasone did not impair the anti-tumor efficacy of CAR-T cells *in vivo*. Local intracranial delivery of CAR-T cells has also been reported to have greater anti-tumor activity than intravenous administration. In another investigation, the antigen-binding domain of newly created IL13Rα2-specific CARs was mutated forms of IL13. Although these CARs target IL13Rα2, they also recognize IL13Rα1, which is broadly expressed. Giedre Krenciute et al. (31) created a set of IL13Rα2-specific CARs with IL13Rα2-specific scFv 47 as antigen-binding domains, short or long spacer regions, transmembrane domains, and intracellular domain molecules derived from co-stimulation and CD3.ζ. In co-culture and cytotoxicity studies, IL13Rα2-CAR T cells detect IL13Rα2-positive target cells but do not cross-react with IL13Rα1. Only IL13Rα2-CAR T cells with a short spacer region, on the other hand, generated IL2 in an antigen-dependent way. T cells expressing IL13Rα2-CAR with a short spacer region and the internal domains CD28.ζ, 41BB.ζ, and CD28.OX40.ζ demonstrated significant anti-glioma activity *in vivo*. Overall, CAR-T cell therapy has the potential to become an effective approach for the clinical management of brain tumors.

2.1.2 EGFRvIII

The epidermal growth factor receptor (EGFR), as the first tyrosine kinase receptor to be cloned, is still at the leading edge of targeted cancer therapy. Being the most common variant of EGFR, EGFRvIII is usually expressed in GBM (32) and is also detected in many epithelial cancers, but not in normal tissues. It is caused by an in-frame deletion in exons 2 to 7 of the EGFR gene and the creation of a new glycine residue at the junction of exon 1 and exon 8. This mutant receptor has constitutive activity in tumors and can lead directly to the cancer phenotype because of its oncogenic nature.

Overexpression of EGFRvIII is considered as a poor prognostic marker, independent of other factors such as age and extent of resection, and may be partly due to its oncogenic nature conferring stability and a persistent tumorigenic signal. Peptide vaccine strategies (rindopepimut) targeting EGFRvIII mutant oncoproteins is a therapeutic approach (33), and secondary immunotherapy using redirected T cells does not require the presentation of antigens and stimulation of primary immune responses and may have more favorable kinetics as compared to vaccines. A neoepitope of EGFRvIII is induced by an in-frame loss of portion of the extracellular structural domain. Based on the success of mouse scFv-based CARs in a GBM xenograft model, Laura A. Johnson et al. (34) chose a vector backbone encoding second-generation CARs. In xenograft subcutaneous and *in situ* models of human EGFRvIII+GBM, EGFRvIII-targeted CAR T cells were also able to suppress tumor development. They also planned a phase I clinical research utilizing humanized scFv-transduced CAR T cells targeting EGFRvIII in patients with residual or recurrent GBM based on these findings (ClinicalTrials.gov, NCT02209376) (35). A first-of-its-kind human study was conducted by Donald M. O'Rourke et al. (35) in which a single dose of autologous T cells was redirected to EGFRvIII mutations by CAR for intravenous infusion. The result showed that single dose of peripherally infused EGFRvIII-directed CAR-T cells mediates antigen loss and induces adaptive resistance in patients with relapsing GBM. However, the major challenges to clinical success of this treatment are the heterogeneity of EGFRvIII expression and the suppressive tumor milieu, which is increasingly immunosuppressive after CAR-T cells. The former requires new antigens to be targeted, while the latter may be circumvented by current medications that target immunosuppressive molecules. Animal studies have indicated that an additional 4-1BB co-stimulatory signaling promotes tumor persistence and localization (31), hence the third-generation construct was chosen for clinical trials. Stephanie L. Goff et al. (36) used a third-generation chimeric antigen receptor construct produced from a human antibody in a dose-escalation phase I study in patients with recurrent GBM expressing EGFRvIII (ClinicalTrials.gov, NCT01454596). Anti-EGFRvIII-CAR+ T cells were treated with infusion products in 18 patients. All patients experienced the expected transient hematological toxicity from preparations of chemotherapy, and the median overall survival of patients was 6.9 months, with two patients surviving for more than a year and a third patient surviving for 59 months. The persistence of CAR+ cells correlated with cell dose, but there was no objective response. However, the Administration of anti-EGFRvIII CAR-transduced T cells in this phase I pilot trial did not show a clinically meaningful effect in patients with polymorphic GBM.

2.1.3 EphA2

The EphA2 receptor is a member of the Eph family of receptor tyrosine kinases. Although EphA2 overexpression is a crucial antigen in the maintenance of the malignant GBM phenotype, EphA2 is not expressed in normal brain tissue (37). Targeting

EphA2 could prevent tumor immune escape, and Jill Wykosky et al. proposed that EphA2 could be a novel molecular marker and target for GBM (38). CAR-T treatment for GBM must guarantee that such antigens are abundantly expressed on most tumor cells but are generally lacking in normal tissues. Therefore, EphA2 has already proven to be a successful target antigen for CAR-T immunotherapy for GBM. A development of an EphA2-specific CAR was reported by Kevin KH Chow et al. (39) to redirect T cells to EphA2-positive GBM *in vitro* with the aim of identifying and killing EphA2-positive glioma cells and glioma-initiating cells, as well as inducing tumor induction in an *in situ* xenografted GBM with Severe Combined Immunodeficiency (SCID) mouse model of regression. However, such manipulations are still carried out through highly artificial means and may not better predict future clinical effectiveness. H.T. Lin et al. (40) conducted the first human study of EphA2-redirected CAR T cells in EphA2-positive recurrent GBM patients. A single intravenous infusion of EphA2-redirected CAR T cells was combined with a lymphocyte clearance regimen of fludarabine and cyclophosphamide. Two patients had grade 2 cytokine release syndrome with pulmonary edema, which was entirely cured with dexamethasone medication therapy, with most cytokines reverting to normal as the edema dropped. The pulmonary edema observed in these patients may be due to an "on-target, off-tumor" effect. However, the possibility of "off-target, off-tumor" lung organ cytotoxicity cannot be completely ruled out in the study. There were no additional organ toxicities, including neurotoxicity. They detected CAR T cell growth in peripheral blood and cerebral fluid for more than four weeks. The tumour shrank metastatically in one patient. One patient had stable disease, while the other two had progressing disease, with an overall survival of 86 to 181 days. At the dose level tested, the intravenous infusion of EphA2 redirected CAR T cells was initially tolerated with transient clinical efficacy. Future research will be needed to modify the dose and frequency of CAR T cell infusions. Zhongzhen Yi et al. (41) showed the anti-tumour effectiveness of third-generation CAR-T cells targeting distinct EphA2 epitopes against GBM. While there have been substantial advances in the clinical effectiveness of EphA2 redirected CAR-T cells for GBM, the anti-tumour effects of CAR-T cells generated in different labs or by different methods remain uneven. Several parameters, including target antigen affinity, off-target toxicity and terminal differentiation, could influence the anti-tumour effects of CAR-T cells, therefore future research on CAR-T cells against GBM are still in its early stages.

2.1.4 HER2

HER2, an epidermal growth factor receptor protein encoded by the ERBB2 gene, has been found to be over-expressed as a tumour-associated antigen in 80% of GBM cells, signaling a poor prognosis, but not in normal neurons or glial cells (42). HER2 is now being aggressively targeted as a cell surface protein in GBM-directed CAR-T cell treatments in preclinical models. Nabil Ahmed et al. (43) identified HER2-specific T cells to target primary GBM stem cells and induce autologous experimental tumour regression. In a phase I clinical trial, Nabil Ahmed et al. (44) revealed that the infusion of autologous HER2-specific embedded CAR-modified virus-specific T cells (VST) is safe and is potentially linked to clinical benefit in patients with progressive GBM (ClinicalTrials.gov, NCT01109095). These cell lines are enriched with cytomegalovirus, Epstein-Barr virus and adenovirus. In this study, they determined the safety of autologous HER2-CAR VST in 17 patients with progressive GBM, with no serious adverse events. 8 patients showed clinical benefit, with a median overall survival of 11.1 months after T-cell infusion and 24.5 months after diagnosis, and 3 patients were alive at last follow-up with no disease progression. However, the efficacy of HER2-CAR VST as a single agent or in combination with other immunomodulatory approaches for the treatment of GBM needs to be further evaluated in phase 2b studies.

2.1.5 Multi-antigen targeted CAR-Ts

Regardless of the major breakthroughs in clinical efficacy, every CAR-T therapy has certain drawbacks when it comes to treating GBM (30, 35, 41, 44). This is due in large part to the fact that safe, specific and homogeneously expressed targets are more difficult to identify, which suggests that there are few antigens that are truly tumour-specific and consequently the cross-reactivity of engineered T cells with normal tissues for targeting/non-tumour can lead to lethal toxicity (45–48). Rather, these targets are often heterogeneously expressed even when antigens with high tumour specificity are identified, and selective CAR targeting can allow antigen-negative tumour cells to escape (35). GBM is a prime example of this dual challenge, and several of these issues that have impeded the efficacy of CAR-T cells need to be addressed during the treatment of GBM. Therefore, in recent years, more and more CAR-T therapies targeting multiple antigens have been proposed, thus avoiding the problems of tumour specificity and heterogeneity associated with single CAR-T therapies. As revealed by Masasuke Ohno et al. (49), expression of MicroRNA (miR) -17-92 augments the anti-tumour activity of T-cells transduced with the anti-EGFRvIII chimeric antigen receptor in mice bearing human GBM xenografts. Meenakshi Hegde et al. (50) used two glioma antigens, HER2/IL-13Rα2 bivalent T-cell products, both of which counteracted antigen escape and enhanced T-cell effector function. However, site-specific antigen pairs are variably different between patients and therefore require the generation of permutations of bivalent T-cell products, which would make the successful clinical translation of this approach challenging. Kevin Bielamowicz et al. (51) created for the first time a trivalent T-cell product, i.e. a single CAR-T cells product using 3 targetable glioma antigens (HER2, IL13Rα2 and EphA2) for broader application. Trivalent CAR-T cells have the potential to overcome antigen heterogeneity in GBM and improve treatment results. Furthermore, Joseph H. Choe et al. (52) hypothesized that T cells recognizing various antigen combinations give a potential solution to the issue of maximizing tumour identification specificity and killing integrity at the same time. SynNotch receptors that identify particular priming antigens in GBM (53), such as the highly tumour-specific GBM neoantigen EGFRvIII or the CNS tissue-specific antigen myelin oligodendrocyte glycoprotein (MOG), can be employed to homogeneously trigger CAR production in tumors. EGFRvIII

expression in tumors is extremely selective, and while CAR-T cells successfully destroy EGFRvIII+ tumour cells, EGFRvIII- tumour cells can escape and thrive (34, 35, 49). EGFRvIII has specificity but is heterogeneous, as opposed to EphA2 and IL13Ra2, both of which are more homogenous but only partially specific for tumors. Due to their unique flaws, it is possible that these antigens be coupled to form a multi-antigen circuit with both high specificity and the ability to cause death more comprehensively. In addition, because they lacked co-localized priming antigens, EGFRvIII synNotch-EphA2/IL13R2 CAR-T cells were able to efficiently and completely eliminate GBM tumors without destroying surrounding normal tissue or EphA2 or IL13R2-positive cells elsewhere in the body. They also discovered that T cells carrying α-MOG synNotch receptor may be effectively and selectively activated in the CNS body by endogenously produced MOG (54, 55). If these cells are driven to produce α-EphA2/IL13R2 CAR, they will only kill CAR-expressing cells in the CNS, not those transplanted outside the CNS. Ultimately, through the use of circuits incorporating recognition of multiple imperfect but complementary antigens, the specificity, integrity and persistence of the T cells targeted to GBM were improved, and therefore, they managed to provide a general recognition strategy applicable to other solid tumors.

2.2 NEs therapy

Neutrophils (NEs), the most prevalent leukocyte population in the blood, may be rapidly recruited to sites of inflammation, and are thought to be a powerful platform for tumour-targeted drug delivery, similar to mesenchymal stromal cells (MSCs) (56). More importantly, NEs can also penetrate the BBB/BTB and specifically brain tumour sites. Inflammation can activate NEs and is often accompanied by a local inflammatory response in the brain after surgical resection of GBM, with massive release of inflammatory factors. Therefore, the inflammatory TME may be a promising therapeutic strategy for GBM. Nanoparticle-based drug delivery systems (DDSs) are seen as a promising prospective technique for brain-targeted medication delivery (57). Although it has demonstrated the capacity to improve tumour targeting, these DDSs cannot accomplish the full therapeutic potential of postoperative glioma therapy because the predominant distribution of particles is in the perivascular region of recurring tumors and because intratumoural drug concentrations are low (58, 59). Xue Jingwei et al. (60) created a cell-based anti-cancer DDS that uses the physiological features of natural NEs to improve the efficacy of postoperative glioma therapy. Unlike conventional nanoparticles, their accumulation at the tumour site is based on passive targeting, i.e. increased permeability and retention effects, or active targeting *via* ligand-receptor interactions. NE-mediated DDS have the ability to recognize post-operative inflammatory signals such as IL-8 and CXCL1/KC and deliver chemotherapeutic agents to infiltrating glioma cells in a spontaneous and on-demand manner. They used cationic liposomes carrying paclitaxel (PTX) as a delivery vehicle based on NEs to effectively deliver PTX to tumour cells and induce cytotoxicity and inhibit post-operative recurrence of GBM (60). Furthermore, highly concentrated inflammatory signals in the brain after surgery triggered NEs to release liposomal PTX, thus allowing the delivery of PTX to the remaining invasive tumour cells. This suggests that this NE-mediated drug delivery is effective in slowing down recurrent tumour growth. Meiying Wu et al. (56) internalized doxorubicin-loaded magnetic mesoporous silica nanoparticles (D-MMSNs) loaded with the antitumor drug Adriamycin (DOX) into inflammation-activatable neutrophils. It provides magnetic resonance imaging (MRI) to track drug-loaded cells and actively target inflamed brain tumors after surgical removal of the primary tumour, releasing D-MMSNs to be taken up by infiltrating GBM cells, so as to maximize the bioavailability of the drug for accurate diagnosis and high anti-glioma efficacy. However, it has been shown that NEs can be polarized into different functional phenotypes in the TME, while it can also be polarized into N1-type anti-tumour or N2-type pro-tumour phenotypes, i.e. the controversy of having both pro- and anti-tumour effects (61–63). The antitumor activity of N1 TAN includes the expression of more immune activating cytokines and chemokines, reduced arginase levels, and a greater ability to kill tumour cells *in vitro*. As blockade of TGF-β facilitates the accumulation of N1 TAN with antitumor activity, TGF-β is the major proximal cytokine within tumors that defines the TAN phenotype and biases differentiation towards the N2 pro-tumorigenic phenotype (62). To address this controversy, Jun Wang et al. (59) mounted DOX into neutrophil exosomes (NEs-Exos), which are extracellular vesicles with characteristics of NEs. It can produce a chemotactic response to inflammatory stimuli and target infiltrating tumour cells in inflamed brain tumors without the risk of tumour promotion. This is an addition to the current research on NEs for GBM.

2.3 Immunotherapy of MSCs

MSCs are pluripotent stem cells, which are normally derived from bone marrow, umbilical cords/placenta, and adipose tissue. MSCs have tissue healing capability and low immunogenicity, and they are not restricted by the BBB/BTB, so they can be intrinsically subsumed into the brain tumour site (64, 65), overcoming the difficulties of conventional therapy being isolated by the BBB/BTB. That is, MSCs exhibit tropism to the cytokines, chemokines and growth factor-mediated TME. Studies have shown that MSCs and their derived soluble factors exhibit inhibitory effects on the growth of GBM cells, revealing a well-established role for MSCs in the treatment of CNS malignancies (66). Nevertheless, there are certain benefits and drawbacks of MSCs generated from different tissues (Figure 3), and we will explore the mechanism by which MSCs derived from diverse tissues prevent the development of GBM in recent years.

2.3.1 Bone marrow-derived MSCs

MSCs were first identified in bone marrow and their tumour tropism has been used for the delivery of anti-cancer therapeutic genes, but MSCs exact mechanisms in the TME remain unknown. Vy A W Ho et al. (67) investigated the biological effects of MSCs from bone marrow on glioma cells. MSCs limit tumour

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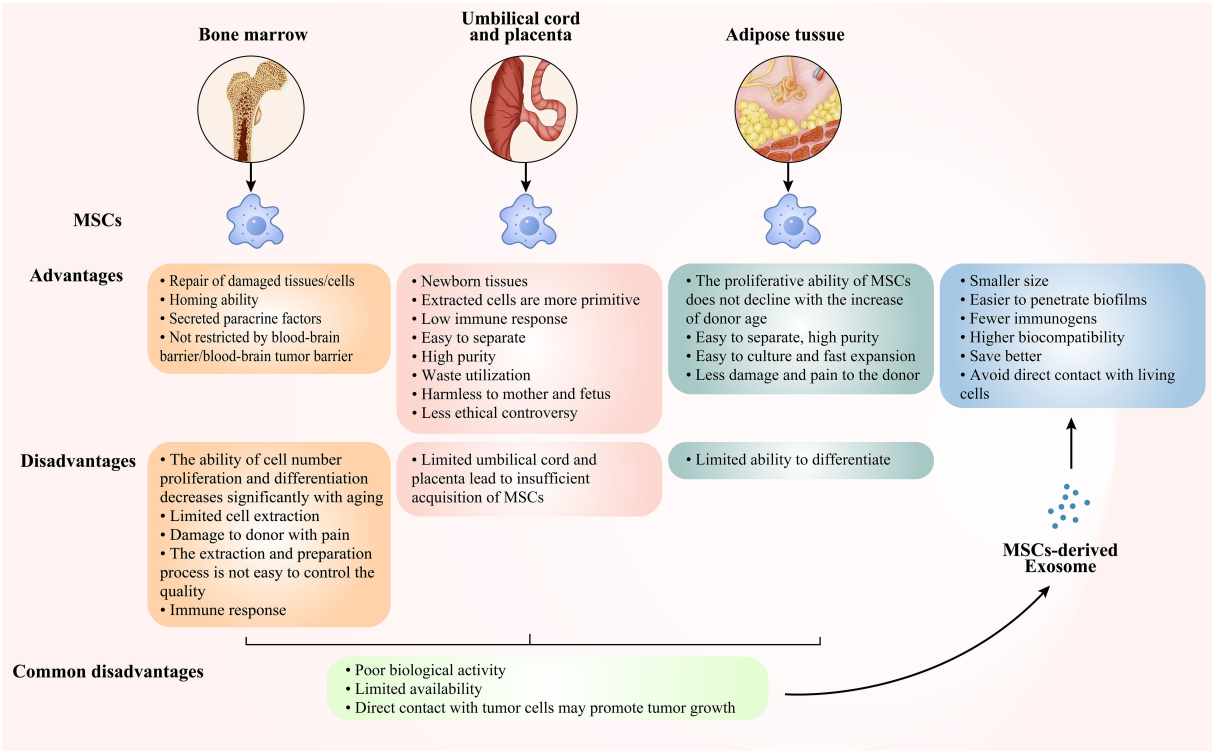

FIGURE 3
Advantages and disadvantages of bone marrow-derived, cord- and placenta-derived, adipose-derived MSCs and MSCs-derived Exo.

angiogenesis by releasing anti-angiogenic factors, according to their findings. Further studies using antibody array technology showed reduced expression of platelet-derived growth factor (PDGF)-BB, IL-1β, phosphorylated Akt and histone B proteins in MSCs/glioma co-cultures. In conclusion, their findings imply that the antitumor actions of MSCs may be mediated by down regulation of the PDGF/PDGFR axis, which is important in glioma angiogenesis. Based on the fact that bone marrow-derived MSCs have been shown to localize to gliomas after intravascular delivery and that these cells are located in inflammatory regions of tissue injury in the TME, Jonathan G. Thomas et al. (68) used ionizing radiation (IR) to increase the tropism of bone marrow MSCs towards GBM. IR is a therapeutic modality that can effectively trigger local damage or inflammation in the TME. According to their results, IR to GSC-derived gliomas increases MSCs tropism, which can be boosted by the chemokine CCL2. Nevertheless, IR can increase vascular permeability by disrupting the BBB (69–72), reduce tight junction proteins (73) or induce endothelial cell damage (74), leaving the mechanism of action of IR in an *in vivo* situation where tumour cells are integrated with supportive cells more unknown. Improving the efficiency of MSCs in the treatment of GBM requires the use of appropriate tools and technical abilities. Extensive research has revealed the promise of suicide genes in the treatment of glioma tumors. Enhancing their effectiveness relies on the ability to apply the right tools and techniques. Saeed Oraee-Yazdani et al. (75) investigated the safety and feasibility of treating patients with primary and secondary polymorphic GBM with lentiviral transduced autologous bone marrow MSC containing herpes simplex virus thymidine kinase (HSV-TK) in combination with intravenous ganciclovir. From the five patients they recruited, it was possible to find that all patients had a 1 year progression-free-survival (PFS) and overall survival (OS) of 60% and 100%, respectively, and two patients had an OS of more than 3 years and a PFS of more than 2 years. This finding suggests that intracerebral injection of bone marrow MSCs expressing the HSV-TK gene, in conjunction with intravenous ganciclovir is safe and practical for treating GBM patients. When recurrent cell infusions are necessary in the same patient, autologous cell sources are frequently used. Some studies have discovered that bone marrow MSCs from healthy donors can be viral carriers (76). However, it is unknown if bone marrow MSCs can be created from chemotherapy-treated glioma patients, or whether such bone marrow MSCs can successfully transmit oncolytic virus. Yuzaburo Shimizu et al. (77) conducted a prospective clinical experiment in which they discovered that bone marrow MSCs could be collected from GBM patients who had previously had chemotherapy and that bone marrow MSCs were efficient carriers of oncolytic virus. Additionally, Nazneen Aslam et al. (78) suggested a possible solution for GSCs and discovered that when actively developing GSCs were treated with paracrine factors from MSCs, the prospective growth capacity and pluripotent of GSCs were disrupted. This effect was mediated by up regulation of the DKK1 gene, which in addition was mediated by up regulation of the Wnt pathway mediated by inhibition of growth factor activity and down regulation of the KITLG gene activated by growth factor and cytokine activity, thus exhibiting antitumor properties. The main

active component of paracrine secretion is extracellular vesicles (EVs), which will be discussed in the Cell-free Immunotherapies for the GBM section. Even so, the proliferation and differentiation capacity of bone marrow-derived MSCs in terms of cell numbers decreases considerably with age, thus leading to limitations in cell extraction. It is also harmful to the donor, the extraction and preparation process is difficult to achieve quality control, and transplantation into humans may trigger an immune response (79, 80). These issues have hampered the therapeutic use of bone marrow MSCs.

2.3.2 Umbilical cord or placental-derived MSCs

The umbilical cord and placenta are novel tissues and the cells removed are primitive. As the cells are young, the functional activity of cell surface antigens is low, making it difficult to stimulate an immune response. It has also been shown to be a waste product that does not cause any harm or damage to the mother or newborn when collected and has a greater capacity for proliferation and differentiation (80–83), so it may be an ideal alternative to bone marrow-derived MSCs. Based on the fact that MSCs exhibit tropism towards cytokines, chemokines and growth factor-mediated TME. Adriana Bajetto et al. (84) examined the effect of umbilical cord MSCs on the growth of GSCs. Umbilical cord MSCs released large amounts of soluble cytokines regarding inflammation, angiogenesis, cell migration and proliferation, such as IL-8, GRO, ENA-78 and IL-6. They regulate GBM cells, either through direct cell-to-cell interactions or indirectly. These cytokine ligands share a receptor, CXC chemokine receptor 2 (CXCR2), so they also assessed the effect of CXCR2 on the proliferation of GSCs induced by umbilical cord MSCs. The results showed that direct (intercellular contact) or indirect (via release of soluble factors) interactions between GSCs and umbilical cord MSCs in co-culture had different effects on anti-GSCs, with the former causing mainly an inhibitory response and the latter a stimulatory response involving paracrine activation of CXCR2. miRs are promising therapeutic targets for GBM, but the difficulties in delivering them to tumour target cells has limited their usage. MSCs can migrate to cancer sites, including GBM, and exert antitumor effects. S Sharif et al. (85) found that delivery of exogenous miR-124 to GBM cells *via* umbilical cord-derived MSCs reduced cell proliferation and migration and conferred sensitivity to the chemotherapeutic agent TMZ. To explore the potential clinical application of gadolinium neutron capture therapy (Gd-NCT) in GBM treatment affected by the rapid clearance and non-specific bio-distribution of gadolinium-based drugs, Yen-Ho Lai et al. (86) developed a stem cell-nanoparticle system (SNS) that actively targets GBM by using gadobisamine-concealed magnetic nanoparticles (Gd-FPFNP) on umbilical cord-derived MSCs was performed to actively target GBM for advanced Gd-NCT. The findings of their study indicate that SNS can potentially overcome the current limitations of Gd-NCT, including off-target effects and rapid metabolism, and that it combines the advantages of cellular therapy and nanotechnology for an alternative strategy to treat brain disorders. However, umbilical cord-derived and placental-derived MSCs are limited in availability, which limits their clinical application (80, 87).

2.3.3 Adipose tissue-derived MSCs

The proliferative potential of adipose tissue-derived MSCs does not reduce with increasing patient age and is less harmful and uncomfortable to the donor (81). Most significantly, because it is easy to extract and has a high potential for self-renewal, adipose tissue-derived MSCs are thought to be a viable alternative source of therapeutic stem cells (87). Mona N. Oliveira et al. (88) emphasized the processes through which adipose-derived MSCs interact with GBM cells, with substantial implications for MSCs in the treatment of GBM. MSC-based gene delivery of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is recognized as a potent therapy for GBM (89, 90). The systemic treatment of TRAIL-secreting stem cells is problematic in that some of these delivery vehicles do not always reach tumour microsatellite nests. Furthermore, as many stem cells are home to normal brain parenchyma and perivascular gaps, TRAIL-laden stem cells are unable to reach tumour microsatellite nests, causing them to remain in normal brain tissue and cause adverse effects such as neuronal cell death. To regulate the expression of suicide inducers and reduce off-target damage, Man Li et al. (91, 92) exploited endogenous tumour signaling pathways to modulate the release of the suicide inducer TRAIL. Findings from their study suggested a significant improvement in the efficacy of adipose MSC-mediated gene delivery for the treatment of GBM. Bahattin Tanrikulu et al. (93), Valentina Coccè et al. (94) also found that the combination of TRAIL-expressing adipose MSCs and multiple drugs (e.g. X-linked apoptosis protein (XIAP) inhibition, XIAP silencing, and octane diamide isohydroxamic acid) or paclitaxel induced GBM cell apoptosis and reduced their proliferation. To improve the effectiveness of adipose-derived MSCs to reach the actual tumour target, Francesco Agostini et al. (95) utilized growth factor-rich supernatant as an additive to adipose MSCs. The results showed that the growth factor-rich supernatant enhanced the specific homing and secretory properties of adipose MSCs towards GBM. However, the ability of adipose-derived MSCs to differentiate into cells is relatively limited.

Despite the numerous benefits of MSCs in the battle against GBM, there are some drawbacks to their use, such as poor biological activity and restricted availability. Additionally, when MSCs come into direct touch with GBM cells, they not only do not operate as tumour suppressors, but instead accelerate tumour development (96). These restrictions add to the inherent dangers of MSCs as live cells.

The ability to homing is critical for MSCs to be employed safely and successfully in therapeutic applications. However, many systemically administered MSCs are lost in patients' substantial organs such as the lungs, liver, and spleen, significantly reducing MSCs' therapeutic usefulness. If they are given a "GPS" to guide them to their final destination, off-target effects can be minimized. To address this problem, research have looked at using pro-inflammatory cytokines (IL-7, IL-12, and TNF-α) and chemokines (CXCR1, CXCR4) to better recruit MSCs to GBM sites (97, 98). In addition to this, the targeting of MSCs to the GBM can be improved by targeting target genes that are specifically highly expressed in the GBM or highly heterogeneous. For

example, Irina V Balyasnikova et al. (99) found that MSCs genetically engineered to express EGFRvIII on the cell surface had increased affinity for GBM target sites. TRAIL is one of the few anti-cancer proteins that selectively causes apoptosis in tumour cells by activating death receptors, while having no effect on healthy cells. Xiang-Jun Tang et al. (100) found that MSCs carrying TRAIL exerted sustained anti-GBM activity. In another preclinical study, MSCs armed with both EGFR-targeting nanoantibodies (ENb) and TRAIL were evaluated to significantly improve the survival of animals in a GBM *in situ* resection model (101). OV selectively replicates and kills cancer cells and spreads within the tumour without harming normal tissue. It also promotes the release of tumour-associated antigens, activates antigen-presenting cells, promotes the activation and aggregation of CD4 + and CD8+ T cells, and directly kills tumors (26). The use of MSCs as a delivery vehicle helps protect the virus from the immune system and improves therapeutic efficiency by enhancing tumour shrinkage (102). Delta-24-RGD is a tumourolytic virus who's binding to MSCs has been shown to selectively target intra-arterially delivered hMSCs-Delta24 to GBM and to deliver and release Delta-24-RGD into tumors, thereby improving survival and tumour eradication in a subpopulation of mice (76). MSCs loaded with oHSV induced significant anti-GBM mechanisms in preclinical models or GBM resection (103). MSCs enhance the tumourolytic effect of Newcastle disease virus on GBM and GSC cells through the secretion of TRAIL (104).

2.4 NK cells therapy

Natural killer (NK) cells play an essential role in the body's anti-infection, anti-tumour, and immunomodulatory processes as recognition and effector cells in the innate immune system. NK cells do not inhibit the killing of their own normal cells, but selectively recognize and kill cells that are low in expression or lack their own major histocompatibility complex (MHC)-I molecules (105). MHC-I molecules are also known as human leukocyte antigen class I (HLA-I) molecules. The binding of killer cell immunoglobulin-like receptors (KIRs) on the surface of NK cells to HLA-I on the surface of target cells induces inhibition of NK cell killing activity (106, 107). When there is a lack of expression of HLA-I on the surface of target cells, the killing activity of NK cells against them can be triggered. The imbalance between KIRs and HLA-I has been shown to trigger NK cells to successfully destroy glioma cells (108). Furthermore, NK cells activity is governed by a variety of signaling factors, and Hila Shaim et al. (109) discovered that GSCs are susceptible to *in vitro* destruction by healthy allogeneic NK cells. Their findings demonstrated that GBM tumour-infiltrating NK cells acquired an altered phenotype associated with impaired lytic function when compared to matching peripheral blood NK cells from GBM patients or healthy donors. This immune evasion approach was attributed to αv integrin-mediated TGF-β activation, which directed interactions between GSCs and NK cells. In contrast, blocking the αv integrin/TGF- β axis can increase NK cell antitumor function. Gregory J Baker et al. (110) found that NK elimination of intracranial neuroGBM was possible in the presence of decreased tumour-derived galactose lectin 1 (gal-1).

The function of monoclonal antibodies like bortezomib and bevacizumab in the antitumor process is getting attention. Andrea Gras Navarro et al., Thi-Anh-Thuy Tran et al. (111, 112) discovered that pretreatment of GBM with the monoclonal antibody bevacizumab increased NK cell cytotoxicity and extended animal life. Relay transfer of CAR-modified NK cells has shown significant anti-glioma activity both *in vitro* and *in vivo*. In contrast to CAR-T cells, which require autologous cells for each patient, NK cells are safe under allogeneic circumstances, which broadens the pool of cell donors capable of producing therapeutically meaningful amounts of CAR-NK cells for therapy (113). In terms of safety, CAR-NK cells outperform CAR-T cells because they operate autonomously on antigen-antibody reactions and do not produce cytotoxic effects, such as cytokine release syndrome, in different studies (114). The inherent characteristics of NK cells make them an appealing option to CAR-engineered effectors in cancer treatment, clearing the way for several clinical studies to further develop the strategy and better its ability to fight glioma cells (114, 115). CAR-NK cells, in brief, identify CAR-targeted antigens and induce NK cell activation, proliferation, and secretion of different inflammatory cytokines and chemokines. When NK cells recognize cancer cells, they establish lytic synapses with them to guide the delivery of lytic granules to susceptible cancer cells while maintaining their normal activating and inhibitory receptors (115). Thus, CAR-NK cells can kill cancer cells that do not exhibit CAR-targeting antigens (CAR non-dependent) as well as cancer cells that do express CAR-targeting antigens (CAR dependent) (116).

2.5 DC cells therapy

Dendritic cells (DC) are now recognized as the most potent and only specialist antigen-presenting cells in the body capable of activating naïve T cells. It is called after the numerous dendritic protrusions that emerge from the cell surface during maturation. Immature and mature dendritic cells perform distinct tasks, with immature dendritic cells being good at antigen differentiation and phagocytosis and mature dendritic cells being good at antigen presentation. DC have MHC class I and II molecules on their surface, which when combined with antigenic peptides trigger CD4 + helper T cells (Th) and CD8+ cytotoxic T lymphocytes (CTL) to elicit specific anti-tumour cellular and humoral immunity, inhibiting tumour cell growth (117). DC also upregulate the expression of cell surface co-stimulatory factors such as MHC-II, CD80, CD86 and CD40, and secrete cytokines such as interleukins and chemokines to stimulate T cell activation, proliferation and aggregation, thereby inducing the activation of an adaptive immune response.

DC vaccines, which are currently being studied extensively in anti-tumour immunotherapy, are based on the powerful antigen-presenting ability of dendritic cells. The key to developing and manufacturing DC vaccines is to allow dendritic cells to carry a marker for the target tumour, which can come from a variety of sources, such as tumour antigen gene modifications (118), synthetic

antigenic peptides, antigen-encoded mRNA or DNA (119), which DC then deliver to T cells. Immune cells such as killer T cells can then accurately and effectively recognize and assault the target tumour cells, significantly decreasing collateral harm to normal cells. According to research, recombinant adenovirus-mediated gene transfer is the most efficient way of changing DC cells (120). GBM secrete a range of immunosuppressive and immune escape factors, such as substantial loss of Fas, to avoid immune killing initiated by the Fas/FasL system (121, 122). Mature dendritic cells improve antigen presentation, activating the Fas/FasL-mediated apoptotic pathway and increasing Fas mRNA, causing a caspase enzyme chain reaction that results in planned cell death. Dendritic cells have no direct killing impact, but they improve immunosurveillance and tumour suppression by improving antigen expression (123). Furthermore, studies have shown that NK and other cells have a glioma-killing impact (124), implying that there is still much space for study into the inhibitory effect of DC-associated killer cells on gliomas. Xin Ma et al. and Haidar A Shamranet al. (125, 126) discovered that glioma cells secreted immunosuppressive factors VEGA and IL-10, which reduced immune cell function, meanwhile Yawen Ma et al. (127) discovered that miR-153 can down-regulate VEGA expression in malignant glioma vessels, inhibiting tumour growth. The first two clinical studies involving DC cell vaccines for the treatment of high-grade gliomas were reported (128). Surasak Phuphanich et al. (129) assessed the findings of a phase I clinical study of the autologous DC cell vaccine ICT107. The vaccine was pulsed with class I peptides from six tumour-associated antigens (TAA) of AIM-2, MAGE1, TRP-2, gp100, HER2/neu and IL-13Rα2, which are expressed on gliomas and over expressed in their cancer stem cell population. The feasibility, safety, and biological efficacy of a TAA-pulsed dendritic cell vaccination in patients with GBM were proven in this phase I study of ICT-107. Based on PFS and OS measures in newly diagnosed GBM patients, AIM2 and MAGE1 antigen expression in pre-vaccination tumors was related to longer survival, whereas HER2 and gp100 expression exhibited a trend toward prolonged PFS and OS. They are conducting a randomized, placebo-controlled phase II study based on these positive results. Patrick Y Wen et al. (130) published the findings of a phase II clinical study in which ICT-107 improved the immunosuppressive microenvironment in newly diagnosed GBM and helped patients overcome tumour heterogeneity, but there was no advantage in terms of total patient mortality (ClinicalTrials.gov, NCT01280552). HLA-A2 primary tumour antigen expression was more frequent than in HLA-A1 patients. HLA-A2 patients had a higher immune response (by Elispot) and patients in the pre-specified subgroups of methylated and unmethylated MGMT achieved meaningful therapeutic benefit with ICT-107. This was the first vaccination study to demonstrate a clinical benefit in GBM, and it paved the way for a phase III trial in patients with HLA-A2+ newly diagnosed GBM. Linda M Liau et al. (131) published interim results from a phase III clinical trial of the autologous tumour cell lysate-loaded dendritic cell vaccine DCVax-L in combination with TMZ dendritic cell vaccine, and the phase III trial results showed promising application (ClinicalTrials.gov, NCT00045968) (Table 2). They later reported that including DCVax-L in standard of care (SOC) resulted in a clinically relevant and statistically significant extension of life for patients with GBM when compared to concurrent (132). The use of autologous DC vaccines pulsed with allogeneic GBM or GBM stem cell line lysates for the therapy of freshly identified and recurring GBM is also safe and well accepted, according to Jethro L Hu et al. and Ian F Parney et al. (133, 134). The above clinical findings contribute to the evidence that immunotherapy may play a part in the treatment of GBM.

2.6 Microglia and Tumour-associated macrophages

Microglia and tumour-associated macrophages (TAMs) are the main components of GBM myeloid cells, which are maintained by self-renewal under physiological conditions and are associated with functions such as CNS inflammation and development (135). Under pathological conditions, especially in GBM, GBM cells release multiple chemokines, such as MCP-1 and CCL2, which allow microglia to activate and accumulate in large numbers around the tumour. At this point, BBB/BTB function is hampered, and monocytes in the blood also penetrate the brain parenchymal *via* the impaired BBB/BTB, and both cells are converted into critical drivers of tumour development by acting as TAMs together to infiltrate at GBM locations (136).

TAMs are the most common immune cells in the TME, and their phenotype is diverse and flexible (137). The bulk of macrophages in tumors are Tumour-promoting TAMs (pTAMs), which interact tightly with tumour cells and thus support tumour growth. pTAMs have the characteristics of M2 macrophages, which are M2 TAMs that support tissue healing and remodeling, Th2 immune response, and tumour progression, and generate Arg-1, IL-10, and TGF-β. pTAMs are the primary factors to the development of an immunosuppressive microenvironment in tumors (138). TAMs are a subset of macrophages in tumors that phagocytose or destroy tumour cells, thereby inhibiting tumour development (139). TAMs have M1 macrophage characteristics, and M1 TAMs exhibit high amounts of pro-inflammatory factors (e.g. TNF-α) and anti-tumour factors IL-12, IL-13, IL-1, and TNF-β, which can boost Th1 responses and tumour-killing capability. Because many malignancies, including GBM and brain metastases, contain significant quantities of tumour growth-promoting pTAMs, recoding pTAMs into sTAMs is a novel approach to successful cancer control and therapy. Wenchao Zhou et al. demonstrated that GSCs can greatly decrease the capacity of TAMs to attract TAMs by silencing periostin (POSTN) secretion, thereby inhibiting tumour development (140). GSC-secreted granulocyte-macrophage colony-stimulating factors (GM-CSFs) produced CD11+ macrophages, a subset of CD11c (high) cells with tumour-promoting activity, according to Yasuhiro Kokubu et al. (141).

TABLE 2 Dendritic cells based clinical studies in GBM patients that have been completed or are ongoing.

Molecular target	Clinical trial NCT number and title	Study phase	Interventions	Study Results	Sponsor/Collaborators
DC	NCT01280552 A Study of ICT-107 Immunotherapy in Glioblastoma Multiforme (GBM)	II	Biological: ICT-107 Biological: Placebo DC	The ICT-107 vaccination was well tolerated, with a 2.2-month improvement in progression-free survival. Overall survival, the primary outcome, was not improved. (doi: 10.1158/1078-0432.CCR-19-0261)	Precision Life Sciences Group
	NCT02546102 Phase 3 Randomized, Double-blind, Controlled Study of ICT-107 in Glioblastoma	III	Biological: ICT-107 Biological: Placebo	Suspended	Precision Life Sciences Group; Medelis Inc.
	NCT03014804 Autologous Dendritic Cells Pulsed With Tumor Lysate Antigen Vaccine and Nivolumab in Treating Patients With Recurrent Glioblastoma	II	Biological: autologous dendritic cells pulsed with tumor lysate antigen Vaccine Other: Laboratory Biomarker Analysis Biological: Nivolumab Other: Quality-of-Life Assessment Other: Questionnaire Administration	Withdrawn	Jonsson Comprehensive Cancer Center; Northwest Biotherapeutics Bristol-Myers Squibb Brain Tumor Funders Collaborative
	NCT00045968 Study of a Drug [DCVax®-L] to Treat Newly Diagnosed GBM Brain Cancer	III	Drug: Dendritic cell immunotherapy	As of this analysis, 223 patients are ≥ 30 months past their surgery date; 67 of these (30.0%) have lived ≥ 30 months and have a Kaplan-Meier (KM)-derived mOS of 46.5 months. Only 2.1% of ITT patients (n = 7) had a grade 3 or 4 adverse event that was deemed at least possibly related to the vaccine. doi: 10.1186/ 2236 s12967-018-1507-6	Northwest Biotherapeutics

3 Cell-free Immunotherapies for GBM

3.1 MSCs-derived exosomes as carriers

Despite the many advantages of MSCs in the fight against GBM, there are some limitations to the use of MSCs, such as low biological activity and limited accessibility (79, 80, 87). Furthermore, in direct contact with GBM cells, MSCs enhance the development of tumors rather than inhibit them (96). This argument highlights the inherent danger of MSCs as live cells. EVs are cell-secreted nanoparticles with a bilateral lipid membrane structure that are actively released by the cell. Based on their biogenesis, size and biophysical properties, the types of EVs can be classified as microvesicles, apoptotic vesicles and exosomes. Microvesicles, approximately 100-1000 nm in diameter, generated by cells directly outwards budding or extruding from cells, containing cell membranes and some cytoplasmic components (142, 143). Apoptotic vesicles, which range in size from 50 nm to 5000 nm, are vesicles shed or burst during apoptosis or death and released outside the cell (144, 145). Distinct from microvesicle formation, exosomes (Exo), which are approximately 30-100 nm in diameter, begin at the cell membrane and bud inwards to produce intracellular vesicles, then undergo early intracellular vesicles, multivesicular complexes, directed assembly and migration, and finally fuse with the cell membrane and depart the cell by exocytosis (146, 147). Exo have the lowest average particle size, the greatest mean content, and the most diversified roles among the three kinds of extracellular vesicles.

MSCs can be an abundant source of Exo, and all Exo express the same group of proteins, such as tetraspanins (e.g. CD63, CD9, CD81), adhesion proteins (e.g. L1CAM, LAMP2), Alix and TSG101 (148–150). Exo vesicle proteins are closely related to proteins in the source cells, such as heat shock proteins (HSP70, HSP90) and cellular skeletal proteins (actin, tubulin, cofilin) (151). Apart from this, Exo carries the same bioactive substances as the source cells, such as nucleic acids, proteins and lipid substances, and can produce a variety of biological effects (152–154).

MSCs exosomes (MSCs-Exo), as paracrine mediators of the therapeutic effect of MSCs, have comparable biological activity to MSCs. However, compared with MSCs, MSCs-Exo are smaller, penetrate biological membranes more easily, are less immunogenic, more biocompatible, and better preserved (155, 156). Previous studies have shown that exosomes are important mediators of intercellular communication. It can be used as carriers of drugs/signaling molecules to efficiently transport cargo to target cells (157, 158). Therefore, MSCs-Exo can be used to safely and effectively deliver drugs to GBM sites in the brain. MSCs-Exo preferentially homed to damaged tissues and sites of

inflammation, including brain malignant gliomas (159, 160). This suggests that these exosomes, like the MSCs from which they are formed, could be used as potential new therapeutics. Furthermore, they provide considerable advantages over uncontrolled cell development and potentially tumour formation in live cells due to their ability to decrease severe side effects and infusion toxicity (96, 161–163). Many studies have demonstrated that microRNAs (miRs) such as miR-93 (164), miR-519a (165), miR-758-5p (166), miR-330-5p (167), miR-139-5p (168), miR-590-3p (169), miR-34a (170, 171) may inhibit GBM production. Unfortunately, the lack of an ideal delivery system has limited the clinical application of miRs in the fight against GBM. Several studies on GBM (including GSCs) have established the transport of GBM-inhibitory miRs to tumour sites through MSCs-Exo to limit tumour development (172–178), suggesting that MSCs-Exo have significant potential for application in the treatment of GBM. Pharmacological delivery to treat GBM has been unsatisfactory, mainly attributed to drug resistance and low targeting efficiency. A combination of selective targeting of GBM cells and synergistic induction of apoptosis using a cocktail of therapeutic agents may help to improve drug delivery. Rana Rahmani et al. (179) found that treating GBM cells with modified MSCs-Exo with two apoptosis-inducing gene therapy agents, cytosine deaminase (CDA) and miR-34a, and targeting the EGFRvIII antigen, enhanced the rate of apoptosis.

3.2 Oncolytic virus

With the advancement of scientific research, not only cellular therapy and Exo are used for tumour immunotherapy but also oncologic viruses (OV) have become effective new therapeutic tools in this field (180–182). OV is a class of naturally occurring or genetically engineered viruses that may infect or kill tumour cells while without harming normal cells. OV has been divided into two types, mildly virulent strain of wild-type OV/natural OV, represented by reovirus, retroviruses and poliovirus (183, 184), and a strain that has been genetically modified to proliferate only within tumour cells, such as adenovirus and herpes simplex virus (185, 186). OV exerts anti-tumour activity *via* several mechanisms. At first, viruses proliferate in tumour cells and directly lyse tumour cells (187). Then, lyses of tumour cells lead to the release of newly generated viral particles that stimulate systemic anti-tumour immune responses through a variety of pathways, such as promoting tumour antigen presentation, improving the immunosuppressive TME, disrupting the tumour vascular system and stimulating adaptive immune responses (188–191). Due to space constraints, this section concentrates on OV therapy of GBM using Reovirus, adenovirus, and herpes simplex virus (Table 3).

3.2.1 Herpes simplex virus-1

Oncolytic herpes simplex virus (oHSV-1) is a neurophilic double-stranded DNA virus. Typically, wild HSV-1 is neurotoxic, so the virus must be genetically modified or greatly attenuated to ensure safety. After genetic modification, OV can still maintain its ability to reproduce while replicating specifically in tumour cells, and therefore OV is widely exploited. Based on previous findings, oHSV was the first state-of-the-art genetically engineered OV to be licensed by the United States FDA for cancer treatment (192) and was approved for the treatment of advanced melanoma (193, 194).

However, as GBM is a primary brain tumour of the human central nervous system, more attention deserves to be paid to the safety of oHSV-1 in the fight against GBM. In past studies, oHSV-1 is encoded by the γ34.5 gene, an ICP34.5 protein, which is neurotoxic (195). To limit neurotoxicity, the double copy γ34.5 gene was knocked out in all oHSV-1 tested in glioma clinical trials, the first generation of oHSV-1. But replication of γ34.5-deficient oHSV-1 is often restricted and severely attenuated, particularly in GSC (196, 197). It is crucial to assure the 34.5 deletion mutant's safety while also ensuring its effective replication in GBM. Hiroshi Nakashima et al. (198) produced the gene HSV-1 OV (NG34), an attenuated HSV-1 with the deletion of the gene encoding the viral ICP6 gene (UL39) and the gene for γ34.5. The UL39 gene encodes the large subunit ICP6 of ribonucleotide reductase, which is essential for postmitotic cell replication. GADD34 gene in humans is expressed by NG34 under the transcriptional regulation of the cellular Nestin gene promoter/enhancer element, which is specifically expressed in GBM. In a GBM mouse model, they discovered that the new oHSV encoding GADD34 was efficacious and generally non-toxic. Another research found that activating MEK in tumour cells boosted replication of γ34.5-deficient HSV-1 (199), but activating MEK in tumour-associated macrophages (TAM) stimulated pro-inflammatory signaling while inhibiting viral replication and propagation (200). Ji Young Yoo et al. (201) investigated the effects of blocking MEK signaling and oHSV-1 binding on brain tumors. It was reported that oHSV treatment facilitated the entry of the MEK kinase inhibitor trametinib into brain tumors and sensitized it *in vivo*.

G207 is a second generation oHSV-1 that inactivates the ICP6 gene by deleting the double copy γ34.5 gene while inserting the lacZ gene at the UL39 locus. During a Phase I clinical trial of genetically engineered oHSV-1 G207 by GK Friedman et al. (202), oHSV-1 G207 was found to establish an effective anti-tumour immune response in pediatric high-grade gliomas (ClinicalTrials.gov, NCT02457845). Subsequently, to extend and confirm the results of this phase I trial, an upcoming multi-institutional phase II clinical trial of G207 in pediatric high-grade glioma (ClinicalTrials.gov, NCT04482933) is still under investigation.

G47Δ is a third generation oHSV-1 with a triple mutation in α47 deleted from the G207 genome. Compared to G207, G47Δ is further attenuated in normal cells, but has enhanced efficacy in anti-tumour, as well as a greater safety profile. oHSV-1 G47Δ showed efficacy and safety in GBM was confirmed by the American Association for Cancer Research in 2016 (203). In subsequent years, Tomoki Todo et al. (204) published their findings of a phase I/II single-arm study in 2022 evaluating the safety of G47Δ for the treatment of recurrent/progressive GBM (ClinicalTrials, UMIN000002661). These findings support and formed the basis of a phase II clinical study in patients with GBM. This was followed by a separate report evaluating G47Δ in residual or recurrent GBM,

TABLE 3 Oncolytic virus based clinical studies in GBM patients that have been completed or are ongoing.

Molecular target	Clinical trial NCT number and title	Study phase	Interventions	Study Results	Sponsor/Collaborators
oHSV-1	NCT03152318 Treatment of Recurrent Malignant Glioma With rQNestin34.5v.2 (rQNestin)	I	Drug: rQNestin Drug: Cyclophosphamide	humans with recurrent GBM treated with rQNestin34.5v.2 has not shown evidence of viral-mediated toxicity or encephalitis (doi: 10.1016/j.omtm.2020.03.028; doi: 10.1158/1078-0432.CCR-21-2347)	Dana-Farber Cancer Institute (National Institutes of Health; Candel Therapeutics, Inc.)
	NCT02457845 HSV G207 Alone or With a Single Radiation Dose in Children with Progressive or Recurrent Supratentorial Brain Tumors	I	Biological: G207 Single dose of HSV-1 (G207) infused through catheters into region(s) of tumor defined by MRI	a total of 4 of 11 patients were still alive 18 months after G207 treatment (doi: 10.1056/NEJMoa2024947)	University of Alabama at Birmingham (Food and Drug Administration; National Center for Advancing Translational Sciences of the National Institutes of Health; et)
	NCT04482933 HSV G207 With a Single Radiation Dose in Children with Recurrent High-Grade Glioma	II	Biological: Single dose of HSV-1 (G207) infused through catheters into region(s) of tumor defined by MRI	Not yet recruiting	Pediatric Brain Tumor Consortium (Treovir, LLC)
	NCT03911388 HSV G207 in Children with Recurrent or Refractory Cerebellar Brain Tumors	I	Single dose of G207 infused through catheters into region(s) of tumor. If G207 is safe in the first cohort of patients, subsequent patients will receive a single dose of G207 infused through catheters into region(s) of tumor followed by a 5 Gy dose of radiation to the tumor within 24 hours of virus inoculation.	Recruiting	University of Alabama at Birmingham
OAds	NCT03896568 MSC-DNX-2401 in Treating Patients with Recurrent High-Grade Glioma	I	Biological: Oncolytic Adenovirus Ad5-DNX-2401 Procedure: Therapeutic Conventional Surgery	The use of perfusion guidance to enhance the precision of endovascular super-selective intra-arterial infusions of mesenchymal stem cells loaded with Delta-24 in the treatment of GBM (doi: 10.1136/neurintsurg-2021-018190)	M.D. Anderson Cancer Center (DNAtrix, Inc.)
	NCT03072134 Neural Stem Cell Based Virotherapy of Newly Diagnosed Malignant Glioma	I	Biological: Neural stem cells loaded with an oncolytic adenovirus	The post-treatment PES and OS of 12 newly diagnosed malignant glioma patients were 9.05 months and 18.4 months, respectively (doi: 10.1016/S1470-2045(21)00245-X)	Northwestern University (National Cancer Institute)
	NCT02197169 DNX-2401 With Interferon Gamma (IFN-γ) for Recurrent Glioblastoma or Gliosarcoma Brain Tumors (TARGET-I)	I	Drug: Single intratumoral injection of DNX-2401 Drug: Interferon-gamma	The addition of IFN did not improve survival, but clinical activity following a single injection of DNX-2401 is encouraging (doi: 10.1200/JCO.2017.35.15_suppl.2002)	DNAtrix, Inc.
Reovirus	NCT02444546 Wild-Type Reovirus in Combination with Sargramostim in Treating Younger Patients with High-Grade Relapsed or Refractory Brain Tumors	I	Biological: Wild-type Reovirus Sargramostim	All patients progressed on therapy after a median of 32.5 days and died a median of 108 days after recruitment (doi: 10.1093/noajnl/vdac085)	Mayo Clinic (National Cancer Institute)

that is, a phase II trial that revealed a survival benefit and a good safety profile, leading to the approval of G47Δ as the first Japanese OV product for the treatment of GBM.

3.2.2 Oncolytic adenovirus

Adenovirus is a non-enveloped virus with an icosahedral capsid containing approximately 38 kb of genomic double-stranded DNA. There are more than 50 serotypes of adenovirus in humans, of which types 2 and 5 have been most frequently studied for the manufacture of lysing viruses (205, 206). In addition to its high genetic stability, high titer production and its ease of purification, the 38kb capacity of the adenovirus capsid allows for the introduction of large transgenes and as a result adenoviruses have been genetically engineered into various types of oncolytic adenovirus (OAds) or conditionally replicating adenovirus (CRAd). Just like other types of OV, OAds are able to replicate relatively specifically in tumour cells and lyse them, releasing progeny viruses that then infect surrounding tumour cells and destroy the tumour through a cascade amplification effect, so that a better outcome can be achieved.

Several treatments for OAds are currently in clinical trials, including Frederick F Lang et al. (207) in a phase I clinical trial of OAds DNX-2401 (Delta-24-RGD), where 20% of patients (5 of 25), survived > 3 years after treatment, three patients had ≥ 95% (12%) reduction in enhancing tumors, and and > 3 years progression-free survival from the start of treatment. This demonstrates that DNX-2401 is safe and has anti-tumour activity in patients with GBM. DNX-2401 is a potential second generation OAds with significant viral replication capacity and the ability to directly destroy tumour cells. DNX-2401 is a potential second-generation OAds with significant viral replication capacity and direct tumour cell destruction. Its mechanism of anti-tumour action is that a 24pb base deletion in the E1A gene that prevents it from binding the retinoblastoma tumour suppressor protein (Rb) protein and thus from replicating in normal tissues, whereas in Rb-deficient tumour cells, where E2F is in a free state, the virus can still replication (208). As the primary mode of entry of adenovirus type 5 into host cells is through binding to coxsackievirus and adenovirus receptors on the cell surface, which are expressed at low levels on the surface of GBM cells (209), DNX-2401 was designed with an RGD peptide insert, Delta-24-RGD, which has an RGD-4C peptide motif inserted into adenovirus fibers, and allows viral entry *via* the integrins avβ3 and avβ5 into tumour cells, which further enhances tumour targeting (210). To increase the anti-glioma immune effect of Delta-24-RGD, in a preclinical study, Yisel Rivera-Molina et al. (211) decided to arm Delta-24-RGD with co-stimulatory ligand glucocorticoid receptor-enhanced T-cell activity (GITRL) with the aim of activating the T-cell population recruited to the tumour after viral infection. From their data, GITRL-armed Delta-24-RGD exhibited enhanced anti-glioma effects, resulting in an increased frequency and activation of T cells. In addition, specific anti-tumour immunity and enhanced central T cell memory encouraged preclinical testing of next generation lysing adenoviruses equipped with immune checkpoint modulators. Given the safety of DNX-2401 in past studies (207), through a phase I clinical trial on convection-enhanced delivery of Delta24-RGD in the tumour and peripheral brain of patients with recurrent GBM, Erik HP van Putten et al. (212) showed that 19 out of 20 enrolled patients received the oncolytic adenovirus Delta24-RGD, which was considered safe and feasible. Four patients demonstrated tumour response on MRI, and one of them regressed completely and is still alive 8 years later. This trial was the first to assess local and regional responses to the injection of OV into the tumour and surrounding brain by serial sampling of interstitial fluid and cerebrospinal fluid (CSF). Analysis of cytokines and chemokines in CSF suggests that IFNγ and TNFα levels may represent potential biomarkers of response in future OV assays. Biomarker testing may ultimately help to identify patients who respond and improve response rates to OV therapy. Their findings show promising clinical responses and indications for anti-tumour immune responses, providing a basis for future testing of (combined) Delta24-RGD treatments in GBM.

ICOVIR17 is an OAds expressing soluble PH20 hyaluronate (HA) that degrades HA and spreads efficiently in the tumour. It has the same mechanism of action as Delta-24-RGD, ICOVIR17 deletion of 24 base pairs in the Rb-binding domain of E1A for tumour-selective replication and RGD modification in the fibril to amplify tropism, except for two additional modifications: insertion of an E2F binding site in the E1A promoter and insertion of the SPAM1 gene encoding PH20 HA after the fibril, which is controlled by the major late promoter control (213). Normally, adenovirus replication is divided into two phases, early (E) and late (L). Early stage expresses adenovirus replication-related genes E1-E4, and late stage expresses adenovirus assembly-related genes L1-L5. However, OAds, as well as most novel targeted therapies, face significant transport barriers in the tumour mesenchyme, in part because they are relatively large (90 nm) and much larger than chemotherapeutic agents. Solid tumors exhibit unique features that impede the transport of large molecules. Among these, the large amount of extracellular matrix present in the tumour mesenchyme and high mesenchymal fluid pressure are the main sources of physical resistance to drug transport. HA is an essential component of the ECM with high expression in most tumour xenografts. Jordi Martinez-Quintanilla et al. (214) revealed for the first time that intratumoural injection ICOVIR17 into nodal GBM mediated HA degradation and enhanced viral dissemination, resulting in significant anti-tumour effects and mouse survival. As much as this work reveals that HA functions in GBM as a physical barrier to effective virus dissemination and tumour killing, it remains unknown whether HA affects the immune response induced by OAds treatment of brain tumors as the mice used in the study were immunodeficient. Therefore, Juri Kiyokawa et al. (215) exploited that degradation of HA would enhance OAds immunotherapy of GBM by overcoming the immunosuppressive function of GBM extracellular matrix. In their study, murine GBM 005 was chosen as a suitable *in vivo* model given that this GBM model encapsulates key features of human disease, including GSC properties and immunosuppressive TME. Their study has shown for the first time that immunomodulatory ICOVIR17 has the dual role of mediating HA degradation in GBM extracellular matrix and subsequently altering the TME immune landscape, and provides a

mechanistic combination of immunotherapy and PD-L1/PD-1 blockers to remodel innate and adaptive immune cells.

CRAd-S-pk7, a type of oncolytic adenovirus, is a promoter doped with survival proteins to drive expression of the replication-essential E1A gene and modifies Ad5 fibronectin by doping with a polylysine sequence (pk7) (216, 217). These modifications enhanced viral replication and targeting of glioma cells, resulting in enhanced antitumor activity and higher survival rates *in vivo*. Jawad Fares et al. (218) conducted the first human phase I dose-escalation trial investigated NSC-CRAd-S-pk7, a CRAd delivered by neural stem cells, for use in patients with newly diagnosed GBM. Their findings support the continuation of the study of NSC-CRAd-S-pk7 in a phase II/III clinical trial. In addition, multi-dose neural stem cell viral therapy (NSC-CRAd-S-pk7) for recurrent high-grade glioma is being investigated (ClinicalTrials.gov, NCT05139056).

3.2.3 Reovirus

Reovirus (RV) belongs to the wild-type OV family of eutheroviruses and is characterized as a staged double-stranded RNA virus. The three prototypical serotypes of RV, first identified in the 1960s, are well characterized as serotype 1 Lang (T1L), serotype 2 Jones (T2J) and serotype 3 Dearing (T3D) (219). RV is widely present in the respiratory and digestive tracts of humans and livestock without causing disease and is only associated with mild flu-like symptoms. RV has been shown to have a tumourolytic effect on a variety of tumour cells and has been used in several clinical trials (220–223). Among these, Peter Forsyth et al. (224) demonstrated for the first time in a single institution phase I clinical trial that intratumoural injection of wild-type eutherian virus in GBM patients was well tolerated. Kimberly P Kicielinski et al. (225) in a preliminary study of direct intratumoural inoculation of the CNS, once again they observed that RV well tolerated: patients had a median progression-free survival of 4.3 weeks and a median survival of 21 weeks. In the GBM study and other previous studies, the tolerability of RV at the dose administered prompted them to design a clinical trial of incremental viral doses intended to achieve higher doses and better distribution of study drug. With the safety and tolerability demonstrated in several phase I clinical trial studies, RV embarked on a phase II study to assess efficacy, particularly in areas where GBM treatment was not effective.

3.3 Immune checkpoint inhibition

GBM generates an immunosuppressive environment through multiple mechanisms, including the programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activating gene 3 (LAG-3) pathway (226). Although some tumors benefit from immune checkpoint inhibition (ICI), GBM does not (24).

The immunosuppressive properties of the GBM microenvironment lead to immune evasion by tumour cells, making inhibition of immune checkpoints such as PD-1 alone ineffective (226, 227). PD-1 inhibition is thought to disrupt the binding of PD-1 to its inhibitory ligands, thereby stimulating cytotoxic T cell-mediated tumour elimination. The Ivy Foundation Early Clinical Trials Consortium conducted a multi-institutional, randomized clinical study to evaluate immunological response and survival in 35 patients with recurrent, surgically resectable GBM after neoadjuvant anti-PD-1 immunotherapy with pembrolizumab (228). Pembrolizumab is an anti-PD-1 monoclonal antibody that has been demonstrated to be effective as a monotherapy in a variety of cancer types, but largely in adjuvant treatment (229). OS was significantly longer in patients randomized to neoadjuvant pembrolizumab and continuing adjuvant therapy after surgery. Neoadjuvant PD-1 inhibition was linked with increased T-cell and interferon-γ (IFN-γ) related gene expression, but decreased intratumor cell cycle-related gene expression, which was not observed in patients receiving adjuvant treatment alone. Local induction of programmed death ligand 1 (PD-L1) in the tumour microenvironment, increased T cell clonal expansion, decreased PD-1 expression in peripheral blood T cells, and decreased monocyte numbers were more frequently observed in the neoadjuvant group in patients treated. These data imply that neoadjuvant PD-1 blocker administration boosts local and systemic antitumor immune responses and may be a more effective therapy for this consistently deadly brain tumour. In the single-arm phase II clinical trial (ClinicalTrials.gov, NCT02550249) by Kurt A Schalper et al. (230), preoperative doses of nivolumab followed by postoperative nivolumab were tested in 30 patients (27 recurrent cases for salvage surgery and 3 newly diagnosed patients for initial surgery) until disease progression or unacceptable toxicity. Neoadjuvant nivolumab resulted in enhanced expression of chemokine transcripts, increased immune cell infiltration and enhanced TCR clonal diversity in tumour-infiltrating T lymphocytes, supporting a local immunomodulatory effect of the treatment, although no clear clinical benefit was demonstrated after salvage surgery.

3.3.1 Simultaneous inhibition of multiple immune checkpoints

Simultaneous inhibition of multiple immune detection sites for anti-GBM treatment may improve treatment outcomes. Antonio Omuro et al. (226) evaluated the anti-PD-1 checkpoint inhibitor nivolumab intravenously in patients with recurrent GBM in a phase I trial, both as monotherapy or in combination with CTLA-4 blocking mAb ipilimumab at different dose levels. Nivolumab as a single agent is well-tolerated, but the combination of nivolumab and ipilimumab is associated with up to 30% of treatment-related adverse events that lead to treatment discontinuation. The tolerability of the combined treatment was determined by the dose of ipilimumab. In two patients who were initially identified as having suspected progression based on neuroradiological assessment and subsequently underwent neurosurgical resection, interestingly, substantial immune cell aggregates were identified by histopathological examination, but no live tumors. John Lynes et al. (231) used cytokine micro dialysis to detect real-time immune assays in GBM patients undergoing PD-1 and LAG-3 checkpoint

inhibition, suggesting that the anti-LAG-3 and anti-PD-1 combination may have a similar immune response side effect profile to other checkpoint inhibitor combinations. E Antonio Chiocca et al. (232) reported an increase in tumour-infiltrating lymphocytes producing IFN-γ and PD-1 in a phase I trial. These inflammatory infiltrates support the immune anti-tumour effects of human interleukin 12. E Antonio Chiocca et al. (233) found a reduction in PD-1 and/or PD-L1 positive cells in four pre- and post-treatment biopsy matched subjects in their trial. This validates the hypothesis that nivolumab reduces GBM cell immune checkpoint signaling induced after treatment with controlled IL-12 gene. In addition, Moreover, recruitment has been completed for a phase II study of controlled IL-12 in combination with neoadjuvant anti-PD-1 (ClinicalTrials.gov, NCT04006119). However, as O^6-methylguanine-DNA methyltransferase (MGMT) renders tumors resistant to TMZ, MGMT promoter status predicts both prognosis and therapeutic response to TMZ chemotherapy. Antonio Omuro et al. (234) conducted CheckMate-498 phase III clinical study comparing nivolumab or TMZ for OS, each in combination with radiotherapy (RT), in patients with newly diagnosed MGMT unmethylated GBM, failed to meet its intended target improvement OS endpoint (ClinicalTrials.gov, NCT02617589). In GBM, the entry of monoclonal antibodies (mAb) is blocked due to CNS is an immune-privileged site. In tumour types, combined treatment with two mAb leads to higher tumour response rates and improved survival compared to monotherapy for the cost of serious immune-related adverse events (235–239). Johnny Duerinck et al. (240) in a phase I clinical trial Intracerebral administration of CTLA-4 and PD-1 immune checkpoint blocking monoclonal antibodies in patients with recurrent GBM. The phase III randomised CheckMate 548 study by Michael Lim et al. (241) identified that nivolumab added to RT+TMZ was not associated with improved survival in newly diagnosed GBM patients with a methylated or indeterminate MGMT promoter. Although these studies failed to demonstrate the clinical benefit of ICI, they could be considered in new combination therapy strategies.

3.3.2 Immune checkpoint inhibition binding to oncolytic virus

Possible complementary effects on tumour killing through combination with OV. Dipongkor Saha and colleagues in 2018 GBM may be treated with oHSV immunoviral therapy in combination with two checkpoint inhibitors (anti-PD-1 and anti-CTLA-4), a triple combination that could assist in curing less immunogenic malignancies such as GBM (31). Also based on the fact that OV and PD-1 inhibitors have become standard immunotherapies against certain cancers, Carmela Passaro et al. (36) conducted preclinical trials in 2019 on GBM, i.e. they investigated *in vitro* and *in vivo* the efficacy of a novel lysine virus (NG34scFvPD-1) of HSV-1 against PD-1, which also expresses a single-chain fragment mutable antibody (scFvPD-1). Irene Appolloni et al. (65) also investigated the specificity, safety and efficacy of EGFRvIII-targeted oHSV-1 for the treatment of human GBM. These studies provide a basis for further exploration of this novel OV in combination with ICI for cancer therapy.

4 Future perspectives and conclusions

There is a deepening perception of what cell-based and cell-free immunotherapy can bring to GBM, which offers new hope for GBM patients, but many details and questions remain to be explored and elucidated. According to recent statistics, adjuvant immunotherapy can prolong survival and significantly improve outcomes for patients with recurrent GBM compared to those treated with only surgery, radiotherapy or chemotherapy. To summarize the lessons learned, the extremely specific and heterogeneous nature of GBM, as well as the complicated immune resistance mechanisms and immunosuppressive TME have resulted in relatively limited response effectiveness and durability of response to treatment drugs. The BBB/BTB is another barrier to medication delivery. These are still tough breakthroughs in GBM therapy. We not only obtained some novel findings on the theoretical study of the local immune characteristics of glioma, but we also provided an experimental basis for the comprehensive diagnosis and treatment of regulating and intervening in the immune microenvironment of glioma. More significantly, “supporting the righteousness” and improving the immune microenvironment, in conjunction with “elimination of evil” by anti-tumour cells, will ideally decrease disease mortality rates, extend patient life, and genuinely help patients. In fact, the treatment of GBM is actually a complex “project” and satisfactory results can hardly be achieved with only one treatment. Immunotherapy research for GBM should be coupled with other treatment modalities in future anti-GBM research, resulting in truly tailored and complete care strategies for patients. It is recommended to develop combined treatment techniques based on immunotherapy, molecular targeted therapy, and radiation to maximize therapeutic efficiency and reduce acquired immunotherapy resistance. The industry therefore needs to innovate, integrate and translate to drive immune combinations forward in a sustained manner. Secondly, assessing therapeutic response to immunotherapy is difficult, and in the future, a standardized imaging and molecular biology evaluation system will be required to reflect and forecast patient outcomes. Cell-based and cell-free immunotherapy are predicted to become an essential component of future glioma treatment when these challenges are solved.

Author contributions

MW conceived this article. MW and XW drafted the manuscript. MW and XW drew the illustrations and tables. MW, XW, XJ, JZho and YZ revised the article. MW, YL and JZha checked and edited the article. YL and YY supervised the article. All authors have read and agreed to the final version of the manuscript.

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Conflict of interest

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

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Case Report: Immunotherapy for low-grade myofibroblastic sarcoma of the pharynx

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Low-grade myofibroblastic sarcoma (LGMS) characterized by the increased proliferation of myofibroblasts is a rare type of malignant myofibroblastic tumor that frequently occurs in the head and neck region. Presently, there is no consensus regarding the treatment of LGMS. Here, we report a rare case of LGMS of the pharynx in a 40-year-old male admitted to our hospital. The patient underwent resection for a right metastatic lesion and parapharyngeal mass. However, he had recurrence and multiple metastases without a surgical indication. Then the patient received the treatment of anlotinib plus pembrolizumab for 4 cycles, and there was a partial response (PR) to the treatment. Due to the adverse reaction of anlotinib, the patient subsequently received monotherapy of pembrolizumab for 22 cycles and achieved a complete response (CR). As the first case report of the immunotherapy for LGMS, our study highlights that this strategy may be of great significance to the treatment of LGMS.

KEYWORDS

low-grade myofibroblastic sarcoma, pharynx, recurrence and multiple metastases, pembrolizumab, immunotherapy

1 Introduction

Low-grade myofibroblastic sarcoma (LGMS) defined as a distinct atypical myofibroblastic tumor is a rare solid infiltrative soft tissue tumor, often with predilection for the head and neck region (1). LGMS is mainly located in deep soft tissues, and it has a high recurrence rate but low metastatic potential (2). In addition, LGMS most commonly occurs in adults, mainly in men with an average age of 40 (3, 4). The diagnosis of LGMS is usually based on the histological and immunohistochemical findings (5). Due to the rarity of LGMS, however, the standardization of its treatment remains unclear. Generally, surgery is the primary treatment for LGMS.

Herein, we present a case of LGMS occurring in the pharynx. After the resection of the metastatic lesion, the patient had recurrence and multiple metastases without a surgical

indication. Thus, he received the combined treatment of pembrolizumab and anlotinib, and subsequent monotherapy of pembrolizumab. To the best of our knowledge, this is the first case report of LGMS with a good response to immunotherapy.

2 Case presentation

In January 2020, a 40-year-old male was admitted to our hospital with obvious right pharyngeal foreign body sensation and pharyngeal discomfort. The patient had no history of other disease and denied the family history. Positron emission tomography and computed tomography (PET/CT) showed a mass in the right lateral wall of the pharynx, along with lung metastases (Figures 1A–C). Magnetic resonance images (MRI) revealed a soft tissue mass in the right parapharyngeal space (7.5 cm × 7.5 cm × 3 cm) (Figure 1D). Furthermore, there were multiple lymphadenopathies in bilateral neck region. The patient underwent a surgical resection of metastatic lesion and parapharyngeal mass of the right pharynx on January 15, 2020. Histological examination showed spindle cells with minimally atypia arranged with fascicular or storiform growth patterns in the fibrous stroma, suggestive of low-grade spindle cell sarcoma (Figures 2A, B). Regarding immunohistochemical staining, there is positive result for smooth muscle actin (SMA), but a negative result for caldesmon and desmin, with a Ki-67 index of approximately 40% (Figures 2C, D). The diagnosis of LGMS was established based on the histological features together with immunohistochemical findings. In March 2020, multiple metastases were observed again in the patient's lymph nodes and lungs (Figures 3A, 4A, E). According to the advice of multi-disciplinary treatment (MDT), the patient did not have a surgical indication.

Starting from April 2020, the patient received 4 cycles of combined therapy with pembrolizumab (200 mg, q21d) and anlotinib (12 mg, qd, d1-14, q3w) and obtained a PR to drug treatment. Considering the hypertensive side effect induced by anlotinib (systolic blood pressure: 180 mmHg; diastolic blood pressure: 110 mmHg), the patient stopped using anlotinib in the follow-up treatment. Subsequently, 22 cycles of pembrolizumab monotherapy (200 mg, q21d) were performed as maintenance therapy from August 2020 to August 2022. Based on the new response evaluation criteria in solid tumors (RECIST) guideline (version 1.1) (6), the patient obtained a PR in August 2020 and February 2022, respectively (50% and 67% decrease in the sum of diameters of target lesions, respectively) (Figures 3B, C, 4B, C, F, G). Not until the last follow-up in August 2022, the patients obtained a CR in August 2022 (disappearance of all target lesions) (Figures 3D, 4D, H). The timeline of treatment administration from the episode of care was presented in Supplementary Figure 1.

3 Discussion

In this report, we presented a successful case of a patient with LGMS who obtained a good response to immunotherapy. The patient was initially diagnosed with LGMS, along with lung metastases. Of note, he had another recurrence and multiple metastases after surgery without a surgical indication. Emerging evidence demonstrated that targeted therapy, immunotherapy or a combination of both were important approaches in treating soft-tissue sarcoma (STS) (7–9). In the present case, we observed a durable effect in the patient treated with immunotherapy, highlighting the importance of the immunotherapy in the treatment of LGMS.

As a rare STS, LGMS is a low-grade malignant tumor derived from mesenchymal myofibroblasts, characterized by its local recurrence and occasional metastasis (1). A previous report revealed that LGMS was mainly located in the head and neck region, especially in the oral cavity, and generalized that local recurrence of LGMS was 26.7%, and distant metastasis was 4.4% (10). As yet, the diagnostic features of LGMS remained challenging. The differential diagnosis for this tumor included nodular fasciitis, low-grade fibrosarcoma, inflammatory myofibroblastic tumor, well-differentiated osteosarcoma, desmoplastic fibroma, leiomyosarcoma, and fibromatosis. The histopathologic resemblance of LGMS to fibromatosis and myofibroma was often a source of diagnostic confusion. In terms of the LGMS in the upper aerodigestive tract, Meng et al. (11) reported that misdiagnosis might occur in small and superficial biopsy samples due to the diverse histologic appearance in the same tumor of myofibroblastic sarcoma. Given that a misinterpretation could result from the specimen being sampled from the tumor surface, Montebugnoli et al. (12) considered that an open incisional biopsy with subsequent histopathological evaluation must be performed. In addition to histologic similarities,

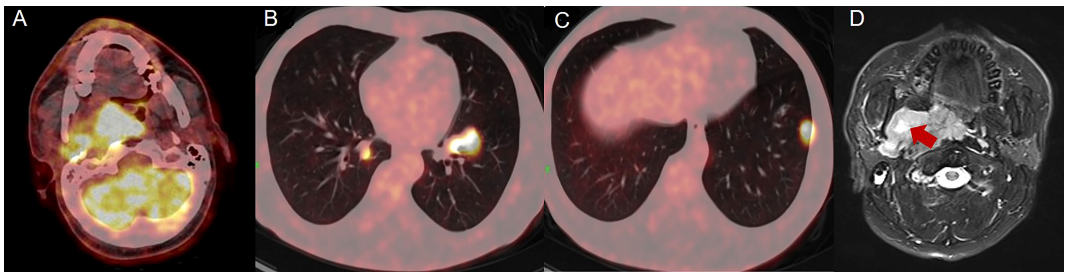

FIGURE 1
Positron emission tomography and computed tomography (PET/CT) and magnetic resonance images (MRI) of lesion and mass. **(A–C)** PET/CT revealed a mass in right lateral wall of the pharynx, along with lung metastases. **(D)** MRI showed a soft tissue mass with a size of 7.5 cm × 7.5 cm × 3 cm.

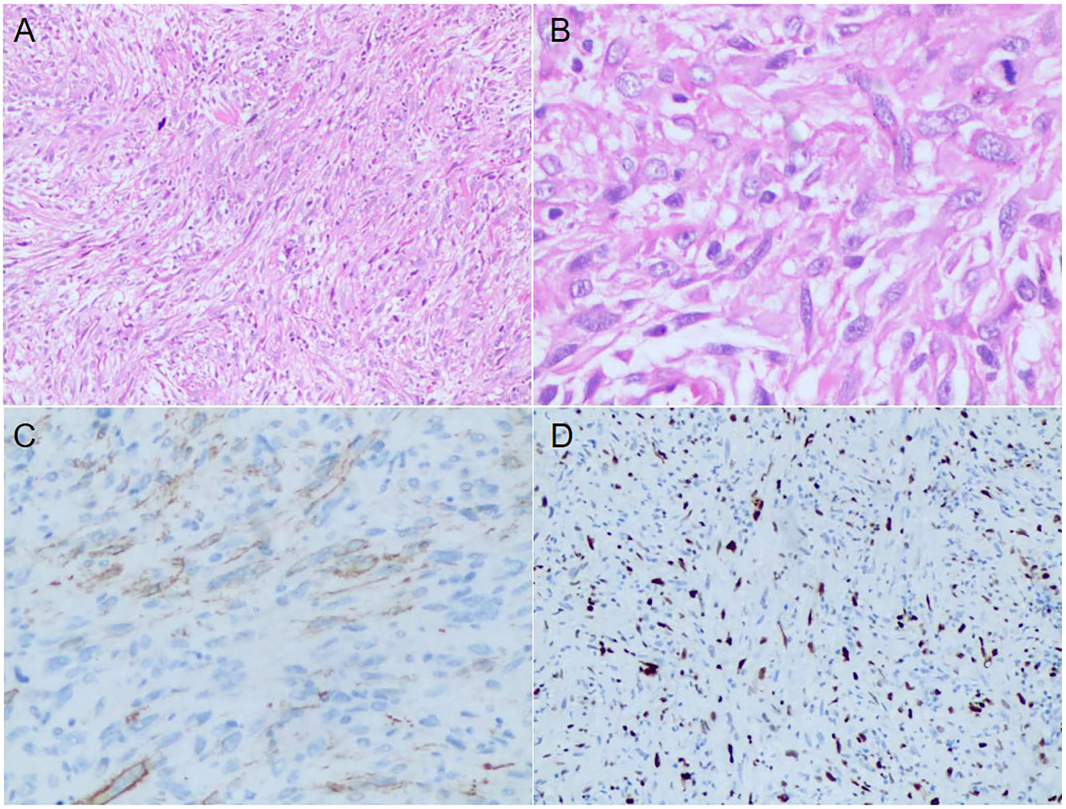

FIGURE 2
Histopathological and Immunohistochemical results of the excisional biopsy. **(A)** The tumor cells were fusiform, arranged in fascicles or storiform growth patterns (HE, ×100). **(B)** A few mitoses and atypical cells with irregular nuclei were observed (HE, ×400). **(C, D)** Immunohistochemical results showed positive staining for α-SMA and Ki-67 with a 40% proliferation index.

LGMS might also be mistaken for nodular fasciitis because of its overlapping immunophenotypes (13). Immunohistochemical results showed that cases with myofibroblastic sarcomas were diffusely positive for at least one myogenic marker and vimentin, including muscle–specific actin and α–SMA (5). Several reports indicated that LGMS might be immunopositive for muscle–specific actin, α–SMA, calponin, fibronectin, and desmin (14, 15). Collectively, although histopathological analysis together with immunohistochemical results were usually considered to confirm the diagnosis of LGMS, the complete clinical features of LGMS were still unclear and needed further investigation.

The primary treatment for LGMS is surgical excision (5, 16). However, several studies suggested surgical excision combined with adjuvant therapy to prevent local recurrence (17, 18). Notably, a

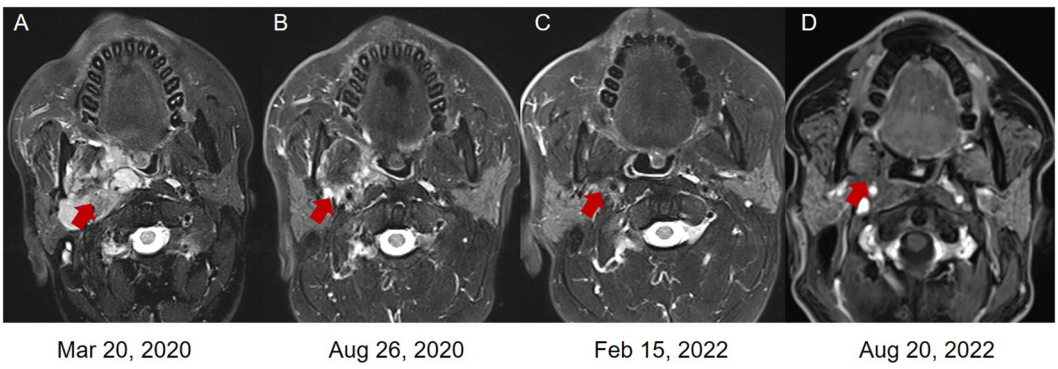

FIGURE 3
The magnetic resonance images (MRI) of the LGMS. **(A)** In March 2020, new multiple lymphatic metastases were observed. **(B)** In August 2020, the patient obtained a PR after the combined treatment of pembrolizumab and anlotinib. The patient obtained a PR **(C)** and CR **(D)** after the monotherapy of pembrolizumab.

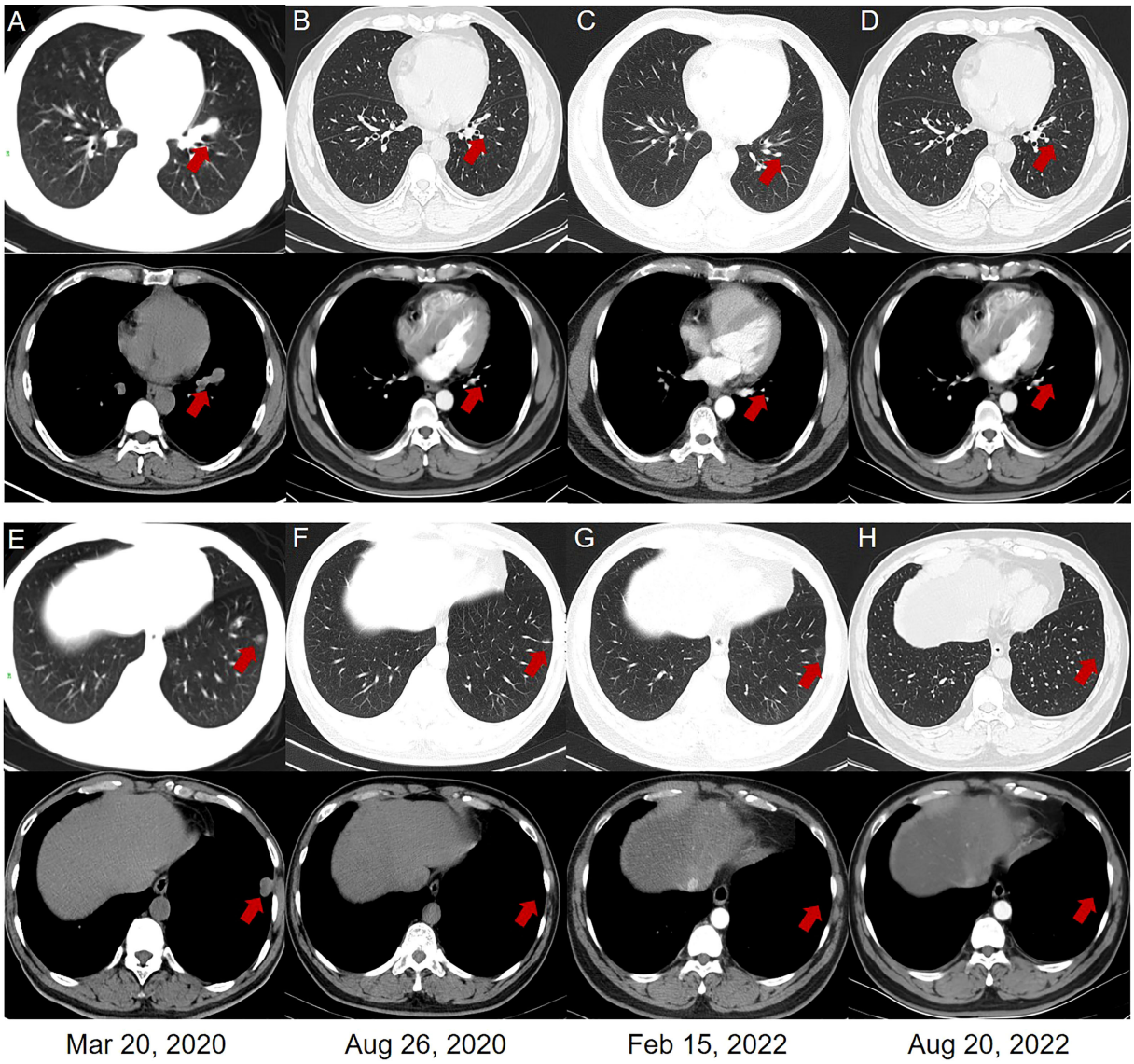

FIGURE 4
Chest computed tomography (CT) scans. **(A, E)** In March 2020, multiple metastases of lung and mediastinal window were observed. **(B, F)** In August 2020, a PR was revealed in the lung and mediastinal window of the patient after the combined treatment of pembrolizumab and anlotinib. The patient obtained a PR **(C, G)** and CR **(D, H)** in the lung and mediastinal window after the monotherapy of pembrolizumab.

recent case series disclosed the association of local recurrence and the tissue invasion of LGMS with the surgical method. Surgical excision with wider safety margins was considered to minimize the risk of recurrence (17). However, wider safety margins were usually more problematic in the oropharynx than in other parts of the body. Besides, there is a controversy in postoperative therapy including radiotherapy and chemotherapy to prevent local recurrence. There was no recurrence for LGMS in the pancreas for five years after surgery and adjuvant chemotherapy (19), while other reports recommended that laryngeal and sacral LGMS were not sensitive to postoperative radiotherapy and chemotherapy (20, 21). Therefore, the selection of postoperative therapy might depend on the invasive region of LGMS and whether the tumor was completely resected.

The National Comprehensive Cancer Network (NCCN) guidelines supported that anthracycline-based chemotherapy was the standard first-line treatment for patients with STS (22). For individuals who failed first-line treatment, antiangiogenesis therapy was the promising strategy in the second-line treatment of advanced or metastatic STS. Anlotinib is a multitarget tyrosine kinase inhibitor (TKI) against tumor angiogenesis and tumor cell proliferation by targeting VEGFR, FGFR, PDGFR, and c-Kit simultaneously. A multi-centered phase IIB trial supported that anlotinib significantly prolonged median progression-free survival (PFS) from 1.47 to 6.26 months in patients with STS as a second-line or subsequent-line treatment (23). Another phase IIB trial conducted in the Chinese population also confirmed the positive efficacy of anlotinib in patients with advanced STS in a real-world setting (24). Furthermore, a recent

clinical trial demonstrated that the combination of anlotinib and epirubicin followed by anlotinib treatment maintenance could serve as the first-line treatment for patients with advanced STS (25). On the other hand, immunotherapy was another possible strategy in the second-line treatment against advanced or metastatic STS. A phase II trial demonstrated that nivolumab combined with ipilimumab showed encouraging objective response rates, PFS and overall survival in certain sarcoma subtypes (8). Importantly, targeted therapy combined with immunotherapy has a synergistic effect on the disease (26). A recent report also showed that the combination of anlotinib and toripalimab was an effective therapy in advanced STS (27). In the present case, the patient could not tolerate anthracycline-based chemotherapy, thus we applied anlotinib combined with pembrolizumab, and the patient obtained a PR. However, the main serious adverse effects of anlotinib were hypertension and hand-foot skin reaction (24, 28, 29). Since the patient experienced hypertension (systolic blood pressure: 180 mmHg; diastolic blood pressure: 110 mmHg) after the combined treatment for 4 cycles, he stopped using anlotinib and subsequently switched to the monotherapy of pembrolizumab for 22 cycles. Ultimately, the patient reached CR.

The exact reason why monotherapy of pembrolizumab was effective in LGMS remained elusive. Pollack et al. identified the detailed overview of the immune microenvironment in sarcoma subtypes and found that high expression levels of genes related to antigen presentation and T-cell infiltration, and T-cell infiltration was significantly correlated with PD-1 and PD-L1 expression levels (30). Therefore, immunotherapy may exert effect through regulating gene expression related to antigen presentation and improving T-cell infiltration. Future studies are warranted to explore underlying cellular and molecular mechanisms of immunotherapy in LGMS.

To our knowledge, this is the first report of the immunotherapy for LGMS. Nevertheless, there exist several limitations in our report. Our report only provides preliminary results but does not figure out the specific reason for the effectiveness of immunotherapy. In addition, the elaborated mechanisms of immunotherapy need to be further clarified in the future.

4 Conclusion

In conclusion, our study sheds light that immunotherapy may be of great significance to the treatment of LGMS.

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

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. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

BS drafted the manuscript. ZL, PL and YH collected materials and prepared figures. WL and SH critically revised the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1190210/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The timeline of treatment administration from the episode of care.

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Efficacy and safety of innate and adaptive immunotherapy combined with standard of care in high-grade gliomas: a systematic review and meta-analysis

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Background: Malignant glioma is the most common intracranial malignant tumor with the highest mortality. In the era of immunotherapy, it is important to determine what type of immunotherapy provides the best chance of survival.

Method: Here, the efficacy and safety of immunotherapy in high-grade glioma (HGG) were evaluated by systematic review and meta-analysis. The differences between various types of immunotherapy were explored. Retrieved hits were screened for inclusion in 2,317 articles. We extracted the overall survival (OS) and progression-free survival (PFS) hazard ratios (HRs) as two key outcomes for examining the efficacy of immunotherapy. We also analyzed data on the reported corresponding adverse events to assess the safety of immunotherapy. This study was registered with PROSPERO (CRD42019112356).

Results: We included a total of 1,271 patients, of which 524 received a combination of immunotherapy and standard of care (SOC), while 747 received SOC alone. We found that immunotherapy extended the OS (HR = 0.74; 95% confidence interval [CI], 0.56–0.99; $Z = -2.00$, $P = 0.0458 < 0.05$) and PFS (HR = 0.67; 95% CI, 0.45–0.99; $Z = -1.99$, $P = 0.0466 < 0.05$), although certain adverse events occurred (proportion = 0.0773, 95% CI, 0.0589-0.1014). Our data have demonstrated the efficacy of the dendritic cell (DC) vaccine in prolonging the OS (HR = 0.38; 95% CI, 0.21–0.68; Z = –3.23; $P = 0.0012 < 0.05$) of glioma patients. Oncolytic viral therapy (VT) only extended patient survival in a subgroup analysis (HR = 0.60; 95% CI, 0.45–0.80; $Z = -3.53$; $P = 0.0004 < 0.05$). By contrast, immunopotentiation (IP) did not prolong OS (HR = 0.69; 95% CI, 0.50–0.96; $Z = -2.23$; $P = 0.0256$).

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Conclusion: Thus, DC vaccination significantly prolonged the OS of HGG patients, however, the efficacy of VT and IP should be explored in further studies. All the therapeutic schemes evaluated were associated with certain side effects.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=112356.

KEYWORDS

glioma, glioblastoma, temozolomide, standard of care, radiotherapy, immunotherapy, high-grade, meta-analysis

1 Introduction

Gliomas are the most common malignant tumor of the central nervous system (CNS) (1). According to the World Health Organization (WHO) classification of CNS tumors, gliomas can be classified into four grades: Grade 1 and Grade 2 define low-grade glioma (LGG), while Grade 3 and Grade 4 define high-grade gliomas (HGG) (2). The 2021 WHO classification further underscores the role of molecular signatures in stratifying glioma patients, in light of their effects on tumor biology. Thus, the classification incorporates criteria from the 2016 fourth edition, to facilitate the accurate diagnosis, prognosis estimation, and management of the patients with gliomas (1, 3). For example, the presence of a homozygous *CDKN2A/B* deletion results in the WHO Grade 4 classification of the isocitrate dehydrogenase (IDH)-mutant astrocytoma, even in the absence of high-grade histological features (3, 4).

HGGs have a dismal prognosis and are typically considered as incurable. In China, the incidence of HGG is about 5–8/100,000 individuals, and the 5-year mortality rate ranks third among solid tumors, after pancreatic cancer and lung cancer (2). In the U.S., HGG accounts for approximately 80.5% of the 24,560 new cases of malignant primary CNS tumors reported each year (5). Glioblastoma (GBM), as the most common type of WHO Grade 4 glioma, accounts for > 50% of HGGs, with a recurrence rate close to 100%, a 5-year survival rate of < 5%, and a median survival duration of ~ 15–17 months (6, 7). Standard of care (SOC) for HGG usually entails maximal surgical resection, followed by radiotherapy plus chemotherapy, administration of temozolomide (TMZ) as a front-line treatment or the PCV (procarbazine plus lomustine plus vincristine) scheme as an alternative strategy. Sometimes, bevacizumab can also be used as a targeted adjuvant therapy.

Immunotherapy, such as dendritic cell (DC) vaccination, chimeric antigen receptor T (CAR T) cell, immune checkpoint inhibitor (ICI), cytokine therapy, and viral therapy (VT) have gained much research attention and achieved great success in cancer treatment. In recent years, more evidence has shown that HGG patients can benefit from immunotherapy (8, 9). Currently, an immunotherapy using autologous, genetically-modified gamma-delta T cells is being investigated in a clinical trial of GBM (NCT05664243). Phase I studies of avelumab recruited six patients to complete the safety study (NCT02968940); a progression-free survival (PFS) of 4.2 months and an overall survival (OS) of 10.1 months was achieved. A phase II study of nivolumab recruited 26 patients to complete the toxicity study, with a PFS of 4.1 months and an OS of 7.3 months (NCT02550249). A phase II study of durvalumab (NCT02336165) showed that bevacizumab-naïve subjects with GBM who received durvalumab had a longer OS (4.5 months) than bevacizumab-refractory subjects (2 months). Meanwhile, compared with the above trials, DC therapies such as ICT-107(NCT01280552) and DCVax®-L were the most effective at improving survival; DCVax®-L has recently been approved for a phase III trial (NCT00045968). In addition, microsatellite instability arises in GBM during TMZ treatment, which induces TMZ resistance but promotes the response to ICIs (10). Therefore, immunotherapy may be a promising adjuvant for alleviating resistance to chemotherapy in HGG.

The immunotherapeutic interventions discussed in the current systematic review are categorized as follows:

1. Boosting adaptive immunity: DC vaccination (11–13) and oncolytic VT (AdvHSV-tk and PVSRIPO) (14–19).
2. Boosting innate immunity: Immunopotentiators (IP) such as transforming growth factor (TGF)-β2 anti-sense oligonucleotide (ODN) and Cpg-ODN (20, 21).

To evaluate the efficacy and safety of the combination of immunotherapy and SOC vs. SOC alone, this meta-analysis utilized patient survival data from published papers. We hope that our findings will help inform clinicians and scientists about the types of immunotherapy of most benefit to HGG patients.

2 Materials and methods

2.1 Search strategy and selection criteria

An electronic search was performed by two authors (B.F. Guo and J.C. Sun) using single terms and phrases through the four databases, Cochrane Library, Embase, PubMed, and Web of Science, for relevant articles published up to January 1st, 2020. Search terms included "high grade glioma", "astrocytoma", "glioblastoma", "immunity", "immunotherapy", "humans" and "randomized". An English language restriction was included. Clinical trials are registered on the website http://ClinicalTrials.gov were also explored.

The following inclusion criteria were adopted: (1) phase II-III clinical trials including at least two arms and its therapeutic intervention restricted to immunotherapy; (2) studies including adult patients (age ≥ 18) with HGG according to standardized diagnostic criteria; (3) studies comparing immunotherapy with SOC treatment. Studies were included when they meet all inclusion criteria. While studies were excluded when they meet any exclusion criteria including (1) studies lacking relevant outcome data; (2) trials without the SOC control arms; (3) phase I trials without NCT numbers; (4) phase II single-arm trials; (5) animal trials or cell assay; (6) abstracts and presentations from all major conference.

2.2 Data extraction and quality assessment

Two investigators (L.B. Xu and S.N. Zhang) extracted relevant information from the included articles. The hazard ratio (HR) has been described as a more suitable measure for analyzing time-to-event outcomes than the odds ratio or relative risk, and thus, the HR data were extracted (22, 23). When reports of HR and 95% confidence interval (CI) were not available, the estimated value was derived directly from Kaplan-Meier curves according to the methodology described by Jayne F Tierney (23). Dot plots of the graphical data were extracted *via* Engauge Digitizer 4.1 software (http://digitizer.sourceforge.net/). Most adverse events (AEs) were collected according to NCI-CTC 2.0/3.0.

2.3 Statistical analysis

Our statistical analyses were performed by R (version 3.6.1 for Windows; https://www.r-project.org/). The specific protocol for operation has been previously published (24). The main endpoints were OS, PFS, and AEs. The HR and 95% CIs were calculated for OS and PFS. The risk ratio (RR) and 95% CIs was calculated for part of AE, while other AEs with single-arm statistic materials were calculated by *Proportion* and 95% CIs. A random-effect model was used when the studies present significant heterogeneity. A fixed-effect model was used for those studies without significant heterogeneity. Heterogeneity across trials was assessed with the I^2 test, and $I^2 > 50\%$ and $P<0.05$ suggested that there was significant heterogeneity. Publication bias was examined by funnel plots. The specific information about types of immunotherapies, lesions, allocation methods, and so on was analyzed and discussed *via* a detailed subgroup analysis. Sensitivity analysis was performed to explore the impact of each individual study by removing one study at a time. Publication bias was examined by funnel plots.

3 Results

3.1 Trial selection

Overall, 2,315 citations were identified by the researchers, and 66 potentially eligible articles were retrieved in full text. 55 of them were excluded but four additional studies were included from another similar meta-analysis, resulting in 11 studies describing the efficacy of immunotherapy between 2004 and 2018. The literature screening process was shown in Figure 1. The Cochrane risk assessment form was used to evaluate the quality of the research. Nine of the 11 studies described the use of randomized control, the random sequence generation method was a random number table method, and two of them used the intent-to-treat (ITT) patients' baseline data to match similar historical patients', so they were evaluated as high-risk. Two of the 11 studies used the double-blind method, three of which were clearly described as open experiments, and the rest of the undescribed evaluations were unclear. Two studies describe the blind method of participants and none of the others does. Four studies had missing data for follow-up, so the incomplete report was evaluated as high risk. Two articles with incomplete reports were evaluated as high risk, and the rest were unclear. The quality of the included studies was evaluated as grade C. The evaluation details are shown in Supplementary Figure 1 and Supplementary Figure 2.

3.2 Main characteristics of the studies

We classified these studies into groups according to the classification of immunotherapy types, such as oncolytic VT (AdvHSV-tk + GCV/PVSRIPO), DC vaccination (autologous DC/mRNA-transfected DC/CD133 specific DC), and IPs (trabedersen/CpG oligonucleotide). A total of 524 participants were subjected to immunotherapy, and 747 participants were provided with SOC (a total of 1,271 participants).

A total of 914 participants from VT studies (393 patients in the experimental arm and 521 patients in the control arm) were included. Three of the studies containing TMZ in the SOC regimen also reported PFS. The details can be found in Table 1. There were three studies on DC therapy. All studies used TMZ in the SOC regimen. 90 participants underwent DC therapy (43 patients in the experimental arm and 47 patients in the control arm) (Table 2). IP was used in two studies that applied TMZ in the SOC regimen (88 patients in the experimental arm and 179 patients in the control arm) (Table 3).

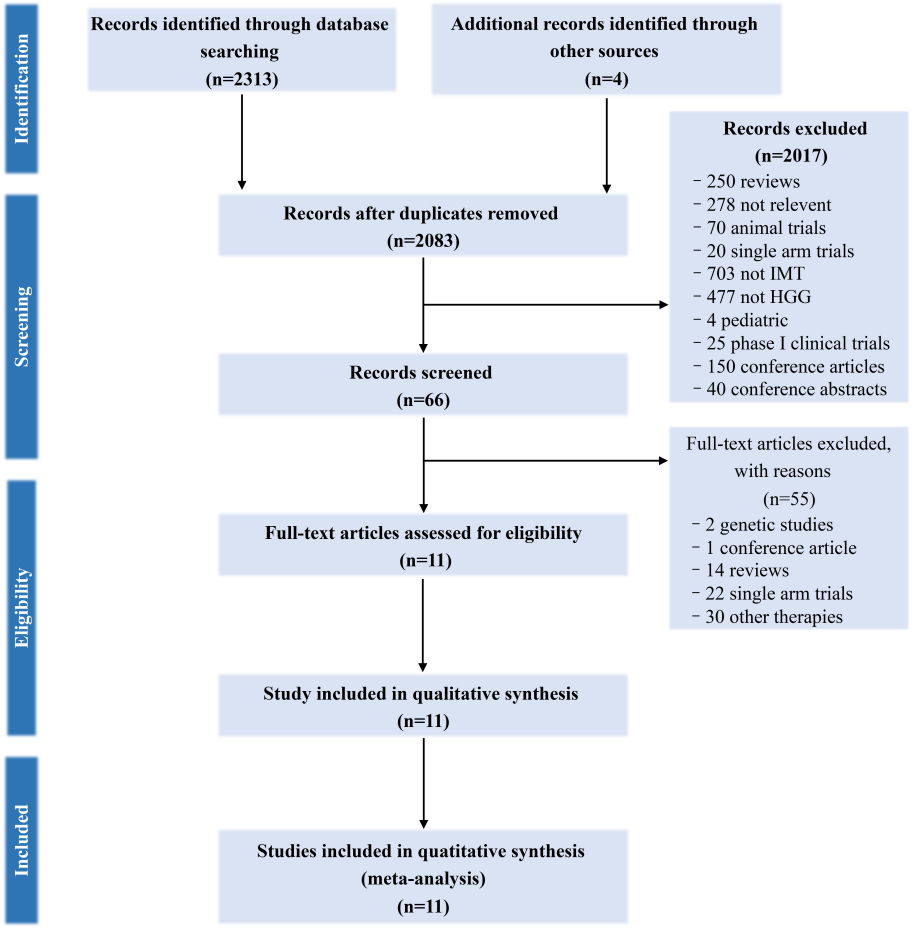

FIGURE 1
PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) flow-chart of search strategy.

3.3 Efficacy and safety analysis of immunotherapy

3.3.1 Overall survival

OS data was extracted from 11 studies with 1,271 participants. However, substantial heterogeneity was found, which shows the variability among the OS data (τ^2 =0.1315; I^2 =65 > 50%; $P < 0.05$). Thus, we employed a random-effect model to assess the efficacy of extending OS. It showed that immunotherapy decreased the risk of death by 26% compared with the SOC (HR = 0.74; 95% CI, 0.56–0.99; $P = 0.0458$; Supplementary Figure 3). Sensitivity analysis was performed to assess how each study influenced efficacy estimates (Supplementary Figure 4), and it showed our result was stable. In the funnel plot, it was found that there was a substantial publication bias, so the trim and fill method was used to adjust publication bias with a new effect estimate by complementing four studies. Unexpectedly, it showed that compared with SOC, immunotherapy did not improve the OS of patients (HR = 0.98; 95% CI, 0.72–1.34; $Z = -2.00$; $P = 0.90 > 0.05$) (Supplementary Figure 5).

3.3.1.1 Subgroup analysis by therapeutic schemes

To further understand the efficacy of immunotherapy for HGGs, subgroup analysis was performed by therapeutic schemes. It was obvious that VT and IP did not prolong the OS [(HR = 0.76; 95% CI, 0.54–1.07; $P > 0.05$); (HR = 1.23; 95% CI, 0.83–1.82; $P > 0.05$)], while DC therapies prolonged OS significantly (HR = 0.38; 95% CI, 0.21–0.68; $P = 0.0012 < 0.05$] (Figure 2). The results were basically consistent with the previous reports (Supplementary Table 1).

3.3.1.2 Subgroup analysis by lesion type

To assess the efficacy of immunotherapy for different types of HGGs, subgroups analysis according to lesion type (recurrent/primary and type of glioma) was performed but showed no significant difference between subgroups, which might attribute to the limited number of studies ($Q = 11.08$; $P = 0.085 > 0.05$) (Supplementary Figure 6).

3.3.1.3 Subgroup analysis by implementary plan of clinical trials

To explore the relationship between the implementary plan of clinical trials and efficacy, subgroup analysis was performed. It

TABLE 1 Main Characteristics of studies that use viral therapy (VT) for the treatment of HGGs.

Study name	NCT number	Pha-se	Patients (E/C)	Age (E)	Age (C)	Intervention	Control	KPS score	Region	Patients Characte-ristics	Follow-up Time	EM OS	CM OS	EM PFS	CM PFS	SOC
Rainov 2000	N/A	III	121/116	60	58	RVHSV-tk+GCV +SG +RT	SG+RT	≥70	Europe	Newly diagnosed GBM	21.6	12.2	11.8			VT+ SOC (SG+RT)
Immonen 2004	N/A	II	17/19	52	56.5	AdvHSV-tk+GCV +SG +RT	SG+RT	≥70	Europe	PR/REC HGG	56	15.5	9.4			VT+ SOC (SG+RT)
Ji 2015	N/A	II	22/22	49	54	AdvHSV-tk +GCV+SG	SG+RT+ chemo	N/A	China	Recurrent HGG	60	10.6	3.3	6.9	2.0	VT+ SOC (SG+RT+ chemo)
Westphal 2013	2004-000464-28[a]	III	124/126	58	57	AdvHSV-tk+GCV +SG +RT+TMZ (48.7%)	SG+RT+ TMZ (65%)	≥70	Europe	Newly diagnosed GBM	46	16.6	15.1			VT+ SOC (SG+RT +partly chemo)
Wheeler 2016	NCT00589875	II	48/134	57	60	AdvHSV-tk+GCV +SG +RT+TMZ	SG+RT+ TMZ	≥70	USA	Newly diagnosed HGG	60	17.1	13.5	8.1	6.5	VT+ SOC (SG+RT+ chemo)
Desjardins 2018	NCT01491893	II	61/104	55	55	PVSRIPO+Bev+SG +chemotherapy+RT	Bev+ SG+ chemotherapy +RT	≥70	USA	Recurrent GBM	27.6	11.7	10.5			VT+ SOC (SG+RT+ chemo)

E, experiment group; C, control group; Age, median age; MOS, median survival; MPFS, median progression-free survival; N/A, not available; SG, surgery; RT, radiotherapy.

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TABLE 2 Main Characteristics of studies that use DC therapy for the treatment of HGGs.

Study name	NCT number	Pha-se	Patient (E/C)	Age (E)	Age (C)	Intervention	Control	KPS score	Region	Patients Characte-ristics	Follow-up Time	EM OS	CM OS	EM PFS	CMPFS	SOC
Cho 2012	N/A	II	16/18	58	58.5	DCV+ SG+RT+TMZ	SG+RT +TMZ	>70	China	Newly diagnosed GBM	56	31.9	15.0	8.5	8.0	DC+ SOC (SG+RT+ chemo)
Vik-mo 2013	NCT00846456	N/A	6/7	57	56	DCV+ SG+RT+TMZ	SG+RT +TMZ	N/A	Europe	Newly diagnosed GBM	33	25.3	19.5	23.1	7.9	DC+ SOC (SG+RT+ chemo)
Yao 2018	NCT01567202	II	21/22	48	50	DCV+ SG+RT+TMZ	SG+RT +TMZ	≥60	China	PR/REC GBM	14	13.7	10.7	7.7	6.9	DC+ SOC (SG+RT+ chemo)

E, experiment group; C, control group; Age, median age; MOS, median survival; MPFS, median progression-free survival; N/A, not available; SG, surgery; RT, radiotherapy.

TABLE 3 Main Characteristics of studies that use immunopotentiator therapy for the treatment of HGGs.

Study name	NCT number	Pha-se	Patient (E/C)	Age (E)	Age (C)	Interven -tion	Con -trol	KPS score	Region	Patients Charac-teristics	Follow-up Time	EMOS	CMOS	EM PFS	CM PFS	SOC
Ursu 2017	NCT00190424	II	39/42	62	59	CpG-ODN+ SG+ RT+TMZ	SG+ RT+ TMZ	≥60	Europe	Recurrent GBM	60.0	15.9	16.8	9.0	8.5	IP+ SOC (SG+RT+ chemo)
Bogdahn 2011	N/A	IIb	40/45	51.5	48	TGF-β2 inhibitor +SG+RT+ TMZ	SG+ PCV + TMZ	≥70	Europe	Recurrent GBM/AA	70.0	39.1	21.7			IP+ SOC (SG+RT+ chemo)

E, experiment group; C, control group; Age, median age; MOS, median survival; MPFS, median progression-free survival; N/A, not available; SG, surgery; RT, radiotherapy.

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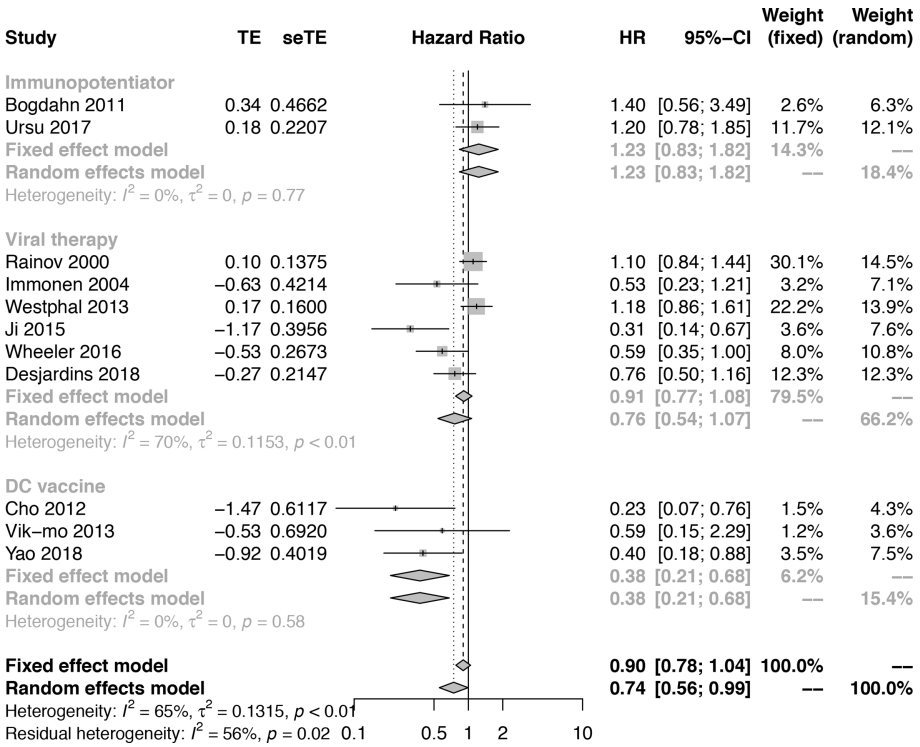

FIGURE 2
Subgroup analysis of the combination of immunotherapy and standard of care compared with standard of care according to therapeutic scheme.

showed that there was no significant difference between the open-label trials and double-blind trials (Q = 1.22; P = 0.2703 > 0.05) (Supplementary Figure 7). Besides, it was noted that the efficacy of observational historical matched studies which match historical patients' baseline information to mimic randomization was better than randomized controlled trial (RCT) studies which also implied the difference between RCT and historical matched studies (Q = 0.18; P = 0.673 > 0.05) (Supplementary Figure 8).

3.3.1.4 Subgroup analysis by recruiting area of clinical trials

According to the recruiting area (multi-center, Europe, China, and the United States), there was a significant difference between the subgroups, and the difference was statistically significant (Q = 23.67, P < 0.0001) (Supplementary Figure 9). The results showed that studies carried out in China and the United States had a better effect on prolonging OS [(HR = 0.33; 95% CI, 0.20–0.54; P < 0.05); (HR = 0.69; 95% CI, 0.50–0.96; P < 0.05)].

3.3.1.5 Subgroup analysis by the intervention of SOC

As the intervention of SOC is also crucial for the efficacy of immunotherapy, the subgroups were divided by the intervention of SOC (TMZ/No TMZ). It showed that there was a significant difference between subgroups (Q = 4.33; P = 0.0374 < 0.05). It suggested that TMZ synergized with immunotherapy (HR=0.63, 95% CI, 0.43–0.94; P = 0.01 < 0.05), (Supplementary Figure 10).

Overall, the influence factors were examined and summarized according to the defined characteristics of interventions, clinical trials, lesions, and study design (Table 4).

3.3.2 Progression-free survival

As six studies involving a total of 397 participants reported PFS, a random-effect model was used to assess the efficacy of immunotherapy versus SOC according to the HR of PFS. Based on the meta-analysis (Supplementary Figure 11) and sensitivity analysis (Supplementary Figure 12), the combination of immunotherapy and SOC significantly improved the PFS (HR = 0.67; 95% CI, 0.45-0.99; Z = −1.99; P = 0.0466 < 0.05). The funnel plot was performed to evaluate the publication bias and the trim and fill method was used. A new combined effect value was obtained by supplementing two studies. The results showed that compared with SOC, immunotherapy did not improve the PFS (HR = 0.83; 95% CI, 0.54−1.28; Z = −0.84; P = 0.399 > 0.05] (Supplementary Figure 13).

3.3.2.1 Subgroup analysis by therapeutic schemes

We wondered if some variables influenced the efficacy of PFS like OS as mentioned before. According to the type of immunotherapy (IP, VT, DC), there was no significant difference between the subgroups (Q = 4.84; P = 0.089 > 0.05) (Supplementary Figure 14).

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TABLE 4 Subgroup analysis of IMT and death incidence for aach variables.

Variable	No. of Studies	No. of Participants		OS, HR(95%CI)	I^2 value(%)	*p* value[c]
		IMT[a]	SOC[b]			
Therapy type						
DC vaccine	3	43	47	0.38 [0.21;0.68]	0	0.004**
Immunopotentiator	2	88	179	1.23 [0.83;1.82]	0	
Viral therapy	6	393	521	0.76 [0.54;1.07]	70	
Lesion Type						
Newly diagnosed GBM	3	143	141	0.60 [0.22;1.64]	71	0.085
Primary and recurrent GBM	1	21	22	0.53 [0.23;1.21]	/	
Newly diagnosed HGG	2	172	260	0.86 [0.44;1.70]	80	
Recurrent HGG	1	22	22	0.31 [0.14;0.67]	/	
Recurrent GBM	2	109	238	0.95 [0.61;1.49]	55	
Primary and recurrent HGG	1	17	19	0.40 [0.18;0.88]	/	
Recurrent AA	1	40	45	1.40 [0.56;3.49]	/	
Label type						
Open-label	8	331	465	0.66 [0.46; 0.96]	63	0.270
Double Blind	3	193	282	0.93 [0.57; 1.54]	70	
Study Design						
Randomized	8	409	502	0.76 [0.52; 1.10]	72	0.673
Historical control	3	115	245	0.68 [0.50; 0.94]	0	
Recruiting area						
Multi-center	2	245	242	1.13 [0.92; 1.39]	0	0.001*
Europe	4	111	205	0.96 [0.62; 1.50]	26	
China	3	59	62	0.33 [0.20; 0.54]	0	
USA	2	109	238	0.69 [0.50; 0.96]	0	
SOC Type						
TMZ	8	262	486	0.63 [0.43; 0.94]	56.8	0.037*
Not TMZ	3	262	261	1.05 [0.80; 1.39]	58.1	

[a]IMT, Immunotherapy combined with SOC; [b]SOC, Standard o Care; [c]p value for subgroup differences (random effects model was applied in first two variables; fixed effects model was applied in other variables); *p < 0.05; **p < 0.01.

3.3.2.2 Subgroup analysis by lesion type

Besides, according to lesion type (recurrent GBM, primary and recurrent GBM, newly diagnosed GBM, recurrent HGG, HGG), the results also showed that there was no significant difference between the subgroups ($Q = 9.46$; $P = 0.051 > 0.05$) (Supplementary Figure 15).

3.3.3 Adverse events of immunotherapy

A total of eight studies reported on the occurrence of AEs of immunotherapy combined with SOC. The toxic and side effects of immunotherapy combined with conventional treatment were evaluated through a single-arm meta-analysis first. The sample size was 397. After the heterogeneity test ($\tau^2 = 1.3055$; I^2 =95.5% > 50%; $P = 0 < 0.05$), random-effect model was adopted. It showed immunotherapy combined with SOC had a risk of AEs (proportion = 0.0773; 95% CI, 0.0589–0.1014) (Supplementary Figure 16). A total of five studies reported the occurrence of AE in both experiment and control arms so the RR value was used to evaluate the toxicity and side effects of immunotherapy. The sample size was 709. After the heterogeneity test ($\tau^2 = 1.3055$; $I^2 = 78.8\% > 50\%$; $P < 0.0001$), a random-effect model was adopted. Meta-analysis results showed that compared with SOC, the risk of AE of immunotherapy combined with SOC increased by 67.37% (RR = 1.67; 95% CI, 1.28–2.19; $Z = 3.76$; $P = 0.0002 < 0.05$), suggesting that immunotherapy had certain toxicity and side effects (Supplementary Figure 17).

3.4 Efficacy analysis of VT, DC therapy and IP

3.4.1 The efficacy of VT

The HR of OS was used to compare the efficacy of VT combined with SOC and SOC alone. A total of six studies, with a sample size of 913, were tested for meta-analysis ($\tau^2 = 0.115$; $I^2 = 70.5\% > 50\%$; $P = 0.0046 < 0.05$). It showed that compared with SOC, the combination of VT and SOC had a trend to prolong the OS of patients, but the difference was not statistically significant (HR = 0.76; 95% CI, 0.54–1.07; $Z = -1.58$; $P = 0.113 > 0.05$) (Supplementary Figure 18). Sensitivity analysis proved our results were stable (Supplementary Figure 19). The funnel plot showed asymmetry so the trim and fill method was used to further evaluate the publication bias. A new combined effect estimate was obtained by supplementing three studies. The results showed that compared with SOC, the combination of VT and SOC did not improve the OS of patients with HGG (HR = 1.03; 95% CI, 0.72–1.48; $Z = -0.17$; $P = 0.869 > 0.05$) (Supplementary Figure 20).

The HR of PFS was used to further evaluate the efficacy of VT combined with SOC. There were only two studies with a sample size of 226 cases. After heterogeneity test ($\tau^2 = 2.12$; $I^2 = 77.7\% > 50\%$; $P = 0.0344 < 0.05$), random-effect model was used for meta-analysis. The results showed that compared with SOC, the combination of VT and SOC did not prolong the PFS (HR = 0.52; 95% CI, 0.22–1.22; $Z = -1.50$; $P = 0.1343 > 0.05$) (Supplementary Figure 21). The progression-free survival HR value was also used for sensitivity analysis which was consistent with the previous result (Supplementary Figure 22).

To explore the potency of VT, subgroup analysis according to the injection method of VT (treatment course, injection volume, multipoint injections) showed that there are significant differences between the subgroups ($Q = 16.27$; $P<0.05$). The treatment of VT with more than two courses of treatment and multipoint injections combined with SOC manifested a better effect on improving the OS (HR = 0.31; 95% CI, 0.14–0.67). However, the use of 9~10 ml injection volume of VT and multipoint injections did not exhibit the treatment effect on prolonging the OS [(HR = 0.53; 95% CI, 0.23–1.21); (HR = 1.13; 95% CI, 0.92–1.39)]. Noteworthily, VT with an injection volume of 1~3.5 ml significantly prolonged the OS compared with VT with an injection volume of 9~10 ml [(HR = 0.69; 95% CI, 0.50–0.96); (HR = 1.13; 95% CI, 0.92–1.39)] (Figure 3).

We pooled all trials that employed the injection method including multiple courses of treatment, multipoint injection, and small injection volume of VT. There were four studies with a sample size of 427. It showed that VT with optimized injection methods combined with SOC significantly prolonged the OS (HR = 0.60; 95% CI, 0.45–0.80; $Z = -3.53$; $P = 0.0004 < 0.05$) (Figure 4). The sensitivity analysis showed our result was stable. The combined effect size was consistent with the previous results (Supplementary Figure 23). However, the funnel plot was asymmetric, suggesting there was a publication bias. Thus, the trim and fill method was used to evaluate the publication bias, and a new combined effect

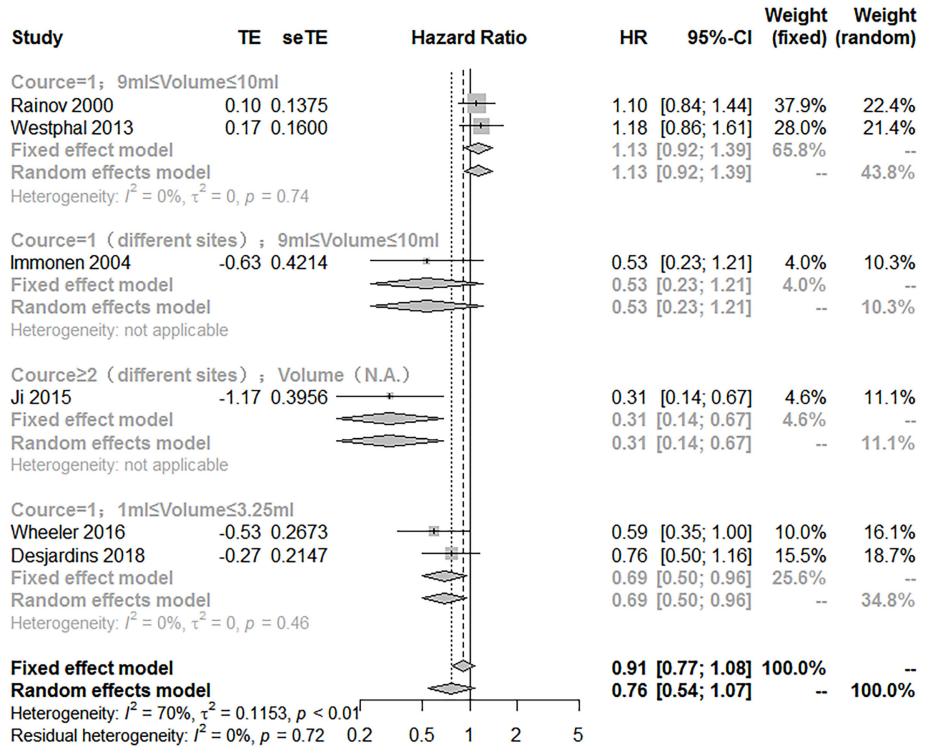

FIGURE 3
Subgroup analysis of the combination of viral therapy and standard of care compared with standard of care according to therapeutic scheme.

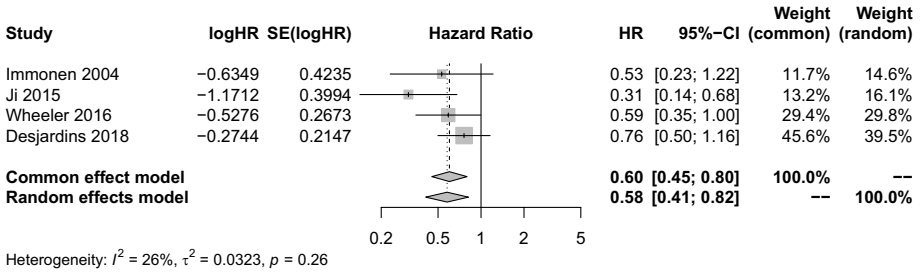

FIGURE 4
Analysis of the OS of the combination of multiple courses of treatment/multi-point injection/small injection volume viral therapy and standard of care compared with standard of care.

value was obtained by supplementing two studies. The results showed VT with optimized injection methods combined with SOC prolong the OS of HGG patients (HR = 0.68; 95% CI, 0.47–0.99; $Z = -2.89$; $P = 0.0039 < 0.05$) (Supplementary Figure 24).

The HR value of PFS was also used to evaluate the efficacy of VT with optimized injection methods combined with SOC. There were two studies with a sample size of 226 cases. It showed that VT with optimized injection methods combined with SOC did not improve the PFS (HR = 0.52; 95% CI, 0.22–1.22; Z = −1.50; $P = 0.1343 > 0.05$) (Supplementary Figure 25). Sensitivity analysis showed the combined effect estimate was consistent with the previous results (HR = 0.52; 95% CI, 0.22–1.22; $Z = -1.50$; $P = 0.1343 > 0.05$) (Supplementary Figure 26).

3.4.2 The efficacy of DC therapy

The HR of OS was used to evaluate the efficacy of DC therapy combined with SOC. There were three studies with a sample size of 90. It showed DC therapy combined with SOC significantly improved the OS of patients with HGGs (HR = 0.38; 95% CI, 0.21–0.68; $Z = -3.23$; $P = 0.0012 < 0.05$) (Supplementary Figure 27). Sensitivity analysis proved our results were stable (Supplementary Figure 28). Funnel plot was symmetric. Notwithstanding, the trim and fill method was still used to evaluate the publication bias, and a new combined effect value was obtained by supplementing two studies. It showed that compared with SOC, the combination of DC therapy and SOC improved the OS of patients with HGGs (HR = 0.38; 95% CI, 0.21–0.68; $Z = -3.23$; $P = 0.0012 < 0.05$) (Supplementary Figure 29).

The HR of PFS was also used to evaluate the efficacy of DC therapy combined with SOC. There were three studies with a sample size of 90. It showed that compared with SOC, the combination of DC therapy and SOC did not improve the PFS (HR = 0.60; 95% CI, 0.35–1.03; $Z = -1.84$; $P = 0.066 > 0.05$) (Supplementary Figure 30). Sensitivity analysis showed the combined effect estimate was consistent with the previous results (Supplementary Figure 31).

3.4.3 The efficacy of IP

The HR value of OS was used to compare the efficacy of IP combined with SOC and SOC alone. There were two studies with a sample size of 166. It showed that compared with SOC, the combination of IP and SOC did not improve the OS of patients with HGGs (HR = 1.23; 95% CI, 0.83–1.82; $Z = 1.05$; $P = 0.2918 > 0.05$) (Figure 5). Sensitivity analysis showed our result was stable. The combined effect estimate was consistent with the previous results (Supplementary Figure 32). The funnel plot was asymmetry so the trim and fill method was still used to evaluate the publication bias, and the results showed that compared with SOC, the combination of IP and SOC did not prolong the OS of patients with HGGs (HR = 1.23; 95% CI, 0.83–1.82; $Z = 1.05$; $P = 0.2918 > 0.05$) (Supplementary Figure 33).

3.5 Adverse events of different type of immunotherapy

3.5.1 The safety of oncolytic VT

According to the type of immunotherapy, the toxic and side effects of VT combined with SOC were evaluated through a single-arm meta-analysis first. A total of three studies (270 participants)

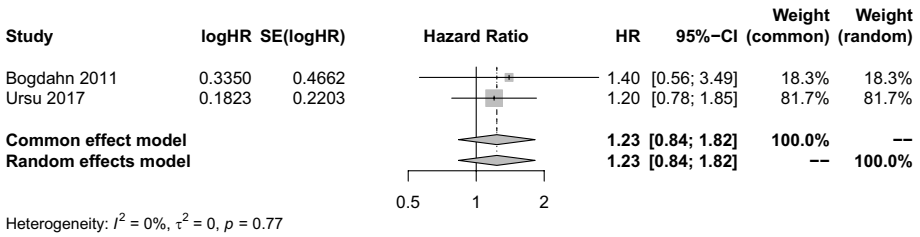

FIGURE 5
Analysis of the OS of the combination of immunopotentiators and standard of care compared with standard of care.

reported the occurrence of AE of VT combined with SOC. After the heterogeneity test (τ^2 = 1.0463; I^2 = 82% > 50%; P < 0.05), a random-effect model was adopted. The meta-analysis results show that there was a risk (3%) of AE with the combination of VT and SOC (proportion = 0.03; 95% CI, 0.02–0.05) (Supplementary Figure 34), suggesting that the combination of VT and SOC had moderate toxicity and side effects. It was noteworthy that compared with the occurrence of AE with the combination of immunotherapy and SOC (proportion = 0.0773; 95% CI, 0.0589–0.1014), the incidence of AE with the combination of VT and SOC was lower. A total of three studies reported the occurrence of AE in double arms. It showed that compared with SOC, the risk ratio of AE with VT combined with SOC increased by 45.33% (RR = 1.45; 95% CI, 1.18–1.79; Z = 3.50; P = 0.0005 < 0.05), suggesting that compared with SOC, the combination of VT and SOC still had certain side effects (Supplementary Figure 35).

3.5.2 The safety of DC therapy

Next, the toxicity and side effects of DC therapy combined with SOC were evaluated through a single-arm meta-analysis. The sample size was 47. After heterogeneity testing (τ^2 = 0.9188; I^2 = 83.4% > 50%; P < 0.05), random-effect model was used. Single-arm meta-analysis results showed that there was a risk (18%) of AE in the combination of DC therapy and SOC [proportion = 0.1800, 95% CI 0.0959–0.3379] (Supplementary Figure 36). It should be noted that with the sample size increasing, the probability of AE can be increased proportionally. Compared with the incidence of AE in immunotherapy and SOC (proportion = 0.0773; 95% CI, 0.0589–0.1014], the incidence of AE in DC therapy and SOC was higher.

3.5.3 The safety of IP

Finally, the toxicity and side effects of IP combined with SOC were evaluated through a single-arm meta-analysis. The sample size was 80. After the heterogeneity test (τ^2 = 0.6718; I^2 = 95% > 50%; P < 0.05), a random-effect model was used. Meta-analysis results showed that there was a risk (18%) of AE in the combination of IP and SOC (proportion = 0.18; 95% CI, 0.13–0.25). Compared with the incidence of AE in the combination of immunotherapy and SOC (proportion = 0.0773; 95% CI, 0.0589–0.1014), the incidence of AE in the combination of IP and SOC was higher (Supplementary Figure 37). A total of two studies reported the occurrence of AE in double arms. The RR value was used to evaluate the side effects of immunotherapy. A sample size was 167. Using random-effect model (τ^2 = 1.0750; I^2 = 91.8% > 50%; P < 0.05), meta-analysis results showed that compared with SOC, the incidence of AE of IP combined with SOC increased by 102.91% (RR = 2.0291; 95% CI, 1.2700–3.2418; Z = 2.96; P = 0.0031 < 0.05) (Supplementary Figure 38).

Based on the above results, we summarized the specific AE of immunotherapy (Table 5). It was noted that HSV-TK viral therapy resulted in cognitive disorder (RR = 5.08; 95% CI, 0.25–104.76), high intracranial pressure (RR = 5.08; 95% CI, 0.25–104.76), extradural hematomas (RR = 7; 95% CI, 0.37–134.11) and catarrhal symptoms (RR = 11; 95% CI, 0.65–187.42). TGF-2 oligonucleotides suffered from infections (RR = 7.67; 95% CI, 0.41–144.19), brain edema (RR = 6.04; 95% CI, 1.42–25.63), depressed level of consciousness (RR = 12.06; 95% CI, 0.69–211.5), hemiparesis (RR = 12.07; 95% CI, 1.63–89.47), and psychiatric disorder (RR = 5.49; 95% CI, 1.63–89.47). Whereas CpG-ODN led to anemia (RR = 5.38; 95% CI, 0.66–44.07) and sepsis (RR = 9.68; 95% CI, 0.54–174.14).

4 Discussion

Immunotherapy is playing an increasingly important role in the treatment of tumors. Various types of immunotherapy are available to patients. Thus, clinicians and researchers alike need to understand which types of immunotherapy will be most effective in a given disease setting. To this end, we performed a systematic review of the efficacy and safety of immunotherapy and SOC in adult HGG patients. Our results showed that immunotherapy combined with TMZ yielded better results than SOC alone.

TABLE 5 Specific Adverse Events of Immunotherapy.

Type of immunotherapy	Symptoms and physical signs.	RR
TGF-β2 oligonucleotides	Infections	7.67[0.41, 144.19]
	Brain edema	6.04[1.42, 25.63]
	Depressed level of consciousness	12.06[0.69, 211.5]
	Hemiparesis	12.07[1.63, 89.47]
	Psychiatric disorder	5.49[0.67, 45.04]
Cpg oligonucleotides	Anaemia	5.38[0.66, 44.07]
	Sepsis	9.68[0.54, 174.14]
HSV-TK viral therapy	Cognitive disorder	5.08[0.25, 104.76]
	Intracranial pressure	5.08[0.25, 104.76]
	Extradural hematoms	7[0.37, 134.11]
	Catarral symptoms	11[0.65, 187.42]

We found that for HGG, the order of efficacy for the immunotherapies evaluated was as follows: DC therapy > VT > IP. DC vaccination triggers *de novo* immune responses against foreign antigens by activating T cells and B cells, which provides a theoretical basis for the development of vaccines against tumor cells. Consistent with our findings, recent results from a large phase III clinical trial (NCT00045968) of an autologous tumor lysate-loaded DC vaccine (DCVax-L) combined with TMZ showed that the median (m)OS was significantly extended in both newly diagnosed patients and those with recurrent GBMs; the therapy also had a good safety profile (25, 26). Notably, because of pseudoprogression and the fact that placebo patients received DCVax-L following crossover, OS, rather than PFS, was considered a feasible endpoint, by comparison to external control populations. The mOS of newly diagnosed GBM patients in the DCVax-L group (N = 232) was 19.3 months (95% CI, 17.5–21.3) from randomization vs. 16.5 months (95% CI, 16.0–17.5) in the control cohort (N = 1,366). For patients with recurrent GBM, mOS was 13.2 months (95% CI, 9.7–16.8) from relapse for patients receiving DCVax-L (N = 64) and 7.8 months (95% CI, 7.2–8.2) for the control group (N = 640). Besides, a meta-analysis also assessed the clinical impact of DC vaccination and VT in comparison to SOC for patients with HGG (27). Eight phase I/II clinical trials of DC vaccines were analyzed and the results showed that OS was markedly improved in both patients with newly diagnosed (HR = 0.65) and recurrent (HR = 0.63) HGG when treated a DC vaccine vs. SOC; however, improvement in PFS was not statistically significant (P = 0.1), which is consistent with our findings. Another meta-analysis performed by Lv et al. included data from six phase II RCTs of DC vaccines in patients with GBM and reported that OS was significantly improved following treatment with a DC vaccine vs. placebo or blank treatment (HR = 0.69; 95% CI, 0.49–0.97; P = 0.03 < 0.05) (28). In this case, however, PFS in GBM patients was somewhat improved as a result of DC vaccination (HR = 0.76; 95% CI, 0.56–1.02; P = 0.07), without significant heterogeneity (I^2 = 0). However, the preparation methods and activation strategy of the DC vaccines differed between studies. Future research needs to determine whether the preparation and activation mode of the DC vaccine affects the efficacy of this immunotherapy.

Our analysis of oncolytic VT trial data showed that, compared with SOC, the combination of VT and SOC did not provide any statistically significant improvement in OS or PFS for patients with HGG. The VT injection specific characteristics were shown here (Table 6). Only the results of a subgroup analysis indicated that VT prolonged OS with optimized injection methods. In accordance, a meta-analysis of four phase I/II/III clinical trials reported that VT did not significantly improve the OS or PFS of patients with newly diagnosed HGGs (27). Currently, there are more than 20 trials registered at *ClinicalTrials.gov* evaluating the efficacy and safety of oncolytic VT in patients with glioma. The modified viral species associated with encouraging results in phase I/II clinical trials include herpes simplex virus, reovirus, vaccinia virus, adenovirus and parvovirus. Although it did not meet the inclusion criteria of the present study, a recent phase II clinical trial evaluated the survival benefits and safety profile of immunotherapy with an intratumoral, oncolytic herpes virus, G47Δ, in residual or recurrent GBM (29). The 1-year survival rate of G47Δ-treated patients was 84.2% (95% CI, 60.4–96.6; 16 of 19) and the mOS was 20.2 months (16.8–23.6 months) vs. 28.8 months (20.1–37.5 months) for patients treated with initial surgery alone. Moreover, patients treated with G47Δ had higher a number of tumor-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes and persistently lower Foxp3 levels in the tumor tissues, than controls. These promising results led to the approval of G47Δ in Japan as the first oncolytic VT product to be used for the treatment of patients with malignant glioma.

For the IP, two trials were analyzed in our study (20, 21). CpG-ODN is a new type of immunostimulating agent, which comprises an immunomodulatory synthetic ODN specifically designed to stimulate Toll-like receptor 9 (30). Currently, there are four phase I/II clinical trials of CpG-ODN involving glioma patients, one of which met our study criteria (21). The study found that IP exhibited poor efficacy, which the authors claimed was unexpected and may have been related to the selection bias of the enrolled patients with recurrent GBM or a difference in the mode of CpG-ODN administration. In accordance, a phase II clinical trial of CpG-ODN, administered intracerebrally to patients with recurrent GBM, did not meet the targeted PFS; however, encouragingly, a few long-term survivors were observed (31). Another IP currently in use is trabedersen (also known as OT-101). It is a synthetic antisense ODN designed to block the production of human TGF-β2. TGF-β2 is reported to exert protumor effects in the tumor microenvironment (TME) *via* different mechanisms, such as by stimulating angiogenesis, promoting T cell exclusion, and preventing helper T (Th)1 effector phenotype differentiation,

TABLE 6 Specific characteristics of viral therapy injections.

Name	Volume	Dose	Cycle	Course	Injection
Desjardins 2018	3.5ml	10^8~10^{10}	1	6.5 hours	/
Immomen 2004	10ml	$3x10^{10}$	1	/	30-70 injections
Ji 2016	/	$1x10^{12}$	≥2	21 Days	/
Rainov 2000	9-10ml	$1x10^8$	1	/	/
Westphal 2013	10ml	$1x10^{12}$	1	/	/
Wheeler 2016	1ml	$3x10^{11}$	1	/	10 injections

/, Not available.

which collectively contribute to tumor immune evasion (32). A recent *post-hoc* analysis of a phase II clinical trial (NCT00431561) data was performed to evaluate the efficacy of trabedersen treatment for recurrent and/or refractory HGG with a poor prognosis (33). The results showed that the intratumorally delivery of trabedersen, *via* a convection-enhanced delivery system, exhibited promising single-agent antitumor activity, resulting in a PFS of > 3 years and an OS of > 3.5 years in over a third of HGG patients. Notably, pseudoprogression was observed in ~10% of patients receiving trabedersen, which was associated with improved survival. To date, only one phase III clinical trial (NCT00761280, initiated in 2008) of trabedersen has involved glioma patients; unfortunately, it was terminated early in 2012 because it did not fulfil its projected patient recruitment figures. Future clinical trials are therefore warranted to further evaluate the efficacy and safety profiles of IP in large-scale patient cohorts.

Due to our stringent criteria, only DC therapy, VT, and IP met the requirements and were included in the study. Notwithstanding, other types of immunotherapy, such as ICIs and CAR (either T or natural killer [NK] type), have shown promise in the treatment of glioma. ICI therapy is one of the earliest forms of cancer immunotherapy. It functions by restoring cytotoxic T cell activity and enhancing antitumoral adaptive immunity (34). Glioma cells express high levels of immunosuppressive factors, such as programmed cell death receptor 1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), which reduce the proliferation and activation ability of T cells and weaken the antitumor immune response. Antibodies against CTLA-4, PD-1, or its ligand, PD-L1, are the most widely studied ICIs in the clinic (35). The success of ICIs in treating various solid tumors has aroused great interest in relation to their application to treat brain tumors (36, 37). Although no ICI trials met our study inclusion criteria, a study that retrospectively analyzed 66 GBM patients who were treated with SOC and the PD-1 inhibitors, pembrolizumab or nivolumab, confirmed that the OS of patients who were responsive to immunotherapy was significantly longer than that of the non-responders (14.3 vs. 10.1 months) (38). Further genomic and transcriptome profiling also revealed multiple genomic alterations and evolutionary patterns in GBM patients undergoing anti-PD-1 immunotherapy. These included enrichment in MARK pathway changes in responders and the increased *PTEN* mutations, which correlated with immunosuppressive expression characteristics, in non-responders. Of note, the CheckMate 143 trial, which was the first randomized phase III study of an ICI in patients with primary brain tumors (NCT02017717) (39), evaluated the efficacy and safety of nivolumab (a PD-1 inhibitor) alone or in combination with bevacizumab (a vascular endothelial growth factor [VEGF] inhibitor) in patients with first relapsed GBM following standard radiotherapy and TMZ treatment. A total of 369 patients were randomized to nivolumab (n = 184) or bevacizumab (n = 185) and no statistically significant difference was found in the risk of death between the groups after treatment (HR = 1.04; 95% CI, 0.83–1.30; P = 0.76). However, a subgroup analysis showed that patients with a methylated *MGMT* promoter and no baseline corticosteroid use may potentially benefit from ICI treatment. Besides, clinical trials are currently underway to evaluate the use of local radiotherapy in combination with anti-PD-1 antibodies in patients with newly diagnosed or recurrent GBM (NCT02648633 and NCT02866747). Currently, the clinical benefits of new immune checkpoint molecules, such as inhibitors of the V-domain immunoglobulin suppressor of T cell activation (VISTA), CD73, and CD38, are being studied. Despite the fact that ICI therapies have improved patient outcomes across numerous cancer types, only a minority (< 10%) of GBM patients achieve a durable response (38, 40, 41). Importantly, current ICI treatment regimens are usually maintained for ~2 years; whether longer-term ICI treatment can improve the curative effect is still under study. Thus, more preclinical and clinical research is needed to further verify the efficacy of ICIs and explore the mechanism underlying their failure in the treatment of HGGs, including GBM.

CAR T cell therapy has shown promising therapeutic effects in patients with hematological malignancies; however, its application in the treatment of GBM is still in the early stages of development. A preliminary study by Brown et al. evaluated the effect of repeated intracranial injection of $CD8^+$ CAR T cells targeting the interleukin (IL)-13 receptor subunit alpha 2 (IL-13Rα2), which is overexpressed in > 50% of GBMs, in three patients with relapsed GBM (42). The treatment was well tolerated and transient antitumor activity was seen in two-thirds of the patients. However, the expression of IL-13Rα2 in residual tumor tissue adjacent to the injection site was significantly reduced, implying that antigen loss occurred as a result of treatment. To address this issue, new CAR T strategies targeting IL-13Rα2 were developed and evaluated in several preclinical and phase I clinical studies (43–45). Notably, the regression of intracranial tumor and spinal metastasis were observed in a patient with recurrent GBM after treatment with IL13Rα2-targeting CAR T cells; moreover, the patient's clinical response lasted for 7.5 months (43). The selection of specific T cell subsets is one of the approaches being used to improve CAR T cell therapy and optimize antitumor efficacy. $CD8^+$ T cells have long been the primary cell population used to develop CAR T cell therapies to treat brain tumors. A recent study compared the antitumor effect of $CD8^+$ and $CD4^+$ CAR T cells targeting IL-13Rα2 in GBM. The authors found that although $CD8^+$ CAR T cells exhibited a potent short-term effect, they were prone to rapid exhaustion, while the $CD4^+$CAR T cell-mediated long-term antitumor response outperformed that of the $CD8^+$ CAR T cells (46). This result demonstrates that $CD4^+$ T cells are an important alternative T cell subset for effective CAR therapy.

In preclinical studies, CAR T cells targeting the epidermal growth factor receptor (EGFR)vIII were efficiently delivered to tumor sites and inhibited the growth of glioma xenografts in murine models (47). EGFRv III is a variant of the EGFR, which is expressed in ~30% of GBM patients and is associated with a poor prognosis. Ten patients with recurrent GBM were adoptively transferred CAR T cells, which were transported in peripheral blood to the intracranial tumor sites, where they exerted an antitumor effect. Interestingly, analysis of pre- and post-treatment tumor samples revealed a decrease in tumor antigen expression and an increase in the presence of inhibitory immune checkpoint molecules and regulatory T cells at the tumor site after treatment, indicating increased tumor resistance (48); other clinical trials are

currently underway to further evaluate the efficacy of these CAR T cells. Human epidermal growth factor receptor 2 (HER2), a receptor tyrosine kinase overexpressed in up to 80% of GBMs, has also recently been recognized as an ideal tumor-associated antigen for CAR targeting in GBM (49, 50). Seventeen patients underwent a phase I trial with peripheral blood infusions of virus-specific T cells modified with a HER2-specific CAR (51). The HER2-CAR T cells did not proliferate but persisted at a low frequency for up to 1 year. Of the 16 evaluable patients, one patient had a partial response lasting more than 9 months, while seven patients had stable disease lasting between 8 weeks and 29 months (three of whom had no progression between the 24- and 29-month follow-up timepoints).

Despite the promising efficacy, the manufacturing time, cost, and in particular, the severe toxicities (e.g., neurotoxicity, immune-effector-cell-associated neurological syndrome, and cytokine release syndrome) associated with CAR T cell therapy highly limit its application. NK cells are a group of unique antitumor effector cells, which have the functions of cytotoxicity, cytokine production, and immune memory, but unlike T cells, are not limited by major histocompatibility complex (MHC)-mediated antigen recognition (52). The favorable safety profile and antitumor potential of NK cells make them promising cells for the implementation of CAR technology. In addition, because NK cells do not require MHC restriction, CAR NK cells can be generated a lot more rapidly than CAR T cells. However, the efficacy of CAR NK therapy needs to be clinically tested and challenges such as the low persistence of NK cells *in vivo* and their limited proliferative potential have to be overcome (53).

Nonetheless, given existing challenges in HGG, such as the high tumor heterogeneity, protumor and anti-inflammatory TME, and blood-brain barrier, patients with HGG are unlikely to benefit from mono-immunotherapy. Recent studies suggest that immunotherapy is an exciting candidate for combination therapy in HGG and many clinical trials are underway to explore suitable combinational strategies. For instance, a single-arm phase II clinical trial (NCT02550249) showed that the use of nivolumab as a neoadjuvant therapy in patients with GBM undergoing surgery resulted in the modulation of the TME (e.g., increased immune cell infiltration and broader T cell receptor [TCR] clonal diversity among the tumor-infiltrating T lymphocytes) (40). Radiotherapy and chemotherapy increase the efficacy of immunotherapy *via* multiple mechanisms, for instance by modulating the TME, increasing the expression and presentation of tumor antigens, and eliminating immunosuppressive cells (54). Results from some preclinical models suggest that chemotherapy can be synergistically used in combination with CpG-ODN to treat tumors, including gliomas. The main reason for this is the abscopal effect caused by radiotherapy and chemotherapy. Radiotherapy and chemotherapy induce the apoptosis of tumor cells. The lysed tumor cells release a large number of immunogenic substances. These substances activate immune cells, thereby triggering a more effective antitumor immune response (55–58). Combining different types of immunotherapy is also considered a promising strategy for HGG treatment (59). For example, ICIs have been proven to improve the antitumor effect of CAR T cell therapy in preclinical studies (60); this combinatory therapy has recently reached the clinical setting for the treatment of GBM (NCT03726515, NCT04003649). The combination of VT and CAR T cell therapy has also shown a synergistic effect by improving survival and tumor regression in a mouse model (61). Besides, the efficacy and safety of DC vaccination combined with ICIs are also being evaluated in phase I/II clinical trials involving GBM patients (NCT03422094, NCT04013672).

Our study has some limitations. First, because the number of incorporated studies was small, our conclusion regarding the efficacy and safety of immunotherapy in the context of glioma should be interpreted with caution. Second, the subgroups based on immunotherapy type were divided into open-label and double-blind groups. However, only one trial was included in the double-blind group. This was mainly due to the insufficient number of studies; we hope that more double-blind trials will be conducted in the future. Third, in the study by Ji et al., the OS of patients in the control arm was only 2.0–3.3 months (17), which was shorter than that reported by all other trials. This short OS may be related to the social attitudes and/or medical conditions in China, whereby a delay to treatment initiation may be caused by the negative connotations associated with seeking medical treatment early and/or barriers to accessing medical treatment. Fourth, we found that glioma patients from China benefited the most from immunotherapy, compared to patients from other countries evaluated. It is possible that racial and/or regional lifestyle differences are also important influencing factors.

In summary, we believe that immunotherapy will become increasingly important in the treatment of patients with glioma, and we hope that it will be considered by clinicians as an adjuvant therapy to chemotherapy and radiotherapy. Besides, our results showed that the efficacy of immunotherapy could be improved by addressing the associated safety concerns. For instance, the IP-mediated stimulation of the innate immune system elicits strong side effects. Thus, when scientists develop new agents for modulating the innate immune system, their safety index should be considered. In addition, we found that DC vaccines were typically injected intradermally into the axilla rather than intracranially, which was a safer method of administration than that used in VT. Thus, our findings indicate that the efficacy and safety of VT may be influenced by injection methods. These methods could not only be optimized by administering multiple courses of treatment, performing multi-point injections, or using small injection volumes, but also by changing to a new genetic vector, such as liposome-, polymer-, or protein-based delivery systems.

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.

Author contributions

BG, SZ conceptualized formal analysis and screened literature and assessed risk of bias. LX and W-LC took part in revision and writing. JS and PZ was responsible for statistical analysis and screened literature. JZ and LZ provided with instructions on methodology. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.966696/full#supplementary-material

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Efficacy and safety of pembrolizumab with preoperative neoadjuvant chemotherapy in patients with resectable locally advanced head and neck squamous cell carcinomas

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Background: This study aimed to explore the efficacy and safety of pembrolizumab combined with chemotherapy as neoadjuvant therapy in patients with resectable locally advanced head and neck squamous cell carcinomas (LA-HNSCCs).

Methods: In this prospective, single-arm, single-centre clinical trial, patients meeting the inclusion criteria were treated with preoperative neoadjuvant therapy with 200 mg pembrolizumab combined with 75 mg/m2 cisplatin and 175 mg/m2 paclitaxel. This was followed by surgery and postoperative adjuvant therapy. The primary endpoint was the postoperative pathological complete response (pCR) rate. All statistical analyses were performed using SPSS 26.

Results: A total of 22 patients were enrolled. The location of primary lesion showed: hypopharynx were 15 (68.2%), oropharynx were 6 (27.3%) and oral cavity was 1 (4.5%). The postoperative pCR rate, was 36.4% (8/22), and there was no delay to surgery due to adverse drug reactions. The rate of laryngeal function preservation was 90.9% (20/22). Delayed wound healing was the main surgical complication, with an incidence of 22.7% (5/22). The median follow-up time was 9.5 months, and only 1 patient (4.55%) suffered a regional recurrence.

Conclusion: Preoperative treatment with pembrolizumab and chemotherapy in resectable LA-HNSCC has a high pCR rate with no significant impact on surgical safety. This treatment was found to increase the rate of laryngeal function preservation. However, the effects of neoadjuvant immunotherapy on long-term prognosis in LA-HNSCCs require further study.

KEYWORDS

Head and neck carcinoma, neoadjuvant, immunotherapy, pembrolizumab, laryngeal function preservation

Research background

Over 60% of head and neck squamous cell carcinomas (HNSCCs) were diagnosed at advanced stage, which raises therapeutic challenge and results in poor prognosis (1, 2). The functional anatomy of the head and neck is complex and important. Either as a direct consequence of the tumour invasion or because of the tumour treatment, patients with LA-HNSCC often suffer from damage to important functions such as respiration, swallowing and speech pronunciation, with significant deleterious effects on their quality of life. Therefore, there is an urgent need to improve both the therapeutic efficacy and the function preservation of LA-HNSCC treatment.

In recent years, the value of immune checkpoint inhibitors, especially anti-programmed cell death-1 (PD-1) and anti-programmed cell death ligand-1 (PD-L1) monoclonal antibodies in the treatment of recurrent/metastatic HNSCCs has received increasing recognition among the academic community. Pembrolizumab is a PD-1 blocker that minimises the inhibitory effects of PD-1 on T cells, thereby enhancing the anti-tumour action of T cells. A phase III clinical trial (KeyNote-048 study) compared treatment with pembrolizumab, both alone and in combination with chemotherapy, versus the EXTREME regimen (combination therapy consisting of a platinum agent, 5-fluorouracil and cetuximab) for recurrent/metastatic HNSCCs. Both alone and in combination with chemotherapy, pembrolizumab was found to be significantly superior to cetuximab combined with chemotherapy and to effectively prolong overall survival (OS) (3). Based on the above results, pembrolizumab is recommended as a first-line treatment for recurrent/metastatic HNSCCs.

The efficacy of immune checkpoint inhibitors promises improved outcomes in the treatment of HNSCCs. The lower tumour heterogeneity, fewer drug-resistant clones and better immune status of patients before surgery makes them more responsive to neoadjuvant immunotherapy, with higher compliance than recurrent/metastatic patients (4). Preliminary results (5–10) have been published from current trials of preoperative immune checkpoint inhibitor and chemotherapy treatment, but these studies focus on pathological downstaging and postoperative pathological responses. The contribution of neoadjuvant immunotherapy to functional preservation during surgery has not previously been reported.

Neoadjuvant immunotherapy is known to improve the postoperative pathological response in a proportion of HNSCC patients. Our previous trial (11) showed that oral cavity cancer patients who achieved pathologic complete response (pCR) following induction chemotherapy had a prolonged survival. Therefore, we speculate that neoadjuvant immunotherapy is likely to have both survival benefits and functional preservation benefits. Thus, this clinical study investigated the effects of preoperative treatment with pembrolizumab and neoadjuvant chemotherapy with cisplatin and paclitaxel in patients with resectable LA-HNSCCs. This paper is a report of our preliminary results.

Materials and methods

Enrolled patients

This was a prospective, single-arm, single-centre clinical trial (registration no.: ChiCTR2200055719). Patients recently treated for HNSCCs at the National Cancer Centre/Cancer Hospital of the Chinese Academy of Medical Sciences between April 2021 and June 2022 were enrolled. The inclusion criteria were aged ≥18 y with histopathologically confirmed squamous cell carcinomas and locally advanced cases of P16-positive oropharyngeal carcinoma at clinical stage II-III, or non-P16-positive oropharyngeal carcinoma and other HNSCCs at stage III-IV suitable for complete surgical resection. The exclusion criteria were previous treatment with PD-1 monoclonal antibodies or similar drugs, radical surgical resection not possible, distant metastasis and allergies to the drugs used in this study. The study protocol was approved by the Ethics Committee of the National Cancer Centre/Cancer Hospital of the Chinese Academy of Medical Sciences (approval no.: 21/056-2727). All enrolled patients gave written informed consent to trial participation.

Research process

The patients were treated with pembrolizumab 200 mg combined with cisplatin 75 mg/m^2 and paclitaxel 175 mg/m^2 as

Abbreviations: LA-HNSCCs, Locally advanced head and neck squamous cell carcinomas; pCR, Pathological complete response; PD-1, Anti-programmed cell death-1; OS, Overall survival; MDT, Multidisciplinary team; CTCAE, Common Terminology Criteria for Adverse Events; MPR, Major pathological response; CPS, Combined positive score.

neoadjuvant therapy. The drugs were administered on the first day of a three-week treatment cycle. Surgery was performed about 4 weeks after the last neoadjuvant treatment. Regardless of any lesion regression after neoadjuvant therapy, the scope of surgical resection was determined according to the baseline pre-treatment assessment. Postoperative adjuvant therapy was determined by a multidisciplinary team (MDT) based on pathological high-risk factors and the preoperative clinical stage of the patient.

Prior to drug treatment and again before surgery, the patients were examined with contrast-enhanced computed tomography of the head, neck and chest to assess the range of their lesions. The response to neoadjuvant therapy was evaluated using the RECIST 1.1 standard. The safety of the neoadjuvant therapy was determined by recording any adverse reactions within 30 d of receiving treatment. These were graded using the Common Terminology Criteria for Adverse Events (CTCAE 5.0). Surgery was defined as delayed if it was not performed within 7 weeks of the last neoadjuvant therapy. Postoperative pathology was evaluated based on the residual tumours in the resected specimens. Based on other solid tumors and KeyNote-689 research design (12–14), pCR was defined as no residual tumour tissue in either the primary lesion or metastatic lymph nodes. The pCR of lymph nodes and primary lesions were established separately. A major pathological response (MPR) was defined as <10% residual tumour of the primary lesion. The expression of PD-1 in primary lesions before neoadjuvant therapy and in residual lesions after surgery was described using the combined positive score (CPS) of 22C3 in immunohistochemical staining to explore any correlation between PD-1 expression and the efficacy of neoadjuvant immunotherapy. CPS is a measure of the ratio of PD-L1 positive cells to total tumour cells.

Data analysis

All statistical analyses were performed using SPSS for Windows version 26 (IBM Corp., Armonk, NY, USA). Categorical variables were described as frequencies (percentages) and analysed with two-tailed Fisher's exact tests. Continuous variables were analysed with two-sample exact Mann–Whitney–Wilcoxon rank sum tests. P-values <0.05 were considered statistically significant. All risk factors found to be significant in univariate analyses were included in multivariate logistic regression analysis.

Results

Enrolment and baseline characteristics of patients

Between April 2021 and June 2022, our research group recruited 28 patients to participate in this trial. Among them, 2 patients did not meet the inclusion criteria. The remaining 26 received neoadjuvant immunotherapy. After therapy, 4 patients refused surgery and chose radiotherapy instead. Finally, 22 patients who received neoadjuvant therapy followed by surgery were included in our data analyses (Figure 1). This comprised 21 men and 1 woman (mean age, 58.1 y). The primary lesions were predominantly classified as hypopharyngeal carcinomas (n = 15), followed by oropharyngeal carcinomas (n = 6) and oral carcinomas (n = 1). Before treatment, the T-stage of most of the primary lesions was T3–4 (n = 14). Of the patients with hypopharyngeal carcinomas, 9 suffered from postcricoid region involvement accompanied by unilateral vocal cord fixation and 2 from oesophageal entrance involvement. None had bilateral vocal cord involvement. The most common initial symptom of the patients was cervical lymph node enlargement. Before treatment, the majority of the patients (n = 19) were in lymph node category N2, with patients' largest metastatic lymph node having an average diameter of 2.74 ± 1.15 cm. The largest metastatic lymph node of 9 patients was >3 cm. The above characteristics indicate late local T and N category in this patient group, and most lesions were at clinical stage IV (n = 16). The 3 patients at clinical stage II were all diagnosed with P16-positive oropharyngeal carcinomas at T-category T2–3 and N- category N2 (Table 1).

Safety and clinical efficacy evaluations

Among the enrolled patients, 17 underwent two cycles of pembrolizumab combined with chemotherapy, and 5 underwent three cycles. These 5 patients received an additional cycle because their surgery was delayed by the COVID-19 epidemic. None of the

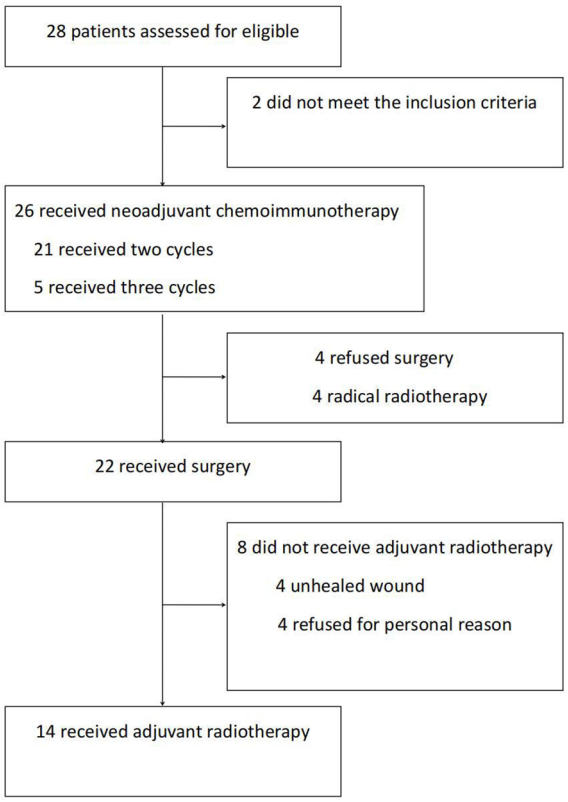

FIGURE 1
Trail flow diagram.

TABLE 1 Patients' demographics features and clinicopathological characteristics before surgery.

Variables	Number of patients	%
Male/Female	21/1	
Age (mean ± SD, years)	58.1 ± 10.49(22~69)	–
Primary site		
Oropharynx	6	27.3
hypopharynx	15	68.2
Oral Cavity	1	4.5
Hypopharynx invasion(N=15)		
Fixed vocal cord	9	60
Extend to esophagus	2	13.3
Tobacco		
YES	16	72.7
NO	6	27.3
Alcohol		
YES	15	68.2
NO	7	31.8
P16 states		
Positive	4	18.2
Negative	18	81.8
T stage		
T1-T2	8	36.4
T3	7	31.8
T4	7	31.8
N stage		
N0-N1	3	13.6
N2	19	86.4
Stage		
II	3	13.6
III	3	13.6
IV	16	72.7
PD-L1 combined positive score		
<5	7	31.8
5-10	2	9.1
≥10	13	59.1

patients had delayed surgery due to adverse drug reactions. The safety evaluation of pembrolizumab combined with preoperative neoadjuvant chemotherapy recorded 2 patients with grade 3 adverse reactions, both of which were leukopenia caused by bone marrow suppression and no grade 4 adverse reactions. The most common grade 1 or 2 adverse reactions were nausea and anorexia (n = 6), followed by rashes (n = 3) and weakness (n = 3) (Table 2).

Our efficacy evaluation found regression of primary lesions or metastatic cervical lymph nodes after neoadjuvant therapy, with partial remission in 18 patients and stable disease in 4 patients. No disease progression (PD) or hyperprogression occurred during treatment.

Surgery and relevant pathological evaluation

The patients underwent surgery about 4 weeks after their final neoadjuvant treatment, with a mean interval of 34.9 d. 8 of the 15 patients with hypopharyngeal carcinomas underwent a pyriform sinus resection or posterior pharyngeal wall resection, 5 underwent a partial laryngectomy and hypopharyngectomy, and 2 underwent a total laryngectomy and hypopharyngectomy. The overall rate of laryngeal preservation was 90.9% (20/22). Among the 7 patients with oropharyngeal carcinomas and oral carcinomas, partial tongue or tongue root resection was performed in 3, partial laryngectomy and tongue root resection in 2 and tonsillectomy in 2 patients. In 12 patients, the primary lesion was closed directly; in 8, it was reconstructed with adjacent flaps; and in 2, it was reconstructed with free flaps. Unilateral cervical lymph node dissection was performed in 10 patients, and bilateral cervical lymph node dissection in 12 patients. Radical unilateral cervical lymph node dissection was only performed in 1 patient, with the rest receiving modified radical dissections. Delayed wound healing was the main postoperative complication, with an incidence of 22.7% (5/22). There were no severe perioperative complications or perioperative deaths. All postoperative complications were cured by conservative treatment.

Eight patients achieved pCR (36.4%). In the remaining 14 patients without pCR, residuals were found in both the primary lesions and the cervical lymph nodes in 5 patients; 1 patient had a pCR only in the primary lesion, and 8 had pCR only in the lymph nodes. The overall cervical lymph node pCR rate was 71.4% (15/21) (cN0 (clinical lymph node negative), n = 1), and the overall pCR rate for primary lesions was 40.6% (9/22). Among the 13 patients with residual primary lesions, 1 presented with only local carcinoma *in situ* and 6 with scattered lesions with shallow depth of invasion (DOI) and severe atypical hyperplasia around it. The rate of MPR was 54.5% (12/22), and 17 patients had a lower pathologic stage, only one patient with a pathology confirmed prevertebral fascia invade reclassified the T4b vs. T2 in pre-surgery. In the 9 patients with hypopharyngeal carcinomas accompanied by unilateral vocal cord fixation, the MPR rate was also high (55.6%, 5/9). Only 2 patients with hypopharyngeal carcinomas underwent total laryngectomies (Figure 2). Postoperative pathological high-risk factors included vascular tumour thrombi (n = 2) and extranodal extensions (n = 2). All surgeries were R0 resections, and postoperative pathology examinations found no positive margins. Three of the 22 patients had residuals at the first intraoperative incisal margin. After extensive resection, no residual tumour was found at the incisal margins.

To identify variables associated with pCR, we performed correlation analyses between pCR and patients' demographic and

TABLE 2 Treatment-related adverse events (TRAEs).

EVENTS	Patients(N=22)		
	Grade1-2(%)	Grade 3(%)	Grade4(%)
Nausea/vomiting	6 (27.3)	0	0
Rash	3 (13.6)	0	0
Fatigue	3 (13.6)	0	0
Leukopenia	2 (9.1)	2 (9.1)	0
Anemia	2 (9.1)	0	0
Neurotoxicity	1 (4.5)	0	0
Diarrhea	1 (4.5)	0	0
Increased aminotransferases	1 (4.5)	0	0
Hypertension	1 (4.5)	0	0
Alopecia	1 (4.5)	0	0

clinical characteristics, condition and disease stage before neoadjuvant therapy. CPS and P16 status were used as markers for statistical analyses. There were 15 (68.2%) patients with CPS >5 in the biopsies of their primary lesions, and this was found to be correlated with pCR (p = 0.015). pCR was also correlated with the pre-treatment tumour stage (p = 0.028). We found no correlations between primary lesions and the diameter of the cervical lymph nodes, the diameter of primary lesions or P16 status (Table 3).

Adjuvant radiotherapy/chemoradiotherapy

In this trial, 14 patients received adjuvant radiotherapy/chemoradiotherapy after surgery, while 4 did not undergo radiotherapy due to the presence of unhealed wounds after surgery, and 4 more with postoperative pCR did not undergo radiotherapy based on comprehensive multidisciplinary team discussions and the patients' wishes. 5 patients received adjuvant chemoradiotherapy due to the presence of IPR and risk factor of distant metastasis, other 9 patients received adjuvant radiotherapy. Adjuvant radiotherapy/chemoradiotherapy was given at 4-6 weeks after surgery, only one patient had an early termination of adjuvant radiotherapy due to infection of COVID-19 during radiotherapy.

Follow-up

At the time of writing, the longest follow-up time has been 19 months, with a median follow-up time of 9.5 months. At present, observation of all patients is ongoing, and 1 patient suffered a

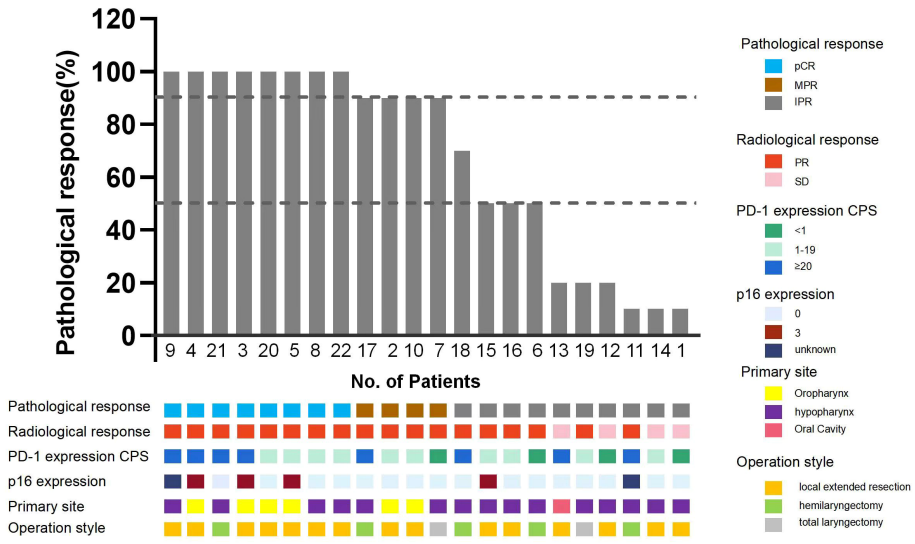

FIGURE 2
Pathological response of the enrolled patients. pCR, Pathological complete response; MPR, Major pathological response; IPR, Incomplete pathological response; p16 expression 0 for negative, 3 for strong positive.

TABLE 3 Correlation Analysis for Pathological Complete Response.

	Pathological Complete Response		P		Pathological Complete Response		P
	YES	NO			YES	NO	
Age, years	56.4 ± 7.5	59.0 ± 12.0	0.585	**N stage**			
Gender				N0-N1	0	3 (21.4)	0.159
Male	7 (87.5)	14 (100)	0.176	N2	8 (100)	11 (78.6)	
Female	1 (12.5)	0		**Stage**			
Primary site				II	3 (37.5)	0	0.028*
Oropharynx	0	1 (7.1)	0.168	III	0	3 (21.4)	
Hypopharynx	4 (50)	11 (78.6)		IV	5 (62.5)	11 (78.6)	
Oral Cavity	4 (50)	2 (14.3)		**CPS>5**			
Tobacco				YES	8 (100)	7 (50)	0.015*
YES	4 (50)	12 (85.7)	0.070	NO	0	7 (50)	
NO	4 (50)	2 (14.3)		**CPS>10**			
Alcohol				YES	7 (50)	6 (21.4)	0.040*
YES	4 (50)	11 (78.6)	0.166	NO	1 (50)	8 (78.6)	
NO	4 (50)	3 (21.4)		**Radiographic evaluation**			
P16 states				PR	8 (100)	10 (71.4)	0.095
Positive	3 (37.5)	1 (7.1)	0.076	SD	0	4 (28.6)	
Negative	5 (62.5)	13 (92.9)		**Primary site diameter**	1.9 ± 0.97	2.5 ± 1.1	0.267
T stage							
T1-T2	4 (50)	4 (28.6)	0.325	**LN diameter**	3.2 ± 0.6	2.5 ± 1.3	0.098
T3	3 (37.5)	4 (28.6)					
T4	1 (12.5)	6 (42.9)		**Time interval for surgery**	37.9 ± 7.8	33.2 ± 10.3	0.281

PR, Partial response; SD, Stable disease; CPS, Combined Positive Score; LN, Lymph nodes.

recurrence of regional lymph node during the follow-up. The recurrence case had a surgical pathology showed an incomplete post-treatment pathological response, and there were >90% clinical residual lesions. After surgery, chemoradiotherapy was performed. Local-regional lymph node lesions recurred 6 months after follow-up. These were treated with chemotherapy, resulting in a one-year disease-free survival rate of 95.5%. The long-term therapeutic effects and the correlations between pathological response rates and patients' condition before treatment and therapeutic effects require validation by further follow-up data (Figure 3).

Discussion

The response rate to neoadjuvant therapy of the patients in our sample was high. The postoperative rate of pCR after surgical resection was 36.4% (8/22). Zinner et al. treated 26 patients with preoperative nivolumab combined with carboplatin and paclitaxel as neoadjuvant therapy and reported a pCR rate of 42% (5), while a recent study of preoperative camrelizumab combined with chemotherapy reported a pCR rate of 37% (7). Our pCR rates were roughly concordant with those of previous reports. Current data in the literature indicate that pCR rates from immunotherapy combined with chemotherapy are higher than those from immunomonotherapy or dual-drug immunotherapy combined with a neoadjuvant regimen (8, 9, 15). When combined with immunotherapy, chemotherapy can have synergistic effects on the induction of immunogenic cell death, the up-regulation of tumour antigen expression and disruption of the immunosuppressive tumour microenvironment (16).

After classification and analysis of the postoperative pathological examinations of the primary lesions and cervical lymph node lesions, we found that, of the 13 patients with postoperative residual lesions, only 1 had only residual lymph node tumour tissue with no residual primary lesion; 7 patients had residual primary lesions, but lymph nodes that were pathologically negative for metastatic tumour tissue and 5 patients had residual malignancy in both the primary lesion and cervical lymph nodes. The responses of the primary lesions and cervical lymph nodes to pembrolizumab combined with neoadjuvant chemotherapy were not synchronous, with lymph nodes showing a higher response rate than primary lesions. The CIAO study also reported a better response to immune checkpoint inhibitors in metastatic lymph nodes than primary lesions (4).

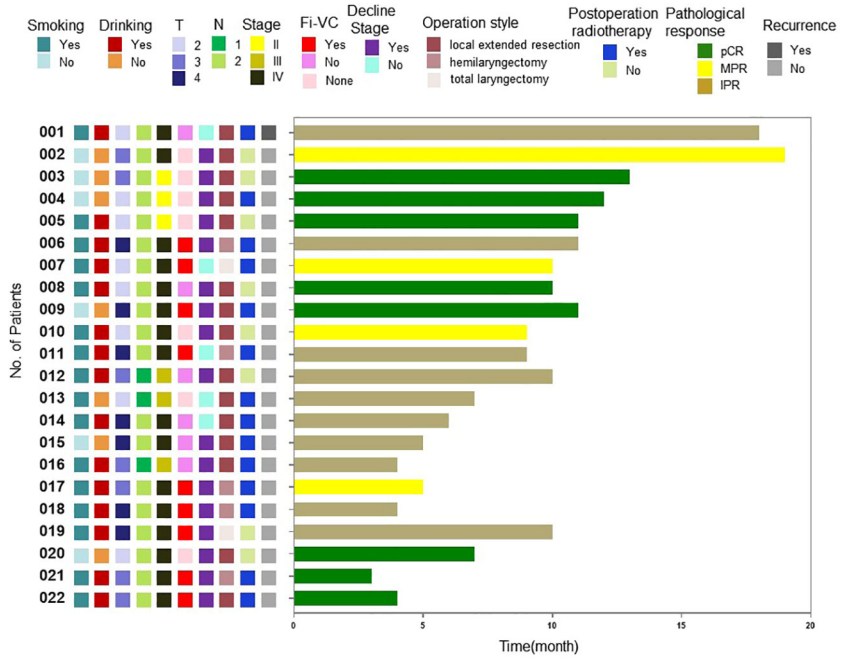

FIGURE 3
Swimming plot of disease-free survival for individual patients.Fi-VC, Fixed vocal cord; pCR, Pathological complete response; MPR, Major pathological response; IPR, Incomplete pathological response.

Although the specific mechanism behind this is unclear, previous research on neoadjuvant chemotherapy for breast cancer has similarly found that metastatic lymph nodes are more likely to achieve a pCR than primary lesions. A pCR in metastatic lymph nodes also showed a higher correlation with improved long-term prognoses in the breast cancer study (17).

CPS is the most widely used marker of the effects of anti-PD-1 drug therapy (18). We found a correlation between pre-treatment CPS levels >5 and pCR, suggesting that CPS also has predictive significance for the effects of pembrolizumab combined with neoadjuvant chemotherapy and immunotherapy. However, previous evaluations of the clinical applicability of CPS have produced differing results. The KEYNOTE-048 study found that patients with CPS between 1 and 20 benefit more from immunotherapy (3), while the CHECKMATE-141 study found that CPS between 5 and 10 were not correlated with positive immunotherapy outcomes (19). This discrepancy warrants further study. Previous research has tested other molecular markers, including tumour mutation burden (TMB), CD8+TiL and IL-6, but the prediction of pCR after neoadjuvant immunotherapy remains poor (20).

Pembrolizumab combined with neoadjuvant chemotherapy provides favourable conditions for functional preservation during surgery. Many of the patients in this study had hypopharyngeal carcinomas and tongue-base carcinomas adjacent to the larynx. However, the overall rate of laryngeal function preservation was 90.9% (20/22). In the past, patients with hypopharyngeal carcinomas at T3 and above accompanied by unilateral vocal cord fixation were usually treated with total laryngectomy (21). Of the 9 patients with postcricoid region involvement accompanied by unilateral vocal cord fixation in this study, only 2 underwent total laryngectomy, while the others were treated with partial laryngectomies and hypopharyngeal resections to achieve radical resection with R0. Previously, platinum-based induction chemotherapy improved the laryngeal function preservation rate but did not affect OS (22). However, the purpose of induction chemotherapy is to improve the proportion of functional preservation through the selection of appropriate concurrent chemoradiotherapy. In contrast, neoadjuvant immunotherapy aims to achieve functional preservation by improving the proportion of partial laryngectomy. Most of the patients in our sample had metastatic lymph nodes with a diameter >3 cm, which were shrunk to varying degrees by neoadjuvant therapy. Only 1 patient underwent radical lymph node dissection; in the rest, the lymph nodes were treated with modified radical dissection, avoiding the functional damage caused by radical dissection.

In terms of surgical safety, no complications that affected surgery resulted from the neoadjuvant therapy. Nevertheless, immunotherapy is not without adverse effects, which include bone marrow suppression, nausea, rashes and hypothyroidism (8). No disease progression occurred in any of the patients after neoadjuvant immunotherapy. However, in previous immunotherapy trials, there have been instances of false progression in imaging-based RECIST assessments after treatment due to the inflammatory infiltration caused by regional immune activation of local lesions (23). During surgery, a variety of

repair methods were attempted, including local skin flaps, free jejunum flaps and free skin flaps. There were no skin flap-related complications in our patients. Compared with preoperative radiotherapy, pembrolizumab combined with neoadjuvant chemotherapy has less influence on the selection of surgical repair methods.

Despite its advantages, pembrolizumab combined with neoadjuvant chemotherapy also presents challenges in surgical treatment. The most common postoperative surgical complications of the patients treated with neoadjuvant therapy were poor wound healing, infection around the tracheal stoma, pharyngeal fistula and lymphatic fistula. Most recovered from these complications with conservative treatment, but postoperative radiotherapy was delayed in several patients because of complications. In addition, it was found during surgery that 3 of the 22 patients had residuals at the first intraoperative incisal margin. Most of the 12 patients with residual lesions also had scattered lesions with shallow DOI (depth of invasion) accompanied by severe peripheral atypical hyperplasia. This suggests that, despite observable lesion regression after neoadjuvant therapy, residual microscopic lesions and atypical hyperplasia in the area of the primary lesion often remain. Therefore, in patients with apparent lesion regression, narrowing of the surgical scope is inadvisable. It is also necessary to standardise the safety boundary reservation and the submission of intraoperative incisal margins to prevent residuals. The problems with incisal margins have received little attention in previous studies on neoadjuvant therapy. This is largely because most previous research has used patients with oral and oropharyngeal carcinomas. In our study, hypopharyngeal carcinomas accounted for the vast majority of the patients, so safety-boundary-related problems were prominent.

At follow-up, all 22 patients were in stable condition. The longest follow-up time was 19 months. One patient suffered local-regional lymph node recurrence. Long-term outcomes will become apparent with further postoperative follow-up. At present, the 3-year recurrence rate of patients with advanced HNSCCs is >50% (2), and the 5-year OS rates of patients at stage III and IVA are 61% and 32%, respectively (24). Whether neoadjuvant therapy affects long-term survival remains to be seen. In most instances, locally advanced HNSCCs usually requires adjuvant radiotherapy after primary surgery. Although many of our patients had negative pathology results and tumour downstaging after surgery, 14 still chose to undergo radiotherapy. Postoperative radiotherapy is an important adjuvant treatment. Given the poor prognosis of advanced HNSCCs, especially hypopharyngeal carcinomas, high treatment intensity is conducive to tumour control. A recent study of the anti-PD-1 immune checkpoint inhibitor camrelizumab combined with neoadjuvant chemotherapy reported a case of postoperative pCR without local recurrence after radiotherapy and death after 14 months, indicating that postoperative adjuvant radiotherapy remains important (7). However, in the CIAO study of simultaneous immunotherapy and dual-drug immunotherapy, 45% of patients received neoadjuvant immunotherapy and surgery only, avoiding the adverse effects of post-oropharyngeal surgery radiotherapy on swallowing, mucosa production, mouth opening and other functions and improving their quality of life (4).

Analysis of the reasons for previous treatment failures in HNSCCs, represented by hypopharyngeal carcinomas, showed that multiple primary mucosal lesions due to physicochemical factors, submucosal invasion and high and occult metastasis of cervical lymph nodes were the main causes of local failure and early and rapid distant metastasis significantly affects long-term survival after surgery. Neoadjuvant therapy has the potential to improve long-term tumour immunity through immune memory. In addition, the response rate to preoperative neoadjuvant therapy was higher than that from anti-recurrence/metastasis treatment. This is likely because of the lower tumour heterogeneity, fewer drug-resistant clones and better immune status of the patients. It is conceivable that neoadjuvant therapy might be more effective to overcome the aforementioned adverse factors in HNSCC treatment and provide long-term tumour control and longer survival.

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

This study was approved by the Ethics Committee Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (approval No.: 21/056-2727). The patients/participants provided their written informed consent to participate in this study.

Author contributions

KW: data curation, software, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. LG: resources, data curation, investigation, methodology, writing-original draft. HL: funding acquisition, resources, data curation, investigation, methodology, writing-original draft. XH: supervision, investigation. DL: resources, data curation. CL: formal analysis, validation, investigation, methodology, writing-original draft, writing-review. SL: supervision, investigation. XW: conceptualization, resources, data curation, supervision, validation, investigation, methodology, writing-original draft, project administration, writing-review and editing. All authors discussed the results and contributed to the final

manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Characteristics of immunotherapy trials for nasopharyngeal carcinoma over a 15-year period

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Background: Immunotherapy has been a hotspot in nasopharyngeal carcinoma (NPC) in recent years. This study aimed to provide a comprehensive landscape of the characteristics of immunotherapy clinical trials in NPC and to determine whether contemporary studies are of sufficient quality to demonstrate therapeutic value.

Methods: This is a cross-sectional analysis of NPC trials registered on ClinicalTrials.gov in the last 15 years (Jan 1, 2008-Nov 20, 2022). Only interventional trials with a primary purpose of treatment were included in the final analysis. Characteristics of immunotherapy trials were compared with those of other NPC trials. Chronological shifts in NPC immunotherapy trials were also analyzed.

Results: Of the 440 NPC studies selected, 161 (36.6%) were immunotherapy trials and 279 (63.4%) were other NPC trials. NPC immunotherapy trials were more likely than other NPC trials to be phase 1-2 (82.6% vs. 66.7%, $P < 0.001$), single-arm (51.3% vs. 39.6%, $P = 0.020$), non-randomized (64.8% vs. 44.4%, $P < 0.001$), and enroll fewer than 50 participants (46.3% vs. 34.4%, $P = 0.015$). Blinding was used in 8.8% of NPC immunotherapy trials. Also, 90.7% of NPC immunotherapy trials were recruited nationally and 82.6% were Asia-centric. Although academic institutions and governments (72.7%) were the major sponsors of NPC trials, immunotherapy trials were more likely to be industry-funded than other NPC trials (34.2% vs. 11.5%, $P < 0.001$). The number of NPC immunotherapy trials increased exponentially after 2017, attributed to the exploration of immune checkpoint inhibitors. Immunotherapy combined with chemotherapy was the most commonly investigated regimen.

Conclusion: NPC immunotherapy trials over a 15-year period were predominantly exploratory. To generate high-quality evidence and advance the clinical application of immunotherapy in NPC, more attention and concerted efforts are needed.

KEYWORDS

nasopharyngeal carcinoma, immunotherapy, Clinicaltrials.gov, clinical trial, immune checkpoint inhibitor

Introduction

Nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV)-related cancer that is particularly prevalent in South East Asia and Southern China (1). Unlike other head and neck cancers, NPC is susceptible to radiotherapy (RT), which has become the mainstay of treatment for this disease. Despite advances in RT techniques and optimization of chemotherapy regimens, about 20% of patients with locally advanced NPC (LANPC) will recur (2). Moreover, recurrent and/or metastatic (R/M) NPC remains the most serious challenge because the median overall survival (OS) of these patients is only 15.7 months (3). Current conventional treatments, including RT, chemotherapy and surgery, are often accompanied by serious adverse effects and limited efficacy (4). Therefore, there is an urgent need for novel treatment strategies to improve the prognosis of patients with NPC.

In recent years, immunotherapy has sparked a revolution in the clinical management of cancer (5, 6). NPC is regarded as a typical "immune-hot" tumor due to the expression of EBV antigen and $CD4^+$/$CD8^+$ T-cell target proteins (7, 8), massive lymphocytic infiltration (9), the expression of programmed death ligand-1 (PD-L1) up to 89-95% (10), and the presence of several key immune molecules (CD40, CD70, CD80, and CD86) that regulate T-cell activation (11). Several clinical studies on NPC immunotherapy have shown early successes (12–14). Nevertheless, aside from individual reports, the overall characteristics of NPC immunotherapy clinical trials and whether contemporary studies are of sufficient quality to demonstrate the therapeutic value of immunotherapy in personalized NPC practice are unclear.

ClinicalTrials.gov, a publicly available registry and results database for human clinical studies, provides the most comprehensive clinical study information worldwide. In 2004, the International Committee of Medical Journal Editors (ICMJE) announced a policy as a prerequisite for publication that requires the registration of clinical trials before enrolling participants (15, 16). As of Nov 20, 2022, ClinicalTrials.gov contains detailed information on more than 430 000 clinical trials conducted in over 200 countries. ClinicalTrials.gov is recognized as a promising information source for facilitating the systematic evaluation of clinical trials (16).

In this study, we examined all of the interventional NPC studies registered on ClinicalTrials.gov in 15 years (Jan 1, 2008-Nov 20, 2022). We compared the fundamental characteristics of NPC trials focusing on immunotherapy with the characteristics of other non-immunotherapy NPC trials, and we evaluated the changes over time.

Materials and methods

Data source and selection criteria

This is a cross-sectional analysis of immunotherapy trials for NPC. We searched ClinicalTrials.gov on Nov 20, 2022 using the keyword "*nasopharyngeal carcinoma*". In total, 803 registered clinical studies were identified and downloaded. We restricted our selection to interventional trials with a primary purpose of treatment that were registered between Jan 1, 2008 and Nov 20, 2022 (n = 440) (Figure 1). This study was considered exempt by the institutional review board of Sun Yat-sen University Cancer Center because it did not involve human participants.

Study data accuracy was ensured by independent verification of all data by three investigators. Two oncologists (H.-G.H. and Y.-Y.Y.) manually and independently reviewed all of the selected trials, and a third author (T.-Y.L.) adjudicated any disagreements. Trials were then categorized according to treatment type, identified by the term "*Intervention/treatment*", "*Brief Summary*", or "*Official Title*". If the treatment type was not clear, other registration information (e.g., "detailed description" and "eligibility") was reviewed.

We defined immunotherapy trials as studies that (1) added immunotherapy to the standard of care (2); compared any treatment regimens with or without immunotherapy; (3) investigated novel immunotherapy regimens, such as new agents, usages, or dosages; (4) compared different immunotherapy regimens; and (5) evaluated interventions for immunotherapy-related complications. Although EBV-specific monoclonal antibody is a type of immunotherapy tool, some of them work in a more targeted way and partly overlap with ICIs and targeted therapy. Therefore, we excluded EBV-specific monoclonal antibody trials from the immunotherapy study.

Immunotherapy is categorized into four types in this study: (1) immune checkpoint inhibitors (ICIs), including but not limited to programmed cell death protein-1 (PD-1)/PD-L1/cytotoxic T-lymphocyte associated antigen-4 (CTLA-4)/lymphocyte activation

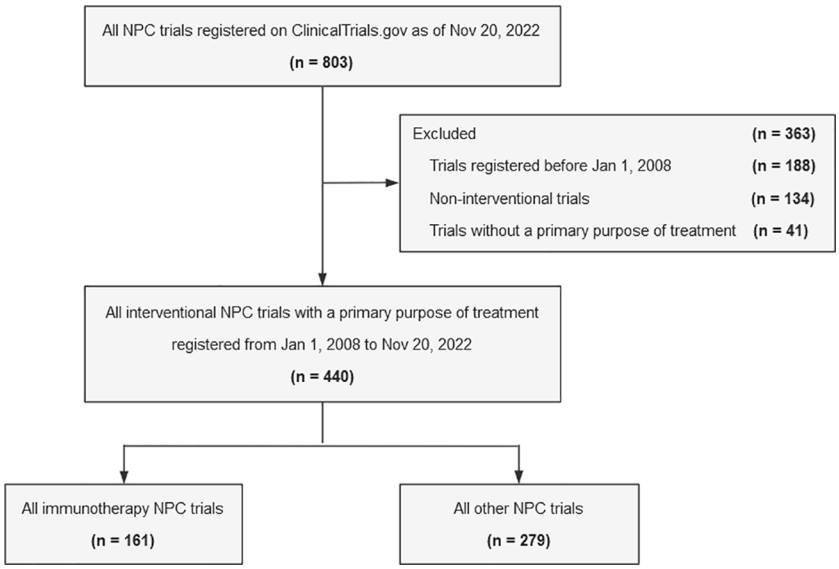

FIGURE 1
Flowchart identifying trials registered on ClinicalTrials.gov from Jan 1, 2008 to Nov 20, 2022. NPC, nasopharyngeal carcinoma.

gene-3 (LAG-3) inhibitors; (2) adoptive cell therapy (ACT), including adoptive cell transfer of autologous cytotoxic T lymphocytes (CTLs), tumor-infiltrating lymphocytes (TILs), cytokine-induced killer (CIK) cells, and genetically modified cellular immunotherapy such as chimeric antigen receptor-modified T (CAR-T) cell therapy and T cell receptor-engineered T (TCR-T) cell therapy; (3) vaccines; and (4) immunomodulators, including cytokines and oncolytic viruses. The remaining eligible studies constituted the other NPC trials, investigating RT, chemotherapy, surgery, targeted therapy, etc.

Study variables

We extracted the following information for each trial: (1) whether the trial was registered before participant enrollment; (2) study phase; (3) sample size; (4) number of arms; (5) masking; (6) allocation methods; (7) number of centers; (8) national or international recruitment; (9) age selection; (10) funding source; (11) site location; and (12) recruitment status. As previously described (17–20), if a trial reported only one treatment arm, the allocation methods (if missing) were classified as non-randomized, and the blinding category (if missing) was classified as open-label.

Funding sources were assigned as an industry, National Institutes of Health (NIH), and other academic institutions or governments based on the recorded lead sponsor and/or collaborator for each clinical trial. A trial was classified as industry-funded if its lead sponsor or one of its collaborators was from the industry with no NIH involvement or NIH-funded if its lead sponsor or one of its collaborators was from the NIH with no industry involvement (21). All other trials were classified as other-funded studies.

Statistical analysis

Descriptive statistics were primarily used to summarize the clinical trial characteristics. Categorical variables were reported as frequencies and percentages. Missing values were excluded from the analyses unless they could be inferred from other relevant data. Trial characteristics were compared using the Pearson χ^2 test, as well as Fisher's exact test, if indicated. The statistical significance level was set at $P < 0.05$ (two-sided). Analyses were undertaken using SPSS, version 25.0 (IBM Corp).

Results

Characteristics of included trials

Of the 440 NPC trials eligible for analysis, 161 (36.6%) were immunotherapy trials and 279 (63.4%) were other NPC trials (Figure 1).

Table 1 shows the trial characteristics of immunotherapy and other NPC trials included in this study. Immunotherapy trials were more likely than other NPC trials to be registered before participant enrollment (116 of 161 [72.0%] vs. 128 of 279 [45.9%], $P < 0.001$). In addition, immunotherapy trials tended to have more phase 1-2 studies (133 of 161 [82.6%] vs. 164 of 246 [66.7%], $P < 0.001$) and less likely to be phase 3 studies (23 of 161 [14.3%] vs. 68 of 246 [27.6%], $P = 0.002$) than the other NPC trials. Furthermore, immunotherapy trials were more likely to be single-arm (80 of 156 [51.3%] vs. 109 of 275 [39.6%], $P = 0.020$), non-randomized (103 of 159 [64.8%] vs. 123 of 277 [44.4%], $P < 0.001$), and enroll fewer than 50 participants (74 of 160 [46.3%] vs. 96 of 279 [34.4%], $P = 0.015$) compared with other NPC trials. Blinding was used in

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TABLE 1 Characteristics of immunotherapy vs. other nasopharyngeal carcinoma trials.

Characteristic	No./Total No. (%)		*P* value[b]
	Immunotherapy NPC trials (n = 161)[a]	Other NPC trials (n = 279)[a]	
Registration before participant enrollment	116/161 (72.0)	128/279 (45.9)	< 0.001
Phase			
Early Phase 1	1/161 (0.6)	2/246 (0.8)	0.002
Phase 1	28/161 (17.4)	26/246 (10.6)	
Phase 1/Phase 2	19/161 (11.8)	14/246 (5.7)	
Phase 2	86/161 (53.4)	124/246 (50.4)	
Phase 2/Phase 3	4/161 (2.5)	7/246 (2.9)	
Phase 3	23/161 (14.3)	68/246 (27.6)	
Phase 4	0/161 (0)	5/246 (2.0)	
Enrollment, No. of patients			
< 50	74/160 (46.3)	96/279 (34.4)	0.040
50 - 100	25/160 (15.6)	61/279 (21.9)	
> 100	61/160 (38.1)	122/279 (43.7)	
No. of study arms			
1	80/156 (51.3)	109/275 (39.6)	0.001
2	59/156 (37.8)	151/275 (54.9)	
≥ 3	17/156 (10.9)	15/275 (5.5)	
Masking			
Open-label	145/159 (91.2)	244/276 (88.4)	0.420
Blind	14/159 (8.8)	32/276 (11.6)	
Allocation			
Randomized	56/159 (35.2)	154/277 (55.6)	< 0.001
Non-randomized	103/159 (64.8)	123/277 (44.4)	
No. of centers			
Single	93/161 (57.8)	183/279 (65.6)	0.102
Multiple	68/161 (42.2)	96/279 (34.4)	
Recruitment			
National	146/161 (90.7)	266/279 (95.3)	0.061
International	15/161 (9.3)	13/279 (4.7)	
Excludes children (aged < 18 y)	150/161 (93.2)	262/279 (93.9)	0.840
Excludes elderly (aged > 65 y)	29/161 (18.0)	65/279 (23.3)	0.227
Funding source			
Industry	55/161 (34.2)	32/279 (11.5)	< 0.001
NIH	9/161 (5.6)	24/279 (8.6)	

(Continued)

TABLE 1 Continued

Characteristic	No./Total No. (%)		*P* value[b]
	Immunotherapy NPC trials (n = 161)[a]	Other NPC trials (n = 279)[a]	
Other[c]	97/161 (60.2)	223/279 (79.9)	
Locations[d]			
US/Canada	32/161 (19.9)	40/279 (14.3)	0.142
Europe	10/161 (6.2)	9/279 (3.2)	0.150
Asia	133/161 (82.6)	236/279 (84.6)	0.593
Other[e]	4/161 (2.5)	4/279 (1.4)	0.472
Recruitment status			
Ongoing[f]	118/161 (73.3)	89/279 (31.9)	< 0.001
Stopped early[g]	6/161 (3.7)	24/279 (8.6)	0.075
Completed	19/161 (11.8)	77/279 (27.6)	< 0.001
Unknown	18/161 (11.2)	89/279 (31.9)	< 0.001

NPC, nasopharyngeal carcinoma; NIH, National Institutes of Health; US, United States.
[a]Different denominators were the number of trials with available data for different variables.
[b]Calculated using the χ^2 test or the Fisher exact test if indicated.
[c]Other Funding sources included individuals, universities, and organizations.
[d]The sum of the percentages may exceed 100% because categories are not mutually exclusive.
[e]Other regions included South America, North America other than US/Canada, Central America, Oceania, and Africa.
[f]This status includes trials that were "not yet recruiting", "recruiting", "enrolling by invitation", "active, not recruiting", or "suspended" in the database.
[g]This status includes trials that were "terminated" or "withdrawn" in the database.

8.8% (14 of 159) of NPC immunotherapy trials and 90.7% (146 of 161) of NPC immunotherapy trials recruited nationally.

Although other funding sources accounted for the highest proportion of immunotherapy and other NPC trials (97 of 161 [60.2%] vs. 223 of 279 [79.9%], $P < 0.001$), immunotherapy trials were more likely to be industry-funded than the other NPC trials (55 of 161 [34.2%] vs. 32 of 279 [11.5%], $P < 0.001$). Asia was the most common study location for the NPC immunotherapy trials (133 of 161 [82.6%]), followed by the United States (US)/Canada (32 of 161 [19.9%]). The most commonly recruited population for NPC immunotherapy trials was distributed in China (120 of 161 [74.5%]), followed by the US (31 of 161 [19.3%]) and Singapore (16 of 161 [9.9%]) (Figure 2)

With regard to recruitment status, immunotherapy trials were more likely to be ongoing (118 of 161 [73.3%] vs. 89 of 279 [31.9%], $P < 0.001$) and less likely to be completed (19 of 161 [11.8%] vs. 77 of 279 [27.6%], $P < 0.001$) than other NPC trials. Despite the marginal difference, immunotherapy trials had a lower proportion of trials that stopped early than the other NPC trials (6 of 161 [3.7%] vs. 24 of 279 [8.6%], $P = 0.075$).

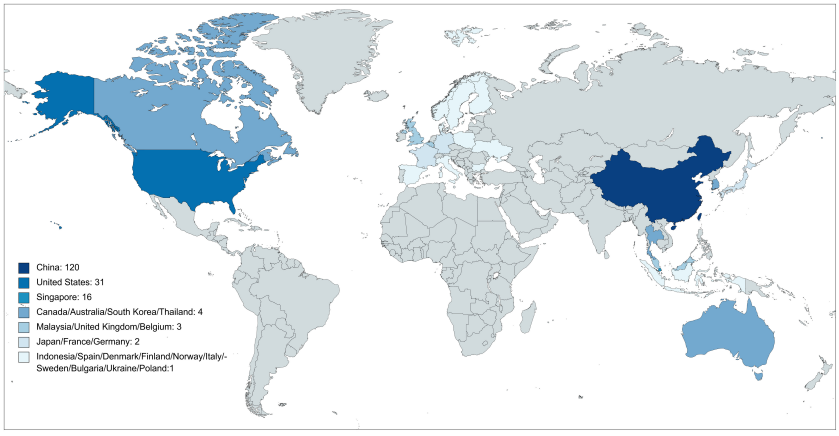

FIGURE 2
Population distribution of nasopharyngeal carcinoma immunotherapy trials.

Chronological shifts in the number of NPC immunotherapy trials

Figure 3A shows chronological shifts in the number of NPC immunotherapy trials. Between 2008 and 2017, the number of NPC immunotherapy trials remained relatively stable, ranging between 2 and 7 annually. The number of NPC immunotherapy trials increased from 15 in 2018 to 32 in 2021 ($P = 0.001$). As of Nov 20, 2022, the number of NPC immunotherapy trials in 2022 had reached 32, the same as in 2021.

Furthermore, we looked at the chronological shifts in the number of different types of immunotherapy trials for NPC (Figure 3B). From 2008 to 2017, the numbers of ICIs, ACT, vaccine and immunomodulator trials were relatively stable (fewer than 5 annually). Notably, the number of ICI trials rapidly increased to 14 in 2018 and 2019 and doubled to approximately 30 from 2020 to 2022. Toripalimab was the most commonly investigated ICIs in NPC (30 of 121 [24.8%]), followed by camrelizumab (20 of 121 [16.5%]) (Figure 4). But the numbers of ACT, vaccine and immunomodulator trials stayed stagnant.

Chronological shifts in the characteristics of NPC immunotherapy trials

Because NPC immunotherapy trials increased exponentially in number after 2017, we analyzed chronological shifts in the characteristics of NPC immunotherapy trials in the two periods Jan 1, 2008 to Dec 31, 2017 (n = 38, 23.6%) and Jan 1, 2018 to Nov 20, 2022 (n = 123, 76.4%) (Table 2). Compared to 2008-2017, a higher proportion of immunotherapy trials were registered before first participant enrollment in 2018-2022 (94 of 123 [76.4%] vs. 22 of 38 [57.8%], $P = 0.038$). Immunotherapy trials were more likely to be phase 2-3 in 2018-2022 than in 2008-2017 (96 of 123 [78.0%] vs. 17 of 38 [44.7%], $P < 0.001$). Despite the marginal difference, more immunotherapy trials had a sample size of more than 100 patients in 2018-2022 than in 2008-2017 (52 of 123 [42.3%] vs. 9 of 37 [24.3%], $P = 0.055$). The two periods' basic trial characteristics remained unchanged (all $P > 0.05$).

The number of industry-funded NPC immunotherapy trials increased marginally from 8 of 38 studies (21.1%) in 2008-2017 to 47 of 123 studies (38.2%) in 2018-2022 ($P = 0.077$), but the number of NIH-funded immunotherapy trials decreased significantly from 7 of 38 studies (18.4%) in 2008-2017 to 2 of 123 studies (1.6%) in 2018-2022 ($P = 0.001$). The proportion of other-funded immunotherapy trials remained stable at approximately 60%. In terms of study locations, there was a significant decrease in US/Canada centric from 16 of 38 studies (42.1%) in 2008-2017 to 16 of 123 studies (13.0%) in 2018-2022 ($P < 0.001$) but a significant increase in Asia centric from 22 of 38 studies (57.9%) in 2008-2017 to 111 of 123 studies (90.2%) in 2018-2022 ($P < 0.001$).

Immunotherapy usage in NPC trials

Among the 161 NPC immunotherapy trials, 46 (28.6%) evaluated single agents and 115 (71.4%) were designed to

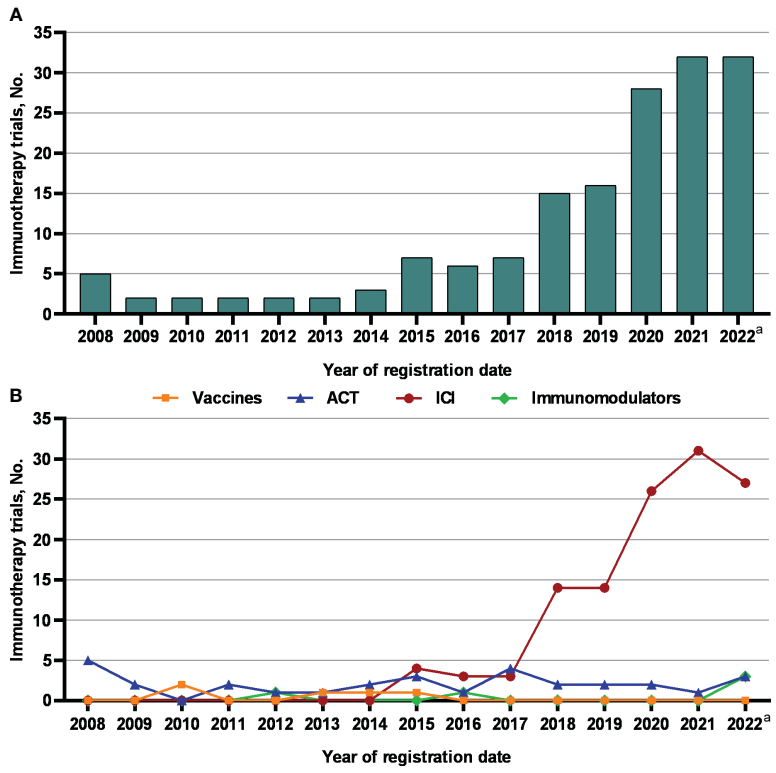

FIGURE 3
The number of **(A)** immunotherapy trials and **(B)** different types of immunotherapy trials for nasopharyngeal carcinoma registered on ClinicalTrials.gov between 2008 and 2022. ACT, adoptive cell therapy; ICIs, immune checkpoint inhibitors. [a] Observed period was Jan 1, 2022 to Nov 20, 2022.

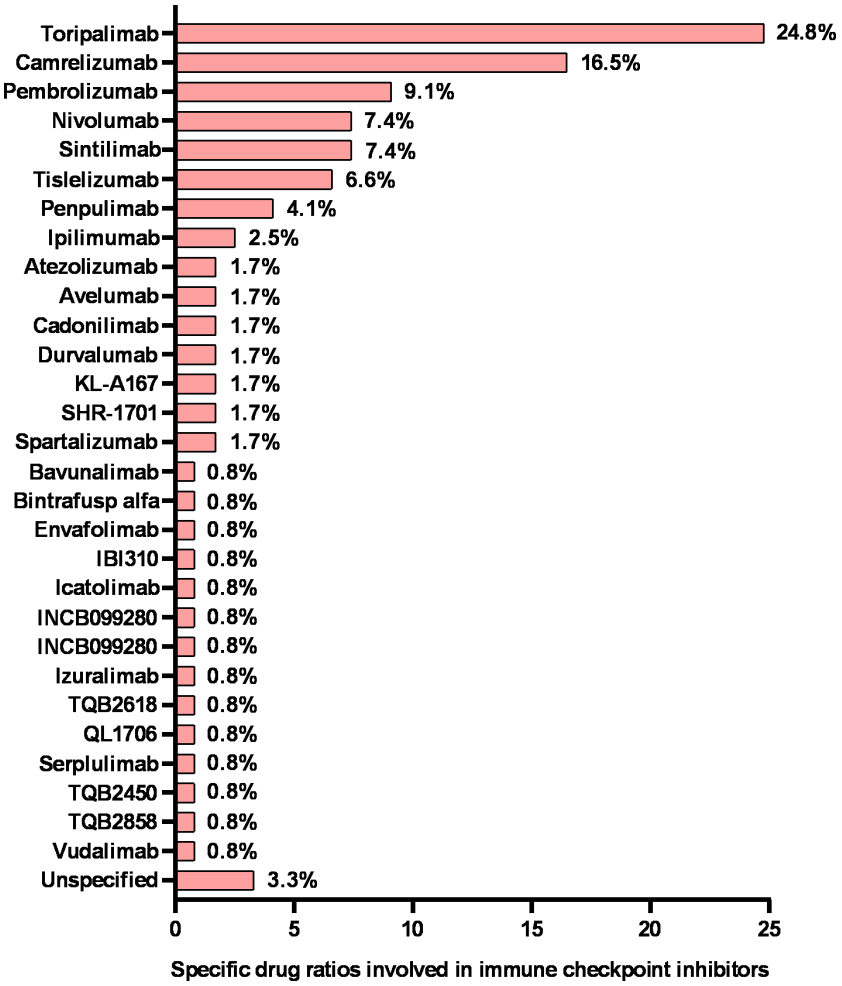

FIGURE 4
Specific drug ratios in nasopharyngeal cancer clinical trials involving immune checkpoint inhibitors. The sum of the percentages may exceed 100% because categories are not mutually exclusive.

investigate immunotherapy combination strategies (Table 3). Monotherapy (34 of 46 [73.9%]) was the most commonly explored immunotherapy regimen in single usage, followed by immunotherapy maintenance after standard treatment (11 of 46 [23.9%]). The highest proportion of immunotherapy combination strategies investigated was combination chemotherapy (39 of 115 [33.9%]), followed by radiochemotherapy (27 of 115 [23.5%]) and targeted therapy (19 of 115 [16.5%]).

Discussion

Well-designed clinical trials are desperately needed to validate the clinical applications of immunotherapy in NPC, given its promising efficacy. However, with an overall low incidence rate worldwide for its unique epidemiology, NPC does not attract much attention from most research. To the best of our knowledge, this is the first study assessing the critical characteristics of NPC immunotherapy trials over a 15-year period. By evaluating a comprehensive landscape, we found that NPC immunotherapy trials were predominantly phase 1-2 trials of limited sample size and tended to be single-arm, non-randomized and industry-funded. Blinding was rarely used. Asia was the major study location and clinical trials with international collaboration were lacking as well. The number of NPC immunotherapy trials increased exponentially after 2017, attributed to the exploration of ICIs. But the progress in trial design over time was slow and the basic trial characteristics largely remained unchanged. These findings raise concerns that trials evaluating the therapeutic role of immunotherapy in NPC may not be received the attention or efforts necessary to generate high-quality data. As a result, this orientation toward a less robust design may compromise evidence-based care for NPC.

As an EBV-associated malignancy, NPC is frequently infiltrated with varied stromal cells, making its microenvironment a highly heterogeneous and suppressive harbor that protects NPC cells from drug penetration and immune attack and promotes tumor progression (22, 23). This general immune landscape of NPC renders patients suitable for immunotherapy. In the past 15 years, immunotherapy trials accounted for 36.6% of all NPC trials. Unfortunately, 82.6% of NPC immunotherapy trials were phase

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TABLE 2 Trend changes in characteristics of immunotherapy trials for nasopharyngeal carcinoma registered on ClinicalTrials.gov between Jan 1, 2008 to Dec 31, 2017, and Jan 1, 2018 to Nov 20, 2022.

Characteristic	No./Total No. (%)		P value[b]
	2008-2017 (n = 38)[a]	2018-2022 (n = 123)[a]	
Registration before participant enrollment	22/38 (57.8)	94/123 (76.4)	0.038
Phase			
Early Phase 1	0/38 (0)	1/123 (0.8)	0.003
Phase 1	13/38 (34.2)	15/123 (12.2)	
Phase 1/Phase 2	8/38 (21.1)	11/123 (9.0)	
Phase 2	15/38 (39.5)	71/123 (57.7)	
Phase 2/Phase 3	0/38 (0)	4/123 (3.2)	
Phase 3	2/38 (5.2)	21/123 (17.1)	
Enrollment, No. of patients			
< 50	25/37 (67.6)	49/123 (39.8)	0.012
50-100	3/37 (8.1)	22/123 (17.9)	
> 100	9/37 (24.3)	52/123 (42.3)	
No. of study arms			
1	18/33 (54.6)	62/123 (50.4)	0.813
2	11/33 (33.3)	48/123 (39.0)	
≥ 3	4/33 (12.1)	13/123 (10.6)	
Masking			
Open-label	35/36 (97.2)	110/123 (89.4)	0.194
Blind	1/36 (2.8)	13/123 (10.6)	
Allocation			
Randomized	9/36 (25.0)	47/123 (38.2)	0.168
Non-randomized	27/36 (75.0)	76/123 (61.8)	
No. of centers			
Single	23/38 (60.5)	70/123 (56.9)	0.693
Multiple	15/38 (39.5)	53/123 (43.1)	
Recruitment			
National	33/38 (86.8)	113/123 (91.9)	0.349
International	5/38 (13.2)	10/123 (8.1)	
Excludes children (aged < 18 y)	32/38 (84.2)	118/123 (95.9)	0.022
Excludes elderly (aged > 65 y)	3/38 (7.9)	26/123 (21.1)	0.089
Funding source			
Industry	8/38 (21.1)	47/123 (38.2)	0.001
NIH	7/38 (18.4)	2/123 (1.6)	
Other[c]	23/38 (60.5)	74/123 (60.2)	

(Continued)

TABLE 2 Continued

Characteristic	No./Total No. (%)		P value[b]
	2008-2017 (n = 38)[a]	2018-2022 (n = 123)[a]	
Locations[d]			
US/Canada	16/38 (42.1)	16/123 (13.0)	< 0.001
Europe	5/38 (13.2)	5/123 (4.1)	0.057
Asia	22/38 (57.9)	111/123 (90.2)	< 0.001
Other[e]	0/38 (0)	4/123 (3.3)	0.574
Recruitment status			
Ongoing[f]	8/38 (21.1)	110/123 (89.4)	< 0.001
Stopped early[g]	2/38 (5.2)	4/123 (3.3)	0.627
Completed	16/38 (42.1)	3/123 (2.4)	< 0.001
Unknown	12/38 (31.6)	6/123 (4.9)	< 0.001

NIH, National Institutes of Health; US, United States.
[a]Different denominators were the number of trials with available data for different variables.
[b]Calculated using the χ^2 test or the Fisher exact test if indicated.
[c]Other Funding sources included individuals, universities, and organizations.
[d]The sum of the percentages may exceed 100% because categories are not mutually exclusive.
[e]Other regions included South America, North America other than US/Canada, Central America, Oceania, and Africa.
[f]This status includes trials that were "not yet recruiting", "recruiting", "enrolling by invitation", "active, not recruiting", or "suspended" in the database.
[g]This status includes trials that were "terminated" or "withdrawn" in the database.

TABLE 3 Immunotherapy usage in nasopharyngeal carcinoma clinical trials.

Characteristics	No./Total No. (%)
Single usage	46/161 (28.6)
Monotherapy	34/46 (73.9)
Versus ST	1/46 (2.2)
Maintenance after ST	11/46 (23.9)
Combined usage[a]	115/161 (71.4)
IT + CT	39/115 (33.9)
IT + RT	7/115 (6.1)
IT + surgery	2/115 (1.7)
IT + TT	19/115 (16.5)
Multiple IT combination	9/115 (7.9)
IT + CT + RT	27/115 (23.5)
IT + CT + surgery	2/115 (1.7)
IT + CT +TT	8/115 (7.0)
IT + CT + RT + TT	2/115 (1.7)

ST, standard treatment; IT, immunotherapy; CT, chemotherapy; RT, radiotherapy; TT, targeted therapy.
[a]Trials combining immunotherapy with other therapies simultaneously.

1-2 studies and tended to be single-arm, non-randomized, and enrolled less than 50 participants. Actually, the high proportion of single-arm, non-randomized, early-phase studies may either be because these studies are exploratory, hypotheses generating to fuel future randomized trials involving more patients, or because they were studying more highly innovative expensive cellular-based studies where funding was often inadequate for larger studies with more patients. In addition, the well-defined geographic distribution of NPC might further limit clinicians from conducting large-scale immunotherapy trials. Similarly, Xu et al. (24) tracked the evolving landscape of global immuno-oncology trials in 2007-2019 and found that most immunotherapy trials worldwide were phase 2 studies. Fortunately, NPC immunotherapy trials in 2018-2022 were more likely to be phase 2-3 (78.0% vs. 44.7%, $P < 0.001$) and had a sample size of more than 100 patients (42.3% vs. 24.3%, $P = 0.055$) than in 2008-2017. However, the other basic trial characteristics did not improve in an obvious manner over time.

Establishing international collaborative groups to foster research networks is an effective way to enroll more participants and improve the power of a study. However, 90.7% of NPC immunotherapy trials were conducted in only one region without sufficient international collaboration. Furthermore, in contrast to the findings that the US leads global immunotherapy research with stable growth (24), Asia (82.6%) is the major study location for NPC immunotherapy trials. And the proportion of US/Canada-centric decreased from 42.1% to 13.0% ($P < 0.001$) while the proportion of Asia-centric increased from 57.9% to 90.2% ($P < 0.001$) over the two periods. It's not surprising because the Asian centricity is concordant with the unique epidemiology of NPC as a predominantly Asian disease. The patterns of NPC (incidence, histology) are different in South East Asia and the rest of the world. It would be helpful if clinical trials could address this discrepancy in future study designs.

Generally, the lengthy duration and high cost of immunotherapy trials may suppress industry enthusiasm. However, our findings showed that NPC immunotherapy trials

were more likely to be industry-funded compared with other NPC trials (34.2% vs. 11.5%, $P < 0.001$) and the proportion has increased over time (21.1% vs. 38.2%, $P = 0.077$). It indicates a large market potential in the field of NPC immunotherapy, thus raising financial interests and industrial enthusiasm for sponsorship of such trials. Moreover, 60.2% of NPC immunotherapy trials were other-funded, and this proportion has remained stable over time. It implies that academic institutions and governments continue to play an important role in supporting immunotherapy clinical research for NPC and shoulder vital public health responsibility. Still, allocating more resources to NPC immunotherapy from all relevant parties is essential to improve the effective leveraging of the constrained resources.

It is noteworthy that there was an increasing number of NPC immunotherapy trials after 2017. Actually, this reflects more recent successes in other major tumor types, and therefore there is an increasing interest in studying this intervention in an EBV-related tumor type like NPC that does not have many mutational targets. However, only the number of ICI trials increased significantly, while the numbers of ACT, vaccine and immunomodulator trials remained stagnant. A potential explanation is that ICIs as pan-cancerous antitumor agents were found to have equally promising efficacy in NPC, thus spurring enthusiasm for research. Furthermore, the recognition of ICI-based immunotherapy by the 2018 Nobel Prize in Physiology or Medicine might have further increased researchers' interest in the exploration of ICIs in NPC. In contrast, the exploration and further application of EBV-specific ACTs and vaccines and immunomodulators were hampered by the lack of specific and effective targets, generally low and transient immune responses, technical limitations and financial shortages. According to the results of published NPC studies (Supplementary Table 1), ICIs monotherapy achieves 17.1-34.0% of the objective response rate in the second or later-line treatment of R/M NPC (14, 25–30). In the first-line treatment, the addition of ICIs to chemotherapy also significantly improved progression-free survival and OS in R/M NPC (31–33). Further studies are needed to assess the therapeutic value of ICIs in LANPC and early-stage disease. Notably, CAR-T/TCR-T cell therapy and antibody-drug conjugates may be another promising immunotherapy modality for NPC, as they have shown promising efficacy in a variety of other cancers (34, 35). Therefore, concerted efforts by oncologists, sponsors and other concerned parties are still needed to advance the development of immunotherapy for NPC.

Integration with conventional treatment modalities is one of the trends in immunotherapy. In this study, we found that the most commonly investigated immunotherapy regimen in NPC was combination chemotherapy, followed by combination radiochemotherapy. A recently published study reported on the promising antitumor activity and a manageable toxicity profile of immunotherapy combined with antiangiogenic therapy in R/M NPC (36). In addition, future NPC studies could consider more novel combination strategies to enhance the clinical responses, for example, ICIs combined with ACT (37) or CAR-T cell therapy combined with the oncolytic virus (38).

Limitations of this study should also be acknowledged. First, not all investigators choose ClinicalTrials.gov to register their projects. There are many alternative registries available around the world (39). Nevertheless, ClinicalTrials.gov is the most robust database to date, accounting for 70–80% of the unique clinical trials recorded by the World Health Organization (39). Second, partial NPC trials have not yet been registered on ClinicalTrials.gov, which hindered us from more fully reflecting current global trends in NPC immunotherapy trials. Third, the National Library of Medicine, which operates ClinicalTrials.gov, is unable to validate all registered data. The accuracy of the data relies on the study sponsor. Fourth, we did not include noninterventional trials in our analysis.

In conclusion, NPC Immunotherapy trials over a 15-year period have been largely exploratory. Advancing the clinical application of immunotherapy in NPC requires more attention and concerted efforts to improve the quality of trials.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://clinicaltrials.gov/.

Author contributions

TL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. HGH, YY, and XD contributed equally to this work. Concept and design: HGH, YY, XD, HH, and TL. Acquisition, analysis, or interpretation of data: HGH, YY, XD, and TL. Drafting of the manuscript: HGH, YY, and XD. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: HGH and XD. Obtained funding: HH and TL. Administrative, technical, or material support: HH and TL. Supervision: HH and TL. 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.

The reviewer YG declared a shared parent affiliation with the authors HGH, YY, HW, ZC, ZW, XF, HH, and TL to the handling editor at the time of review.

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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.2023.1195659/full#supplementary-material

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Machine learning-based radiomics for predicting BRAF-V600E mutations in ameloblastoma

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Background: Ameloblastoma is a locally invasive and aggressive epithelial odontogenic neoplasm. The BRAF-V600E gene mutation is a prevalent genetic alteration found in this tumor and is considered to have a crucial role in its pathogenesis. The objective of this study is to develop and validate a radiomics-based machine learning method for the identification of BRAF-V600E gene mutations in ameloblastoma patients.

Methods: In this retrospective study, data from 103 patients diagnosed with ameloblastoma who underwent BRAF-V600E mutation testing were collected. Of these patients, 72 were included in the training cohort, while 31 were included in the validation cohort. To address class imbalance, synthetic minority over-sampling technique (SMOTE) is applied in our study. Radiomics features were extracted from preprocessed CT images, and the most relevant features, including both radiomics and clinical data, were selected for analysis. Machine learning methods were utilized to construct models. The performance of these models in distinguishing between patients with and without BRAF-V600E gene mutations was evaluated using the receiver operating characteristic (ROC) curve.

Results: When the analysis was based on radiomics signature, Random Forest performed better than the others, with the area under the ROC curve (AUC) of 0.87 (95%CI, 0.68-1.00). The performance of XGBoost model is slightly lower than that of Random Forest, and its AUC is 0.83 (95% CI, 0.60-1.00). The nomogram evident that among younger women, the affected region primarily lies within the mandible, and patients with larger tumor diameters exhibit a heightened risk. Additionally, patients with higher radiomics signature scores are more susceptible to the BRAF-V600E gene mutations.

Conclusions: Our study presents a comprehensive radiomics-based machine learning model using five different methods to accurately detect BRAF-V600E gene mutations in patients diagnosed with ameloblastoma. The Random Forest model's high predictive performance, with AUC of 0.87, demonstrates its potential for facilitating a convenient and cost-effective way of identifying

patients with the mutation without the need for invasive tumor sampling for molecular testing. This non-invasive approach has the potential to guide preoperative or postoperative drug treatment for affected individuals, thereby improving outcomes.

KEYWORDS

ameloblastoma, machine learning, radiomics, LASSO, BRAF-V600E

Introduction

Ameloblastoma is a common benign tumor of dental origin, arising from the epithelial component of the developing dental embryo, and often affecting the mandible or maxilla (1) The BRAF-V600E gene mutation is frequently reported in approximately 70% of ameloblastoma (2). Ameloblastoma typically grows slowly but may show features of local invasion into surrounding tissues or cause bone resorption (3). Surgical excision is the most commonly used treatment approach due to the tumor's complex growth pattern, but it can lead to facial deformities (4) and disease recurrence (5). On the other hand, conservative approaches such as fenestration decompression combined with secondary curettage and local curettage, lead to a high rate of tumor recurrence, and radical excisional surgery remains the preferred treatment option (6, 7). The transformation of ameloblastoma to ameloblastic carcinoma, although rare in clinical practice, is still a priority for clinicians when diagnosing the disease (8). Moreover, the recurrence of ameloblastoma can extend over many years, and a disease-free period of 5 years does not necessarily imply complete recovery (9). Therefore, regular follow-up is required. In summary, there is a pressing need for targeted treatment modalities for this disease to avoid extensive surgery and disease recurrence. The pathogenesis of ameloblastoma at the molecular level is not yet fully understood. However, some studies have identified potential prognostic markers or therapeutic targets, indicating the need for further research (2). Interestingly, genetic molecular alterations in ameloblastoma have been shown to be associated with clinical features and patient prognosis (10, 11). The BRAF-V600E gene mutation has been identified as a crucial factor in the pathogenesis of ameloblastoma (12). The MAPK/ERK pathway has been found to be activated by the BRAF-V600E gene mutation (13), leading to increased cell proliferation and inhibition of apoptosis and promoting the development of ameloblastoma (14). The prevalence of BRAF-V600E gene mutation in aggressive and recurrent ameloblastoma suggests a potential role in the biological behavior of the tumor (15). Other molecular mechanisms, such as the activation of the Wnt/β-catenin pathway, have also been implicated in the pathogenesis of ameloblastoma, leading to increased cell proliferation and inhibition of apoptosis (16). Further investigation into these pathways may provide important insights into the development and progression of ameloblastoma and facilitate the development of targeted therapies.

Radiomics is an increasingly important discipline in the medical field, providing quantitative analysis of medical images using advanced computational techniques to extract multiple features and increase the accuracy of clinical decision making by physicians (17, 18). Radiomics analysis has been applied to predict mutations in tumor somatic cells in various cancers (19). Yang et al. (20) showed good area under the curve (AUC) and specificity in predicting KRAS/NRAS/BRAF gene mutations in colorectal cancer patients based on radiomics features of computed tomography (CT). Radiomics has also been used to predict response to chemotherapy and radiotherapy in non-small cell lung cancer patients, assisting clinicians in making treatment decisions (21). While some studies have explored the relationship between radiomics features and BRAF gene mutation status, the role of CT-based machine learning (ML) for radiomics in identifying BRAF-V600E gene mutations in ameloblastoma remains to be investigated.

Therefore, the objective of this study is to develop a radiomics-based model that predicts the BRAF-V600E gene mutation status in patients with ameloblastoma. This study will employ five ML algorithms based on CT and combine radiomics and clinical features of patients to evaluate the model's effectiveness in predicting BRAF-V600E gene mutations in ameloblastoma. The outcomes of this study may prove valuable in distinguishing patients with ameloblastoma who have developed BRAF-V600E gene mutations, and help clinicians make treatment decisions without resorting to invasive testing.

Methods

Patients

The inclusion criteria and procedures for participant recruitment in this retrospective study were in accordance with the guidelines stipulated in the 1964 Helsinki Declaration. Approval for this study was obtained from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval number: 2023-KY-0140). One hundred and three patients diagnosed with ameloblastoma at the First Affiliated Hospital of Zhengzhou University between January 2012 and December 2022 were included in this study. The clinical information of patients includes age, gender, tumor site, and tumor diameter. The

inclusion criteria were as follows: (1) a pathological diagnosis of ameloblastoma; (2) clear CT images prior to surgery; and (3) detection of the BRAF-V600E gene mutation after surgery. The exclusion criteria (Figure 1) were as follows: (1) CT images cannot be used (n=82); (2) without clinical data (n=15); (3) without BRAF-mutant test (n=135); and (4) accepted prior surgery treatment because of ameloblastoma (n=37). Patients were allocated into two distinct groups, namely a training cohort and a validation cohort, based on the chronological sequence of their surgical procedures. The training cohort comprised 72 patients who underwent surgery within the timeframe spanning January 2012 to January 2020. On the other hand, the validation cohort incorporated 31 patients whose surgical procedures took place between February 2020 and December 2022.

Image acquisition and processing

The CT machine utilized in this study was the Aquilion 16 CT (Toshiba, Japan), located at the First Affiliated Hospital of Zhengzhou University. The following scan parameters were employed: voltage 120kV, current 200 mA, and a slice thickness of 5mm.

Regions of interest (ROI) segmentation and mask dilation

Our radiomics analysis encompassed various stages: lesion segmentation, feature extraction, feature selection, and feature analysis and evaluation (Figure 2). Prior to initiating the lesion segmentation, we implemented a resampling process to standardize the images in accordance with the Image Biomarker Standardization Initiative (IBSI) guidelines (22), which involved resampling voxel sizes of 1 mm × 1 mm × 1 mm. The patient CT images collected for this study were obtained at a resolution of 512 × 512.

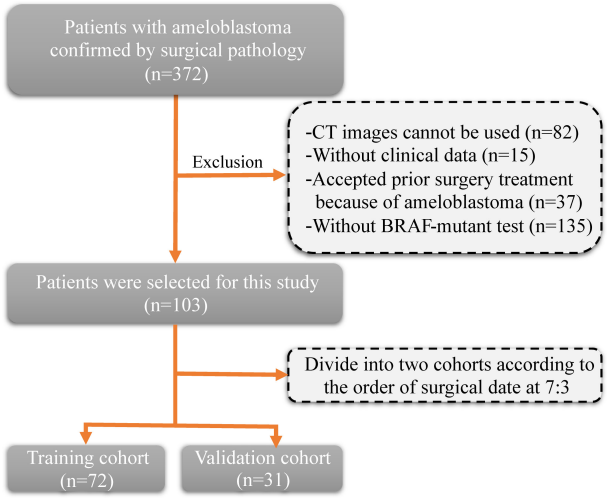

FIGURE 1
Flow diagram of the study population.

ROIs were manually segmented on a slice-by-slice basis along the lesions utilizing ITK-SNAP software (version 3.8.0, www.ITK-SNAP.org). An oral surgeon with over three years of experience undertook this segmentation, with the individual blinded to the clinical information of the patients. Another senior dentist with five years of experience verified all manual delineations. The delineated ROIs were saved in Neuroimaging Informatics Technology Initiative (NII) format for further analysis. Subsequently, quantitative radiomic features were extracted from CT images, using Pyradiomics software (version 2.2.0, http://pyradiomics.readthedocs.io) (23). The intraclass correlation coefficients (ICCs) were calculated to gauge the consistency between the features extracted by the two radiologists. Any features presenting intra-observer or inter-observer ICCs less than 0.75 were excluded, attributable to their comparatively low robustness (24).

Analysis of BRAF-V600E gene mutations

Based on the specimen, BRAF-V600E gene mutations were examined using real-time fluorescent polymerase chain reaction (PCR) and DNA sequencing (ABI Step One/ABI sequence Analyzer) technologies (25). The nucleic acid's original concentration was 184 ng/μl. In the present study, wild-type BRAF-V600E referred to the absence of mutations in those loci.

Radiomics feature extraction

The hand-crafted features can be categorized into three groups: (1) geometric features, (2) intensity features, and (3) texture features. Extracted features comprised 360 first-order features, 440 gray-level co-occurrence matrix (GLCM) features, 280 gray-level dependence matrix (GLDM) features, 320 gray-level run length matrix (GLRLM) features, 320 gray-level size zone matrix (GLSZM) features, 100 neighboring gray tone difference matrix (NGTDM) features and 14 shape features (Supplementary Datasheet 1). In total 1834 radiomics features were extracted from ROIs.

Feature selection

Prior to in-depth analysis, all extracted radiomics features underwent standardization into a normal distribution using z-scores, thereby nullifying potential discrepancies in data value scales. Given the contrast between the relatively low dimensional sample size and the high dimensional radiomics features, feature selection was indispensable to prevent overfitting (26). We conducted a Student's t-test for features adhering to a normal distribution, only considering features with a p-value less than 0.05 for subsequent analysis.

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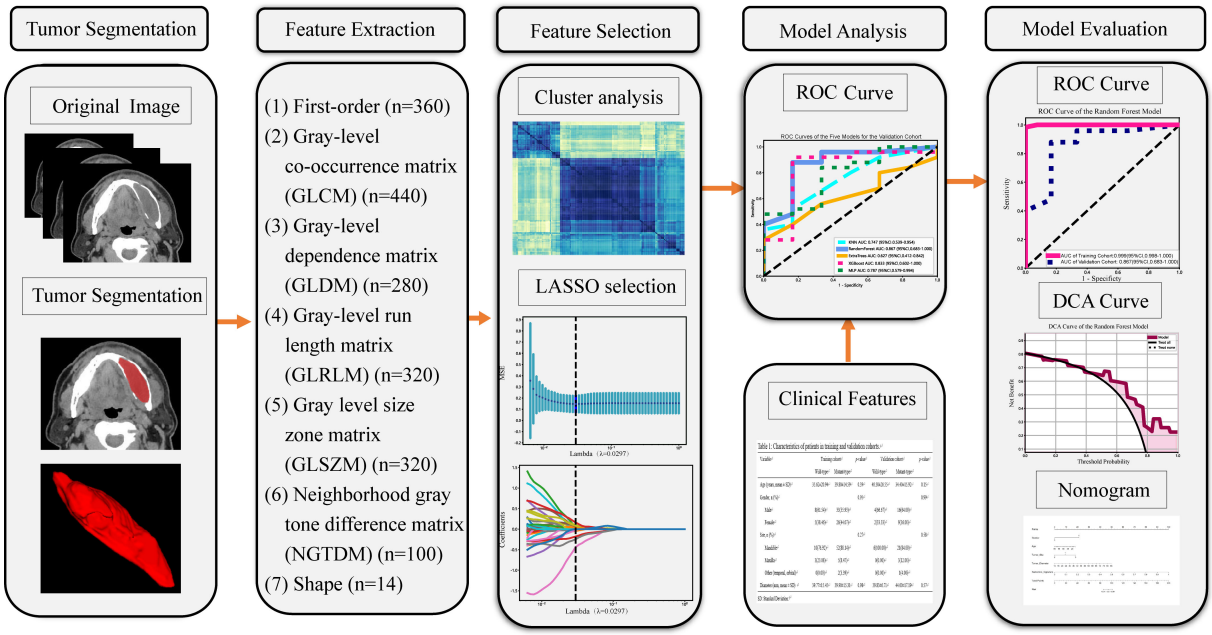

FIGURE 2
Workflow of the study. LASSO, the least absolute shrinkage and selection operator; n, Number of features; ROC, receiver operating characteristic; DCA, decision curve analysis.

Spearman's rank correlation coefficient was used to assess the correlation between features with high repeatability (27). To avoid redundancy, we retained only one feature from any pair with a correlation coefficient greater than 0.9 (28). To maximize the informative value of the feature set, we employed a greedy recursive deletion strategy for feature filtering. This involved removing the feature with the greatest redundancy in the current set until 51 features remained.

We employed the Least Absolute Shrinkage and Selection Operator (LASSO) regression model to construct a signature based on the discovery dataset. LASSO regression shrinks all regression coefficients towards zero and sets many coefficients of uncorrelated features to exactly zero. The optimal regularization weight λ was determined using a minimum criterion and 10-fold cross-validation. Retained features with non-zero coefficients were used to fit the regression model and combined to form radiomics features. A radiomics score was then calculated for each patient by weighting the linear combination of the retained features by their model coefficients. We used the Python *scikit-learn* package (29) for LASSO regression modeling.

Radiomics signature

Radiomics data were balanced using synthetic minority over-sampling technique (SMOTE) algorithm synthesis (30). In this work, we processed hyperparameter optimization using grid search to optimize the parameters of models and apply the best parameters to predict BRAF-V600E gene mutations in ameloblastoma. After LASSO feature screening, the final features were input into various ML models, including K-Nearest Neighbor (KNN), Random Forest, ExtraTrees, eXtreme Gradient Boosting (XGBoost) and Multilayer Perceptron (MLP), for constructing the risk model. To obtain the final radiomics signature, a 5-fold cross-validation approach was adopted. Radiomics-clinical nomogram was developed by combining the radiomics signature and clinical features using the logistic regression algorithm.

Statistical analysis

In an endeavor to ascertain the equivalence of patient attributes across cohorts, we applied differing statistical approaches for data analysis. Student's t-test were utilized for the analysis of normally distributed data, whilst non-normally distributed data were scrutinized using the Mann-Whitney U test. For categorical variables, chi-square tests proved the method of choice for evaluation. Furthermore, we conducted an assessment of the predictive power of three distinctive models using ROC curves. The calculation of the AUC was undertaken, followed by the computation of the balanced sensitivity and specificity of the cut-off point, yielding the maximum value of the Youden index. We calculated the 95% confidence interval (CI) of the AUC utilizing the bootstrap method with 1000 intervals for increased precision. This comprehensive and rigorous analysis approach serves to illuminate the strengths and potential limitations of our study, providing a more robust understanding of the data and underlying patterns therein. The AUC ranged from 0.5 to 1.0. The discriminative test was deemed perfect when the AUC equaled 1.0. An AUC between 0.8 and 1.0 was indicative of a good discriminant test, whereas an AUC ranging from 0.6 to 0.8 represented a moderate test. If the AUC fell within the 0.5 to 0.6 range, the discriminant test was considered poor (31, 32). We performed statistical analyses using SPSS software (version

21.0). A two-sided *p*-value of less than or equal to 0.05 was stipulated as the threshold for statistical significance.

Results

Clinical characteristics

The baseline clinical characteristics of the enrolled patients are presented in Table 1. No significant differences were observed in terms of age, gender, tumor site, and tumor diameter between gene mutation and non-mutation groups (*p*-value > 0.05). There was no statistically significant difference (*p*-value > 0.05) between clinical characteristics and predicted BRAF-V600E mutation on univariate analysis (Supplementary Table 1).

LASSO feature selection

We selected 1834 features for the extraction of ROIs, and the categories of features and the corresponding *p*-values are shown in Figure 3A and in Supplementary Datasheet 1. We kept 155 features that the *p*-value was less or equal to 0.05. The correlation coefficients for each feature were visualized and can be seen in Supplementary Datasheet 2. Subsequently, six nonzero coefficient features were selected to create radiomics-scores with a LASSO logistic regression model (λ = 0.0297) (Figures 3B, C). The histograms of the feature scores are shown in Figure 3D.

Diagnostic performance among radiomics model and nomogram

For the validation cohort, the AUC value for each classifier across the different ML algorithms are presented in Figure 4 (more details in Supplementary Table 2). When the analysis was based on radiomics signature, Random Forest performed better than the others, with AUC of 0.87 (95%CI, 0.68-1.00) (Figure 5A). The performance of XGBoost model is slightly lower than that of Random Forest, and its AUC is 0.83 (95% CI, 0.60-1.00). These two models have a good performance. The other three models performed moderately. In this study, we evaluated the model through decision curve analysis (DCA). The DCA for Random Forest model is presented in Figure 5B.

The nomogram combined the clinical features (gender, age, tumor site and diameter) and radiomics signature (Figure 5C). It is evident from Figure 5C that among younger women, the affected region primarily lies within the mandible, and patients with larger tumor diameters exhibit a heightened risk. Additionally, patients with higher radiomics signature scores are more susceptible to the BRAF-V600E gene mutation.

Discussion

Ameloblastoma is a common tumor of dental origin, and its biological behavior is complex and not yet fully understood. This disease is prone to recurrence and has a tendency to become malignant, often classified as borderline tumors. Pulmonary metastases have also been reported in some cases of ameloblastoma (33). The conventional treatment for ameloblastoma is surgical resection, which depends on various factors such as tumor location, size, histological type, patient's age, and general health (34). The goal is to achieve complete removal of the tumor while preserving the patient's physical function and aesthetic appearance as much as possible. However, due to the slow growth of ameloblastoma, patients often present with large tumors at the time of consultation, which may result in facial deformities after resection and impair oral and maxillofacial function, leading to physical and mental health issues (35). Therefore, early detection, diagnosis, and treatment of ameloblastoma are crucial. It is also important to explore new adjuvant treatments in combination with surgery to reduce the recurrence rate of this disease. In the realm of ameloblastoma research, the focus of existing radiomics studies has largely been on the differential diagnosis of the disease. For instance, Liu et al. (36) utilized a Convolutional Neural Network (CNN) methodology to

TABLE 1 Characteristics of patients in training and validation cohorts.

Variable	Training cohort		*p*-value	Validation cohort		*p*-value
	Wild-type	Mutant-type		Wild-type	Mutant-type	
Age (years, mean ± SD)	35.62±20.94	39.80±14.59	0.39	46.50±26.55	34.40±15.92	0.15
Gender, n (%)			0.95			0.99
Male	8 (61.54)	33 (55.93)		4 (66.67)	16 (64.00)	
Female	5 (38.46)	26 (44.07)		2 (33.33)	9 (36.00)	
Site, n (%)			0.27			0.58
Mandible	10 (76.92)	52 (88.14)		6 (100.00)	21 (84.00)	
Maxilla	3 (23.08)	5 (8.47)		0 (0.00)	3 (12.00)	
Other (temporal, orbital)	0 (0.00)	2 (3.39)		0 (0.00)	1 (4.00)	
Diameter (mm, mean ± SD)	39.77±15.45	39.90±15.31	0.98	39.83±6.71	44.00±17.19	0.57

SD, Standard Deviation.

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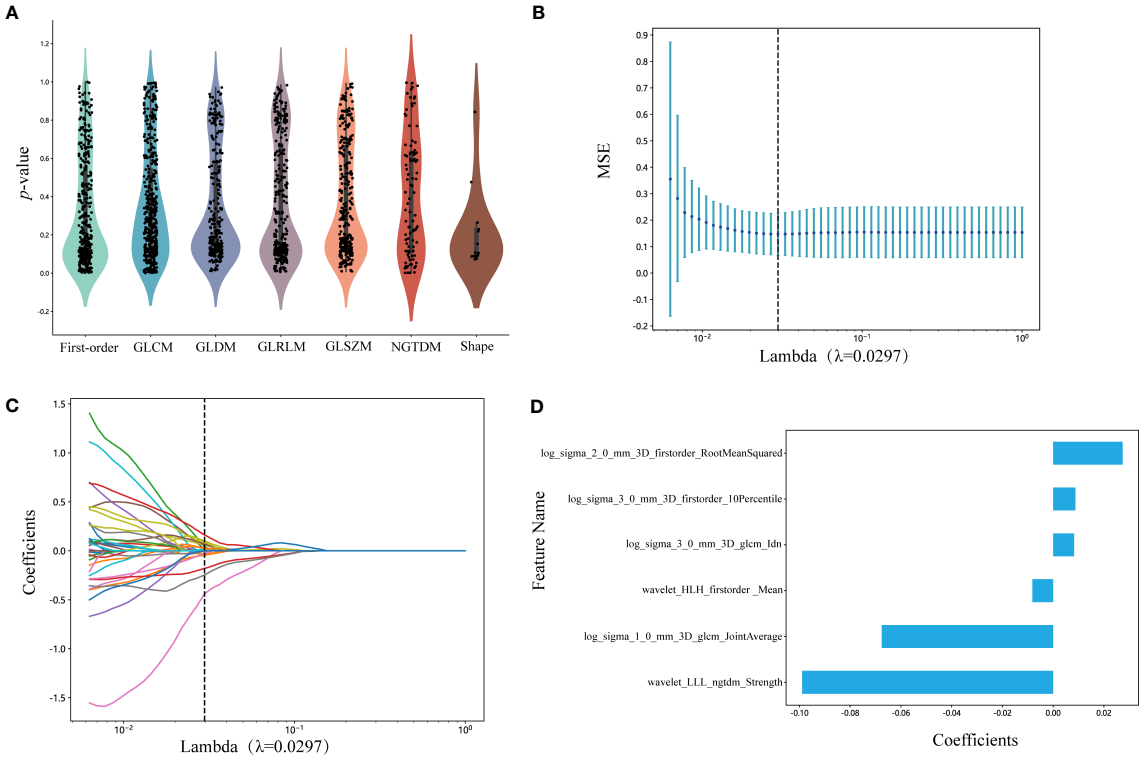

FIGURE 3
(A) Statistics of radiomic features. Points represent features. GLCM, gray-level co-occurrence matrix; GLDM, gray-level dependence matrix; GLRLM, gray-level run length matrix; GLSZM, gray-level size zone matrix; NGTDM, neighboring gray tone difference matrix. **(B)** Mean square error of cross-validation of LASSO model. The optimal λ value is 0.0297. MSE: mean square error. **(C)** LASSO coefficient solution path of features. The optimal λ value is 0.0297. **(D)** The histogram of the feature score. The y-axis indicates the selected six radiomics features, and the x-axis represents the coefficients of LASSO model. LASSO, the least absolute shrinkage and selection operator; LLL, low-low-low-pass filtered image; HLH, high-low-high-pass filtered image.

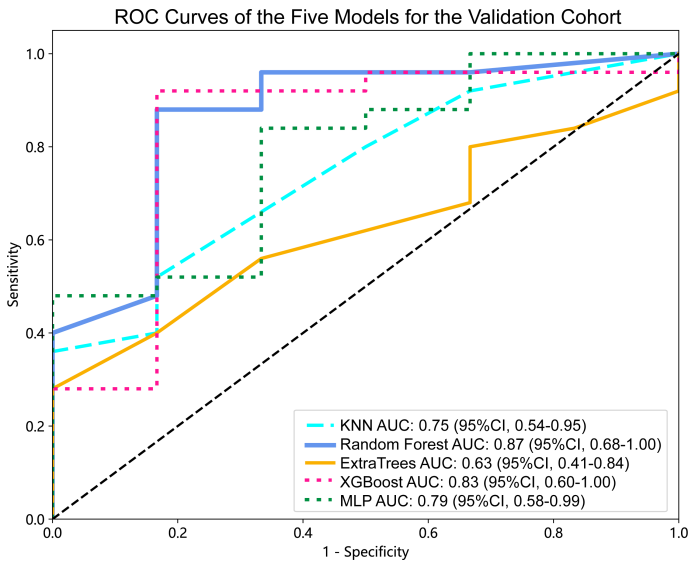

FIGURE 4
ROC curves of the five models for the validation cohort. AUC, area under ROC curve; ROC, receiver operating characteristic; CI, confidence interval; KNN, K-Nearest Neighbor; XGBoost, eXtreme Gradient Boosting; MLP, Multilayer Perceptron.

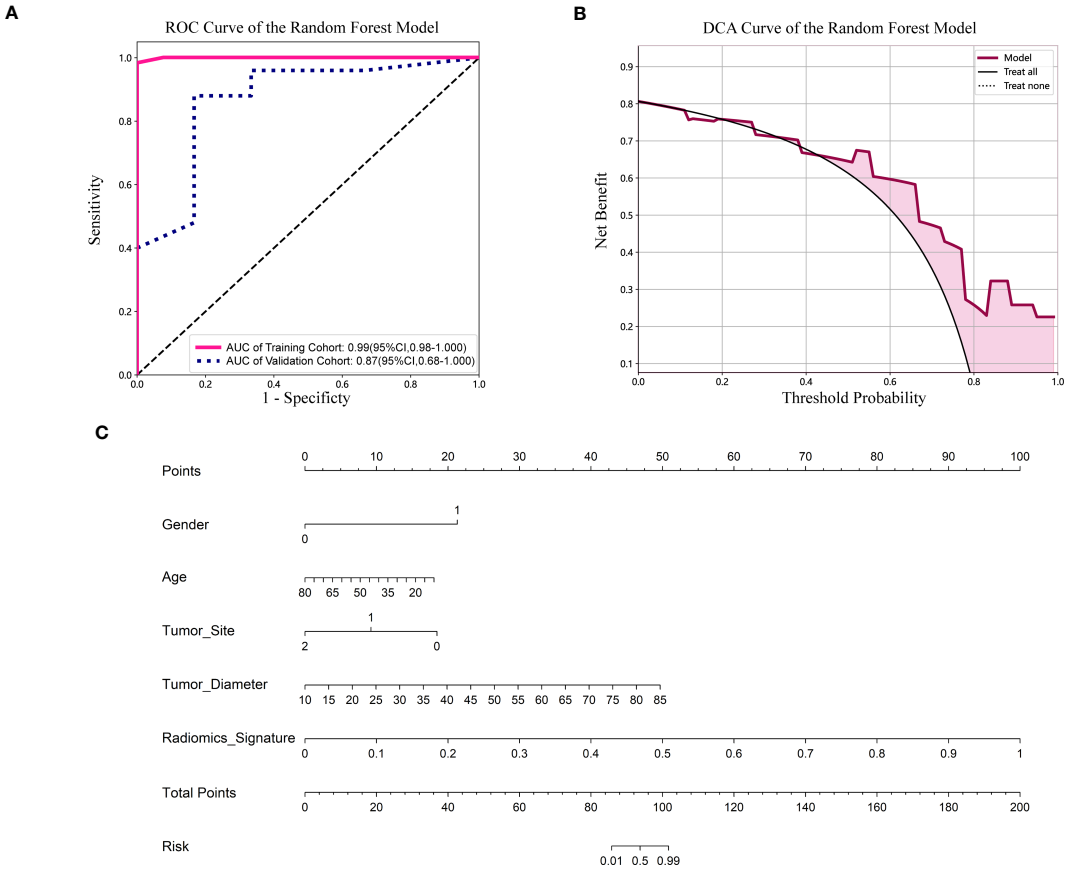

FIGURE 5
(A) ROC curve of the Random Forest model. CI, confidence interval; ROC, receiver operating characteristic; AUC, area under ROC curve. **(B)** DCA of the Random Forest model. DCA: decision curve analysis. **(C)** Nomogram based on the clinical and radiomics features prediction model to predict the risk of BRAF-V600E gene mutation. Gender, 0; male, 1; female. Tumor site, 0; mandible, 1; maxilla, 2; other (temporal, orbital).

distinguish between ameloblastoma and odontogenic keratocyst, drawing on the patients' panoramic radiographs for their analyses. Alternatively, Chai et al. (37) adopted a similar CNN modeling approach, but their study was distinctive in that it relied on patients' cone beam computed tomography (CBCT) data to differentiate between the two conditions.

Ameloblastoma is a heterogeneous tumor that can be classified into different subtypes based on their histological characteristics, including conventional, unicystic, and desmoplastic types, among others. Each subtype exhibits distinct biological behaviors and treatment responses (38). Meanwhile, it has been found that ameloblastoma carrying mutations in the BRAF gene tend to occur more frequently in the mandible and in younger patients (39). In the era of precision medicine, it is crucial to identify the molecular features of different disease subtypes and develop targeted therapies accordingly. Previous studies have used radiomics features as the primary investigative tool to identify the molecular subtypes of various gene mutations present in low-grade gliomas (40). Multiple studies have demonstrated that the BRAF-V600E gene mutation contributes to the activation of the MAPK signaling pathway and plays a crucial role in the pathogenesis of ameloblastoma (41). Therefore, the development of BRAF-V600E-specific inhibitors represents a promising approach to improve the treatment of ameloblastoma in addition to surgery. Currently, BRAF-V600E inhibitors such as Zelboraf and Dabrafenib are approved for the treatment of melanoma (42), while their use in ameloblastoma is still under investigation. However, some studies have shown promising clinical outcomes with the use of Dabrafenib in ameloblastoma (43). As research into the BRAF-V600E gene mutation continues, the use of targeted therapeutic agents for this mutation in ameloblastoma is expected to become a valuable adjuvant treatment to reduce the recurrence rate of patients.

In this study, we propose a predictive model based on a combination of non-invasive CT images and patient clinical features to predict BRAF-V600E gene mutation status in patients with ameloblastoma. We included clinical information such as age, gender, tumor location, and tumor diameter of patients with ameloblastoma to establish a correlation between this clinical information and the BRAF-V600E gene mutation. ML algorithms were used to identify patterns and relationships in the data. Five ML models were trained on 72 patients, and their performance was validated with 31 patients. The Random Forest model performed good predictability with AUC of 0.87 (95%CI, 0.68-1.00) in the validation cohort, indicating that it may handle noisy data in CT images more effectively than other tree-based models. The performance of XGBoost model is slightly lower than that of Random Forest, and its AUC is 0.83 (95% CI, 0.60-1.00). These two models have a good performance.

The nomogram showed the importance ranking of individual features, with radiomics features having greater importance than patient clinical information, which is consistent with the results derived from the Random Forest model. This study provides evidence of a clear association between CT image features and BRAF-V600E genotype, and demonstrates the ability of radiomics to identify BRAF-V600E gene mutation status. The prediction of BRAF-V600E gene mutations based on Random Forest models has the potential to replace conventional invasive biopsies. To our knowledge, this is the first study to build a ML model to predict BRAF-V600E mutation status in patients with ameloblastoma. Therefore, this study makes a significant contribution to existing research in this field.

Supervised learning and unsupervised learning are the two main ML methods. While supervised learning has been the primary method in the field of data mining (44), all five ML models used in this study are supervised learning methods. Our retrospective study demonstrated that the Random Forest models were viable for predicting BRAF-V600E gene mutation status.

There are several limitations to this study that should be acknowledged. Firstly, being a retrospective study, it may have some inherent limitations such as data selection bias. Secondly, the sample size of patients included in the study was relatively small after a rigorous screening process. However, we believe that the inclusion of more than 100 patients for radiological analysis is desirable in the current study (45). In future studies, we plan to expand the sample size to assess the stability and clinical application of the Random Forest models. Simultaneously, we will persist in our patient follow-up efforts and utilize radiomics features to prognosticate their progression-free survival, particularly concerning recurrence of ameloblastoma, drawing inspiration from the research trajectory established by Le et al. (46). Additionally, we aim to employ semi-automated or automated radiological methods in future studies to enhance the robustness of the prediction models used in this study.

Conclusion

In conclusion, this study demonstrated that a combination of radiomics signatures and clinical features can accurately predict BRAF-V600E gene mutation status in patients with ameloblastoma. While these findings require validation with a larger sample size, the use of machine learning models provides a non-invasive and cost-effective approach for predicting BRAF-V600E gene mutations. This approach could potentially aid in screening patients before resorting to invasive sampling and in developing personalized treatment plans to optimize outcomes for patients with ameloblastoma.

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

Approval for this study was obtained from the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (approval number: 2023-KY-0140). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

WL and YaL contributed equally to this study. WL and YaL designed and wrote the main manuscript text. XL and LW collected information and analyzed data on the patients included in the study. WC, XQ and XZ edited and revised the manuscript text. JC, YiL and LL presented the research oversaw its implementation. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1180908/full#supplementary-material

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