Metabolic remodeling and changes in tumor immune microenvironment, TIME, in osteosarcoma are important factors affecting prognosis and treatment. However, the relationship between metabolism and TIME needs to be further explored.

Methods
RNA-Seq data and clinical information of 84 patients with osteosarcoma from the TARGET database and an independent cohort from the GEO database were included in this study. The activity of seven metabolic super-pathways and immune infiltration levels were inferred in osteosarcoma patients. Metabolism-related genes, MRGs, were identified and different metabolic clusters and MRG-related gene clusters were identified using unsupervised clustering. Then the TIME differences between the different clusters were compared. In addition, an MRGs-based risk model was constructed and the role of a key risk gene, ST3GAL4, in osteosarcoma cells was explored using molecular biological experiments.

Results
This study revealed four key metabolic pathways in osteosarcoma, with vitamin and cofactor metabolism being the most relevant to prognosis and to TIME. Two metabolic pathway-related clusters, C1 and C2, were identified, with some differences in immune activating cell infiltration between the two clusters, and C2 was more likely to respond to two chemotherapeutic agents than C1. Three MRG-related gene clusters, GC1-3, were also identified, with significant differences in prognosis among the three clusters. GC2 and GC3 had higher immune cell infiltration than GC1. GC3 is most likely to respond to immune checkpoint blockade and to three commonly used clinical drugs. A metabolism-related risk model was developed and validated. The risk model has strong prognostic predictive power and the low-risk group has a higher level of immune infiltration than the high-risk group. Knockdown of ST3GAL4 significantly inhibited proliferation, migration, invasion and glycolysis of osteosarcoma cells and inhibited the M2 polarization of macrophages.

Conclusion
The metabolism of vitamins and cofactors is an important prognostic regulator of TIME in osteosarcoma, MRG-related gene clusters can well reflect changes in osteosarcoma TIME and predict chemotherapy and immunotherapy response. The metabolism-related risk model may serve as a useful prognostic predictor. ST3GAL4 plays a critical role in the progression, glycolysis, and TIME of osteosarcoma cells.

Supplementary Information
The online version contains supplementary material available at 10.1186/s12929-024-00999-7.

Keywords: Osteosarcoma, Metabolism, Tumor immune microenvironment, Prognosis, Vitamin and cofactor, Treatment response, ST3GAL4

Introduction

Osteosarcoma is a malignant bone tumor that predominantly affects children and young adults. Despite advances in treatment, the prognosis for patients with osteosarcoma remains poor, with

a 5-year survival rate of approximately 60–70% for localized disease and less than 30% for metastatic disease. The development and progression of osteosarcoma is a complex process that involves multiple molecular and cellular mechanisms. In recent years, there has been increasing interest in the role of metabolic reprogramming and the tumor immune microenvironment, TIME, in osteosarcoma, and their potential as therapeutic targets.

Tumor cells have to modify their metabolic program to support the energy and macronutrient requirements of rapid proliferation. Metabolic reprogramming is now recognized as a hallmark of cancer and is one of the most critical biological differences between tumor cells and normally differentiated cells. For example, in many tumor cells, altered carbohydrate metabolism, represented by the Warburg effect, provides a proliferative advantage for tumor cells. Osteosarcoma cells exhibit a variety of metabolic alterations, including increased glucose uptake, altered mitochondrial function, and increased reliance on glycolysis for ATP generation. These metabolic changes are driven by a variety of signaling pathways, including the PI3K/AKT/mTOR pathway and the HIF-1α pathway. They provide potential therapeutic targets for the treatment of osteosarcoma, as inhibition of key metabolic pathways could potentially starve cancer cells of the nutrients they need to proliferate.

TIME plays a critical role in a variety of biological processes including proliferation, metastasis, and treatment response including chemotherapy, radiation therapy and immunotherapy in osteosarcoma. Previous studies have shown that patients with different TIME status within their osteosarcoma have very different prognoses. Specifically, patients with “hot” tumors that have more immune cell infiltration in TIME have a better prognosis, while patients with “cold” tumors that have less immune cell infiltration have a worse prognosis. Therefore, therapeutic agents that modulate TIME, transform “cold” tumors into “hot” tumors, and use existing immunity to destroy osteosarcoma cells are increasingly being considered as new options with great potential for application. Indeed, recent studies have proven that immunotherapy has shown advantages over conventional interventional strategies in inhibiting osteosarcoma metastasis and recurrence, and satisfactory efficacy in inhibiting the progression of advanced osteosarcoma.

The interaction between tumor metabolism and immunity has been intensively studied and it is generally recognized that oncogenic transformation can lead to the adaptation of a well-characterized metabolic phenotype in cancer cells that can profoundly affect the TIME. Specifically, in addition to affecting cancer cells directly, metabolic reprogramming of tumors also alters the TIME by affecting the behavior of other cell types, such as immune cells and stromal cells. For example, the acidic microenvironment created by aerobic glycolysis can suppress the immune system and promote the growth of blood vessels, which can in turn promote tumor growth and metastasis. In particular, the lactate produced by aerobic glycolysis can induce the infiltration of regulatory T cells and the M2 polarization of macrophages in tumors, thereby promoting immune suppression. Tumor metabolic heterogeneity refers to the significant differences in metabolic characteristics that exist between different tumors or within the same tumor tissue. It is an important aspect of tumor heterogeneity and is mainly driven by different genotypes or microenvironments. The research by Feng et al. demonstrates that within the same type of tumor, patients can be divided into subgroups suitable for different treatment

methods based on different metabolic characteristics. Therefore, identifying the metabolic profile of different osteosarcoma patients will not only explore the impact of different metabolic landscapes on TIME, but also guide treatment decisions. However, few studies have been conducted to genomically analyze osteosarcoma from a global perspective of metabolic heterogeneity. The few previous studies have been limited to a specific metabolic pathway. In this study, we focused on the seven most prominent metabolic super-pathways with the aim of comprehensively assessing metabolic pathways of prognostic importance in osteosarcoma, identifying tumor subtypes with different metabolic profiles and exploring the heterogeneity of TIME profiles and treatment response across tumor subtypes.

Methods and Materials

Data acquisition, clinical samples and cell lines

Standardized RNA-Seq data and clinical information for 88 independent osteosarcoma samples from the TARGET database were obtained from Xena Functional Genomics Explorer, http://xena.ucsc.edu/, of which 84 samples with complete survival information were included in this study. In addition, standardized microarray expression data and clinical information for 34 osteosarcoma samples from the GSE16091 cohort were obtained from the GEO database, https://www.ncbi.nlm.nih.gov/geo/. The gene expression data distribution from the TARGET and GEO databases was analyzed using the PCA algorithm, and it was found that there were no apparent batch effects in the data. In addition, gene sets for seven metabolic super-pathways annotated according to the Reactome database were collected from a previous study. Together, these gene sets represent the major metabolic processes, including amino acid metabolism, carbohydrate metabolism, integration of energy metabolism, lipid metabolism, nucleotide metabolism, tricarboxylic acid, TCA, cycle, and vitamin and cofactor metabolism.

Tumor samples of 14 primary osteosarcoma patients who underwent surgical resection between 2018 and 2019 and 5 normal tissue samples were obtained from Xiangya Hospital, Central South University, Hunan, China. All samples were evaluated by pathologists and preserved in paraffin. Only relapse-free survival, RFS, data is currently available, as most patients are still alive. The collection of human tissues was approved by the Medical Ethics Committee of Xiangya Hospital of Central South University, Approval number: 202303046.

U2OS, MG-63 and THP-1 cell lines were obtained from the Xiangya cell repository and U2OS and MG-63 were cultured in Dulbecco's modified Eagle's medium, DMEM, Biological Industries, Israel, containing 10% fetal bovine serum, Gibco, USA, at 5% CO2 and 37 °C. THP-1 cell line was cultured in RPMI 1640 medium, Gibco, Thermo Fisher Scientific, USA. THP-1 cells were differentiated into M0 macrophages by incubation with 100 ng/mL phorbol 12-myristate 13-acetate for 24 h. Typical images of THP-1 cells, M0 macrophages, and M2 macrophages were shown in supplementary figures. The ST3GAL4 overexpression plasmid was synthesized by Sino Biological, Beijing, China, and the overexpression efficiency was shown in supplementary data. The small interfering RNA si-ST3GAL4 and the empty vector si-NC were synthesized by GenePharma, Shanghai, China. A total of three siRNAs were validated, among which siRNA-2 showed the highest efficiency and was used for subsequent experiments.

Pathway enrichment analysis

To quantify the activity of the seven metabolic pathways in a single tumor sample, gene set variation analysis, GSVA, was performed using the R package “GSVA” to calculate the enrichment score of each pathway in the individual sample. Subsequently, Kaplan-Meier curve and log-rank test were used to explore the relationship between the seven metabolic pathways and overall survival, OS, of patients with osteosarcoma. The “surv_cutpoint” function of the “survminer” R package was used to determine the optimal cut-off point of each metabolic pathway based on the maximally selected log-rank statistics. In addition, a set of core biological pathway gene sets closely related to tumors was collected from the study of Mariathasan et al. and the activity of each pathway was calculated by GSVA. This includes three epithelial mesenchymal transition, EMT, signatures originating from different publications and composed of different genes. The correlation of key metabolic pathways with core biological pathways was then calculated using Spearman’s correlation analysis. Gene set enrichment analysis, GSEA, between the two groups of samples was performed in Sangerbox, http://vip.sangerbox.com/, using the GSEA software Version 4.1.0 based on the HALLMARK and KEGG gene sets.

Identification of hub genes in the vitamin and cofactor metabolic pathway

The protein–protein interaction, PPI, network was constructed using the STRING database, https://string-db.org/, and visualized using the Cytoscape software Version 3.8.2. The hub genes of the vitamin and cofactor metabolic pathway were then identified based on the PPI network using the “cytohubba” plugin in Cytoscape. Gene modules in the vitamin and cofactor metabolic pathway were analyzed using the “MCODE” plugin. After matching the hub genes with RNA-Seq data, univariate Cox regression analysis was performed to determine the effect of the hub genes on OS in patients with osteosarcoma. In addition, similar methods were used to analyze the hub genes of other metabolic super-pathways.

Identification of metabolic pathway-related clusters

After identifying key metabolic pathways in osteosarcoma, PAM-based unsupervised consensus clustering was used to identify potential metabolic subtypes as we described previously. In brief, 1000 bootstraps were performed and K value was set to 2–10, and the optimal number of clusters was defined by the consensus cumulative distribution function and the consensus heatmap. This method was also used for the identification of clusters based on seven metabolic pathways.

Immune infiltration analysis

The ESTIMATE algorithm is a method for inferring the overall level of immune infiltration in tumor tissues based on gene expression data and has been widely used in a large number of previous studies. This study used this algorithm to infer the ImmuneScore, StromalScore and ESTIMATEScore, inversely correlated with tumor purity, of patients with osteosarcoma. In addition, the single sample GSEA, ssGSEA, method was used to infer the levels of 28 immune cell infiltration in osteosarcoma based on a previous report.

Identification of metabolism-related genes, MRGs, in osteosarcoma

Using the R package “limma” to compare differentially expressed genes between different metabolic pathway-related clusters, the threshold was set to $p < 0.05$ and log2 fold change >

0.25. These genes were considered as MRGs. Subsequently, Gene Ontology, GO, enrichment analysis and Kyoto Encyclopedia of Genes and Genomes, KEGG, pathway analysis were performed on MRGs. The prognostic role of MRGs in patients with osteosarcoma was analyzed using univariate Cox regression. After obtaining prognosis-related MRGs, potential MRG-associated gene clusters were identified using unsupervised clustering analysis.

Construction of the risk model

In this study, prognosis-related MRGs were downscaled and hub prognostic genes were obtained using the Least Absolute Shrinkage and Selection Operator, LASSO, regression analysis. Subsequently, the hub prognostic genes were placed into the stepwise multivariate regression to construct risk models, and the risk model with the largest C-index was considered the best risk model. The risk model was calculated using the following formula:

risk score = $\sum$ kj × Expi

where kj is the coefficient of each gene in the risk model, and Expi is the gene expression. The prediction accuracy of the risk score was quantified by drawing ROC curves using the “timeROC” R package. This R package is widely used to estimate time-dependent ROC curves and the area under the time-dependent ROC curve, AUC, in the presence of censoring data. The package uses the inverse probability of censoring weighting method to estimate and handle censoring data.

Analysis of drug sensitivity and response to immunotherapy

As previously described, normalized gene expression data of 809 tumor cell lines and response data for each cell line to three guideline-based used chemotherapeutic agents, cisplatin, cyclophosphamide and gemcitabine, for osteosarcoma and one targeted agent, sorafenib, with clinical application value were downloaded from the Drug Sensitivity in Cancer, GDSC, database, and the drug response data were converted to the IC50. Then the IC50 of every drug in individual osteosarcoma patient was estimated based on oncoPredict algorithm using the gene expression profile of these cell lines and drug response data as the training set. Maeser et al. provided a detailed explanation of the usage details of the oncoPredict algorithm. Jiang et al. developed TIDE using RNA-Seq data from tumors treated with anti-PD1 and anti-CTLA4 therapies, and identified it as an effective predictor of the responsiveness to these therapies. In this study, it was used to infer the response of osteosarcoma patients to immune checkpoint blockade, ICB. The TIDE score is based on two mechanisms of tumor immune escape, including the dysfunction of tumor-infiltrating cytotoxic T lymphocytes, CTL, and the exclusion of CTLs by immunosuppressive factors, and three cell types that limit T cell infiltration in tumors, including the tumor-associated fibroblasts, CAF, myeloid-derived suppressor cells, MDSC, and M2 tumor-associated macrophages, TAM_M2.

Single-cell RNA-sequencing scRNA-seq analysis

The scRNA-seq dataset GSE152048 containing 11 osteosarcoma samples was downloaded from the GEO database. The dataset was processed and analyzed according to the standard procedure of the R package “Seurat” v.4.3.0, and a total of 26,175 genes and 123,322 cells were included in the study. After performing data downscaling and clustering, the clusters were annotated using the previously reported cellular markers. In addition, the expression of ST3

beta-galactoside alpha-2,3-sialyltransferase 4 ST3GAL4 in the scRNA-seq dataset GSE162454 was also analyzed using the Tumor Immune Single-cell Hub TISCH http://tisch.comp-genomics.org/.

Quantitative reverse transcription-PCR RT-qPCR

Adding 1 ml TriPure, chloroform, isopropanol and 75% anhydrous ethanol to the 6-well plate to extract cell RNA. After quantification, RNA reverse transcription and RT-qPCR were performed as described previously. The PCR primers used are listed in Additional file 3: Table S2.

Cell proliferation assay

After cell transfection using the Lipofectamine® 3000 Invitrogen, Carlsbad, CA, USA according to the manufacturer's instructions, the transfected cells were cultured for 24 h and inoculated in 96-well plates with 2000 cells per well. After cell walling, cells were incubated for different time periods 0, 1, 2 and 3 days and 10 μl of CCK-8 reagent was added to each well. After incubation at 37 °C for 3 h, absorbance was measured at 450 nm to determine cell viability.

Cell invasion and migration assays

Cell invasion ability was measured using the Transwell assay and cell migration ability was measured using the scratch assay. The Transwell and scratch assays were carried out as described previously. The scratch assay was incubated for 36 h. For Cell invasion assay, the cells were resuspended in serum-free medium and placed in the upper chamber of the Transwell system. Culture medium with 10% serum was added to the lower chamber and was used as a chemoattractant. After 24 h of incubation, the cells were stained and counted.

Colony formation assay

MG-63 and U2OS cells with knocked down or overexpressed ST3GAL4 were seeded in 6-well plates at a density of 1000 cells per well. After 10 days of cultivation, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. Subsequently, the cells were stained with 0.1% crystal violet, and the number of colonies in each well was counted.

Seahorse assays

10,000 tumor cells were seeded in a Seahorse 96-well assay plate and incubated overnight. The probe plates were pretreated and the calibration solution was prepared following the manufacturer's protocol. Subsequently, the probe plates were placed in a CO2-free incubator overnight. After the overnight incubation, the detection solution was prepared as per the instructions of the Glycolysis Stress Test kit Agilent Technologies, #103020-100 and the reagents were added sequentially. Real-time metabolic changes in cells were detected using the Agilent Seahorse XFe96 Agilent Technologies.

Co-culture experiment and flow cytometry assay

Co-culture was performed using the Boyden chamber, M0 macrophages were seeded at upper chamber and tumor cells were seeded at lower chamber. After 48 h, cells from the upper chamber were collected. For flow cytometry assay, cells were prepared for single cell suspension and were fixed with 2% paraformaldehyde solution in PBS.

Then, cells were fixed and permeabilized with the FIX & PERM Kit MultiSciences Biotech, Hangzhou, China and stained with CD206 321104; Biolegend. A FACS flow cytometer BD FACS LSRFortessa, USA was used for the flow cytometry analysis.

Immunohistochemistry IHC

IHC was carried out as described previously. The rabbit polyclonal antibody to ST3GAL4 was purchased from Invitrogen PA5-62056, 1:200 dilution. Two blinded pathologists scored the intensity and percentage of positive cells for ST3GAL4 staining. The intensity was scored as follows: 0 negative, 1 weakly positive, 2 moderately positive, and 3 strongly positive. The percentage of ST3GAL4-positive cells was scored as follows: 0 0%, 1 1–25%, 2 26–50% and 3 > 50%. The IHC score was defined as the sum of the intensity score and the percentage score of positive cells.

Statistical analysis

Differences between two groups were compared using unpaired Student's t-test or Wilcoxon rank sum test. For comparisons between more than two groups, differences were compared using one-way ANOVA or Kruskal–Wallis test. The correlation between two groups was calculated using Sperman’s correlation analysis. Unless otherwise indicated, statistical significance was set at two-sided $p < 0.05$. All statistical calculations were performed using R software Version 4.2.1.

Results

Identification of key metabolic pathways in osteosarcoma

Metabolic heterogeneity may lead to differences in clinical outcomes, and we are committed to exploring the key metabolic pathways associated with clinical outcomes. After quantifying the activity of the seven metabolic super-pathways, Kaplan–Meier curve analysis identified four key metabolic pathways that were significantly associated with prognosis. Higher levels of carbohydrate $p = 0.038$, energy $p = 0.017$, lipid $p = 0.010$, and vitamin & cofactor metabolism $p = 0.009$ were associated with better OS in osteosarcoma. Among them, vitamin & cofactor metabolism has the highest significance. These four key metabolic pathways were first explored in relation to the overall level of immune infiltration. As shown in Fig. 1A, only vitamin & cofactor metabolism is significantly positively correlated with overall immune and stromal infiltration and negatively correlated with tumor purity. Further analysis of immune cell infiltration revealed a potential positive correlation between vitamin & cofactor metabolism and infiltration of most immune cells, including activated CD8 T cells and activated dendritic cell. Consistently, in the subgroup analysis, only the high vitamin & cofactor metabolism group showed higher levels of immune cell infiltration compared to the low vitamin & cofactor metabolism group. Due to the positive correlation with vitamin & cofactor metabolism, carbohydrate and lipid metabolism were also positively correlated with infiltration of some immune cells. Immune checkpoint analysis only found a potential positive correlation between hepatitis A virus cellular receptor 2 HAVCR2 and metabolic pathways. Moreover, a robust positive correlation prevails among diverse

immune characteristics, encompassing immune checkpoints and the infiltration of immune cells. Core biological pathway analysis revealed that vitamin & cofactor metabolism was potentially associated with immune-related biological pathways, such as CD8 T effector, antigen processing and immune checkpoint. The results of the subgroup analysis further confirmed these findings. Lipid metabolism was associated with some immune pathways, but also with cell cycle and mismatch repair. Overall, vitamin & cofactor metabolism is the important metabolic pathway affecting prognosis and TIME in osteosarcoma.

Identification of hub genes in the vitamin & cofactor metabolic pathway

Given the importance of the vitamin & cofactor metabolic pathway, we constructed a PPI network of pathway genes and found extensive interactions Fig. 2A. In addition, the top 10 hub genes in the vitamin & cofactor metabolic pathway were identified according to the PPI network Fig. 2B. Notably, most of the hub genes belongs to the apolipoprotein APO family, indicating the central role of APO family genes in vitamin & cofactor metabolism. Eight hub genes were subsequently matched in the RNA-Seq data of the TARGET cohort, and correlation analysis showed some positive correlations among the APO family genes in the eight hub genes Fig. 2C. Univariate Cox regression analysis showed that APOB and APOE were significantly associated with OS in patients with osteosarcoma Fig. 2D. Furthermore, two gene modules were also identified from the PPI network, one of which contains many APO family genes Fig. 2E. Furthermore, hub genes were identified in three other prognostic-related metabolic super-pathways Additional file 1: Figure S8-S10. Among them, the top 10 hub genes in the carbohydrate metabolism pathway are not associated with prognosis. The top 10 hub genes in the energy metabolism pathway are mainly composed of the G protein family, and GNG4 and GNG10 have prognostic significance. The top 10 hub genes in the lipid metabolism pathway are mainly composed of the mediator complex family, and only CD36 among the top 10 genes has prognostic significance. Moreover, we have also constructed interaction networks and identified hub genes in three additional non-prognostic metabolic super-pathways Additional file 1: Figure S11-S13.

ST3GAL4 is highly expressed in malignant cells and is closely associated with the TIME of osteosarcoma. In the above results we identified 17 core MRGs to construct a risk model. The advent of scRNA-seq has enabled researchers to investigate the activity of genes across diverse cell types. The activation of genes in malignant cells can significantly impact their biological behavior, consequently influencing tumor progression. To delve deeper into the expression patterns of the 17 core MRGs across distinct cell types, we initially identified 11 major cell types using characteristic gene expression in the scRNA-seq cohort GSE152048. Subsequently, ST3GAL4 was found to not only have a high positive coefficient in the risk model, but also to be predominantly expressed in malignant cells, specifically osteoblastic and chondroblastic osteosarcoma cells, compared to other core MRGs. Notably, ST3GAL4, rather than other MRGs, was specifically highly expressed in proliferating osteoblastic osteosarcoma cells, suggesting the potentially important role of ST3GAL4 in the proliferation of osteosarcoma cells. Importantly, it was also verified in the scRNA-seq cohort GSE162454 that ST3GAL4 was predominantly expressed in malignant cells.

Further, osteosarcoma patients with high ST3GAL4 were found to have significantly worse OS and RFS. C2 samples had significantly higher ST3GAL4 expression compared to C1 samples, and, although not statistically significant, GC2 samples had relatively lower ST3GAL4 expression compared to GC1 and GC3 samples. Immune checkpoint analysis revealed that samples with high ST3GAL4 had significantly lower expression of CD274, CTLA4, HAVCR2, and PDCD1LG2. Samples with high expression of ST3GAL4 also had lower ImmuneScore, StromalScore, and higher tumor purity. Analysis based on the TIDE algorithm revealed that ST3GAL4 expression was positively correlated with MDSC score and TAM-M2 score, but not correlated with CAF score, TIDE score, and dysfunction and exclusion of CTLs. Taken together, samples with high ST3GAL4 may have difficulty responding to ICB treatment. The response prediction based on the TIDE algorithm also demonstrated that patients with high ST3GAL4 had a relatively low ICB response rate. However, there was no significant association between ST3GAL4 and sensitivities to cisplatin, cyclophosphamide, gemcitabine, and sorafenib. ST3GAL4 expression was potentially positively correlated with cell cycle and mismatch repair and potentially negatively correlated with immune checkpoint and Pan-F-TBRS.

The ST3GAL family consists of six members (ST3GAL1-6), and it is necessary to further analyze the other members of this family. ScRNA-seq analysis showed that other ST3GAL members were not specifically highly expressed in proliferating malignant cells. In addition, survival analysis showed that only ST3GAL1 was associated with shorter OS and RFS in osteosarcoma, but its prognostic value was not as significant as ST3GAL4. Immune-related analysis found that only ST3GAL2 was associated with overall immune infiltration in osteosarcoma, but immune checkpoint analysis failed to find a widespread correlation. Furthermore, it was found that ST3GAL5 and ST3GAL6 were negatively correlated with TIDE score, MDSC score, CAF score, exclusion of CTLs, and some core biological pathways including EMT. In summary, only ST3GAL4 in the ST3GAL family is associated with both the prognosis and immune characteristics of osteosarcoma.

ST3GAL4 is a potential prognostic marker and associated with tumor progression, glycolysis, and the M2 polarization of macrophages in osteosarcoma. To verify the clinical application, IHC staining was performed on osteosarcoma and normal tissue samples. The protein expression of ST3GAL4 was found to be significantly higher in tumor tissue than in normal tissue. Survival analysis indicated that patients with high ST3GAL4 protein expression had shorter RFS. We knocked down and overexpressed ST3GAL4 in the osteosarcoma cell lines MG-63 and U2OS to explore its effect on the malignant phenotype of osteosarcoma cells. After the knockdown of ST3GAL4, the proliferation, invasion, migration, and the ability of colony formation of MG-63 and U2OS were all inhibited. After overexpressing ST3GAL4, the malignant phenotypes mentioned above were all enhanced.

Although no correlation was found between ST3GAL4 and the four metabolic super-pathways, based on previous studies, we speculate that ST3GAL4 may have a potential association with glycolysis. To further explore the relationship between ST3GAL4 and glycolysis, seahorse assay was conducted. As expected, the knockdown of ST3GAL4 reduced the basal glycolysis level and maximal glycolysis level in osteosarcoma cells. Furthermore, through the co-culture system, we explored the impact of ST3GAL4 on macrophage polarization. RT-PCR analysis showed that

the knockdown of ST3GAL4 significantly decreased the expression of the M2 macrophage marker CD206. Interestingly, the expression of PD-L1 in macrophages was also reduced in the ST3GAL4 knockdown group. Flow cytometry analysis further confirmed a lower proportion of M2 macrophages in the ST3GAL4 knockdown group compared to the control group, confirming the regulation of ST3GAL4 on macrophage polarization in osteosarcoma.

Metabolic reprogramming is considered to be one of the hallmarks of cancer. The metabolic activity of cancer is extremely complex and needs to be systematically characterized. However, several previous studies have demonstrated considerable heterogeneity in the expression of genes involved in various metabolic pathways, and thus, metabolic gene expression alone cannot accurately reflect the metabolic changes in tumors. Based on studies with parallel metabolomics data as well as transcriptomics data, metabolic pathway-based expression patterns reflect the true metabolic status well. Therefore, the investigation of osteosarcoma metabolism from metabolic pathways is equally promising. Current studies on the metabolic profile of osteosarcoma tend to focus only on a specific metabolic pathway, and there are no known studies that have yet examined the impact of different metabolic pathways on osteosarcoma from a holistic perspective. In addition, metabolic activity greatly influences the formation of TIME, therefore it is necessary to further reveal the relationship between metabolism and TIME in osteosarcoma.

In this study, we first explored the impact of enrichment levels of the seven most prominent metabolic super-pathways on the prognosis of osteosarcoma. Unexpectedly, four of the seven metabolic super-pathways (carbohydrate, lipid, energy, and vitamin & cofactor) were all associated with better OS in osteosarcoma. This appears to be a departure from previous knowledge that cancer cells have an increased need for glucose and energy uptake. However, in agreement with our study, lipid metabolism was associated with a better prognosis for a variety of tumors in a pan-cancer analysis, and energy metabolism showed a heterogeneous prognostic correlation. Notably, carbohydrate and vitamin & cofactor metabolism were associated with worse prognosis in tumors, which is different to our results in osteosarcoma. They are highly heterogeneous tumors containing multiple subtypes including osteoblastic and chondroblastic osteosarcoma. The exact characteristics of osteosarcoma metabolism remain to be elucidated, which may result in a different metabolic pattern from other tumors as well as clinical relevance. It should not be overlooked that a large number of previous studies have focused on carbohydrate metabolism in osteosarcoma. In this study, the most significant difference was found between OS of osteosarcoma with different levels of vitamin & cofactor metabolism, implying that vitamin & cofactor metabolism may largely influence the prognosis of osteosarcoma. Importantly, vitamin & cofactor metabolism was strongly correlated with immune and stromal cell infiltration in the TIME of osteosarcoma, and carbohydrate and vitamin & cofactor metabolism were also correlated with infiltration levels of various antitumor immune cells such as effector memory CD8 T cells. Previous studies have demonstrated that higher immune cell infiltration in osteosarcoma is associated with better prognosis, which may partially explain why osteosarcoma patients with high levels of carbohydrate and vitamin & cofactor metabolism have better clinical outcomes. Given the importance of vitamin & cofactor metabolism in osteosarcoma prognosis and TIME, further in-depth study of its mechanism in

osteosarcoma and development of therapeutic strategies targeting the vitamin & cofactor metabolism may be promising.

Further, we identified the hub genes in the vitamin & cofactor metabolic pathway. Remarkably, most of the hub genes belonged to the APO family. APOs are proteins that bind to lipids in the blood, forming lipoproteins and transporting them through the bloodstream to cells and tissues. Lipoproteins play an important role in vitamin metabolism. For example, APOA is the main component of HDL and is directly correlated with the level of vitamin E in the blood, promoting its absorption in the intestines. In colon cancer cells, APOB also participates in the transport of vitamin E. In addition, APOE largely affects the concentration of fat-soluble vitamins in plasma. Recent studies have found that APOC1 promotes osteosarcoma progression through binding to MTCH2, and preoperative APOB/APOA1 has been identified as an independent prognostic factor for osteosarcoma in children and adolescents. In addition, APOD induced the osteoblastic differentiation of the osteosarcoma cell line Sao-2. This suggests that the APO family may regulate osteosarcoma vitamin & cofactor metabolism and affect the prognosis of osteosarcoma.

Based on the activity levels of the four clinically relevant key metabolic super-pathways, two distinct metabolic pathway-related clusters (C1 and C2) were identified in a cluster analysis. C1 is mainly characterized by energy metabolism, while C2 is characterized by lipid and vitamin metabolism. Although C1 and C2 do have distinct metabolic characteristics, there was no significant difference in survival between them due to the limitation in sample size. It is necessary to explore the prognostic differences between them in larger cohorts in the future. It is noteworthy that C2 patients were more sensitive to cisplatin and gemcitabine. Therefore, this classification may be appropriate to identify osteosarcoma patients with different metabolic profiles and to guide the dosing of cisplatin and gemcitabine.

To further explore the clinical significance of the metabolic profile of osteosarcoma, we identified MRGs based on metabolic pathway-related clusters. MRGs were enriched not only in metabolism-related pathways, such as oxidative phosphorylation, but also in immune-related pathways, including antigen processing and presentation. The results further emphasize the importance of the metabolism in the immune regulation of osteosarcoma. Many metabolic genes are also important immunomodulatory genes. Hexokinase, involved in glycolysis, is an important regulator of innate immunity, demonstrating the overlap between cellular energy metabolism and the immune system. PDCD1 has been identified to interact with arginine biosynthesis or fatty acid degradation and elongation.

According to the identified MRGs, we defined MRG-related gene clusters (GC1-3), which may have more important clinical translational implications than metabolic pathway-related clusters. The significantly different clinical outcomes among GCs suggest that these GCs reflect essential aspects of tumor development and can be used as potential prognostic predictors. Different GCs had significantly different TIME; GC2 and GC3 had higher immune infiltration and were more likely to respond to ICB treatment. This classification approach may facilitate the development of personalized ICB treatment strategies for osteosarcoma. Differences in sensitivity to cisplatin, gemcitabine, and sorafenib among GCs also suggest their potential to

guide clinical dosing. Mechanistically, GC3 with the worst prognosis exhibits higher activity in the MYC and mTOR pathways, both known for promoting tumor progression. Although GC1 also showed high Wnt pathway activity, its better prognosis might be due to higher immune response activity.

We downscaled the MRGs by multiple algorithms and identified 17 core MRGs and constructed a risk model. The model showed good efficacy and was validated in an independent cohort, suggesting potential for clinical application. It reflects different immune status for osteosarcoma; patients with higher risk score had lower immune infiltration. ScRNA-seq-based analysis revealed that ST3GAL4, one of the 17 core MRGs, was highly expressed in proliferating malignant cells. Mechanistically, ST3GAL4 was associated with cell cycle and mismatch repair. The gene encodes an enzyme involved in protein glycosylation and sialyl Lewis x synthesis, known to influence tumor behaviors. Knockdown of ST3GAL4 in MG-63 and U2OS cells suppressed malignant behaviors. Clinically, high ST3GAL4 protein expression was correlated with poor prognosis. While no association was found with four metabolic super-pathways, ST3GAL4 was confirmed to promote glycolysis. Lactate produced by glycolysis can induce M2 macrophage polarization, linking ST3GAL4 with immunosuppression. ST3GAL4 also promotes the synthesis of ligands for Siglec-7 and Siglec-9, which further enhances macrophage M2 polarization.

Although patients with high ST3GAL4 expression showed lower ICB response, this did not fully align with risk group or C1/C2 subtypes due to population heterogeneity. Overall, ST3GAL4 is a promising prognostic marker and therapeutic target.

However, further investigation is needed due to limitations like small sample size and lack of parallel metabolomics-transcriptomics datasets. Further studies on vitamin & cofactor metabolism and ST3GAL4 in osteosarcoma are warranted.

Vitamin & cofactor metabolism plays an important role in the prognosis and TIME of osteosarcoma. MRG-related gene clusters can reflect the immune status of osteosarcoma and facilitate the development of personalized immunotherapy and chemotherapy strategies. The metabolism-related risk model may serve as a useful prognostic predictor. ST3GAL4 plays a critical role in the progression, glycolysis, and TIME of osteosarcoma and affects the prognosis.

ST3GAL4 as a Prognostic Marker in Osteosarcoma

The prognostic significance of ST3GAL4 has been explored in multiple studies, which have established that higher expression levels correlate with worse overall survival (OS) and relapse-free survival (RFS) in osteosarcoma patients. This is consistent with the established role of ST3GAL4 in tumor cell proliferation and immune evasion. Specifically, osteosarcoma patients with high ST3GAL4 expression exhibited significantly reduced survival outcomes, emphasizing its potential as a novel biomarker for risk stratification.

Mechanisms of ST3GAL4-Mediated Immune Modulation

Sialylation, a biochemical modification catalyzed by sialyltransferases like ST3GAL4, plays a critical role in modulating immune responses within the TIME. High expression of ST3GAL4 results in the overproduction of sialylated glycans on tumor cell surfaces, which significantly affects the immune system's ability to mount an effective attack on malignant cells.

Key mechanisms include:

- Inhibition of T cell activation: Sialic acid residues on tumor cell surfaces may engage inhibitory receptors like Siglec-7 and Siglec-9 on immune cells, resulting in T cell exhaustion and impaired cytotoxicity.

- Macrophage polarization: As discussed earlier, ST3GAL4 promotes M2 macrophage polarization, which facilitates tumor immunosuppression, angiogenesis, and metastasis. The presence of M2 macrophages contributes to immune tolerance and enhances the tumor's resistance to immune checkpoint blockade (ICB) therapies.

- Impairment of antigen presentation: Sialylation may suppress the expression of major histocompatibility complex (MHC) molecules, thereby hindering the efficient presentation of tumor antigens to T cells, further inhibiting immune-mediated tumor clearance.

The immune landscape of ST3GAL4-high tumors is marked by reduced immune cell infiltration, including a lower proportion of cytotoxic CD8+ T cells, and an overall immune-suppressive environment.

ST3GAL4 and Glycolysis in Osteosarcoma

In addition to its immune-modulatory role, ST3GAL4 has been implicated in the metabolic reprogramming of osteosarcoma cells, particularly in relation to glycolysis. Cancer metabolism, often referred to as the Warburg effect, is characterized by enhanced glycolytic activity even in the presence of oxygen. This metabolic shift supports rapid cell proliferation and contributes to tumor progression.

In osteosarcoma, ST3GAL4 overexpression enhances glycolytic activity by promoting the extracellular acidification rate (ECAR). This is crucial for providing the energy required to sustain the rapid growth and migration of osteosarcoma cells. Conversely, ST3GAL4 knockdown reduces glycolysis, inhibiting tumor cell proliferation, migration, and invasion.

Furthermore, the glycolytic intermediates produced in osteosarcoma cells can polarize macrophages to the M2 phenotype, which in turn suppresses anti-tumor immunity. This connection between metabolism and immune modulation underscores the importance of targeting metabolic pathways like glycolysis for therapeutic intervention in osteosarcoma.

Targeting ST3GAL4 in Osteosarcoma Therapy

Given its dual role in immune evasion and metabolic reprogramming, ST3GAL4 presents a promising target for therapeutic strategies in osteosarcoma. Potential approaches to target ST3GAL4 include:

1. Inhibition of ST3GAL4 Activity

- Enzyme inhibitors targeting ST3GAL4 could block its sialyltransferase activity, preventing the synthesis of sialylated glycan structures on tumor cells. This would restore immune recognition and enhance the efficacy of immune checkpoint inhibitors (ICIs) such as anti-PD-1/PD-L1 or anti-CTLA4 therapies.

- Small molecule inhibitors designed to block the glycosylation process could potentially reverse the immunosuppressive tumor microenvironment, making it more responsive to existing therapies.

2. Immune-Based Therapies

- Given the strong correlation between ST3GAL4 expression and M2 macrophage polarization, macrophage-targeted therapies could potentially reprogram M2 macrophages into M1 macrophages, enhancing the immune response against osteosarcoma.

- Combination therapies involving ST3GAL4 inhibition alongside ICIs could significantly improve patient outcomes by overcoming the immune evasion mechanisms associated with high ST3GAL4 expression.

3. Metabolic Inhibition

- Targeting the metabolic pathways influenced by ST3GAL4, such as glycolysis, could be another therapeutic strategy. Glycolytic inhibitors like 2-deoxyglucose (2-DG), metformin, or newer drugs targeting glycolytic enzymes could reduce the tumor's metabolic flexibility and impair its ability to thrive in a hypoxic environment.

- Combining metabolic inhibitors with ST3GAL4-targeted therapies might disrupt both the tumor’s metabolic reprogramming and its immune evasion, creating a multi-pronged attack on osteosarcoma.

Clinical Implications and Future Directions

The discovery of ST3GAL4 as a prognostic biomarker has profound implications for personalized treatment strategies in osteosarcoma. By integrating ST3GAL4 expression with

other clinical parameters, a more refined and individualized approach to therapy can be developed.

- Risk stratification models incorporating ST3GAL4 expression could help identify high-risk patients who are less likely to respond to traditional therapies, guiding the choice of more aggressive or targeted treatments.

- Early-stage clinical trials investigating ST3GAL4 inhibitors or metabolic reprogramming therapies are needed to validate its therapeutic potential in osteosarcoma.

Further studies on the molecular mechanisms of ST3GAL4 in osteosarcoma, including its role in epigenetic regulation and its interactions with other metabolic pathways, will provide deeper insights into how this enzyme influences tumor progression and treatment response. Moreover, the development of ST3GAL4-targeted drugs could expand therapeutic options for patients with advanced osteosarcoma, a malignancy with poor prognosis and limited treatment options.