

Genes Such as IRS2 Differ from Ewing Sarcoma and Normal Tissue-specific Expression for Osteosarcoma

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Abstract: *Objective:* To screen and validate the expression of other key genes specific to osteosarcoma that distinguish normal tissue from Ewing sarcoma using a bioinformatics approach. *Methods:* GSE73166 for osteosarcoma associated with Ewing sarcoma and GSE42352 for osteosarcoma associated with normal tissue were obtained from the GEO database, and these two datasets were screened for differential genes (DEGs) and subjected to corresponding GO enrichment and KEGG enrichment with GSEA validation, and then correlation analysis, PCA and immuno-infiltration analysis were performed on the two datasets, and these two datasets were intersected to derive 28 differential genes, and the corresponding heat map analysis and protein interaction network analysis were performed for these 28 genes, and then the expression statistics of these 28 genes in GSE21257 for different graded genes and survival analysis in R2 for osteosarcoma in TARGET. *Results:* TNFRSF11B, COL12A1, PLOD2, PTGES, MEF2C, IRS2 and MMP13 were found to have an effect on the staging of osteosarcoma, while CCND1 (P=0.021), IRS2 (P=0.015), OLFML2B (P=0.035), CPE (P=0.037), MAMDC2 (P=0.044) and MMP13 (P=0.013) had an impact on the survival prognosis of osteosarcoma. *Conclusions:* IRS2 and 11 other genes differ from Ewing sarcoma and normal tissues, producing a specific expression on the staging or survival prognosis of osteosarcoma.

Keywords: Osteosarcoma; Ewing Sarcoma; GEO Database; Enrichment; Tumor Staging; Survival Analysis.

1. Introduction

Osteosarcoma is the most common primary bone malignancy in adolescents, and the main reason for treatment failure and poor prognosis is mostly the high propensity of osteosarcoma to metastasize [1], with 15% to 20% of patients with osteosarcoma having pulmonary metastases at the early stage of diagnosis [2]. Osteosarcoma is usually thought to originate from primitive mesenchymal-derived bone-forming cells and usually occurs in rapidly growing bones [3], and is currently treated with a preoperative-surgical- postoperative treatment approach [4], and although the advent and application of neoadjuvant chemotherapy has led to further developments in the treatment of osteosarcoma, there has been no significant change in overall patient survival [5]; Therefore, we need to look for some genes related to osteosarcoma for targeted therapy. The publicly funded TCGA project and the GEO database include various functional genomics datasets for different tumors [6], from which we can perform genetic difference studies, although in previous studies, people often simply compared normal populations with bone tumor populations to derive difference genes, which are usually more numerous and have many similar cancer properties [7], and Certain genes play a similar role in most other cancers [8]. In this case, we screened out cancer genes related to sarcoma by adding a differential gene expression for osteosarcoma and Ewing sarcoma, and took out genes that only work for osteosarcoma but not for all cancers by taking intersections. We analyzed these genes for their protein interaction network interactions and validated these genes in a series of staging and survival analyses in a new dataset that may provide new insights into the pathogenesis and treatment strategies of osteosarcoma.

2. Methods

2.1. Processing of Data for Screening

We retrieved and obtained the gene expression data of GSE42352 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42352>) and GSE73166 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73166>) from the GEO database [9], in which GSE42352 contained gene expression information for 103 osteosarcoma samples and 15 normal group samples, and GSE73166 contained gene expression information for 10 osteosarcoma samples and 8 Ewing sarcoma cases.

2.2. Differential Analysis of Expression

We used the limma package in R software [10] to homogenize the data of GSE42352 and GSE73166, respectively, transform the gene probe IDs and perform differential analysis to derive DEGs and plot volcanoes, and screen DEGs by using $|\log_2FC| > 1.5$ and P-value < 0.01 as thresholds, and obtain by Venn diagram [11] intersection of GSE42352 and GSE73166 and heat map these intersecting genes by ggplot [12].

2.3. Correlation and PCA Analysis

Correlation analysis was performed by the pearson method in R and mapped by heatmap, followed by PCA analysis using R.

2.4. Immunological Infiltration Analysis

R software GSVA, GSEABase and reshape2 packages were used for immune correlation functional analysis and pheatmap package was used for visualization.

2.5. Enrichment Analysis

Enrichment analysis was performed on GSE42352 and GSE73166 by DAVID (<https://david.ncifcrf.gov/>) database[13] and KOBAS2 (<http://kobas.cbi.pku.edu.cn/>) database[14], respectively, to determine the enrichment of the two sets of data, and then enrichment analysis was performed on the genes that were taken from the intersection. We performed enrichment analysis of Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) by GPlot[15]

where GO functional annotation includes biological processes involved (BP), cellular components (CC) and molecular functions of genes (MF)[16], while KEGG enriches its pathways and constructs intermolecular interrelationships and reciprocal networks [17], GSEA enrichment was then performed for validation.

2.6. Construction of Protein-Protein Interaction Networks

In order to obtain the interaction relationships between genes, we performed the construction of protein-protein interaction networks by STRING database (<https://cn.string-db.org/>) for 28 genes that have taken intersections [18, 19] and derived the corresponding interaction network maps by Cytoscape software[20]. The derived genes were then validated by other datasets.

2.7. Analysis of Genetic Differences and Survival in Osteosarcomas of Different Stages

We obtained a total of 53 groups of osteosarcoma case data from GSE21257 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21257>) for validation, and we organized the patient data to remove six groups of osteosarcoma patients with unknown grouping information based on the four stages of tumor expression, and the remaining 47 cases were analyzed for gene content, and we performed another survival analysis of the 26 genes we obtained using the microarray dataset for osteosarcoma from the R2 database (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) [21].

3. Results

3.1. Screening and Enrichment Analysis of Differential Genes between Osteosarcoma and Normal Tissue and Differential Genes between Osteosarcoma and Ewing Sarcoma

We obtained 370 differential genes from GSE42352, including 183 up-regulated genes and 167 down-regulated genes, by differential expression analysis, and we obtained 353 differential genes from GSE73166, including 168 up-regulated genes and 185 down-regulated genes, and mapped the volcanoes using the ggplot package in R (Figure 1 a,b).

We then performed enrichment analysis of these two sets of differential genes by DAVID database and KOBAS database, respectively. In GSE42352, we performed enrichment analysis of differential genes in tumor and normal tissues with Count>10 for GO-enriched string plot annotation, and found that these genes mostly function in extracellular region, cell membrane, cell adhesion, regulation of cell morphology, heparin binding, signaling and acute phase response (Figure 1c); in GSE73166, because the enrichment of functional annotations and the number of genes were too many for string plots, so we made bubble plots showing $p<0.001$ enriched IDs (Figure 1d), we took $p<0.001$ and Count top three in BP, CC and MF respectively. For these significant enriched functional annotations (Table 1), in terms of biological processes involved, they were mainly focused on cell adhesion, positive regulation of cell proliferation and differentiation of osteoblasts, and cellular components in the plasma membrane, extracellular regions and extracellular space, the molecular functions of genes mainly include calcium binding, homogenization activity of proteins and sequence-specific double-stranded DNA binding. We then enriched all genes in both datasets by GSEA and found that in GSE42352 mostly in antigen processing and expression of exogenous peptide antigens and MHC protein complex binding (Figure 1e), and in GSE73166 most genes were enriched in collagen fibril tissue, external encapsulated structural tissue, extracellular structural tissue and extracellular matrix tissue (Figure 1f).

Table 1. Annotation of significant enrichment function for GSE73166GO enrichment.

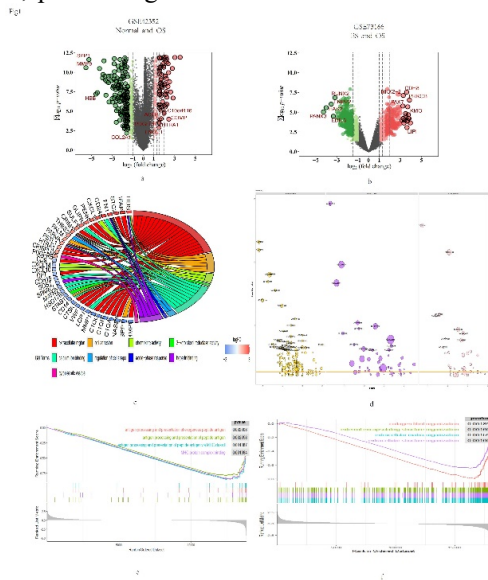
ID	term	Category	PValue	Count
GO:0007155	cell adhesion	GOTERM_BP_DIRECT	9.80E-10	33
GO:0008284	positive regulation of cell proliferation	GOTERM_BP_DIRECT	8.52E-04	21
GO:0001649	osteoblast differentiation	GOTERM_BP_DIRECT	2.51E-12	19
GO:0005886	plasma membrane	GOTERM_CC_DIRECT	1.32E-04	113
GO:0005576	extracellular region	GOTERM_CC_DIRECT	1.56E-10	75
GO:0005615	extracellular space	GOTERM_CC_DIRECT	4.46E-09	67
GO:0005509	calcium ion binding	GOTERM_MF_DIRECT	1.63E-11	42
GO:0042803	protein homodimerization activity	GOTERM_MF_DIRECT	2.08E-05	29
GO:1990837	sequence-specific double-stranded DNA binding	GOTERM_MF_DIRECT	1.47E-04	23

We next performed KEGG pathway enrichment on 370 genes in GSE42352 and identified the top three enriched pathways as metabolic pathway, pathway in cancer and human papillomavirus infection in order of Count (Figure 2a), and then KEGG enrichment by GSEA yielded antigen processing and presentation, asthma, autoimmune thyroid disease and type I diabetes (Figure 2c). We then placed the

obtained KEGG information into Cytoscape to derive a reticulated interplay network map of genes and their enriched KEGG pathways, where blue represents the ID of the enriched pathway, while red represents its upregulated genes and green its downregulated genes (Figure 2e). In GSE73166, we found the role of these genes in KEGG enrichment through DAVID (Figure 2b), making the same to obtain the

first three pathways (Table 2), MAPK signaling pathway, calcium ion conduction pathway and protein digestion and uptake, respectively, and enrichment through GSEA to obtain ECM-receptor interaction, local adhesion, protein digestion

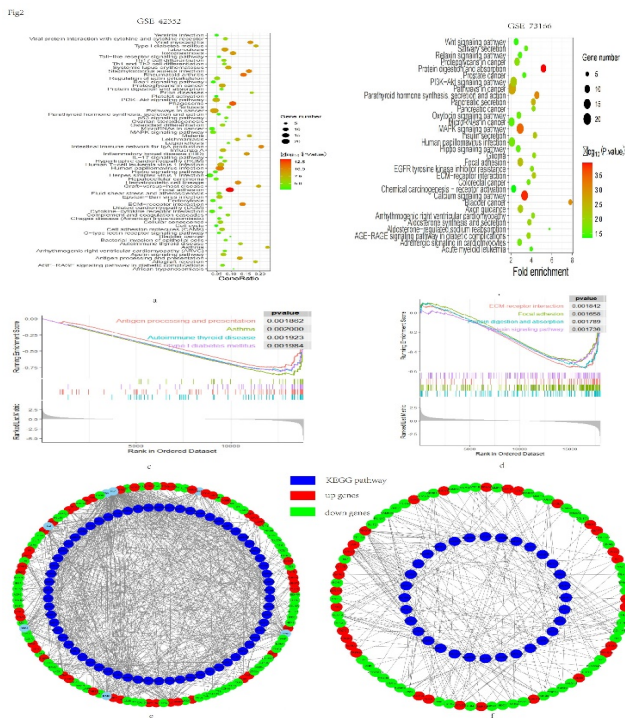
and uptake and relaxin signaling pathway (Figure 2d). The obtained KEGG pathway was placed in Cytoscape to yield Figure 2f.



GO: Gene Ontology Functional Annotation; BP: Biological Processes; CC: Cellular Component; MF: Molecular Function of Genes
Fig 1. Volcano map and GO enrichment of differential genes in osteosarcoma and normal, Ewing sarcoma. (a)GSE42532 differential genes;(b) GSE73166 differential genes;(c) GO-enriched string map of GSE42352 differential gene;(d) GO-enriched bubble map of GSE73166 differential gene;(e) GSEA enriched by GSE42352GO; (f) GSEA enriched by GSE73166GO.

Table 2. The first three pathways enriched by GSE73166KEGG

ID	Term	Category	Count	PValue
hsa04010	MAPK signaling pathway	KEGG_PATHWAY	16	3.67E-04
hsa04020	Calcium signaling pathway	KEGG_PATHWAY	15	1.47E-04
hsa04974	Protein digestion and absorption	KEGG_PATHWAY	10	1.21E-04



KEGG: Kyoto Encyclopedia of Genes and Genomes;
Fig 2. KEGG enrichment of GSE42352 and GSE73166 and GSEA and KEGG pathway gene-related reticulation. (a) KEGG pathway bubble map of GSE42352 differential gene; (b) KEGG pathway bubble map of GSE73166 differential gene; (c)GSEA enriched by GSE42352KEGG;(d) GSEA enriched by GSE73166KEGG;(e) Gene mesh map of GSE42352 pathway; f: Gene mesh map of GSE73166 pathway reticulation map

3.2. Correlation and PCA Analysis

To further verify the reproducible rows of data within groups, we used Pearson correlation test and principal component analysis (PCA). Based on the Pearson correlation test, we found that the correlation between samples in the control group was strong in GSE42352, and the correlation between samples in the osteosarcoma group was also strong (Figure 3a), and the within-group data reproducibility based on PCA was acceptable in GSE42352. The distance between samples in the control group was close, and the distance between samples in the osteosarcoma group was also close (Figure 3c). Based on Pearson correlation test, we found that GSE73166 was more correlated for Ewing sarcoma samples and more correlated for osteosarcoma group samples (Figure 3b). PCA showed that the intra-group data reproducibility at

GSE73166 was acceptable. The distance between samples in the Ewing sarcoma group was far from PC1, and the distance between samples in the osteosarcoma group was close to PC1 (Figure 3d).

3.3. Immuno-infiltration Analysis

Immune infiltration analysis of both datasets revealed that the proportion of normal cell T-cell regulatory and T-cell follicular helpers was significantly greater in GSE42352 than in osteosarcoma cells, while the proportion of CD4 T cells was greater in osteosarcoma cells than in normal cells (Figure 3e), and the proportion of activated NK cells was significantly greater in Ewing sarcoma cells than in osteosarcoma cells in GSE73166, while the CD4 T cells accounted for significantly more than Ewing sarcoma cells (Figure 3f).

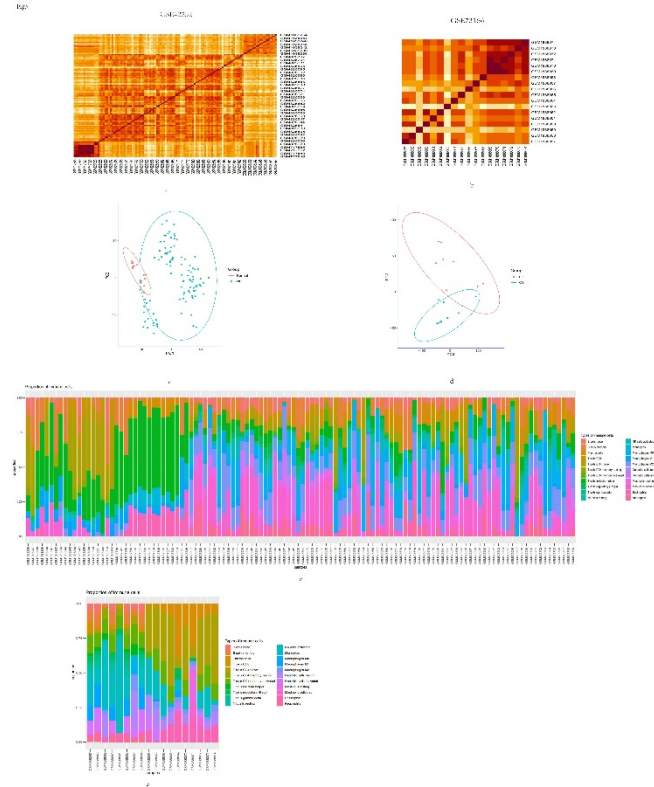


Fig 3. Correlation PCA analysis and immuno-infiltration analysis. (a) GSE42352 Correlation Heat Map; (b) GSE73166 Correlation Heat Map; (c) GSE42352 PCA diagram; (d) GSE42352 PCA diagram; (e) GSE42352 immuno-infiltration map; (f) GSE73166 immuno-infiltration map

3.4. Identification of DEGs

We took the intersection of differential genes between osteosarcoma and normal tissue and differential genes between osteosarcoma and Ewing sarcoma to derive 28 differential genes and made a Wayne diagram (Figure 4a). These genes are both specifically expressed between osteosarcoma and normal tissues and differ within the larger group of sarcomas. We next performed differential analysis using making heat maps in 28 genes again GSE42352 and GSE73166, respectively (Figure 4 b, c).

3.5. Functional Enrichment Analysis of DEGs

We performed enrichment analysis of these 28 genes and in GO enrichment we found that these genes were more tolerated by normal cells in GSE42352 than osteosarcoma cells in GO: 0043627 (response to estrogen), GO: 0009410

(response to foreign body stimulation) and GO: 0042493 (response to drugs), while in GO: 0030282 (bone mineralization), GO: 0001958 (endochondralization) and GO: 0001649 (osteoblast differentiation) were significantly lower than osteosarcoma cells (Figure 4d). Similarly, in GSE73166, the Ewing sarcoma population was more tolerant than osteosarcoma only in GO: 0043627 (response to estrogen), while in GO: 0030282 (bone mineralization), GO: 0030198 (extracellular matrix), GO: 0001649 (osteoblast differentiation), GO: 0007155 (cell adhesion), GO: 0031012 (extracellular matrix), GO: 0005615 (extracellular space) and GO: 0005576 (extracellular compartment) were all significantly lower than in osteosarcoma (Figure 4e). While in KEGG enrichment there were mainly local adhesions, human papillomavirus infection and PI3K-Akt signaling pathways (Table 3).

Table 3. Top three pathways of 28 genes in KEGG enrichment

Term	Database	ID	Input number	Background number	P-Value
Focal adhesion	KEGG PATHWAY	hsa04510	3	199	0.000399465
Human papillomavirus infection	KEGG PATHWAY	hsa05165	3	330	0.001691328
PI3K-Akt signaling pathway	KEGG PATHWAY	hsa04151	3	354	0.002061614

3.6. Construction of PPI Interaction Network

We imported the data of 28 genes selected in STRING through Cytoscape, and we ranked them according to the size of the mediator centrality (BC value) and filtered out the top seven genes among them as LOX, TNFRSF11B, COL12A1, IBSP, MMP13, CCND1, and SULF1 (Figure 4f).

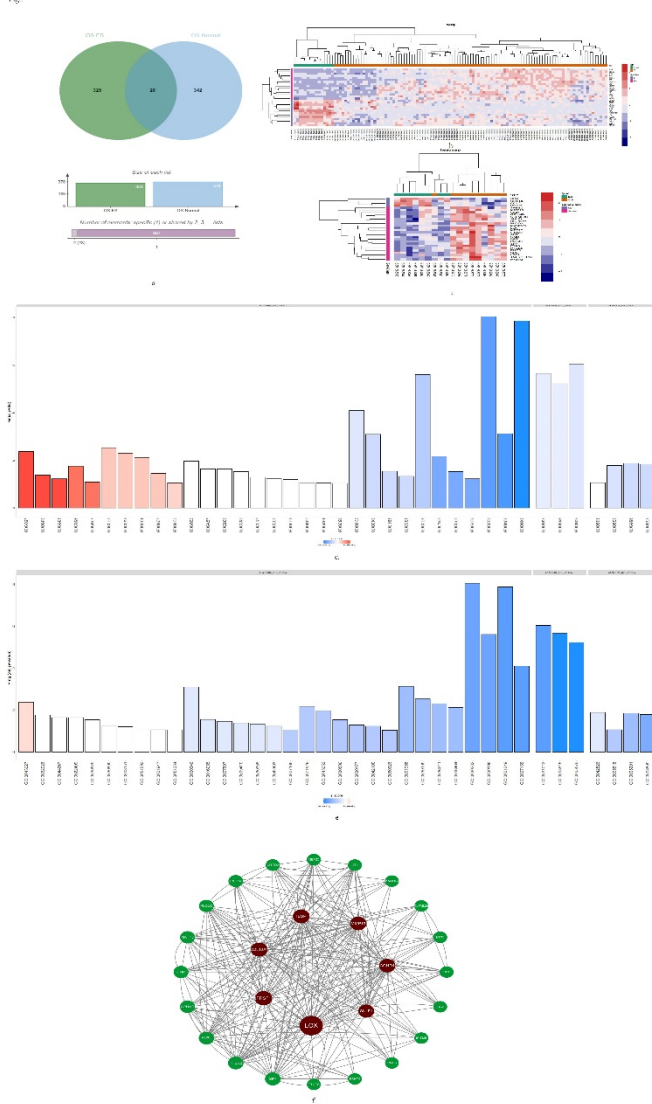


Fig 4. Screening of differential genes and heat map analysis of related genes, Enrichment analysis and protein interaction network map of 28 genes in two data sets. (a) Venn diagram of the intersection of GSE42352 and GSE73166 differential genes; (b) Heat map difference analysis of 28 genes in GSE42352; (c) Heat map difference analysis of 28 genes in GSE73166; (d) Bar chart of 28 genes enriched in GSE42352GO; (e) Bar chart of 28 genes enriched in GSE73166GO; (f) Protein-related interactions of 28 genes.

3.7. Gene Expression in Different Grades of Osteosarcoma and Associated Survival Analysis

We took the expression of 28 genes from 47 patients with

known grouping information in GSE21257 for statistical analysis, and two-by-two comparisons were made during I, II, III and IV. We found that TNFRSF11B stage III expression was more than stage IV ($P=0.026$), COL12A1 stage III expression was significantly more than stage I ($P=0.032$) stage IV ($P=0.037$) all increased, PLOD2 phase III expression was higher than phase II ($P=0.026$), PTGES phase IV expression was higher than phase I ($P=0.041$), MEF2C phase III expression was higher than phase I ($P=0.024$) phase IV ($P=0.028$), IRS2 phase IV expression was significantly lower than phase II ($P=0.022$) phase III ($P=0.046$), MMP13 phase III expression was higher than phase II ($P=0.019$) (Figure 5a). We further performed survival analysis by R2 of a dataset of 88 osteosarcoma samples and found that patients with high expression of CCND1 ($P=0.021$), IRS2 ($P=0.015$) and OLFML2B ($P=0.035$) had a better prognosis, while for CPE ($P=0.037$), MAMDC2 ($P=0.044$) and MMP13 ($P=0.013$) patients with low expression of these genes tended to have a better prognosis (Figure 5b).

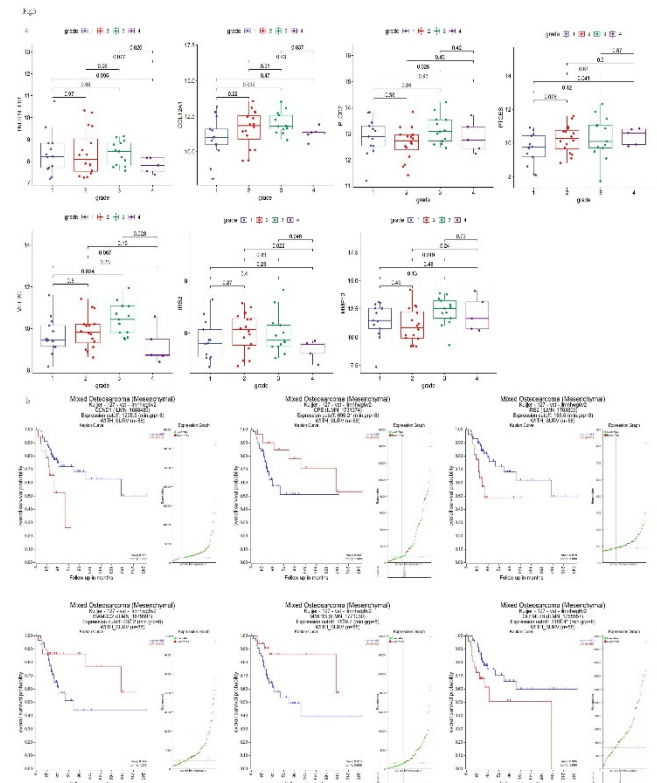


Fig 5. Staged expression and survival analysis of the screened differential genes. (a) Expression of 28 genes in GSE21257 at different stages of tumor; (b) Survival prognosis map of 88 patients with different genes in R2.

4. Discussion

Osteosarcoma, the most common primary bone tumor, mainly involves children, adolescents and young adults, with the second highest incidence in the elderly [22], and is characterized by a high risk of metastatic progression and

recurrence after treatment [23]. The overall survival of osteosarcoma has stabilized over the past decades, and it is now imperative to establish new targets and develop relatively effective treatment strategies [24]. In previous studies, differential genes were often obtained by taking two different datasets of tumor tissue and normal tissue[25]; however, it is not yet known whether these genes are common to osteosarcoma rather than to all sarcomas. It is well known that sarcomas include a heterogeneous group of mesenchymal tumors divided into soft tissue sarcomas and primary osteosarcomas [26], while osteosarcoma and Ewing sarcoma in primary osteosarcoma are often compared[27]without a corresponding comparison with normal cells. Therefore, we selected 28 differential genes that are relatively unique to osteosarcoma by using the intersection of differential analysis of GSE42352 normal and osteosarcoma cells and differential gene analysis of GSE73166 osteosarcoma population and Ewing sarcoma population.

We started by first enriching the differential genes in both datasets separately, and when enriching the differential genes in normal and osteosarcoma cells, we came to the same conclusion as our predecessors that in GO enrichment for extracellular regions, cell membranes, cell adhesion, regulation of cell morphology, heparin binding, signaling and acute phase response, while in KEGG metabolic pathways, pathways in cancer and human papillomavirus infection play a role[28]. We enriched differential genes in osteosarcoma and Ewing sarcoma and found that in BP the main focus is on cell adhesion, positive regulation of cell proliferation and osteoblast differentiation, while CC is in the plasma membrane, extracellular regions and extracellular space, MF mainly includes calcium binding, protein homogenization activity and sequence-specific double-stranded DNA binding, while pathways are mainly for MAPK signaling pathway, calcium ion conduction pathways and protein digestion and uptake, the differential genes of the two sarcomas apparently did not exhibit differences in cancer-related pathways. We again validated this by GSEA enrichment analysis and we found that in GSE42352 the cells were mostly genes for antigen processing and expression of exogenous peptide antigens and MHC protein complex binding, in GSE73166 the cells were more inclined towards collagen fibril organization, external encapsulated structural organization, extracellular structural organization and extracellular matrix organization; while in KEGG genes in GSE42352 were more inclined to antigen processing and presentation, asthma, autoimmune thyroid disease and type I diabetes, and in GSE73166 to ECM-receptor interactions, local adhesions, protein digestion and uptake and relaxin signaling pathways. We also performed correlative PCA validation of these two datasets and found grouping specificity, with significantly more normal cell T-cell regulatory and T-cell follicular helpers than osteosarcoma cells in immunity, and more CD4 T cells than normal cells in osteosarcoma cells, and a significantly greater proportion of activated NK cells in Ewing sarcoma cells than in osteosarcoma cells, while CD4 T cells in osteosarcoma cells accounted for significantly more than Ewing sarcoma cells.

We next took the 28 differential genes from the intersection of these two data sets, and the heat map showed that these genes were indeed differentially expressed between the samples, and we only enriched these 28 genes and found that unlike the enrichment of differential genes in the above two data sets, these genes mainly showed more specific

differences, for example, in response to estrogen, foreign body stimulation and drugs than the osteosarcoma cells. tolerance than osteosarcoma cells, while being inferior to osteosarcoma cells in bone mineralization, endochondralization and osteoblast differentiation. Similarly, the Ewing sarcoma population was stronger than the osteosarcoma population in terms of response to estrogen, while significantly lower than osteosarcoma in terms of bone mineralization, extracellular matrix, and osteoblast differentiation. In contrast, local adhesions, human papillomavirus infection, and PI3K-Akt signaling pathways play a major role in KEGG enrichment.

In the protein interaction network construction of 28 genes, we screened 7 genes, namely LOX, TNFRSF11B, COL12A1, IBSP, MMP13, CCND1, SULF1. We then performed gene expression statistics for different tumor stages in GSE21257 and found that TNFRSF11B, COL12A1 PLOD2, PTGES, MEF2C, IRS2, and MMP13 expressions were significantly different in osteosarcoma stages, especially stage III. We further performed survival analysis of these genes in 88 osteosarcoma patients selected inside by survival analysis in R2 and found that patients with high expression of CCND1 ($P=0.021$), IRS2 ($P=0.015$) and OLFML2B ($P=0.035$) had a better prognosis, while for CPE ($P=0.037$), MAMDC2 ($P=0.044$) and MMP13 ($P=0.013$) patients with low expression of these genes tended to have a better prognosis. In contrast, IRS2 and MMP13 were correlated with both the staging and prognosis of osteosarcoma.

In summary, we first performed GO enrichment and KEGG enrichment for the differential genes in the two datasets, respectively, and found that the results were not satisfactory, for example, the metabolic pathways and pathways in cancer were too broad. We further enriched the 28 genes after taking the intersection for analysis and found a series of more specific enrichment contents such as bone mineralization and endochondralization, and we further plotted the genes in different stages of osteosarcoma expression as well as survival analysis, we found the role of TNFRSF11B, COL12A1, PLOD2, PTGES, MEF2C, CCND1, OLFML2, CPE, MAMDC2, IRS2 and MMP13 genes in osteosarcoma, which provide new ideas for our future targeted therapy.

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Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

Data openly available in a public repository.

Author Contributions

Conceptualization, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Formal analysis, Xiang-

Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Investigation, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Methodology, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Project administration, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Software, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Validation, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan; Writing – original draft, Xiang-Long ZHAO, Mingxuan Li, Zhe Yang, Wu Chen and Changgong Lan.

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