Original Research

12 Survival-related differentially expressed genes based on the TARGET-osteosarcoma database

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Impact statement

Osteosarcoma (OS) is the most common primary malignant bone tumor and the third most common cancer among children and young adults. It frequently spreads to the lung, and has a five-year survival rate of 70%. A better understanding of the molecular mechanisms, genetic regulation, and prognostic indicators of OS will support the development of more effective and less toxic therapies.

Abstract

The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) project aims to determine molecular changes that drive childhood cancers, including osteosarcoma. The main purpose of the program is to use the open-source database to develop novel, effective, and less toxic therapies. We downloaded TARGET-OS RNA-Sequencing data through R studio and merged the mRNA expression of genes with clinical information (vital status, survival time and gender). Further, we analyzed differential gene expressions between dead and alive patients based on TARGET-OS project. By this study, we found 5758 differentially expressed genes between deceased and alive patients with a false discovery rate below 0.05; 4469 genes were upregulated in deceased patients compared to alive, whereas 1289 genes were downregulated. The survival-related genes were obtained using Kaplan–Meier survival analysis and Cox univariate regression (KM < 0.05 and Cox P-value < 0.05). Out of 5758 differentially expressed genes, only 217 have been associated with overall survival. Eight survival-related downregulated genes (*ERCC4*, *CLUAP1*, *CTNNBIP1*, *GCA*, *RAB40C*, *SIRPA*, *USP11*, and *TCN2*) and four survival-related upregulated genes (*MUC1*, *COL13A1*, *JAG2* and *KAZALD1*) were selected for further analysis as potential independent prognostic candidate genes. This study may help to discover novel prognostic markers and potential therapeutic targets for osteosarcoma.

Keywords: Osteosarcoma, sarcoma, TARGET, RNA sequencing, survival analysis, differential gene expression

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Introduction

Osteosarcoma (OS) is a primary malignant bone tumor with a high incidence rate in children and adolescents.[1] An initial peak has been observed between the ages of 10 and 19 years during the pubertal growth spurt and a secondary peaks after the age of 65 years associated with Paget's disease.[2] There is a predilection for the metaphyseal area of long bones with approximately 75% of all cases occurring in distal femur (41.6%), proximal tibia (16.9%), proximal humerus (9.2%), and proximal femur (7.7%).[3]

Although osteosarcoma has been associated with p53 and retinoblastoma protein dysfunction respectively, our understanding of the prognostic indicators and the genetic mechanisms of disease progression are incomplete.[4] OS is an aggressive, invasive sarcoma that frequently metastasizes, most commonly to the lung, with a five-year survival rate of 70%.[5] Multi-agent chemotherapy was introduced in the 1970s and improved the prognosis from a dismal 15–20% survival to a 60–70% five-year survival rate, but outcomes have not significantly improved since then. Currently, neo-adjuvant chemotherapy using doxorubicin, methotrexate, cisplatin, or ifosfamide to reduce the size of a tumor and eradicate micro-metastases followed by surgery and further chemotherapy is the standard of care for OS.[6] We appreciate that tumor size, stage at presentation and response to chemotherapy are significant prognostic

indicators, but our understanding of the molecular mechanisms of disease progression is poor.[7,8]

Genome-wide RNA sequencing (RNA-Seq) provides a quantitative resolution of the transcriptome, can discover novel transcripts, identify alternative splicing, and detect gene expression changes between different samples.[9,10] This functional analysis of the genome improves our understanding of the underlying mechanisms of complex diseases, especially in cancer.[11,12] We have used RNA-Seq data to identify potential novel prognostic markers for OS using survival prediction methods based on differential gene expression profiles.

The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) is an open database provided by the National Cancer Institute (NIH, https://ocg.cancer.gov/programs/target). We have analyzed the molecular changes in OS with respect to clinical outcome to better understand the gene regulatory networks, mechanisms of disease progression, and key survivorship determinants.

Materials and methods

Data information

The results presented here are based upon data generated by the TARGET-OS and the data used for the analysis are available at https://portal.gdc.cancer.gov/projects.

TARGET-OS RNA-Seq data were downloaded by R studio (https://www.r-project.org/) through Bioconductor packages of BiocManager, TCGAbiolinks, TCGAWorkflowData, DT, genomic data commons (GDC), and summarized experiment (Bioconductor version of 3.12, https://www.bioconductor.org/). The data category, data type, and workflow type were determined as transcriptome profiling, gene expression quantification, and HTSeq-counts respectively through the TCGAbiolinks package. The RNA-Seq data were merged with clinical information of the patients' such as vital status, survival time, and gender. Samples without vital status, survival time, and gender were excluded from the data.

Differential gene expression analysis

The genes with missing expression values were removed from the data. Statistical data and differential expression of digital gene expression analyses were performed through edgeR (Empirical Analysis of Digital Gene Expression Data in R) package for R.[13,14] Differential gene expression levels between dead and alive patients' were obtained by the package through investigating the log-2-change of the genes (logFC, the cut-off value of 0.5). The edgeR package also provides the Benjamini–Hochberg method to control the false discovery rate (FDR, the cut-off value of 0.05).[15] The gene expression levels between dead and alive patients were compared with *t*-test and visualized using R and GraphPad Prism software version 8 (GraphPad software, San Diego, CA, USA). The significant level was set at both FDR < 0.05 and $P < 0.05$.

Survival analysis

Patients' survival time and vital status were merged with differentially expressed genes. R's survival packages; survival, survminer, and survdiff were used for survival analysis. Further, Kaplan–Meier (K–M) survival plots were generated and combined with the Xena Functional Genomics Explorer-GDC TARGET-OS.[16] The cut-off criterion was set to K–M < 0.05 and Cox *P*-value < 0.05 for screening of the survival-related gene at overall survival.

Pathway enrichment analysis and gene–gene functional interactions

The biological process and pathway enrichment analyses were performed through The gene ontology (GO)[17,18] and The NCATS BioPlanet[19] datasets, respectively. Further, gene–gene functional interactions of differentially expressed genes were performed using the Cytoscape software (version of 3.8.2) and GeneMANIA[20] datasets.

Results

Sample characteristics

The RNA-Seq data were downloaded from TARGET-OS project and the data were merged with vital status, survival time, and gender. After removing the samples without vital status, survival time, and gender information, the patient sample number was 86. Age ranged from 3 to 34 years (mean 15 years) and there were 37 (43%) females and 49 (57%) males. Patient demography and tumor characteristics are presented in Tables 1 and 2.

Differential expression analysis between dead and alive patients

Differential gene expression between dead and alive patients was compared with the edgeR package using R studio. By this analysis, 5758 genes were differentially expressed between dead and alive patients (both *P*-value and FDR <0.05) and 50,690 genes were not significant (both *P*-value and FDR >0.05). Of the 5758 genes, 4469 were upregulated, whereas 1289 genes were downregulated in dead versus living patients.

The survival-related genes in OS patients

Genes associated with survival were identified using Cox regression. Out of 5758 differentially expressed genes, 217 were found to be associated with overall survival ($P < 0.05$). Further, the survival-related genes were selected depending on their *P*-values (preferably lowest). The overall survival-related genes of dead and alive patients were validated and visualized using violin plots (Figures 1 and 2).

Table 1. The characteristics of the patients.

Vital status	Female ($n = 37$)	Male ($n = 49$)	Total
Alive	23	34	57
Dead	14	15	29
			86

Table 2. The characteristics of the tumors.

Site of tumor	Number	Disease at diagnosis and metastasis site
Femur	39	25 non-metastatic and 14 metastatic (8 lung only, 5 lung and bone, 1 bone only)
Tibia	21	17 non-metastatic and 4 metastatic (3 lung only, 1 bone and lung)
Fibula	8	7 non-metastatic and 1 metastatic (lung only)
Humerus	4	3 non-metastatic and 1 metastatic (lung only)
Pelvis	2	1 non-metastatic and 1 metastatic (lung only)
Pelvis–ilium	1	1 non-metastatic
Pelvis/sacrum	1	1 non-metastatic
Leg NOS[a]	6	5 non-metastatic and 1 metastatic (lung only)
Foot NOS[a]	1	1 non-metastatic
Radius	1	1 non-metastatic
Ilium	1	1 metastatic (lung only)
Arm NOS[a]	1	1 non-metastatic
Total	86	63 non-metastatic and 23 metastatic (16 lung only, 6 lung and bone, 1 bone only)
Summary		15 dead metastatic and 15 dead non-metastatic
		8 alive metastatic and 48 alive non-metastatic

[a]Not otherwise specified.

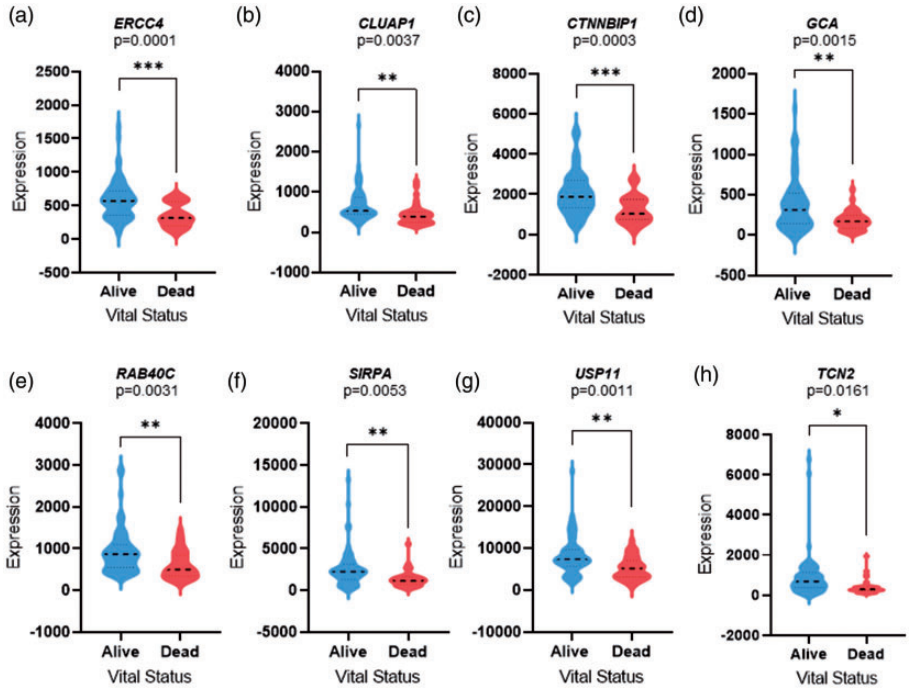

Figure 1. Violin plots displaying the downregulated survival-related genes expression levels; (a) *ERCC4*, (b) *CLUAP1*, (c) *CTNNBIP1*, (d) *GCA*, (e) *RAB40C*, (f) *SIRPA*, (g) *USP11*, and (h) *TCN2*. The violin plots are filled in blue (left) and red (right) represent alive and dead patients, respectively. The y-axis highlights the raw expression of the genes, whereas x-axis vital status of the patients. A dashed line in the violin plots represent the median value of the gene expression. *P*-value was calculated using Student's *t*-test, $P < 0.05$ considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.00001$). (A color version of this figure is available in the online journal.)

The survival-related genes that were significantly downregulated in the dead compared with living patients include; ERCC excision repair 4 (*ERCC4*), Clusterin-associated protein 1 (*CLUAP1*), catenin beta interacting protein 1 (*CTNNBIP1*), grancalcin (*GCA*), member RAS oncogene family (*RAB40C*), signal regulatory protein alpha (*SIRPA*), ubiquitin-specific peptidase 11 (*USP11*), and transcobalamin 2 (*TCN2*) (Table 3). K–M plots of the downregulated genes showed the association between downregulated genes and poor survival outcome of the patients with the significant value of $P < 0.05$ (Figure 3).

Significantly upregulated genes in the dead patients include; mucin 1-cell surface associated (*MUC1*), collagen type XIII alpha 1 chain (*COL13A1*), jagged canonical notch ligand 2 (*JAG2*), and Kazal type serine peptidase inhibitor domain 1 (*KAZALD1*) (Table 4). K–M plots highlighted upregulation of these genes and the positive correlation with poor patient survival (Figure 4).

GO and BioPlanet pathway enrichment analyses and gene–gene functional interactions of the survival-related genes

To examine the characteristics of the survival-related genes, functional classification and pathway enrichment analyses of downregulated and upregulated genes were performed using the GO tool and BioPlanet, respectively.

Figure 5(a) highlights the most significantly overrepresented GO terms of biological process of downregulated genes in dead patients. The top six biological process of GO terms are; negative regulation of meiotic nuclear division (GO: 0045835, *P*-value = 0.002398), negative regulation of chromosome organization (GO: 2001251, *P*-value = 0.002398), negative regulation of telomere capping (GO: 1904354, *P*-value = 0.0028), positive regulation of monocyte differentiation (GO: 0045657, *P*-value = 0.0032), telomere maintenance via recombination (GO: 0000722, *P*-value = 0.003993), and regulation of mesenchymal cell proliferation (GO: 0010464, *P*-value = 0.004790). Further, Figure 5(b) shows the pathway enrichment analysis of the downregulated genes, including, signal regulatory protein (SIRP) family interactions (*P*-value = 0.0051), dual incision reaction in GG-NER (*P*-value = 0.0079), dual incision reaction in TC-NER (*P*-value = 0.011), and association of TriC/CCT with target proteins during biosynthesis (*P*-value = 0.0115). In Figure 5(c), gene–gene interaction of the downregulated genes was shown.

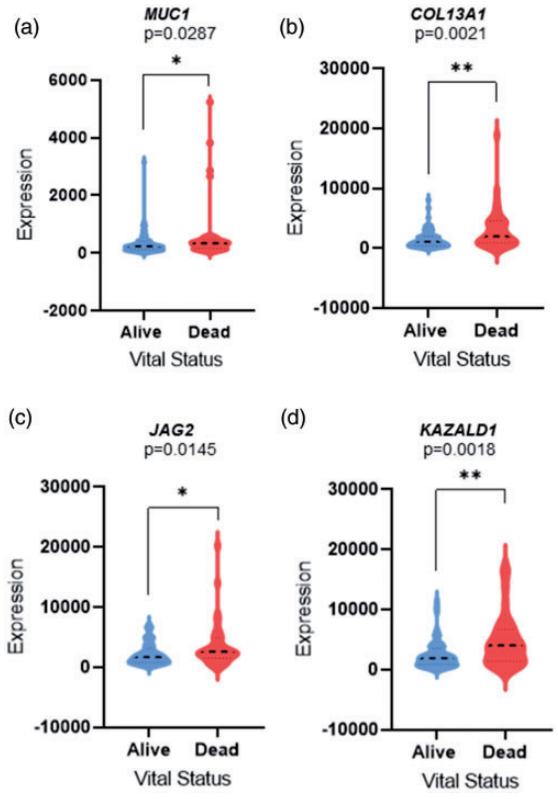

Figure 2. Violin plots displaying the upregulated survival-related genes expression levels; (a) *MUC1*, (b) *COL13A1*, (c) *JAG2*, and (d) *KAZALD1*. The violin plots are filled in blue (left) and red (right) represent alive and dead patients, respectively. The y-axis highlights the raw expression of the genes, whereas x-axis vital status of the patients. A dashed line in the violin plots represent the median value of the gene expression. *P*-value was calculated using Student's *t*-test, $P < 0.05$ considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.00001$). (A color version of this figure is available in the online journal.)

Figure 6(a) highlights the most significantly overrepresented GO terms of biological process of upregulated genes in dead patients such as positive regulation of histone H4 acetylation (GO: 0090240, *P*-value = 0.0014), negative regulation of cell adhesion mediated by integrin (GO: 0033629, *P*-value = 0.0014), auditory receptor cell differentiation (GO: 0042491, *P*-value = 0.0014), regulation of histone H4 acetylation (GO:0090239, *P*-value = 0.0016), endochondral ossification (GO: 0001958, *P*-value = 0.0016), and negative regulation of transcription by competitive promoter binding (GO: 0010944, *P*-value = 0.0018). Figure 6(b) performs the pathway enrichment analysis of the upregulated genes, including, signalling by NOTCH2 (*P*-value = 0.0027), initiation of the second proteolytic cleavage of Notch receptor by receptor-ligand binding (*P*-value = 0.0028), interleukin-11 pathway (*P*-value = 0.0045), termination of O-glycan biosynthesis (*P*-value = 0.0051), and activated NOTCH1 signalling in the nucleus (*P*-value = 0.0061). Lastly, Figure 6(c) highlights the gene–gene interaction of the upregulated genes.

Discussion

Our limited understanding of the genetic regulation and molecular mechanisms of disease progression in osteosarcoma is a barrier to the development of novel precision therapies. Transcriptome analysis is central to the discovery and understanding of prognostic biomarkers, a research priority in OS.

In this study, we analyzed differential gene expression by survival based on 86 TARGET-OS project patients. Of the 5758 differentially expressed genes, 217 were associated with survival (FDR <0.05 and $P < 0.05$). Based on *P* values, we selected eight downregulated genes (*ERCC4*, *CLUAP1*, *CTNNBIP1*, *GCA*, *RAB40C*, *SIRPA*, *USP11*, and *TCN2*) and four upregulated genes (*MUC1*, *COL13A1*, *JAG2*, and *KAZALD1*) in patients who died of their disease for further analysis (Tables 3 and 4).

The associations between differentially regulated (both upregulated and downregulated) genes and OS have already been analyzed (except *RAB40C*, *USP11*,

Table 3. Survival-related downregulated genes in dead patients.

Symbol	Description	logFC	logCPM	F	*P*-Value	FDR
ERCC4	ERCC excision repair 4	−0.604331065	3.6044199	21.15321871	1.35E−05	0.000711
CLUAP1	Clusterin associated protein 1	−0.506755578	3.835841364	15.51321235	0.000159425	0.004417889
CTNNBIP1	Catenin beta interacting protein 1	−0.535497627	5.404612024	12.20243352	0.000736136	0.01311661
GCA	Grancalcin	−0.977336188	2.918813582	13.50950687	0.000399133	0.008618337
RAB40C	Member RAS oncogene family	−0.526285629	4.284398674	14.18739992	0.000291816	0.006857791
SIRPA	Signal regulatory protein alpha	−0.695679783	5.630441654	10.89208674	0.001375861	0.020650984
USP11	Ubiquitin-specific peptidase 11	−0.526261302	7.42223499	14.5888379	0.000242739	0.005965627
TCN2	Transcobalamin 2	−0.745119819	4.055907039	9.088352903	0.003325462	0.037655142

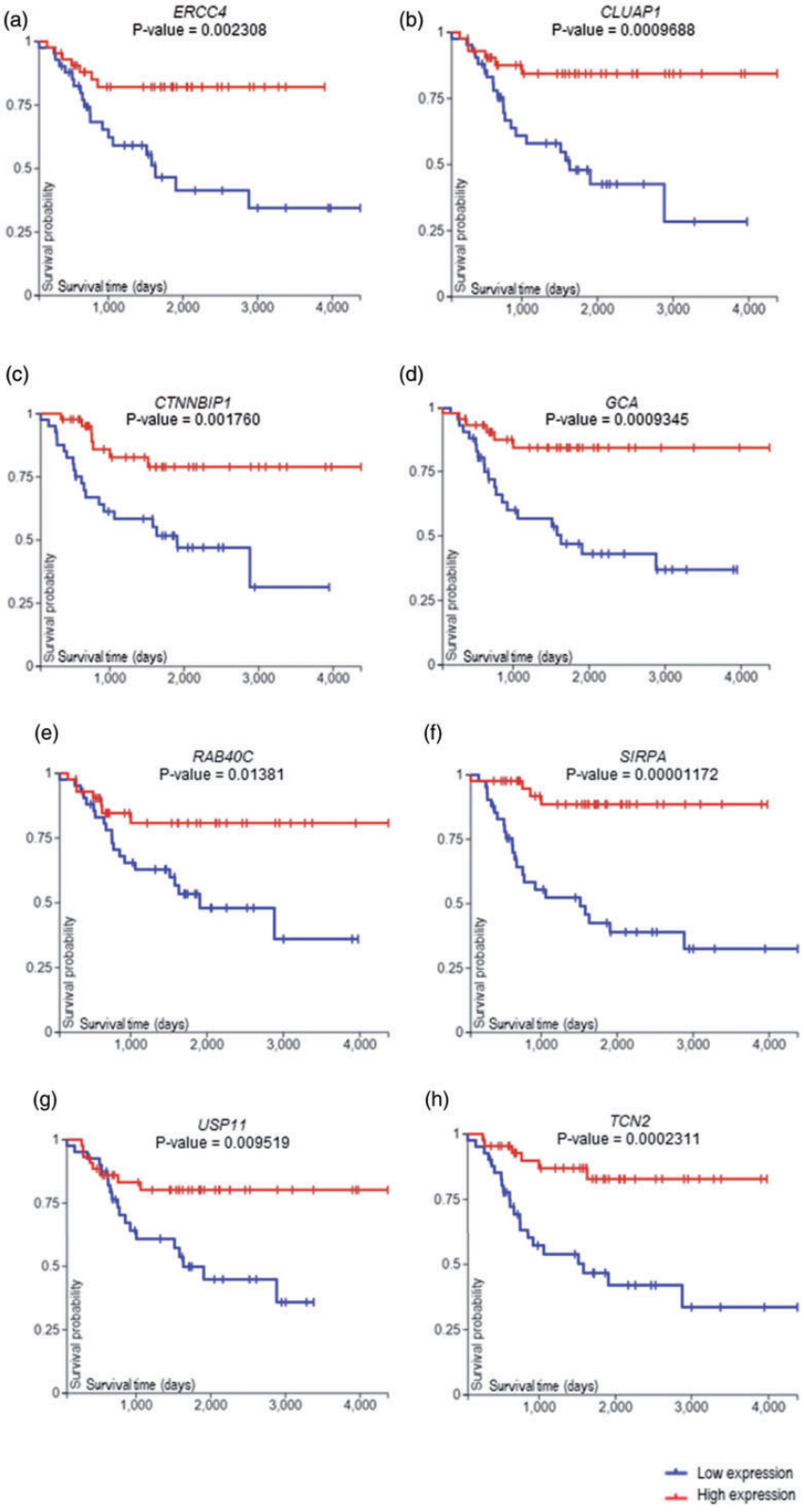

Figure 3. Kaplan–Meier survival analysis of (a) *ERCC4*, (b) *CLUAP1*, (c) *CTNNBIP1*, (d) *GCA*, (e) *RAB40C*, (f) *SIRPA*, (g) *USP11* and (h) *TCN2*. Blue color represents genes with low expression, whereas red, genes with high expression. *P*-value was computed by log-rank test, $P < 0.05$ considered statistically significant. (A color version of this figure is available in the online journal.)

Table 4. Survival-related upregulated genes in dead patients.

Symbol	Description	logFC	logCPM	F	*P*-Value	FDR
MUC1	Mucin 1, cell surface associated	1.351910472	3.516411994	18.43384428	4.36E-05	0.001702678
COL13A1	Collagen type XIII alpha 1 chain	1.1977585	5.747573231	14.90975716	0.000209656	0.00541287
JAG2	Jagged canonical Notch ligand 2	0.955088787	6.021108153	16.09517412	0.000122657	0.003634496
KAZALD1	Kazal type serine peptidase inhibitor domain 1	1.002413796	6.419848871	12.11332211	0.000767825	0.013540203

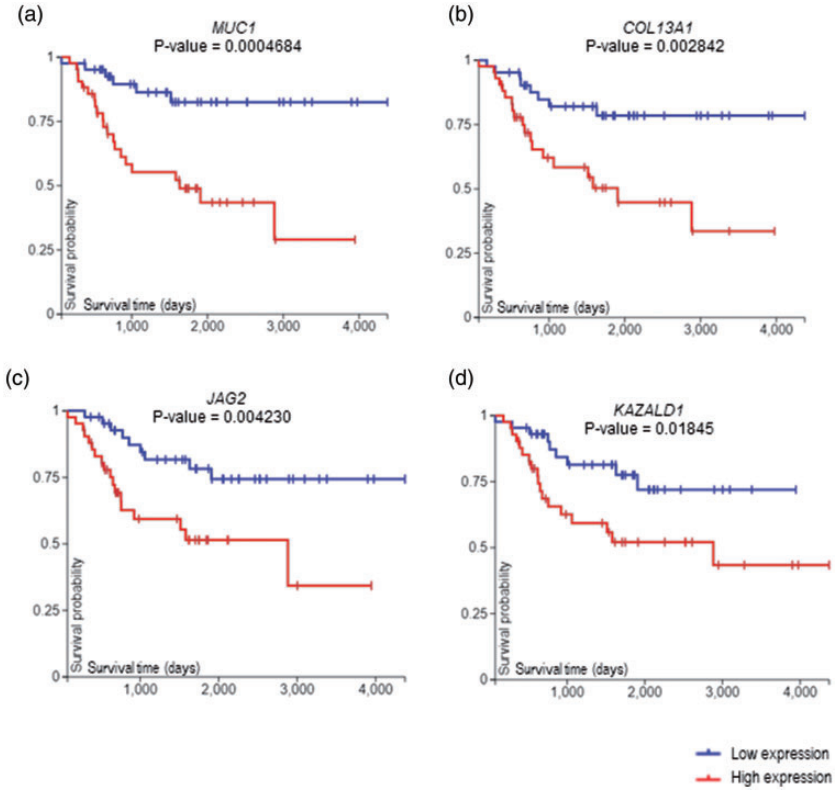

Figure 4. Kaplan–Meier survival analysis of (a) *MUC1*, (b) *COL12A1*, (c) *JAG2*, and (d) *KAZALD1*. Blue color represents genes with low expression, whereas red, genes with high expression. *P*-value was computed by log-rank test, $P < 0.05$ considered statistically significant. (A color version of this figure is available in the online journal.)

CTNNBIP1, TNC2, GCA, JAG2, and *KAZALD1*).[21–26] However, in these studies, the prognostic values of the genes have not been investigated.

ERCC4, also known as XPF, gene encodes proteins that can play an important role in DNA repair and chromosome stability.[27] The gene also has been involved in fanconi anemia (FA) which leads to bone marrow failure, congenital malformations, chromosome fragility, and cancer susceptibility including OS.[28,29] According to several studies, patients with low expression of *ERCC4* were associated with worse overall survival in hepatocellular carcinoma.[30] Our study also highlighted that OS patients with low expression of *ERCC4* have displayed worse survival outcome of the patients (Figure 3(a)).

There is an increased expression of *CLUAP1* in cell growth and the S phase of cell-cycle progression.[31] Further, over expression of the gene was observed in OS tissue samples and cell lines, including HuO3N1, OS2000, Saos-2, HuO9N2, HOS, and MG63. Noteworthy, overexpression of *CLUAP1* has also been observed in ovarian, colon, and lung cancers.[22]

This is the first report of an association between *CTNNBIP1* and survival in OS, but the gene is known to be a prognostic indicator in lung adenocarcinomas.[32] According to the same study, *CTNNBIP1* expression correlated with longer overall survival in lung adenocarcinomas patients.[32]

GCA is a protein that has been identified in human neutrophils and the monocytes/macrophage lineage.[33] The function of this protein is not completely understood, but the gene was downregulated in metastatic OS in *in vitro* studies.[34]

Breast cancer patients with high expression CD47 expression (cluster of differentiation 47) and active CD47/SIRPA (SIRPα) pathway have a poor prognosis.[35] Furthermore, one study determined that the CD47/SIRPA pathway is activated in OS. CD47 is known as a "do not eat me" signal that binds to SIRPA in the surface of macrophages which leads to escape from phagocytosis and cell death.[36] Notably, knockout of SIRPA in macrophages enhances phagocytosis of OS cells.[25]

Unfortunately, there are no established studies to highlight the direct association between *USP11* and OS.

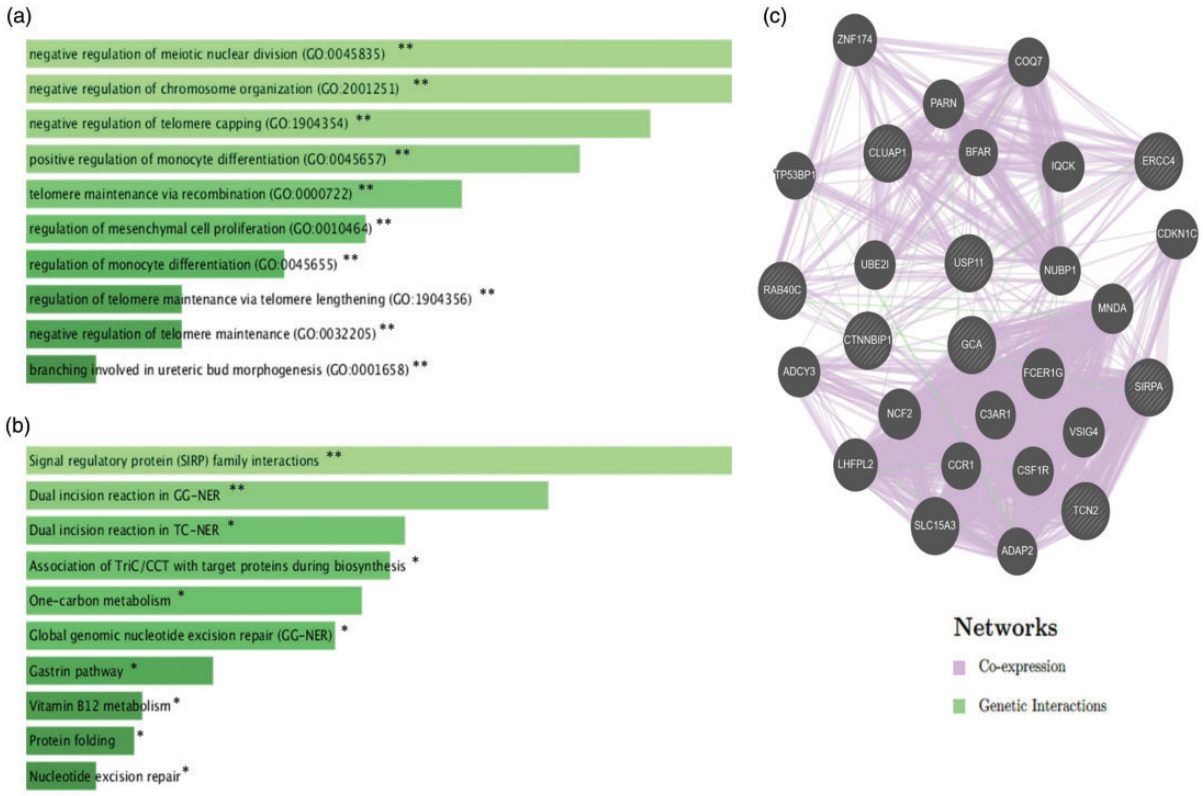

Figure 5. Enrichment analysis of eight survival-related downregulated genes; functional gene ontology analysis of biological process (a), pathway enrichment analysis (b) and gene–gene interaction network analysis (c). *P*-values less than 0.05 were considered statistically significant, $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

Figure 6. Enrichment analysis of four survival-related upregulated genes; functional gene ontology analysis of biological process (a), pathway enrichment analysis (b) and gene–gene interaction network analysis (c). *P*-values less than 0.05 were considered statistically significant, $^{*}P < 0.05$, $^{**}P < 0.01$. (A color version of this figure is available in the online journal.)

However, low expression of *USP11* was correlated with better survival outcome in breast cancer patients.[37] Interestingly, a study suggested that over-expression of *USP11* promotes growth and metastasis of colorectal cancer.[38]

No clear association that has been found yet between *TCN2* gene and any cancer type, including OS. Intriguingly, mutation (loss of function) of this gene result in transcobalamin deficiency that leads to an abnormal immunity in the individuals.[39] Our result showed that down regulation of *TCN2* is associated with worse survival outcome (Figure 3(h)).

MUC1 has been associated with metastatic progression both *in vivo* and *in vitro* in several cancer types through O-glycosylated serine/threonine repeat region (MUC1-ECD), as well as through activities of its intracellular domain (MUC1-CD).[40] It is already established that over-expression of *MUC1* predicts poor prognosis in breast cancer patients.[41] Interestingly, high expression level of *MUC1* in sarcoma metastasis was correlated with worse overall survival in sarcoma.[26] Our result also correlated a high expression of *MUC1* with low survival of OS patients (Figure 4(a)).

Although, no associations have been found regarding to *COL13A1* and OS, the gene strongly correlated with poor clinical outcome in bladder cancer.[42] Another study mentioned that over expression of *COL13A1* is associated with high risk of disease progression and aggressive invasion in urothelial carcinoma of the bladder.[43] We also found that *COL13A1* was upregulated in dead OS patients compared to alive, further high expression of the gene is associated with poor survival outcome (Figure 4(b)). Nevertheless, the functional role of *COL13A1* in cancer especially in OS needs to be extensively studied.

Over-expression of *JAG2* has been associated with poor survival outcome in oral squamous cell carcinoma.[44] Another study also highlighted that high expression level of *JAG2* increases chemo-resistance of colorectal cancer cells.[45] Interestingly, the positive correlation of *MYC* (MYC proto-oncogene, bHLH transcription factor) and *JAG2* are critical pro-survival factors in childhood medulloblastoma.[46,47] There are no published studies to highlight the relationship between *JAG2* and OS. However, one study suggested that *JAG2* may be involved in OS progression and initiation because the gene interacts with both p53 signalling and Notch signalling pathways, which play an essential role in development of bone tumors.[48,49]

There is no published clear association between *KAZALD1* gene and OS. According to one study, high expression of *KAZALD1* promotes glioma malignant progression by invasion and high proliferation of tumor cells. The authors also suggested that low expression of *KAZALD1* confers better overall survival.[50] Our result also showed that high expression of *KAZALD1* is correlated with worse overall survival in OS patients (Figure 4(d)).

In conclusion, in the present study we have identified 5758 differentially expressed genes between dead and alive patients in which 217 of genes showed an association with overall survival. Out of 217 genes, 8 survival-related down-regulated (*ERCC4*, *CLUAP1*, *CTNNBIP1*, *GCA*, *RAB40C*, *SIRPA*, *USP11*, and *TCN2*) and 4 survival-related upregulated genes (*MUC1*, *COL13A1*, *JAG2*, and *KAZALD1*) were selected. These genes have not been adequately studied in cancer, especially in OS. Consequently, further research with the candidate genes is required to characterize their roles in OS progression, invasion, and metastasis to provide specific and successful prognostic markers for the disease.

AUTHORS' CONTRIBUTIONS

All authors made significant contributions to the work presented in this paper. ER contributed to the data collection, data analysis and writing the manuscript. JX and DW both involved in project conception and reviewing the manuscript. SK contributed to the study design, data analysis and reviewing the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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