Analysis of the molecular mechanism of osteosarcoma using a bioinformatics approach

JIANXUN YANG[1] and NING WANG[2]

Departments of [1]Traumatology Five and [2]Children's Rehabilitation, Linyi People's Hospital, Linyi, Shandong 276000, P.R. China

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Abstract. The aim of this study was to explore the underlying molecular mechanism related to the process and progression of osteosarcoma (OS). The differentially expressed genes (DEGs) were downloaded from the Gene Expression Omnibus database. The pathway and gene ontology (GO) enrichment analysis, as well as transcription factor, tumor-associated gene and tumor suppressor gene analyses were performed to investigate the functions of DEGs. Next, the protein-protein interaction (PPI) network was constructed and module analysis was further assessed by cluster analysis with the overlapping neighborhood expansion (Cluster ONE) cytoscape plug-in. A total of 359 upregulated and 614 downregulated DEGs were identified to be differentially expressed between OS samples and normal controls. Pathways significantly enriched by DEGs included the focal adhesion and chromosome maintenance pathways. Significant GO terms were cell adhesion, cell cycle and nucleic acid metabolic processes. The upregulated PPI network was constructed with 170 nodes and the downregulated PPI network was constructed with 332 nodes. Breast-ovarian cancer gene 1 (*BRCA1*), melanocyte-stimulating hormone 2 (*MSH2*), cyclin D1 (*CCND1*) and integrin α5 (*ITGA5*) were identified to be hub proteins in PPI. In conclusion, the dysregulated genes played key roles in the progression of OS. Cell adhesion is a significant biological process in OS development, and the genes *BRCA1, MSH2, CCND1* and *ITGA5* may be potential targets in the therapy of OS.

Introduction

Osteosarcoma (OS) is an aggressive malignant neoplasm. It arises from primitive transformed cells of mesenchymal origin (and thus a sarcoma), exhibits osteoblastic differentiation and produces malignant osteoid (1). The main symptoms of OS are local pain and swelling (2,3). More than half of all OS cases arise in the long bones of the limbs, particularly the knee (4). Although OS is a rare tumor, with annual age-adjusted rates of 1.0 per 100,000 males and 0.6 per 100,000 females, it is the most common histological form of primary bone cancer (5). Furthermore, OS is the eighth most commonly occurring childhood cancer (5). In addition, the prognosis is poorer (with a cure rate of ~30%) for tumors located in the axial skeleton and in patients with metastasis at onset (6). OS has become a significant health concern.

Great achievements have been obtained in exploring the pathological mechanism of OS development. Certain genes have been identified to exert key roles in OS progression. Single-nucleotide polymolphisms of insulin-like growth factor 2 receptor are associated with an increased risk of OS (7). The phosphoprotein p53 gene as an anti-oncogene is associated with controlling the cell cycle in OS (8) and mutations in the p53 gene correlate significantly with the presence of high levels of genomic instability, which lead to cancerization in OS (9). Furthermore, microRNAs (miRs) play a significant role in cancer cell growth and migration; for example, miR-199a-3p decreases cell growth and migration in OS cell lines significantly (10). In addition, significant pathways have also been determined to exhibit critical roles in OS. The phosphoinositide 3-kinase (PI3K/Akt) pathway is well known to be a major cell survival pathway in OS (11-13). Akt regulates several downstream targets resulting in cell growth, survival and cisplatin resistance. The activity of the receptor activator of nuclear factor κB ligand (RANKL) pathway is essential for tumorigenic osteolysis and creates a suitable context for tumor expansion as they increase tumor cell proliferation (14). Achievements in understanding the mechanism of OS contribute to the therapy of this cancer. However, the present knowledge of the molecular mechanism of OS development remains insufficient.

In this study, a biological informatics approach was applied to analyze the gene expression profiles in OS and a functional analysis of differentially expressed genes (DEGs) between OS cells and matched normal tissues was performed. Furthermore, relevant transcription factor (TF) genes, tumor suppressor genes (TSGs) and tumor-associated genes (TAGs) were analyzed. In addition, the protein-protein interaction (PPI) network and modules were constructed. The aim of the present study was to provide a systematic perspective to

Correspondence to: Dr Ning Wang, Department of Children's Rehabilitation, Linyi People's Hospital, 27 East Jiefang Road, Linyi, Shandong 276000, P.R. China
E-mail: wnwangn@163.com

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understanding the mechanism and identifying new therapeutic targets for OS.

Materials and methods

Affymetrix microarray data. The gene expression profile of GSE11414 was obtained from the National Center of Biotechnology Information (NCBI) Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). Expression profiles were derived from six samples including two samples of human OS cell lines U2OS (ATCC no. HTB-96), two MG63 cell lines (ATCC no. CRL-1427) and two normal human osteoblast cell lines. In this study, the raw data and annotation files were downloaded based on the platform of GPL6244 (Affymetrix Human Gene 1.0 ST array, Affymetrix, Inc., Santa Clara, CA, USA).

Data pre-processing and analysis of DEGs. The pre-processing of raw expression data, including background correction, quantile normalization and probe summarization, was performed using the robust multiarray average (15) algorithm with application of the Affymetrix package in the R statistical software program (Bell Laboratories Inc., Madison, WI, USA) (16) and Affymetrix annotation files supplied by the Brain Array Lab (Microarray Lab, University of Michigan, Ann Arbor, MI, USA). If multiple probes corresponded to the same gene, the mean value was calculated as the expression value of this gene. For the GSE11414 dataset, the paired t-test of the limma package (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) (17) was used to identify DEGs. The multiple testing correction was performed to control the false discovery rate (FDR) with the application of the significance analysis of the microarray procedure. Genes with FDR <0.05 and |log fold change (FC)| >2.0 were considered as significantly differentially expressed.

Pathway and gene ontology (GO) functional enrichment analysis of DEGs. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base for systematic analysis of gene functions and enzymatic pathways and linking genomic information with higher order functional information (18). Reactome is an open-source, manually curated database of reactions, pathways and biological processes (19). GO analysis is a commonly used approach for functional studies of genomic or transcriptomic data (20). The Database for Annotation, Visualization and Integrated Discovery (DAVID) (21) provides exploratory visualization tools that promote identification via functional classification and biochemical pathway maps, and conserve protein domain architectures (22). In order to analyze the DEGs at the functional level, GO annotation and KEGG pathway enrichment analysis was performed for DEGs by DAVID, and reactome pathways were also identified. P<0.01 was selected as the cut-off criterion.

Functional annotation of DEGs. The TSG database (TSGene) provides detailed annotations for each TSG, including cancer mutations, gene expressions, methylation sites, TF regulations and PPIs (23). The TAG database was designed to utilize information from well-characterized oncogenes and TSGs to facilitate cancer research (24). According to the data on TFs, the enriched DEGs for transcription regulation were selected based on the GO, KEGG and reactome annotation terms. Additionally, the selected DEGs were mapped onto the TSGene and TAG databases to extract the oncogenes and cancer suppressor genes.

PPI network construction and network module analysis. The search tool for the retrieval of interacting genes (STRING) is an online database which provides uniquely comprehensive coverage and ease of access to PPI information (25). In this study, the PPIs of upregulated and downregulated DEGs were analyzed by STRING with the cut-off criterion of a combined score >0.4. Then the network was visualized using cytoscape software (Institute for Systems Biology, Seattle, WA, USA) (26). Furthermore, the connectivity degree of the network was analyzed by the network statistics method and used to obtain the hub protein in the PPI network. The node degree threshold was set to 15 (degree ≥15). Finally, the modules of the PPI network were constructed using cluster analysis with the overlapping neighborhood expansion (Cluster ONE) cytoscape plug-in. P<0.01 was selected as the cut-off criterion.

Table I. KEGG pathway enrichment analysis for DEGs.

Pathway	Gene count	P-value
Upregulated DEGs		
Axon guidance	12	2.45E-03
Downregulated DEGs		
Focal adhesion	19	6.08E-05
Lysosome	14	7.18E-05
ECM-receptor interaction	11	1.59E-04
Mucin type O-Glycan biosynthesis	6	4.96E-04
Arachidonic acid metabolism	7	4.18E-03
Rheumatoid arthritis	9	4.25E-03
Hypertrophic cardiomyopathy	8	8.05E-03
Malaria	6	8.19E-03

KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene; ECM, extracellular matrix. Gene count refers to the number of DEGs; P-value was obtained by expression analysis systemic explorer test.

Results

Identification of DEGs. Using the cut-off criteria of FDR <0.05 and |log FC| >2.0, we finally obtained 973 DEGs, comprising 359 up- and 614 downregulated genes.

Pathway enrichment analysis. The KEGG pathway analysis revealed that the upregulated DEGs were only enriched in the axon guidance pathway (Table I). The downregulated DEGs were significantly enriched in eight KEGG pathways, and are listed in Table I. Cyclin D1 (*CCND1*) was involved in the focal adhesion pathway, and integrins α5 (*ITGA5*) and α7 (*ITGA7*) were involved in the focal adhesion and extracellular matrix (ECM)-receptor interaction pathways.

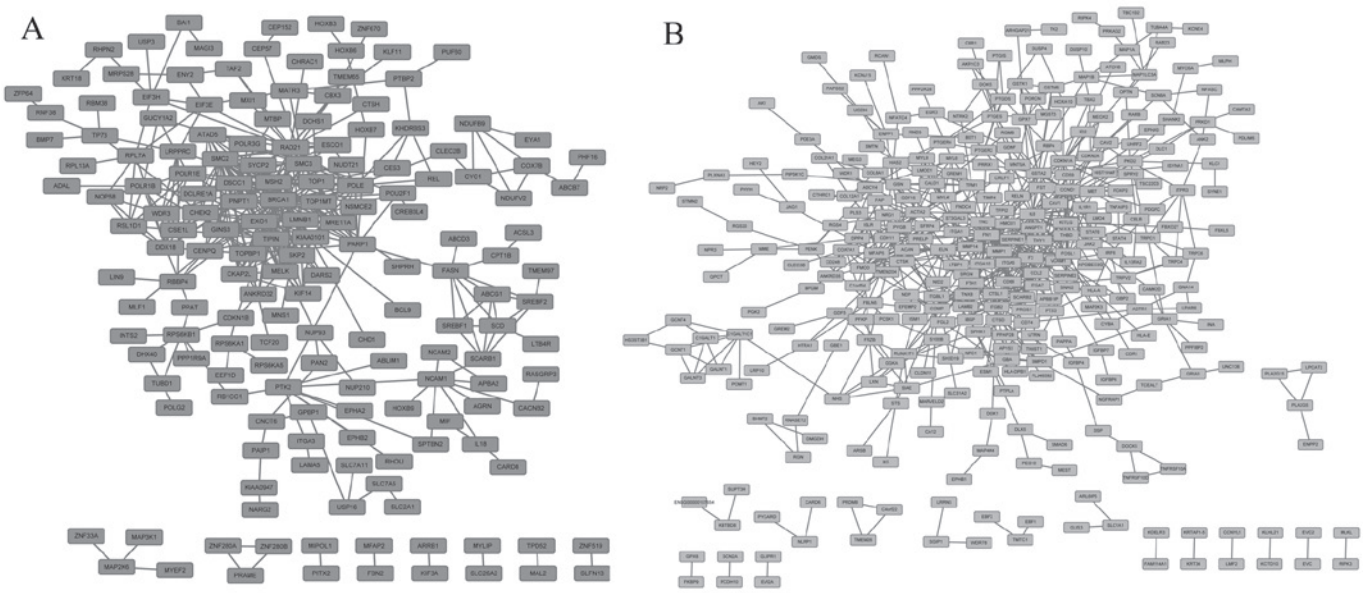

Figure 1. Protein-protein interaction (PPI) networks for differentially expressed genes (DEGs). The protein is represented by the name of gene which encodes the protein. (A) PPI for upregulated DEGs. (B) PPI for downregulated DEGs.

Reactome pathway analysis revealed 14 pathways in the upregulated DEGs, and the top five pathways were chromosome maintenance, establishment of sister chromatid cohesion, neural cell adhesion molecule signaling for neurite outgrowth, cell cycle and meiotic synapsis. The downregulated DEGs were enriched in 22 pathways in the reactome pathway analysis and the top five were ECM organization, smooth muscle contraction, elastic fibre formation, integrin cell surface interactions, and synthesis of prostaglandins and thromboxanes.

GO enrichment analysis. The top five functional nodes in each GO category of up- and downregulated DEGs are listed in Table II. GO annotation indicated that the upregulated DEGs were primarily enriched during the cell cycle and the proliferation process. The downregulated DEGs predominantely participated in the process of cell adhesion, regulation of cell proliferation and extracellular region.

Function analysis of DEGs. There were 17 upregulated and 15 downregulated DEGs mapped onto the TF database (Table III). The DEGs mapped onto the TAG database analysis indicated that 61 downregulated DEGs were detected, comprising 12 oncogenes, 42 TSGs and 7 genes with an unclear impact on tumors, while 27 upregulated DEGs were identified, comprising 6 oncogenes, 18 TSGs and 3 genes with an unclear impact on tumors (Table III).

PPI network analysis. By submitting the upregulated and downregulated genes into STRING, respectively, we obtained PPIs associated with the DEGs. Fig. 1A demonstrates that the upregulated PPI network was constructed with 170 nodes and 362 edges. The nodes of breast-ovarian cancer gene 1 (*BRCA1*, degree=27) and melanocyte-stimulating hormone 2 (*MSH2*, degree=26) were hub proteins. Fig. 1B reveals that the downregulated PPI network was constructed with 332 nodes and 679 edges. Fibronectin 1 (*FN1*, degree=55), *CCND1* (degree=18) and *ITGA5* (degree=16) were hub proteins in the network.

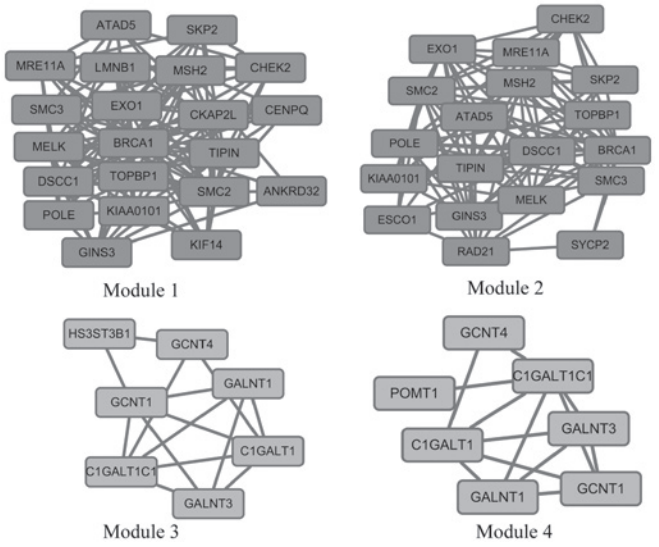

Figure 2. Modules in protein-protein interaction network. Module 1 and 2 show upregulated differentially expressed genes (DEGs), and module 3 and 4 show downregulated DEGs.

Module analysis. A total of 18 modules were identified in upregulated PPI network, among which the best one was module 1 (Fig. 2). Module 1 was composed of 21 nodes and 120 edges; P=2.771E-7. The hub proteins of *BRCA1* (degree=20) and *MSH2* (degree=14) were involved in module 1.

The downregulated PPI network contained 46 modules, of which the best one was module 4 (Fig. 2). It was constructed with 7 nodes and 13 edges; P=0.02. The hub proteins of core 1 β3-Gal-T-specific molecular chaperone (*C1GALT1C1*, degree=5) and core 1 β3 Galactosyltransferase (*C1GALT1*, degree=5) were involved in it.

Table II. GO enrichment analysis for DEGs.

Category	Term	Gene count	P-value
Upregulated DEGs			
GOTERM_BP_FAT	GO:0007049~cell cycle	52	1.56E-06
GOTERM_BP_FAT	GO:0090304~nucleic acid metabolic process	116	6.80E-06
GOTERM_BP_FAT	GO:0006139~nucleobase-containing compound metabolic process	131	7.64E-06
GOTERM_BP_FAT	GO:0034641~cellular nitrogen compound metabolic process	138	8.94E-06
GOTERM_BP_FAT	GO:0022402~cell cycle process	41	1.11E-05
GOTERM_MF_FAT	GO:1901363~heterocyclic compound binding	129	7.52E-06
GOTERM_MF_FAT	GO:0005003~ephrin receptor activity	5	9.42E-06
GOTERM_MF_FAT	GO:0097159~organic cyclic compound binding	129	1.62E-05
GOTERM_MF_FAT	GO:0003676~nucleic acid binding	89	2.20E-05
GOTERM_MF_FAT	GO:0019900~kinase binding	19	1.97E-04
GOTERM_CC_FAT	GO:0005634~nucleus	161	1.56E-11
GOTERM_CC_FAT	GO:0043227~membrane-bounded organelle	214	3.58E-07
GOTERM_CC_FAT	GO:0005622~intracellular	255	3.91E-07
GOTERM_CC_FAT	GO:0044464~cell part	283	4.79E-07
GOTERM_CC_FAT	GO:0005623~cell	283	4.86E-07
Downregulated DEGs			
GOTERM_BP_FAT	GO:0007155~cell adhesion	74	1.93E-11
GOTERM_BP_FAT	GO:0022610~biological adhesion	74	2.13E-11
GOTERM_BP_FAT	GO:0030198~extracellular matrix organization	36	1.16E-10
GOTERM_BP_FAT	GO:0043062~extracellular structure organization	36	1.27E-10
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	83	3.29E-10
GOTERM_MF_FAT	GO:1901681~sulfur compound binding	20	3.46E-07
GOTERM_MF_FAT	GO:0005539~glycosaminoglycan binding	20	4.60E-07
GOTERM_MF_FAT	GO:0005520~insulin-like growth factor binding	8	5.68E-07
GOTERM_MF_FAT	GO:0097367~carbohydrate derivative binding	21	6.04E-07
GOTERM_MF_FAT	GO:0008201~heparin binding	17	6.55E-07
GOTERM_CC_FAT	GO:0005576~extracellular region	146	0
GOTERM_CC_FAT	GO:0044421~extracellular region part	91	4.44E-16
GOTERM_CC_FAT	GO:0005615~extracellular space	72	3.29E-14
GOTERM_CC_FAT	GO:0031012~extracellular matrix	43	2.01E-11
GOTERM_CC_FAT	GO:0043202~lysosomal lumen	13	4.80E-07

Top 5 GO terms in different categories are listed. GO, gene ontology; DEG, differentially expressed gene; BP, biological process; MF, molecular function; CC, cellular component.

Discussion

Although OS is a relatively uncommon cancer, it is the most common primary bone malignancy in children and young adults (27). In previous studies, gene expression profiling has been used to identify TFs and pathways associated with OS (27) as well as genome-wide changes in OS cell lines (28). In this study, GO and PPI analyses indicated that DEGs including *ITGA5* were significantly dysregulated and were also involved in ECM-receptor interaction and the focal adhesion pathway. Module analysis revealed that *BRCA1* and *MSH2* were hub proteins in module 1 of the upregulated PPI network. In addition, *CCND1* was a hub protein in the downregulated PPI network.

ITGA5 is an essential member of the integrin family, which belongs to cell adhesion receptors targeting cell adhesion to the ECM (29). *ITGA5* combines with integrin β1 to form heterodimers to mediate cell adhesion on fibronectin (29). In human OS cells, *ITGA5* is regulated by transforming growth factor β, and is involved in the adhesion of tumor cells to laminin (30). It has been reported that a decrease in adhesion of intact cells to fibronectin is correlated with a decrease in the ability of α5β1 integrin in human OS (31). Furthermore, the upregulation of α5β1 integrin contributes to tumor invasion and metastatic potential (32). In the present study, ITGA5 was also found to be associated with pathways involved in focal adhesion and ECM-receptor interaction. Focal adhesion connects the cell cytoskeleton and the ECM through integrins (33). Accordingly, *ITGA5* exhibited a crucial role in the OS development via cell adhesion.

BRCA1 is a TSG (34) identified in all humans. It is responsible for repairing DNA damage (35) and is involved in cell cycle control (36). This corresponded with the

Table III. Functional analysis of DEGs.

	TF genes	TAG-tumor suppressor	TAG-oncogene	TAG-other
Upregulated DEGs	ZNF33A, ZNF146, ZNF124, TP73, TGIF2, SREBF1, REL, POU2F1, PITX2, MXI1, KLF11, HOXB9, HOXB7, HOXB6, HOXB3, EHF, BRCA1	ANP32A, ARID2, BAI2, BRCA1, CDKN1B, CHEK2, EHF, EPHB2, EPHB4, JUP, LIN9, MSH2, MXI1, NDRG2, RASSF2, RB1CC1, SHPRH, TP73	CSE1L MERTK MLF1 REL TPD52 ZNF146	BCL9 CHD1 SKP2
Downregulated DEGs	ZNF43, TWIST1, TBX2, STAT6, STAT4, SMAD6, NFATC4, MEOX2, LMO3, IRF6, ID2, HOXA10, HEY2, FOXP2, EGR3	ADPRH, ARHGAP20, CAV2, CDH11, CDKN1A, CDKN2A, DLC1, DOK1, DPP4, GLIPR1, HTRA1, IGFBP3, IGFBP4, IGFBP7, ITGA5, ITGA7, LXN, MEG3, WNT5A, UHRF2, MIR125B1, MIR145, MIR29A, MTSS1, PCDH10, PYCARD, RARB, TPM1, RARRES1, RNASET2, RND3, SLIT2, SPRY2, TCEAL7, TCTA, TFPI2, TGFBR3, THBD, THSD1, THY1, TNFAIP3, TNFRSF10A	CCND1 FOSL1 HOXA10 METCDR1 MME MRAS RAB23 RUNX1T1 SERTAD2 SPHK1 TWIST1 WISP2	CAV1 CBLB CCL2 MAP1A SFRP4 WISP3

DEG, differentially expressed gene; TF, transcription factor; TAG, tumor-associated genes.

function analysis and pathway analysis in the present study. The overexpression of *BRCA1* repairs the damage in OS cell lines by enhancing the global genomic repair pathway, whereas knockdown of *BRCA1* inhibits nuclear localization of the vitamin D receptor which is present in osteoblasts and affects osteoblast functions, including proliferation, apoptosis and mineralization (37). Furthermore, induction of *BRCA1* triggers apoptosis in OS via the activation of the c-Jun N-terminal kinase/stress-activated protein kinase signaling pathway (38). Additionally, the TF analysis revealed that *BRCA1* belongs to the TFs, and this has been verified in OS (39). The carboxyl terminus of *BRCA1*, when fused to the heterologous Gal4 DNA binding domain, activates transcription, which is inhibited when cancer predisposing mutations are introduced in the *BRCA1* component (40,41). The present study revealed that the *BRCA1* gene was upregulated in the progression of OS and it was a hub protein with degree score of 20 in module 1. The *BRCA1* gene was a key regulator in the progression of OS.

MSH2, as a DNA mismatch repair (MMR) protein, is a tumor suppressor. This corresponded with the present analysis of TSGs. It forms a heterodimer with *MSH6* or *MSH3* to form the DNA repair complex. A previous study demonstrated that *MSH2* promotes methylation, microsatellite instability and various mutational events associated with development of OS (42). It also distinguishes OS cancer stem cells from normal cells by mediating DNA repair (43). Furthermore, the increased expression of *MSH2* enhances the drug resistance of OS by mediating MMR when chemotherapeutic agents cause DNA damage critically or interfere with cellular metabolism (44). In addition, *MSH2* associates with *BRCA1* to form a large complex which is involved in the recognition and repair of aberrant DNA structures (45). Conversely, *CCND1,* which belongs to the TAGs, is characterized by a significant periodicity in protein abundance throughout the cell cycle. It has been reported that D-type cyclin exhibits a role in the progression of OS through the cell cycle (46). A previous study confirmed that the suppression of *CCND1* caused by miR-15a and miR-16-1 reduces OS cell proliferation via cell cycle arrest, and that the activated *CCND1* may have the ability to induce apoptosis (47). Therefore, *MSH2* and *CCND1* are considered to exhibit key roles in OS development.

In conclusion, the present study observed that a number of genes with altered expression were associated with the development and progression of OS. The genes of *ITGA5*, *BRCA1, MSH2* and *CCND1*, which are involved in cell adhesion, DNA repair or cell cycle progression, exhibit significant roles in the development of OS. The current study indicates that these genes may present potential targets in the treatment of OS. However, further evaluation of the potential applications is required.

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