Abstract. Osteosarcoma (OS) is the most common primary malignancy of the bone. The aim of the present study was to identify the key genes to uncover the novel mechanism of OS at a molecular level. The differentially-expressed genes (DEGs) between the OS and control groups were identified by analyzing the GSE32395 microarray data using Student’s t-test. The Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology enrichment analyses, including biological process, cellular component and molecular function categories, were performed using the online Database for Annotation, Visualization and Integrated Discovery. The protein-protein interaction (PPI) network was constructed using Search Tool for the Retrieval of Interacting Genes and was visualized by Cytoscape. The 100 upregulated and 83 downregulated DEGs were significantly enriched in the glycosaminoglycan biosynthesis-chondroitin sulfate and cell adhesion molecules pathways, respectively. A PPI network with 51 nodes and 84 interactions was constructed. DEGs in the PPI network were significantly enriched in the glycosaminoglycan biosynthesis-chondroitin sulfate pathway, in the regulation of mitosis process and in microtubule motor activity function. Centromere-associated protein E (CENPE), protein regulator of cytokinesis 1 (PRC1), phosphotyrosine picked threonine-protein kinase (TTK) and polo-like kinase 4 (PLK4) exhibited a high degree of connectivity in the PPI network. The glycosaminoglycan biosynthesis-chondroitin sulfate pathway, the regulation of mitosis biology process and microtubule motor activity are crucial for the progression of OS. The interaction between CENPE and PRC1 may play a pivotal role in the progression of OS. The interaction of TTK with PLK4 may play a key role in the proliferation of OS cells. These genes may be potential biomarkers for OS diagnosis and therapy.

Introduction

Osteosarcoma (OS) is the most common malignant tumor of the bone originating from osteoblasts, which mainly affects children or young adults (1). The incidence rate of OS worldwide is 4 cases per million individuals per year, and ~60% occur in patients <20 years of age (2). OS can arise in any bone, with typical symptoms including a history of pain, followed by localized swelling and limitations of joint movement, and the most common sites of primary tumors are the distal femur, proximal tibia and proximal humerus (3). The prognosis for patients with metastatic OS is poor, with a <20% survival rate (4). The cancer-related mortality caused by OS in adolescents is due to development of fatal metastasis, usually in the lungs (5). Currently, the main therapy for OS is conventional chemotherapy, but metastatic OS exhibits resistance to this (6-8). Consequently, a study of the molecular mechanisms of OS may provide further data for the development of novel targeted therapies for OS patients.

Currently, studies have found that glycogen synthase kinase-3β (GSK-3β), a serine/threonine protein kinase, has an oncogenic effect on OS cells, and that the inhibition of GSK-3β can result in the inhibition of the nuclear factor-κB (NF-κB) pathway, which can lead to the apoptosis of OS cells (9,10). The NF-κB transcription factor (TF) family plays an important role in the innate and adaptive immune responses, and is involved in cancer development (11). In an in vitro study, Tang et al showed that NF-κB inhibitors can suppress the growth of OS in human osteosarcoma (U2OS) cell lines (12). In addition, cyclin-dependent kinases (CDKs) are essential for cell cycle regulation and cell division, and play important roles in the development of OS by affecting numerous pathways, including those of cell cycle control (9,13). Moreover, it has been shown that CDK1 and CDK2 are required for the
duplication of the centrosome and spindle pole body, and the inhibition of CDK2 by CDK inhibitor can induce the apoptosis of OS cell lines (14-16). Collectively, the pathogenesis of OS is multifactorial and more attention should be focused upon it.

In the present study, in order to gain better insight into OS, differentially-expressed genes (DEGs) in OS were screened. Meanwhile, the functions of the DEGs were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). In addition, a protein-protein interaction (PPI) network of DEGs was constructed to investigate the key DEGs associated with the progression of OS in-depth. These key DEGs could be beneficial for uncovering the novel mechanism of OS at a molecular level and could be potential targets for the treatment of OS.

Materials and methods

Data sources. The gene expression profile of the GSE32395 dataset was obtained from the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) based on the GPL6244 [HuGene-1.0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version]. A total of 10 chips, including 8 OS cell lines (OS group) and 2 osteoblasts (control group) were evaluated in the dataset.

Data preprocessing and DEG screening. The gene expression matrix was obtained by pre-processing the raw data as implemented in the Bioconductor AFFY package (17). The data was preprocessed through a Robust Multichip Averaging algorithm, including background correction, normalization and probe summarization (18). Next, two-tailed Student’s t-tests were used to identify the DEGs between the OS and control groups (P<0.05; log2 fold change (FC)|≥2).

Gene functional annotation. The functional annotation of the DEGs was conducted to identify the TF based on the TRANSFAC database. In addition, oncogenes and tumor suppressor genes (TSGs) were also identified based on the TSG (19) and Tumor-Associated Genes (TAG) (20) database.

KEGG pathway and GO functional enrichment analyses of DEGs. KEGG pathway (21) and GO functional (22) analyses were performed to identify significantly enriched pathways and the functional terms of the DEGs, respectively, using the online tool of the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/) (23) with a P-value of <0.05. GO terms were identified under the categories of biological process (BP), cellular component (CC) and molecular function (MF).

PPI network construction. DEGs were submitted to the Search Tool for the Retrieval of Interacting Genes (STRING), version 9.1 (24). All interactions in STRING were provided with a probabilistic confidence score (combined score), and in the present analysis, only interactions with a combined score of >0.4 were retained. A PPI network was constructed using STRING and visualized in Cytoscape. The proteins in the network served as nodes and the degree of a node corresponded to the number of interactions with other proteins. The proteins with a high degree of interactions were considered as the hub nodes.

Results

DEGs between the OS and control groups. A total of 183 DEGs were identified, including 100 upregulated genes and 83 downregulated genes. Among these DEGs, there were 5 upregulated TFs (ZHX1, PBX1, NR1D1, MAFB and LEF1) and 2 downregulated TFs (SIM2 and HMGB2), as well as 8 upregulated TAGs (6 TSGs and 2 other genes that were uncertain on tumor development) and 7 downregulated tumor suppressor. The upregulated TSGs were UNCS5B, PARK2, MTSS1, MIR181B1, FBXO32 and ADAMTS9; the other upregulated genes were MAFB and CDR. The downregulated TSGs were TPM1, RBL1, PTPRJ, PPP2CB, PCDH10, IGFBP7 and DAB2.

KEGG pathway enrichment analysis of upregulated and downregulated DEGs. The upregulated DEGs were enriched in 2 pathways, namely the glycosaminoglycan biosynthesis-chondroitin sulfate and arrhythmogenic right ventricular cardiomyopathy pathways. Meanwhile, the downregulated DEGs were enriched in 12 pathways, which mainly involved pathways such as those of cell adhesion molecules (CAMs), pentose phosphate, allograft rejection, type I diabetes mellitus and the cell cycle. The significantly enriched pathways of upregulated and downregulated DEGs are listed in Table I.

GO term enrichment analysis of upregulated and downregulated DEGs. GO enrichment analyses were performed for the upregulated and downregulated DEGs. The top five GO terms are listed in Table II. The upregulated DEGs were significantly enriched in 92 BP terms, 8 CC terms and 28 MF terms. For the BP terms, the significantly enriched processes were mainly associated with extracellular matrix organization and cell adhesion. For the CC terms, the significantly enriched components were mainly associated with vesicles and collagen type XI. For the MF terms, the significantly enriched functions were mainly associated with peptide binding and tubulin binding. Meanwhile, the downregulated DEGs were significantly enriched in 58 BP terms, 18 CC terms and 21 MF terms. For the BP terms, the significantly enriched processes were mainly involved in cell division. For the CC terms, the significantly enriched components were mainly associated with the 6-phosphofructokinase complex and the oncostatin-M receptor complex. For the MF terms, the significantly enriched functions were mainly associated with peptide antigen binding and microtubule binding.

PPI network of DEGs. The PPI network, including 51 nodes and 84 interactions, was constructed (Fig. 1). The connectivity degree of each node in this PPI network was calculated and the nodes with a degree of ≥2 are listed in Table III. According to the degrees of the nodes, centromere-associated protein E (CENPE; also known as centromere protein E or kinesin-related protein), protein regulator of cytokinesis 1 (PRC1), phosphotyrosine picked threonine-protein...
Table I. KEGG pathway enrichment analysis of differentially-expressed genes.

| KEGG pathway                                      | Gene counts | P-value       |
|---------------------------------------------------|-------------|---------------|
| **Upregulated**                                   |             |               |
| Glycosaminoglycan biosynthesis-chondroitin sulfate| 2           | 6.21x10^-3    |
| Arrhythmogenic right ventricular cardiomyopathy   | 3           | 7.34x10^-3    |
| **Downregulated**                                 |             |               |
| Cell adhesion molecules                           | 5           | 2.9x10^-4     |
| Pentose phosphate pathway                         | 2           | 6.66x10^-3    |
| Allograft rejection                               | 2           | 1.2x10^-2     |
| Graft-vs.-host disease                            | 2           | 1.50x10^-2    |
| Type I diabetes mellitus                          | 2           | 1.64x10^-2    |
| Cell cycle                                        | 3           | 1.86x10^-2    |
| Autoimmune thyroid disease                        | 2           | 2.35x10^-2    |
| Natural killer cell-mediated cytotoxicity         | 3           | 2.37x10^-2    |
| Phagosome                                         | 3           | 3.21x10^-2    |
| Viral myocarditis                                 | 2           | 4.06x10^-2    |
| Adherens junction                                 | 2           | 4.38x10^-2    |
| Antigen processing and presentation               | 2           | 4.71x10^-2    |

KEGG, Kyoto Encyclopedia of Genes and Genomes; Gene counts, number of genes.

Figure 1. Protein-protein interaction network of DEGs in osteosarcoma. Red nodes represent upregulated DEGs; green nodes represent downregulated DEGs; grey lines stand for the interactions between two proteins. DEGs, differentially-expressed genes.
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kinase (TTK) and polo-like kinase 4 (PLK4; also known as serine/threonine-protein kinase 18 or serine/threonine-protein kinase Sak) were selected as the hub nodes, as they interacted with >10 nodes in the PPI network, suggesting their crucial roles in the network.

The DEGs in the PPI network were significantly enriched in 14 pathways, which included pathways involving glycosaminoglycan biosynthesis-chondroitin sulfate and natural killer cell-mediated cytotoxicity (Table IV). In addition, the DEGs in the PPI network were also significantly enriched in 81 BP terms, 22 CC terms and 30 MF terms. The top five GO terms for each are listed in Table V. For the BP terms, the significantly enriched processes were mainly associated with the regulation of mitosis and cell division. For the CC terms, the significantly enriched components were mainly associated with the spindle and major histocompatibility complex class I protein complex. For the MF terms, the significantly enriched functions were mainly associated with microtubule motor activity and chondroitin-glucuronate 5-epimerase activity.

Discussion

According to the gene expression profile analysis between the OS and control groups, 100 upregulated and 83 downregulated DEGs were obtained. The upregulated DEGs were significantly enriched in the glycosaminoglycan biosynthesis-chondroitin sulfate pathway and the multicellular

| GO ID             | Term                                      | Gene counts | P-value     |
|-------------------|------------------------------------------|-------------|-------------|
| GO:0044236_BP     | Multicellular organismal metabolic process| 5           | 5.24x10^-4 |
| GO:0035108_BP     | Limb morphogenesis                        | 5           | 7.66x10^-4 |
| GO:0007156_BP     | Homophilic cell adhesion                  | 5           | 8.18x10^-4 |
| GO:0048703_BP     | Embryonic viscerocranium morphogenesis    | 2           | 1.19x10^-3 |
| GO:0009636_BP     | Response to toxic substance               | 4           | 4.60x10^-3 |
| GO:0005794_CC     | Golgi apparatus                           | 16          | 3.90x10^-4 |
| GO:0070195_CC     | Growth hormone receptor complex           | 1           | 5.16x10^-3 |
| GO:0005592_CC     | Collagen type XI                          | 1           | 1.03x10^-2 |
| GO:0005899_CC     | Insulin receptor complex                  | 1           | 1.54x10^-2 |
| GO:0031982_CC     | Vesicles                                  | 11          | 2.13x10^-2 |
| GO:0005520_MF     | Insulin-like growth factor binding        | 3           | 2.58x10^-4 |
| GO:0008907_MF     | Integrase activity                        | 1           | 5.03x10^-3 |
| GO:0005509_MF     | Calcium ion binding                       | 9           | 6.23x10^-3 |
| GO:0043014_MF     | α-tubulin binding                         | 2           | 6.39x10^-3 |
| GO:0042277_MF     | Peptide binding                           | 4           | 7.32x10^-3 |
| GO:0010862_BP     | Positive regulation of pathway-restricted SMAD protein phosphorylation | 3 | 2.42x10^-4 |
| GO:0060391_BP     | Positive regulation of SMAD protein import into nucleus | 2 | 9.63x10^-4 |
| GO:0035335_BP     | Peptidyl-tyrosine dephosphorylation       | 3           | 1.32x10^-3 |
| GO:0007088_BP     | Regulation of mitosis                     | 4           | 1.52x10^-3 |
| GO:0051301_BP     | Cell division                             | 8           | 2.20x10^-3 |
| GO:0005819_CC     | Spindle                                   | 9           | 9.35x10^-7 |
| GO:0042612_CC     | MHC class I protein complex               | 2           | 9.44x10^-4 |
| GO:0005865_CC     | Striated muscle thin filament             | 2           | 2.03x10^-3 |
| GO:0005900_CC     | Oncostatin-M receptor complex             | 1           | 1.26x10^-2 |
| GO:0005945_CC     | 6-Phosphofructokinase complex             | 1           | 1.26x10^-2 |
| GO:0050699_MF     | WW domain binding                         | 2           | 4.91x10^-3 |
| GO:0042801_MF     | Polo kinase kinase activity               | 1           | 5.02x10^-3 |
| GO:0047757_MF     | Chondroitin-glucuronate 5-epimerase activity | 1           | 5.02x10^-3 |
| GO:0008017_MF     | Microtubule binding                       | 4           | 6.70x10^-3 |
| GO:0042605_MF     | Peptide antigen binding                   | 2           | 8.64x10^-3 |

GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; Gene counts, number of genes.
organismal metabolic process. The downregulated DEGs were significantly enriched in CAMs and the positive regulation of the pathway-restricted SMAD protein phosphorylation process. Next, the PPI network was constructed, and the pathways and functions of DEGs were enriched in the network. CENPE, PRC1, TTK and PLK4 were selected as the hub nodes, as they interacted with >10 nodes. DEGs in the PPI network were significantly enriched in the glycosaminoglycan biosynthesis-chondroitin sulfate pathway, regulation of mitosis process and microtubule motor activity functions.

These results showed that the upregulated DEGs and the DEGs of the PPI network were significantly enriched in the glycosaminoglycan biosynthesis-chondroitin sulfate pathway. Previous evidence has shown that glycosaminoglycan biosynthesis-chondroitin sulfate has relevance with cell proliferation and growth in human OS cells (25,26). The downregulated DEGs were significantly enriched in CAMs and positive regulation of pathway-restricted SMAD protein phosphorylation process in the present study. A previous study indicated that CAMs play an important role in the process of the metastasis of OS (27). The SMAD signaling pathway usually participates in a wide range of cancer cellular processes, including growth, proliferation, differentiation and apoptosis (28). In addition, the present results showed that the regulation of the mitosis biology process and microtubule motor activity molecular function were significantly enriched in the PPI network. Studies have indicated that microtubule motor activity plays an important role in the mitosis of OS cells to promote OS cell proliferation (29,30). The results of the present study were consistent with those of previous studies, which have demonstrated that the glycosaminoglycan biosynthesis-chondroitin sulfate pathway, regulation of mitosis biology process and microtubule motor activity are crucial for the progression of OS.

In the PPI network, the present study found that CENPE was the top node and interacted with 11 proteins, including PRC1. The two were closely associated with the biological process of cell division. CENPE is a mitotic motor whose inactivation disrupts spindle checkpoint function (31). CENPE localization has been found in U2OS cells (32). In addition, the incidence of lymphomas and lung adenomas is elevated in mice with a CENPE+/− background, indicating that CENPE plays an important role in tumorigenesis (33). PRC1, a microtubule-binding and -bundling protein, is essential for the formation of the central spindle and in cytokinesis (34). A previous study testified that PRC1 may play critical roles in tumor cell growth and be a promising target for the development of anticancer drugs for breast cancer (35). However, it has not been reported whether PRC1 could be a target for OS treatment. In the present study, we speculated that the interaction between CENPE and PRC1 may play a pivotal role in the progression of OS.

The PPI network in the present study also showed that TTK was a hub node with a higher degree that directly interacted with PLK4. TTK, also known as monopolar spindle-1 kinase, plays critical roles in centrosome duplication, the proper execution of mitosis and tumor cell proliferation (36-38). In addition, TTK, is a key regulator of the spindle assembly checkpoint, with significant overexpression found in a wide range of human tumors (39). Caldarelli et al (40) showed that selective TTK inhibitors can inhibit the proliferation of U2OS cells by promoting mitotic override. Moreover, TTK has been observed to affect the NF-κB signaling pathway (41,42), and NF-κB activation is prevalent in carcinomas, mainly driven by inflammatory cytokines within the tumor microenvironment (43). PLK4 is a member of the polo-like kinase family and a key regulator of centriolar duplication (44). In vitro evidence has demonstrated that the depletion of PLK4 with small interfering RNA contributes to the decrease in the cell proliferation of U2OS cells (43). In addition, previous studies have shown that the overexpression of PLK4 results in amplification of centrosomes, while its deletion reduces the centriole number in U2OS cells (45,46). Furthermore, PLK4 is a direct NF-κB target gene and NF-κB is absolutely required for cell proliferation in U2OS cells (47,48). For OS, Tang et al (12) indicated that the combination of NF-κB inhibitors and chemotherapy drugs can increase the effectiveness of the chemotherapy drugs in vitro and in vivo. Therefore, we considered that the interaction between TTK and PLK4 may play a crucial role in the centrosome duplication and proliferation of OS cells via the NF-κB pathway activation in OS.

### Table III. Differentially-expressed genes with >2 degrees of connectivity in the protein-protein interaction network.

| Gene     | Degrees |
|----------|---------|
| CENPE    | 11      |
| PRC1     | 11      |
| TTK      | 10      |
| KIF23    | 10      |
| ANLN     | 10      |
| PLK4     | 10      |
| FAM64A   | 8       |
| DBF4     | 8       |
| KIF20B   | 8       |
| DIAP3    | 7       |
| HMGB2    | 6       |
| HLA-B    | 5       |
| PARK2    | 4       |
| HLA-A    | 4       |
| TUBA4A   | 4       |
| SEPT5    | 3       |
| DSE      | 3       |
| TPM1     | 3       |
| NCAM1    | 3       |
| CDRI     | 3       |
| UST      | 2       |
| KDM6A    | 2       |
| COL4A1   | 2       |
| MYL9     | 2       |
| TAGLN    | 2       |
| GHR      | 2       |
| MICB     | 2       |

The PPI network in the present study showed that TTK was a hub node with a higher degree that directly interacted with PLK4. TTK, also known as monopolar spindle-1 kinase, plays critical roles in centrosome duplication, the proper execution of mitosis and tumor cell proliferation (36-38). In addition, TTK, is a key regulator of the spindle assembly checkpoint, with significant overexpression found in a wide range of human tumors (39). Caldarelli et al (40) showed that selective TTK inhibitors can inhibit the proliferation of U2OS cells by promoting mitotic override. Moreover, TTK has been observed to affect the NF-κB signaling pathway (41,42), and NF-κB activation is prevalent in carcinomas, mainly driven by inflammatory cytokines within the tumor microenvironment (43). PLK4 is a member of the polo-like kinase family and a key regulator of centriolar duplication (44). In vitro evidence has demonstrated that the depletion of PLK4 with small interfering RNA contributes to the decrease in the cell proliferation of U2OS cells (43). In addition, previous studies have shown that the overexpression of PLK4 results in amplification of centrosomes, while its deletion reduces the centriole number in U2OS cells (45,46). Furthermore, PLK4 is a direct NF-κB target gene and NF-κB is absolutely required for cell proliferation in U2OS cells (47,48). For OS, Tang et al (12) indicated that the combination of NF-κB inhibitors and chemotherapy drugs can increase the effectiveness of the chemotherapy drugs in vitro and in vivo. Therefore, we considered that the interaction between TTK and PLK4 may play a crucial role in the centrosome duplication and proliferation of OS cells via the NF-κB pathway activation in OS.
In conclusion, the present study analyzed the gene expression profile of OS using bioinformatics analysis and found that the glycosaminoglycan biosynthesis-chondroitin sulfate pathway, the regulation of mitosis biology process and microtubule motor activity are crucial for the progression of OS. The interaction between CENPE and PRC1 may play a pivotal role in the progression of OS. The interaction between TTK and PLK4 may play key roles in the centrosome duplication and proliferation of OS cells via NF-κB pathway activation in OS. These genes may all be potential biomarkers for OS diagnosis and therapy. However, further studies with a larger sample size and primary experiments with OS tissues and cells are required to confirm these results.
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