Identification of gene expression profiles associated with doxorubicin resistance in paired doxorubicin-resistant and doxorubicin-sensitive osteosarcoma cell lines

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Abstract: By investigating the differences in the gene expression patterns among paired doxorubicin-resistant and doxorubicin-sensitive human osteosarcoma cell lines and seeking novel associated genes using a human mRNA microarray, we identified that 3,278 mRNAs (1,607 up-regulated and 1,671 down-regulated) were aberrantly expressed in three sets of doxorubicin-resistant MG63/DXR cells and their corresponding parental MG63 cells (fold-change >2.0, P<0.05 and FDR<0.05). Eleven randomly selected mRNAs were confirmed by qRT-PCR and WB detection in three paired doxorubicin-resistant and doxorubicin-sensitive osteosarcoma cell lines (MG63 vs MG63/DXR, KH-OS vs KH-OS/DXR, U2-OS vs U2-OS/DXR), and the results were consistent with our microarray data. Bioinformatics analysis identified some novel genes and pathways related to the development of chemoresistance, including the RUNDC3B, ADAM22, ARMCX2, CRYAB and NOD-like receptor signaling pathway, the RIG-I-like receptor signaling pathway and more classical genes and pathways such as ABCB1, apoptosis-related pathways, the TNF signaling pathway, and chemokine signaling pathways. In addition, we found that RUNDC3B was distinctly increased in specimens of OS patients with a poor response to chemotherapy and that patients with reduced expression may survive longer than those with elevated expression, which suggests that RUNDC3B may be a possible biomarker to predict chemotherapeutic response and prognosis of osteosarcoma patients. These results provided valuable clues for future studies to discover possible novel targets to combat multi-drug resistance.

Keywords: Osteosarcoma, mRNA, drug-resistant, chemoresistance

Introduction

Osteosarcoma (OS) is the primary malignant bone tumor which has the highest incidence among children and adolescents [1]. The introduction of multi-agent chemotherapy followed by surgical resection and postoperative chemotherapy has improved the long-term survival of osteosarcoma patients from 20% to nearly 70%. However, the survival of patients who develop local recurrence, metastasis or multi-drug resistance has largely dropped to less than 20% [2]. Of them, patients who are resistant to common multi-agent chemotherapy regimens always present chemoresistance to the second-line chemotherapy agents and targeted agents, such as Sorafenib and Apatinib [3]. To clarify the underlying mechanism of chemoresistance in osteosarcoma, multiple genetic and molecular analyses have been performed. Considering the complex mechanism of multi-drug resistance in patients with OS, it is very important to select an appropriate model in vitro to elucidate the underlying mechanism [4, 5]. Thus, many types of drug-resistant osteosarcoma cell lines were established in various labs around the world. Two methods are commonly used—the first is a step-wise manner in which drug-sensitive cells are exposed to increasing doses of chemotherapy drugs, such as doxorubicin (DXR), cisplatin (DDP), methotrexate (MTX), and ifosfamide (IFO); the other method is to directly obtain primary cells from patients who developed terrible drug resistance. In addition, an increasing number of
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Table 1. Clinical parameters of osteosarcoma patients enrolled in this study

<table>
<thead>
<tr>
<th></th>
<th>Chemosensitive group</th>
<th>Chemoresistant group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>24</td>
<td>0.08</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>18.6 ± 0.4 (6-40)</td>
<td>19.2 ± 0.8 (8-36)</td>
<td>0.1</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>Proximal of</td>
<td>8</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Distal of Femur</td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Proximal of Tibia</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lung Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>18</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Follow-up time (m)</td>
<td>25.0 ± 1.2 (6-96)</td>
<td>22.4 ± 1.4 (3-80)</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Researchers have reported the presence of cross-drug resistance in established single-drug-resistant cell lines, which laid the foundation of multi-drug resistance (MDR) [6-8]. Previous studies have focused on the most important molecules, such as the ABC transporter family which controls the entry and efflux of drugs [9-12] or other genes and pathways related to the vital cell processes, including Bcl-2, CyclinD1 [13], IL-6 [14] or the MAPK, Notch, Wnt, and mTOR signaling pathways [15-17]. However, few studies have focused on the comprehensive changes of gene expression profile in cell lines during the process of acquiring multi-drug resistance.

The development of advanced technologies, including serial analysis of gene expression using microarrays or second-generation sequencing, have provided the means to identify integral gene expression patterns for a large number of tumor tissues and cell lines [18]. These approaches have been used to characterize genes whose altered expression is key in tumor development and behavior. In addition, extensive GO analysis can be used to identify the molecular signature of the differentially expressed genes based on the gene expression array profile. Subsequent pathway analyses with the resulting gene lists can reveal distinct signaling pathways that might account for the observed biological properties [19, 20].

In this study, we conducted a comprehensive analysis of mRNAs in the human doxorubicin-resistant osteosarcoma cell line MG63/DXR and its corresponding MG63 parental cell line using microarray analysis. We then validated the expression of some randomly selected differentially expressed genes and predicted their possible functions or molecular mechanism using bioinformatics. These findings may provide critical understanding to the mechanism of drug-resistance in osteosarcoma, and may reveal new therapeutic targets.

Materials and methods

Cell lines and cell culture

The MG63 human osteosarcoma cell line (American Type Culture Collection, ATCC, No. CRL-1427) was cultured in Dulbecco’s modified Eagle’s medium (advanced DMEM, Gibco, Cat. 12491-015, California, USA) supplemented with 10% fetal bovine serum (Gibco, Gran Island, NY, USA). The doxorubicin-resistant osteosarcoma cell line MG63/DXR, which was kindly provided by Dr. Yoshio Oda [21], was selected in a step-wise manner by exposing drug-sensitive MG63 cells to increasing doses of doxorubicin (DXR). The paired KH-OS and KH-OS/DXR cells were kindly donated by Dr. Efstathios S. Gonos (National Hellenic Research Foundation, Athens, Greece), which used the same method for selection. The paired U2-OS and U2-OS/DXR were a generous gift from Dr. Duan ZF [22] (Massachusetts General Hospital and Harvard Medical School, Boston, USA) and were produced using the same selection method. The surviving cells were then cultured in the conditioned medium containing with 1 μg/ml DXR (Sigma-Aldrich, Cat. No. D1515, Missouri, USA) to retain their drug-resistant phenotype.

Collection and histological evaluation of clinical samples

A total 60 of patients’ specimens were used in this study (Table 1). All patients received the same multidrug chemotherapy before surgery. The 60 primary osteosarcoma tissues were obtained from patients who underwent complete resection at the Shanghai Tenth People’s Hospital between 2006 and 2014. Informed consents were obtained from all patients. After preoperative chemotherapy, the tumors were
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CCK-8 assay

Cell suspensions were prepared, and the number of cells was counted using a cell counting board. Cells were plated in 96-well plates at a density of 2,000 cells per well. After 24 hours, doxorubicin (DXR), cisplatin (DDP), methotrexate (MTX), or ifosfamide (IFO) was added at different concentrations. Each concentration was repeated in triplicate. Meanwhile, the same volume of conditioned medium was added to the control wells. After a 48-h incubation at 37°C, 10 μL of CCK-8 was added to each well for another 2-h. The absorbance value (OD) at 450 nm was measured using an enzyme-linked immunological detector to calculate the viability rate and draw the related curves. The results shown are representative results from three independent experiments (Table 3).

RNA isolation, labeling and array hybridization

Total RNA was extracted from the three groups of paired doxorubicin-resistant osteosarcoma MG63/DXR cells and the parental MG63 cells with TRizol reagent (Invitrogen) according to the manufacturer’s protocols. The concentration, purity, and integrity of the RNA samples were determined by the ratio of UV absorbance at 260 nm to 280 nm and by electrophoresis. Total RNA was repurified with an RNaseasy MinElute Kit according to manufacturer’s protocols (https://www.arraystar.com). The microarray analysis was performed by KangChen Biotech (Shanghai, China).

Bioinformatics analysis

GO analysis was applied to analyze the primary functions of the differentially expressed genes according to the GO database (www.geneontology.org). Pathway analysis was used to determine the significant pathways of the different genes according to the KEGG database (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg).

Real-time RT-PCR

Total RNA from cultured cell lines and human tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocols. Primers (Table 2) for real-time RT-PCR were designed using Primer Express v 2.0 software (Applied Biosystems, Foster, CA, USA). RT was performed using the SuperScript First-Strand Synthesis System for RT-PCR.

Table 2. Primers used for PCR validation

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward and Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNDC3B</td>
<td>F: 5’ ACCAGTTATCAGCAGAAGTTAGC 3’ R: 5’ GGCCAAGTATTAGGGAGGATATCT 3’</td>
</tr>
<tr>
<td>ABCB1</td>
<td>F: 5’ GGAGTTGTCCAGTATGTGGGA 3’ R: 5’ GCATCTGTCGGTGATATCTGTTA 3’</td>
</tr>
<tr>
<td>RASGRP2</td>
<td>F: 5’ CAGCTAACGACATGACAGCG 3’ R: 5’ GGAATTTCTGTTGTTTTCTG 3’</td>
</tr>
<tr>
<td>AMX2</td>
<td>F: 5’ GCCCGGATGTTGAGGAGGT 3’ R: 5’ GCTACGTGATGTTCTGTTA 3’</td>
</tr>
<tr>
<td>CCK-8</td>
<td>F: 5’ AGGTGTGGGAGGATGTTGTA 3’ R: 5’ GGAATTTCTGTTGTTTTCTG 3’</td>
</tr>
<tr>
<td>ABCB1</td>
<td>F: 5’ AGGCTGCTGAGTCCCTGCTGTCAC 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>C3orf14</td>
<td>R: 5’ GAGTCGCGGTAAAGCTGTCAC 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>C8orf48</td>
<td>F: 5’ CACAGAAGGTCTGGTTGCTGTCT 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>MAGEH1</td>
<td>R: 5’ AGGATCGGCAAGTTGCTGTCAC 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>LSP1</td>
<td>R: 5’ AGGACAGCTGACCTAACCAGG 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>FABP5</td>
<td>R: 5’ CTGGCTGTATTGCTGCTGCTGTCAC 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>ADAM22</td>
<td>R: 5’ GCAGAGCGGTCACTGGGATGAGG 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>R: 5’ ACATGAGATGCAACAGACGCT 3’ R: 5’ GCTAGGTTGCTGCTGCTGCTGTCAC 3’</td>
</tr>
</tbody>
</table>

Table 3. IC50 for MG63 and MG63/DXR

<table>
<thead>
<tr>
<th>IC50 (ug/ml)</th>
<th>MG63</th>
<th>MG63/DXR</th>
<th>R factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (DXR)</td>
<td>0.4</td>
<td>10.2</td>
<td>23.2</td>
</tr>
<tr>
<td>Cisplatin (DDP)</td>
<td>0.4</td>
<td>5.2</td>
<td>13</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>1.2</td>
<td>4.4</td>
<td>3.7</td>
</tr>
<tr>
<td>Ifosfamide (IFO)</td>
<td>1.5</td>
<td>8.6</td>
<td>5.7</td>
</tr>
</tbody>
</table>
Gene expression profiles associated with doxorubicin resistance in osteosarcoma

According to the manufacturer’s protocol. Real-time PCR was performed using SYBR Green I (Applied Biosystems). The data were normalized to the arithmetic mean of housekeeping gene GAPDH and calculated using the 2-ΔΔCT method. The primer sequences are listed in Table 2.

Western blot

Cells were collected, and cell lysis buffer (ThermoFisher Scientific, California, USA) supplemented with the appropriate protease inhibitors (Invitrogen Inc, CA, USA) was used according to the manufacturer’s protocol to extract total protein. After the protein concentration was determined using a NanoDrop 2000 microspectrophotometer, 5 × loading buffer was added and mixed to the appropriate volume of sample. Then, the samples were boiled for 10 min at 100°C until the protein was fully denatured, after which samples were stored at -20°C. Equal quality of proteins as well as protein markers was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime Biotechnology Co., Shanghai, China), after which the proteins in the gel were transferred onto a poly-vinylidene fluoride (PVDF) membrane (Merck Millipore Corporation, Billerica, Massachusetts, USA). After blocking with 5% skim milk for 1 h, the PVDF membranes were incubated with primary antibodies (Abcam, MA, USA) overnight at 4°C. The following day, the supernatant was removed, and the samples were washed three times with Tris-buffered saline containing tween (TBST). Then, the membranes were incubated with secondary antibodies (Abcam, MA, USA) at room temperature for 1 h and washed three times with TBST. Finally, the PVDF membranes were incubated with an enhanced chemiluminescence (ECL) working solution in a darkroom. The light emission was detected by X-ray to determine the presence of a protein band, which can reflect the relative expression of the protein.

Figure 1. CCK-8 assay. The CCK-8 assay was performed to verify that MG63/DXR cells were more resistant to doxorubicin, cisplatin, methotrexate, and ifosfamide than MG63 cells. The IC50 values of these compounds in MG63/DXR cells were 10.2 μg/ml, 5.2 μg/ml, 4.4 μg/ml, and 8.6 μg/ml respectively, whereas those in MG63 cells were 0.44 μg/ml, 0.4 μg/ml, 1.2 μg/ml, and 1.5 μg/ml respectively.
Statistical analysis

The expression levels of mRNAs that were differentially expressed between human doxorubicin-resistant osteosarcoma MG63/DXR cells and the parental MG63 cells were compared by the paired, two-tailed t-test using the SPSS 20.0 software package (SPSS, Chicago, IL). Overall survival was calculated by Kaplan-Meier survival analysis and compared using the log-
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rank test. All of the data are shown as the means ± SD of three independent experiments. P<0.05 was considered statistically significant.

Results

CCK-8 assay

Drug resistance of the MG63/DXR cell line was identified by comparing the IC50 values in MG63/DXR cells with those in the MG63 cell line. As shown in Figure 1, when exposed to doxorubicin for 48 h, the IC50 value in MG63/DXR cells was 10.2 μg/ml, whereas that in MG63 cells was 0.4 μg/ml. In addition, when exposed to cisplatin, methotrexate or ifosfamide for 48 h, the IC50 values in MG63/DXR cells were 5.2 μg/ml, 4.4 μg/ml, and 8.6 μg/ml respectively, whereas those values in MG63 cells were 0.4 μg/ml, 1.2 μg/ml, and 1.5 μg/ml respectively. The resistance factors (R factor) were 23.2, 13, 3.7, and 5.7 for doxorubicin, cisplatin, methotrexate, and ifosfamide, respectively (Table 3). Obviously, the MG63/DXR cells were more resistant to the four common chemotherapeutic drugs than MG63 cells, laying a solid foundation for further study.

Differentially expressed mRNAs and their chromosome distribution

In total, 3,278 mRNAs in the paired MG63/DXR and MG63 cells showed statistically significant differences in expression (P<0.05; fold-change >2). Among these differentially expressed mRNAs, 1,607 mRNAs were found to be up-regulated more than 2-fold in MG63/DXR cells than in MG63 cells, while 1,671 were down-regulated more than 2-fold in MG63/DXR cells (P<0.05). We used a hierarchical clustering analysis to arrange the samples into groups based on their expression levels, which allowed us to hypothesize the relationships among the identified genes (Figure 2A). The resulting volcano map shows the relationships of the differently expressed mRNAs (Figure 2B) between the samples. TSPYL5 (fold change: 1217.0948879) was the most up-regulated mRNA, and ARMCX2 (fold change: 1624.4826-651) was the most down-regulated mRNA.

Chromosomal localization was established to determine the chromosomal patterns of the overall differentially expressed mRNAs based on the microarray data (Figure 2C and 2D). The differentially regulated 3,278 mRNAs were distributed to every chromosome. Among them, chromosome 14 had the most up-regulated mRNAs (214) and chromosome 1 had the most down-regulated mRNAs (262). However, chromosomes Y and 8 contained the fewest number of up-regulated and down-regulated mRNAs, respectively (2 and 8), which implies that mRNAs transcribed from these chromosomes could play a vital role in the occurrence of chemoresistance in OS.

GO and pathway analysis

Based on the primary data, GO enrichment analysis further classified the differentially expressed mRNAs into three categories: biological processes, cellular components and molecular functions. We found that the dysregulated transcripts were associated with heart development and extracellular matrix organization (ontology: biological processes), intracellular molecules and cytoplasm (ontology: cellular components), and catalytic activity and oxidoreductase activity (ontology: molecular function) (Figure 3). There were 278 dysregulated genes involved in biological processes, 152 genes involved in cellular components and 125 genes involved in molecular functions.

Further pathway analysis, which is based on the differential genes compared to the KEGG database, found that 31 pathways were significantly enriched among the up-regulated mRNAs and that 25 pathways were enriched among the down-regulated mRNAs. ‘Glutathione metabolism-Homo sapiens (human)’ and ‘Transcriptional dysregulation in cancer-Homo sapiens (human)’ were the most enriched networks, respectively. In addition, some of these pathways, such as the apoptosis-related pathway, TNF signaling pathway, and chemokine signaling pathway, have been reported to be involved in the development of drug resistance in osteosarcomas [27-30] (Figure 4). In addition, some novel pathways have been correlated to the occurrence of chemoresistance, including the NOD-like receptor signaling pathway and the RIG-I-like receptor signaling pathway.

Real-time RT-PCR validation and Western blot validation

To further verify the reliability of the microarray data, 11 mRNAs were randomly selected and subjected to real-time PCR and Western blot in three sets of doxorubicin-resistant and doxorubicin-sensitive osteosarcoma cell lines (MG63
Figure 3. GO analysis of the dysregulated mRNAs. The results of GO analysis of up-regulated mRNAs from our dataset are shown in (A). The results of GO analysis of down-regulated mRNAs are in (B).
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Figure 4. Pathway analysis of the differentially expressed profiles. The ‘Apoptosis related pathway’ (A) and “NOD-like receptor signaling pathway” (B) are associated with chemoresistance of osteosarcoma. The genes labeled “yellow” are differentially expressed between MG63/DXR and MG63 cells and are involved in the processes.
Figure 5. Differential expression of mRNAs was validated by qPCR and WB. Comparison of the microarray data and quantitative real-time PCR results. A. Eleven differentially expressed mRNAs (four up-regulated and seven down-regulated) were verified by qPCR in three sets of drug-resistant and drug-sensitive osteosarcoma cell lines, and the results are shown in the histogram. The qPCR results were in accordance with the microarray data. B. Three up-regulated genes (RUNDC3B, ABCB1, and ADAM22) and two down-regulated genes (ARMCX2 and CRYAB) were verified by WB in the same three paired cell lines. The results are in accordance with the microarray and qPCR data.
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Figure 6. RUNDC3B expression in OS tissue and its potential clinical significance. A. The expression of RUNDC3B in specimens from OS patients who had a poor chemotherapeutic response was approximately five fold that of specimens from patients with a good chemotherapeutic response (7.2 ± 0.3 vs 1.4 ± 0.1). B. The survival time of the patients in the RUNDC3B-high group was 22.2 ± 1.8 months and those in the low expressing group was 52.6 ± 1.4 months, which may suggest the potential significance of this gene.

Western blotting was used to verify the accuracy of the microarray at the protein level. We randomly choose up-regulated mRNAs, including RUNDC3B, ABCB1, and ADAM22, and down-regulated mRNAs, including ARMCX2 and CRYAB. The results showed that the expression of the five selected genes (including the classical multi-drug resistance related gene ABCB1) were all significantly dysregulated in the paired resistant and sensitive osteosarcoma cell lines, which reflects the results of the microarray (Figure 5B).

Potential clinical significance of RUNDC3B in osteosarcoma

To explore the potential clinical significance of these selected genes in osteosarcoma, the expression level of RUNDC3B was examined using real-time RT-PCR in 60 primary osteosarcoma tissue samples, which were divided into two groups: the chemosensitivity group (N=30) and chemoresistance group (N=30). The baseline between the two groups was comparable (Table 1). We used the median expression levels of RUNDC3B as a cut-off to stratify the 60 patients into the RUNDC3B-high group (n=38) and the RUNDC3B-low group (n=22). As shown in Figure 6A, our data indicated that RUNDC3B expression in the chemoresistance group was approximately five fold greater than that in the chemosensitivity group. In addition, Figure 6B shows that patients with lower expression of RUNDC3B may survive longer than those with higher expression (52.6 ± 1.4 months vs 22.2 ± 1.8 months), which suggests that RUNDC3B may be a biomarker to predict the chemotherapeutic response and prognosis of osteosarcoma patients. However, this theory should still be examined and verified with more methods and more clinical samples of osteosarcoma.

Discussion

Chemotherapy has been a very important adjuvant therapy in treating osteosarcoma. However, the development of multi-drug resistance has greatly impeded its clinical application [23, 24]. In recent decades, with the help of established multidrug-resistant models, the underlying mechanism of chemoresistance in
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Osteosarcoma has been extensively investigated from different aspects in molecular biology [3]. Multidrug resistance mechanism in OS include the following [25]: 1) increased efflux of drugs through ATP-binding cassette transporters, 2) decreased drug accumulation in the cell mediated by lower RFC, 3) contribution of PKC in drug efflux, 4) APE1- or ERCC-mediated repair of DNA damage, 5) inhibition of the activity and structural changes of DNA topoisomerase II, 6) detoxification in the cell by GSTP1, 7) inhibition of apoptosis by p53, Bcl-2, or miRNAs, and 8) osteosarcoma stem cells.

Many important genes have been reported to be involved in the drug resistance of osteosarcoma. For example, Jia M et al. [26] found that Trps1 was associated with the multidrug resistance of osteosarcoma by regulating MDR1 gene expression. He Z et al. [27] found the overexpression of MRP4 was related to multidrug resistance in osteosarcoma cells. Roncuzzi L et al. [28] found that HIF-1α activation was involved in the doxorubicin resistance of human osteosarcoma cells. Wang JJ et al. [29] reported a relationship between RFC gene expression and the intracellular drug concentration in methotrexate-resistant osteosarcoma cells. Wang D et al. [30] found that human apurinic endonuclease 1 (APE1) could enhance the sensitivity of osteosarcoma to DNA-damaging agents.

To overcome and reverse multidrug resistance in OS, many methods targeting key molecules or signal transduction pathways have been established, including siRNA, small bioactive molecules, and extracts based on Chinese medicine. For example, Fanelli M et al. [31] found that targeting ABCB1 and ABCC1 with their specific inhibitor CBT-1 can overcome drug resistance in osteosarcoma. Duan Z et al. [32] found that A-770041, a small biological molecule, could reverse paclitaxel and doxorubicin resistance in osteosarcoma cells. Wang D et al. [30] found that human apurinic endonuclease 1 (APE1) could enhance the sensitivity of osteosarcoma to DNA-damaging agents.

To explore changes in the comprehensive gene profile between the doxorubicin-resistant and doxorubicin-sensitive human osteosarcoma cell lines MG63/DXR and MG63, the mRNA expression profiles of these cells were investigated using a microarray analysis. We analyzed three sets of human primary MG63/DXR osteosarcoma drug-resistant cells and their MG63 parental drug-sensitive cells and identified (fold change >2.0) that a total of 3,278 mRNAs were differently expressed in drug-resistant MG63/DXR cells, including 1,607 up-regulated and 1,671 down-regulated mRNAs relative to the expression in MG63 cells. Additionally, the 3,278 differentially regulated mRNAs were distributed along every chromosome. There were 11 mRNAs (4 up-regulated and 7 down-regulated) randomly chosen to undergo qRT-PCR and Western blotting to validate the consistency of our results in three paired doxorubicin-resistant and doxorubicin-sensitive osteosarcoma cell lines (MG63 vs MG63/DXR, KH-OS vs KH-OS/DXR, U2-OS vs U2-OS/DXR). In addition, we examined the expression of RUNDC3B in 30 pairs of osteosarcoma patient samples to explore the potential clinical significance of this gene.

Among the 11 validated genes that were differentially expressed in the paired cell lines, we found that RUNDC3B and ADAM22 showed markedly higher expression in the drug-resistant osteosarcoma cell lines, which was similar to previous reports of another critical gene related to multi-drug resistance-ABCB1. There are no reports describing either RUNDC3B or ADAM22 and osteosarcoma. Actually, RUNDC3B is also known as RPIB9 or RPIP9, and its expression has been found to be closely related to ABCB1 and dual regulation of P-glycoprotein expression by trichostatin A in cancer cell lines [35]. In addition, ADAM22 plays an important role in insulin sensitivity and endocrine-resistance [36, 37]. Bolger JC et al. [38] found that ADAM22 was a prognostic and therapeutic drug target in the treatment of endocrine-resistant breast cancer. Furthermore, the other two down-regulated genes validated by qRT-PCR and WB, ARMCX2 and CRYAB, have also been reported in the development of drug resistance in other tumors. For example, Zeller C et al. [39] found that ARMCX2 exhibited acquired methylation in drug-resistant ovarian cancer cells and may be a clinical biomarker of drug resistance. Wittig R et al. [40] found that CRYAB is among the 14 genes.
that were differentially expressed in three different drug-resistant malignant melanoma cell lines and that CRYAB is a strong influence in developing resistance against DNA-damaging drugs. However, there are few reports about the function of these genes in the chemoresistance of osteosarcoma. In our study, we found that these genes were stably dysregulated in the paired drug-resistant and drug-sensitive osteosarcoma cell lines and further revealed the potential clinical significance of RUNDC3B in osteosarcoma patients. It is possible that these genes are also important for the development of drug resistance in OS.

By using bioinformatics analyses such as GO and pathway analyses, we identified the biological functions that are enriched among the differentially expressed mRNAs. GO analysis revealed that these genes were primarily involved in cell components and basic metabolic processes, which may suggest the importance of metabolic processes in regulating the chemoresistance of osteosarcoma. Pathway analysis showed that there were 31 pathways corresponding to all the up-regulated transcripts and 25 pathways corresponding to all the down-regulated transcripts. Among these pathways, classical signaling pathways such as apoptosis-related pathways, the TNF-signaling pathway, and chemokine signaling pathways have been reported to be involved in the acquisition of drug resistance in osteosarcoma, which may indicate the reliability of our results. In addition, we have identified some obscure pathways that may be involved in drug resistance of osteosarcoma, such as the NOD-like receptor signaling pathway and RIG-I-like receptor signaling pathway, which may provide novel directions in researching the underlying mechanism of resistance.

Our present study shows a set of mRNAs with differential expression in the human doxorubicin-resistant osteosarcoma cell line MG63/DXR and its sensitive parental cell line MG63. Furthermore, our study revealed that the up-regulated genes RUNDC3B and ADAM22 as well as the down-regulated genes ARM CX2 and CRYAB may be involved in the regulation of drug sensitivity of osteosarcoma cell lines. In addition, we identified some novel pathways related to the development of chemoresistance, including the NOD-like receptor signaling pathway. Furthermore, RUNDC3B may be a potential biomarker to predict the chemotherapeutic response and prognosis of osteosarcoma patients. These results provide the groundwork for future studies. A deeper understanding of these transcripts and their role in the formation of osteosarcoma chemoresistance are necessary to discover possible novel targets for reversing multi-drug resistance.

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Disclosure of conflict of interest

None.

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