Potential Molecular Mechanisms of AURKB in the Oncogenesis and Progression of Osteosarcoma Cells: A Label-Free Quantitative Proteomics Analysis

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Abstract
Our previous study indicated that knockdown of Aurora-B inhibit the proliferation of osteosarcoma cells. But the function and molecular mechanisms of Aurora-B in osteosarcoma cells growth and metastasis remains unclear. The aim of this study was to investigate the molecular mechanisms of Aurora-B in the progression of osteosarcoma. Osteosarcoma cells (U2-OS and 143B) were treated with specific Lentivirus-Vectors (up or downregulation Aurora-B). The ability of cells proliferation, migration, and invasion was measured using Cell-Counting Kit-8, wound healing and transwell invasion assays. Furthermore, based on label-free quantitative proteomic analysis of potential molecular mechanisms of Aurora-B in human 143B cells. A total of 25 downregulated and 76 upregulated differentially expressed proteins were screened in terms of the change in their expression abundance. We performed functional annotation and functional enrichment analyses. Gene ontology enrichment, KEGG analysis, and protein–protein interaction networks were constructed and analyzed. We found that the PTK2 may play an important role in the progression of osteosarcoma cells. Finally, Western blot revealed that expression of PTK2, AKT, PI3K, and nuclear factor-kappaB increased after over expression of Aurora-B. Overall, these data highlight that Aurora-B may promote the malignant phenotype of osteosarcoma cells by activating the PTK2/PI3K/AKT/nuclear factor-KappaB pathway.

Keywords
osteosarcoma, AURKB, proteomics

Abbreviations
AURKB, Aurora-B; BP, biological process; DEPs, differentially expressed proteins; GO, gene ontology; HCA, hierarchical cluster analysis; MF, molecular function; OS, osteosarcoma; PPI, protein–protein interaction.

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Introduction
Osteosarcoma (OS) is the most frequent human primary malignant bone tumor, occurring frequently in children and adolescents. The 5-year survival rate for OS patients has been recorded at 55% to 80% after effective chemotherapy. In addition, the analysis of previous studies showed that the 5-year survival rate of metastatic patients is still less than 20%. Pulmonary metastasis is the main cause of death for the patients with extremities OS. Therefore, identifying the molecular mechanisms of metastasis of OS is necessary for the development of operational the therapeutic strategies.
Aurora-B (AURKB), a serine/threonine kinase, ensures the proper execution and fidelity of mitosis. Aurora-B is a member of the chromosomal passenger complex, which has been involved in various mitotic processes, including chromosome–microtubule interactions, mitotic spindle assembly/checkpoint, sister chromatid cohesion.5,6 Recent studies indicates that AURKB, an important antitumor target, is strongly associated with metastasis of numerous tumor types.7,8 Our previous studies revealed that knockdown of AURKB suppressed OS cells migratory, proliferation and invasion in vitro.9,10 However, whether AURKB takes a role in OS metastasis and what is the mechanism is not yet clear.

In the present study, we confirmed that AURKB promote malignant phenotype of OS cells in vitro. Furthermore, we analyzed proteome alterations in 143B cells of upregulated AURKB group (U) and negative group (C). For this purpose, we utilized label-free quantitative proteomics technology11 to quantify/visualize the changes in proteins expression abundance and identify differentially expressed proteins (DEPs) in the 2 groups of 143B cells. We performed functional annotation and functional enrichment analyses. Finally, gene ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, and protein–protein interaction (PPI) networks were constructed and analyzed. We sought to explore the potential genes and pathways related to AURKB in osteosarcoma and to improve the role of AURKB in osteosarcoma, so as to provide some new ideas for the study of osteosarcoma. The findings of this study are likely to play a momentous role in osteosarcoma genesis and may provide new biomarkers for the diagnosis and treatment of osteosarcoma.

Materials and Methods

Cell Culture

The U2-OS and 143B cell lines were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc, Waltham, Massachusetts) and were cultured at 37°C in 5% CO2.

Plasmids and Transfection

The Lentivirus-Vectors were compound by GENECHEM (Shanghai, China). Osteosarcoma cells (U2-OS and 143B) were transfected with Lentivirus-Vectors of Lentivirus-Vectors of downregulating AURKB (LV/ShAURKB), upregulating AURKB (LV/AURKB), negative Lentivirus-Vectors (NC-LV/AURKB and NC-LV/ShAURKB).

Western Blot Analysis

Total protein from 143B cells and U2-OS cells were extracted using BBproExtraTM (BB-3101). And protein concentration was determined using the Bicinchoninic acid assay (BCA) (Applygen Technologies Inc, Beijing, China). Proteins were separated by 8% SDS-PAGE (10 µg/lane) and transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk (Solarbio Science & Technology Co, Ltd., Beijing, China) for 1 hour at room temperature, the membranes were incubated overnight with the following specific/primary antibodies: rabbit monoclonal AURKB (EP1009Y; 1:50 000; Abcam, Cambridge, Massachusetts), rabbit monoclonal PI3K (EPR18702; 1:1000; Abcam), rabbit monoclonal PI3K (Tyr458; 1:1000; Cell Signaling Technology Inc, Boston, USA), rabbit monoclonal AKT (EPR18405; 1:2000; Abcam), rabbit monoclonal AKT (phospho T308; abb38449; 1:1000; Abcam), rabbit monoclonal nuclear factor-Kappa B (NF-κB, E379; 1:50 000; Abcam), rabbit monoclonal PTK2 (EP695Y; 1:1000; Abcam), and monoclonal mouse β-actin (TA-09; 1:2000; ZSGB-BIO, Inc., Beijing, China) at 4°C. The membrane was washed 3 times with TBST. The membranes were then incubated with secondary antibody for 1 hour at room temperature. The expression of indicated proteins were visualized using an EasySee Western Blot kit (Transgen, Inc., Beijing, China). The intensity of bands was measured using ImageJ version 1.48 software (National Institutes of Health, Bethesda, Massachusetts).

RNA Isolation and Quantitative Polymerase Chain Reaction

Trizol (Invitrogen) method was used to extract the total RNA from OS cells. The expression level of AURKB was evaluated by quantificational real time-PCR. And GAPDH was used as the endogenous reference genes. Primer sequences used to amplify the containing were as follows: AURKB sense 5-AGAAGGAGA-GAATCTCCTACCCCT-3’, AURKB antisense 5-CGCCTAAGAGATGCAGGGTG-3’, GAPDH sense 5-CAGGGGCTGC-3, GAPDH antisense 5-GTATTTGGGAGGG ATCTCGCT-3’. The amplification reaction was performed using StepOne Real-Time PCR System for 40 cycles. The data were analyzed using the 2−ΔΔCt method.

Cell-Counting Kit-8 Assay

The proliferation of OS cells was evaluated by a Cell-Counting Kit-8 (CCK-8, TransGen, Inc., Beijing, China) assay. Osteosarcoma cells (4000/well) were seeded out in 96-well plates for 0 hour, 24 hours, and 48 hours, respectively. Then, 10 µL of CCK-8 solution was added to each well, and incubated for an additional 2 hours at 37°C. The absorbance of each wells was measured at 450 nm with a microplate reader. Three independent experiments were performed over multiple days.

Transwell Invasion Assays

We used the BD BioCoat BD Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, New Jersey) to analyze the cell’s invasion ability according to the manufacturer’s protocol. The images of OS cells were captured at 24 hours. The invaded OS cells were then fixed and stained with 0.1% crystal violet. Three randomly selected fields per membrane were photographed, and
cell counting was measured by Image J software. Three independent experiments were conducted over multiple days.

**Migration Assays**

Osteosarcoma cells were grown to confluence in 6-well plates to a density of approximately $5 \times 10^6$ cells/well. Osteosarcoma cells were denuded by dragging a rubber policeman (Fisher Scientific, Hampton, New Hampshire). Then we added fresh quiescent medium alone or containing 10% FBS into the dishes and put the dishes into the incubator ($37^\circ$C for 24 hours). Images were captured at 0 hours and 24 hours using an ECLIPSE TS 100 microscope (magnification, $\times 200$; Nikon, Tokyo, Japan), and the migrated distance was measured by...
Table 1. Representative DEPs (>1.50-fold/<0.67-fold) Identified by Label-Free Quantitative Proteomic in 143B Cells.

| Gene Name | Accession | KEGG | STRING | Fold Change | P Value |
|-----------|-----------|------|--------|-------------|---------|
| PTK2      | Q05397/FAK1_HUMAN | hsa:5747 | 9606.ENSP00000341189 | 2.51 | .024717241 |
| NFKB2     | Q00653/NFKB2_HUMAN | hsa:4791 | 9606.ENSP00000358983 | 2.51 | .000680994 |
| CAT       | P04040/CATA_HUMAN | hsa:847 | 9606.ENSP00000241052 | 1.66 | .07164393 |
| ALB       | P02768/ALBU_HUMAN | hsa:213 | 9606.ENSP00000295897 | 4.41 | .002070141 |
| SH3PXD2B  | A1X283/SPD2B_HUMAN | hsa:285590 | 9606.ENSP00000309714 | 3.48 | .014927944 |
| UBR5      | Q95071/UBR5_HUMAN | hsa:51366 | 9606.ENSP00000429084 | 3.44 | .010616956 |
| MMS19     | Q96726/MMS19_HUMAN | hsa:64210 | 9606.ENSP00000359818 | 1.97 | .019678863 |
| CCNB1     | P14635/CCNB1_HUMAN | hsa:891 | 9606.ENSP00000256442 | 1.91 | .015066071 |
| ARF3      | P61204/ARF3_HUMAN | hsa:377 | 9606.ENSP00000256682 | 1.54 | .060280584 |
| SRSF2     | Q01130/SRSF2_HUMAN | hsa:6427 | 9606.ENSP00000353089 | −1.58 | .006471426 |
| SRSF1     | Q07955/SRSF1_HUMAN | hsa:6426 | 9606.ENSP00000258962 | −1.73 | .0204472 |
| SRSF5     | Q13243/SRSF5_HUMAN | hsa:6430 | 9606.ENSP00000377892 | −1.5 | .014723125 |
| SRRM1     | Q81YB3/SRRM1_HUMAN | hsa:10250 | 9606.ENSP00000326261 | −1.56 | .045394162 |
| RBM22     | Q9NW64/RBM22_HUMAN | hsa:55696 | 9606.ENSP00000199814 | −1.63 | .025822602 |

**Protein Preparation**

Lysis buffer (2% SDS, 7 M urea, 1× Protease Inhibitor Cocktail (Roche Ltd, Basel, Switzerland) was used to lyse cells and extract proteins. The BCA protein assay was used to determine the protein concentration. Add 11 μL of 1 M DTT and incubate sample at 37°C for 1 hour. 100 Mm of TEAB was added into the samples to remove urea. After adding 120 μL of 55 mM iodoacetamide, the sample was incubated for 20 minutes protected from light at room temperature. And then, the above proteins were digested by sequence-grade modified trypsin (Promega, Madison, Wisconsin).

**Nano-HPLC-MS/MS Analysis**

After resuspended with 30 μL of buffer A (0.1% formic acid in water), the peptides were separated by nano-LC. And this process is analyzed by on-line electrospray tandem mass spectrometry. All experiments were performed on a Nano ACQUITY UPLC system (Waters Corporation, Milford, Massachusetts), including a Q-Exactive mass spectrometer (Thermo Fisher Scientific) equipped with an online nano-electrospray ion source. A total of 10 μL of peptide sample, with a flow of 10 μL/min for 3 minutes, was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm × 2 cm). And then, the sample, from 3% B (B: 0.1% formic acid in ACN) to 32% B in 120 minutes, was separated on the analytical column (Acclaim PepMap C18, 75 μm × 15 cm) with a linear gradient. The column was reequilibrated for 10 minutes under initial conditions (flow rate = 300 nL/min). The electrospray voltage used was 2 kV, corresponding to the inlet of the mass spectrometer. Within data-dependent acquisition mode, the mass spectrometer was automatically switched under MS and MS/MS mode. The MS/MS resolution was maintained at 17.5K under HCD mode. MS1 mass resolution was maintained at 70K with m/z 300 to 1800 (dynamic exclusion time =10 seconds).

**Data Analysis**

PEAKS Studio version 8.5 (Bioinformatics Solutions Inc, Waterloo, Ontario, Canada) was used to process Tandem mass spectra. PEAKS DB were searched (fragment ion mass tolerance = 0.05 Da; parent ion tolerance = 7 ppm) by Swiss-Prot human database assuming the digestion enzyme Trypsin. Carbamidomethylation was setted as a fixed modification. Acetylation (Protein N-term), Oxidation (M), and Deamidation (NQ), were setted as variable modifications. Indicators for filtering proteins: 1% FDR and 1 unique peptide and protein abundance calculation were performed using analysis of variance (ANOVA). Normalization was used for on averaging the abundance of all peptides. Conditions for filtering different expressed proteins: fold change >1.5 and contained at least 2 unique peptides with P < .05.

**Function Method Description**

Hierarchical cluster analysis (HCA), as an algorithmic approach, was used to find discrete groups with varying degrees of (dis)similarity in a data set represented by a (dis)similarity matrix. This analysis is processed with pheatmap package (https://CRAN.R-project.org/package=pheatmap). The volcano plot is a type of scatter-plot which plots significance versus fold-change on the x- and y-axes. The volcano plot drawn by using ggplot2 package (http://ggplot2.org) is used to quickly identify changes in large data sets composed of replicate data. Functional annotation was processed by Blast2GO version 4. Whole protein sequence database was analyzed by BlastP using whole database and mapped, annotated with gene ontology database. Statistically altered functions of different expressed proteins were
calculated by Fisher exact test in BLAST2GO. KOBAS was used for KEGG analysis (http://kobas.cbi.pku.edu.cn/). Protein–protein interaction network was processed by STRING version 10 (www.string-db.org).

**Statistical Analysis**

Statistical significance was analyzed using Student t test and ANOVA test using SPSS version 18.0 software (SPSS, Inc, Chicago, Illinois). Statistically significant difference is expressed as *P < .05. The data were presented as the mean (standard deviation).

**Results**

**Aurora-B Promote Malignant Phenotype of OS Cells In Vitro**

In order to investigate the biological function caused by AURKB in osteosarcoma cells, OS cells (U2-OS and 143B) were used to treat with specific Lentivirus-Vectors (up or downregulation AURKB). Transwell invasion, Wound Healing, and CCK-8 were performed to evaluate the invasion, migration, and proliferation of OS cells, respectively. Results showed that the invaded cells, migration rate and the proliferation of OS cells were significantly increased in cells upregulated AURKB than those of in negative group, and significantly reduced in cells downregulated AURKB compared to negative control group. Those results indicated inhibition AURKB repress OS cells malignant phenotype in vitro (Figure 1).

**Identification of DEPs.** To investigate the molecular mechanisms of AURKB in the progression of Osteosarcoma, we utilized label-free quantitative proteomics technology to quantify/visualize the changes in proteins expression abundance and identify DEPs in the 2 groups (LV-AURKB and NC-AURKB) of 143B cells. We identified 101 DEPs. Among them, 76 proteins were upregulated in U (LV-AURKB) group compared to C (NC-AURKB) group and 25 were downregulated. Representative DEPs mentioned in the article identified by label-free quantitative proteomic in 143B cells are shown in Table 1. Hierarchical cluster analysis are illustrated in Figure 2A. The color depth of HCA indicates the size of the protein expression. In the vertical axis, the sample may be submitted to cluster analysis and the protein may be clustered in the horizontal direction. The volcano plot is shown in Figure 2B. The red dots mean the upregulated proteins. The blue dots mean downregulated proteins. And the green dots mean those proteins without change.

**Gene ontology enrichment analysis.** Following GO enrichment analyses for upregulated DEPs, the GO BP (biological process) terms enriched were mainly related to actin filament-based process, actin cytoskeleton organization, localization, response to nutrient, cytoskeleton organization. The GO MF (molecular function) terms enriched were mainly associated with actin-dependent ATPase activity, microfilament motor activity, protein binding, protein complex binding, actin binding. The GO CC (Cellular Component) terms enriched were mainly associated with vesicle, extracellular exosome, extracellular vesicle, extracellular organelle, and cytoplasm. Alternatively, The GO BP terms enriched by downregulated DEPs were mainly related to RNA processing, nucleocytoplasmic transport, nuclear transport, nucleic acid metabolic process, and nuclear export. The identified proteins separated according to MF mainly include RNA binding, poly(A) RNA binding,
nucleic acid binding, heterocyclic compound binding, and organic cyclic compound binding. Cellular component of the proteins was classified and large groups were found to be nuclear lumen, intracellular organelle lumen, membrane-enclosed lumen, organelle lumen, and nuclear part (Figure 3).

**KEGG Analysis.** The pathways enriched by upregulated DEPs were mainly related to regulation of actin cytoskeleton, and Fc gamma R-mediated phagocytosis. The downregulated DEPs were mainly related to spliceosome and herpes simplex infection. The top 20 pathways terms are shown in Table 2.

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**Figure 3.** Representative GO enrichment analysis of (A) up and (B) downregulated DEPs. Significantly changed GO of predicted DEPs were illustrated. The left y-axis titled with percentage of proteins and the right y-axis presented number of proteins, while the x-axis showed GO category. DEPs indicate differentially expressed proteins; GO, gene ontology.
Protein–protein interaction network construction. Based on data from the STRING database, the PPI network was constructed (Figure 4). All nodes represent proteins and edges indicate connections between proteins. For upregulated DEPs, 10 edges were selected as hub proteins (degree ≥ 9), including ALB (degree = 17), PTK2 (degree = 9), CAT (degree = 9), AFR3 (degree = 10). For downregulated DEPs, 5 edges were selected as hub proteins (degree ≥ 5), including SRSF5 (degree = 5), SRSF1 (degree = 5), SRRM2 (degree = 5), SRRM1 (degree = 5), and RBM22 (degree = 5).

Western Blot Analysis. To validate the results of bioinformatics speculation, the proteins of PTK2, AKT, p-AKT, PI3 K, p-PI3 K, and NF-κB were further confirmed by western blot. As showed in Figure 5, the expression of AURKB enhanced protein expressions of PTK2, AKT, p-AKT, PI3 K, p-PI3 K, and NF-κB in 143B cells and U2-OS cells.

Discussion

Various evidences indicated AURKB, as a oncogene, promotes malignant tumor growth and metastasis. In the present study, We confirmed that AURKB promote invasion, migration and proliferation of OS cells in vitro. Furthermore, label-free quantitative proteomics technology was used to identify the protein difference between upregulated AURKB group and negative group in 143B cells. Finally, we explored the molecular mechanism of AURKB promote malignant phenotype of osteosarcoma.

For the downregulated proteins, enriched pathways were mainly related to Spliceosome. These proteins are hub proteins in the PPI network, such as SRSF1, SRSF2, SRSF5, RBM22 and SRRM1 (Figure 5B). Current research shows that alternative splicing may play a positive role in tumor genesis and development. The effects of RNA alternative splicing on tumors include long-lasting proliferative signals, activation of invasion and metastasis, escape of growth-suppressing genes, resistance to apoptosis, and so on. It is suggest that AURKB may have a certain relationship with alternative splicing. For the upregulated proteins, regulation of actin cytoskeleton pathway was noted to be enriched by several upregulated proteins, including PTK2, which were also hub proteins in the PPI network (Figure 5A). It has been reported that cytoskeletal reorganization affects cell migration, whereas invasion and metastasis of tumors are mainly dependent on cell migration. The activation of NF-κB can be influenced by many physiopathological events including phagocytosis, pathogen invasion, cellular adhesion and chemotaxis governed by actin-based cytoskeleton. Our previous study also confirmed that knockdown of AURKB alters osteosarcoma cell malignant phenotype via decreasing NF-κB signaling.

What fascinated us was that the major hub proteins about PTK2 in upregulated proteins. PTK2, also known as FAK, is a cytosolic non-receptor tyrosine kinase that is an important regulator of integrin-regulated signaling and plays an important regulatory role in tumor proliferation, migration, invasion, metastasis, and angiogenesis. PTK2 is highly expressed in breast cancer, lung cancer, prostate cancer, liver cancer, pancreatic cancer, colorectal cancer and other malignant tumors.
Ren et al\textsuperscript{24} reported that siRNA-based knockdown of PTK2 remarkably reduced the malignant phenotype of osteosarcoma cells. Sonody et al\textsuperscript{25} conclude that PTK2 activates the PI3K/Akt/NF-KB signaling pathway with the concomitant induction of IAPs, which finally reduce apoptosis by inhibiting caspase-3 cascade. In addition, PTK2 has been shown to be significant for epithelial-to-mesenchymal transition (EMT).\textsuperscript{26} It has been reported that downstream signaling pathways of Src\textsuperscript{27} and

\textbf{Figure 4}. The protein–protein interaction network of the differentially expressed proteins identified. A, upregulated DEPs (B) downregulated DEPs. Nodes represent proteins and edges indicate connections between proteins, differentially expressed proteins. DEPs indicate differentially expressed proteins.
PI3K are important for PTK2-mediated cell motility. Zaytsev et al. reported that inhibition of FASN attenuated the activation of MET, Akt, PTK2, and paxillin, which are known to regulate adhesion, migration, and invasion. These results collectively suggest that PTK2 overexpression and phosphorylation might predict more aggressive biologic behavior in osteosarcoma and may be an independent predictor of poor prognosis. In addition, confirmed in our previous study that knockdown of AURKB inhibits OS cells malignant phenotype via modulating PI3K/Akt/NF-κB signaling pathway. We speculate that AURKB may promote the malignant phenotype of osteosarcoma cells by activating the PTK2/PI3K/Akt/NF-κB pathway. Finally, we confirmed by Western blot analysis that expression of PTK2, AKT, PI3K, p-AKT, p-PI3K, and NF-κB increased after overexpression of AURKB.

Although bioinformatics techniques of proteomics have the potential to enrich and identify disease-related potential mechanisms, there are still some limitations in this study, which lacks complete experimental validation and does not adequately demonstrate the direct relevance of all mechanisms. Further experimental studies of a larger sample size, most importantly, are still needed in the future to confirm the results. However, proteomics analysis, based on our previous studies, provides a direction for further research on the molecular mechanism of AURKB in promoting the malignant phenotype of osteosarcoma.

Conclusions
In summary, our results indicated that upregulated AURKB can enhance the malignant phenotype of OS cells, and AURKB may promote the malignant phenotype of OS cells by activating the PTK2/PI3K/Akt/NF-κB pathway. Finally, we confirmed that expression of PTK2, AKT, PI3K, p-AKT, p-PI3K, and NF-κB increased after overexpression of AURKB. Thus, target AURKB and PTK2/PI3K/Akt/NF-κB pathway may be a promising strategy.

Authors’ Note
Zhi-li Liu and Shang-Hu Huang designed the study. Jia-Ming Liu, Ai-Fen Peng, Wen-zhao Chen and Jiang-Wei Chen, carried out the experiment. Wen-Sen Pi, interpretation of the data. Wen-Sen Pi and Zhi-Yuan Cao, wrote the manuscript. Wen-Sen Pi and Zhi-Yuan Cao contributed equally to this work.

Declaration of Conflicting Interests
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