Identification of Differentially Expressed Genes in two Types of Osteosarcoma cell lines upon Zinc Oxide Nanoparticles Treatment Using the RNA-seq Technique

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Research Article

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Abstract

Osteosarcoma (OS) predominantly occurs in adolescents, and more often in males than females with characteristics of local invasive growth and early pulmonary metastases. Owing to highly selective and effectiveness, nanoparticles (NPs) have been a new alternative for traditional chemotherapeutic drugs. Previous studies have proved that zinc oxide nanoparticles (ZnO NPs) is one of a promising inorganic NPs in treatment of various tumors besides OS. In this study, we use RNA-seq analysis to deeply explore the potential biological mechanism in the process of ZnO NPs-treated different types of OS cell lines. We detected that 928 genes (DEGs) differentially expressed both in 143B and MG-63 cells, and the eight highest DEGs were verified by RT-qPCR. Gene Ontology (GO) categories analysis displayed regulation of transcription factor on nucleic acid binding in molecular function term, and extracellular space in cellular components term in both OS cell lines. Kyoto Encyclopedia of Genes and Genomes (KEGG) classification analysis found that the MAPK, Toll-like receptor and NF-κB pathways were co-enriched in both OS cell lines. The Protein-protein Interaction (PPI) revealed that HMOX1, MAFB, CXCL10 and CXCL11 were most involved in multiple aspects of biological events in OS cells under ZnO NPs treatment. Furthermore, the key protein molecules in differential signal pathways in both OS cell lines were detected and confirmed by Western Blot (WB). In conclusion, our findings unveiled a range of potential antitumor mechanisms and exploitable bioeffects of ZnO NPs treatment on OS.

Introduction

Osteosarcoma (OS) is a malignant bone tumor in children and adolescents, which genetically displays highly aggressive and early systemic metastasis[1, 2]. Although surgical resection combined with neoadjuvant chemotherapy has greatly improved the 5-year survival rate of OS patients, a high risk of local relapse or distant metastasis are still a tough problem as drug resistance hampers the curative effect of clinical therapies [3, 4]. To improve the outcome for OS patients, it is urgently needed to identify reliable and more efficient treatment of OS. Thus, new type of comprehensive and multidimensional treatments, such as monoclonal antibody therapy, tyrosine kinase inhibitor and nano-material drugs have been investigated and gradually applied in OS patients[5].

Currently, nanoparticles have emerged as a promising and widely applied drug systems for cancer treatment which can destroy tumor cells with less toxic to normal cells[6]. Zinc oxide nanoparticles (ZnO NPs) are regarded as multi-functional nanoparticle material with broad application prospects in various field including chemotherapy[7–12]. On various malignant cells, the endocytosed ZnO NPs can induce cytotoxicity in malignant cells by excessive oxidative stress followed by producing intracellular reactive oxygen species (ROS), which could be attributed in part via the intracellular release of Zn^{2+} ions[13, 14]. In HeLa cells, the synthesized ZnO NPs can induce apoptosis by producing oxidative damage and modulating the expression of pro-apoptotic proteins[15]. In the malignancy triple negative breast tumor cells, ZnO NPs display significant anti-proliferative activity as a promising agent for further treatment[16]. ZnO NPs can also induce cellular apoptosis through the mitochondrial oxidative damage via p70S6K
signaling pathway in human GSCC[17]. These studies provided an experimental basis for ZnO NPs to be considered as a promising novel anti-tumor agent for cancer treatment.

Currently, researches on the tumor of ZnO NPs treatment mainly focuses on major functional signaling pathways in tumor cells while there are no relevant researches on the effect of ZnO NPs on tumor cells to profile the whole gene expression changes. Our previous study have firstly proved that ZnO NPs induced OS cell death through several related pathway, while the whole biological effects remains elusive[9, 18–20]. Therefore, in this study, we further investigated the effects of ZnO NPs on OS genome-wide RNA expression profile. We found that multiple genes displayed significant change upon ZnO NPs exposure, and further molecular biological functions and signaling pathways detection displayed consistence with results of RNA-Seq. Summarily, our study may contribute to understanding the potential molecular targets and application value of ZnO NPs on OS treatment.

**Materials And Methods**

**Cell Culture**

The human osteosarcoma cell lines 143B and MG-63 were purchased from American Type Culture Collection (ATCC). Cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin- streptomycin (10,000 U/mL) (Gibco, USA) routinely incubated at 37°C in a 5% CO2, 95% humidity in cell culture incubator (Thermo Fisher Scientific, USA).

**Reagents and Antibodies**

ZnO NPs (<100nm) were purchased from Sigma-Aldrich. Antibodies against ERK, p38 MAPK, c-Jun TLR4, MyD88 and TRAF6 were obtained from Abcam (UK). Antibodies against p-IκBα, p-p65 and GAPDH were purchased from Cell Signaling Technology (CST, USA).

**Generation and Normalization of RNA-sequencing Data** Total RNA was extracted from OS cells treated with non-Zn0 NPs and Zn0 NPs treatment using the TRizol reagent according to instruction. All RNA samples with acceptable quality assessment were submitted for RNA libraries generation using NEBNext Ultra RNA Library Prep Kit (NEB, USA) and sequenced on the Illumina HiSeq 4000 platform. Paired-end reads were mapped to the human reference genome by Salmon. Transcript abundances were summarized at the gene level and were normalized based on transcripts-per-million by Shanghai OE Biotech company (Shanghai, China).

**Transcriptome Data Analysis**

Raw reads of the FASTQ format were processed using in- house Perl scripts to obtain clean reads, and all of the downstream analyses were based on the high-quality clean reads. Reference genome and annotation files were downloaded from the NCBI website and the reads to each gene were mapped by HISAT. The gene expression levels were estimated according to FPKM (fragments per kilobase of exon...
per million fragments mapped). The differential expression genes between the non-ZnO NPs and ZnO NPs treatment were obtained using DESeq2.14. The P value was adjusted by Benjamini and Hochberg's approach, and padj <0.05 and fold change >1.5 were set as the thresholds for significant differential expression.

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyses**

Gene Ontology (GO) functional analysis can categorize DEGs into the three structured modules: biological processes (BP), cellular components (CC) and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) can identify metabolic pathways and signal transduction pathways related to the DEGs. To compare differences in cell functions and signal pathway in the transcriptomes between non-ZnO NPs and ZnO NPs treatment, GO and KEGG analysis were performed to functionally characterize the identified DEGs by the Cluster Profiler visualized with the GO plot and Cytoscape software.

**Network Construction of Protein-protein Interaction**

The protein interactions prediction database BioGRID was utilized to screen interacting protein pairs based on DEGs. PPI networks of up- and down-regulated DEGs were visualized using Cytoscape software (http://cytoscape.org/). In the networks, nodes represent proteins and edges represent interactions between two proteins.

**Real-time qPCR Verification**

Cellular total RNA was extracted by Trizol reagent (Sigma-Aldrich, USA), and quantified using the Nano-one (Thermo Fisher Scientific, USA). The cDNA was synthesized using Reverse Transcription kit (QIAGEN, Germany), and used as templates for qPCR. The relative expression level of each related gene was calculated using the $2^{\Delta\Delta CT}$ method. The primers of selected DEGs and the house-keeping gene GAPDH were designed by Primer Premier 7 software. The sequence of primers was listed in Table1.

**Western Blot Assay**

Protein lysates were extracted from cells samples treated with non-ZnONPs and ZnO NPs using RIPA lysis buffer containing protease and phosphatase inhibitor tablets (Sigma, USA). The concentrations of protein lysates were quantified by BCA assay (Thermo Fisher Scientific, USA). Then samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. Moreover, membranes were incubated with primary antibodies at 4 °C overnight followed by the secondary antibody incubation at room temperature for 1h. The membranes were visualized by ECL (Millipore, USA), and the quantification of band density was determined by Quantity one.

**Statistical Analysis**
All data were analyzed by IBM SPSS Statistics 20.0 software and expressed as mean ± standard deviation (SD). Statistical analyses of cell surviving were performed using Student’s t test and one-way analysis of variance (ANOVA). The value of P < 0.05 was considered statistically significant.

Results

ZnO NPs treatment induces an extensive transcriptional response

The high throughput RNA-sequencing was performed on the 143B and MG-63 OS cells treated with or without ZnO NPs. Then, total RNA were extracted from different groups of cells and pooled for RNA-seq. All of the sequencing reads were aligned to the NCBI human reference genome. To observe the gene expression patterns, cluster analysis of all the DEGs based on the log10 (FPKM+1) was conducted with heat maps for the 143B cells (Figure 1A) and MG-63 cells (Figure 1B). In 143B cells, 2544 significantly DEGs were identified in ZnO NPs treated OS cells compared with control, which consisted of 1628 up- and 916 down-regulated genes. In MG-63 cells, the results obtained 2768 significantly DEGs in two groups treated like 143B cell, Among the 2768 DEGs, the expression of 1756 were increased upon ZnO NPs intervention, and 1012 genes were down-regulated (Figure 1C). Of all the genes that differently regulated between the two cells, 928 overlapping genes existed between these two cellular lines (Figure 1D). These data demonstrated that ZnO NPs treatment induces an extensive transcriptional response in OS cells. The full list of DEGs is shown in Supplementary Table S1 and S2.

Functional gene ontology (GO) classification analysis of DEGs upon ZnO NPs treatment

To analysis DEGs and obtain the biological functional influence of ZnO NPs treatment on OS cells, we employed GO analysis to evaluate the cellular components, biological processes, and molecular functions correlated with DEGs between non-ZnO NPs and ZnO NPs-treated groups. The functions of three structured vocabularies were significant differences between the two cell lines (Figure 2A, 2B). In 143B cell line, we found functions of biological processes (BP) focused on tissue development- and interleukin-7-mediated signaling pathway. In MG-63 cells, however, functions mainly focused on the innate immune pathway and cell adhesion. In the cellular components (CC) aspect, functions concentrated on extracellular space in both cellular lines. In specific cells, nucleosome assembly and Wnt signalosome were focus on 143B cells, whereas integrin complex and chromatoid body were specific located in MG-63 cells. In the last section of molecular function (MF), the nucleic acid binding related pathways were enriched in both cell lines. However, hormone activity was enriched in 143B cell line while specific enrichment in MAP kinase tyrosine phosphatase pathway in MG-63 cell line. These data suggested that ZnO NPs treatment triggers multiples responses in OS cell functions.

Functional KEGG: Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs upon ZnO NPs treatment

To get further understanding of the metabolic and signal transduction pathways involved in ZnO NPs treatment, KEGG analysis was conducted between non-ZnO NPs and ZnO NPs-treated groups in two OS
cell lines. In both 143B and MG-63 cells, total of seven pathways were significantly enriched in both cell lines, including MAPK, Toll-like receptor (TLRs) and NF-kappa B signaling pathways which were related to tumor growth and inflammation functions (Figure 3A, 3B). Moreover, the analysis displayed that multiple pathways were different in these two cellular lines. The TNF and p53 signaling pathways were enriched in 143B cells (Figure 3A). The PI3K-AKT and RIG-like receptor signaling pathways were specifically abundant in MG-63 cells (Figure 3B). Interestingly, the result also revealed the alcoholism regulated pathway was enriched in 143B cells. However, in MG-63 cells, the changed microRNA related pathways were enriched. These KEGG terms exhibited some pathway similarity in both cell lines as well as certain cell-specific pathways.

**PPI networks of DEGs between non-ZnO NPs and ZnO NPs-treated OS cellular lines**

The protein–protein interaction (PPI) analysis provided a novel approach for the discovery of novel gene interaction and identify neighbors of known mediators. To investigate the PPI network in ZnO NPs treated OS cell lines, we used mapping tool of NetworkAnalyst3.0)/Cytoscape to conduct PPI analysis (Figure 4A) with the top 20 genes classified as hub genes with the greatest differences in expression of each cellular lines. In the PPI network, the most significantly proteins were HMOX1 and MAFB in 143B cell line. On the other hand, CXCL10 and CXCL11 proteins were the most significantly regulated hub proteins in MG-63 cells (Figure 4B). These proteins are involved in multiple aspects of biological events in OS cells during ZnO NPs treatment.

**Verification of the expression level of DEGs by RT-qPCR**

To verify the DEGs data of RNA-seq analysis, the RT-qPCR assay was conducted to verify the expression levels of 8 genes displayed the most significant changes upon ZnO NPs treatment in two cellular lines (Table 1). As shown in Figure 5A, RT-qPCR verified that MT1M, HSPA6, RASD1 and CLDN6 were significantly increased while HOXA6, SERTM2 and TNFRSF10D were significantly downregulated in ZnO NPs-treated 143B cells. In Figure 5B, HSPA6, XIRP1, C9orf152 and HMOX1 were significantly up-regulated while TNFSF10, CXCL10, SLFN12L and CHL1 obviously downregulated. Taken together, these results indicated that data of most DEGs obtained from RNA-seq are well identified by RT-qPCR detection.

**Verification of ZnO NPs stimulation of pathways by Western Blot**

Based on the KEGG results from RNA-seq, multiple signaling pathways were influenced by ZnO NPs treatment in both 143B and MG-63 cellular lines. We choose to verify pathways important for cancer biological, cancer treatment and drug resistance aspects, including MAPK, Toll-like receptor, NF-κB pathway. As shown in Figure 6A, the MAPK signaling pathway displayed overall repression, as the expression of p-ERK, p-p38 MAPK and p-c-Jun were all downregulated to some degree upon ZnO NPs treatment. Next, TLR4, MyD88 and TRAF6 which are the key proteins of the Toll-like receptor pathway were significantly upregulated upon ZnO NPs treatment in both cellular lines (Figure 6B). Similarly, the NF-κB pathway showed a significant inhibitory characterized by p-IκBα and p-p65 upon ZnO NPs
treatment (Figure 6C). These above changes resembled responses to multiple aspects of signaling pathway in ZnO NPs-treated OS cells, which further disturbed cellular functions.

Discussion

To date, the molecular mechanism of OS development and metastases remains unclear. Previous studies have focused on treatment of conventional antitumor drugs on genes expression involved in the progression and metastasis of OS and overlooked the landscape of new type of antitumor drugs with promising application\[21, 22\]. Recent studies have demonstrated that ZnO NPs participate in interfering and blocking process of OS cell functions, including cell growth, cell apoptosis and inhibited cell proliferation, migration, invasion and metastasis\[23\]. In this study, we employed RNA-Seq combined with molecular biology experiments to profile the transcriptome of OS cells under ZnO NPs treatment. The transcriptome analysis revealed that ZnO NPs triggered extensive transcriptional responses, including the up- and down-regulation of several genes in multiple cellular progression.

Several genes (MT1M, HSPA6, HMOX1, Emilin1 and TNFRSF) showed overlapped between the two cell lines in the top 30 DEGs. The stimulation of MT1M encodes member of the metallothionein superfamily, which bind various heavy metals and secreting zinc, which suggested that successful absorption and transport of ZnO NPs into the cell and may be target for drug resistance. The HSPA6 gene enables enzyme binding activity involved in cellular response to heat and protein refolding, which were also triggered by ZnO NPs treatment. HMOX1 gene, an important functional gene in hypoxia-induced HIF-1 pathway\[24\], was obviously upregulated by ZnO NPs which was consist with our previous study\[20\]. In contrast, TNFRSF downregulation by ZnO NPs may suggest that the TNF family proteins play an important role in the fate of OS cells. Emilin1, an important protein component of extracellular matrix\[25\], plays a key role in maintaining the extensibility of arterial wall to support the normal structure and elastic retraction function of blood vessels, especially arteries, which was also significantly downregulated by ZnO NPs.

In multiple other cancer cell lines, NPs also triggered dramatic transcriptional changes. In the model of OS-732 cellular, the effects of hydroxyapatite nanoparticles were conducted by RNA-seq, and discovered the downregulated the FAK/PI3K/Akt signaling pathway, which further inhibit OS cell growth, migration and invasion in vitro and vivo\[26\]. The RNA-seq analysis displayed that Artemisia-AgNPs treatment showed dose-dependent growth inhibition of HeLa and MCF-7 cells by inducing cell apoptosis and inhibited cell proliferation through cell-cycle arrest and DNA damage response\[27\]. The combination of RNA-seq and bioinformatics analysis revealed that a series of genes involved in cancer cell death could be regulated by Nano-SiO2\[28\]. Further study showed that Nano-SiO2 treatment leads to cell cycle arrest, apoptosis enhancement and necroptosis induction in the HCC cells\[29\]. In our current study, our KEGG analysis detected that ZnO NPs triggered overlapping activation signal pathways (MAPK, NF-κB and TLRs) associated with cellular specific functions both in two type of OS cell lines. The MAPK signaling controls several fundamental biological processes of cells, including cellular growth, proliferation, survival, autophagy\[30\], and the NF-κB pathway regulated multiple important cellular functions,
especially inflammatory responses, such as interleukin-6 (IL-6), IL-8, and tumor necrosis factor-α (TNF-α), and inhibited the proliferation and metastasis of OS cells[31, 32], which were both obviously downregulated by ZnO NPs. The TLR pathway play crucial roles in many aspects of the innate immune responses through the recognition of specific conserved pathogen-associated molecular patterns (PAMPs) from various microbes, leading to activation of the interferons or IFN-inducible genes and pro-inflammatory cytokines, which participate in tumor immune monitoring and play an important role in tumor growth, resulting in enhancing antibody-dependent cell-mediated cytotoxicity[33]. Our current study provided evidence of regulation effect on TLR pathway by ZnO NPs, which was consist with the previous study reported[34]. To a certain extent, this effect may provide new ideas and directions for the therapeutic use of ZnO NPs in the treatment of osteosarcoma.

**Conclusion**

Although multiple new strategies and drugs are developed and used in cancer treatments, OS is still a major public health problem. In this study, we demonstrated the potential anticancer targets by RNA-seq in two OS cell lines. These outcomes provided new insights into molecular mechanisms of ZnO NPs on OS cells, and open doors for future investigations aimed at developing innovative anti-cancer drugs.

**Declarations**

**Author contributions**

X-FG contributed to this work. G-PH conceived and designed the project. X-FG and L-BW performed the experiments and analyzed the data. X-FG and G-PH wrote the manuscript.

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**Conflicts of interest**

The authors declare no conflict of interest.

**References**


Table

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

The DEGs analysis between non-ZnO NPs and ZnO NPs-treated OS cell lines

Hierarchical clustering of DEGs in 143B (A) and MG-63 (B) cells based on log10(FPKM+1) values (Blue color indicated a decreased expression of genes and red color indicated an increased expression. (C) Venn diagram showed the overlapping DEGs between 143B and MG-63 cell lines. (D) The expression of shared genes in top 30 DEGs between 143B and MG-63 cell lines. Fold Change >1.5 and padj < 0.05 were set as the threshold for significant differential expression of genes.
Figure 2

GO analysis of DEGs data upon ZnO NPs treatment in 143B(A) and MG-63(B) OS cell lines. The top ten terms of biological processes (BP), cellular components (CC) and molecular function (MF) are shown. GO terms with padj < 0.05 were considered significantly enriched.
Figure 3

KEGG analysis of DEGs upon ZnO NPs treatment in 143B(A) and MG-63(B) OS cell lines. The DEGs enriched in top 20 pathways upon ZnO NPs treatment analyzed by KEGG. The KEGG pathway enrichment analysis for DEGs with a threshold FDR value < 0.05.

Figure 4

The PPI network analysis upon ZnO NPs treatment in in 143B(A) and MG-63(B) OS cell lines. The PPI network with top hub DEGs. The nodes represent the differential genes and the connections between the
nodes reveal the interactions between the genes.

Figure 5

The expression levels of DEGs upon ZnO NPs treatment in 143B(A) and MG-63(B) OS cell lines were detected by RT-qPCR. Total RNA were extracted from cells different treated with ZnO NPs and control cells, and RNA qPCR were utilized to quantify the mRNA expression of targeted genes in 143B (A) and MG-63 (B) cells. The expression of GAPDH was used as control.

Figure 6

The expression of shared cellular signaling pathways in both 143B and MG-63 OS cell lines with ZnO NPs treatment were verified by Western blot. The represented proteins of p-p38 MAPK and p-c-jun in the MAPK
signaling pathway were significantly downregulated in both two types of OS cell lines (A); the represented proteins of TLR4, Myd88 and TRAF6 in Toll-like receptor signaling pathway were significantly upregulated in both two types of OS cell lines (B), the represented proteins of p-IκBα and p-p65 in NF-κB signaling pathway were significantly downregulated in both two types of OS cell lines (C).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- SupplementaryTableS1.xlsx
- SupplementaryTableS2MG63NPsvsMG63.xlsx