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RESEARCH ARTICLE

Preliminary screening of differentially expressed genes involved in methyl-CpG-binding protein 2 gene-mediated proliferation in human osteosarcoma cells

Gang Meng · Yi Li · YangFan lv · Huanzi Dai · Xi Zhang · Qiao-Nan Guo

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Abstract Methyl-CpG-binding protein 2 (MeCP2) is essential in human brain development and has been linked to several cancer types and neuro-developmental disorders. This study aims to screen the MeCP2 related differentially expressed genes and discover the therapeutic targets for osteosarcoma. CCK8 assay was used to detect the proliferation and SaOS2 and U2OS cells. Apoptosis of cells was detected by flow cytometry analysis that monitored Annexin V-APC/7-DD binding and 7-ADD uptake simultaneously. Denaturing formaldehyde agarose gel electrophoresis was employed to examine the quality of total RNA 18S and 28S units. Gene chip technique was utilized to discover the differentially expressed genes correlated with MeCP2 gene. Differential gene screening criteria were used to screen the changed genes. The gene up-regulation or down-regulation more than 1.5 times was regarded as significant differential expression genes. The CCK8 results indicated that the cell proliferation of MeCP2 silencing cells (LV-MeCP2-RNAi) was significantly decreased compared to non-silenced cells (LV-MeCP2-RNAi-CN) ($P<0.05$). MeCP2 silencing could also induce significant apoptosis compared to non-silenced cells ($P<0.05$); 107 expression changed genes were screened from a total of 49,395 transcripts. Among the total 107 transcripts, 34 transcripts were up-regulated and 73 transcripts were down-regulated. There were five significant differentially expressed genes, including IGFBP4, HOXC8, LMO4, MDK, and CTGF, which correlated with the MeCP2 gene. The methylation frequency of CpG in IGFBP4 gene could achieve 55 %. In conclusion, the differentially expressed IGFBP4, HOXC8, LMO4, MDK, and CTGF genes may be involved in MeCP2 gene-mediated proliferation and apoptosis in osteosarcoma cells.

Keywords Methyl-CpG-binding protein 2 · Osteosarcoma · Proliferation · Differentially expressed genes

G. Meng · Y. lv · X. Zhang · Q.-N. Guo (✉)
Department of Pathology, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China
e-mail: qiaonian885@yeah.net

Q.-. Guo
e-mail: qiaonan85@263.net

Y. Li
Department of Oncology, Kunming General Hospital of Chengdu Military Command, Kunming 650032, China

H. Dai
Department of Nephrology, Daping Hospital, Third Military Medical University, Chongqing 400042, China

Introduction

In recent years, morbidity of osteosarcoma significantly increased in the whole world [1, 2]. Osteosarcoma is the most common primary malignant bone tumor and usually arises from primitive transformed cells of mesenchymal origin, such as the distal femur, proximal tibia, and proximal humerus during the second decade of life [3, 4]. DNA hypermethylation plays an important role in silencing the tumor suppressor genes of human cancers [5]. DNA methylation has emerged as an attractive target for the cancer therapeutics [6, 7]. The inhibitive effects of DNA methylation are mediated by the methyl-CpG-binding proteins (MBDs) [8]. Methyl-CpG-binding protein 2 (MeCP2) is the most commonly discussed MBD protein in previous studies. MeCP2 plays a very important role in tumor proliferation, migration, and invasion of osteosarcoma [9]. MeCP2 is a basic chromosomal protein that binds to symmetrical methylated 5′-CpG dinucleotide sequences [10, 11]. MeCP2 is essential in human brain development and has been linked to several cancer types and neuro-developmental disorders [12].

Gene chip technique, also called gene microarray analysis, is a kind of Frontier biology technology, which developed in the recent years [13, 14]. Gene chip technique acts many more

functions in the basic research or clinical study, such as gene expression profile analysis, gene diagnosis, drug screening, and sequence analysis [15]. In this study, the gene differential expression was detected in human osteosarcoma cell lines SaOS2 and U2OS and in silenced MeCP2 gene cell lines by using gene chip technique. This study aims to screen the MeCP2-related genes and discover the therapeutic targets for osteosarcoma.

Materials and methods

Cell lines, culture, and transfection

Human osteosarcoma cell lines, SaOS2 and U2OS, were obtained from American Type Culture Collection (CRL-6253) and were cultured in Dulbecco's modified Eagle's medium (Hyclone, CA, USA), which includes 10 % fetal bovine serum (Gibco, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, MO, USA). The SaOS2 and U2OS cells were cultured at 37 °C with 5 % CO_2. The viral vector was transfected into and infected the SaOS2 and U2OS monolayer cell.

CCK8 assay

Cell growth was measured using the cell proliferation reagent WST-8 (Roche Biochemicals, Mannheim, Germany). After plating cells in 96-well microtiter plates (Corning Costar, Corning, NY) at 1.0×10^3/well, 10 μl of CCK8 was added to each well at the time of harvest, according to the manufacturer's instructions. One hour after adding CCK8, cellular viability was determined by measuring the absorbance of the converted dye at 450 nm.

Flow cytometry assay for apoptosis

Apoptosis of cells was detected by flow cytometry analysis that monitored Annexin V-APC/7-DD binding and 7-ADD uptake simultaneously according to the manufacturer's instruction (Biovision, San Francisco, CA, USA). Harvested cells (1×10^5) were washed three times with PBS and then resuspended in 200 μl binding buffer containing 5 μl Annexin V-FITC (20 g/ml) and 5 μl 7-AAD. After incubation for 15 min at room temperature, the stained cells were subsequently analyzed by flow cytometry (FACscalibur; Becton Dickinson, Franklin Lakes, NJ).

Total RNA extraction and cDNA synthesis

The total RNA was extracted from SaOS2 cell cultures using RNA extraction kit (Tiangen, Bejing, China), according to the manufacturer's instruction. Complementary DNAs (cDNAs) were synthesized by a reverse transcription kit (Invitrogen, CA, USA). The detailed protocol of the RNA extraction and cDNA synthesis were performed according to the report of Xu et al. [16].

Silencing

Small interfering RNA (siRNA) targeted to human MeCP2 mRNA was designed following the procedure described by Reynolds et al. [17]. Selected siRNAs were inserted into the Lentiviral vector (Ambion, Austin, TX, USA). The Lv-MeCP2-siRNAs (the two targeting sequence are as the following: CCACCTAAGAAGCCCAAT and GCTGGACACG GAAG CTTAA) and controls (Lv-MeCP2-siRNA-CON) were synthesized by Jikai Biological Company (Jikai Biology, Shanghai, China). Once we obtained Lv-MeCP2-siRNAs targeting MeCP2 Lv-MeCP2-siRNA-CON, SaOS2 cell cultures were transduced at varying multiplicities of infection (MOIs) to obtain a considerable silencing effect.

Gene hybridization and gene chip assay

The gene chip (Affymetrix GeneChip® PrimeView™, CA, USA) was pre-hybridized for 10 min (60 r/min) in the hybridization oven. The pre-hybridized chip was also treated with the made-up gene hybridization solution for 16 h (60 r/min). The hybridized gene chip was washed, stained, and scanned according to the instruments of GeneChip Scanner 3000 (Affymetrix, CA, USA).

Statistical analysis

The chip was scanned by Affymetrix scanner, and the obtained data were read and analyzed by GCOS1.2 software. The criteria of differential gene screening were acted as an "increase" or "marginal increase," log ratio ≥1, which was also regarded as up-regulation genes. The criteria of differential gene screening were acted as an "decrease" or "marginal decrease," log ratio ≤−1, which was also regarded as down-regulation genes. The gene up-regulation or down-regulation more than 1.5 times was regarded as differential expression gene.

Results

LV-MECP2-RNAi inhibits the proliferation of cells

The CCK8 assay results indicated that the cell proliferation of LV-MeCP2-RNAi-transfected SaOS2 and U2OS was significantly decreased compared to the LV-MeCP2-RNAi-CN group (Fig. 1, $P<0.05$). These results suggest that the MeCP2

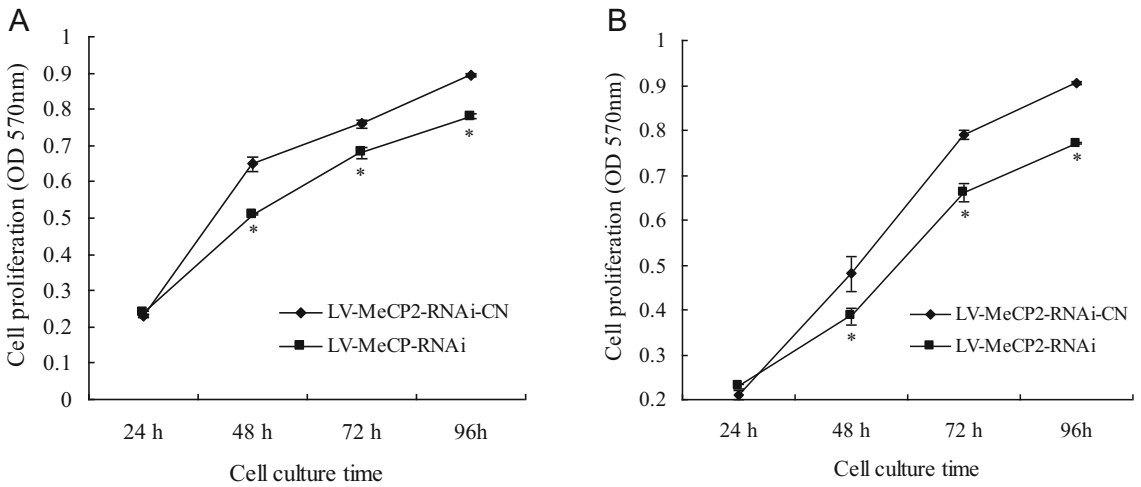

Fig. 1 SaOS2 and U2OS cell proliferation changes detected by XXT assay. **a** Cell proliferation in SaOS2 cells. **b** Cell proliferation in U2OS cells. *$P<0.05$ represents the cell proliferation of LV-MeCP2-RNAi compared to LV-MeCP2-RNAi-CN group in both cells

gene may participate in the proliferation of the human osteosarcoma cells.

LV-MECP2-RNAi triggers U2OS and SaOS2 cell apoptosis

Cell apoptosis of U2OS and SaOS2 cells was detected by using cytometry analysis. Figure 2a showed that LV-MECP2-RNAi transfection could induce significant apoptosis compared with the LV-MECP2-RNAi-CN transfection in the SaOS2 cells ($P<0.05$). Meanwhile, in the U2OS cells, the apoptosis was also triggered significantly compared with the control group (Fig. 2b, $P<0.05$).

Quality examination of total RNA and gene chip

The total RNA was detected by spectrophotometer at A260 and A280 nanometer wavelength to identify the concentration and purity. The values of A260/A280 of total RNA in LV-MECP2-RNAi-CN and LV-MECP2-RNAi were 2.04 and 2.03, respectively. Denaturing formaldehyde agarose gel electrophoresis results indicated that the 18S and 28S bands clearly appeared in the gel, and both bands have not been degraded (Fig. 3). These results suggest that the quality of total RNA was satisfied, which could be included in the gene chip assay. The gene chip quality examination results showed that the signal intensity achieved the experimental criteria, and the value of 5′/3′ was appropriate.

Differentially expressed MeCP2-related gene

LV-MECP2-RNAi-CN and LV-MECP2-RNAi groups hybridized with 49,395 transcripts, respectively. The average signal intensity of transcripts was indicated in Fig. 4.

One hundred seven differentially expressed genes were screened from the total of 49,395 transcripts according to the differential significance criteria. Among the total 107

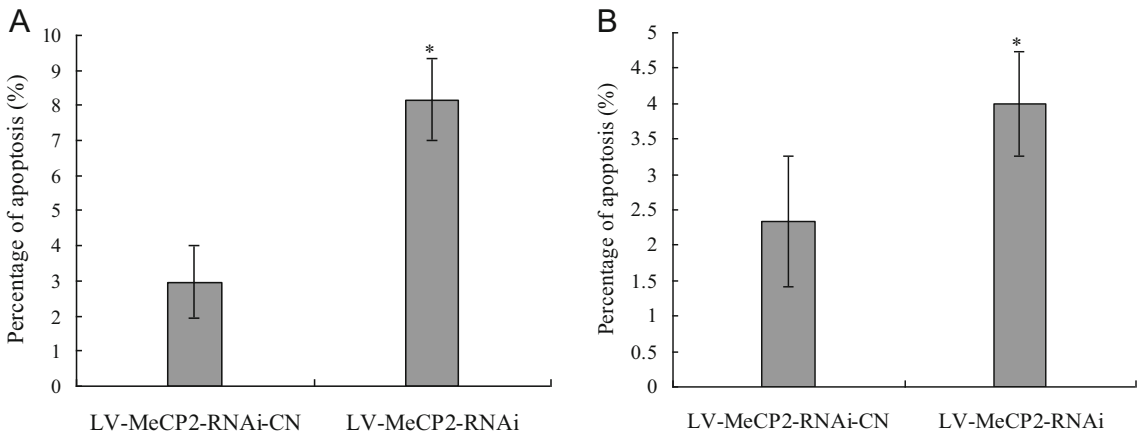

Fig. 2 Apoptosis rate of SaOS2 or U2OS cells in LV-MeCP2-RNAi and LV-MeCP2-RNAi-CN group. **a** Apoptosis rate of SaOS2 cells. **b** Apoptosis rate of U2OS cells. *$P<0.05$ represents the cell apoptosis rate of LV-MeCP2-RNAi compared to LV-MeCP2-RNAi-CN group in both cells

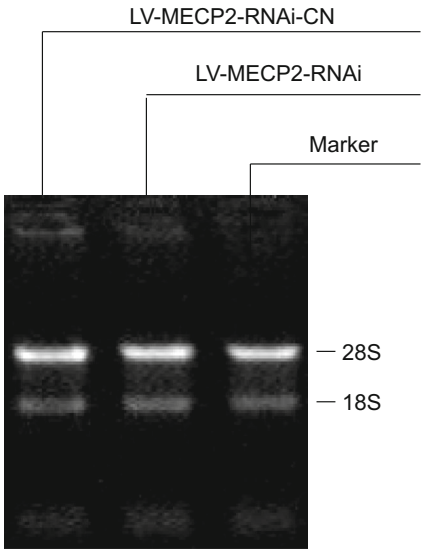

Fig. 3 Denaturing formaldehyde agarose gel electrophoresis image of the total RNA

transcripts, 34 transcripts were up-regulated and 73 transcripts were down-regulated. There were five significant differentially expressed genes, including IGFBP4, HOXC8, LMO4, MDK, and CTGF, which correlated with the MeCP2 gene (Table 1).

CpG was highly methylated in IGFBP4 gene

The CpG methylation of IGFBP4 gene was examined by using Bisulfite Sequencing PCR (BSP) method. The results

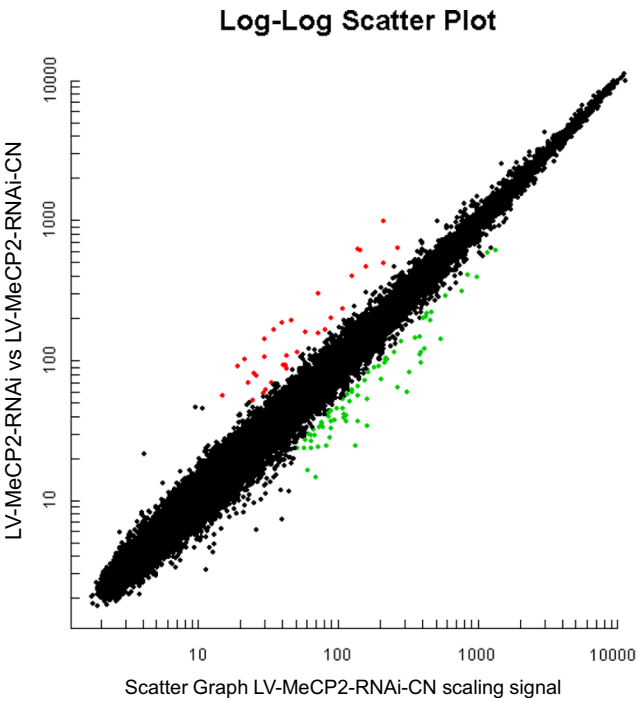

Fig. 4 Scatter graph of the differentially expressed genes

Table 1 Screening of McCP2-related gene

Gene	Changes	Localization in chromosome
IGFBP4	Up-regulation 2.14 times	chr17q12-121.1
HOXC8	Down-regulation 2.11 times	chr12q13.3
LMO4	Down-regulation 2.31 times	chr1p22.3
MDK	Down-regulation 1.89 times	chr11p11.2
CTGF	Down-regulation 1.89 times	chr6q23.1

indicated that among the four pair of premiers (Table 2), only the Premier 1 (P1) amplified the significantly methylated CpG in TGFBP4 gene sequence (Fig. 5). The methylation frequency of P1 was 55 %, P2 was 0.7 %, P3 was 0.4 %, and P4 was 0.7 % in IGFBP4 gene (Fig. 5).

Discussion

The previous studies reported that the epigenetic genomic related factor, MeCP2, could be triggered through DNA methylation at CpG dinucleotides [18]. Epigenetic genomic regulation and DNA methylation also have been recognized to play an important role in the etiology of tumor and tumor development [19, 20]. Therefore, we explored for the first time to discover the MeCP2 related differentially expressed genes in this study. Regulating the differential genes in the tumor cells or tissues, we could indirectly regulate the proliferation, invasion, migration, and development of the tumors.

The formation and development of tumors is a complex, multi-step, and multi-factor process, which is also a result of abnormal gene expression or inhibitive gene inactivation of the tumor-related genes [21]. Therefore, the changes of tumor growth-related genes or genomes may play an important role in the development of tumors. Following with the accomplishment of human genome project, gene chip technique has been extensively applied in the molecular biology and tumor biology. Gene chip technique has many merits, including high flux, high efficiency, and little sample.

Table 2 Premiers for the CpG amplification in IGFBP4 gene

Premiers	Sequences
P1-F	TTAGGAGGTTTGGTTTTTAGYG
P1-R	TAAACCRAAACCCCRAAA
P2-F	GAAAGGGGGTTTYGTAGTA
P2-R	ATCCCTAACCACACCCCC
P3-F	GGTTAGGGATYGGTATAAAGTT
P3-R	ACCAACAACAAAACRACC
P4-F	GGYGGTTATGTTGTTTTTTT
P4-R	ACTCCATACACACRCCTTACC

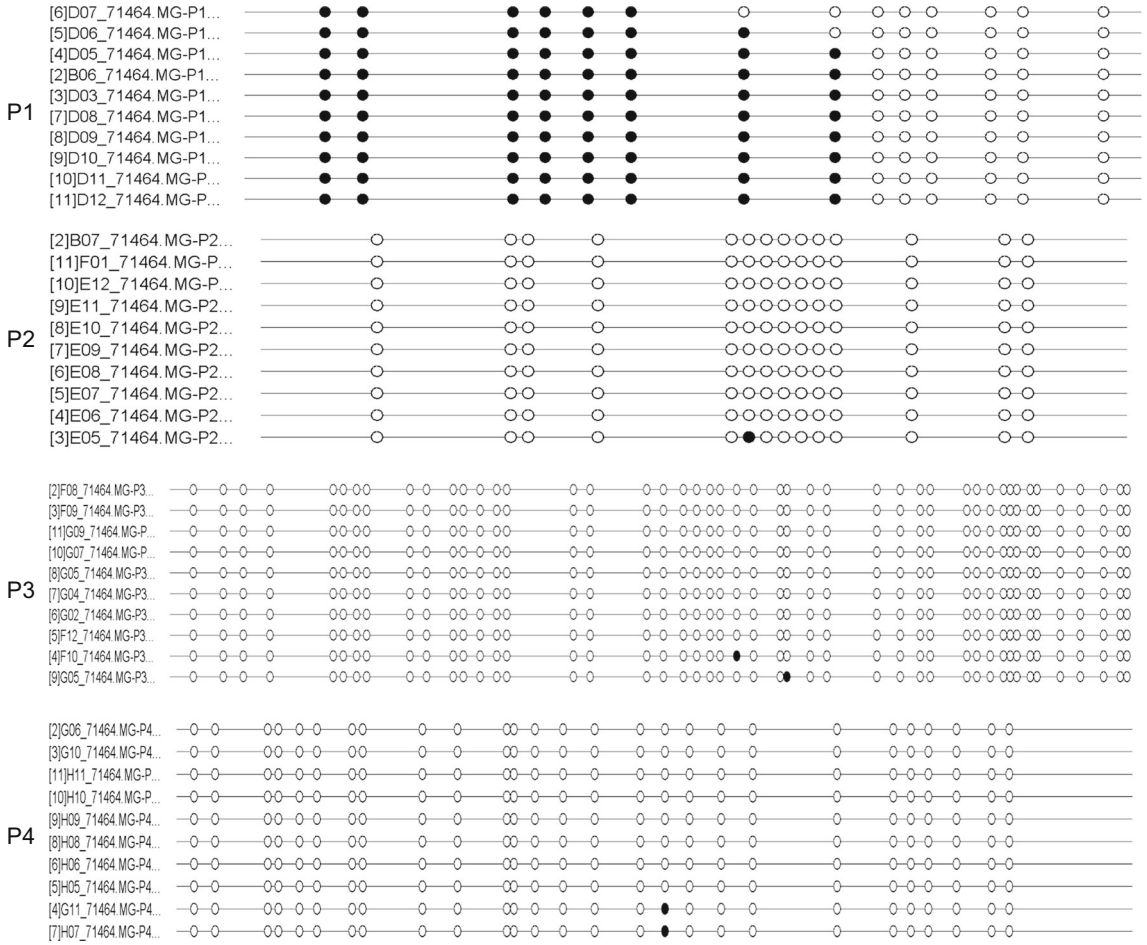

Fig. 5 CpG in IGFBP4 gene sequence was highly methylated

In this study, lentiviral vector was used to carry the silencing gene, MeCP2 siRNA (Lv-MeCP2-siRNA), to infect the U2OS and SaOS2 cells. Our previous study has been indicated that the MeCP2 siRNA could inhibit the MeCP2 expression both in U2OS and SaOS2 cells (data not shown). The cell proliferation assay (CCK8 method) indicated that the LV-MeCP2-RNAi could significantly decrease cell proliferation compared to the LV-MeCP2-RNAi-CN group ($P<0.05$). This proliferation assay results suggest that the MeCP2 gene may participate in or regulate the proliferation and growth of human osteosarcoma. This function of MeCP2 in tumor growth is consistent with that of a previous report. Zhao et al. [22] also found that MeCP2 could promote the human hepatocellular carcinoma HePG2 cell proliferation by activating ERK1/2 and inhibiting p38 activity. Bernard et al. [23] also proved that MeCP2 is a required factor for the proliferation and growth of prostate cancer. Our study also illustrated that the MeCP2-caused proliferation of cells was triggered by the activation of apoptosis. The results showed that the MeCP2 gene silencing could significantly increase the apoptosis rate, which suggest that the MeCP2 inhibits cell apoptosis. We discovered this result in osteosarcoma for the first time. In other tumors, also, only Yaqinuddin et al. [9] found that the MeCP2 enhances the tumor proliferation by blocking the apoptotic pathway. However, whether the MeCP2 mediates the proliferation and inhibits the apoptosis in the other tumors also need to be explored.

This study screened 107 differentially expressed genes were screened from the total 49,395 transcripts used the U133 plus 2.0 gene expression chip. All of the 107 transcripts, including 34 up-regulated transcripts and 73 down-regulated transcripts. Among these up- and down-regulated transcripts, only 5 transcripts significant differentially expressed genes correlated with the MeCP2 gene were screened, including IGFBP4, HOXC8, LMO4, MDK, and CTGF.

IGFBP4 could inhibit the activity of insulin-like growth factor 1 (IGF1) and does not enhance the actions of IGF1 under any conditions [24]. IGFBP4 is produced mainly in the liver, but many other tissues, especially neoplastic tissue, produce it [25]. Ryan et al. [26] found that the protease-resistant IGFBP4 blocks tumor growth and tumor angiogenesis. Our results also indicated that when the MeCP2 inhibited, the proliferation was inhibited and the IGFBP4 level was up-regulated. Furthermore, we also found that the methylation

frequency of CpG in IGFBP4 gene was significantly high. HOXC8 is one of the 39-member HOX family proteins [27]. Recent studies have also linked HOXC8 into the tumorigenesis of various cancer types. For example, HOXC8 expression is selectively turned on in human cervix cancer cells [28] and is associated with the loss of tumor differentiation in human prostate cancer cells. Li et al. [29] also found that HOXC8 is elevated in invasive/metastatic breast tumor cell lines, and its presence is required for breast cancer cell migration and metastasis. LIM-only protein 4 (LMO4) belongs to the LIM-only family of transcriptional coregulatory proteins characterized by the presence of two tandem LIM domains [30]. Increased LMO4 expression is observed in several epithelial cancers including squamous cell carcinomas of oral cavity, prostate, pancreas, and breast cancer [31]. Midkine (MDK) is a heparin-binding growth factor that is highly expressed in many malignant tumors, including lung, esophageal, stomach, colon, hepatocellular, breast, renal, and pancreatic carcinoma [32–34]. Hao et al. [35] found that inhibition of MDK with iMDK provides a potential therapeutic approach for the treatment of lung cancers that are driven by MDK. CTGF is a cysteine-rich, matrix-associated, heparin-binding protein and is widely expressed in a variety of human tissues. The previous study indicated that CTGF associates with tumor cell proliferation, adhesion, and angiogenesis [36]. CTGF has been shown that CTGF is an oncogenic factor promoting tumor progression in pancreatic cancer, prostate cancer, liver cancer, breast cancer, and sarcoma [37–39]. All of the above factor, including HOXC8, LMO4, MDK, and CTGF, could enhance the cell proliferation by mediating the tumor cell angiogenesis and development. In this study, by silencing the MeCP2 gene, the HOXC8 gene down-regulated 2.11 times, the LMO4 gene down-regulated 2.31 times, the LDK gene down-regulated 1.89 times, and the CTGF gene down-regulated 1.89 times. Our results also indirectly indicated that the above differentially expressed genes are also correlated with osteosarcoma cell proliferation and growth. The differentially expressed IGFBP4, HOXC8, LMO4, MDK, and CTGF genes may also participate in the process of cell cycle regulation, signal transduction, transcriptional control, and translational control, which would be discussed in our future studies.

In this study, five differentially expressed genes were discovered correlated with MeCP2 gene in osteosarcoma cells. However, this is only a preliminary screening, and real-time PCR, Western blot, and electrophoretic mobility assay must be performed to identify these MeCP2-related genes.

In conclusion, MeCP2 gene correlated osteosarcoma cell proliferation is a multi-gene and multi-pathway process. The differentially expressed IGFBP4, HOXC8, LMO4, MDK, and CTGF genes may be involved in the MeCP2 gene-mediated proliferation in osteosarcoma cells.

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Conflicts of interest None

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