JBUON 2020; 25(6): 2708-2713
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Methylation of WWOX gene promotes proliferation of osteosarcoma cells

Yang Liu[1], Qingqing Wang[2], Pengjie Yu[2], Wei Miao[2], Chuanchuan Liu[3], Yongqiang Pu[2], Chengwu Zhang[2]

[1]Department of Orthopedics, Affiliated Hospital of Qinghai University, Xining City, Qinghai 810000, China. [2]Department of Surgical Oncology, Affiliated Hospital of Qinghai University, Xining City, Qinghai 810000, China. [3]Qinghai University, Xining City, Qinghai 810000, China.

Summary

Purpose: *To explore the effects of WW domain-containing oxidoreductase (WWOX) gene methylation on proliferation and apoptosis of osteosarcoma cells.*

Methods: *A total of 51 patients with osteosarcoma confirmed by pathological examinations were enrolled as the observation group, while 49 cases with non-osteosarcoma diagnosed and treated in our hospital were randomly selected as the control group. Osteosarcoma cell lines MG63 and HOS were selected as observation group, while those added with methylation inhibitor were set as control group, of which genomic methylation level was determined via HPLC. Proliferation of the two cell lines was compared via cell counting kit-8 (CCK-8) assay at 12 h, 24 h, 36 h and 48 h. Invasion rate of cells in each group was tested via Transwell assay at 24 h.*

Results: *The average methylation rate of WWOX gene was remarkably higher in osteosarcoma tissues in comparison in normal adjacent tissues and control group ($p<0.05$). A higher methylation rate was found in MG63 cell line compared with in HOS cell line ($p<0.05$). More cells were observed in MG63 cell line than in HOS cell line and control group from 24h ($p<0.05$). Besides, 24-h invasion rate was higher in MG63 cell line than in HOS cell line and control group ($p<0.05$). Moreover, MG63 cell line prompted a lower 24-h apoptotic rate in comparison with HOS cell line and control group ($p<0.05$).*

Conclusions: *Methylation level of WWOX gene is intimately associated with the occurrence and progression of osteosarcoma, which is able to promote the proliferation of cancer cells to a certain extent, thus accelerating the development of disease.*

Key words: *osteosarcoma cells, WWOX gene, methylation, proliferation, apoptosis*

Introduction

Osteosarcoma is one of the most common primary malignant bone tumors, which mainly occurs in children and adolescents, with high malignancy and poor prognosis. More than 80% of patients have micrometastases (mainly in the lungs) when they are definitely diagnosed with osteosarcoma, and the postoperative 5-year survival rate is approximately 20% [1-6]. WW domain-containing oxidoreductase (WWOX) gene is a gene isolated by Bednarek via shotgun gene sequencing technology, which is mainly located on chromosome 16q23.3-q24, composed of 9 exons and able to encode 414 amino acids [7]. A relevant study indicates that WWOX gene functions as a tumor-suppressor that is capable of inhibiting the proliferation of tumor cells, while its transcription and expression levels may be associated with the occurrence and progression of tumors [8]. A study has been confirmed that there is a declining expression of WWOX in patients with various tumors such as osteosarcoma,

Corresponding author: Chengwu Zhang, MM. Department of Surgical Oncology, Affiliated Hospital of Qinghai University, No. 29 Tongren Road, Xining, Qinghai 810000, China.
Tel: +86 13519710998, Email: 952068360@qq.com
Received: 08/10/2020; Accepted: 20/11/2020

colon cancer and breast cancer, which is correlated with the poor prognosis [9]. Recent studies have demonstrated a close association between abnormal methylation of deoxyribonucleic acid (DNA) and the occurrence and progression of tumors. Therefore, the methylation levels of WWOX gene promoter in human osteosarcoma and osteochondroma cells were compared in this study to further explore the relationship between methylation of WWOX gene promoter and osteosarcoma, so as to investigate the effect of WWOX gene methylation on human osteosarcoma cells

Methods

General data

A total of 51 patients with osteosarcoma pathologically confirmed from September 2017 to November 2018 were enrolled as the observation group (28 males and 23 females aged 23.5±7.8 years on average), while 49 cases with non-osteosarcoma and non-malignancy diagnosed in our hospital were randomly selected as the control group (27 males and 22 females, with an average age of 25.7±8.1 years). No statistically significant differences were found in gender and age between the two groups ($p>0.05$). This study was approved by the Ethics Committee of Affiliated Hospital of Qinghai University. Signed informed consents were obtained from all participants before the study entry.

Materials

MG63 and HOS cell lines (American Type Culture Collection (ATCC) (Manassas, VA, USA)), methylation inhibitor (Decitabine, Isoreag, Shanghai, China), DNA methylation modification kit (EpiTect Bisulfite kit, QIAGEN, Hilden, Germany), high-glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone, South Logan, UT, USA), fetal bovine serum (FBS, Gibco, Rockville, MD, USA), phosphate buffered saline (PBS, Hyclone, South Logan, UT, USA), Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan), and Apoptosis Detection Kit (Solarbio, Beijing, China).

Extraction of DNA

About 25 mg of osteosarcoma cells and normal osteoblast samples were routinely collected and placed into powder with liquid nitrogen to extract DNA using Ezup Column Animal Genomic DNA Purification Kit [Sangon Biotech (Shanghai, China) Co., Ltd.] in accordance the instructions.

Detection of WWOX gene methylation

According to the instructions of the DNA methylation modification kit, 15 μL of DNA was added with 5 μL of RNase-free water, 85 μL Bisulfite Mix and 35 μL DNA Protect Buffer in turn for modification for 5 h. Subsequently, the modified DNA was purified twice by desalting, followed by methylation-specific polymerase chain reaction (PCR) in a 50 μL system, including 25 μL of EpiTect Master Mix, 4 μL of modified DNA, 2 μL of forward primer and 2 μL of reverse primer and 17 μL of RNase-free water. WWOX gene methylation primer sequences are shown in Table 1. PCR was performed under the following conditions: methylation reaction (M): pre-denaturation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, as well as extension at 72°C for 10 min, and unmethylation reaction (U): pre-denaturation at 95°C for 10 min, (94°C for 30 s, 53°C for 45 s and 72°C for 45 s) ×40 cycles and extension at 72°C for 10 min. The obtained product was purified, added into the BDSH of high-performance liquid chromatography (HPLC) instrument through a micro-injector, and eluted with the mixture of methanol, sodium pentanesulfonate and triethylamine as the mobile phase at a low temperature (flow rate: 1 L/min, ultraviolet wavelength: 273 nm, sensitivity: 0.01 AUPS). The DNA methylation level in the samples was measured with deoxycytosine and methyldeoxycytosine standards as controls. Each sample to be determined was repeatedly measured three times and the average value was calculated.

Table 1. WWOX gene methylation primer sequences

	Primer sequence
WWOX	Forward: 5'-CAAGGGCGAGTGAAGCAGT-3'
	Reverse: 3'-GGCGGAGGGTGGTATTTTGT-5'

Cell culture and grouping

Osteosarcoma cell lines MG63 and HOS were subcultured using high-glucose DMEM containing 10% FBS, and transfected with lentivirus carrying an RFLP-labeled plasmid, followed by screening via puromycin to obtain recombinant cell lines with a purity of more than 90%. Later, the MG63 cell line processed by methylation inhibitor Decitabine was as assigned into MG63 + Dec group, the treated HOS cell line was enrolled as HOS + Dec group, while the two cell lines without treatment of Decitabine were MG63 group and HOS group, respectively.

Determination of cell proliferation via CCK-8 assay

Cells in the four groups were inoculated into 96-well plates at a density of 1×10^5 per well. Five replicates were tested at each time point. The absorbance at 12 h, 24 h, 36 h and 48 h with a wavelength of 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Then, the average values of five duplicate wells were compared.

Examination of cell migratory ability via transwell assay

Three replicate wells were set for each group of cells, and the cells (1×10^6) were inoculated into the upper chamber according to the instructions of the Transwell kit. After 24 h, the number of cells entering the lower chamber was recorded using a fluorescence microscope under a field of view of 100×. Cells from three differ-

ent positions randomly selected were counted in each well, and the average number of cells invading the lower chamber was calculated.

Detection of cell apoptosis using flow cytometry

Firstly, Binding Buffer (5×) was diluted into 1×Binding buffer working solution for the subsequent experiment [4 mL sterile deionized water was added into 1 mL Binding Buffer (5×)]. Secondly, cells were digested using ethylene diamine tetraacetic acid (EDTA)-free trypsin for 3 min until the adherent cells could be gently pipetted down. After trypsin digestion solution was removed, DMEM was added. Next, cells were pipetted down and transferred to the centrifuge tube, centrifuged at 500 g for 5 min, and collected. Subsequently, cells were pipetted and washed with PBS, and counted. Cells (5×10^5) were collected and centrifuged at 500 g for 5 min, and then the supernatant was discarded. After that, about 195 μL of Binding Buffer working solution was added to gently resuspend the cells. Then, 5 μL of Annexin V-FITC was added into the cells, slightly mixed, and incubated at room temperature for 10 min in the dark. Finally, 10 μL of propidium iodide (PI) (20 μg/mL) was added, slightly mixed, and incubated at room temperature for 5 min in the dark, followed by detection using a flow cytometer.

Statistics

SPSS 19.0 (IBM, Armonk, NY, USA) was applied for statistical analyses. Data were presented as mean ± SD. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test, followed by *Post Hoc* test (least significant difference). The survival curves were plotted using the Kaplan-Meier method, and statistical significance between the two groups was detected using log-rank test. P<0.05 suggested that the difference was statistically significant.

Results

Comparison of methylation rate of WWOX gene among three different tissues

The average methylation rate of WWOX gene was remarkably higher in osteosarcoma tissues in comparison with that in normal adjacent tissues and control group (p<0.05), and no difference in methylation was notable between normal adjacent tissues and control group (p>0.05) (Figure 1).

Comparison of methylation rate of WWOX gene among four groups of cells

A higher methylation rate was found in MG63 cell line compared with HOS cell line and two groups treated with methylation inhibitor Decitabine (MG63 + Dec group and HOS + Dec group), while it was higher in HOS cell line than in MG63 + Dec group and HOS + Dec group (p<0.05) (Figure 2).

Proliferation in four groups of cells determined via CCK-8 assay

More cells were observed in MG63 cell line than in HOS cell line and MG63 + Dec group and HOS + Dec group at 24 h, 36 h and 48 h (p<0.05), while the proliferation of HOS cell line was greater than in MG63 + Dec group and HOS + Dec group (p<0.05) (Figure 3).

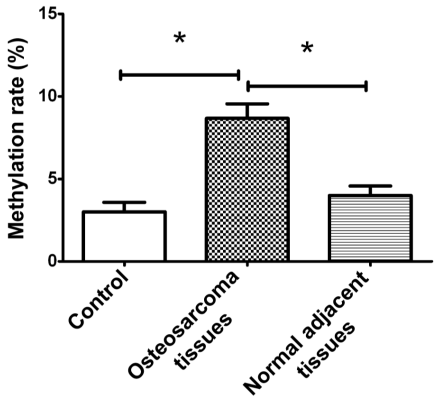

Figure 1. Comparison of methylation rate of WWOX gene among three different tissues. *p<0.05 *vs.* osteosarcoma tissues.

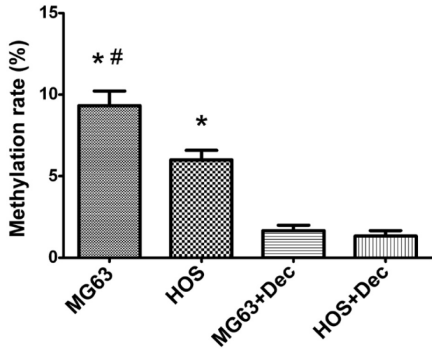

Figure 2. Comparison of methylation rate of WWOX gene among four groups of cells. *p<0.05 *vs.* HOS + Dec group, #p<0.05 *vs.* HOS group.

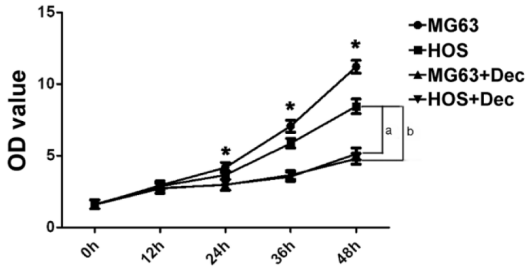

Figure 3. Proliferation in four groups of cells determined via CCK-8 assay. * The number of cells is significantly increased in MG63 cell line compared with that in HOS group (p<0.05), **a:** Cells are markedly increased compared with that in MG63 + Dec group, **b:** Cells are notably raised compared with that in HOS + Dec group.

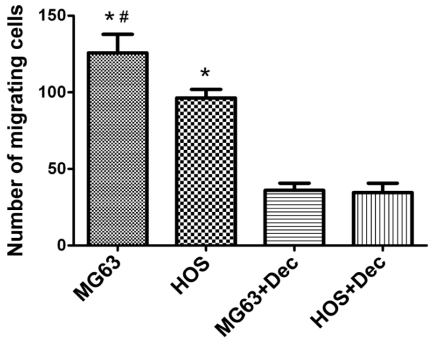

Figure 4. Number of migrating cells in each group. *p<0.05 *vs.* HOS + Dec group, #p<0.05 *vs.* HOS group.

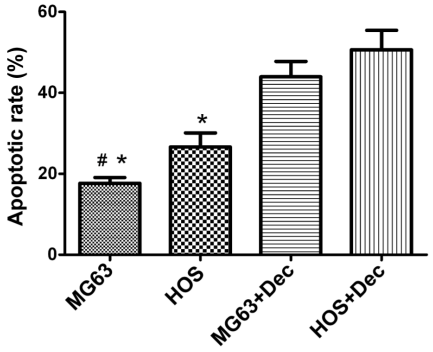

Figure 5. Comparison of apoptotic rate among four groups of cells. *p<0.05 *vs.* HOS + Dec group, #p<0.05 *vs.* HOS group.

Comparison of migratory ability among four groups of cells

The number of migrating cells within 24 h was larger in MG63 cell line than in HOS cell line, MG63 + Dec group and HOS + Dec group (p<0.05), while it was greater in HOS cell line in comparison with that in MG63 + Dec group and HOS + Dec group (p<0.05) (Figure 4).

Comparison of apoptotic rate among four groups of cells

MG63 cell line prompted a lower 24-h apoptotic rate in comparison with HOS cell line and control group (p<0.05), while it was lower in HOS cell line in contrast with that in MG63 + Dec group and HOS + Dec group (p<0.05) (Figure 5).

Discussion

Osteosarcoma is a malignant tumor characterized by strong invasiveness, which is prone to metastasis, and mainly occurs in the fast-growing bone tissues [10-13]. However, the pathogenesis of osteosarcoma remains unclear, which can be diagnosed only based on clinical symptoms and imaging examinations, and most patients bear distant metastases when diagnosed. At present, the diagnosis of osteosarcoma is undergoing a bottleneck period owing to the limitations of medical technology and high malignancy of the disease itself. However, epigenetics has become a significant diagnostic and therapeutic approach with the in-depth research on epigenetics (DNA methylation, histone modification and nucleosome recombination) and the emergence of drugs targeting genetically related enzymes and new detection technologies. Investigating the occurrence and progression of tumors from the perspective of epigenetics is conducive to the early diagnosis and timely prevention of malignant tumors (such as bone tumors) and may provide new ideas for the treatment of bone tumors.

WWOX gene is a new tumor-suppressor gene found on human common chromosomes, which is called WW domain-containing oxidoreductase due to the oxidoreductase domain [14,15]. WWOX is able to induce cell proliferation or apoptosis by interacting with various carcinogens. It has been reported in the literature that WWOX gene is abnormally expressed in solid tumors such as gastric and ovarian cancer [16-18]. In related studies, the ablation of WWOX gene in mice has been found to be highly associated with the progression of osteosarcoma [19-21]. Subsequent human osteosarcoma genome-wide analysis reveals that WWOX expression is weakened in osteosarcoma cells [22-25]. At the molecular level, the promoter inhibits the transactivation and reverses its expression after being methylated [26,27]. The expression of WWOX gene is suppressed due to its epigenetic changes, thus reducing its pro-apoptotic effect. In particular, the promoter methylation may play an important role. This study aimed to explore the association between WWOX gene methylation and osteosarcoma cells.

The average methylation rate of WWOX gene was compared between patients with osteosarcoma and normal tissues in this study, which displayed that it was remarkably higher in osteosarcoma tissues (p<0.05), demonstrating that the average methylation level of WWOX gene is lower in normal cells than in patients with malignant bone tumors. In addition to research at the tissue level, *in vitro* studies at the cellular level indicated that MG63 cell line exhibited a higher methylation rate than HOS cell line, so the two groups of cells represent the level of methylation according to the cell lines, that is, MG63 cell line represents high methylation group while HOS cell line represents low methylation group. In terms of the cell proliferation, more proliferating cells were detected in MG63 cell line in the high methylation group than in the low methylation group,

MG63 + Dec group and HOS + Dec group, revealing that high methylation of WWOX gene is capable of promoting cell growth. Meanwhile, highly methylated MG63 cells also have advantages in migratory ability and apoptosis over lowly methylated HOS cells.

Conclusions

In summary, osteosarcoma has a high grade of malignancy and poor prognosis, and its pathogenesis (progression of disease) has not been fully illuminated through the current research, so a lot of further research is needed. It is revealed in this study that the methylation of WWOX gene is inextricably linked to the progression of osteosarcoma, indirectly confirming the tumor suppressive effect of WWOX gene. The inhibitory effect on cancer cells disappears after the methylation of WWOX gene, which promotes cell proliferation and disease progression, and contributes to the gene therapy or biological therapy for tumors in the future.

Funding acknowledgements

This study was supported by The Key Research & Development and Transformation Project of Qinghai Province for 2018 (No.2018-SF-113).

Conflict of interests

The authors declare no conflict of interests.

References

1. Meng Q, Tang B, Qiu B. Growth inhibition of Saos-2 osteosarcoma cells by lactucopicrin is mediated via inhibition of cell migration and invasion, sub-G1 cell cycle disruption, apoptosis induction and Raf signalling pathway. JBUON 2019;24:2136-40.
2. Li W, Zhang S. Survival of patients with primary osteosarcoma and lung metastases. JBUON 2018;23:1500-4.
3. Chen Z, Zhao G, Zhang Y, Ma Y, Ding Y, Xu N. MiR-199b-5p promotes malignant progression of osteosarcoma by regulating HER2. JBUON 2018;23:1816-24.
4. Zhang Y, Ma R, Cheng S, Gu G. Marrubenol inhibits osteosarcoma cancer cell growth by inducing autophagic cell death and inhibiting cancer cell migration and invasion. JBUON 2018;23:729-34.
5. Wang C. Therapeutic effects of adriamycin combined with high-intensity focused ultrasound on osteosarcoma. JBUON 2019;24:826-31.
6. Jiang K, Zhang J, Ji M, Gai P, Lv Q. Inhibitory effect of 5-Fluorouracil on the proliferation of human osteosarcoma cells in vitro. JBUON 2019;24:1706-11.
7. Wittig JC, Bickels J, Priebat D et al. Osteosarcoma: a multidisciplinary approach to diagnosis and treatment. Am Fam Physician 2002;65:1123-32.
8. Bednarek AK, Laflin KJ, Daniel RL, Liao Q, Hawkins KA, Aldaz CM. WWOX, a novel WW domain-containing protein mapping to human chromosome 16q23.3-24.1, a region frequently affected in breast cancer. Cancer Res 2000;60:2140-5.
9. Paige AJ, Taylor KJ, Taylor C et al. WWOX: a candidate tumor suppressor gene involved in multiple tumor types. Proc Natl Acad Sci U S A 2001;98:11417-22.
10. Zhu W, Zhu L, Bao Y, Zhong X, Chen Y, Wu Q. Clinical evaluation of neoadjuvant chemotherapy for osteosarcoma. JBUON 2019;24:1181-5.
11. Du Y, Zhang J, Yan S et al. Oridonin inhibits the proliferation, migration and invasion of human osteosarcoma cells via suppression of matrix metalloproteinase expression and STAT3 signalling pathway. JBUON 2019;24:1175-80.
12. Zhu J, He T, Wei Z, Wang Y. Retrospective analysis of the effect of treatment of osteosarcoma complicated by pathological fracture by neoadjuvant chemotherapy combined with limb salvage surgery. JBUON 2018;23:1809-15.
13. Chen Y, Wang S, Geng B, Yi Z. Pelargonidin induces antitumor effects in human osteosarcoma cells via autophagy induction, loss of mitochondrial membrane potential, G2/M cell cycle arrest and downregulation of PI3K/AKT signalling pathway. JBUON 2018;23:735-40.
14. Yan H, Tong J, Lin X, Han Q, Huang H. Effect of the WWOX gene on the regulation of the cell cycle and apoptosis in human ovarian cancer stem cells. Mol Med Rep 2015;12:1783-8.
15. Xie B, Zen Q, Wang X et al. ACK1 promotes hepatocellular carcinoma progression via downregulating WWOX and activating AKT signaling. Int J Oncol 2015;46:2057-66.
16. Aqeilan RI, Trapasso F, Hussain S et al. Targeted deletion of Wwox reveals a tumor suppressor function. Proc Natl Acad Sci U S A 2007;104:3949-54.
17. Del MS, Kurek KC, Stein GS, Lian JB, Aqeilan RI. Role of the WWOX tumor suppressor gene in bone homeostasis and the pathogenesis of osteosarcoma. Am J Cancer Res 2011;1:585-94.
18. Kurek KC, Del MS, Salah Z et al. Frequent attenuation of the WWOX tumor suppressor in osteosarcoma is associated with increased tumorigenicity and aberrant RUNX2 expression. Cancer Res 2010;70:5577-86.
19. Yang J, Cogdell D, Yang D et al. Deletion of the WWOX gene and frequent loss of its protein expression in human osteosarcoma. Cancer Lett 2010;291:31-8.
20. Diniz MG, Borges ER, Pimenta FJ et al. Evidence of molecular alterations in the tumour suppressor gene

WWOX in benign and malignant bone related lesions of the jaws. Oncol Rep 2011;25:499-502.

21. Kurek KC, Del MS, Salah Z et al. Frequent attenuation of the WWOX tumor suppressor in osteosarcoma is associated with increased tumorigenicity and aberrant RUNX2 expression. Cancer Res 2010;70:5577-86.
22. Aqeilan RI, Hassan MQ, de Bruin A et al. The WWOX tumor suppressor is essential for postnatal survival and normal bone metabolism. J Biol Chem 2008;283:21629-39.
23. Sakamoto A, Iwamoto Y. Current status and perspectives regarding the treatment of osteo-sarcoma: chemotherapy. Rev Recent Clin Trials 2008;3:228-31.
24. Yang C, Gao R, Wang J, Yuan W, Wang C, Zhou X. High-mobility group nucleosome-binding domain 5 increases drug resistance in osteosarcoma through upregulating autophagy. Tumour Biol 2014;35:6357-63.
25. Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 2006;32:423-36.
26. Petrilli AS, de Camargo B, Filho VO et al. Results of the Brazilian Osteosarcoma Treatment Group Studies III and IV: prognostic factors and impact on survival. J Clin Oncol 2006;24:1161-8.
27. Bacci G, Mercuri M, Longhi A et al. Grade of chemotherapy-induced necrosis as a predictor of local and systemic control in 881 patients with non-metastatic osteosarcoma of the extremities treated with neoadjuvant chemotherapy in a single institution. Eur J Cancer 2005;41:2079-85.