

Original Article

Increased HE4 mRNA levels are related with advanced stage in osteosarcoma

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Received September 26, 2017; Accepted March 3, 2018; Epub July 15, 2018; Published July 30, 2018

Abstract: Objective: To detect the expression patterns of human epididymis protein 4 (HE4) mRNA in osteosarcoma tissues and cell lines and determine the biological role of HE4 in osteosarcoma. Method: Serum and tissues were collected from osteosarcoma patients and healthy donors. MG63 and U2OS cells were cultured and MG63 cells were transfected with HE4-siRNA. Enzyme-linked immunosorbent assays (ELISA), MTT assays, flow cytometry, Real-time quantitative polymerase chain reaction (qPCR) and western blotting (WB) were performed to determine the proliferation, apoptosis, and expression levels of HE4 mRNA and protein, respectively. Result: Osteosarcoma patients had higher levels of HE4 mRNA and protein than patients with other bone disease or healthy donors. The advanced clinical stage had higher levels of HE4 in both tissue and serum samples. Knock down the expression of HE4 inhibited the proliferation and induced the apoptosis of osteosarcoma cell lines, decreased the mRNA and protein levels of Bax, PI3K, AKT, DNMT1 and HDAC1, but increased the mRNA and protein levels of bcl-2. Conclusions: the expression level of HE4 in osteosarcoma cell lines is higher than normal bone cell lines. HE4 may be a biomarker for early diagnosis of osteosarcoma and may act as a treatment target for osteosarcoma.

Keywords: Human epididymis protein 4, osteosarcoma, quantitative real-time RT-PCR, enzyme-linked immunosorbent assay

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in the world, which is a highly malignant mesenchymal-based tumor that has a high incidence in children [1, 2]. Approximately 6 million cases were diagnosed around the world every year [3]. The incidence of male versus female is 1.5:1 [4]. Major clinical symptoms of osteosarcoma were mitochondrial dysfunction, pain, and anemia [5]. Comprehensive therapeutic technologies have obtained great improvements in OS. The overall survival and progression-free survival are also improved greatly recently. However, approximately 20-30% of patients still suffered from recurrence [6, 7]. Therefore, it is necessary to clarify the underlying mechanisms of pathogenesis and find a novel therapeutic target for osteosarcoma.

HE4 is a member of the whey-acidic-protein (WAP) domain family, which shares 50 well-conserved amino acids. They could encode leu-

kocyte protease inhibitors to secrete leukocyte protease inhibitor (SLPI) and elafin [8, 9]. It has been reported that SLPI and elafin are expressed in various carcinomas and may play important roles in cancer development or progression [8-11]. Some studies showed that the HE4 level in serum is higher in patients with lung cancer compared with the healthy donors, which means the HE4 could act as a biological marker in lung cancer [12, 13]. Therefore, whether the HE4 can act as a biological marker and the relationship between the expression levels of HE4 and advanced clinical stages of patients was investigated in this study.

DNA methylation and histones modifications are two main mechanisms, which lead to gene silencing that could affect the gene promoter region DNA methyltransferases (DNMTs), including DNMT1, DNMT2, DNMT3a and DNMT3b [14]. The histone deacetylation is also maintained by histone deacetylases (HDACs) [15, 16]. They could take an important role in cancer due to abnormal gene methylation.

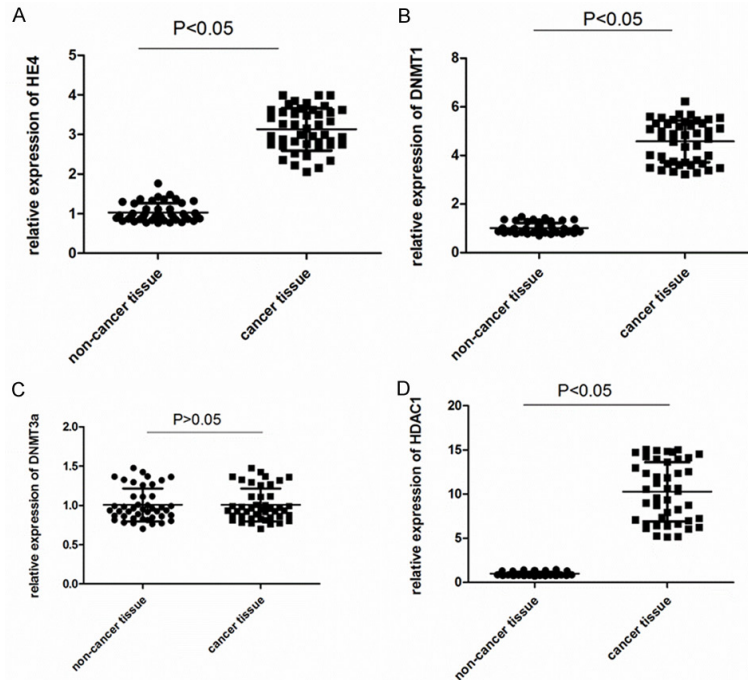


Figure 1. mRNA level of HE4, DNMT1, DNMT3a and HDAC1 were detected by using RT-PCR. Compared with non-cancer tissue, the HE4, DNMT1, and HDAC1 mRNA level were higher in cancer tissue, where there was no difference between two groups in DNMT3a level ($P < 0.05$).

Moreover, the expressions of phosphatidylinositol 3'-kinase (PI3K), v-akt murine thymoma viral oncogene homolog (Akt), B-cell CLL/lymphoma 2 (bcl-2), and bcl-2 associated X (BAX) also play important functions in the tumor formation. Therefore, those key elements could be utilized in this study to analyze the main functions and roles of HE4 in osteosarcoma.

Materials and methods

Specimen collection

The study was approved by the ethical committees of the third department of orthopedics, Cangzhou central hospital. All patients agreed to participate in this clinical study. Healthy donor groups also took part in this study. We collected tissues and serum samples at the time of diagnosis from 50 osteosarcoma stage I cases, 50 osteosarcoma stage II cases, 50 osteosarcoma stage III cases, 50 osteosarcoma stage IV cases, 50 healthy donors from May 20, 2014 to March 05, 2017. The disease aggravates from the stage I (1) to IV (4). The patients got none treatment before the collection of samples. The National Comprehensive Cancer Network (NCCN) criteria were utilized to

determine the clinical stages of patients with osteosarcoma.

Enzyme-linked immunosorbent assay

Serum samples were collected and undisturbed for one hour at normal atmospheric temperature. The samples were isolated by centrifugation (1500 g), and then the supernatants were collected and stored at -80°C . Human ELISA kit (Protein Sample USA) was used for the examination of serum levels of HE-4. The kits had the sensitivity of 5 pg/mL for markers. The plasma was taken after being centrifuged at 1600 g for 10 min. The plasma level of HE4 was detected by the ELISA kit. The operation was performed strictly according to the kit instruction.

Cell culture and transfection

The human OS cell lines MG63 and U2OS, as well as normal human osteoblast hFOB1.19 cells, were purchased from Shanghai hongshun cells bank. Cells were cultured in RPMI-1640 medium (Sigma, USA). The medium was replenished with 10% fetal bovine serum (FBS), two antibiotics namely penicillin and streptomycin (all from Sigma, USA) at 37°C in a 5% CO_2 atmosphere. MG63 were seeded into six wells and transfected with HE4-siRNA by lipofectamine 2000.

MTT assay

MG63 were seeded onto 96 wells. The proliferation of MG63 cells was determined by MTT assay. Briefly, following cell culture, 10 μL MTT (Sigma, USA) was added to each well. The 96 well plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 h. The resulting sample was dissolved in 100 μL isopropanol and evaluated by absorbance at 490 nm by FlexStation 3 (Molecular Device, USA). Each experiment was repeated for at least three times.

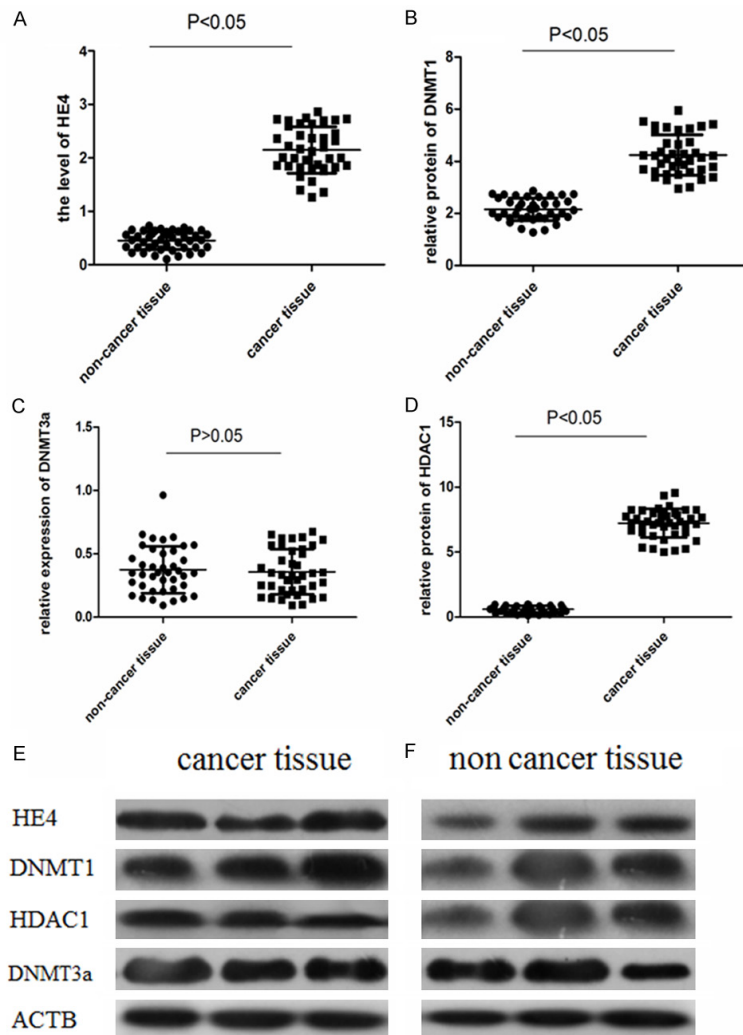


Figure 2. The protein level of HE4, DNMT1, DNMT3a and HDAC1 were detected by using WB. Compared with non-cancer tissue, the HE4, DNMT1, and HDAC1 protein level were higher in cancer tissue, where there was no difference between two groups in DNMT3a level ($P < 0.05$).

Apoptosis assay

MG63 cells were seeded onto six wells after transfected with siRNA. Then, MG63 cells were treated according to manufacturer's instructions. The cells were stained with AnnexinV-APC and PI. After that, the stained cells were analyzed with BD FACSC auto II analyzer.

RT-PCR for HE4

Quantitative real-time RT-PCR was performed using SYBR Green Kit (life Invitrogen, USA) with the ABI 7500 real-time rotary analysis (ABI, Life). Real-time PCR primers were shown in table I HE4, forward 5-AGUAACTCCTTCTCG-GGGCG-3 and reverse 5-UC-UAATTCGGTGA-

AATTGG-3; 18SrRNA, forward 5-CGGCGACGACCCATTGCAAC-3 and reverse 5-GAATCG-AACCCTGATTCCCCGTC-3. The qRT-PCR was performed under this condition: denaturation at 95°C for 5 minutes, next by 45 cycles of 95°C for 15 s, 60°C for 35 s, 72°C for 20 s. The 2- $\Delta\Delta$ ct method was used to calculate gene expression ratio. The expressions of HE4 were normalized to that of 18SrRNA.

Western blotting

Cells were lysed by using radioimmune precipitation assay (RIPA) buffer to obtain total protein. The concentration of protein was determined by using the BCA protein assay kit (Thermo, USA). The protein was separated by 8-15% SDS-PAGE and the separated bands were electrotransferred to PVDF membrane (Amersham Pharmacia, USA). PVDF membrane was blocked with 5% non-fat skimmed milk at a room temperature. The membrane was probed with antibodies Bax (1:1000), HE4 (1:1000), bcl-2 (1:1000), PI3K (1:1000), AKT (1:1000), ACTB (1:5000), DNMT1 (1:1000), HDAC1 (1:1000) and DNMT3a (1:1000) (Abcam

USA). The diluted corresponding secondary antibody was incubated at room temperature for 1 hour, rinsed with TBS for 5 minutes for a total of 3 times and analyzed by chemiluminescence method. After the protein bands were scanned by the scanner, the relative quantification of the gray bands of the protein bands was carried out by Imagequant 5.1 software. The β -actin band was used as a reference. The target protein bands were utilized to compare the relative gray-scale ratios of β -actin.

Statistical analysis

Data were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were presented as the mean $\pm$ standard

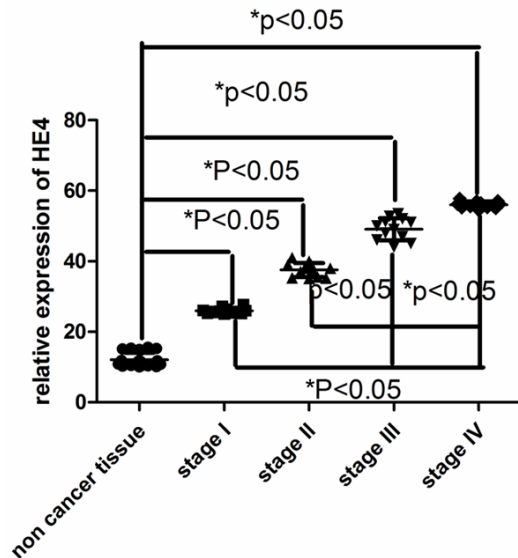


Figure 3. The expression level of HE4 was detected by performing RT-PCR in non-cancer tissue and stage I, stage II, stage III and stage IV groups, data are means $\pm$ SD of three independent experiments ($P<0.05$).

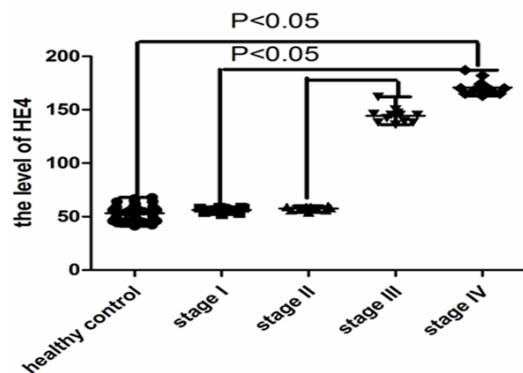


Figure 4. The serum level of HE4 was detected by performing Elisa in healthy control (n=50) and stage I (n=50), stage II (n=50), stage III (n=50) and stage IV (n=50) groups, values are means $\pm$ SD of three independent experiments ($P<0.05$).

deviation (SD). Comparisons among multiple groups were determined using one-way analysis of variance (ANOVA) or Kruskal-Wallis. $P<0.05$ was considered statistically significant.

Results

The mRNA and protein expression levels in osteosarcoma tissues and non-osteosarcoma tissues

The mRNA expression level of HE4, DNMT1, and HDAC1 in osteosarcoma tissues were high-

er than that in non-osteosarcoma tissues (**Figure 1A** $P=0.0026$, **Figure 1B** $P=0.0019$, **Figure 1D** $P=0.0001$). However, the DNMT3a mRNA level had no significant difference between osteosarcoma tissues and non-osteosarcoma tissues (**Figure 1C** $P=0.126$). The protein levels of HE4, DNMT1, HDAC1, and DNMT3a were determined in osteosarcoma tissues and non-osteosarcoma tissues of osteosarcoma patients. The result showed that HE4, DNMT1, and HDAC1 protein levels were higher in osteosarcoma tissues than non-osteosarcoma tissues (**Figure 2A** $P=0.0012$, **Figure 2B** $P=0.0011$, **Figure 2D** $P=0.0006$, **Figure 2E** and **2F**). However, DNMT3a protein level had no significant difference between osteosarcoma tissues and non-osteosarcoma tissues (**Figure 2C** $P=0.329$, **Figure 2E** and **2F**).

The expression levels of HE4 in different clinical stages of osteosarcoma tissues and serum samples

After that, the mRNA expression levels of HE4 in different clinical stages of osteosarcoma tissues were detected by RT-PCR. The result showed that higher levels of HE4 were found in advanced stages (**Figure 3**), which indicated that the level of HE4 associated with clinical stages. The serum levels of HE4 in stage 1 and 2 OS patients have no statistically significant difference compared with the donors (**Figure 4**, $P=0.126$). However, in patients at clinical stage 3 and 4, the levels of HE4 were statistically higher than from healthy donors group ($P<0.05$) (**Figure 4**, $P<0.05$).

The molecular mechanism investigation of HE4 expression using MG63 cells

Then, the HE4-siRNA were synthesized and transfected into MG63 cells. The mRNA and protein expression levels of HE4 were decreased significantly in siRNA-HE4 group (**Figure 5**). FCM and MTT assays were performed to detect the biological roles of HE4 in osteosarcoma. The result showed that silencing the expression of HE4 inhibited the proliferation and promoted the apoptosis of MG63 cells (**Figure 6**).

The mRNA and protein expression levels of Bax, bcl-2, PI3K, and AKT in HE4 knock-down group were detected. The result showed that the mRNA and protein expression levels of Bax, PI3K, and AKT were decreased in HE4 knock-

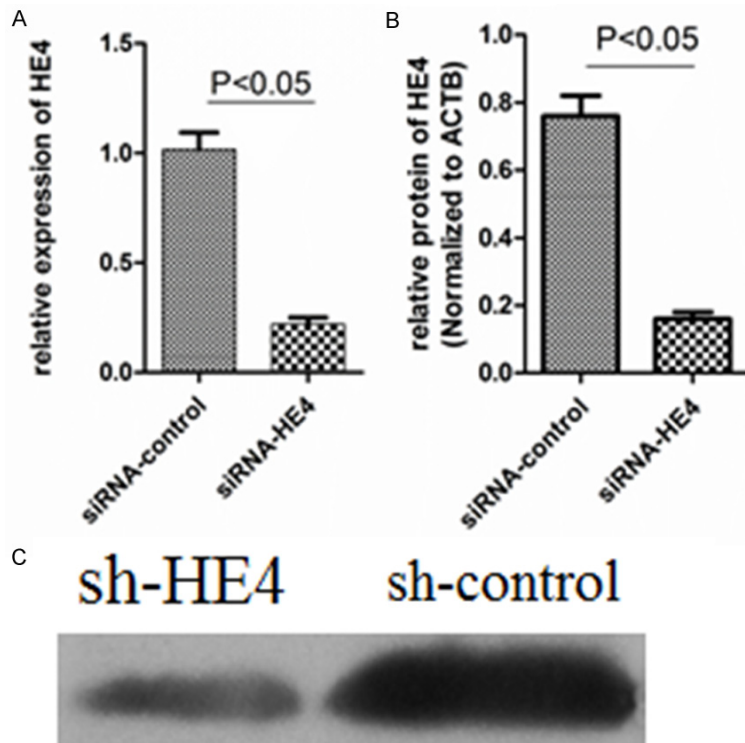


Figure 5. The knock down expression of HE4 by transfecting siRNA. The mRNA and protein level of HE4 were detected by performing PCR and WB ($P<0.05$).

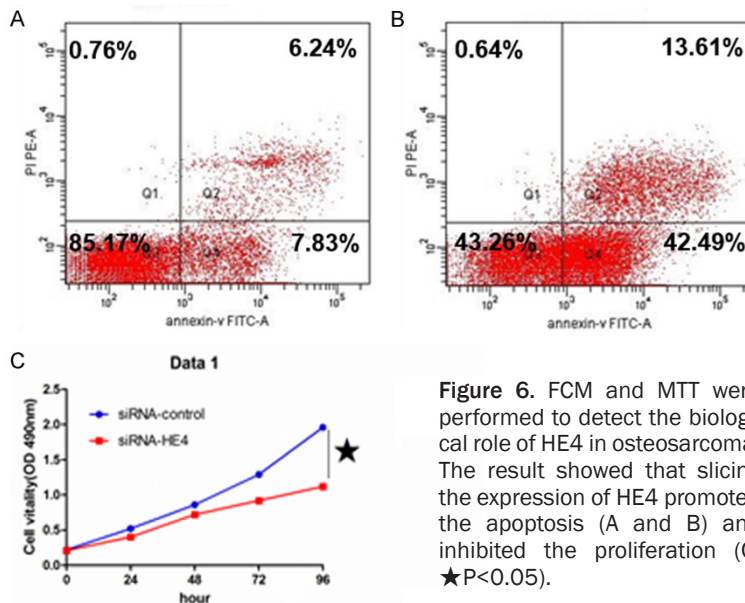


Figure 6. FCM and MTT were performed to detect the biological role of HE4 in osteosarcoma. The result showed that slicing the expression of HE4 promoted the apoptosis (A and B) and inhibited the proliferation (C, ★ $P<0.05$).

down groups compared with control groups. However, the bcl-2 mRNA and protein expression levels were increased in HE4 knock-down group (Figure 7). As shown before, the expression levels of DNMT1 and HDAC1 in osteosar-

coma tissues were higher than that in non-osteosarcoma tissues, which indicated that the epigenetic regulation could take part in the occurrence and development of osteosarcoma. Therefore, after knock down the expression of HE4, the mRNA and protein expression levels of DNMT1 and HDAC1 were also determined. The result showed that knock down the expression of HE4 could inhibit the mRNA and protein expression levels of DNMT1 and HDAC1 (Figure 7).

Discussion

This study confirmed that the HE4 level is up-regulated not only in OS tissues and OS cell lines but also in the serum of OS patients. According to world health organization, OS is a very common bone cancer [4]. The OS robs around 600 thousands of lives of people every year. Although the incidence of osteosarcoma is relatively low (about 6% of all childhood cancers) compared with other malignant tumors, the five-year survival rate is significantly poor. There are many reasons that can lead to OS such as gene mutation, cytokines and epigenetic regulation of gene [6].

In the previous study, the HE4 has been reported as a biomarker in many types of cancers. For example, the expression of HE4 is higher in the serum of lung cancer patients [17]. Some reports have shown that the HE4 level had consistency with the level of CA199 in ovarian cancer [9, 11, 18], which were consistent with this study. In endometrial cancer patients, HE4 is a sensitive diagnostic serum biomarker for the detection of EC patients, which have been demonstrated for a better diagnostic performance compared to

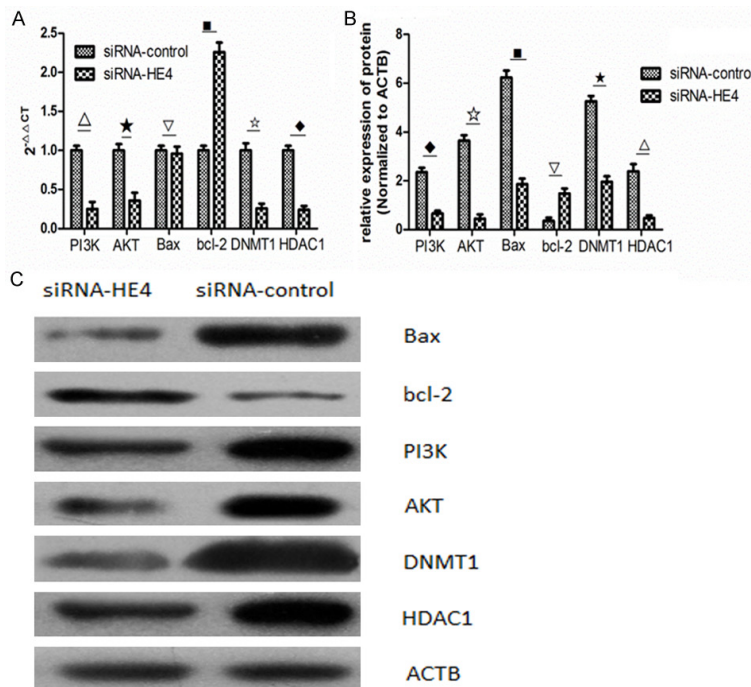


Figure 7. The mRNA and protein level of Bax, PI3K, AKT, bcl-2, DNMT1, and HDAC1 were detected after knock down the expression of HE4. The mRNA and protein level of DNMT1, HDAC1, PI3K and AKT were decreased in HE4 knock-down group compared with control group. The bcl-2 mRNA and protein level was increased in HE4 knock-down group. The Bax protein level was decreased in knock down the group (A, $\triangle$ $\star$ ∇ $\blacksquare$ $\star$ $\blacklozenge$ $P < 0.05$; B, $\blacklozenge$ $\star$ ∇ $\star$ $\triangle$ $P < 0.05$). C. Protein expression in siRNA-control and siHE4 cells, Protein expression level was determined by immunoblotting with ACTB (β -actin) as a control.

CA-125. Good performance of HE4 in the diagnosis of early stages EC also indicated its usefulness as a prognostic biomarker and monitoring therapy and detecting the early recurrence [19, 20]. In endometrial cancer, HE4 was also utilized as prognostic biomarkers [21]. The mRNA and protein expression levels of HE4 in osteosarcoma tissue and non-osteosarcoma tissue were also detected. The result showed that the HE4 levels were higher in osteosarcoma tissue than in non-osteosarcoma tissue. Furthermore, the highest expression level of HE4 was found in clinical stage IV, which could be utilized to speculate that the high level of HE4 might relate with advanced clinical stages.

After knocking down the expression of HE4, the results stated that the down-regulation of HE4 could inhibit the proliferation and induce the apoptosis of OS cell lines. Then, the expression levels of bcl-2 mRNA and protein levels were also detected after knock down the expression

of HE4. The result indicated that silencing the expression of HE4 inhibited the expression of bcl-2. Therefore, this study could speculate that silencing HE4 expression could promote the apoptosis of OS cells by decreasing the expression levels of bcl-2 mRNA and protein levels.

The occurrence and development of cancer are partly due to a failure of inducing cell apoptosis, bcl-2 and bax have important roles in regulating apoptosis and cell cycle progression [22-25]. In this study, knock down the expression levels of HE4 increased the expression of Bax protein levels, but had no influence on the expression levels of Bax mRNA. Therefore, it could be speculated that HE4 may regulate Bax expression in post-transcriptional levels. PI3K and AKT signal ways also take important functions in many types of cancer [26]. In this study, knock down the expres-

sion of HE4 decreased the expression levels of mRNA and protein of PI3K and AKT. Therefore, the experimental results of this study could state that HE4 may interact with PI3K, further regulate the expression of PI3K and AKT.

The concept of epigenetic is opposite to the concept of the gene. The epigenetic refers to genetic changes in gene expression without any changes in DNA sequences. The epigenetic regulation of gene takes an important role in occurrence and progression of cancers [14]. The DNMT1, DNMT3a, and HDAC1 were detected in osteosarcoma tissues. The result indicated that the mRNA and protein expression levels of DNMT1 and HDAC1 were higher compared to non-osteosarcoma tissues. Therefore, it could be speculated that the epigenetic regulation of gene may take part in the occurrence of osteosarcoma. Moreover, the expression levels of DNMT1 and HDAC1 after silencing HE4 expression were also detected. The results showed that the DNMT1 and HDAC1 expression were inhibited in knock-down group, which fur-

ther confirmed that the HE4 may interact with methylation related genes.

In conclusion, serum HE4 was higher in patients with osteosarcoma compared with healthy donors. The HE4 level was higher in the advanced clinical stage not only in tissue levels but also in serum. The epigenetic regulation could take part in the osteosarcoma.

Disclosure of conflict of interest

None.

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