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ORIGINAL ARTICLES

Evaluation of the expression level of Sine oculis homeobox homolog 1 in cervical cancer tissue in comparison with healthy adjacent tissue

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Abstract: *Background and Aim: This study aimed to investigate the expression level of Sine oculis homeobox homolog 1(SIX1) in normal and cancerous cervical tissues. Methods: Real-time PCR was used to evaluate SIX1 expression level in fresh surgically obtained cervical biopsies. Also, cervical specimens were tested for HPV DNA detection by MY09/11, GP5+/6+ nested PCR method. Results: The expression level of SIX1 mRNA in Cervical cancer was markedly higher than in normal cervical tissue (p<0.0001). Moreover, HPV DNA was detected in 100% of cases and 21.05% of controls (P<0.0001). Conclusions: This finding indicates that increased SIX1 expression might be of relevance to the pathogenesis of cervical cancer. Perhaps, studies to find agents that specifically target SIX1, develop novel anti-cancer and anti-metastasis drugs. This idea has to be investigated in future clinical studies.*

Keywords: *cervical cancer, SIX1, molecular mechanism, pathogenesis, expression level*

INTRODUCTION

Cervical cancer (CC) is the fourth most common cancer in women with worldwide estimated 527,000 cases and 266,000 deaths in 2012 [1, 2]. Persistent infection with high-risk Human papillomaviruses (HPVs) known as the fundamental cause of CC; although, most of the infected women usually recover spontaneously and only a small proportion progress to CC [2, 3]. HPV E6 and E7 oncoproteins not only inactivate the tumor suppressors p53 and Rb respectively but also modulate a plethora of cellular proteins and signalling pathways related to DNA replication, cell survival, apoptosis, anoikis, epithelial to mesenchymal transition (EMT), anchorage-independent growth (AIG) and angiogenesis. Accordingly, alteration of these cellular cascades leads to cell immortalization, transformation and finally occurrence/development of CC and metastasis [4, 5]. Among these cellular factors, Sine oculis homeobox homolog 1 (SIX1) play a crucial role in CC onset and metastasis [6]. SIX1 is one of the important members of SIX family. This family, including a series of transcription factors, are homolog of the Drosophila sine oculis (so) gene and have a total of six members (SIX1 to SIX6) in vertebrates. Among them, SIX1 has attracted the attention of many researchers recently. SIX1 gene is located on human chromosome 14q23 and its structure composed of two conserved domains, the homeodomain (HD) and the SIX domains (SD). Many studies have shown that SIX1 is essential for the development of

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various human organs in physiological conditions and overexpressed in multiple human cancers, also associated with metastatic phenotype [7, 8]. It is indicated that SIX1 stimulates DNA replication, cell proliferation and maintains survival conditions in CC cells. In the other hand, HPV E7 oncoprotein can induce SIX1 overexpression. Consequently, HPV can obtain opportunity of effectively persistent infection in cervical cells and develop CC [9, 10]. Advanced stages of CC have not effective treatments and most of patients with metastatic CC die. Thus, the better understanding of HPV-induced CC molecular mechanism is indispensable and can assist to introduce more effective novel therapeutic approaches for HPV-induced CC prevention and treatment. In the present study, we evaluate the expression level of SIX1 mRNA by real-time PCR in CC biopsies in comparison with healthy adjacent tissues.

MATERIAL AND METHOD

2.1. Specimens

A total of 38 histopathologically confirmed fresh cervical biopsies consist of 19 cancer samples and 19 normal samples were surgically collected from the Department of gynecology at Imam Khomeini hospital in Tehran, Iran. Normal subjects were women referring to gynecology department of hospital for any cause (for example ovarian cancer), except for cervical involvement. No subjects received preoperative radiotherapy, chemotherapy, or other treatment. The study was approved by the medical ethics committee from Tehran University of Medical Sciences and written informed consent was obtained from all patients before enrollment (approval number 1395.1301).

2.2. DNA Extraction

DNA was extracted from biopsies by the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to manufacturer's instructions. A Nanodrop spectrophotometer (Nanodrop Technologies) was used to determine DNA concentration and purity.

2.3. PCR Analysis

First, we confirmed the integrity of extracted DNA by amplification of a housekeeping gene β-globin (act as an internal control) using PCO3/PCO4 primers. The reaction conditions were as follows: 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 7 min. Then, the presence of HPV DNA was examined by nested PCR assay in all of the positive specimens for the β-globin gene. For this purpose, we used MY09/11 outer followed by GP5+/6+ inner primers (are located within the L1 region of the HPV genome. The Sequences (5 to 3) of all PCR primers are listed in table 1. The temperature cycling program in the first round (MY09/11) and second round (GP5+/6+) PCR was identical as follows: 94°C for 4 min, 40 cycles of 94°C for 1 min, 42°C for 1 min, 72°C for 30 s and a final extension at 72 °C for 4 min [11].

2.4. RNA Extraction

The total RNA from tissue specimens was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted RNA was eluted in 50 µl Diethylpyrocarbonate (DEPC) water. The concentration and purity of RNA were determined by Nanodrop spectrophotometer (Nanodrop Technologies). In order to the prevention of genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.5. Real-Time PCR

One microgram of RNA samples was reverse transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the kit's instructions. Real-time PCR reactions were carried out in duplicate with Applied Biosystems 7500 Real-Time PCR system using SYBRs Premix ExTapTM II (Takara, Japan) and specific primers that listed in Table 1 [12]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied as an internal amplification control. The $2^{-\Delta\Delta CT}$ method was used for relative quantification of SIX1 expression levels and results were normalized to GAPDH, as a housekeeping gene.

2.6. Statistical analysis

Statistical analysis was performed using the SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Variables were compared between groups using the Fisher's exact test or student's t-test as appropriate. Numerical results are expressed as the mean ± SEM. The p-values of <0.05 were considered statistically significant.

RESULTS

3.1. Demographic characteristics

The mean age of the women in the CC group and control group was 61.3 ± 10.2 and 36.0 ± 8.3 years, respectively. Based on statistical analysis, the mean age in the CC subjects was significantly higher than the control group (p-value <0.0001).

Table 1: Sequences of primers used for real-time PCR reactions in current study

Primers	Sequence (5'–3')
β-globin	PCO3 (Forward) ACACAACTGTGTTCACTAGC PCO4 (Reverse) CAACTTCATCCACGTTCACC
HPV	MY09 (Forward) CGTCC(A/C)A(A/G)(A/G)GGA(A/T)ACTGATC MY11 (Reverse) GC(A/C)CAGGG(A/T)CTATAA(C/T)AATGG GP5+ (Forward) TTTGTTACTGTGGTAGATACTAC GP6+ (Reverse) AAAAATAAACTGTAAATCATATTC
GAPDH	(Forward) GGAGCGAGATCCCTCCAAAAT (Reverse) GGCTGTTGTCATACTTCTCATGG
*SIX*1	(Forward) AAGGAGAAGTCGAGGGGTGT (Reverse) TGCTTGTTGGAGGAGGAGTT

HPV, Human papilloma virus; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; SIX1, Sine oculis homeobox homolog 1

3.2. Prevalence and type of HPV infection

HPV DNA was detected in 100% (19/19) of the cancer cases and 21.1% (4/19) of normal cervical specimens (Table 2). There was a significant difference between the two studied groups (p<0.0001). After sequencing analysis of HPV-positive specimens, we identified HPV-16 in all of the CC samples. Also genotyping of 4 HPV-positive specimens in normal group was HPV-16: 2 cases, HPV-66 and HPV-68: each one was one case (Table 2).

Table 2: Demographic and clinico-pathological characteristics in patients with cervical cancer and without cervical cancer

Variables	Cases (n = 19)	Controls (n = 19)	p-value
Age (mean years)	61.3 ± 10.2	36.0 ± 8.3	<0.0001, t-test
HPV positivity	19 (100%)	4 (21.1%)	<0.0001, Fisher'sisher's exact test
HPV type	HPV-16 = 19	HPV-16 = 2, HPV-66 = 1, HPV-68 = 1	

HPV, Human papilloma virus

3.3. SIX1 mRNA expression

Relative real-time PCR results showed that the mean SIX1 mRNA expression in the CC patients was 2.61 ± 0.09 and in the normal subjects was 1.12 ± 0.03 (Table 3).

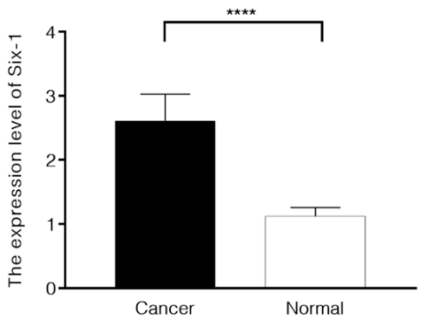
Table 3: Expression level of SIX1 in patients with cervical cancer and without cervical cancer

Gene		Cases	Controls
*SIX*1	Mean ± SE	2.61 ± 0.09	1.12 ± 0.03
	CI	2.42 - 2.79	1.06 - 1.18
	Minimum expression	1.94	0.92
	Maximum expression	3.47	1.39
	p-value	<0.0001 t-test	

SIX1, Sine oculis homeobox homolog 1; SE, standard error; CI, confidence interval

Then, statistical analysis revealed that SIX1 mRNA levels were significantly (p<0.0001) higher in CC tissue compared to the normal cervical tissue (Figure 1).

Figure 1: The expression level of SIX1 in CC and normal cervical group. Data are presented as mean ± SEM and the fold increase of transcripts for SIX1 mRNA normalized by GAPDH housekeeping gene. ****p<0.0001.



DISCUSSION

The sine oculis homeodomain (SIX) proteins are a family of evolutionarily conserved transcription factors that play a pivotal role in both embryogenesis and carcinogenesis. The

homeoprotein transcription factor SIX1 is one of the members of this family that has attracted much attention from the scientific community in recent years [13]. Based on the previous studies, SIX1 upregulated in several human cancers such as breast, ovarian, cervical, liver, rhabdomyosarcoma and Wilms' tumor, also contribute to initiation and metastatic development of these malignancies. Researches have also observed the SIX1 overexpression strongly correlated with poor prognosis and shortened overall survival in cancer patients [14-16]. Consistent with previous studies, we demonstrated the SIX1 mRNA levels is significantly higher in CC than normal cervical tissues. The results of previous studies showed that SIX1 can drive cancer stem cell phenotype and EMT, also lymphangiogenesis, consequently metastasis via stimulation of the TGF-β signaling pathway and VEGF-C, respectively [17-19]. EYA2 act as a coactivator for SIX1, thus mediate its transcriptional activation. Previously it has been shown that inhibition of SIX1-EYA2 complex formation, may be a promising and more effective approach for treatment of cancer and tumor metastasis [20]. In CC cells, SIX1 act as principal regulator of DNA replication, as well as a key factor in metastasis process. Therefore, SIX1 upregulation can lead to malignant proliferation of cervical cells, also metastasis through stimulation of DNA replication-related genes and a5b1 integrin, respectively [10, 21]. Interestingly, high-risk HPV employ various mechanisms to promote metastasis in CC [4]. Anoikis, meaning homelessness, is a detachment-induced cell death. Anoikis resistance, EMT, AIG, and angiogenesis are important cellular events that closely associate with cancer metastasis [22]. Hr-HPV E7 oncoprotein can stimulate SIX1 overexpression in CC. SIX1 consider as an EMT-activating factor, thus its upregulation result in EMT through increasing of ZEB1 and decreasing of E-cadherin. Indeed, SIX1-induced EMT, increase cell survival in detached cancerous cells and drive anoikis overcoming and finally, metastatic dissemination [9]. In the present study, among the total of 38 fresh surgically obtained cervical biopsies, 100% (19/19) and 21.1% (4/19) were positive for HPV DNA, in CC and normal adjacent groups, respectively. Then, sequencing analysis revealed all of HPV-positive specimens in CC group were HPV type 16 that highlighted the indispensable role of HPV-16 in CC onset and progression. The previous studies demonstrated the high risk HPV-16 and HPV-18 account for about 70% of CCs [23]. In current study, four HPV-positive specimens in normal cervical group were: two samples of HPV-16 and one sample of HPV-66 and HPV-68. Based on international agency for research on cancer (IARC) classification, HPV-66 belongs to 2B category (probably carcinogenic). Also, HPV-68 belongs to 2A category (possibly carcinogenic) [24]. Our outcomes corroborated previous reports specifying that the SIX1 involved in the tumorigenesis of cervical cancer [13, 25]. The main limitation of our work was low sample size. In conclusion, SIX1 upregulated significantly in CC, which in turn induces TGF-β, VEGF-C and ZEB1 that collectively can have an association in the tumorigenesis and metastasis of CC.

CONCLUSIONS

We demonstrated SIX1 expression levels was frequently expressed in CC tissues. Also, HPV DNA was detected in 100% (19/19) of the cancer cases and 21.1% (4/19) of normal cervical specimens. HPV can upregulate SIX1 in CC. In addition, SIX1 can contribute to the development of CC and lymph node metastasis of cervical tumors. Nevertheless, the precise biologic role of SIX1 in CC is unclear yet and future works should study on the larger number of patients.

List of abreviation:
CC, Cervical cancer; HPV, Human papillomavirus; SIX1, Sine oculis homeobox homolog 1; EMT, Epithelial to mesenchymal transition; AIG, Anchorage independent growth; so, Drosophila sine oculis; HD, Homeodomain; SD, SIX domains; DEPC, Diethylpyrocarbonate; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IARC, International agency for research on cancer.

Contribution Note:
EK, EF, MFP, TMA designed the study, EK, EF, MFP performed laboratory works, MFP, MF, MY statically analyzed the data, EK, EF, MFP, MF, MY, TMA wrote and revised the manuscript.

All authors read and approved the final manuscript.

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