Original Article

SIX1 overexpression in diffuse-type and grade III gastric tumors: Features that are associated with poor prognosis

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Abstract

Background: Gastric cancer is the second most common cancer worldwide. In Iran, the incidence of gastric cancer is well above the world average, and is the first common cancer in Iranian men and the third one in women. Located at chromosome 14q23, *SIX1* is a homolog of the Drosophila 'sine oculis' (so) gene and is highly conserved in numerous species. In addition to the role of SIX1 in the development, its expression is frequently dysregulated in multiple cancers. This study aimed to evaluate the clinicopathological features of the expression of *SIX1* gene in gastric adenocarcinoma.

Materials and Methods: Thirty pairs of gastric tissue samples from patients with gastric adenocarcinoma were evaluated for *SIX1* gene expression using quantitative real-time polymerase chain reaction. A paired *t*-test or one-way ANOVA with *post hoc* multiple comparisons were used to analyze the differences between groups. Statistical significance was defined as $P \leq 0.05$.

Results: *SIX1* expression was decreased in tumoral samples. However, its expression increased significantly in diffuse-type gastric cancer. Furthermore, there was a trend toward statistical significance in increasing *SIX1* gene expression with higher grades. Of note, the difference was significant between grades I and III.

Conclusions: The results suggest that *SIX1* gene expression might be used in the future as a potential biomarker to predict the outcome of the disease as diffuse-type and grade III of gastric tumors are associated with poor prognosis.

Key Words: Diffuse-type gastric cancer, gene expression, poor prognosis *SIX1*, tumor grades

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INTRODUCTION

Gastric cancer is the second most common cancer worldwide, with an estimated 900,000 new cases and 700,000 gastric cancer-related deaths in the world.[1] In Iran, the incidence rate of gastric cancer is well above the world average, and is the first common cancer in Iranian men and the third one in women.[2] Because of the lack of trustworthy early diagnostic methods and effective treatment, more than 80% of patients with advanced gastric cancer die of the disease or recurrent disease within 1 year after diagnosis. The majority of patients with gastric cancer are being diagnosed in advanced stages of the disease such that usual treatment protocols are ineffective in a remarkable number of cases.[3]

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Therefore, elucidation of the molecular characteristics of gastric tumors is an essential need to develop methods of early cancer detection and reduce its mortality.

Located at chromosome 14q23,[4] *SIX1* (sineoculis homeobox homolog 1), a member of the Six gene superfamily, is a homolog of the Drosophila 'sine oculis' (so) gene and is highly conserved in numerous species from Drosophila to human.[5,6] The *SIX1* gene product functions in concert with Eya1,[6] Pax and Dac in DNA binding[7] and regulates the expression of many downstream target genes.[8] Therefore, the *SIX1* homeoprotein organizes a variety of cellular processes during normal development of some tissues and organs such as promoting progenitor cell population proliferation and their invasion before cell differentiation and specification, survival, migration and apoptosis. However, after development, expression of *SIX1* changes and decreases in most normal adult tissues.[9-11] Furthermore, SIX1 indirectly affects cell movement and adhesion between cells and the extracellular matrix (ECM) by regulating the expression of Ezrin.[7]

In addition to the role of *SIX1* in the development, its expression is frequently dysregulated in multiple cancers including breast cancer,[12] Wilms' tumors,[13] ovarian cancer,[14] hepatocellular carcinoma,[15] alveolar rhabdomyosarcomas,[16] and cervical cancer.[17] The misexpression of *SIX1* in cancer can enhance cancer cell proliferation and survival and lead to tumor inception and progression.[8,18,19] These findings explain that unsuitable *SIX1* expression in adult differentiated tissues results in cell proliferation stimulus and, in turn, leads to initiation and progression of numerous cancers.[11]

Considering the dysregulation of *SIX1* gene expression in various tumors, in this study, we studied the expression of *SIX1* gene in gastric tumors.

MATERIALS AND METHODS

Tumor and non-tumor tissues

Thirty pairs of gastric tissue samples (tumor and their adjacent non-tumor tissues) from patients with gastric adenocarcinoma were provided from the Iran Tumoural Bank (Tehran, Iran) as described previously.[20] The clinicopathological characteristics of the specimens are shown in Table 1. Written informed consent from all subjects was obtained by the Iran Tumoral Bank. The experimental procedures were approved by the Ethics Committee of the Isfahan University of Medical Sciences. The samples were frozen in liquid nitrogen and kept at -80°C until analysis.

RNA isolation and reverse transcription

Total RNAs from frozen gastric cancer tissue samples were extracted using Qiazol reagent and RNeasy columns (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA integrity was examined by running on a 1% agarose gel and total RNA concentrations determined spectrophotometrically. Two micrograms of total RNA were reverse transcribed using random hexamer primers (TAG Copenhagen) and MMLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions.

Quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR)

Synthesized cDNAs were subjected to quantitative real-time PCR using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) and specific primers for *SIX1* and *TBP*[21] as an endogenous control in a total volume of 20 μL reaction mixture and were run on the Rotor-gene 6000 system. The following primers were used to amplify *SIX1*:
SIX1 forward primer:
5'- TAAGAACCGGAGGCAAAGAG -3'
SIX1 reverse primer:
5'- AGTTTGAGCTCCTGGCGTG -3'

The amplification conditions for *SIX1* were as follows: An initial denaturation step at 95°C for 10 min followed by 45 amplification cycles consisting of denaturation at 95°C for 20 s, annealing for 20 s at 55°C and an extension at 72°C for 20 s. For each sample, measurements were performed at least in triplicate. The standard curve method was used to calculate relative gene expression. For further verification of the identity of the PCR products, agarose gel electrophoresis was performed.

Statistical analysis

Data are represented as means ± standard error of mean (SEM) from at least three separate experiments.

Table 1: Clinicopathological parameters of gastric cancer samples

Characteristics	Numbers (%)
Sex	
Male	18 (60)
Female	12 (40)
Age (years)	
≥70	15 (50)
<70	15 (50)
Tumor grades	
Grade I	10 (33.3)
Grade II	8 (26.6)
Grade III	12 (40)
Tumor types	
Diffuse	15 (50)
Intestinal	15 (50)

To compare the gene expression levels between the tumor and non-tumor tissues and associated clinicopathological characteristics with gene expression, Student's *t* test and ANOVA statistical tests were performed. The SPSS program, version 20.0, was utilized for statistical analyses, and differences were considered significant if $P < 0.05$.

RESULTS

SIX1 is underexpressed in human gastric adenocarcinoma tissues

Relative quantitation of the expression levels of *SIX1* in gastric adenocarcinomas showed that the relative levels of *SIX1* transcripts were significantly decreased (around 1.5-fold, *P* value = 0.018) in cancerous tissues compared with adjacent non-cancerous tissues: 0.48 ± 0.03 versus 0.64 ± 0.06, respectively, as shown in Figure 1.

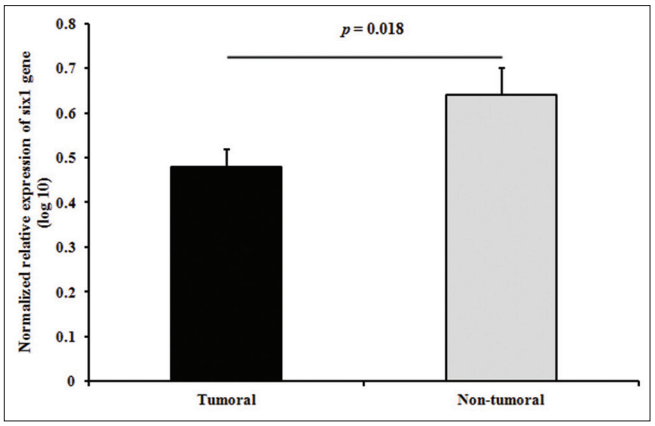

Figure 1: The relative expression levels of *SIX1* in tumoral versus non-tumoral gastric samples. Error bars represent standard error of mean (SEM)

Association of *SIX1* expression with clinicopathological parameters in gastric adenocarcinoma tissues

Next, we analyzed the association of *SIX1* relative gene expression with the reported clinicopathological characteristics of the tumors (histological classifications and grade). As shown in Figure 2, the expression level of *SIX1* was different in both diffuse- and intestinal-type tumors. We found that *SIX1* overexpressed in diffuse-type gastric tumors (mean: 0.56) compared with intestinal-type tumors (mean: 0.40) (*P* value: 0.025). Furthermore, there was no significant association between the expression levels of *SIX1* and different grades of the tumors (*P* value: 0.09). However, *SIX1* was overexpressed in grade III of gastric tumors compared with grade I (*P* value: 0.03) [Figure 3].

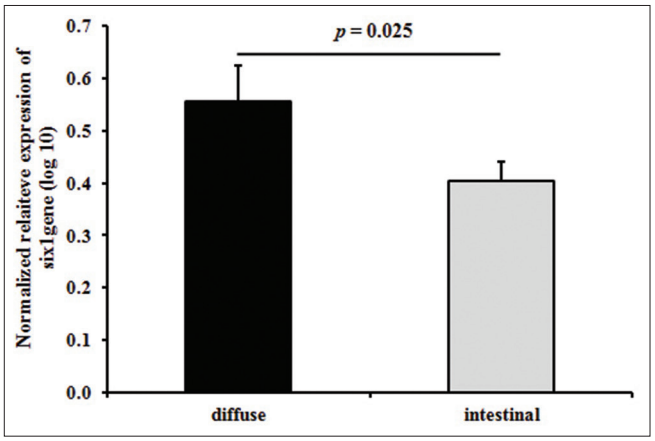

Figure 2: Relationship of the relative expression levels of *SIX1* with the histological classifications of the gastric tumors (diffuse vs. intestinal types)

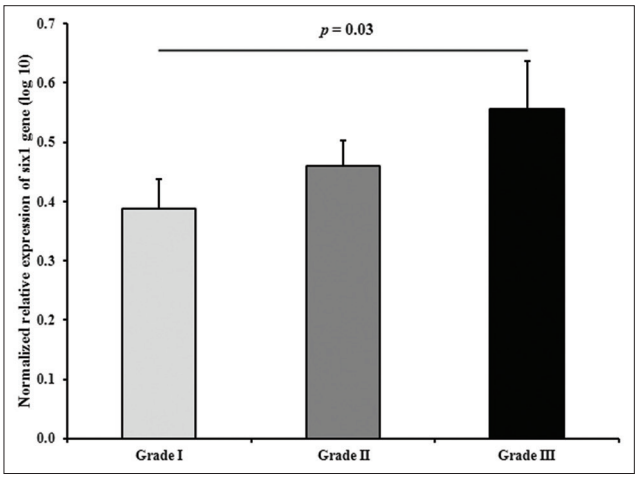

Figure 3: The *SIX1* relative expression stratified according to different tumor grades. The difference between grades I and III was statistically significant (*P* = 0.03)

DISCUSSION

To the best of our knowledge, this is the first study that evaluates the expression of *SIX1* gene in gastric adenocarcinoma using quantitative real-time RT-PCR. Our study showed that the relative expression of *SIX1* is significantly downregulated in tumoral tissues compared with the adjacent non-tumoral tissues ($P = 0.018$). However, our results showed that expression of *SIX1* increased significantly in diffuse-type gastric tumors in comparison with the intestinal-type gastric tumors ($P = 0.025$). Furthermore, *SIX1* expression significantly increased in grade III gastric tumors in comparison with grade I gastric tumors ($P = 0.03$).

SIX1 is an important developmental regulator in several diverse tissues/organs,[10,22-25] and induces the expression of diverse genes (e.g. *Cyclin D1*, *Cyclin A1* and *c-myc*) in various cell types.[8] It has been postulated that dysregulation of *SIX1* leads to cancer.[7,26]

Overexpression of *SIX1* has been documented in several types of cancer, including breast cancer,[12] Wilms' tumors,[13] ovarian cancer,[14] hepatocellular carcinoma,[15] alveolar rhabdomyosarcomas[16] and cervical cancer,[17] where it facilitates proliferation and metastasis of the cancerous cells.[26] In the same vein, we observed that *SIX1* expression increased significantly in diffuse type and grade III gastric cancer. Of note, microarray analyses have also shown that *SIX1* overexpresses in diffuse-type gastric tumors versus intestinal-type gastric tumors.[27,28] However, Matsusaka *et al*. recently reported that EYA1, a Six 1 coactivator, is often methylated in both EBV + and EBV-/high methylation gastric cancers.[29] As EYA1 interacts with and functions upstream of the homeobox gene *Six 1* in the development of some organs including ear and kidney,[24,30] it is plausible that *SIX1* was co-underexpressed with *EYA1* in gastric cancer. Furthermore, microarray analysis performed by Cui *et al*. showed that *SIX1* expression decreases in gastric tumors versus normal gastric tissues.[31]

Located within a critical interval on chromosome 14q23, Ruf *et al*. identified a 3-bp deletion in the *SIX1* gene in branchio-otic syndrome.[32] Furthermore, a loss of 14q23 has been reported in breast cancer,[33] gastrointestinal stromal tumors[34] and neuroblastomas.[35] In the same vein, deletions have been observed in 14q in gastric cancer.[36] Moreover, Gümüs-Akay *et al*. recently reported that the most common losses in gastric adenocarcinomas were found on arms 18q (26%), 5q (21%) and 14q (21%).[37] Taken together, the overall underexpression of *SIX1* in gastric cancer may be attributed to the loss of 14q.

In conclusion, this is the first report that evaluates the expression of *SIX1* in gastric cancer. Our results showed that *SIX1* is significantly downregulated in gastric tumors. However, our results showed that expression of *SIX1* increased significantly in diffuse-type and grade III gastric tumors. Taken together, this gene might be used in the future as a potential biomarker to predict the outcome of the disease as diffuse-type and grade III gastric tumors are associated with poor prognosis.[38] Further studies should be carried out to elucidate the mechanisms which cause *SIX1* underexpression in gastric tumors and to find out how SIX1 and EYA1 function in gastric cancer.

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