**Original Article**
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# Role of SIZN1 in Esophageal Squamous Cell Carcinoma

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## Abstract

**Background:** Bone morphogenetic proteins are a family of cytokines and growth factors that are involved in tumorigenesis. ZCCHC12 (SIZN1), as a transcriptional co-activator of bone morphogenetic protein signaling, is identified as a positive regulator of central nervous system development during embryogenesis. It positively regulates the CREB and AP1 transcription factors that cooperate with the bone morphogenetic protein signaling pathway. In the present study, SIZN1 mRNA expression was assessed in esophageal squamous cell carcinoma patients.

**Methods:** The levels of SIZN1 mRNA expression in tumor tissues from 50 patients with esophageal squamous cell carcinoma were compared with their corresponding normal margins by using real-time polymerase chain reaction.

**Results:** We observed that 10 out of 50 (20%) cases overexpressed SIZN1, whereas 40 out of 50 (80%) cases showed either normal or under expression of SIZN1. There was a significant correlation between the levels of SIZN1 mRNA expression and tumor depth of invasion ($P$=0.040). Furthermore, a significant correlation between lymph node metastasis and SIZN1 mRNA expression was observed in esophageal squamous cell carcinoma patients ($P$=0.036).

**Conclusion:** This study is the first report that has assessed SIZN1 expression in esophageal squamous cell carcinoma patients. SIZN1 can be a potential therapeutic target for primary esophageal squamous cell carcinoma because of its role in the early stages of tumor progression and metastasis.

***Keywords:*** BMP signaling, Transcriptional co-activator, Esophageal cancer, Zinc finger protein

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## Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer in Asian countries, with a poor prognosis and 5-year survival rate

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between 20-30%.[1-3] Therefore, it is very important to introduce new diagnostic or therapeutic markers in the early stages of ESCC. The bone morphogenetic protein (BMP) pathway originates from BMP ligands that bind to a type I/II receptor complex, which activates the type I receptor kinase and leads to the phosphorylation of receptor Smads.[4] Subsequently, phosphorylated R-Smad binds to Smad4 and enters into the nucleus where it interacts with nuclear factors such as p300/CBP, Runx2, Hoxc-8, and GATA[2-5] to stimulate or suppress the transcription of target genes.[5] Bone morphogenetic protein signaling is involved in the development of central nervous system.[6] Zinc finger proteins, as the most abundant eukaryotic protein family, are involved in different processes such as DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, and protein folding.[7] Zinc finger motifs are categorized based on the number of bound zinc ions and characteristics of binding amino acids.[8] The most common Zinc finger motifs are C2H2 and CX2CX4HX4C.[9] Many eukaryotic proteins play roles through the CCHC zinc finger, from which the CCHC-type zinc finger nucleic acid binding protein (CNBP) binds to the sterol regulatory element (SRE) during the process of transcriptional regulation.[10] ZCCHC9 inhibits the MAPK pathway through binding to the NF-kB promoter sequence.[11] ZCCHC8 is also involved in RNA degradation and processing.[12] ZCCHC12 (SIZN1), as a transcriptional co-activator in BMP signaling, is a positive regulator of central nervous system development during embryogenesis. It encodes a 402 amino acid protein that includes a single CCHC zinc finger and a nuclear localization domain.[13] It has been shown that SIZN1 positively regulates CREB and AP1 transcription factors, which cooperate with the BMP signaling pathway.[14] SIZN1 is known as a co-activator for c-Jun. SIZN1 is a transcriptional co-activator that regulates BMP signaling through Smad family members and subsequent recruitment of CREB-binding protein (CBP) to the transcription machinery.[13] This study is in line with our recent project in which we have assessed the role of EVX1 as one of the BMP target genes in ESCC patients.[15] In the current study we have aimed to assess the role of BMP signaling through SIZN1 as one of the components of transcriptional machinery of this pathway in ESCC patients.

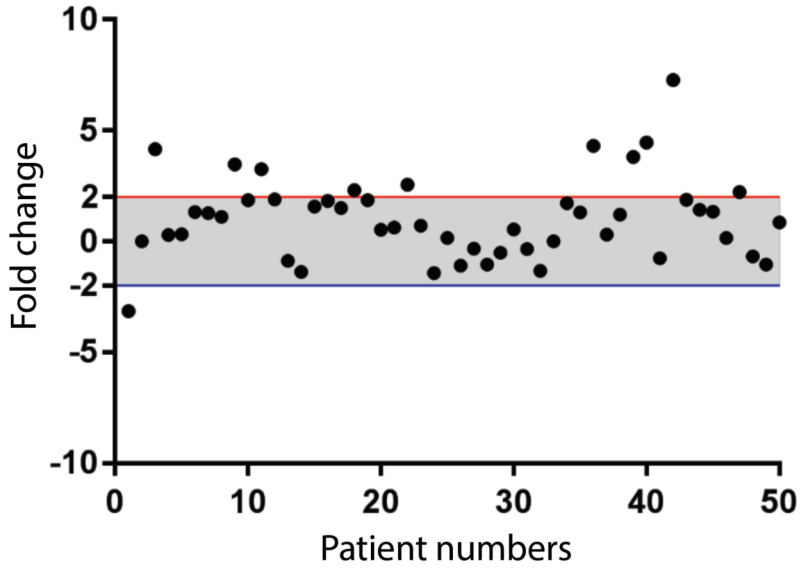


**Figure 1.** Scatter plot represents a descriptive analysis of relative gene expression of SIZN1. The thresholds for the over- and under expressed cases are shown by red and blue lines, respectively. The gray area indicates cases with normal levels of SIZN1 mRNA expression.

**Table 1.** Correlation between level of SIZN1 mRNA expression and different clinicopathologic features in esophageal squamous cell carcinoma (ESCC) patients.

| | SIZN1 mRNA expression | | |
|---|---|---|---|
| | Normal expression/ underexpression | Overexpression | *P*-value |
| **Patients (n)** | 40 (80%) | 10 (20%) | |
| **Age (years, mean±SD)** | 61.63 ± 1.87 | 60.78 ± 4.63 | 0.852 |
| **Size (Cm, mean±SD)** | 4.22 ± 0.32 | 3.85 ± 0.45 | 0.584 |
| **Fold changes (mean±SD)** | 0.34 ± 0.19 | 3.78 ± 0.47 | |
| **Sex** | | | 0.877 |
| Male | 19 (79.2%) | 5 (20.8%) | |
| Female | 21 (80.8%) | 5 (19.2%) | |
| **Location** | | | 0.399 |
| Upper | 2 (100%) | - | |
| Middle | 19 (73.1%) | 7 (26.9%) | |
| Lower | 19 (86.4%) | 3 (13.6%) | |
| **Grade*** | | | 0.954 |
| P.D. | 5 (83.3%) | 1 (16.7%) | |
| M.D. | 26 (78.8%) | 7 (21.2%) | |
| W.D. | 9 (81.8%) | 2 (18.2%) | |
| **Lymph node metastasis** | | | 0.036** |
| Yes | 17 (77.3%) | 5 (22.7%) | |
| No | 23 (82.1%) | 5 (17.9%) | |
| **Stage of tumor progression** | | | |
| 0.770 | | | |
| I/II | 25 (80.6%) | 6 (19.4%) | |
| III/IV | 15 (78.9%) | 4 (21.1%) | |
| **Depth of tumor invasion (T)** | | | 0.040** |
| T I/II | 8 (88.9%) | 1 (11.1%) | |
| T III/IV | 32 (78.0%) | 9 (22.0%) | |

* P.D.: Poorly differentiated, M.D.: Moderately differentiated, W.D.: Well-differentiated; ** Statistically significant

## Materials and Methods

### *Tissue samples*

We enrolled 50 ESCC cases (24 males and 26 females) who underwent tumor resections in hospitals affiliated with Mashhad University of Medical Sciences. The cases had a mean age of 61.47±12.10 years. Each patient signed an informed consent form that had been previously approved by the Ethic Committee of Mashhad University of Medical Sciences. We recruited new cases that did not have any chemotherapy or radiotherapy modalities prior to the tumor resections. Eligible tumor tissues contained at least 70% of tumor cells, as verified by histopathologic analysis. All fresh tissues were transferred to an RNA solution (Qiagen, Hilden, Germany) after the resection and maintained at -20°C.

### *RNA extraction, cDNA synthesis, and comparative RT-PCR*

We used the RNeasy Mini Kit (Qiagen, Hilden, Germany) for RNA extraction and the First-strand Synthesis Kit (Fermentas, Lithuania) for cDNA synthesis from the normal and tumor tissues, as previously described.[16,17] Comparative relative RT-PCR (SYBR Green method, Ampliqon, Denmark) was assessed by Stratagene Mx-3000P (Stratagene, La Jolla, CA, USA). Reactions were

performed in triplicate to assess the levels of SIZN1 mRNA expression in the tissues.

### *Statistical analysis*

Probable correlation between SIZN1 mRNA expression and clinicopathologic features of tumors were studied by Pearson and Spearmans' tests, ANOVA, and the t-test using SPSS 16.0 (SPSS, Chicago, IL, USA). A $P$ value <0.05 was considered statistically significant.

### *Ethical Approval*

All procedures performed in this study that involved human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee, the 1964 Declaration of Helsinki, and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

## Results

### *Levels of SIZN1 mRNA expression*

Relative comparative real time PCRwas used to measure the levels of SIZN1 mRNA expression in the ESCC tumors and their corresponding normal margins (Figure 1). We noted that 10 out of 50 (20%) ESCC cases overexpressed SIZN1, whereas the other tissues showed normal expression or under expression of this gene. Fold change levels of SIZN1 mRNA expression ranged between -3.15 to 7.28 with a mean±SD of 1.03±1.86. Mean±SD fold changes for SIZN1 were 0.34±0.19 for underexpressed tumors and 3.78±0.47 for overexpressed tumors.

### *Clinicopathologic features and SIZN1 mRNA expression*

The majority of cases, 28 out of 50 (56%), did not have lymph node metastasis, 39 out of 50 (78%) had T3 tumor invasion, and 31 out of 50 (62%) had stages I/II tumors. With the exception of 2 cases, all samples were located in the lower/middle esophagus (96%) and most cases were moderately differentiated (33/50, 66%) as seen in table 1. Although we did not observe any significant correlation between fold changes of SIZN1 expression levels and sex, males had higher levels of expression (1.12±0.37) in comparison with females (0.94±0.38). The cases with lymph node involvement had significantly higher levels of SIZN1 mRNA expression (1.11±0.49 fold change) compared with those without lymph node involvement (0.97±0.27 fold change; $P$=0.036). There was a significant correlation between depth of invasion and levels of SIZN1 mRNA expression in which 32 out of 41 (78%) cases with TIII/TIV depth of invasion had either normal or under expression of SIZN1 ($P$=0.040). There was an interesting correlation observed between the pattern of SIZN1 expression and depth of invasion. There was a declining trend in expression with higher T depth of invasion, from 2.58±0.69 fold change in T1 tumors to -0.86±0.51 fold change in T4 tumors. The majority of SIZN1 overexpressed cases were stages I/II (6/10, 60%). As with the pattern of SIZN1 mRNA expression based on tumor depth of invasion, the SIZN1 mRNA expression also showed a declining trend with higher tumor stages, from 1.25±0.64 fold change for stage I tumors to 0.98±0.55 fold change for stage III tumors. In contrast to tumor depth of invasion and surgical staging, there was no clear pattern of SIZN1 mRNA expression based on tumor grade. Moderately differentiated tumors had the highest levels of SIZN1 mRNA expression (1.10±0.35 fold change), whereas well-differentiated tumors had the lowest levels of SIZN1 mRNA expression (0.84±0.45 fold change). Most SIZN1 overexpressed cases were moderately differentiated (7/10, 70%). There was no pattern observed for tumor location. The tumors located in the middle esophagus had the highest level of SIZN1 mRNA expression (1.47±0.38 fold change), whereas those located in the lower esophagus had the lowest levels of SIZN1 mRNA expression (0.51±0.37 fold change). We noted that 7 out of 10 (70%) SIZN1 overexpressed tumors were located in the middle esophagus. All of the upper esophageal cases had normal expression or underexpression of SIZN1 mRNA. There was no significant correlation between

tumor size and levels of SIZN1 mRNA expression; however, the mean size in overexpressed cases was smaller (3.85±0.45 cm) compared to the other cases (4.22±0.32 cm). There was no significant correlation between age and levels of SIZN1 mRNA expression. The ages of patients with normal or under expressed SIZN1 mRNA compared to overexpressed cases were almost equal (61.63±1.87 vs. 60.78±4.63 years).

## Discussion

Bone morphogenetic protein signaling plays important roles during development, differentiation, and tumorigenesis.[18,19] SIZN1, as a transcriptional co-activator, is associated with Smads upon BMP signaling activation. It recruits CBP (histone acetyl transferase) to the transcription machinery to trigger signaling. Moreover, CBP is introduced as a co-activator for AP-1[20] and CREB,[21] which are the main transcription factors in various processes such as cell proliferation, death, and differentiation.[22] It has been reported that BMP activation is associated with tumor progression and inhibition of tumor migration and angiogenesis. Dose effects and cell context are variables to be considered to explain these discrepancies. The inhibitory role of BMPs has been reported in colon, breast, thyroid, and gastric malignancies.[23,24] Recent reports discussed the stimulatory role of the BMP pathway in colorectal cancer progression.[25] Therefore, for the first time, we have examined the probable role of SIZN1 as one of the components of transcription machinery in this pathway in ESCC patients. BMP exerts its inhibitory role through the formation of a RUNX3/β-catenin/TCF4 complex, which attenuates recruitment of β-catenin/TCF4 to the c-Myc promoter. Subsequently, the cells are released from the proliferative effect of the Wnt signaling pathway. This interaction between WNT and BMP signaling as stimulatory and inhibitory pathways is important during the regulation of intestinal homeostasis.[26] Recently, we have also reported the role of EVX1 as one of the BMP target genes in ESCC patients, where we observed a significant correlation between EVX1 under expression and lymph node metastasis.[15] In the present study we observed an inverse significant correlation between tumor depth of invasion and levels of SIZN1 mRNA expression. We observed a declining trend for SIZN1 fold changes with increased levels of tumor invasion. The majority of T3/4 cases had either normal or under expression of SIZN1. We observed a decreased trend for SIZN1 mRNA expression with the advanced tumor stages. In contrast with the tumor depth and stage, the levels of SIZN1 mRNA expression in metastatic lymph nodes were significantly higher compared with the normal and under expressed cases. Generally, assessment of transcription factor and target gene of BMP pathway revealed that the BMP signaling probably has an inhibitory role during ESCC progression and a probable triggering function in the early tumor stages. A whole transcriptome sequencing study in thyroid cancer patients has shown remarkable up-regulation of SIZN1 in papillary thyroid cancer in comparison with normal tissues.[27] They observed a significant correlation between SIZN1 overexpression and lymph node metastasis, which was similar to our results in ESCC patients.

In conclusion, SIZN1 appears to be involved in the primary steps of ESCC progression and metastasis, which emphasizes its role in primary stages of this disease. Although SIZN1 expression is important in lymph node metastasis, its expression does not have a significant role during the advanced tumor stages. Therefore, SIZN1 may be introduced as an early detection marker as well as probable therapeutic target for ESCC.

## Conflict of Interest

None declared.

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