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# *SOX1 and DIDO1 Co-Under-Expression is Correlated with Poor Prognosis in Esophageal Squamous Cell Carcinoma*

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**Abstract**

**Background:** Bone morphogenetic proteins (BMPs) are broadly involved in normal embryo development and abnormal pathological processes such as cancer. SOX1, an HMG-box protein related to SRY, and death-associated transcription factor 1, DIDO1, are introduced as two transcription factors involved in cell fate decision. Our aim in this study was to assess the clinicopathological significance of SOX1 and DIDO1 gene expression in Esophageal Squamous Cell Carcinoma (ESCC).

**Method:** Relative comparative real time PCR was performed in 50 ESCCs compared to corresponding margin normal tissues to survey the expression of SOX1 and DIDO1 mRNA expression.

**Results:** Expression levels of SOX1 and DIDO1 mRNA in ESCC were significantly downregulated. The expression of the genes was correlated to each other ($p<0.01$). Co-underexpression of the genes was significantly associated with different indices of poor prognosis including lymph node metastasis, stage of tumor progression, and depth of tumor invasion ($P<0.05$).

**Conclusion:** The pattern of SOX1 and DIDO1 gene expression in ESCC may suggest a tumor suppressor role for the genes in the disease and introduce their concomitant downregulation as a new marker for ESCC poor prognosis.

**Keywords:** SOX1; DIDO1; ESCC; Prognosis; Marker.

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## Introduction

Esophageal Carcinoma (EC), as a high peril cancer, has a variety of disadvantageous specifications including difficult early diagnosis, rapid tumor growth and metastasis, relatively low response to drug treatment, and poor 5-years survival [1]. Great efforts have been made to explore the mechanisms of epigenetic and genetic changes during esophageal tumorigenesis. Although the molecular biology of EC is still too complicated to understand thoroughly, activation of oncogenes and flawed signaling pathways, such as c-Myc, VEGF and TGF-β, are participated in the development of the disease, and correspondingly, molecular therapies targeted to abnormal signaling pathways are widely designed and tested [2].

Bone morphogenetic proteins (BMPs) are members of the transforming group factor-beta (TGF-β) superfamily. This family of homologous signaling proteins has a diverse number of functions and plays an important role in cell proliferation, migration, apoptosis, and differentiation [3,4]. BMPs, as ligand, exert functions by coupling with two groups of BMP receptors, named type I and II receptors of serine/ threonine kinase, to activate Smad-dependent and -independent pathways, respectively [5]. Type I receptors include active receptor-like kinase-2, -3 and -6 (ALK-2, ALK3, and ALK-6), whereas type II receptors comprise BMPRII, ActRIIA, and ActRIIB. Through Smad signaling, phosphorylated Smad1/5/8 (R-Smads) by the activated type I receptor, interacts with Smad4 to induce target gene expression in the nucleus [6-8]. More than twenty different BMPs have been known to date [9]. The DIDO-1 (death inducer-obliterator-1) gene is a BMP4-specific Smad-regulated target gene [10] which can triggers apoptosis in the cell [11]. DIDO-1 apoptotic induction is prevented by caspase inhibitors and Bcl-2 overexpression [12,13]. BMP signaling genes are abnormally expressed in different cancers, such as prostate, breast and ovarian malignancies [8,14,15].

Sox families of sex-determining region Y (SRY)-box transcription factors contain a highly conserved high-mobility group (HMG) DNA-binding domain, and play vital roles in both embryonic and postnatal development [16-18]. Sox1 belongs to the SoxB1 subgroup that encodes transcription factor implicated in the regulation of embryonic development and the designation of cell destiny [19]. SOX1 plays role in several physiological processes including cell development, proliferation, migration, apoptosis, and invasion, in both normal and malignant tissues [20].

Since SOX1 and DIDO1 play role in cell fate decision, our aim in this study was to investigate the clinicopathological significance of SOX1 and DIDO1 gene expression in ESCC patients.

## Materials and methods

### Clinical specimens

Fifty primary (without pre-operative chemo- or radio-therapy) esophageal cancer tissue and related margin non-tumor specimens were collected from ESCC patients who had undergone surgery at Omid Oncology Hospital of Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. The study was approved by the Ethics Committee of MUMS and written informed consent was obtained from all patient. Tumor samples were carefully examined histopathologically to ensure containing more than 70% tumor cells, and related pathological staging was determined based on the International Union Against Cancer (UICC) TNM classification [21].

### Relative comparative real-time-PCR

Total RNA was obtained from tumor and margin normal esophagus tissues of 50 ESCC patients by TriPure reagent (Roche, Nutley, NJ) according to the manufacturer's instructions. Following cDNA synthesis using Synthesis kit (Takara, Japan), Real-time PCR was accomplished using a (Mx-3000P) with specific primer sets (Table 1), and SYBR Green Master Mix. ROX was used as the reference dye. Thermal cycler conditions used after heating at 95 C for 10 min involved 40 cycles of denaturation at 95 C for 30 s, 58 C for 15 s and 72 C for 30 s. The relative expression level of SOX1 and DIDO1 were computed by using the ΔΔCt method and GADPH was used as internal control. Fold change of genes expression was analyzed as described before [22,23]. All experiments were conducted in triplicate.

### Statistical analysis

All statistical analysis was performed with SPSS version 25 (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± SD. T-test, v2 or Fisher exact test, and ANOVA were used to determine the correlation between gene expression and various clinicopathological factors. Pearson's correlation was used to determine the correlation between SOX1 and DIDO1 expressions. $P < 0.05$ was considered to be significant.

## Results

Relative comparative qRT-PCR was conducted on 50 ESCC samples in comparison with their margin normal tissues to analyze SOX1 and DIDO1 gene expression. The clinicopathological features of the patients are described in Table 2. The average age (±SD) of the recruited patients was 61.46 (±12.09) with the age ranged between 30 and 87. Male/female ratio was 0.92 (24:26). Tumor samples size ranged from 1.50 to 12.0 cm (mean ± SD; 4.14 ± 1.89). While 31 of 50 tumor tissues were diagnosed at stages I and II of tumor progression, 19 of 50 ESCC samples were in advanced stages III, IV. The majority of tumor samples (41 of 50) were invaded to the adventitia of the esophagus presenting T3 and T4 depth of tumor invasion. Furthermore, 33 out of 50 patients ESCCs were moderately differentiated, while 11 and 6 tumors were well and poorly differentiated, respectively.

### SOX1 and DIDO1 were downregulated in ESCC

The expression pattern of SOX1 and DIDO1 was analyzed at mRNA level in ESCCs compared to margin normal tissues. The state of SOX1 and DIDO1 genes expression in different clinicopathological features of 50 patients is presented in Table 2. Based on the results, SOX1 was underexpressed in 12 out of 50 (24%) ESCC samples, while its expression in the remained 38 of 50 (76%) samples was majorly unchanged. The minimum and maximum of SOX1 fold changes were -3.6 and 1.9, respectively. Furthermore, the mean ± SD of SOX1 fold change in samples was 0.00 ± 1.55. While, DIDO1 was overexpressed in 14 of 50 (28%) ESCCs, its underexpression was detected in 36 of 50 (72%) tumor samples. The minimum and maximum of DIDO1 fold changes were -5.70 and 7.11, respectively. Furthermore, the mean ± SD of DIDO1 fold change in samples was 0.64 ± 2.82. Interestingly, there was a significant correlation between SOX1 and DIDO1 gene expression in ESCCs ($P<0.0001$). As shown as a regression plot in Figure 1, tumor samples with low level of SOX1 mRNA expression show lower amount of DIDO1 mRNA expression in comparison with other

samples. Indeed, the simultaneous expression of the genes and amount of their expression in tumor samples were correlated to each other.

**SOX1 and DIDO1 co-underexpression was associated with ESCC indices of poor prognosis**

The correlation of SOX1 and DIDO1 expression with clinicopathological factors of ESCC patients was analyzed (Table 2). Interestingly we found that co-underexpression of SOX1 and DIDO1 was significantly correlated with different indices of ESCC poor prognosis including advanced stages of tumor progression (stages III and IV) (P = 0.037), lymph node metastasis of tumor cells (P<0.01), and invaded tumors to the adventitia (T3 and T4 depth of tumor invasion, P<0.01).

**Table 1:** The sequence of primer sets used for quantitative real-time RT-PCR.

| Gene | Forward | Reverse |
|---|---|---|
| **SOX1** | TGAACGCCTTCATGGTGTGGTC | ATTACAAGTACCGGCCGCGC |
| **DIDO1** | TTTGTTGGTCCAGTTTCGCCTTC | ACGACAAGCAAGAGACTGTTTCAC |
| **GAPDH** | GGAAGGTGAAGGTCGGAGTCA | GTCATTGATGGCAACAATATCCACT |

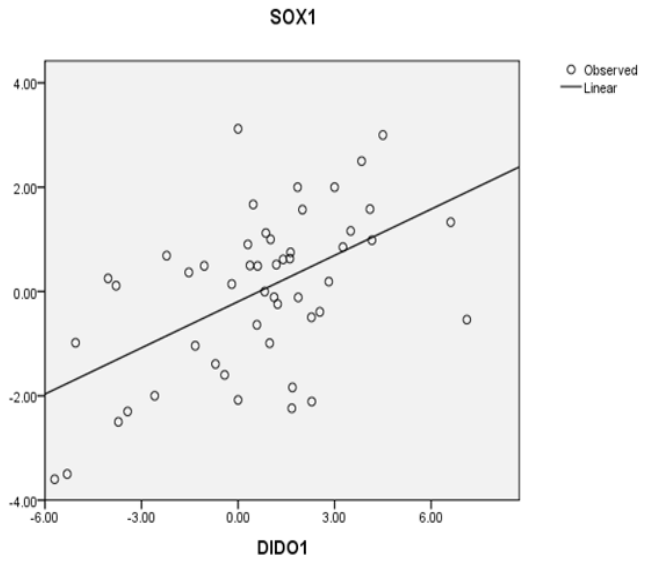


**Figure 1:** Regression plot illustrating the significant correlation between SOX1 and DIDO1 mRNA expression. X and Y axis present log2 fold change of gene expression. DIDO1 expression is decreased in samples with lower amount of SOX1 gene expression, compared to other samples.

**Table 2:** The state of SOX1 and DIDO1 genes expression in different clinicopathological features of ESCC patients.

| Clinicopathological factors | | N | S0X1 expression | | DIDO1 expression | | Co-under expression of the genes( P value) |
|---|---|---|---|---|---|---|---|
| | | | Under | Normal | Under | Over | |
| **Sex** | Female | 26 | 7 | 19 | 18 | 8 | 0.254 |
| | Male | 24 | 5 | 19 | 18 | 6 | |
| **Lymph node metastasis** | No metastasis | 28 | 6 | 22 | 20 | 8 | 0.001* |
| | Node metastasis | 22 | 6 | 16 | 16 | 6 | |
| **Depth of tumor invasion** | T1, 2 | 9 | 1 | 8 | 7 | 2 | 0.001* |
| | T3, 4 | 41 | 11 | 30 | 29 | 12 | |
| **Stage of tumor progression** | Stage I, II | 31 | 6 | 25 | 22 | 9 | 0.037* |
| | Stage III, IV | 19 | 6 | 13 | 14 | 5 | |
| **Grade of tumor differentiation** | P.D** | 6 | 1 | 5 | 5 | 1 | 0.084 |
| | M.D | 33 | 8 | 25 | 24 | 9 | |
| | W.D | 11 | 3 | 8 | 7 | 4 | |
| **Tumor location** | Lower | 22 | 8 | 14 | 17 | 5 | 0.178 |
| | Middle | 26 | 3 | 23 | 18 | 8 | |
| | Upper | 2 | 1 | 1 | 1 | 1 | |

**PD poorly differentiated, MD moderately differentiated, WD well differentiated

* Significant correlation

## Discussion

BMP signaling pathway plays role in tumor cell growth, invasion, metastasis, as well as angiogenesis, and its components are found in different cancers such as prostate, breast, ovarian, melanoma, lung and esophageal malignancies [24-26]. Since the molecular mechanisms involved in ESCC development have not yet been understood, identifying new molecular markers which accurately illustrate biological characteristics of the disease can help for a clinically appropriate treatment.

Here we investigated the expression pattern of two involved genes in cell fate decision SOX1 and DIDO1 in ESCCs and analyzed its correlation with different clinicopathological features of the patients. We found underexpression of the genes in ESCCs in significant correlation to each other. Co-underexpression of the genes was significantly correlated with different indices of poor prognosis including lymph node metastasis, stage of tumor progression, and depth of tumor invasion. These data may emphasize the importance of these genes as tumor suppressor in ESCC progression.

SOX proteins family is known as a group of transcriptional regulators defined by a highly conserved HMG domain which mediates DNA binding [27]. Sox genes play momentous roles in sex determination, chondrogenesis, hematopoiesis, neural crest development and neurogenesis [17,28,29]. SOX1 is introduced as a tumor suppressor in a manifold of cancers. It has been reported that SOX1 can interfere with the Wnt/β-catenin signaling in cervical cancer and reverses the malignant phenotype [30,31]. SOX1 is epigenetically silenced in most of lung cancers and its restoration inhibited cell migration through regulating actin cytoskeletal remodeling [32]. While SOX1 downregulation is associated with poor prognosis and tumor growth in hepatocellular cancer [33], its expression plays a critical role in the inhibition of ESCC invasiveness and aggressiveness especially in advanced stages of the disease [34]. It inhibits angiogenesis by direct regulation of IGFBP1/MIF's pathways in the Non-small cell lung cancer [35]. SOX1 can inhibit proliferation and induce apoptosis by regulating Wnt/β-catenin signaling pathway in gastric cancer, serving it as a potential therapeutic target in treatment of the disease [36]. In line with these reports, we also found SOX1 underexpression in ESCC.

DIDO1 is reported as an involved gene in regulation of apoptosis [37], and its deregulation can cause chromosomal instability through a defective mitotic checkpoint [38].

In this study, SOX1 and DIDO1 were underepressed in 24% and 72% of ESCC samples, respectively. These results may highlight a tumor suppressor role for both genes in the disease. Since a direct correlation was observed between the genes, it may be extrapolated that reduction of one gene may result in underexpression of another. SOX1 is underexpressed in ESCC in significant correlation with poor prognosis criteria of the disease including Stage of tumor cell progression and depth of tumor invasion [34]. However, here we found its significant correlation with DIDO1 underexpression in ESCC, where co-downregulation of the genes was associated with different indices of ESCC poor prognosis. Co-underexpression of SOX1 and DIDO1 was correlated with advanced stages of tumor cell progression (stage III and IV) which may suggest a role for these genes in suppressing ESCC growth and progression. Tumor invasion happens along with tumor cell progression through advanced stages. Therefore, association of SOX1 and DIDO1 co-down regulated ESCCs with increased depth of tumor cell invasion (T3and T4) may not be out of mind. As our results shown, this association is not only confirmed in ESCC but also extended to local lymph node metastasis of tumor cells. The association of co-underexpression of SOX1 and DIDO1 with different indices of poor prognosis may introduce the concomitant downregulation of the genes as a marker of ESCC poor prognosis. These results may indicate that SOX1 and DIDO1 play an important role in inhibition of the disease progression and confirm a tumor suppressor role for the genes in ESCC.

BMP signaling pathway is involved in esophageal squamous cell carcinoma progression. Hu et al demonstrated that BMP signaling pathway is potentially activated in esophageal squamous cancer cells in correlation with tumor growth, invasion and metastasis [9]. In addition, it has been recently reported that BMP signaling genes are significantly expressed in ESCC, and a significant correlations was existence between BMP signaling genes and the stemness state maintenance factor SALL4 especially in invaded tumors to the adventitia (T3/T4) [39]. Therefore, SOX1 may play role in BMP signaling pathway modulation and its downregulation in ESCC may probably inhibit apoptosis in esophageal squamous carcinoma cells through downregulation of death inducer gene DIDO1.

Interestingly, the crosstalk between SOX1 and BMP signaling was revealed in different pathways. It has been shown that levels of SOX1 gene expression is related to BMP signaling inhibition during neurogenesis [40,41]. A correlation is also shown between TGF-$\beta$/BMP pathways and SOX1 mRNA expression in neural development [42,43]. Although activation of SOX1 expression and suppressing endogenous BMP signaling is require for transition of ES cell differentiation from the epiblast into the neuroectoderm state [44]. These evidences confirm the linkage between SOX1 and BMP signaling pathway, and our finding in this study may extend this crosstalk to the ESCC progression. Although it should be investigated in details to be confirmed in the disease.

## Conclusion

In conclusion, we confirmed underexpression of SOX1 and DIDO1 in ESCC in significant correlation with each other. To the best of our knowledge, this is the first report highlighting the clinical significance of SOX1 and DIDO1 co-downregulation in ESCC, which was associated with different indices of poor prognosis of the disease including depth of tumor invasion, lymph node metastasis, and stage of tumor cell progression. Therefore, this pattern of SOX1 and DIDO1 gene expression may suggest a tumor suppressor role for the genes and introduce co-underexpression of the genes as a new marker for ESCC poor prognosis.

## Declarations

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**Ethics approval and consent to participate and Consent for publication:** The study was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran, and written informed consent was obtained from all recruited patients to participate in the study and related publications.

**Availability of data and materials:** All raw data are available in case of request.

**Authors' contributions:** SS performed the majority of the work presented in this manuscript and drafted the manuscript. MMF designed the concept and conducted the experiments, analyzed data and edited the manuscript. All of the authors read and approved the final manuscript.

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