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ORIGINAL ARTICLE

Contribution of EVX1 in Aggressiveness of Esophageal Squamous Cell Carcinoma

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Abstract Homeobox genes play an overruling role in the regional cell fate determination during development. *EVX*1 is known as a new target gene of BMP signaling pathway, a group of morphogens which are making the largest subset within the transformation growth factor beta (TGF-β) superfamily. In this study, we aimed to enlighten the expression level of *EVX1* in esophageal squamous cell carcinoma (ESCC) and to disclose its apparent roles in maintenance and progression of the disease. The expression level of *EVX1* was analyzed in fresh tumoral tissues in comparison with distant tumor-free tissues of 50 ESCC patients using relative comparative real-time PCR. The importance of EVX1 in development and cancer was also reviewed. *EVX1* was underexpressed in 70 % of tumor samples. There was a significant correlation between down-regulation of *EVX1* and lymph node metastasis of tumor cells ($p = 0.027$). Furthermore, *EVX1* underexpression was significantly correlated with depth of tumor cell invasion ($P = 0.037$). To the best of our knowledge, this is the first report highlighting *EVX1* expression in ESCC to date. The clinicopathological relevance of *EVX1* mRNA expression in ESCC targeted this gene as a new independent molecular marker for advanced tumor, which determine the characteristics and behavior of aggressive ESCC.

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Introduction

Homeobox genes play a pivotal role in the regional cell fate determination during development [1–3]. Provoking uncontrolled cell proliferation, hampering cell differentiation and maturation process, followed by abnormal expression of homeobox genes eventuates in tumor formation. Homeobox genes contain a trihelical DNA-binding motif, termed homeodomain. On the basis of structure and function, numerous proteins which are involved in transcriptional regulation during development comprise the highly conserved homeodomain. Such proteins function as a pattern formation director [4], cell mitosis conductor and synthesis manager of hormones and various molecules taken part in information transmitting due to harmonizing growth and differentiation of the embryo [5].

The even-skipped like homeobox gene *Xhox3* (the Xenopus *EVX1*ortholog), is responsive to activin and bone morphogenetic proteins (BMPs). It is expressed in ventral and posterior mesoderm during gastrulation and functions as a ventro-posteriorizing factor.

*EVX*1 is known as a new target gene of BMP signaling pathway, a group of morphogens which are making the largest subset within the transformation growth factor beta (TGF-β) superfamily. BMPs play essential roles during embryonic development, pattern formation and pathogenesis of a variety of diseases. Moreover, BMPs pertain to regulation of cell proliferation, differentiation, apoptosis, migration, extracellular matrix (ECM) remodeling, immune functions, and tumor invasion/metastasis [6]. In adults, BMP signaling partakes in tissue remodeling and regeneration for homeostasis maintenance in regulation of stem cell behavior thoroughly [7].

Esophageal cancer is the seventh leading cause of cancer-related death worldwide. The incidence of esophageal squamous cell carcinoma (ESCC) may be as high as 800 cases per 100,000 populations in "Asian ESCC belt" [8]. Unlike in the United States, squamous cell carcinoma is responsible for 95 % of all esophageal cancers worldwide [9]. Innumerable advances in diagnosis and treatment of ESCC have been achieved recently; nevertheless, the survival rate has not been significantly developed. The most operative reasons are the advanced stage at diagnosis and the lack of a utilitarian method both in understanding its carcinogenic mechanism and clinical evaluation, especially the lack of sensitive and specific molecular markers for early detection.

It is known that the processes of normal embryogenesis and tumorigenesis share many of the cellular pathways, and tumorigenesis is known as an aberrant form of organogenesis [10, 11]. Homeobox genes like *EVX1* are classic links between embryogenesis and tumorigenesis. The mammalian HOX (homeobox) genes encode a subset of transcription factors regulating axial regional specification during embryonic development and have recently been shown to be aberrantly expressed in a variety of solid tumors [12, 13]. Therefore, our aim in this study was to elucidate mRNA expression pattern of *EVX1* in ESCC patients and investigate its probable clinical relevance. Likewise, we reviewed different roles of EVX1 in embryonic stem cells (ESCs) development to highlight its probable similar functions in ESCC maintenance and aggressiveness.

Materials and Methods

Tissue Samples

A total of 50 fresh tumoral and corresponding tumor-free tissues of esophagus were acquired from the ESCC patients after the therapeutic esophagectomy at Omid Hospital of Mashhad University of Medical Sciences (MUMS), the referral oncology hospital of northeastern Iran. The intervened patients had no preoperative chemo- or radio-therapy. All tissue samples were transferred to RNA *later* solution (Qiagen, Hilden, Germany) at −20 °C until RNA extraction. Based on microscopy, all the tumoral samples contained more than 75 % tumor cells, with rare or no infiltrating cells. This procedure supported the precise evaluation of gene expression in tumor cells compared to normal. The study protocol was approved by the ethics committee of the MUMS and the informed consents of all patients were obtained. Histopathological features were defined based on the latest Union International Cancer TNM classification guidelines [14].

cDNA Synthesis and Quantitative RT-PCR

RNA extraction from normal and tumoral ESCC tissues was accomplished using RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription of total RNA with oligo dT was enacted by first-strand synthesis kit (Fermentas, Lithuania) based on the manufacturer's protocols. cDNA amplification was done through designed specific primer sets (Table 1) in StratageneMx-3000P real-time thermocycler (Stratagene, La Jolla, CA). Relative comparative Real-time PCR was performed by using SYBR green PCR Master Mix (Fermentas, Lithuania), compromising ROX as a reference dye. The thermal cycling program was as described before [14]. Through manipulating comparative threshold cycle method, data were normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

The PCR efficiency for GAPDH and EVX1 was measured by utilizing standard curves yielded by the serial dilution of cDNA. All experiments were performed in duplicates. A more than two-fold fluorescence intensity of mRNA expression in tumor tissue, compared with corresponding normal tissues, was regarded as overexpression. Less than minus two-fold was considered as underexpression, and the range in between was defined as no change in expression.

Statistical Analysis

The statistical analysis was performed using SPSS 19.0 statistical package (SPSS, Chicago, IL). The correlations between the expression levels of the gene and variable histopathological factors were measured by X^2 test or the Fisher exact test, independent-sample *t* test and ANOVA depending on the categorical data.

Results

Study Population

Gene expression analysis was scrutinized by reverse transcription relative comparative real-time PCR. The enrolled patients were comprised of 24 males and 26 females (sex ratio: 0.92) with a wide spectrum of age ranged from 30 to 87 year. The mean (±SD) age of the patients was 61.46 (±12.09). The clinicopathological features of the recruited ESCC patients are presented in Table 2.

Downregulation of *Evx1* mRNA Expression in ESCC

Having analyzed the expression of *EVX1* mRNA in ESCC samples, we crystallized the significant underexpression of *EVX1* in the majority of tumor tissues. Although expression of *EVX1* in ESCC samples was very variable showing a wide range of fold changes from −4.08 to 9.70, *EVX1* was underexpressed in 70 % (35 of 50) of tumor samples. The mean (±SD) of *EVX1* mRNA expression fold changes in tumor samples was 1.12 (±3.16).

Table 1 Primer sequences used for quantitative real-time RT-PCR

Gene	Forward primer sequence	Reverse primer sequence
EVX1	GCTGCGGGTTTCCTTTCATCTTC	AGCCCCATTGCCCTCTTCTTTC
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT

Association of Evx1 Expression with Clinicopathological Variables

Following analysis of the correlations between *EVX1* mRNA expression and various clinicopathological variables of ESCC patients, we discerned significant correlation of *EVX1* underexpression with lymph node metastasis of tumor cells ($p = 0.027$). Node metastasis was detected in 13 of 35 cases (37 %) with *EVX1* underexpression. Furthermore, there was a significant correlation between *EVX1* underexpression and depth of tumor invasion ($P = 0.037$). Indeed, 26 of 35 (74 %) *EVX1* underexpressed ESCC samples were invaded to the adventitia (T3, 4). Correlations between *EVX1* mRNA expression and different clinicopathological features are summarized in Table 2.

Discussion

Homeobox genes code a family of transcription factors playing crucial roles in either embryogenesis or differentiation of adult cells [15]. Most of the family members are scattered around the genome, but a subgroup of the homeobox genes, HOX, are organized into clusters. HOX genes were originally detected in *Drosophila* as engaged factors in homeotic transformations. Supporting by an evolutionary scenario, an ancestor of *Evx* was linked to the ProtoHox cluster, and a tandem duplication of a large genomic region developed the Hox and ParaHox clusters, plus the cluster-neighbor *Evx* [15]. The conciderable 'coupled' Hox-like cluster *Eux*Hox was subsequently broken, thus assembling the *Evx* and the Hox clusters, and isolating the ParaHox cluster. During mammalian evolution, the ancient HOX cluster sustained duplications followed by gene losses which eventually led to the development of current 39 present HOX genes in classified four clusters [15]. HOX proteins are not only prerequisite switches of developmental stage- and cell-specific gene regulation, but they are also key principles of cell identity and potential targets during tumorigenesis [16].

Xhox3 (the Xenopus *EVX1* ortholog) is the downstream target of BMP signaling, a subgroup of TGFβ superfamily [17, 18]. In BMP signaling pathway, BMPs bind to the two types of serine/threonine receptors through Smad-dependent and independent machinery. The simple core cascade of BMP signaling consists of ligands promoting type I receptor phosphorylation through type II receptor which leads to carboxy-terminal phosphorylation of regulatory Smads (R-Smads) including Smad1, 5 and 8 which interact with Smad4 as common partner of Smad (Co-Smad) in cytosol. Through ligand stimulation, a heterodimeric complex of Co-Smads and R-Smads will be

Table 2 Clinicopathological features of the 50 ESCC patients and their correlations with EVX1 mRNA expression

Features		EVX1 gene expression				
		Underexpression		Overexpression		*P. value*
Sex	Male	14	40.0 %	10	66.7 %	0.841
	Female	21	60.0 %	5	33.3 %	
Node metastasis	No metastasis	22	62.9 %	6	40.0 %	0.027*
	Node metastasis	13	37.1 %	9	60.0 %	
Tumor invasion	T1, T2	9	25.7 %	0	0.0 %	0.200
	T3, T4	26	74.3 %	15	100.0 %	
Stage of progression	Stage 1,2	25	71.4 %	6	40.0 %	0.037*
	Stage 3,4	10	28.6 %	9	60.0 %	
Grade of differentiation	P.D**	4	11.4 %	2	13.3 %	0.797
	M.D	22	62.9 %	11	73.3 %	
	W.D	9	25.7 %	2	13.3 %	
Location	Lower	15	42.9 %	7	46.7 %	0.969
	Middle	19	54.3 %	7	46.7 %	
	Upper	1	2.9 %	1	6.7 %	

*Statistically significant

**PD Poorly differentiated; MD Moderately differentiated;WD Well differentiated

assembled resulting in Smad translocation into the nucleus and regulation of related genes expression [6, 18].

In this study we illustrated the significant underexpression of *EVX1* in ESCC tissues. Likewise, we pictured the clinical relevance of *EVX1* in ESCC and evidenced that low levels of gene expression was associated with lymph node metastasis and depth of tumor invasion, suggesting critical role of *EVX1* down-regulation in ESCC aggressiveness and metastasis.

EVX1 (Human homeobox protein Even-skipped homeobox 1) is a developmental homeobox domain protein located at 7p15 which is closely related to the Drosophila gene even-skipped (eve). Even-skipped homeobox 1 protein is expressed in the visceral endoderm and is required for normal blastocyst development and differentiation [19]. *EVX1* belongs to the pair-rule class of segmentation genes [4] which functions as a cogent repressor protein of gene transcription plays an important role during mouse embryogenesis. On the other hand, it is involved in development of a variety of tumors including malignant melanoma, cervical carcinoma, leukemia, mammary and prostate cancers [4].

BMP4- and activin induce gene expression paradigm captaining cell fate decisions in response to TGF-ß signals in human ESC (Fig. 1). The only treatment of hESC with BMP4 was ended to a prompt and transient induction of the posterior streak/mesoderm markers *EVX1*, *T* and *BAMBI* [20–22], and induction of the mesoderm marker *TWIST1*, succeedingly [23, 24].

On the contrary, it has been declared that hESC treatment with activin alone was terminated to the induction of goosecoid (*GSC*), *SOX17*, *LIM1*, and *CXCR4* markers of anterior streak and DE [25, 26]. As discerned in other preceding systems [27, 28], the posteriorizing activity of BMP4 was prevalent over the anteriorizing activity of activin when both growth factors were added. Anterior streak/DE markers were suppressed over the conclusive differentiation period whereas expression of posterior streak/mesoderm markers was sustained [29].

The role of GSC and EVX1 in ACTIVIN- and BMP-induced A–P patterning of streak-like ES cell descendants was revealed by *GSC* knockdown [29]. Indeed, expression of pluripotency markers in undifferentiated GSCKD-hESC lines was not enough significant to affect pluripotency characteristic of hESC [29]. It was indicated that ACTIVIN and/or BMP4 treatment of GSCKD-hESC lines led to an increase of *EVX1* and *T* transcripts, and decrease of *SOX17* [29]. Consistent with this results, differentiation after ACTIVIN and/or BMP4 treatment on hESC lines through *GSC* overexpression have been demonstrated repression of *EVX1* and *T*, and conversely upregulation of *SOX17* [29]. In the core of this gene regulatory network, function of *EVX1* was analyzed by shRNA knockdown. The analysis of A-P marker demonstrated down-regulation of *T* and strong up-regulation of ACTIVIN-induced *GSC* and *SOX17* in EVX1KD-hESC [29].

GSC is a paired-like homeobox gene expressed in the vertebrate organizer. GSC plays desperate roles in gastrulation [30, 31] and neural crest development. Expression of *GSC* is correlated with the EMT process and collective migration. Cumulated evidence has shown that GSC induces morphogenetic movements during gastrulation and administrates cell migration in Xenopus embryos, which illustrates that the GSC DNA-binding protein may supervise the engaged genes in intercellular signaling, cell motility, and cell adhesion. In Xenopus embryos, GSC-governed cell movement affects migration of cell groups supremely, but not the individual cells,

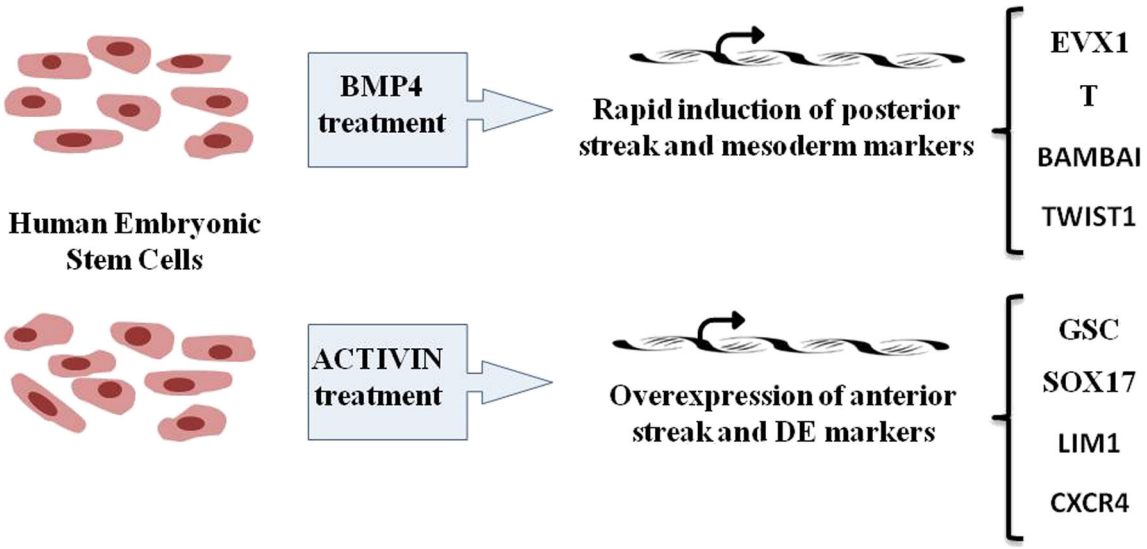

Fig. 1 **a.** Treatment of hESC with BMP4 alone was earned to a rapid and transient induction of the posterior streak/mesoderm markers EVX1, T and BAMBI, and a later induction of the mesoderm marker TWIST1. **b.** hESC treatment with activin alone induced expression of GSC, SOX17, LIM1, and CXCR4 markers of anterior streak and DE

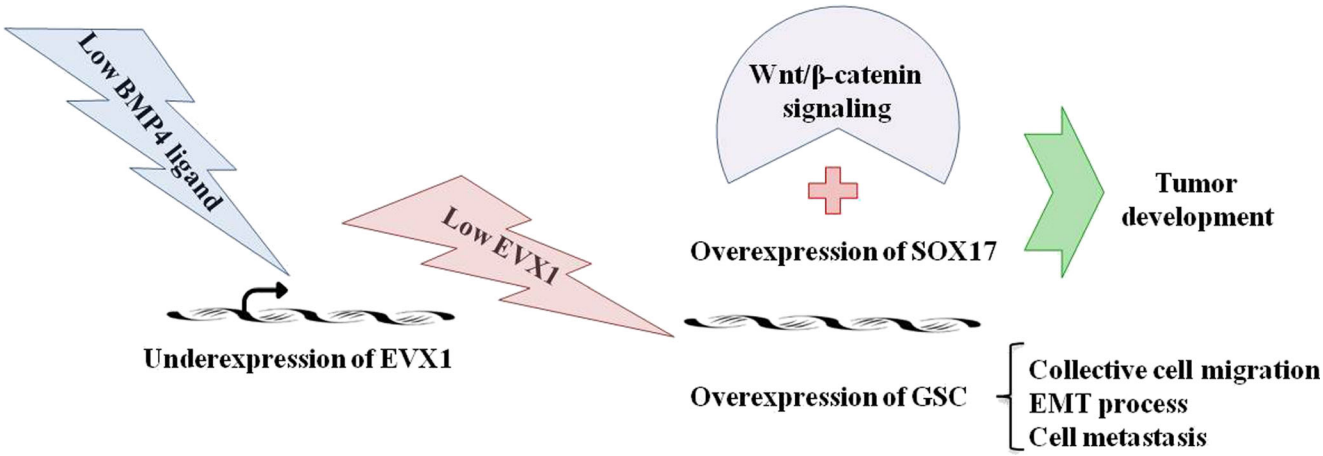

Fig. 2 Evx1 downregulation is developed by low presence of BMP4 which triggers GSC and SOX17 overexpression. GSC-controlled cell movement inspires migration of groups of cells but not of individual cells. Despite, It develops the overexpression of major regulators of EMT, TWIST1 and Mastermind-like 1 (MAML1) which leads to metastasis. On the other hand, SOX17 together with Wnt/β-catenin signaling performs in tumor development through induction of target genes

although it is proposed that GSC has role in promoting metastasis of human breast tumor cells through initiation of EMT [32].

GSC and EVX1 interact with each other to mediate cell fate choices in response to TGFβ family signaling during streak-like development of hESC, where EVX1 acts through direct repression of *GSC* [29]. Since the increased expression of *GSC* was in result of *EVX1* knockdown, we supposed that the consequence of decreased expression of *EVX1* may promote EMT and collective migration (Fig. 2). Conversely, co-overexpression of major regulators of *EMT*, *TWIST1* and *MAML1*, has been reported in esophageal squamous cell carcinoma (ESCC) in correlation with lymph node metastasis, tumor depth of invasion, and ultimately development of tumoral cells through advanced stages of the tumor [33].

Therefore, underexpression of *EVX1* may develop the overexpression of major regulators of EMT, TWIST1 and Mastermind-like 1 (MAML1). The mesodermal oncogenic marker, TWIST1 is a basic helix-loop-helix transcription factor with oncogenic characteristics. It induce epithelial mesenchymal transition (EMT) leading to migration of tumor cells [34]. This process is believed to play a critical role in tumor aggressiveness [35, 36]. [37]. It has been signified that upregulation of *TWIST1* enhances invasion and metastatic ability of various types of tumor cells [38, 39]. Interestingly, MAML1 is an integral component of the transcriptional activation complex of Wnt target genes such as *cyclin D1*, *C-MYC* and *TWIST1*.

Conversely, overexpression of *SOX17* as another consequence of *EVX1* downregulation was supported by Du et al. Expression of *SOX17* is revealed at the early stages of tumorigenesis following Wnt/β-catenin activation, and these may play roles in tumor development [40]. Nonetheless, SOX17 may play a preventive role against malignant progression through repression of Wnt activity. It was reported that induction and down-regulation of *SOX17* expression in gastrointestinal tumorigenesis are important for tumor initiation and malignant progression, respectively [40].

In conclusion, we elucidated clinicopathological relevance of *EVX1* mRNA expression in ESCC, and introduced *EVX1* as a new independent molecular marker for advanced tumor, which determine the characteristics and behavior of aggressive ESCC. To the best of our knowledge, this is the first report highlighting *EVX1* expression in ESCC and its impact on clinicopathological features. The oncogenic mannerism of *EVX1* may be certified by its down-regulation in ESCC and its correlation with the lymph node metastasis and depth of tumor invasion.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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