Contribution of TWIST1-EVX1 Axis in Invasiveness of Esophageal Squamous Cell Carcinoma; a Functional Study

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Background: Epithelial-mesenchymal transition (EMT) is a biological process in embryonic development and cancer progression, and different gene families, such as HOX genes, are closely related to this process.

Objectives: Our aim in this study was to investigate the correlation between TWIST1 and EVX1 mRNA expression in ESCC patients and also examine the probable regulatory function of TWIST1 on EVX1 expression in human ESCC cell line.

Materials and Methods: TWIST1 and EVX1 gene expression patterns were assessed in ESCC patients by relative comparative Real-time PCR then correlated with their clinical characteristics. In silico analysis of the EVX1 gene was conducted. KYSE-30 cells were transduced by a retroviral system to ectopically express TWIST1, followed by qRT-PCR to reveal the correlation between TWIST1 and EVX1 gene expression.

Results: The expression of TWIST1 and EVX1 was correlated to each other significantly (p=0.005) in ESCC. Of 28 patients with under/normal expression of TWIST1, 22 samples (78.57%) had over/normal expression of EVX1. TWIST1 overexpression was correlated with advanced stages of the tumor (III, IV) (P = 0.019) and lymph node metastasis. However, EVX1 under expression was associated with lymph node metastasis (p = 0.027) and invasiveness of the disease (P = 0.037) in ESCC. Furthermore, retroviral transduction enforced significant overexpression of TWIST1 in GFP-hTWIST-1 approximately 9-fold compared to GFP control cells, causing a − 8.83- fold reduction in EVX1 mRNA expression significantly.

Conclusions: Our results indicated the repressive role of TWIST1 on EVX1 gene expression in ESCC. Therefore, our findings can help dissect the molecular mechanism of ESCC tumorigenesis and discover novel therapeutic targets for ESCC invasion and metastasis.

Keywords: EVX1, Epithelial-mesenchymal transition (EMT), Esophageal squamous cell carcinoma (ESCC), TWIST1

1. Background

EMT is considered as a critical event during embryogenesis; however, a number of its characteristics are displayed in tumor behavior, including decreased cell adhesion, enhanced resistance to programmed cell death mechanisms, and functional abilities related to the homeodomain, such as expression of homeobox (HOX) genes, which conceivably can implicate in the metastatic cascade (1). These properties are usually associated with upregulation of several genes, including N-cadherin, vimentin, central contact proteins, ECM proteins, and transcription factors such as TWIST, E47, SNAIL 1, and SNAIL 2 (SLUG) that repress E-cadherin as well as nuclear localization of beta-catenin (2).

Homeobox (HOX) genes encode HD (homeodomain) transcription factors play essential roles in multiple cellular processes, including cell identity, maintenance, proliferation regulation, cell growth, cell-cell interaction, and organogenesis (3). Many of these functions were established in cooperation with other factors and molecules (3). Numerous homeodomain genes indicate close evolutionary relations with the HOX cluster, such as EMX, HLXB9, GBX, MOX, HESX1, EN, and EVX genes (4). HOX genes are associated with the progression of several malignancies (5). Investigating how HOX genes have altered expression in cancers can consequently shed light on tumor risk identification. Nonetheless, the mechanisms involved in the deregulation of HOX genes are not well understood.

The even-skipped homeobox 1 (EVX1) is regarded as a novel target gene of the bone morphogenetic protein (BMP) signaling pathway, which belongs to the transforming growth factor-β (TGF-β) superfamily. EVX1 is a DNA-binding transcription factor with sequence-specific DNA binding activity. Several studies have reported a transcriptional repressor role for an even-skipped gene product in both embryogenesis and tumorigenesis (6, 7). It is under expressed in the majority of ESCCs. Furthermore, its downregulation in ESCC is associated with poor prognosis indices such as invasion and lymph node metastasis (8).

The Twist Family BHLH Transcription Factor 1 (TWIST1) is a vital key regulator of EMT progression triggers EMT and promotes reorganization of the cellular cytoskeleton and extracellular matrix. Homodimeric and heterodimeric structures of TWIST1 function as transcriptional activators and repressors, respectively, through binding to conserved E-box consensus sequences (CAGGTG) in the promoter of target genes (9-11).

2. Objectives

Since previous studies have reported the oncogenic function of TWIST1 and EVX1 in the tumorigenesis, progression, and aggressiveness of ESCC (8, 12), our aim in the current study is to determine the existence of a possible functional correlation between TWIST1 and EVX1 expression in human ESCC cell line.

3. Materials and Methods

3.1. Tissue Samples
Tumoral and margin normal esophagus tissues were freshly collected from 44 ESCC treatment-naive patients who were referred to Oncology Omid Hospital, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran. The study was approved by the ethics committee of MUMS. Informed consent was obtained from enrolled patients. The histopathological features of tumor tissues were determined based on the seventh edition of the Union International Cancer TNM classification guidelines (13).

3.2. RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR
RNA extraction from normal and tumor tissue of esophageal samples and cDNA synthesis were performed as described before. The gene expression analyses of TWIST1 and EVX1 were performed by relative comparative real-time PCR using the SYBR Green method (Fermentas, Lithuania) in a Stratagene Mx3000P detection system (Stratagene, La Jolla, CA). ROX was used as a reference dye. The optimal thermal condition of TWIST1 and EVX1 gene amplification is described before (8). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a normalizer, and fold change of gene expression was calculated based on $\Delta\Delta CT$ (14). All experiments were run in triplicate.

3.3. In Silico Analysis of Sequence of EVX1
Related sequences for EVX1 mRNA, and gene, were obtained from Genbank (accession numbers NM_ 131249.2, NC_ 007130.6, and AF071238.1, respectively). The sequences were analyzed using CLC Main Workbench version 5.6 (CLC bio, Aarhus, Denmark).

3.4. Cell Lines and Culture Condition
The KYSE-30 and the HEK293T cells were maintained in RPMI-1640 and DMEM/F-12 mediums (Gibco), respectively, improved with 10 % FBS, 100 µg. mL^{-1} streptomycin, and 100 U.mL^{-1} penicillin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Both cell lines were purchased from the Pasteur Institute Cell Bank, Tehran, Iran (http://ncbi.pasteur.ac.ir/).

3.5. Retroviral Transduction and Overexpression Study
The retrovirus generation was executed according to the manufacturer's instructions (15). Briefly, to produce the recombinant retroviral viruses, plasmids, such as Pruf-IRES-GFP-hTWIST-1, pMD2 (containing env), and pGP

(containing, gag-pol) were transiently co-transfected into HEK293T cell line, as well as GFP control plasmid. Pruf-IRES-GFP-hTWIST-1 and Pruf-IRES-GFP plasmids were gifted from Dr. Stan Gronthos (Mesenchymal Stem Cell Group, Division of Haematology, Institute of Medical and Veterinary Science, University of Adelaide, SA, Australia). Then, the retroviral particles were harvested at 24, 48, and 72 h post-transfection, concentrated by ultracentrifugation and filtered with a 0.45 μm filter. Finally, the recombinant viruses were transduced to KYSE-30 cells with the calcium phosphate transfection method.

3.6. Expression Study in KYSE-30 Cell Line
RNA extraction, DNase treatment, cDNA synthesis, and real-time PCR expression analysis of TWIST1 and EVX1 were performed on the GFP control and GFP-hTWIST1 induced cells using specific primer sequences indicated in **Supplemental 1**.

3.7. Bioinformatics Analysis
To identify the most potential functional targets among the genes associated with TWIST1 and EVX1, protein interaction network (PIN) analysis was carried out by GeneMAINA (http://www.genemania.org) database and visualized with the Cytoscape plugin.

3.8. Statistical Analysis
The correlation between genes was analyzed using Pearson's correlation test. The correlation between gene expression and different histopathological features was measured using ANOVA and independent-sample t-test. A P value <0.05 was statistically significant. The statistical package SPSS 19.9 (SPSS, Chicago, IL, USA) was used for statistical analysis.

4. Results

4.1. Study Population and Clinical Demographic Data
Table 1 presents the clinicopathological features of recruited patients. All samples were treatment-naive; therefore, their histopathological features were not affected by therapeutic interventions. Enrolled samples included 24 males and 20 females. The patients' age was ranged from 30 to 83 years (mean age ± SD: 62 ± 12). Expert pathologists confirmed the tumor and margin of normal tissues histologically.

Table 1. Correlation of TWIST1 and EVX1 gene expression with different clinicopathological features of the recruited patients

		TWIST1		P-value	EVX1		P-value
		N/Under	Over		N/Under	Over	
Sex	**Male**	14	10	0.611	14	10	0.514
	Female	14	6		16	4	
Grade	**P.D**	4	3	0.949	6	1	0.985
	M.D	16	11		19	8	
	W.D	8	2		5	5	
location	**Lower**	12	9	0.353	16	5	0.359
	Middle	16	7		14	9	
	Upper	0	0		0	0	
Tumor invasion	**T1**	0	0	0.479	0	0	0.037*
	T2	4	2		4	2	
	T3	24	14		26	12	
Node metastasis	**No metastasis**	21	6	0.013*	17	10	0.027*
	Node metastasis	7	10		13	4	
Stage	**I,/ II**	21	6	0.019*	17	10	0.261
	III/ IV	7	10		13	4	

*Correlation is significant at the 0.05 level

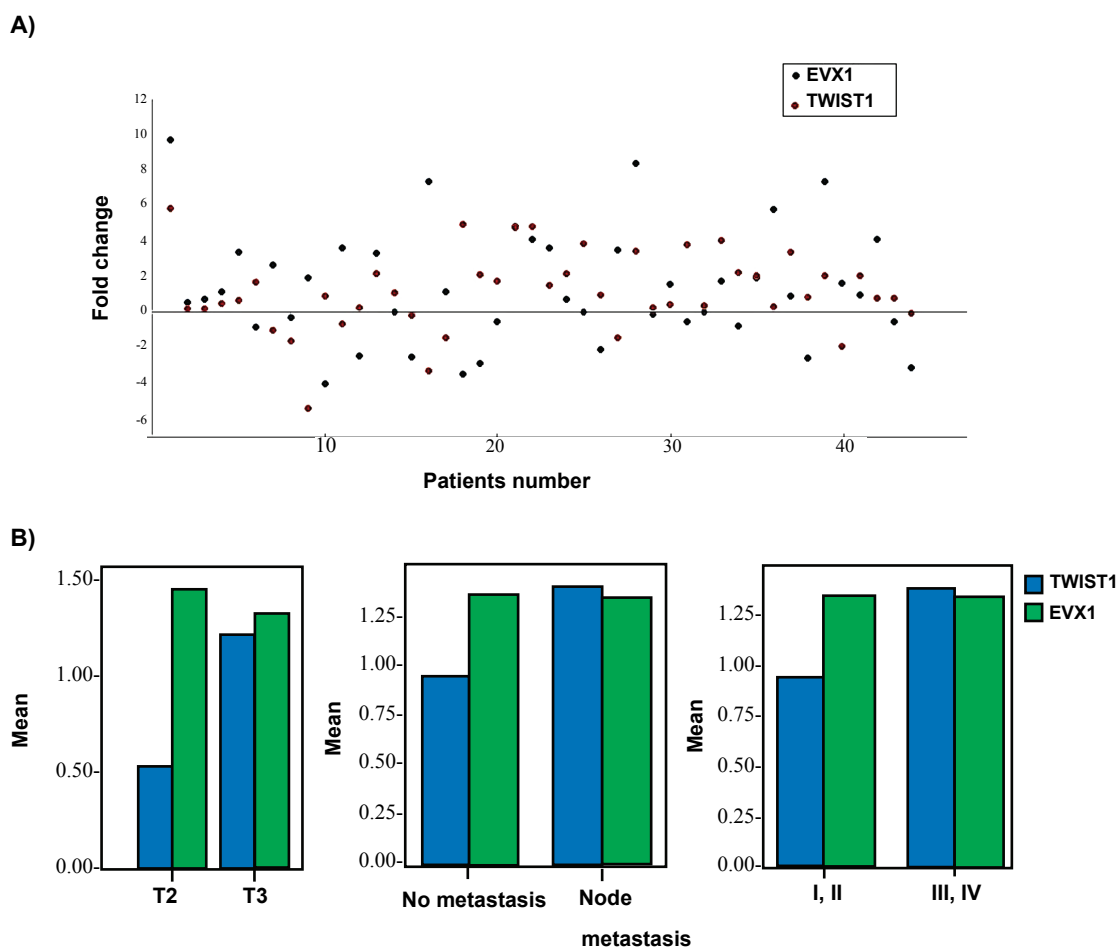*4.2. Levels of TWIST1 and EVX1 mRNA Expressions in ESCC*

We compared TWIST1 and EVX1 mRNA expression levels in 44 tumors and the margin normal esophageal epithelium, using relative comparative real-time PCR. Results showed the overexpression of TWIST1 in 36.4% (16 of 44) of ESCC patients. 68.2% of samples (30 of 44) did not have overexpression of EVX1. The mean (±SD) of TWIST1 fold changes in ESCCs was 3.34 (±1.28), with minimum and maximum of 2.01 and 5.85, respectively. Also, the minimum and maximum fold changes of EVX1 expression were -3.52 and 9.7, respectively (mean ± SD: 2.25± 8.82). **Figure 1A** represents the gene expression patterns of TWIST1 and EVX1 in ESCCs as a scatter plot. The means of TWIST1 and EVX1 gene expression are depicted as a bar chart in different pathological states, including

depth of tumor invasion (T2 and T3), lymph node metastasis, and stage of tumor progression (**Fig. 1B**).

4.3. Association of TWIST1 and EVX1 Expression with Indices of ESCC Poor Prognosis

TWIST1 overexpression was significantly associated with ESCC poor's prognosis. It was correlated to the stage of tumor cell progression (P = 0.019). 58.82% of tumors (10 of 17) with advanced stages of tumor (III, IV) show overexpression of TWIST1. While 75% of the cases (21 of 28) with normal or underexpression of TWIST1 were in lower tumor stages (I, II). Moreover, it was associated with lymph node metastasis (P = 0.013). 17 of 44 specimens (38.63%) had lymph node metastasis, and TWIST1 overexpression was detected in 62.5% (10 out of 17) of these specimens. On the other hand, 27 of 44 patients showed no metastasis of tumor cells to lymph

Figure 1. A) Scatter plot of TWIST1 and EVX1 expression in the ESCC patients. The y-axis indicates the fold change of gene expression, and the x-axis represents the number of patients. Relative mRNA expression of more than 2-fold in tumor tissue is considered as overexpression; less than minus 2-fold as underexpression, and the range in between is defined as normal. **B)** The bar chart presents the association between TWIST1/EVX1 mean expression levels and different clinicopathological features of ESCC.

nodes which upregulation of TWIST1 was not detected in 77.7% of them (21 of 27). The expression of EVX1 was also related to the different indices of poor prognosis (**Table 1**). Underexpression of EVX1 in ESCCs was also associated with lymph node metastasis (p = 0.027) and invasiveness of the disease (P = 0.037) (8).

4.4. Correlation between TWIST1 and EVX1 in ESCCs
The expression of TWIST1 and EVX1 was correlated to each other significantly (p=0.005, correlation coefficient: 0.375) in ESCC. Indeed, in patients with downregulation of TWIST1 mRNA expression, an elevated level of EVX1 expression occurs. Of 28 patients with under/normal expression of TWIST1, 22 cases (78.57%) had over/normal expression of EVX1 (**Table 2**).

4.5. DNA Sequence Analysis of EVX1 Promoter Region
Three kb of the genomic sequences immediately

Table 2. Correlation between expression profiles of TWIST1 and EVX1 in ESCC

Expression pattern	TWIST1		P-value
	Overexpression	Under/normal expression	
EVX1			
Overexpression	6	8	0.05*
Normal/under expression	10	20	

* Significant correlation (correlation coefficient = 0.502)
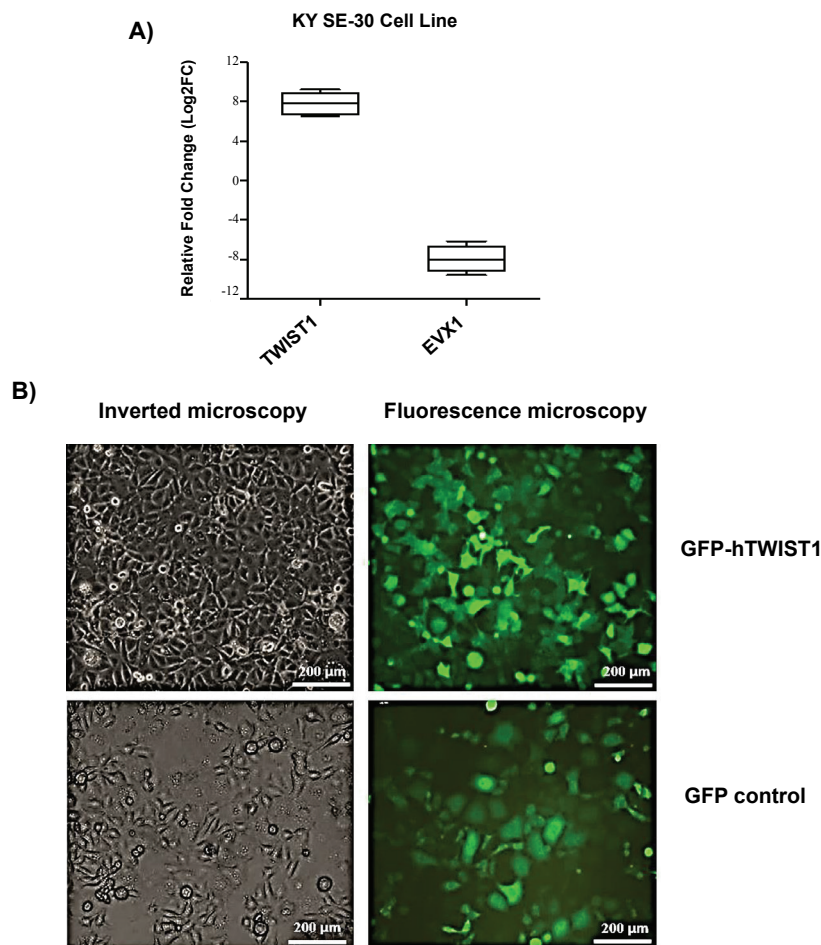
Figure 2. Enforced TWIST1 expression down-regulates EVX1 mRNA expression in KYSE-30 ESCC cells. **A)** Retroviral transduction enforced significant overexpression of TWIST1 in GFP-hTWIST-1 approximately 9-fold compared to GFP control cells causing a − 8.83-fold reduction in EVX1 mRNA expression significantly. Asterisk indicates statistical significance. **B)** Inverted and fluorescence microscope images of the GFP-hTWIST1 and control cells.

upstream of the transcription start site (TSS) of the EVX1 gene as well as its coding region were analyzed for finding the E-boxes consensus sequence (CANNTG). Eight E-box sequences were found upstream of TSS, where two of them were placed close to the TSS at positions −12 and −232. Other E-boxes are located from −1151 to −2767. Furthermore, 25 potentially active E-boxes were found in the transcription unit of the EVX1 gene. 15 of these 25 are placed in intronic regions, while others are exonic (**Supplemental 2**).

4.6. Ectopic Expression of TWIST1 Causes Downregulation of EVX1 in KYSE-30 ESCC Cell Line
The functional study was performed to examine whether TWIST1 ectopic expression can affect EVX1 mRNA expression. The retroviral system enhanced TWIST1 expression approximately 9-fold in GFP-hTWIST1 compared to control cells. Remarkably, this

level of the gene overexpression downregulated EVX1 expression nearly -8.83-fold (**Fig. 2A**, P < 0.01). The images of GFP-hTWIST1 and GFP control ESCC cells are illustrated by both Inverted and fluorescence microscopy (**Fig. 2B**).

4.7. Protein Interaction Network Analysis
Gene MANIA database was applied to create the protein-protein interaction (PPI) network and predictions. The findings displayed that multiple markers were interacting with TWIST1, including ATOH1, HOXA5, and GMNN, as well as interacting with EVX1, such as BMP signaling pathway-related markers (VENTX and ATOH1) and other markers (SOX17, HOXA5, HOXA9, GSC, and PROP1) (**Fig. 3**). These interaction networks were co-expressed, physically and genetically interacted, and pathway connected.
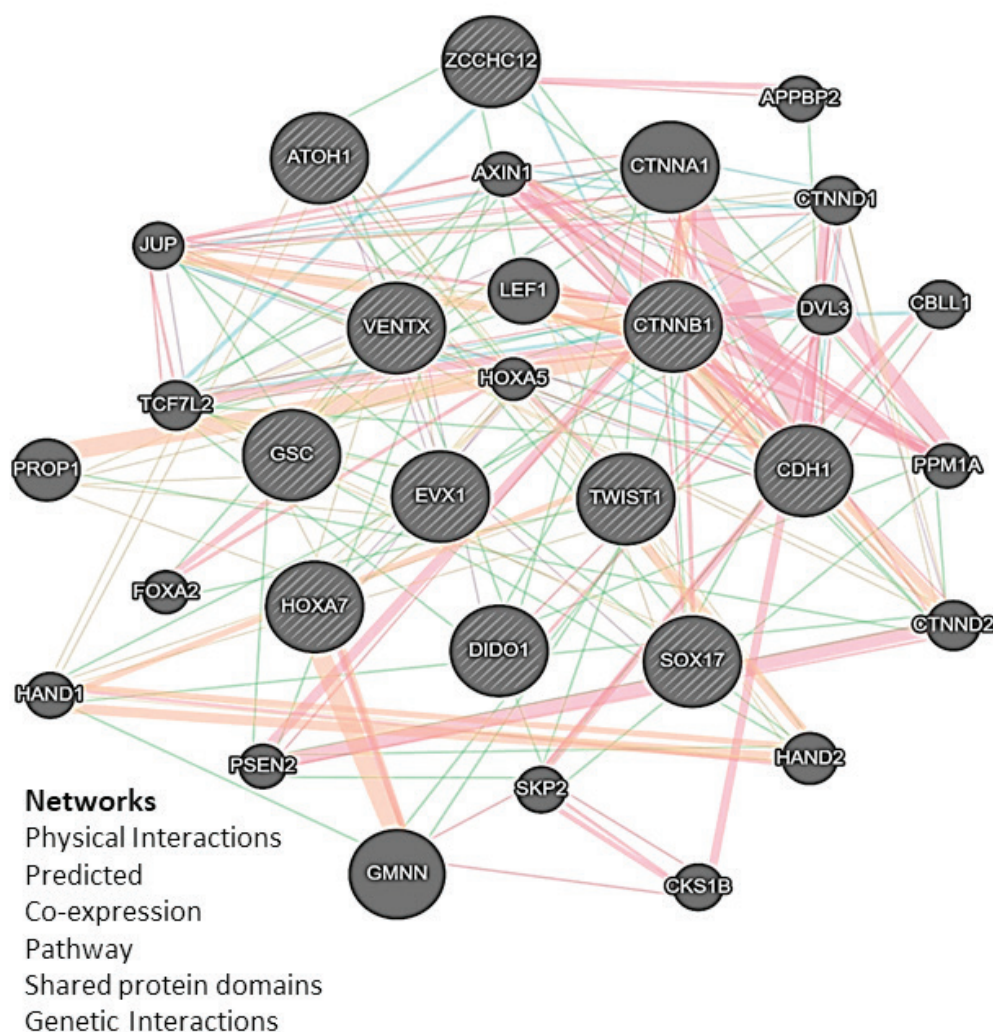
Figure 3. Protein-Protein Interaction (PPI) network was conducted using the GeneMANIA database.

5. Discussion

EMT devotes migratory and invasion traits to epithelial cells through losing cell polarity and cell-cell adhesion in both normal development and cancer (16). Since the capability of cancer dissemination and metastasis is tightly correlated with re-enabling developmental phenotypes, including EMT in different types of malignancies. EMT inducers are consisting of diverse transcription factors, including TWIST1, GOOSECOID, and SNAIL, which these factors can trigger the EMT spontaneously in multiple cell lines (16, 17). Besides, there is emerging evidence that HOX genes are closely related to the EMT in development and cancer (18, 19).

In the current study, the up-regulation of TWIST1 mRNA expression was correlated to the reduced level of EVX1 expression in ESCC patients. Significantly, gene expression of TWIST1 and EVX1 was correlated with poor prognosis clinicopathologic variables, including tumor cell progression and lymph node metastasis which introduce these genes as valuable markers for ESCC poor prognosis. Additionally, to examine the effect of EVX1 on the expression of TWIST1-induced KYSE-30 ESCC cell line, cells stably expressing TWIST1 were established by a retroviral system. The results revealed that EVX1 mRNA expression in TWIST1-overexpressing cells was significantly downregulated compared with control cells (**Fig. 2A**).

The HOX genes network, from yeast to humans, regulates a variety of cellular events. In mammals, at least 39 Hox genes are categorized into four different clusters (HoxA, HoxB, HoxC, and HoxD) , which are localized at four distinct chromosomal loci (20, 21). Deregulated homeobox gene expression has been associated with several malignancies such as oral, ESCC, lung, bladder, prostate, and breast malignancies (8, 21-24). A downstream gene of the HoxA cluster, the EVX1 transcription factor, plays both transcriptional inducer and repressor roles during embryogenesis (25). In mouse embryonic stem cells (mESCs), W9.5, Evx1 regulates anterior-posterior (AP) profiling during gastrulation by acting as a downstream effector of BMP and Wnt/beta-catenin signaling pathways (25).

Induced expression of HOXA7 enhanced expression of E-cadherin (26) in the IOSE- 29 (immortalized ovarian surface epithelial) cell line. Moreover, HOXA10 enforced expression induces E-cadherin expression, inhibits vimentin expression, and suppresses the invasive behavior of endometrial carcinoma cell lines (KLE and SPEC2) (27). On the other hand, bHLH transcription factors (TWIST1 and TWIST2), the bHLH factor E2.2, and homeobox genes (GSC and SIX1) are considered as indirect repression of E-cadherin (28). Although the TWIST1 gene is generally recognized as an indirect suppressor of E-cadherin, it can directly bind to the existent E-boxes 2 and 3 in the E-cadherin promoter to suppress its expression (29, 30). TWIST1 induces metastatic phenotype and cell motility in different human breast cancer cell lines such as MDA-MB-231, MDA-MB-435, and SUM1315 (31).

Contributed transcription factors in cancer metastasis play an essential role through homeobox genes modulation (32,33). The under expression of Evx1 induces GOOSECOID (GSC) and SOX17 overexpression, which leads to collective cell migration, EMT progress, and cell metastasis (34).

Accordingly, to pinpoint the downstream effector markers affected by TWIST1 and those responsible for ESCC tumorigenesis, the protein interactions network was realized by bioinformatic analysis in which TWIST1 and EVX1 were involved. The evidence-based GeneMANIA created protein network discovered high connectivity among TWIST1 and downstream target proteins (**Fig. 3**). According to increasing evidence, the TWIST1-EVX1 axis is supposed to be a critical hallmark in ESCC progression.

The TWIST1 transcriptional function is regulated by phosphoregulation, dimer choice, and spatial-temporal expression (35). While homodimers of TWIST1 usually induce transcription of target genes, TWIST1 heterodimers typically suppress transcription (36, 37). It has been indicated that TWIST1 can form heterodimers with the SNAIL and negatively regulate gene expression in ESCC (17). Such regulatory structure may be responsible for EVX1 downregulation in invasive ESCC cell line KYSE-30. To further extend this result, we analyzed the promoter region and transcription unit of the EVX1 gene, and numerous E-boxes were found. There were not only 11 E-boxes in the 3Kb upstream region of the TSS of EVX1 gene, but 25 potentially E-box were also identified within the EVX1 gene transcription unit (10 exonic and 15 intronic). It has been explained that intronic and intergenic E-boxes are the major TWIST attachment sequences (37, 38). Interestingly, our data revealed the inverse relationship between TWIST1 and EVX1

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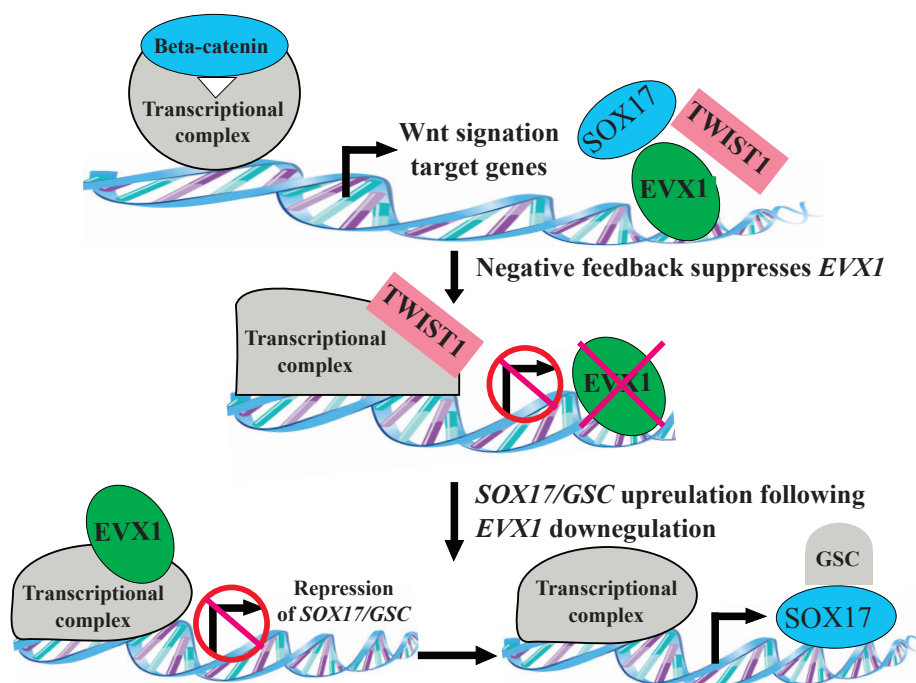
Figure 4. Probable interaction between TWIST1 and EVX1 and subsequently involved cascades in ESCC invasion and metastasis. Activation of the Wnt/beta-catenin pathway could enhance the expression of Wnt target genes such as TWIST1, EVX1, and SOX17. TWIST1 may suppress EVX1 transcriptionally in the negative feedback of the Wnt pathway. Finally, EVX1 downregulation induces GSC and SOX17 upregulation, which can lead to EMT progress, collective cell migration, and cell metastasis.

in ESCC patients, as well as the KYSE-30 cell line. Thus, TWIST1 can potentially bind to each sequence of E-box localized either upstream region of TSS or downstream of the transcription unit to under-express the EVX1 gene.

In cancer cells, a complex interplay of multiple signaling pathways leads to EMT induction. Members of the TGF-beta family play fundamental roles in initiating and maintaining EMT through both embryonic development and cancer metastasis processes (39). The antagonistic cross-regulation between EVX1 and GSC controls cell fate decisions in response to TGFβ signaling in hES (human embryonic stem) cells, where EVX1 directly represses GSC (40). Another study indicated that TWIST1 represses BMP signaling pathway by recruiting HDAC1 to Smad4 (41). Moreover, the inhibitory effects of TWIST1 on BMP/Smad signaling were dominated through Id1 (Inhibitor of differentiation/DNA binding) by the promotion of TWIST1 degradation (41). According to our findings, TWIST1 can effectively function as a BMP signaling inhibitor through eliminating downstream target genes,

which lead to promoting malignancy and metastasis potentials. Therefore, our observations propose a novel mechanism by which the BMP pathway can be potentially regulated through the binding of TWIST1 to the E-box sequences of the EVX1 promoter. Since TWIST1 is a predominant activator of EMT and KYSE-30 cells resemble an invasive phenotype, this inverse correlation can play a vital function in the KYSE-30 invasiveness cascade.

Wnt/beta-catenin signaling directly activates EVX1 through the binding of beta-catenin to the promoter of EVX1 and plays a role in hESCs differentiation (42). SOX17 expression is stimulated through beta-catenin activation in the initial stage of the tumor, which results in EVX1 downregulation (34). Since TWIST1 is known as a Wnt signaling target gene, we hypothesize that Wnt signaling upregulates TWIST1 by transcriptional repression of EVX1, which causes SOX17 and GSC upregulation and leads to EMT progress (**Fig. 4**). Collectively, these valuable results suggested a regulatory role for TWIST1 in EVX1 expression, which can be a distinctive hallmark in ESCC progression.

6. Conclusions

In the current study, a correlation between TWIST1 and EVX1 expression was demonstrated in ESCC patients, and also transcriptional downregulation of EVX1 by TWIST1 was confirmed in ESCC cell line KYSE-30. The gene expression of TWIST1 and EVX1 was correlated with ESCC clinic pathology, including the stage of tumor cell progression and lymph node metastasis which may introduce these genes as valuable markers for ESCC poor prognosis. This is the first report that functionally described the repressive role of TWIST1 on EVX1 expression in ESCC cell line and patients.

Conflicts of interest

The authors declare no conflicts of interest.

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