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High expression of *ZEB1* is associated with EMAST & metastasis in colorectal cancer patients

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***Background & objectives*: Transforming growth factor-beta (TGF-β) signalling pathway has been reported to be involved in metastasis and at the same time has been considered compellingly an important mediator of epithelial-to-mesenchymal transition (EMT). Besides, EMT process is maintained by zinc-finger E-box-binding homeobox 1 (*ZEB1*) gene which is induced by TGF-β pathway. TGF-β has been shown to be associated with elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) phenomenon, which is one of the prognostic biomarkers of colorectal cancer (CRC). This study was conducted to determine the link among *ZEB1*-induced TGF-β, EMAST status and metastasis.**

***Methods*: The expression level of *ZEB1* was evaluated using quantitative reverse transcription (qRT) real-time PCR in 122 formalin fixed paraffin-embedded tissues of CRC sample with known EMAST status and TGF-β/Smad-dependent pathways. The association among *ZEB1* expression, TGF-β signalling pathway, EMAST status and metastatic behaviour was examined.**

***Results*: *ZEB1* gene expression level was higher in tumour tissues as compared to normal samples ($P<0.045$). In addition, *ZEB1* positive expression level was associated significantly with metastasis ($P=0.05$), EMAST$^+$ status ($P=0.052$) and activated TGF-β signalling pathway ($P=0.002$).**

***Interpretation & conclusions*: Our results validated significant association between activated TGF-β signalling pathway and EMAST$^+$ phenotype with higher expression of *ZEB1* and higher level of metastasis.**

Key words Colorectal cancer - EMAST repeats - metastasis - TGF-β signalling pathway - ZEB1

A raft of molecular pathways appeared to be involved in tumour invasion convoluted process which results inevitably in the dissemination of carcinoma cells from their primary site[1]. Multiple studies have revealed that a key initial step in tumour metastasis is a molecular programme called

epithelial-to-mesenchymal transition (EMT) which has been reported to be a chief event in cancer malignancy including colorectal cancer (CRC)[2-4] whereby, as mentioned in studies, a clinically heterogeneous disease[5-7]. This well-defined system provides a mechanism for epithelial cells to acquire a mesenchymal phenotype by cell-cell junction's dissolution and cell motility required for invasion. Consequently, cells with stem cell qualities are highly relevant issues to CRC metastasis, which leads to CRC patient's death[8]. A number of research studies have revealed that EMT process is regulated by plenty of cell signalling pathways. An illustration of this is the transforming growth factor-β (TGF-β) signalling, which is considered a compelling mediator of EMT and is also an acute factor involved in tumour progression[9,10]. TGF-β is a dual-functional cytokine that participates either in tumour suppression by abrogating proliferation or in cancer propagation through a complicated network of canonical and non-canonical pathways in various cancer types, especially in CRC[10-12]. This catastrophic switching from a tumour inhibitor to promoter relies upon the cell type or tissue context[13]. Canonical TGF-β signalling pathway is accompanied by the activation of SMAD proteins through phosphorylation[14]. Afterwards, this complex translocates into the nucleus, wherein it regulates the expression of target genes such as *SNAIL*, *TWIST* and zinc-finger E-box-binding homeobox 1 (*ZEB1)*[15], which are known deliberately as EMT programming inducers in order to maintain migration, proliferation and invasion capacity and eventually adopt a mesenchymal characteristic[16]. Amongst these EMT-related transcription proteins, *ZEB1* is fundamentally responsible for EMT and is at the crossroad of several stages of CRC involving EMT which is a critical player in transcriptional downregulation of epithelial markers such as E-cadherin as an adherent junction and activation of mesenchymal genes[17]. Besides, *ZEB1* gene, which is induced by TGF-β, cooperates closely with SMAD proteins throughout TGF-β signalling commencement in order to maintain EMT process[18]. On the other hand, the potential of many elements as prognostic biomarkers for CRC patients has been evaluated in recent studies including microsatellite instability (MSI)[19] and elevated microsatellite alterations at selected tetranucleotide repeats (EMAST)[20]. A failure at the mismatch repair system leads to MSI and EMAST, which leads to problems like tumour progression due to the propensity for metastatic behaviour[21,22]. Interestingly, previous studies have examined TGF-β signalling pathway mediated by SMAD proteins as one of the overriding causes of EMAST marker, which could be responsible for poor clinical outcome in CRC cases along with EMAST-positive ($EMAST^+$) phenotype[23]. Nonetheless, there is no reported link between *ZEB1* transcriptional factor and EMAST biomarker, and consequently, this relation has remained enigma. The goal of this study was to elucidate the precise correlation among EMAST phenotype, *ZEB1* induced by TGF-β signalling pathway and metastasis.

Material & Methods

This study was conducted in 2017 at the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, after obtaining approval from the Medical Ethics Committee of the Institute. One hundred and twenty two CRC patients who underwent surgery at Taleghani Hospital and Shohada Tajrish Hospital, Shahid Beheshti University of Medical Sciences, from September 2010 to March 2017 were included. In these patients, EMAST status and TGF-β/Smad-dependent pathway were determined in our previous studies[23,24]. The expression of *ZEB1* was examined in formalin-fixed paraffin-embedded (FFPE) tumour, and normal adjacent tissues (NATs) and its association with CRC patient's survival, EMAST marker and TGF-β/Smad-dependent pathways were investigated.

RNA isolation and gene expression profiling: Whole RNA extraction was carried out using RNeasy® FFPE kit (QIAGEN GmbH, Germany), by following the manufacturer's instruction. RNA yield and purity were measured spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The reverse transcription reaction was carried out using PrimeScript RT Master Mix (Takara Bio Inc., Otsu, Japan) as well as Random Hexamer Primers in accordance with the procedure referred. cDNA samples were stored at −20°C till further use. *ZEB1* gene expression level was detected through quantitative reverse transcription (qRT)-PCR using the LightCycler ABI 7500 Real-Time PCR System and Maxima® SYBR Green/Rox with MicroAMP optical 96-well reaction (Applied Biosystems, USA); qRT-PCR was carried out as per the following conditions: five seconds of pre-denaturation at 95°C accompanied by 45 cycles containing denaturation at 95°C for five seconds, annealing for 34 sec at 60°C and a final extension at 72°C for 30 sec. Accurate measurements of each sample were made in duplicate. A final volume

of 20 µl including 0.4 µl of each primer, 10 µl Maxima SYBR Green/Rox and 4 µl cDNA was used for real-time PCR analysis. The $2^{-\Delta\Delta CT}$ (threshold cycle) approach was applied for gene expression quantification, by which, Ct values were normalized to the housekeeping gene, β-actin and relative gene expression values were determined. Forward and reverse primers used were as follows: *ZEB1* (forward primer: GAATTCACAGTGGAGAGAAGCC, reverse primer: GGAGCCAGAATGGGAAAAGCG) and β-actin (forward primer: CACCATTGGCAATGAGCGGTTC, reverse primer: AGGTCTTTGCGGATGTCCACGT). LinReg Software (Linreg version 2017.1, Amsterdam, the Netherlands) was applied in order to assess the productiveness of the afore-mentioned primers. The relative quantitation (RQ) values were included in statistical analysis.

Statistical analyses: The data was analyzed using the SPSS statistical software version 16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, USA). The Chi-squared method was applied to assess the disparities among variables. The information consistent with normal distribution was stated as mean ± standard deviation and examined with paired t test. Likewise, Student's t test was applied with the aim of comparing gene expression levels between two separate variables. Kaplan-Meier curves for overall survival were made using GraphPad Prism software. In addition, a log-rank test was used to approach the contrast through survival curve groups.

Results

Forty nine (40%) of the 122 patients with CRC had a tumour with EMAST^{+} phenotype and the rest of them (59.8%, n=73) had a tumour with EMAST^{-} phenotype. The expression level of *ZEB1* gene was of significantly higher in tumour tissues as compared to NATs (P<0.045, Fig. 1). Among 122 patients, the mean value of RQ for *ZEB1* was 2.70±2.56 in CRC tumours (median, 2.22).

Overexpression of *ZEB1* was observed in tumour specimens in 68 per cent (83/122) of all analyzed patients. The difference in the mean values of RQ for each marker is outlined in Table in accordance with the clinicopathological features and EMAST phenotype. As depicted, the mean values of RQ for *ZEB1* were not significant in terms of stage and differentiation. Patients characterized with metastasis compared to non-metastatic indicated a significant higher mean value of RQ for *ZEB1* (P=0.05). Moreover, *ZEB1* overexpression was significantly higher in tumours associated with activated TGF-β signalling pathway

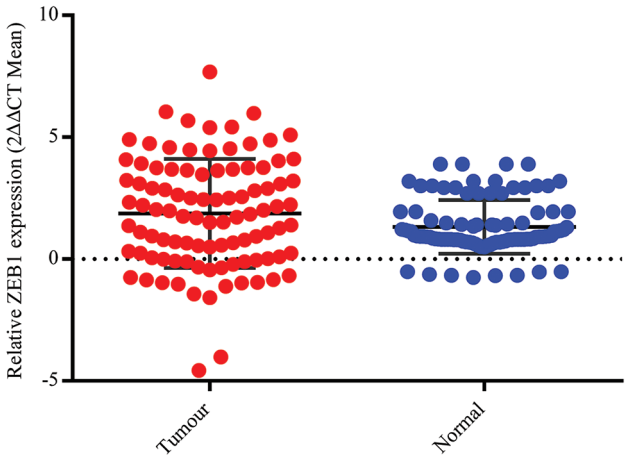

Fig. 1. Expression of ZEB1 in CRC; in collation between normal adjacent tissue and ZEB1, ZEB1 expression was significantly higher in tumour specimens (P<0.045). *ZEB1,* zinc-finger E-box-binding homeobox 1; CRC, colorectal cancer.

Table. Clinicopathological features of 122 patients according to zinc-finger E-box-binding homeobox 1 (*ZEB1*) expression

Characteristics	Total, n (%)	*ZEB1* expression Mean±SD
Tumour stage		
I-II	70 (85.4)	2.33±2.31
III-IV	52 (14.6)	3±2.81
Differentiation		
Well	40 (48.8)	3.23±3.14
Moderate/poor	82 (51.2)	2.32±2.15
Metastasis		
Yes	52 (14.6)	2.96±2.47*
No	70 (85.4)	2.36±2.59
EMAST		
Negative	73 (89.06)	2.4±2.65
Positive	49 (10.94)	2.94±2.37†
TGF-β signalling		
Negative	88 (72.13)	2.27±2.48
Positive	34 (27.86)	3.5±2.54$^{\delta}$

$^{*}P$<0.05 compared to no metastasis; $^{\dagger}P$<0.05 compared to EMAST^{-}; $^{\delta}P$ <0.01 compared to TGF-β signalling negative. *ZEB1*, zinc-finger E-box-binding homeobox 1; SD, standard deviation; EMAST, elevated microsatellite alterations at selected tetranucleotide repeats; TGF-β, transforming growth factor-β

(*P*=0.002). Similarly, the mean values of RQ for *ZEB1* found to be higher in tumours marked with EMAST$^+$ status (*P*=0.052). Thirty nine patients (32%) died during 50 months of follow up (range, 8-82 months). The mean overall survival (OS) time for all the patients was 50 months, with nominal 1-, 3- and 5-yr survival of 96, 88 and 49 per cent, respectively. Information thus gleaned indicated that increased expression of *ZEB1,* which is shown in Figure 2, was not linked significantly with reduced OS (*P*=0.140), in comparison to patients with low *ZEB1* expression. The TGF-β/Smad-dependent pathway was active in 27.9 per cent (n=34) of patients, and inactive in 72.1 per cent (n=88) of the patients.

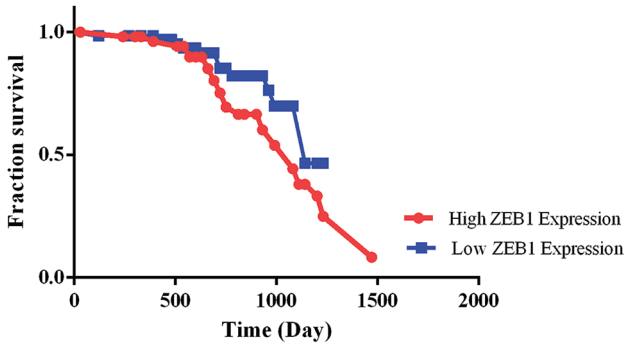

Fig. 2. Kaplan-Meier curve of 122 CRC patients according to ZEB1 expression. There was no significant distinction in overall survival among patients with higher expression of ZEB1 and those with lower expression of ZEB1. *ZEB1,* zinc-finger E-box-binding homeobox 1; CRC, colorectal cancer.

Discussion

A shred of genetic evidence which is characterized as a distinct model of MSI is called EMAST and has been observed in 60 per cent of CRC patients[25]. It is claimed that a large number of CRCs harbour EMAST$^+$ status, which means CRC tumours are strongly associated with EMAST$^+$ phenomenon[26]. It has now been proposed that there is a significant correlation between EMAST$^+$ status and metastatic cascade; nonetheless, the mechanism operating behind this association has not been ascertained yet. The most striking result emerging from the present study was higher gene expression of *ZEB1* in tumour specimens compared with normal samples. Besides, *ZEB1* gene expression was at a higher level in patients with metastasis as compared to non-metastatic patients. With this in mind, our results drew attention towards that patients characterized with activated TGF-β signalling pathway and EMAST$^+$ phenotype indicating higher expression of *ZEB1* and a significantly higher rate of metastases. It is notable that the prevalence of EMAST status and its link with clinical features as well as CRC progression is not widely known; nonetheless, both the prevalence and the predictive role and impact of EMAST are highly controversial due to the limited number and inconsistencies of studies. Moreover, the present study concentrated on the role of TGF-β signalling cascade in CRC. TGF-β/Smad signalling pathway has both crucial and heterogeneous roles in CRC. Moreover, the stated signalling pathway is an indispensable element in EMT programming. TGF-β ligand leads to enhancement of TRI and TRII receptor's dimerization which engenders Smad protein phosphorylation. Eventually, Smad2 and Smad3 are activated and relocated to the nucleus, bounding to Smad4 protein so as to act as a transcriptional regulatory factor[27]. Importantly, advanced CRC tumours showed an activated TGF-β signalling pathway. Lou *et al*[28] revealed that overexpression of TGF-β 1 was higher in tumours with higher stages (III and IV) rather than lower stages (I and II). Furthermore, a multistep complicated discrete process in which tumour cells travel from an initial tumour site to a different organ is called tumour metastasis and this feature makes it the principal cause of failed chemotherapy[29]. On the basis of published studies[30-32], numerous mechanisms underlying tumour metastasis have been elucidated, namely EMT, which is a decisive step in cancer metastasis and is strongly regulated by several signalling pathways including TGF-β/Smad pathway. The best transcriptional activators of EMT are *Snail1, Twist, Zeb1* and *Slug* which are instrumental in tumour progression[11]. Furthermore, in a previous analysis[33], a significant correlation between EMT-activator *Snail1* gene expression and EMAST$^+$ phenotype was highlighted. Once the TGF-β signalling pathway is activated, *ZEB1* transcription factor's expression increases which, in turn, regulates EMT-related gene expression[18,29]. Watson *et al*[34] reached a conclusion that EMAST$^+$ phenotype is associated with advanced stages in CRC. Furthermore, the stated association was confirmed by Devaraj *et al*[20] in another study. There is a shred of emerging evidence that this biomarker, EMAST, is a metastasis modulator, which can generate genetic pathways that transform normal cells into cancer cells and potentially cause metastasis through inflammation rather than oncogenic transformations[35]. Nevertheless, Garcia *et al*[36] suggested that HIF-1-induced hypoxia links EMAST$^+$ biomarker to recurrent metastasis. On the other hand, Gonzalez *et al*[37] unveiled that in CRC cells, TGF-β induces EMT,

invasion and endothelial migration through SMAD2, SMAD3 and SMAD1/5/8 activation. Consistent with other studies[23,38], an activated TGF-β/Smad canonical signalling pathway had a strong correlation with the existence of EMAST$^+$ phenotype. Zhang *et al*[39] outlined that the expression rate of *ZEB1* in colorectal tissue cells was at a higher level than NATs and accordingly, higher *ZEB1* expression was significantly associated with hepatic metastasis. It has now been demonstrated that the positive expression level of *ZEB1* has been associated with differentiation, meaning that higher expression of *ZEB1* had a greater frequency in poorly differentiated CRC tumours than in highly or moderately differentiated CRC tumour tissues[40]. There is some evidence to strongly suggest that TGF-β signalling pathway causes *ZEB1* transcriptional factors to increase through Smad4-dependent pathways leading to EMT response[41]. The present research was undertaken in order to figure out the significant association between *ZEB1* expression and metastasis with TGF-β signalling pathway activation and appearance of EMAST phenomenon. A consensus molecular subtype (CMS) classification system is the most reliable system for classifying CRC, which maintains a link between distinct molecular characteristics and biological subcategories. The CMS signature is divided into four subclasses from CMS1 to CMS4 and each subtype has a biological and molecular framework[42]. Previous studies have defined a panel for CMS4 subtype in which CMS4 is hallmarked by EMT-related gene expression and TGF-β signalling pathway activation[43]. Based on the combined data analysis of the present study, which suggested a significant association between TGF-β activation and EMAST status with *ZEB1* expression, it is hypothesized that EMAST biomarker can also be a feature characteristic of CMS4.

Moderate sample size (122 samples) is one of the main limitation of the present study, and therefore, this should be further investigated using a greater sample size in future studies. Additional studies are therefore required to further investigate the associations between TGF-β activation and EMAST status with ZEB1 expression.

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***Conflicts of Interest*:** None.

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