Original Article

Investigation of the Correlation between Androgen Receptor and ZEB1 and its Value in Progression of Gastric Cancer

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Abstract

Background: Zinc-finger Enhancer Binding protein (ZEB1) acts as a transcription factor to promote cancer progression through regulating Epithelial to Mesenchymal Transition (EMT). It is well-known that ZEB1 mRNA expression is directly induced by both Estrogen Receptor (ER) and Progesterone Receptor (PR). Moreover, Androgen Receptor (AR) and PR could bind to the same regulatory element. Since it has been shown that AR overexpresses in Gastric Cancer (GC) as a male-predominant tumor, the goal of this study was to evaluate whether AR could regulate ZEB1 expression in GC.

Methods: The expression profile of ZEB1 in 60 fresh GC and adjacent non-tumor tissues and 50 normal gastric specimens was assessed by qRT-PCR, and the association of ZEB1 expression with clinicopathological features was investigated. Furthermore, possible correlation between ZEB1 and AR was evaluated to elucidate a novel prognostic marker using Kaplan-Meier method and Cox regression model. Finally, molecular interaction of ZEB1 and AR was assessed using a potent AR antagonist in GC cells.

Results: Among GC patients, 70.2% (40/57) overexpressed ZEB1 and 64.91% (37/57) overexpressed AR relative to normal gastric tissues. ZEB1 overexpression was significantly correlated with the AR overexpression in GC patients. Moreover, ZEB1 overexpression was remarkably associated with lower overall survival; however, it was not an independent prognostic factor. Evidence shows that simultaneous evaluation of ZEB1 and AR expression could independently predict survival of GC patients (HR= 2.193, p=0.047).

Conclusion: These findings have clinical importance suggesting simultaneous evaluation of ZEB1 and AR expression as a potential prognostic marker. Moreover, AR may regulate ZEB1 expression in GC cells proposing a possible promising targeted therapy for GC patients.

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Keywords: Androgen receptor, Enzalutamide, Gastric cancer, Prognostic marker, Targeted therapy, ZEB1

Introduction

Although the incidence of Gastric Cancer (GC) shows a decreasing trend in the last decade, most GC patients are initially diagnosed at late TNM (Tumor, Node, Metastasis) stages or with distant metastases who have poor prognosis [1]. Therefore, there is a crucial need to introduce proper treatment strategies which could improve the survival of these Advanced Gastric Cancer (AGC) patients. In contrast to new chemotherapy protocols for GC, the 5-years survival rate for AGC patients is about 5 to 20% with a median Overall Survival (OS) of about 12 months [2]. Regarding this fact, numerous studies have been devoted to find pivotal molecular pathways to target specific inhibitors. These targeted therapies can be applied alone or in

combination with standard chemotherapies. Moreover, the identification of proper markers that precisely predict aggressiveness of GC is urgently warranted. Therefore, the molecular factors responsible for aggressiveness of GC should be assessed profoundly.

It is well-known that EMT has a critical role in the progression and aggressiveness of GC [3]. There are numerous studies which investigated the genes essential to the EMT process [3-5].

Zinc finger E-box Binding homeobox 1 (ZEB1) is one of these genes which has been shown to promote metastasis and develop invasion in various cancer types including breast, prostate, colorectal, ovarian and gastric tumors [6-10]. Previously, Murai *et al* indicated that GC patients with ZEB1 overexpression had significantly poorer survival than those with ZEB1 underexpression [11]. In a recent study, ZEB1 rs431073 polymorphism has been demonstrated as a prognostic marker of GC survival [10]. Moreover, in 2019, Xue *et al* revealed that ZEB1 regulates proliferation and EMT of GC *via* modulating Wnt5a and related mechanisms [12].

A member of the evolutionarily conserved nuclear receptor superfamily, androgen receptor is a transcription factor which regulates the expression of several genes [13]. It is indicated that Androgen Receptor (AR) could act as an oncoprotein and modulate metastasis and progression of several cancer types [14-16]. Recently, some studies have been devoted to assessment of the role of AR in GC as a male-predominant tumor [17,18]. They showed that AR has a pivotal role in progression of GC through interacting with EMT-related genes such as E-cadherin.

Besides, some studies have investigated the interaction between ZEB1 and AR in breast and prostate cancer [19-21]. Therefore, an attempt was made to investigate any interaction between these two EMT-related genes in GC.

The aim of this study was assessing the ZEB1 expression in GC and normal gastric tissues, its association with clinicopathological characteristics and the potential correlation between *ZEB1* and *AR* genes expression in GC patients. Finally, using an AR antagonist in GC cell lines, the possible interaction between ZEB1 and AR signaling pathways was evaluated aiming to introduce a novel promising therapeutic agent for AGC patients.

Materials and Methods

Patients and clinicopathological data

In the present cohort study, 60 fresh tissue samples were collected from GC patients who underwent surgical resection at Madaen, Kasra or Imam Khomeini Hospitals, Tehran, Iran, between June 2016 and June 2017. All patients were pathologically and clinically diagnosed with GC; moreover, patients who received chemotherapy or radiotherapy before surgery or patients with double primary tumors were excluded. Fresh tumor tissue specimens and adjacent non-tumor tissues were prepared within 15 *min* of excision, stabilized in RNA later solution (RNA later RNA stabilization Reagent, QIAGEN, Germany) at 4°*C* overnight and preserved at -20°*C* until RNA extraction. The patients were followed up until death or the end of the study (Sept. 2018). OS refers to the time (months) between the date of surgery and the date of death or at the end of follow-up.

Furthermore, 50 fresh samples were obtained from normal cases who underwent endoscopy procedure in Digestive Diseases Research Institute, Shariati hospital, Tehran, Iran.

The informed consents were signed by all participating patients or their first family members. The Clinical Research Ethics Committee of Tehran University of Medical School (TUMS) has approved our research. This study complies with the ethical principles of the HORC-SCT, Shariati Hospital and the Helsinki Declaration of 1964 and later versions. Ethics committee approval code: ir.TUMS.horcsct.rec.1394.103.10.

Human GC cell lines

Human GC cell lines were acquired from National Cell Bank of Iran (NCBI; Tehran, Iran). These cells included KATO III, NCBI Code: C640 and MKN45, and NCBI Code: C615. Authentication of these cell lines was carried out by STR (Short Tandem Repeat) profiling using Cell IDTM system (Promega). The cell lines were obtained in July 2017 and all *in vitro* experiments were accomplished fewer than 6 months after receipt. Moreover, cellular morphology was periodically checked. GC cell lines were cultured in RPMI 1640 containing 10% FBS and maintained at 37°*C* under humidified atmosphere with 5% CO_2.

Chemicals

Enzalutamide (MDV3100) (An androgen-receptor antagonist with greater affinity to AR than Bicalutamide) was purchased from Selleck chem (Houston, TX, USA) and was dissolved in DMSO. The final concentrations of DMSO did not exceed 0.1% (*v/v*) in all the treatments. MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfo-phenyl)-2H-tetrazolium] was purchased from SIGMA-ALDRICHs, Steibeim, Germany. RNA later (RNA Stabilization Reagent) was obtained from QIAGEN, Germany.

Cytotoxicity assays

KATO III and MKN45 cells in logarithmic growth phase were plated at a density of 2500 cells per well in 96-well plates. The cells were exposed to different concentrations of ENZ (0.1-50 μM). The viability was assessed 24, 48 and 72 *hr* post treatment by MTT assay. Vehicle-treated cells were used as the control group.

Total RNA preparation and reverse transcription-PCR

Total RNA was extracted from the RNA later-stabilized tissues or cell line lysates using 1 *ml* RiboEx reagent (Gene All Biotechnology Co, South Korea).

cDNA was synthesized using PrimeScriptTM RT

reagent Kit (TaKaRa, Japan). The reaction vessel was incubated in an ABI Veriti Thermocycler (Applied Biosystems). The control gene used in this study was human Beta-2-microglobulin (B2M). RT-PCR was performed with ABI Veriti Thermocycler (Applied Biosystems) using Taq DNA polymerase master mix red (Ampliqon, Copenhagen, Denmark). All PCR products were visualized by 1% agarose gel electrophoresis.GelQuant.NET was used to investigate the intensity of each band.

Real-time quantitative PCR

A Light Cycler 96 instrument (Roche Molecular Diagnostics) was applied to perform the quantitative reverse transcription-PCR (qRT-PCR) analysis using SYBR Green Real Q-PCR Master Mix kit (Ampliqon, Copenhagen, Denmark) as described by the manufacturer. Thermal cycling condition consisted of an activation step for 15 *min* at 95°*C* followed by 40 cycles of denaturation step (15 *s* at 95°*C*) and a combined annealing/extension step for 1 *min* at 60°*C*. Water instead of cDNA was included in the PCR reaction as negative controls. In the present study, two different housekeeping genes [hypoxanthine phosphoribosyl transferase1 (HPRT) and B2M)] were used for normalization of target genes expression levels. However, B2M proved to be the more stable one among the evaluated genes, and showed no variation between tissues.

mRNA expression levels were quantified as ΔCt values by comparing it with the mean Ct values of B2M taken as reference/endogenous control gene (ΔCt=Ct target-Ct reference) to normalize the possible differences in the amount of total RNA. The relative expression levels were calculated using the 2−(ΔΔCT) method according to the following formula: ΔΔCT= ΔCt tumor–ΔCt normal [22].

In the present study, one sample from normal cases which had the highest ΔCt value was used as a calibrator for each specific gene. All other samples from three different groups (tumor tissues, non-tumor adjacent tissues and normal tissues) were compared with the calibrator to calculate the fold change in gene expression. Next, the cut off value was determined using Receiver Operating Characteristic curve (ROC curve) for all mentioned genes. Values higher than cut off point were considered as overexpression and the values lower than cut off point were considered as under expression. The sequences of primers used in the present study are listed in table 1.

Statistical analysis

Difference in expression of ZEB1 between gastric tumors and adjacent non-tumor tissues or normal tissues was compared by independent samples Mann-Whitney U test. Correlation was computed using Spearman rank test. The associations between expression of ZEB1 and clinicopathological characteristics were evaluated using Chi-square or Fisher's exact tests.

The survival rate was analyzed by Kaplan-Meier method (Log-rank test). Univariate and multivariate survival analysis was performed by the Cox proportional hazards model to evaluate the prognostic value of known categorical variables and ZEB1 expression. All significant factors ($p<0.05$) in the univariate analysis were used for multivariate evaluation. Stepwise backward elimination was used till only significant variables were maintained in multivariate model. Functional experiments analyzed by two-tailed Student's t-test and the data are presented as the mean ± standard deviation from three independent experiments. Computerized statistical analyses were performed by the IBM SPSS® statistics 22 software. $p<0.05$ was considered statistically significant.

Results

Clinicopathological characteristics

Clinicopathological characteristics of GC patient is listed in table 2. Fifty normal cases including 25 females and 25 males (age median and range 51 and 19-83 years, respectively) were also collected.

ZEB1 overexpression has been shown to have a statistically significant correlation with lymphovascular invasion, advanced TNM stages and *AR* gene overexpression (Table 2). Among 37 GC patients overexpressing AR, only 4 patients underexpressed ZEB1. Moreover, ZEB1 overexpression was marginally significant when GC tumor size is concerned. 31 patients out of 40 cases who had tumor bigger than 5 *cm*, overexpressed ZEB1 gene (77.5%). No remarkable association was found between ZEB1 expression and age or gender. The analysis is based on comparing tumors tissues which showed increased ZEB1 expression with normal gastric tissues.

ZEB1 expression in GC and normal tissues

Quantitative real-time PCR was used to detect the relative ZEB1 mRNA expression in gastric samples (Figure 1).

The results showed significantly higher values of

Table 1. Nucleotide sequences of the primers used for QRT-PCR

Gene	Accession number	Forward Primer	Reverse Primer
B2M	NM_004048	GATGAGTATGCCTGCCGTGT	CTGCTTACATGTCTCGATCCCA
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA
AR	NM_000044	TTGTCCATCTTGTCGTCTTCGG	GCCTCTCCTTCCTCCTGTAGT
ZEB1	XM_017016597.1	CTACAACAACAAGACACTGCTGT	TGTTCTTTCAGAGAGGTAAACCG

Table 2. Association between ZEB1 expression and clinicopathological characteristics of patients with gastric cancer

Clinical variables	Total patients: n (%)	Evaluable patients: n (%)		p
	57 (100)	Overexpressed No. (%)	Underexpressed No. (%)	
Age (years) median, range	63, 33-83			
n <63	28 (49.1)	19 (33.3)	9 (15.8)	0.707
n ≥63	29 (50.9)	21 (36.8)	8 (14)	
Sex				
Male	37 (64.9)	24 (42.1)	13 (22.8)	0.233
Female	20 (35.1)	16 (28.1)	4 (7)	
Tumor size (*cm*)				
n <5	17 (29.8)	9 (15.8)	8 (14)	**0.054**
n ≥5	40 (70)	31 (54.4)	9 (15.8)	
Lauren's classification				
Intestinal	53 (93)	37 (64.9)	16 (28.1)	0.657
Diffuse	4 (7)	3 (5.3)	1 (1.8)	
Tumor grade				
Poorly differentiated	30 (52.6)	22 (38.6)	8 (14)	0.917
Moderately differentiated	21 (36.8)	14 (24.6)	7 (12.3)	
Well differentiated	6 (10.5)	4 (7)	2 (3.5)	
Tumor type				
Adenocarcinoma	44 (77.2)	31 (54.4)	13 (22.8)	0.592
Signet ring cell carcinoma	13 (22.8)	9 (15.8)	4 (7)	
Lymphovascular invasion				
YES	40 (70.2)	31 (54.4)	9 (15.8)	**0.046**
NO	17 (29.8)	9 (15.8)	8 (14)	
Perineural invasion				
YES	47 (82.5)	34 (59.6)	13 (22.8)	0.464
NO	10 (17.5)	6 (10.5)	4 (7)	
Tumor shape				
Ulcerated flat	46 (80.7)	32 (56.1)	14 (24.6)	0.537
Linitis plastica	4 (7)	3 (5.3)	1 (1.8)	
Polypoid	7 (12.3)	5 (8.8)	2 (3.5)	
Tumor location				
Proximal	26 (45.6)	17 (29.8)	9 (15.8)	0.474
Middle	22 (38.6)	16 (28.1)	6 (10.5)	
Distal	6 (10.5)	4 (7)	2 (3.5)	
Diffuse	3 (5.3)	3 (5.3)	0 (0)	
T classification				
pT1	0 (0)	0 (0)	0 (0)	0.610
pT2	12 (21.1)	8 (14)	4 (7)	
pT3	19 (33.3)	12 (21.1)	7 (12.3)	
pT4	26 (45.6)	20 (35.1)	6 (10.5)	
N classification				
N0	19 (33.3)	10 (17.5)	9 (15.8)	0.222
N1	10 (17.5)	7 (12.3)	3 (5.3)	
N2	16 (28.1)	13 (22.8)	3 (5.3)	
N3	12 (21.1)	10 (17.5)	2 (3.5)	
M classification				
M0	41 (71.9)	28 (49.1)	13 (22.8)	0.438
M1	16 (28.1)	12 (21.1)	4 (7)	
TNM stage				
I + II	22 (38.6)	12 (21.1)	10 (17.5)	**0.041**
III + IV	35 (61.4)	28 (49.1)	7 (12.3)	
AR Expression				
Underexpressed	20 (35)	7 (12.3)	13 (22.7)	**0.037**
Overexpressed	37 (64.9)	33 (57.9)	4 (7)	

ZEB1, Zinc-finger E-box-binding homeobox factor 1. AR, Androgen Receptor. # The 8th TNM Classification of Malignant Tumors proposed by the AJCC/UICC.

ZEB1 expression in GC tissues compared to adjacent non-tumor tissues and normal tissues (median of fold change expression, 17.95 *vs.* 4.23, p=0.009; 17.95 *vs.* 2.81, p<0.001, respectively).

Correlations between mRNA expression of ZEB1 and androgen receptor

Spearman rank test was applied to determine the correlation between expression of ZEB1 and AR. efficient was detected between these two genes expression (r=0.536, p<0.001) (Figure 2). Among groups of patients overexpressing ZEB1, higher values of AR mRNA expression were observed .

ZEB1 expression correlates with OS of GC patients

In the present study, GC patients were followed up for 26 months after their surgery. Three patients who failed to contact were lost for follow up. Kaplan-Meier analysis revealed a significant correlation between ZEB1 mRNA expression and OS of GC patients (Figure 3A). Among patients who overexpressed ZEB1, 62.5% and among patients underexpressed ZEB1,

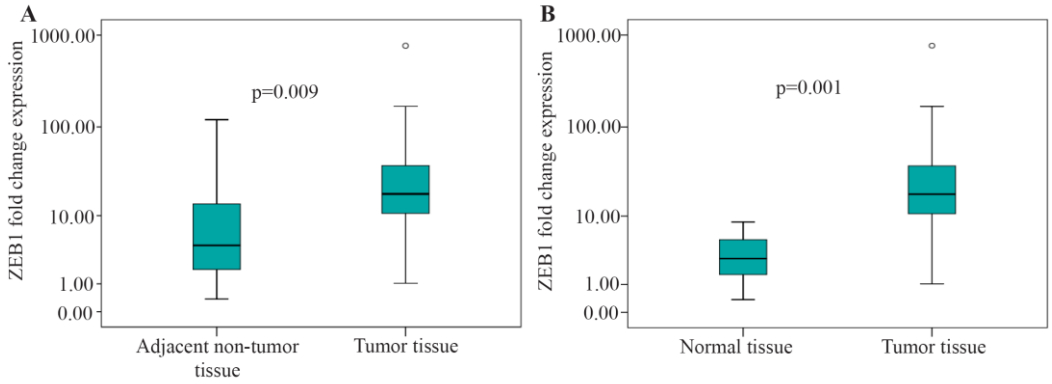

Figure 1. Graphical box-plot expression profile at transcriptome level. Comparing ZEB1 expression in gastric tumor tissues with (A) adjacent non-tumor and (B) normal tissues. Results are the mean of three independent experiments±SD ($p<0.05$).

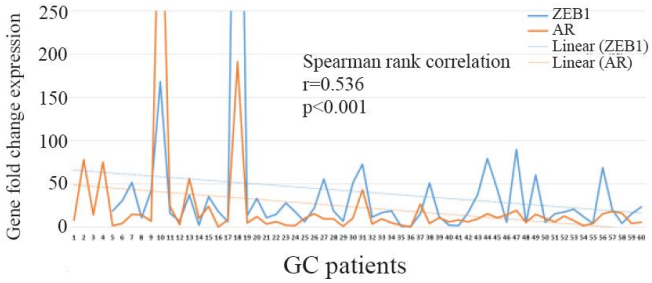

Figure 2. Relationship between AR and ZEB1 expression in GC samples using Spearman rank test.

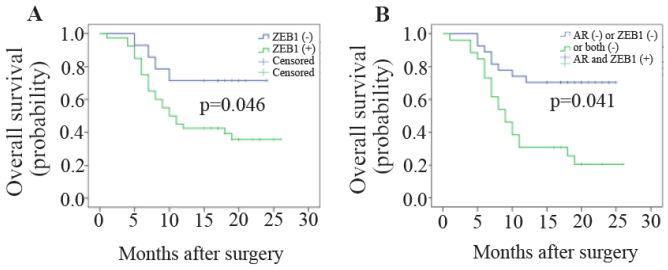

Figure 3. Kaplan-Meier curves of OS for GC patients according to (A) ZEB1 expression, (B) OS for GC patients who simultaneously overexpressed ZEB1 and AR (Log-rank test).

28.6% passed away during this study. Moreover, GC patients who simultaneously overexpressed *ZEB1* and *AR* genes were asked whether they had worse outcome (lower OS) than other GC patients (Figure 3B). Interestingly, survival analysis revealed the higher rate of death among these GC patients (76.9%).

Additionally, univariate and multivariate Cox regression analysis was applied to measure the prognostic role of clinicopathological characteristics and ZEB1 expression in GC patient (Table 3).

It was recently detected that *AR* gene overexpression associates with poor prognosis of GC patients. In the present study, among all clinicopathological characteristics, T classification, N classification, M classification, advanced TNM stages, *ZEB1* gene overexpression and simultaneous overexpression of ZEB1 and AR were significantly correlated with survival of GC patients. Tumor size was marginally significant. However, multivariate analysis showed that after adjustment with other variables, only M classification, TNM stage and simultaneous overexpression of ZEB1 and AR were independent prognostic factors.

Alteration in AR signaling affects ZEB1 gene expression

In the present study, two GC cell lines (MKN45 and KATO III) were used for further assessment of the possible crosstalk between ZEB1 and AR. A novel AR antagonist (Enzalutamide) [23] was applied to assess whether an AR signaling inhibitor could affect ZEB1 mRNA expression in GC cells. The concentrations used mostly in similar studies were up to 50micro molar. Therefore, GC cells were treated with concentrations ranging from 0.1 to 50 μM. It was revealed that treatment of GC cells with ENZ for 48 *hr* could significantly decrease ZEB1 mRNA expression in a concentration-dependent manner in both cell lines (Figures 4A and B). However, no significant difference was observed before 48 *hr* in fold change gene expression in any concentration. Moreover, higher concentrations of ENZ (more than 50 μM) had killed GC cells in only 24 *hr*, thus there were no cells for extracting their mRNA and assessing ZEB1 expression.

RT-PCR results were found to be in accordance with the quantitative real-time PCR data (Figure 4C). Analyzing the RT-PCR band intensity showed that MKN45 cells were more sensitive to ENZ treatment.

Discussion

Metastasis, a hallmark of all types of tumors, is the leading cause of approximately 90% of cancer patients' deaths [3]. During EMT process, cancer cells lose adhesion and then expand their motility and aggressiveness dramatically. Various cell signaling pathways are known to trigger the induction and maintenance of EMT such as Wnt/beta-catenin, TGF-beta, E-cadherin, Snail, Twist, STAT3, and ZEB1 [3,24].

ZEB1 is a family member of E-box binding transcription factor that contains seven zinc fingers, and can activate or repress its target genes [25]. Many studies

Table 3. Univariate and multivariate Cox regression analysis of overall survival in patients with gastric cancer

Variable	Univariate cox			Multivariate cox		
	HR	95% CI	p	HR	95% CI	p
Sex						
Male	1.000					
Female	0.771	0.378-1.574	0.475			
Age (years)	0.999	0.472-1.942	0.903			
Tumor size (*cm*)						
n <5	1.000			1.000		
n ≥5	2.588	0.993-6.742	**0.052**	1.547	0.535-4.471	0.421
Lauren's classification						
Intestinal	1.000					
Diffuse	2.388	0.741-7.912	0. 142			
Tumor grade						
Well differentiated	1.000					
Moderately differentiated	1.184	0.272-5.150	0.822			
Poorly differentiated	1.337	0.296-6.040	0.705			
Perineural invasion						
No	1.000					
Yes	3.019	0.736-12.665	0.181			
Lymphovascular invasion						
NO	1.000			1.000		
YES	2.587	0.990-6.759	0.052	1.291	0.411-4.056	0.662
T classification						
pT1						
pT2	1.000			1.000		
pT3	2.173	0.576-7.9	0.245	2.457	0.516-11.709	0.259
pT4	3.782	1.085-12.829	**0.033**	1.502	0.315-7.171	0.610
N classification						
N0	1.000			1.000		
N1	4.924	1.268-19.116	**0.024**	2.344	0.470-11.687	0.299
N2	7.435	2.080-26.573	**0.002**	2.056	0.363 -11.652	0.261
N3	9.801	2.661-36.664	**0.001**	2.714	0.846-15.482	0.128
M classification						
M0	1.000			1.000		
M1	3.178	1.566-6.471	**0.001**	2.323	1. 345-4.013	**0.023**
TNM stage						
I + II	1.000			1.000		
III + IV	8.119	2.429-26.411	**0.001**	5.690	1.641-19.725	**0.006**
ZEB1						
Underexpressed	1.000			1.000		
Overexpressed	2.743	1.009-6.420	**0.048**	1.130	0.256-4.105	0.455
AR						
Underexpressed	1.000			1.000		
Overexpressed	4.147	1.582-10.874	**0.004**	2.425	0.898-6.547	0.070
ZEB1 and AR						
ZEB1 or AR or both under expressed	1.000			1.000		
Both overexpressed	3.598	1.641-11.116	**0.002**	2.193	1.02-5.136	**0.047**

HR, Hazard Ratio; CI, Confidence Interval. # The 8th TNM Classification of Malignant Tumors proposed by the AJCC/UICC.

have reported ZEB1 as a pivotal player in cancer progression by regulating EMT in gastric, breast, prostate, ovarian and colorectal cancers [9,24-27]. Moreover, some studies have demonstrated the prognostic role of ZEB1 in GC [24,28].

It is indicated that AR can independently work with EMT-related transcription factors and function as oncogene resulting in aggressive phenotype [21]. Recently, the oncogenic role of AR in GC by crosstalk with EMT-related genes such as E-cadherin and β-catenin was demonstrated which was in accordance with some new studies [17,29].

In the present study, the clinical importance of ZEB1 expression and its correlation with AR expression were assessed in GC using tumor and adjacent non-tumor tissues from GC patients, normal gastric tissues from normal cases and two GC cell lines.

It was revealed that *ZEB1* gene overexpression significantly associates with lymphovascular invasion, advanced TNM stages and AR overexpression. This result is consistent with previous study investigating ZEB1 expression in GC [12,24,30]. However, Yabusaki *et al* reported that ZEB1 overexpression is only correlated with T classification [26].

Despite several studies evaluating the role of ZEB1 in GC prognosis, their results are controversial. It was demonstrated that *ZEB1* gene expression in GC tissues is higher than adjacent non-tumor or normal gastric tissues. Furthermore, patients who overexpressed ZEB1 had significantly lower OS. Although these results are

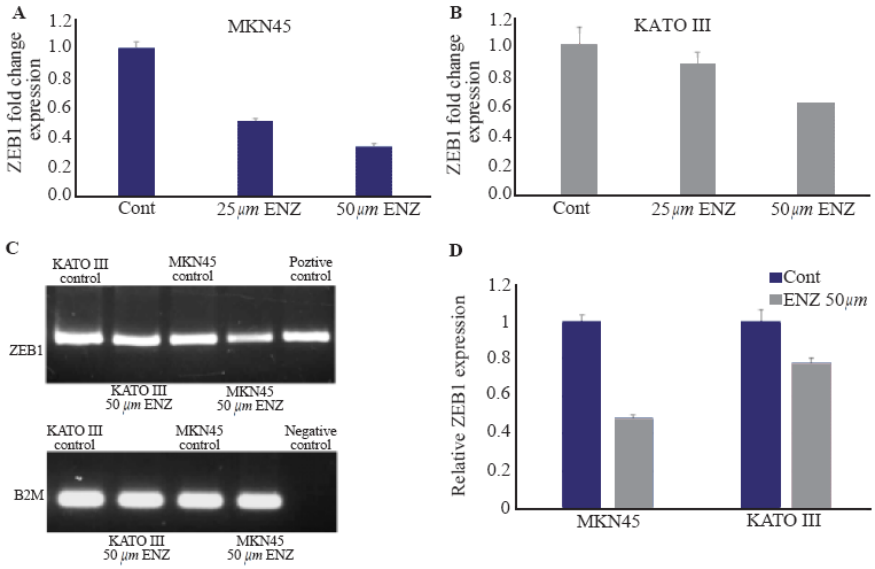

Figure 4. The effects of ENZ on GC cell lines. (A, B). The effect of ENZ on ZEB1 gene expression. After 48 *hr* of treatment with ENZ (25 and 50 *μM*), the GC cells were harvested for quantitative real-time PCR test. Gene expression levels were normalized to B2M. Data are given as mean±SD. Statistically significant values of *p<0.05, **p<0.01, and ***p<0.001 were determined compared with the control. (C) ZEB1 mRNA expression in GC cell detected by RT-PCR after 48 *hr* of treatment with ENZ (50 *μ*). Digital images of the gels were captured by a bio-Rad gel documentation system using Image Lab Software. The samples derived from the same experiment and gels were processed in parallel. DDW was used for negative control and genes expression in LNCaP prostate cancer cells for positive control (D) Analysis of the RT-PCR band intensity using GelQuant. NET.

in concordance with some previous studies [11,26,30], Xue *et al* showed that patients with low ZEB1 expression have lower OS [12].

Also, Cox regression analysis was used to find out whether *ZEB1* gene expression could be used as an independent prognostic factor in GC. It has been disclosed that although ZEB1 overexpression was significantly associated with OS of GC patients (HR=2.743, p=0.048), it cannot be used as an independent prognostic factor after adjustment for other variables entered to the model. Our results are consistent with previous studies [25,26]. However, Okugawa *et al* claimed that ZEB1 overexpression is an independent factor for predicting the outcome of GC patients (HR:2.93, p<0.001) [28]. Because of these contradictory results, a novel prognostic marker was introduced which could precisely forecast GC patients' prognosis .

Since the oncogenic role of AR in gastric carcinogenesis by interacting with EMT-related genes has been revealed recently, whether it has crosstalk with ZEB1 as another EMT-related signaling pathway was an issue of interest. The reasoning behind this hypothesis is rooted in the following evidence.

Firstly, many studies have indicated that ZEB1 and AR both interact with some common signaling pathways including E-cadherin, β-catenin, Twist and Snail pathways which are involved in invasion of tumors [31-37]. For instance, as revealed recently, a study demonstrated that AR can attach to regulatory sequence of *E-cadherin* gene which results in reducing E-cadherin expression and enhancing metastasis in breast and colon cancer cells [35]. Similarly, it has been shown that ZEB1 could attach to *CDH1* gene promoter and reduce the expression of E-cadherin [38].

Secondly, several previous researches have demonstrated the interplay between ZEB1 and AR signaling in various tumor types [9,20,21]. Graham *et al* showed that ZEB1 and AR crosstalk promotes metastasis and cell migration in triple negative breast cancer.

In the present study, for the first time, the association of *ZEB1* and *AR* genes expression in GC patients was evaluated. It is revealed that GC patients overexpressing ZEB1 significantly overexpressed *AR* gene at the transcriptional level (p=0.037). Moreover, Spearman rank test demonstrated significant correlation coefficient (r=0.536) between these two genes expression in GC patients.

Additionally, this study provides a clue about the functional interaction between ZEB1 and AR signaling pathways in gastric cancer. We recently indicated the anti-tumor effects of ENZ on the proliferation of GC cells. Here we showed that inhibition of AR signaling using ENZ could alter the mRNA expression of ZEB1 in GC cell lines in a dose-dependent manner. These results are in accordance with previous studies on prostate cancer cells which demonstrated the alteration of ZEB1 expression on mRNA and protein levels [20,21].

Our results have provided the evidence suggesting that simultaneous evaluation of ZEB1 and AR mRNA expression attained a more precise prognostic marker for GC patients' outcomes. Fascinatingly, simultaneous over-expression of *ZEB1* and *AR* genes, as a single variable, turned out to be independent unfavorable factor for OS of GC patients adjusted for other variables us-

ing multivariate Cox regression model (HR=2.193, p= 0.047).

Thus, our data indicate that ZEB1 and androgen receptor signaling pathways have indisputable promising clinical potentials to design novel targeted therapy and use as new prognostic marker for GC patients.

Conclusion

In summary, this is the first attempt proposing a role for crosstalk between ZEB1 and AR pathways in GC progression and metastasis. Up to our knowledge, no reports have indicated clinical significance associated with regulation of ZEB1 expression by AR in GC. Our study provided evidence explaining a possible encouraging marker, simultaneous assessment of ZEB1 and AR expression, which could appropriately forecast prognosis of GC patients. Moreover, our preliminary results have indicated that ZEB1 mRNA levels could decrease in response to a potent AR antagonist, Enzalutamide, in GC cell lines confirming our observations in GC samples which have shown a significant association between these two genes. However, the use of ZEB1/AR pathways as a prognostic marker and therapeutic target in GC patients warrants further investigation to explore the exact mechanism of interaction between them besides assessment of anti-AR therapy in GC patients *via* larger cohort studies and randomized clinical trials.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Availability of Data and Materials

The authors confirm that the data supporting the findings of this study are available within the article supplementary materials.

References

1. Catalano V, Labianca R, Beretta GD, Gatta G, de Braud F, Van Cutsem E. Gastric cancer. Crit Rev Oncol Hematol 2009;71(2):127-164.
2. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastroesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010;376(9742): 687-697
3. Huang L, Wu R-L, Xu AM. Epithelial-mesenchymal transition in gastric cancer. Am J Translational res 2015; 7(11):2141-2158.
4. Klymkowsky MW, Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. Am J Pathol 2009;174(5):1588-1593.
5. Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer Res 2006;66(17): 8319-8326.
6. Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, et al. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene 2005;24(14):2375-2385.
7. Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A, Merkel S, et al. A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. Gastroenterology 2006;131(3):830-840.
8. Graham TR, Zhau HE, Odero-Marah VA, Osunkoya AO, Kimbro KS, Tighiouart M, et al. Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res 2008;68(7):2479-2488.
9. Graham TR, Yacoub R, Taliaferro-Smith L, Osunkoya AO, Odero-Marah VA, Liu T, et al. Reciprocal regulation of ZEB1 and AR in triple negative breast cancer cells. Breast Cancer Res Treat 2010;123(1):139-147.
10. Romero S, Musleh M, Bustamante M, Stambuk J, Pisano R, Lanzarini E, et al. Polymorphisms in TWIST1 and ZEB1 Are Associated with Prognosis of Gastric Cancer Patients. Anticancer Res 2018;38(7):3871-3877.
11. Murai T, Yamada S, Fuchs BC, Fujii T, Nakayama G, Sugimoto H, et al. Epithelial-to-mesenchymal transition predicts prognosis in clinical gastric cancer. J Surg Oncol 2014;109(7):684-689.
12. Xue Y, Zhang L, Zhu Y, Ke X, Wang Q, Min H. Regulation of Proliferation and Epithelial-to-Mesenchymal Transition (EMT) of Gastric Cancer by ZEB1 via Modulating Wnt5a and Related Mechanisms. Med Sci Monit 2019;25:1663-1670.
13. Ma WL, Hsu CL, Wu MH, Wu CT, Wu CC, Lai JJ, et al. Androgen receptor is a new potential therapeutic target for the treatment of hepatocellular carcinoma. Gastroenterology 2008;135(3):947-955, 55 e1-5.
14. Li Y, Izumi K, Miyamoto H. The role of the androgen receptor in the development and progression of bladder cancer. Japanese J Clinical Oncology 2012;42(7):569-577.
15. Konduri S, Schwarz MA, Cafasso D, Schwarz RE. Androgen receptor blockade in experimental combination therapy of pancreatic cancer. J Surg Res 2007;142(2): 378-386.
16. Feng H, Cheng AS, Tsang DP, Li MS, Go MY, Cheung YS, et al. Cell cycle-related kinase is a direct androgen receptor-regulated gene that drives beta-catenin/T cell factor-dependent hepatocarcinogenesis. J Clin Invest 2011;121(8):3159-3175.

17. Tang W, Liu R, Yan Y, Pan X, Wang M, Han X, et al. Expression of estrogen receptors and androgen receptor and their clinical significance in gastric cancer. Oncotarget 2017.
18. Tian Y, Wan H, Lin Y, Xie X, Li Z, Tan G. Androgen receptor may be responsible for gender disparity in gastric cancer. Med Hypotheses 2013;80(5):672-674.
19. Miao L, Yang L, Li R, Rodrigues DN, Crespo M, Hsieh JT, et al. Disrupting Androgen Receptor Signaling Induces Snail-Mediated Epithelial-Mesenchymal Plasticity in Prostate Cancer. Cancer Res 2017;77(11):3101-3112.
20. Cottard F, Asmane I, Erdmann E, Bergerat JP, Kurtz JE, Ceraline J. Constitutively active androgen receptor variants upregulate expression of mesenchymal markers in prostate cancer cells. PLoS One 2013;8(5):e63466.
21. Anose BM, Sanders MM. Androgen Receptor Regulates Transcription of the ZEB1 Transcription Factor. Int J Endocrinol 2011;2011:903918.
22. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3(6): 1101-1108.
23. Mateo J, Smith A, Ong M, de Bono JS. Novel drugs targeting the androgen receptor pathway in prostate cancer. Cancer Metastasis Rev 2014;33(2-3):567-579.
24. Jia B, Liu H, Kong Q, Li B. Overexpression of ZEB1 associated with metastasis and invasion in patients with gastric carcinoma. Mol Cell Biochem 2012;366(1-2): 223-229.
25. Zhang GJ, Zhou T, Tian HP, Liu ZL, Xia SS. High expression of ZEB1 correlates with liver metastasis and poor prognosis in colorectal cancer. Oncol Lett 2013;5 (2):564-568.
26. Yabusaki N, Yamada S, Murai T, Kanda M, Kobayashi D, Tanaka C, et al. Clinical significance of zinc-finger E-box binding homeobox 1 mRNA levels in peritoneal washing for gastric cancer. Mol Clin Oncol 2015;3(2): 435-441.
27. Zhou YM, Cao L, Li B, Zhang RX, Sui CJ, Yin ZF, et al. Clinicopathological significance of ZEB1 protein in patients with hepatocellular carcinoma. Ann Surg Oncol 2012;19(5):1700-1706.
28. Okugawa Y, Toiyama Y, Tanaka K, Matsusita K, Fujikawa H, Saigusa S, et al. Clinical significance of Zinc finger E-box Binding homeobox 1 (ZEB1) in human gastric cancer. J Surg Oncol 2012;106(3):280-285.
29. Zhang BG, Du T, Zang MD, Chang Q, Fan ZY, Li JF, et al. Androgen receptor promotes gastric cancer cell migration and invasion via AKT-phosphorylation dependent upregulation of matrix metalloproteinase 9. Oncotarget 2014;5(21):10584-10595.
30. Chen H, Lu W, Huang C, Ding K, Xia D, Wu Y, et al. Prognostic significance of ZEB1 and ZEB2 in digestive cancers: a cohort-based analysis and secondary analysis. Oncotarget 2017;8(19):31435-31448.
31. Wong TS, Gao W, Chan JY. Transcription regulation of E-cadherin by zinc finger E-box binding homeobox proteins in solid tumors. Biomed Res Int 2014;2014:921564.
32. Zhou L, Hu YL, Wu SW, Yu L, Cheng ZN, Zhu B. [Expressions of Slug, ZEB1 and KISS-1 in gastric adenocarcinoma and their clinical significance]. Nan Fang Yi Ke Da Xue Xue Bao 2016;36(4):532-537. Chinese.
33. Pena C, Garcia JM, Silva J, Garcia V, Rodriguez R, Alonso I, et al. E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations. Hum Mol Genet 2005;14(22): 3361-3370.
34. Han J, Xie C, Pei T, Wang J, Lan Y, Huang K, et al. Deregulated AJAP1/beta-catenin/ZEB1 signaling promotes hepatocellular carcinoma carcinogenesis and metastasis. Cell Death Dis 2017;8(4):e2736.
35. Liu YN, Liu Y, Lee HJ, Hsu YH, Chen JH. Activated androgen receptor downregulates E-cadherin gene expression and promotes tumor metastasis. Mol Cell Biol 2008;28(23):7096-7108.
36. Chiurillo MA. Role of the Wnt/beta-catenin pathway in gastric cancer: An in-depth literature review. World J Exp Med 2015;5(2):84-102.
37. Ware KE, Somarelli JA, Schaeffer D, Li J, Zhang T, Park S, et al. Snail promotes resistance to enzalutamide through regulation of androgen receptor activity in prostate cancer. Oncotarget 2016;7(31):50507-50521.
38. Aghdassi A, Sendler M, Guenther A, Mayerle J, Behn CO, Heidecke CD, et al. Recruitment of histone deacetylases HDAC1 and HDAC2 by the transcriptional repressor ZEB1 downregulates E-cadherin expression in pancreatic cancer. Gut 2012;61(3):439-448.