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Expression profile of *ZFX* isoform3/variant 5 in gastric cancer tissues and its association with tumor size

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ABSTRACT

Objective(s): Previous studies demonstrate that changes in pre-mRNA splicing play a significant role in human disease development. Furthermore, many cancer-associated genes are regulated by alternative splicing. There are mounting evidences that splice variants which express predominantly in tumors, have clear diagnostic value and may provide potential drug targets. Located on the X chromosome, *ZFX* gene functions as a transcription regulator for self-renewal of stem cells. This gene has 5 splice variants that encode 3 isoforms. In the present study, we evaluated the clinicopathological relevance of the expression of *ZFX isoform 3/variant 5* gene in gastric carcinoma.

Materials and Methods: A total of 60 tumoral and non-tumoral gastric specimens were evaluated for *ZFX* isoform 3/variant 5 gene expression using quantitative real-time PCR.

Results: Our results showed that the expression of *ZFX* isoform 3/variant 5 transcript was heterogeneous in gastric specimens. We further showed that there was a positive correlation between the variant expression and tumor size, but not with other clinicopathological features of gastric tumors.

Conclusion: This report shows that the expression of *ZFX* isoform 3/variant 5 transcript was heterogeneous in gastric specimens. Furthermore, there was no significant association between *ZFX* isoform 3/variant 5 expression and most of clinicopathological features of gastric tumors except for a positive correlation with tumor size. The elucidation of the precise molecular mechanisms governed by the *ZFX* isoforms/variants needs further investigation.

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Introduction

As a key element in the eukaryotic gene expression, alternative splicing increases the coding capacity of the human genome that leads to the generation of numerous mRNA variants which yield different polypeptides from a unique coding gene (1-3). It is estimated that more than 88% of human protein-coding genes are affected by this process (4). Several studies demonstrate that changes in pre-mRNA splicing play a significant role in human disease development (5-7). There are mounting evidences that many cancer-associated genes are regulated by alternative splicing. Furthermore, the patterns of alternative splicing are changed in human cancers (8, 9). The cancer-associated genes involved in the key aspects of human malignancies including angiogenesis, invasion, differentiation, resistance to apoptosis (10) and metastasis are affected by alternative splicing (8, 11, 12).

As the second most frequent cause of cancer death and a main public health concern, gastric cancer is a common disease worldwide affecting about one million people per year (13-15). It is more common in men than in women (the ratio is about 2:1). According to the national cancer registry data, gastric cancer is one of the first leading cause of Iranian cancer-related deaths in men and the second one in women (16). An unhealthy lifestyle including a high concentration of salt in diet and smoking is the major environmental risk factors of this cancer in Iran (17).

The *ZFX* gene on the human X chromosome is a member of *ZFY* family that encodes a 90 KDa zinc finger protein (C2H2-type) (18-20). This protein is a transcriptional regulator, identified as a putative stemness gene in embryonic and adult hematopoietic stem cells (HSCs) (21). Furthermore, ZFX over expresses in various types of human malignancies including prostate adenocarcinoma (21), esophageal

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carcinoma (22), follicular lymphoma and diffuse large B-cell lymphoma, (23) and gliomas (24). Therefore, it might be regarded as a cancer stem cell marker in the examined cancers as it confers a self-renewal-like property to cancer cells (25).

ZFX has five different variants that encode 3 different isoforms (26). Recently, we showed that *ZFX* (variant 1 and 3) overexpresses in the diffuse type of gastric cancer (27). Due to the crucial role of alternative splicing and the lack of data concerning the expression of *ZFX* isoform 3/variant 5 in gastric cancer, in the present study, we evaluated the clinicopathological relevance of the expression of *ZFX isoform 3/variant 5* gene in gastric tissue samples. This variant lacks two exons in the 5' UTR and has an additional segment in the 3' CDS, as compared to variant 1. Furthermore, The deriving isoform is shorter and has a distinct C-terminus, as compared to isoform 1 (26).

Materials and Methods

Experimental subjects

Gastric tissue samples (tumoral and non-tumoral) were collected from Iran Tumoral Bank (Tehran, Iran) as described previously (27). In brief, 30 paired tissue samples were examined for gene expression, of which 30 were adjacent non-tumoral and 30 were tumoral specimens of gastric from the same patients. The experiments were approved by The Ethics Committee of Isfahan University of Medical Sciences. All patients provided written informed consent to the Iran Tumoral Bank prior to the participation. The study conforms to the code of Ethics of the World Medical Association (Declaration of Helsinki).

Gene expression analyses

Total cellular RNAs from tumoral and adjacent non-tumoral tissue specimens were isolated with Qiazol reagent and RNeasy Mini kit, following manufacturer's instructions. (Qiagen, Hilden, Germany). For the omission of any genomic DNA, DNase treatment was performed on-column using DNase set (Qiagen, Hilden, Germany). Agarose gel electrophoresis was used to assess the quality of the RNA. The RNA concentration was determined by optical density at 260 nm using nanodrop (Biochrome, USA). Two microgram of total RNA from each sample was reverse transcribed to single-stranded cDNA using MMLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania) with random hexamer primers (TAG Copenhagen). Quantification of expression of *ZFX* isoform 3/variant 5 was done by quantitative real-time RT-PCR (qRT-PCR) using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) with specific primers for *ZFX* isoform 3/variant 5 as follows: GGCAGCAGCTTATGGTAATAATTC and CATGGAACTCGTGCGCCCTCA and TBP (28). Reaction mixes were subsequently run on the Rotor-gene 6000 (Qiagen, Hilden, Germany). The conditions of the PCR for *ZFX* isoform 3/variant 5 consist of an initial denaturation step at 95°C for 10 min, followed by 40 amplification cycles consisting of denaturation at 95°C for 25 sec, annealing at 60.5°C for 30 sec and an extension at 72°C for 30 sec. To further verify the identity of the *ZFX* isoform 3/variant 5 PCR product, agarose gel electrophoresis was performed. Relative gene expression was calculated using the ΔΔct method.

Statistical analyses

Triplicate reactions were done for each independent preparation and the results were statistically analyzed by SPSS software, version 16 using Student`s t-test and ANOVA. Differences were considered significant if $P<0.05$.

Results

Optimization of RT-PCR reaction

Optimization of conventional and real-time RT-PCR for *ZFX* isoform 3/variant 5 was performed on MCF7 cell line and a tumoral gastric tissue sample using specific primers. A single specific band with the expected size for the amplified *ZFX* isoform 3/variant 5 (177 bp) was obtained using agarose gel electrophoresis (Figure 1a). Furthermore, real-time RT-PCR reaction for the examined gene showed a unique melting curve without primer dimers (Figure 1b).

Expression of ZFX isoform 3/variant 5 in tumoral and non-tumoral gastric tissues

qRT-PCR was performed to examine whether there is a significant difference in the expression of *ZFX* isoform 3/variant 5 mRNA in 30 paired cancerous and non-cancerous gastric tissue samples. Fold changes in the expression of target gene was

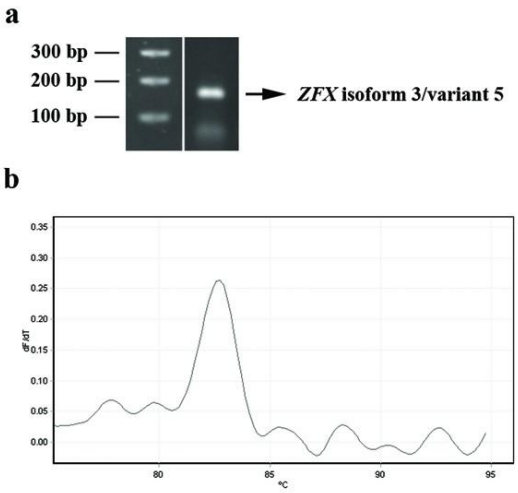

Figure 1. Optimization of conventional and real-time RT-PCR. (a) Electrophoresis of *ZFX* isoform 3/variant 5 PCR products on the agarose gel. (b) A unique melting curve without primer dimers showing specific amplification of *ZFX* isoform 3/variant 5 on real-time RT-PCR

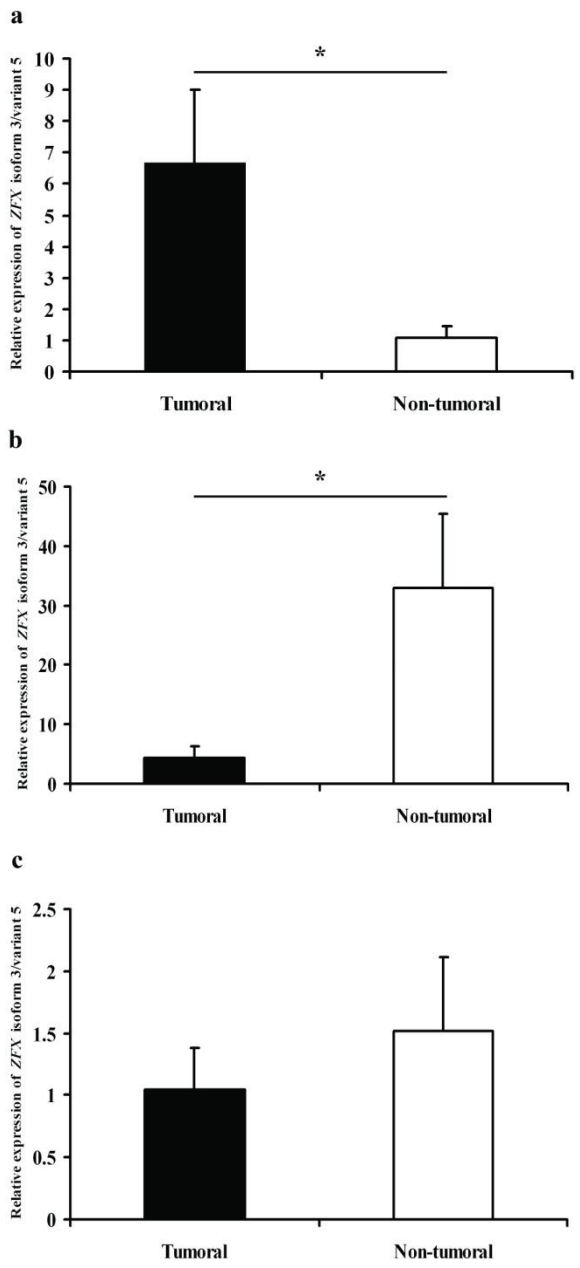

Figure 2. Relative expression of *ZFX* isoform 3/variant 5 in gastric tissue samples. Charts comparing the relative gene expression of *ZFX* isoform 3/variant 5 as determined by qRT-PCR in (a) a subset of paired tissue samples in which *ZFX* isoform 3/variant 5 relative expression was higher in tumoral ones. (b) a subset of paired tissue samples in which *ZFX* isoform 3/variant 5 relative expression was higher in non-tumoral ones. (c) a subset of paired tissue samples in which *ZFX* isoform 3/variant 5 relative expression did not change significantly between tumoral and non-tumoral ones. Values shown represent the mean ± SEM. The asterisk shows statistical significant differences

determined using the ΔΔct method. Real-time qRT-PCR results demonstrated that there was no significant difference in *ZFX* isoform 3/variant 5 expression between tumoral and non-tumoral tissues (*P*-value: 14×10^{-2}). Regarding fold changes in the tumoral and non-tumoral specimens, it was possible to categorize them into 3 classes: tumor tissues with significantly increased expression of the transcript (33.33%, *P*-value: 18×10^{-3}) (Figure 2a), tumor samples with significantly decreased expression of the transcript (36.66%, *P*-value: 14×10^{-3}) (Figure 2b), and the tissues without significant change in the transcript expression (30%) (Figure 2c). Of note, 60% of the first tissue category was high-grade diffuse-type gastric tumors.

Table 1. Relationship between *ZFX* isoform 3/variant 5 expression levels and clinicopathological parameters of gastric cancer samples

Characteristics	Numbers (%)	*ZFX* iso3/v5 relative expression (Mean±SEM)	*P*-value
Sex			
Male	18 (60)	3.030± 1.11	0.08
Female	12 (40)	12.20± 7.96	
Age (years)			
≥70	15 (50)	2.94± 0.84	0.13
<70	15 (50)	10.45± 6.46	
N classification			
N0	9 (30)	2.35± 0.81	0.10
N1-N3	21 (70)	8.56± 4.64	
M classification			
Mx	15 (50)	10.02± 6.42	0.30
M0	11 (36.6)	3.69± 1.65	
M1	4 (13.3)	2.47± 2.31	
Lymphatic invasion			
Negative	16 (55.17)	8.24± 5.96	0.32
Positive	13 (44.82)	5.28± 2.13	
Tumor size (cm)			
≥5	20 (68.7)	2.11± 0.61	0.01
<5	9 (31. 3)	17.58± 10.33	
Tumor grades			
Grade I	10 (33.3)	2.29± 0.90	0.14
Grade II	8 (26.6)	15.26± 11.97	
Grade III	12 (40)	4.65± 1.80	
Tumor types			
Diffuse	15 (50)	4.08± 1.46	0.22
Intestinal	15 (50)	9.31± 6.43	

Association of ZFX isoform 3/variant 5 expression with clinicopathological features of gastric carcinoma

To examine the clinical importance of the *ZFX isoform 3/variant 5* expression, we investigated the correlation between clinicopathological status of gastric tumoral samples and the gene expression. Analyses showed no significant association between the expression of *ZFX isoform 3/variant 5* and sex, age, N classification, lymphatic invasion, grade and type, except for a statistically significant correlation with tumor size (Table 1).

Discussion

ZFX has five different variants that encode 3 different isoforms (26). In the present study, for the first time we assessed and quantified the expression of *ZFX* isoform 3/variant 5 transcript in cancerous and non- cancerous gastric tissues using qRT-PCR. Our results demonstrated that the expression of this

transcript was heterogeneous in gastric specimens, while its expression was significantly increased in some specimens (33.33%), and it was underexpressed in the others (36.66%). Of note, 60% of overexpressed class was high-grade diffuse-type gastric tumors. However, there was a positive correlation between the *ZFX isoform 3/variant 5* gene expression and tumor size, but not with other clinicopathological features of gastric tumors.

Recently, we demonstrated that *ZFX* (variant 1 and 3, almost exclusively isoform 1) was differentially expressed in tumoral and non-tumoral tissues, in different tumor types (intestinal vs diffuse) and grades (27). Comparing the expression of *ZFX* isoform 3/variant 5 transcript in gastric tissue samples with *ZFX* isoform 1, it appears that these two isoforms expressed differentially in different tumor types, while isoform 1 expression increased significantly in diffuse-type, and *ZFX* isoform 3/variant 5 expression increased in intestinal-type gastric cancer, although not statistically significant.

Pre-mRNA alternative splicing is the flexible point in the control of gene expression in human. This process creates protein isoforms with different, even contradicting, functions from a unique gene. Cancer cells often exploit this process to produce proteins that promote survival and growth (29). Notably, alternative splicing can shift in the expression of a proapoptotic isoform to an antiapoptotic one, and vice versa. This alteration in the balance of isoforms can affect cell survival. For example, the pre-mRNA of Bcl-x can alternatively splice and produce two isoforms: Bclx (s), which promotes apoptosis and Bcl-x (L), which has anti-apoptotic effects (30). Furthermore, alternative splicing may affect other tumor characteristics. For instance, Gunthert *et al* showed that two variants of CD44 express exclusively in a metastasizing pancreatic carcinoma cell line, but not in the parental tumor (31). Moreover, overexpression of serine/arginine-rich protein SF2/ASF can alter the ratios between isoforms of key regulators of cell growth and results in the transformation and tumor formation (32). Collectively, our results may provide a preliminary clue to the function of various ZFX isoforms/variants during tumorigenesis that elucidation of the precise molecular mechanisms governed by the ZFX isoforms/variants needs further investigation.

Conclusion

This report shows that the expression of *ZFX* isoform 3/variant 5 transcript was heterogeneous in gastric specimens. Furthermore, there was no significant association between *ZFX* isoform 3/variant 5 expression and most of clinicopathological features of gastric tumors except a positive correlation with tumor size. Taken together, our results call for further studies to precisely define the role of various *ZFX* isoforms/variants during tumorigenesis.

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