Original Article

Expression of ZFX gene correlated with the central features of the neoplastic phenotype in human brain tumors with distinct phenotypes

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Abstract

Background: The zinc finger transcription factor zinc finger protein, X-linked (*ZFX*) acts as an important director of self-renewal in several stem cell types. Moreover, *ZFX* expression abnormally increases in various cancers and relates to tumor grade. We performed this study, to examine its role in the pathogenesis of astrocytoma and meningioma.

Materials and Methods: We used real-time reverse transcription polymerase chain reaction method for evaluation of *ZFX* expression in 25 astrocytoma tumoral tissue and 25 meningioma tumoral tissues with different WHO grades. Furthermore, the association of gene expression with various clinic-pathological characteristics was examined.

Results: We found that there is a significant association between gene expression and different tumor grades, the presence or absence of invasion, forming and nonforming of glomeruloid vessels, the age over or under 50 and the presence or absence of calcification in astrocytomas. This is the first report that shows that *ZFX* was directly correlated with the central features of the neoplastic phenotype, including the growth of cancer cells, angiogenesis, and invasion.

Conclusion: Regarding all the above-mentioned studies, it is highly plausible that silencing the expression of *ZFX* gene in gliomas has a major role in the therapeutic interventions of the disease in future.

Key Words: Astrocytoma, brain tumor, expression, meningioma, zinc finger protein, X-linked

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INTRODUCTION

Zinc finger protein, X-linked (*ZFX*) is a zinc finger transcription factor that is very conserved in vertebrates. *ZFX*, a key factor that controls the self-renewal of stem cells[1] and it's gene is situated on the mammalian X chromosome.[2] Studies in mouse embryonic and adult hematopoietic stem cells showed that *ZFX* acts as a transcriptional regulator for self-renewal of both stem cell types. Furthermore, to balance between self-renewal and differentiation in human embryonic stem cells, *ZFX* plays a role as a molecular rheostat.[3] Recently, it has been shown that *ZFX* is up-regulated in cancer stem-like cells in esophageal carcinoma cell lines.[4] Furthermore,

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ZFX is overexpressed in gastric[5] and prostate adenocarcinoma,[6] diffuse large B-cell lymphoma, follicular lymphoma,[7] and glioma tissues and cell line.[8,9]

Neuroepithelial and other tissues in the brain are the source of a variety of tumors. Brain tumors are the growth of abnormal cells in the tissues of the brain ("primary brain tumors") or from cancer cells that have metastasized from other organs or tissues ("secondary brain tumors"). The terms of "grade" is used to describe the most primary brain tumors. Low-grade tumors grow slowly and frequently remain dormant for long-time while high-grade tumors show the rapid ability of growth and spread.[10,11] Meningiomas and astrocytomas are two common types of human brain tumors. Meningioma tumors are usually benign in nature, whereas astrocytoma tumors are more malignant. Meningiomas account for about 20% of all primary intracranial tumors.[12] Glioblastoma multiforme (GBM) is the grade IV of astrocytomas and second only to meningioma as the most common brain tumor. However, it is considered as the most frequent malignant primitive brain tumors. Fifty-two percent of all functional tissue brain tumors and 20% of all intracranial tumors are glioblastoma.[13]

Cancer stem-like cells have been characterized in gliomas which play a role in the origin and progression of the disease. The cancer stem cell (CSC) theory states that gliomas are maintained and repopulated by a small subpopulation of self-renewing stem cells within each tumor. Tumors enriched in the CSC subpopulations exhibit greater self-renewal capacity, as well as angiogenesis, aggressiveness and resistance to radiation. Moreover, non-CSCs can also develop into CSCs if the right genetic alterations occur.[14]

Due to the crucial role of the *ZFX* gene in stem cell self-renewal and carcinogenesis, and lack of evidence concerning its expression in brain tumors with different origins, we evaluated its expression in 25 tumoral meningioma and 25 astrocytoma tissue samples by using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Patients and samples

Totally, 25 astrocytoma samples and 25 meningioma samples were examined for *ZFX* gene expression. The tumoral tissue samples were obtained from Alzahra Hospital (Isfahan, Iran). The experimental procedures were approved by the Ethics Committee of Isfahan University of Medical Sciences. Prior to participation, the informed consents were obtained from patients. All samples were verified by pathological analyses and classified according to the WHO classification standard.

RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA concentration determined by spectrophotometer and then samples stored at –80°C. cDNA was synthesized using RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania).

Primers and reverse transcription polymerase chain reaction procedure

Quantitative real-time RT-PCR was performed using specific primers for *ZFX* messenger RNA (mRNA). Furthermore, GUSB gene considered as a house-keeping gene.[15] The sequences of primers used for RT-PCR of *ZFX* and GUSB mRNA were, respectively, as follows: (1) Forward primer, 5'-TGG GCA GCA GCT TAT GGT AAT-3', and reverse primer, 5'-TGT TTA GCC AGT CTG CCG AG-3'; (2) forward primer, 5'-CAC GAC ACC CAC CAC CTA CAT C-3', and reverse primer, 5'-GAC GCA CTT CCA ACT TGA ACA G-3'. The SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (Takara, Tehran, Iran) was used according to protocol of manufacture and the reactions were performed in the Rotor-gene 6000 (Qiagen, Hilden, Germany). The PCR cycling conditions for the genes included an initial denaturation step at 95°C for 5 min, followed by 45 amplification cycles consisting of denaturation at 94°C for 40 s, annealing at 60°C for 30 s and an extension at 72°C for 30 s. The identity of PCR products was further verified on a 1.5% agarose gel.

Moreover, to be ensured that the actual gene of interest (*ZFX*) is getting amplified, some of the PCR products were sequenced (Macrogen, Seoul, Korea).

All measurements were done in at least triplicates and LinRegPCR version 7.5, Biogazelle, the Netherlands, LinRegPCR@amc.uva.nl (software for analysis of real-time PCR data) was used and also REST 2009 (Relative Expression Software Tool V 2.0.13, Biogazelle, the Netherlands, LinRegPCR@amc.uva.nl) were used for the calculation of relative expression. Relative *ZFX* mRNA levels were normalized to GUSB. The comparative Ct (ΔΔCt) method was used to calculate fold changes in gene expression. In addition, the results were statistically analyzed using independent sample *t*-test. The SPSS program version 20, IBM, NY, USA was utilized for statistical analyses and $P < 0.05$ was considered as statistically significant.

RESULTS

Clinical-pathologic variables

We managed to do a case series study on 50 brain tumor samples and the clinical-pathologic variables of patients are listed in Table 1.

Expression of zinc finger protein, X-linked gene in samples

Electrophoresis of the PCR products on agarose gel demonstrated single band with the expected size for the *ZFX* (110 bp) and GUSB (121 bp) transcripts. Analysis of gene expression using real-time PCR showed a unique melting curve without primer dimers for each of the examined genes. Further verification of the *ZFX* PCR product by sequencing demonstrated that *ZFX* transcript amplified specifically and its BLAST against human transcripts showed 100% identity with all five variants of *ZFX* transcript.

The relationship between *ZFX* gene expression with different grades, gender, age, tumor size, mitosis, necrosis, invasion, angiogenesis, and the gelomeruloeid formation were investigated.

Table 1: A brief description of patients with astrocytoma and meningioma

Characteristics	Number (%)*	
	Astrocytoma	Meningioma
Sex		
Male	16 (64)	9 (36)
Female	9 (36)	16 (64)
Grade		
Low grade (WHO grade I and II)	6 (24)	22 (88)
High grade (WHO grade III and IV)	19 (76)	3 (12)
Mitosis		
Positive	9 (36)	4 (16)
Negative	16 (64)	21 (84)
Necrosis		
Positive	14 (56)	4 (16)
Negative	11 (44)	21 (84)
Invasion		
Positive	2 (8)	8 (32)
Negative	23 (92)	17 (68)
Size		
≥5 cm^3	15 (60)	22 (88)
<5 cm^3	10 (40)	3 (12)
Age		
≥50 years	12 (48)	20 (80)
<50 years	13 (52)	5 (20)
Glomeruloid vessel formation		
Positive	13 (52)	NA
Negative	12 (48)	
Calcification		
Positive	2 (8)	NA
Negative	23 (92)	

*Values in parentheses are percentages. NA: Not available

There was a significant association between gene expression and different tumor grades (P value: 8×10^{-3}) [Figure 1a], invasion (P value: 1×10^{-3}) [Figure 1b], glomeruloid vessel formation (P value: 2×10^{-3}) [Figure 1c], age (P value: 1×10^{-3}) [Figure 1d], and calcification (P value: 1×10^{-3}) of astrocytomas [Figure 1e]. However, there was no significant association between *ZFX* gene expression and tumor size (P value: 5.9×10^{-2}) [Figure 1f], presence or absence of mitosis (P value: 1.23×10^{-2}) [Figure 1g], and presence or absence of necrosis (P value: 6.42×10^{-2}) in astrocytoma tumors [Figure 1h].

There was a significant association between gene expression and different tumor grades (P value: 28×10^{-3}) [Figure 2a] and gender (P value: 19×10^{-3}) in meningioma samples [Figure 2c]. Furthermore, there was no significant association between gene expression and tumor size (P value: 821×10^{-3}) [Figure 2b] and age (P value: 56×10^{-3}) [Figure 2d] in meningiomas.

Average gene expression was different between astrocytoma and meningioma. Expression of *ZFX* gene in astrocytoma was significantly greater than those in meningioma (P value: 44×10^{-3}) [Figure 3].

DISCUSSION

Zinc finger protein, X-linked has an important function in the tumorigenesis.[16] In hepatocellular carcinoma, overexpression of *ZFX* confers self-renewal and chemoresistance properties.[17] Furthermore, *ZFX* acts as an oncogene in the malignant proliferation process in osteosarcoma.[18] Moreover, *ZFX* overexpresses abnormally in the prostate,[19] breast[20] and gastric[5,21] adenocarcinomas, nonsmall cell lung cancer[22] and glioma.[8] In summary, *ZFX* promotes the growth and migration of cancer cells,[23] regulates cancer cells proliferation and survival[9] and plays an important role in cell cycle progression.[22]

In this study, we found that there was a direct correlation between *ZFX* gene expression and important clinico-pathological features in astrocytomas including Grades, invasion, glomeruloid vessels, age and an inverse correlation with calcification. Furthermore, performing *ZFX* gene expression analysis in meningioma for the 1st time showed that there is an inverse correlation between the gene expression and different tumor grades. Of note, *ZFX* increased significantly in females affected with meningioma.

Zhou *et al*. reported that *ZFX* gene expression increases in glioma tissues compared with noncancerous brain tissues.[8] In the same vein, our results are consistent with their results. However, the primers designed in

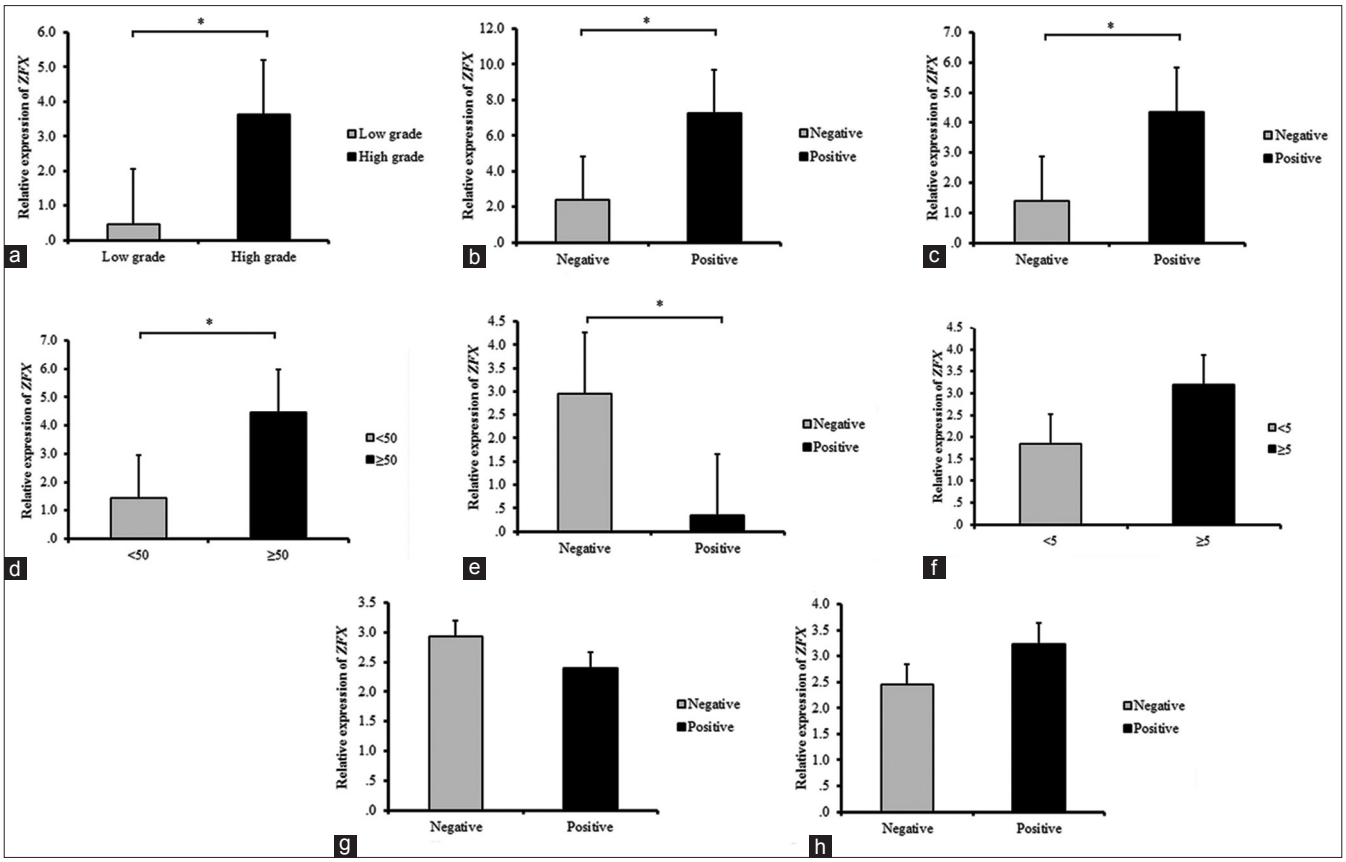

Figure 1: The relative expression of zinc finger protein, X-linked (*ZFX*) in astrocytoma tissue samples. Charts demonstrating the association of relative E expression of *ZFX* with various clinico-pathological features including. (a) Different tumor grades, (b) the presence or absence of invasion, (c) the presence or absence of glomeruloid vessles, (d) age, (e) the presence or absence of calcification, (f) tumor size (g) the presence or absence of mitosis, and (h) the presence or absence of necrosis. *Statistical significant differences

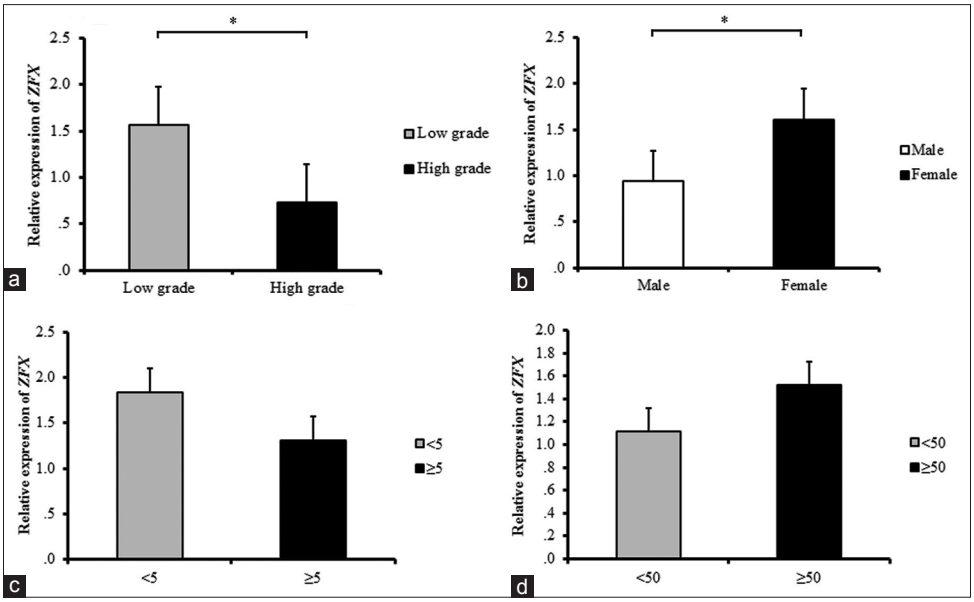

Figure 2: The relative expression of zinc finger protein, X-linked (*ZFX*) in meningioma tissue samples. Charts demonstrating the association of relative E expression of *ZFX* with various clinico-pathological features including. (a) Different tumor grades, (b) gender, (c) tumor size, and (d) age. *Statistical significant differences

that study did not specifically and uniquely recognize the five variants of *ZFX* gene. Their primers recognize the three variants of ZFY gene as well. Recently, Zhu *et al*. showed that *ZFX* upregulates in human gliomas

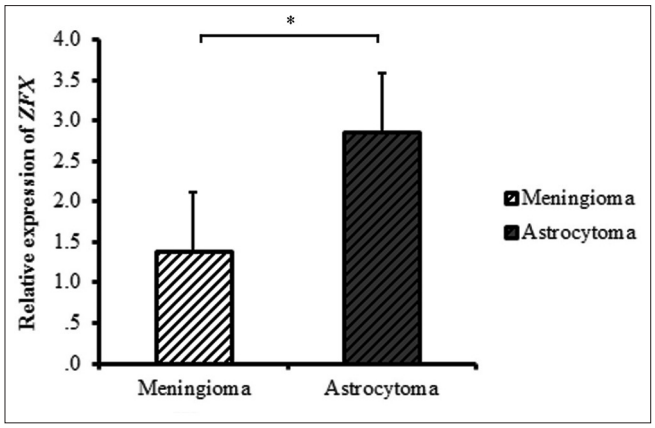

Figure 3: The relative expression of zinc finger protein, X-linked in astrocytoma and meningioma tissue samples. *Statistical significant differences

in which *ZFX* protein were positively correlated with human glioma grades. Altogether, these results are in accord with each other and show that at both transcriptional and translational levels, *ZFX* positively correlated with glioma grades.

Regarding microarray data from Oncomine database, Nutt *et al*. have been studied the expression of 12,000 genes in a series of 50 glioma samples, 28 glioblastoma samples and 22 anaplastic oligodendroglioma samples by microarray analysis. Their results showed that *ZFX* expression increases significantly in glioblastoma.[24] Furthermore, Kotliarov *et al*. used microarray analysis for a more detailed examination of the glioma genome in a large number of primary tumor samples, including 33 samples of astrocytoma, 82 glioblastoma samples, 52 oligodendroglioma samples and 11 oligoastrocytoma samples. They also found that *ZFX* overexpresses in glioblastoma samples.[25] Collectively, these data are consistent with each other revealing that *ZFX* overexpresses in glioblastoma samples.

In this study, we found that there was an inverse correlation between the *ZFX* gene expression and calcification. Calcification is largely a sign of slow growth in gliomas.[26,27] Furthermore, the incidence of calcification decreases in the spectrum from low-grade to high-grade astrocytoma (GBM). In the same vein, our results indicated that there is a direct relationship between the increased expression of *ZFX* gene and the invasion in gliomas. Regarding the major role of stem cells in GBM invasion and the role of *ZFX* as a transcriptional regulator for self-renewal of stem cells, it is highly plausible that *ZFX* contributes to the central features of the neoplastic phenotype.[28] Furthermore, our results demonstrated that *ZFX* gene expression positively correlated with the formation of glomeruloid vessels. Glomeruloid bodies are the hallmark of neo-vascularization. Of note, angiogenesis is a hallmark of neoplastic phenotype, leading to the invasion.[29] Therefore, it seems that *ZFX* contributes to the invasion of neoplastic cells, at least in part, by neo-vasculogenesis.

CONCLUSION

This is the first report that shows that *ZFX* was directly correlated with the central features of the neoplastic phenotype, including the growth of cancer cells, angiogenesis, and invasion, in malignant brain tumors. Comprehensively, it is highly plausible that silencing the expression of *ZFX* gene in gliomas has a major role in the therapeutic interventions of the disease in future.

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