Research in Molecular Medicine

Original Article

2016, Vol: 4, Issue: 1, Pages: 45-49

DOI: 10.7508/rmm.2016.01.007

ZFX Overexpression in Breast Cancer Positively Correlates with Metastasis

Mahboube Ganji Arjenaki [1], Modjtaba Emadi-Baygi [1, 2], Hossein Teimori [3*], Maryam Moradi-Chaleshtori [3], Valliollah Mehrzad [4]

[1] Department of Genetics, Faculty of Basic Sciences, Shahrekord University, Shahrekord, Iran.
[2] Research Institute of Biotechnology, Shahrekord University, Shahrekord, Iran.
[3] Cellular and Molecular Research Center, School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran.
[4] Department of Internal Medicine and Division of Hematology, Isfahan University of Medical Sciences, Isfahan, Iran.

Received: 27 Apr 2015

Revised : 18 May 2015

Accepted: 10 Jun 2015

Corresponding Author:
Hossein Teimori
Cellular and Molecular Research Center, School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran.
Phone: +9838-33331471
E-mail: hosseintimm@yahoo.com

Abstract

Background: As the third most frequent cause of cancer death, breast cancer is a common disease worldwide. Most of the patients are being diagnosed in the stage that conventional treatments are not effective, and invasion and metastases lead to death. Therefore, identification of novel molecular markers to improve early diagnosis, prognosis and treatment of the breast cancer is a necessity. Zinc finger X-linked (ZFX) gene is a member of ZFY family, which they upregulation has been demonstrated in several types of cancer. The aim of this study was to assess ZFX gene expression in Formalin-fixed, paraffin-embedded (FFPE) tissues of the breast cancer invasive ductal carcinoma and to investigate its correlation with clinicopathological parameters.

Materials and Methods: A total of 52 tumor and non-tumor breast specimens were evaluated for ZFX gene expression using quantitative real-time RT-PCR. Total RNA extraction was performed using RNeasy FFPE kit (Qiagene). complementary DNA (cDNA) synthesis was performed using PrimeScript-RT Master Mix (Takara). The PCR mixture containing SYBR® Premix Ex Taq ™ II (Takara Bio Inc., Otsu, Japan), was run on the Rotor-gene 3000 (Qiagen, Hilden, Germany)

Results: The ZFX expression increased significantly in breast tumor tissues compared with non-tumor breast tissues. We further showed that there was a positive correlation between the ZFX gene expression level and lymphatic invasion.

Conclusion: ZFX might be used as a potential biomarker to monitor breast carcinoma progression. Further studies to determine the mechanism of action of ZFX is needed to unravel the role of this gene in breast cancer pathogenesis.

Keywords: Breast cancer; Gene expression; ZFX; FFPE

Please cite this article as: Ganji Arjenaki M, Emadi-Baygi M, Teimori H, Moradi-Chaleshtori M, Mehrzad V. ZFX Overexpression in Breast Cancer Positively Correlates with Metastasis. Res Mol Med. 2016; 4 (1): 45-49

Introduction

Breast cancer is the second most common cancer and fifth cause of death from cancer (1). Among Iranian women, breast cancer is the fifth leading cause of cancer-related death (2). It is a heterogeneous disease characterized by genetic diversity which causes qualitatively and quantitatively aberrant gene expression that can be grouped into various histopathological subtypes according to the following features: histological type, grade, estrogen receptor and tumoral size. Breast cancer stem cells initiate and sustain tumor growth, increase invasion, and overexpress genes that promote metastasis (3-6). Zfx is a shared transcriptional regulator of embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs) (7) and it is up-regulated in cancer stem-like cells in esophageal carcinoma cell lines (8). Furthermore, ZFX is overexpressed in several types of fresh frozen (FF) malignancies specimens like prostate cancer (9), gastric cancer (10), gliomas (11), diffuse large B-cell and follicular lymphoma (12). FF tissues are not routinely available in clinical practice. Thus, gene-expression profiling methods could not have been applied to all the patients in clinical practice. Formalin-fixed paraffin-embedded (FFPE)

tissues are routinely used for diagnosis of disease because they are stable at room temperature, easily stored and constitute a widely available archive of clinical samples linked to clinical and follow-up databases (13, 14).

FFPE tissues represent an invaluable resource for the validation of differentially expressed genes as novel therapeutic targets or prognostic indicators. These gene-expression profiles are now being emphasized as an important tool for clinical decision on the primary therapy of early breast cancer (15, 16).

In this study, we aimed to quantify ZFX gene-expression in breast FFPE tissue samples using real-time qRT-PCR.

Materials and Methods

Archival FFPE tissue samples

Fifty two breast FFPE tissue blocks (from 2007until 2008) were retrieved from the archives of the Alzahra and Seyd Al-Shohada Hospitals in Isfahan.

Sample preparation

Three consecutive 10-µm sections were cut from each block, including 26 non-tumor and 26 tumor tissues on a standard microtome (Reichert-Jung Hn40; Leica Instruments) and placed into individual 1.5-mL Microcentrifuge tubes for extraction.

Deparaffinization Method

Prior to nucleic acid purification from FFPE samples, paraffin must be removed to enable exposure of the sample to proteinase K. FFPE sections deparaffinized with heptan and methanol in accordance with manufacturer's instruction for the RNeasy FFPE kit (Qiagen, Hilden, Germany).

Total RNA isolation and cDNA synthesis

Extraction of total cellular RNA from tumor and adjacent non-tumor tissue specimens was performed using the RNeasy FFPE kit (Qiagen), according to the manufacturer's instruction (Qiagen, Hilden, Germany).

RNA was used for complementary DNA (cDNA) synthesis using PrimeScript - RT Master Mix (Takara Bio Inc., Otsu, Japan) and random hexamer primers.

Quantitative real-time PCR

Quantification of ZFX gene expression was performed by quantitative real-time RT-PCR. Specific primers to amplify the ZFX gene were as follows: 5′-TGTTGCTGAAATCGCTGACG-3′ and 5′-CATTGTCATCCATTTGCTGCT-3′. Primers for glucuronidase, beta (GUSB), as a reference gene, was described elsewhere (17). The PCR mixture containing SYBR® Premix Ex Taq ™ II (Takara Bio Inc., Otsu, Japan), was run on the Rotor-gene 3000 (Qiagen, Hilden, Germany). The conditions of the PCR amplification included an initial denaturation at 95 ℃ for 10 min, followed by 40 amplification cycles consisting of denaturation at 95 ℃ for 30 s, annealing for 30 s at 55 ℃ and 60℃ for ZFX and GUSB genes, respectively; and finally an extension at 72 ℃ for 30 s. For each sample, measurements were taken at least in duplicate. The identity of PCR fragments was further confirmed by agarose gel electrophoresis. The $2^{-\Delta\Delta c}$ method was used for relative gene expression analysis (18).

Statistical analyses

Statistical analyses were performed using SPSS version 16.0. T test was applied for the analysis of the differences between groups. A p value of <0.05 was considered statistically significant.

Results

Optimization of PCR amplification

In order to gain a specific band for ZFX gene, optimization was done by conventional PCR. Electrophoresis of the PCR product showed a specific band on agarose gel with the expected size (108bp). Furthermore, melting curve analysis of real-time PCR showed that a single product was amplified (data not shown).

Expression of ZFX gene in breast FFPE specimens

To analyze the ZFX gene expression profile in breast tissues, quantitative real-time PCR were carried out in 52 tumor and non-tumor FFPE breast carcinoma tissues using specific primers for both ZFX and GUSB (as internal control) genes. The results of real-time qRT-PCR experiments demonstrated a significant increase of the relative expression of the ZFX gene in tumor tissues compared to non-tumor tissues ($p = 0.007$, Figure 1).

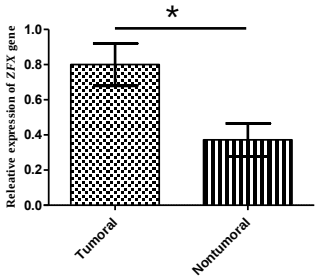

Figure 1. Relative expression of ZFX in breast tumor and non-tumor tissues (mean ± SEM).

Association between ZFX gene expression and clinicopathological parameters

A total of 26 tumor samples were collected from two centers. All the samples had been histologically diagnosed as invasive ductal carcinoma (DIC) and positive estrogen receptor (ER+).
We investigated the correlation between ZFX gene expression and the reported clinicopathological parameters in our samples (Table 1).

Table 1. Relationship between ZFX expression levels and clinicopathological parameters of breast cancer samples.

Characteristics	Numbers	ZFX relative expression (mean ± SEM)	p-value
Age (years)			
≤50	14	0.79± 0.2	0.2
>50	12	0.74 ± 0.15	
Tumor size (cm)			
≤3	16	1.2 ± 0.5	0.1
>3	10	1.3 ± 1.4	
Vein invasion			
Negative	16	0.68± 0.14	0.06
Positive	7	1 ± 0.37	
Lymphatic invasion			
Negative	22	0.1 ± 0.3	0.01
Positive	4	0.9 ± 0.01	

Bold values are statistically significant ($p<0.05$)

Collectively, there was a significant association between ZFX expression and lymphatic and vein invasion.

Discussion

Zinc finger X-linked (ZFX) gene encode transcription factor promotes the transcription of oncogenes. Domains of ZFX proteins are the cause of specific functionality of this protein (19-22).
ZFX protein is the transcriptional regulator that plays an important role in self renewal and differentiation mechanisms in human embryonic and hematopoietic stem cells (7, 23). ZFX has an important role in cell cycle progression and cell growth control (24). Our pilot study showed that the relative expression of ZFX significantly increased in tumor tissues compared to non-tumor ones ($p = 0.007$). Furthermore, the level of ZFX transcript was upregulated in tumors with lymphatic invasion ($p = 0.01$). Moreover, there was a marginal correlation between ZFX expression and vein invasion ($p= 0.06$). However, no significant correlation was found between the expression levels of ZFX and age and tumor size. Taken together, current results indicate that ZFX may play an important role in breast cancer metastasis and survival as there was a significant correlation between ZFX expression level and lymph node invasion and invasive ductal carcinoma specimens. As a result, this gene might be used as a potential prognostic factor in breast cancer survival (25, 26).
Until now, upregulation of ZFX gene has been reported in different types of cancers such as prostate cancer (9), gastric cancer, in which we found a positive correlation between ZFX isoform 3/variant 5 transcript and gastric tumor size (27, 28), and gliomas in which Afzali *et al*. showed a significant correlation between ZFX gene expression and central features of the neoplastic phenotype, including the growth of cancer cells, angiogenesis, and invasion.(11, 29);diffuse large B-cell and follicular lymphoma(12). Furthermore, silencing of ZFX in Hep-2, U251 and PC-3 human cancerous cell lines showed the importance of this gene in the proliferation and apoptosis of the cells (11). Therefore, ZFX plays an important role(s) in various biological and/or pathological processes such as cell growth, survival, differentiation, cell cycle and apoptosis. To our knowledge, the expression profile of ZFX gene in archived breast tissues has not been previously investigated using qRT-PCR. However, Yang *et al*. showed that the expression of ZFX was high in invasive breast cancer specimens using immunohistochemical analysis. Interestingly, they showed that ZFX expression was upregulated in metastatic breast cancer. Moreover, the age, and tumor size had no significant impact on ZFX expression as reported by Yang *et al*.
To further validate our results, we retrieved the corresponding data from the Oncomine cancer profiling database (30). Upregulation of ZFX in tumor samples, especially ductal carcinoma, have been reported in some researches (31-35). Furthermore, many studies showed a correlation between ZFX expression and metastasis (36-38). Taken together, current results are in accordance with microarray analyses indicating that ZFX overexpresses in breast cancer and positively correlates with metastasis. However, since we extracted RNA from FFPE tissues, the procedure of the tissue preparation and their storage may affect the yield of the extracted RNA that in turn influence the amplification and quantification of the genes.

Conclusion

Our results showed that ZFX is overexpressed in invasive ductal carcinoma breast cancer samples, and it positively correlates with lymphatic and vein invasion. As a result, this gene might be used as a potential biomarker for monitoring breast carcinoma progression and survival rate. Further studies to

determine the mechanism of action of ZFX are needed to unravel the role of this gene in breast cancer pathogenesis.

Acknowledgements
We thank the staffs at Cellular and Molecular Research Center in Shahrekord University of Medical Sciences and the Alzahra and Seyd Al-Shohada Hospitals for technical support.

Author contributions
MGA and MEB make substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; All authors participate in drafting the article or revising it critically for important intellectual content; and all authors give final approval of the version to be submitted and any revised version.

Support/Funding
This research is partially funded by the Shahrekord University of Medical Sciences.

Conflict of Interest
Authors declare no conflict of interest.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015 1; 136(5):E359-86. PMID: 25220842

2. Akbari A, Razzaghi Z, Homaee F, Khayamzadeh M, Movahedi M, Akbari ME. Parity and breastfeeding are preventive measures against breast cancer in Iranian women. Breast cancer. 2011; 18(1):51-5. PMID: 20217489.

3. Liu H, Patel MR, Prescher JA, Patsialou A, Qian D, Lin J, *et al*. Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. Proc Natl Acad Sci U S A. 2010; 107(42):18115-20. PMID: 20921380

4. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, *et al*. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001; 98(19):10869-74. PMID: 11553815.

5. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, *et al*. Molecular portraits of human breast tumours. Nature. 2000; 406(6797):747-52. PMID: 10963602.

6. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, *et al*. Molecular definition of breast tumor heterogeneity. Cancer cell. 2007; 11(3):259-73. PMID: 17349583.

7. Galan-Caridad JM, Harel S, Arenzana TL, Hou ZE, Doetsch FK, Mirny LA, *et al*. Zfx controls the self-renewal of embryonic and hematopoietic stem cells. Cell. 2007; 129(2):345-57. PMID: 17448993.

8. Huang D, Gao Q, Guo L, Zhang C, Jiang W, Li H, *et al*. Isolation and identification of cancer stem-like cells in esophageal carcinoma cell lines. Stem Cells Dev. 2009; 18(3):465-73. PMID: 18680391

9. Tricoli JV, Bracken RB. ZFY gene expression and retention in human prostate adenocarcinoma. Genes Chromosomes Cancer. 1993; 6(2):65-72. PMID: 7680890

10. Nikpour P, Emadi-Baygi M, Mohammad-Hashem F, Maracy MR, Haghjooy-Javanmard S. Differential expression of ZFX gene in gastric cancer. J Biosci. 2012; 37(1):85-90. PMID: 22357206

11. Zhou Y, Su Z, Huang Y, Sun T, Chen S, Wu T, *et al*. The Zfx gene is expressed in human gliomas and is important in the proliferation and apoptosis of the human malignant glioma cell line U251. J Exp Clin Cancer Res. 2011; 30:114. PMID: 22185393.

12. Sakhinia E, Glennie C, Hoyland JA, Menasce LP, Brady G, Miller C, *et al*. Clinical quantitation of diagnostic and predictive gene expression levels in follicular and diffuse large B-cell lymphoma by RT-PCR gene expression profiling. Blood. 2007; 109(9):3922-8. PMID: 17255358.

13. Takano EA, Mikeska T, Dobrovic A, Byrne DJ, Fox SB. A multiplex endpoint RT-PCR assay for quality assessment of RNA extracted from formalin-fixed paraffin-embedded tissues. BMC biotechnology. 2010; 10:89. PMID: 21162754

14. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, *et al*. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. The New England journal of medicine. 2004; 351(27):2817-26. PMID: 15591335.

15. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol. 2001; 158(2):419-29. PMID: 11159180

16. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ, *et al*. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Annal Oncol. 2011; 22(8):1736-47. PMID: 21709140

17. Emadi Baygi M, Soheili ZS, Schmitz I, Sameie S, Schulz WA. Snail regulates cell survival and inhibits cellular senescence in human metastatic prostate cancer cell lines. Cell Biol Toxicol. 2010; 26(6):553-67. PMID: 20397042.

18. Livak KJ, Schmittegen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4): 402-8. PMID: 11846609

19. Lai KP, Chen J, He M, Ching AK, Lau C, Lai PB, *et al*. Overexpression of ZFX confers self-renewal and chemoresistance properties in hepatocellular carcinoma. Int J Cancer. 2014; 135(8):1790-9. PMID: 24585547

20. Wu S, Lao XY, Sun TT, Ren LL, Kong X, Wang JL, *et al*. Knockdown of ZFX inhibits gastric cancer cell growth in vitro and in vivo via downregulating the ERK-MAPK pathway. Cancer Lette. 2013; 337(2):293-300. PMID: 23587796.

21. Schneider-Gadicke A, Beer-Romero P, Brown LG, Mardon G, Luoh SW, Page DC. Putative transcription activator with alternative isoforms encoded by human ZFX gene. Nature. 1989; 342(6250):708-11. PMID: 2512506

22. Schneider-Gadicke A, Beer-Romero P, Brown LG, Nussbaum R, Page DC. ZFX has a gene structure similar to ZFY, the putative

human sex determinant, and escapes X inactivation. Cell. 1989; 57(7):1247-58. PMID: 2500252.

23. Harel S, Tu EY, Weisberg S, Esquilin M, Chambers SM, Liu B, *et al*. ZFX controls the self-renewal of human embryonic stem cells. PloS one. 2012; 7(8):e42302. PMID: 22879936.

24. Jiang M, Xu S, Yue W, Zhao X, Zhang L, Zhang C, *et a*l. The role of ZFX in non-small cell lung cancer development. Oncol Res. 2012; 20(4):171-8. PMID: 23461064

25. Oh, Julia L. "Multifocal or multicentric breast cancer: understanding its impact on management and treatment outcomes." Methods of cancer diagnosis, therapy and prognosis. Springer Netherlands, 2008: 583-7.

26. Ustaalioglu BO, Bilici A, Kefeli U, Seker M, Oncel M, Gezen C, *et al*. The importance of multifocal/multicentric tumor on the disease-free survival of breast cancer patients: single center experience. Am J Clin Oncol. 2012; 35(6):580-6. PMID: 21926901

27. Nikpour P, Emadi-Baygi M, Mohammad-Hashem F, Maracy MR, Haghjooy-Javanmard S. Differential expression of ZFX gene in gastric cancer. J Biosci. 2012; 37(1):85-90. PMID: 22357206

28. Rahmati S, Emadi-Baygi M, Nikpour P, Emadi-Andani E. Expression profile of ZFX isoform3/variant 5 in gastric cancer tissues and its association with tumor size. Iran J Basic Med Sci. 2014; 17(10):767-71. PMID: 25729545

29. Afzali A, Emadi-Baygi M, Nikpour P, Nazemroaya F, Kheirollahi M. Expression of ZFX gene correlated with the central features of the neoplastic phenotype in human brain tumors with distinct phenotypes. Adv Biomed Res. 2015; 4:179. PMID: 26605218

30. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, *et al*. ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia. 2004; 6(1):1-6. PMID: 15068665

31.Ma ZS, Wang DW, Sun XB, Shi H, Pang T, Dong GQ, *et al*. Quantitative analysis of 3-Tesla magnetic resonance imaging in the differential diagnosis of breast lesions. Exp and Ther Med. 2015; 9(3):913-8. PMID: 25667653

32. Richardson AK, Currie MJ, Robinson BA, Morrin H, Phung Y, Pearson JF, et al. Cytomegalovirus and Epstein-Barr virus in breast cancer. PloS one. 2015; 10(2):e0118989. PMID: 25723522.

33. Tabchy A, Hennessy BT, Gonzalez-Angulo AM, Bernstam FM, Lu Y, Mills GB. Quantitative proteomic analysis in breast cancer. Drugs Today (Barc). 2011; 47(2):169-82. PMID: 21431104

34. Lu YM, Zhong JB, Wang HY, Yu XF, Li ZQ. The prognostic value of intermedin in patients with breast cancer. Dis Markers. 2015; 2015:862158. PMID: 25694747

35. Zhao H, Morimoto T, Sasa M, Tanaka T, Izumi K. Immunohistochemical expression of uPA, PAI-1, cathepsin D and apoptotic cells in ductal carcinoma in situ of the breast. Breast cancer. 2002; 9(2):118-26. PMID: 12016391

36. Landemaine T, Jackson A, Bellahcene A, Rucci N, Sin S, Abad BM, *et al*. A six-gene signature predicting breast cancer lung metastasis. Cancer Res. 2008; 68(15):6092-9. PMID: 18676831

37. Perou CM, Borresen-Dale AL. Systems biology and genomics of breast cancer. Cold Spring Harbor perspectives in biology. 2011; 3(2). PMID: 21047916

38. Radvanyi F. [Molecular events involved in the metastatic process]. Prog Urol. 2008; 18 Suppl 7:S167-72. PMID: 19070788