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Research Paper

Upregulation of the double-stranded RNA binding protein DGCR8 in invasive ductal breast carcinoma

Hedieh Fardmanesh [a], Mohammad Shekari [a,*], Abolfazl Movafagh [b], Shohreh Alizadeh Shargh [c], Ali Akbar Poursadegh Zonouzi [a], Samira Shakerizadeh [a], Ahmad Poursadegh Zonouzi [d], Asghar Hosseinzadeh [e]

[a] Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[b] Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Medical Laboratory Science Department, Midwifery-Nursing Institute, Islamic Azad University of Chalous Branch, Chalous, Iran
[d] Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[e] Department of Biology ,Tabriz Branch, Islamic Azad University, Tabriz, Iran

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ABSTRACT

High-throughput experimental studies have indicated that the miRNAome is globally downregulated in various types of malignancy, and dysregulation of miRNAs processing component(s) is one possible mechanism for this phenomenon. Despite the progression in identifying cellular functions of Digeorge Syndrome Critical Region 8 (DGCR8) in miRNAs biogenesis, the role of altered expression of DGCR8 in the pathogenesis of invasive ductal breast carcinoma (IDC) has not yet been fully investigated. The objective of the present study was to evaluate DGCR8 mRNA expression in seventy fresh invasive ductal breast carcinomas and matched adjacent non-neoplastic tissues using quantitative real-time PCR and to assess the value of clinicopathological parameters on its expression. Our findings revealed that DGCR8 mRNA expression is upregulated in more than two-thirds of the cancerous specimens (68.66%) when compared to adjacent non-neoplastic tissue. This difference is statistically significant ($P < 0.05$). We found that DGCR8 mRNA levels were increased in the high-grade and metastatic compared with those of both low-grade and non-metastatic. We demonstrated that there is not significant correlation between DGCR8 mRNA expression levels and clinicopathological parameters. In conclusion, our study suggested that upregulation of DGCR8 may be involved in tumorigenesis and aggressiveness of IDC and may serve as future therapeutic target.

1. Introduction

Breast cancer is the most common malignancy among women, accounting for 23% of the total cancer cases (Escalona et al., 2010; Salimi et al., 2012). According to Cancer Facts and Figures, by the American Cancer Society, in 2015, 231,840 estimated new cases of breast cancer will be diagnosed, and 40,290 deaths will occur. (Siegel et al., 2015). In spite of remarkable advances in treatment strategies, breast cancer still ranks 2nd as the leading cause of cancer-related deaths in women in the United State (Siegel et al., 2015). Up to now, widespread researches have been conducted to disclose the molecular mechanisms involved in pathogenesis of breast cancer. However, most puzzling facets of breast cancer are still remained enigmatic.

Over the last decade, microRNAs (miRNAs), a class of endogenous, non-coding small RNAs, has been considered as an interesting issue in cancer researches (Iorio and Croce, 2012). MiRNAs orchestrate gene expression through degradation of the target mRNA or translation repression at the post-transcriptional level (Lu and Clark, 2012). Transcripts which are targeted by miRNAs play a fundamental role in various cellular processes such as proliferation, cell differentiation and programmed cell death (Mulrane et al., 2013). So, dysregulation of miRNAs expression contributes to development and progression of various human malignancies. Globally downregulation of miRNAome has been documented in various types of cancer using high-throughput profiling techniques (Melo and Esteller, 2011; Jansson and Lund, 2012). Dysregulation of miRNA biogenesis machinery components, single nucleotide polymorphisms and epigenetic changes have been suggested as possible molecular causes of miRNAome global downregulation (Cochrane et al., 2010). The miRNA genes initially are transcripted into the pri-miRNAs by RNA polymerase II (Melo and Esteller, 2011). To form pre-miRNAs, pri-miRNAs are partially processed in the nucleus by the microprocessor machinery, composed of Drosha and DGCR8, and then the pre-miRNA transferred into cytoplasm by exportin 5/RanGTP complex (Jansson and Lund, 2012; Iorio and Croce, 2012). In cytoplasm,

Abbreviations: miRNA, MicroRNA; DGCR8, DiGeorge Syndrome Critical Region 8; IDC, Invasive Ductal Breast Carcinoma; TRBP, Transactivating Response RNA Binding Protein; dsRBDs, double-stranded RNA Binding Domains; cDNA, complementary DNA; PCR, Polymerase Chain Reaction; mRNA, Messenger RNA; 5′ UTR, 5′ Untranslated Region; HER2, Human Epidermal Growth Factor Receptor 2; CEA, Carcinoembryonic Antigen; BMI, Body Mass Index.

* Corresponding author.
E-mail address: mshekari_ch@yahoo.com (M. Shekari).

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Dicer processes pre-miRNA to generate the ~22 nt mature miRNAs through composition with transactivating response RNA binding protein (TRBP) (Melo and Esteller, 2011; Iorio and Croce, 2012). DGCR8 is an essential miRNA processing factor and stabilizes the Drosha protein through protein–protein interaction (Han et al., 2006). DGCR8 contains two double-stranded RNA binding domains (dsRBD) which act as a "molecular ruler" by recognizing the cleavage site within the pri-miRNA (Han et al., 2006; Grund et al., 2012). Moreover, Herbert et al. recently suggested that DGCR8 acts as a critical link between extracellular proliferative cues and reprogramming of the cellular miRNA profile (Herbert et al., 2013). Knocking out of DGCR8 gene in mice disrupts proliferation and differentiation of embryonic stem cells and leads to embryonic lethality (Wang et al., 2007). Similarly, it has been demonstrated that DGCR8 plays an important function in development of vascular, neural and cardiac cells by regulating the apoptosis and differentiation of them, and DGCR8 deficiency results in malformations in the affected organs (Fénelon et al., 2011; Chen et al., 2012; Chapnik et al., 2012).

Altered expression of DGCR8 has been reported in several malignancies such as colorectal (Kim et al., 2014), skin (Sand et al., 2012), gastric (Jafari et al., 2013) and breast (Kwon et al., 2014) cancers. However, DGCR8 mRNA expression in IDC has been poorly understood, and it raises the question whether the DGCR8 dysregulation could be involved in the pathogenesis of IDC. Since deciphering of the link between DGCR8 dysregulation and cancer, will likely provide insight of cancer controls' mechanisms, so we investigated the mRNA levels of DGCR8 in IDC and matched tumor-adjacent normal tissues in the present study.

2. Materials and methods

2.1. Sample preparation

Specimens were collected from seventy patients diagnosed with IDC (a set of 70 paired tissues, cancerous versus tumor-adjacent normal tissue) undergoing radical surgery at the Nour-nejat hospital, Tabriz, Iran during 2013–2015. The mean age of patients was 47.27 ± 8.09 years (ranging between 33 and 71 years). Patients who received any neoadjuvant chemotherapy or radiotherapy before surgery were excluded from the study. The histological grade, tumor size and lymph node metastasis of all resected samples were determined by histopathological analysis. The present study was approved by the Ethics and Human Rights Committee of Tabriz University of medical sciences, Tabriz, Iran and the informed consents were filled out by all participants.

2.2. RNA extraction and cDNA synthesis

All tissue samples were stored in RNAlater solution (Qiagen, Germany) at −80 °C until RNA extraction. Total RNA was isolated from tissue samples using Tri-Pure® Isolation Reagent (Roche, Germany) and RNA yield was treated with RNase-free DNase I (Thermo Scientific, USA), according to the manufacturer's instructions. The quality and quantity of extracted RNA were confirmed by agarose gel electrophoresis and NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA), respectively. Subsequently, total RNA (1 μg) was reversely transcribed to cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) with random hexamer primer, following the manufacturer's protocol.

2.3. Quantitative real-time –PCR

Real-time PCR was conducted in triplicate using gene-specific primers and Syber Green-I dye in AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer, Korea) by the Rotor-Gene™ 6000 system (Corbett Research, Australia) according to the manufacturer's instructions. The primer pairs used for DGCR8 were 5′-CAAGCAGGAGACATCGGACAAG-3′ and 5′ -CACAATGGACATCTTGGGCTTC-3′, and for β-actin 5′-CAGCCA TGTACGTTGCTATCCAGG-3′ and 5′-AGGTCCAGACGCAGGATGGCATG-3′. Each reaction mixture contained 100 ng cDNA, master mix 2X, ROX dye 50×, and 10 pmol of each primer pairs for DGCR8 and β-actin in a final volume of 25 μl. Initial denaturation at 94 °C for 5 min was followed by 50 cycles (for DGCR8) and 35 cycles (for β-actin) of denaturation at 94 °C for 10 s, annealing temperatures at (61 °C for DGCR8 and 59 °C for β-actin) for 15 s, extension at 72 °C for 20 s. Standard curves were plotted using serially diluted cDNA and the expression levels of DGCR8 in the samples were normalized through the mean expression of the house keeping gene β-actin. We also used no template negative control.

2.4. Statistical analysis

Analysis of the distribution of DGCR8 expression levels around cutoff points using Kolmogorov–Smirnov test showed that the data were not normally distributed and thus, non-parametric statistical tests were used to compare the data. Two-tailed Mann–Whitney U test was used to compare the expression levels of DGCR8 between the cancerous and tumor-adjacent normal tissues. The associations between various clinicopathological characteristics and the expression levels of DGCR8 were analyzed through one way ANOVA and Fisher's exact tests. Spearman's correlation test was used to analyze the correlation between DGCR8 expression and clinicopathological parameters. Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Expression of DGCR8 in cancerous and tumor-adjacent normal tissues

We used quantitative real-time PCR procedure to evaluate the mRNA expression levels of DGCR8 in seventy cancerous and tumor-adjacent normal tissues and the ratio of DGCR8 mRNA expression analyzed by the $2^{-\Delta\Delta ct}$ method. Initial analysis showed that samples could be divided into two groups with low and high expression of DGCR8 when the cut-off value was set at 0.0 of the $\log_2$ ratio of DGCR8 expression (Table 1). The mRNA expression levels of DGCR8 varied among samples and 68.66% of cancerous tissues had a significantly upregulated expression when compared to tumor-adjacent normal tissues ($P < 0.05$) (Table 1) (Fig. 1).

3.2. Relation between DGCR8 expression level and clinicopathological parameters

To determine the influence of the clinical parameters on expression levels of DGCR8, patients were subdivided according to each clinicopathological features (Table 1). With regard to age of patients, DGCR8 upregulated in 69.77% (≤50 years) and 66.67% (>50 years) of patients (Table 1). The mean of DGCR8 mRNA expression level in ≤50 years patients was higher than >50 years (Fig. 2a); however, this difference was not statistically significant ($P = 0.995$) (Table 1). Regarding histopathological grade, upregulation of DGCR8 expression was observed in 63.64% (grade I), 71.43% (grade II) and 66.67% (grade III) (Table 1). Comparison of DGCR8 expression between different histological grades using one way ANOVA test showed that the mean expression of DGCR8 in patients with high grade (II, III) were higher than low grade (I), however, there was no significant differences among patients considering to grade ($P = 0.711$) (Table 1) (Fig. 2b). Considering tumor size, DGCR8 expression was upregulated in 64.29% (≤2 cm), 71.06% (2–5 cm) and 66.67% (>5 cm) of samples. The DGCR8 expression between different tumor sizes was compared using one way ANOVA test. The mean expression of DGCR8 in cancerous samples with larger size was higher than samples with smaller size, but this difference was not statistically significant ($P = 0.619$) (Table 1) (Fig. 2c). In addition, upregulation of DGCR8