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RESEARCH

Dysregulated Expression of Dicer and Drosha in Breast Cancer

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Abstract Large-scale profiling approaches have revealed global down-regulation of microRNAs (miRNAs) in several human cancer types including breast cancer. Altered expression of Dicer and Drosha, two key enzymes in the miRNA maturation, is believed to be one of the most important mechanisms. By using quantitative real-time RT-PCR (QT-PCR), we examined the expression of Dicer and Drosha in 49 pairs of matched human breast cancer tissues. Decreased expression was observed in 53.1% (Dicer), 51.9% (Drosha) and 75.5% (Dicer plus Drosha) breast cancer tissues. In conclusion, the decreased expression of Dicer and Drosha may play a role in down-regulation of miRNAs in breast cancer.

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Introduction

Breast cancer represents the most common cancer in women with more than 1,000,000 new cases and approximately 373,000 deaths each year in the world [1, 2]. With ever-improving chemotherapy, radiation, hormonal treatments, as well as targeted therapy, an improvement in overall survival has been observed. However, the treatment of breast cancer is currently far from being optimal with patients suffering from recurrence and distant metastases [3, 4]. The better understanding of the mechanism of genesis and procession of breast cancer will contribute to the optimal treatment of the disease.

Breast cancer is a complex genetic disease in which oncogene amplification and/or tumor suppressor gene mutation lead to step-wise deregulation of cell proliferation and death. Rapidly accumulating evidence has revealed that miRNA dysregulation is closely associated with development and progression of a variety of cancers including breast cancer [5, 6]. In most cases, down-regulation of miRNAs is observed frequently in cancer samples as revealed by large-scale expression profile analysis [7–9], however, the underlying mechanisms were poorly understood, to which more and more oncologists have paid their attention.

Generally the biogenesis of miRNAs can be divided into two steps: transcriptional and post-transcriptional steps. Most miRNA genes are firstly transcripted into the pri-miRNAs which were subsequently cleaved by Drosha endonuclease the RNAase III family to produce precursor miRNAs (pre-miRNAs) in nuclear, pre-miRNAs can be further cleaved by Dicer, another type III endonuclease, to

generate mature miRNAs following being transported into cytoplasm by exportin5 [10]. Contribution of transcription alteration to changed miRNA's expression in cancers had been extensively addressed, while the roles of post-transcriptional regulation in aberrant expression of miRNA were much less explored [11]. Since Drosha and Dicer are two critical enzymes required for post-transcriptional miRNA processing, we investigate here whether the altered expressions of Dicer and Drosha are associated with the development of breast cancer .

Materials and Methods

Study Population

This study was conducted at Henan cancer hospital, Zhengzhou, Henan province, China. The protocols were approved by the Institutional Review Board of Henan tumor hospital. Tissue samples came from 49 patients who underwent radical surgery for primary breast cancer at the Breast diseases Center on 2009. All the samples were collected in compliance with the local ethics regulations with fully informed patient consent. The pathological diagnoses were performed by the specialists of the hospital. The patients enrolled in this study were with operable breast cancer without primary therapy before surgery. The median age of patients at surgery was 52 (range 18–84) years-old. Most patients were pre menopausal and with hormone receptor positive and Her-2 negative ductal invasive carcinoma (Table 1).

RNA and cDNA Preparation

Tissue samples were stored at −80°C until RNA isolation. Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, California, USA) based on the suggested protocol. RNA quality was assessed by 1% agarose gel electrophoresis in the presence of ethidium bromide. Reverse transcription PCR (RT-PCR) was performed as described [12] using the mRNA selective PCR kit (DRR025A, TaKaRa, Dalian, China) according to the manufacturer's instruction. Briefly, 2 μg total RNA was reversely transcribed by AMV reverse transcriptase XL with the oligo dT primer.

Quantitative Real-Time RT-PCR

The QT-PCR was performed as described [13] by using LightCycler system (Roche) with the FastStart DNA Master

Table 1 Patients characteristics of the study cohort (N=49)

Median age	52		
Age range	(18–84)		
Menopausal status	pre	36 (73.5%)	
	Post	13 (26.5%)	
pT Histologicaltype:	Ductal	39 (79.6%)	
	Lobular	2 (4.1%)	
	Mixed	3 (6.1%)	
	Others	5(10.2%)	
Histological grade(SBR)	1		10(20.4%)
	2		31 (63.3%)
	3		8 (16.3%)
N status	N 0		23 (46.9%)
	N1-3 N		13 (26.5%)
	≥4		13(26.5%)
Estrogen receptor	ER-(<10%)		19 (38.8%)
(%:marked cells)	ER+(≥10%)		30(61.2%)
Progesterone receptor	PR- (< 10%)		16 (32.7%)
(%: marked cells)	PR+(≥ 10%)		33 (67.3%)
HER2 status	-		35(71.4%)
	+		14 (28.6%)
Cancer subtype	Luminal A		28(57.1%)
	Luminal B		6 (12.2%)
	Basal-like		8 (16.3%)
	HER2+		7(14.3%)

SYBR Green. Primer pairs were used as following: Dicer (5′-AGCCCCAGCCCAGCGATGAA-3′, ′-GTCCAG GATTGGGGCCAAGAGTCC-3′); Drosha (5′-AG CCCTGGTGCCTGAGGAGGAGAT-3′, 5′-TGC AGGGCGTATCCCAAAGTGGAC-3′); Hprt (5′-AGGC CATCACATTGTAGCCCTCTGT-3′, 5′-TACTGCCTGAC CAAGGAAAGCAAAGT-3′).The standard curve method of quantification was used to calculate the expression of target genes relative to the housekeeping gene Hprt. The final mRNA levels were converted to ratios of decreased expression (≤1) or increased expression (>1) relative to levels of Dicer and Drosha mRNA in normal breast tissues. Each reaction was performed in triplicate and was repeated in at least three separate experiments. To rule out DNA contamination in the RNA preparations, the QT-PCR controls were performed with RNA templates which did not show any amplification.

Statistical Analysis

All values are reported as means±SD. Differences were assessed by two tailed Student t test. $P<0.05$ was considered to be statistically significant. Because our Dicer and Drosha Ratio data didn't follow normal distribution, we take the median of ratios as cut-off value. Spearman method was used for correlation analysis. All analysis was performed by using SPSS software (v11.0).

Results

The Specificity of QT-PCR

Because we use SYBR green dye incorporation methods for QT-PCR, it's very important to select optimal primer pairs for QT-PCR of Dicer and Drosha as well as Hprt as internal control. To achieve this, we designed at least two primer pairs for each gene, only primer pairs by which amplified products display single unique peak in melting curve analysis were chosen for further experiments. As a result, we got ideal primer pairs for all three genes, and representative results for each reaction were shown in Fig. 1, both of the melting temperatures for Dicer and Drosha are peaked closing to 85, while melting temperature for Hprt is peaked around 89.

Expression of Dicer and Drosha in Breast Cancer

We examined the mRNA expression level of Dicer and Drosha in 49 pairs of breast cancer tissue and their respective non-cancerous breast tissues by using qRT-PCR and calculated the ratios between cancerous and non-cancerous tissues. The distribution of Dicer and Drosha mRNA level in the cancerous tissues was not statistically normal Thus we use 1.8467 (median) as the cut off value for high and low Dicer and 1.0493 (median) for Drosha mRNA levels in subsequent analyses.

Levels of mRNA varied among the cancerous tissues from different patients. 53.1% had decreased Dicer mRNA and 51.0% had decreased Drosha mRNA. In 26.5% of the specimens, there were decreased levels of both Dicer and Drosha mRNA. For 77.6% of the specimens the decreased level of Dicer or Drosha was observed. The median ratio of Dicer expression in cancer tissues with decreased Dicer mRNA levels was 0.5325 (range, 0.1293 to 1.7583) and in those with decreased Drosha mRNA levels was 0.4307 (range, 0.157 to 0.911). Specimens with increased mRNA levels had a median ratio for Dicer of 7.1093 (range, 2.1417 to 81.9157) and a median ratio for Drosha of 4.754 (range, 1.2357 to 23.6367).

As reported in recent study that 1.0 was used as the cut-off value for high and low Dicer and Drosha mRNA levels in ovarian cancer. With 1.0 as the cut-off value 42.9% had decreased Dicer mRNA and 75.5% had decreased Dicer or Drosha mRNA.

Clinical Associations

Table 1 lists the baseline characteristics of all 49 patients. Up to now no disease progression or death was observed although the follow up was performed every 3 months. The data about the DFS (Disease free survival) and OS (Overall survival) are unavailable now. The associations between Dicer or Drosha mRNA levels and clinical characteristics such as hormonal receptor status and Her-2 status were investigated. We performed an univariate analysis, and found that neither Dicer nor Drosha mRNA levels were significantly associated with age, tumor grade, hormonal receptor status, Her-2 status or subtype in our breast tumors (Table 2).

Discussion

MiRNAs are functional RNA molecules that are transcribed from the DNA sequence of RNA genes, but not translated into protein. Several hundred genes in our genome have been shown to encode miRNAs. Using computational target predictions, Lewis et al. have shown that 5,300 human genes, or 30% of the human gene set, are implicated as miRNA targets, making miRNA one of the most abundant classes of regulatory genes in humans [14]. Given dysregulation in a variety of cancer samples [7], miRNAs have been implicated in the causation of human cancers extensively.

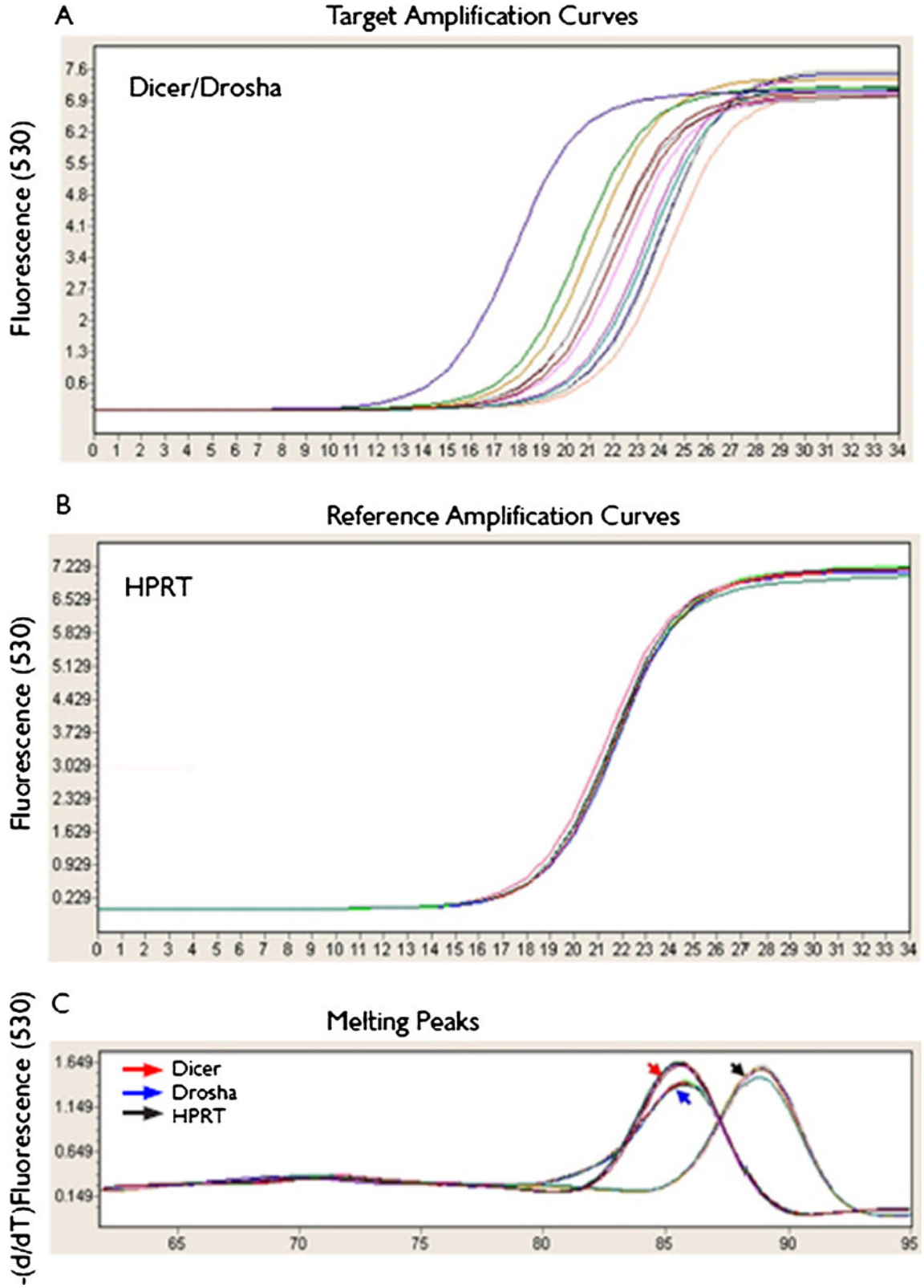

Fig. 1 Amplification and melting curves of Dicer, Drosha and Hprt products. The representative pictures of the amplification curves over 40 cycles for Dicer/Drosha (A), and Hprt (B), and the melting curves for corresponding products (C)

MiRNA expression is regulated at transcriptional and post-transcriptional levels like the regulation of protein expression [10, 11]. During the maturation of miRNAs, Dicer and Drosha are important elements and involved in

Table 2 Association between clinical data and% of Dicer/ DROSHA expression

Dicer or DROSHA*		Intensity <1 (n=38)	Intensity>1 (n=11)	P value
Age<50 years	17 (80.9%)	4 (19.1%)	0.882	
≥ 50 years	21 (75%)	7(25%)		
pT<= 20 mm	14(87.5%)	2(12.5%)	0.376	
[20–50] mm	19 (76.0%)	6 (24.0%)		
> 50 mm	5 (62.5%)	3(37.5%)		
Histological type	Ductal	32 (76.2%)	10 (23.8%)	0.702
Lobular	1 (50.0%)	1 (50.0%)		
Mixed	3 (100.0%)	0 (0.0%)		
Others	2(100.0%)	0(0.0%)		
Histological grade	1	6(75%)	2(25.0%)	1
(SBR) 2	29(76.3%)	9 (23.7%)		
3	3(100%)	0(0%)		
N status	N0	20(76.9%)	6 (23.1%)	0.904
N1-3	10(83.3%)	2 (16.7%)		
N≥4	8(72.7%)	3(27.3%)		
Estrogen receptor	ER- (< 10%)	16(84.2%)	3(15.8%)	0.591
(%: marked cells) ER+(≥ 10%)	22(73.3%)	8 (26.7%)		
Progesterone receptor	PR- (< 10%)	13(81.3%)	3 (18.7%)	0.947
(%: marked cells) PR+(≥ 10%)	25(75.8%)	8 (24.2%)		
HER2 status	-	28 (77.8%)	8 (22.2%)	1
+	10(76.9%)	3(23.1%)		
Luminal A	Yes	22 (78.6%)	6 (21.4%)	0.602
No	26 (83.9%)	5 (16.1%)		
Cancer subtype	Luminal A	22 (78.6%)	6(21.4%)	0.866
Luminal B	4(66.7%)	2 (33.3%)		
Basal-like	6 (75%)	2(25%)		
HER2+	6 (85.7%)	1 (14.3%)		

*Ratios of Dicer divided by 1.8467 and Drosha divided by 1.049

post-transcriptional regulation. Theoretically the reduced expression or dysfunction of Dicer or Drosha may result in the reduced amount of mature miRNA. In fact, the reduced expression of Dicer or Drosha was detected in various tumors. William M et al. reported that levels of Dicer and Drosha mRNA were decreased in 60% and 51% of ovarian-cancer specimens and the levels of Dicer and Drosha mRNA in ovarian-cancer cells have associations with outcomes in patients with ovarian cancer [15]; Sand M et al. reported a dysregulation of Dicer and Drosha expression in human skin cancer samples [16]; Recently, the reduced expression of Dicer in breast cancer has also been reported to be significantly associated with hormone receptor status and cancer subtype in breast cancer and have an independent prognostic impact disease [4]. However, a study to compare both Dicer and Drosha expression in strictly pair-matched samples of cancerous and adjacent non-cancerous tissues was missed.

The aim of this study was to elucidate the expression patterns of both Dicer and Drosha by qRT-PCR method in pair-matched breast specimens and analyze their association with different clinical parameters. Our results indicate that the variable expression of both Dicer and Drosha were observed in different breast cancer tissues and in most cases the mRNA level of Dicer was reduced in cancerous tissues as compared with corresponding non-cancerous tissues, which is in line with the literature [4]. Apart for Dicer, the down-regulation of Drosha mRNA was also observed in more than half of the cancerous tissues. At the same time we found that the expression pattern of Dicer and Drosha was not parallel and the mRNA level of Dicer and Drosha were inconsistent in 57.1% of the tumor tissues. For more than three quarters (77.6%) of the specimens the decreased level of Dicer or Drosha was observed. The result suggested not only Dicer but also Drosha could play an important role in tumorgenesis of breast cancer and that in case of contradiction of Dicer and Drosha expression they may work complementally.

We failed to find the significant association between Dicer or Drosha and clinical characteristics although the

association between Dicer expression and hormone receptor or subtype in breast cancer has been reported [4]. A small and unbalanced sample analysis and different sample context may account for the non-statistic significance. The prognostic value of Dicer in breast cancer and ovarian cancer was revealed in different study team. However, in our study the prognosis impact of Dicer or Drosha was not analyzed because all the patients with operable breast cancer performed surgery six months ago and the data about DFS and OS was unavailable temporarily. Therefore the follow up will resume and prognostic value of Dicer and Drosha will be analyzed in the future.

In conclusion, our findings indicated that, in addition to Dicer, Drosha was also frequently down-regulated in breast cancer tissues, which may implicate its involvement in the genesis of breast cancer. A combined decreasing in Dicer and Drosha expression in majority of examined samples was helpful to explain globally decreased miRNA expression in breast cancer.

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Competing interests The authors declare that they have no competing interests.

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