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Downregulation of *Caspase 8* in a group of Iranian breast cancer patients – A pilot study

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ABSTRACT

Purpose: It is now well known that evading apoptosis, as a cancer hallmark, can lead to tumour initiation, progression and metastasis. As a result of genome wide association studies, an initiator protease in this pathway, caspase 8 (CASP8), has been found to be an important gene regarding breast cancer susceptibility. The alterations of the expression of this gene have been reported in breast cancer cell lines. Given that in previous studies expression analysis of this gene had only been done in breast cancer cell lines, in this study we aimed to evaluate the expression of this gene in breast cancer tissues versus adjacent normal tissues, using real-time quantitative method.

Methods: Caspase 8 mRNA expression was quantified using comparative RT-qPCR in 27 fresh frozen breast tumours and 27 adjacent normal tissues. Moreover, relationship between the expression changes of CASP8 in tumour tissue and various clinical and pathological features were evaluated in an Iranian population.

Results: The present study showed that expression of CASP8 was significantly reduced in tumour tissues compared to neighbouring normal tissues ($p = .004$). CASP8 expression was significantly correlated with the status of hormone receptors (ER and PR).

Conclusion: To the best of our knowledge, this study is the first report on reduced expression of CASP8 in breast cancer versus adjacent normal tissues. Our data support previous results obtained from cell lines and therefore highlights the seminal role of the induction of CASP8 expression, as a novel therapeutic approach, in order to sensitize tumour cells to apoptotic stimuli.

Introduction

Breast cancer is the most prevalent female malignancy and the second cause of cancer death in women worldwide [1]. The incidence of breast cancer is increasing in Iran and it is still at the top of the list of female neoplasms similar to other parts of the world [2,3]. Different cellular pathways are involved in carcinogenesis of cancers, one of which is apoptosis. It is over 16 years that apoptosis has been known as one of the main pathways in carcinogenesis. Evading apoptosis, as a cancer hallmark, was first introduced by Hanahan et al. in 2000 as one of the main biological skills acquired during the multistep development of human tumours [4]. This pathway can be triggered by a variety of intra- and extracellular factors during tumour development including radiation, loss of cell-matrix interaction, hypoxia, DNA damages and telomere malfunction [5]. Cells that may lack apoptosis can survive and initiate tumour development or clonal expansion within the tumour or invade other tissues [6]. Additionally, anti-

Abbreviations: CASP8, Caspase 8; ER, Estrogen Receptor; PR, Progestrone Receptor; RT-qPCR, Quantitative Reverse Transcription Polymerase Chain Reaction; HER-2, Human Epidermal growth factor Receptor-2; HNSCC, Head and Neck Squamous Carcinoma; LNM, Lymph Node Metastasis.

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cancer agents mostly work through apoptosis induction. Thus, any defect in the process of apoptosis can result in treatment failure, tumour relapse and/or metastasis [7]. Alterations in this cellular pathway occur due to genetic and epigenetic changes in cancer cells. Transcriptome is one of the levels in which the results of such changes can be assessed. Gene expression profiling has changed the face of breast cancer classification and management**.** By use of quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and cDNA microarray expression technology, tumours with specific gene expression patterns have been identified. This has led to the development of a new classification strategy, categorization of breast cancers into molecular subtypes, including luminal A, luminal B, basal-like, *HER-2/neu*-overexpressing, claudin low and finally normal-like breast tumours [8]. The discovery of the molecular breast cancer subtypes, helped to differentiate groups of cancers that were morphologically similar but were widely different, especially with respect to response to different therapeutic strategies [9]. On the other hand, over the last several years, multiple genome-wide association studies and candidate-gene association studies have shown that some common variants of *CASP8* - initiator caspase in apoptosis pathway- are associated with breast cancer risk. This evidence also shows that some variants of *CASP8* may affect its expression. *CASP8* – 652 6 N Del (rs3834129) – which is located in the promoter region of the *CASP8* gene – is associated with decrease in expression of *CASP8* mRNA [10–12]. Negative prognostic impact of this *CASP8* polymorphism along with another SNP of this gene, Asp302His (rs1045485), has been recently shown in breast cancer [13]. In general, caspases work as conserved cysteine endoproteinases and play a crucial role in "programmed cell death". By activation of death receptors, *CASP8* initiates apoptosis process in normal cells. Since signalling via the death receptor (extrinsic) pathway critically depends on *CASP8*, the disturbance of *CASP8* function or reduction of its expression may contribute to human diseases [14]. The alterations in the expression of this gene have been reported in a variety of tumours and also in breast cancer cell lines [15–18]. Understanding changes in apoptosis pathway not only gives clues about the pathogenesis of cancer, but also can result in finding therapeutic strategies. Since loss of expression or function of caspase-8 has an obvious effect on the ability of cancer cells to undergo apoptosis, different strategies have been studied to upregulate *CASP8* expression in order to restore its function in tumours lacking this essential apoptosis regulator [19,20]. This fact shows that *CASP8* can be a specific therapeutic target for different cancers in the near future.

In previous studies, expression analysis of this gene had only been done in breast cancer cell lines by qualitative methods. Thus, in this study we aimed to evaluate the expression of this gene in breast cancer tissues versus adjacent normal tissues, using real-time quantitative methods. Another goal of this study was to investigate relationships between expression changes of *CASP8* with other factors such as HER2, HR markers, grade of tumour and other demographic factors in an Iranian population.

Materials and methods

Sample collection

Thirty patients with primary diagnosis of breast cancer were enrolled in this pilot study. According to our exclusion criteria, three patients were excluded based on the pathological examination of tissues which showed no tumour involvement. Twenty-seven primary breast tumour and normal adjacent tissues were obtained from patients who had undergone breast surgery at Omid and Qaem hospitals in Mashhad. All patients had not received preoperative chemotherapy. The samples were collected after obtaining patients' written consent following approval by the ethical committee of Mashhad University of Medical Sciences. The samples were directly collected in RNAlater® Thermo Fisher (Carlsbad, CA, USA) for immediate isolation of total RNA and stored at -80 °C for later use.

RNA Extraction and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentrations of isolated RNA samples were read using Nanodrop at 260 and 280 nm. Analysis of RNA by agarose gel electrophoresis showed sharp 18 s and 28 s rRNA bands that were indicative of intact RNA. Reverse transcription of the RNA (1 μg) into cDNA was performed using RevertAid First Strand cDNA Synthesis kit (Fermentas, Burlington, USA) based on the manufacturer's protocols. In order to confirm the cDNA synthesis, using GAPDH primers, PCR reaction was initiated at 94 °C for 3 min and amplified during 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s and followed by a final extension step at 72 °C for 5 min according to the manufacturer's instructions. Finally, the PCR products were visualized by gel electrophoresis on a 2% agarose gel.

Real-Time Polymerase Chain Reaction (PCR)

The CASP8 gene expression levels in tumour and adjacent normal tissues were measured by quantitative real time PCR in Roche LightCycler® 96 instrument using SYBR Premix Ex Taq II (TAKARA, Japan). The total 15 μl reaction volume contained 7.5 μl SYBR Premix, 1.5 cDNA, 0.3 μM of each primers and 4 μl ddH_2O. Thermal cycling consisted of an initial denaturation step 95 °C for 5 min followed by an amplification program repeated for 40 cycles. The amplification program was 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s with a single fluorescence acquisition at the end of the elongation step. *ACTB* gene was used as internal control. All qPCR reactions were done in triplicate. The primer sequences for real time PCR are shown in Table 1. All primers were selected from the cited articles [21,22].

Statistical analysis

To assess distribution of ΔCT, Shapiro-Wilk normality test was done in both healthy and tumour groups. Since the test results showed not normally distributed data, "Wilcoxon Signed Rank" test was used to compare the ΔCT in the two paired groups (tissues of tumour and normal tissues are for the same patients). Also, for testing the significance of changes in the *CASP8* expression, Wilcoxon Signed Rank Test was used. Associations between *CASP8* expression and patients' clinicopathological features, including age, menstrual status, family history of breast cancer, tumour size and grade, lymph node metastasis (LNM), status of HR, and HER-2, were assessed by Fisher's exact test. The power of this study was calculated based on the current sample size (27 in each group) as well as difference between delta Ct and related standard deviation in two groups, considering α level at 0.05. The statistical analysis was done using by SPSS v22.0 (IBM, Chicago, IL)

Results

Patients' characteristics

Analyses for the expression of *CASP8* were performed for tumour and matched normal tissue of 27 patients with sporadic breast cancer. The mean age of the patients was 48.15 years old. A total of 16 patients (59.2%) were premenopausal, and 11 patients

Table 1
Real-time PCR primers.

Gene	Sequence (5′->3′)	Length	Tm	GC%	Length of product
CASP8 Primer Forward	CCAGAGACTCCAGGAAAAGAGA	22	58.83	50	185
CASP8 Primer Reverse	GATAGAGCATGACCCTGTAGGC	22	60.03	54.5	
β-Actin Primer Forward	TCCCTGGAGAAGAGCTACGA	20	59.38	55	194
β-Actin Primer Reverse	AGCACTGTGTTGGCGTACAG	20	60.88	55	

(40.8%) were postmenopausal. Ten patients (37%) had lymph node involvement while 17 patients (63%) had normal report. All patients participating in this research had invasive ductal carcinoma (IDC) according to the pathology reports. The majority of patients (18, 66.7%) were classified as grade II, 3 (11.1%) grade I and 6 (22.2%) grade III (Tables 2 and 3).

Real-time PCR results

The present study showed that expression of *CASP8* was reduced in tumour tissues compared to neighbouring normal tissues in our patients. The Ct mean in tumour group was 9.3 (SD = 1.66) and in normal group was 7.83 (SD = 2.44). Based on Wicoxon test, the ΔCt difference of *CASP8* between the tumour group and the normal group was significant (p = .004) indicating that expression of this gene in cancerous tissues was significantly lower than that in adjacent normal tissues. However, comparison of the results based on fold change in all specimens showed a 44% non-significant reduction (p = .06) in expression of *CASP8* in tumour tissue. CASP8 was down regulated in 19 of 27 tumours while in 8 tumours this gene was upregulated (Table 4).

Table 2
Demographic characteristics of patients.

Demographic factor	Number (%)
Age	
<50	15 (55.6)
≥50	12 (44.4)
Tumor Size	
≤2cm	11 (40.7)
2–5cm	7 (26)
≥5cm	5 (18.5)
Not available	4 (14.8)
Menstrual status	
Menopause	11 (40.8)
Non-menopause	16 (59.2)
Family history of breast cancer	
Positive	5 (18.5)
Negative	18 (66.7)
Not available	4 (14.8)
History of pregnancy	
Positive	20 (74.1)
Negative	3 (11.1)
Not available	4 (14.8)
Age at first pregnancy	
≤20	11 (55)
20–30	8 (40)
≥30	1 (5)
History of breastfeeding	
Positive	18 (66.7)
Negative	5 (18.5)
Not available	4 (14.8)
History of abortion	
Positive	4 (14.8)
Negative	19 (70.4)
Not available	4 (14.8)
BMI	
≤20	3 (11)
20–25	3 (11)
≥25	14 (52)
Not available	7 (26)

Table 3
Summary of the Pathological Features of Breast Cancer Patients.

Immunohistochemical characteristics	No. of cases
HR Status	
HR +	13 (48.2)
HR -	5 (18.5)
Not available	9 (33.3)
HER-2 status	
HER-2+	4 (14.9)
HER-2–	9 (33.3)
HER-2 equivocal	5 (18.5)
Not available	9 (33.3)
Ki67 status	
Ki67 +	18 (66.7)
Ki67–	0
Not available	9 (33.3)
Tumor grade	
Grade I	3 (11.1)
Grade II	18 (66.7)
Grade III	6 (22.2)
Lymph node involvement	
Positive	10 (37)
Negative	17 (63)

Table 4
Summary of *CASP8* relative expression.

Relative expression	Number (%)	Mean of *CASP8* ΔΔCT ($\Delta CT_{Tumour} - \Delta CT_{Normal}$)	Mean of *CASP8* fold change ($2^{-\Delta\Delta CT}$)
Upregulated in tumour (↑)	8 (30)	−1.05	2.22
Down regulated in tumour (↓)	19 (70)	2.43	0.57
Unchanged	0 (0)	–	–

Relationship between expression of CASP8 and clinicopathological features

We analyzed the associations between levels of *CASP8* expression and a series of clinicopathological characteristics, including age, menstrual status, family history of breast cancer, BMI, OCP consumption, tumour size, LNM and the status of HR, and HER-2, in breast cancer patients (Table 5). We detected downregulated *CASP8* expression more in HR positive tumour; 84% of HR positive tumours were clustered in downregulated group while this proportion was only 20% for HR negative tumours (p = .022). Other factors were not significantly associated with *CASP8* expression, for instance *CASP8* down regulation was observed more in patients with negative family history of breast cancer (13 of 18 patients, 72.2%) than patients who had positive family history of breast cancer (2 of 5 patients, 40%) but this difference was not statistically significant (P = .297).

Discussion

The aim of this study was firstly to evaluate the expression of *CASP8* in breast cancer tissue compared to normal tissue of the same patients; and secondly, we aimed to investigate relationships

Table 5
Assiciation between *CASP8* expression and clinicopathological features.

Characteristics	Down regulation	Up regulation	*p*-Value
Age			
<50	10 (66.7)	5 (33.3)	
≥50	9 (75)	3 (25)	0.696
Menstrual status			
Menopause	8 (72.7)	3 (27.3)	1.000
Non-menopause	11 (69)	5 (31)	
Family history of breast cancer			
Positive	2 (40)	3 (60)	0.297
Negative	13 (72.2)	5 (27.8)	
BMI			
≤20	2 (66.7)	1 (33.3)	0.368
20–25	3 (100)	0 (0)	
≥25	8 (57)	6 (43)	
OCP consumption			
Yes	5 (71.5)	2 (28.5)	1.000
No	10 (62.5)	6 (37.5)	
Tumor size			
≤2 cm	9 (82)	2 (18)	0.765
2–5 cm	5 (71.5)	2 (28.5)	
≥5cm	3 (60)	2 (40)	
LNM			
Positive	8 (80)	2 (20)	0.666
Negative	11 (64.7)	6 (35.3)	
Tumor grade			
I	2 (66.7)	1 (33.3)	0.970
II	13 (72.2)	5 (27.8)	
III	4 (66.7)	2 (33.3)	
HR Status			
Positive	11 (84.6)	2 (15.4)	0.022*
Negative	1 (20)	4 (80)	
HER-2 status			
Positive	3 (75)	1 (25)	0.894
Negative	6 (66.7)	3 (33.3)	
Equivocal	3 (60)	2 (40)	

* significant correlation.

between expression changes of *CASP8* and other factors including biochemical markers, clinical specificities and demographic determinants. As it was expected based on *CASP8's* mechanism of action, the main result of this study has shown a significant reduction (p = .004) in the expression of *CASP8* in tumour tissue, compared with normal neighbouring tissues. On the other hand, the result of fold change calculations showed a reduction of 44% in the expression of *CASP8* in tumour tissues, though this difference was not statistically significant (p = .06).

The role of apoptotic defects in formation of malignancies, their progression and invasion is well documented today [6]. *CASP8* is one of the potential genes the inactivation of which can disrupt apoptosis. This protease is one of the main initiators of programmed cell death [14]. As an external validity, other studies on different types of human cancer have shown significant reduction of *CASP8* expression in tumour cells, which can be caused by a variety of genetic, epigenetic or post-translational mechanisms. These changes were previously reported, particularly in breast cancer cell lines [15,17,18]. It has also been recently shown that *CASP8* plays a role in the suppression of oncogenic transformation, regardless of its role in apoptosis. The absence of caspase-8 can facilitate other oncogenic mutations and/or accumulation of spontaneous oncogenic changes. For example, there is evidence showing that phosphorylation of tyrosine 380 in *CASP8* can result in increase of cell migration. This report suggests that the absence of caspase-8 may lead not only to decreased apoptosis, but also to enhanced motility of affected cells [23,24]. Furthermore, other studies show that a reduction in expression of *CASP8* is associated with *MYCN* oncogene amplification and increase of its related protein [25]. All these evidences emphasize on CASP8 involvement in carcinogenesis.

According to results of recent genome wide association studies, finding common variants in this gene which are associated with breast cancer risk brought *CASP8* name one more time to the attention. Expression of *CASP8* can be dysregulated by some of its polymorphisms, an example of such gene variation is 652 6Ndel which shows a reduction in expression and also has a negative impact on prognosis of breast cancer patients [23,26]. Previous studies also found association between expression of *CASP8* and stage of tumour or prognosis of the disease. For instance, lack of *CASP8* expression in medulloblastoma is correlated with undesirable prognosis. Also, a significant relationship between the decrease in *CASP8* expression and the stage of head and neck squamous cell carcinoma (HNSCC) has been reported [27,28]. Patient follow up in the future can help us find out about the effect of *CASP8* alterations on the survival rate of our patients and their prognosis.

Another aim of this study was to investigate the relationship of *CASP8* expression level with Her2 and hormone receptors expression status and other patients' characteristics. Based on these analyses, we found significant relationships between CASP8 expression and status of HR in breast cancer patients.

One of the main points of this study is the reasonable statistical power (>80%) which was calculated based on the difference between delta Ct means (1.48) and related standard deviation (1.7) in both groups.

To the best of our knowledge, this study is the first report on reduced expression of *CASP8* in breast cancer versus adjacent normal tissues. Since the induction of *CASP8* expression – as a way to sensitise tumour cells to apoptotic stimuli – had been done successfully, this induction can be regarded as a new therapeutic approach in this type of cancer as well [19]. More studies are currently being done on these samples to assess *CASP8* protein expression and its relation with the genetic background of the population. Evaluation of the outcome of expression reduction of this gene in the apoptosis process and studying the mechanism of this reduction are also necessary to elucidate the potential role of this gene in therapeutic measurements.

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Author contributions: MA performed related laboratory work, helped with sample collection, analysed the data and drafted the manuscript. ND and FAJ helped with sample collection and laboratory work. ASF and MG confirmed the diagnosis and provided the appropriate specimens. AP conceived and designed the study, supervised the project and edited the manuscript.

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Conflict of interest

The authors have no conflict of interest to declare.

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