

RESEARCH ARTICLE

Bcl-2 Gene Expression in Human Breast Cancers in Iran

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Abstract

Background: Breast cancer is among the five most common cancers and ranks first among cancers diagnosed in Iranian women. Screening and treatment of this disease with molecular methods, especially regarding high incidences at early age and advanced stage, is essential. Several genes with altered expression have been identified by cDNA microarray studies in breast cancer, with the Bcl-2 gene indicated as a likely candidate. In this study, we studied Bcl-2 gene expression levels in parallel tumor and non-tumor breast tissues. **Materials and Methods:** Forty samples including 21 tumor, 16 non tumor (marginal) and 3 benign breast tissues which were all pathologically diagnosed, were subjected to RNA extraction and polyA RT-PCR with the expression level of Bcl-2 quantified using real-time PCR. **Results:** There is higher expression levels of the Bcl-2 gene in tumor samples compared with marginal samples, but not attaining significance ($p > 0.05$). Bcl-2 expression in 14 (66.7%) of the cases of tumor samples and 9 (56.3%) cases of the marginal samples were positive. Comparison of the expression of the Bcl-2 gene in histological grade showed that a high expression of Bcl-2 was associated with a high histological grade ($p < 0.41$). **Conclusions:** Our data suggests that dysregulated Bcl-2 gene expression is potentially involved in the pathogenesis of breast cancer. Using gene expression analysis may significantly improve our ability for screening cancer patients and will prove a powerful tool in the diagnosis and prognostic evaluation of the disease whilst aiding the cooperative group trials in the Bcl-2 based therapy project.

Keywords: Breast Cancer - Bcl-2 - over expression - real-time quantitative PCR

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Introduction

Breast cancer is a heterogeneous disease which is characterized by the proliferation and abnormal differentiation of malignant immature cells that often carry aberrations that deregulate hundreds or even thousands of genes (Harirchi et al., 2011; Ghojzadeh et al., 2013). Breast cancer is amongst the five most common cancers and ranks first among cancers diagnosed in Iranian women, comprising 24.4% of all malignancies with the mean age ranging from 47.1 to 48.8 years (Ferlay et al., 2012; Roder et al., 2012). Although the incidence of breast cancer is still relatively low when compared with western countries (Iran: 23.65 per 100,000 and United States: 140.8 per 100,000 Caucasian women), the number of patients with newly diagnosed breast cancer are increasing (Harirchi et al., 2011). As mentioned above, the life-time risk of developing breast cancer for American women is 1 in 8 whilst in Iran this figure is reduced to one in thirty five. Almost half of the patients presenting with the disease are below 50 years of age (Das et al., 2012) and present in

an advanced stage of the disease. It would be beneficial to suggest that the best choice of breast cancer screening and treatment should be age group oriented, especially amongst Iranian patients, as mammographic screening results in women between 40-49 years of age are worse than those in women older than 50 years of age due to the density of their breasts. This can be an important cause in the reduction in accuracy, effectiveness, and power of mammography as a breast cancer screening tool in younger Iranian women (Houssami et al., 2011; Ghojzadeh et al., 2012).

The importance of the early detection of breast cancer through screening on mortality and morbidity has been shown in several large trials (Razavi et al., 2009; Hoerger et al., 2011; Ghojzadeh et al., 2012). Early detection of breast cancer plays the leading role in reducing mortality rates and improving the patient's prognosis (Gyorffy et al., 2010). The heterogeneous nature of breast cancer has resulted in an overwhelming interest in the search for prognostic markers to identify patients who might benefit the most from the therapeutic modalities available. The

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prognosis varies, not only with the state of disease, but also with its biological behavior (Shen et al., 2004; Reddy et al., 2012).

A main focus of old reports studied the events driving malignant transformation and tumor progression was cell proliferation. However, a major shift has recently occurred in this focus, by realization that tumor growth and its aggressiveness may be determined not only by the proliferative rate, but also by the rate of cell death (Amirnia et al., 2012). Assessment of apoptosis and individual components of the apoptosis pathway might therefore be relevant in determining the prognosis in an individual patient. There are two principal pathways leading to apoptosis, one begins in the plasma membrane with the presence of cell-surface death receptors and the other begins in the mitochondria. The mitochondrial pathway is regulated by members of the Bcl-2 protein family (Cleland et al., 2010; Martinou et al., 2011). Several prognostic markers have been identified in breast cancer, however, among biologic variables presently under investigation, apoptosis markers, in particular Bcl-2 and Bax expression, are receiving much attention for their relationship with the cellular response to genotoxic damage in experimental tumors (Chipuk et al., 2010). To follow on, Bcl-2 was detected predominantly in differentiated tumors (Sadjadi et al., 2009). Expression of the Bcl-2 gene in human breast cancer tissues has been reported in 32-85.9% of the cases (Won et al., 2011). The recent availability of reagents able to detect the expression of Bcl-2 and related genes has substantially contributed to the understanding of some of the genetically controlled mechanisms that regulate active cell death and to the investigation of the role of pro- and anti-apoptotic proteins in determining the cellular response to cytotoxic drugs, hormonal agents and radiation. Bcl-2 expression has been shown to inversely parallel cell turnover or the modeling of tissues by apoptosis (Yan et al., 2009; Moul 2012; Smerage et al., 2013). The main goal of this study was to explore the Bcl-2 expression pattern in tumor samples from the respective breast tissues and compare the results to marginal samples as a control.

Materials and Methods

Samples acquisition

Using a descriptive study design, tissue samples from 40 patients were obtained through either the use of a biopsy or following a surgical mastectomy. All samples were separated in to three groups including 21 tumors, 16 marginal and 3 benign samples tissues which were all pathologically diagnosed. Sample data details are present in Table 1.

RNA extraction and cDNA synthesis

All samples were subjected to total RNA extraction using RNeasy™ mini kit (Qiagen), as recommended by the manufacturer, then a cDNA library was constructed using polyA RT-PCR as previously reported (Lou et al., 2012). First-strand cDNA was synthesized from 4 µg of total RNA using an Oligo (dT) primer and Reverse Transcriptase. These were then tailed with a homopolymer (dA), using terminal deoxynucleotidyltransferase (TdT).

Table 1. Characteristics of Clinicopathological the Breast Cancer Specimens

Variable	Level	Cases (N=24)
Age	Median	46.82
	≤50	8 (47.05)
	>50	9 (52.94)
	Missing	23
Cancer Type	Infiltrating Ductal Cancer (IDC)	17 (94.44)
	Infiltrating Lobular Cancer (ILC)	0
	Ductal Carcinoma in Situ (DCIS)	1 (5.55)
	Others	6
Breast	Left	9 (56.25)
	Right	7 (43.75)
	Missing	8
Tumor Grade	1	4 (30.76)
	2	4 (30.76)
	3	5 (38.46)
	Missing	11
Axillary Lymph Nodes	Positive	12 (66.66)
	Negative	6 (33.33)
	Missing	6
Metastasis	Positive	10 (55.55)
	Negative	8 (44.44)
	Missing	6
Mastectomy	Left	9 (52.94)
	Right	7 (41.17)
	Total	1 (5.88)
	Missing	7

*Data was shown as N (%) pattern

Global amplification of cDNA corresponding to all expressed genes was performed with all samples by following protocol. Briefly, for each sample, 10µl of red master mix, 1µl of oligodT primer (NOT 1-16) and 7µl H₂O were added in 2µl of cDNA. The PCR master mix was placed in a thermal cycler with the following protocol: 94°C for 30s, 42°C for 1min, 72°C for 2min. All samples were stored at -70°C (Logan et al., 2009).

Specific RT PCR

PCR primer pairs were designed for mRNA sequence within 300 bp of the 3' end of each gene (Table 2). Specific PolyA PCR for GAPDH and Bcl-2 genes was carried out with all samples. Briefly, for each sample, 2µl of cDNA was added to 6µl of red master mix and 2µl of GAPDH primer was then placed in a thermal cycler (Eppendorf) with the following setting; 5min at 94°C, followed by 30 cycles of 95°C for 30s, 56°C for 30s, 72°C for 1 min.

Standards as gene specific quantity marker

A dilution series of human genomic standards for calibration of real-time PCR was generated using human genomic DNA (hgDNA). hgDNA (Promega) was homogenized by sonication and serially diluted in Tris-EDTA (TE) buffer to produce standards in which the number of DNA molecules ranged from 1/10 to 1/100,000. Each standard was then aliquot into 1ml amounts and stored at -20°C.

Real-time quantitative PCR using Taqman™ probes

Primers and probes for Taqman™ PCR were designed for each gene using Primer Express Software (Perkin Elmer/Applied Biosystems), in which all are detailed in Table 2. For each gene, Taqman™ real-time PCR was

Table 2. List of Primers and Used Probes

Gene (human)	Accession No.	Primers
Housekeeping genes		
Gap	J04038	1=CATGGCCTCCAAGGAGTAAG 2=GGGACTCCCCAGCAGTGA 3=ACCAGCCCCAGCAAGAGCAAGA 4=CATGGCCTCCAAGGAGTAAG 5=GGTTGAGCACAGGGTACTTTA
Ribosomal	U14971	1=GAGACGACGAGGAGGAGGATTA 2=GCAGGAAAACGAGACAATCCA 3=TCCACCTGTCCCTCCTGGGCTG
Breast Cancer Gene		
Bcl-2	M14745	1=TGAGTAAATCCATGCACCTAAACCTTT 2=CACTGTCACTCTTGCAAATCTACCT 3=TGGGCCCTCCAGATAGCTCATTTTCATT 4=GAGTGACAGTGGATTGCAT 5=CAGAATATCAGCCACCTCTT

*1=TaqMan primer 1 (5'-3'), 2=TaqMan primer 2 (5'-3'), 3=TaqMan [6-carboxyfluorescein (FAM)]-probe-[6-carboxyteramrthylrhodamine(TAMARA)] (5'-3'), 4=Forward primer (5'-3'), 5=Reverse primer (5'-3')

performed in 20µl target volume using 5µl of PolyAcDNA from each sample, 10µl master mix, and 0.5µl of gene-specific forward primer, 0.5µl of gene-specific reverse primer, 0.1µl of TaqMan probe and 3.9µl of ddH₂O. All real-time PCRs were performed using the ABI Prism 7500 system with the following settings: 50 cycles at 95°C, denaturing process for 15s, annealing at 60°C, chain elongation and detection for 60s. All samples, as well as the five serial human genomic standards were measured in each gene. Negative controls (NTC) were prepared each time. Samples were analyzed using an ABI Prism 7700 sequence detection system, (PE Applied Biosystems) as recommended by the manufacturer.

Normalization

To measure the expression level of the Bcl-2 gene, GAPDH and human ribosomal protein S9 (RBS9) genes were used for normalization. The expression levels of the two housekeeping genes: GAPDH, S9 and the Bcl-2 genes were measured by Real-time PCR in each sample. The mean Ct (Cycle threshold) values and the copy number of each gene were determined by reference. The mean copy number of the two housekeeping genes (Mhouse) were calculated and then divided by the highest Mhouse in all samples, resulting in a normalization correction factor. Following real-time PCR amplification and quantification of the selected genes, this factor was used for the normalization of the expression levels for the Bcl-2 genes measured. The standard curves, which were plotted, demonstrated the ability of the method to accurately measure the expression levels of the Bcl-2 gene.

Statistical analysis

Initial analysis indicated that the data was not normally distributed; therefore, non-parametric tests were used. Statistical analysis of the expression levels of the genes were performed in each of the diagnostic groups using the following non-parametric tests: test of median and Kruskal-Wallis (K-W) using p<0.05 for calculation of the statistical significance. All the tests were performed using the statistical package from the Social Science (SPSS) software.

Results

To investigate the expression pattern and quantification of the Bcl-2 gene, all samples were separated in to three groups: tumor, margin and benign. After RNA extraction, a cDNA library was constructing from each sample. To confirm that the PolyA cDNA produced from all of the samples can be used to detect the Bcl-2 gene expression in real-time PCR, specific PCR was performed for GAPDH and Bcl-2 genes. GAPDH PCR was positive in all of the samples and bands of appropriate sizes were present in all of the samples; demonstrating the presence of the target transcript in the polyA cDNA. Bcl-2 PCR in some of samples was negative (Figure 1). Briefly, 2µl of globally amplified PolyA cDNAs prepared from tumor, marginal and benign breast tissue samples were subjected to gene-specific PCR using gene specific primers. For each reaction, 5µl of PCR products were loaded onto 2% agarose gel, electrophoresied and visualised with EtBr. GAPDH PCR was positive in all samples, however, Bcl-2 PCR was negative in some of the samples (Figure 1).

Taqman™ real-time quantitative PCR

For each gene, Taqman™ PCR was applied in a real-time PCR ABI Prism 7700 system. Negative template controls (NTC) and human genomic standards were prepared each time. Standard curves were plotted using Ct values of the five-fold serial dilutions of the human genomic DNA for the optimization and efficiency of real-time PCR reactions. The efficiency of the real-time PCR reactions was calculated from the slope of the standard curve. Accordingly, the efficiency of the GAPDH, S9 and Bcl-2 PCR reactions was calculated as 85%, 91%, and 87.71%, respectively. A representative example of a real-time PCR amplification plot and standard curve of the Bcl-2 gene is shown in Figure 2. Real-time PCR amplifications were performed using Bcl-2 specific primers and probes. Standard curve is showing amplification efficiencies of five-fold serial dilutions of the human genomic DNA (Figure 2).

Comparison of the housekeeping genes (GAPDH and S9) in the expression of the tumor, margin and benign tissues

The expression of each housekeeping gene was

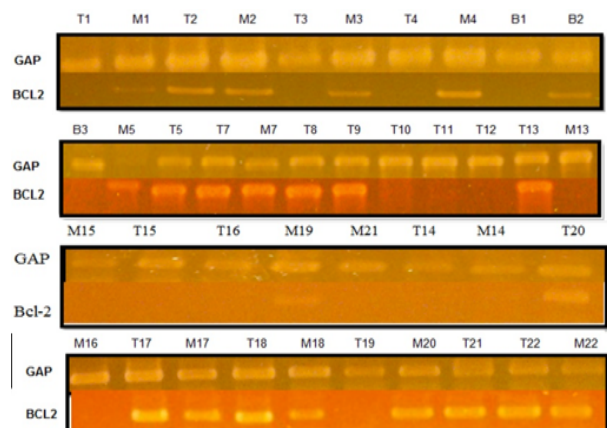


Figure 1. GAPDH and Bcl-2 PCR in All Samples of Breast Cancer (T=Tumor; M=Margin; B=Benign)

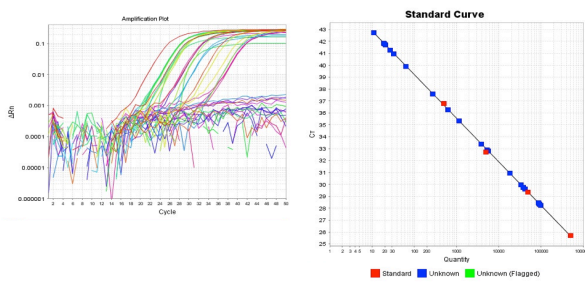


Figure 2. Real-Time RT-PCR Quantification of Bcl-2 mRNA

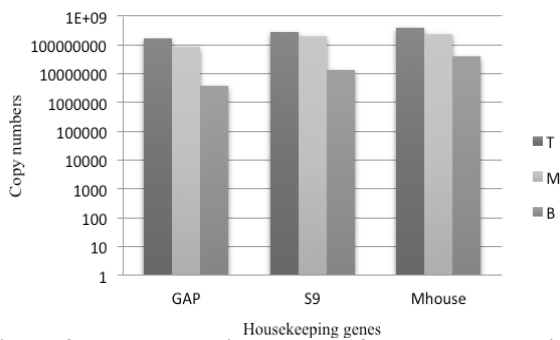


Figure 3. The Expression Levels of Two Housekeeping Gene (GAPDH and S9) for Each Set (T=Tumor; M=Margin; B=Benign; Mhouse=Mean of the housekeeping genes)

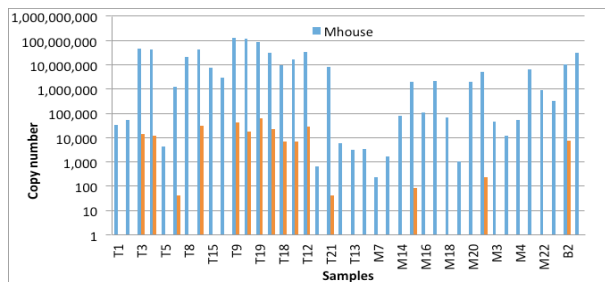


Figure 4. Distribution of the Bcl-2 Gene in Tumor, Marginal and Benign Samples

compared in the three sample groups: tumor, margin and benign. The expression of the two housekeeping genes: GAPDH and S9 in tumor and marginal samples were almost equal, thus these housekeeping genes were useful for the normalization and quantification of the Bcl-2 gene in the respective samples. The expression variety of the Bcl-2 gene resulted in a tumor nature, with the estimation of error in the testes very low.

Distribution of the Bcl-2 gene in all samples

Distribution of the expression levels of the Bcl-2 gene in all of the samples together with Mhouse, are presented in Figure 4. Both of the two housekeeping were genes expressed in all of the samples, with no correlation between the expression of the Bcl-2 gene and the two housekeeping genes. Thus, the expression variety of the Bcl-2 gene resulted in the biological nature of the tumor.

Comparison of the Bcl-2 gene expression in tumor and marginal samples

In order to assess the expression pattern and quantification of the Bcl-2 gene in tumor and marginal samples, comparison of the Bcl-2 gene expression was

performed in tumor and margin samples. The results indicated that there were significant differences between the expression levels of the Bcl-2 gene in tumor and marginal samples. The Bcl-2 gene expression level in tumor samples is higher than marginal samples.

Comparison of the Bcl-2 gene expression between the different grades of tumors

The expression level of the Bcl-2 gene was correlated with other clinic pathological variables such as the grade of tumors. The results showed in grade III of tumor samples, Bcl-2 gene was expressed significantly higher than grade I and II. The results illustrated that the Bcl-2 gene was expressed significantly higher in grader III breast tumor samples, in comparison to group I and II.

Discussion

Comparison of tumor and marginal samples identified the Bcl-2 gene expression rates as being significantly different between the two groups. Real-time PCR results showed higher expression levels of the Bcl-2 gene in tumor samples compared with marginal samples, however, this was not a significant difference ($p>0.05$). The greatest difference appeared in tumor sample 8 and marginal sample 14. Bcl-2 expression in 14 (66.66%) of the cases of tumor samples and 9 (56.25%) cases of the marginal samples were positive. Two out of three benign samples were negative for the Bcl-2 expression. Several cross-sectional and meta-analysis studies verify Bcl-2 as a favorable independent prognostic biomarker in breast cancer. A recent study by showed that Bcl-2 expression is highly associated with an increased risk of a local recurrence in patients within the early stages of breast cancer. However, other data sets indicate that a high Bcl-2 expression rate by tumor cells had no predictive value in breast cancer patients (Jaberipour et al., 2010). In the study conducted by Slooten, relationships between Bcl-2 expression, response to chemotherapy and a number of pathological and biological tumor parameters were assessed in 441 premenopausal, lymph node-negative breast cancer patients using immunohistochemistry. A positive correlation was found between high Bcl-2 expression and estrogen and progesterone receptor positivity and low tumor grade. However, high Bcl-2 expression was negatively correlated with p53 and c-erb-B-2 positivity, high Ki-67 index, mitotic index and large tumor size. Patients with tumors expressing high levels of Bcl-2 had a significantly better disease-free and overall survival rates. High levels of Bcl-2 are preferentially expressed in well-differentiated tumors and are associated with favorable prognosis (Van Slooten et al., 1996; Dawson et al., 2010). However, Bcl-2 expression is not an independent prognostic factor in this patient series (Carlquist and Anderson, 2011). The Bcl-2 gene has thus been reported to be a marker of good prognosis and responsiveness to tamoxifen (Rajpu et al., 2013).

For the purposes of the application of the Bcl-2 indicator gene to routine diagnosis, it is also important that the possible differences in gene expression between the different diagnostic groups for the different fractions

are identified in order to establish, if at all, the fractions which are diagnostically most useful and if so, which Measurements of the mean expression of the two housekeeping genes in the different fractions indicated equal expression, although not significant, in the two fractions. This illustrates the absence of any correlation between the Bcl-2 gene expression and the two housekeeping genes in this purified fraction.

Comparison of the expression of the Bcl-2 gene in histological grade showed that a high expression of Bcl-2 was associated with a high histological grade ($p=0.41$).

Tang et al. (2013) also showed the inverse correlation between Bcl-2 expression and nuclear grade 3 and a positive correlation between Bcl-2 expression and metastasis to the lymph nodes. In another study which was immunohistochemically analysed the expression of the Bcl-2 protein in 202 female breast carcinomas, They found that the intensity of Bcl-2 expression was inversely related to tumor grade, tumor necrosis, oestrogen and progesterone receptor content, However heterogeneous expression of the Bcl-2 protein was associated with high grade (Moul, 2012). It should however, be noted that there is a clear need to quantify the results by the majority of specimens.

To follow on, the comparison is essentially between that of breast carcinoma versus non-neoplastic breast tissue. This comparison confirms the ability of the method and gene selected to distinguish these two states and supports possible use of the method to detect residual or recurrent disease. In addition, establishing this gene expression method for use of surgeons in the operating theatre may help surgeons to distinguish tumor and non-tumor tissues and remove all of the tumoral tissue, which would decrease the recurrence and relapse of the disease.

The method used was able to distinguish between tumor and none tumor samples on the basis of the Bcl-2 gene, extending their diagnostic use. The method used is simple, relatively inexpensive, robust and rapid, allowing translation to routine clinical use, whilst initial sample amplification by polyA PCR allows use of very small samples, further increasing clinical utility. This is likely to be more important for other tumor types to which, given the generic nature of this technique and of the principle of microarray gene signature identification, the method can also be applied, potentially forming a platform for novel molecular diagnostics over all cancer types. Finally, the results suggest that Bcl-2 may be involved in tumor genesis and the development of breast carcinoma with different biological effects and that it can be used in the diagnosis and treatment of breast cancer patients.

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