BRCA1 Gene Expression in Breast Cancer in Kuwait: Correlation with Prognostic Parameters

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Key Words

Breast cancer · *BRCA1* gene · Estrogen receptor · Progesterone receptor · C-erbB-2

Abstract

Objectives: This study assessed the *BRCA1* gene expression in breast cancer in Kuwait, and compared it with other known prognostic factors for the disease. **Materials and Methods:** Forty-eight random samples of archival paraffin-embedded breast cancer tissues were studied for *BRCA1* gene expression. Immunohistochemical method utilizing antibodies against different epitopes on the *BRCA1* protein was used to study *BRCA1* protein expression. In addition, for 29 patients, reverse transcription-polymerase chain reaction was used to detect *BRCA1* mRNA expression. *BRCA1* expression was correlated with age, histological type and grade of breast cancer, estrogen and progesterone receptor status, and C-erbB-2 expression. **Results:** No demonstrable *BRCA1* mRNA and protein expression was found in 79 and 83% of the breast cancer tissues, respectively. A positive relationship was demonstrated between lack of *BRCA1* (mRNA and protein) expression and high histological grade, negative estrogen and progesterone receptor status, and overexpression of C-erbB-2 in the breast cancer tissues. **Conclusions:** The study demonstrated lack of *BRCA1* gene expression (mRNA and protein) in the ma-

jority of breast cancers in Kuwait and confirmed the inverse relationship between *BRCA1* expression and parameters that determine poor prognosis in breast cancer.

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Introduction

Breast cancer is a major cause of cancer-related mortality in women [1]. Although this tumor generally appears in sporadic form, between 5 and 10% of cases can be considered hereditary, attributable to inherited mutations in several susceptibility genes [1, 2]. About 40–50% of hereditary breast cancers and most of hereditary breast-ovarian syndromes are thought to be due to mutations in the breast cancer susceptibility gene 1 (*BRCA1*) [3]. The *BRCA1* gene, identified by positional cloning in 1994, consists of 24 exons, 22 of which encode for a protein of 220 kDa consisting of 1,863 amino acids [4]. *BRCA1* mRNA is induced at late G₁/early S phase before DNA synthesis [5], and the expression of the *BRCA1* protein closely follows that of its mRNA [6]. Recent studies have shown that *BRCA1*-related breast cancers tend to have clinicopathological features that are usually associated with a poor prognosis, such as high-grade, estrogen (ER)- and progesterone (PR)-negative status, and overexpression of C-erbB-2 [7, 8].

Table 1. Description of four different anti-BRCA1 antibodies used

Anti-BRCA1 antibody	Mono/ polyclonal	Epitope	Dilution used	Source
AB-1 (MS110)	monoclonal	N-terminus (exon 1–10)	1/50	Oncogene Research Products (Calif., USA)
AB-D20	polyclonal	N-terminus	1/500	Santa Cruz Biotechnology (Calif., USA)
AB-8F7	monoclonal	exon-11	1/10	Genetex (Tex., USA)
AB-C-Terminus (hBRCA1)	polyclonal	C-terminus (exon 12–24)	1/100	Pharmingen (Calif., USA)

Table 2. Sequences of *BRCA1* and *GAPDH* primers and probes

<i>BRCA1</i> primers	forward 5'-ACAGCTGTGTGGTGCTTCTGTG-3' reverse 5'-CATTGTCTCTGTCCAGGCATC-3'
<i>BRCA1</i> probe labeled with FAM	5'-CATCATTCACCCCTTGGCACA GGTGT-3'
<i>GAPDH</i> primers	forward 5'-TCATTGACCTCAACTACATGGTTT-3' reverse 5'-GAAGATGG TGATGGGATTTC-3'
<i>GAPDH</i> probes labeled with JOE	5'-CAAGCTTCCCGTTCTCAGCC-TAMRA

Epidemiological studies in Kuwait show that breast cancer is the most common cancer in women [9]. Currently, the genetic basis of breast cancer in Kuwait is unknown, and no study has been done on *BRCA1* gene expression in breast cancer in Kuwait. This study compares the expression of *BRCA1* (mRNA and protein) in breast cancers in Kuwait with prognostic parameters such as histological type and grade of cancer, hormone receptor status, and C-erbB-2 expression.

Materials and Methods

Forty-eight random archival formalin-fixed paraffin-embedded breast cancer tissues obtained from the pathology files of Mubarak Al-Kabeer Hospital, Kuwait were studied. Sections of 5 μ m thickness were cut from each tissue. One section of each was stained with hematoxylin and eosin and used to confirm the histological type, and grading of the tumor. The remaining sections were stored for use in immunohistochemistry (IHC) and for RNA extraction. Histological grading of the carcinomas was done according to the system of Scarff-Bloom-Richardson [10]. Cells from MCF-7 cell line grown in Roswell Park Memorial Institute medium (Gibco BRL) and known to express *BRCA1* [11] were processed into paraffin and used as positive controls for both IHC and real-time reverse transcription-polymerase chain reaction (RT-PCR).

Immunohistochemistry

To study *BRCA1* protein expression, 5- μ m sections were deparaffinized, rehydrated, and treated with 3% H₂O₂ in methanol for 30 min to block endogenous peroxidase. Epitope retrieval was carried out in 0.01 M citrate buffer at 95°C in water bath for 20 min. Four antibodies were used (table 1). Nonspecific immunostaining

was eliminated using rabbit antimouse serum before monoclonal antibodies (AB-1, AB-8F7), and swine antirabbit serum before polyclonal antibodies (AB-D20, AB-C-Terminus) for 30 min. After overnight incubation at 4°C with the primary antibody in a humidified chamber, biotinylated antibody (link antibody) was added, followed by streptavidin (dilution 1/500). Diaminobenzene was used as chromogen. Nuclear with or without cytoplasmic staining of 10% or more cancer cells was considered positive. To study ER, PR, and C-erbB-2, 5- μ m-thick sections were treated as above except that the sections were incubated in primary antibody for 90 min at the appropriate dilution (monoclonal (ID5) ER: 1/50, polyclonal PR: 1/50, polyclonal C-erbB-2: 1/50; Dako A/S, Denmark).

RNA Extraction and Real-Time RT-PCR

Of the 48 breast cancer tissues studied by IHC, 29 samples were considered suitable for RNA extraction, because 70% or more of the tissue section contained tumor. The rehydrated sections were scraped off the glass slide into an Eppendorf tube containing RNase inhibitor, proteinase K buffer (pH = 8.3), 1% Tween surfactant, and proteinase K (10 mg/ml). The tubes were kept at 60°C in a water bath overnight (16 h), then mixed with 400 μ l Trizol and chloroform to precipitate the RNA. To control for RNA degradation, *GAPDH* RNA had to be amplified in RNA samples extracted from breast tissue sections before *BRCA1* expression in that tissue is deemed negative. There was no need to treat the samples with DNase because *BRCA1* probe and *GAPDH* primers were spanning exon/exon junction (table 2). RNA was extracted from MCF-7 cells and used as a positive control for RT-PCR. RT-PCR Master mixes were prepared according to Invitrogen ThermoScript RT-PCR System protocols. Samples for no-RT and no-template were also included in each test to detect any DNA interference or RNA contamination. The ABI7000 real-time machine (Applied Biosystems, Calif., USA) was used. Amplification conditions were 60°C for 60 min, 95°C for 5 min, and 45 PCR cycles at 95°C for 15 s, 60°C for 1 min.

Fig. 1. Amplification curves of RNA extracted from breast cancer MCF7 cell line (dotted arrow), and paraffin-embedded breast cancer tissues (black arrows) using BRCA1 primers. X-axis represents the PCR cycle in which the cDNA started the amplification, y-axis represents delta Rn, which is the normalized reporter signal minus the baseline signal.

Table 3. Comparison between *BRCA1* mRNA and protein expression in breast cancer tissue

<i>BRCA1</i> mRNA expression (RT-PCR)	<i>BRCA1</i> protein expression (IHC)							
	AB-1		AB-8F7		AB-D20		AB-C-Terminus	
	negative	positive	negative	positive	negative	positive	negative	positive
Positive (n = 6)	2	4	0	6	2	4	4	2
Negative (n = 23)	21	2	7	16	3	20	15	8
Correlation	p = 0.002		p = 0.11		p = 0.59		p = 0.33	

BRCA1 mRNA expression was compared to *BRCA1* protein expression and both were correlated with hormone receptor and C-erbB-2 expression as well as histological type and grade of tumor and age of the patient. The data were analyzed using the Statistical Package for Social Sciences software (SPSS version 11.01). Relationships among the parameters were analyzed using the χ^2 test. A p value less than 0.05 was considered significant.

Results

BRCA1 Expression in Breast Cancer Tissue

No demonstrable *BRCA1* mRNA expression was found in 79% of the breast cancer tissues. Of the 29 breast cancer tissues analyzed, only 6 (21%) showed detectable

BRCA1 mRNA expression (fig. 1). Lack of *BRCA1* protein expression varied between 35 and 83% in breast cancer tissues depending on the type of anti-*BRCA1* antibody used. Thus, positive IHC staining for *BRCA1* was 16.7, 33.3, 62.5, and 64.6% with AB-1, AB-C-Terminus, AB-D20, and AB-8F7, respectively. *BRCA1* mRNA expression in breast cancer tissues showed a significant association with protein expression using only one of the antibodies, AB-1 (p = 0.002). Thus, 72% of breast cancers, which did not demonstrate *BRCA1* mRNA, also did not show *BRCA1* protein expression by IHC using AB-1. There was no significant association between *BRCA1* mRNA expression and any of the remaining three antibodies used in this study (table 3). Therefore, in addition

Fig. 2. BRCA1 nuclear (arrows) and cytoplasmic staining of breast cancer using monoclonal AB-1. Magnification $\times 250$.

Table 4. Relationship between BRCA1 mRNA and protein (AB-1) expression and type of breast cancer

Histological type	BRCA1 mRNA		BRCA1 protein (AB-1)	
	positive	negative	positive	negative
Infiltrating ductal	5	16	6	29
Medullary	0	5	1	6
Infiltrating lobular	0	1	1	2
Intraduct	1	0	0	1
Tubulolobular	0	1	0	2
	$p = 0.16$		$p = 0.80$	

to BRCA1 mRNA, only results of BRCA1 protein obtained using AB-1 (fig. 2) were used for further analysis in this study.

Histological Types of Breast Cancer Tissue

Five main histological types were present in this study. The most common histological type was infiltrative ductal carcinoma (35 cases), which accounted for 72.8% of cases. The rest were medullary/atypical medullary carcinoma (7 cases), infiltrative lobular carcinoma (3 cases), mixed ductal-lobular carcinoma (2 cases), and intraductal carcinoma (1 case). There was no significant association between BRCA1 expression (both mRNA and protein) and histological type of breast cancer (table 4).

Histological Grades of Breast Cancer Tissue

In this study, cancers with total scores of 3–5 were considered as low grade while those with scores of 6–9 were considered high grade. Of the 48 breast cancer cases the majority (77%) were of high histological grade. There was a significant association between BRCA1 mRNA expression and histological grade of breast cancer. Of 23 breast cancers, 18 (78%) which did not show any BRCA1 mRNA expression were of high histological grade ($p = 0.05$). This association could not be demonstrated with protein expression (table 5).

ER and PR Expression in Breast Cancer Tissue

ER expression was positive in 51% of breast cancers, while PR expression was positive in 49% of cancers. The majority of breast cancers with no BRCA1 expression also showed no ER or PR expression, although these associations were not statistically significant (table 5).

C-erbB-2 Expression in Breast Cancer Tissue

The majority (87%) of breast cancers studied had overexpression of the proto-oncogene C-erbB-2. Most of the breast cancers with no BRCA1 expression also showed overexpression of C-erbB-2, although this association was not statistically significant (table 5).

Age Distribution of Breast Cancer Cases Studied

The patients' ages ranged between 29 and 65 years with a mean of 54.3 years. Seven cases (14.6%) were early-onset breast cancer (<40 years), but the majority (39.6%) were in the 40- to 49-year age group. There was no statistically significant relationship between BRCA1 expression and age of breast cancer patients.

Discussion

Over the past decade, much progress has been made in understanding the molecular biology of breast cancer. The use of molecular and immunohistochemical techniques is providing insights that will allow us to tailor the management of patients with breast cancer. BRCA1 is becoming an important prognostic factor for breast cancer. The morphological features of tumors from patients with breast cancers associated with BRCA1 mutation include higher grade, with an excess of medullary/atypical medullary carcinoma [7]. Tumors associated with BRCA1 mutation are more likely to be steroid hormone receptor (ER and PR)-negative [12]. The present study showed lack of BRCA1 mRNA expression, as well as lack of

Table 5. Relationship between *BRCA1* mRNA and protein (AB-1) expression and various prognostic factors

Prognostic factor		BRCA1 mRNA		BRCA1 protein (AB-1)		p
		positive	negative	positive	negative	
ER	positive	3	9	5	15	0.37
	negative	2	12	3	16	0.42*
PR	positive	4	17	6	13	0.10
	negative	1	14	2	18	0.08*
c-erb B2	positive	3	20	8	26	0.29
	negative	1	2	0	5	0.40*
Histological grade	positive	2	18	6	31	0.59
	negative	4	5	2	9	0.05*

* p values for *BRCA1* mRNA vs. prognostic factor.

BRCA1 protein expression (using AB-1) in the majority of breast cancer tissues studied, with a statistically significant relationship between *BRCA1* mRNA expression and the poor prognostic factor, high histological grade ($p = 0.05$). This finding is similar to that of Lee [13], who also demonstrated that loss of *BRCA1* expression significantly correlates with higher breast cancer histological grade. Grade is an independent prognostic indicator and is inversely related to outcome [14].

Several reports have indicated that a specific histological phenotype can be recognized in breast carcinoma occurring in women with germ line mutations in *BRCA1* [14, 15]. One of these studies reported an excess of ductal carcinoma in breast cancers associated with *BRCA1* mutations [14]. An excess of tumors with medullary and atypical medullary pattern within the group of ductal carcinomas was also reported in association with *BRCA1* mutations [7, 15]. The findings of the present study do not support the findings of these workers. Although 83% of breast cancers with negative *BRCA1* mRNA expression in the study were infiltrative ductal carcinoma, the relationship was not statistically significant. This may be due to the fact that the numbers studied were rather small.

This study also showed an association between lack of *BRCA1* expression and negative ER and PR expression. Although not statistically significant, the trend was noticeable. Previous reports also showed that breast cancers associated with *BRCA1* mutation were significantly more often ER- and PR-negative [7, 16]. ER has become one of the most important prognostic and predictive markers for breast cancer [16]. ER-positive tumors tend to grow more slowly, are better differentiated, and are associated

with slightly better overall prognosis [17]. PR status is also a good predictor for tumor responsiveness to therapy. Approximately 75% of ER/PR-positive tumors respond positively to endocrine therapy [18].

In addition to the above relationship with hormone receptors, the present study also demonstrated a positive relationship between overexpression of the proto-oncogene C-erbB-2 in breast cancers which lack *BRCA1* mRNA and protein expression. Our findings agree with those of Yoshikawa et al. [8], who recently reported that breast cancers with reduced *BRCA1* protein expression had a tendency toward overexpression of C-erbB-2. This proto-oncogene is a biological marker for breast cancer, overexpression of which indicates poor prognosis [19]. C-erbB-2 is amplified in approximately 20% of invasive cancers and has received interest because of its association with lymph node metastasis, short relapse time, poor survival, and decreased response to endocrine and chemotherapy [19]. Antibodies directed against the C-erbB-2 protein have attracted a lot of attention recently because of the availability of the monoclonal antibody Herceptin for treatment of breast cancer [20].

Some studies have reported that breast cancers associated with *BRCA1* mutations develop at a relatively early age [14]. However, results of this study have shown no significant association between *BRCA1* expression and age of breast cancer patients. Once again, this may be due to the small numbers in this study.

Conclusions

To our knowledge, this is the first attempt to study *BRCA1* expression at both mRNA and protein levels in archival breast cancer tissues in Kuwait. The present study demonstrates lack of *BRCA1* expression in the majority of breast cancers investigated. Negative *BRCA1* mRNA and/or protein expression correlated with high histological grade, negative ER and PR status of the breast cancers, and with overexpression of C-erbB-2. These find-

ings confirmed the relationship between lack of *BRCA1* expression and parameters that determine poor prognosis in breast cancer.

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