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Quantitative Analysis of BRCA1 and BRCA2 mRNA Expression in Sporadic Breast Carcinomas and Its Relationship with Clinicopathological Characteristics

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BRCA1 and BRCA2 mRNA expression in sporadic breast cancers was quantified by a real-time reverse transcriptase-polymerase chain reaction (RT-PCR), and the relationship of their expression with various clinicopathological factors was studied. BRCA2 mRNA levels (0.993±1.395, mean±SD (BRCA2/β-glucuronidase mRNA ratios)) were significantly ($P<0.01$) higher than BRCA1 mRNA levels (0.519±0.570 (BRCA1/β-glucuronidase mRNA ratios)), and a weak but significant ($r=0.390$, $P<0.01$) correlation was observed between BRCA1 and BRCA2 mRNA expression levels. There was no significant association between BRCA1 mRNA expression and clinicopathological factors such as menstrual status, tumor size, lymph node status, estrogen and progesterone receptor status, and histological grade. On the other hand, there was a significant association between higher BRCA2 mRNA expression and estrogen receptor (ER) negativity ($P<0.01$) or progesterone receptor (PR) negativity ($P<0.01$) or high histological grade ($P<0.01$). These results suggest a differential contribution of *BRCA1* and *BRCA2* in the pathogenesis of sporadic breast cancers. BRCA2 mRNA is speculated to be up-regulated in response to proliferation and genomic instability in high histological grade tumors.

Key words: Breast cancer — BRCA1 — BRCA2 — mRNA expression — RT-PCR

BRCA1 and *BRCA2* are well-established breast cancer susceptibility genes, which were cloned through linkage analysis using large breast cancer families.[1, 2] Germline mutations of these two genes account for 40–50% of site-specific breast cancer families,[3, 4] and life-time risk of breast cancers among *BRCA1* or *BRCA2* germline mutation carriers is reported to be 56%–85%.[5–7] Both behave as classical tumor suppressor genes, loss of both alleles being required for carcinogenesis. Although the functions of BRCA1 and BRCA2 are far from clear, recent studies have revealed that BRCA1 and BRCA2 form a complex with Rad51 and are involved in the repair of double-strand DNA breaks as well as mitotic and meiotic recombination. In addition, BRCA1 was shown to inhibit cell growth when it was transfected to breast cancer cells *in vitro* and *in vivo*.[8]

Somatic mutations of *BRCA1* and *BRCA2* seem to be a very rare event,[9–12] but loss of heterozygosity (LOH) is frequently observed at 17q12–q21 and 13q12–q13, suggesting implication of these two genes in the pathogenesis of breast cancers through not a structural, but a regulatory mutation or through hypermethylation of a promoter region, leading to attenuated transcription. It has been shown that BRCA1 mRNA levels are down-regulated in sporadic breast cancers as compared with the normal breast tissues, and hypermethylation of the promoter region of *BRCA1* explains this down-regulation in some sporadic breast cancers.[13, 14] On the other hand, BRCA2 mRNA levels do not show a consistent tendency, and may be either up-regulated or down-regulated among sporadic breast cancers.[15] Unlike BRCA1, the promoter region of BRCA2 has been shown not to be hypermethylated.[16] Thus, the contributions of BRCA1 and BRCA2 to the pathogenesis of sporadic breast cancers might be different, and still remain to be established.

In order to clarify further the roles of BRCA1 and BRCA2 in breast carcinogenesis, the association of BRCA1 and BRCA2 mRNA expression with various clinicopathological factors of sporadic breast cancers needs to be studied. Although a few reports are available on this issue,[15, 17] BRCA1 and BRCA2 mRNA expression levels were mostly examined by a semiquantitiatve reverse transcriptase-polymerase chain reaction (RT-PCR) assay,[15, 17] and BRCA1 and BRCA2 mRNA expression levels were never examined together in these reports. Thus, in the present study, we have attempted to quantify both BRCA1 and BRCA2 mRNA levels with high accuracy, taking advantage of a real-time PCR, and to compare their expression levels with various clinicopathological factors in sporadic breast cancers.

MATERIALS AND METHODS

Surgical specimens Breast tumor tissues were obtained at surgery from 107 female patients with invasive ductal carcinoma during the period from April 1997 to February 2000. The median age of the patients was 51 years old,

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ranging from 27 to 87 (59 premenopausal and 48 postmenopausal). Thirty-two and 75 patients had tumors ≤2 cm and ≥2 cm, respectively, and 58 and 43 patients had histological grade I+II and III tumors, respectively. Lymph node metastases were observed in 41 patients, and estrogen receptor (ER) positivity and progesterone receptor (PR) positivity were 62% and 56%, respectively. The surgical specimens were snap-frozen in liquid nitrogen and kept at −80°C until use. Informed consent was obtained from each patient.

RNA extraction Total cellular RNA was extracted from the surgical specimens and cultured cells using TRIZOL reagent according to the protocol provided by the manufacturer (Molecular Research Center, Cincinnati, OH). A 3 μg aliquot of total RNA was reverse-transcribed to single-strand cDNA using oligo-(dT)15 primer and Superscript II (Life Technologies, Inc., Rockville, MD) and scaled up to a final volume of 50 μl. RT reaction was performed at 42°C for 90 min, followed by heating at 70°C for 10 min.

Primers, probes, and real-time PCR The primers and probes for the *BRCA1* and *BRCA2* target genes were determined with the assistance of the computer programs Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA) which selected the theoretically optimized sequences for this system. Primer pairs were selected so that they were located in different exons (exons 22 and 23 for BRCA1 and exons 25 and 26 for BRCA2) in order to prevent the amplification of contaminating genomic DNA. The sequences of probes for BRCA1 and BRCA2 were 5′-CATCATTCACCCTTGGCACAGGTGT-3′, and 5′-TGATCCCAAGTGGTCCACCCCAAC-3′, respectively. Both probes were labeled with FAM as a fluorescent reporter. Amplification primer pairs were 5′-ACAGCTGTGTGGTGCTTCTGTG-3′ and 5′-CATTGTCCTCTGTCCAGGCATC-3′ for BRCA1; and 5′-CTTGCCCCTTTCGTCTATTTG-3′ and 5′-TACGGCCCTGAAGTACAGTCT-T-3′, for BRCA2, respectively. PCR reactions were carried out using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) in a 50 μl reaction mixture containing 1 μl of cDNA template, 25 μl of TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems), 0.1 μM probe, and 0.3 μM of each primer. The PCR conditions for BRCA1 and BRCA2 were as follows; after incubation at 50°C for 2 min and denaturing at 95°C for 10 min, 40 cycles of 95°C for 15 s, 61°C for 1 min. To quantify transcripts of the genes precisely, we monitored the β-glucuronidase transcripts as a quantitative control, and each sample was normalized with respect to its β-glucuronidase transcript content. The primer probe mixture for β-glucuronidase was purchased from Perkin-Elmer Applied Biosystems and the method followed the manufacturer's protocol. Briefly, a 50 μl reaction mixture containing 1 μl of cDNA template, 25 μl of TaqMan Universal PCR Master Mix, and 2.5 μl of primer probe mixture was amplified according to the following program: after incubation at 50°C for 2 min and denaturing at 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min.

The standard curves for BRCA1, BRCA2 and β-glucuronidase mRNA were generated using serially diluted solutions (10^{-4}–10^{-8}) of plasmid clones bearing inserted BRCA1, BRCA2 or β-glucuronidase cDNA as templates (Fig. 1A). The parameter C_t is the fractional cycle number at which the induced fluorescence signal resulting from cleavage of the probe exceeded the threshold level. The amount of target gene expression was calculated from the

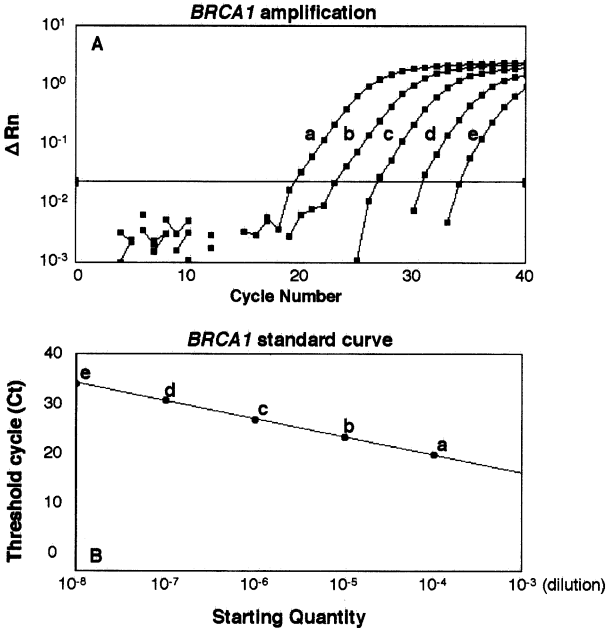

Fig. 1. BRCA1 standard curve obtained by real-time RT-PCR. A: Amplification plots. Samples containing five different dilutions (a: 10^{-4}, b: 10^{-5}, c: 10^{-6}, d: 10^{-7}, e: 10^{-8}) of standard plasmids for BRCA1 were subjected to real-time PCR. Cycle number is plotted versus change in normalized reporter signal (ΔR_n). For each reaction tube, the fluorescence signal of the reporter dye (the dye for BRCA1 and BRCA2 was FAM, and that for β-glucuronidase was VIC) was divided by the fluorescence signal of the passive reference dye (TAMRA), to obtain a ratio defined as the normalized reporter signal (R_n). ΔR_n represents the normalized reporter signal (R_n) minus the baseline signal established in the first 15 PCR cycles. ΔR_n increases during PCR as BRCA1 PCR product copy number increases until the reaction reaches a plateau. C_t represents the fractional cycle number at which a significant increase in R_n above the baseline (horizontal black line) can first be detected. Two replicates for each standard curve point sample were performed, but the data for only one are shown here. B: Standard curve plotting log starting copy number vs. C_t. Black dots represent data obtained from standard curve point samples.

standard curve (Fig. 1B), and quantitative normalization of cDNA in each sample was performed using the expression of *β-glucuronidase* gene as an internal control. Finally, BRCA1 and BRCA2 mRNA levels were shown as ratios to β-glucuronidase mRNA levels. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for the calculation of mRNA expression levels.

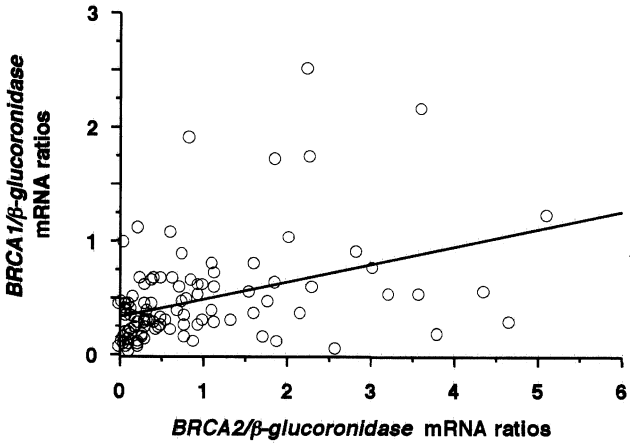

Fig. 2. Correlation between BRCA1 and BRCA2 mRNA levels. $y=0.328+0.153x$.

ER and PR assay Enzyme immunoassay was conducted for the measurement of ER and PR protein levels in breast cancers using the kit provided by Abbott Research Laboratories, Chicago, IL according to the manufacturer's instructions. The cut-off value for ER and PR was 5 fmol/mg protein.

Statistical methods BRCA1 and BRCA2 mRNA expression levels among various groups were compared by using Student's *t* test. Correlation between BRCA1 and BRCA2 mRNA levels was analyzed by a simple curve fit test. Statistical significance was assumed for $P<0.05$.

RESULTS

Relationship between BRCA1 and BRCA2 mRNA expression and clinicopathological factors of breast cancers BRCA2 mRNA levels (0.993±1.395, mean±SD) were significantly ($P<0.01$) higher than BRCA1 mRNA levels (0.519±0.570) in 107 breast cancers. A weak but significant correlation was observed between BRCA1 and BRCA2 mRNA levels ($r=0.390$, $P<0.01$) (Fig. 2).

There was no significant association between BRCA1 mRNA levels and any of the clinicopathological factors in Table I. BRCA2 mRNA levels of ER-negative and PR-negative tumors were significantly ($P<0.01$) higher than those of ER-positive and PR-positive tumors, respectively. In addition, histological grade III tumors showed a significantly ($P<0.01$) higher BRCA2 mRNA levels than histo-

Table I. Relationship between BRCA1 or BRCA2 mRNA Expression and Clinicopathological Factors

	BRCA1 mRNA levels	BRCA2 mRNA levels	BRCA1/BRCA2 mRNA ratios
Menstrual status			
Premenopausal (59)[a]	0.475±0.053[b]	1.031±0.145	1.249±0.335
Postmenopausal (48)	0.575±0.104	0.945±0.244	2.021±0.548
Tumor size			
≤2 cm (32)	0.395±0.070	0.896±0.225	1.867±0.740
>2 cm (75)	0.572±0.072	1.046±0.169	1.237±0.201
Lymph node status			
Negative (66)	0.482±0.059	0.881±0.184	2.047±0.479
Positive (41)	0.579±0.108	1.172±0.189	0.860±0.178
Estrogen receptor			
Negative (40)	0.505±0.093	1.451±0.294[c]	1.265±0.566
Positive (65)	0.523±0.071	0.701±0.117	1.825±0.366
Progesterone receptor			
Negative (46)	0.590±0.096	1.521±0.274[c]	1.384±0.504
Positive (59)	0.458±0.065	0.570±0.089	1.790±0.395
Histological grade			
I+II (58)	0.493±0.075	0.582±0.088[c]	2.215±0.543
III (43)	0.606±0.089	1.593±0.289	0.970±0.186

a) Number of patients.
b) Mean±SE.
c) $P<0.01$.

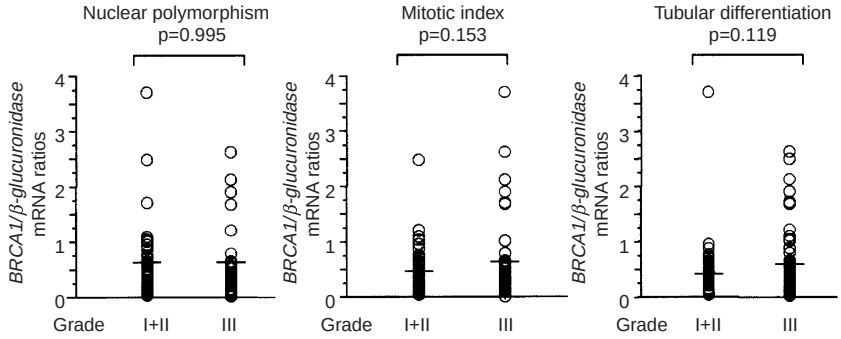

Fig. 3. Relationship between BRCA1 mRNA expression and three components (nuclear polymorphism, mitotic index, tubular differentiation) of histological grade.

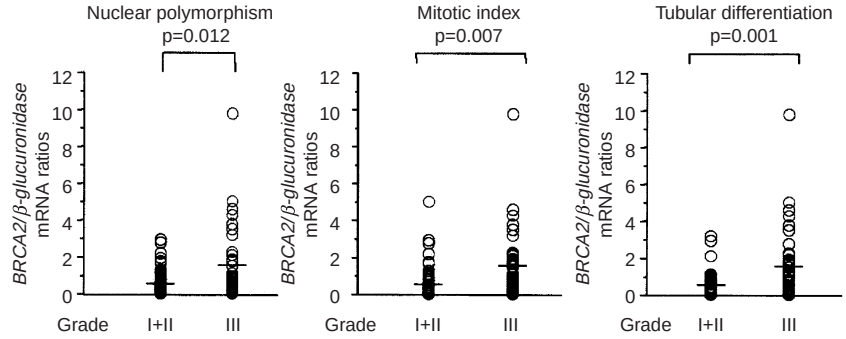

Fig. 4. Relationship between BRCA2 mRNA expression and three components (nuclear polymorphism, mitotic index, tubular differentiation) of histological grade.

logical grade I and II tumors. There was no significant association between BRCA1/BRCA2 mRNA ratios and any of the clinicopathological factors.

Relationship between BRCA1 and BRCA2 mRNA expression and three components of histological grade

Since a significant association was observed between BRCA2 mRNA expression and histological grade, a further analysis was done to elucidate which of the three components (nuclear polymorphism, mitotic index, tubular differentiation) used for the determination of histological grade was most related to BRCA2 mRNA expression. BRCA1 mRNA expression was not significantly related to any of the three components (Fig. 3) but BRCA2 mRNA expression was significantly related to every component (Fig. 4).

DISCUSSION

BRCA1 is a typical tumor suppressor gene which requires the inactivation of both alleles for carcinogenesis. Actually, it has been shown that a wild allele is lost in almost all breast tumors arising in *BRCA1* germline mutation carriers.[18] Several studies including our own[19] have shown that *BRCA1*-associated hereditary breast cancers have some distinctive pathobiological phenotypes, being characterized by low ER positivity and high histological grade.[20, 21] This phenotype is considered to result from the complete loss of BRCA1 function in the tumors. Although somatic mutation of *BRCA1* is a very rare event, down-regulation of BRCA1 mRNA as well as BRCA1 protein has been reported in majority of sporadic breast cancers,[13, 22, 23] suggesting a possible involvement of BRCA1 in pathogenesis of these cancers.

Phenotype of sporadic breast cancers according to the expression status of BRCA1 mRNA has rarely been reported. One distinctive histological characteristic of *BRCA1*-associated hereditary breast cancers is a high incidence of histological grade III tumors.[19] However, we could not demonstrate such an association in sporadic breast cancers in the present study based on BRCA1 mRNA levels, or in the previous study based on BRCA1 protein levels examined by immunohistochemistry.[24] Seery *et al.* also failed to demonstrate an association between lower BRCA1 mRNA expression and high histological grade in sporadic breast cancers.[17] Since a very

high proportion of *BRCA1*-associated hereditary breast cancers have p53 abnormality, we speculate that down-regulation of BRCA1 mRNA alone might not be enough for the development of high histological grade tumors, and additional gene alterations such as p53 abnormality might be necessary. Recently, we have been able to demonstrate that BRCA1 down-regulation and p53 abnormality work synergistically to induce chromosomal instability in sporadic breast cancers.[25)]

BRCA2 is also a typical tumor suppressor gene, and loss of a wild allele has been reported in almost all breast tumors arising in *BRCA2* germline mutation carriers.[26)] As opposed to *BRCA1*-associated hereditary breast cancers, no distinctive phenotypes of *BRCA2*-associated hereditary breast cancers have been reported except for a slight increase in the incidence of lobular carcinomas,[27, 28)] but we could not confirm such an increase in our previous study.[19)] Although BRCA1 mRNA expression is reported to be down-regulated in almost all sporadic breast cancers as compared with the normal breast tissues, BRCA2 mRNA expression is reported to be up-regulated in a significant proportion of sporadic breast cancers.[15)] Our present observation that BRCA2 mRNA levels were significantly higher than BRCA1 mRNA expression levels seems to be consistent with this report. Bieche *et al.* (1999) showed that up-regulation of BRCA2 mRNA was significantly associated with high histological grade tumors, and we obtained the same result. Each of three components, including mitotic index, used for determination of histological grade was significantly associated with higher BRCA2 mRNA expression (Fig. 4). These results are consistent with a model in which BRCA2 exerts a positive effect on proliferation. Such a model, however, is very unlikely to be reconcilable with the fact that *BRCA2* is a tumor suppressor gene which should inhibit proliferation. One possible explanation for this inconsistency is that BRCA2 mRNA expression is induced by proliferation. BRCA2 mRNA expression was shown to be induced by proliferation,[29, 30)] and, thus, it is possible that, if the regulation of BRCA2 mRNA expression is intact in tumors, a high proliferation might result in the up-regulation of BRCA2 mRNA levels. Association between nuclear polymorphism and higher BRCA2 mRNA expression can also be explained by the similar thesis that genomic instability (nuclear polymorphism) induces BRCA2 mRNA expression due to the need for BRCA2 for DNA repair.

BRCA1 and BRCA2 mRNA expression was shown to be induced by estrogens through ER in cell culture.[31)] However, we found that BRCA1 mRNA expression was not associated with ER positivity and BRCA2 mRNA expression was significantly associated with ER negativity, suggesting that estrogen-dependent regulation of BRCA1 and BRCA2 mRNA expression might be impaired or some other regulatory mechanism of these genes might be predominantly operative. Another possible explanation is that an association between BRCA2 mRNA expression and ER negativity merely reflects an association between BRCA2 mRNA expression and high histological grade, since ER-negative tumors are more likely to be high histological grade tumors.

The relationship of BRCA1 and BRCA2 mRNA (or protein) expression with LOH at *BRCA1* and *BRCA2* loci, respectively, has been studied by several investigators.[15, 22, 32–34)] Although consistent results have not been obtained, most studies have failed to show a clear relationship between BRCA1 and BRCA2 mRNA (or protein) expression and LOH,[15, 32–34)] suggesting that some other regulatory mechanism, such as methylation of the promoter region of *BRCA1*, might play a more important role. It is well established that frequency of LOH of the *BRCA2* locus is increased in histological grade III tumors as compared with grade I and II tumors.[35)] Thus, if LOH plays an important role in down-regulation of BRCA2 mRNA levels, BRCA2 mRNA levels in histological grade III tumors should be lower than those in histological grade I and II tumors. However, on the contrary, we found that BRCA2 mRNA levels in histological grade III tumors were significantly higher than those in grade I and II tumors. These findings seem to indicate that LOH is unlikely to play an important role in the regulation of BRCA2 mRNA expression in sporadic breast tumors.

In conclusion, our results have suggested a differential contribution of *BRCA1* and *BRCA2* in the pathogenesis of sporadic breast cancers. BRCA2 mRNA is speculated to be up-regulated in response to proliferation and genomic instability in high histological grade tumors. The present results on *BRCA2* need to be confirmed at the protein level after suitable antibodies become available, and the genetic (epigenetic) mechanism of dysregulation of BRCA1 and BRCA2 mRNA expression also remains to be studied.

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