BRCA1 Expression Status in Relation to DNA Methylation of the *BRCA1* Promoter Region in Sporadic Breast Cancers

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To understand the biological role of BRCA1 in sporadic breast cancers, the relationship between DNA methylation of the *BRCA1* promoter region and BRCA1 expression was studied using molecular biological and immunohistochemical methods. Furthermore, BRCA1 expression was compared with the expression of various cell cycle regulatory proteins and the morphological nuclear grade of cancer cells. Of 32 sporadic breast cancers investigated in this study, 10 (31%) revealed DNA methylation of the *BRCA1* promoter region. The expression of BRCA1 was observed in the nuclei of cancer cells and 18 (56%) of 32 cancers were positive for BRCA1 immunoreactivity. Breast cancers with *BRCA1* methylation lacked BRCA1 expression, except for only three cancers, and there was a significant inverse relationship between *BRCA1* methylation and its expression in sporadic breast cancers ($P=0.043$). Compared with the expression of various cell cycle regulatory proteins, breast cancers with *BRCA1* methylation showed decreased expression of estrogen receptor ($P=0.016$) and p27 ($P=0.018$) and increased expression of p21 ($P=0.011$). Furthermore, breast cancers without BRCA1 expression or with *BRCA1* methylation had a tendency to contain nuclei with higher grade. These findings indicate that *BRCA1* methylation might greatly influence its expression and BRCA1 expression might play an important role in cell cycle regulation and influence the grade of malignancy of sporadic breast cancers.

Key words: BRCA1 — Expression — Immunohistochemistry — DNA methylation — Sporadic breast cancers

BRCA1 has been identified by positioning cloning methods as a strong candidate for the 17q-linked gene for familial breast cancers and has been linked to more than 45% of familial breast cancers and 80% of families with breast and ovarian cancer.[1] *BRCA1* is a putative tumor suppressor gene located on chromosome 17q21 and spans 100 kb of genomic DNA, which encodes a protein of 220 kD consisting of 1863 amino acids.[1] The BRCA1 protein is mainly localized in cell nuclei and is phosphorylated in a cell cycle-dependent manner.[2] As regards subcellular nuclear localization, the BRCA1 protein has been reported to colocalize with Rad51, a homologue of bacterial RecA, in S phase cells,[3] which suggests a role for BRCA1 in the control of recombination and of genome integrity. However, the exact biological function of BRCA1 still remains unclear.

More than 300 germline mutations have been identified so far in patients with familial breast and/or ovarian cancer.[1, 4] These mutations are distributed across the entire coding region of the *BRCA1* gene, and the majority is predicted to result in truncated proteins or loss of a BRCA1 transcript. Therefore, mutations in the *BRCA1* gene may play a significant role in the tumorigenesis of familial breast cancer.[5, 6] Though it has been shown that BRCA1

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mRNA levels are reduced or absent in both sporadic and familial breast cancer,[7, 8] a few somatic mutations in the *BRCA1* gene have been identified in sporadic breast cancers.[9] From these studies, the decreased expression of BRCA1 in sporadic breast cancer is thought to be caused by mechanisms other than somatic mutation of *BRCA1*.

Recently, it has become clear that gene expression is regulated by CpG methylation in the promoter region, for instance, of several tumor suppressor genes *Rb*, *p15*, *p16*, and *VHL*.[10–14] In 1997, Dobrovic and Simpfendorfer first reported methylation of the *BRCA1* gene in sporadic breast cancers.[15] The following year, Mancini *et al*. reported that CpG methylation of the *BRCA1* gene was tumor-specific and involved a putative CREB binding site.[16] These findings have suggested that decreased expression of BRCA1 in sporadic breast cancer might be attributed to down-regulation by CpG methylation in the 5′ regulatory region of the *BRCA1* gene. However, there is still controversy over the occurrence of inactivation of BRCA1 by hypermethylation of its promoter.[17]

In the present study, the relationship between BRCA1 protein expression and methylation status of the *BRCA1* gene was investigated in 32 cases of sporadic breast cancer. In order to know the function of BRCA1 protein, the expression status of several cell cycle regulatory proteins was immunohistochemically studied in these breast can-

cers.[18] Then the expression of the BRCA1 protein was compared with immunohistochemical findings of cyclin D1, p53, p16, p21 and p27, in addition to the expression of estrogen receptor and the histological nuclear grade of breast cancer.

MATERIALS AND METHODS

Materials Thirty-two breast cancer patients without any family history of breast cancer were randomly selected and used in this study. Breast cancer patients underwent surgery at Gunma University Hospital and Motojima General Hospital (Ohta, Gunma), and both cancerous and non-cancerous breast tissues were freshly obtained at operation and kept frozen at $-80°C$ until the *BRCA1* gene methylation analysis. The remaining breast cancer tissues were fixed in 15% formalin and embedded in paraffin for routine pathological examination and various immuno-histochemical studies. Using hematoxylin eosin-stained slides, the nuclear grade of all breast cancers was re-evaluated based on the histological criteria for breast carcinoma of the Japanese National Surgical Adjuvant Study of Breast Cancer (NSAS-BC), Pathology Section.[19, 20]

DNA methylation analysis of the *BRCA1* gene From 32 frozen cancerous and non-cancerous tissues, each DNA was purified using the conventional phenol-chloroform method after homogenization and proteinase K digestion. The amount of DNA was quantified by absorbance measurements at 260 nm (UV). For the detection of methylation status in the *BRCA1* gene, each DNA obtained from both cancerous and non-cancerous tissues was digested with *Hha*I (TaKaRa, Otsu, Shiga) overnight, followed by amplification of the *BRCA1* gene using polymerase chain reaction (PCR), as illustrated in Fig.1.[21] As a negative control of DNA methylation in the *BRCA1* gene, purified DNA from MCF7 breast cancer cells was used in this study. The primer pair for the amplification of the *BRCA1* gene (Fig. 1) was as follows: sense primer (1695 to 1675) 5′-AATCCAGAGCCCCGAGAGACGC-3′; anti-sense primer

(1436 to 1456) 5′-GGTTTCCGTGGCAACGGAAAAGCG-CG-3′. Total volume of the PCR mixture was 10 μl, containing 10 ng of template DNA, 200 μM of each deoxyribonucleoside triphosphate, 1 μM of each primer, 1 μl of 10× PCR buffer (Perkin Elmer, Branchburg, NJ) and 1 μl (5 U) of AmpliTaq DNA Polymerase (Perkin Elmer) and the PCR reaction was carried out for 28 cycles under the following conditions: 94°C/30 s for denaturing, 60°C/30 s for annealing and 72°C/30 s for extension after the first denaturation at 94°C for 1 min, and final extension was performed at 72°C for 7 min. The PCR products were electrophoresed on 8% polyacrylamide gel and the bands were visualized using ethidium bromide staining. This DNA fragment of 259 bp contained three methylation-sensitive *Hha*I sites (Fig. 1). To examine whether the amplified PCR product was exactly the same as the 5′ prime region of the *BRCA1* gene, the amplified 259 bp fragment was digested by two restriction enzymes, *Sac*I and *Afa*I, whose sites are located in this DNA region. The PCR products digested by *Sac*I and *Afa*I showed 168 and 205 bp bands on electrophoresis, respectively (Fig. 2). The methylation status in the *BRCA1* gene was determined by observing whether the band specific to the *BRCA1* gene could be amplified by PCR after digestion of breast cancer DNA with *Hha*I restriction enzyme.

Immunohistochemistry For the immunohistochemical study, 3 μm thick sections were cut from paraffin blocks which contained representative histology of the breast cancer. Paraffin sections on silane-coated slides were dewaxed with xylene and rehydrated through a graded alcohol series. Then, endogenous peroxidase activity was blocked in absolute methanol solution containing 0.3% hydrogen peroxide for 30 min and the slides were washed in 10 mM

Fig. 2. PCR amplification of the promoter region and exon 1a of *BRCA1* containing *Afa*I and *Sac*I sites. PCR product was shown to contain the promoter region and exon 1a of *BRCA1*. M: DNA size marker (ψ174/*Hae*III). Lane 1: The PCR product of the promoter region and exon 1a of *BRCA1* was 259 bp in size. Lane 2: After digestion of the 259 bp PCR product by *Afa*I, the product was shown to be 205 bp in size. Lane 3: After digestion of 259 bp PCR product by *Sac*I, the product was shown to be 168 bp in size.
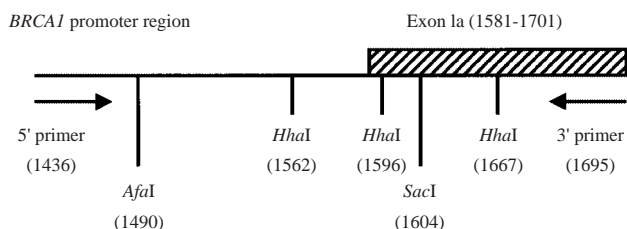

Fig. 1. CpG and restriction sites in the promoter region and exon 1a of *BRCA1*. Cytosine methylation was analyzed in this region by *Hha*I digestion and PCR amplification. ▨ exon 1a, → ← 5′ and 3′ primers.
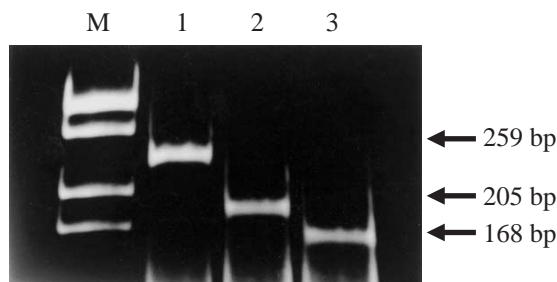
phosphate-buffered saline (PBS), pH 7.4. For antigen retrieval, they were immersed in appropriate solutions such as 1 mM EDTA, 0.1 mM citrate-phosphate buffer or 20% zinc sulfate solution, and microwaved at 90°C for 10 min (Bio-Rad Laboratories, Hercules, CA). After the buffer had cooled, normal horse serum was reacted with the slides for 30 min to eliminate non-specific immuno-staining. Then the slides were reacted with various pri-mary antibodies overnight at 4°C in a humidified chamber. The details of the primary antibodies used in this study were as follows; monoclonal antibody (mAb) Ab-1 for BRCA1 with a dilution of 1:50 (Oncogene Research Products Calbiochem, Cambridge, UK), mAb for estro-gen receptor (ER) with a dilution of 1:8 (Immunotech, Marseille, France), mAb DO7 for p53 protein with a dilu-tion of 1:100 (Novocastra, Newcastle, UK), mAb NCL-WAF1 for p21 with a dilution of 1:40 (Novocastra), mAb cyclin D1 with a dilution of 1:50 (Novocastra), mAb p27 with a dilution of 1:5000 (Transduction Lab., Lexington, KY) and mAb JC8 for p16 with a dilution of 1:500 (kindly provided by Dr. J. Koh, Massachusetts General Hospital). After the reaction of each primary antibody, the slides were reacted with biotinylated anti-mouse antibody for 30 min at room temperature followed by streptavidin with a dilution of 1:500 (Dako, Glostrup, Denmark). Finally, the slides were stained using the 3,3′-diaminobenzidine tet-rahydrochloride solution and were counterstained lightly using hematoxylin.

For immunohistochemical evaluation of BRCA1, nuclear immunostaining of tumor cells was compared with that of normal mammary duct cells. If more than 50% of tumor cell nuclei showed the same or stronger immunore-activity than that of normal mammary duct cells, it was evaluated as positive (+). For each immunohistochemical evaluation of p53, p21, p27, the frequency of nuclear immunostaining, negative or less than 5%, 5% to 50% and more than 50% were evaluated as negative (−), positive (+) and strongly positive (++), respectively. In p16 and cyclin D1, more than 50% of tumor cells with nuclear staining were evaluated as positive (+) and in ER, more than 10% of tumor cells with nuclear staining were evalu-ated as positive (+).

Statistical analysis All statistical analyses were per-formed using the χ^2 test. A P value less than 0.05 was considered to be statistically significant.

RESULTS

DNA methylation analysis of the *BRCA1* gene As shown in Fig. 1, the PCR system could amplify a 259 bp DNA fragment containing the 5′ prime region of the *BRCA1* gene, including its promoter area.

The PCR system amplified the 259 bp fragment band from all DNA samples purified from cancerous and non-

Fig. 3.   Methylation analyses of the promoter region and exon 1a of *BRCA1* using PCR after *Hha*I digestion. Lanes 1–2: DNAs from MCF7, digested with *Hha*I (C) and not digested with *Hha*I (U). Lanes 3–6: Case without *BRCA1* methylation (case No.22). Lanes 4, 6: DNAs digested with the methylation-sensitive enzyme *Hha*I (C). Lanes 3, 5: DNAs not digested with *Hha*I (U). Lanes 3, 4: Normal breast tissue (N). Lanes 5, 6: Breast cancer tissue (T). Lanes 7–10: Case with *BRCA1* methylation (case No.5). Lanes 8, 10: DNAs digested with *Hha*I (C). Lanes 7, 9: DNAs not digested with *Hha*I (U). Lanes 7, 8: Normal breast tis-sue (N). Lanes 9, 10: Breast cancer tissue (T). PCR products were present at 259 bp in lanes 1, 3, 5, 7, 9 and 10. M: DNA size marker (ψ174/*Hae*III).

cancerous breast tissues without digestion by *Hha*I (Fig. 3). To detect the methylation status of the *BRCA1* gene, the PCR was carried out on DNAs purified from cancer-ous and non-cancerous breast tissues after *Hha*I digestion. The PCR failed to detect any 259 bp band using DNAs from non-cancerous lesions in all cases and DNAs from cancerous lesions in 22 cases after *Hha*I digestion. In 10 breast cancer cases, however, the PCR amplified a 259 bp band using *Hha*I-digested DNA purified from only cancer tissues (Fig. 3 and Table I). Using DNA samples from the MCF7 cell line, the PCR failed to amplify the 259 bp band after *Hha*I digestion (Fig. 3).

Results of immunohistochemical studies and histologi-cal nuclear grade All immunohistochemical findings are summarized in Table I. Using Ab-1 antibody, immunore-activity for BRCA1 was observed in cell nuclei of normal mammary ducts and glands. In breast cancer cells, BRCA1 immunoreactivity was also seen in cancer cell nuclei and the intensity varied from completely negative to strongly positive (Fig. 4, a and b). According to the criteria for BRCA1 immunohistochemical evaluation, 18 of 32 breast cancers (56%) were evaluated as positive for BRCA1 expression. The immunoreactivity for ER, p53, p21 and p27 was exclusively seen in breast cancer nuclei and the frequencies of immunopositivity for these parameters were 59%, 34%, 47% and 69%, respectively. However, immu-

Table I. Methylation Status of the *BRCA1* Promoter Region and Immunohistochemical Expression of BRCA1, p21, p53, p27, ER, Cyclin D1, p16 and Nuclear Grade

Case number	Methylation of the *BRCA1*	BRCA1 expression	p53	p21	p27	ER	Cyclin D1	p16	Histology/ nuclear grade
1	+	−	−	++	++	+	+	−	IDC3
2	+	−	−	+	+	+	+	−	IDC3
3	+	−	−	+	++	−	−	+	IDC3
4	+	−	+	−	−	−	−	+	IDC3
5	+	−	−	−	−	−	+	−	IDC3
6	+	−	−	+	−	−	+	−	IDC2
7	+	−	+	+	−	−	+	+	IDC2
8	+	+	−	+	+	+	+	−	IDC3
9	+	+	−	+	−	+	−	−	IDC3
10	+	+	−	+	−	+	+	−	IDC3
11	−	+	−	+	+	−	−	+	IDC3
12	−	+	+	+	+	−	+	+	IDC3
13	−	+	−	−	+	+	+	−	IDC3
14	−	+	−	−	+	+	−	−	IDC2
15	−	+	−	+	+	+	−	+	IDC2
16	−	+	−	+	++	+	+	+	IDC2
17	−	+	+	−	−	−	−	+	IDC2
18	−	+	−	−	++	+	−	+	IDC2
19	−	+	−	−	++	+	+	+	IDC2
20	−	+	+	+	+	−	−	−	IDC2
21	−	+	+	−	++	+	+	−	IDC2
22	−	+	−	−	−	+	+	−	IDC2
23	−	+	+	−	++	+	+	+	IDC2
24	−	+	−	−	+	+	−	−	IDC1
25	−	+	−	−	++	+	+	−	IDC1
26	−	−	+	−	−	−	−	+	IDC3
27	−	−	−	−	+	−	−	+	IDC3
28	−	−	+	−	++	+	+	−	IDC3
29	−	−	−	+	+	+	+	+	IDC3
30	−	−	−	−	−	−	−	−	IDC3
31	−	−	+	−	+	−	−	+	IDC2
32	−	−	+	+	+	+	+	+	IDC2

++, strongly positive; +, positive; −, negative; IDC, invasive ductal carcinoma.

noreactivity for cyclin D1 and p16 was observed in both the cytoplasm and nuclei of the tumor cells with frequencies of 56% and 50%, respectively.

Of 32 breast cancers, 15 were evaluated as histological nuclear grade 3, and the remaining ones were composed of 13 cancers with nuclear grade 2 and two with nuclear grade 1.

Relation among *BRCA1* methylation, its expression, the status of ER, and various cell cycle associated proteins and the morphological nuclear grade As shown in Table I, *BRCA1* methylation was inversely related to its expression in breast cancer cases investigated in this study ($P=0.043$). According to the combination of *BRCA1* methylation and its expression, breast cancer cases were roughly subdivided into three groups: the first group was positive for *BRCA1* methylation and negative for BRCA1 expression, the second was negative for *BRCA1* methylation and positive for BRCA1 expression and the third was negative for both *BRCA1* methylation and its expression. However, three exceptional cancers in the first group were positive for *BRCA1* methylation and expression. Breast cancers negative for *BRCA1* methylation tended to contain less atypical nuclei than nuclear grade 2, and a similar tendency was observed in breast cancers with BRCA1 expression. Moreover, *BRCA1* methylation was related to the expression of p21 and inversely related to p27 expression, and BRCA1 expression was related to ER expression ($P=0.011$, 0.018 and 0.016, respectively). The status of cyclin D1 and p16 expressions showed an inverse relation, but p53 expression showed no significant relationship with

BRCA1 methylation or expression, or with the expression of other cell cycle regulatory proteins.

DISCUSSION

Although *BRCA1* mutations are responsible for nearly half of familial breast cancers, *BRCA1* mutation is rarely detected in sporadic breast cancers or is only rarely found in ovarian cancers.[7, 9] However, BRCA1 expression has been reported to be markedly reduced in sporadic breast cancers, suggesting that BRCA1 expression might be down-regulated by mechanisms other than point mutation. In 1997, Dobrovic and Simpfendorfer first reported hypermethylation of the *BRCA1* gene in two of 7 sporadic breast cancers using Southern blot analysis after methylation-sensitive restriction enzyme treatment.[15] In a large group of sporadic breast cancers, Catteau *et al.* found the frequency of *BRCA1* methylation to be 11% using Southern blot analysis.[22] In 1998, aberrant CpG methylation within the 5′ regulatory region of the *BRCA1* gene was confirmed by new methodology using PCR amplification and sequencing after sodium bisulfate modification.[16, 23] Moreover, Mancini *et al.* reported that CpG methylation of the *BRCA1* gene was tumor-specific and was located in a putative CREB binding site of the gene.[16] In the present study using molecular biological techniques different from Southern blot hybridization, *BRCA1* methylation was detected in 10 (31%) of 32 sporadic breast cancers, which is the highest frequency so far reported.

However, the *BRCA1* mRNA levels were significantly down-regulated in sporadic breast cancers compared with normal breast tissues.[8, 17] To clarify the expression of the *BRCA1* gene at the protein level in sporadic breast cancers, several immunohistochemical studies have been performed,[2, 24, 25] but no conclusion has yet been drawn. Even the subcellular localization of the BRCA1 protein is still controversial, i.e., whether it is localized in the cytoplasm or in nuclei of normal and neoplastic breast tissues. In 1998, Jarvis *et al.* reported that loss of nuclear BRCA1 expression in breast cancers is associated with a more aggressive tumor phenotype.[25] Very recently, Wilson *et al.*[24] comprehensively characterized 19 anti-BRCA1 antibodies and concluded that Ab-1 antibody is the only reliable antibody when working on paraffin sections with antigen retrieval and an amplified staining procedure. According to their immunohistochemical study using various antibodies for BRCA1, immunoreactivity specific for BRCA1 is likely to be localized exclusively in the nuclei of normal and neoplastic breast tissues. Of 79 formalin-fixed, paraffin-embedded breast cancers, 52 tumors (65.8%) were positively stained using Ab-1 antibody for BRCA1. Our immunohistochemical findings using 32 paraffin-embedded breast cancers and Ab-1 antibody corroborate Wilson's report.[24] In addition, a previous study
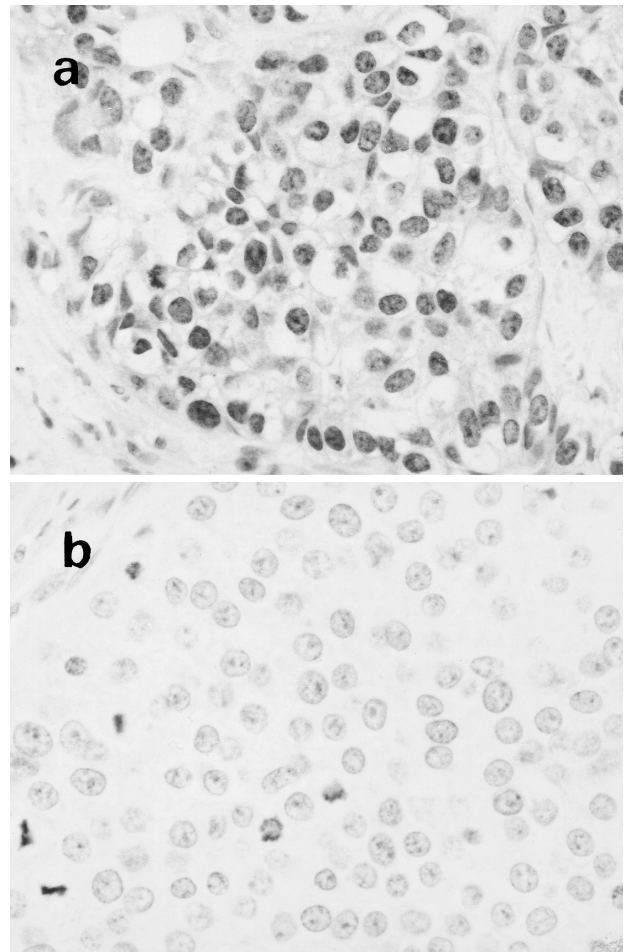
Fig. 4. Immunohistochemistry for BRCA1 in sporadic breast cancers. a) Most tumor cells showed diffuse nuclear staining for Ab-1 (case No. 13). ABC method ×400. b) None of the tumor cells showed immunoreactivity for Ab-1 (case No. 4). Many mitotic figures are apparent. ABC method ×400.

attempted to detect BRCA1 immunoreactivity using paraffin sections and commercially available antibodies (D-20, C-20 and 17F8), but the immunohistochemical findings were unreliable, unstable and inconclusive (data not shown).

Magdinier *et al.* attempted to detect the relationship between *BRCA1* methylation and its expression in 37 sporadic breast cancers. They found marked down-regulation of *BRCA1* mRNA, but failed to detect any hypermethylation patterns in the *BRCA1* gene.[17] Using breast cancer cell lines, Rice *et al.* reported decreased *BRCA1* mRNA owing to aberrant methylation of the *BRCA1* CpG island promoter.[23] Our study first revealed the inverse relationship between *BRCA1* methylation and its expression in a large sample of sporadic breast cancers. As shown in

Table I, sporadic breast cancers can be subdivided into three groups according to the status of *BRCA1* methylation and its expression. In the first group, expression of the *BRCA1* gene is down-regulated by hypermethylation of the putative promoter region, as in tumor suppressor genes such as *Rb*, *p16*, *p15* and *VHL*. Exceptionally, three cancers were positive for both *BRCA1* methylation and its expression, but this phenomenon may be explained by differences of aberrant CpG methylation in the promoter region. In the second group, the *BRCA1* gene was biologically intact. In the third group, point mutation of the *BRCA1* gene may be responsible for decreasing BRCA1 expression, as seen in familial breast cancers. However, *BRCA1* somatic mutations are rarely found in sporadic breast cancers, so that other mechanisms may account for the reduced expression of the *BRCA1* gene. Recently, Ozcelik *et al.* reported that deletion at the BRCA1 locus and preferential allelic expression were observed in some sporadic breast cancers with low *BRCA1* mRNA levels.[26] It remains for future investigations to clarify the true mechanisms that reduce or silence BRCA1 expression in the third breast cancer group.

Several previous studies have revealed that decreased BRCA1 expression is closely related to highly proliferative or aggressive phenotypes in sporadic breast cancers. In invasive ductal carcinomas, Wilson *et al.* have clearly shown a significant inverse relationship between immunohistochemical grades of BRCA1 expression and histopathological grades of cancers.[24] Comparing the state of *BRCA1* methylation and its expression with nuclear grade of cancer cells in this study, we found that decreased BRCA1 expression in cancer cells is closely related to higher tumor nuclear grade, which corroborates previous reports.[22, 24, 27] In breast cancers, the majority of aggressive tumors is also devoid of ER expression. Evidence for estrogen regulation of BRCA1 is obvious in the mammary gland during puberty, pregnancy and lactation.[28] Recent advances in molecular endocrinology have revealed that estrogen may regulate BRCA1 expression via protein-protein interaction between ER and the c-Fos·Jun complex at the AP1 site, because there are many transcriptional regulatory sites, including the AP1 binding site, in the *BRCA1* promoter region. Therefore, it is reasonable that several studies, including the present study, show related changes of expression of the *BRCA1* and *ER* genes in sporadic breast cancers.[18, 21, 28]

BRCA1 is a putative tumor suppressor protein and is known to be expressed and phosphorylated in a cell cycle-dependent manner, suggesting that BRCA1 might be closely associated with cell cycle regulation.[2, 28, 29] In the present study, the possible relationship between BRCA1 and several cell cycle regulatory proteins was investigated. The findings of the present study show that *BRCA1* methylation tends to be proportionally and inversely related to p27 and p21, respectively. From the viewpoint of cell cycle arrest for breast cancers, the associated expression of BRCA1 and p27 reflects less aggressive phenotypes. The relationship between *BRCA1* methylation and decreased expression of p27 in this study might reflect BRCA1 participation in cell cycle regulation by Cdk inhibitors. However, p21 expression has been frequently found in breast cancers with *BRCA1* methylation. In sporadic breast tumors, Sourvinos and Spandidos have already shown that decreased BRCA1 expression levels are associated with over-expression of both p21 and MDM-2, which results in up-regulation of p21, leading to cell cycle arrest of tumor cells.[18] The present study failed to find relationships between BRCA1, p53, cyclin D1 and p16, but an inverse relation between cyclin D1 and p16 expression was clearly demonstrated, as previously reported.[30, 31] More studies are necessary to clarify the relationship between BRCA1 and Cdk inhibitors.

In conclusion, the present study confirms that the *BRCA1* promoter region is abnormally methylated in sporadic breast cancers and an inverse correlation was found between methylation and protein expression of *BRCA1*. Moreover, decreased BRCA1 expression is closely related to the aggressive phenotype of sporadic breast cancers.

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