**Advances in Brief**

**Reduction of BRCA1 Protein Expression in Japanese Sporadic Breast Carcinomas and Its Frequent Loss in BRCA1-associated Cases**

Kiyotsugu Yoshikawa, Kazuo Honda, Takashi Inamoto, Hisashi Shinohara, Akira Yamauchi, Kenji Suga, Takazo Okuyama, Toshihide Shimada, Hiroshi Kodama, Shinzaburo Noguchi, Adi F. Gazdar, Yoshiro Yamaoka, and Rei Takahashi

Departments of Pathology and Tumor Biology [K. Y., H. S., K. S., T. O., T. S., R. T.] and Gastroenterological Surgery [K. Y., H. S., A. Y., Y. Y.], and Division of Science of Nursing [T. I.], Kyoto University Graduate School of Medicine, Konoe-cho, Yoshida, Sakyo-ku, Kyoto 606, Japan; First Department of Surgery, Ehime University Graduate School of Medicine, Onsen-gun, Ehime 791-02, Japan [K. H.]; Kodama Breast Clinic, Kitanokamihakubai-cho, Kita-ku, Kyoto 606, Japan [H. K.]; Department of Surgical Oncology, Osaka University Graduate School of Medicine, Yamadagaoka, Suita, Osaka 565, Japan [S. N.]; and Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas 75235 [A. F. G.]

**Abstract**

**BRCA1** is a tumor suppressor gene that is responsible for hereditary breast and ovarian cancer syndrome. To clarify the possible involvement of the BRCA1 protein in mammary carcinogenesis in sporadic and hereditary forms, we have analyzed the BRCA1 protein expression pattern in five breast epithelial cell lines, including a BRCA1-deficient cell line, and 162 breast cancer tissue samples [including 108 sporadic, 35 hereditary (BRCA1 status unknown), and 19 BRCA1-associated cases] from Japanese women. Twelve anti-BRCA1 antibodies were tested by fixation conditions, in which nuclear localization of BRCA1 protein was preserved, and by specificity of the antibodies, which was evaluated in BRCA1-deficient cancer cells. Using monoclonal antibodies applicable to immunohistochemical analysis of paraffin-embedded tissue sections, we found high-level expression of BRCA1 protein in normal mammary epithelium and various degrees of reduced expression in breast cancer cells. Of the 19 BRCA1-associated breast cancer tissues, 15 (79%) showed reduction (8 cases) or complete loss (7 cases) of nuclear expression. Thirty (28%) of 108 sporadic and 6 (17%) of 35 hereditary carcinomas showed reduced BRCA1 protein expression. Reduction of BRCA1 protein expression in sporadic carcinomas was associated with solid-tubular phenotype, with poor tubular differentiation, and with an overexpression of c-erbB-2 protein, which is one of the prognostic factors in breast cancer. Our data suggest that reduced expression of BRCA1 protein may play an important role in mammary carcinogenesis, not only in BRCA1-associated breast carcinomas, but also in sporadic carcinomas, and also suggest that mechanisms other than mutation may be involved in its reduced expression.

**Introduction**

Breast cancer is one of the most common malignancies among women, and its cumulative risk by age 85 is 1 in 8 women in the United States and 1 in 40 women in Japan (1). The BRCA1 gene, which is responsible for hereditary breast and ovarian cancer, has been identified by positional cloning (2). The frequency of BRCA1 mutations in serial Japanese breast cancer patients has been estimated to be about 1%, which is less than that of United States patients (3, 4). Many tumors with germ line BRCA1 mutations display loss of heterozygosity at this locus, which uniformly involves loss of the wild-type BRCA1 allele (5), indicating the role of the BRCA1 gene as a tumor suppressor gene. Although no somatic mutation has been detected, loss of heterozygosity, decreased levels of the BRCA1 mRNA (6, 7), and methylation of the BRCA1 promoter region (8–10) have been demonstrated in some sporadic breast cancers, indicating the involvement of BRCA1 even in sporadic forms.

Function of the BRCA1 protein is not fully elucidated, but it has been reported that the BRCA1 gene has RING finger domain in its NH2 terminus (2) and BRCT motif in its COOH terminus (11) that functions as a transactivator (12, 13). BRCA1 interacts directly with p53 and transcriptionally activates p21Waf1 (14, 15). It also interacts with Rad51, a recA homologue of Escherichia coli that is involved in repair of double-strand breaks in DNA (16, 17), suggesting that BRCA1 may play a pivotal role in maintaining genomic stability.

Significance of BRCA1 protein expression in mammary tissues remains to be clarified because several groups of investigators (18–21) have reported different results on its subcellular localization. Chen et al. (18) reported that BRCA1 protein is localized in nuclei in normal mammary epithelial cells, but is aberrantly located in the cytoplasm in malignant breast and ovarian cells. On the basis of the presence of a granin consensus sequence, Jensen et al. (19) reported that BRCA1 is located in both the cytoplasm and the cellular membrane and secreted via the Golgi apparatus. Scully et al. (20) demonstrated that the BRCA1 protein is located in the nuclei of both normal and malignant mammary cells. Coen et al. (21) proposed that BRCA1 protein is present in cytoplasmic tube-like invaginations in the nucleus. Controversy on the subcellular localization
of BRCA1 is, at least, attributable to specificity of the antibody (22, 23), fixation methods used (20, 21), and the presence of splice variant isoforms lacking exon 11 along with NLSs2 (24, 25).

To date, there have been a few studies on immunocytochemical and immunohistochemical analyses of BRCA1 protein in sporadic, hereditary, and BRCA1-associated breast carcinomas. It is important to determine the expression level of nuclear form of BRCA1 protein in normal and malignant breast tissues in light of the functional significance of nuclear form of BRCA1 protein.

Herein, we evaluate the applicability of 12 anti-BRCA1 antibodies from various sources to immunostaining analysis of BRCA1 protein by using eight fixatives and a BRCA1-deficient cell line, as well as various human tissues as controls. We further analyze expression level of BRCA1 protein in 143 malignant tissues from Japanese patients with sporadic and hereditary breast cancers. Our results show nuclear expression of BRCA1 protein is reduced in about one-third of Japanese sporadic breast cancers. Frequent loss of BRCA1 protein expression in Japanese BRCA1-associated tumors suggests that immunohistochemical analysis may be a useful method for prescreening tumors to detect mutations in the BRCA1 gene. Our data also suggest that reduced expression of BRCA1 protein may play an important role in mammary carcinogenesis in Japanese sporadic cancers and that mechanisms other than mutation may be involved in the reduced expression of BRCA1 protein.

Materials and Methods

Cell Lines. Three breast cancer cell lines (MCF7, MDA-MB-468, and MDA-MB-231) were obtained from the American Type Culture Collection (Manassas, VA). HBL-100, a human breast epithelial cell line immortalized with SV40 T antigen, was obtained from Riken Cell Bank (Tsukuba, Japan). The HCC1937 cell line was derived from tumor cells that contain 5382 insC mutation with a loss of wild-type allele in the BRCA1 gene (26). The MCF7 and HBL-100 cells were cultured under standard condition (see above). The HCC1937 cells were cultured in RPMI 1640 supplemented with antibiotics and 5% FBS in a 100% humidified atmosphere. The HCC1937 cells were cultured in RPMI 1640 supplemented with antibiotics and 5% FBS under standard condition (see above).

Xenograft Tumor of MDA-MB-468 Cells in a Nude Rat. MDA-MB-468 cells (1 × 107) were injected s.c. into the back of a female, athymic nude rat (Clea, Tokyo, Japan), 3 weeks of age. The rat was sacrificed after 3 weeks, and the tumor was excised, fixed in neutral-buffered formalin, and embedded in paraffin.

Patients and Tumor Specimens. Paraffin sections of 27 hereditary breast cancer tissues with a germline mutation of the BRCA1 gene were collected with the cooperation of the familial cancer research group in the Japanese Ministry of Health and Welfare. Screening for BRCA1 mutations of these cases and its DNA sequencing were described elsewhere (3, 4, 27–29). Eight of the 27 tissues that showed very weak staining of BRCA1 protein in lymphocytes and in normal mammary epithelium were excluded from the immunohistochemical analysis. Also analyzed were the tissues of 143 breast cancer patients who were treated surgically at the Kodama Breast Clinic (Kyoto, Japan) from 1995 through 1997. These 143 patients were grouped into two cohorts. The first group of patients (hereditary cases, n = 35) were those who fulfilled at least one of the following criteria: (a) three or more cases of breast cancer in first- or second-degree relatives; (b) two cases of breast or ovarian cancer in first- or second-degree relatives, with at least one case occurring before age 50, or (c) at least one case of bilateral breast tumors among first- or second-degree relatives. The second group of patients (sporadic cases, n = 108) were those who did not meet any of the criteria described above. The criteria we used for the hereditary group were similar to those used by Katagiri et al. (27), but were modified slightly, taking into consideration the fact that BRCA1-associated breast cancers in Japanese women are characterized by a higher proportion of bilateral tumors and later onset of disease than they are in United States women (3, 4). For each patient in our study, we obtained frozen sections of malignant and adjacent normal breast tissues. From among the 143 frozen tissue specimens, we randomly selected 30 and used the corresponding paraffin-embedded sections for immunohistochemical analysis to confirm consistency in the staining pattern between the frozen and paraffin-embedded sections. The tumors were histologically subtyped according to the General Rule for Clinical and pathological Record of Mammary Cancer (Japanese Mammary Cancer Society) and were also histologically graded by using the criteria of Scarff-Bloom-Richardson, as described previously (30). The level of PgR in the breast cancer tissues was determined using the enzyme immunoassay by Otsuka Pharmaceuticals (Tokyo, Japan). Paraffin-embedded sections of tissues of an autopsied male infant and nonmalignant liver tissues of four adult patients who underwent hepatic surgery were also used for immunohistochemical analysis of BRCA1 protein.

Primary Antibodies. The locations of the immunogens for 12 anti-BRCA1 antibodies and the proposed functional motifs of the BRCA1 protein are depicted in Fig. 1. The immunogen and the isotype of each primary antibody, the fixative used in either immunocytochemical or immunohistochemical analysis of frozen sections, the working dilution or concentration used in immunostaining, and the source of each primary antibody to BRCA1 are summarized in Table 1. MAbs other than those against BRCA1 that were used for immunohistochemical analysis are p53 (mouse MAb, IgG2h, DO-7; DAKO, Tokyo, Japan), ER (IgG1, ER1D5; MBL, Nagoya, Japan), PCNA (IgG2a, PC10; DAKO), and c-erbB-2 (IgG1, Ab-3; Calbiochem, La Jolla, CA). The working concentrations or dilution

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2 The abbreviations used are: NLS, nuclear localization signal; PAb, polyclonal antibody; MAb, monoclonal antibody; PCNA, proliferating-cell nuclear antigen; ER, estrogen receptor; PgR, progesterone receptor; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; ICC, immunocytochemistry; FBS, fetal bovine serum; NuMa, nuclear matrix; H+L, heavy chain + light chain.
of the antibodies were 0.5 μg/ml (p53), 1:1000 dilution (ER), 0.5 μg/ml (PCNA), and 2 μg/ml (c-erbB-2). The antibodies used for the immunoblot analysis were MS110, 17F8, C-20, α-tubulin (MAb, IgG1, Ab-1; Calbiochem), NuMA protein (MAb, IgG1, Ab-1; Calbiochem), and EGFR (MAb, IgG2a, NCL-EGFR; Novocastra, Newcastle, United Kingdom). The working concentrations or dilution of these antibodies were 2 μg/ml (MS110), 2 μg/ml (17F8), 1 μg/ml (C-20), 0.5 μg/ml (α-tubulin), 2.5 μg/ml (NuMA), and 1:50 dilution (EGFR). The last three antibodies were used as markers for the cytoplasmic, nuclear, and membrane fractions, respectively.

**Immunoblotting.** Cell extracts containing 100 μg of protein were separated electrophoretically by using either 5% or 4–20% SDS-PAGE (Daichi Pure Chemicals, Tokyo, Japan). The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan) for 2 h at 200 mA. After blocking the membrane with 5% nonfat dried milk in PBS-T (PBS/0.1% Tween 20), primary antibody was allowed to react with the protein for 1 h at room temperature. The secondary antibody used was horseradish peroxidase-conjugated donkey anti-rabbit IgG (H+L) for the PAb or sheep anti-mouse IgG (H+L) for the MAb (Amer sham, Buckinghamshire, United Kingdom). The antibody complexes were detected by using an enhanced chemiluminescence system (Amersham).

**Immunoprecipitation.** The lysates were precleared and immunoprecipitated with a preincubated complex composed of either PAb (1–2 μg) and protein G PLUS/Protein A-Agarose (30 μl; Calbiochem) or MAb and Sepharose-conjugated antimouse IgG (H+L; 20 μl; Zymed, South San Francisco, CA) for 2 h at 4°C. The beads were microcentrifuged and washed four times with radioimmunoprotein assay buffer. The immunoprecipitates were eluted by boiling the beads for 5 min in SDS-loading buffer. The Immunoblot analysis to detect BRCA1 protein was performed as described above.

**Subcellular Fractionation.** HBL-100 and MDA-MB-468 cells were fractionated into subcellular components as described previously (31). The protein of each fraction was analyzed by immunoblotting or by immunoprecipitation, followed by immunoblotting.

**Fixatives.** The eight fixatives that were used in the immunocytochemical and immunohistochemical analyses were acetone, methanol, acetone/methanol (1:1), Zamboni solution (0.2% buffered picric acid and 2% paraformaldehyde solution), 4% paraformaldehyde, periodate-lysine-paraformaldehyde, neutral-buffered formalin, and alcoholic formalin, which is composed of 10% buffered formalin diluted into 70% ethanol.

**ICC.** Cells were cultivated at subconfluence in a 4-well Lab-Tek chamber slide (Nunc, Naperville, IL). After being fixed as described in Table 1, the cells were permeabilized for 5 min in PBS containing 0.1% Triton X-100. The cells were then incubated at 37°C for 20 min in Blockace (Dainihon Pharmaceuticals, Osaka, Japan). If immunoperoxidase staining was used, the endogenous peroxidase activity was blocked by incubating the cells for 10 min in 3% H2O2 containing 0.1% sodium azide. The cells were then incubated at room temperature for 2 h with the anti-BRCA1 antibody in PBS containing 1% BSA (see Table 1 for the working dilutions or concentrations of the primary antibodies) and for 30 min with biotinylated goat anti-rabbit IgG for the PAb (1:500 dilution) or with goat anti-mouse IgG for the MAb (1:200 dilution, both antibodies; Vector Laboratories, Burlingame, CA). The chamber slide was then incubated for 30 min with the avidin-biotin peroxidase complex reagent (Vecstatin ABC kit, Vector Laboratories). Diaminobenzidine (0.05%) was used as the final chromogen, and hematoxylin was used as the nuclear counterstain.

For immunofluorescence staining, the cells, after being incubated with anti-BRCA1 antibody, were further incubated for 1 h with FITC-conjugated goat anti-rabbit IgG (H+L) for the PAb or with goat anti-mouse IgG (H+L) for the MAb (Amersham, Buckinghamshire, United Kingdom). The antibody complexes were visualized with a confocal microscope (Bio-Rad, Tokyo, Japan).

**IHC.** Frozen tissue sections, 5 μm thick, were mounted on silanized slides and fixed for 12 min, as described in Table 1. In the analysis using the MS110, 8F7, anti-PCNA, or anti-c-erbB-2 antibody, the tissue sections were subjected to heat-induced epitope retrieval by placing them in 10 mM sodium citrate buffer (pH 6) and heating them in a microwave oven at 500 W for 8 min. In the analysis of paraffin-embedded tissue sections using the MS110, 8F7, or SG11 antibody, the tissue sections were subjected to heat-induced epitope retrieval, as described above, for the frozen sections, except that paraffin-embedded sections were heated for 20 min. Immunohistochemical staining was performed as described previously (32). For BRCA1 protein, the overall staining intensity in cancer cells was scored on a 0 to +3 scale. A tissue section that did not exhibit any brown nuclear reactivity in any tumor cells was scored as 0. When an occasional tumor cell showed positive nuclear staining but the total percentage of positivity in the tissue section was <20%, a score of +1 was given. A tissue section in which 20–80% of tumor cells exhibited positive nuclear staining was scored as +2. When >80% of tumor cells in a tissue section...
displayed positive nuclear staining, a score of +3 was given. For p53 expression, absent, focal and diffuse staining was scored as 0, +1, and +2, respectively, as described previously (33). For ER expression, staining was scored as 0, +1, +2, and +3, as described previously (34), and patients whose tumors were scored as +1, +2, or +3 were considered ER positive. The PCNA-labeling index for each patient was calculated as a percentage of PCNA-positive tumor cells among 500 randomly chosen tumor cells. For c-erbB-2 expression, only those tissue sections that had distinct membrane staining in 80% of the tumor cells were considered positive for overexpression.

As a negative control of BRCA1 immunostaining, preimmune or isotype-matched irrelevant IgG was used instead of each primary antibody. The specificity of SG11, C-20, D-20, I-20, K-18, and D-9, was further confirmed by an antibody blocking procedure in which the antibody was incubated with an excess amount of cognate peptide.

All tissue sections were independently reviewed for anti-BRCA1 immunostaining, preimmune or isotype-matched irrelevant IgG was used instead of each primary antibody. The specificity of SG11, C-20, D-20, I-20, K-18, and D-9, was further confirmed by an antibody blocking procedure in which the antibody was incubated with an excess amount of cognate peptide.

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ixed BRCA1 Protein Expression in Japanese Breast Cancers

Table 1  Primary antibodies to BRCA1 protein

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Location of immunogen (amino acid)</th>
<th>Isotype</th>
<th>Fixative used</th>
<th>Working dilution or concentration used in IHC</th>
<th>Source of antibody</th>
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<tr>
<td>NH¿-terminal antibodies</td>
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<tr>
<td>MS110 1–304 MAb (IgG 1)</td>
<td>NBF/Et superscript c</td>
<td>NBF, MW superscript d</td>
<td>1 µg/ml</td>
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<tr>
<td>MS13 1–304 MAb (IgG 1)</td>
<td>NBF/Et (PLP)</td>
<td>1:1000</td>
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<td>D-20 2–21 PAb</td>
<td>NBF/Et PLP</td>
<td>0.1 µg/ml</td>
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<tr>
<td>K-18 70–89 PAb</td>
<td>NBF/Et (PLP)</td>
<td>0.5 µg/ml</td>
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<td>Exon 11 antibodies</td>
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<tr>
<td>8F7 341–748 MAb (IgG 1)</td>
<td>Zamboni NBF, MW superscript d</td>
<td>2 µg/ml</td>
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<tr>
<td>17F8 762–1315 MAb (IgG 1)</td>
<td>Zamboni (–)</td>
<td>5 µg/ml</td>
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<tr>
<td>COOH-terminal antibodies</td>
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<tr>
<td>AP16 1313–1863 MAb (IgG 1)</td>
<td>NBF/Et (PLP)</td>
<td>0.5 µg/ml</td>
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<tr>
<td>I-20 1823–1842 PAb</td>
<td>(NBF/Et) (PLP)</td>
<td>0.5 µg/ml</td>
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<tr>
<td>C-20 1843–1862 PAb</td>
<td>(NBF/Et) (PLP)</td>
<td>0.1 µg/ml</td>
<td>m</td>
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<tr>
<td>D-9 1843–1862 MAb (IgG 1)</td>
<td>NBF/Et (–)</td>
<td>1 µg/ml</td>
<td>n</td>
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<tr>
<td>SG11 1847–1863 MAb (IgG 2b)</td>
<td>(NBF/Et) (NBF, MW)</td>
<td>0.2 µg/ml</td>
<td>o</td>
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<tr>
<td>VALY 1842–1844 PAb</td>
<td>(Zamboni) (NBF)</td>
<td>1:100</td>
<td>p</td>
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* Parentheses indicate which COOH-terminal antibodies were not applicable to ICC because diffuse nuclear staining was observed in HCC1937 cells.

* IHC of frozen section. Parentheses indicate which antibodies were not applicable to IHC because there was no nuclear staining (17F8 and D-9), because only an extremely small number of scattered cancer cells showed nuclear staining (MS13 and AP16), or because there was nonspecific nuclear staining in BRCA1-associated breast tumors that contained the BRCA1 protein-truncating mutation (I-20, C-20, SG11, and VALY).

* NBF/Et, alcoholic formalin; PLP, periodate-lysine-paraformaldehyde; NBF, neutral-buffered formalin; MW, microwave treatment.

* Applicable to paraffin sections.

* Provided by Dr. Ralph Scully.

* Santa Cruz Biotechnology (Santa Cruz, CA).

* GeneTex (San Antonio, TX).

* BioGenex (San Ramon, CA).

Statistical Analyses. The association of BRCA1 protein expression with age, histological typing, primary tumor status, lymph node metastasis, tubular differentiation, histological grading, menopause status, expression of p53, c-erbB-2, ER and PgR, and the PCNA-labeling index was analyzed by using the χ² test, one-way ANOVA, and multivariate analysis by the stepwise method.

Results

Application of BRCA1 Antibodies to ICC. To learn whether a panel of anti-BRCA1 antibodies recognize endogenous BRCA1, a combination of immunoprecipitation and immunoblotting analysis was performed using the HBL-100 mammary epithelial cell line. Ten of the 12 antibodies detected p220BRCA1 (Fig. 2A, a). In addition, p120BRCA1 and p100BRCA1 were coimmunoprecipitated by the antibodies (MS110, MS13, I-20, SG11, and C-20) that react with the NH¿- and COOH-termini of the BRCA1 protein (Fig. 2A, a). The band of p220BRCA1 was also detected by straight immunoblotting analysis (data not shown). However, additional nonspecific bands were recognized, suggesting that prudent evaluation should be necessary in immunostaining by using these BRCA1 antibodies.

Previous studies on detailed analysis of subcellular localization of BRCA1 isoforms demonstrated that full-length BRCA1 protein is localized predominantly in nuclei and that exon 11-spliced form of BRCA1 is localized in cytoplasm (24, 25). In line with these studies, we verified that p220BRCA1 exists in the nuclear fraction of both nonmalignant (HBL-100) and malignant (MDA-MB-468) mammary epithelial cells (Fig. 2A, b). Immunoblot analysis of fractionated and immunoprecipitated MDA-MB-468...
Fig. 2. Evaluation of BRCA1 antibodies in immunocytochemistry. A, a, immunoblot of the HBL-100 cell lysate immunoprecipitated with a panel of anti-BRCA1 antibodies. Membrane was probed with antibody MS110. b, subcellular fractionation analysis of BRCA1 protein in HBL-100 and MDA-MB-468 cells. The fractionated lysates were analyzed by immunoblotting with antibodies to BRCA1 (17F8 and C-20), α-tubulin (cytoplasmic marker), NuMA (nuclear marker), and EGFR (membrane marker). p220BRCA1 was detected in the nuclear fraction of both cell lines. An intense 180-kDa band that was recognized in the membrane fraction of the MDA-MB-468 cells by using C-20 corresponds to EGFR, with which C-20 cross-reacts (22, 23). c, subcellular localization of p120BRCA1. MDA-MB-468 cell lysates were fractionated into their cytoplasmic, nuclear, and membrane components, were immunoprecipitated by using antibody SG11, and were analyzed by immunoblotting with antibody MS110. b and c, Lanes T, C, N, and M represent total, cytoplasmic, nuclear, and membrane fractions, respectively. B, effects of fixatives on the immunoperoxidase staining of MDA-MB-468 cells. MDA-MB-468 cells were fixed by Zamboni solution, alcoholic formalin (NBF/Et), or acetone and stained by using the 17F8 or MS110 antibody. C, confocal immunofluorescence staining of MDA-MB-468 cells by using the MS110 or 8F7 antibody. Top panels, indirect immunofluorescence staining for BRCA1. Bottom panels, propidium iodide (P.I.) staining for DNA. D, immunoperoxidase staining of HCC1937 cells using four COOH-terminal anti-BRCA1 antibodies: SG11, C-20, I-20, and VALY. B, C, and D, preimmune or isotype-matched irrelevant IgG was used instead of each primary antibody as a negative control of immunostaining. E, laser scanning cytometric analysis showing the staining profile by using anti-BRCA1 antibodies (8F7 and SG11) during the cell-cycle phases in MDA-MB-468 cells. The solid lines represent the threshold for positive immunoreactivity, based on the isotype-matched negative controls (mouse IgG1 for 8F7 and mouse IgG2b for SG11).
MB-468 cell lysate revealed that p120BRCA1 was detected in cytoplasmic fraction (Fig. 2A, c). Due to proposed functional importance of BRCA1 protein in DNA repair and transactivation (12–17), it is essential to investigate fixatives that are suitable for preserving nuclear BRCA1 protein in immunostaining procedures. It is well known that the immunostaining pattern may differ, depending on the fixative used. Suspecting this could be true for BRCA1 protein, we compared acetone, methanol, acetone/methanol, 4% paraformaldehyde, periodate-lysine-paraformaldehyde, neutral buffered formalin, alcoholic formalin, and Zamboni solution. The ICC column in Table 1 lists the combinations of antibodies and fixatives under which nuclear staining was observed in both HBL-100 and MDA-MB-468 cells. Representative immunostaining patterns achieved with the different fixatives are shown in Fig. 2B. In MDA-MB-468 cells, antibody 17F8 showed immunoreactivity predominantly to the nuclei when the cells were fixed in Zamboni solution, but immunoreactivity to the cytoplasm when fixed in alcoholic formalin. Nuclear staining was observed with antibody MS110 when the cells were fixed either in acetone or in alcoholic formalin, which gave more intense staining. Such fixative-dependent alterations in staining pattern were also observed in HBL-100 cells (data not shown).

A characteristic nuclear dot pattern of BRCA1 protein localization was demonstrated both in MDA-MB-468 cells (Fig. 2C) and in HBL-100 cells (data not shown) by immunofluorescence staining with MAb, MS110, 8F7, and 17F8 (data not shown), under the specific fixation conditions.

Although all of the anti-BRCA1 antibodies exhibited immunoreactivity to the nuclei of mammary epithelial cells when the fixative was used, as indicated in Table 1, some of the antibodies showed nonspecific immunostaining. Using BRCA1-deficient cell line HCC1937, which should lack the COOH-terminal portion of BRCA1 protein with which COOH-terminal antibodies react, diffuse nuclear staining was observed with COOH-terminal antibodies SG11, C-20, I-20, and VALY (Fig. 2D), suggesting that these antibodies cross-react with a protein(s) other than BRCA1. Cross-reactivity of SG11 was also revealed by laser scanning cytometric analysis of asynchronized MDA-MB-468 cells by using 8F7 and SG11 MAb (Fig. 2E). Cytometric BRCA1 staining profile by using SG11 showed two unusual peaks during the cell cycle, one in the G1 phase and the other in the G2-M phase, whereas those two peaks were not found in the profile by using 8F7.

Expression of BRCA1 Protein in Breast Cell Lines. All of the four breast cell lines, except HCC1937, expressed all BRCA1 isoforms in various quantities (Fig. 3A, a). In addition to the MDA-MB-468 cells, MCF7, HBL-100, and MDA-MB-231 cells showed predominantly nuclear and weak cytoplasmic staining with antibody MS110 (Fig. 3B). HCC1937 cells carry 5382 insC mutation with a loss of wild-type allele in the BRCA1 gene (26). The deduced mutant protein should have amino acid sequence altered from codon 1755 through to stop codon at 1829, and its expected size is ~200 kDa. No band was detected in the immunoprecipitant of COOH-terminal MAb SG11 as expected (Fig. 3A, b). A very faint 200-kDa band was recognized in the immunoprecipitant of NH2-terminal MS110 only after a long exposure, suggesting an extremely low level of truncated protein expression. Immunoperoxidase staining of HCC1937 cells demonstrated weak cytoplasmic staining when antibodies MS110 (Fig. 3B) were used. Immunofluorescence staining, however, still demonstrated a nuclear dot pattern in these cells (data not shown).

Selection of Anti-BRCA1 Antibodies for Immunohistochemical Analysis. On the basis of the results described above, we used the fixation methods in IHC that fulfilled the following criteria: (a) nuclear staining was predominant; and (b) background staining was minimal. In addition, we excluded any COOH-terminal antibody that showed immunoreactivity to the nuclei of BRCA1-associated cancer cells that had a protein-truncating mutation. The different fixation methods gave drastically different immunostaining results, depending on the antibody used. These results are summarized in the IHC column of Table 1. Antibodies 17F8 and D-9, which showed no immunoreactivity to the nuclei but some immunoreactivity to the cytoplasm with any fixation method, were excluded from immunohistochemical analysis. Although the other 10 antibodies showed reactivity to the nuclei of breast cancer cells, we excluded MS13 and AP16 and four of the COOH-terminal antibodies (I-20, C-20, SG11, and VALY) from further study for the following reasons. We excluded them because only an extremely small number of scattered cancer cells showed nuclear staining with MS13 and AP16, and because false-positive nuclear staining was observed with I-20, C-20, SG11, and VALY in BRCA1-associated breast cancer cells that should lack the COOH terminus of the BRCA1 protein. Of the remaining four antibodies, we considered the MAbS MS110 and 8F7 appropriate for staining paraffin-embedded tissue sections for the following two reasons: (a) the immunostaining pattern the two antibodies produced was consistent between the frozen and the corresponding paraffin-embedded tissue sections; and (b) immunostaining patterns produced by these antibodies in formalin-fixed, paraffin-embedded xenograft tumor of MDA-MB-468 cells formed in a nude rat were consistent with those obtained in immunocytochemical analysis (data not shown). Using these two monoclonals, we confirmed ubiquitous but characteristic tissue distribution of the BRCA1 protein. The cortical region of the infant thymus (Fig. 4A) and the basal cells of adult epidermal squamous epithelium (Fig. 4C) showed intense nuclear staining, whereas hepatocytes in adult liver showed weak nuclear staining (Fig. 4B). These results were consistent with previous studies (2, 35) on BRCA1 mRNA expression. Normal breast tissues showed intense nuclear staining in the epithelial cells of mammary ducts and lobules with positive staining in the myoepithelium of the mammary ducts, in fibroblasts, and in lymphocytes (Fig. 4D). The overall intensity of nuclear staining in the cancerous tissues varied from strong (+3) to negative (0), whereas the surrounding lymphocytes invariably showed moderate to strong staining (Fig. 4, E–H). Of note, we found no cytoplasmic staining either in the cancer cells or in normal mammary epithelial cells.

Analysis of Japanese Patients with BRCA1-associated Breast Cancer. We used IHC with antibody MS110 to analyze BRCA1 protein expression in 19 Japanese patients whose germ-line mutations of the BRCA1 gene had been detected (44–48; Table 2). The BRCA1 protein expression level was reduced (+1 or 0) in 7 of the 11 carcinomas that had truncating mutations and in all 8 carcinomas that had intronic or missense
mutations, including the 5 carcinomas that had complete loss of BRCA1 protein expression. Although it is unknown whether intronic or missense mutations really have functional significance, these results indicate an association between the gene alterations and reduced expression of the gene products. Altogether, 15 (79%) carcinomas, including 7 (37%) carcinomas with complete loss of BRCA1 protein expression, showed reduced nuclear expression. Representative immunostaining of a carcinoma with complete loss of BRCA1 protein expression is shown in Fig. 4H.

The presence of functional NLSs in exon 11 has been reported (25, 36). Of the four carcinomas that contained a mutant BRCA1 gene, the product of which lacks NLSs, three still showed positive nuclear staining (Table 2, cases 2–4). None of the four carcinomas showed cytoplasmic expression of BRCA1 protein.

BRCA1 Expression Level in Hereditary and Sporadic Breast Cancer Cells. We used IHC to analyze BRCA1 protein expression in the tumors of 143 patients with hereditary (group 1, n = 35) or sporadic (group 2, n = 108) breast...
carcinomas (Table 3). There was no case in either group of patients in which tumor cells showed complete loss of BRCA1 expression. Thirty (28%) of the 108 sporadic and 6 (17%) of the 35 hereditary breast tumors showed reduced expression of BRCA1 protein. There was no significant difference in BRCA1 protein expression between the two patient cohorts. In contrast to the tumors of the hereditary and sporadic breast cancer groups, the tumors of patients with BRCA1-associated breast cancers showed reduced expression of BRCA1 protein, a reduction that was statistically significant (Table 3; χ² test, P < 0.01).

These results suggest that a significant number of hereditary cases in our study are not attributable to alterations of the BRCA1 gene product. Indeed, no mutation of BRCA1 in exon 11 was identified by the protein truncation test when tumor or blood DNA samples were analyzed in 11 hereditary cases (data not shown).

Correlation of BRCA1 Scoring with Clinicopathological Parameters and Immunostainings of p53, ER, PCNA, and c-erbB-2 in Patients with Sporadic Breast Cancer. We compared the expression levels of BRCA1 protein with the

Fig. 4. BRCA1 protein expression in various normal and breast cancer tissues. A, thymus; B, liver; C, skin; D, normal mammary gland; E–H, representative immunostaining of breast carcinomas showing a BRCA1 score of +3, +2, +1, and 0, respectively. H, a case of BRCA1-associated breast cancer with an A to T transition at the first nucleotide of codon 1085 (Table 2, Case 10). Note the weak staining in hepatocytes (B) and negative staining in the BRCA1-associated cancer cells (H), in contrast to the intense nuclear staining in adjacent lymphocytes (B, F, G, and H, arrowheads) and in biliary epithelial cells (B, arrow).
BRCA1 scoring: see the footnote to Table 2.

Differentiation (BRCA1 protein expression had a tendency toward poor tubular staining in 20 to 80% of tumor cells; solid-tubular carcinomas in all types of tumors with erbB dependency, PCNA-labeling index for cell proliferation, and in sporadic breast cancers, included ER and PgR for hormone Biological markers, whose importance has been well established profile included age, histological typing, primary tumor status, markers of the 108 patients with sporadic breast cancer. The clinicopathological profiles and immunostaining of biological markers, whose importance has been well established in sporadic breast cancers, included ER and PgR for hormone dependency, PCNA-labeling index for cell proliferation, and p53 and c-erbB-2 for prognostic indicators. The percentage of solid-tubular carcinomas in all types of tumors with +1 BRCA1 scoring (14 of 30, 47%) was higher than that with +3 BRCA1 score (3 of 17, 18%), which was statistically significant (2 test, this scoring was statistically significant (2 test, one-factor ANOVA (Table 4), or multivariate analysis (data not shown). Carcinomas with reduced BRCA1 protein expression had a tendency toward poor tubular differentiation (x^2 test, P = 0.124) and toward an overexpression of c-erbB-2 protein (x^2 test, P = 0.158). Because regulation of BRCA1 expression in response to estrogen has been reported (37), we analyzed the association between level of BRCA1 protein expression and menopause status in 78 ER-positive cases and the association between level of BRCA1 protein expression level and menstrual cycle in 36 ER-positive premenopausal cases. We found no statistically significant associations from these two analyses (data not shown).

**Discussion**

Confusion about the size and subcellular localization of the BRCA1 protein may be derived, in part, from differences in immunostaining methodology, especially the type of fixative used, and from a lack of high specificity of anti-BRCA1 antibodies. Because of the drastic change in immunostaining results that occur under different fixation conditions and is extremely dependent on antibodies, we determined the fixation method based on biochemical results and a further analyzed nuclear form of BRCA1 protein. We found that a specific fixative may be needed for each antibody to preserve the immunoreactivity of its epitope. A single antibody often proxied nuclear form of BRCA1 protein. We found that a

### Table 2 Summary of mutation patterns and IHC scoring of BRCA1 protein in 19 Japanese patients with BRCA1-associated breast cancer

<table>
<thead>
<tr>
<th>Case number</th>
<th>Mutation site</th>
<th>Codon</th>
<th>Base change</th>
<th>Size of truncated protein (amino acid)</th>
<th>BRCA1 scoring a by IHC</th>
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<tr>
<td>1</td>
<td>Exon 2</td>
<td>22</td>
<td>TTA→TGA</td>
<td>(21)_b</td>
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<td>(27)</td>
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<td>2</td>
<td>Exon 5</td>
<td>63</td>
<td>TTA→TAA</td>
<td>(62)_b</td>
<td>+2</td>
<td>(28)</td>
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<tr>
<td>3</td>
<td>Exon 5</td>
<td>63</td>
<td>TTA→TAA</td>
<td>(62)_b</td>
<td>+1</td>
<td>(28)</td>
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<tr>
<td>4</td>
<td>Exon 11</td>
<td>445</td>
<td>2 bp(AA) del'</td>
<td>(453)_b</td>
<td>+2</td>
<td>(29)</td>
</tr>
<tr>
<td>5</td>
<td>Exon 11</td>
<td>797</td>
<td>2 bp del</td>
<td>(798)</td>
<td>+1</td>
<td>(3, 4)</td>
</tr>
<tr>
<td>6</td>
<td>Exon 11</td>
<td>852</td>
<td>1 bp(T)del</td>
<td>(891)</td>
<td>+1</td>
<td>(3, 4)</td>
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<tr>
<td>7</td>
<td>Exon 11</td>
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<td>CAG→TAG</td>
<td>(933)</td>
<td>+2</td>
<td>(29)</td>
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<tr>
<td>8</td>
<td>Exon 11</td>
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<td>CAG→TAG</td>
<td>(933)</td>
<td>+1</td>
<td>(29)</td>
</tr>
<tr>
<td>9</td>
<td>Exon 11</td>
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<td>CAG→TAG</td>
<td>(933)</td>
<td>+2</td>
<td>(29)</td>
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<tr>
<td>10</td>
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<td>AGA→TGA</td>
<td>(1084)</td>
<td>0</td>
<td>(29)</td>
</tr>
<tr>
<td>11</td>
<td>Exon 11</td>
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<td>(3, 4)</td>
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Intronic mutations

<table>
<thead>
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<th>BRCA1 scoring a by IHC</th>
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<td>12</td>
<td>+1</td>
<td>(27)</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>(27)</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>(27)</td>
</tr>
<tr>
<td>15</td>
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<td>(27)</td>
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<td>16</td>
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<td>(27)</td>
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Missense mutations

<table>
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<th>BRCA1 scoring a by IHC</th>
<th>References</th>
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<td>17</td>
<td>0</td>
<td>(27)</td>
</tr>
<tr>
<td>18</td>
<td>+1</td>
<td>(3, 4)</td>
</tr>
<tr>
<td>19</td>
<td>+1</td>
<td>(3, 4)</td>
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</table>

### Table 3 Expression level of BRCA1 protein in hereditary, sporadic, and BRCA1-associated breast carcinomas

<table>
<thead>
<tr>
<th>Type of breast cancer</th>
<th>Number of patients</th>
<th>BRCA1 scoring a</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Hereditary</td>
<td>35</td>
<td>0 +1 +2 +3</td>
<td></td>
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<tr>
<td>Sporadic</td>
<td>108</td>
<td>25 61 17</td>
<td></td>
</tr>
<tr>
<td>BRCA1-associated</td>
<td>19</td>
<td>15 0 4 0</td>
<td></td>
</tr>
</tbody>
</table>

a By the x^2 test, this scoring was statistically significant (P < 0.01).

b Seven of the 15 BRCA1-associated breast cancers were scored as 0.
BRCA1 Protein Expression in Japanese Breast Cancers

The cytoplasmic fraction in both normal and malignant mammary epithelial cell lines. Because p120BRCA1 seems to correspond to the product of the NLS-deleted mutations was unexpected. We should take into consideration the following possibilities. One possibility is that the expression level of BRCA1 mRNA is directly related to the number of wild-type alleles. The relatively high level of BRCA1 protein expression (+2) we observed in some of our protein-truncating tumors may possibly result from retention of the wild-type allele, the high stability of this mutant protein. All of the antibodies stained the nuclei of mammary cells and in Japanese BRCA1-associated breast cancers by using COOH-terminal antibodies SG11, C-20, I-20, and VALY, two of which (C-20 and SG11) immunoprecipitated BRCA1 protein. All of the antibodies showed the nuclei of mammary epithelial cells. Consequently, results obtained by using COOH-terminal antibodies should be carefully evaluated, even if the cells show nuclear staining.

The NH2-terminal antibody MS110 is useful in prescreening tumors for the BRCA1 mutation because of the amino acid difference in high detection rate (7 of 19, 37%) of alterations in the BRCA1 gene product in breast cancers. Of particular interest in our study is the finding that immunostained breast tumors with intronic or missense mutations showed a markedly reduced or no expression of BRCA1 protein, and extent of the staining reduction was more pronounced than those with protein-truncating mutations. Although the functional significance of the intronic and missense mutations is not known, our observations suggest that its DNA alterations are tightly linked to down-regulation of BRCA1 protein expression and resultant development of disease. Kainu et al. (40) demonstrated by in situ hybridization analysis that expression level of BRCA1 mRNA in BRCA1-associated breast cancers (39). The BRCA1-deficient cell line HCC1937 enabled us to examine the specificity of the COOH-terminal antibodies. False-positive immunostaining was unexpectedly demonstrated in HCC1937 cells and in Japanese BRCA1-associated breast cancer tissue by using COOH-terminal antibodies SG11, C-20, I-20, and VALY, two of which (C-20 and SG11) immunoprecipitated BRCA1 protein. All of the antibodies stained the nuclei of mammary epithelial cells. Consequently, results obtained by using COOH-terminal antibodies should be carefully evaluated, even if the cells show nuclear staining.

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proteins such as BARD1 (43) or BAPI (44), which bind to the NH2 terminus of BRCA1 protein, may transport this mutant protein to the nucleus. Another possibility is that the wild-type BRCA1 allele may not be lost in these cancer cells, which was not confirmed in our study because of the limited tissue samples. However, we could not rule out the potential cross-reactivity of the wild-type MS110. Although the NH2-terminal antibody proved to be useful in prescreening tumors for the BRCA1 mutation, further development of a highly specific COOH-terminal antibody is anticipated to improve the specificity and sensitivity of immunohistochemical detection of mutations in the BRCA1 gene, especially protein-truncating mutations.

The frequency of reduced BRCA1 protein expression (28%) in our sporadic breast cancer cases is similar to that found in a BRCA1 mRNA in situ hybridization study by Kainu et al. (40). Lack of somatic mutations in sporadic breast carcinomas in other studies may suggest a possible involvement of other mechanisms, such as allelic loss, large deletion, or methylation of the BRCA1 promoter region (8–10) in reduced expression of BRCA1 protein.

BRCA1-associated breast carcinomas have a high mitotic index (45), a high histological grade (46), a high S phase fraction (45, 47), a low ER-positive rate (47, 48), and a high rate of p53 overexpression (49). BRCA1 directly reacts with p53 (15), and activation of the p53 checkpoint in response to loss of BRCA1 function has been demonstrated in mouse embryos during embryogenesis (50). Interestingly, we observed reduced expression of the BRCA1 protein expression in breast cancer cells in comparison with that in normal mammary epithelial cells, and association between the reduction of the BRCA1 protein expression and poor tubular differentiation or an overexpression of c-erbB-2 protein in sporadic breast cancers. Altogether these findings suggest that reduced BRCA1 expression in breast cancers may somehow be related to their malignant phenotype and may also indicate a crucial role of the BRCA1 protein in the development of sporadic breast cancers. Analysis of the association between BRCA1 protein expression and the disease-free or overall survival rates of patients with breast cancer will clarify the significance of reduced expression of BRCA1 protein in sporadic breast tumors. Finally, additional efforts toward full elucidation of the function of BRCA1 protein and the mechanism by which BRCA1 expression is reduced in both hereditary and sporadic breast carcinomas should lead to a better understanding of mechanism of mammary carcinogenesis, as well as the development of diagnostic, therapeutic, and preventive strategies.

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