Screening of BRCA1 Mutation Using Immunohistochemical Staining with C-Terminal and N-Terminal Antibodies in Familial Ovarian Cancers

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We examined the subcellular localization of BRCA1 proteins using immunohistochemical staining with C-terminal (GLK-2 antibody) and N-terminal (Ab-2 antibody) monoclonal antibodies in 44 familial ovarian cancers. Among these, 24 cases were associated with 13 independent germ-line mutations of BRCA1, and loss of heterozygosity (LOH) at one or more BRCA1 microsatellite markers was found in all 21 informative tumors tested. With GLK-2 antibody, cytoplasmic staining was observed in 15 of 16 tumors (93.8%) with mutation in exon 11, and BRCA1 staining was absent in 8 of 8 tumors (100%) with mutation in exons other than exon 11. When immunohistochemical staining was performed with Ab-2 antibody, both nuclear and cytoplasmic staining were observed in 14 of 16 tumors (87.5%) with mutation in exon 11. Interestingly, nuclear staining was observed in 3 of 3 tumors (100%) with mutation downstream of exon 11, even though no staining was detected in 5 of 5 tumors (100%) with mutation upstream of exon 11. On the other hand, in familial ovarian cancers without BRCA1 mutations, nuclear staining or both nuclear and cytoplasmic staining was observed in 18 of 20 specimens (90%) and 20 of 20 specimens (100%) with GLK-2 antibody and with Ab-2 antibody, respectively. These results suggest that an immunohistochemical assay in combination with employing the C-terminal and the N-terminal antibodies appears to have potential as a reliable and useful technique for the screening of BRCA1 mutations, at least to predict the status of mutation, upstream or downstream of exon 11.

Key words: Familial ovarian cancer — BRCA1 — Subcellular localization — Splice variant — Immunohistochemistry

Since the cloning of the BRCA1 gene on chromosome 17q21 in 1994,1) many collaborative laboratories have reported a good many different germ-line mutations of BRCA1, in which 85% of the mutations are frameshifts or nonsense mutations predicted to result in protein truncation.1–11) The BRCA1 mutation database established by the Breast Cancer Information Core contains nearly 700 mutations. Germ-line mutations of BRCA1 are predicted to be responsible for 45% of breast cancer families and 80% of breast-ovarian cancer families.12) The lifetime risk for mutation carriers is reported to be 85% for breast cancer and 60% for ovarian cancer.4, 13) Recently, we have reported that the expected lifetime risk of ovarian cancer is 80% for Japanese women with germ-line mutations of BRCA1.14) Although new methodologies for genetic testing have been developed, they are still laborious and expensive because the BRCA1 gene is very large and does not have any mutation hot spots. Considering the high frequency of truncating mutations, we have focused our attention on the possibility that a C-terminal antibody may be unable to

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detect the truncated proteins. Therefore, we used immunohistochemical assay for the screening of BRCA1 mutations.

Regarding the subcellular localization of BRCA1 proteins, some groups have reported that BRCA1 is detected in the nucleus with both N-terminal and C-terminal antibodies.\(^\text{15-21}\) Furthermore, it has been demonstrated that nuclear localization signals (NLSs) exist in exon 11, and that a BRCA1 splice variant lacking exon 11 (BRCA1-\(\Delta\) exon 11) is localized in the cytoplasm.\(^\text{19, 20}\) Although various germ-line mutations of \(\textit{BRCA1}\) have been reported, little is known about the intracellular location of BRCA1 proteins in the tumors with \(\textit{BRCA1}\) mutation. We performed loss of heterozygosity (LOH) analysis at the \(\textit{BRCA1}\) locus to determine whether the wild-type allele had been lost in the tumors with \(\textit{BRCA1}\) mutation. Furthermore, in order to investigate the correlation between the subcellular localization and germ-line mutations of \(\textit{BRCA1}\), we examined the BRCA1 localization with the C-terminal and the N-terminal monoclonal antibodies using an immunohistochemical technique in Japanese familial ovarian cancers with or without \(\textit{BRCA1}\) mutation.

**MATERIALS AND METHODS**

**Patients and tumor specimens** We examined 44 epithelial familial ovarian cancer patients in 20 site-specific ovarian cancer families and 10 breast-ovarian cancer families in Japan. The criteria for a site-specific ovarian cancer family, a breast-ovarian cancer family, and the \(\textit{BRCA1}\) mutation analysis used have been described in detail previously.\(^\text{10}\) In 24 patients from 20 families, we found 13 independent germ-line mutations of \(\textit{BRCA1}\), consisting of 9 frameshifts, 3 nonsense mutations, and one missense mutation (G to T substitution at nucleotide 5451). This missense mutation, which presumably leads to a splice aberration, was not observed in healthy women in this family or in a number of healthy volunteers without a family history of ovarian and/or breast cancers, indicating that the abnormality could be diagnosed as a pathogenic mutation, but not as a polymorphism. Germ-line mutations of \(\textit{BRCA1}\) in these tumors are shown in Table I. Furthermore, 20 epithelial ovarian cancer patients, who had no known family history of ovarian and/or breast cancers, were examined in this study. Histological subtypes using the

| Table I. Germ-line Mutations of \(\textit{BRCA1}\) in Familial Ovarian Cancers |
|---|
| **Family** | **Case No.** | **Histology** | **BRCA1 mutation** |
|---|---|---|---|
| Exon | Codon | Nucleotide | Amino acid change | Predicted effect |
|---|---|---|---|---|
| 2 (Ov)\(^\text{a}\) | 6 | Serous | 11 | 934 | C 2919 T | Gln to stop | Protein truncation |
| 14 (Ov) | 231 | Serous | 11 | 652 | 2073 del A | Frameshift | Protein truncation |
| 26 (Br/Ov)\(^\text{b}\) | 26-1 | Serous | 11 | 1133 | 3516 del TT | Frameshift | Protein truncation |
| 31 (Br/Ov) | 31-1 | Undifferentiated | 11 | 871 | 2730 del CC | Frameshift | Protein truncation |
| 32 (Br/Ov) | 32-1 | Serous | 11 | 692 | 2194 del AT | Frameshift | Protein truncation |
| 34 (Br/Ov) | 34-1 | Serous | 11 | 796 | 2507 del AG | Frameshift | Protein truncation |
| 34 (Br/Ov) | 34-2 | Serous | 11 | 796 | 2507 del AG | Frameshift | Protein truncation |
| 36 (Ov) | 36-1 | Serous | 11 | 934 | C 2919 T | Gln to stop | Protein truncation |
| 36 (Ov) | 36-2 | Serous | 11 | 934 | C 2919 T | Gln to stop | Protein truncation |
| 54 (Br/Ov) | 54-1 | Serous | 11 | 1309 | 4046 del TACA | Frameshift | Protein truncation |
| 54 (Br/Ov) | 54-3 | Serous | 11 | 1309 | 4046 del TACA | Frameshift | Protein truncation |
| 55 (Br/Ov) | 55-1 | Serous | 11 | 934 | C 2919 T | Gln to stop | Protein truncation |
| 70 (Br/Ov) | 70-1 | Endometrioid | 11 | 934 | C 2919 T | Gln to stop | Protein truncation |
| G15 (Ov) | G15-1 | Serous | 11 | 934 | C 2919 T | Gln to stop | Protein truncation |
| H117 (Br/Ov) | H117-2 | Serous | 11 | 1125 | 3494 del TC | Frameshift | Protein truncation |
| H117 (Br/Ov) | H117-3 | Serous | 11 | 1125 | 3494 del TC | Frameshift | Protein truncation |
| 1 (Ov) | 16 | Serous | 3 | 41 | 241 del A | Frameshift | Protein truncation |
| 15 (Ov) | 241 | Serous | 5 | 63 | T 307 A | Leu to stop | Protein truncation |
| 33 (Br/Ov) | 33-1 | Endometrioid | 5 | 63 | T 307 A | Leu to stop | Protein truncation |
| 65 (Ov) | 65-1 | Serous | 5 | 63 | T 307 A | Leu to stop | Protein truncation |
| 71 (Br/Ov) | 71-1 | Serous | 8 | 169 | C 624 T | Gln to stop | Protein truncation |
| 21 (Ov) | 290 | Serous | 12 | 1373 | 4237 del AG | Frameshift | Protein truncation |
| 30 (Ov) | 30-2 | Serous | 12 | 1373 | 4237 del AG | Frameshift | Protein truncation |
| 48 (Ov) | 48-1 | Serous | 21 | 1778 | G 5451 T | Asp to Tyr | Splice aberration |

\(^{a}\) Ov: Site-specific ovarian cancer family.

\(^{b}\) Br/Ov: Breast-ovarian cancer family.
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classification of the World Health Organization are as follows: 9 serous adenocarcinomas, 6 mucinous adenocarcinomas, 3 clear cell carcinomas and 2 endometrioid adenocarcinomas.

Formalin-fixed, paraffin-embedded specimens from the patients were collected after obtaining informed consent. Familial ovarian cancer specimens were obtained at several hospitals in Japan, and sporadic ovarian cancer specimens were obtained at Niigata University Hospital between 1995 and 1998. In addition, normal ovarian tissues were obtained from the patients with ovarian dermoid cysts, uterine cervical cancer, or ovarian cancer at Niigata University Hospital.

**DNA extraction** For LOH experiments, normal and tumor DNAs were extracted from peripheral blood or formalin-fixed, paraffin-embedded non-cancerous tissues and formalin-fixed, paraffin-embedded tumor tissues by using standard phenol-chloroform extraction procedures, respectively.

**LOH analysis** We performed LOH analysis at three microsatellite markers, D17S855, D17S1322 and D17S1323, located on the BRCA1 gene. The polymerase chain reaction (PCR) products were mixed with 95% formamide, denatured and separated on 6% denaturing polyacrylamide gels. DNA fragment analysis was done on an automated sequencer (ALF Sequencer II, ALF Express) using Fragment Manager software (Pharmacia Biotech in Japan, Tokyo). Cases were considered informative when heterozygosity was detected in the normal control tissues. LOH was defined as a reduction of 50% or more in the peak area of one of the tumor sample alleles as compared to the normal sample. All positive samples for LOH at individual loci were analyzed at least twice in independent LOH assays.

**Antibody** The GLK-2 monoclonal antibody (Phenopath Laboratory, Seattle, WA) was directed against peptides corresponding to C-terminal amino acids 1839 to 1863 of the human BRCA1 protein. The Ab-2 monoclonal anti-

| Case No. | LOH a) | IHC b) | Ab-2 | GLK-2 |
|----------|--------|--------|------|-------|
|          | D17S855 | D17S1322 | D17S1323 |       |
| 6        | UI     | UI     | UI   | Both c) | Cytoplasm |
| 231      | •      | •      | ND   | Both    | Cytoplasm |
| 26-1     | •      | UI     | ◯    | Cytoplasm | Cytoplasm |
| 31-1     | •      | •      | •    | Both    | Cytoplasm |
| 32-1     | UI     | •      | •    | Both    | Cytoplasm |
| 34-1     | UI     | •      | •    | Both    | Cytoplasm |
| 34-2     | UI     | •      | •    | Both    | Cytoplasm |
| 36-1     | •      | •      | •    | Both    | Cytoplasm |
| 36-2     | ND     | ND     | ND   | Both    | Cytoplasm |
| 54-1     | UI     | •      | ◯    | Both    | Cytoplasm |
| 54-3     | •      | •      | ◯    | Both    | Cytoplasm |
| 55-1     | •      | •      | ◯    | Both    | Cytoplasm |
| 70-1     | •      | •      | ◯    | Both    | Cytoplasm |
| G15-1    | ◯      | •      | •    | Both    | Cytoplasm |
| H117-2   | UI     | •      | UI   | Both    | Cytoplasm |
| H117-3   | ND     | ND     | ND   | Both    | Absence |
| 16       | •      | •      | •    | Absence | Absence |
| 241      | •      | •      | •    | Absence | Absence |
| 33-1     | UI     | •      | ◯    | Absence | Absence |
| 65-1     | •      | •      | •    | Absence | Absence |
| 71-1     | •      | •      | •    | Absence | Absence |
| 290      | •      | •      | •    | Nucleus | Absence |
| 30-2     | •      | •      | •    | Nucleus | Absence |
| 48-1     | •      | •      | •    | Nucleus | Absence |

a) LOH: Loss of heterozygosity.
b) IHC: Immunohistochemistry.
c) Both: Nucleus and cytoplasm.
•: loss of heterozygosity; ◯: retention of heterozygosity; UI, uninformative; ND, no data.
body (Oncogene Research Products, Cambridge, MA) was raised against epitopes of N-terminal amino acids 1 to 304 of the human BRCA1 protein.

**Immunohistochemical assay of BRCA1** Immunohistochemical studies were performed on 5 μm sections, placed on polylysine-coated slides. After deparaffinization in xylene and rehydration through graded alcohol, each section was treated with 1% hydrogen peroxide for 20 min, and then blocking serum was applied for 20 min. The slides were incubated overnight with the primary antibody (GLK-2 at 1:10000 and Ab-2 at 1:10) at 4 °C in a closed chamber. Immunohistochemical staining was performed by the streptoavidin-biotin-peroxidase complex technique (Histofine SAB-PO(M) Kit; Nichirei Corp., Tokyo). Staining was done with 3,3′-diaminobenzidine followed by light counterstaining with methyl green and dehydration.

We classified the results of immunohistochemical assay as follows: nuclear staining, cytoplasmic staining, both nuclear and cytoplasmic staining, and absence of BRCA1 staining. Immunohistochemical stainings were analyzed by two independent observers.

**Statistical analysis** Fisher’s exact test was used to evaluate associations between the subcellular localization and germ-line mutations of BRCA1.

To assess the screening potential of immunohistochemical assay for predicting germ-line mutations of BRCA1, the sensitivity, specificity and positive predictive values were calculated for each criterion of immunohistochemical staining (nucleus, cytoplasm, both nucleus and cytoplasm, and absence of staining).

**RESULTS**

**LOH analysis** In familial ovarian cancers with germ-line mutation of BRCA1, LOH at the markers D17S855, D17S1322 and D17S1323 was detected in 93% (14 of 15 informative cases), 100% (20 of 20 informative cases) and 68% (13 of 19 informative cases) of tumors, respectively (Table II). LOH in at least one of the BRCA1 microsatellite markers was detected in all 21 informative tumors tested. In addition, in familial ovarian cancers without BRCA1 mutations, LOH was found in 8/14 (57%) of informative tumors at D17S855, 7/14 (50%) of informative tumors at D17S1322 and 9/14 (64%) of informative tumors at D17S1323.

### Table III. Results of LOH Analysis and Immunohistochemical Assay in Familial Ovarian Cancers without Germ-line Mutations of BRCA1

| Family Case No. Histology | LOH a) | Immunohistochemical Assay b) |
|--------------------------|--------|-----------------------------|
| 4 (Ov) 91               | D17S855 | Serous                      |
| 4 (Ov) 92               | D17S1322 | Serous                      |
| 6 (Ov) 71               | D17S1323 | Endometrioid                |
| 6 (Ov) 72               | Ab-2    | Clear cell                  |
| 11 (Ov) 210             | GLK-2   | Serous                      |
| 11 (Ov) 211             |         | Endometrioid                |
| 13 (Ov) 220             |         | Serous                      |
| 13 (Ov) 221             |         | Endometrioid                |
| 16 (Ov) 250             |         | Clear cell                  |
| 16 (Ov) 251             |         | Clear cell                  |
| 27 (Ov) 27-1            |         | Mucinous                    |
| 27 (Ov) 27-2            |         | Brenner                      |
| 60 (Ov) 60-3            |         | Serous                      |
| 60 (Ov) 60-4            |         | Endometrioid                |
| 61 (Ov) 61-1            |         | Endometrioid                |
| 61 (Ov) 61-2            |         | Serous                      |
| 75 (Ov) 75-1            |         | Serous                      |
| 75 (Ov) 75-2            |         | Serous                      |
| 78 (Ov) 78-1            |         | Serous                      |
| 78 (Ov) 78-2            |         | Serous                      |

a) LOH: Loss of heterozygosity.
b) IHC: Immunohistochemistry.
c) Ov: Site-specific ovarian cancer family.
d) Both: Nucleus and cytoplasm.

. loss of heterozygosity; . retention of heterozygosity; UI, uninformative; ND, no data.
The screening of BRCA1 mutation with immunohistochemistry in 20 sporadic ovarian cancers was performed. The frequencies of allelic losses found at the markers D17S855, D17S1322, and D17S1323 were 53% (8 of 15 informative cases), 36% (5 of 14 informative cases), and 31% (5 of 16 informative cases), respectively (Table IV).

Expression of BRCA1 protein in familial ovarian cancer tissues with germ-line mutation of BRCA1

First, we employed GLK-2 antibody in order to study the expression of the BRCA1 gene product in 24 familial ovarian cancer specimens with germ-line mutations of BRCA1. In 15 of 24 tumor specimens, GLK-2 antibody had only cytoplasmic staining, but not nuclear staining, and in 9 tumors, no staining was observed at all. When the relationship between GLK-2 staining mode and the position of germ-line mutations of BRCA1 was analyzed, GLK-2 cytoplasmic staining (Fig. 1A) was detected in 15 of 16 (93.8%) specimens with mutation in exon 11, and no staining (Fig. 1, C and E) was seen in 8 of 8 (100%) specimens with mutation in exons other than exon 11. Exceptionally, one case with mutation in exon 11 showed no staining with GLK-2 antibody (Table II). By the use of GLK-2 antibody, specific immunostaining identifying BRCA1 protein was visualized in the majority of tumor cells in all areas of the specimens, and the intensity of cytoplasmic staining was homogeneous and relatively high. In addition, nuclear staining was observed in the stromal cells and in the lymphocytes of the sections where the tumor cells had no BRCA1 staining.

Secondly, when Ab-2 antibody was employed to detect the BRCA1 protein in ovarian cancer specimens, both the nucleus and cytoplasm (Fig. 1B) were stained in 14 of 16

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**Table IV. Results of LOH Analysis and Immunohistochemical Assay in Sporadic Ovarian Cancers and Normal Ovarian Tissues**

| Case No. | Histology   | LOH  | IHC  | Ab-2 | GLK-2 |
|----------|-------------|------|------|------|-------|
|          | D17S855     | D17S1322 | D17S1323 |      |       |
| Sporadic ovarian cancers |              |      |      |      |       |
| S-1      | Serous      | ●     | UI   | ●    | Both  |
| S-2      | Serous      | UI    | UI   | ●    | Nucleus |
| S-3      | Serous      | ○     | ○    | ○    | Nucleus |
| S-4      | Serous      | UI    | ○    | ○    | Nucleus |
| S-5      | Serous      | UI    | ●    | ●    | Both  |
| S-6      | Serous      | ●     | UI   | ●    | Both  |
| S-7      | Serous      | ●    | ●    | UI   | Both  |
| S-8      | Serous      | ●    | ●    | ○    | Both  |
| S-9      | Serous      | UI    | ●    | ●    | Both  |
| S-10     | Mucinous    | ○     | UI   | ○    | Nucleus |
| S-11     | Mucinous    | ○    | ○    | ○    | Nucleus |
| S-12     | Mucinous    | ○    | UI   | UI   | Nucleus |
| S-13     | Mucinous    | ○    | ○    | UI   | Nucleus |
| S-14     | Mucinous    | ●    | ●    | ○    | Cytoplasm |
| S-15     | Mucinous    | ●    | ○    | UI   | Absence |
| S-16     | Clear cell  | UI    | ○    | ○    | Nucleus |
| S-17     | Clear cell  | ○    | ○    | ○    | Nucleus |
| S-18     | Clear cell  | ○    | UI   | ○    | Both  |
| S-19     | Endometrioid| ●    | ○    | ○    | Nucleus |
| S-20     | Endometrioid| ●    | ○    | ○    | Both  |
| Normal ovarian epithelium |              |      |      |      |       |
| N-1      |             |      |      | Nucleus | Nucleus |
| N-2      |             |      |      | Nucleus | Nucleus |
| N-3      |             |      |      | Nucleus | Nucleus |
| N-4      |             |      |      | Both  | Both |
| N-5      |             |      |      | Both  | Both |

a) LOH: Loss of heterozygosity.
b) IHC: Immunohistochemistry.
c) Both: Nucleus and cytoplasm.
●, loss of heterozygosity; ○, retention of heterozygosity; UI, uninformative.
tumor specimens (87.5%) with mutation in exon 11. This staining was homogeneous and detected in most of the tumor cells in the sections. In contrast to the staining with GLK-2 antibody, nuclear staining (Fig. 1F) by Ab-2 antibody was observed in 3 of 3 (100%) tumor tissues with mutation downstream of exon 11, and no staining (Fig. 1D) was seen in 5 of 5 (100%) tumor tissues with mutation upstream of exon 11 (Table III). Nuclear staining by Ab-2 antibody was noted in over 70% of the tumor cells and the surrounding lymphocytes within the sections, and the intensity in the nucleus was different in each tumor cell.

Expression of BRCA1 protein in familial ovarian cancer tissues without BRCA1 mutation We examined the subcellular localization of BRCA1 proteins with GLK-2 antibody and Ab-2 antibody in 20 familial ovarian cancer specimens without BRCA1 mutations. Although ovarian cancer tissues with BRCA1 mutation were homogeneous in terms of histological subtypes (21 of 24 (88%) samples were serous adenocarcinomas), tumor tissues without
BRCA1 mutations varied as to histological subtypes: 11 serous adenocarcinomas (55%), 4 endometrioid adenocarcinomas, 3 clear cell carcinomas, one mucinous adenocarcinoma and one malignant Brenner tumor, as shown in Table III.

In 17 tumors, the staining patterns with GLK-2 antibody and Ab-2 antibody were almost identical. Nuclear staining (Fig. 1, G and I) was observed in 8 tumor specimens (40%), and both nuclear and cytoplasmic staining (Fig. 1, H and J) were detected in 9 tumor specimens (45%). Different staining patterns were found in 3 cases. Although cytoplasmic staining in 2 cases (case No. 71 and 221) and nuclear staining in one case (case No. 211) were observed with GLK-2 antibody, both nuclear and cytoplasmic staining were detected by Ab-2 antibody (Table III). These expressions were found in over 90% of the tumor cells in the specimens, and the intensity of the nuclear dot pattern varied in each tumor cell.

Expression of BRCA1 protein in sporadic ovarian cancer tissues In 20 sporadic ovarian cancers, staining patterns by GLK-2 and Ab-2 antibody were almost identical. Nuclear staining (Fig. 1, G and I) was observed in 8 tumor specimens (40%), and both nuclear and cytoplasmic staining (Fig. 1, H and J) were detected in 9 tumor specimens (45%). Different staining patterns were found in 3 cases. Although cytoplasmic staining in 2 cases (case No. 71 and 221) and nuclear staining in one case (case No. 211) were observed with GLK-2 antibody, both nuclear and cytoplasmic staining were detected by Ab-2 antibody (Table III). These expressions were found in over 90% of the tumor cells in the specimens, and the intensity of the nuclear dot pattern varied in each tumor cell.

Table V. Screening Validity of Immunohistochemical Assay with GLK-2 Antibody for Predicting Germ-line Mutations of BRCA1

| Criteria of IHC | BRCA1 mutation | Sensitivity (%) | Specificity (%) | Positive predictive value (%) |
|---------------|----------------|----------------|----------------|-----------------------------|
| Cytoplasm     | Exon 11        | 93.8           | 92.9           | 88.2                        |
| Absence       | Others<sup>b</sup> | 100.0          | 97.2           | 88.9                        |
| Nucleus or Both<sup>c</sup> | Negative | 90.0           | 100.0          | 100.0                       |

<sup>a</sup>) IHC: Immunohistochemistry.
<sup>b</sup>) Others: Exons other than exon 11.
<sup>c</sup>) Both: Nucleus and cytoplasm.

DISCUSSION

We analyzed the subcellular localization of BRCA1 proteins using immunohistochemical assay in 44 familial ovarian cancers with or without BRCA1 mutations. With GLK-2 antibody, nuclear staining was completely absent in all 24 cases carrying germ-line mutation of BRCA1, and cytoplasmic staining appeared in 15 of 16 specimens (93.8%) in which frameshift or nonsense mutation exists in exon 11 (Table II). In this study, we analyzed only one case with missense mutation, G to T at nucleotide 5451 in exon 21, which leads to aberration of a splicing site of exon 21. In this case, no signal at all was seen with GLK-2 antibody, suggesting the possibility that this splicing aberration involved exon 24, containing the GLK-2 epitopes. Previous studies have well documented that a splice variant protein lacking most of exon 11 (BRCA1-Δ exon 11) exists in a number of normal or malignant tissues, including ovarian tissues. In fact, we observed cytoplasmic staining, presumably due to splice variants, in normal ovarian tissues. Therefore, cytoplasmic staining may represent the detection of BRCA1-Δ exon 11 protein by GLK-2 antibody. In addition, nuclear staining with GLK-2 antibody was found in the majority of tumor samples from familial ovarian cancer patients without BRCA1 mutations (90%) and sporadic ovarian cancer patients (85%), as well as normal ovarian tissues (100%) as shown in Tables III and IV.
Thus, by utilizing GLK-2 antibody, significant correlations are obtained between the immunohistochemical staining pattern and the mutational position of the \textit{BRCA1} gene; \textit{BRCA1} mutation in exon 11 is indicated by cytoplasmic staining, mutation in exons other than exon 11 is indicated by absence of \textit{BRCA1} staining, and the absence of \textit{BRCA1} mutation is indicated by nuclear staining \((P<0.01)\). The difference of localization should allow us to predict whether familial ovarian tumors have \textit{BRCA1} mutation or not with more than 90% sensitivity and specificity (Table V).

On the other hand, with Ab-2 antibody, nuclear staining was observed in 17 of 19 tumors with mutation downstream of the NLSs localized in exon 11, whereas \textit{BRCA1} staining was absent in the tumors with mutation upstream of the NLSs in exon 11 (Table II).

Based on the present results, we predict the correlation between germ-line mutations and immunohistochemical staining patterns of \textit{BRCA1} protein with Ab-2 and GLK-2 antibodies shown in Fig. 2. These findings are consistent with the notion that full-length \textit{BRCA1} protein is present in the nucleus, whereas aberrant proteins may exist in two forms; first as truncated protein in the nucleus in cases in which the NLSs are retained, and secondly, as splice variant protein lacking most of exon 11 (including the NLSs) in the cytoplasm. Thakur \textit{et al.} have reported that two putative NLSs: amino acids 501 to 507 (NLS1; KCK-RKRR) and 607 to 614 (NLS2; KKNRLRRK) are present in exon 11, and only NLS1 is necessary for nuclear transport.\(^{19}\) Although there was no ovarian cancer with \textit{BRCA1} mutation upstream of the NLSs in exon 11 in this study, these tumors may show only cytoplasmic staining with Ab-2 antibody.\(^{20}\) Since nuclear staining with Ab-2 antibody indicates that the mutation exists downstream of the NLSs in the tumors with \textit{BRCA1} mutation, Ab-2 antibody may be useful for distinguishing whether the mutation exists up- or downstream of the NLSs in exon 11.

Furthermore, in all 21 informative cases with \textit{BRCA1} mutation, LOH was detected at least in one of the markers D17S855, D17S1322 and D17S1323 on the \textit{BRCA1} gene.
This finding clearly rules out the possibility that the antibody could detect products from wild-type allele in addition to those from mutated allele.

In one case (No. 221) of familial ovarian tumor without germ-line mutations of BRCA1, both cytoplasmic staining with GLK-2 antibody and LOH at the BRCA1 region were observed. Several explanations might be possible for the result obtained in this case, such as intronic mutation causing splicing aberration, lower sensitivity of single-strand conformation polymorphism (SSCP) analysis, somatic cell mutation detected only in tumor tissues but not in normal cells, and independent LOH of BRCA1 mutation.

In addition, in the analysis of sporadic ovarian cancer tissues, GLK-2 antibody detected cytoplasmic staining in two cases (S-5, 14) and no BRCA1 staining in one case (S-15), whereas LOH at the BRCA1 locus was observed in all three cases (Table IV). Two interpretations could be placed on these three cases. First, they may carry germ-line mutation in the BRCA1 gene, although we could not obtain a positive family history of ovarian and/or breast cancers from hospital records or record data from patients. Secondly, these tumors may have somatic mutation in the BRCA1 gene. The detection of BRCA1 somatic mutations in these tumor tissues, which showed cytoplasmic staining by GLK-2 antibody, would strengthen the results of this experiment. However, we could not successfully perform the direct sequence analysis of the entire coding region of BRCA1, since insufficient PCR products were obtained from DNAs extracted from paraffin-embedded samples.

In addition, Wilson et al. have demonstrated that over-expression of the full-length BRCA1 protein results in cellular toxicity, whereas the BRCA1-Δ exon 11 protein is not toxic.²⁰ Hence, we compared the clinical features of patients with mutation in exon 11 to those with mutation in exons other than exon 11. However, no clear difference was observed between the two groups of patients in terms of the outcome assessed as survival rate or disease-free interval (data not shown).

According to the present results, immunohistochemical assay with C-terminal antibody may have significant value in screening for BRCA1 mutations. It has been shown that BRCA1 mutation carriers have a lifetime risk of 85% for breast cancer and 60% for ovarian cancer.⁴,¹³ In Japan, preliminary analysis suggests that the expected lifetime risk of ovarian cancer is about 80% for women with germ-line BRCA1 mutations.¹⁹ Additionally, although the risk of breast cancer following ovarian cancer is unknown, it has been reported that some ovarian cancers with BRCA1 mutations subsequently develop breast cancer.⁵,²⁵ Therefore, two possible advantages of this analysis exist in clinical practice. First, a positive test might indicate that other family members are at risk for ovarian and/or breast cancers. Secondly, a positive antibody test might alert a woman with ovarian cancer that she also has an increased susceptibility to breast cancer. However, since immunohistochemical assay requires tumor materials, only women who have ovarian cancer can be examined and the analysis can not identify individuals at risk for ovarian cancer. Nevertheless, since a definite advantage is afforded by an immunohistochemical technique, the prospect of a project which screens ovarian cancer tissues, including those of hereditary ovarian cancer families, offers an attractive model for further investigation.

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REFERENCES

1) Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. K., Norris, F. H., Helvering, L., Morrison, P., Rosteck, P., Lai, M., Barrett, J. C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A. and Skolnick, M. H. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science, 266, 66–71 (1994).

2) Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., Miki, Y., Eddington, K., McClure, M., Frye, C., Weaver-Feldhaus, J., Ding, W., Gholami, Z.,
Soderkvist, P., Terry, L., Jhanwar, S., Berchuck, A., Iglehart, J. D., Marks, J., Ballinger, D. G., Barrett, J. C., Skolnick, M. H., Kamb, A. and Wiseman, R. BRCA1 mutations in primary breast and ovarian carcinomas. Science, 266, 120–122 (1994).

3) Castilla, L. H., Couch, F. J., Erdos, M. R., Hoskins, K. F., Calzone, K., Garber, J. E., Boyd, J., Lubin, M. B., Deshano, M. L., Brody, L. C., Collins, F. S. and Weber, B. L. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. Nat. Genet., 8, 387–391 (1994).

4) Simard, J., Tonin, P., Durocher, F., Morgan, K., Rommens, J. H., Couch, F. J., Erdos, M. R., Hoskins, K. F., Wolfe, J., G. M. A high incidence of BRCA1 mutations in 20 breast-cancer patients. Cancer Res., 55, 2998–3002 (1995).

5) Friedman, L. S., Ostermeyer, E. A., Szabo, C. I., Dowd, P., Lynch, E. D., Rowell, S. E. and King, M.-C. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. Nat. Genet., 8, 399–404 (1994).

6) Shattuck-Eidens, D., McClure, M., Simard, J., Labrie, F., Narod, S., Couch, F., Hoskins, K., Weber, B., Castilla, L., Erdos, M., Brody, L., Friedman, L., Ostermeyer, E., Szabo, C., King, M.-C., Jhanwar, S., Offit, K., Norton, L., Gilewski, T., Lubin, M., Osborne, M., Black, D., Boyd, M., Steel, M., Inglis, S., Haile, R., Lindblom, A., Olsson, H., Borg, A., Bishop, D. T., Solomon, E., Radice, P., Spatti, G., Gayther, S., Ponder, B., Warren, W., Stratton, M., Liu, Q., Fujimura, F., Lewis, C., Skolnick, M. H. and Goldgar, D. E. A collaborative survey of 80 mutations in the BRCA1 breast and ovarian cancer susceptibility gene. Implications for pre-symptomatic testing and screening. JAMA, 273, 535–541 (1995).

7) Takahashi, H., Behbakt, K., McGovern, P. E., Chiu, H.-C., Couch, F. J., Weber, B. L., Friedman, L. S., King, M.-C., Furusato, M., LiVolsi, V. A., Menzin, A. W., Liu, P. C., Benjamin, I., Morgan, M. A., King, S. A., Rebane, B. A., Cardonick, A., Mikuta, J. J., Rubin, S. C. and Boyd, J. Mutation analysis of the BRCA1 gene in ovarian cancers. Cancer Res., 55, 2998–3002 (1995).

8) Matsushima, M., Kobayashi, K., Emi, M., Saito, H., Saito, J., Suzumori, K. and Nakamura, Y. Mutation analysis of the BRCA1 gene in 76 Japanese ovarian cancer patients: four germline mutations, but no evidence of somatic mutation. Hum. Mol. Genet., 4, 1953–1956 (1995).

9) Serova, O., Montagna, M., Torchard, D., Narod, S. A., Tonin, P., Sylla, B., Lynch, H. T., Feunteun, J. and Lenoir, G. M. A high incidence of BRCA1 mutations in 20 breast-ovarian cancer families. Am. J. Hum. Genet., 58, 42–51 (1996).

10) Takano, M., Aida, H., Tsuneki, I., Takakuwa, K., Hasegawa, I., Tanaka, H., Saito, M., Tsuji, S., Sonoda, T., Hatae, M., Chen, J.-T., Takahashi, K., Hasegawa, K., Toyoda, N., Saito, N., Yukishiji, M., Araki, T. and Tanaka, K. Mutational analysis of BRCA1 gene in ovarian and breast-ovarian cancer families in Japan. Jpn. J. Cancer Res., 88, 407–413 (1997).

11) Stratton, M. F., Gaway, S. A., Russell, P., Dearden, J., Gore, M., Blake, P., Easton, D. and Ponder, B. A. J. Contribution of BRCA1 mutations to ovarian cancer. N. Engl. J. Med., 336, 1125–1130 (1997).

12) Easton, D. F., Bishop, D. T., Ford, D., Crockford, G. P. and the Breast Cancer Linkage Consortium. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. Am. J. Hum. Genet., 52, 678–701 (1993).

13) Ford, D., Easton, D. F., Bishop, D. T., Narod, S. A., Goldgar, D. E. and the Breast Cancer Linkage Consortium. Risks of cancer in BRCA1-mutation carriers. Lancet, 343, 692–695 (1994).

14) Aida, H., Takakuwa, K., Nagata, H., Tsuneki, I., Takano, M., Tsuji, S., Takahashi, T., Sonoda, T., Hatae, M., Takahashi, K., Hasegawa, K., Mizunuma, H., Toyoda, N., Kamata, H., Torii, Y., Saito, N., Tanaka, K., Yukishiji, M., Araki, T. and Tanaka, K. Clinical features of ovarian cancer in Japanese women with germ-line mutations of BRCA1. Clin. Cancer Res., 4, 235–240 (1998).

15) Chen, Y., Chen, C.-F., Riley, D. J., Alreed, D. C., Chen, P.-L., Hoff, D. V., Osborne, C. K. and Lee, W.-H. aberrant subcellular localization of BRCA1 in breast cancer. Science, 270, 789–791 (1995).

16) Sully, R., Ganasan, S., Brown, M., de Caprio, J. A., Cannista, S. A., Feunteun, J., Schnitt, S. and Livingston, D. M. Location of BRCA1 in human breast and ovarian cancer cells. Science, 272, 123–126 (1996).

17) Chen, Y., Chen, P.-L., Riley, D. J., Lee, W.-H., Alreed, D. C. and Osborne, C. K. Location of BRCA1 in human breast and ovarian cancer cells—response. Science, 272, 125–126 (1996).

18) Chen, Y., Farmer, A. A., Chen, C.-F., Jones, D. C., Chen, P.-L. and Lee, W.-H. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. Cancer Res., 56, 3168–3172 (1996).

19) Thakur, S., Zhang, H. B., Peng, Y., Le, H., Carroll, B., Ward, T., Yao, J., Farid, L. M., Couch, F. J., Wilson, R. B. and Weber, B. L. Localization of BRCA1 and a splice variant identifies the nuclear localization signal. Mol. Cell. Biol., 17, 445–452 (1997).

20) Wilson, C. A., Payton, M. N., Elliott, G. S., Buaas, F. W., Cuijulis, E. E., Grosshans, D., Ramos, L., Reese, D. M., Slamon, D. J. and Calzone, F. J. Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-Δ11b. Oncogene, 14, 1–16 (1997).

21) Wilson, C. A., Ramos, L., Villasenor, M. R., Andersen, K. H., Press, M. F., Clarke, K., Karlan, B., Chen, J.-J., Scully, R., Livingston, D., Zuch, R. H., Kanter, M. H., Cohen, S., Calzone, F. J. and Slamon, D. J. Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nat. Genet., 21, 236–240 (1999).

22) Sambrook, J., Fritsch, E. F. and Maniatis, T. Isolation of...
high-molecular-weight DNA from mammalian cells. In “Molecular Cloning—A Laboratory Manual,” pp. 9.14–9.23 (1989). Cold Spring Harbor Laboratory Press, New York.

23) Neuhausen, S. L., Swensen, J., Miki, T., Liu, Q., Tavtigian, S., Shattuck-Eidens, D., Kamb, A., Hobbs, M. R., Gingrich, J., Shizuya, H., Kim, U.-J., Cochran, C., Futreal, P. A., Wiseman, R. W., Lynch, H. T., Tonin, P., Narod, S., Cannon-Albright, L., Skolnick, M. H. and Goldgar, D. E. A P1-based physical map of the region from D17S776 to D17S78 containing the breast cancer susceptibility gene BRCA1. Hum. Mol. Genet., 3, 1919–1926 (1994).

24) Lu, M., Conzen, S. D., Cole, C. N. and Arrick, B. A. Characterization of functional messenger RNA splice variants of BRCA1 expressed in nonmalignant and tumor-derived breast cells. Cancer Res., 56, 4578–4581 (1996).

25) Frank, T. S., Manley, S. A., Olopade, O. I., Cummings, S., Garber, J. E., Bernhardt, B., Antman, K., Russo, D., Wood, M. E., Mullineau, L., Isaacs, C., Peshkin, B., Buys, S., Venne, V., Rowley, P. T., Loader, S., Offit, K., Robson, M., Hampel, H., Brener, D., Winer, E. P., Clark, S., Weber, B., Strong, L. C., Rieger, P., McClure, M., Ward, B. E., Shattuck-Eidens, D., Oliphant, A., Skolnick, M. H. and Thomas, A. Sequence analysis of BRCA1 and BRCA2: correlation of mutations with family history and ovarian cancer risk. J. Clin. Oncol., 16, 2417–2425 (1998).