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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.[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-Δ exon 11) is localized in the cytoplasm.[19, 20] Although various germ-line mutations of *BRCA1* have been reported, little is known about the intracellular location of BRCA1 proteins in the tumors with *BRCA1* mutation. We performed loss of heterozygosity (LOH) analysis at the *BRCA1* locus to determine whether the wild-type allele had been lost in the tumors with *BRCA1* mutation. Furthermore, in order to investigate the correlation between the subcellular localization and germ-line mutations of *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 *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 *BRCA1* mutation analysis used have been described in detail previously.[10] In 24 patients from 20 families, we found 13 independent germ-line mutations of *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 *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 *BRCA1* in Familial Ovarian Cancers

Family	Case No.	Histology	*BRCA1* mutation				
			Exon	Codon	Nucleotide	Amino acid change	Predicted effect
2 (Ov)[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)[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.

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,[22] respectively.

LOH analysis We performed LOH analysis at three microsatellite markers, D17S855, D17S1322 and D17S1323, located on the *BRCA1* gene.[1, 23] 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).[10] 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-

Table II. Results of LOH Analysis and Immunohistochemical Assay in Familial Ovarian Cancers with Germ-line Mutations of *BRCA1*

Case No.	LOH[a]			IHC[b]	
	D17S855	D17S1322	D17S1323	Ab-2	GLK-2
6	UI	UI	UI	Both[c]	Cytoplasm
231	●	●	ND	Both	Cytoplasm
26-1	●	UI	○	Cytoplasm	Cytoplasm
31-1	●	●	●	Both	Cytoplasm
32-1	UI	●	●	Cytoplasm	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.

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)]			IHC[b)]	
			D17S855	D17S1322	D17S1323	Ab-2	GLK-2
4 (Ov)[c)]	91	Serous	○	○	○	Nucleus	Nucleus
4 (Ov)	92	Serous	○	○	○	Nucleus	Nucleus
6 (Ov)	71	Endometrioid	UI	○	○	Both[d)]	Cytoplasm
6 (Ov)	72	Clear cell	UI	○	○	Nucleus	Nucleus
11 (Ov)	210	Serous	●	○	●	Both	Both
11 (Ov)	211	Endometrioid	●	●	●	Both	Nucleus
13 (Ov)	220	Serous	UI	UI	UI	Both	Both
13 (Ov)	221	Serous	●	●	●	Both	Cytoplasm
16 (Ov)	250	Clear cell	○	○	ND	Both	Both
16 (Ov)	251	Clear cell	UI	○	○	Nucleus	Nucleus
27 (Ov)	27-1	Mucinous	○	UI	UI	Nucleus	Nucleus
27 (Ov)	27-2	Brenner	○	UI	UI	Nucleus	Nucleus
60 (Ov)	60-3	Serous	●	UI	●	Both	Both
60 (Ov)	60-4	Endometrioid	●	●	●	Both	Both
61 (Ov)	61-1	Endometrioid	UI	UI	●	Nucleus	Nucleus
61 (Ov)	61-2	Serous	●	●	●	Nucleus	Nucleus
75 (Ov)	75-1	Serous	○	●	●	Both	Both
75 (Ov)	75-2	Serous	UI	UI	UI	Both	Both
78 (Ov)	78-1	Serous	●	●	●	Both	Both
78 (Ov)	78-2	Serous	●	●	UI	Both	Both

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.

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

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

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

tumors at D17S1323 (Table III). Furthermore, we performed LOH analysis in 20 sporadic ovarian cancers. 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

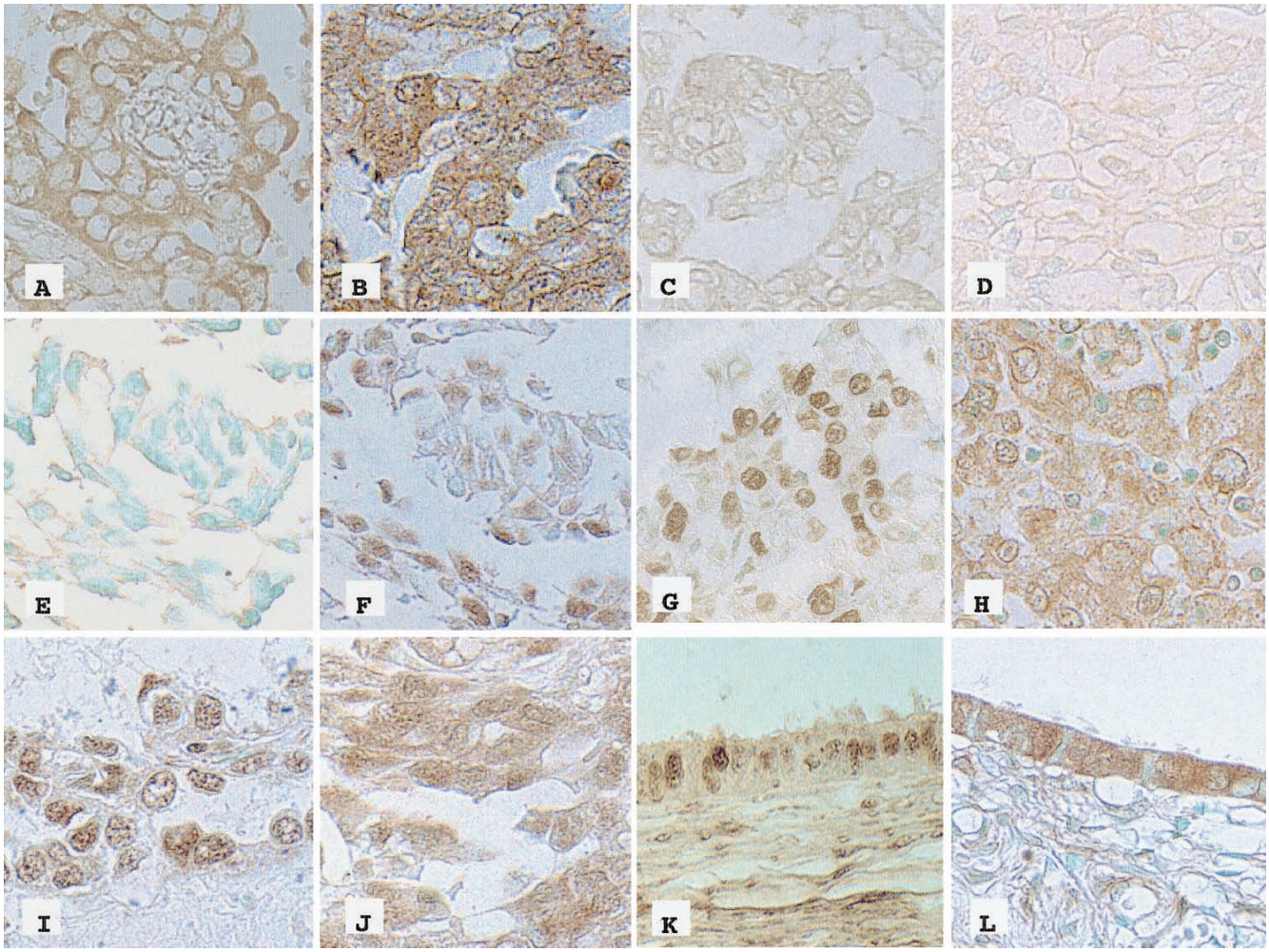

Fig. 1. Immunohistochemical staining of BRCA1 proteins with GLK-2 antibody or Ab-2 antibody in formalin-fixed, paraffin-embedded sections. Representative cases are shown. A and B: No. 54-1 tumor with *BRCA1* mutation in exon 11. Cytoplasmic staining is observed with GLK-2 antibody (A), whereas both nuclear and cytoplasmic staining is observed with Ab-2 antibody (B). C and D: No. 241 tumor with *BRCA1* mutation in exon 5. BRCA1 staining is absent with GLK-2 antibody (C) and Ab-2 antibody (D). E and F: No. 30-2 tumor with *BRCA1* mutation in exon 12. BRCA1 staining is absent with GLK-2 antibody (E), whereas nuclear staining is observed with Ab-2 antibody (F). G–J: tumors without *BRCA1* mutations. No. 72 tumor, nuclear staining with GLK-2 antibody (G). No. 75-1 tumor, both nuclear and cytoplasmic staining with GLK-2 antibody (H). No. 61-2 tumor, nuclear staining with Ab-2 antibody (I). No. 78-1 tumor, both nuclear and cytoplasmic staining with Ab-2 antibody (J). K and L: normal ovarian tissues. Nuclear staining with GLK-2 antibody (K). Both nuclear and cytoplasmic staining with Ab-2 antibody (L). ×100.

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

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

Criteria of IHC[a)]	*BRCA1* mutation	Sensitivity (%)	Specificity (%)	Positive predictive value (%)
Cytoplasm	Exon 11	93.8	92.9	88.2
Absence	Others[b)]	100.0	97.2	88.9
Nucleus or Both[c)]	Negative	90.0	100.0	100.0

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

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 detected as identical in 16 tumor specimens; in the nucleus in 9 samples (45%), both in the nucleus and the cytoplasm in 6 samples (30%) and in the cytoplasm in one sample. There was a difference in 4 cases; both nuclear and cytoplasmic staining by Ab-2 antibody in contrast to nuclear staining by GLK-2 antibody (cases No. S-1 and S-2), both nuclear and cytoplasmic staining by Ab-2 antibody in contrast to cytoplasmic staining by GLK-2 antibody (case No. S-5), and nuclear staining by Ab-2 antibody with no staining by GLK-2 antibody (case No. S-15) (Table IV). The intensity of staining in the cytoplasm was homogeneous in all samples tested, whereas the intensity of nuclear staining varied in each tumor cell, as noted in familial ovarian cancers without *BRCA1* mutations.

Expression of BRCA1 protein in normal ovarian tissues The expression of BRCA1 protein was also analyzed in normal ovarian epithelium. In 5 normal ovarian tissues, BRCA1 was detected in the nucleus (Fig. 1K) or in both the nucleus and the cytoplasm (Fig. 1L) in epithelial cells and in underlying connective tissues with GLK-2 or Ab-2 antibody (Table IV). Nuclear expression was observed in about 70% of normal cells and stromal cells within the sections, and the intensity of the immunoreactivity was relatively low.

Screening validity of immunohistochemical assay with GLK-2 antibody for predicting germ-line mutations of *BRCA1* The sensitivity, specificity and positive predictive values of cytoplasmic staining for predicting *BRCA1* mutation in exon 11, absence of BRCA1 staining for predicting *BRCA1* mutation in exons other than exon 11, and nuclear staining for predicting no *BRCA1* mutations are shown in Table V.

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.[19, 20, 24)] Although the function of these transcripts remains elusive, it has been shown that they are localized in the cytoplasm.[19, 20)] 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.

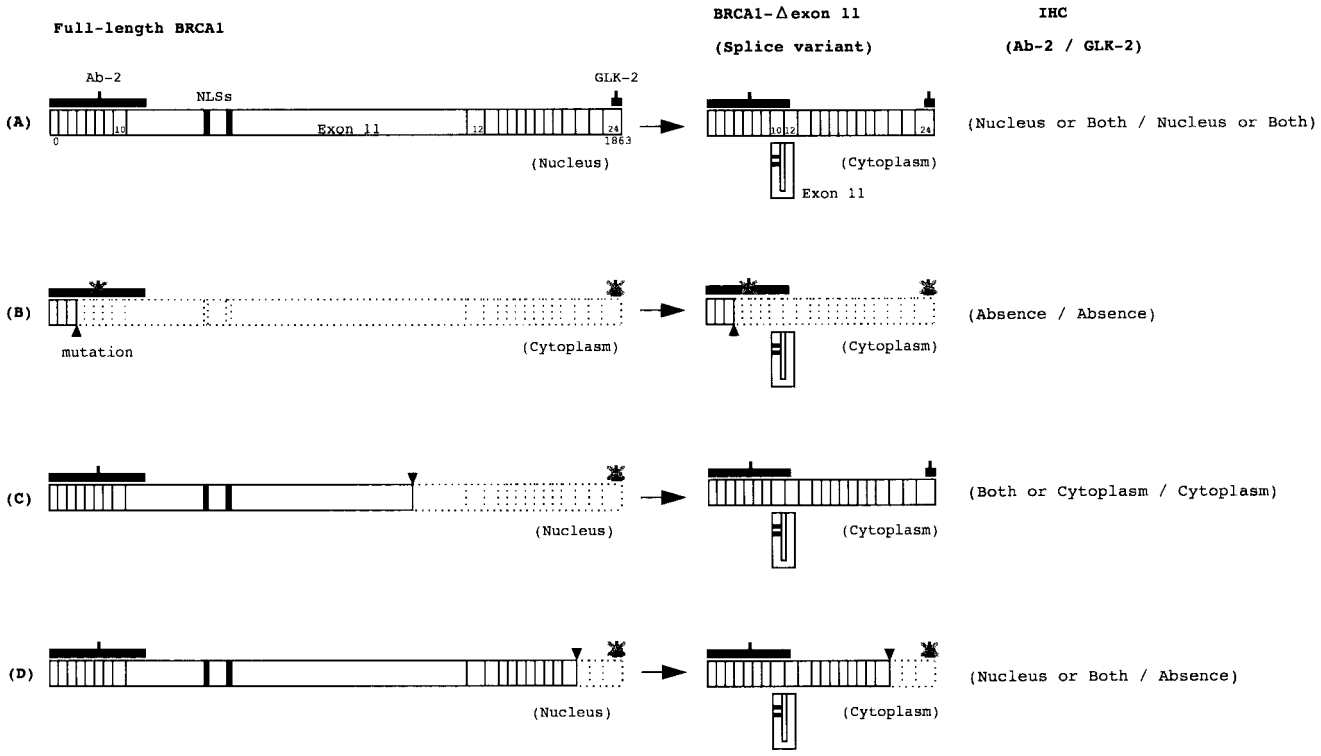

Fig. 2. Schematic representation of the correlation between germ-line mutations (frameshift or nonsense mutation) and immunohistochemical staining patterns of BRCA1 protein (full-length BRCA1 and BRCA1-Δ exon 11) with Ab-2 antibody and GLK-2 antibody. The predicted subcellular localization of the protein is indicated in brackets (lower right). The predicted results of the immunohistochemical assay with Ab-2 antibody and GLK-2 antibody are shown to the right of the figure. The position of the two putative nuclear localization signals (NLSs) are marked by black bars. IHC, immunohistochemistry; Both, nucleus and cytoplasm. (A) Tumor without *BRCA1* mutations. (B) Tumor with mutation in exons upstream of exon 11. (C) Tumor with mutation in exon 11 downstream of the NLSs. (D) Tumor with mutation in exons downstream of exon 11.

Thus, by utilizing GLK-2 antibody, significant correlations are obtained between the immunohistochemical staining pattern and the mutational position of the *BRCA1* gene; *BRCA1* mutation in exon 11 is indicated by cytoplasmic staining, mutation in exons other than exon 11 is indicated by absence of BRCA1 staining, and the absence of *BRCA1* mutation is indicated by nuclear staining ($P<0.01$). The difference of localization should allow us to predict whether familial ovarian tumors have *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 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 BRCA1 protein with Ab-2 and GLK-2 antibodies shown in Fig. 2. These findings are consistent with the notion that full-length 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 *et al.* have reported that two putative NLSs: amino acids 501 to 507 (NLS1; KCKRKRR) 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 *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 *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 *BRCA1* mutation, LOH was detected at least in one of the markers D17S855, D17S1322 and D17S1323 on the *BRCA1* gene

(Table II). 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 overexpression of the full-length BRCA1 protein results in cellullar 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* mutaions. 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 germline *BRCA1* mutations.[14] 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.[7, 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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