Immunolocalization of BRCA1 protein in tumor breast tissue: prescreening of BRCA1 mutation in Tunisian patients with hereditary breast cancer?

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BRCA1 is a tumor suppressor gene which is inactivated by mutation in familial breast and ovarian cancers. Over 300 different disease causing germ-line mutations have been described; 60% are unique to an individual family. This diversity and the large size of the gene lead us to search for a prescreening method for BRCA1 mutations. Since BRCA1 is a nuclear protein in normal cells, but reported by some authors to be cytoplasmic in breast tumor cells of patients with BRCA1 mutation, we evaluated immunohistochemistry as a prescreening technique to identify BRCA1 mutations in patients with familial presentation of breast cancer. Using a monoclonal antibody against the carboxy-terminal region of BRCA1, we performed immunohistochemistry on 18 tumor samples from patients with hereditary breast cancer. Cytoplasmic staining of BRCA1 was observed in 10 cases. Of the 18 tumors, 12 (66%) showed either BRCA mutation or BRCA1 accumulation or both, indicating that BRCA1 function might be lost in breast tumor cells not only through mutation, but also via abnormal cytoplasmic location. The immunohistochemical test used in this study would not be efficient as a pre-screening method of deleterious mutations, but it appeared useful to investigate tumor physiology.

Key Words: BRCA1, BRCA2, breast cancer, immunohistochemistry, DHPLC, sequencing, Tunisian population.

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Breast cancer is the most common type of cancer affecting women in the western world. Approximately 10 % of cases exhibit a familial pattern of inheritance (Nathanson *et al.* 2001). Thirty to fifty percent of familial cases involve mutations in one of the breast cancer susceptibility genes BRCA1 or BRCA2, (Miki *et al.* 1994; Wooster *et al.* 1995) that confer a high risk of breast and ovarian cancer (Arnold *et al.* 2002; Brose *et al.* 2002; Thompson *et al.* 2002).

Starting from the index patient with breast or ovarian cancer, associated criteria have been proposed to classify breast cancer families according to their BRCA1 or BRCA2 suggestivity (Kennedy *et al.* 2002): (i) two first or second degree relatives from the same side of the family diagnosed with breast or ovarian cancer at an average age of less than 60 years; (ii) three first or second degree relatives from the same side of the family diagnosed with breast or ovarian cancer at any age; (iii) mother or sister diagnosed with breast or ovarian cancer before the age of 35 years; (iv) one first or second degree relative with bilateral breast cancer, first cancer diagnosed before the age of 50 years; (VI) father or brother diagnosed with breast cancer before the age of 60 years. In the last case, screening is recommended for BRCA2 and not for BRCA1 (Kennedy *et al.* 2002).

BRCA1 consists of 23 exons over approximately 80 Kb with a coding region of 5.5 Kb. Two carboxyl-terminal BRCT domains are shared with several cell cycle checkpoint proteins, such as p53-binding protein 1 (Koonin *et al.* 1996). This structural feature, combined with the reported binding of BRCA1 to Rad50 (Zhong *et al.* 1999)

and Rad51 (Scully *et al.* 1997) as well as its ability to facilitate transcription-coupled DNA repair (Abbott *et al.* 1999), involves BRCA1 in the cellular response pathway for repair of DNA damage. BRCA1 also has an acidic carboxyl terminus that acts as a transactivation domain (Frankish 2001). Although BRCA1 is located predominantly in the nucleus, it contains an NH2-terminal nuclear export signal (NES) and can undergo dynamic shuttling between nucleus and cytoplasm (Rodriguez *et al.* 2000).

Over 300 different disease causing germ-line mutations of BRCA1 are reported in the breast cancer information core (BIC) (http://www.nhgri.nih.gov/Intramural_research/lab_transfer/bic). Because of its large size, the absence of widely available functional tests for the screening of BRCA1 mutations and the absence of mutational hot spots in the coding region, genetic testing for mutations is expensive and time consuming.

It was reported by Kashima et al that monoclonal antibody anti-BRCA1 produced against a peptide corresponding to amino-acids 1839-1863 of the C-terminus immunostained the cytoplasm of ovarian carcinoma cells with a mutation in BRCA1 exon 11 (Kashima *et al.* 2000). This antibody did not stain tumors with a mutation outside exon 11. This suggests that anti-BRCA1 antibody may give information about the location of mutations in BRCA1 in breast tumors.

We analyzed 18 samples from Tunisian patients highly suggestive of BRCA1 or BRCA2 mutation using an immunohistochemical test with a monoclonal antibody against BRCA1 protein to detect its accumulation in tumor cells, with the aim of using this test as a pre-screening method for BRCA1 mutations. The immunohistochemical analysis was followed by direct sequencing of the entire BRCA1 gene to confirm the nature and location of the expected mutations in selected families.

Material and Methods

Patients

Samples were obtained from 18 unrelated patients with hereditary breast and/or ovarian cancer (17 females and one male). All patients gave informed consent using a standardized written form. They were identified through the genetic consultation program of ISA institute (Salah Azaiez Institute of Carcinology). All patients fulfilled the selection criteria of genetic testing listed above. Tumor size, axilary lymph node status and the histological grade according to modified Bloom-Richardson (Page *et al.* 1987) were assessed for all index cases. Three histological types were identified, infiltrative ductal carcinoma (16/18 cases), lobular ductal carcinoma (one case) and in situ ductal carcinoma (one case).

Tumor size ranged from 8 mm to 80 mm. The axilary lymph node was positive for eight cases (8/18). Modified Bloom-Richardson histological grade was determined for 17 patients: 7 were grade III, 7 were grade II and 3 were grade I. These clinical features fit well with epidemiological data on Tunisian breast cancer patients.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed, paraffin wax embedded samples. Routine sections of 3 µm thick were cut onto DAKO Capillary Gap slides (S2024) (DAKO Corp, Carpinteria, CA), fixed in 10 per cent buffered formalin and dried at 60°C overnight. Slides were dewaxed in xylene transferred to absolute alcohol, and incubated in 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase. The slides were then transferred to running tap water before being transferred to 3 L of boiling citrate buffer pH 6.0 in a 15-lb pressure cooker. Slides were then rinsed in Tris-buffered saline (TBS) pH 7.4 and incubated in normal goat serum (1:10) for 10 minutes. The serum was

Table 1. Details of the primary antibodies.

Specificity	*Clonality*	*Mono/polyclonal*	*Dilutions*	*Code No*	*Source*
BRCA1	GLK-2	Monoclonal	1/50	M3606	DakoCytomation
Estrogens Receptor	1D5	Monoclonal	Prediluated	N1575	DakoCytomation
Progesterone Receptor	PgR636	Monoclonal	Prediluated	N1630	DakoCytomation
P53	DO-7	Monoclonal	1/50	M7001	DakoCytomation

tipped off and the sections incubated in primary antibody for 60 minutes at the appropriate dilution. Four monoclonal antibodies were used, anti-BRCA1 (GLK-2), anti-p53 (DO-7), anti-ER (1D5) and anti-PR (PgR636) (Table 1). After incubation, the slides were rinsed in TBS and incubated in DAKO Duet (K0492) biotinylated goat antimouse/rabbit secondary reagent (1:100) for 35 minutes. After rinsing in TBS, the slides were then incubated in DAKO Duet (K0492) streptavidin-biotin–horseradish peroxidase complex for 35 minutes, rinsed in TBS, and treated with DAB (3,3′ diaminobenzidine chromogen: 896102, Kem-En-Tec, Copenhagen, Denmark) for 10 minutes. The slides were then rinsed in tap water, counterstained in Mayer's hematoxylin and mounted.

Molecular analysis

DNA was first extracted by Proteinase K digestion from peripheral blood (10 mL) mononuclear cells isolated from each sample and then column purified (QIAGEN Inc, Chatsworth, CA, USA).

PCR conditions: Polymerase chain reactions amplifying BRCA1 or BRCA2 exons were performed in 50-µL containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5–4.5 mM MgCl2, 50 mM dNTPs, with 10 µM of each primer (designed by Centre Jean Perrin, sequences available on demand), 100 ng of genomic DNA, and 1 unit of either Taq polymerase or AmpliTaq Gold (PE Biosystems, Foster City, CA, USA). PCR cycling comprised an initial denaturation at 95°C for 10 min and 30 cycles of 94°C for 20 s, annealing at specific temperatures for each primer pair and extension at of 72°C for 45 s.

DHPLC: Denaturing high-performance liquid chromatography has been previously described (Oefner *et al.* 1997) on an automated HPLC instrument equipped with a DNASep column (Transgenomic Inc., San Jose, CA, USA).

PCR products are subjected to 3 min of denaturing at 95°C followed by gradual reannealing from 95 to 65°C over 30 min, prior to analysis by DHPLC. Elution was performed with a linear acetonitrile gradient (9017-03, J.T.Baker, Phillipsburg, NJ) at a flow rate of 0.9 mL/min. The start- and end-points of the gradient as well as the temperature required for successful resolution of heteroduplex molecules were adjusted according to algorithms provided by the WAVEmaker system control software (Transgenomic Inc., San Jose, CA, USA).

Sequencing: Amplicons were purified by solid-phase extraction by QIAquick column gel purification (QIAGEN Inc). The product was sequenced in forward or reverse reactions with the PE Biosystems Taq DyeDeoxy terminator cycle sequencing kit according to the manufacturer's instructions. Cycle sequencing consisted of 25 cycles of 96°C for 30 s and 60°C for 30 s. Sequence analysis was performed using the Seqman (DNA Star) and seqScape V2.5 (Applied Biosystems) software.

Results

Familial and Epidemiological data

Our aim was to evaluate the immunohistochemical technique as a pre-screening test for BRCA1 mutation in a selected sample of breast cancer patients with familial presentation of the disease and well-established suggestivity of BRCA1 or BRCA2 mutation.

Index cases from eighteen Tunisian families with breast cancer were selected by familial inquiry. Most cases (15/18) were suggestive of BRCA1 mutation (Table 2). These cases included one case with three first and second degree affected relatives from the same side of the family with breast cancer; six cases with two first degree relatives from the same side of the family with breast or ovarian cancer, all of them having an average age younger than 60 years; three cases with mother or sister diagnosed with breast cancer at an age younger than 60 years; and four cases with bilateral breast cancer or breast and ovarian cancer in the same individual and one first or second degree relative with breast or ovarian cancer. The remaining three index cases suggestive of BRCA2 mutations included one case with male bilateral breast cancer and two cases of female breast cancer related in the first degree to a man with breast cancer. Figure 1 illustrates a typical family suggestive of BRCA1 and BRCA2 mutations.

Immunohistochemical results

We performed immunohistochemical analysis to distinguish tumor status between patients according to their suggestivity of BRCA1 mutation or BRCA2 mutation. Table III presents results using four monoclonal antibodies: anti-oestrogen receptors (ER), anti-progesterone receptor (PgR), anti-

Table 2. Summary of family history of cancer in 18 index cases with breast cancer and the corresponding first and second degree relatives with breast and or ovarian cancer.

Breast cancer patient	*Index case*	*First degree relatives*	*Second degree relatives*	*Suggestivity*
Case 1	Breast cancer Bilatéral	0	1 Breast cancer	BRCA1
Case 2	Breast cancer	2 Breast cancer	0	BRCA1
Case 3	Breast cancer	2 Breast cancer	1 Breast cancer	BRCA1
Case 4	Breast cancer	1 Ovarian cancer	1 breast cancer	BRCA1
Case 5	Breast and Ovarian cancer	0	1 Breast cancer	BRCA1
Case 6	Breast cancer	Father Breast cancer	0	BRCA2
Case 7	Male breast cancer Bilatéral	0	0	BRCA2
Case 8	Breast cancer Bifocal	1 Breast cancer	0	BRCA1
Case 9	Breast and Ovarian cancer	0	0	BRCA1
Case 10	Breast cancer	1 Breast cancer	0	BRCA1
Case 11	Breast cancer	0	2 breast cancer	BRCA1
Case 12	Breast cancer	Brother breast cancer	0	BRCA2
Case 13	Breast and Ovarian cancer	1 breast cancer	0	BRCA1
Case 14	Breast cancer	0	1 Ovarian cancer	BRCA1
Case 15	Breast cancer	1 Breast cancer	1 Breast cancer	BRCA1
Case 16	Breast cancer Bifocal	2 Breast cancer	0	BRCA1
Case 17	Breast cancer Bifocal	1 breast cancer	1 breast cancer	BRCA1
Case 18	Breast cancer	1 breast cancer	0	BRCA1

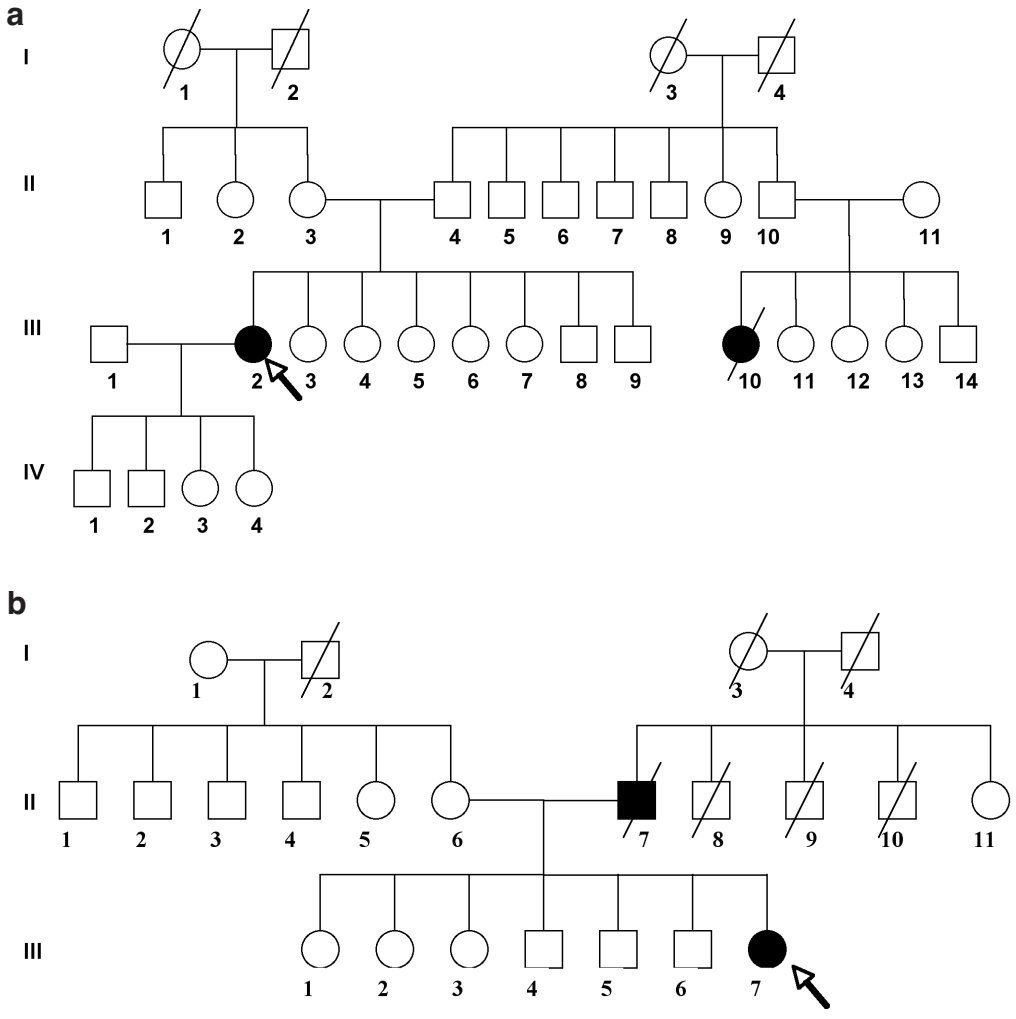

Figure 1. Pedigree of breast cancer families suggestive of BRCA1 and BRCA2 mutation. Circles and square denote female and male individuals, respectively, while the slash marks indicate the deceased. Blackened shapes are affected individuals and proband is designed with the right arrow. (a) BRCA1 c.5263dupC mutation pedigree. The proband (III-2) presented with an association in the same individual of breast and ovarian cancer at age 50. Individual (III-10) developed ovarian cancer and died in her 37s. (b BRCA2 c.1309_1312del4 mutation pedigree. The proband (III-7) presented with breast cancer at age 39. Her father (II-7) developed breast cancer and died in his 60s.

Table 3. Overexpression of Estrogen Receptor (ER), Progesterone Receptor (PgR), p53 and BRCA1 tumor suppressor proteins in Invasive ductal Carcinoma of 18 index cases (proband) with familial breast cancer.

Breast cancer patient	*ER*	*PR*	*P53*	*BRCA1*
Case 1	+	+	-	+
Case 2	+	+	-	-
Case 3	+	+	-	-
Case 4	+	+	+	-
Case 5	-	+	+	-
Case 6	-	+	-	+
Case 7	+	+	-	+
Case 8	+	-	-	+
Case 9	-	-	+	+
Case 10	-	-	-	+
Case 11	+	+	+	+
Case 12	+	+	-	+
Case 13	+	+	+	-
Case 14	-	-	-	+
Case 15	+	+	-	-
Case 16	-	+	-	+
Case 17	+	+	+	-
Case 18	-	-	-	-

p53 and anti-BRCA1.

We used a monoclonal antibody directed against a peptide corresponding to amino-acids 1839-1863 of C-terminus of BRCA1. Ten of the eighteen cases showed positive cytoplasmic immunostaining.

Different combinations of protein expression were observed. In half of the cases where it was accumulated, BRCA1 was expressed along with ER and PgR (Table 3). BRCA1 and p53 expression, as well as BRCA1 and hormonal receptors expression, seemed not to be related (Table 4).

BRCA1 accumulation in hereditary breast cancer tumors seemed not to be associated with the suggestivity of BRCA1 or BRCA2 mutations established on the basis of clinical and familial presentation. Three cases suggestive BRCA2 mutations and seven of fifteen cases suggestive of BRCA1 mutations were positive for BRCA1 cytoplasmic accumulation.

Molecular results: DHPLC and sequencing

On the basis of the familial presentation of the 18 cases, we sequenced the suggested gene which is most likely being mutated.

Four of fifteen cases suggestive of BRCA1 mutation were found mutated, one in exon 11 (c.4041_4042delAG), one in exon 20 (c.5263dupC) and two presenting the same mutation in exon 5 (c.211dupA). For the three cases suggestive of BRCA2 mutation, one carried c.1309_1312del4 in exon 10. The remaining thirteen cases did not show any deleterious mutation in the expected gene. The possibility of a mutation in the other gene was analysed by DHPLC for six cases, with no positive results.

Table 4. comparison between BRCA1 protein accumulation and p53, RH expressions in breast cancer tissues.

	BRCA1+	*BRCA1 -*	*p value*
P53 +	2	4	*p*=0,21
P53 -	8	4	
HR +	7	7	*p*=0,37
HR -	3	1	

The immunohistochemical and molecular analysis showed that among the eleven cases with no mutation in BRCA1 gene, five accumulated BRCA1 protein in the cytoplasm of the tumor cells (Figure 2a). Two out of four BRCA1 mutated cases (c.5263dupC and c.211dupA) and one case mutated in BRCA2 (c.1309_1312del4) (Figure 2b) accumulated BRCA1 protein in the cytoplasm of their tumor cells. Two out of four cases, including two mutated in BRCA1 (c.4041_4042delAG and c.211dupA), did not show any positive cytoplasmic immunostaining (Figure 2c).

Discussion

One major goal of this study was to choose an appropriate and reliable prescreening method for BRCA mutation analysis. We performed immunohistochemical analysis targeting BRCA1 protein in tumor cells. This protein is predicted to accumulate in cytoplasm when the mutation is in exon 11 (Kashima *et al.* 2000). Our aim was to detect the cytoplasmic form of BRCA1 protein, considering that this abnormal location could be an indicator for BRCA1 mutation (at least in exon 11).

Since several anti-BRCA1 antibodies are available, we chose a monoclonal antibody that has been shown to detect only the cytoplasmic form of BRCA1 protein (Kashima *et al.* 2000).

There are major discrepancies concerning the usefulness of various antibodies in detecting

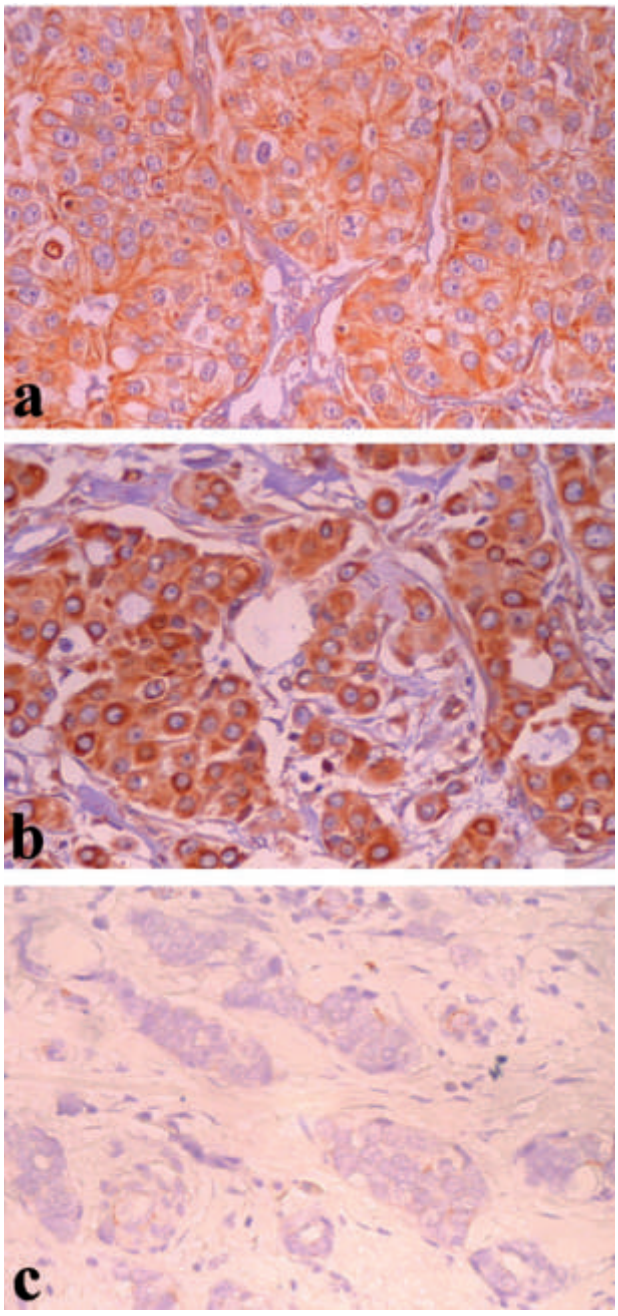

Figure 2. (a) Cytoplasmic staining pattern with the M3606 antibody in a familial invasive ductal breast carcinoma from patient without BRCA1 mutation. Brown cytoplasmic staining represents detection of BRCA1 protein using DAB as chromogen (100 % of the tumor cells expressed 3+ staining). (b) Cytoplasmic staining pattern with the M3606 antibody in a familial invasive ductal breast carcinoma from patient with c.1309_1312del4 BRCA2 mutation (100 % of the tumor cells expressed 2+ staining). (c)Negative cytoplasmic staining pattern with M3606 antibody in familial invasive ductal breast carcinoma from patient with c.4041_4042delAG BRCA1 mutation.

BRCA1 protein expression and localization (Al-Mulla *et al.* 2005). Some authors reported BRCA1 protein in the nuclei of normal cells, but aberrantly located in the cytoplasm of malignant mammary cells (Chen *et al.* 1995), while others found BRCA1 protein in the nuclei of both normal and malignant cells (Scully *et al.* 1996). Still others reported that BRCA1 is located in the cytoplasm and at the cell membrane (Jensen *et al.* 1996), or in the cytoplasm in tube-like invaginations into the nucleus (Coene *et al.* 1997). Others (Al-Mulla *et al.* 2005) confirmed that BRCA1 is expressed as a nuclear and cytoplasmic antigen in breast cancer tissues. These contradictory results about the location of the BRCA1 protein probably result from the quality of the archival paraffin-embedded breast cancer tissues affecting protein detection and the quality of the antibodies. The staining pattern in these paraffin-embedded tissues could also depend on tissue fixation conditions (Scully *et al.* 1996; Coene *et al.* 1997); immunostaining methodology, antibody concentration, specificity (Wilson *et al.* 1996; Bernard-Gallon *et al.* 1997) and undefined cross-reactivity (Yoshikawa *et al.* 1999).

In the present study, we report positive BRCA1 immunostaining only in the cytoplasm of tumor cells of some breast cancer patients. No immunostaining was proven either in the nucleus of tumor cells or in both the cytoplasm and nucleus of normal cells (Figure 2a and 2b). The monoclonal antibody used in this study, directed against the C-terminal region of BRCA1, seemed to reveal only the cytoplasmic form of this protein. Since several authors have described this protein in the nucleus, we suggest that the epitope recognized by this monoclonal antibody might be hidden in the nuclear form of BRCA1, either by its conformation or due to interactions with other nuclear molecules. Indeed, Kashima et al (Kashima *et al.* 2000), who used the same antibody, did not find any nuclear immunostaining of tumor cells.

BRCA1 protein accumulation did not correlate with p53 or with hormone receptor expression. However, among p53 negative tumors, 66 % were BRCA+ and 33 % were BRCA-, and among p53 positive tumors, 33 % were BRCA+ and 66 % were BRCA-. Moreover, among BRCA1- tumors, 50 % were p53+ and 50 % were p53- while only 20 % of the BRCA1+ tumors expressed p53. This might indicate that association of BRCA1 cytoplasmic accumulation and p53 expression is not frequent. However, this result should be confirmed by further studies.

Our purpose was to explore the usefulness of immunohistochemistry as a prescreening method for BRCA1 analysis, using 18 patients selected on the basis of their suggestivity of BRCA1 or BRCA2 mutation. All were tested by direct sequencing. We found five deleterious mutations; four in BRCA1 and one in BRCA2.

Twenty six percent of the cases suspected to be mutated in BRCA1 were mutated in this gene. Likewise, 33% of the familial cases suspected to be mutated in BRCA2 were mutated in this gene. In total, 27% of families suggestive of BRCA mutation showed deleterious mutations at the expected locus. This result leads us to consider the possibility of large deletions in BRCA1 or BRCA2 or of mutation in other genes responsible for the familial risk of breast cancer.

Mutation of BRCA1 exon 11 has been associated with positive immunostaining in tumor cells using monoclonal antibodies against amino acids 1839-1863 of BRCA1 (i.e. the C-terminus) (Kashima *et al.* 2000). In contrast to this work, our case mutated in exon 11 (c.4041_4042delAG) was not associated with staining with this same antibody in the cytoplasm of tumor cells. Taking into account the frameshift mutation and the specificity of the antibody used, this lack of positive staining was expected. The immunohistochemical test was also negative for one case with a frameshift mutation in exon 5 (c.211dupA) leading to severely truncated protein that would not be detected by the C-terminal antibody even if located in cytoplasm.

Three of five mutated cases accumulated BRCA1 in the cytoplasm of tumor cells: two BRCA1 cases and one BRCA2 case. Both BRCA1 mutations were frameshifts that cause translation to stop before the C-terminal epitope recognized by the antibody, suggesting that the observed immunohistochemical staining could not be due to the mutant protein. In fact, the monoclonal antibody used should reveal the abnormal location of the wild-type form of the BRCA1 protein. As BRCA1-mutated patients are heterozygous, our results indicate that in the tumor cells of these patients, rather being lost through LOH (lost of heterozygosity), the wild-type form of BRCA1 is abnormally located in the cytoplasm, abolishing its nuclear function. The molecular mechanism explaining this accumulation is unknown. In the case with BRCA2 gene mutation, the abnormal BRCA2 protein may retain less Rad51 in the nucleus and, due to the interaction between BRCA1 and Rad51 proteins, this may result in cytoplasmic BRCA1 protein accumulation too (Scully *et al.* 1997).

In conclusion, among the 18 analyzed tumors, 12 showed either BRCA mutation or BRCA1 accumulation or both indicating that BRCA1 function might be lost in breast tumor cells not only through mutation and LOH, but also via abnormal cytoplasmic location. The immunohistochemical test used in this study was not be efficient at revealing deleterious BRCA1 mutations, but it appeared useful to investigate tumor cell physiology and supports the involvement of BRCA1 protein in breast tumor cells. Whether this implication is related to hereditary breast cancer, should further be investigated in the sporadic form.

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