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Background: Ductal carcinoma *in situ* (DCIS) of the breast is known to possess characteristics of the pre-invasive stage of breast cancer and is the precursor to invasive ductal carcinoma (IDC). However, the natural history of the progression from DCIS to IDC remains unknown at the molecular level.

Methods: We investigated the loss of heterozygosities (LOHs) in tumors of seven patients with a history of breast biopsy. The seven specimens were diagnosed as DCIS on histopathological re-examination. These patients were diagnosed with ipsilateral breast cancer a few years after biopsy. We used thirteen selected microsatellite markers that were mapped to and/or very close to the tumor suppressor genes or regions with frequent LOHs in breast cancer. DNA isolated from microdissected formalin-fixed, paraffin-embedded tissues was subjected to a PCR-LOH analysis for these chromosome loci, and the pattern of LOHs was compared between the two asynchronous lesions for the seven cases.

Results: In all patients except one, the LOHs were concordant at 91% as the informative chromosome loci in cases 1 to 6 were 56, and the concordance in LOH pattern between DCIS and IDC was detected at 50 loci. The LOHs had accumulated in accordance with the tumor progression from DCIS to IDC. The recurrent lesion occurred at or near the site of the primary biopsy and had similar or identical histopathologic features.

Conclusions: These recurrences observed were probably residual disease rather than true recurrences. Our results suggest the following: (i) genetic alternations accumulate during cancer progression from DCIS to IDC, (ii) DCIS is a lesion that has a high risk of developing invasive transformation and (iii) after approximately 5 years without treatment, DCIS may develop into IDC.

Key words: breast cancer – LOH – DCIS – IDC – natural history

INTRODUCTION

Genetic alternations, including activation of oncogenes and inactivation of tumor suppressor genes, are involved in the development of human breast cancer (1,2). Several studies have reported the loss of heterozygosities (LOHs) in invasive ductal carcinoma (IDC) and ductal carcinoma *in situ* (DCIS) (3–6). LOHs is also frequently found on several chromosome arms in IDC and DCIS. To date, specific allelic losses have been reported in chromosome arms 6q, 8p, 11q, 13q, 16q, 17p and 17q.

Many IDCs have histopathologically spread as a result of intraductal components (DCIS) (7). IDC is believed to generally arise from DCIS. Therefore, it is important to understand the genetic alternations that lead to the transformation of DCIS into IDC. We had previously analyzed the LOHs in patients harboring synchronous atypical ductal hyperplasia (ADH), DCIS and IDC and found that higher frequencies of LOHs were observed in parallel with the tumor progression from ADH to DCIS and then to IDC (8). Fujii et al. analyzed LOH in patients harboring synchronous DCIS and IDC and found that LOH in DCIS was also observed in synchronous IDC (9). The data supported the idea of a stepwise progression from DCIS to IDC.

An understanding of the natural history of DCIS is thus the basis of prognostication and therapeutic recommendation. Despite the abundant molecular data on breast cancer in general, the natural history of the progression from DCIS to IDC remains unknown at the molecular level because it is difficult to follow the pathological progression from DCIS to IDC in the

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Table 1. Summary of the clinical characteristics and pathologic data

Case	Site	Lesion	Date of biopsy	DCIS	Surgical margin	Date of operation	Intraductal components	Interval time (months)
				Necrosis			Necrosis	
				Nuclear grade			Nuclear grade	
				Architectural pattern			Architectural pattern	
Case 1	Right	SL	1977/12/01	necrosis (–)	positive	1980/04/09	necrosis (–)	28
				NG 1			NG 1	
				sol, crib			sol, crib	
Case 2	Left	SL	1982/08/20	necrosis (–)	positive	1985/05/14	necrosis (–)	33
				NG 2			NG 2	
				pap, crib			pap, crib	
Case 3	Right	SL	1986/11/14	necrosis (–)	positive	1992/02/07	necrosis (–)	63
				NG 2			NG 2	
				low pap			low pap	
Case 4	Right	SL	1988/02/29	necrosis (–)	positive	1995/07/10	necrosis (–)	89
				NG 1			NG 1	
				crib, sol			crib, sol	
Case 5	Left	SL	1989/11/17	necrosis (–)	unknown	1994/11/09	necrosis (–)	60
				NG 2			NG 2	
				crib			crib	
Case 6	Left	SL	1992/11/18	necrosis (–)	unknown	1997/06/24	necrosis (–)	55
				NG 2			NG 2	
				sol, pap			sol, pap	
Case 7	Right	AL	1987/04/10	necrosis (–)	unknown	1992/11/18	necrosis (–)	67
				NG 2			NG 1	
				low pap			sol	

SL, same lesion; AL, another lesion; NG, Nuclear Grade; NG 1, low nuclear grade; NG 2, intermediate nuclear grade; NG 3, high nuclear grade; sol, solid; crib, cribriform; pap, papillary; low pap, low papillary.

same patient. The diagnosis of DCIS cannot be established without first removing a breast lesion for microscopic examination. A follow-up of such patients after the biopsy would be the best way to predict the clinical outcome of the disease. We, therefore, decided to evaluate and compare the molecular and clinicopathologic features of a selected number of patients with primary DCIS, who were treated only by biopsy and later had recurrent IDC lesions (asynchronous lesions) of the breast, using the LOH analysis to characterize the relationship between the two events at the molecular level.

PATIENTS AND METHODS

Samples Analyzed

We selected patients with a history of breast biopsy, which was histopathologically diagnosed as benign. These patients were diagnosed with ipsilateral breast cancer, IDC, approximately 2 years after the biopsy. In each case, paraffin-embedded, hematoxylin–eosin stained sections were re-examined by three authors (MA, TM, NO) in accordance with the Consensus conference on the classification of DCIS (10). In this study, we examined samples from seven patients diagnosed with DCIS (non-comedo type).

The seven cases comprised Japanese patients with sporadic breast cancer. Three of them were from Tohoku University Hospital in Sendai, two from other hospitals in Sendai and two were from Kurashiki. Informed consent regarding the usage of their samples for genetic experiments was obtained from the patients who were treated in Tohoku University Hospital. All the tumor specimens (primary DCIS and recurrent IDC) were fixed in formalin and embedded in paraffin. These paraffin-embedded tissues were sectioned at 3 μm, stained with hematoxylin and eosin and then re-examined histopathologically. In recurrent IDCs, intraductal components of IDC were also examined and evaluated and then compared with primary DCISs. The clinical characteristics and pathologic data of the seven cases are summarized in Table 1.

Microdissection and DNA Extraction

In each case, the formalin-fixed and paraffin-embedded tissues were sectioned at 10 μm. *In situ* cancer (primary DCIS) cells,

Table 2. Results of LOH analysis of primary DCIS and recurrent IDC

	Case 1		Case 2		Case 3		Case 4		Case 5		Case 6		Case 7	
	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC	DCIS	IDC
D3S1300	+	+	+	+	+	+	–	–	+	–	n	n	–	+
D6S262	+	+	+	+	n	n	–	–	+	+	n	n	n	n
D8S137	n	n	n	n	–	–	–	–	n	n	+	–	–	+
D8S339	+	+	+	+	–	–	+	–	+	+	n	n	+	–
D9S126	n	n	+	+	+	+	–	–	–	–	+	+	+	+
D11S899	n	n	+	+	+	+	+	+	n	n	+	+	+	+
D11S528	n	n	+	+	+	+	–	–	–	–	n	n	–	–
D13S267	–	–	+	+	+	+	+	+	n	n	+	+	–	+
D16S514	n	n	+	–	n	n	+	+	–	–	n	n	–	–
D16S422	+	+	n	n	–	–	–	–	n	n	–	–	+	–
D17S928	+	+	+	+	–	–	+	+	+	+	n	n	n	n
TP53	+	–	+	+	n	n	+	+	+	+	+	+	+	+
D17S800	+	+	+	+	n	n	n	n	+	–	+	+	+	+

+, retained heterozygosity; –, loss of heterozygosity; n, not informative due to homozygosity.

invasive cancer cells (recurrent IDC) and normal cells were collected by microdissection using the Laser Captured Microdissection system, LM100 (Olympus, Japan) (11). DNA was extracted by a standard procedure, according to methods described previously (12).

LOH ANALYSIS

Thirteen selected microsatellite markers, which were mapped to and/or very close to the tumor suppressor genes or regions with frequent LOHs in breast cancer, were used in this study. Primers for PCR amplification of these markers were designed based on the nucleotide sequences obtained from the GenBank database. Nucleotide sequences of the primers and annealing temperatures of PCR amplifications have been described previously (8). One out of each pair of primers was labeled with Cy 5 (Pharmacia Biotech, Uppsala, Sweden), and PCR amplifications and electrophoreses were performed according to methods described previously (13) using an ALFred DNA Sequencer with Fragment Manager software (Pharmacia Biotech, Piscataway, NJ). An allelic loss was defined as a more than a 50% reduction in the area of a peak calculated in the tumor as compared to that of a corresponding normal tissue. PCR reactions were performed twice per marker to confirm LOHs in both primary DCIS and recurrent IDC.

RESULTS

All the seven primary lesions were histopathologically diagnosed as a benign proliferative disease, but the re-examined diagnosis for all revealed DCIS (non-comedo type), van Nuys classification Group 1 (14). A total of seven cases with primary DCIS and recurrent IDC were analyzed using 13 selected microsatellite markers that are the predicted loci for localization of tumor suppressor genes (TSGs) for breast cancer. Microscopic examination of the biopsy specimens for primary DCIS revealed that the surgical margins of these specimens were clearly positive in cases 1 to 4. However, the surgical margins in cases 5 to 7 were undetermined because only one slice of the specimen was used and other aspects of the surgical edge were unclear. The time interval between the data of the first biopsy (primary DCIS) and that of the curative operation (recurrent IDC) was 28~89 months; the mean time was 55 months. None of the women, who had a positive family history of breast cancer, received any form of radiation therapy, hormonal therapy or chemotherapy after the biopsy of the primary lesions.

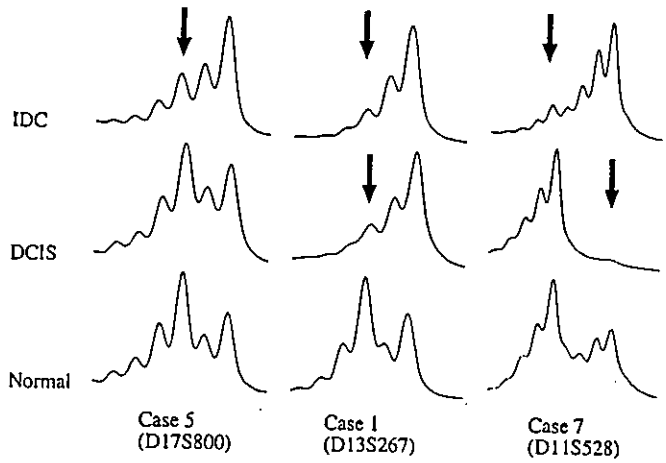

Figure 1. Examples of microsatellite analyses. Case 5 showed no allelic loss in ductal carcinoma *in situ* (DCIS) and showed a new allelic loss in invasive ductal carcinoma (IDC). Case 1 showed allelic loss in both DCIS and IDC. Case 7 showed the loss of a shorter allele in IDC, and a longer allele in DCIS. Arrows indicate lost alleles.

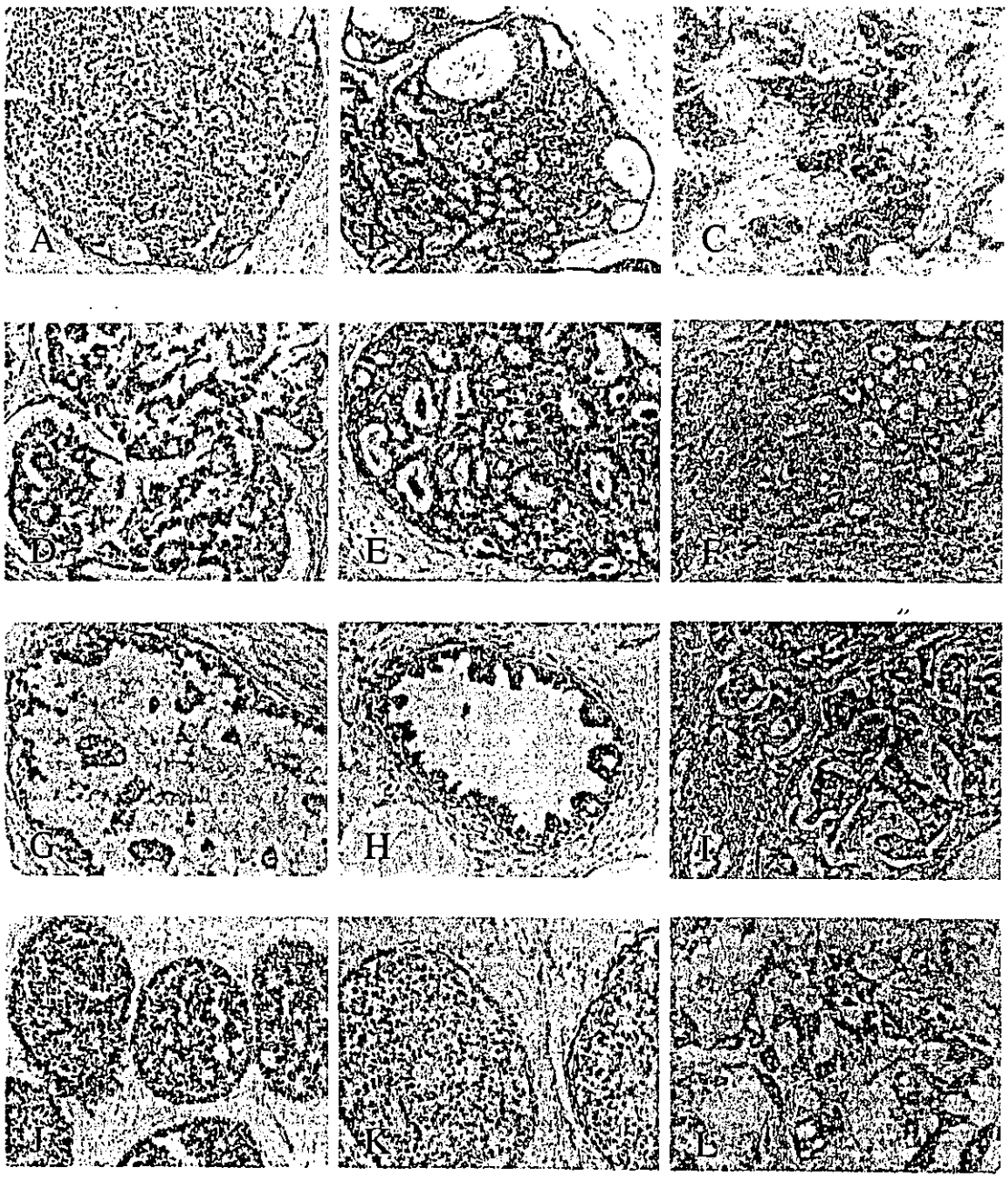

Figure 2. Histopathological appearances of primary DCIS and recurrent IDC. Case 1 (DCIS: A, intraductal component of IDC: B, IDC: C), Case 2 (DCIS: D, intraductal component of IDC: E, IDC: F), Case 3 (DCIS: G, intraductal component of IDC: H, IDC: I), Case 4 (DCIS: J, intraductal component of IDC: K, IDC: L). Cases 1 to 4 showed similar nuclear grades and architectural patterns for primary DCIS and recurrent IDC. LOHs in the primary DCIS were also observed in the recurrent IDC.

LOHs were observed at one or more of the tested loci in all seven cases. The incidence of LOH in each locus is shown in Table 2. Typical examples of LOH analyses are shown in Fig. 1. In all cases except one (case 7), the LOHs were concordant at 91% (50/56) as the informative chromosome loci in cases 1 to 6 were totally 56, and the concordance in LOH pattern between DCIS and IDC was detected at 50 loci. Even if the LOH pattern differed, the LOHs had accumulated in accordance with the tumor progression from DCIS to IDC. The recurrent lesion occurred at or near the site of the primary biopsy and had similar or identical histopathologic features.

Case 1 showed one LOH at 13q in primary DCIS and an additional LOH at p53 in recurrent IDC. Recurrent IDC appeared at the edge of the primary operation scar; the time interval from progression of primary DCIS to recurrent IDC was 28 months. Comedo-necrosis was not observed, and the nuclear grade was low (NG 1) in both primary DCIS and recurrent IDC. Similarly, the architectural pattern of DCIS was cribriform + solid in both primary DCIS (Fig. 2A) and recurrent IDC (Fig. 2B).

Case 2 showed no allelic loss in primary DCIS, however, a new allelic loss was observed at 16q in recurrent IDC. Recur-

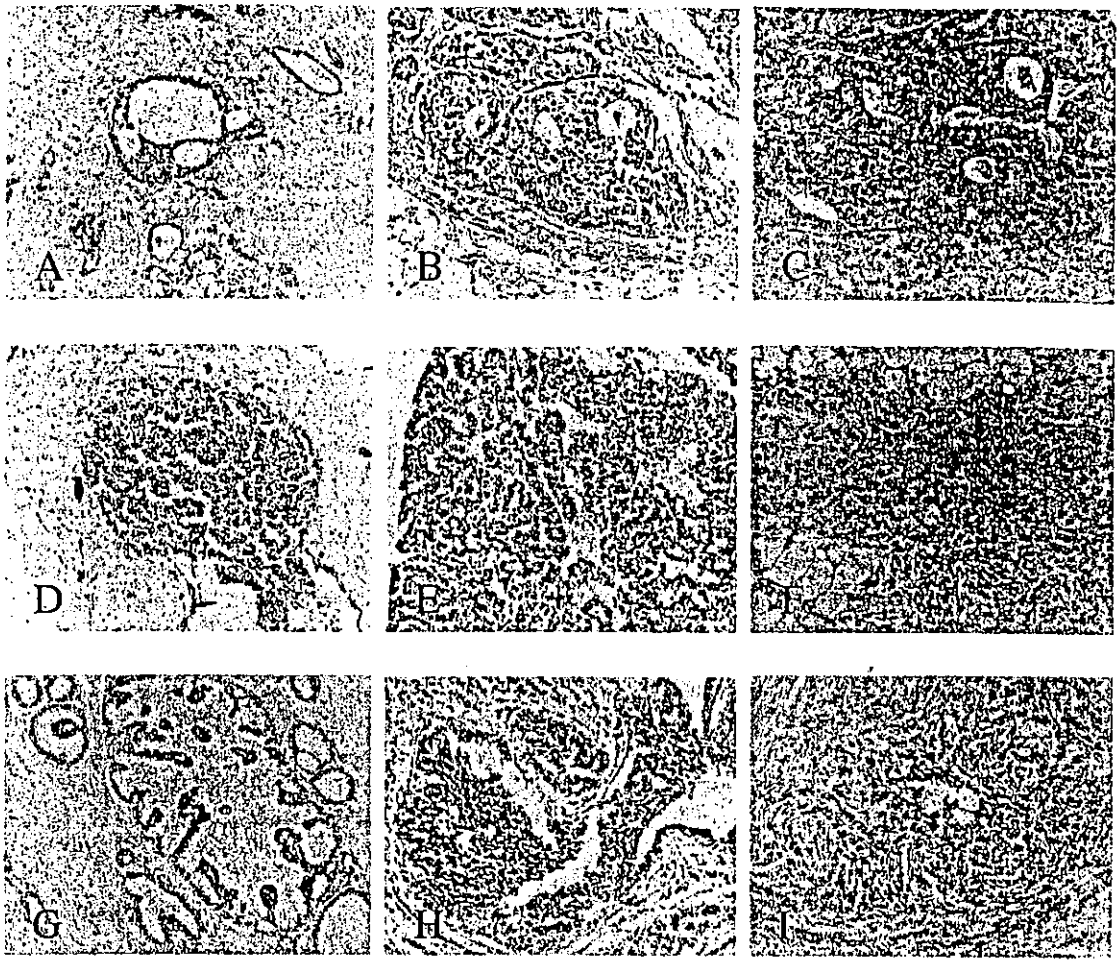

Figure 3. Histopathological appearances of primary DCIS and recurrent IDC. Case 5 (DCIS: A, intraductal component of IDC: B, IDC: C), Case 6 (DCIS: D, intraductal component of IDC: E, IDC: F), Case 7 (DCIS: G, intraductal component of IDC: H, IDC: I). Cases 5 and 6 showed similar nuclear grades and architectural patterns for primary DCIS and recurrent IDC. LOHs in the primary DCIS were also observed in the recurrent IDC. Case 7 showed different nuclear grades, architectural patterns, and allelic losses between DCIS and IDC.

rent lesions appeared at the edge of the biopsy scar, and the time interval was 33 months. Comedo-necrosis was not observed, the nuclear grades were intermediate (NG 2), and the architectural patterns were papillary + cribriform in both lesions (Fig. 2D, E).

Case 3 showed identical LOHs at 8p, 16q and 17p and no new LOHs in the recurrent lesions. Recurrent lesions appeared at the biopsy scar, and the time interval was 63 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 2G, H). Case 4 showed identical allelic losses at 3p, 6q, 8p, 9p, 11q and 16q and a new loss at 8p in the recurrent lesion. The recurrent lesion appeared near the primary lesion, and the time interval was 89 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 2J, K).

Case 5 showed identical allelic losses at 9p, 11q and 16q and new losses at 3p and 17q in the recurrent lesion. The recurrent lesion appeared at the primary scar, and the time interval was 60 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 3A, B).

Case 6 showed LOH at 16q and a new LOH at 8p in recurrent IDC. The recurrent lesion appeared at the edge of the primary lesion, and the time interval was 55 months. Comedo-necrosis was not observed, and the nuclear grades and architectural patterns were similar in both lesions (Fig. 3D, E).

Case 7 showed LOHs at 3p, 8p, 11q, 13q and 16q in primary lesions, however, LOHs were observed at 8p, 11q and 16q in the recurrent lesion. The recurrent and primary lesions appeared in different quadrants. Comedo-necrosis was not observed in both lesions. The nuclear grade was NG II in primary DCIS but NG I in IDC. The architectural pattern was micropapillary in DCIS (Fig. 3G) and solid in IDC (Fig. 3H). This case is considered to have different clonality in IDC and DCIS because the appearance of lesions; the nuclear grades, architectural patterns and allelic losses for DCIS and IDC were all different.

In all the cases, the architectural pattern of DCIS associated with IDC was non-comedo, and the nuclear grade was low or intermediate. Cases 1 to 6 not only showed the same clonality,

but also exhibited similar nuclear grades, architectural patterns and appearance of lesions between the primary DCIS and recurrent IDC. Furthermore, all the allelic losses observed in the primary DCIS were also observed in the recurrent IDC. In all these cases, the time interval between diagnosis of primary DCIS and the recurrent IDC was 28~89 months, and the mean time interval was 55 months.

DISCUSSION

The natural history of breast carcinogenesis, progression from DCIS to IDC, has important prognostic and therapeutic implications. This is the first report on longitudinal molecular analysis from primary DCIS (non-comedo type) to recurrent IDC. We investigated LOHs using 13 microsatellite markers in seven cases of breast cancer. These chromosomal loci were selected on the basis of various previous studies (3–6). In all cases except one, LOHs were observed in parallel with the tumor progression from DCIS to IDC, suggesting a common genetic pathway for the development of both lesions and the continuous proliferation of the residual disease. These cases occurred at or near the site of the primary biopsy and had similar or identical histopathologic features. The observed recurrences were probably residual disease rather than true recurrences because surgical margins were clearly positive in four of the six cases.

According to the data obtained from this study, it is important to discuss the genetic criteria for differentiating between locally recurrent tumors and metachronous multicentric tumors. All the tumors except that of case 7 were considered to originate near the primary lesion, suggesting continuous genetic accumulation from the residual disease. They carried a maximum of two out of nine chromosomal loci (22%, case 5) with different LOH patterns at the asynchronous lesions. However, the lesion in case 7, which originated in another quadrant with different histological characteristics, showed a different LOH pattern at five (45%) of 11 chromosomal loci, suggesting that the threshold of discordance should lie between 22% and 45%.

In conclusion, hypothetically, recurrent IDC develops from residual primary DCIS that has accumulated one or more additional genetic alternations. Lininger et al. reported a comparison of LOH in primary DCIS and recurrent DCIS, in three cases with ipsilateral recurrent DCIS. LOH observed in primary DCIS was also observed in recurrent DCIS, and at least one additional LOH was observed in recurrent DCIS (15). Page et al. reported that the natural history of small, non-comedo DCIS lasts for at least two decades, with invasive carcinoma developing at the site of biopsy (16). However, our results showed that the natural history of small, non-comedo DCIS developed into IDC after approximately 5 years of treatment by biopsy only.

These results suggest that the following: (i) genetic alternations accumulate during cancer progression from DCIS to IDC, and (ii) DCIS is a lesion that has a high risk of developing invasive transformation, and (iii) non-comedo DCIS may develop into IDC after approximately 5 years without radiation therapy, hormonal therapy or chemotherapy.

BRCA1 and *BRCA2* are breast cancer susceptibility genes on chromosome bands 17q21 and 13q12-q13, respectively (18,19). LOH on the *BRCA1* locus is observed in IDC (case 5). On the other hand, LOH at the *BRCA2* locus is observed in DCIS and IDC (case 1). In our previous study (8), inactivation of *BRCA1* played an important role in the early stage of breast carcinogenesis, and the inactivation of *BRCA2* was a late event.

The loss of D16S422 at the locus for *CDH13* was high in DCIS and IDC (cases 3, 4, 6). This gene, located on chromosome bands 16q24, encodes the adhesion molecule H-cadherin (*CDH13*) and is recognized as a TSG responsible for breast cancer. The expression of *CDH13* is significantly reduced in breast cancer (20), and its frequent inactivation in lung cancer has also been reported (21). Further, the introduction of wild-type DNA to breast cancer cells inhibited cell growth (20). Our previous study (8) and results suggested that H-cadherin might play an important role even in the early stages of breast carcinogenesis.

The *CDH1* gene, located on chromosome 16q22, encodes the adhesion molecule E-cadherin, which suppresses invasion *in vitro*. A decreased expression in breast carcinomas correlates with the presence of invasion and a shorter disease-free survival (22). Our previous study (8) suggested that E-cadherin might be a rate event of breast carcinogenesis. In our results, LOH on the E-cadherin locus is observed in IDC (case 1) and in DCIS and IDC (case 5).

The loss of chromosome 8p is also frequently observed not only in IDC, but also in DCIS. Putative TSG in this region probably plays an important role in the early stages of breast carcinogenesis (cases 3, 4). The linkage analysis indicated that a positive LOD score at D8S137 was observed in some familial breast cancer pedigrees that were neither linked to 13q nor to 17q (23). There may be a third breast cancer susceptibility gene (*BRCA3*) on this chromosome arm, and our results may provide an important clue for identifying the putative *BRCA3* gene.

ATM, the gene responsible for ataxia telangiectasia, is located on chromosome 11q22-q23 and the LOH of this gene (cases 4, 5) might be an early event. The frequent LOH of *p53* has been reported in a variety of tumors (24). In this study, however, the LOH of *p53* was not very frequent (case 1). Many investigators have analyzed the mutations of *p53* in breast cancer tumors and have found that this gene was not frequently mutated in this disease (25). Our present study is in agreement with these results.

Though the data on genetic progression of breast cancer is increasing, the mechanisms of carcinogenesis (natural history) from DCIS to IDC at the molecular level are still unknown. The genome-wide search for LOH showed 56 regions with consistent LOH (17). In an LOH analysis of 75 different breast cancers at multiple chromosome loci, every cancer showed a different pattern of deletions. In our previous study on LOH analysis in 70 DCIS lesions at the same chromosome loci, frequent LOH was observed at 8p, 16q and 17q, but the inci-

dences of LOH at loci on 3p, 6q, 9p, 11p, 11q and 17p were marginal. It is not confirmed whether the mutations of genes on these loci play a major role in the genesis of breast cancer. The results of this study also show a different pattern of deletions in each case (Table 2). However, we could not completely exclude these regions as the loci for the responsible genes since the number of samples used in this study were limited.

To summarize, the progression from DCIS to IDC may involve a pathway consisting of stepwise genetic alterations. DCIS is likely to have a high risk of developing invasive transformation. Further studies are necessary to identify the genetic alterations of breast tumor progression from DCIS to IDC in order to develop appropriate clinical management of breast cancer patients.

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Original Articles

Pathological Assessment of Intraductal Spread of Carcinoma in Relation to Surgical Margin State in Breast-conserving Surgery

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Background: Spreading of carcinoma has been considered to be a prognostic factor for local failure after breast-conserving therapy. The extensive intraductal component (EIC) was defined as when the component of intraductal carcinoma constitutes more than 25% of the primary tumor with intraductal foci. However, the definition of EIC was based on the predominance of intraductal component surrounding the invasive lesions and not on the segmental anatomy. We designated carcinoma extension as the intraductal spread of carcinoma (ISC) along with the duct–lobular system by three-dimensional (3-D) reconstruction analysis. This study was initiated to simplify the method of two-dimensional (2-D) pathological examination based on 3-D mapping.

Methods: Thirty-four specimens from breast cancer patients were subjected to 3-D reconstruction. We investigated the correlation between actual extension of intraductal carcinoma and EIC defined by 2-D examination or ISC grading defined by 3-D reconstruction. Furthermore, using another 62 histological mappings, we investigated how correctly the simplified 2-D method using several paraffin blocks reflected the actual carcinoma spread and margin state.

Results: Carcinoma extension over 2 cm was observed in 64% specimens that were EIC positive and 26% specimens that were EIC negative. In contrast, according to the ISC grading defined by 3-D reconstruction, none of the specimens with a low grade of ISC demonstrated carcinoma extension over 2 cm. Carcinoma extension over 2 cm was observed in 71% of specimens with a high grade of ISC, thus demonstrating a correlation between carcinoma extension and ISC grading. In addition, the simplified 2-D method using only several blocks reflected both the 3-D ISC grading and surgical margin state.

Conclusions: We conclude that ISC grading correlates with carcinoma extension and surgical margin state. From a clinical point of view, the simplified 2-D examination using paraffin blocks may contribute to routine surgical pathology in evaluating the degree of carcinoma extension in breast-conserving therapy.

Key words: intraductal spread of carcinoma (ISC) – extensive intraductal component (EIC) – three-dimensional examination – breast-conserving therapy

INTRODUCTION

Breast-conserving treatment has become one of the standard therapies for early breast cancer. The results of several randomized prospective trials such as the Milan trial (1,2) and NSABP trial (3,4) have demonstrated that no detrimental effects in terms of survival and distant metastasis rates were observed in patients treated by breast-conserving surgery and radiation compared with those treated by modified radical mastectomy. The cosmetic effect is well evaluated as it improves the quality of life in breast-conserving therapy. The extension of carcinoma component, however, is occasionally higher than the preoperative prediction. Several groups have noted that a high degree of intraductal carcinoma extension and multicentricity are due to cancer residues after breast-

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conserving therapy (5–11). Not only survival issues but also cosmetic, economic and psychological demerit from the local recurrence within the breast occur.

Schnitt and co-workers (12,13) designated intraductal extension of carcinoma as extensive intraductal component (EIC) when intraductal carcinoma occupies more than 25% of the primary tumor with intraductal foci separate from the main tumor mass. EIC is a very simple and easy detection method by two-dimensional (2-D) pathological examination to identify the intraductal carcinoma extension. It is based, however, on the predominance of intraductal component in the main tumor associated with invasive component and not on the segmental anatomy. We think that it is necessary to define the intraductal carcinoma extension based on the segmental anatomy, proposing the term intraductal spread of carcinoma (ISC) (14,15). ISC was defined as a state in which ductal carcinoma *in situ* (DCIS) extends beyond the terminal duct–lobular unit (TDLU) and into large ducts. We classified four ISC grades based on the extent in the duct–lobular system and showed how closely it correlated with carcinoma residues after breast-conserving treatment (14,15).

The definition of ISC is based on 3-D pathological examination. Serial slices 5 μm thick were made of the specimens from breast cancer patients receiving quadrantectomy and computer-assisted 3-D mapping of the tumors was constructed along with the duct–lobular system. This 3-D examination can detect the detail of carcinoma extension and the surgical margin state. It is very difficult, however, to do the 3-D pathological examination in all patients receiving breast-conserving surgery because about 2500–5000 hematoxylin and eosin-stained preparations are needed for one patient. This study was initiated to simplify the method of 2-D pathological examination based on 3-D pathological mapping. First, we investigated how correctly the EIC judgement accepted as an easy method by 2-D examination and ISC grading by 3-D examination show the actual carcinoma spread. Then the same was done with simulation based on the simplified 2-D method using several paraffin blocks.

MATERIALS AND METHODS

MATERIALS

INVESTIGATION OF CORRELATION BETWEEN ACTUAL INTRADUCTAL CARCINOMA EXTENSION AND EIC JUDGEMENT BY 2-D EXAMINATION OR ISC GRADING BY 3-D EXAMINATION

Thirty-four specimens from stage I and II breast cancer patients receiving quadrantectomy in Tohoku University Hospital from 1990 to 1994 were fixed in 10% formalin neutral buffer solution and embedded in paraffin. The patients ranged in age from 25 to 73 years (median, 54 years). The histopathology of all the cases was invasive ductal carcinoma (IDC) and included IDC with predominant intraductal component cases.

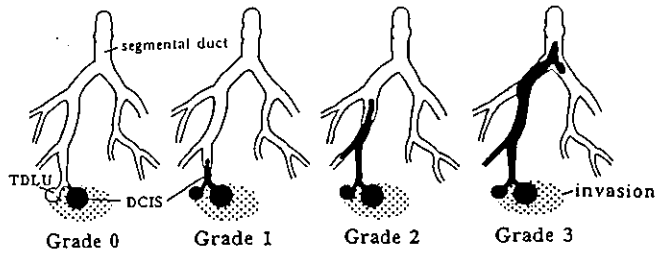

Figure 1. A schematic diagram of intraductal spread of carcinoma (ISC). Grade 0: carcinoma is confined within a terminal duct–lobular unit (TDLU). Grade 1: carcinoma extends beyond TDLU, but is confined within a single large duct or its periphery. Grade 2: carcinoma extends more than two large ducts, but is confined within subsegmental ducts. Grade 3: carcinoma involves segmental duct with diffuse intraductal growth.

SIMPLIFIED METHOD FOR DETECTING ISC GRADING BY 2-D PATHOLOGICAL EXAMINATION USING SEVERAL PARAFFIN BLOCKS

Sixty-two specimens from stage I and II breast cancer patients receiving quadrantectomy in Tohoku University Hospital from 1990 to 1996 were fixed in 10% formalin neutral buffer solution and embedded in paraffin. The patients ranged in age from 29 to 83 years (median, 52 years). The histopathology of all the cases was invasive ductal carcinoma (IDC) and included IDC with predominant intraductal component cases.

METHODS

INVESTIGATION OF CORRELATION BETWEEN ACTUAL INTRADUCTAL CARCINOMA EXTENSION AND EIC JUDGEMENT BY 2-D EXAMINATION OR ISC GRADING BY 3-D EXAMINATION

The tissues were subjected to serial sectioning and 3-D reconstruction using a workstation. First, the specimens were sequentially sliced to 3 mm thickness using a ham slicer and made into 5 μm thick sections. Sections 100 μm thick were stained with hematoxylin and eosin. From these, graphic 3-D reconstruction was performed with the aid of a computer system developed on a workstation (Hewlett-Packard, Model 300) and OZ software (Rise, Sendai, Japan).

In this study, we defined the high degree of intraductal carcinoma extension group as when the intraductal carcinoma component exists more than 2 cm distant from the edge of invasive lesions. We investigated how the EIC judgment by 2-D or ISC grading by 3-D examination method can determine the spread of intraductal carcinoma lesions.

EIC judgement

Schnitt and co-workers (12,13) designated intraductal extension of carcinoma as EIC when intraductal carcinoma constitutes more than 25% of the primary tumor with intraductal foci separate from the main tumor mass.

ISC definition and classification

We designated intraductal extension as intraductal spread of carcinoma (ISC) and classified ISC into four grades, i.e., grade

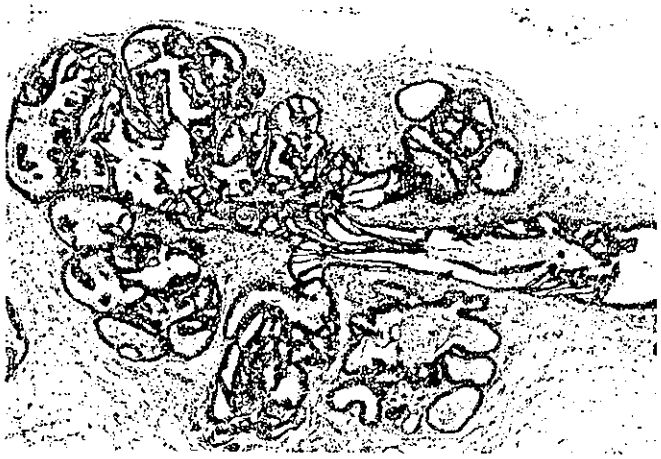

Figure 2. A photomicrograph of an ISC grade 1 case. Carcinoma cells extend beyond ductules, intralobular terminal ducts (ITD) and extralobular terminal ducts (ETD). TDLU comprises ductules, ITD and ETD.

0–3 based on the extent of carcinoma along with the duct–lobular system (15) (Fig. 1).

ISC was defined as the state in which DCIS was present clearly extending beyond the TDLU or present prominently within the large ducts. The predominance of an invasive or an intraductal component was not considered (15). A photomicrograph of an ISC grade 1 case is presented in Fig. 2.

Simplified Method for Detecting ISC Grading by 2-D Pathological Examination Using Several Paraffin Blocks

Serial slices 5 mm thick were made from the specimens and used to map the pathological results. We then tried to establish a simplified 2-D pathological examination method using several pathological paraffin blocks (Fig. 3). We simulated the several paraffin blocks such as 4 cm long, 5 mm thick, parallel to the nipple-tumor line and chose the main tumor site, both bilateral 10 mm sites. For example, the main tumor site was by two paraffin blocks when the tumor size was 1 cm and two sets of bilateral blocks, total six blocks.

We then investigated how the simplified 2-D pathological examination method using several paraffin blocks can correctly determine the intraductal carcinoma extension and the surgical margin status.

Definition of ISC high degree group by simplified 2-D pathological examination method

The correlation between the distance of intraductal spread of carcinoma from the edge of the invasive lesion and ISC grading by 3-D examination is shown in Table 1. The distances were 0–2 mm in ISC grade 0 group, 0–4 mm in ISC grade 1 group, 6–27 mm in ISC grade 2 group and 24–53 mm in ISC grade 3 group. The distances of intraductal spread of carcinoma from the edge of invasive lesion were all less than 4 mm in ISC grade 0 and 1 groups and all more than 6 mm in ISC grade 2 and 3 groups. In this study, we defined the ISC high degree group by the simplified 2-D pathological examination method when the intraductal carcinoma component existed more than 5 mm distant from the edge of the invasive lesions.

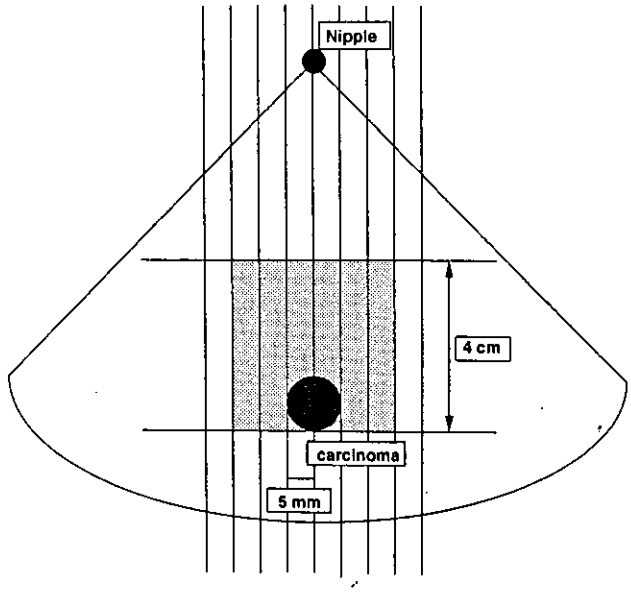

Figure 3. Schematic diagram of the simplified method for detecting ISC grading by 2-D pathological examination using several paraffin blocks.

Judgement of positive surgical margin

Quadrantectomy and complete axillary dissection (levels I and II with or without level III) were performed in all breast-conserving therapy patients. The surgical margin was judged positive when the carcinoma component existed within 5 mm from the stump.

Pathological judgement

Two pathologists performed the pathological judgment, one from the Department of Pathology, Tohoku University Hospital and the other from the Department of Pathology, Institute for Differentiation, Aging and Cancer, Tohoku University.

Statistics

Results were compared by the chi-squared test. Differences were considered statistically significant when the P value was <0.05.

Table 1. Correlation between distance of intraductal spreading and ISC grading

Grade	No. of cases	Distance of intraductal spreading from invasive carcinoma (mean ± SD) (mm)	Range (mm)
0	6	0.3 ± 0.7	0–2
1	11	1.4 ± 2.1	0–4
2	9	17.2 ± 12.7	6–27
3	8	40.8 ± 11.8	24–53

Table 2. Correlation of EIC judgement and ISC grading with intraductal spreading of carcinoma examined by 3-D mapping

Judgement	No. of cases	Intraductal carcinoma extension	
		<2 cm	≥2 cm
EIC (−)	23	17 (74%)	6 (26%)*
EIC (+)	11	4 (36%)	7 (64%)*
ISC 0, 1	17	17 (100%)	0 (0%)**
ISC 2, 3	17	5 (29%)	12 (71%)**

*$P < 0.05$. **$P < 0.001$.

RESULTS

Investigation of Correlation Between Actual Intraductal Carcinoma Extension and EIC Judgement by 2-D Examination or ISC Grading by 3-D Examination

The results of the correlation between actual intraductal carcinoma extension and EIC judgement by 2-D examination or ISC grading by 3-D examination are presented in Table 2. Intraductal carcinoma extension over 2 cm from the edge of the invasive lesion was observed in seven of 11 (64%) specimens that were EIC positive and in six of 23 (26%) specimens that were EIC negative. On the other hand, intraductal carcinoma extension less than 2 cm was observed in 17 of 23 (74%) specimens that were EIC negative and in four of 11 (36%) specimens that were EIC positive. The difference in intraductal carcinoma extension between the EIC positive and negative groups was statistically significant ($P < 0.05$). EIC judgement by 2-D examination is able to detect a high degree of intraductal carcinoma extension in the EIC positive group, but it also appeared that a high degree of intraductal carcinoma extension was observed in 26% of the EIC negative group.

In contrast, according to the ISC grading defined by 3-D reconstruction, none of 17 specimens with low grade (0, 1) ISC demonstrated carcinoma extension over 2 cm and in 12 of 17 (71%) specimens that were high grade (2, 3) ISC. Intraductal carcinoma extension less than 2 cm was observed in five of 17 (29%) specimens that were high grade ISC, but in 17 of 17 (100%) specimens that were low grade ISC. The difference in intraductal carcinoma extension between ISC grade 0, 1 group and ISC grade 2, 3 group was statistically significant ($P < 0.001$). Furthermore, all of the ISC grade 0, 1 group actually showed intraductal carcinoma extension less than 2 cm. ISC grading by 3-D examination was able to detect a high degree of intraductal carcinoma extension in the grade 2, 3 group, but there were no false negatives in the grade 0, 1 group.

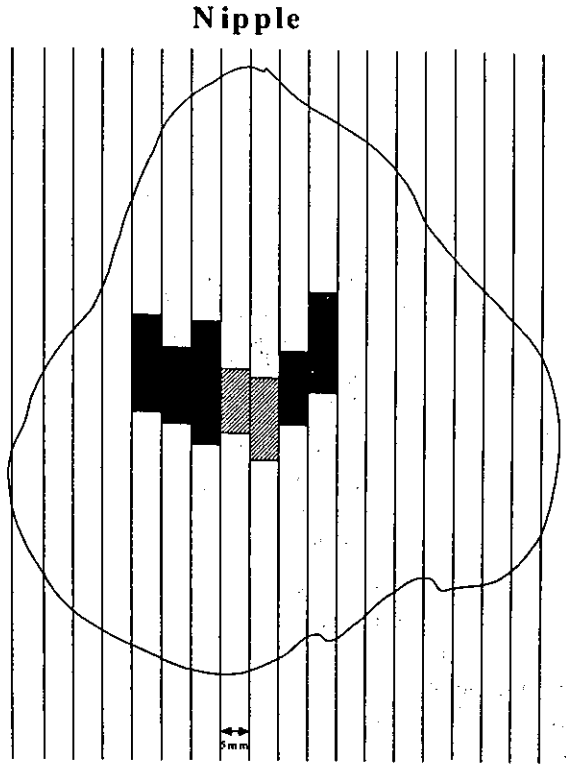

Figure 4. Histopathological cancer map of a quadrantectomy specimen. The hatched zone denotes invasive ductal carcinoma and the black zone denotes ductal carcinoma *in situ*. The ISC grading by 3-D examination is grade 2 and judgement of 2-D examination is ISC high degree group.

There was no significant relationship between ISC grade and clinicopathological findings, such as invasive tumor size, histological grade, hormone receptor status or lymph node metastasis.

Simplified Method for Detecting ISC Grading by 2-D Pathological Examination Using Several Paraffin Blocks

One case of histopathological cancer mapping of a quadrantectomy specimen is presented in Fig. 4. The results of corre-

Table 3. Correlation between ISC judgement by 2-D examination and distance of intraductal spreading or positive rate of surgical margin

Judgement	No. of cases	Intraductal spreading of carcinoma		Positive rate of surgical margin (within 5 mm)
		<2 cm	≥2 cm	
ISC low degree group	28	27 (96%)	1 (4%)*	0 (0%)**
ISC high degree group	34	8 (24%)	26 (76%)*	18 (53%)**

*$P<0.001$. **$P<0.001$.

lation between ISC judgement by simplified 2-D examination and actual distance of intraductal carcinoma extension, positive rate of surgical margin are presented in Table 3. Intraductal carcinoma extension over 2 cm was observed in 26 of 34 (76%) specimens that were in the ISC high degree group and 18 of 34 (53%) specimens appeared to be surgical margin positive. On the other hand, one of 28 (4%) specimens that were in the ISC low degree group demonstrated intraductal carcinoma extension over 2 cm and none of this group appeared to be surgical margin positive. The differences in intraductal spreading of carcinoma, positive rate of surgical margin between ISC low degree group and ISC high degree group were statistically significant ($P < 0.001$). The simplified method for detecting ISC grading by 2-D pathological examination using several paraffin blocks was able to detect correctly a high degree of intraductal carcinoma extension and positive surgical margin in the ISC high degree group and a high degree of intraductal carcinoma extension and surgical margin positive case is rarely observed in the ISC low degree group.

DISCUSSION

Breast-conserving treatment has become a standard therapy for early breast cancer with the aim of improving the quality of life of the patient. However, the problem of local failure in the operated breast still remains unresolved. Several groups have noted that invasive cancers accompanied by EIC positive features are associated with higher local recurrence rates within the breast after breast-conserving therapy than EIC negative invasive cancers (16–20). The definition of EIC is very simple and it is easy to presume the existence of intraductal carcinoma extension. However, it is not based on the segmental anatomy, which we think is necessary to define the intraductal carcinoma extension. Wellings et al. proposed the use of anatomical terms, the duct–lobular system (21). Large ducts and TDLU comprise a duct–lobular system. Large ducts include collecting duct, lactiferous sinus, segmental duct and subsegmental duct. TDLU consists of an extralobular terminal duct, intralobular terminal ducts and ductules. We have conducted 3-D reconstruction analyses of breast tissue using subserial sections. The studies demonstrated that breast carcinoma and peripheral papilloma originated from the TDLU (14,15,22).

We proposed the name ISC and classified ISC into four grades (15). ISC was defined as the state in which DCIS extends beyond TDLU and into large ducts. In our previous study, all EIC positive cases showed a high degree of ISC; however, 28% of EIC negative cases also showed a high degree of ISC. We proposed ISC grades based on its extent in ductal anatomy and showed how closely it correlated with carcinoma residues after breast-conserving surgery (15).

In this study, it was demonstrated that ISC grading by 3-D pathological examination is able to detect a high degree of intraductal carcinoma extension and surgical margin positive cases correctly. The definition of ISC by 3-D pathological examination appears to be correct for detecting a high degree of intraductal carcinoma extension without false negatives because no cases with a high degree of intraductal carcinoma extension were found in the ISC grade 0, 1 group. The definition of EIC is able to presume intraductal carcinoma extension from 1–3 hematoxylin and eosin-stained preparations for one patient; however, for ISC definition with serial slices 50–100 μm thick and computer-assisted 3-D mapping a total of about 2500–5000 hematoxylin and eosin-stained preparations are necessary for one patient. Hence it is very difficult to do this 3-D pathological examination for all patients receiving breast-conserving surgery. Therefore, in this study, we tried to define the simplified 2-D pathological examination method using several paraffin blocks for detecting a high degree of intraductal carcinoma extension based on 3-D examination.

In this simplified method for detecting ISC grading by 2-D pathological examination, we can presume a high degree of intraductal carcinoma extension and surgical margin status using the following blocks with each main tumor size: 6 to 1 cm, 8 to 2 cm and 10 to 3 cm. In our previous study, it needed 30–93 blocks (average 52.5 blocks) for one patient receiving breast-conserving surgery to make serial slices 5 mm thick. In this simplified method, the number of examination blocks was able to be reduced to 1/5–1/8. We made blocks 4 cm long, 5 mm thick parallel to the nipple-tumor line. In the blocks across this line, the duct is cut into round slices and its level is almost indiscernible. However, in the blocks parallel to this line, one can easily observe how cancer grows in the periphery spread towards large ducts (13–15,21–24). At first fearing that many high degree ISC might be missed with slices 5 mm thick, we began with slices 2 mm thick. As in fact, however, ISC did not always follow parallel to the nipple-tumor line, almost all ISC could be detectable with slices 5 mm thick.

From our 3-D pathological examination, it was proved that the higher the degree of intraductal carcinoma extension, the greater is the multicentricity. The frequencies of multiplicity were 0% in ISC grade 0, 11% in grade 1, 54% in grade 2 and 100% in grade 3 (15) defined by 3-D reconstruction. The coexistence of a high degree of intraductal carcinoma extension and multicentricity is an important factor for cancer residues after breast-conserving therapy. Six of 62 cases that were examined by the simplified method subsequently developed ipsilateral breast cancer. Three of six cases were positive for surgical margin and five were patients with high ISC grade. There may be greater benefit of ISC assessment for predicting ipsilateral breast cancer recurrence. The extension of carcinoma component correctly and easily obtained from resected specimens is of use in deciding adjuvant therapy including postoperative radiotherapy and predicting ipsilateral breast recurrence after breast-conserving therapy.

If the patient has a tumor with high ISC grade, without positive surgical margin status, irradiation is adopted in our hospital to prevent local recurrence. Endocrine therapy should also be adopted for patients with ER and/or PgR positive tumors.

Furthermore, to evaluate ultrasonography, 3-D MRI and helical CT as preoperative approaches, a careful comparative

study with pathological data is needed. From these data, we have to evaluate the entry criteria and adequate resection range of breast-conserving therapy individually.

We conclude that ISC grading correlates with carcinoma extension and surgical margin state. From a clinical point of view, the simplified 2-D examination using paraffin blocks may contribute to routine surgical pathology in evaluating the degree of carcinoma extension in breast-conserving therapy.

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ORIGINAL ARTICLE

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Evaluation of the diagnostic accuracy of the stop codon (SC) assay for identifying protein-truncating mutations in the *BRCA1* and *BRCA2* genes in familial breast cancer

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Abstract Screening for protein-truncating mutations of the *BRCA1* and *BRCA2* genes is useful in genetic testing for familial breast cancer because, first, the methods are usually simple and not expensive, and second, the detected mutations indicate pathogenic mutations in general. We evaluated the diagnostic accuracy of the stop codon (SC) assay for detecting protein-truncating mutations in the *BRCA1* and *BRCA2* genes by comparing the results with DNA sequencing in samples from 29 patients with breast cancer from 24 Japanese families with a history of breast cancer. Protein-truncating mutations were detected in 5 of the 24 families (20.8%; two in the *BRCA1* gene and three in the *BRCA2* gene). Among the 176 DNA fragments examined using the SC assay, the existence of three protein-truncating mutations (one in the *BRCA1* gene and two in the *BRCA2* gene) was predicted correctly by the assay. Only one reverse transcriptase-polymerase chain reaction fragment was positive for the SC assay but was negative using DNA sequencing. Our study showed clearly that the SC assay is sensitive (3 of 3, 100%) and specific (172 of 173, 99%) for detecting pathogenic protein-truncating mutations in the *BRCA1* and *BRCA2* genes, and that it could be useful for screening larger populations.

Key words Stop codon assay · Familial breast cancer · *BRCA1* · *BRCA2* · Genetic testing

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Introduction

Mutations in the *BRCA1* and *BRCA2* genes have been linked with susceptibility to breast and ovarian cancer, and patients who carry germline mutations of these genes are at high risk of developing these cancers. Accumulating evidence has shown that the *BRCA1* and *BRCA2* genes together are likely to account for the majority (~80%) of familial breast and ovarian cancers with at least four patients with breast cancer (Ford et al. 1998), and for approximately 50% of those with three or more female patients with breast or ovarian cancer, although the proportion of these mutations varies among populations (Szabo and King 1997). Although the cumulative risk of breast cancer in female carriers in the families with multiple cancer cases selected for linkage analysis was estimated to be >80% by age 70 years (Easton et al. 1993), recent studies have shown that cumulative breast cancer risk in *BRCA1* or *BRCA2* mutation carriers varies widely depending on the population studied (*BRCA1*, 47%–87%; *BRCA2*, 37%–84%) (Anglian Breast Cancer Study Group 2000; Ford et al. 1998; Narod et al. 1995; Neuhausen 1999; Rebbeck 1999; Struewing et al. 1997; Thorlacius et al. 1998). These observations have been derived mainly from Western countries; thus, the data are not always thought to be applicable to the Japanese population. Several Japanese studies have shown that the contribution of the *BRCA1* and *BRCA2* genes to Japanese familial breast and ovarian cancers (10%–30%) seems to be the same as that in Caucasian and Ashkenazi Jewish populations, although fewer patients have undergone *BRCA* testing (Anglian Breast Cancer Study Group 2000; Ikeda et al. 2001; Inoue et al. 1995; Inoue et al. 1997; Shih et al. 2002; Takano et al. 1997).

A rapid, simple, and accurate screening method is needed to accelerate genetic testing for familial breast and ovarian cancers because the two *BRCA* genes have large coding sequences that consist of 48 exons to be sequenced (Miki et al. 1994; Tavtigian et al. 1996; Wooster et al. 1995); therefore, detection of mutations using DNA sequencing analysis is labor intensive and expensive. Furthermore, most of the current screening methods, such as single-strand conformation polymorphism and denaturing high-performance liquid chromatography, detect not only pathogenic mutations but also several single-nucleotide polymorphisms (SNPs) (Kuklin et al. 1997; Orita et al. 1989; Xiao and Oefner 2001). In general, protein-truncating mutations of the *BRCA1* and *BRCA2* genes only provide reliable information for cancer-risk evaluation of patients and their family members because protein-truncating mutations theoretically eliminate the functional domain(s) of the gene product, although there is a carboxy-terminal nonsense mutation (K3326X) that is not disease associated (Mazoyer et al. 1996). None of the current methods discriminate missense mutations from nonpathogenic SNPs. Furthermore, more than 80% and 90% of reported *BRCA1* and *BRCA2* mutations, respectively, are protein-truncating mutations that were mapped broadly to the large (*BRCA1*, 5.6kb; *BRCA2*, 10.3kb) coding sequences (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Based on these findings, methods that only detect protein-truncating mutations have been used to screen *BRCA1* and *BRCA2* mutations. These methods include the protein truncation test (PTT) (Hogervorst et al. 1995; van der Luijt et al. 1994), also called the in vitro synthesized-protein assay (Powell et al. 1993), and the yeast-based stop codon (SC) assay (Ishioka et al. 1997). These techniques are used widely for genetic testing of other genes, including the *APC* gene, especially when most of the mutations are protein-truncating mutations (FitzGerald et al. 1997; Ishioka et al. 1997; Powell et al. 1993; van der Luijt et al. 1994). Although many studies have shown that such methods have technical advantages for saving time and cost (Hogervorst et al. 1995; Ishioka et al. 1997; Powell et al. 1993; van der Luijt et al. 1994), none of the reports evaluated the diagnostic accuracy (sensitivity and specificity) of the methods. According to one report (http://www.nhgri.nih.gov/ELSI/TFGT_final/index.html), the analytical validity (analytical sensitivity and specificity) of a new genetic test must be assessed by comparing it to the most definitive or "gold standard" method before it is made available for clinical use. In the present study, we focused on a small number of Japanese breast cancer patients with a family history of breast cancer, who have not previously undergone genetic testing. We examined mutations in the *BRCA1* and *BRCA2* genes both by SC assay and by DNA sequencing (the "gold standard") in a blind manner and compared the methods. We confirmed previous reports indicating that the frequency of *BRCA* mutations in Japanese patients with familial breast cancer is low (10%–30%) (Ikeda et al. 2001; Inoue et al. 1995; Inoue et al. 1997; Takano et al. 1997), and we also showed that the SC assay is sufficiently sensitive and specific to screen protein-truncating mutations in the *BRCA1* and *BRCA2* genes, even in a population with a lower frequency of *BRCA* mutations.

Patients and methods

Patients

Twenty-nine patients with a history of breast cancer, who were also from 24 familial breast cancer pedigrees, were selected according to the criteria defined by the Tohoku Familial Cancer Society as having one or more of the following: (1) at least three relatives with breast or ovarian cancer with one being a first-degree relative of the other two; (2) at least two relatives with breast or ovarian cancer who are first-degree relatives of each other and at least one of them should (a) be diagnosed before the age of 40 years, (b) have bilateral breast cancer, or (c) have cancer of other organs; (3) early-onset bilateral breast cancers and at least one cancer diagnosed before the age of 40 years. Informed consent was obtained from all the patients participating in this study. Blood samples were collected, labeled with coded numbers, and analyzed without the patients' clinical information being known to the analyst. Three technicians performed the two genetic analyses; one performed the SC assay and two performed direct DNA sequencing. The patients' clinical information and the results of each assay were not disclosed to the examiners until all assays were complete. Approval was obtained from the Ethics Committee of Tohoku University Graduate School of Medicine for analysis of the *BRCA1* and *BRCA2* genes.

Extraction of DNA and RNA

Approximately 2 × 7 ml of peripheral blood was collected in a 10-ml tube containing RPMI 1640 cell culture medium and transferred to the laboratory at 4°C. Genomic DNA was extracted directly from 3 ml of the blood using a QIAamp 8 DNA Blood BioRobot Kit (Qiagen, Valencia, CA, USA) equipped with a BioRobot 9604 (Qiagen). We used a Micro-Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA, USA) to extract poly A^+ RNA from mononuclear cells (approximately 3×10^7 cells separated from 5 ml blood using a lymphoprep tube) from 14 of the patients. The remaining 7 ml of blood was used to immortalize lymphoblastoid cells using Epstein-Bar virus infection, and these were used for DNA and/or RNA extraction in some cases.

Polymerase chain reaction (PCR)

First-strand cDNA was synthesized from the poly A^+ RNA using a first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden), and this was used to amplify DNA fragments: BRCA1a (0.8kb); BRCA1c (1.6kb) (Ishioka et al. 1997); and BR2a (2.2kb), BR2d (1.9kb), and BR2e (1.9kb) (Fig. 1A). The DNA fragments BRCA1b (3.4kb) (Ishioka et al. 1997), BR2b (2.7kb), BR2c (2.1kb), and BR2/ex10 (1.1kb)

(Fig. 1A) were amplified from genomic DNA. The primers used for amplification of the *BRCA1* fragments BRCA1a–c have been described previously (Ishioka et al. 1997). The primers for amplification of the *BRCA2* fragments were 5′-ATGCCTATTGGATCCAAAGAGAG-3′ and 5′-TGACAGAGAATCAGCTTCTGGGG-3′ for BR2a, 5′-TCTTCTGTGAAAAGAAGCTGTTCAC-3′ and 5′-CCCGCTAGCTGTATGAAAACCC-3′ for BR2b, 5′-AGAAAGAACAAAATGGACATTCTAAG-3′ and 5′-TTGTTGAAATTGAGAGAGATATGGAG-3′ for BR2c, 5′-TCTGAGCATAGTCTTCACTATTCACCTAC-3′ and 5′-TCTTAAGAGGGGAGGATCTAACTGG-3′ for BR2d, 5′-TACAGATATGATACGGAAATTGATAG-3′ and 5′-TAAAGGCAGTCTACTCAAGAAATCC-3′ for BR2e, and 5′-TATAAAATATTAATGTGCTTCTGTT-3′ and 5′-AAGGAATCGTCATCTATAAAACTA-3′ for BR2/ex10. All PCR fragments were obtained using ExTaq or LA Taq DNA polymerase (Takara Shuzo, Tokyo, Japan). Information on the PCR parameters are available at our website (http://www.idac.tohoku.ac.jp/dep/co/data/saka/brca01.htm).

Plasmids and gap vectors

For the SC assay of *BRCA2*, six novel plasmids were constructed (Fig. 1B): pCI-BR2a, pCI-BR2b, pCI-BR2c, pCI-BR2d, pCI-BR2e, and pCI-BR2/ex10. They were obtained by inserting a PCR-derived cDNA fragment of the *BRCA2*-containing nucleotide (nt) 1–2130 (the first letter of the initiation codon was defined as nt 1), nt 1921–4587, nt 4411–6480, nt 6265–8211, nt 7972–9837, and nt 794–1897, into a *Bam*HI site of pCI-HA(URA3)-2 (Ishioka et al. 1997). All plasmids exhibited the Ura$^+$ phenotype, confirming an in-frame fusion of each *BRCA2* fragment and the yeast *URA3* gene. To make linearized gap vectors, we digested pCI-BR2a, pCI-BR2b, pCI-BR2c, pCI-BR2d, pCI-BR2e, and pCI-BR2/ex10 by the restriction enzymes *Pst*I/*Xba*I, *Xba*I/*Spe*I, *Spe*I/*Pst*I, *Pst*I/*Spe*I, *Spe*I/*Nsi*I, and *Spe*I, respectively, and purified them by a GFX PCR DNA and Gel Band Purification Kit (Pharmacia) after separating the longer DNA fragment by agarose gel electrophoresis. The preparation of the gap vectors used for the stop codon assay for *BRCA1* has been described previously (Ishioka et al. 1997).

SC assay

The yeast strain used in this study was YPH499 (*MATa*, *ura3-52*, *lys2-801amber*, *ade2-101ocher*, *trp1Δ63*, *his3Δ200*, *leu2Δ1*) (Stratagene, La Jolla, CA, USA). The preparation of frozen competent cells and the method for transformation has been described previously (Ishioka et al. 1997). In brief, each *BRCA1* or *BRCA2* PCR fragment (~200 ng) was cotransformed with the corresponding linearized gap vector (~30 ng) and transformants were selected on a synthetic complete medium lacking leucine (SC-Leu). The 25–50 transformants were assayed further for either the Ura$^+$ phenotype, by growing them on synthetic complete

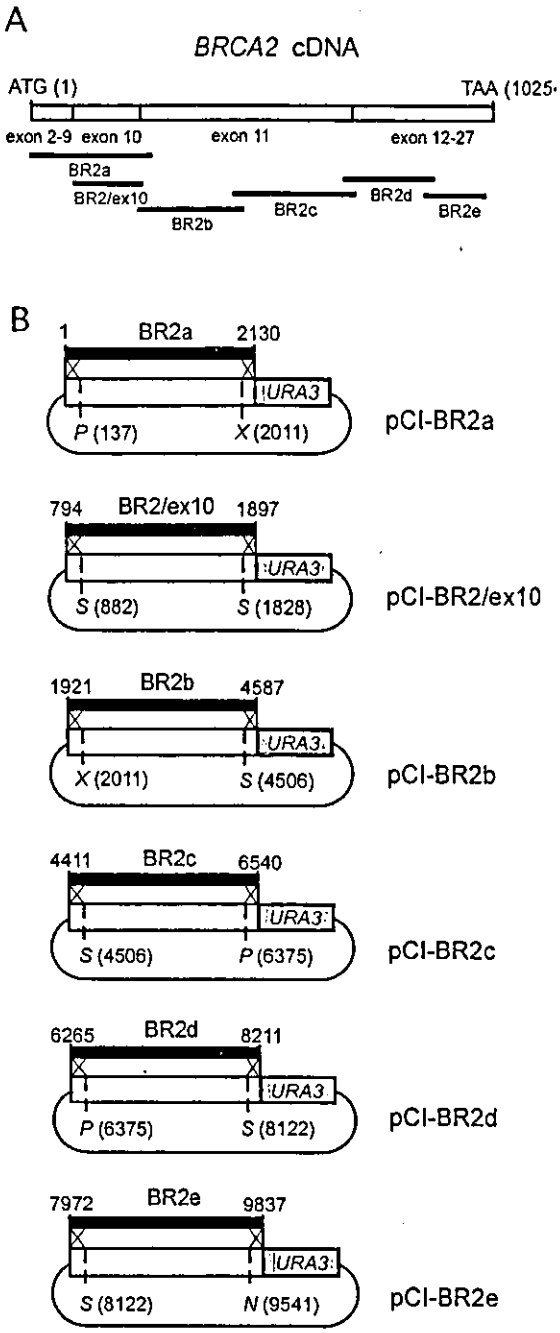


Fig. 1A,B. Schematic representation of the stop codon (SC) assay of the *BRCA2* gene. **A** Sequences of *BRCA2* cDNA and six polymerase chain reaction (PCR) fragments were chosen for the SC assay. **B** Plasmids for the SC assay of the *BRCA2* gene and the corresponding PCR fragments. cDNA (BR2a, BR2d, and BR2e) or genomic DNA (BR2/ex10, BR2b, and BR2c) fragments shown in **A** were inserted in-frame into the *Bam*HI site of pCI-HA(URA3)-2 (Ishioka et al. 1997), producing plasmids pCI-BR2a, pCI-BR2d, pCI-BR2e, pCI-BR2/ex10, pCI-BR2b, and pCI-BR2c, respectively. Digestion of the plasmids by the indicated restriction endonucleases removed the central portion of the *BRCA2* sequences and generated gap vectors. Cotransformation of a gap vector with a corresponding PCR fragment into yeast recircularizes the plasmid by homologous recombination in vivo. The *numbers* indicate nucleotide positions downstream of the first nucleotide of ATG in the *BRCA2* coding region. *P*, *Pst*I; *X*, *Xho*I; *S*, *Spe*I; *N*, *Nsi*I

medium that lacked leucine and uracil (SC-Leu-Ura), or for 5FOA resistance, by growing them on synthetic medium containing 5FOA (1mg/ml). If 80% or more of transformants were $Ura^+/5FOA^S$, the sample was scored as negative for the protein-truncating mutation, indicating a homozygous wild type. If less than 80% of transformants were $Ura^+/5FOA^S$, we amplified the PCR fragment again and repeated the SC assay to confirm the final result. If the result showed less than 80% $Ura^+/5FOA^S$ again, the sample was positive for the presence of the heterozygous protein-truncating mutation.

DNA sequencing

We sequenced approximately 23.0kb of the *BRCA1* and *BRCA2* genes that contained all the coding exons and their flanking exon–intron boundaries. Except for exon 11 of both the *BRCA* genes, each coding exon and the flanking intron was amplified with a set of upstream and downstream primers and was sequenced by 1–3 sequence primer(s). Exon 11 of each of the *BRCA1* and *BRCA2* genes was sequenced using 16 and 18 primers, respectively. All of the primer sequences are listed at our website (http://www.idac.tohoku.ac.jp/dep/co/data/saka/brca01.htm) and all sequencing reactions were performed using a CEQ DTCS Kit or CEQ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, CT, USA), and then processed by an automated capillary sequencer CEQ2000 or CEQ2000EX (Beckman Coulter).

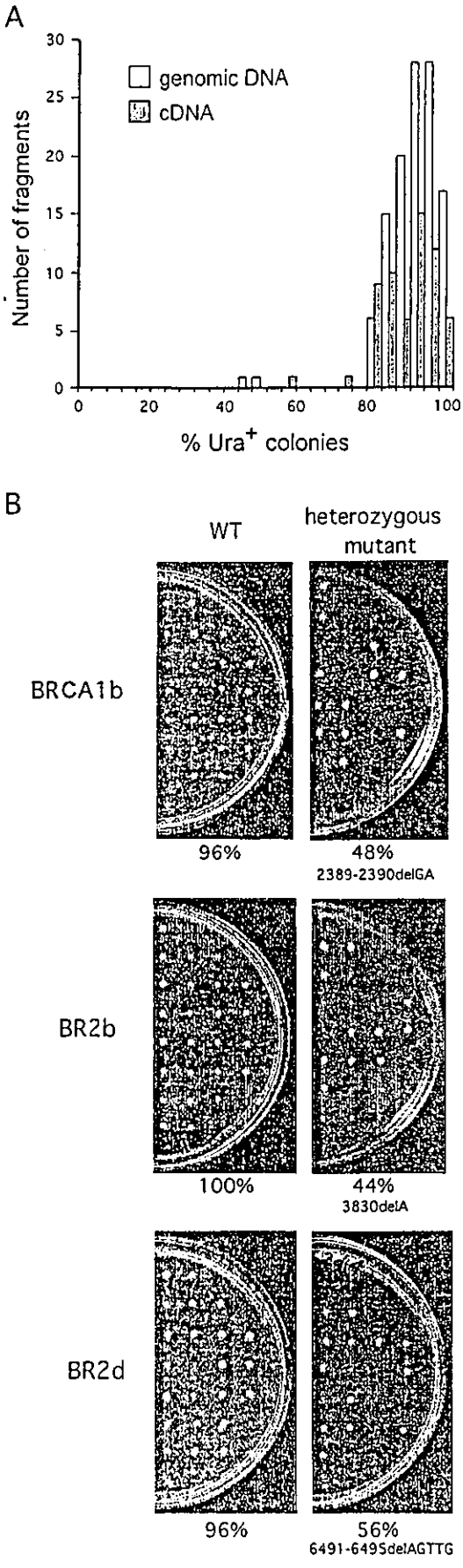
Results and discussion

We have described previously the SC assay for the *BRCA1* gene and the *APC* gene, a gene that is responsible for familial adenomatous polyposis (Ishioka et al. 1997). In the SC assay, a PCR-amplified DNA fragment derived from patient cDNA or genomic DNA was recombined into a specific gap vector harboring the yeast *URA3* gene, and the yeast transformant was selected as a colony using an appropriate selection medium (SC-Leu). The inability to express the URA3-fusion protein depends on the existence of a protein-truncating mutation within the inserted PCR fragment. Therefore, most of the colonies (≥80%) are able to grow on a medium lacking uracil (Ura^+) when the patient DNA fragment has neither nonsense nor frame-shift mutations. If the patient's DNA contains a heterozygous protein-truncating mutation, approximately half of the colonies cannot grow in the same medium (Ura^-). So far, the same or a similar yeast-based assay has been applied to other genes (Ishioka et al. 1997; Suzuki et al. 1998).

In this study, we constructed a plasmid system for an SC assay of the *BRCA2* gene (Fig. 1B), which divides the open reading frame of the *BRCA2* gene into six fragments (Fig. 1A) and inserts each of them into a *Bam*HI fragment of the pCI-HA(URA3)-2 (Ishioka et al. 1997; Suzuki et al. 1998) (Fig. 1B). When these plasmids are introduced into *ura3*$^-$ yeast cells, the yeast cells can be positively selected on the plate lacking uracil (Ura^+) or negatively selected on the plate containing 5FOA ($5FOA^S$) (data not shown). These vectors were digested by one or two restriction endonucleases (Fig. 1B) and used as gap vectors. We confirmed previously that cotransformation of each of the gap vectors with corresponding PCR fragments with or without a known protein-truncating mutation resulted in a $Ura^-/5FOA^R$ or $Ura^+/5FOA^S$ yeast phenotype, respectively.

Screening protein-truncating mutations by SC assay

Using the SC assay of *BRCA1* (Ishioka et al. 1997) and *BRCA2* (earlier), we screened a total of nine fragments covering the coding sequences of both *BRCA1* (three fragments) and *BRCA2* (six fragments) in 29 breast cancer patients (24 families) whose *BRCA* status had not been examined. A technician who was not informed about the patients' clinical background and the results of the DNA sequencing performed the SC assay. In 12 cases, both genomic DNA and RNA were available and, therefore, we examined all nine DNA fragments. In the remaining 17 cases, we examined only four (BR1b, BR2/ex10, BR2b, and BR2c) fragments that covered approximately 57% of the coding sequences (Fig. 1A) because RNA was not available and it was impossible to perform reverse transcriptase (RT)-PCR to amplify the remaining fragments. Among the 176 fragments we analyzed, the majority (172) were negative for the SC assay; 80% or more of the colonies were Ura^+ (mean ±SD = 90.0 ± 6.5%), which showed that there were no protein-truncating mutations in these fragments (Fig. 2A). The remaining four fragments that were derived from different patients were positive for the SC assay because a significantly lower number of Ura^+ colonies were found (Ura^+ fraction <80%) (Fig. 2A). To confirm the results, we reexamined the SC assay using independently amplified PCR fragments. Three of the four fragments (cases BR007, BR005, and BR001) again were positive, and, therefore, were thought to have retained a protein-truncating mutation within each fragment. Representative results are shown in Fig. 2B. The remaining fragment, which was derived from cDNA (BR2a fragment) and previously had a 72% Ura^+ fraction, had a negative score (88%) in the second experiment. Although we did not pursue the cause of low fidelity in this case, we had found previously that the RT reaction sometimes caused unexpectedly lower fidelity and that this issue was usually resolved by repeating the experiment. Therefore, we concluded finally that the BR2a fragment was negative for the SC assay. Because, in general, it is impossible to check and control the quality of RNA strictly, we stress that the repeat analysis and confirmation of the mutations by DNA sequence analysis are important, especially when the PCR fragment is derived from RT-PCR and the Ura^+ fraction is close to the cut-off value (80%). So far, 424 *BRCA1* or *BRCA2* fragments have been screened using the SC assay, and the Ura^+ fraction (mean ± SD) was 52.4 ± 8.9% (n = 17) or 89.3 ± 7.1% (n = 407) in fragments



with or without a heterozygous mutation. To evaluate the cut-off value (80%) of the SC assay, we drew the ROC (receiver operating characteristic) plots (Zweig and Campbell 1993) based on the individual data of this study. The plots indicated that any values from 60% to 80% of the rational cut-off value warranted good sensitivity (=1.00) and specificity (>0.99). Therefore, the value of 80% should be a reasonable cut-off value and may minimize possible false-negative cases in future studies.

Detection of sequence variations in the coding sequences by direct DNA sequencing

To confirm the sequence variations, we sequenced the full-length coding sequences and the flanking introns of the *BRCA1* and *BRCA2* genes using genomic DNA in all of the cases by automated direct sequencing, a standard method for mutation detection. Two technicians who were blind to the patients' clinical backgrounds and the results of the SC assay performed the DNA sequencing. We found five protein-truncating mutations, including three frameshift mutations (2389-2390delGA in exon 11 of *BRCA1*, 3830delA in exon 11 of *BRCA2*, and 6491-6495delAGTTG in exon 11 of *BRCA2*) and two nonsense mutations (C1372X in exon 12 of *BRCA1* and S2835X in exon 20 of *BRCA2*) (Table 1). During the sequencing analysis, we also found 40 other sequence variants (16 in *BRCA1* and 24 in *BRCA2*) within the coding regions and their flanking introns (data not shown). We concluded that most of these variants are common polymorphisms because they are found repeatedly in other patients and because some of them have been reported in an SNP database. However, they also contain 12 SNPs (5 in *BRCA1* and 7 in *BRCA2*) that were found only once in this study and not reported previously (Table 1). In the case of the five SNPs that substitute a single amino acid, there is a possibility that they contain pathogenic missense mutations, but there is no reliable functional assay at present.

Diagnostic accuracy of the SC assay for detecting protein-truncating mutations

To examine the diagnostic accuracy of the SC assay, we compared the results of the SC assay with the DNA sequencing data (Fig. 3). We found no protein-truncating

Fig. 2A,B. Distribution of Ura$^+$ fractions (Ura$^+$ colonies/total colonies assayed). **A** The result of the SC assay of both *BRCA1* and *BRCA2* genes is shown for a total of 176 fragments derived from specimens (116 genomic DNA and 60 cDNA) of this study. **B** Representative results of the SC assay showing heterozygous mutations. *Right panel*, The yeast transformants (25 clones) derived from the BR1b fragment of cases BR007 (*top*), BR2b fragment of BR005 (*middle*), and BR2d fragment of BR001 (*bottom*) were evaluated for their ability to grow in synthetic media lacking uracil. Approximately one half of the transformants (44%–56%) did not grow. A specific mutation shown *under each panel* was finally detected by DNA sequencing. *Left panel*, Negative controls for the corresponding DNA fragments derived from patients without any protein-truncating mutation. More than 80% of colonies grew. *WT*, Wild type

Table 1. Sequence variants (polymorphism and mutation) of *BRCA1* and *BRCA2* genes

Case number	Allele ID	Location	Nucleotide change	Sequence (5' to 3')	Interpretation
5 protein-truncating mutations					
BR007	BRCA1-7	Exon 11	2389-2390delGA	ggcaaaaaca GA/-accaaataaa	Frame-shift mutation
BR017	BRCA1-11	Exon 12	4116T>A	catctgggtg T/A gagagtgaaa	Nonsense mutation (C1372X)
BR005	BRCA2-18	Exon 11	3830delA	aagatagaaa A/- tcataatgat	Frame-shift mutation
BR001	BRCA2-22	Exon 11	6491-6495delAGTTG	gacaaacaac AGTTG/- gtattaggaa	Frame-shift mutation
BR018	BRCA2-26	Exon 20	8504C>A	gagaagacat C/A atctggatta	Nonsense mutation (S2835X)
12 sequence variations found once in this study					
BR028	BRCA1-4	Exon 11	814G>A	gagccatgtg G/A cacaaatact	G275D
BR027	BRCA1-13	Intron 14	IVS14+14A>G	agaaacatca A/G tgtaaagatg	Close to exon–intron boundary
BR013	BRCA1-15	Exon 16	4883T>C	tataatgcaa T/C ggaagaaagt	M1628T
BR027	BRCA1-17	Intron 22	IVS22+31A>T	gagagggagg A/T cacaatattc	Close to exon–intron boundary
BR006	BRCA1-18	Intron 23	IVS23+8G>T	atggtaaggt G/T cctgcatgta	Close to exon–intron boundary
BR017	BRCA2-2	Intron 2	IVS2-16T>A	taaggtggga T/A ttttttttta	Close to exon–intron boundary
BR026	BRCA2-3	Intron 2	IVS2-9T>G	ggatttttt T/G ttaaatagat	Close to exon–intron boundary
BR028	BRCA2-8	Exon 10	798T>C	gtcatggatt T/C ggaaaaacat	F266 silent
BR025	BRCA2-12	Exon 10	1744A>C	tttaatatcc A/C ctttgaaaaa	T582P
BR020	BRCA2-17	Exon 11	3420T>C	tgcagaagag T/C acatttgaag	S1140 silent
BR023	BRCA2-19	Exon 11	4566G>T	ctactctgtt G/T ggttttcata	L1522F
BR030	BRCA2-20	Exon 11	6131G>T	tcccaaaaag G/T cttttcatat	G2044V

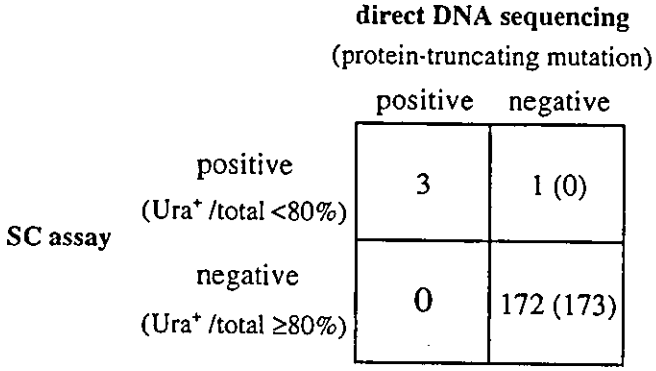


Fig. 3. Diagnostic accuracy of the SC assay for detection of protein-truncating mutations in both *BRCA1* and *BRCA2* genes. The results of the SC assay were compared with that of direct DNA sequencing. *Values in the box* indicate the number of fragments used in the SC assay. *Values in parentheses* indicate the results after reassay of fragments that were positive in the first screening

mutation in the DNA sequences of the 172 fragments that were negative for the SC assay, indicating that the negative predictive value was 100%. Of the remaining four fragments that were positive for the SC assay, three fragments contained protein-truncating mutations (Fig. 2B, Table 1). Only one fragment that was positive in the first screening had no protein-truncating mutation (false-positive case), but this was eventually scored as negative after repeating the assay (see earlier). Among the five protein-truncating mutations detected by DNA sequencing, two mutations could not be compared with the SC assay because no RNA was available. Therefore, the SC assay detected all protein-truncating mutations within the examined DNA fragments (sensitivity, 100%). Because there was one false-positive case in the first screening, the specificity of the assay was 99% (172 of 173) but the value finally became 100%. These results indicate that the SC assay is sufficiently sensitive and specific to screen protein-truncating mutations of the *BRCA1* and the *BRCA2* genes, even in a population that contains small fractions of *BRCA* mutations.

We have reported previously on the analytical validity of the SC assay for the *BRCA1* gene using blood samples with or without known *BRCA1* truncating mutations, and we have also discussed the technical advantages of the SC assay over the PTT (Ishioka et al. 1997). In this study, we expanded the application of the SC assay to the *BRCA2* gene, and confirmed the diagnostic accuracy of the SC assay for both *BRCA1* and *BRCA2* genes by analyzing specimens with unknown *BRCA* status in a blind manner. Once an examined specimen is positive in the SC assay, only 0.8–3.4kb DNA sequencing is needed to detect a protein-truncating mutation. We conclude that the SC assay is a useful and reliable screening method to detect pathogenic protein-truncating mutations in both *BRCA* genes. Apart from the technical advantages, we also note that the SC assay has an economical advantage. We have calculated the net cost (labor cost not included) for detecting protein-truncating mutations with or without the use of the SC assay. The cost of the SC assay for screening plus DNA sequencing only for SC assay-positive fragments was significantly lower than that for full-length DNA sequencing in all specimens; e.g., if the fraction of BRCA-truncating mutation is 20% in a population, the former costs only 11.4% of the latter. The advantage of the SC assay over the PTT should be validated by comparing the two assays in a large number of clinical samples.

We also showed that the frequency of the *BRCA1* and *BRCA2* gene mutations in our Japanese familial breast cancer patients was similar to that among similar patients in Western countries, which confirms previous reports (Ikeda et al. 2001; Inoue et al. 1995; Inoue et al. 1997; Takano et al. 1997). However, care should be taken in interpreting the data because there were some differences, for example, the number of breast cancer patients in the families of the two populations. Furthermore, there may be mutations that could not be detected by the current screening system and