to disuse atrophy.

Breast conserving surgery with primary volume replacement with LTF has advantages because, 1) patients can avoid foreign prosthesis like silicone bags, 2) LTF maintains its volume for a long period and 3) patients can avoid poly-surgery. The disadvantage of volume replacement with LTF is that the thickness of the LTF depends on the thickness of subcutaneous adipose tissue. It does not always meet the demand for the thickness of breast tissue. In cases with thin LTF for volume replacement, the central area of the breast under the nipple should be covered by existing mammary gland to maintain thickness with a partial suture and the peripheral area would be covered by LTF. Using this method, the difference in volume between the breasts is not conspicuous.

In conclusion, volume replacement with LTF is a reasonable and useful method to achieve both local control and good cosmesis in primary surgical breast cancer treatment.

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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.

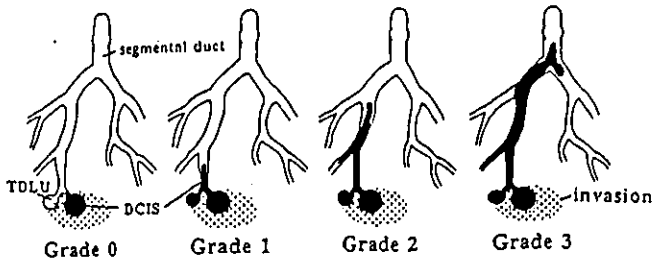

Figure 1. A schematic diagram of intraductal spread of carcinoma (ISC). Grade 0: carcinoma is confined within a terminal duct–lobular unit (TDLU). Grade 1: carcinoma extends beyond TDLU, but is confined within a single large duct or its periphery. Grade 2: carcinoma extends more than two large ducts,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

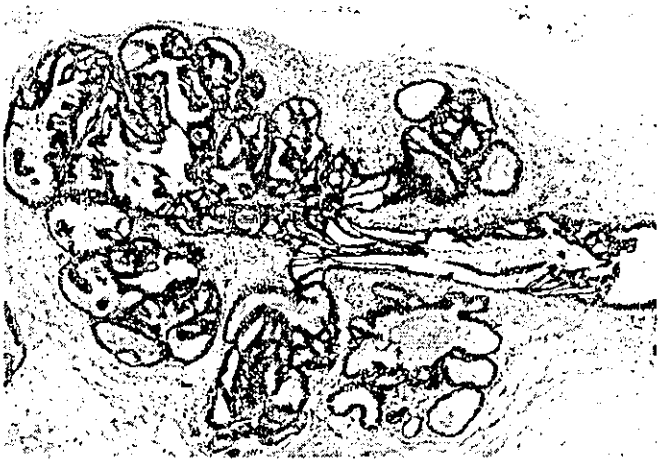

Figure 2. A photomicrograph of an ISC grade 1 case. Carcinoma cells extend beyond ductules, intralobular terminal ducts (ITD) and extralobular terminal ducts (ETD). TDLU comprises ductules, ITD and ETD.

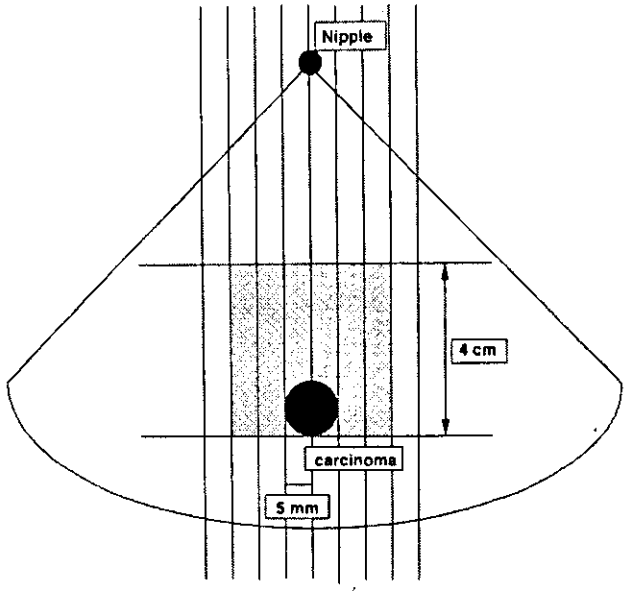

Figure 3. Schematic diagram of the simplified method for detecting ISC grading by 2-D pathological examination using several paraffin blocks.

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.

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.

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.

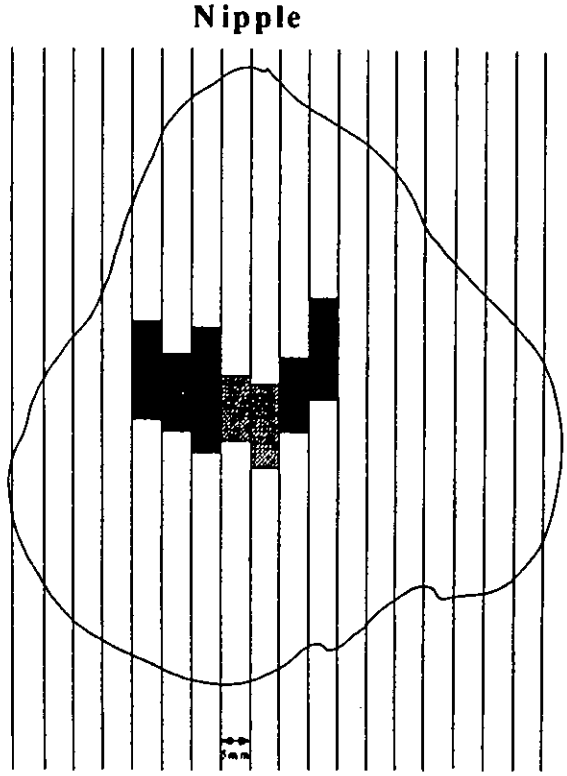

Figure 4. Histopathological cancer map of a quadrantectomy specimen. The hatched zone denotes invasive ductal carcinoma and the black zone denotes ductal carcinoma *in situ*. The ISC grading by 3-D examination is grade 2 and judgement of 2-D examination is ISC high degree group.

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

Microinvasive ductal carcinoma (T1mic) of the breast. The clinicopathological profile and immunohistochemical features of 28 cases

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Microinvasive ductal carcinoma of the breast, namely ductal carcinoma *in situ* with microinvasion (T1mic) as defined by the American Joint Committee on Cancer (AJCC) Staging Manual, is a rare disease, although it is increasing because of widespread use of mammography. The aim of the present study was to describe the clinicopathological and immunohistochemical features of this entity. Twenty-eight patients who were diagnosed as T1mic from January 1997 to August 2002 were studied by using 3–5 mm-thick serial sections with hematoxylin–eosin staining. Immunohistochemical staining for the estrogen receptor (ER), progesterone receptor (PR), p53, KI-67, and HER-2 were performed. All 28 patients were female, with a mean age of 48.8 years. Twenty-six patients (93%) revealed mammographic abnormalities on routine examination. All foci of the invasions were measured using an ocular micrometer. Invasive foci consisted of isolated cells or cell clusters, or appeared as a tongue-like projection of tumor through the basement membrane of the duct of ductal carcinoma *in situ* (DCIS). The mean number of invasive foci was 3, and the mean size was 0.6 mm. We found that high nuclear grade and predominant comedo subtype of DCIS components were 57.1% and 46.4%, respectively. Twenty-four cases (86%) demonstrated necrosis of DCIS components. Microinvasion was often associated with periductal stromal reaction (71.5%) and/or a lymphocytic infiltration (78.6%). All patients, excluding two, received axillary resection (the mean number of lymph nodes examined per case was 12), and none had lymph node metastasis. The positive expression of ER and PR strongly related to low grade nuclei and non-comedo subtype; however, the positive expression of HER-2 and P53 related to high grade nuclei and comedo subtype ($P < 0.01$). KI-67 expression was significantly higher in the high grade nuclei group than in the low grade group ($P < 0.01$). Our study suggested that high nuclear grade and comedo DCIS were more aggressive and more common with microinvasion, and that microinvasion is more likely to be multifocal.

Key words: breast carcinoma, ductal carcinoma *in situ*, immunohistochemistry, microinvasion, T1mic

The mammographic screening program has increased the detection of ductal carcinoma *in situ* (DCIS) and also DCIS with microinvasion. One of the most important goals in the histological examinations of DCIS is whether any focal invasion exists within the stroma surrounding the DCIS, because the presence of invasion may be associated with metastatic focus. Although the term 'microinvasion' has been used for many years, until now, there is no standard or consensus definition in the world.

Since this term was used, microinvasion has been defined in various ways, such as: DCIS with evidence of stromal invasion;[1] DCIS with limited microscopic stromal invasion below the basement membrane, but not invading more than 10% of the surface of the histological sections examined;[2] one or two microscopic foci of possible invasion not >1 mm in greatest dimension;[3] a single focus of invasive carcinoma <2 mm, or up to three foci of invasion each not more than 1 mm in greatest dimension;[4] and the maximal extent of invasion is not more than 2 mm or comprising <10% of the tumor, with >90% (DCIS).[5]

The lack of a uniform definition for microinvasion has clearly contributed to the confusion regarding this entity. The exact clinicopathological nature of patients who have DCIS with microinvasion is not well defined, and clinical management of these patients is controversial.

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In the 6th edition of the *American Joint Committee on Cancer (AJCC) Staging Manual*, microinvasion is defined as 'the extension of cancer cells beyond the basement into the adjacent tissues with no focus more than 0.1 cm in greatest dimension'.[6] Lesions that fulfil this definition are staged as T1mic, a subset of T1 breast cancer. The staging manual further stated that 'when there were multiple foci of microinvasion, the size of only the largest focus is used to classify the microinvasion' and that the size of the individual foci should not be added together.

Until now, this entity has not been studied in detail in Japan. The present study aims to describe the histopathologic and immunohistochemical features of T1mic according to the latest definition of the AJCC (1997). Based on this definition, we retrospectively studied 28 cases of DCIS with microinvasion.

MATERIALS AND METHODS

We have reviewed the case files of patients from September 1998 to August 2002 in Tohoku University Hospital and Tohoku Kousai Hospital, and from January 1997 to August 2002 in Chugoku Chuo Hospital. A total of 30 patients were considered as T1mic. None of these patients were treated with chemotherapy or radiotherapy before their operation, and all patients received surgical therapies, including partial mastectomy (lumpectomy), quadrantectomy, and mastectomy. Twenty-six patients received axillary lymph node dissection. The clinical data, including patient age, menopausal status, stage according to the Union Internationale Contre le Cancer TNM classification,[7] tumor size, and lymph nodes status, were obtained from the patients' charts. Two patients were excluded from our study because of the size of focus more than 1 mm in the greatest diameter, at the time of re-evaluation. Thus, ultimately 28 cases were reviewed in this study. During the same periods, we have experienced 1216 cases of primary breast carcinomas including 172 pure DCIS. Thus, the overall incidence of T1mic was 2.3% among all breast carcinomas and about one sixth of pure DCIS.

The tissue was obtained by partial mastectomy, quadrantectomy, or mastectomy. The specimen was fixed in formalin, serially sectioned in its entirety in numbered slices 3–5 mm thick. Each numbered slice was put in as many numbered separate cassettes as necessary and paraffin-embedded in sequence. The mean number of blocks was 22 (from 6 to 35). All blocks were cut 2 μm thick, and stained with hematoxylin–eosin (HE). All slides of breast tissue were reviewed for each case. For the lymph nodes, we performed routine HE examinations. The mean number of 12 axillary lymph nodes (range 0–25 lymph nodes per case) were reviewed.

The diagnosis of T1mic was according to Prasad's criteria,[8] namely: (i) cytologically malignant epithelial cells in the stroma; (ii) the greatest dimension of the largest focus of invasion no more than 1 mm, as measured by ocular micrometer; (iii) demonstrable absence of basement or myoepithelial layer around the invasive cells; (iv) *in situ* carcinoma in the vicinity of microinvasive carcinoma.

Each focus of microinvasion was measured by ocular micrometer (10x; Nikon Labophot, Tokyo, Japan) and the number of the focus was also calculated. The architectural type, nuclear grade, and the presence of necrosis in the associated DCIS were recorded. Each case was subclassified into one of the histopathological subtypes. If more than 70% of the duct profiles containing carcinoma were composed of a single pattern, the case was categorized in that subtype,[9] the remaining cases were classified as mixed. For the classification of *in situ* components, we adopted the Van Nuys classification system.[10] According to that system, cases were defined in three groups: non-high nuclear grade without necrosis (Group 1); non-high nuclear grade with necrosis (Group 2); and high nuclear grade (Group 3). All available slides were reviewed by two authors (MY and TM) together, and a consensus diagnosis was reached.

Immunohistochemistry, including estrogen receptor (ER), progesterone receptor (PR), HER-2, p53, and Ki-67, was performed on formalin-fixed, paraffin-embedded tissue sections which were cut at 2 μm using the avidin–biotin complex-horse radish peroxidase diaminobenzidine technique. The primary antibodies are shown in Table 1. Tumors were recorded as positive for ER and PR if more than 10% of the tumor cell nuclei showed a moderate degree of brown staining. HER-2 overexpression was considered positive when the malignant cells showed cytoplasmic membrane staining of at least 2+ on a scale of 0 to 3+. p53 staining was considered positive when more than 10% of the cells of interest showed distinct nuclear staining.[11] Scoring of Ki-67 in cancer cells was performed on high-power fields (×400) using a standard

Table 1 Immunohistochemical reagents and methods used

Antibody	Source	Clone	Dilution	Pretreatment
Ki-67	Immunotech, France	MIB-1	1:300	Autoclave
p53	Biomeda Corp., Foster City, CA, USA	B20.1	1:40	Microwave
HER-2	DAKO, Glustrup, Denmark	CB-11	1:3000	Autoclave
ER	Immunotech, France	1D5	1:400	Autoclave
PR	CHEMICON International, Temecula, CA, USA	1A6	1:30	Autoclave

ER, estrogen receptor; PR, progesterone receptor.

light microscope. In each case, approximately 1000 carcinoma cells were counted independently by two authors (MY and TM), and the percentage of immunoreactivity (i.e. labeling index (LI)) was determined. The mean of the two values was obtained.

For statistical analysis, all comparison between groups and/or parameters were performed using Chi-square test χ^2 or Fisher test. Differences between categories were considered significant when the *P*-value was less than 0.05.

RESULTS

Clinical characteristics

All 28 patients were Japanese women ranging in age from 26 to 79 years (mean 48.8 years), 16 of them were premenopausal at the time of diagnosis. In 17 patients the tumor was located in the left side, and in 11 patients the tumor was located in the right side. Twenty-six patients presented with a palpable mass. Of the 28 cases, 26 (93%) presented mammographically-detected lesions such as calcification, mass, or architectural distortion. Twenty-two cases (78%) showed a microcalcification with or without other associated abnormalities. Seven were found by screening examination (25%), but no syndrome. Five of the 28 (18%) had a discharge from the nipple. Only one had a family history of breast carcinoma. Twelve were treated with mastectomy, eight with quadrantectomy, and eight had received a partial mastectomy. There were eight patients who had initially received a lumpectomy, and also received further surgery because of positive margins. These included mastectomy ($n = 1$), partial mastectomy ($n = 3$) and quadrantectomy ($n = 4$). Tumors ranged in size from 0.5 to 6 cm, with a mean of 2.7 cm. One patient had Paget's disease.

Histopathological features

Sixteen patients had a Grade 3 nuclei of DCIS, nine had a Grade 2, and three had a Grade 1 tumor. Thirteen tumors had pure comedo patterns, three had solid and comedo growth patterns, four had solid patterns, four had cribriform patterns, one had papillary patterns, one had papillary and solid growth patterns, one had micropapillary patterns, and one had micropapillary and cribriform patterns. According to the criteria of Moriya and Silverberg,[9,12] high grade DCIS and comedo DCIS were 57.1% and 46.4%, respectively. Twenty-four patients (85.7%) demonstrated necrosis within DCIS at least focally. Using the Van Nuys system, there were 16 cases of high nuclear grade (Group 3), seven cases of non-high nuclear grade with necrosis (Group 2), and five cases of non-high nuclear grade without necrosis (Group 1).

The foci of microinvasion consisted of irregular clusters or a small aggregate of malignant cells, or isolated tumor cells, devoid of a surrounding myoepithelial cell layer (Figs 1,2). In some cases, the microinvasive foci appeared as a tongue-like projection of tumor through the basement membrane of a duct of DCIS, maintaining continuity with the *in situ* carcinoma (Fig. 3). The degree of nuclear atypia in microinvasive focus was generally the same with that of adjacent DCIS. Seven of the tumors had only one focus of microinvasion, and 21 had multiple foci. The number of microinvasions ranged from 1 to 7, with a mean of 3.

Periductal inflammation was present in 21 of the 28 (75%) patients, and periductal stromal reactions were found in 20 of the 28 (71.5%) patients (Fig. 4). Microinvasion associated with a lymphocytic infiltration was seen in 22 cases (78.6%; Fig. 4).

The mean number of lymph nodes examined per case was 12 (range 0–25), and no lymph node metastasis was found.

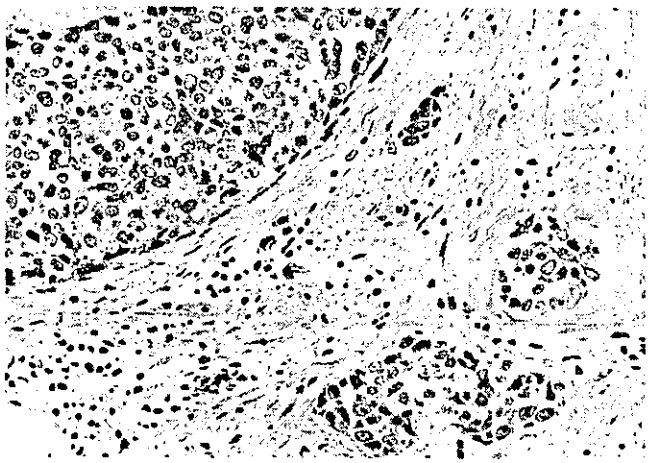

Figure 1 The focus of microinvasion showing irregular clusters or a small aggregate of malignant cells. HE, ×200.

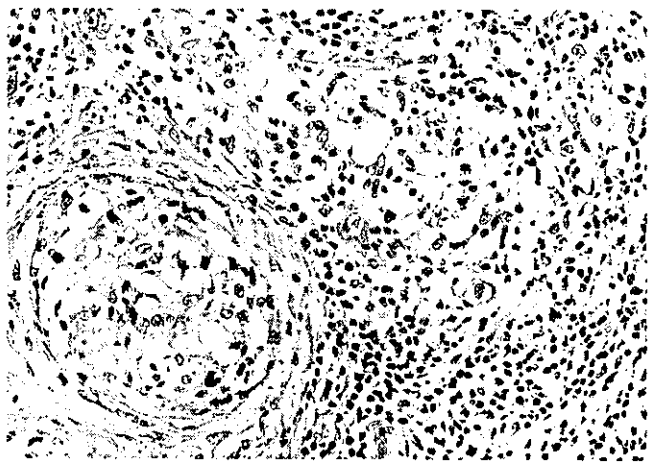

Figure 2 The focus of microinvasion showing isolated carcinoma cells within the stroma. Note chronic inflammation within the focus of microinvasion. HE, ×200.

Immunohistochemical features

Expression of ER, PR HER-2, p53, and Ki-67 (Fig. 5) in the DCIS area (but inexclusively similar in microinvasive focus) differed among the subgroups according to the Van Nuys system shown in Table 2.

In the present study, the positive expressions of ER, PR, p53 and HER-2 were 17 (60.7%), 16 (57.1%), 10 (35.7%) and 15 (53.6%), respectively. The mean LI of Ki-67 was 13.9% in all cases, 19.3% in the high grade group, and 7.8% in the non-high grade group (Fig. 5). HER-2, p53 positive expression (Figs 6,7), and Ki-67 high expression were seen in the high nuclear grade group and less frequently in the group with non-high nuclear grade. The expression of ER and PR showed an opposite trend, being higher in the group with non-high nuclear grade without necrosis. The comedo type showed a high expression of p53, HER-2 and Ki-67 and low expression of ER and PR compared with the non-comedo type ($P < 0.01$).

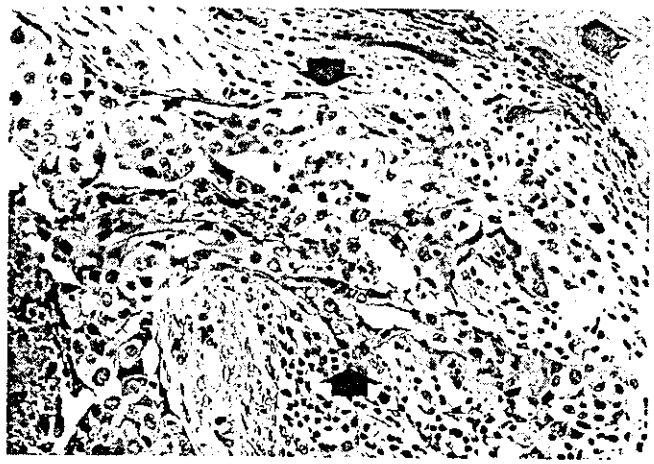

Figure 3 The focus of microinvasion showing a tongue-like projection of tumor through the basement membrane of a duct of DCIS. Note disruption of the ordinal duct profile (arrows). HE, ×200.

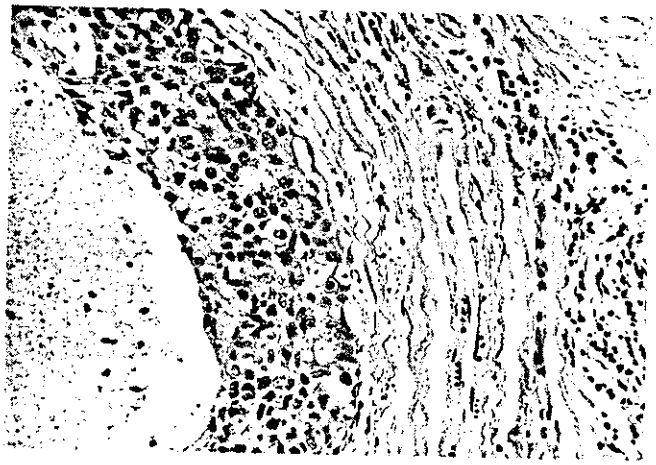

Figure 4 Periductal concentric fibrosis and chronic inflammatory cell infiltrates in DCIS with high grade nuclei. HE, ×200.

DISCUSSION

Over the past two decades, the widespread use of mammographic screening for breast carcinoma has increased the incidence of DCIS, as well as increased the detection of earlier stage invasive carcinoma, including microinvasive carcinoma.

In the present study, using the latest definition of microinvasive carcinoma, we have characterized the clinicopathological features and immunohistochemical features associated with this lesion, which is a rare disease. The incidence of microinvasive carcinoma of the breast has been reported as less than 1% of all breast cancers,[4,13] 1.06% of infiltrating carcinoma, and 5.1% of *in situ* carcinoma.[13] Our incidence, 2.3% of primary breast carcinomas and about one sixth of DCIS, was higher than in previous reports. This

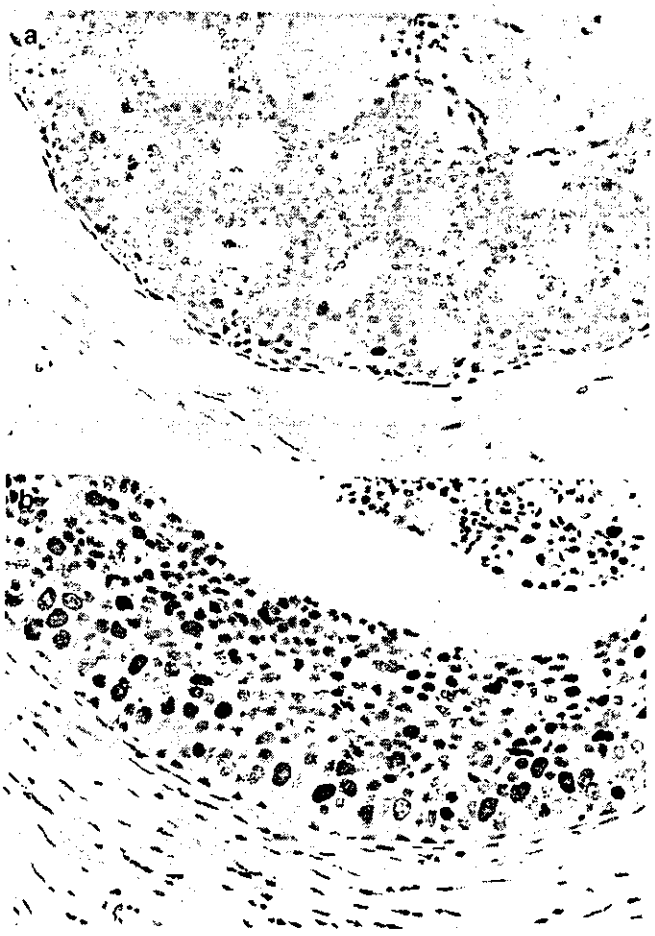

Figure 5 Ki-67 immunohistochemistry in (**a**) low grade and (**b**) high grade DCIS. ABC, ×200.

Table 2 Tumor immunohistochemical characteristics according to Van Nuys Classification ($n = 28$)

	Van Nuys Group 1 Non-high grade DCIS without necrosis	Van Nuys Group 2 Non-high grade DCIS with necrosis	Van Nuys Group 3 High grade DCIS	*P*-value Group 1 + Group 2 versus Group 3
No. patients	5 (17.9%)	7 (25%)	16 (57.1%)	
ER				
Positive	5 (100%)	6 (85.7%)	4 (25%)	
Negative	0 (0%)	1 (14.3%)	12 (75%)	<0.01
PR				
Positive	5 (100%)	6 (85.7%)	6 (37.5%)	
Negative	0 (0%)	1 (14.3%)	10 (62.5%)	<0.01
HER-2				
Positive	1 (20%)	1 (14.3%)	13 (81.2%)	
Negative	4 (80%)	6 (85.7%)	3 (18.8%)	<0.01
p53				
Positive	1 (20%)	0 (0%)	10 (62.5%)	
Negative	4 (80%)	7 (100%)	6 (37.5%)	<0.01
Ki-67	4.78%	9.58%	19.31%	<0.01

DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor.

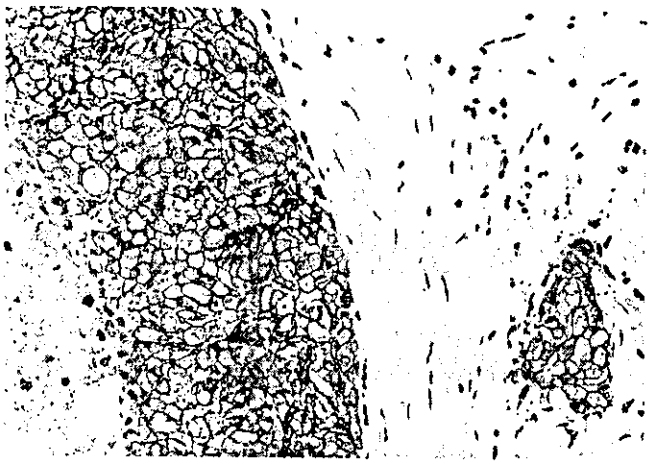

Figure 6 HER-2 overexpression in high grade DCIS. ABC, x200.

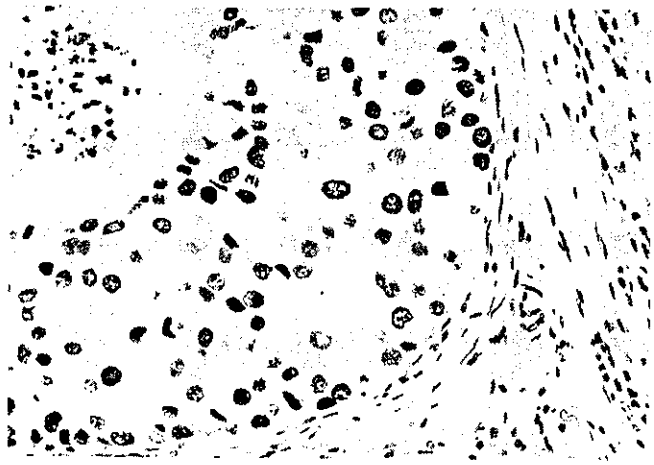

Figure 7 p53 positivity in high grade DCIS. ABC, x200.

seems to be due to more precise analysis, because the pathological analysis was done by 3–5 mm-thick serial sections for all of the lesions. The data was taken from three institutes and depicts the real incidence in Japanese women.

The majority of patients in the current study were discovered on routine mammograms, and 22 of the 28 (78.6%) patients had abnormal calcifications. The studies by Prasad *et al.*[13] on 21 cases of microinvasive carcinoma of the breast showed that 60.9% of patients were discovered on routine mammograms, and all but one patient had abnormal calcifications, which was similar to our results. This points to the seminal role of the mammogram in the detection of microinvasion.[14]

The mean age of the patients in our study was 48.8 years, which was younger than the others with 56.4–60.9 years,[4,5,13] suggesting that we should pay attention to younger patients who were diagnosed as DCIS, whether or not there is a stromal microinvasion.

Silverstein and Lagios[15] reported that patients of DCIS with microinvasion tended to have palpable lesions and involved margins after their initial markers, a reflection of the fact that DCIS with microinvasion are larger than pure DCIS without microinvasion. Our series showed 26 of the 28 cases had a palpable mass, and eight patients had an involved margin after the initial operation. The *in situ* component of a microinvasive lesion is often large, averaging 38 mm in Silverstein's report.[15] In the studies by Lagios *et al.*, the average *in situ* component of a microinvasive lesion was greater than 50 mm in diameter.[16] Our results showed that the average diameter of the DCIS component was 27 mm, less than that of Silverstein[15] and Lagios *et al.*,[16] but more than Prasad *et al.*'s[17] result in which the mean size of the associated DCIS was 13 mm. We found that seven patients had one focus of microinvasion and 21 patients had multiple foci (range 1–7, mean 3), suggesting that microinvasion tended to be multifocal.

Several authors reported[3–5,18,19] that high grade nuclei and comedo carcinoma were the most frequent histological subtype of DCIS associated with microinvasion. In the present study, high grade nuclei and comedo DCIS accounted for 57.1% and 46.4%, respectively. Comparable findings have been reported by others, with comedo DCIS comprising 66.7–81.6%. Our results were less than that of others, perhaps because the criteria of classification subtypes we used had comedo subtype as DCIS with more than 70% of the duct profiles showing comedonecrosis, solid cell nests and predominant high-grade nuclei.[12] In any way, the incidence of comedo/high nuclear grade DCIS lesions is seen more frequently in T1mic than pure DCIS. Papillary DCIS has accounted for 6.2–18.4% of reported microinvasive carcinomas,[3–5,17] compared with 14.3% in our study. In the current study, 20 of the 28 patients showed periductal fibrosis, and 21 of the 28 patients showed inflammaotory cell responses surrounding DCIS.

It has been reported that microinvasion is more often associated with high grade and comedo subtype intraductal carcinoma, but it may occur in other types of intraductal carcinoma, such as cribriform, papillary, micropapillary, and solid types,[20,21] and low nuclear grade does not provide protection against invasion.[13] In the present study, we found that high grade nuclear and comedo were the most common subtype, but three of the 28 (10.7%) were low grade, and 15 (53.6%) were non-comedo subtypes. These findings may suggest that high grade and comedo DCIS were more aggressive, but non-high grade or comedo DCIS can probably become invasive directly and does not need to evolve through high grade or comedo carcinoma. It is probably true that the characteristics of invasive carcinoma are determined in the preinvasive stage, with low-nuclear grade DCIS leading to well-differentiated infiltrating ductal carcinomas and high grade DCIS leading to poorly differentiated infiltrating ductal carcinomas.[13,22]

There has been considerable interest in the role of the oncoprotein HER-2 and the tumor suppressor protein p53 in the early stage of breast cancer. In the current study, the percentages of positive expression of HER-2 and p53 were 53.6% and 35.7% in all cases, and 80% and 60% in the high nuclear grade group. We also measured tumor cell proliferation in DCIS accompanying microinvasion by calculating the LI using Ki-67 immunostaining. We observed that high grade nuclei significantly associated with a higher mean LI (19.3%), while non-high grade nuclei associated with a lower mean LI (7.8%). The positive expression of ER and PR strongly related to low grade nuclear and non-comedo subtype. This all suggests that high grade DCIS is more aggressive, and HER-2 and p53 expression may play a role in the breast cancer invasion process.

The surgical management of microinvasive carcinoma, particularly the management of the axilla, has been controversial. Some believe that axillary lymph node dissection is not necessary for DCIS with microinvasion because it is generally not associated with lymph node metastasis and has a favorable natural history.[23,24] Other groups report rates of lymph node metastasis ranging from 2.7% to 20% and advocate complete axillary lymph node resection.[2,16,25] None of our cases were associated with lymph node metastases, indicating this lesion might have a favorable prognosis.

Silverstein recommended that treatment of the breast for both DCIS and DCIS with microinvasion should be similar, and should be based on the size of the DCIS component relative to breast size, margin status, the ability to follow the patient mammographically, and patient preferences.[15] However, the local recurrence-free survival was greater for patients with pure DCIS when compared to DCIS with microinvasion, although the difference was not statistically significant.[15] Thus, it is suggested that DCIS with microinvasion should be treated with postoperative radiation therapy if the patient elects breast conservation.

The current study described the clinicopathologic profiles and biological features of 28 cases of T1mic. Further study should explore more cases and long-term follow-up of patients with T1mic of the breast.

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Dossier: Breast cancers

Intracrine mechanism of estrogen synthesis in breast cancer

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Abstract

It has been demonstrated that biologically active estrogens are locally produced from circulating inactive steroids in an intracrine mechanism in the breast carcinoma. The in situ production of estrogens is considered to play an important role in the proliferation of breast cancer cells, especially in the postmenopausal women. Therefore, the total blockade of this pathway may lead to an improvement in the prognosis in breast cancer patients due to the inhibition of estrogenic actions. In this review, we describe the recent studies of enzymes related to intracrine mechanism of estrogen synthesis, including aromatase, steroid sulfatase (STS), and 17β-hydroxysteroid dehydrogenase, in human breast carcinoma tissues, and discuss the biological significance of local production of estrogens in human breast cancer.

Keywords: Breast carcinoma; Estrogen; Intracrine

1. Introduction

Estrogens are well known to contribute immensely to the development of hormone-dependent breast carcinomas. However, there is no consistent evidence of increased serum estrogen concentrations or other systemic estrogen abnormalities in women with breast cancer. Miller et al. [11] have shown that tissue concentrations of bioactive estrogen estradiol were more than 10-times higher in breast carcinomas than in plasma, and demonstrated that human breast neoplasms can produce estradiol in vitro [11]. Inactive steroids in plasma are locally converted to the bioactive estrogens in the breast carcinoma, where biosynthesis takes place without release into the extracellular space, as "intracrine activity". Intratumoral production of estrogens plays an important role in the proliferation of breast cancer cells, especially in postmenopausal women, and it is suggested that the blockade of this pathway may inhibit the growth of breast tumors.

2. Intracrine mechanism of estrogen synthesis in human breast carcinoma

The intracrine mechanism of estrogen production in human breast carcinoma tissues, which have been proposed by previous investigators, is summarized in Fig. 1. High concentrations of circulating inactive steroids, including androstenedione and estrone sulfate, are considered to be major precursor substrates of local estrogen production [9,21]. Androstenedione is inactive androgen secreted from the adrenal cortex or gonads, and it is catalyzed into estrone by aromatase (*CYP19* gene) [11,23]. On the other hand, estrone sulfate is a major circulating form of plasma estrogen, and is hydrolyzed to estrone by steroid sulfatase (STS) [4,15]. Estrone is subsequently converted to potent estrogen estradiol by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD type 1), and acts on breast cancer cells through estrogen receptor (ER) α and/or β. Therefore, it is very important to examine these enzymes in human breast carcinoma to obtain

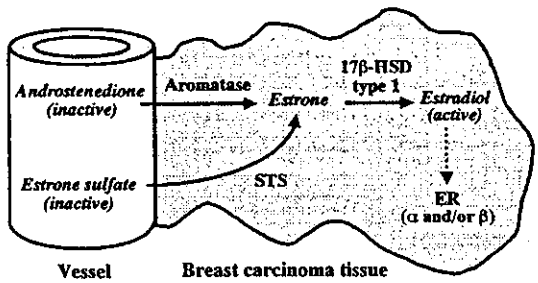

Fig. 1. Scheme representing intracrine mechanism of estrogen synthesis in human breast cancer. STS, steroid sulfatase; 17β-HSD type 1, 17β-hydroxysteroid dehydrogenase type 1; and ER, estrogen receptor.

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a better understanding of the local regulation of estrogenic actions in the breast cancer.

2.1. Aromatase

Aromatase is a key enzyme in the estrogen synthesis, mainly aromatization of androgen androstenedione to estrone. More than 70% of breast carcinoma specimens had aromatase activity comparable with or greater than, that found in other tissues, and mRNA level of aromatase in the breast carcinomas, was significantly increased compared with that in non-malignant tissues [10]. Aromatase immunoreactivity was reported in stromal cells and adipocytes adjacent to the carcinoma [24], suggesting the relationship between aromatase over-expression and carcinoma invasion. The correlation between aromatase expression and clinicopathological parameters remains unclear [8,24].

The increased aromatase expression is associated with a switch in promoter usage from the normal adipose-specific promoter I.4 to the cAMP-responsive promoter II [1,7]. Very recently, Clyne et al. [3] have reported the expression of liver receptor homologue-1 (LRH-1), which is a nuclear receptor that activates transcription of various steroidogenic genes, in breast carcinoma tissues, and LRH-1 activated the promoter II of aromatase in 3T3L1 preadipocyte cells [3]. Aromatase expression was also induced by cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) α, and prostaglandin E_2 [18,23].

2.2. STS

STS is a single enzyme, that hydrolyzes several sulfated steroids such as estrone sulfate, dehydroepiandrosterone (DHEA) sulfate, and cholesterol sulfate [2,5]. Estrone sulfate is quantitatively the most important form of circulating estrogen in both cycling and postmenopausal women. Estrone sulfate has a relatively long half-life in the peripheral blood, where serum levels of estrone sulfate are known to be 10-fold higher than those of unconjugated estrogens. STS catalyzes estrone sulfate to estrone in breast carcinoma, which contributes to local estrogen production. The enzymatic activity of STS has been reported to be higher in breast cancer tissues, than that in normal breast tissues [6,22]. STS immunoreactivity was detected in carcinoma cells in 70–90% of breast carcinoma cases [20,29], and its immunoreactivity was positively correlated with tumor size and was significantly associated with an increased risk of recurrence [29]. In addition, STS inhibitors have been demonstrated to be effective for depressing the proliferation of estrogen-dependent MCF-7 cells when estrone sulfate was the source of estrogen [25]. No significant correlation has been reported between STS immunoreactivity and ER status in breast cancer tissues [29].

In contrast to the aromatase, little is known about the regulatory mechanism of STS expression and activity. In breast cancer cells, IL-6 and TNFα stimulated STS activity and acted synergistically to increase enzyme activity, possibly via a post-transcriptional modification of the enzyme [13]. There is also evidence that progestins may inhibit the expression and activity of STS in breast cancer cells [16].

2.3. 17β-HSD type 1

17β-HSD catalyzes the reversible interconversion of estrogens or androgens. To date, 11 isozymes of 17β-HSD have been cloned, and type 1 17β-HSD is considered to play an important role in the conversion of estrone to potent estrogen estradiol. Immunoreactivity of 17β-HSD type 1 was detected in carcinoma cells in about 60% of invasive ductal carcinoma cases [17,26], and the mRNA levels of 17β-HSD type 1 and intratumoral estradiol/estrone ratios were significantly higher in postmenopausal than premenopausal breast cancers [12]. 17β-HSD type 1 immunoreactivity was significantly correlated with ERα and progesterone receptor (PR), suggesting that estradiol, synthesized by 17β-HSD type 1 in carcinoma cells, acts on these cells locally in breast carcinomas [26].

The expression of 17β-HSD type 1 mRNA was induced by retinoic acid in T-47D breast cancer cells [19]. Retinoic acid receptor (RAR) α and retinoid X receptor (RXR) α are localized in carcinoma cells in 80% of breast carcinomas [28], and significant correlation was detected between RARα LI and 17β-HSD type 1 immunoreactivity, and between RARα or RXRα LI and ERα LI [28].

3. Regulation of local estrogen synthesis in breast carcinomas as an endocrine therapy

The biological effects of estrogens are mediated through ER, a member of the superfamily of nuclear receptors. Therefore, antiestrogens, which block ER have been used as endocrine therapies in breast carcinoma for many years. Tamoxifen has been administered to patients with breast cancer, which has resulted in a 30–35% reduction in clinical symptoms, and a 20–25% reduction in mortality [14]. Recent investigations have demonstrated the importance of intracrine mechanism of estrogen production in breast carcinomas, and it is suggested that the inhibition of this pathway could be clinically useful for reducing the progression of breast tumors in postmenopausal women. Aromatase inhibitors have been used in breast carcinoma as an endocrine therapy, however, even complete regulation of aromatase does not fully decrease the estrogen levels of either tumor or plasma in breast cancer patients. This may be partly due to the involvement of other enzymes such as STS and/or 17β-HSD type 1. No association was detected among the expression of aromatase, STS, and 17β-HSD type 1 in breast carcinoma tissues [27], which suggests that inhibition of STS and/or 17β-HSD type 1 may also have possible important therapeutic potential in addition to the aromatase in the breast cancer patients.

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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′-TGAC AGAGAATCAGCTTCTGGGG-3′ for BR2a, 5′-TCTT CTGTGAAAAGAAGCTGTTCAC-3′ and 5′-CCCGCT AGCTGTATGAAAACCC-3′ for BR2b, 5′-AGAAAGA ACAAAATGGACATTCTAAG-3′ and 5′-TTGTTGAA ATTGAGAGAGATATGGAG-3′ for BR2c, 5′-TCTGA GCATAGTCTTCACTATTCACCTAC-3′ and 5′-TCTT AAGAGGGGAGGATCTAACTGG-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 *PstI*/*XbaI*, *XbaI*/*SpeI*, *SpeI*/*PstI*, *PstI*/*SpeI*, *SpeI*/*NsiI*, and *SpeI*, 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 (~200ng) was cotransformed with the corresponding linearized gap vector (~30ng) 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

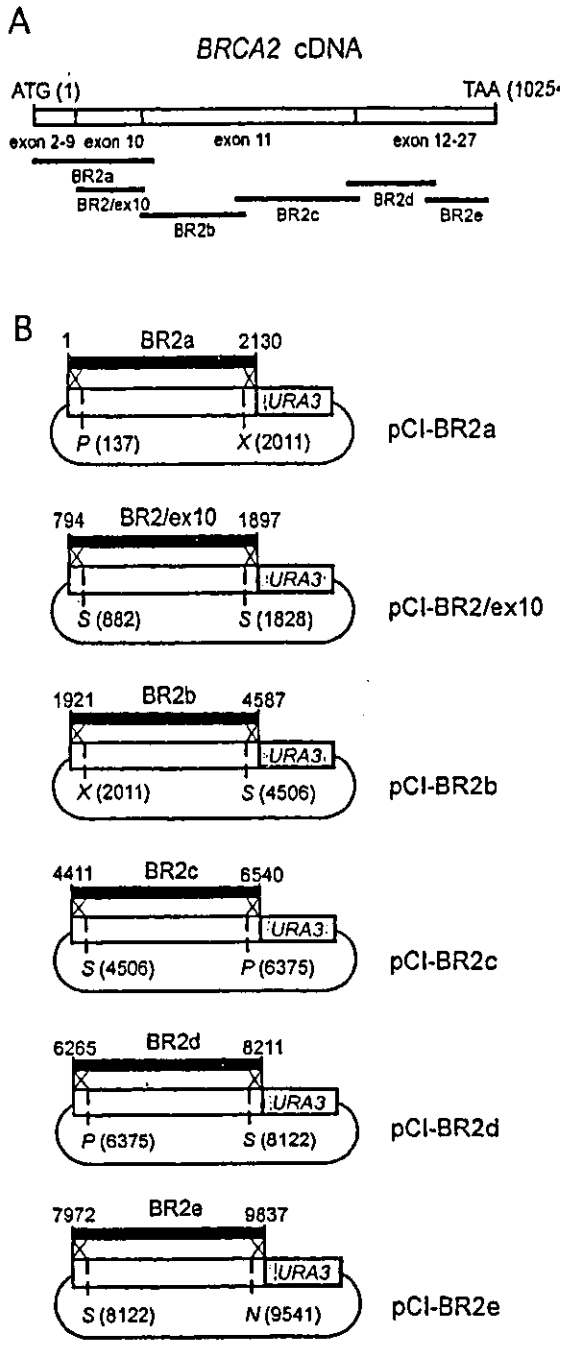

Fig. 1A,B. Schematic representation of the stop codon (SC) assay of the *BRCA2* gene. **A** Sequences of *BRCA2* cDNA and six polymerase chain reaction (PCR) fragments were chosen for the SC assay. **B** Plasmids for the SC assay of the *BRCA2* gene and the corresponding PCR fragments. cDNA (BR2a, BR2d, 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*, *PstI*; *X*, *XhoI*; *S*, *SpeI*; *N*, *NsiI*