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ORIGINAL ARTICLE

Clinicopathological analyses of triple negative breast cancer using surveillance data from the Registration Committee of the Japanese Breast Cancer Society

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Abstract
Background Triple negative (TN) breast cancer is defined as a subtype that is negative for estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). To clarify the characteristics of TN breast cancer, surveillance data of the Registration Committee of the Japanese Breast Cancer Society were analyzed.
Method Of 14,748 cases registered in 2004, 11,705 (79.4%) were examined for ER, PgR, and HER2. Of these, the most prevalent (53.8%) was a hormone-responsive subtype with ER positive/PgR positive/HER2 negative, followed by TN subtype (15.5%).
Results The proportion of postmenopausal patients was relatively high in the TN subtype. This cancer was diagnosed at a slightly advanced stage and with more cases positive for lymph node metastases than other subtypes. Morphologically, the TN subtype was more frequently classified as solid-tubular carcinoma. Mucinous, tubular, or secretary carcinomas were frequently found in the hormone receptor positive/HER2 negative subtype, while squamous cell carcinoma, spindle cell carcinoma, and metaplastic

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carcinoma with bone/cartilage metaplasia were very frequently found in the TN group. Apocrine carcinoma was also found very frequently in the TN group. Selection of chemotherapy was not based on receptor subtypes, but was determined by the degree of tumor progression.

Conclusions Although TN types are similar to basal-like breast tumor, as determined by gene profiling, their diagnosis needs verification by determination of the level of epidermal growth factor receptor or cytokeratin 5/6 expression. TN type should be examined further for immunohistochemical features and analyzed for prognostic details in this cohort.

Keywords Triple negative tumor · Breast cancer · Surveillance data

Introduction

Triple negative (TN) breast cancer represents a subtype that is negative for the three main prognostic/predictive receptors for breast cancer, namely, estrogen receptor (ER), progesterone receptor (PgR), and HER2 (human epidermal growth factor receptor type 2) [1]. ER and/or PgR positive cancer, which means hormone receptor (HR)-positive cancer, usually responds to endocrine therapy. Cancers scored immunohistochemically as 3+ or 2+ and that are 'fluorescence in situ hybridization' (FISH)-positive are regarded as HER2-positive and are targets for treatment with trastuzumab and other agents aimed at HER2. However, currently no targeted therapeutic agents have been identified specifically for TN breast cancer, and the only option at present is conventional systemic chemotherapy. In this context, it is essential to be familiar with the biological features of TN breast cancer in order to develop the best therapeutic strategy [1–3].

An alternative approach to subtyping breast cancers has been developed by Sørlie et al. [4, 5], who classified breast cancer into four or more intrinsic subtypes on the basis of gene profiling acquired from microarray analyses of a large number of breast cancer tissue specimens. In their classification, the first was called a basal-like subtype; it shared some characteristics with basement membrane cells and had a high proliferative capability [6]. The second was the HER2 (ErbB2) subtype, in which HER2 and related genes were overexpressed and ER-related genes were under expressed. This subtype was also relatively highly proliferative and expected to respond to trastuzumab. The third subtype had normal epithelium (normal-like subtype), but its other significant features have yet to be established. The fourth was called the luminal subtype, which expressed various amounts of ER-related genes that could be further subclassified into luminal A or B. If a connection can be found between the intrinsic subtypes and 'classic' breast cancer subtypes based on receptor status, the correlation is best understood by contrasting the basal-like subtype to TN breast cancer, as the former is positive for cytokeratin 5/6 or epidermal growth factor receptor (EGFR) (HER1).

The basal-like subtype accounts for 15–20% of breast cancers, irrespective of the method of analysis or ethnic group [6]. However, premenopausal African–American patients have a significantly higher incidence of this subtype compared to other patients [7]. It is well known that the pathological and biological characteristics of breast cancer are significantly worse in young African–American patients and that they show a clinically poor prognosis. In contrast, the basal-like subtype is relatively uncommon in breast cancers diagnosed in Japanese women; in 793 breast cancer patients, only 8% were this genetic subtype [8]. A significant overlap has been repeatedly demonstrated between the biological and clinical characteristics of sporadic TN breast cancers and basal-like subtypes, and breast carcinomas arising in BRCA1 mutation carriers [5].

In the receptor subtype determination, there is an ongoing debate on how to determine what to take as the cutoff value for deciding the positive/negative expression levels of hormone receptors. For example, there is no agreement at present on whether 'negative' should be based on: (1) no expression, (2) a score of 0 or 2 on the Allred Score [9], which takes into account the number of positive cells and the intensity of staining for the receptor in question, or (3) the proportion of receptor-positive cells less than 10% [10].

The purpose of this study was to disclose clinicopathological features of TN breast cancer. With the support of the Registration Committee of the Japanese Breast Cancer Society, we analyzed about 11,000 cases registered in 2004 in order to classify them by receptor subtypes based on expression levels of ER/PgR/HER2 and to analyze the clinicopathological characteristics of TN tumors.

Materials and methods

Basic data of patients

Comprehensive data on breast cancer patients diagnosed in Japan in 2004 were registered by the Registration Committee of the Japanese Breast Cancer Society, who reported the final registry data in 2008, although patient outcome data have not been published yet. The registrations were made by 352 institutions and included 14,749 cases. The data collected were: age, clinicopathological features of the tumor including size, presence of lymph node metastases, and receptor status (ER, PgR, and HER2), surgical techniques, and regimens of chemotherapy.

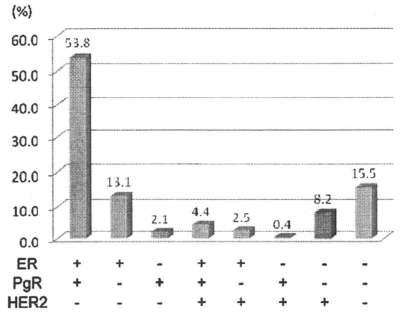

Fig. 1 Breast cancer surveillance data reported by the Japanese Breast Cancer Society

Individual participating institutions determined ER, PgR, and HER2 status by their own in-house method, as well as the other criteria for the registration. In 2004 the status of ER and PgR was being determined by the immunohistochemical (IHC) technique using monoclonal antibodies. Additionally, the cutoff level was mainly adopted to a score of between 2 and 3 on the Allred Score [9], or 10% as a staining proportion [10]. Tumors that were immunohistochemically scored as 3+, or scored 2+ with FISH-positive, were regarded as HER2-positive in a majority of individual participating institutions.

Subanalysis of receptors

Subanalysis was performed by permission of the Registration Committee and the Board of the Japanese Breast Cancer Society. Status of ER, PgR, and Her2 had been determined in 11,705 cases (79.4% of all registered cases), of which 1,819 cases (15.5%) were registered as negative for any one of ER/PgR/HER2. The most prevalent subtype was ER+/PgR+/HER2− (53.8%), followed by TN breast cancer (15.5%) (Fig. 1).

Receptor subtypes were divided according to their ER/PgR/HER2 profiles: the HR+/HER2− subtype was positive for ER and/or PgR and negative for HER2; the HR+/HER2+ subtype was positive for ER and/or PgR and positive for HER2; the HR−/HER2+ subtype was negative for both ER and PgR and positive for HER2; the triple negative (TN) subtype was negative for all three receptors, ER, PgR, and HER2 (Table 1)

Statistical processing

Fischer's exact test was used to compare various prevalence rates among the groups. Unpaired t test was employed to make inter-group comparisons in the number of cases and mean values. A significance level was set at less than 0.01 when multiple comparisons were required between four groups.

Table 1 Receptor subtype and status

Subtype	ER/PgR/HER2 status	
	Receptor profile	ER/PgR/HER2
HR+/HER2−	ER+ and/or PgR+, HER2−	+/+/−, +/−/−, −/+/−
HR+/HER2+	ER+ and/or PgR+, HER2+	+/+/+, +/−/+, −/+/+
HR−/HER2+	ER− and PgR−, HER2+	−/−/+
Triple negative	ER−, PgR− and HER2−	−/−/−

+, Positive; −, negative

Results

Patient backgrounds

The relative proportions of the four cancer subtypes were: HR+/HER2− subtype, 68.7%; HR+/HER2+ subtype, 7.6%; HR−/HER2 subtype, 8.3%; and TN subtype, 15.4%. There was no difference in mean age between the groups. The incidence of bilateral breast cancer was significantly lower in HER2-positive subtypes than in HER2-negative subtypes ($P = 0.040$). The proportion of premenopausal patients was significantly greater in HR-positive groups (i.e., HR+/HER2− and HR+/HER2+ subtypes) than in the HR-negative groups (i.e., HR−/HER2+ or TN subtype) ($P < 0.001$). There were no significant differences between subtypes with respect of family history of breast cancer, height, body weight, or body mass index (BMI) (Table 2). Regarding disease stage, 37.2% of the HR+/HER2− subtype were diagnosed at stage I, indicating relatively early initiation of therapy, whereas the prevalence of stage I at diagnosis in HER2 was only 14.2%, which meant these patients received their first treatment at the slightly advanced stages of II–IV (Fig. 2).

Clinical findings

HR+/HER2− subtype was detected at an earlier stage than the other subtypes, that is, when the tumor was somewhat smaller in diameter, and compared advantageously in the incidence of node metastases especially with the ER−/HER2+ subtype. There was a tendency for the incidence of distant metastases to be higher in the HER2-positive groups (i.e., ER+/HER2+ and ER−/HER2+ subtypes) and for breast-conserving therapy to be less frequently performed in patients with the HR−/HER2+ subtype (Table 2).

Table 2 Patient background and clinicopathological data

	Receptor subtype			
	HR+/HER2−	HR+/HER2+	HER2	TN
Number of patients (%)	8,039 (68.7)	892 (7.6)	977 (8.3)	1,797 (15.4)
Age median (range)	56 (NR–100)	54 (23–93)	56 (22–95)	57.5 (NR–94)
Ratio of bilateral breast cancer (%)	6.6[b]	5.9[a]	4.8[a]	6.2[b]
Incidence of breast cancer family history (%)	8.6	8.4	24.1	28.1
Ratio of premenopausal patients (%)	37.1[c]	38.8[c]	24.1[d]	28.1[d]
Height (cm) mean ± SD	154.3 ± 6.3	154.9 ± 6.1	154.0 ± 6.2	153.8 ± 6.3
Weight (kg) mean ± SD	54.7 ± 9.0	54.5 ± 8.8	53.9 ± 8.6	54.2 ± 9.0
BMI mean ± SD	23.0 ± 3.7	22.7 ± 3.5	22.7 ± 3.3	22.9 ± 3.5
Tumor size (cm) mean ± SD	2.6 ± 2.1[e]	3.2 ± 2.2	3.5 ± 2.6[f]	3.4 ± 2.7
Incidence of positive lymph node involvement (%)	20.6	34.9	38.5	32.2
Incidence of distant metastasis (%)	2.5	5.6	5.6	3.2
Incidence of breast-conserving surgery (%)	53.9	41.3	35.1	45.0

NR no record

[a] HER2 positive versus [b]HER2 negative according to ratio of bilateral breast cancer; $P = 0.040$, Fisher's exact probability test

[c] Hormone receptor-positive group versus [d]hormone receptor-negative group according to the ratio of premenopausal patients; $P < 0.0001$, Fisher's exact probability test

[e] HR+/HER2− subtype versus [f]TN subtype according to tumor size; $P < 0.00001$, standard t test of mean and standard deviation (SD)

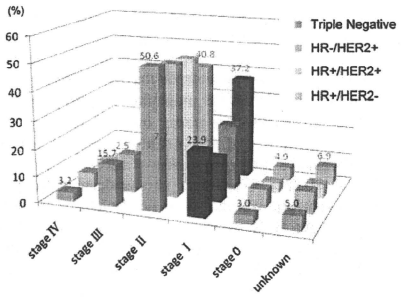

Fig. 2 Stage at diagnosis by receptor subtype

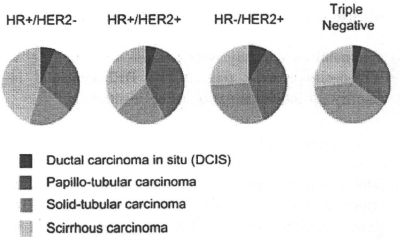

Fig. 3 DCIS and histological subtypes of invasive ductal carcinoma (IDC) by receptor subtype

Pathological findings

From a viewpoint of morphologic classification, whereas scirrhous carcinoma was most frequently found in ER+/HER2− subtype, solid-tubular carcinoma prevailed in TN breast cancer (Fig. 3). As to breast cancers of special types, mucinous carcinoma occurred rarely in the TN group, but was quite frequent among ER+/HER2− subtype patients (Fig. 4a). Invasive lobular carcinoma was found reasonably frequently in the HR+/HER2− type (Fig. 4b). Tubular and secretary carcinomas were mostly found in patients with a HR+/HER2− subtype (Fig. 4c).

Medullary carcinomas were observed frequently in the TN group (Fig. 4d). Squamous cell carcinoma, spindle cell carcinoma, or metaplastic carcinoma with bone/cartilage metaplasia was likewise very common in patients in the TN subtype (Fig. 4e). This group also included the highest percentages of apocrine carcinomas (Fig. 4f).

Selection of chemotherapeutic regimens

Two main chemotherapeutic regimens were administered: (1) anthracycline-containing regimens (ACR), which included: doxorubicin plus cyclophosphamide (AC), epirubicin plus C (EC), C plus A plus 5-fluorouracil (CAF) and CEF, and (2) taxane (paclitaxel or docetaxel)-containing

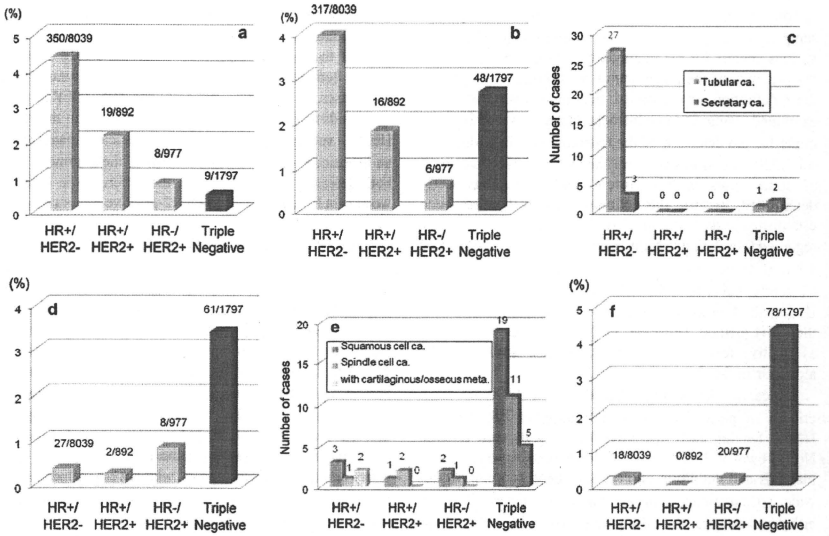

Fig. 4 **a** Incidence of mucinous carcinoma by receptor subtype. **b** Incidence of invasive lobular carcinoma by receptor subtype. **c** Incidence of tubular or secretary carcinomas by receptor subtype. **d** Incidence of medullary carcinoma by receptor subtype. **e** Incidence of metastatic carcinoma by receptor subtype. **f** Incidence of apocrine carcinoma by receptor subtype

Table 3 Chemotherapy according to receptor subtype

	Subtype			
	HR+/HER2−	HR+/HER2+	HR−/HER2+	TN
Number of cases	8,039	892	977	1,797
Node positive cases (%)	20.6	34.9	38.5	32.2
Number of patients treated by chemotherapy	3,913	696	940	1,563
Incidence of patients treated by ACR (%)	28.3	45.0	53.7	49.5
Incidence of patients treated by taxanes (%)	16.9	29.9	36.7	29.2
Incidence of neoadjuvant chemotherapy (%)	25.0	33.9	27.2	25.0

ACR anthracycline-containing regimen

regimens. In one patient there was a possibility that these two main regimens might have been administered concomitantly. ACR was administered to 28.3% of HR+/HER2− subtype tumors and taxane-based regimens to 16.9%. However, the incidence of axillary lymph nodes metastases was 20.6% in this subtype, which was smaller compared to other subtypes. In the other three subtype groups, as shown in Table 3, patients invariably received ACR or taxane regimens. Although the incidence of neoadjuvant chemotherapy was almost identical in each subtype group, the HR+/HER2+ group tended to be treated with this type of chemotherapy a little more frequently (Table 3).

Discussion

Of 14,749 breast cancer cases in Japan in 2004, 11,705 (79.4%) were examined for their ER, PgR, and HER2 status. TN tumors, defined as negative for all three receptors, accounted for 15.5% (1,819 cases). This was the largest collection of data on the prevalence rate of TN

tumors in Japan and was gathered from a large patient sample. Although there were no restrictions placed on the various centers for the methods they used for determining the presence of the receptors, or the criteria employed for their definition, we assumed that most cases had been examined using immunohistochemistry, since this technique for the detection of the receptors was in widespread use in 2004. It is possible that the hormone receptor status of some cases in this current study were incorrectly determined, because the definition criteria had not been established at that time in 2004. Most Japanese institutions regarded 0 or 2 on the Allred score as negative; others used a cutoff value of 10% for the determination of ER/PgR. Currently, the criteria for HER2 positivity are: 3+ with the IHC method, or 2+ with the IHC method and positive with the FISH method. However, in 2004, a tumor was defined as positive for HER2 if the IHC method resulted in 3+ alone, or in 2+ 3+.

Hence, in the present analyses, there were no strict criteria in place for the determination of ER, PgR, or HER2, leaving each institution to apply its own criteria. Now, however, it is considered that standardized analytical methods and definition criteria have been adopted nationwide, so that future analyses will be more reliable. Nevertheless, despite this limitation, we consider that the results of this population study are clinically very significant because of the large number of cases (over 11,000) and participating institutions (over 350).

The basal-like subtype accounts for 15–20% of breast cancers, irrespective of the method of analysis or ethnic group [6]. However, premenopausal African–American patients have a significantly higher incidence of this subtype compared to other patients [7, 11]. It is well known that the pathological and biological characteristics of breast cancer are significantly worse in young African–American patients and that they show a clinically poor prognosis. Therefore, the high incidence of basal-like subtype in young African–American patients correlates with the high histological grade of the tumors and the poor prognosis of the disease in this specific patient subgroup. On the other hand, it has been reported that this basal-like subtype is comparatively rare in Japanese women, with an incidence of only 8% documented in a recent study of 793 breast cancer cases in Japan [8]. We were interested in a possible familial nature of TN tumors, because of suggestions of a link to BRCA1 mutation. In the present study, however, we could not find any evidence of a family history of breast cancer in this subtype or that it affected younger women than other subtypes. This may have been due to the fact that various subtypes of breast cancer are included in the definition of 'TN,' although the basal-like subtype is thought to comprise 40–80% of cases [7, 12].

On the other hand, HR+/HER2− subtype tumors tended to be smaller and the patients freer of lymph node metastases at the time of diagnosis, while the HER2 subtypes were often positive for regional or distant lymph node metastases. This finding indicates the HR+/HER2− subtype tends to be detected at an earlier stage than the HR−/HER2+ subtype, which was characteristically diagnosed at an advanced stage. However, even if these tumors were both detected at an early stage, the difference in outcome would not be affected since HER2-positive tumors progress more rapidly.

Regarding histological subtypes, scirrhous carcinoma and solid-tubular carcinoma tended to be found more frequently in the HR+/HER2− and the TN subtypes, respectively. Although invasive lobular carcinoma was also found in the TN type, its true incidence is unclear as it is rarely difficult to distinguish from scirrhous carcinoma. This TN subgroup also included many cases of medullary and metaplastic carcinomas. Spindle cell and squamous cell carcinomas of the TN tumors showed metaplasia derived from invasive ductal carcinoma and exhibited characteristics of basal-like tumors [12]. However, medullary and apocrine carcinomas, which were included in the metaplastic carcinomas, have a better prognosis than the common type and, among TN breast cancers, should be regarded as different from the more common basal-like breast cancer.

There was no apparent correlation between the choice of chemotherapeutic regimen and tumor receptor subtype, both an anthracycline-containing regimen (ACR) and taxanes were used depending on the degree of progression. Neoadjuvant therapy was used in 27.2 and 25% of HR−/HER2+ and TN tumors, respectively, indicating that in 2004 this therapy was being used in large resectable tumors.

In conclusion, we analyzed data from a large number of breast cancer cases registered by the Japanese Breast Cancer Society in order to characterize and advance our understanding of the TN subtype of breast cancer. The present study demonstrated that it was important to establish standard analytical methods and criteria for detection of ER, PgR, and HER2 and that in particular it was necessary to define TN breast cancer more carefully. In the future, we need to follow up the prognosis and response to chemotherapy in these TN breast cancer cases in an attempt to characterize the subtype in more detail [2]. TN breast cancer simulates basal-like tumor, which has been classified from gene profiles. The basal-like type of TN breast cancer is diagnosed by IHC methods based on the expression of EGFR and cytokeratin 5/6. We are looking into further analyzing the cases from the 2004 registry from the perspectives of immunohistochemistry, prognosis, and use of adjuvant chemotherapy.

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CASE REPORT

Intracystic invasive papillary carcinoma of the male breast with analyses of loss of heterozygosity on chromosome 16q

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Abstract A 64-year-old man noticed a right subareolar mass in May 2005. On physical examination, an oval-shaped, well-circumscribedthe tumor (6.0 × 5.5 cm in size) was located just beneath the right nipple. The tumor was elastic, firm and freely movable. Neither axillary nor supraclavicular lymph nodes were palpable. Mammography demonstrated a 5 × 5-cm, relatively distinct and dense mass without microcalcifications or spiculations. There were no findings of concurrent gynecomastia. Ultrasonography revealed a large multilocular cyst with a mural hypoechoic protruding lesion exhibiting wide-based morphology with an irregular margin. On contrast-enhanced computed tomography, the inner lesion enhanced, but direct invasion of the tumor to the major pectoral muscle was not found. An intracystic papillary lesion, possibly papillary carcinoma, was suspected. In December 2007, wide excision of the tumor was performed. On histopathological examination, the tumor had a papillary pattern with a small cribriform component in the cystic wall with microinvasion of the stroma. Marginal status was negative. The final diagnosis of the disease was a microinvasive intracystic papillary carcinoma of low grade without axillary lymph node metastases. Immunohistochemically, estrogen receptor and progesterone receptor were both positive, but negative for HER-2 protein. No LOH on 16q could be detected. The prognosis of the disease was unclear; however, the malignant potential of this condition may be more clearly determined by studying the LOH on chromosome 16q.

Keywords Intracystic papillary carcinoma · Male breast cancer · Loss of heterozygosity

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Introduction

Intracystic papillary tumors of the breast account for less than 1% of all breast lesions biopsied [1], whereas the majority of male papillary carcinomas are intracystic, and approximately 40–50% of the cases are noninvasive. Invasive intracystic carcinomas in male patients are extremely rare. In addition, most cases of intracystic papillary carcinoma of the male breast have a single cystic lesion with a malignant intracystic component. In order to differentiate papillary carcinomas from papillomas in females, analysis of loss of heterozygosity (LOH) on 16q has been reported to be a useful tool for correct diagnoses [2]. We report a case of intracystic invasive papillary carcinoma with analyses of LOH on 16q in a male patient.

Case report

A 64-year-old man noticed a right subareolar mass in May 2005. The patient consulted the family doctor and was

diagnosed as having right gynecomastia. A regular check-up was scheduled, but in August 2007 the tumor enlarged rapidly and become painful. He did not notice any nipple discharge. He was referred to our hospital for further examination.

The patient had a history of hypertension, hyperlipemia, diabetes mellitus and brain infarction. There was no family history of breast malignancy.

On physical examination, the tumor (6.0 × 5.5 cm in size) was located just beneath the right nipple with an oval-shaped, smooth surface and clear margins (Fig. 1). The tumor was also elastic, firm and freely movable. Neither axillary nor supraclavicular lymph nodes were palpable. The left breast was unremarkable. Mammography demonstrated a 5 × 5-cm, relatively distinct and dense mass without microcalcifications or spiculations (Fig. 2). There were no findings of concurrent gynecomastia. Ultrasonography revealed a large multilocular cyst with a mural protruding lesion exhibiting wide-based morphology with an irregular margin (Fig. 3). On contrast-enhanced computed tomography, a large irregular mass with an inner enhanced lesion was detected on the right chest wall (Fig. 4). Direct invasion of the tumor to the major pectoral muscle was not found. Aspiration showed bloody fluid, but cytological examination of this fluid failed to reveal malignancy. Three tumor markers of the serum, including CEA, NCC-ST-439 and CA15-3, were all within normal ranges.

From these findings, the tumor was suspicious for being an intracystic papillary lesion, possibly papillary carcinoma. In December 2007, a wide excision of the lump was performed. Grossly, the size of the lesion was 5.0 × 4.0 cm. The small protruding lesion was 1.5 × 1.0 cm. On histopathological examination, the tumor had a papillary or cribriform pattern in the cystic wall. The cellular composition of the tumor was monomorphic with prominent nucleoli. Microinvasion (less than 0.1 cm) of the stroma was found; the final diagnosis of the disease was a microinvasive intracystic papillary carcinoma (Fig. 5). Marginal status of the resected specimen was negative. Axillary dissection was added later, but there were no lymph node metastases. Estrogen receptor (ER) and progesterone receptor (PgR) were both positive. On immunohistochemical staining, the cancer cells were

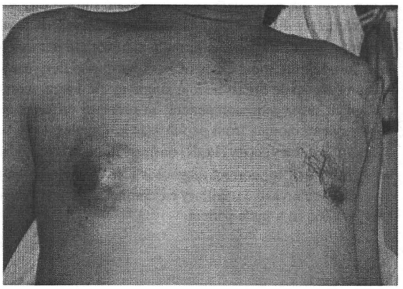

Fig. 1 On physical examination, the tumor (6.0 × 5.5 cm in size) was located just beneath the right nipple with an oval shape, smooth surface and clear margins

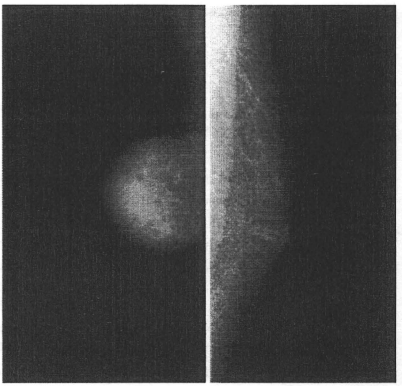

Fig. 2 Mammography demonstrated a 5 × 5-cm, relatively distinct and dense mass without microcalcifications or spiculations

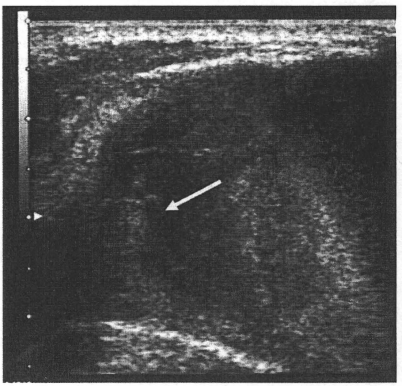

Fig. 3 Ultrasonography revealed a large multilocular cyst with a mural hypoechoic protruding lesion exhibiting wide-based morphology with an irregular margin (*arrow*)

negative for HER-2 protein. Adjuvant hormone therapy with 20 mg/day of tamoxifen was started 2 weeks after the axillary dissection. The patient was disease-free 12 months after surgery.

LOH of 16q

LOH on chromosome 16q has been shown to occur frequently in intracystic papillary carcinoma, but not in intraductal papilloma in the female breast [2]. We conducted LOH analysis in this case in order to determine if the examination of LOH on 16q was helpful for the differential diagnosis of male breast papillary tumors. The details of the procedure have been reported previously [2].

Tissue microdissection and DNA extraction

Cancerous cells and non-cancerous tissues were microdissected, and the microdissected cells were placed in proteinase K solution and incubated. The proteinase K was inactivated by incubation, followed by standard phenol–chroloform extraction and ethanol precipitation in the presence of glycogen.

PCR and analysis of alleic pattern

PCR amplification of genomic DNA was performed. Non-cancerous DNA samples with two different amplified bands were defined as informative cases for LOH analysis. The presence of LOH was determined in accordance with the manufacturer's criteria. LOH was considered to exist if the ratio of peak heights calculated by the following formula was lower than 0.67 or greater than 1.35: [Peak height of the affected allele (allele A) of the tumor × Peak height of the unaffected allele (allele B) of normal cells]/(Peak height of allele A of normal cells × Peak height of allele B of tumor cells). Results were considered non-informative when the alleles of normal tissue were homozygous, when the tissue lysate failed to be amplified or when the results could not be interpreted unambiguously.

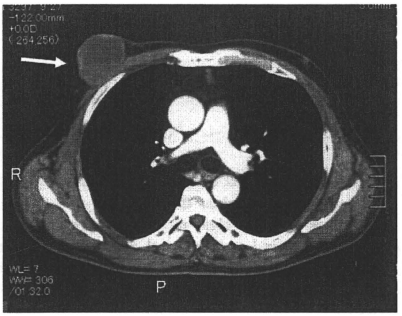

Fig. 4 On contrast-enhanced computed tomography, a large irregular mass with the enhanced inner lesion was detected on the right chest wall (*arrow*). Direct invasion of the tumor to the major pectoral muscle was not found

Results

Using five markers (D16S409: 16q12.1, D16S308: 16q12.1, D16S514: 16q21, D16S508: 16q21, D16S520: 16q24: 16q24, D16S413: 16q24), no LOH on 16q could be detected, even though one marker (D16S413) failed to be amplified (Table 1). The other five markers were all heterozygous (Fig. 6).

Discussion

The clinical findings of intracystic carcinoma were tumors and nipple discharge. Eighty percent of the discharge was bloody.

Mammography is not particularly useful for diagnosing intracystic lesions, whereas ultrasonography can detect intracystic papillary lesions on the wall of the cyst [3].

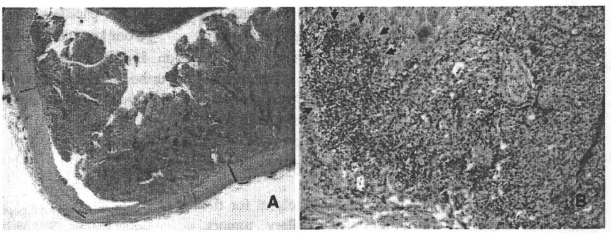

Fig. 5 On histopathological examination, the tumor was found to have a papillary or cribriform pattern in the cystic wall (**a**). Isolated invasive carcinoma cells were obscured by a lymphocytic reaction (*arrow*) in the stroma (**b**)

Table 1 Results of heterozygosity on 16q

Locus	Marker	F-primer	R-primer	LOH analysis
16q12.1	D16S409	TGAATCTTACATCCCATCCC	AGTCAGTCTGTCCAGAGGTG	Heterozygous
16q12.1	D16S308	CAGCCAGGGTAGTAAGGCTAGACCT	TGGGTGGCAGAGTGAGACCCTGTCT	Heterozygous
16q21	D16S514	CTATCCACTCACTTTCCAGG	TCCCACTGATCATCTTCTC	Heterozygous
16q21	D16S508	CAGGAAAATAAATCTAACACACATA	CCTGTGGGCACTGATAAATA	Heterozygous
16q24	D16S520	GCTTAGTCATACGAGCGG	TCCACAGCCATGTAAACC	Heterozygous
16q24	D16S413	ACTCCAGCCCGAGTAA	GGTCACAGGTGGGTTC	Not amplified

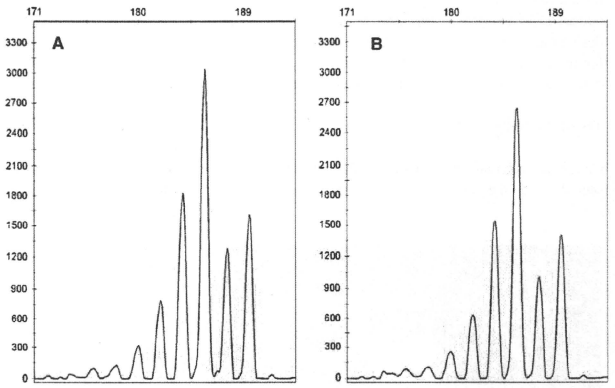

Fig. 6 No LOH on 16q could be detected (D16S520). **a** Cancer tissue DNA. **b** Non-cancerous tissue DNA. In both **a** and **b**, alleles 1 and 2 were preserved

Aspiration biopsy cytology made a correct diagnosis in 35% of the cases [4]. For the cytological feature revealed by aspiration biopsy, cytology might be misleading because cellular atypia is slight in intracystic papillary lesions. The diagnosis should be made by core needle biopsy [5]. Ultrasound-guided core needle biopsy is therefore the most valuable method for a correct preoperative diagnosis. Excisional biopsy is sometimes needed to confirm the diagnosis, because invasive cases have been reported previously [6–8].

In the Japanese literature reviewed, the average age of the patients with intracystic male breast carcinoma was 69 years (range 41–91), suggesting that the patients with the disease were older than for the common-type breast cancers. The mean tumor diameter was 3.0 cm (range 1.7–7.0) [7, 9]. There is no consensus regarding the surgical procedure of intracystic papillary carcinoma. Approximately 90% of the patients were treated with mastectomy; however, excisional biopsy with a negative margin can be also recommended in this condition [7–10]. However, the etiology still remains unknown.

Examination of LOH on 16q could be helpful for the differential diagnosis of intracystic papillary tumors, because such LOH was detected in approximately 70% of the cases. In addition, the incidence of LOH on chromosome 16q is high in intracystic carcinomas, regardless of their low histological grade of atypia and of no or only relatively limited extent of invasion in general. In this case, however, no LOH on 16q could be detected.

The prognosis of the disease is also unknown except for non-invasive cases; however, the malignant potential of the disease may be more clearly determined by studying the LOH on chromosome 16q. In this case, it is possible that another LOH, such as LOH on 17p or 11p, is also related to intracystic papillary carcinoma.

To our knowledge, this is the first report to analyze LOH on 16q in intracystic male papillary breast carcinoma. Further studies will be required to clarify the etiology and biological behavior of this condition.

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Predictors of recurrence in breast cancer patients with a pathologic complete response after neoadjuvant chemotherapy

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BACKGROUND: Although a pathologic complete response (pCR) after neoadjuvant chemotherapy is associated with favourable outcomes, a small proportion of patients with pCR have recurrence. This study was designed to identify factors predictive of recurrence in patients with pCR.

METHODS: A total of 449 breast cancer patients received neoadjuvant chemotherapy, and 88 evaluable patients had a pCR, defined as no evidence of invasive carcinoma in the breast at surgery. The clinical stage was II in 61 patients (69%), III in 27 (31%). All patients received taxanes and 92% received anthracyclines. Among 43 patients with HER2-positive tumours, 27 received trastuzumab. Cox regression analyses were performed to identify predictors of recurrence.

RESULTS: Median follow-up was 46.0 months. There were 12 recurrences, including 8 distant metastases. The rate of locoregional recurrence was 10.4% after breast-conserving surgery, as compared with 2.5% after mastectomy. Multivariate analysis revealed that axillary metastases (hazard ratio (HR), 13.6; $P<0.0001$) and HER2-positive disease (HR, 5.0; $P<0.019$) were significant predictors of recurrence. Five of six patients with both factors had recurrence. Inclusion of trastuzumab was not an independent predictor among patients with HER2-positive breast cancer.

CONCLUSION: Our study results suggest that HER2 status and axillary metastases are independent predictors of recurrence in patients with pCR.

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Keywords: breast cancer; pathologic complete response; neoadjuvant chemotherapy; predictive factor; trastuzumab

Neoadjuvant chemotherapy is a widely accepted treatment not only for locally advanced breast cancer, but also for earlier-stage operable disease (van der Hage *et al*, 2001; Bonadonna *et al*, 1998; Bear *et al*, 2003). Mauri *et al* (2005) performed a meta-analysis of clinical trials comparing patients who received preoperative chemotherapy with those who received postoperative chemotherapy. Death, disease progression, and distant recurrence were equivalent in both the arms. The main advantages of neoadjuvant chemotherapy included the evaluation of the *in vivo* chemosensitivity of tumours in individual patients; minimisation of micrometastases; and surgical downstaging of tumours, allowing breast-conserving surgery (BCS) to be performed in patients who might have otherwise required a mastectomy. However, the survival advantage of neoadjuvant chemotherapy appears to be negligible (Fisher *et al*, 1997; Bonadonna *et al*, 1998; Kuerer *et al*, 2001; Wolmark *et al*, 2001).

In several studies, a pathologic complete response (pCR), defined as the absence of invasive tumour in the breast only or in the breast and axilla, correlates with a far lower risk of subsequent recurrence, as well as with improved overall survival (Fisher *et al*, 1997, 1998; Bonadonna *et al*, 1998; Morrell *et al*, 1998; Kuerer *et al*, 1999; Chollet *et al*, 2002). Thus, efforts have been made to increase pCR rates by using more effective drugs and treatment regimens (Smith *et al*, 2002; Buzdar *et al*, 2005); the achievement of pCR has become the primary end point of many clinical studies.

Although a pCR is associated with favourable outcomes in most patients, some patients with pCR have disease recurrence. Previous studies have reported 5-year recurrence rates of 13–25% (Fisher *et al*, 1998; Morrell *et al*, 1998; Kuerer *et al*, 2001; Wolmark *et al*, 2001). Only a few studies have examined predictors of recurrence in patients who have a pCR to neoadjuvant treatment (Ring *et al*, 2004; Gonzalez-Angulo *et al*, 2005; Guarneri *et al*, 2006). We therefore retrospectively analysed predictive factors of recurrence in patients with breast cancer who achieved a pCR after neoadjuvant chemotherapy.

PATIENTS AND METHODS

Patients

This was a retrospective study of 88 evaluable patients with primary breast carcinoma who had a pCR after receiving neoadjuvant chemotherapy at National Cancer Center Hospital, Tokyo between 1996 and 2006. The follow-up period was completed

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in December 2008. The locoregional or distant recurrences were evaluated on physical examination, or by radiological imaging.

Histopathology

All patients were confirmed to have invasive carcinoma histologically by core needle biopsy. Surgical specimens were sectioned at 7- to 10-mm thick slices, and the pathological response was evaluated by pathologists specialised in breast pathology. The histologic type of the primary tumour was classified according to the *General Rules for Clinical and Pathological Recording of Breast Cancer*, The Japanese Breast Cancer Society (2004). The histologic grade of the tumours was classified according to the Elston–Ellis classification system (Elston and Ellis, 1991). The patients' levels of oestrogen receptor (ER, 1D5; Dako, Glostrup, Denmark), progesterone receptor (PgR, 1A6; Novocastra, Newcastle Upon Tyne, UK), and HER2 (HercepTest, Dako) were measured by immunohistochemical (IHC) analysis of paraffin-embedded tissue specimens. Oestrogen receptor and PgR were classified as positive if more than 10% of cancer cell nuclei were stained, regardless of the staining intensity. HER2-positive status was defined as IHC (3+); more than 10% of cancer cells markedly positive, or positive results of fluorescence *in situ* hybridisation (FISH) for HER2 amplification, that is, a HER2/CEP17 signal ratio of 2.0 (Vysis Pathvysion; Abbott, Chicago, IL, USA). IHC (2+) tumours, in which more than 10% of cancer cells were moderately positive, were excluded from the analysis without performing FISH test.

A wide range of criteria have been used to define pCR, and a consensus has yet to be reached. In this study, pCR was defined as no evidence of invasive carcinoma in the breast at the time of surgery in line with the criteria of the National Surgical Adjuvant Breast and Bowel Project B-18 (Wolmark *et al*, 2001) and the recommendations of Sataloff *et al* (1995). Because the presence or absence of residual ductal carcinoma *in situ* (DCIS) after preoperative therapy does not influence long-term rate of local recurrence or overall survival (Mazouni *et al*, 2007), we included patients with residual DCIS in the category of pCR.

Treatment

Neoadjuvant chemotherapy was indicated in patients with clinical stage II or III primary breast cancer whose tumours were larger than 3 cm. Although the potential benefits of adding taxanes to anthracycline-based regimens remain controversial in terms of long-term outcomes (Bear *et al*, 2006), regimens combining anthracyclines with taxanes, either sequentially or concomitantly, are widely used. In this study, neoadjuvant chemotherapy regimens included (1) four cycles of doxorubicin (DOX, 50 mg m^{-2}) and docetaxel (DTX, 60 mg m^{-2}) (AT), followed by additional adjuvant treatment with two cycles of AT or four cycles of intravenous cyclophosphamide, methotrexate, and 5-fluorouracil (CMF); (2) four cycles of fluorouracil (500 mg m^{-2})/epirubicin (100 mg m^{-2})/cyclophosphamide (600 mg m^{-2}) (FEC) along with 12 weekly cycles of paclitaxel (80 mg m^{-2}); (3) four cycles of doxorubicin (60 mg m^{-2})/cyclophosphamide (600 mg m^{-2}) (AC) along with 12 weekly cycles of paclitaxel (80 mg m^{-2}); (4) twelve weekly cycles of paclitaxel (80 mg m^{-2}) only; and (5) four cycles of AC along with four cycles of DTX (60 mg m^{-2}). After November 2002, patients with HER2-positive tumours received trastuzumab (initially 4 mg kg^{-1} followed by 2 mg kg^{-1} weekly) in combination with paclitaxel for 12 weeks. Trastuzumab was not administered post-operatively because it had not been approved for use in an adjuvant setting in Japan until 2007.

As for breast surgery, patients underwent either mastectomy ($n=40$) or BCS ($n=48$). Axillary lymph node dissection or sentinel lymph node biopsy alone was additionally performed. The decision to perform BCS was based on the ability to remove residual disease completely with optimal cosmetic results; patient preference was also considered. Twenty-one patients (24%) received adjuvant endocrine therapy including tamoxifen, anastrozole, or both drugs for 5 years if either the pre-treatment biopsy specimen or the surgical specimen obtained after chemotherapy was positive for ER or PgR. We defined surgical margin positive if the tumour cells were directly exposed to the margin.

Postoperative radiotherapy was administered to 60 patients (68%) who had either undergone BCS or had locally advanced disease. The radiotherapy protocol was as follows: after mastectomy, patients with clinical stage III disease received radiotherapy, delivered in 2 Gy fractions to chest wall and axilla (total dose 50 Gy). After BCS, all patients received radiotherapy, delivered in 2 Gy fractions to the breast (total dose 50 Gy). A booster dose was delivered to the tumorectomy bed if the surgical margin was positive. Regardless of the surgical methods, patients with four or more positive axillary lymph nodes received radiotherapy, delivered in 2 Gy fractions to subclavicular region (total dose 50 Gy).

Clinical significance of locoregional recurrence after neoadjuvant chemotherapy

The impact of locoregional recurrence (LRR) survival after neoadjuvant chemotherapy on survival remains poorly understood. However, patients with LRR after adjuvant chemotherapy, especially those with ER-negative tumours, have substantially worse outcomes regardless of axillary node status (Wapnir *et al*, 2006; Anderson *et al*, 2009). Among patients who achieved a pCR in neoadjuvant setting in our study, the ER-negative rate was 73% and higher than that of patients in adjuvant settings. This suggests the LRR after neoadjuvant chemotherapy might be a negative prognostic factor.

Statistical analysis

Statistical analyses were performed using SAS, version 9.2 (SAS Institute Inc., Cary, NC, USA). The log-rank test was used to identify predictive factors associated with recurrence after the achievement of pCR. Then, variables with *P*-values of $\leqslant 0.20$ on univariate analysis were included in the multivariate models. Multivariate analysis with a Cox proportional-hazards model was used to identify independent predictors in all 88 patients. Models were selected by stepwise forward analysis, retaining variables significant at the $\alpha = 0.05$ level for the final model. The Kaplan–Meier product-limit method was used to compute recurrence-free survival according to the number of predictive factors. Recurrence-free survival was measured from the date of initial diagnosis to the date of recurrence (including LRR) or the last follow-up visit. In addition, the relations of recurrence to clinicopathological factors in the 43 patients with HER2-positive tumours were also evaluated. A Cox proportional-hazards model including variables with *P*-values of $\leqslant 0.05$ on univariate analysis was used to identify independent predictors of recurrence.

RESULTS

Characteristics of patients with relapse

Of 449 patients with breast cancer who received neoadjuvant chemotherapy, 88 (20%) evaluable patients were identified as having a pCR. The median follow-up was 46 months (range, 8–115). Table 1 shows the patient and tumour characteristics. The median age was 54.5 years (range, 29–78). The median diameter of the primary breast tumour was 45.0 mm (range, 25–130). All patients received taxane-based chemotherapy, and 92% also received anthracycline-based therapy.

A total of 12 patients (13.6%) had tumour recurrence (Table 2). All recurrences were diagnosed within 32 months after initial diagnosis. Seven patients died of breast cancer within the follow-up

period. Among the six patients who had LRR, five had received BCS as primary surgery, and four had DCIS after neoadjuvant chemotherapy. LRR occurred in 5 of 48 patients (10.4%) after BCS, as compared with only 1 of 40 patients (2.5%) after mastectomy.

Predictive factors for recurrence in all 88 patients with pCR

The results of univariate analysis of predictive factors for recurrence are shown in Table 3. Variables tested for inclusion in the multivariate model were axillary lymph node metastasis at surgery, HER2 status (positive *vs* negative) and stage (III *vs* II). After controlling for these factors, axillary lymph node metastasis (hazard ratio (HR), 13.6; 95% CI, 4.6–63.3; $P<0.0001$) and HER2-positive disease (HR, 5.0; 95% CI, 1.3–19.3; $P<0.019$) remained significant independent predictors of recurrence (Table 4). According to the number of independent risk factors (HER2-positive disease and axillary lymph node metastasis) for recurrence, the 5-year recurrence-free rate varied between 94.4% for no factor ($n=36$), 89.1% for 1 factor ($n=46$), and 0% for 2 factors ($n=6$).

Table 1 Patient characteristics

Characteristic	All patients ($N=88$) No. of patients
Age, years	
⩽50/>50	33/55
Clinical stage	
II/IIIA/IIIB,IIIC	61/18/9
Pre-treatment pathology	
Invasive ductal/lobular/mucinous/others	85/1/1/1
Nuclear grade	
1/2/3/unknown	2/24/61/1
Hormone receptor status	
ER or PgR positive/both negative	23/65
HER2 status	
Positive/Negative	43/45
Neoadjuvant chemotherapy	
FEC→weekly paclitaxel (±trastuzumab)	31 (16 with trastuzumab)
AC→weekly paclitaxel (±trastuzumab)	30 (8 with trastuzumab)
AT (doxorubicin + docetaxel)	19
Weekly paclitaxel (± trastuzumab)	7 (3 with trastuzumab)
AC→docetaxel	1
Surgery	
Mastectomy/Breast-conserving surgery	40/48

Abbreviations: FEC = fluorouracil + epirubicin + cyclophosphamide; AC = doxorubicin + cyclophosphamide; PgR = progesterone receptor.

Predictive factors for recurrence among 43 patients with HER2-positive disease

Among 43 patients with HER2-positive breast cancer who had a pCR, 27 received trastuzumab. The results of the univariate analysis of predictive factors for recurrence are shown in Table 3. Variables tested for inclusion in the multivariate model were axillary lymph node metastasis at surgery, inclusion of trastuzumab, and stage (III). After controlling for these factors, only axillary lymph node metastasis (HR, 74.6 (8.0–692.9); $P<0.0001$) remained a significant independent predictor of recurrence.

DISCUSSION

Because a small proportion of patients with breast cancer have recurrence after achievement of a pCR, prediction of the risk of recurrence has an important role in postoperative management. Our multivariate analysis of all 88 patients with a pCR showed that axillary lymph node metastasis and HER2-positive disease were independent predictors of recurrence. Five of the six patients with both of these factors had recurrence after achieving a pCR in our study. Such patients may benefit from additional postoperative therapy and not be optimal candidates for clinical trials with pCR as the primary end point.

Although pCR in this study was defined as no evidence of invasive carcinoma only in the breast, the trial of the University of Texas MD Anderson Cancer Center pCR criteria requires not only complete response of the primary lesion but also the disappearance of axillary metastasis (Green *et al*, 2005). We also performed Cox regression model analysis of 73 patients who satisfied the MD Anderson pCR criteria (results not shown). On univariate analysis, tumour diameter (>50 mm) and grade (3) had *P*-values of ⩽0.20. However, no factor was independently significant in the multivariate analysis. The reasons for the differences in the results according to the definitions of pCR were the smaller sample size, the smaller number of recurrences (only five recurrences), and the elimination of the large influence of axillary lymph nodes on recurrence.

Table 2 Characteristics of patients with recurrence

		Initial diagnosis			Operative information			State at recurrence			
No.	Age	Tumour diameter	HER2	ER or PgR	Ax. M.	DCIS	BCS	LRR	Distant M.	Brain M.	RFS
1	39	90	–	–	–	–	–	–	+	+	8
2	33	52	–	+	–	–	+	–	+	–	26
3	62	55	+	–	–	–	+	+	+	–	26
4	29	35	+	+	–	+	+	+	+	–	30
5	58	42	+	–	–	–	+	+	+	–	32
6	55	65	+	–	+	+	–	–	+	–	32
7	63	49	+	–	+	+	–	+	–	–	18
8	36	34	–	+	+	-	–	–	+	–	20
9	49	30	+	–	+	+	–	–	+	–	21
10	56	25	+	–	+	+	+	+	–	–	21
11	50	55	+	–	+	-	+	–	+	+	29
12	71	60	–	–	+	+	+	+	–	–	32

Abbreviations: Ax. M. = axillary lymph node metastasis; M. = metastasis; BCS = breast-conserving surgery; RFS = recurrence-free survival (months); LRR = locoregional recurrence; ER = oestrogen receptor; PgR = progesterone receptor; HER2 = human epidermal growth factor 2; DCIS = ductal carcinoma *in situ*.

As expected, histopathological lymph node status was a strong predictor of recurrence in patients who had a pCR of their primary tumours. In contrast, HER2 status was found to be a predictor of recurrence for the first time. Gonzalez-Angulo *et al* (2005) studied predictive factors for distant metastasis in 226 patients with pCR. Although HER2 positivity was not a significant predictor of distant metastasis, HER2 status was unknown in 58% of the patients, and only 5% received taxane-based chemotherapy. Interactions between HER2 status and paclitaxel have been reported in an adjuvant setting, especially among patients with ER-negative tumours (Hayes *et al*, 2007). In our exploratory study, HER2 status was assessed by IHC or FISH analyses in all patients, the ER- or PgR-positive rate was low (26%), and all the patients received taxane-based therapy. The combination of these factors may have contributed to the identification of HER2 positivity as a significant independent predictor of recurrence after the achievement of a pCR.

Buzdar *et al* (2005, 2007) and Gianni (2008) reported the results of randomised trials of trastuzumab given with neoadjuvant chemotherapy to patients with HER2-positive breast cancer, and the pCR rate was significantly higher than that in the control arm. However, there are only a few, small randomised trials

Table 4 Multivariate analysis of predictors of recurrence (all 88 patients)

Characteristic	HR	*P*-value	95% CI
Axillary lymph node metastasis	13.6	<0.0001	4.6–63.3
HER2-positive disease	5.0	0.019	1.3–19.3

Abbreviations: HR = hazard ratio; CI = confidence interval; HER2 = human epidermal growth factor receptor 2.

Table 3 Univariate analysis of predictive factors for recurrence

	All patients ($N = 88$)			HER2 positive ($N = 43$)		
Characteristic	**No.**	**Patients with recurrence (%)**	***P*-value**	**No.**	**Patients with recurrence (%)**	***P*-value**
Age						
>50 years old	55	10.9		28	17.9	
⩽50 years old	33	18.2	0.28	15	20	0.83
Tumour diameter						
> 50 mm	30	20.0		12	25.0	
⩽50 mm	58	10.3	0.22	31	16.1	0.44
Clinical stage						
II	61	9.8		30	13.3	
III	27	22.2	0.09	13	30.8	0.11
ER or PgR						
Positive	23	13.0		9	11.1	
Negative	65	13.8	0.87	34	20.6	0.45
HER2						
Positive	43	18.6				
Negative	45	9.1	0.19			
Nuclear grade						
3	61	14.5		28	21.4	
1–2	26	11.5	0.71	15	13.3	0.49
Type of chemotherapy						
Anthracycline + taxane	81	13.4		39	18.0	
Taxane based	7	28.6	0.38	4	25.0	0.91
Type of chemotherapy						
With trastuzumab	27	7.4		27	7.4	
Without trastuzumab	61	16.4	0.28	16	37.5	0.015
Surgery						
Mastectomy	40	12.5		21	23.8	
BCS	48	14.6	0.84	23	13.6	0.48
Residual DCIS						
Present	39	15.4		23	21.7	
None	49	12.2	0.65	20	15.0	0.50
No. of LNs examined						
⩽10	15	14.7		7	14.3	
>10	73	13.7	0.93	36	19.4	0.79
Axillary LN status						
Node positive	15	46.7		6	83.3	
Node negative	73	6.9	<0.001	37	8.1	<0.001

Abbreviations: ER = oestrogen receptor; PgR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; pCR = pathological complete response; BCS = breast-conserving surgery; DCIS = ductal carcinoma *in situ*; LN = lymph node.

of neoadjuvant trastuzumab, and so far no study has shown that neoadjuvant trastuzumab can improve overall survival (Rowan, 2009). Indeed, in our study, the pCR rate in patients with HER2-positive breast cancer who received neoadjuvant chemotherapy with trastuzumab was 50% (27 out of 54), which was much higher than that for the study group as a whole (20%, 88 out of 449). However, the inclusion of trastuzumab was not a significant predictor of recurrence on multivariate analysis. This is partly because trastuzumab was not administered post-operatively. The optimal duration of trastuzumab in neoadjuvant and adjuvant setting should be confirmed prospectively in randomised trials.

The demand for BCS is expected to rise as the reported rate of pCR after BCS increases. However, LRR rates after BCS in patients who received neoadjuvant chemotherapy in previous studies have varied from 2.6 to 22.6% (Mauriac *et al*, 1999; Rouzier *et al*, 2001; Peintinger *et al*, 2006). This wide variability has led to uncertainty, and the benefits of BCS have been questioned. Objective evaluation of the safety and effectiveness of BCS has been precluded by the small numbers of patients who have achieved a pCR, different criteria for determining whether BCS is indicated, and different treatment regimens. Mauri *et al* (2005) performed a meta-analysis of clinical trials comparing preoperative with postoperative chemotherapy. Although the proportion of patients with distant recurrence was equivalent in both arms, LRR was more frequent in the preoperative chemotherapy arm, with an HR of about 1.2. In our study, most cases of LRR occurred after BCS, and the proportion of patients with LRR was 10.4% after BCS, as compared with only 2.5% after mastectomy. Our study results suggest that even after achieving a pCR, patients should be carefully followed up for LRR after BCS.

This study was retrospective and lacked a sufficient number of patients with recurrence after the achievement of a pCR to allow us to make firm recommendations for a given treatment option. Despite these limitations, some tentative conclusions can be drawn. First, our retrospective analysis showed that HER2-positive disease and axillary metastasis were independent predictors of recurrence after the achievement of a pCR at the primary site in response to neoadjuvant chemotherapy. This finding suggests that patients with HER2-positive disease and axillary metastasis may be candidates for more aggressive adjuvant therapy even after the achievement of a pCR, but this assumption must be confirmed in future clinical trials. Second, the inclusion of trastuzumab in regimens for neoadjuvant chemotherapy might not be predictive of recurrence, even though the rate of pCR among patients who received trastuzumab was much higher than that among all patients who received neoadjuvant chemotherapy. Third, the rate of LRR was higher after BCS than after mastectomy. Patients who undergo BCS should thus be closely followed up for LRR.

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PRECLINICAL STUDY

Expression pattern of stromal cell-derived factor-1 chemokine in invasive breast cancer is correlated with estrogen receptor status and patient prognosis

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Abstract Chemokine receptor CXCR4 is known to be crucially involved in tumor progression, but the role of its ligand, stromal cell-derived factor-1 (SDF-1), remains unclear. The present study was conducted to clarify the clinicopathological and prognostic impact of SDF-1 expression in invasive breast cancers. Expression of SDF-1 mRNA and protein was examined in five breast cancer cell lines with or without estradiol treatment. In 52 surgically resected breast cancers, the level of SDF-1 mRNA in frozen samples and the pattern of SDF-1 protein immunoreactivity in formalin-fixed paraffin-embedded tissue sections were compared. In another cohort of 223 breast cancers, the correlation between SDF-1 immunoreactivity and clinicopathological parameters was examined using a tissue microarray. Estradiol treatment markedly increased the expression of SDF-1 mRNA and protein in the estrogen receptor (ER)-positive cell lines, MCF-7 and T47D. Among the 52 resected breast cancers, those with a cytoplasmic-dominant pattern of SDF-1 expression showed higher SDF-1 mRNA levels (median 27.4) than those with a membrane-dominant or negative pattern (median 13.6, $P = 0.0017$). Accordingly, the cytoplasmic-dominant pattern was defined as "high SDF-1 expression," and other patterns were defined as "low SDF-1 expression." Among the cohort of 223 tumors, "high SDF-1 expression" was detected in 158 (70.9%) and was significantly correlated with ER positivity ($P < 0.0001$), HER2 negativity ($P = 0.021$), and lower grade ($P < 0.0001$). Univariate analysis demonstrated that "high SDF-1 expression" was a significant indicator of better clinical outcome in both the entire patient cohort ($P = 0.017$) and the 133 patients with ER-positive tumors ($P = 0.036$), but not in the 90 patients with ER-negative tumors. Multivariate analysis showed that SDF-1 status was an independent factor related to overall survival in patients with ER-positive tumors ($P = 0.046$). SDF-1 status is a significant prognostic factor and may be clinically useful for assigning adjuvant therapy to patients with ER-positive invasive breast cancers.

Keywords SDF-1 (CXCL12) · Breast cancer · Estrogen receptor · Estrogen-regulated genes · Immunohistochemistry

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Abbreviations

SDF-1	Stromal cell-derived factor-1
ERG	Estrogen-regulated gene
TMA	Tissue microarray
ER	Estrogen receptor alpha
PR	Progesterone receptor

Introduction

Breast cancer is one of the most serious and prevalent diseases affecting women worldwide. Although age-adjusted

rates of breast cancer incidence are decreasing in the United States and the United Kingdom, they are still increasing in Asian countries including Japan [1]. The clinical outcome of patients with breast cancer in Japan has improved over the last 50 years [2], and this has been partly attributable to advances in adjuvant endocrine therapy and chemotherapy [3].

Approximately 70% of breast cancers are known to express estrogen receptor alpha (ER) and are considered to be hormone-dependent. Estrogens regulate the expression of various genes, and these estrogen-regulated genes (ERGs), including those encoding progesterone receptor (PR), transforming growth factor (TGF)-alpha, cyclin D1, bcl-2, and estrogen-responsive finger protein (Efp), have been shown to play various roles in estrogen signaling. Furthermore, ERGs are considered to be potential predictive biomarkers of response to hormonal therapy [4]. Currently, however, PR is the only ERG whose expression is used routinely to determine whether endocrine therapy is needed [5]. Recently, Hall and Korach [6] have revealed that the transcriptional activation and protein expression of stromal cell-derived factor-1 (SDF-1), also known as CXCL12, are regulated by estradiol in ER-positive breast cancer and ovarian cancer cell lines.

SDF-1, which was initially cloned from murine bone marrow stromal cells and characterized as a pre-B-cell growth-stimulating factor [7, 8], is a small chemotactic cytokine belonging to the CXC chemokine family. In cooperation with its cognate receptor, CXCR4 [chemokine (C-X-C motif) receptor 4], the CXCR4/SDF-1 axis plays various roles in many normal and pathological processes including embryogenesis, hematopoiesis, immunological homeostasis, human immunodeficiency virus infection, and the progression of rheumatoid arthritis. Furthermore, in various types of cancer, including breast cancer, CXCR4 on tumor cells has been shown to be critically involved in tumor progression [9]. In contrast, only a limited number of studies have investigated tumor-derived SDF-1, and the significance of this molecule in tumor biology is not fully understood [10].

In order to elucidate the clinicopathological role of tumor-derived SDF-1 in breast cancer, we examined the expression of SDF-1 protein using immunohistochemistry and that of SDF-1 mRNA by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis in surgically resected specimens of invasive breast cancer. In parallel, we also examined SDF-1 expression in five human breast cancer cell lines with and without estradiol treatment to acquire supportive evidence for SDF-1 expression in breast cancer tissues.

Materials and methods

Cell lines

The human breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-435, and MDA-MB-436 were purchased from the American Type Culture Collection (Rockville, MD, USA). Experiments using these cell lines were performed within 6 months. The cells were cultured in Dulbecco's MEM (DMEM) containing 10% fetal bovine serum and penicillin–streptomycin (100 U/ml). Before all of the experiments, the cells were cultured for 48 h in phenol red-free DMEM with 10% charcoal/dextran-treated fetal bovine serum.

Quantitative RT-PCR

Total RNA was extracted by using RNeasy Mini (Qiagen, Hilden, Germany) from the five cell lines, 52 samples of frozen tumor tissue, and 13 samples of frozen mammary gland tissue without any apparent histological abnormality. Each RNA sample was subjected to complementary DNA synthesis, and quantitative RT-PCR was performed with the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Scoresby VIC, Australia). The TaqMan® Gene Expression Assay used was **Hs00171022_m1** for SDF-1. The expression of 18s-rRNA was assessed as an internal control in order to verify mRNA integrity. In quantitative RT-PCR analysis using frozen tissue samples, the expression level of SDF-1 mRNA was calculated by the delta-delta Ct method for each sample.

Enzyme-linked immunosorbent assay (ELISA)

For quantification of SDF-1 secreted into the supernatant of the cell lines, a DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA) was used in accordance with the manufacturer's instructions. Each experiment was performed three times, and mean values were calculated.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by a wash with 0.5% Tween 20 in phosphate-buffered saline (PBS). The cells were then incubated with mouse monoclonal anti-human SDF-1 antibody (MAB350, R&D Systems) or murine IgG isotype control monoclonal antibody (MAB002, R&D systems), and SDF-1 protein and murine IgG background staining were visualized using fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse antibody as a secondary antibody (AP192F, Chemicon, Temecula, CA, USA).