DNA sequencing, such as a largely deleted mutant allele that has been observed in other ethnic populations (Petrij-Bosch et al. 1997; Puget et al. 1999a; Puget et al. 1999b; Puget et al. 1997; Swensen et al. 1997), and that could not be described as missense mutations because there is no functional assay available. In fact, it has been reported that at least 10%–15% of deleterious *BRCA1* mutations are missense mutations. These issues need to be resolved by developing other methods, including a reliable, rapid, and accurate functional assay.

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Extended Abstracts for
the 33rd International Symposium of
the Princess Takamatu Cancer Research Fund

Innovative Achievements
in Cancer Imaging

November 12-14, 2002
Tokyo, Japan

Edited by **Pablo R. Ros**
        Tadao Kakizoe
        Yuji Itai
        Noriyuki Moriyama

Princess Takamatsu Cancer Research Fund

ADVANCES IN DIAGNOSIS OF BREAST CANCER: MAMMOGRAPHY FOR SCREENING AND MRI FOR BREAST-CONSERVING SURGERY

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Introduction

Breast cancer superseded gastric cancer as the leading incident in Japan in 1994; the age standardized incidence rate to the world population raised from 17.0 in 1975 to 30.1 in 1996 per 100,000, and 9,652 women died of breast cancer in 2001. As incidence of breast cancer became the leading neoplasm, innovation in breast imaging has evolved an important issue in the lights of mortality reduction by screening as well as quality of life of the patients recieving treatment.

Advances in diagnosis: Early detection using screening mammography

In Japan under the Health and Medical Service Law for the Elderly clinical breast examination (CBE) was introduced into public health services without any evidence on its effectiveness in 1987. It was based on the presumption that any early detection is always beneficial. Our recent case control study has demonstrated that CBE lacks effectiveness, although it might be effective in an asymptomatic women[1].

To establish mammography screening, the Grant-in-aid for Cancer Research "Quality control of creast cancer screening with mammography" was organized in 1995. The essential issues were analyzed including sensitivity, cost-effectiveness to create new guideline. The experience of the Miyagi Trial helped to shape the planned national guideline. An improved detection rate was obtained, as compared to that by CBE alone. The false negative rate was 2.8%, lower than those of HIP and BCDDP in USA, indicating an improved sensitivity and effectiveness of mammography screening in Japan[2].

Since 1997, we moved on to establish nationwide screening system. We performed

quality assessment of mammography, and generated training programs for photographers and interpreting physicians. We should know that surgeons, gynecologists other than radiologists have been involved in the CBE program. Therefore, it was essential to design an education program before starting mammography screening. Interpreting physicians were required to have adequate levels of sensitivity and specificity for imaging categorization. We drafted the Japanese guidelines for quality assurance, leading the Ministry of Health and Welfare to change the national guideline to include mammography for women aged 50 and over in 2000.

Because breast cancer incidence rate is highest at their forties, and mortality rate is highest at their fifties, recruitment of women aged under 49 remains a big challenge. Remarkable progress in imaging technologies has contributed to an improvement in the usefulness of mammography screening. It is therefore possible that the past randomized mammography trials targeted women aged 40-49 might have underestimated the effectiveness [3]. We evaluated the performance parameters including recall rate, detection rate, and sensitivity of screening mammography with CBE in women aged 40-49, and compared the data with those obtained from screening in women aged 50-69. From 1995 to 1998 we gave one-view (MLO) mammography for breast cancer screening to 15,271 subjects aged 40-49 and 17,755 subjects aged 50-69 in Miyagi prefecture.

The recall rate, detection rate and sensitivity for women aged 40-49 were 10.4%, 0.20% and 93.8%, respectively. The data for women aged 50-69 were 7.2%, 0.21% and 95.0%. The recall rate for women aged 40-49 was higher than that of women aged 50-69, but the sensitivities and detection rates were almost equal. Node-negative rate in women aged 40-49 and those aged 50-69 were 80% and 84%. These data suggest that mammography screening with CBE may be an appropriate modality for women aged 40-49, although strict quality control should be required to optimize the recall rate in women aged forties who have higher breast tissue density than women aged fifties.

Advances in MRI for breast conserving surgery

Magnetic resonance imaging (MRI) for breast cancer diagnosis has been utilized prior to surgery. We have recently analyzed the radiologic-pathologic correlations regarding three-D cancer extension on the basis of detailed histopathologic analyses by sub-serial sectionings of the specimens. MRI correlated more faithfully with pathological findings as compared to the conventional mammography and ultrasonography, indicating that the 3-D MRI was more appropriate to estimate carcinoma distribution prior to surgery [4].

We have developed a novel breast-conserving surgery consisting of quadrantectomy and immediate volume replacement using lateral tissue-flap (LTF) [5]. The quadrantectomy

Table 1. Comparison of the data provided by mammography-combined screening with CBE alone

	Mammography + CBE	CBE alone
Subjects	12,515	50,105
Recall rate	3.58%	4.47%
Cancers detcted	35	44
Detection rate	0.28%	0.09%
False negatives	1	8
Sensitivity	97.2%	84.6%
Positive predictive value	7.8%	2.0%

(December, 1989 to June, 1992, Miyagi Cancer Society)

Table 2. Comparison of the data of mammography-combined screening according to age groups

	40-49 years old	50-69 years old
Subjects	15,271	17,755
Recall rate	10.4%	7.2%
Cancers detcted	30	38
Detection rate	0.20%	0.21%
False negatives	2	2
Sensitivity	93.8%	95.0%
Specificity	89.8%	93.0%
Positive predictive value	1.6%	3.0%

(April, 1995 to March, 1998, Miyagi Cancer Society)

was employed on the basis of segmental anatomy of duct-lobular system in which breast carcinoma originates[6]. Excellent cosmetic outcome as well as local control have been obtained. The principle of the surgery is to obtain negative surgical margins; quadrantectomy is a radical procedure in the sense that it aims at removal of all the carcinoma cells of the primary tumor. Our strategy is to identify patients in whom irradiation might be safely omitted after breast-conserving surgery.

Discussion

Since 1894 when Halsted reported a radical procedure, mastectomy has been employed as a standard operation over the century. However, the concept shifted to limited surgery, because of great change in the characteristics of patient population. Better education, more extensive information, refined diagnostic tools, and expanding screening campaigns all contribute to earlier detection of breast cancer. Breast conserving therapy becomes an important issue in the light of QOL. It is essential for surgeons and radiologisits to understand origin and extending pattern of breast cancer, and make the surgical margin negative. The quadrantic distribution of breast cancers influences extent of excision

Table 3. Evaluation of effectiveness for breast cancer screening in Japan (October, 2001, the Research Group supported by the Ministry of Health, Welfare and Labour)

Screening modality	Strength of recommendation*		Quality of evidence**
CBE alone	All ages	1-c	3
CBE with mammography	50y +	1-a	1
	40-49y	1-b	1
CBE with ultrasound	All ages	II	None

*Strength of recommendations
I-a: Good evidence to support the recommendation specifically considered in a periodic health examination. I-b: Fair evidence to support the recommendation specifically considered in a periodic health examination. I-c: Fair evidence to support the recommendation excluded from a periodic health examinatin. I-d: Good evidence to support the recommendation excluded from a periodic health examination.
II: Insufficient evidence to recommend for or against the inclusion of the condition.
**Quality of evidence
1: RCT, 2: Control trial without randomization, 3: Cohort or Case control study, 4: Ecological, time series study, 5: Others

and may predict pattern of recurrence when tumor clearance is incomplete even if radiation is applied as an adjuvant therapy. Surgery should reduce the chance of local recurrence.

The role of radiologists becomes more important in diagnosis and treatment of breast cancer. Especially, the great contribution will be required for the radiologists into mammography screening in terms of quality control as experienced in westren countries.

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Specialty and Present Interest:

Surgical oncology, Genetics, Cancer screening, Bio-nanotechnology

Oral Session

Collaboration of Breast Cancer Clinic and Genetic Counseling Division for BRCA1 and BRCA2 Mutation Family in Japan

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Background: BRCA1 and BRCA2 mutations cause high breast cancer incidence rates as high as 80%. Although prophylactic therapy is still controversial, several prophylactic therapies have been proposed and tried for BRCA1 and BRCA2 mutation carriers. Prophylactic surgery, chemo-prevention and precise screening have been proposed as prophylactic therapy. All BRCA1 and BRCA2 mutation carriers need knowledge about their disease and the countermeasures that are used to protect against onset of disease. Counseling plays an important role in this regard for people with genetic diseases. Therefore, collaboration between breast cancer clinics and genetic counseling services is the most important issue in clinical practice. Our group consists of three national universities and a general hospital. In this article we describe our trial to construct a clinical system against hereditary breast cancer as an interim report for the Japanese Ministry of Health, Labour and Welfare.

Patients and Methods: Twenty familial breast cancer patients were registered in this study. The whole sequence of BRCA1 and BRCA2 were analyzed. If pathological mutations were detected, their first degree families were introduced to the counseling division at each institute when candidates visited counseling divisions.

Results and Discussion: Four cases of a deleterious mutation in BRCA1 or BRCA2 were detected among 20 cases. Their first degree relatives are now under consideration for visiting counseling divisions. The clinical system described in this study should play a role to protect BRCA1 or BRCA2 mutation carriers in Japan.

Breast Cancer 11:30-32, 2004.

Key words: Hereditary breast cancer, Familial breast cancer, BRCA1, BRCA2

Breast cancer is the most common malignancy observed in Japan and western countries. Treatment of groups at high-risk for breast cancer is also a challenging problem. Familial breast cancer is an important factor impacting the early and bilateral onset of breast cancer. Five to 10% of breast cancer patients meet the definition of familial breast cancer[1,2]. BRCA1 and BRCA2 mutations have been reported as major causes of familial breast cancer. Twenty to thirty percent of Japanese familial breast cancer patients show deleterious BRCA1 or BRCA2 mutation[3]. Further, it has been reported that 80-90 percent of BRCA1 and BRCA2 mutation carriers suffer from breast cancer in their lives[4]. This high risk of breast cancer incident suggests the importance of screening for BRCA1 and BRCA2 mutations and preventive measures for familial breast cancer. Counseling and prophylactic therapies are of importance in prevention of such genetic diseases. Familial breast cancer patients and their first degree relatives are anxious about their genetic status. There is no standard clinical system and method for prophylactic therapy for BRCA mutation carriers in Japan. Hospitals or medical systems that provide proper knowledge of familial breast cancer and preventive therapies are needed. A larger number of patients are required to establish interventions for Japanese BRCA1 or BRCA2 mutation carriers. In this study we aimed to construct a multi-center
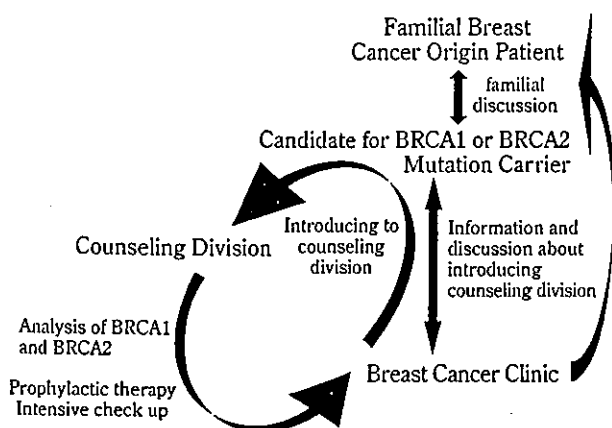
Fig 1. Counseling system for BRCA1 or BRCA2 mutation careers.

and inter-division cooperative system to smoothly introduce breast cancer clinic patients to the counseling division. This is an interim report for the grant of Japanese Health, Labour and Welfare.

Patients and Methods

Twenty familial breast cancer patients were registered between March 2002 and March 2003. Blood sampling and BRCA1/BRCA2 mutation analysis were done with informed consent according to the guidelines of the Japanese Ministry of Health, Labour and Welfare. Whole sequences of BRCA1 and BRCA2 were analyzed by the direct sequence method at Myriad Genetic Laboratories, Inc.

Clinical System

If obvious pathological mutations were detected, the patients were informed about the genetic counseling division at breast cancer clinics. Their first degree relatives were also informed about their genetic background and invited to visit genetic counseling services. When the first degree relatives visited genetic counseling divisions, they were informed about familial breast cancer and given the option to undergo genetic examination at breast cancer clinics.

Results

Deleterious Mutations of BRCA1 and BRCA2

Four cases of apparent deleterious BRCA1 and BRCA2 mutations were detected from among the total 20. These four carriers of deleterious mutations are now undergoing counseling to discuss along with their first degree relatives options for BRCA1 and BRCA2 examination and prophylactic treatments at the counseling divisions (Fig 1). An uncertain mutation thought to be a normal variant was also found in four cases.

Discussion

Treatment for BRCA1 and BRCA2 mutation carriers is a controversial issue. Prophylactic interventions for pathological BRCA1 and BRCA2 mutation carriers include prophylactic surgery, medication, irradiation and intensive follow-up. Prophylactic surgery consists of mastectomy and oophorectomy. Prophylactic mastectomy shows a prevention rate of 90%[5], while prophylactic oophorectomy shows a 50% prevention rate[6, 7]. Although prophylactic mastectomy shows a high rate of prevention as high as 90%, prophylactic surgical treatments are invasive interventions not only physically but also mentally for healthy people. Contant et al. proposed prophylactic mastectomy accompanied by immediate breast reconstruction (IBR). IBR can be one of solution for those hesitant to undergo prophylactic mastectomy[8]. Medication and intensive check-up as conservative treatments show relatively low prevention rate. Treatment with tamoxifen has an approximately 50% prevention rate[9]. Intensive check-ups may also allow BRCA mutation carriers to undergo breast-conserving surgery and have a better prognosis as they can be diagnosed at an earlier clinical stage.

A nonsense mutation of BRCA1 that was reported to be a Japanese founder mutation was detected as reported by Sekine et al.[10]. In addition, a variation in BRCA2 detected in four cases may be related to breast cancer onset.

There have been very few reports of prophylactic therapy in Japan. Proper prophylactic therapy has to be established for Japanese BRCA mutation carriers and prognosis and outcome of prophylactic therapy needs to be clarified. Our study group consists of two national university hospitals and a general hospital that cover each area. We have also been studying BRCA1 and BRCA2 mutations before this study. Together with the data from the former study, we are now beginning to analyze potential for BRCA1 and BRCA2 mutation carriers. Each institute in our study group has

a breast cancer clinic and a counseling division. If BRCA1 and BRCA2 mutation carriers decide to visit our breast cancer clinics and receive counseling about their genetic status, they can accept our introduction to clinicians in the counseling division (Fig 1). When the candidates decide to undergo genetic examination, we analyze their BRCA1 and BRCA2 sequences. The results of the examinations are reported to us. The results are given to the breast cancer clinics in each institute and the patient is informed. If they have a deleterious mutation, they visit the genetic counseling division again and receive information about familial breast cancer and prophylactic treatments.

Prophylactic mastectomy, oophorectomy, irradiation, medication and intensive check-ups have been proposed for the treatment of deleterious BRCA1 and BRCA2 mutation carriers. Prophylactic mastectomy may seem to be a radical therapy even if they show the highest prevention rate. Since the general prognosis of breast cancer occurring in BRCA mutation carriers is not different from that of sporadic breast cancer patients. Diagnosis before the onset of breast cancer should positively influence outcomes. In addition, Hoogerbrugge *et al.* reported that the breast tissue of BRCA mutation carriers has atypical hyperplastic change. Careful follow-up for BRCA1 and BRCA2 carriers is required unless they undergo prophylactic surgery[11].

Long term observation should be continued to clarify the actual onset rate and clinical outcome of breast cancer among Japanese BRCA1 and BRCA2 mutation carriers. Eventually this counseling system may be available for all Japanese families with deleterious BRCA1 and BRCA2 mutation carriers.

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Original Article
Invasive micropapillary carcinoma of the breast: Clinicopathological and immunohistochemical study

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Invasive micropapillary carcinoma (IMPCa) of the breast refers to a unique variant of invasive ductal carcinoma, but its biological behavior has not been elucidated well. We analyzed 16 IMPCa cases (10 pure type, six mixed type). The incidence of IMPCa was 1.0% of all primary breast carcinoma. High nuclear grade (75.0%), as well as poorly differentiated histological grade (81.3%), was frequently seen. Lymph node metastases were evident in 92.9% of the examined cases, and about half of them showed more than 10 positive nodes. Comparison between serially experienced invasive ductal carcinoma, not otherwise specified (IDC-NOS), revealed that both high nuclear grade and poor histological grade were significantly more frequent ($P < 0001$), there was a lower frequency of positive estrogen receptor/progesterone receptor ($P < 0.05$, $P < 0.01$), a higher frequency of HER-2 overexpression ($P < 0.025$), and more frequent lymph node metastases ($P < 0.05$) in IMPCa. The comparison between lymph node positive IDC-NOS did not show any statistically significant differences in frequency for positive p53, matrix metalloproteinase protein-2 (MMP-2), vascular endothelial growth factor (VEGF) or E-cadherin. However, IMPCa showed a significantly increased number of blood vessels counted by CD34 immunostains ($P < 0.05$). These results suggest that IMPCa is, at least, the same or more aggressive than lymph node positive cases of IDC-NOS. Hence, not only the high incidence of lymph node metastases but also distant, blood-borne metastases may be important.

Key words: breast carcinoma, ductal carcinoma, immunohistochemistry, invasive micropapillary carcinoma, pathology

Invasive micropapillary carcinoma (IMPCa) of the breast is considered to be a recently recognized, unusual type of invasive ductal carcinoma with unique morphology. Characteristically, this variant of carcinoma shows tumor cells arranged in small clusters with a central lumen usually present, and an image of a micropapillae within clear spaces, which appear to be empty, but in some instances mucinous materials have been seen with special stains.[1] Siriaungkul and Tavassoli identified nine cases of IMPCa, and came to the tentative conclusion that the behavior was not significantly different from that of invasive ductal carcinoma, not otherwise specified (IDC-NOS).[2] However, further investigations revealed that this tumor has a highly malignant potential because IMPCa had a high incidence of lymph node metastases, and tended to recur earlier.[3–7] Additionally, Paterakos and colleagues mentioned that the survival rate of patients with IMPCa was similar to patients with carcinoma with equivalent numbers of lymph node metastases.[8]

Immunohistochemical studies had been performed in some reports.[3,4,8,9] However, the precise clinicopathological characteristics have not been elucidated well, especially in Japanese women. Thus, we examined IMPCa, clinicopathologically and immunohistochemically, and compared them with IDC-NOS.

MATERIALS AND METHODS

We reviewed the case files from September 1998 to December 2001 in Tohoku University Hospital and Tohoku Kousai Hospital, and from January 1997 to December 2001 in Chugoku Chuo Hospital. Re-examination of the glass slides was done by two of the authors (CDLC and TM). To identify IMPCa cases, we followed the criteria: 'epithelial tufts forming

micropapillae without a fibrovascular core located within clear spaces, which are usually empty, and epithelial cells exhibiting reverse polarity with serrated peripheral borders' (Fig. 1).[1,3,8] The cases with obvious mucin within the empty space, which is sometimes allowed by some authors,[3] were eliminated from the series in this study.

Eleven patients were considered as IMPCa: six cases were considered as pure type IMPCa, with more than 90% of the invasive micropapillary carcinoma composed of characteristic features (Fig. 2); and five cases were considered as mixed type, with 33% to 90% of invasive carcinoma composed of IMPCa. A characteristic pattern of less than 33% invasive components was included in invasive ductal carcinoma, not otherwise specified (IDC-NOS). During the same periods, we have experienced 1056 cases of primary breast carcinomas. Thus, the overall incidence of all IMPCa cases in our series was 1.0%, and the pure IMPCa was 0.6% of all primary breast carcinomas. An additional five cases (four pure and

Figure 1 Invasive micropapillary carcinoma showing a micropapillary pattern of carcinoma cells floating within the empty space. HE, ×20.

Figure 2 Pure-type invasive micropapillary carcinoma with an extensive characteristic histological pattern. HE, ×100.

one mixed) were added from the previous files and, finally, a total of 16 IMPCa cases were analyzed. For comparison, 150 cases of serially experienced IDC-NOS from the files of the Pathology Department of Tohoku University Hospital in 2002 were used. For immunohistochemical analysis, another 23 cases with positive lymph node metastases at the initial operation, with available follow-up data (43–149 months, mean 108 months, with eight cases (34.8%) dead from disease), were selected as control cases.

All specimens were fixed in 10% formalin and embedded in paraffin, and 3 μm-thick sections were cut and mounted on glass slides. On the hematoxylin–eosin (HE) slides, the maximum diameter of the invasive carcinoma, presence or absence of intraductal components, presence or absence of comedonecrosis within the intraductal carcinoma, the nuclear/histological grading, and lymph node status, were evaluated. For nuclear grading, the criteria of the Japan National Surgical Adjuvant Study of Breast Cancer (NSAS-BC) Pathology Section[10] was used, and for histological grading, a modified Bloom & Richardson's method (Nottingham's classification)[11] was used.

Immunohistochemical staining for estrogen receptor, progesterone receptor, HER-2, and p53 was performed on the Ventana Bench Mark Automated Staining System. Manual immunostaining was used for matrix metalloproteinase protein-2 (MMP-2), vascular endothelial growth factor (VEGF), E-cadherin, Ki-67, CD34, Factor VIII related antigen, and type IV collagen. The source of the primary antibodies, dilution, and methods of pretreatment are listed in Table 1. The primary antibodies for manual staining were kept overnight at 4°C. After that, the Histofine SAB-PO kit (Nichirei, Tokyo, Japan) was applied. The positive staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB), and lightly counterstained with hematoxylin.

Estrogen and progesterone receptors were evaluated with a proportion score (PS), which represents the estimated proportion of positive tumor cells (range 0–5), and an intensity score (IS), which estimates the average staining intensity of positive tumor cells (range 0–3). The PS and IS were added to obtain a total score (TS) (range 0–8).[12] A TS greater than 4 was considered positive, and a TS less than 4 was considered negative. For VEGF, the cytoplasm of the carcinoma cells was compared with background staining to decide if they were positive or negative. To evaluate p53, more than 10% positive cells was considered as weakly positive (+), between 30% and 70% was considered moderately positive (++), and more than 70% was considered strongly positive (+++). For HER-2, the Hercep Test (DAKO) scoring criteria was used.[13] A weak to moderate complete membrane staining in more than 10% of tumor cells (score 2+) and a strong complete membrane staining in more than 10% of tumor cells (score 3+) were considered positive. The Ki-67 index was calculated as the number of Ki-67 positive cells per 100
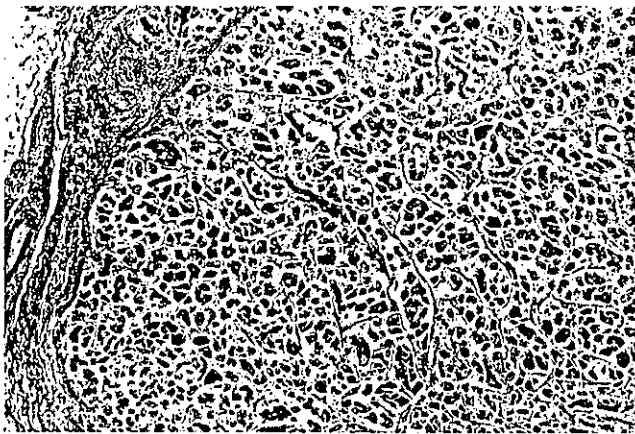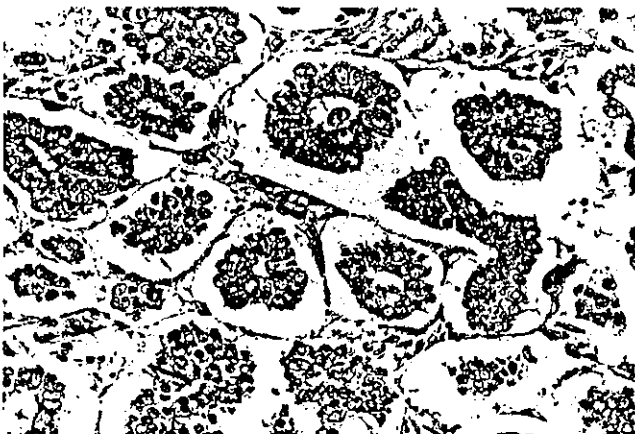
Table 1 Immunohistochemical reagents and methods used

Antigen	Antibody	Source	Dilution	Antigen retrieval
ER	6F11	Novocastra, Newcastle upon Tyne, UK	1:50	Heating†
PR	MAB429	Chemicon, Temecula, CA, USA	1:30	Heating†
HER-2	(Polyclonal)	DAKO, Glostrup, Denmark	1:800	Heating†
p53	DO-7	Biomeda, Foster City, CA, USA	1:40	Heating†
Ki-67	MIB-1	Immunotech, Marseille, France	1:300	Autoclave
CD34	NV4A1	Nichirei, Tokyo, Japan	1:100	None
VEGF	(Polyclonal)	R&D Systems Inc., Minneapolis, MN, USA	1:100	Autoclave
E-cadherin	4A2C7	Zymed, San Francisco, CA, USA	1:400	Autoclave
MMP-2	42–5D11	Fuji, Tokyo, Japan	1:30	None
Factor VIII-R Ag	(Polyclonal)	DAKO, Glostrup, Denmark	1:200	Protease
Type IV collagen	CIV22	DAKO, Glostrup, Denmark	1:100	Pepsin

ER, estrogen receptor; PR, progesterone receptor; R Ag, related antigen; VEGF, vascular endothelial growth factor. †Performed by the Ventana Bench Mark Automated Staining System.

Table 2 Clinicopathological features of 16 invasive micropapillary carcinoma cases

Case	Age	Distribution	Size (mm)†	Nuclear grade	Histological grade‡	Comedo necrosis§	LN status	Follow up
1	39	Pure	24	High	III (Poor)	−	0/8	18 months, NED
2	43	Pure	30	Intermediate	III (Poor)	+	1/7	54 months, NED
3	35	Pure	26	High	III (Poor)	+	6/9	17 months, NED
4	37	Pure	8	High	III (Poor)	−	27/27	34 months, DOD
5	59	Pure	84	High	III (Poor)	+	29/29	216 months, AWD
6	50	Pure	35	High	III (Poor)	−	2/15	16 months, NED
7	44	Pure	14	Intermediate	II (Moderate)	−	27/29	3 months, NED
8	43	Pure	25	High	III (Poor)	−	25/34	83 months, DOD
9	38	Pure	32	High	III (Poor)	−	18/21	56 months, DOD
10	42	Pure	27	Intermediate	II (Moderate)	−	3/19	24 months, NED
11	65	Mixed	37	Intermediate	II (Moderate)	+	8/19	61 months, DOD
12	55	Mixed	25	High	III (Poor)	−	3/16	88 months, AWD
13	67	Mixed	17	High	III (Poor)	+	NA	46 months, AWD
14	44	Mixed	12	Intermediate	II (Moderate)	−	NA	30 months, NED
15	56	Mixed	45	High	III (Poor)	+	12/24	14 months, NED
16	38	Mixed	28	High	III (Poor)	−	5/22	30 months, NED

AWD, alive with disease; DOD, dead of disease; LN, lymph node; NA, data not available; NED, no evidence of disease. †Microscopic maximum diameter of invasive component. ‡Modified Bloom–Scharf–Richardson scoring system.[11] §Presence (+) or absence (−) within intraductal components.

tumor cells (expressed as a percentage). E-cadherin was considered as positive when staining was present in at least 10% of the tumor cells' membranes. For other markers, the presence of a single positive cell was considered a positive result. The number of blood vessels was counted by CD34 immunostains in a 1 mm² area, at least 4 times, and then a percentage promedium was made.

Statistical analysis to compare IMPCa and IDC-NOS were done by either the chi-squared test or standard *t*-test.

RESULTS

Clinical and pathological findings of 16 IMPCa are listed in Table 2. The age distribution at initial operation was between 38 and 67 years, with the average 50.9 years. Ten cases (62.5%) were pure type, and six were mixed type (37.5%). The tumor size, calculated by the maximum diameter of the invasive component on microscopy, was 7–84 mm (average 31.0 mm). The nuclear grade was high in 11 cases (68.8%) and intermediate in five cases (31.2%). Histological grade

was III (poorly differentiated) in 12 cases (75.0%), and grade II (moderately differentiated) in four cases. Generally, both nuclear and histological grading was identical between IMPCa and the IDC-NOS area in mixed-type cases. Associated intraductal components was revealed in 10 cases, and, among them, comedonecrosis was seen in five cases. Extensive intraductal components were not evident. Lymphatic invasion was seen in 15 cases (93.4%), and was mostly extensive. A total mastectomy was performed for 10 cases, quadrantectomy for three cases, and lumpectomy for three cases. Lymph node dissection at the initial operation was performed in 14 cases. Lymph node metastases were seen in 13 cases (92.9%), and six of them (46.2%) showed more than 10 positive nodes. After the operation, chemotherapy was used for 13 cases, irradiation was used for five cases, and hormonal therapy was used for six cases as the adjuvant therapy. Follow up after the operation was evident for 2–204 months (mean 38 months); four cases (25.0%) were dead of disease (at 34, 56, 61, and 83 months), and three cases were alive with disease. During follow up, metastases was seen in the pleura (four cases), skin (three cases), bone

(two cases), chest wall (one case), axillary lymph node (one case), and pericardium (one case).

Table 3 shows the comparison with serially obtained IDC-NOS cases. The incidences of both high nuclear grade and poor histological grade were significantly higher in IMPCa (both $P < 0001$, respectively). The ratio of cases with positive hormone receptors was significantly low ($P < 0.05$, $P < 0.01$), but HER-2 positive cases were more frequent ($P < 0.025$). Lymph node metastases were more frequently seen, significantly, in IMPCa (13/14; 92.9%) than IDC-NOS (94/150; 65.3%) ($P < 0.05$). Additionally, half of the node positive IMPCa cases showed more than 10 positive lymph nodes, and the frequency was significantly higher than IDC-NOS ($P < 0.001$).

The results of immunohistochemistry can be seen in Figs 3,4,5,6, and the comparison between node positive control cases is summarized in Table 4. CD34 was positive in the endothelial cells of blood vessels (Fig. 3). However, neither micropapillary nests or the inner surface of empty space were positive for CD34, Factor VIII related antigen, nor type IV collagen. Invasive micropapillary carcinoma cases were more frequently positive for p53, but not statistically significant. CD34 showed a significantly increased number of blood vessels within the area of IMPCa ($P < 0.05$). Blood vessel counts by VEGF, E-cadherin MMP-2, and the Ki-67 index did not show any significant differences between the two groups. Immunohistochemical

Figure 3 Invasive micropapillary carcinoma. Vascular endothelial growth factor was positive in the cytoplasm. LSAB, ×150.

Figure 4 E-cadherin was positive in most of the cell membrane of carcinoma cells in the invasive micropapillary carcinoma cases. LSAB, ×150.

Table 3 Comparison of histopathological features between IMPCa and IDC-NOS

	IMPCa (16 cases)	IDC-NOS (150 cases)
Age (average)	50.9 years	54.1 years
Nuclear grade		
1 (Low)	0	5 (3.3%)
2 (Intermediate)	4 (25.0%)	86 (57.3%)
3 (High)*	12 (75.0%)	59 (39.3%)
Histological grade		
I (Well differentiated)	0	27 (18.0%)
II (Moderately differentiated)	3 (18.8%)	71 (47.3%)
III (Poorly differentiated)**	13 (81.3%)	52 (34.7%)
ER***		
Positive	8 (50.0%)	112 (74.3%)
Negative	8 (50.0%)	38 (24.7%)
PR****		
Positive	5 (31.2%)	97 (64.7%)
Negative	11 (68.8%)	53 (35.3%)
HER-2*****		
Positive	8 (50.0%)	33 (20.3%)
Negative	8 (50.0%)	117 (79.7%)
LN status***		
Positive	13 (92.9%)	94 (65.3%)
Negative	1 (7.1%)	50 (34.7%)
10 or more +******	7/14 (50.0%)	10/114 (6.9%)

ER, estrogen receptor; IMPCa, invasive micropapillary carcinoma; IDC-NOS, invasive ductal carcinoma, not otherwise specified; LN, lymph node; PR, progesterone receptor. *Comparison between high and non-high grade, $P < 0.001$; **comparison between III (poor) and non-III grade, $P < 0.001$; ***$P < 0.05$; ****$P < 0.01$; *****$P < 0.025$; ******$P < 0.001$.
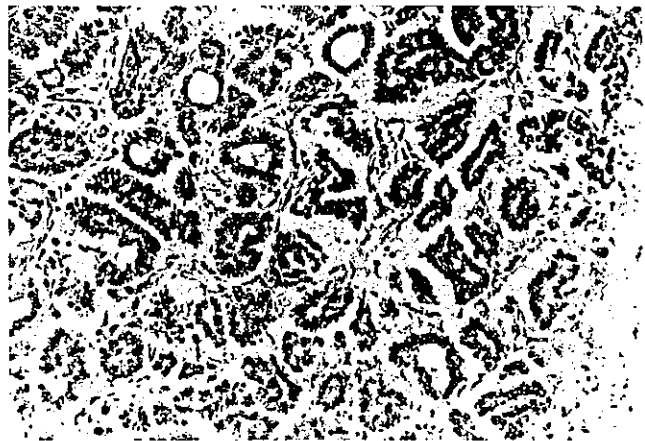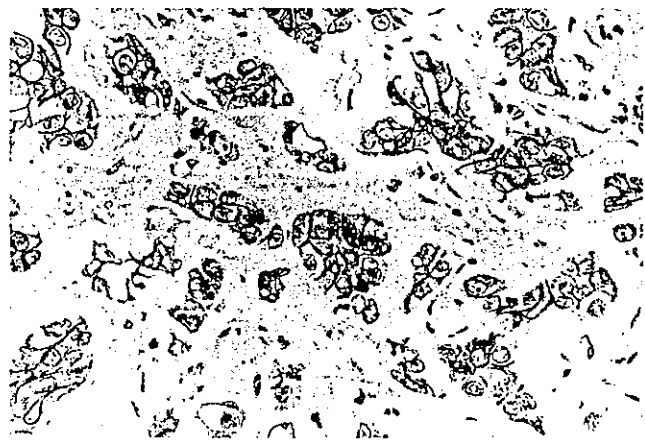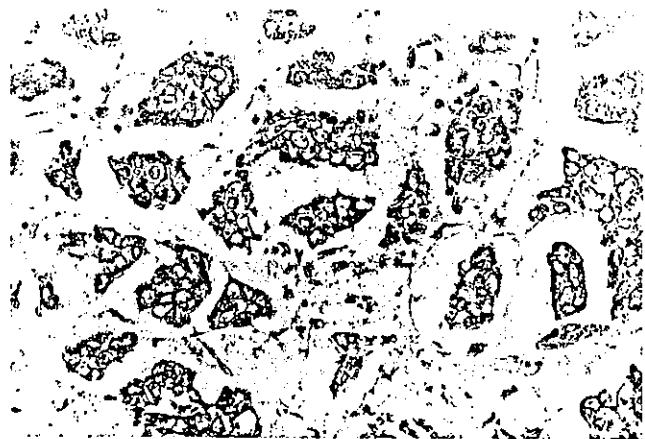

Figure 5 Matrix metalloproteinase-2 was positive in the cytoplasm of carcinoma cells in the invasive micropapillary carcinoma cases. LSAB, ×150.
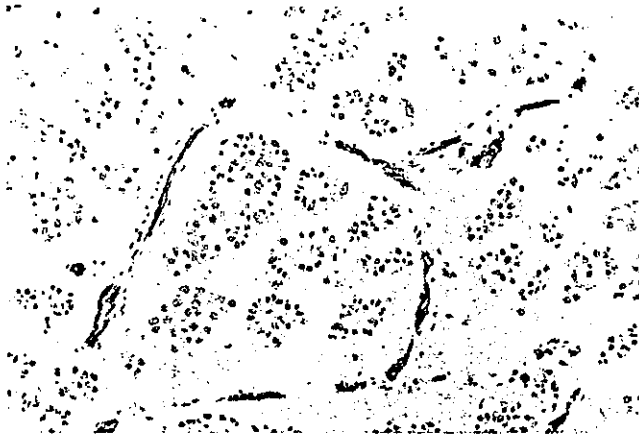
Figure 6 CD34 revealed abundant capillary-sized vessels within the intervening stroma, but the empty spaces were not surrounded by endothelial cells. LSAB, ×80.

Table 4 Immunohistochemical findings of IMPCa and node positive control cases

	IMPCa (16 cases)	n+ IDC-NOS (23 cases)
p53		
Positive	9 (56.3%)	7 (30.4%)
Negative	7 (43.7%)	16 (69.6%)
Ki-67(%)	25.1 ± 14.1	23.5 ± 14.2
CD34 (MVC/mm^2)*	63.1 ± 36.0	33.6 ± 27.8
VEGF		
Positive	16 (100%)	22 (95.7%)
Negative	0	1 (4.3%)
E-cadherin		
Positive	16 (100%)	22 (95.7%)
Negative	0	1 (4.3%)
MMP-2		
Positive	15 (93.8%)	22 (95.7%)
Negative	1 (6.2%)	1 (4.3%)

IMPCa, invasive micropapillary carcinoma; IDC-NOS, invasive ductal carcinoma, not otherwise specified; MMP-2, matrix metalloproteinase protein-2; MVC, microvessel count; n+, node positive; VEGF, vascular endothelial growth factor. *$P < 0.05$.

features were generally identical in the IMPCa and IDC-NOS areas in mixed cases.

DISCUSSION

The incidence of IMPCa in our series was 1.0% of all primary breast carcinomas. It was much lower than any other previous studies, which showed 3.4%, 6%, and 7.6%, respectively.[6,7,14] One of the possible reasons for this low incidence is that the minimal requirement of the diagnostic criteria, namely the proportion of IMPCa in mixed-type cases, is different among the reported articles. Indeed, the incidence of pure type was reported as 1.7%,[8] and 0.8%,[14] which was not much different from the present study (0.6%). Additionally, several studies have stated that the presence of the

IMPCa pattern within the invasive breast carcinoma, regardless of the proportion, shows the unfavorable nature of the tumor.[7,14,15]

Histologically, it is not difficult to notice this characteristic subtype of breast carcinoma. They are a variant of invasive ductal carcinoma, and a frequent association of intraductal carcinoma (10 of 16 in our series). E-cadherin, a marker of ductal carcinoma,[16] was consistently positive. Furthermore, they showed a high incidence of high nuclear/histological grade, both of which were more frequent, and statistically significant, than IDC-NOS, as in previous studies.[4,6,8,14] Immunohistochemical findings supported these features, with a tendency for a lower incidence of estrogen receptor (ER)/ progesterone receptor (PR) positivity and a higher incidence of HER-2 positivity, compared to IDC-NOS. Previous studies reported a relatively high frequency of hormone receptor positivity in IMPCa (i.e. approximately 70% are ER positive by two authors[3,14]), but that might be associated with the staining procedure and/or counting methods. The proportions of the positive cases for c-erbB-2 (36.3%) and p53 (12.1%) were reported in one manuscript.[3]

One of the unique characteristics of this tumor type is a frequent association with lymph node metastases, especially with a large number of positive nodes.[7,15] Frequent (15/16 in the present series) and massive lymphatic vessel invasion is also revealed.[15] Lymph node metastases were not associated with the proportion of the IMPCa area within the tumor,[2,14,15] and frequently seen in cases of smaller tumors,[6] most likely because the events of lymphatic invasion occur earlier. Empty spaces, surrounding the micropapillae of carcinoma cell nests, were not surrounded by endothelial cells (CD34 and Factor VIII related antigen were totally negative in our series), a basement membrane (type IV collagen was totally negative), or epithelial cells. They were surrounded by fibrocollagenous stroma with spindle shaped stromal cells.[2,7] The empty spaces were not lymphatic vessels, and not seen in frozen sections,[5,7] and, thus, considered as an artifact at the time of fixation. The spaces may resemble peudoangiomatous stromal hyperplasia (PASH).[16] However, the association of steroid hormones (progesterone), frequently detected in the cases of PASH, were not seen considerably in IMPCa because the incidence of the presence of hormone receptors was relatively low. Additionally, IMPCa will occur in any ages (average 50.9 years old), and it is not like premenopausal deviation in PASH cases. Although the empty spaces themselves may exist in the same areas, the pathogenesis will be totally different between the two diseases.

These findings may suggest that IMPCa morphology is correlated with aggressive behavior of tumors, especially for metastatic potential. As IMPCa in general show a high frequency of lymph node metastasis, we have compared IMPCa with node-positive IDC-NOS without an IMPCa pattern. However, there were no significant differences of staining results

for p53, Ki-67, VEGF, E-cadherin and MMP-2 between the two groups. Hence, it is still unclear whether IMPCa histology is one of the significant unfavorable features among carcinomas with lymph node metastases. However, IMPCa showed significantly large numbers of small vasculatures within the stroma between the empty spaces, by microvessel densities using CD34 immunostains. Large numbers of small vasculatures will be associated with blood-borne distant metastases. In actual fact, IMPCa frequently express bone, lung and/or liver metastases.[7] Hence, the large numbers of small vasculatures may be one of the strong prognostic indicators of IMPCa, which has not been previously elucidated.

Finally, it is very interesting to investigate what kinds of findings are strongly associated with a poorer prognosis in these patients. As mentioned above, the proportion of the IMPCa area in a single mass may not affect the prognosis. Even the smaller tumors, less than 1 cm or even less than 0.5 cm, may show extensive lymph node metastases,[6] as in the present study, case 4 (8 mm in maximum diameter, lymph node status 27/27). Blood-borne metastases may also be important. Cases that died from IMPCa in the present series were of various ages (37, 38, 43 and 65 years) and sizes (8, 25, 32 and 37 mm), with high nuclear grade and poor differentiation. Positive lymph nodes for metastases were surprisingly high in number (8, 18, 25, and 27). Immunohistochemical profiles were variable, and specific features were not evident (data not shown). Some authors have estimated that negativity for estrogen receptors, more than four positive nodes, and high mitotic activity were of prognostic significances.[8,9] Although we did not analyse pure and mixed subtypes separately, there were three pure and one mixed IMPCa cases that were dead of disease. The significant differences between pure and mixed type, and whether the presence of a minor proportion of IMPCa is an unfavorable factor, still seems to be controversial. There are several possibilities for the explanation of the significant biological differences according to the proportion of IMPCa (within the tumor). One is that the aggressive behavior is associated with the total volume of IMPCa, regardless of the proportion. Another is that the non-IMPCa area of mixed cases may have the same aggressive manner with IMPCa areas, and the presence of IMPCa in any area is an unfavorable sign. However, the number of analyzed cases and the periods of follow up were limited, and uni- or multivariate analysis was not always significant, so further investigations are necessary for final conclusions. Tentatively, we consider that the IMPCa histology itself will be a strong indicator of the aggressive behavior of the carcinoma.

In conclusion, IMPCa itself, in any amount, should be considered as a poor prognostic sign of invasive breast carcinoma. The IMPCa may at least be more aggressive than IDC-NOS, and show significantly higher vasculature than node-positive IDC-NOS, according to the results of the present study. As these tumors show distant, blood-borne metastases, high vasculature in the intervening stroma is important, as well as their extensive lymphatic spread.

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Isolation of Temperature-sensitive p53 Mutations from a Comprehensive Missense Mutation Library*

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Temperature-sensitive (ts) mutations have been used as a genetic and molecular tool to study the functions of many gene products. Each ts mutant protein may contain a temperature-dependent intramolecular mechanism such as ts conformational change. To identify key ts structural elements controlling the protein function, we screened ts mutants from a comprehensive mutation library consisting of 2,314 p53 missense mutations for their sequence-specific transactivity through p53-binding sequences in *Saccharomyces cerevisiae*. We isolated 142 ts p53 mutants, including 131 unreported ts mutants. These mutants clustered in β-strands in the DNA-binding domain, particularly in one of the two β-sheets of the protein, and 15 residues (Thr^{155}, Arg^{158}, Met^{160}, Ala^{161}, Val^{172}, His^{214}, Ser^{215}, Pro^{223}, Thr^{231}, Thr^{253}, Ile^{254}, Thr^{256}, Ser^{260}, Glu^{271}, and Glu^{285}) were ts hot spots. Among the 142 mutants, 54 were examined further in human osteosarcoma Saos-2 cells, and it was confirmed that 89% of the mutants were also ts in mammalian cells. The ts mutants represented distinct ts transactivities for the p53 binding sequences and a distinct epitope expression pattern for conformation-specific anti-p53 antibodies. These results indicated that the intramolecular β-sheet in the core DNA-binding domain of p53 was a key structural element controlling the protein function and provided a clue for finding a molecular mechanism that enables the rescue of the mutant p53 function.

p53 tumor suppressor is a 393-amino acid transcription factor that activates the transcription of a number of downstream genes through p53 binding to two copies of the specific consensus DNA sequence 5'-RRRC(A/T)(T/A)GYYY-3' (in which R is a purine nucleoside and Y is a pyrimidine nucleoside) in their regulatory regions (1). These molecular switches are activated by post-translational modifications, including phosphorylation, acetylation, and prolyl isomerization (2–5) of p53 in response to genotoxic or non-genotoxic stresses. The resulting biological effects are cell cycle arrest, apoptosis, DNA repair, and angiogenesis (6–10). A growing number of p53 downstream genes have been isolated, and p53 has been structurally and func-

tionally divided into three portions, namely the NH$_2$-terminal portion containing the transactivation domain, the central core portion corresponding to the DNA-binding domain, and the COOH-terminal portion containing the oligomerization domain. The evolution of the DNA-binding domain is highly conserved in p53 orthologues (11) and also in the conserved human homologues p63 and p73 (12, 13).

The structure of the DNA-binding domain (residues 94–312) was resolved by x-ray crystallography (14). The domain consists of two α-helixes (H1 and H2) and 11 β-strands (S1, S2, S2', and S3–S10) that were interconnected by loops (long L1–L3 loops and other short loops). Two anti-parallel β-sheets containing four (S1, S3, S5, and S8) and five (S4, S6, S7, S9, and S10) β-strands make up a large β-sandwich that serves as a scaffold for a loop-sheet-helix (LSH) motif (L1, S2, S2', S10, and H2) and two large loops (L2 and L3). The loop-sheet-helix consists of two separate regions as follows: (i) the L1 loop (residues 113–123) and the S2-S2' β-hairpin (residues 124–135) that correspond to evolutionary conserved region II (residues 117–142) (11); and (ii) the end of the S10 strand (residues 264–274) and the H2 helix (residues 278–286) that correspond to conserved region V (residues 270–286). In the loop-sheet-helix, the L1 loop and the H2 helix contact with a DNA major groove formed by the RRRC region of the consensus sequence. One of the large loops, the L2 (residues 164–194), is interrupted by a short helix (H1) and contains conserved region III (residues 171–181). Another large loop, L3 (residues 237–250), coincides with conserved region IV (residues 234–258) and makes contact with the DNA minor groove formed by the A/T rich region of the consensus sequence. The L2 loop stabilizes the L3 loop by packing through a side-chain interaction and a zinc atom tetrahedrally coordinated on residues Cys^{176}, His^{179} of the L2 loop and Cys^{238} and Cys^{242} of the L3 loop.

Mutations in the *TP53* gene are the most frequent genetic alterations in the various human tumors (15). According to the latest TP53 mutation databases (16, 17), more than 15,000 somatic mutations have been reported to date. The mutations are clustered in the DNA-binding domain, and the majority (~80%) are missense mutations. Among tumor-derived mutations, those at residues Arg^{175}, Gly^{245}, Arg^{248}, Arg^{249}, Arg^{273}, and Arg^{282} have frequently been reported, and all missense mutations were unable to bind the specific p53 binding sequences and the inactive transactivation for downstream genes. These are structurally important residues, because they directly involve DNA binding or stabilization of the L2 and L3 loops of the protein. However, the majority of remaining missense mutations have not yet been examined. Recently, we constructed 2,314 missense mutations that covered almost all of the tumor derived missense mutations, as well as a number

This paper is available on line at http://www.jbc.org

of previously unreported missense mutations, and examined their ability to transactivate marker genes through distinct p53 binding sites when the mutants were expressed in yeast. We determined the functional effect of each mutant p53 and found that the p53 function correlated well with the structure and mutations (18).

Temperature-sensitive (ts)[1] p53 mutations have been reported and used as tools for conditional p53 expression in mammalian cells. We identified previously four distinct ts p53 mutations in eight of the 91 human tumor cell lines using a yeast-based transcription assay and predicted that 5–10% of the tumor-derived missense mutations should be ts mutations (19). To date, 61 p53 ts mutations have been isolated by using several different methods, including a yeast-based functional assay (Table I). Among these, the V272M ts mutant was reactivated by a small molecule, aminothiol WR1065 (20), at a non-permissive temperature, suggesting that ts mutants may be functionally rescued by small molecules.

The purpose of this study was the screening and isolation of a large number of ts mutations from a comprehensive missense mutation library, mapping them to the p53 structure, and considering the function-structure relationship through the ts mutants. To isolate a number of ts p53 mutations, we screened the p53 library containing 2,314 p53 missense mutations using a yeast-based p53 functional assay and found 142 ts p53 mutants, including previously unreported 131 mutants. We confirmed that most were also ts in p53-less mammalian cells. The ts mutants were preferentially mapped on one of the β-sheets, and there were hot spot sites for ts mutations. Because a fairly significant fraction of the p53 mutants in the *TP53* mutation databases were ts mutants, these ts p53 mutant proteins may be novel molecular targets through the ts mechanism and structure-dependent restoration of p53 function.

EXPERIMENTAL PROCEDURES

p53 Missense Mutation Library—2,314 p53 missense mutations were constructed recently through a 96-well, formatted, site-directed mutagenesis and stably expressed in a haploid yeast strain harboring a p53-responsive *p21[WAF1]* reporter plasmid (pAS03G) (21) or in diploid yeast strains harboring p53-responsive reporter plasmids with a *MDM2* promoter or p53 binding sequences derived from *BAX* (pKS07R), *14-3-3σ* (pKS09R), *p53AIP1* (pKS11R), *GADD45* (pKS13R), *Noxa* (pKS15R), and *p53R2* (pKS17R) as described previously (18).

Screening ts p53 Mutants Using a Yeast Assay—The 2,314 yeast clones expressing the mutant p53 were grown on 25 96-well formatted plates containing synthetic complete (SC) media lacking leucine and tryptophane (SC −Leu −Trp) in the case of the haploid strains, or SC media lacking leucine, tryptophane and histidine (SC −Leu −Trp −His) in the case of the diploid strains.

Fluorescent Intensity—To evaluate the transactivity of each mutant p53 quantitatively, the yeast clones (haploid cells) were replicated on SC −Leu −Trp solid media using a 96-pin replicator and grown at 37 or 32 °C for 2 days. The plates were then directly processed in a 96-well formatted fluorometer (Fluoroskan Ascent FL, Labsystems) to measure the fluorescent intensity (excitation, 485 nm; emission, 538 nm) of p53-dependent enhanced green fluorescent protein expression through a human *p21[WAF1]*-derived p53 binding sequence. The diploid cells, selected by mating reaction, were incubated on SC −Leu −Trp −His plates at 37 or 30 °C for 2 days, and the fluorescent intensity of Ds-Red was measured using the same fluorometer (excitation, 544 nm; emission, 590 nm) to evaluate the p53-dependent Ds-Red expression through other p53-binding sequences. At least two independent experiments were performed for each reporter, and the fluorescence intensities were averaged. The averaged values were standardized in each p53 binding sequence, clustered, and visualized using the CLUSTER and TREEVIEW programs. The standardized data were also spotted on a two-dimensional graph for 30 and 37 °C. We defined the following criteria to select ts mutants from the p53 mutant library, namely $M_{30}/W_{30} \geq 0.7$, $M_{37}/W_{37} \leq 0.5$, and $M_{30}/M_{37} \geq 2$, where M_{30} and M_{37}

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[1] The abbreviations used are: ts, temperature-sensitive; SC, synthetic complete; HRP, horseradish peroxidase.

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indicate the fluorescent intensities of the p53 mutants at 30 and 37 °C, respectively, and W_{30} and W_{37} indicate the fluorescent intensities of the wild-type p53 at 30 and 37 °C, respectively.

Cell Culture and Transfection—A TP53-deficient human osteosarcoma cell line, Saos-2, was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (JRH Biosciences) in the presence of 5% CO_2. For luciferase assays, the cells were grown to 60–90% confluence in 96-well tissue culture plates at 37 °C and then cultured at 32 or 37 °C for another 24 h. For immunoprecipitation, the cells were grown in 90 × 20-mm tissue culture plates at 37 °C in the presence of 5% CO_2 and further incubated at 32 or 37 °C for another 18 h. Transient transfections were performed using the Effectene (Qiagen) transfection reagent. For luciferase assays, the cells were co-transfected with 12.5–50 ng of the expression vector (pCR259-p53WT, pCR259-p53MT, or a p53-less control pCR259 vector) (18) and 50–87.5 ng of the p53-responsive luciferase plasmid (p21Ps-luc, pMDMPs-luc, pB-AXPs-luc, pSIGMAPs-luc p53R2Ps-luc, or p53GADD45Ps-luc) (18, 21) and incubated for a further 24 h. For immunoprecipitation, the cells were transfected with 2 µg of the expression vector (pCR259-p53WT, pCR259-p53MT, or a control pCR259 vector) and further incubated for 36 h.

Luciferase Assay—After 24 h of transfection, luciferin (Steady-Glo luciferase assay system, Promega), a substrate of luciferase, was added to the culture media and further incubated for 60–120 min according to the manufacturer's instructions. The fluorescent intensity was measured using the Fluoroskan Ascent FL (see above). The relative fluorescent intensity to the wild-type control was calculated from three sets of independent experimental data at 32 and 37 °C. The value differences at the two temperatures were statistically evaluated by *t* test. The ts mutants were defined when the *p* value was <0.001.

Immunoprecipitation and Immunoblotting of p53—Saos-2 cell lysates were prepared in 100 µl of NET buffer (150 mM NaCl, 50 mM Tris-HCl (pH8.0), 5 mM EDTA, and 1% Nonidet P-40) containing 0.1 µg/µl phenylmethylsulfonyl fluoride. Fifty microliters of the cell lysates were immunoprecipitated with 10 µl of the PAb1620 (Ab-5; Oncogene) or the PAb240 (Ab-3; Oncogene) monoclonal antibody against human p53. The lysates, with 8 µl of the crude lysate, were fractionated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to Optitran BA-S83 membranes (Schleicher & Schuell), and the expressed p53 mutants were detected using a HRP-conjugate anti-p53 antibody (p53(FL393)HRP, Santa Cruz Biotechnology). The proteins were visualized and quantitatively analyzed using an ECL Western blotting detection system (Amersham Biosciences), a lumino-image analyzer (LAS1000, Fuji Film) and 1D image analysis software (Kodak Digital Science).

Drawing p53 Peptide Structures—To map the ts p53 mutants on the p53 core domain, the NCBI structure file, 1TUP, was customized for our purpose and visualized using Cn3D 4.0 software (22).

RESULTS

Clustering of 2,314 Mutations on Transactivities at Two Distinct Temperatures—An unsupervised, hierarchical one-dimensional cluster analysis allowed us to cluster the 2,314 p53 mutants on the basis of similar measured transactivities for eight distinct p53 binding sequences (p53 binding sites) at 30 and 37 °C (Fig. 1A). The mutants are divided into two major clusters. In one of these clusters the mutants retain transactivities; in the other they lose activity, and these clusters are mostly temperature-independent. Notably, there is one temperature-dependent sub-cluster within the latter cluster (Fig. 1B). The cluster consists of 64 p53 mutants, and the transactivities of the mutants are inactive on almost all p53 binding sites at 37 °C but active on some p53 binding sites at 30 °C, indicating that a large number of mutants are ts for transactivation in yeast cells.

Isolation of ts p53 Mutants in Yeast—Although the cluster analysis found the typical ts mutants that represent temperature sensitivity for most p53 binding sites, there are mutants that show temperature sensitivity on limited types of p53 binding sites and, therefore, are not clustered. To also isolate such clones, the transactivities of the 2,314 mutant clones at 30 and 37 °C were standardized and overviewed by a scatter plot for each p53 binding site (Fig. 2). Among the 18,512 data points (8 × 2,314 clones), the majority had similar transcriptional