Prognostic Index (VNPI), which is the sum of grade, extent, and margin status, correlates well with the risk for local recurrence[50]. However, the overall survival, at least 5- or 10-years later, were not significantly different. Additionally, the ratio of invasive recurrences among all recurrent cases (almost half of the cases, as in similar results in Japanese patients[51]) was not be influenced by the VNPI.

Other pathological parameters are detected by special techniques, but they are not always performed in routine practice. The Ki-67 index, hormone receptor status (ER/PgR), HER-2/neu status, and p53 expression status are shown in Table 4. Many articles have examined the overexpression of c-erbB-2 (HER-2/neu) in high-grade DCIS, and the frequency of overexpression was higher than that of invasive ductal carcinomas.

Finally, lymph node metastases of DCIS are reported in almost 0%, or 1-2% of the cases, as mentioned previously. The method of pathological examination, rather than underestimation of microinvasion, may be the cause of the unexpected metastasis. Most node positive DCISs are higher-grade lesions.

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Symposium

Breast Conserving Surgery with Primary Volume Replacement using a Lateral Tissue Flap

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Breast conserving surgery (BCS) is now a standard surgical treatment for early breast cancer. The number of patients with tumors under 3 cm who underwent breast conserving surgery overtook the number of patients who underwent total mastectomy for the first time in Japan in 2003. We have been employing breast conserving surgery with primary reconstruction using a lateral tissue flap (LTF), and have performed breast conserving surgery for 266 patients from 1990 to 2002. The incidence of local relapse was 5.6%. Although we did not irradiate a low risk group of 101 patients, our method is not inferior to other reports in which all cases underwent irradiation. Primary reconstruction with LTF has three advantages. The first is that we can avoid poly-surgery for breast reconstruction. The second is that the volume of the graft is maintained longer than reconstruction with a musculo-cutaneous flap. The third is that patients can avoid allergic reactions or granulomas as seen with artificial prosthesies. In conclusion, breast conserving surgery with immediate volume replacement with a LTF is a reasonable surgical procedure and has the advantage of avoiding unnecessary surgical procedures for reconstruction and surgical invasion without delaying the diagnosis of local relapse. Moreover, an adequate assessment of risk can spare low risk groups irradiation.

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Key words: Breast conserving surgery, Lateral tissue flap

The incidence of breast cancer is the greatest of all female malignancies since 1991 and is still increasing in Japan. The mortality rate reached the third highest after lung cancer and gastric cancer in 1998. Patient demand helped develop breast conserving surgery (BCS) as a local treatment to maximize prognosis and cosmesis.

Breast conserving surgery was established as a standard surgical procedure in United States and Europe in the late 1980s[1]. The number of patients with breast cancer under 3 cm in size who underwent breast conserving surgery overtook the number of patients who was underwent total mastectomy in 2003 for the first time in Japan. The coexistence of good prognosis and cosmesis is the most important advantage of BCS. To achieve these two aims, we have started quadrantectomy (Bq) based on the anatomical structure of the mammary gland since 1990[2]. Compensating for the large volume defect generated by quadrantectomy was a problem. Quadrantectomy generally requires more breast volume be excised than partial mastectomy. We employed lateral tissue flap (LTF) reconstruction to compensate for the defect resulting from quadrantectomy. LTF is composed of adipose tissue placed caudal to the axillary arch.

Primary volume replacement for BCS has the risk of delaying the diagnosis of local recurrence. Since most of local recurrences occur within 5 years, intensive local examination and periodic observation is required. In this article, we describe the method for breast conserving surgery with immediate reconstruction with a LTF, and the clinical outcomes concerning local recurrence after breast conserving surgery with primary volume replacement using LTF.

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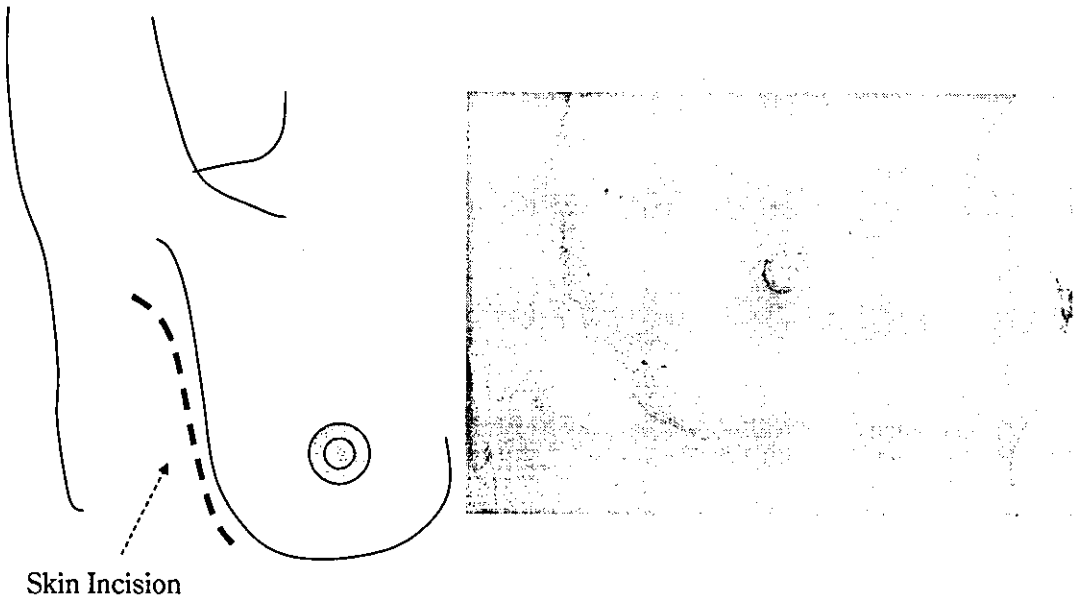

Fig 1. Lateral incision of breast conserving surgery.

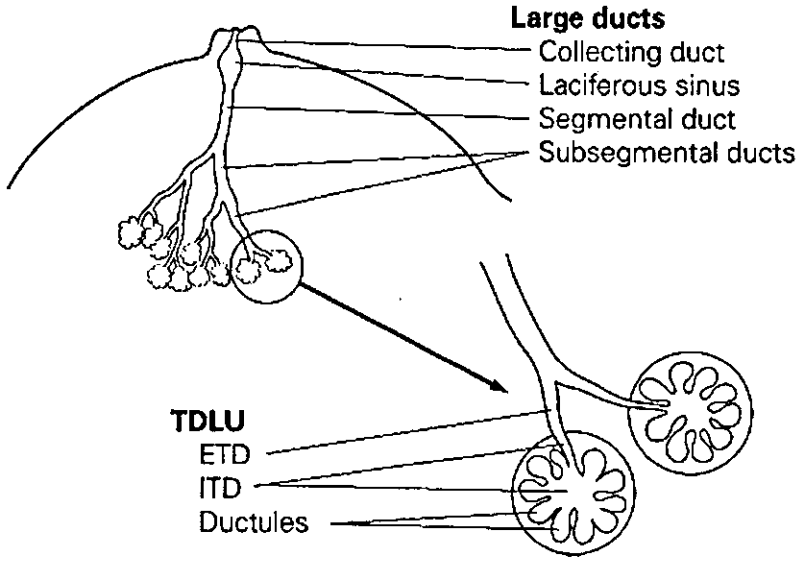

Fig 2. Duct-lobular system of the mammary gland.

Surgical Procedures

Skin Incision and Abrasion

In our surgical treatment of BCS, a sigmoid skin incision is made on the middle axillary line (lateral incision) to avoid an operative scar which can be seen from an anterior view (Fig 1). A lateral incision is made to excise tumors under 3 cm in diameter at any location to perform the quadrantectomy, volume replacement with LTF and axillary lymph node dissection.

The skin is widely peeled at the subcutaneous layer with a radio knife to allow quadrantectomy, axillary dissection and volume replacement with a single incision. Skin over the caudal area of the axillary region is also peeled back to make the LTF to correct the volume defect.

Quadrantectomy

We excise tumors using quadrantectomy for BCS. Quadrantectomy is performed based on the

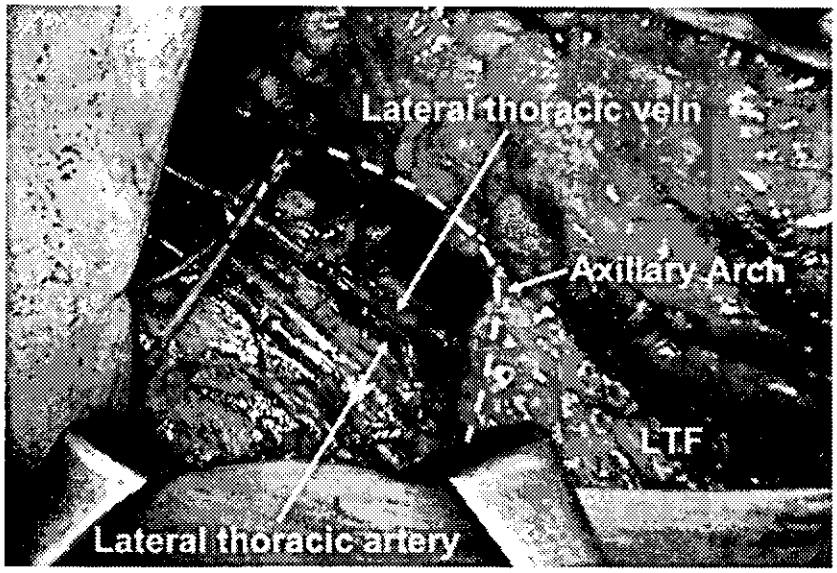

Fig 3. Lateral thoracic vein, axillary arch and the lateral tissue flap.

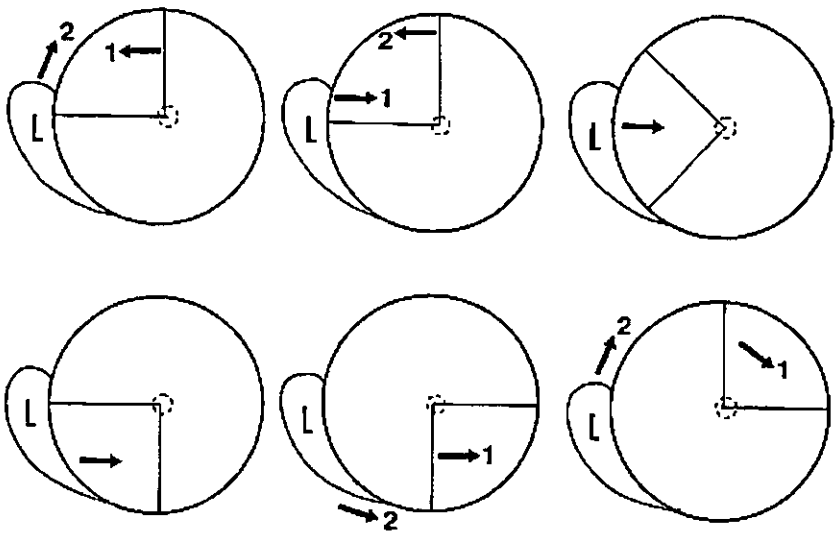

Fig 4. Rotation of the lateral tissue flap.

anatomical extension and structure of the duct-lobule system of the mammary gland to achieve complete resection of the tumor and its extension to the ductal structure (Fig 2)[2]. We perform partial mastectomy if there is no finding of tumor extension in the mammary duct. Tumor extension is evaluated by X-CT, MRI and ultrasonography.

Volume Replacement with LTF

LTF is made with adipose tissue located caudal to the axillary arch. The axillary arch is the caudal border of the axillary region, and is composed of connective tissue related to axillary fascia (Fig 3). The blood supply and drainage of the LTF is maintained by lateral thoracic vessels and communicating vessels from the latissimus dorsi. When we prepare the LTF, we preserve the communicating vessels from the latissimus dorsi and/or lateral thoracic vessels (Fig 3). To preserve these vessels is the key point to prevent necrosis of the LTF. Volume replacement is performed by rotation of the LTF. Indirect volume replacement is performed for medial volume defect in areas of A and B (Fig 4). Direct volume replacement is performed for lateral volume defect in areas of C and D (Fig 4).

Fig 5. Long-term anterior view of the breast after breast conserving surgery.

Irradiation Group and Non-irradiation Group

Irradiation of the conserved breast was performed for 165 patients. We avoided irradiation for 101 of patients at low risk of local recurrence. Low risk group was defined as pathologically n0, ly (− or 1+), without an extended intra-ductal component, with negative margins (cancer free within 5 mm of the surgical margin), without multi-centric lesions and without a contra-lateral lesion.

Incidence of Local Recurrence

We performed breast conserving surgery for 266 patients from Sep. 1990 to Dec. 2003. Fifteen patients (5.6%) had local relapse. The median observation period was 72 months. Irradation of the conserved breast was performed for 165 patients. We avoided irradiation for 101 low risk patients as noted above. In the non-irradiated patients the incidence of local relapse was 6.9%. The incidence of local relapse in the group undergoing irradiation was 4.8%. There was no statistically significant difference between irradiated and non-irradiated cases.

Cosmesis

Fig 5 shows the cosmetic features of BCT with volume replacement using LTF for the right breast. There is no conspicuous difference between either the breast. The operation scar is not seen from the front.

Discussion

We started breast conserving surgery in 1989. One of the advantages of using LTF for volume replacement is long-term maintenance of the graft. LTF does not shrink like a musculo-cutaneous flap (MC flap) using the latissimus dorsi, which shrinks because of disuse atrophy.

Volume replacement with LTF is a minimally invasive breast reconstruction technique compared with breast reconstruction with a saline bag.

The incidence of local recurrence is not inferior to that of ordinary breast conserving surgery. In our department, local recurrence is found in early stages without distant metastasis in almost all cases by careful and frequent follow up by mammography and ultrasonography. For most patients, local recurrence occurs within 5 years, we perform ultrasonography every 3-6 months for 5 years. Annual examination by mammography is also performed. The incidence of local recurrence is 5.6%. This is not inferior to the incidence reported in western countries[1, 6-9]. This is due to volume resection of the mammary gland sufficient to achieve negative margins and appropriate irradiation after estimation of risk. As Voogd *et al.* reported that an age younger than 35 years, extended intraductal component, and vascular invasion are the factors that contribute to local recurrence[10], our data suggests that appropriate risk evaluation can avoid irradiation in the low-risk group. On the other hand, local recurrence in high-risk cases with is well controlled by irradiation. We were able to reduce the rate of local relapse to as low as 4.8%. Recently, we estimated tumor extension by CT and MRI[3, 4]. When there is very limited extent of tumor, we employ partial mastectomy. These modalities also assist us in improving negative margin rates.

With breast conserving surgery followed by volume replacement with LTF, blood flow of the LTF should be well maintained. We preserve communicating branches from the latissimus dorsi and lateral thoracic vessels when we prepare the LTF. Careful lymph node dissection should be done with complete removal of lymph nodes and adipose tissue around the lateral thoracic vessels when we preserve lateral thoracic vessels so as to completely remove cancer cells (Fig 3).

The long term volume stability of the LTF is good, because adipose tissue does not shrink due

to disuse atrophy.

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

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

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Original 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

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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).[1,3,6] 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 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.

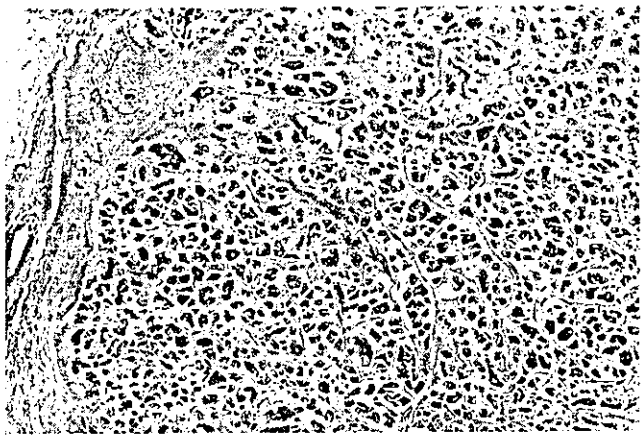

Figure 1 Invasive micropapillary carcinoma showing a micropapillary pattern of carcinoma cells floating within the empty space. HE, ×20.

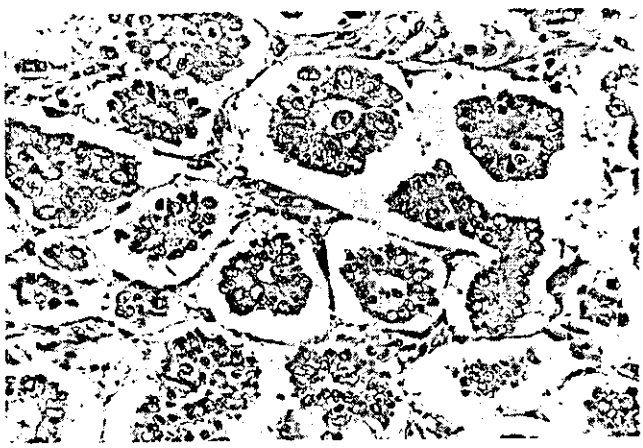

Figure 2 Pure-type invasive micropapillary carcinoma with an extensive characteristic histological pattern. HE, ×100.

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 mm^2 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

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, $P < 0.001$; ***$P < 0.05$; ****$P < 0.01$; *****$P < 0.025$; ******$P < 0.001$.

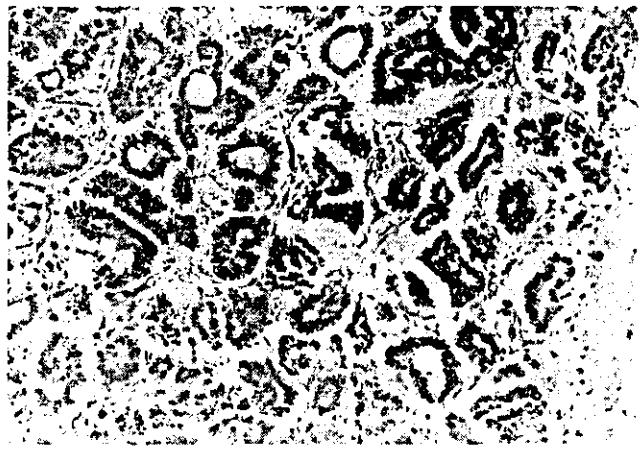

Figure 3 Invasive micropapillary carcinoma. Vascular endothelial growth factor was positive in the cytoplasm. LSAB, ×150.

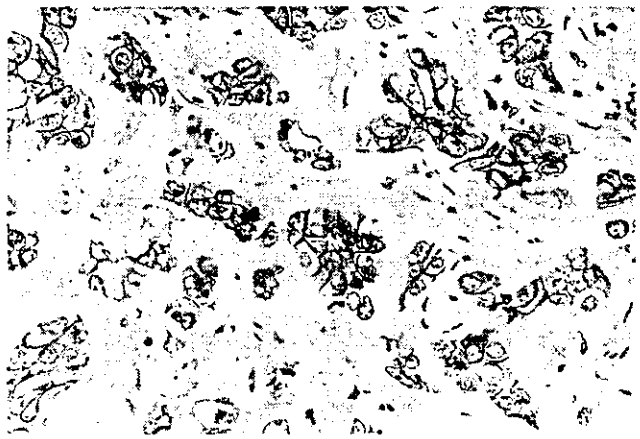

Figure 4 E-cadherin was positive in most of the cell membrane of carcinoma cells in the invasive micropapillary carcinoma cases. LSAB, ×150.

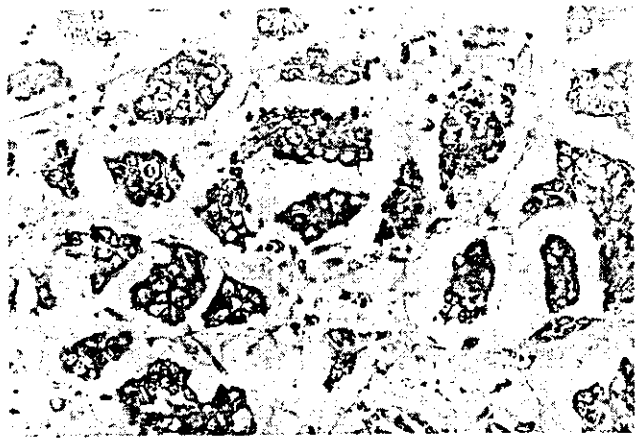

Figure 5 Matrix metalloproteinase-2 was positive in the cytoplasm of carcinoma cells in the invasive micropapillary carcinoma cases. LSAB, ×150.

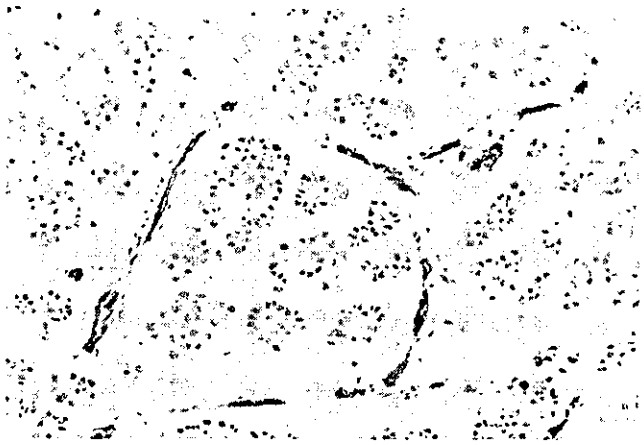

Figure 6 CD34 revealed abundant capillary-sized vessels within the intervening stroma, but the empty spaces were not surrounded by endothelial cells. LSAB, ×80.

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 p53 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 functionally 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

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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} 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 Bioscience) 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, pBAXPs-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 ID 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. 1*A*). 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. 1*B*). 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

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

TABLE I
Summary of the 61 reported p53 mutations

p53 mutant[a]	Experimental system[b]	p53BS or promoter[c]	Reference
S99F***	1	BAX, CDKN1A, PIG3	27
A119V	1	BAX, CDKN1A, PIG3	27
Y126S	1	BAX, CDKN1A, PIG3	27
Y126D	1	BAX, CDKN1A, PIG3	27
K132N	3	CON	28
K132R	3	CON	28
M133T	1	RGC	29
V135* (mouse p53)	2	CDKN1A	30, 31
A138V***	2	BAX, BCL2, CDKN1A, MDM2	32
T140Y	3	CON	28
V143A	1, 2	BAX, GADD45A, CCNG1, CDKN1A, CON, IGFBP3, MDM2, RGC	33–35
P152L***	1	BAX, CDKN1A, PIG3	27
P152T**	1	BAX, CDKN1A, PIG3	27
G154V**	2	GAL4	36
T155I**	1	BAX, CDKN1A, PIG3	27
M160I/A161T*	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
I162F	1	BAX, CDKN1A, PIG3	27
T170R	1	BAX, CDKN1A, PIG3	27
V172F***	1	RGC	29
R175K*	2	BAX, FOS, IGFBP3	37
R175I*	2	BAX, FOS, IGFBP3	37
R175P***	2	BAX, FOS, IGFBP3	37
R175Q*	2	BAX, FOS, IGFBP3	37
R175S***	2	BAX, FOS, IGFBP3	37
R175M*	2	BAX, FOS, IGFBP3	37
H179Q	2	GAL4	36
E180K	1	BAX, CDKN1A, PIG3, RGC	27, 38
R181G	1	BAX, CDKN1A, PIG3	27
R181H	1	BAX, CDKN1A, PIG3	27
H193R***	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
V197L***	1, 2	BAX, CDKN1A, FOS, TGFA, RGC	39, 40
Y205N***	1	BAX, CDKN1A, PIG3	27
H214R**	1	RGC	19
P219L***	1	BAX, CDKN1A, PIG3, RGC	27, 38
Y220C	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
Y220H	1	BAX, CDKN1A, PIG3	27
E224K	1	BAX, CDKN1A, PIG3	27
D228V	1	BAX, CDKN1A, PIG3, RGC	27, 38
Y234C**	1	RGC	19
Y234H***	1	BAX, CDKN1A, PIG3	27
M237R**	2, 3	CON, FOS, RGC	28
N239S	3	CON	28
M246V**	3	CON	28
N247I***	2, 3	CON, GAL4	28, 36
R248W	3	CON	28
P250L**	1	BAX, CDKN1A, PIG3, RGC	27, 38
L252F***	1	BAX, CDKN1A, PIG3	27
I254F**	1	BAX, CCNG1, CDKN1A, CON, GADD45A, IGFBP3, MDM2, RGC	35
T256A**	3	CON	28
D259N	1	BAX, CDKN1A, PIG3, RGC	27, 38
G266E	1	BAX, CDKN1A, PIG3	27
V272M***	1–3	BAX, CDKN1A, CON, GADD45A, MDM2, PIG3, RGC	19, 28, 38, 41
R273H	3	CON	28
R273L	1, 3	BAX, CDKN1A, CON, PIG3	27, 28
A276G	1	BAX, CDKN1A, PIG3	27
D281Y	3	CON	28
R283H***	1	BAX, CDKN1A, PIG3, RGC	38
E285K**	1	RGC	19
E286G***	1	BAX, CDKN1A, PIG3	27
E286K***	1	BAX, CDKN1A, PIG3	27
286K/287D*	1	BAX, CDKN1A, PIG3	27

[a] The meaning of the asterisk symbols used in this column is as follows: *, ts mutants not constructed in this study; **, ts mutants also isolated in this study; ***, distinct substitution(s) at the same residue were ts mutants in this study.

[b] The meaning of the numbers used in this column is as follows: 1, yeast system; 2, mammalian cell system; 3, cell-free system.

[c] All but three of the gene names used in this column refer to those used in the Online Mendelian Inheritance in Man (OMIM) site (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). The three exceptions are: CON, p53-binding consensus sequence; GAL4, yeast GAL4-binding sequence; and RGC, human ribosomal gene cluster sequence. The study on the GAL4 was performed by GAL4-binding domain and p53 fusion protein.

activity (either active or inactive) at both 30 and 37 °C, indicating that they were not ts. Obviously, there were significant numbers of p53 mutant clones that represented higher transactivity at 30 °C than at 37 °C, showing ts mutants for the transactivation function (*circled spots* in Fig. 2). On the other hand, only a limited number of clones represented higher transactivity at 37 °C than at 30 °C, showing cold-sensitive mutants. As there is no clear boundary between ts and non-ts mutants, we defined the borders for convenience as described under "Experimental Procedures." According to the definition, 142 p53 mutants were selected as ts for yeast transactivation assay (Fig. 3*A*), indicating that 6.1% (142 of 2,314) of the p53 mutants were ts for at least one of the p53 binding sites. The 142 mutants, including 131 previously unreported ts mutants,

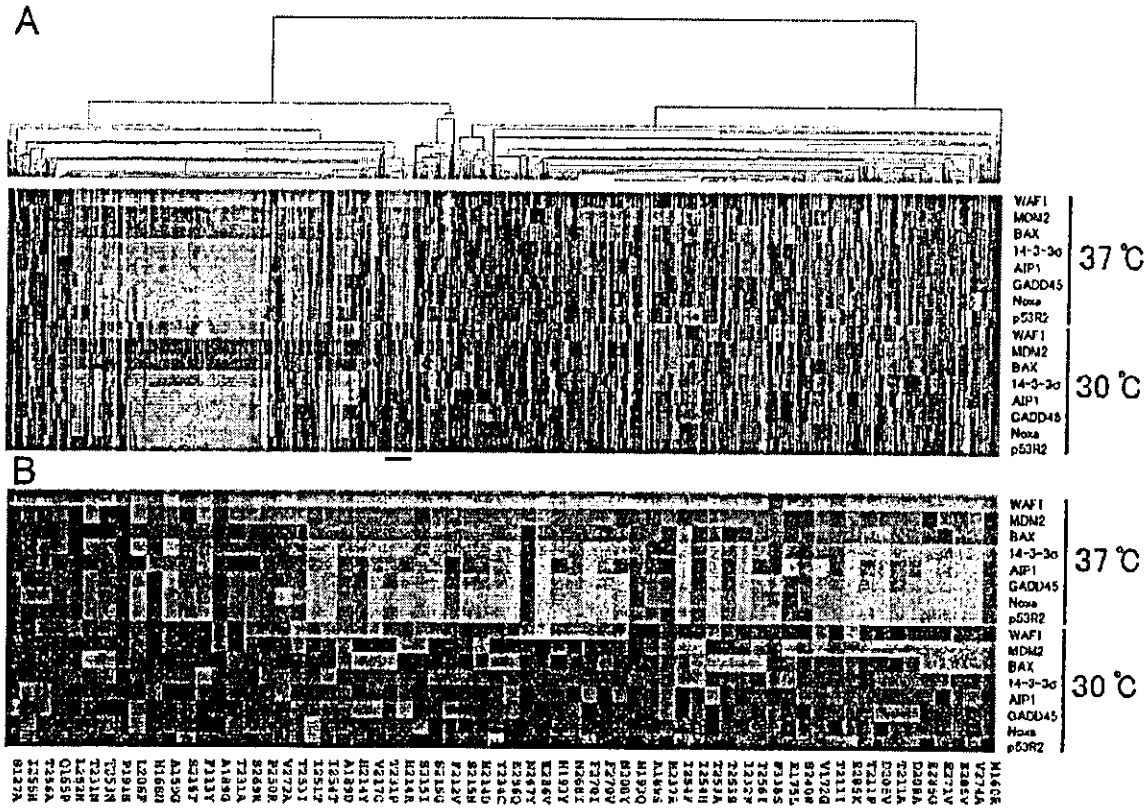

FIG. 1. **Cluster analysis of mutant p53 transactivities at 30 and 37 °C.** *A*, one-dimensional hierarchical cluster analysis of 2,314 p53 mutants. Standardized data for the indicated p53 binding sites and temperatures were shown as the color gradation of *red* (high), *green* (low), and *black* (intermediate). A ts sub-cluster was *underlined*. *B*, the ts sub-cluster containing 64 p53 mutants was magnified.

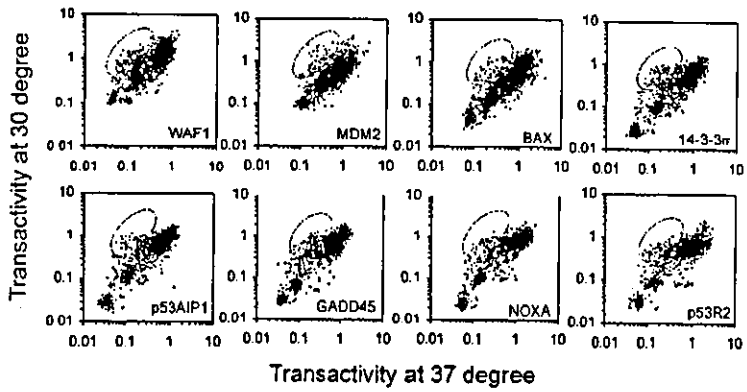

FIG. 2. **Scatter plot of the transactivities of the 2,314 p53 mutants for eight distinct p53 binding sequences at 30 and 37 °C.** Standardized data for the indicated p53 binding sites and temperatures were used. *Circled spots* contained ts clones.

were mapped on 82 residues of p53 (82 of 393; 20.9%) and 131 (92%) were within the core DNA-binding domain (residues 97–286).

Mapping of ts p53 Mutants on p53 Core DNA-binding Domain—To characterize the isolated ts mutants in the structural context, we mapped the 131 mutants on the core DNA-binding domain (Fig. 4, *A* and *B*). Among these, 70 mutants (53.4%) were mapped on the β-strands. The frequency of ts mutants in the constructed mutants differed among the 10 β-strands; the frequency was relatively higher in S4 (14 of 49, 28.6%), S7 (11 of 34, 32.3%), S9 (16 of 49, 32.7%), and S10 (15 of 61, 24.6%), whereas it was lower in S1 (1 of 17, 5.9%), S2 (1 of 24, 4.2%), S3 (1 of 33, 3.0%), S5 (2 of 22, 9.1%), S6 (2 of 24, 8.3%), and S8 (7 of 43, 16.3%). In particular, residues 158 to 161 (S4), 211 to 217 (S7), 251 to 256 (S9), and 268 to 272 (S10) were hot areas for ts mutants because there were 10 or more mutants in every four contiguous residues. Three or more ts mutants were observed in residues Thr^{155}, Arg^{158}, Met^{160}, Ala^{161}, Val172, His^{214}, Ser^{215}, Pro^{223}, Thr^{231}, Thr^{253}, Ile^{254}, Thr^{256}, Ser^{260}, Glu^{271}, and Glu^{285}, and those residues should be designated ts hot spots.

Amino Acid Substitutions of the ts Mutants—Amino acid residues before and after substitution of the ts mutant are summarized in Table II. Isoleucine (21.4%), threonine (21.1%), and tryptophane (20%) were the most frequent residues among the original p53 residues. Glycine (20%), isoleucine (18%), alanine (16.6%), leucine (16.2%), and proline (13.2%) were the most frequent residues among the residues after substitution,

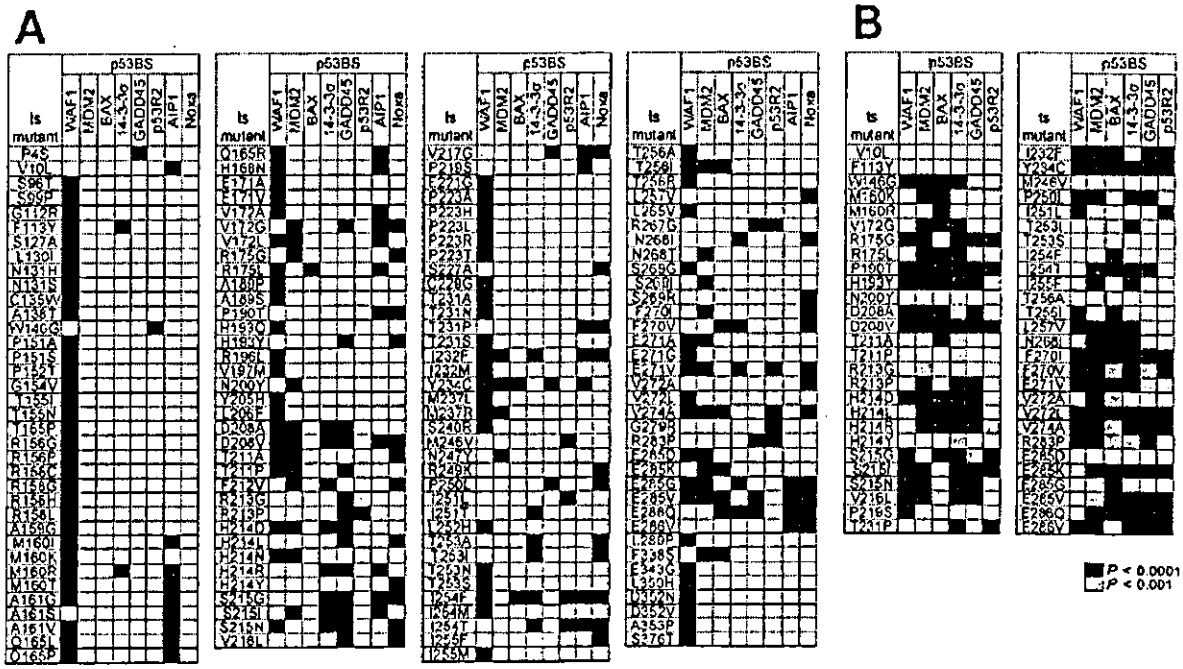

FIG. 3. **Panels of ts p53 mutants.** *A*, the ts p53 mutants isolated by yeast-based functional assay. 142 mutants were listed from the NH_2 terminus to the COOH terminus of p53. *Filled boxes* represent temperature sensitivity that satisfied the defined criteria (see "Experimental Procedures"). *B*, 54 of 142 ts mutants were examined for ts transactivities in Saos-2 cells. When, according to *t* test, the luciferase activities at 32 and 37 °C were statistically different with a *p* value <0.001 or <0.0001, the corresponding *boxes* in the panel were colored *gray* or *black*, respectively.

whereas aspartic acid (3.5%) and glutamic acid (0%) were found less frequently.

Evaluation of the ts p53 Mutants in Mammalian Cells—To evaluate whether the isolated p53 mutants in yeast were also ts for sequence-specific transactivation in mammalian cells, we randomly chose 54 p53 mutants from the 142 ts mutant p53 cDNA clones (Fig. 3*B*), and constructed expression vectors for mammalian cell experiments. Each mutant p53 was expressed in a p53-deficient human osteosarcoma cell line, Saos-2, and examined for the sequence-specific transactivation at both 32 and 37 °C by luciferase assay. When the values of the three independent experiments relative to the wild-type p53 at 32 °C were significantly ($p < 0.001$; *t* test) different from those at 37 °C in at least one of the six promoters ($p21^{WAF1}$, *MDM2*, *BAX*, *14-3-3σ*, *p53R2*, and *GADD45*), the mutant clone was defined as a ts mutant in mammalian cells. Among the 54 mutants, 48 (89%) were ts mutants in at least one of the six promoters. The results indicated that most ts mutants isolated in the yeast assay are also ts mutants in mammalian cells, suggesting that many of the remaining 88 clones may also be ts mutants in mammalian cells. Among the 48 clones, 16 were ts in all 6 promoters, whereas 32 clones were ts in a limited number of promoters, although many retained weak ts phenotypes for other promoters (data not shown).

Epitope Analyses of the p53 Protein Expressed in Saos-2 Cells Using Conformation-sensitive Antibodies—To examine whether the ts mutants display ts changes in their epitopes against conformation-sensitive antibodies, PAb1620 for wild-type-like conformation and PAb240 for denatured mutant conformation, six randomly selected ts mutants, M160R, H193Y, T211A, P219S, T253I, and V274A, were expressed in Saos-2 cells at both 32 and 37 °C. The cell lysates were immunoprecipitated using the two antibodies, detected by Western blot analysis using an HRP-conjugated anti-p53 antibody, and quantitatively analyzed using a lumino-image analyzer. In the case of wild-type p53, the PAb1620 epitope was exclusive, and only a trace of the PAb240 epitope was detected (Fig. 5*A*). Similar to wild-type p53, the PAb1620 epitope was dominant in R273H, although the PAb240 epitope was also detected. On the other hand, the PAb240 epitope was dominant, and the PAb1620 epitope was less abundant in R175H. The ratios of the epitope expressions of PAb1620 to PAb240 are shown in Fig. 5*B*. R175H and R273H were not ts because there were no significant differences in the ratios between 32 and 37 °C. Among the ts mutants, P219S and T253I showed an obvious ts increase in ratio. The remaining ts mutants showed no change or only a slight change in ratio.

DISCUSSION

Comparing 142 ts p53 Mutants with the Previously Reported p53 Mutant—Among the 142 ts mutants, 131 were previously unreported mutants. In our survey of previous papers, including our own, 61 human ts p53 mutants have been reported (Table I). These obviously include ts mutants not isolated in our system. We speculate that there are two reasons for the discrepancy. First, they were isolated using experimental systems different from those in our study, including a reporter assay for sequence-specific transactivation in mammalian cells, similar yeast assays with different p53 binding sites, an electrophoretic mobility shift assay (EMSA) in a cell-free system, and monitoring changes in structure-sensitive antibody reactivity. Therefore, it is possible that there are many potential ts mutants not isolated by the method adopted in this study. For example, a known ts mutant, V143A, did not appear as ts in the yeast cells because the ts phenotype may be mediated by ts interaction with human ASPP2 (p53BP2), a positive modulator of p53 transactivation (23, 24) that does not exist in yeast cells. Obviously, there may be mechanisms not directly affecting p53 binding to DNA. We are now planning to screen such novel ts mutants by using protein-protein interactions that may modify