**Table 1** Univariate analyses for identifying factors that are significantly different among patients with IDC, ILC, or MPC

| No. of patients (%) | | | |
|---|---|---|---|
| Factors | IDC | ILC | *P* values; a, b, c |
| | | | MPC |
| Age, y | | | a, .002; b, .605; c, .033 |
| ≤39 | 655 (11) | 15 (5) | 6 (13) |
| >39 | 5481 (89) | 286 (95) | 40 (87) |
| Total | 6136 | 301 | 46 |
| Neoadjuvant therapy | | | a, .572; b, .023; c, .019 |
| No | 1572 (77) | 83 (75) | 42 (91) |
| Yes | 467 (23) | 28 (25) | 4 (9) |
| Total | 2039 | 111 | 46 |
| Adjuvant therapy | | | a, .097; b, <.001; c, <.001 |
| No | 547 (24) | 21 (17) | 28 (61) |
| Yes | 1756 (76) | 101 (83) | 18 (39) |
| Total | 2303 | 122 | 46 |
| ER | | | a, .085; b, <.001; c, <.001 |
| Negative | 615 (28) | 25 (21) | 21 (100) |
| Positive | 1577 (72) | 95 (79) | 0 |
| Total | 2192 | 120 | 21 |
| PR | | | a, .725; b, <.001; c, <.001 |
| Negative | 715 (33) | 41 (34) | 21 (100) |
| Positive | 1477 (67) | 79 (66) | 0 |
| Total | 2192 | 120 | 21 |
| HER2 category (0, 1 *vs* 2, 3) | | | a, .017; b, .052; c, .313 |
| 0 or 1 | 1799 (81) | 107 (90) | 25 (96) |
| 2 | 189 (9) | 6 (5) | 0 |
| 3 | 226 (10) | 6 (5) | 1 (4) |
| Total | 2214 | 119 | 26 |
| Invasive tumor size (mm) | | | a, <.001; b, .090; c, .804 |
| ≤20 | 2214 (41) | 83 (30) | 13 (28) |
| >20 | 3242 (59) | 193 (70) | 33 (72) |
| Total | 5456 | 276 | 46 |
| Skin invasion | | | a, .069; b, .037; c, .292 |
| Absent | 5002 (92) | 247 (89) | 37 (84) |
| Present | 407 (8) | 29 (11) | 7 (16) |
| Total | 5409 | 276 | 44 |
| Lymph vessel invasion | | | a, <.001; b, <.001; c, .001 |
| Absent | 2848 (47) | 178 (60) | 41 (89) |
| Present | 3160 (53) | 118 (40) | 5 (11) |
| Total | 6008 | 296 | 46 |
| Blood vessel invasion | | | a, .051; b, .230; c, .597 |
| Absent | 5589 (93) | 285 (96) | 45 (98) |
| Present | 393 (7) | 11 (4) | 1 (2) |
| Total | 5983 | 296 | 46 |
| Lymph node metastasis | | | a, .963; b, .461; c, .499 |
| Absent | 3716 (60) | 183 (61) | 29 (66) |
| Present | 2430 (40) | 119 (39) | 15 (34) |
| Total | 6147 | 302 | 44 |

NOTE. *P* value a, IDC *vs* ILC; *P* value b, IDC *vs* MPC; *P* value c, ILC *vs* MPC.

were completed by 2 or 3 pathologists per case at the time of treatment: (1) skin invasion (absent, present), (2) lymph vessel invasion (absent, present), (3) blood vessel invasion (absent, present), and (4) lymph node metastasis (absent, present).

## 2.4. Histologic examination of MPCs

Serial sections of each MPC tumor were cut from paraffin blocks. One section from each tumor was stained with hematoxylin and eosin and examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following 14 histologic features of primary invasive MPCs were evaluated, and several of these histologic features (numbers 7 to 14) were evaluated according to the WHO classification [1] : (1) invasive tumor size (≤20, >20 to ≤50, >50 mm), (2) skin invasion (absent, present), (3) histologic grade (1, 2, 3; only for carcinoma component) [20], (4) number of mitotic figures in 10 high-power-fields, (5) lymph vessel invasion (absent, present), (6) blood vessel invasion (absent, present), (7) tumor necrosis (absent/≤30%, >30%), (8) MPC type (epithelial, mixed), (9) squamous cell carcinoma versus other types of carcinoma (Fig. 1A), (10) adenocarcinoma with spindle cell differentiation versus other types of carcinoma, (11) adenosquamous carcinoma versus other types of carcinoma (Fig. 1B), (12) carcinoma with chondroid metaplasia versus other types of carcinoma (Fig. 1C), (13) carcinoma with osseous metaplasia versus other types of carcinoma, and (14) carcinosarcoma versus other types of carcinoma (Fig. 1E). The following 7 histologic features of MPCs metastasizing in lymph nodes were evaluated: (1) histologic grade (1, 2, 3; only for

carcinoma component) [20], (2) extranodal invasion (absent, present), (3) squamous cell carcinoma in lymph node-metastatic tumors (absent, present) (Fig. 1D), (4) adenocarcinoma with spindle cell differentiation in lymph node-metastatic tumors (absent, present), (5) adenosquamous cell carcinoma in lymph node-metastatic tumors (absent, present), (6) carcinosarcoma in lymph node-metastatic tumors (absent, present), and (7) tumor stroma in lymph node-metastatic tumors (none, mild, moderate, severe). *Extranodal invasion* was defined as the extension of tumor cells through the capsule of at least one lymph node into the perinodal adipose tissue. Nuclear atypia, structural atypia, and the number of mitotic figures were evaluated in the same manner as for the primary invasive tumors.

One author (N. O.) assessed all the characteristics of the primary tumors and the nodal metastatic tumors, and another author (T. H.) identified the characteristics of all the IDCs to confirm the tumor cell characteristics in these tumor components recorded by N. O. without knowledge of the outcome of the patients with MPC. Whenever a discrepancy occurred, the authors reexamined the slides to reach a consensus.

### 2.5. Immunohistochemistry

We used the immunohistochemistry records for estrogen receptor (ER), progesterone receptor (PR), and HER2 for the IDCs, ILCs, and MPCs diagnosed by 2 or 3 pathologists at the time of routine examination. A tumor with nuclear staining for ER or PR in 10% or more of its tumor cells was assessed as ER-positive or PR-positive. HER2 cell membrane expression was categorized as follows: (1) HER2 category 0, negative; (2) HER 2 category 1, weakly positive (faintly stained cell membrane and $\leq$10% of overall tumor area); (3) HER2 category 2, moderately positive (moderately stained cell membrane and >10% of overall tumor area); and (4) HER2 category 3, strongly positive (strongly stained cell membrane and >10% of overall tumor area). Tumors classified as HER2 category 0 or 1 were considered negative for HER2 expression. All the MPCs were immunohistochemically studied using commercially available monoclonal antibodies to keratins (AE1/3) (Fig. 1F) and vimentin (Fig. 1G) and were confirmed to be positive for both keratins and vimentin.

### 2.6. Patient outcome and statistical analysis

Survival was evaluated using a median follow-up period of 153 months (range, 1-304 months) until February 2007. Of the 6138 IDC patients, 1019 developed tumor recurrences; and 771 died of their disease. Of the 302 ILC patients, 55 developed tumor recurrences; and all of them died of their disease. Of the 46 MPC patients, 15 developed tumor recurrences; and 11 died of their disease. The recurrence-free and overall survival periods were determined beginning at the time of surgery. Tumor relapse was considered to have occurred whenever evidence of metastasis was first observed.

The $\chi^2$ test was used to analyze whether significant differences existed in the frequencies of the clinicopathologic factors among the patients with IDC, ILC, or MPC.

We analyzed the outcome predictive power of tumor histology (IDC, ILC, MPC) and clinicopathologic factors for tumor recurrence and tumor-related death using multivariate analyses performed according to the Cox proportional hazard regression model as follows: model 1 included tumor histology, age, invasive tumor size, skin invasion, lymphatic invasion, blood vessel invasion, and nodal status; and model 2 included the above 7 factors plus neoadjuvant therapy, adjuvant therapy, ER/PR expression, and HER2 expression.

For the MPCs, the 14 histologic factors examined in the primary MPCs plus the 7 histologic factors examined in the MPCs located in the lymph nodes as well as age, neoadjuvant therapy, adjuvant therapy, and HER2 expression were entered into the univariate analyses; the factors that were significantly associated with tumor recurrence or tumor-related death were then entered into the multivariate analyses performed using the Cox proportional hazard regression model.

The multivariate analyses were performed using a case-wise and step-down method that was applied until all the remaining factors were significant at a $P$ value $< .05$. Survival curves were drawn using the Kaplan-Meier method. All the analyses were performed using Statistica/Windows software (StatSoft, Tulsa, OK).

## 3. Results

### 3.1. Univariate analyses of factors with significant differences among patients with IDC, ILC, or MPC

Patients with MPC showed significantly lower frequencies of neoadjuvant therapy, adjuvant therapy, and lymph vessel invasion than patients with IDC or ILC and a significantly higher frequency of skin invasion than patients with IDC (Table 1). Furthermore, all the patients with MPC exhibited negative immunostaining for ER and PR. Patients with ILC were significantly older than patients with IDC or MPC and had a significantly larger tumor size, a significantly lower HER2 category, and a significantly lower frequency of lymph vessel invasion than patients with IDC (Table 1). No significant differences in any other factor were observed among the 3 groups.

### 3.2. Multivariate analyses of outcome among patients with IDC, ILC, or MPC

In model 1 and model 2, the patients with MPC had significantly higher hazard rates (HRs) for tumor recurrence

(model 1: HR, 5.5; 95% confidence interval [CI], 3.2-9.6; model 2, HR, 6.6; 95% CI, 2.5-17.1) and tumor death (model 1: HR, 4.2; 95% CI, 2.2-8.1; model 2, HR, 12.4; 95% CI, 3.2-46.2) (Fig. 2A) than the patients with IDC in the multivariate analyses, although no significant differences in the HRs for tumor recurrence and tumor death were observed between patients with IDC and those with ILC in the multivariate analyses (data not shown). Furthermore, the patients with MPC had significantly higher HRs for tumor recurrence and tumor death that the patients with IDC independent of nodal metastasis in the multivariate analyses (Table 2). No significant differences in the HRs for tumor recurrence and tumor death were observed between patients with IDC and those with ILC among patients with or without nodal metastasis in the multivariate analyses (Table 2). Meanwhile, among patients not older than 39 years, the patients with MPC had significantly higher HRs for tumor recurrence and tumor death in model 1 of the multivariate analysis (Table 3); but model 2 could not be examined because of the small numbers of patients with ILC (2 cases) and MPC (3 cases). In patients with triple-negative carcinomas, the patients with MPC and the patients with ILC had significantly higher HRs for tumor recurrence and tumor death than the patients with IDC in multivariate analyses (Table 3).

### 3.3. Outcome predictive factor for patients with MPC

A patient age not exceeding 39 years (Fig. 2C), the use of neoadjuvant therapy, the presence of skin invasion (Fig. 2B), the presence of squamous cell carcinoma in a lymph node (Fig. 2D), and the International Union Against Cancer

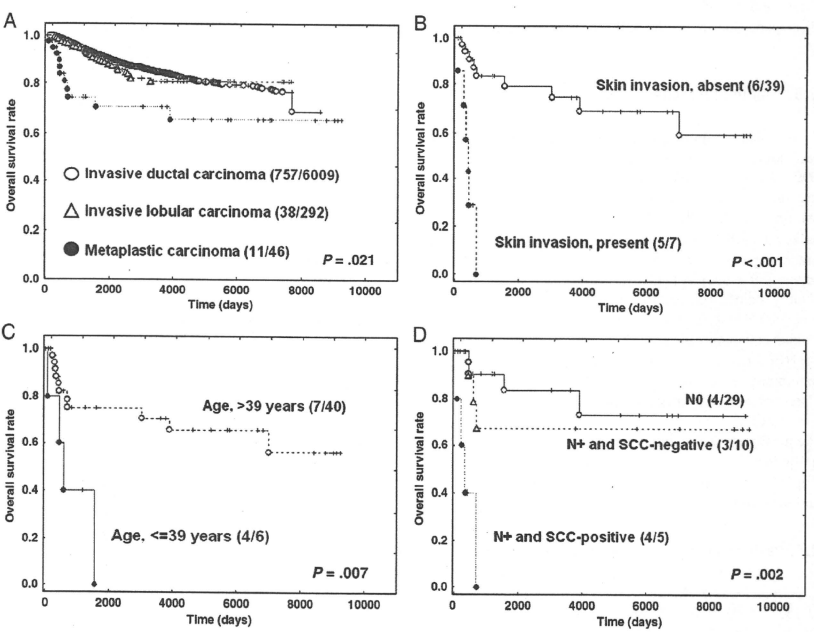


**Fig. 2** Overall survival curves. A, Patients with MPC show a significantly shorter overall survival period than patients with IDC and patients with ILC, and no significant difference in overall survival period is present between patients with IDC and patients with ILC. B, MPC patients with skin invasion show a significantly shorter overall survival period than those without skin invasion. C, MPC patients 39 years and younger show a significantly shorter overall survival period than those older than 39 years. D, MPC patients with squamous cell carcinoma in lymph nodes show a significantly shorter overall survival period than those without nodal metastasis or those with nodal metastasis but with no squamous cell carcinoma in their lymph nodes.

**Table 2** Multivariate analyses for tumor recurrence and tumor-related death in patients with IDC, ILC, or MPC according to nodal status

| No. of patients (%) | | | | | |
|---|---|---|---|---|---|
| Model 1 | | | | | |
| *Patients without nodal metastasis (n = 3915)* | | | | | |
| | | Tumor recurrence | | Tumor-related death | |
| | Cases | Cases | HR (95% CI) *P* value | Cases | HR (95% CI) *P* value |
| IDC | 3703 | 403 (11) | Referent | 261 (7) | Referent |
| ILC | 183 | 22 (12) | 1.2 (0.7-2.1) .425 | 12 (7) | 1.0 (0.5-2.0) .943 |
| MPC | 29 | 7 (24) | 6.0 (2.8-12.9) <.001 | 4 (14) | 3.5 (1.3-9.8) .016 |
| *Patients with nodal metastasis (n = 2558)* | | | | | |
| IDC | 2424 | 614 (25) | Referent | 510 (21) | Referent |
| ILC | 119 | 33 (28) | 1.2 (0.8-1.9) .336 | 29 (25) | 1.3 (0.9-2.0) .163 |
| MPC | 15 | 7 (47) | 4.9 (2.3-10.5) <.001 | 7 (47) | 4.0 (1.8-9.2) <.001 |
| Model 2 | | | | | |
| *Patients without nodal metastasis (n = 1852)* | | | | | |
| IDC | 1737 | 163 (9) | Referent | 42 (2) | Referent |
| ILC | 101 | 10 (10) | 1.2 (0.6-2.4) .690 | 3 (7) | 1.4 (0.4-4.6) .627 |
| MPC | 14 | 4 (29) | 5.2 (1.2-22.7) .028 | 4 (29) | 4.4 (1.2-15.9) .023 |
| *Patients with nodal metastasis (n = 412)* | | | | | |
| IDC | 391 | 94 (24) | Referent | 30 (8) | Referent |
| ILC | 16 | 5 (31) | 2.0 (0.7-5.9) .187 | 3 (19) | 3.0 (0.7-13.9) .164 |
| MPC | 5 | 3 (60) | 8.6 (2.3-32.9) .001 | 3 (60) | 28.9 (4.6-123.5) <.001 |

NOTE. Patients without nodal metastasis—Model 1 (tumor recurrence and tumor-related death): adjusted for tumor histology, age, skin invasion, lymphatic invasion, blood vessel invasion, and tumor size. Model 2 (tumor recurrence and tumor-related death): adjusted for the above factors (in model 1) as well as neoadjuvant therapy, adjuvant therapy, HER2 category, and ER and PR statuses. Patients with nodal metastasis—Model 1 (tumor recurrence and tumor-related death): adjusted for tumor histology, age, skin invasion, lymphatic invasion, blood vessel invasion, and tumor size. Model 2 (tumor recurrence and tumor-related death): adjusted for the above factors (in model 1) as well as neoadjuvant therapy, adjuvant therapy, HER2 category, and ER and PR statuses.

Abbreviation: n, number of cases that were examined in the multivariate analyses.

(UICC) pTNM stage were significantly associated with tumor recurrence and tumor-related death in the univariate analyses (Table 4). A tumor necrosis percentage of more than 30% of the primary tumors, the UICC pN category, the histologic grade of the tumors in the lymph nodes, the presence of extranodal invasion, the presence of adenocarcinoma with spindle cell differentiation in tumors in the lymph nodes, and the presence of tumor stroma in tumors in the lymph nodes were significantly associated with tumor-related death in the univariate analyses (Table 4). Other clinicopathologic factors, including MPC subtype, were not significantly associated with tumor recurrence or tumor death in the univariate analyses (Table 4).

In the multivariate analyses, the presence of skin invasion and an age not exceeding 39 years significantly increased the HRs for tumor recurrence and tumor death, whereas the presence of squamous cell carcinoma in tumors in the lymph nodes significantly increased the HR for tumor death (Table 5).

## 4. Discussion

In this study, none of the MPCs was positive for ER and PR; and only one MPC was positive for HER2. Furthermore, the presence of lymph vessel invasion, the presence of blood vessel invasion, and the UICC pN status did not exhibit any prognostic significance in patients with MPC, confirming the results of previous studies [7,9,14]. Because these factors are well-known outcome predictors

**Table 3** Multivariate analyses for tumor recurrence and tumor-related death in patients with IDC, ILC, or MPC according to age and triple-negative status

| No. of patients (%) | | | | | |
|---|---|---|---|---|---|
| Model 1 (n = 674) | | | | | |
| *Patients ≤39 y old* | | | | | |
| | | Tumor recurrence | | Tumor-related death | |
| | Cases | Cases | HR (95% CI) *P* value | Cases | HR (95% CI) *P* value |
| IDC | 654 | 159 (24) | Referent | 114 (17) | Referent |
| ILC | 15 | 2 (13) | 1.0 (0.3-6.3) .952 | 1 (7) | 0.7 (0.1-5.0) .712 |
| MPC | 6 | 4 (67) | 32.4 (11.1-99.2) <.001 | 4 (67) | 55.5 (17.1-173.5) <.001 |
| *Patients whose carcinomas were negative for ER, PR, and HER2 (triple-negative IDC) (n = 304)* | | | | | |
| IDC | 271 | 42 (16) | Referent | 19 (7) | Referent |
| ILC | 14 | 4 (29) | 3.6 (1.2-11.1) .023 | 2 (14) | 4.6 (0.9-21.9) .059 |
| MPC | 19 | 6 (32) | 9.4 (1.8-15.0) .002 | 3 (16) | 5.1 (1.3-19.4) .017 |

NOTE. Patients not older than 39 years—Model 1: adjusted for tumor histology, skin invasion, lymphatic invasion, blood vessel invasion, tumor size, and nodal status. Triple-negative IDC patients—Tumor recurrence: adjusted for tumor histology, age, skin invasion, lymphatic invasion, and nodal status. Tumor-related death: adjusted for tumor histology, age, skin invasion, and nodal status.

Abbreviation: n, number of cases that were examined in the multivariate analyses.

**Table 4** Association of clinicopathologic factors with tumor recurrence and tumor-related death in patients with MPC

| Factors | Cases | No. of patients (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | 46 | Cases with tumor recurrence | | *P* value | Cases with tumor-related death | | *P* value |
| Age, y | | | | | | | |
| ≤39 | 6 | 4 | (67) | .009 | 4 | (67) | .007 |
| >39 | 40 | 11 | (28) | | 7 | (18) | |
| Neoadjuvant therapy | | | | | | | |
| No | 42 | 11 | (26) | <.001 | 8 | (19) | .002 |
| Yes | 4 | 4 | (100) | | 3 | (75) | |
| Adjuvant therapy | | | | | | | |
| No | 37 | 10 | (27) | .505 | 7 | (19) | .474 |
| Yes | 9 | 5 | (56) | | 4 | (44) | |
| Invasive tumor size (mm) | | | | | | | |
| ≤20 | 13 | 3 | (23) | .352 | 1 | (8) | .072 |
| >20 to ≤50 | 26 | 9 | (35) | | 7 | (27) | |
| >50 | 7 | 3 | (43) | | 3 | (43) | |
| Skin invasion | | | | | | | |
| Absent | 39 | 9 | (20) | <.001 | 6 | (13) | <.001 |
| Present | 7 | 6 | (86) | | 5 | (71) | |
| Histologic grade | | | | | | | |
| Grade 1 | 1 | 0 | | .558 | 0 | | NA |
| Grade 2 | 5 | 1 | (20) | | 0 | | |
| Grade 3 | 40 | 14 | (35) | | 11 | (28) | |
| No. of mitotic figures in 10 high-power fields. | | | | | | | |
| ≤32 | 24 | 8 | (33) | .878 | 5 | (21) | .483 |
| >32 | 22 | 7 | (32) | | 6 | (27) | |
| Lymph vessel invasion | | | | | | | |
| Absent | 41 | 12 | (29) | .398 | 9 | (22) | .498 |
| Present | 5 | 3 | (60) | | 2 | (40) | |
| Blood vessel invasion | | | | | | | |
| Absent | 45 | 15 | (33) | NA | 11 | (24) | NA |
| Present | 1 | 0 | | | 0 | | |
| Area (%) occupied by of tumor necrosis within the tumor | | | | | | | |
| Absent/≤30 | 38 | 11 | (29) | .119 | 7 | (18) | .031 |
| >30 | 8 | 4 | (50) | | 4 | (50) | |
| Types of MPC | | | | | | | |
| Epithelial | 34 | 12 | (35) | .813 | 8 | (24) | .828 |
| Mixed | 12 | 3 | (25) | | 3 | (25) | |
| Squamous cell carcinoma *vs* other types of carcinoma | | | | | | | |
| Squamous | 7 | 4 | (57) | .134 | 3 | (43) | .136 |
| Other types | 39 | 11 | (28) | | 8 | (21) | |
| Adenocarcinoma with spindle cell differentiation *vs* other types of carcinoma | | | | | | | |
| Adenoca with spindle | 8 | 4 | (50) | .422 | 2 | (25) | .938 |
| Other types | 38 | 11 | (29) | | 9 | (23) | |
| Adenosquamous carcinoma *vs* other types of carcinoma | | | | | | | |
| Adenosquamous ca | 19 | 4 | (21) | .150 | 3 | (16) | .264 |
| Other types | 27 | 11 | (41) | | 8 | (30) | |
| Carcinoma with chondroid metaplasia *vs* other types of carcinoma | | | | | | | |
| Ca with chondroid | 4 | 1 | (25) | .659 | 1 | (25) | .835 |
| Other types | 42 | 14 | (33) | | 10 | (24) | |
| Carcinoma with osseous metaplasia *vs* other types of carcinoma | | | | | | | |
| Ca with osseous | 1 | 0 | | NA | 0 | | NA |
| Other types | 45 | 15 | (33) | | 11 | (24) | |
| Carcinosarcoma *vs* other types of carcinoma | | | | | | | |
| Carcinosarcoma | 7 | 2 | (29) | .660 | 2 | (29) | .432 |
| Other types | 39 | 13 | (33) | | 9 | (23) | |

(*continued on next page*)

**Table 4** (*continued*)

| Factors | Cases | No. of patients (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | 46 | Cases with tumor recurrence | | *P* value | Cases with tumor-related death | | *P* value |
| UICC pN category (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .255 | 4 | (14) | .049 |
| N1 | 11 | 4 | (36) | | 4 | (36) | |
| N2 | 2 | 2 | (100) | | 2 | (100) | |
| N3 | 2 | 1 | (50) | | 1 | (50) | |
| Histologic grade of lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .195 | 4 | (14) | .032 |
| Grade 1 | 1 | 0 | | | 0 | | |
| Grade 2 | 2 | 1 | (50) | | 1 | (50) | |
| Grade 3 | 12 | 6 | (50) | | 6 | (50) | |
| Extranodal invasion of lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .214 | 4 | (14) | .035 |
| Absent | 7 | 3 | (43) | | 3 | (43) | |
| Present | 8 | 4 | (50) | | 4 | (50) | |
| Squamous cell carcinoma in lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .024 | 4 | (14) | .002 |
| Absent | 10 | 3 | (30) | | 3 | (30) | |
| Present | 5 | 4 | (80) | | 4 | (80) | |
| Adenocarcinoma with spindle cell differentiation in lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .159 | 4 | (14) | .020 |
| Absent | 14 | 6 | (43) | | 6 | (43) | |
| Present | 1 | 1 | (100) | | 1 | (100) | |
| Adenosquamous carcinoma in lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .554 | 4 | (14) | .163 |
| Absent | 12 | 6 | (50) | | 6 | (50) | |
| Present | 3 | 1 | (33) | | 1 | (33) | |
| Carcinosarcoma in lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .610 | 4 | (14) | .199 |
| Absent | 13 | 7 | (54) | | 7 | (54) | |
| Present | 2 | 0 | | | 0 | | |
| Tumor stroma in lymph node-metastatic tumors (n = 44) | | | | | | | |
| N0 | 29 | 7 | (24) | .061 | 4 | (14) | .032 |
| None | 7 | 3 | (42) | | 3 | (43) | |
| Mild | 1 | 1 | (100) | | 1 | (100) | |
| Moderate | 3 | 0 | | | 0 | | |
| Severe | 2 | 2 | (100) | | 2 | (100) | |
| UICC pTNM stage (n = 44) | | | | | | | |
| I | 11 | 2 | (18) | .044 | 0 | | .003 |
| IIA | 16 | 4 | (25) | | 3 | (19) | |
| IIB | 6 | 2 | (33) | | 2 | (33) | |
| IIIA | 4 | 1 | (25) | | 1 | (25) | |
| IIIB | 5 | 4 | (80) | | 4 | (80) | |
| IIIC | 2 | 1 | (50) | | 1 | (50) | |

Abbreviations: NA, not available; Squamous, squamous cell carcinoma; Adenoca with spindle, adenocarcinoma with spindle cell differentiation; Adenosquamous ca, adenosquamous carcinoma; Ca with chondroid, carcinoma with chondroid metaplasia; Ca with osseous, carcinoma with osseous metaplasia; pN, pathologic regional lymph node; N0, no nodal metastasis; N1, 1 to 3 nodal metastases; N2, 4 to 9 nodal metastases; N3, 10 or more nodal metastases; pTNM, pathologic TNM.

for patients with IDC or patients with ILC, these findings strongly suggest that the biological characteristics of MPCs are quite different from those of IDCs or ILCs [16,21-25]. Four previous studies have investigated whether a significant difference in the survival period exists between patients with MPC and those with IDC [8,15,18,19]. The statistical analyses for survival in these studies, which produced controversial results regarding the survival of patients with MPC, were performed using a matched control case analysis, not a consecutive case analysis; and

**Table 5** Multivariate analyses for tumor recurrence and tumor-related death in patients with MPC

| | Tumor recurrence | | Tumor-related death | |
|---|---|---|---|---|
| | HRs 95% CI | *P* value | HRs 95% CI | *P* value |
| Skin invasion | | | | |
| Absent | Referent | | Referent | |
| Present | 24.8 5.4-112.1 | <.001 | 39.1 5.0-309.2 | <.001 |
| Age, y | | | | |
| >39 | Referent | | Referent | |
| ≤39 | 14.1 3.1-65.3 | <.001 | 34.4 4.4-269.9 | <.001 |
| Squamous cell carcinoma in lymph node-metastatic tumors | | | | |
| N0 and absent | Referent | | Referent | |
| Present | 2.2 0.9-5.3 | .087 | 5.6 1.6-19.4 | .006 |

NOTE. Tumor recurrence: adjusted for skin invasion, age, neoadjuvant therapy, and squamous cell carcinoma in lymph node-metastatic tumors. Tumor-related death: adjusted for skin invasion, age, squamous cell carcinoma in lymph node-metastatic tumors, neoadjuvant therapy, occupied area of tumor necrosis, UICC pN category, histologic grade of lymph node-metastatic tumors, extranodal invasion, adenocarcinoma with spindle cell differentiation in lymph node-metastatic tumors, and tumor stroma in lymph node-metastatic tumors.

the periods during which the patients with MPC and the patients with IDC were operated on also differed [8]. The results of the present study were obtained using consecutive cases treated during the same period; our findings clearly demonstrated that MPCs are associated with a significantly higher rate of tumor recurrence or tumor death than IDCs or ILCs, independent of the nodal status, age not exceeding 39 years, adjuvant therapy status, or neoadjuvant therapy status. Thus, we can conclude that MPCs have a greater malignant biological potential than IDCs or ILCs. Furthermore, the triple-negative MPCs observed in this study had more aggressive characteristics than the triple-negative IDCs and the triple-negative ILCs, whereas the triple-negative ILCs had greater malignant biological characteristics than the triple-negative IDCs; these findings strongly suggest that studies on outcome predictors or targeted therapies for triple-negative breast carcinoma should be performed according to the specific type of triple-negative breast carcinoma. Because some genes are selectively expressed in patients with MPC but not in patients with other types of breast carcinomas [13,16,24,25], the development of neoadjuvant therapy or adjuvant therapy targeting such genes may improve the outcome of patients with MPC.

At the beginning of this study, we speculated that the MPC type, such as epithelial versus mixed or squamous versus others, might be significantly associated with the outcome of patients with MPC. However, the results of this study clearly demonstrated that the MPC subtype had no significant effect on the outcome of patients with MPC, confirming the results of previous studies [8,11,13,26]; instead, the most important outcome predictors for patients with MPC were the presence of skin invasion, an age not exceeding 39 years, and the presence of a squamous cell carcinoma component in tumors in the lymph nodes. Consequently, these 3 factors appear to be important prognostic factors for patients with MPC; and the results of this study confirm that the WHO classification for MPC, which contains both epithelial and mixed types of MPC [1], is a reasonable classification for patients with MPC from the viewpoint of patient outcome. Because of the relatively small number of cases of each MPC subtype, however, this study was unable to investigate whether important clinicopathologic predictors of outcome exist for specific MPC subtypes, such as low-grade adenosquamous carcinoma versus high-grade adenosquamous carcinoma and fibromatosis-like low-grade carcinosarcoma versus high-grade carcinosarcoma. Therefore, the clinicopathologic outcome predictors for each MPC subtype should be separately investigated in the future.

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MODERN PATHOLOGY (2010) 23, 662–672


# p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci in invasive ductal carcinoma of the breast

Takahiro Hasebe[1], Motoki Iwasaki[2], Sadako Akashi-Tanaka[3], Takashi Hojo[3], Tatsuhiro Shibata[4], Yuko Sasajima[5], Takayuki Kinoshita[3] and Hitoshi Tsuda[5]

[1]*Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Service, National Cancer Center, Chuo-ku, Tokyo, Japan;* [2]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Chuo-ku, Tokyo, Japan;* [3]*Department of Breast Surgery, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan;* [4]*Cancer Genomics Project, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan and* [5]*Clinical Laboratory Division, National Cancer Center Hospital, Chuo-ku, Tokyo, Japan*

**The purpose of this study was to determine whether p53 protein expression in tumor-stromal fibroblasts forming fibrotic foci is a significant outcome predictor, similar to p53 protein expression in tumor-stromal fibroblasts not forming fibrotic foci, and whether the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci served as an important outcome predictor among 1039 patients with invasive ductal carcinoma of the breast. We analyzed the outcome predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci using multivariate analyses with well-known clinicopathological factors. The Allred score risk classifications for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci were superior to the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci alone for accurately predicting the tumor-related death of patients with invasive ductal carcinoma when examined using multivariate analyses. The Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci significantly increased the hazard rates for tumor recurrence and tumor-related death independent of the UICC pTNM stage in the multivariate analyses. These results indicated that the Allred score risk classification based on the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci is a very useful outcome predictor among patients with invasive ductal carcinoma.**
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**Keywords:** fibroblast; fibrotic focus; p53; tumor cell–stromal cell interaction; breast

Along with others, we have already reported that a fibrotic focus, a characteristic histological feature of tumor stroma, is a very useful histological tumor-stromal indicator for accurately predicting the outcome of patients with invasive ductal carcinoma (IDC),[1–5] and the proliferative activity of tumor-stromal fibroblasts forming and not forming fibrotic foci has a very important function in nodal metastasis and distant organ metastasis by IDCs.[6,7] Because it has recently been reported that the gene expression profile and protein expression profile of the tumor stroma have a very important function in tumor progression in carcinoma[8,9] and that the interactions between tumor cells and stromal cells also are very important in tumor progression in carcinomas,[10,11] these findings strongly suggest that the tumor stroma has a significant function in tumor progression in IDCs. Mutations of the p53 tumor suppressor gene have been described in the stromal fibroblasts of breast and prostate carcinomas in

Correspondence: Dr T Hasebe, MD, PhD, Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: thasebe@ncc.go.jp
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humans and experimental animals,[12–14] and p53 mutations in breast cancer stromal cells have been reported to be closely associated with nodal metastasis.[15] However, some studies have reported that p53 mutations are not observed in the tumor stroma of breast cancer,[16,17] and the possibility of technical problem, eg polymerase chain reaction artifacts for the p53 gene abnormality, has been suggested by Campbell *et al*.[18] We recently showed that p53 expression in tumor-stromal fibroblasts not forming fibrotic foci was a very important outcome predictor for IDC patients who had or had not received neoadjuvant therapy.[19,20] On the basis of the above findings, the p53 status of tumor-stromal fibroblasts not forming fibrotic foci probably has a very important function in tumor progression in IDCs.

We also previously reported that our newly devised grading system for lymph vessel tumor emboli is a very useful histological grading system for accurately predicting the outcome of patients with IDC who have not received neoadjuvant therapy; furthermore, this grading system can be used to classify the prognosis of IDC patients with lymph vessel invasion into low-risk, intermediate-risk, and high-risk groups.[21] In addition, we recently confirmed that this grading system for lymph vessel tumor emboli was a very important outcome predictor for patients with IDC in a different patient group.[22]

The purpose of this study was to determine whether the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci served as an important outcome predictor among patients with IDC of the breast using multivariate analyses with well-known prognostic factors and our grading system for lymph vessel tumor emboli. The results indicated that a score classification based on the combined assessment of p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci was a very useful outcome predictor among patients with IDC of the breast.

## Materials and methods

### Cases

The subjects of this study were 1039 consecutive patients with IDC of the breast who did not receive neoadjuvant therapy and who were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (almost the same case series as that used in our previous study).[19,22] The IDCs were diagnosed preoperatively using needle biopsy, aspiration cytology, a mammography, or ultrasonography. All the patients were Japanese women, ranging in age from 23 to 72 years (median, 55 years). All had a solitary lesion; 497 patients were premenopausal and 542 were postmenopausal. A partial mastectomy had been performed in 455 patients, and a modified radical mastectomy had been performed in 584. A level I and level II axillary lymph node dissection had been performed in all the patients, and a level III axillary lymph node dissection had been performed in some of the patients with IDC.

Of the 1039 patients, 873 received adjuvant therapy, consisting of chemotherapy in 218 patients, endocrine therapy in 281 patients, and chemoendocrine therapy in 374 patients. The chemotherapy regimens used were anthracycline-based with or without taxane and non-anthracycline-based, and the endocrine therapy regimens consisted of tamoxifen with or without a gonadotropin-releasing hormone agonist, tamoxifen, with or without an aromatase inhibitor, an aromatase inhibitor alone, or a gonadotropin-releasing hormone agonist alone. No cases of inflammatory breast cancer were included in this series. All the tumors were classified according to the pathological UICC-TNM (pTNM) classification.[23] The protocol of this study (20-112) was reviewed by the institutional review board of the National Cancer Center.

For the pathological examination, we fixed the surgically resected specimens in 10% formalin, and the size and gross appearance of the tumors were recorded. The tumor size was confirmed by comparison with the tumor size on the histological slides; if more than one invasive focus was present, the size of the largest invasive focus was recorded as the invasive tumor size, based on a previously reported definition for determining the size of microinvasion in IDC with multiple microinvasive foci[23] in this study.

### Histological Examination

Serial sections of each tumor area were cut from paraffin blocks. One section from each tumor was stained with hematoxylin and eosin and was examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following eight histological factors and the grading system for lymph vessel tumor emboli[21,22] were evaluated: (1) invasive tumor size ($\leq 20$, $>20$ to $\leq 50$, $>50$ mm); (2) histological grade (1, 2, 3);[24] (3) tumor necrosis (absent, present);[25] (4) fibrotic focus (absent, fibrotic focus diameter $\leq 8$ mm, fibrotic focus diameter $>8$ mm) (Figure 1);[1,2] (5) blood vessel invasion (absent, present); (6) adipose tissue invasion (absent, present); (7) skin invasion (absent, present); and (8) muscle invasion (absent, present).

### Immunohistochemistry

Immunohistochemical staining for estrogen receptors, progesterone receptors, p53, and HER2 products was performed using an autoimmunostainer (Optimax Plus; BioGenex, San Ramon, CA, USA). The antigen retrieval device for Optimax Plus was

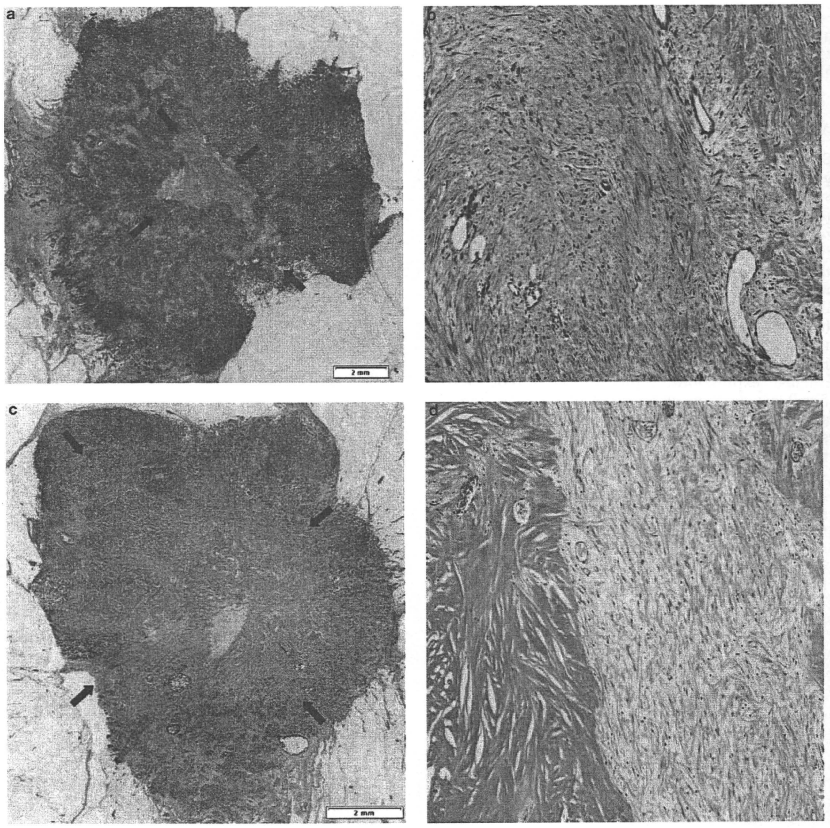



**Figure 1** Invasive ductal carcinomas with fibrotic foci (**a–d**). (**a**) A fibrotic focus measuring 6.4 × 3.3 mm is visible within the tumor (panoramic view, arrows). The fibrotic focus shows a scar-like feature and is surrounded by invasive ductal carcinoma cells. (**b**) The fibrotic focus area consists mainly of fibroblasts arranged in a storiform pattern. (**c**) A fibrotic focus measuring 10.2 × 7.3 mm is visible within the tumor (panoramic view, arrows). The fibrotic focus has a fibrosclerotic core and is surrounded by invasive ductal carcinoma cells. Small residual tumor islands are present within the fibrotic focus. (**d**) The fibrotic focus consists of fibroblasts and hyalinized collagen fibers in a storiform arrangement.

an autoclave, and each specimen was immersed in citrate buffer and incubated at 121°C for 10 min. Immunoperoxidase staining was performed using a labeled streptavidin biotin staining kit (BioGenex) according to the manufacturer's instructions. The antibodies used were the anti-estrogen receptor mouse monoclonal antibody ER88 (BioGenex), the anti-progesterone receptor mouse monoclonal antibody PR88 (BioGenex), the anti-HER2 mouse monoclonal antibody CB11 (BioGenex), and the p53 mouse monoclonal antibody DO7 (Dako, Glostrup, Denmark). ER88, PR88, and CB11 were previously diluted, and DO7 was applied at a dilution of 1:100. After immunostaining, the sections were counterstained with hematoxylin. Sections of the IDCs that were positive for estrogen receptor, progesterone

receptor, HER2, and p53 were used each time as a positive control. As a negative control, the primary antibody was replaced with normal mouse immunoglobulin.

### Assessment of ER, PR, p53, and HER2 Expression

Slides of the tumor cells immunostained for estrogen receptor, progesterone receptor, and p53 were scored using the Allred scoring system, as described previously,[26–28] and the Allred scores for estrogen receptor, progesterone receptor, and p53 expression in the tumor cells were classified into the following three categories[19]: (1) Allred score for estrogen receptor in tumor cells (0 or 2, 3–6, and 7 or 8); (2) Allred score for progesterone receptor in tumor cells (0 or 2, 3–6, and 7 or 8); and (3) Allred scores for p53 in tumor cells (0 or 2 or 3, 4–6, and 7 or 8). We modified the Allred scoring system to assess the nuclear expression of p53 in the tumor-stromal fibroblasts forming and not forming fibrotic foci,[19,20] and the Allred scores for p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci were classified into the following categories: (1) Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci (0, 2, 3, and 4–8); and (2) Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci (0 or 2, 3, and 4–8) (Figures 2 and 3). Of the 1039 IDCs, 373 IDCs had fibrotic foci; we could not assess the Allred scores for p53 in tumor-stromal fibroblasts forming a fibrotic focus in 97 of the 373 IDCs with fibrotic foci because the immunohistochemistry examinations for these specimens were performed using tumor tissue sections that did not contain a fibrotic focus at the time of routine examination. The HER2 status of the tumor cells was semiquantitatively scored on a scale of 0–3 according to the level of HER2 protein expression,[29] and it was classified into three categories: 0 or 1, 2, and 3.

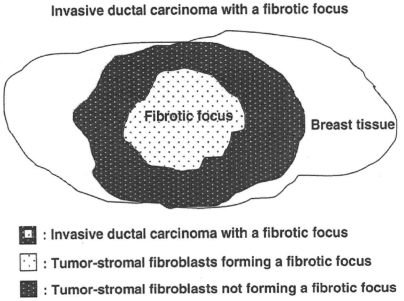



**Figure 2** Schematic illustration of an invasive ductal carcinoma with a fibrotic focus.

### Patient Outcome and Statistical Analysis

Survival was evaluated using a median follow-up period of 52 months (range: 18–102 months) until February 2009. Of the 1039 IDC patients, 910 patients were alive and well, 129 had developed tumor recurrences, and 58 had died of their disease. The tumor recurrence-free survival and overall survival periods were calculated using the time of surgery as the starting point. Tumor relapse was considered to have occurred whenever evidence of metastasis was found.

The Mann–Whitney test was used to compare the Allred scores for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci, and the correlation analyses were performed using Cochran-Mantel-Haenszel statistics.

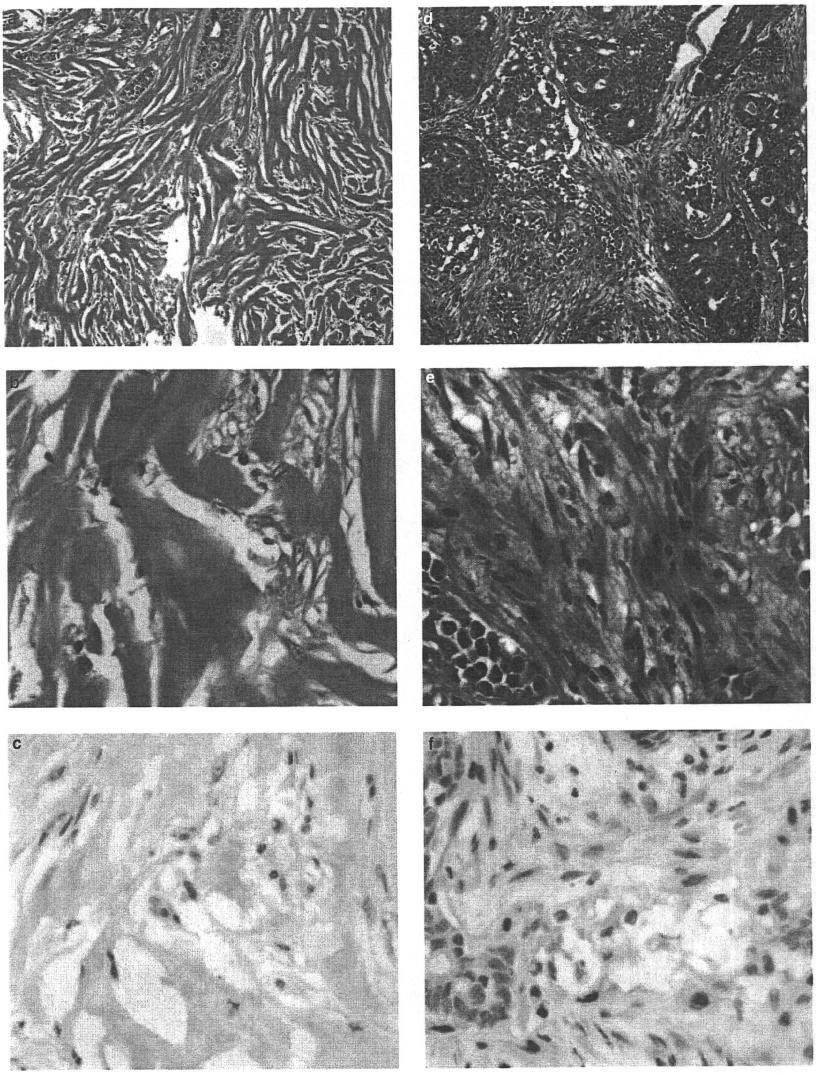
We analyzed the outcome predictive power of the eight histological factors, the grading system for lymph vessel tumor emboli;[21,22] the Allred scores for estrogen receptor; progesterone receptor, and p53 in tumor cells; the category of HER2 expression in tumor cells; the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci, adjuvant therapy (yes or no); age ($\leq$39 years and $>$39 years); and the UICC-pathological nodal status (N factor, ie, no nodal metastasis, N0; 1–3 nodal metastases, N1; 4–9 nodal metastases, N2; and 10 or more nodal metastases, N3)[23] for tumor recurrence, and tumor-related death in univariate analyses using the Cox proportional hazard regression model. The factors significantly associated with outcome in the univariate analyses were then entered together into the multivariate analyses using the Cox proportional hazard regression model according to the UICC pTNM stage. The case-wise and step-down method was applied until all the remaining factors were significant at a *P*-value of below 0.05. Because fewer than 10 tumor-related deaths occurred among the UICC stage I IDC patients (Table 2), it was impossible to perform multivariate analyses for tumor-related death in this group. All the analyses were performed using Statistica for Windows software (StatSoft, Tulsa, OK, USA).

## Results

### Allred Scores for p53 in Tumor-Stromal Fibroblasts Forming and Not Forming Fibrotic Foci

Although a significant association was observed between the Allred scores for p53 in tumor-stromal fibroblasts forming and those not forming fibrotic foci ($P<0.001$; Figure 4a), the latter value (mean value, 2.2; standard deviation, 2.1) was significantly higher than the former (mean value, 1.6; standard deviation, 2.0; $P=0.001$). The Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were also significantly associated with the fibrotic focus diameter, and in IDCs with a fibrotic focus

diameter >8 mm, the number of IDCs with Allred scores of 4–8 for p53 in tumor-stromal fibroblasts forming fibrotic foci was larger than that of IDCs with Allred scores of 0, 2, or 3 for p53 in tumor-stromal fibroblasts forming fibrotic foci (Figure 4b).



### Allred Score Risk Classification for p53 in Tumor-Stromal Fibroblasts Forming and not Forming Fibrotic Foci in Patients with Invasive Ductal Carcinoma with and without Fibrotic Foci

We devised an Allred score risk classification for p53 in tumor-stromal fibroblasts in IDCs based on the combined Allred scores for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci (Table 1). This classification was successfully used to classify IDC patients with or without fibrotic foci into three risk classes (low risk, intermediate risk, and high risk) according to the ratios for tumor recurrence and tumor-related death (Table 2; Figure 5). Among the UICC pTNM stage I IDC patients, the patients in the intermediate- and high-risk classes showed a significantly higher tumor recurrence rate than the patients in the low-risk class (Table 2). Among the UICC pTNM stage II IDC

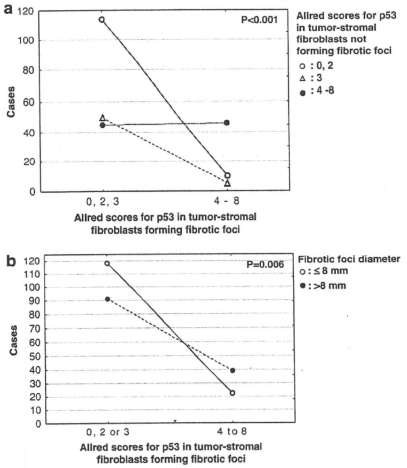



**Figure 4** (**a**) Associations between the Allred scores for p53 in tumor -stromal fibroblasts forming and not forming fibrotic foci; the scores were significantly associated with each other ($P<0.001$). (**b**) Associations between the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci and the diameter of the fibrotic foci. Invasive ductal carcinomas with fibrotic foci >8 mm in diameter had a significantly higher Allred score for p53 in tumor-stromal fibroblasts forming fibrotic foci than those with fibrotic foci ≤8 mm in diameter ($P=0.006$).

**Table 1** Overall Allred score classification of p53 in tumor-stromal fibroblasts forming and not forming a fibrotic focus

| | |
|---|---|
| *Invasive ductal carcinoma with a fibrotic focus* | |
| A) The Allred scores of p53 in tumor-stromal fibroblasts forming a fibrotic focus | Score class |
| 0, 2, or 3 | 0 |
| 4–8 | 2 |
| B) The Allred scores of p53 in tumor-stromal fibroblasts not forming a fibrotic focus | Score class |
| 0 or 2 | 0 |
| 3 | 1 |
| 4–8 | 2 |
| Total (A+B) | 0–4 |
| *Invasive ductal carcinoma without a fibrotic focus* | |
| The Allred scores of p53 in tumor-stromal fibroblasts not forming a fibrotic focus | Score class |
| 0 or 2 | 0 |
| 3 | 1 |
| 4–8 | 2 |
| Total | 0–2 |
| The Allred score risk classes for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci | Score class |
| Low-risk class | 0 and 1 |
| Intermediate-risk class | 2 and 3 |
| High-risk class | 4 |

**Table 2** Tumor recurrence and tumor-related death rates according to the Allred score risk classes for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci in patients with invasive ductal carcinoma with or without a fibrotic focus

| *Risk classes* | *Cases* | *TRR (%)* | *P-value* | *MR (%)* | *P-value* |
|---|---|---|---|---|---|
| *Invasive ductal carcinoma patients as a whole* | | | | | |
| Low-risk | 648 | 36 (6) | | 9 (1) | |
| Intermediate-risk | 232 | 52 (22) | <0.001 | 24 (10) | <0.001 |
| High-risk | 46 | 24 (52) | <0.001 | 15 (33) | 0.001 |
| Total | 926 | 112 (12) | | 48 (5) | |
| *UICC pTNM stage I invasive ductal carcinoma patients* | | | | | |
| Low-risk | 239 | 5 (2) | | 0 | |
| Intermediate-risk | 69 | 10 (15) | <0.001 | 4 (6) | <0.001 |
| High-risk | 6 | 2 (33) | 0.295 | 0 | 0.454 |
| Total | 314 | 17 (5) | | 4 (1) | |
| *UICC pTNM stage II invasive ductal carcinoma patients* | | | | | |
| Low-risk | 309 | 18 (6) | | 5 (2) | |
| Intermediate-risk | 120 | 23 (19) | <0.001 | 7 (6) | 0.045 |
| High-risk | 24 | 11 (46) | 0.041 | 6 (25) | 0.012 |
| Total | 453 | 52 (12) | | 18 (4) | |
| *UICC pTNM stage III invasive ductal carcinoma patients* | | | | | |
| Low-risk | 100 | 13 (13) | | 4 (4) | |
| Intermediate-risk | 43 | 19 (44) | <0.001 | 13 (30) | <0.001 |
| High-risk | 16 | 11 (69) | 0.054 | 9 (56) | 0.042 |
| Total | 159 | 43 (27) | | 26 (16) | |

TRR, tumor recurrence rate; MR, mortality rate.

**Figure 3** Tumor-stromal fibroblasts forming (**a, c, e**) and not forming a fibrotic focus (**b, d, f**). A fibrotic focus consists of tumor-stromal fibroblasts and hyalinized collagen fibers (**a** and **c**) and many tumor-stromal fibroblasts show a moderately intense nuclear staining pattern for p53. The Allred score for p53 in these tumor-stromal fibroblasts forming a fibrotic focus is 7 (intensity score, 2; proportion score, 5) (**e**). Carcinoma cells invade in irregular-shaped nests with a tubular structure (**b**) and tumor-stromal fibroblasts with oval nuclei not forming a fibrotic focus are seen (**d**). Many tumor-stromal fibroblasts not forming a fibrotic focus show a faint, moderate or strong intense nuclear staining pattern for p53, whereas tumor cells showing a faint intense nuclear staining pattern for p53 are visible (**f**). The Allred score for p53 in these tumor-stromal fibroblasts not forming a fibrotic focus is 8 (intensity score, 3; proportion score, 5).

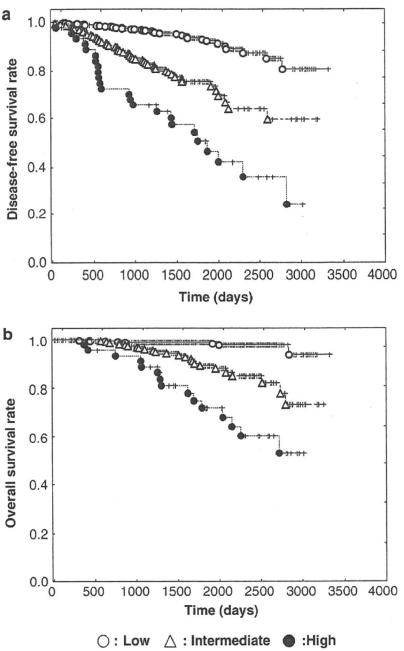



**Figure 5** Disease-free survival curves and overall survival curves of invasive ductal carcinoma (IDC) patients overall (**a** and **b**) according to the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming a fibrotic focus (FF). The disease-free survival time (**a**) and the overall survival time (**b**) of the IDC patients significantly decrease with the risk class of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming FF.

patients, the tumor recurrence rate and the mortality rate for each risk class were significantly increased according to the risk classes of the classification (Table 2). Among the UICC pTNM stage III IDC patients, the patients in the intermediate-risk class showed a significantly higher tumor recurrence rate and mortality rate than the patients in the low-risk class, and the patients in the high-risk class showed a marginally significantly higher tumor recurrence rate and a significantly higher mortality rate than the patients in the intermediate-risk class (Table 2).

Overall, the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci (trend hazard rate, 2.9; trend 95% confidence interval, 1.6–5.2; *P*-value, <0.001) was superior to the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci alone (trend hazard rate, 1.5; trend 95% confidence interval, 0.8–2.6; *P*-value, 0.172) for accurately predicting tumor-related death among patients with IDC, as shown in a multivariate analysis.

### Factors Significantly Associated with Tumor Recurrence and Tumor-Related Death

Among the patients with UICC pTNM stage I IDC, an intermediate-risk class (hazard rate, 6.2; 95% confidence interval, 2.1–18.5; *P*-value, 0.001) and a high-risk class (hazard rate, 11.6; 95% confidence interval, 2.1–63.8; *P*-value, 0.005) for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci and a histological grade of 3 (hazard rate, 2.9; 95% confidence interval, 1.1–7.6; *P*-value, 0.034) significantly increased the hazard rates for tumor recurrence in a multivariate analysis.

Among the patients with UICC pTNM stage II IDC, an intermediate-risk class and a high-risk class for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analyses (Table 3). Grades 2 and 3 lymph vessel tumor emboli and the presence of blood vessel invasion significantly increased the hazard rates for tumor recurrence in the multivariate analysis (Table 3). A UICC pN1 category and a fibrotic focus diameter >8 mm significantly increased the hazard rates for tumor-related death and an Allred score of 7 or 8 for the progesterone receptors in the tumor cells significantly decreased the hazard rate for tumor-related death in the multivariate analyses (Table 3).

Among the patients with a UICC pTNM stage III IDC, an intermediate-risk class and a high-risk class for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci, grade 3 lymph vessel tumor emboli and a UICC pN3 category significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analysis (Table 4). A fibrotic focus diameter >8 mm significantly increased the hazard rate for tumor recurrence and an Allred score of 7 or 8 for estrogen receptor in the tumor cells significantly decreased the hazard rate for tumor-related death in the multivariate analysis (Table 4).

## Discussion

This study clearly showed that the values of the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci were significantly higher than those in tumor-stromal fibroblasts forming fibrotic foci. Fibrotic foci are fibrotic scar-like lesions that mainly consist of tumor-stromal fibroblasts admixed with various numbers of tumor cells; some fibrotic foci do not contain any tumor cells.[1,2] In contrast, tumor-stromal fibroblasts not forming fibrotic foci commonly admix with many tumor cells that show stromal invasion. This difference

**Table 3** Multivariate analyses for tumor recurrence and tumor-related death in UICC pTNM stage II invasive ductal carcinoma patients ($n=453$)

| *Factors* | *Tumor recurrence* | | *Tumor-related death* | |
|---|---|---|---|---|
| | *HR (95% CI)* | *P-value* | *HR (95% CI)* | *P-value* |
| *p53 Allred score risk classes of tumor-stromal fibroblasts forming and not forming a fibrotic focus* | | | | |
| Low-risk | Referent | | Referent | |
| Intermediate-risk | 3.5 (1.4–4.4) | 0.003 | 3.3 (1.0–10.5) | 0.043 |
| High-risk | 5.2 (1.8–6.5) | <0.001 | 4.7 (1.3–17.3) | 0.021 |
| *Grading system for lymph vessel tumor emboli* | | | | |
| Grade 0 | Referent | | Referent | |
| Grade 1 | 1.5 (0.8–3.0) | 0.226 | 0.5 (0.1–2.5) | 0.421 |
| Grades 2 and 3 | 2.5 (1.4–4.4) | 0.003 | 2.0 (0.6–6.3) | 0.275 |
| *Blood vessel invasion* | | | | |
| Absent | Referent | | Referent | |
| Present | 2.1 (1.1–3.8) | 0.017 | 1.1 (0.3–3.8) | 0.914 |
| *The Allred scores for progesterone receptors in tumor cells* | | | | |
| 0 or 2 | Referent | | Referent | |
| 3–6 | — | | 0.8 (0.2–3.0) | 0.729 |
| 7 or 8 | — | | 0.2 (0.07–0.7) | 0.009 |
| *UICC pN category* | | | | |
| pN0 | Referent | | Referent | |
| pN1 | — | | 14.7 (1.9–113.1) | 0.010 |
| *Fibrotic focus, diameter* | | | | |
| Absent | Referent | | Referent | |
| ≤8 mm | — | | 1.3 (0.2–8.5) | 0.763 |
| >8 mm | — | | 3.4 (1.2–9.8) | 0.025 |

HR, hazard rate; CI, confidence interval; —, not significance in univariate analysis.
The multivariate analysis for tumor recurrence was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, blood vessel invasion, histological grade, and age.
The multivariate analysis for tumor-related death was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, blood vessel invasion, the Allred scores for progesterone receptors in tumor cells, UICC pN category, fibrotic focus diameter, and age.

strongly suggests that the tumor cell–stromal cell interaction occurs more frequently in the outer area of a fibrotic focus than in the inner area of a fibrotic focus within IDCs,[10,11] probably resulting in the higher Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci. However, the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were significantly associated with those for p53 in tumor-stromal fibroblasts not forming fibrotic foci. Thus, the tumor cell–stromal cell interaction probably occurs more frequently in IDCs with fibrotic foci than in IDCs without fibrotic foci.

We and others have already reported that the fibrotic focus diameter is a significant outcome predictor among patients with IDC who have fibrotic foci,[1–5] and our previous study showed that a fibrotic focus diameter of greater than 8 mm, similar to the Allred score for p53 in tumor-stromal fibroblasts not forming a fibrotic focus, was a significant outcome predictor for patients with IDC independent of the UICC pTNM stage.[19] In this study, a fibrotic focus diameter was also a significant outcome predictor for IDC patients of UICC pTNM stage II and IDC patients of UICC pTNM stage III, and IDCs with fibrotic foci greater than 8 mm in diameter showed a significantly higher Allred score for p53 in tumor-stromal fibroblasts forming fibrotic foci than IDCs with fibrotic foci of 8 mm or less in diameter. Thus, one can conclude that p53-expressing tumor-stromal fibroblasts located in both the inner and outer regions of fibrotic foci heighten the malignant potential of IDCs, probably accounting for the prognostic value of the fibrotic focus diameter. In addition, the grading system for lymph vessel tumor emboli significantly increased the hazard rates for tumor recurrence or tumor-related death in multivariate analyses performed for IDC patients with UICC pTNM stage II and UICC stage III. Therefore, the fibrotic focus diameter and the grading system for lymph vessel tumor emboli are likely to be very important histological outcome predictors for patients with IDC.

The results of this study clearly show that the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci had a greater outcome predictive power than the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci alone. Furthermore, the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci is a very important outcome predictor for patients with IDC

**Table 4** Multivariate analyses for tumor recurrence and tumor-related death in UICC pTNM stage III invasive ductal carcinoma patients

| *Factors* | *Tumor recurrence* | | *Tumor-related death* | |
|---|---|---|---|---|
| | *HR (95% CI)* | P-*value* | *HR (95% CI)* | P-*value* |
| *p53 Allred score risk classes of tumor-stromal fibroblasts forming and not forming a fibrotic focus* | | | | |
| Low-risk | Referent | | Referent | |
| Intermediate-risk | 2.9 (1.3–6.3) | 0.009 | 5.2 (1.6–17.2) | 0.007 |
| High-risk | 6.0 (2.6–13.9) | <0.001 | 20.1 (5.8–69.0) | <0.001 |
| *Grading system for lymph vessel tumor emboli* | | | | |
| Grade 0 | Referent | | Referent | |
| Grade 1 | 0.6 (0.2–1.8) | 0.340 | 0.5 (0.1–3.1) | 0.480 |
| Grade 2 | 0.6 (0.2–1.6) | 0.281 | 1.7 (0.5–5.8) | 0.426 |
| Grade 3 | 6.5 (2.9–14.4) | <0.001 | 2.6 (1.0–6.7) | 0.045 |
| *UICC pN category* | | | | |
| pN0 | Referent | | Referent | |
| pN1 | 6.3 (0.5–81.3) | 0.166 | 8.8 (0.4–203.7) | 0.171 |
| pN2 | 6.9 (0.6–70.2) | 0.108 | 5.0 (0.3–80.1) | 0.256 |
| pN3 | 2.8 (1.5–5.3) | 0.001 | 3.3 (1.4–7.8) | 0.005 |
| *Fibrotic focus, diameter* | | | | |
| Absent | Referent | | Referent | |
| ≤8 mm | 1.6 (0.6–4.3) | 0.383 | 1.3 (0.2–8.6) | 0.777 |
| >8 mm | 2.8 (1.3–6.2) | 0.009 | 2.1 (0.5–9.5) | 0.337 |
| *The Allred scores for estrogen receptor in tumor cells* | | | | |
| 0 or 2 | Referent | | Referent | |
| 3–6 | 0.7 (0.3–1.9) | 0.488 | 1.2 (0.3–5.0) | 0.836 |
| 7 or 8 | 0.6 (0.2–1.5) | 0.257 | 0.4 (0.2–0.9) | 0.033 |

HR, hazard rate; CI, confidence interval; pN, pathological regional lymph node; N0, no nodal metastasis; N1, 1–3 nodal metastases; N2, 4–9 nodal metastases; N3, 10 or more nodal metastases.
The multivariate analysis for tumor recurrence was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, UICC pN category, fibrotic focus diameter, the Allred scores for estrogen receptors in tumor cells, the Allred scores for progesterone receptors in tumor cells, the Allred scores for p53 in tumor cells, invasive tumor size, tumor necrosis, and histological grade.
The multivariate analysis for tumor death was performed using the p53 Allred score risk classes in tumor-stromal fibroblasts forming and not forming a fibrotic focus, grading system for lymph vessel tumor emboli, UICC pN category, fibrotic focus diameter, the Allred scores for estrogen receptors in tumor cells, the Allred scores for p53 in tumor cells, HER2 category in tumor cells, age, invasive tumor size, and histological grade.

and an intermediate-risk or high-risk classification significantly increased the hazard rates for tumor recurrence and tumor-related death independent of the UICC pTNM stage in multivariate analyses that included well-known prognostic factors. Thus, we can conclude that the Allred score risk classification based on the Allred score for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci appears to be an excellent histological predictor of outcome among patients with IDC with or without fibrotic foci. However, as we could not analyze the outcome predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci among patients with IDC according to the types of adjuvant therapy (chemotherapy, endocrine therapy, and chemoendocrine therapy) in detail, the predictive power of the Allred score risk classification for p53 in tumor-stromal fibroblasts forming and not forming fibrotic foci should be analyzed separately among IDC patients treated with chemotherapy, endocrine therapy, and chemoendocrine therapy in the future.

In this study, we did not investigate the associations of the Allred scores for p53 with the presence of *p53* gene abnormalities in tumor-stromal fibroblasts. Although *p53* mutations in tumor-stromal fibroblasts are relatively common among primary breast cancers and other cancers and have been reported to exert a positive effect on cancer growth,[12–15] some studies have not shown any p53 mutations in the tumor-stroma of breast cancer.[16–18] We have already reported that fibroblasts forming fibrotic foci show significantly higher proliferative activities than those not forming fibrotic foci and found that no significant association exists between the proliferative activity of fibroblasts forming fibrotic foci and the fibrotic foci diameter.[7] In contrast, the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci were significantly lower than the Allred scores for p53 in tumor-stromal fibroblasts not forming fibrotic foci, and a significant association between the increase in the Allred scores for p53 in tumor-stromal fibroblasts forming fibrotic foci and the fibrotic foci diameter was observed in this study. Thus, although the mechanism that increases the malignant potential of IDCs through the expression of p53 in tumor-stromal fibroblasts should be investigated from the viewpoint of

*p53* gene abnormalities, p53 immunoreactivity in tumor-stromal fibroblasts produced by tumor cell–stromal cell interactions inside and outside fibrotic foci might in fact reflect specific reactive changes other than the proliferative activity of fibroblasts forming fibrotic foci within the stroma that might be correlated with the prognosis.

In conclusion, this is the first study to show clearly that p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci is strongly associated with the outcome of IDC patients. Because p53 expression in tumor-stromal fibroblasts forming and not forming fibrotic foci might be important in tumor progression in IDCs, p53 expression could be a very important target for tumor gene therapy for IDCs, suppressing tumor cell–stromal cell interactions arising from *p53* gene abnormalities or p53-related tumor microenvironment reactions.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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Original article

# Evaluation of sentinel node biopsy by combined fluorescent and dye method and lymph flow for breast cancer

Takashi Hojo*, Tomoya Nagao, Mizuho Kikuyama, Sadako Akashi, Takayuki Kinoshita

*National Cancer Center Hospital, Department of Surgery and Division of Breast Cancer, 5-1-1 Tsukiji Chuo-ku, Tokyo, Japan*

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ABSTRACT

*Background:* Conservative breast resection with subsequent sentinel lymph node biopsy (SNB) is an increasingly popular initial approach for the treatment of breast cancer due to decreased invasiveness. SNB is a shorter procedure with fewer side effects than more substantial surgical procedures, but it sometimes fails to identify metastatic disease. Therefore, a highly sensitive and convenient method is needed to identify sentinel lymph nodes (SLN) with a high probability of containing disease in SNB. We compared the combination of radioisotope or dye with a fluorescence compound to analyze lymph flow to identify targets for SNB.

*Materials and methods:* We examined patients with breast cancer lacking metastases in the axillary lymph node (ALN). Two methods for targeted SNB were developed: (1) Indocyanine Green (ICG) and Patent blue were injected into the skin overlying the tumor and sub-areolar region just before the surgical procedure. (2) ICG and radiocolloid were injected into the skin overlying the tumor and sub-areolar region. The draining fluorescent lymphatic duct was visualized using a Photodynamic Eye (PDE). We removed the SLNs that were identified by the dye and fluorescence imaging methods. Method 1 was applied to 113 patients undergoing SNB, and 29 patients were treated with Method 2. In our study, patients were grouped by lymph flow into two types: Type C demonstrated convergence to one lymph duct. Type S demonstrated separate lymph ducts.

*Results:* Using the fluorescence imaging method, 99.3% of SLNs were identified, and 3.8 SLNs per patient were seen. The SLN identification rates for Patent blue dye and radiocolloid were 92.9% and 100%, respectively, while 1.9 and 2.0 SLNs per patient, respectively, were seen with these methods. We classified two types of lymph flow based on the pattern of lymphatic drainage. Type C converged to a single lymph duct, while Type S drained to separate ducts. Type S lymph drainage was seen in 29/142 patients (20.4%), and Type C drainage was found in 113/141 patients (79.6%). Of the patients with Type S drainage, there were 4.1 SLNs per patient, but only 3.4 SLNs per patient were seen in individuals with Type C drainage. Forty cases had metastases found in the ALNs, and five of these cases were dye-negative and fluorescence-positive. Among these cases, the average number of SLNs identified was one.

*Conclusion:* The combination of fluorescence with a visible dye is a highly sensitive method for SLN identification. When SNB is guided by only the dye method, there is a risk of missing appropriate SLNs in patients with Type S lymph drainage or weak dye staining. The use of a fluorescence method together with dye could increase sensitivity of detection in these cases. Furthermore, fluorescent methods are ideal for hospitals that cannot use conventional radioactive measures.



## Introduction

Recent efforts in the surgical treatment of breast cancer have focused on breast conserving procedures, and ALN resection has become progressively less invasive with the implementation of sentinel lymph node biopsy (SNB). However, SNB can fail to identify lymph node metastases, and it is important to identify the optimal sentinel lymph node (SLN) for biopsy. This is a critical step in the evaluation of ALN status in patients with early breast cancer. Several methods are currently used to identify sentinel nodes including the dye method, the gamma probe-guided method, or a combination of these two, and there are many reports describing the successful use of these methods.[1] The combined use of a dye and gamma probe is more accurate compared to the dye method

* Corresponding author.
*E-mail address:* tahojo@ncc.go.jp (T. Hojo).

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