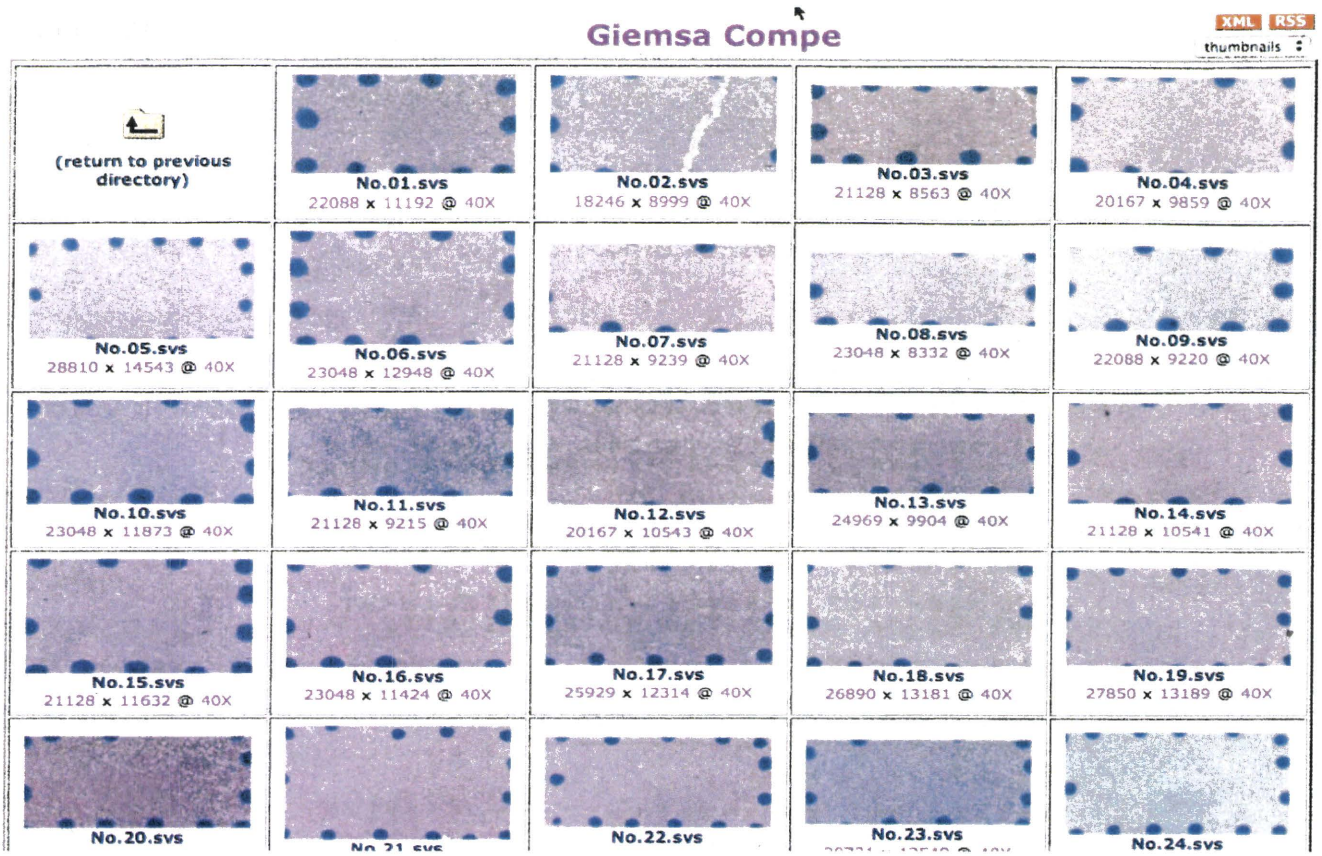

図 4　染色像一覧画面

画像へアクセスすると，まず画像一覧画面が展開される．自施設と他施設の染色像を対比することが容易となる．

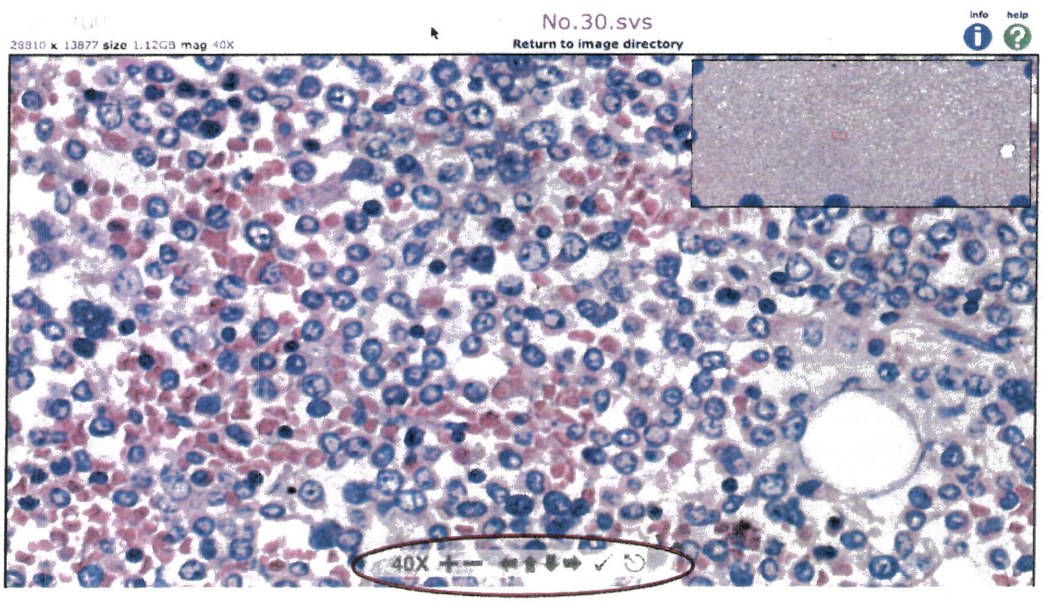

図 5　染色像供覧画面

下段のコントロールパネル(赤枠)で任意の倍率に変えることが可能で，画像内の移動も容易である．

ことにより詳細を確認したい箇所への移動も可能である．また，通常の印刷操作によって目的とする箇所の染色画像を印刷することも可能となる．

同時に，各参加施設へはこの方法による外部精度管理報告に対するアンケート調査を実施した．

Ⅱ 結　果

この方法による外部精度管理報告に対するアンケート調査の内容と集計結果を図**6**および図**7**に示す．

参加33施設中，職場でインターネット環境の利用が可能である施設は30施設(91%)で，3施設がインターネットの使用が不可能であり(図6)，DVDに画

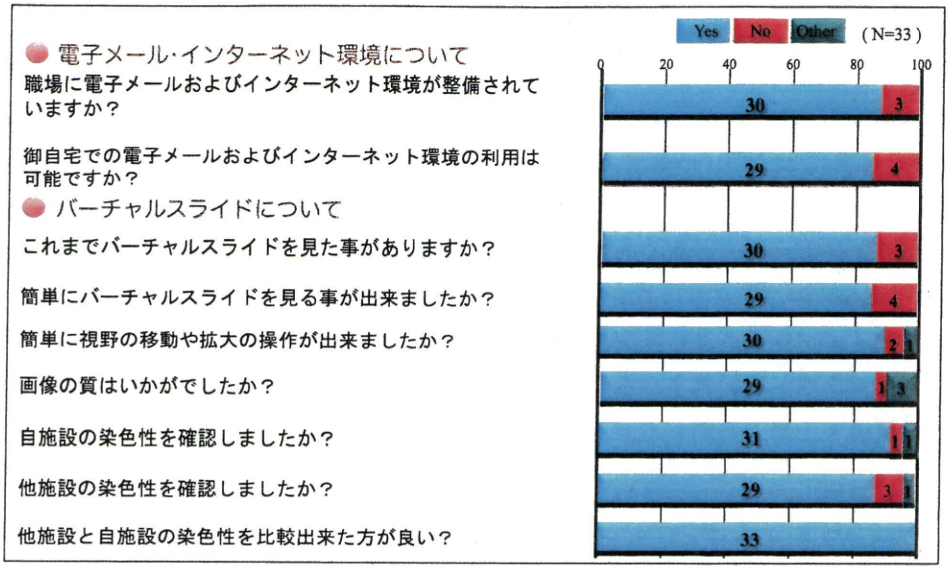

図6　アンケート調査(1)

インターネット環境の整備とバーチャルスライドに対する印象についてのアンケート調査結果を示す．概ね良好な反応を得ている．

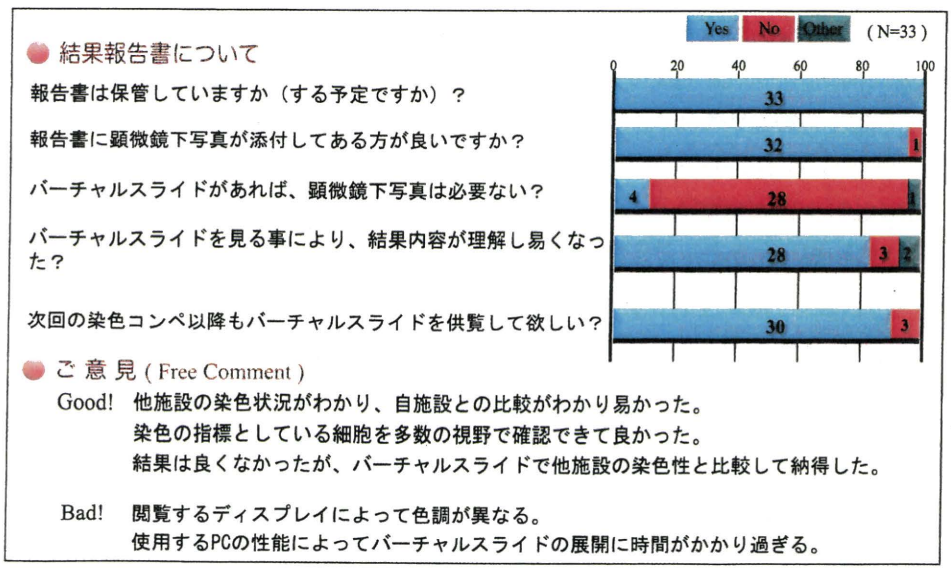

図7　アンケート調査(2)

バーチャルスライドを使った精度管理報告についてのアンケート結果を示す．多くの施設が結果内容が理解しやすくなったと回答している．グラフ下には，自由記載とした会員からの主な意見を記す．

像ファイルを入れたものを送付し，染色像の確認をしてもらった．

バーチャルスライドに対する印象や画像の取り扱いおよび画像の質に対しても，大半の施設からおおむね良好な反応を得た(図6)．

報告書の保管は，全施設において実施されておりバーチャルスライドの供覧とともに，従来通りに各施設ごとの顕微鏡下写真を添付してほしいという意見が多くあった(図7)．

33施設中28施設(85%)の精度管理担当者が，バーチャルスライドの供覧によって結果内容が理解しやすくなったと回答し，次回からもこの方法を採用してほしいとした施設は30施設(91%)であった(図7)．

自由記載の欄の『良かった』とする意見では，「報告書の内容が理解しやすくなった」とする主旨の意見が多く，この他に「経験の浅い技師への教育に利用できる」等の意見もあった．『悪かった』とする意見には，使用するディスプレイにより色調が異なることや，バーチャルスライドを閲覧する際の展開に時間がかかる等の使用する端末に関する意見があった．

Ⅲ 考　察

近年，インターネット環境の整備によって多くの情報がより簡単に収集でき，それを共有するツールとして活用することが可能となった[1~5]．今回試行した病理組織染色外部精度管理の報告方法はこの利便性に着目し活用したものであり，染色像の共有により精度管理評価報告書への理解が高まることを期待したものである．

現在，他地域の臨床衛生検査技師会でおのおの企画し，実施されている病理組織染色の外部精度管理は，指定した染色を各施設の染色プロトコルによって実施後返送してもらうため，報告書が参加施設へ届くころには各施設の手元に染色実施標本が残らず，報告書の内容とともに改めて自施設の染色性を確認することが不可能である．北臨技形態部門ではこの不便さを解消し，報告書への理解を高めるために過去の染色コンペ評価報告書には，実際の各施設の染色像とともに標準的染色性を示した施設の染色像と，評価判定基準を示す数枚の顕微鏡下写真を添付してきた．しかし，この方法ではいくつかの改良すべき問題点があった．①他施設の染色状況と対比することができない．②一部分の顕微鏡下写真のみでは，染色不良箇所を十分にチェックすることができない．③参加全施設分の顕微鏡下写真を撮影する部門員の負担と，印刷にかかるコストが大きいことなどが挙げられる．

今回，バーチャルスライドによる染色像の供覧を顕微鏡下写真に変わり評価報告書へ付随することにより，広範囲な染色像の閲覧が可能となり，染色像一覧画面ではほぼルーペ像に近い状態で提示することができるため(図4)，自施設のみならず参加全施設の染色性を容易に対比確認することが可能となった．

同一組織切片を用いて統一した染色テーマによる多施設の染色像を一度に比較提示できるこの方法は，インターネット環境を介する利点でもあり[3]，施設間の染色性統一化へ向けて大変有意義なものである．また，数視野だけでなく広範囲な染色画像を供覧することにより施設ごとの評価を明確化することができ，評価報告書への理解も得やすくなることが期待できる．

これまで実施してきた染色コンペに提出されたガラス標本は，後の検討資料とするために標本保管ケースに入れ保存してきた．この場合，経年的な褪色を避けることができなかったが，バーチャルスライド画像として保管することで，提出されたままの染色状況で半永久的な染色像の保存が可能となることも，デジタル化することの利点の一つに挙げられる[1]．

バーチャルスライド画像を作製する労力については，今回作製した5.0×8.0 mmの染色範囲では，およそ1施設分に対して10分程度の処理時間があれば十分であり，供覧する染色画像作製の簡易化と画像はそのままインターネットを経由して各施設へ配信されるため，印刷費用の軽減を図ることができる．しかしながら，バーチャルスライドの画像配信があれば印刷物としての染色像の配布が必要なくなるか？というアンケート調査の問いに対しては，「いいえ」と答えている施設が多く(図7)，配布されたものをそのままファイリングして保管したいという手間の問題や各施設保有のプリンターの性能に対する結果であると考える．

今回，この方法を試行したことでいくつかの短所

も明らかとなった．その一つとして，閲覧する際に使用する端末の能力については，施設ごとにさまざまであり統一化が困難である．実際にアンケート調査の自由記載の欄には，画像展開する際の処理スピードが遅いとの意見もあった．これに関しては，バーチャルスライドの画像情報量が膨大であることに起因するものであるが[2,3]，バーチャルスライド画像の圧縮技術が各ベンダーにより開発され，ブロードバンド時代の昨今，通信速度の改善によりこの問題を解決し，各施設の使用機器の性能が向上されることに期待したい[1~5]．また，使用するPCモニターによって色具合が異なることも確認した．今回は，染色コンペ参加施設の標本を北臨技形態部門員4名による同時顕微鏡下観察での染色性評価に先行して，バーチャルスライドによる評価を実施しており，細胞質内顆粒等の詳細な判定を必要とするギムザ染色に関しては，顕微鏡下での観察が当然ながら勝る結果となり，両者間で判定結果に乖離が生じた染色標本もみられた．このことは，近年バーチャルスライドをみる機会が増えつつあるものの，判定時のバーチャルスライド画像の取り扱いや視点の慣れに起因するものと考える．

鷲谷は，モニター上で染色性の善し悪しを議論するには，明るさと色具合を忠実に再現する必要があるとし，その詳細な画像の設定基準について報告している[6,7]．特に，バーチャルスライドを作製する際，染色種による色調の再現性について，今後種々の染色方法での検討が必要となり，モニターのRGB色調バランス設定やホワイトバランスあるいは輝度調整方法についての仕様を報告書に付記して，染色手技のみならず閲覧使用モニターの施設間統一化を図っていきたい．

現在，バーチャルスライド作製装置の普及に伴い，遠隔病理診断や多地点におけるカンファランス等，バーチャルスライド技術を用いたさまざまな活用方法が導入されつつある[1~5]．しかし，この方法による画像供覧を付随させた病理組織染色の外部精度管理報告を採用している検査技師会は，国内をみても他にない．デジタル化の気運上昇とともに，今後この方法が発展することが期待される．

Ⅳ 結　語

新たな外部精度管理事業の報告方法として，評価報告書にバーチャルスライドによる染色画像を供覧する方法を提案した．この方法は，多施設を対象とした病理組織染色の外部精度管理報告に有用であることをアンケート調査結果より確認した．今後いくつかの問題点を解消し，さらに精度の高い外部精度管理事業として定着させていきたい．

本論文の要旨は，第58会日本医学検査学会(2009年7月，横浜)および，第8回日本テレパソロジー・バーチャルマイクロスコピー研究会(2009年8月，仙台)において報告した．

■文献

1) 澤井高志：遠隔病理診断(テレパソロジー)の展望，機器・試薬 2009；32：326-336.
2) 澤井高志：バーチャルスライドの特集にあたって．進化するバーチャルスライド，Medical Technology 2008；36：792-795.
3) 東福寺幾夫：バーチャルスライドの原理と特徴．進化するバーチャルスライド，Medical Technology 2008；36：796-800.
4) 松野吉宏：バーチャルスライドの応用-厚生労働省が目指すもの．進化するバーチャルスライド，Medical Technology 2008；36：801-803.
5) 山城勝重：テレパソロジー/バーチャルスライドシステムの遠隔医療への応用，Medical Technology 2008；36：824-827.
6) 鷲谷清忠：1．細胞診のためのデジタル画像の正しい取り扱い方法，日本臨床細胞学会秋田県支部会誌 2009；15：17-22.
7) 鷲谷清忠：2．形態学のためのデジタル画像の取り扱い方法，日本臨床細胞学会秋田県支部会誌 2009；15：23-27.

MODERN PATHOLOGY (2010) 23, 581–592

Grading system for lymph vessel tumor emboli: significant outcome predictor for patients with invasive ductal carcinoma of the breast who received neoadjuvant therapy

Takahiro Hasebe[1], Nobuko Tamura[2], Motoki Iwasaki[3], Nao Okada[2], Sadako Akashi-Tanaka[2], Takashi Hojo[2], Chikako Shimizu[4], Masashi Adachi[4], Yasuhiro Fujiwara[4], Tatsuhiro Shibata[5], Yuko Sasajima[6], Histoshi Tsuda[6] and Takayuki Kinoshita[2]

[1]*Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo, Japan;* [2]*Department of Breast Surgery, National Cancer Center Hospital, Tokyo, Japan;* [3]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan;* [4]*Division of Breast and Medical Oncology, National Cancer Center Hospital, Tokyo, Japan;* [5]*Cancer Genomics Project, National Cancer Center Research Institute, Tokyo, Japan and* [6]*Clinical Laboratory Division, National Cancer Center Hospital, Tokyo, Japan*

The purpose of this study was to confirm that the grades of lymph vessel tumor emboli in biopsy specimens obtained before neoadjuvant therapy and in the surgical specimens obtained after neoadjuvant therapy according to the grading system we devised are significant histological outcome predictor for invasive ductal carcinoma (IDC) patients who received neoadjuvant therapy. The subjects of this study were the 318 consecutive IDC patients who had received neoadjuvant therapy in our institution. The lymph vessel tumor embolus grades in the biopsy specimens and in the surgical specimens were significantly associated with the increases in mean number of nodal metastases. Multivariate analyses with well-known prognostic factors and p53 expression in tumor-stromal fibroblasts clearly showed that the lymph vessel tumor embolus grade based on the biopsy specimens and based on the surgical specimens significantly increased the hazard rates for tumor recurrence and tumor-related death in all the IDC patients as a whole, in the IDC patients who did not have nodal metastasis, and in the IDC patients who had nodal metastasis, and the outcome-predictive power of the lymph vessel tumor embolus grades based on the surgical specimens was superior to that of the lymph vessel tumor embolus grades based on the biopsy specimens. The grades in the grading system for lymph vessel tumor emboli were significantly associated with nodal metastasis, and the histological grading system is an excellent system for accurately predicting the outcome of patients with IDC of the breast who have received neoadjuvant therapy.
Modern Pathology (2010) **23,** 581–592; doi:10.1038/modpathol.2010.3; published online 29 January 2010

Keywords: lymph vessel; histology; breast cancer; prognosis; p53

Lymphatic invasion in breast cancer patients with invasive ductal carcinoma (IDC) has been reported to have prognostic significance.[1–5] We have already reported that the grading system for lymph vessel tumor emboli that we devised is a very useful histological grading system for accurately predicting the outcome of patients with IDC who did not receive neoadjuvant therapy, and that the grading system can be used to classify IDC patients with lymph vessel invasion into a low-, intermediate-, and high-risk groups for outcome.[6] Furthermore, we have recently reported finding that the lymph vessel tumor embolus grades based on the biopsy specimens and based on the surgical specimens are also

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
Received 15 September 2009; revised 27 November 2009; accepted 7 December 2009; published online 29 January 2010

an important outcome-predictive factor for IDC patients who received neoadjuvant chemotherapy and had nodal metastasis.[7]

The purpose of this study was to confirm that the grading system for lymph vessel tumor embolus is a significant outcome predictor for IDC patients who received neoadjuvant therapy according to nodal status, by multivariate analysis with well-known clinicopathological factors and p53 protein expression in tumor-stromal fibroblasts in IDCs.[8] p53 protein expression in tumor-stromal fibroblasts, but not in tumor cells, in IDCs has been recently demonstrated to be a very important outcome predictor for IDC patients who received neoadjuvant therapy.[8] The results of this study clearly showed that the grading system for lymph vessel tumor emboli is an excellent histological outcome predictive of the histological grading system for IDC patients who received neoadjuvant therapy independent of nodal metastasis.

Materials and methods

Cases

The subjects of this study were the 318 consecutive patients with IDC of the breast who received neoadjuvant therapy and were surgically treated at the National Cancer Center Hospital between January 2000 and December 2005 (the same series of patients as in an earlier study we conducted[8] and 88 patients of the 318 patients in this series were included among the subjects of our previous study[7]). Their IDCs were diagnosed preoperatively by needle biopsy, aspiration cytology, mammography, or ultrasonography. Clinical information was obtained from the patients' medical records after complete histological examination of all IDCs. All patients were Japanese women, and they ranged in age from 23 to 77 years old (median, 55 years). All had a solitary lesion; 127 patients were premenopausal, and 191 were postmenopausal. Partial mastectomy had been performed in 152, and modified radical mastectomy in 166. Level I and Level II axillary lymph node dissection had been performed in all patients, and Level III axillary lymph node dissection had been performed in some of the IDC patients.

Of the 318 subjects, 37 (12%) had shown a pathological complete response to neoadjuvant therapy (34, no residual tumor and no nodal metastasis; 3, residual ductal carcinoma *in situ* and no nodal metastasis).

The neoadjuvant therapy consisted of chemotherapy in 235 patients, endocrine therapy in 43 patients, and chemoendocrine therapy in 3 patients, and 214 out of the 281 patients who had received neoadjuvant therapy had also received adjuvant therapy, which consisted of chemotherapy in 47 patients, endocrine therapy in 116 patients, and chemoendocrine therapy in 51 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. There were no cases of inflammatory breast cancer in this series. All tumors were classified according to the pathological UICC-TNM (pTNM) classification system.[9] The protocol of this study (20–112) was reviewed by the institutional review board of the National Cancer Center, and all patients provided written informed consent.

For the pathological examination, biopsy specimens obtained before neoadjuvant therapy and surgically resected specimens obtained after neoadjuvant therapy were fixed in 10% formalin and subsequently examined. The size and gross appearance of the surgically resected tumor specimens were recorded as the residual invasive tumor size. The tumor size of the surgically resected specimens was confirmed by comparison with the tumor size on histological slides; if more than one invasive focus was present, the size of the largest invasive focus was recorded as the residual invasive tumor size in this study.

Histological Examination

Serial sections of the biopsy specimens obtained before neoadjuvant chemotherapy and of the tumor area in the surgically resected specimens obtained after neoadjuvant therapy were cut from paraffin wax blocks. One section of each biopsy specimen and surgical specimen was stained with hematoxylin and eosin and examined histologically to confirm the diagnosis, and another section was subjected to immunohistochemistry. The following eight histological features of the primary-invasive tumors were evaluated in the biopsy specimens obtained before neoadjuvant therapy and the surgical specimens obtained after neoadjuvant therapy: (1) residual tumor size (no residual tumor or residual ductal carcinoma *in situ*, residual tumor $\leq$20 mm, >20 to $\leq$50 mm, >50 mm), (2) histological grade (1, 2, 3),[10] (3) tumor necrosis (absent, present),[11] (4) fibrotic focus (biopsy specimen: absent, present; surgical specimen: absent, fibrotic focus diameter <8 mm, fibrotic focus diameter >8 mm),[12,13] (5) blood vessel invasion (absent, present), (6) adipose tissue invasion (absent, present), (7) skin invasion (absent, present), and (8) muscle invasion (absent, present). We also evaluated the outcome-predictive power of Fisher's neoadjuvant therapy effect classification for surgical specimens obtained after neoadjuvant therapy.[14,15]

Grading System for Lymph Vessel Tumor Emboli in IDCs

We have already stated the histological criteria of the grading system for lymph vessel tumor emboli in

our previous study.[6,7] Briefly, we first examined all slides of IDCs that contained both tumor areas and nontumor areas to identify lymph vessel tumor emboli. Next, we selected the lymph vessel tumor emboli, for example, large lymph vessel tumor emboli far from the stroma-invasive tumor margin to examine for the presence and number of mitotic figures and apoptotic figures of lymph vessel tumor emboli in an IDC (Figures 1a and c). We classified all IDCs into the following four grades according to the number of mitotic and apoptotic figures in tumor cells in lymph vessels in one per high-power field: grade 0, no lymph vessel invasion; grade 1, absence of no mitotic and or apoptotic figures, or presence of any number of mitotic figures and absence of but no apoptotic figures, or absence of mitotic figure and presence of any number of apoptotic figures but no mitotic figures; grade 2, 1–4 mitotic figures and ≥ 1 apoptotic figures, or ≥ 1 mitotic figures and 1–6 apoptotic figures; and grade 3, >4 mitotic figures and >6 apoptotic figures. We selected the lymph vessel tumor emboli in which to count the mitotic figures and apoptotic figures and then recorded the numbers of mitotic figures and apoptotic figures among the tumor cells making up the lymph vessel tumor emboli of the IDC in the high-power field that contained the largest number of mitotic figures, and/or the largest number of apoptotic figures were recorded as the number of mitotic figures and apoptotic figure in the lymph vessel tumor emboli of the IDC. For the biopsy specimens, we examined the presence or absence of lymph vessel tumor

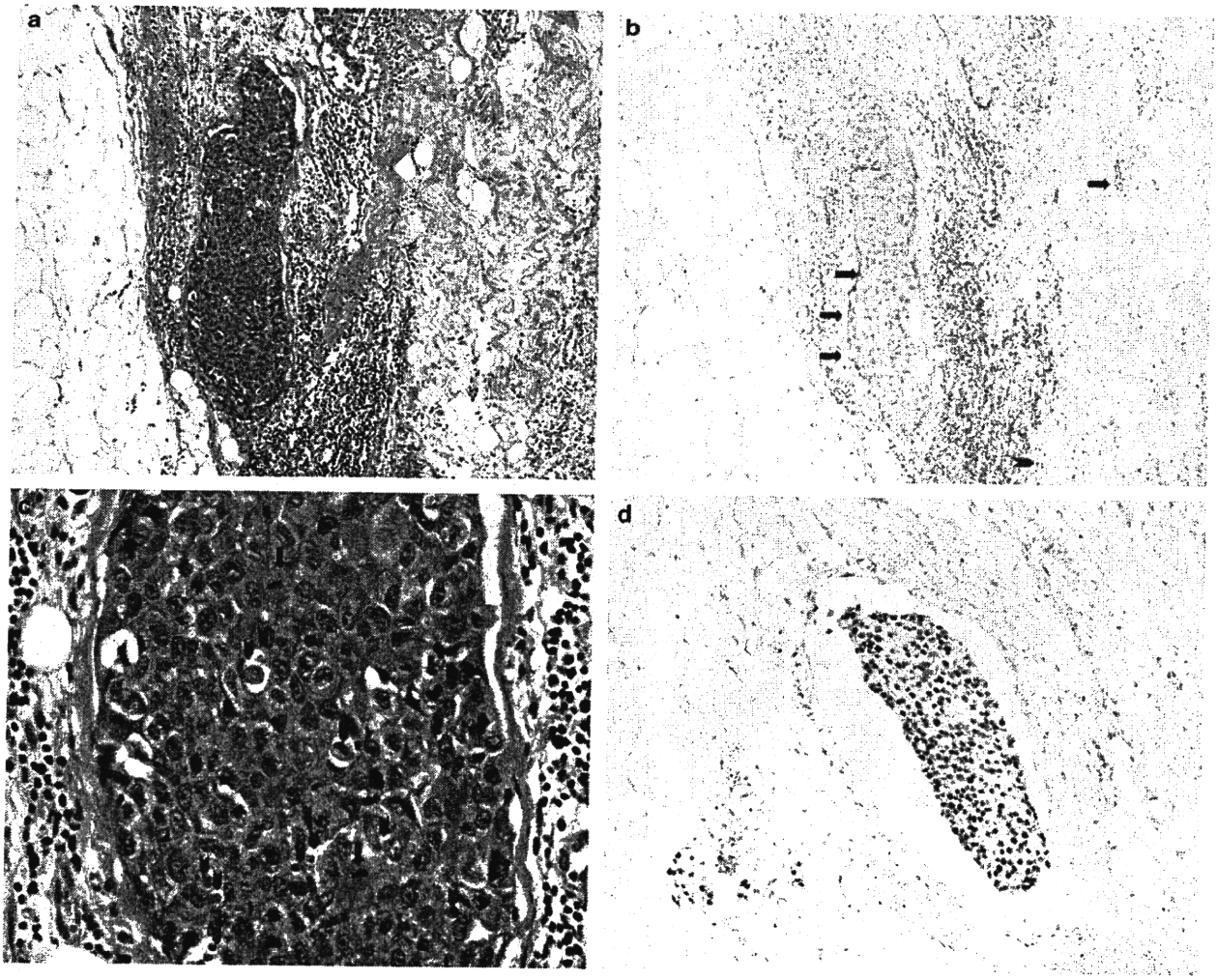

Figure 1 (**a**) Two lymph vessel tumor emboli are observed. (**b**) Vessel walls positive for D2-40 around two lymph vessel tumor emboli can be seen (arrows). One small lymph vessel is also positive for D2-40 (arrow), and one artery is negative for D2-40 (arrowhead). (**c**) Several apoptotic bodies and apoptotic tumor cells are observed (arrowheads), and there are five mitotic tumor cells (arrows) in tumor embolus in the lymph vessel. Apoptotic bodies are small, variously shaped pyknotic bodies that resemble sesame seeds, and apoptotic tumor cells were identified as tumor cells that contained eosinophilic or amphophilic cytoplasm and an irregularly shaped pyknotic nucleus. (**d**) Three lymph vessel tumor emboli with an Allred score of 8 for p53 can be seen. More than 90% of the tumor cells comprising of the lymph vessel tumor emboli show an intense nuclear staining for p53.

embolus or lymph vessel tumor emboli; when lymph vessel tumor embolus or lymph vessel tumor emboli were observed in the biopsy specimen, an assessment similar to that described above was performed. Some IDCs contained large lymph vessel tumor emboli, especially in IDCs containing a grade 2 or 3 lymph vessel tumor emboli, and it was difficult to determine whether they were true lymph vessel tumor emboli or a non-IDC component by hematoxylin and eosin staining alone. We therefore performed immunohistochemical staining with D2-40 antibody (monoclonal mouse antibody, Signet, Dedham, MA, USA, 1:200) to confirm that the lymph vessel tumor emboli identified by hematoxylin and eosin staining in some of the IDCs with grade 2 or 3 lymph vessel tumor emboli were true tumor emboli (Figure 1b). The D2-40 antibody was generated against an O-linked sialoglycoprotein having a molecular weight of 40 kDa and had been shown to be a selective marker of the lymphatic endothelium.[16,17]

Immunohistochemistry

Immunohistochemical staining for estrogen receptors, progesterone receptors, p53, HER2 products, and D2-40 was performed with an autoimmunostainer (Optimax Plus: BioGenex, San Ramon, CA, USA). The antigen retrieval device used for the Optimax Plus was an autoclave, and each specimen was immersed in citrate buffer and incubated at 121°C for 10 min. Immunoperoxidase staining was performed by using a labeled streptavidin biotin staining kit (BioGenex) according to the manufacturer's instructions. The antibodies used were an anti-estrogen receptor mouse monoclonal antibody (mAb), ER88 (BioGenex), an anti-progesterone receptor mAb, PR88 (BioGenex), and an anti-HER2 mAb, CB11 (BioGnex), and a p53 mAb, DO7 (Dako, Glostrup, Denmark). ER88, PR88, and CB11 were already diluted, and DO7 was applied at a 1:100 dilution. After immunostaining, the sections were counterstained with hematoxylin. Sections of IDCs positive for estrogen receptor, progesterone receptor, p53, HER2, and D2-40 were used each time as positive controls. As for a negative control, the primary antibody was replaced with normal mouse immunoglobulin.

Slides immunostained for estrogen receptor, progesterone receptor, and p53 in stroma-invasive tumor cells, and for p53 in tumor-stromal fibroblasts were scored by the Allred scoring system as previously described.[8,18–22] The highest intensity score, not the average intensity score, for nuclear expression of p53 was assigned for in tumor-stromal fibroblasts, and the highest p53 nuclear expression proportion score and intensity score were then to be evaluated in one high-power field ($\times 40$ objective and $\times 10$ ocular).[8] The Allred scores for estrogen receptor, progesterone receptor, and p53 expression in stroma-invasive tumor cells and tumor-stromal fibroblasts were classified into the following three categories: (1) Allred score for estrogen receptor in stroma-invasive tumor cells, 0 or 2, 3–6, and 7 or 8; (2) Allred score for progesterone receptor in stroma-invasive tumor cells, 0 or 2, 3–6, and 7 or 8; (3) Allred scores for p53 in stroma-invasive tumor cells, 0 or 2 or 3, 4–6, and 7 or 8; and (4) Allred scores for p53 in tumor-stromal fibroblasts, 0 or 2, 3, and 4–8. HER2 expression in stroma-invasive tumor cells was classified into the three categories: 0 or 1, 2, and 3.[23] We also assigned Allred scores for estrogen receptor, progesterone receptor, and p53 (Figure 1d), and HER2 category in lymph vessel tumor emboli by the similar manner as in stroma-invasive tumor cells in 26 of the 82 IDCs with lymph vessel invasion. We were unable to assign Allred scores for estrogen receptor, progesterone receptor, and p53, and HER2 category in the other IDCs with lymph vessel invasion, because immunohistochemistry for these was performed in tumor tissue sections that did not containing an lymph vessel tumor embolus.

One author (TH) assessed all the immunohistochemical parameters, and one of four other authors (NT, HT, TS, or YS) reviewed the immunohistochemical parameters to confirm the IDC immunohistochemical characteristics recorded by TH. Discordant results were reevaluated jointly to reach until a consensus was reached. The histological examination and immunohistochemical examination were performed without knowledge of the patient's outcome.

Patient Outcome and Statistical Analysis

Survival was evaluated by follow-up for a median period of 62 months (range: 38–105 months) until February 2009. As of the end of February 2009, 191 of the 281 patients were alive and well, 90 had developed tumor recurrence, and 53 had died of their disease. The measurements of tumor-recurrence-free survival, and overall survival started at the time of surgery. Tumor relapse was considered to have occurred whenever there was evidence of metastasis.

Multiple regression analysis was used to perform the statistical analyses for associations between lymph vessel tumor embolus grade and number of lymph node metastases, and the correlation analyses were performed by the correlation statistics of Cochran–Mantel–Haenszel statistics. We analyzed the outcome-predictive power for tumor recurrence and tumor-related death by the univariate and multivariate analyses using the Cox proportional hazard regression model. The factors analyzed were the mentioned eight factors, age (≤ 39, >39 years), type of neoadjuvant therapy (endocrine therapy, chemotherapy and chemoendocrine therapy), adjuvant therapy (no, yes), and the factors that were significantly associated with outcome in the uni-

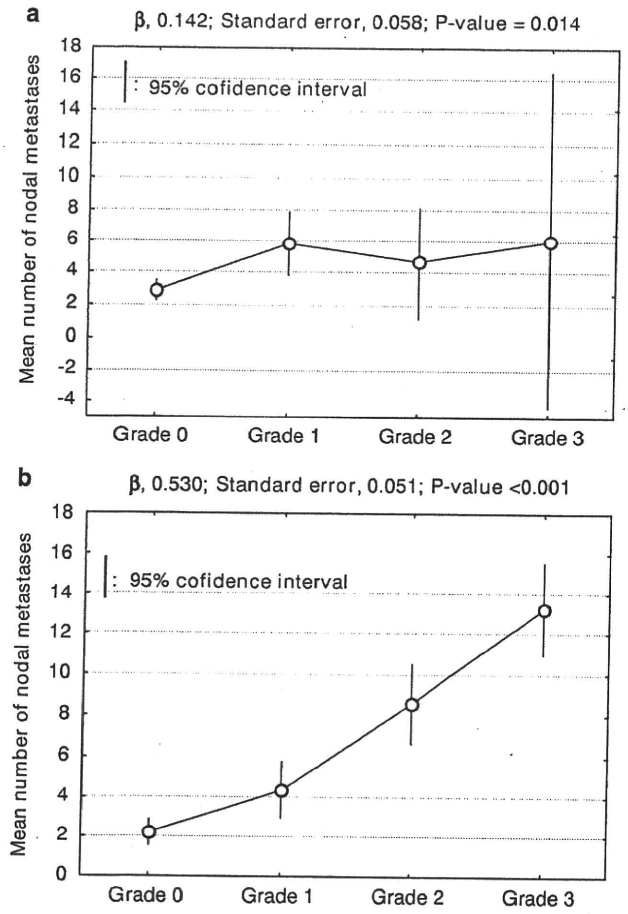

Figure 2 Graphs showing associations between mean nodal metastasis values and the grades of lymph vessel tumor emboli in the biopsy specimens (**a**) and in the surgical specimens (**b**). The mean number of nodal metastases increases significantly with the grade of lymph vessel tumor emboli in the biopsy specimens and in the surgical specimens.

variate analyses were then entered together into the multivariate analyses according to nodal status. As the eight factors were examined using both biopsy specimens obtained before neoadjuvant therapy and surgical specimens obtained after neoadjuvant chemotherapy, to accurately assess the prognostic value of each of these factors in multivariate analyses, their mutual influence on outcome was avoided by conducting separate analyses of the prognostic predictive power of the findings in the biopsy specimens obtained before neoadjuvant therapy and the surgical specimens obtained after neoadjuvant therapy (model 1, factors examined based on biopsy specimens obtained before neoadjuvant therapy; model 2, factors examined based on surgical specimens obtained after neoadjuvant therapy). The case-wise and step-down method was applied until all the remaining factors were significant at a *P*-value below 0.05. As there were fewer than 10 tumor deaths among the patients who did not have nodal metastasis, we were unable to perform multivariate analyses for tumor death in this groups.

Table 1 Association between grading system for lymph vessel tumor emboli and the Allred scores for p53 in stroma-invasive tumor cells, the Allred scores for p53 in tumor-stromal fibroblasts and the Allred scores for p53 in lymph vessel tumor emboli assessed in the surgical specimens

	Grades of lymph vessel tumor emboli				
Case ($n=271$)	*Grade 0* 191	*Grade 1* 42	*Grade 2* 22	*Grade 3* 16	*P-value*
Allred scores for p53 in stroma-invasive tumor cells					
0 or 2 or 3	63 (33)	14 (32)	6 (27)	0	0.001
4–6	75 (39)	22 (54)	9 (41)	3 (19)	
7 or 8	53 (28)	6 (14)	7 (32)	13 (81)	
Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	110 (58)	27 (64)	6 (27)	1 (6)	<0.001
3	26 (13)	6 (14)	2 (9)	5 (31)	
4–8	55 (29)	9 (22)	14 (64)	10 (63)	
Allred scores for p53 in lymph vessel tumor emboli					
0 or 2 or 3		6 (55)	2 (50)	1 (9)	0.005
4–6		2 (18)	0	0	
7 or 8		3 (27)	2 (50)	10 (91)	

Survival curves were drawn by the Kaplan–Meier method. All analyses were performed with Statistica/Windows software (StatSoft, Tulsa, OK, USA).

Results

Associations Between the Lymph Vessel Tumor Embolus Grades and Factors

Although the lymph vessel tumor embolus grades based on the biopsy specimens and based on the surgical specimens were significantly associated with the increases in mean number of nodal metastases (Figure 2), the value of β for the correlation between lymph vessel tumor embolus grades in the surgical specimens and mean number of nodal metastases was higher than between the lymph vessel tumor embolus grades in the biopsy specimens and the mean number of nodal metastases.

The results of the univariate analyses showed that the lymph vessel tumor embolus grades assessed in the surgical specimens were significantly associated with the Allred scores for p53 in stroma-invasive tumor cells and in tumor-stromal fibroblasts assessed in the surgical specimens (Table 1) and that they were also significantly inversely associated with Allred score for estrogen receptor in stroma-invasive tumor cells assessed in the surgical specimens in the univariate analyses (data not shown). There was no significant association between lymph vessel tumor embolus grades in the surgical specimens and progesterone receptor in stroma-invasive tumor cells in the surgical specimens, and between lymph vessel tumor embolus grades in the surgical specimens and HER2 category in tumor cells in the surgical specimens (data not shown). The Allred scores for p53 in lymph vessel tumor emboli

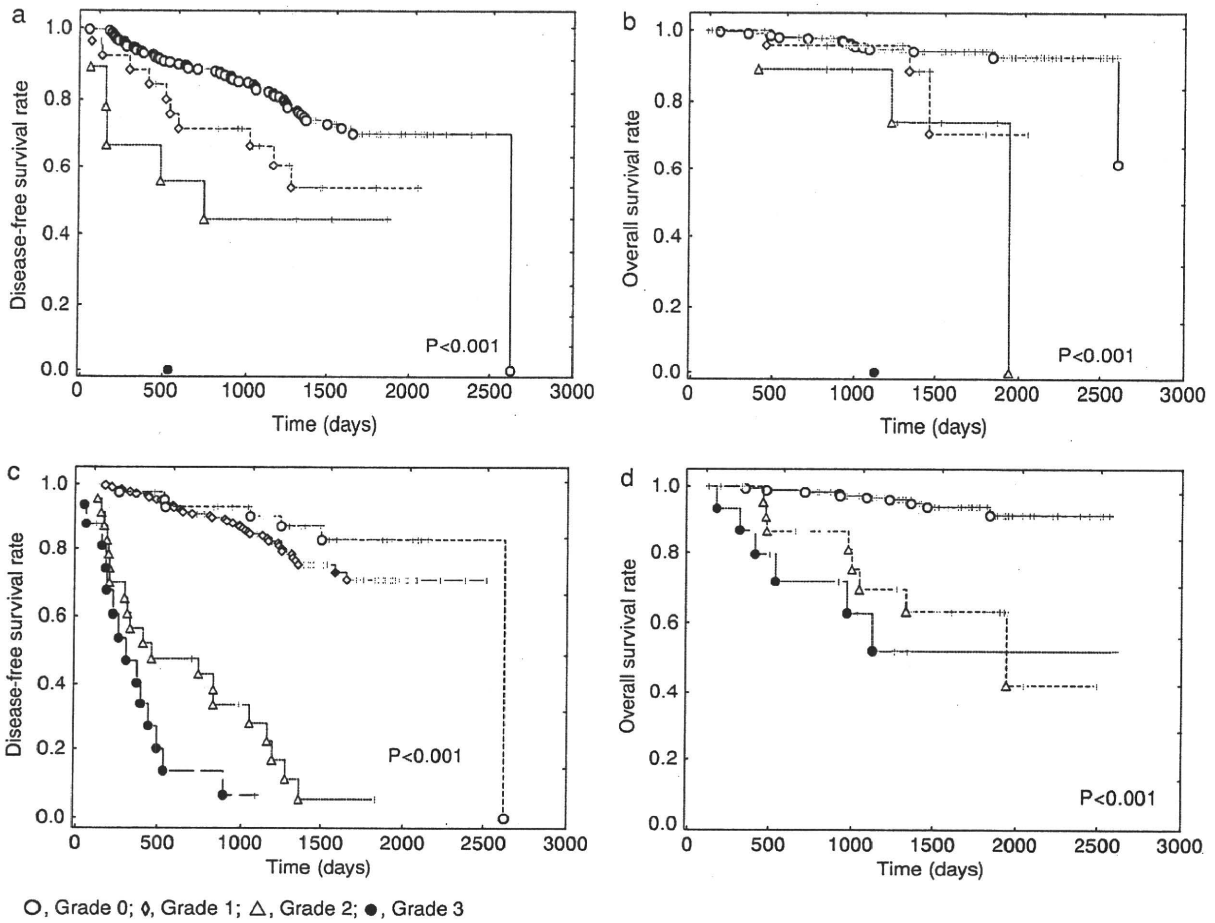

Figure 3 Disease-free survival curves (**a** and **c**) and overall survival curves (**b** and **d**) of all of the invasive ductal carcinoma (IDC) patients who received neoadjuvant therapy as a whole. (**a** and **b**) The disease-free survival time and the overall survival time of the IDC patients classified by grade of lymph vessel tumor emboli in the biopsy specimens obtained before neoadjuvant therapy become significantly shorter as the grades of lymph vessel tumor emboli increased. (**c** and **d**) The disease-free survival time and the overall survival time of the IDC patients classified by the grade of lymph vessel tumor emboli in the surgical specimens become significantly shorter as the grades of lymph vessel tumor emboli increased. None of the IDC patients had grade 1 lymph vessel tumor emboli show tumor-related death.

assessed in the surgical specimens were significantly associated with the grades of the lymph vessel tumor emboli assessed in the surgical specimens (Table 1), but there were no significant associations between the Allred scores for estrogen receptor or progesterone receptor in lymph vessel tumor emboli, or the HER2 categories in the lymph vessel tumor emboli and grades of lymph vessel tumor embolus assessed in the surgical specimens (data not shown).

In our previous study, although the multivariate analysis clearly showed that negative nodal status and HER2 category 3 in tumor cells were significantly associated with pathological complete response,[8] the lymph vessel tumor embolus grades based on the biopsy specimens were not significantly associated with pathological complete response in the univariate analysis (data not shown).

Factors Significantly Associated with Outcome

The univariate analyses of all of the cases as a whole showed that the lymph vessel tumor embolus grades in the biopsy specimens (Figures 3a and b) and the surgical specimens (Figures 3c and d) were significantly associated with tumor recurrence and tumor-related death (Table 2). In the multivariate analyses using model 1, UICC pTNM-pN1, N2, and N3 categories significantly increased the hazard rates for tumor recurrence, and UICC pTNM-pN2 and N3 also significantly increased the hazard rates for tumor-related death (data not shown). The Allred score of 3 for p53 in tumor-stromal fibroblasts and Allred scores of 4–8 for p53 in tumor-stromal fibroblasts significantly increased the hazard rates for tumor recurrence, and lymph vessel tumor embolus grades 2 and 3 also significantly increased the hazard rate

Table 2 Outcome rates of patients with invasive ductal carcinoma according to grade of lymph vessel tumor emboli, and according to nodal status

	Grade of lymph vessel tumor emboli							
Grade	*Assessed in the biopsy specimens*				*Assessed in the surgical specimens*			
	Cases	*TRR (%)*	*MR (%)*	*P-value*	*Cases*	*TRR (%)*	*MR (%)*	*P-value*
Invasive ductal carcinoma patients as a whole								
0	260	61 (23)	16 (6)	TRR <0.001	199	42 (21)	10 (5)	TRR <0.001
1	26	10 (38)	3 (12)	MR <0.001	43	7 (16)	0	MR <0.001
2	9	5 (56)	3 (33)		23	20 (87)	8 (35)	
3	1	1 (100)	1 (100)		16	14 (88)	6 (38)	
Total	296	77	23		281	83	24	
Invasive ductal carcinoma patients who did not have nodal metastasis								
0	113	10 (9)	3 (3)	TRR 0.003	90	12 (13)	2 (2)	TTR 0.010
1	12	4 (33)	0	MR 0.130	7	0	0	MR 0.006
2	2	1 (50)	1 (50)		3	3 (100)	2 (67)	
3	0				0			
Total	127	15	4		100	15	4	
Invasive ductal carcinoma patients who had nodal metastasis								
0	147	51 (35)	13 (9)	TTR 0.007	109	30 (28)	8 (7)	TTR <0.001
1	14	6 (43)	3 (21)	MR 0.001	36	7 (19)	0	MR <0.001
2	7	4 (57)	2 (29)		20	17 (85)	6 (30)	
3	1	1 (100)	1 (100)		16	14 (88)	6 (38)	
Total	169	62	19		181	68	20	

MR, mortality rate; TRR, tumor recurrence rate.

for tumor recurrence in the multivariate analyses (data not shown). Allred scores of 7 or 8 for estrogen receptors in tumor cells significantly decreased the hazard rate for tumor-related death, and HER2 category 3 in tumor cells significantly increased the hazard rate for tumor-related death in the multivariate analyses (data not shown). When model 2 was used, lymph vessel tumor embolus grade 2 and lymph vessel tumor embolus grade 3 significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analyses (data not shown). The Allred score of 3 in tumor-stromal fibroblasts, Allred scores of 4–8 for p53 in tumor-stromal fibroblasts, and histological grade 3 significantly increased the hazard rates for tumor recurrence, and the Allred scores of 4–8 in tumor-stromal fibroblasts and histological grade 3 also significantly increased the hazard rate for tumor-related death in the multivariate analyses (data not shown). Residual invasive tumor size >50 mm significantly increased the hazard rate for tumor recurrence and the presence of skin invasion significantly increased the hazard rate for tumor-related death in the multivariate analyses (data not shown).

In the group of IDC patients without nodal metastasis, the univariate analyses showed that the lymph vessel tumor embolus grades in the biopsy specimens were significantly associated with tumor recurrence, but not with tumor-related death, and that the lymph vessel tumor embolus grades in the surgical specimens were significantly associated with both tumor recurrence and tumor-related death (Table 2). In the multivariate analyses, lymph vessel tumor embolus grades 1 and 2 in the biopsy specimens, Allred scores of 4–8 for p53 in tumor-stromal fibroblasts in the biopsy specimens, and age ≤38 years significantly increased the hazard rates for tumor recurrence in the multivariate analyses (Table 3, model 1), and lymph vessel tumor embolus grade 2 in the surgical specimens, Allred score of 3 for p53 in tumor-stromal fibroblasts in the surgical specimens, and Allred scores of 4–8 for p53 in tumor-stromal fibroblasts in the surgical specimens significantly increased the hazard rates for tumor recurrence in the multivariate analysis (Table 3, model 2).

In the group of IDC patients with nodal metastasis, the univariate analyses showed that the lymph vessel tumor embolus grades in the biopsy specimens and the surgical specimens were significantly associated with tumor recurrence and tumor-related death (Table 2). In the multivariate analyses of model 1, lymph vessel tumor embolus grades 2 and 3, an Allred score of 3 for p53 in tumor-stromal fibroblasts, and Allred scores of 4–8 for p53 in tumor-stromal fibroblasts significantly increased the hazard rate for tumor recurrence, and lymph vessel tumor embolus grade 1 significantly increased the hazard rate for tumor-related death (Table 4). The Allred scores of 7 or 8 for estrogen receptors in tumor cells significantly decreased the hazard rate

Table 3 Multivariate analysis for tumor recurrence in invasive ductal carcinoma patients who did not have nodal metastasis

Factors	*Cases*	*Number of patients (%) Tumor recurrence*			
		Present	*HRs*	*95% CI*	P-*value*
Model 1					
Grading system for lymph vessel tumor emboli					
Grade 0	113	10 (9)	Referent		
Grades 1 and 2	12	4 (33)	4.2	1.3–13.0	0.014
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	40	0	Referent		
3	27	3 (11)	Referent		
4–8	57	12 (21)	3.9	1.1–14.3	0.037
Age (years)					
⩽39	21	7 (33)	Referent		
>39	114	11 (10)	0.3	0.1–0.9	0.040
Model 2					
Grading system for lymph vessel tumor emboli					
Grade 0	90	12 (13)	Referent		
Grade 1	7	0	Referent		
Grade 2	3	3 (100)	10.2	2.5–40.9	0.001
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	64	3 (5)	Referent		
3	12	4 (33)	6.4	1.4–29.6	0.017
4–8	19	7 (37)	9.8	2.2–34.9	0.002

CI, confidence interval; HR, hazard rate.
Model 1: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli and the Allred scores for p53 in tumor-stromal fibroblasts assessed in biopsy specimen obtained before neoadjuvant therapy, and age.
Model 2: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli and the Allred scores for p53 in tumor-stromal fibroblasts assessed in surgical specimen obtained after neoadjuvant therapy, and age.

for tumor recurrence, and Allred scores of 3–6 for estrogen receptors in tumor cells significantly decreased the hazard rate for tumor death in the multivariate analyses (Table 4). UICC pN3 category significantly increased the hazard rate for tumor recurrence, and HER2 category 3 in tumor cells, histological grade 3, and absence of adjuvant therapy significantly increased the hazard rates for tumor-related death in the multivariate analyses (Table 4). In model 2, lymph vessel tumor embolus grade 2, lymph vessel tumor embolus grade 3, Allred scores of 4–8 for p53 in tumor-stromal fibroblasts, and histological grade 3 significantly increased the hazard rates for tumor recurrence and tumor-related death in the multivariate analyses (Table 4). Residual invasive tumor size >50 mm and Allred scores of 7 or 8 for p53 in tumor cells significantly increased the hazard rates for tumor recurrence, and the presence of skin invasion significantly increased the hazard rate for tumor-related death in the multivariate analysis (Table 4).

Discussion

The results of this study clearly showed significant associations between increases in grade of lymph vessel tumor embolus assessed in the biopsy specimens and surgical specimens and the number of nodal metastases. We have also found a significant association between grade of lymph vessel tumor embolus and number of nodal metastases in a different no-neoadjuvant therapy IDC group in another study.[6] Thus, the grading system for lymph vessel tumor embolus can be concluded to be a very useful histological grading system for accurately predicting lymph node metastasis by IDCs in the no-neoadjuvant therapy group and in the neoadjuvant therapy group.

In a previous study, we found that the grading system for lymph vessel tumor emboli can be used to classify IDC patients with lymph vessel invasion into a low-, intermediate-, and high-risk groups for outcome, and that IDCs with grade 0 lymph vessel tumor embolus and IDCs with grade 1 lymph vessel tumor emboli were almost equally malignant in a different no-neoadjuvant therapy IDC group.[6] Although those findings were clearly confirmed in this study again, the results of this study clearly showed that lymph vessel tumor embolus grade 2 in the surgical specimens was an important outcome-predictive factor for IDC patients independent of nodal status. It can be therefore concluded that lymph vessel tumor embolus grade 2 is an

Table 4 Multivariate analyses for tumor recurrence and tumor-related death in invasive ductal carcinoma patients who had nodal metastasis

Factors	*Cases*	*Number of patients (%)*			
		Tumor recurrence		*Tumor-related death*	
		Present	*HRs (95% CI) P-value*	*Present*	*HRs (95% CI) P-value*
Model 1					
Grading system for lymph vessel tumor emboli					
Grade 0	147	51 (35)	Referent	13 (9)	Referent
Grade 1	14	6 (43)	1.8 (0.7–5.0) 0.249	3 (21)	4.3 (1.1–17.7) 0.044
Grades 2 and 3	8	5 (63)	3.6 (1.4–9.5) 0.008	3 (38)	0.5 (0.1–3.3) 0.458
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	47	7 (15)	Referent	0	Referent
3	38	14 (36)	(1.6–9.1) 0.003	6 (16)	Referent
4–8	81	39 (48)	2.4 (1.4–4.1) 0.002	11 (14)	1.8 (0.6–5.7) 0.324
The Allred scores for estrogen receptors in tumor cells					
0 or 2	62	28 (45)	Referent	14 (23)	Referent
3–6	22	11 (50)	1.4 (0.5–3.7) 0.550	3 (14)	0.1 (0.02–0.6) 0.009
7 or 8	82	21 (26)	0.4 (0.2–0.6) <0.001	0	
UICC pN category					
N1	97	27 (28)	Referent	6 (6)	Referent
N2	55	25 (45)	1.7 (0.8–3.5) 0.158	11 (20)	—
N3	31	17 (55)	2.6 (1.4–4.7) 0.002	4 (13)	—
HER2 category in tumor cells					
0 or 1	104	35 (34)	Referent	5 (5)	Referent
2	28	8 (29)	0.6 (0.3–1.4) 0.244	2 (7)	0.6 (0.06–7.1) 0.703
3	35	18 (51)	1.5 (0.7–3.4) 0.317	11 (31)	14.5 (3.9–53.1) <0.001
Histological grade					
1	43	7 (16)	Referent	0	Referent
2	104	46 (44)	2.3 (0.9–5.9) 0.082	13 (13)	Referent
3	22	9 (41)	2.0 (0.6–6.6) 0.281	6 (27)	6.2 (1.8–21.0) 0.003
Adjuvant therapy					
No	36	16 (44)	Referent	7 (19)	Referent
Yes	147	53 (36)	— —	14 (10)	0.2 (0.06–0.7) 0.014
Model 2					
Grading system for lymph vessel tumor emboli					
Grade 0	109	30 (28)	Referent	8 (7)	Referent
Grade 1	36	7 (19)	0.3 (0.1–0.9) 0.027	0	Referent
Grade 2	20	17 (85)	5.7 (2.9–11.0) <0.001	6 (30)	4.2 (1.4–12.6) 0.010
Grade 3	16	14 (88)	6.8 (3.1–14.8) <0.001	6 (38)	8.1 (2.5–25.7) <0.001
The Allred scores for p53 in tumor-stromal fibroblasts					
0 or 2	80	16 (20)	Referent	4 (5)	Referent
3	27	9 (33)	1.4 (0.5–4.0) 0.484	0	Referent
4–8	69	41 (59)	2.5 (1.5–4.3) 0.001	16 (23)	5.2 (1.9–14.4) 0.002

Table 4 Continued

Factors	*Cases*	*Number of patients (%)*			
		Tumor recurrence		*Tumor-related death*	
		Present	*HRs (95% CI)* P-*value*	*Present*	*HRs (95% CI)* P-*value*
Histological grade					
1	43	5 (12)	Referent	0	Referent
2	86	32 (37)	1.9 (0.7–5.7) 0.232	5 (6)	Referent
3	52	31 (60)	2.2 (3.1–14.8) <0.001	15 (29)	5.4 (1.9–15.9) 0.002
Residual invasive tumor size (mm)					
⩽20	42	8 (19)	Referent	3 (7)	Referent
>20–⩽50	92	29 (32)	1.0 (0.4–2.4) 0.943	8 (9)	—
>50	47	31 (66)	3.0 (1.8–5.3) <0.001	9 (19)	—
The Allred scores for p53 in tumor cells					
0 or 2	48	12 (25)	Referent	2 (4)	Referent
3–6	79	25 (32)	1.9 (0.8–4.6) 0.142	6 (8)	3.0 (0.5–17.5) 0.224
7 or 8	48	29 (60)	2.1 (1.1–4.2) 0.023	12 (25)	1.2 (0.2–8.0) 0.817
Skin invasion					
Absent	131	40 (31)	Referent	9 (7)	Referent
Present	50	28 (56)	1.9 (0.9–3.6) 0.068	11 (22)	2.9 (1.1–7.5) 0.025

CI, confidence interval; HR, hazard rate; —, not significant in univariate analysis.
Model 1: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, the Allred scores for estrogen receptors in tumor cells, HER2 category in tumor cells, the Allred scores for progesterone receptors, and the Allred scores for p53 in tumor cells assessed in biopsy specimens obtained before neoadjuvant therapy, and UICC pN category assessed in surgical specimens obtained after neoadjuvant therapy and type of neoadjuvant therapy. Tumor-related death was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, the Allred scores for estrogen receptors in tumor cells, HER2 category in tumor cells, and the Allred scores for progesterone receptors in tumor cells assessed in biopsy specimens obtained before neoadjuvant therapy, and adjuvant therapy.
Model 2: Tumor recurrence was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, residual invasive tumor size, the Allred scores for p53 in tumor cells, skin invasion, the Allred scores for estrogen receptors in tumor cells, HER2 category in tumor cells and UICC pN category assessed in surgical specimens obtained after neoadjuvant therapy, and type of neoadjuvant therapy. Tumor-related death was adjusted for grading system for lymph vessel tumor emboli, the Allred scores for p53 in tumor-stromal fibroblasts, histological grade, residual invasive tumor size, the Allred scores for p53 in tumor cells, skin invasion, the Allred scores for estrogen receptors in tumor cells, and HER2 category in tumor cells assessed in surgical specimens obtained after neoadjuvant therapy, and adjuvant therapy.

important outcome predictor for IDC patients who have received neoadjuvant therapy, the as same as lymph vessel tumor embolus grade 3 is. The results of this study also clearly showed that lymph vessel tumor embolus grades based on biopsy specimens or surgical specimens are a very important outcome-predictive factor for IDC patients who have received neoadjuvant therapy independent of nodal status, but the outcome-predictive power of lymph vessel tumor embolus grade in the surgical specimens was superior to that of lymph vessel tumor embolus grade in the biopsy specimens. Thus, we can conclude that evaluation of lymph vessel tumor embolus grade in surgical specimens should be used to predict outcome.

Although we have already reported that lymph vessel tumor embolus grade is an important outcome predictor for IDC patients who have received neoadjuvant therapy, the outcome-predictive power of the lymph vessel tumor embolus grade for IDC patients who received neoadjuvant therapy and did not have nodal metastasis could not be assessed.[7] This study clearly showed that lymph vessel tumor embolus grades based on biopsy specimens and surgical specimens are very important outcome predictors for IDC patients who have received neoadjuvant therapy and do not have nodal metastasis. Furthermore, the outcome-predictive power of lymph vessel tumor embolus grade is almost the same as that of p53 expression in tumor-stromal fibroblasts, and superior to that of histological grade.

The lymph vessel tumor embolus grading system is therefore concluded to be an excellent histological grading system for accurately predicting the outcome of IDC patients who have received neoadjuvant therapy that is independent of their nodal status.

The results of this study clearly showed that lymph vessel tumor embolus grades are significantly associated with both the Allred scores for p53 in lymph vessel tumor emboli, as well as the Allred scores for p53 in stroma-invasive tumor cells, and in tumor-stromal fibroblasts, this strongly suggesting that p53 protein expression in lymph vessel tumor emboli, in tumor-stromal fibroblasts, and in stroma-invasive tumor cells is a very important key factor for evaluating the malignant potential of IDCs with lymph vessel tumor emboli. Especially, as lymph vessel tumor embolus grades are based on the numbers of mitotic figures and apoptotic figures in tumor cells in lymph vessels, p53 protein expression in lymph vessel tumor embolus probably accelerates the turnover rate of tumor cells comprising lymph vessel tumor emboli, and increases the malignancy of IDCs as lymph vessel tumor embolus grade rises. As we did not investigate for the presence of p53 gene abnormalities, the mechanism that is responsible for the increase in the malignant potential of IDCs according to grades of lymph vessel tumor embolus from the standpoint of p53 gene abnormalities in lymph vessel tumor emboli, as well as in tumor-stromal fibroblasts, or in stroma-invasive tumor cells should be investigated. In addition, as some studies have reported some identifying genes that closely regulate the cell cycle of tumors,[24–26] such genes should be investigated to determine whether they are candidates for p53 in regulating tumor cell cycle of lymph vessel tumor emboli.

In conclusion, the grading system for lymph vessel tumor emboli is significantly associated with nodal metastasis, and is an excellent histological grading system for accurately predicting the outcome of patients with IDC of the breast who received neoadjuvant therapy. Pathologists can most accurately assess the true malignant potential of IDCs by using this grading system as a histological prognostic classification for IDCs of the breast.

Acknowledgement

This study was supported by a Grant-in-Aid for Scientific Research (KAKENHI) (C) (19590378, 21590393) from the Japan Society for the Promotion of Science and was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare (20–16, H21-006).

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Lauria R, Perrone F, Carlomagno C, *et al.* The prognostic value of lymphatic and blood vessel invasion in operable breast cancer. Cancer 1995;76:1772–1778.
2 Nime FA, Rosen PP, Tzvi Thaler H, *et al.* Prognostic significance of tumor emboli intramammary lymphatics in patients with mammary carcinoma. Am J Surg Pathol 1977;1:25–30.
3 Hasebe T, Sasaki S, Imoto S, *et al.* Characteristics of tumors in lymph vessels play an important role in the tumor progression of invasive ductal carcinoma of the breast: a prospective study. Mod Pathol 2002; 15:904–913.
4 Hoshida T, Isaka N, Hagendoorn J, *et al.* Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. Cancer Res 2006;66:8065–8075.
5 Yamauchi C, Hasebe T, Iwasaki M, *et al.* Accurate assessment of lymph vessel tumor emboli in invasive ductal carcinoma of the breast according to tumor areas, and their prognostic significance. Hum Pathol 2007;38:247–259.
6 Hasebe T, Yamauchi C, Iwasaki M, *et al.* Grading system for lymph vessel tumor emboli for prediction of the outcome of invasive ductal carcinoma of the breast. Hum Pathol 2008;39:427–436.
7 Tamura N, Hasebe T, Okada N, *et al.* Tumor histology in lymph vessels and lymph nodes for the accurate prediction of outcome among breast cancer patients treated with neoadjuvant chemotherapy. Cancer Sci 2009;100:1823–1833.
8 Hasebe T, Tamura N, Okada N, *et al.* p53 expression in tumor stromal fibroblasts is closely associated with the nodal metastasis and the outcome of patients with invasive ductal carcinoma who received neoadjuvant therapy. Hum Pathol 2009;41:262–270.
9 Sobin LH, Wittekind Ch. UICC International Union Against Cancer. TNM classification of malignant tumors. In: Sobin LH and Wittekind Ch (eds). 6th edn. Wiley-Liss: Geneva, 2002, pp 131–141.
10 Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. Br J Cancer 1957; 11:359–377.
11 Gilchrist KW, Gray R, Fowble B, *et al.* Tumor necrosis is a prognostic predictor for early recurrence and death in lymph node-positive breast cancer: a 10-year follow-up study of 728 eastern cooperative oncology group patients. J Clin Oncol 1993;11:1929–1935.
12 Hasebe T, Tsuda H, Hirohashi S, *et al.* Fibrotic focus in infiltrating ductal carcinoma of the breast: a significant histopathological prognostic parameter for predicting the long-term survival of the patients. Breast Cancer Res Treat 1998;49:195–208.
13 Hasebe T, Sasaki S, Imoto S, *et al.* Prognostic significance of fibrotic focus in invasive ductal carcinoma of the breast: a prospective observational study. Mod Pathol 2002;15:502–516.
14 Fisher B. Biological and clinical considerations regarding the use of surgery and chemotherapy in the treatment of primary breast cancer. Cancer 1977; 40:574–587.
15 Fisher B. Adjuvant chemotherapy in the primary management of breast cancer. Med Clin North Am 1977;61:953–965.

16 Fukunaga M. Expression of D2-40 in lymphatic endothelium of normal tissues and in vascular tumours. Histopathology 2005;46:396–402.
17 Niakosari F, Kahn HJ, Marks A, *et al.* Detection of lymphatic invasion in primary melanoma with monoclonal antibody D2-40: a new selective immunohistochemical marker of lymphatic endothelium. Arch Dermatol 2005;141:440–444.
18 Allred DC, Harvey JM, Berardo MD, *et al.* Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998;11:155–168.
19 Harvey JM, Clark GM, Osborne K, *et al.* Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 1999;17:1474–1481.
20 Mohsin S, Weiss H, Havighurst T, *et al.* Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. Mod Pathol 2004;17:1545–1554.
21 Badve SS, Baehner FL, Gray RP, *et al.* Estrogen- and progesterone-receptor status in ECOG2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. J Clin Oncol 2008;26:2473–2481.
22 Allred DC, Clark GM, Elledge R, *et al.* Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993;85:200–206.
23 Wolff AC, Hammond ME, Schwartz JN, *et al.* American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med 2007;131:18–43.
24 Westbrook L, Manuvakhova M, Kern FG, *et al.* Cks1 regulates cdk1 expression: a novel role during mitotic entry in breast cancer cells. Cancer Res 2007;67:11393–11401.
25 Nakamura Y, Tanaka F, Haraguchi N, *et al.* Clinicopathological and biological significance of mitotic centromere-associated kinesin overexpression in human gastric cancer. Br J Cancer 2007;97:543–549.
26 Budhram-Mahadeo VS, Irshad S, Bowen S, *et al.* Proilferation-associated Brn-3b transcription factor can activate cyclin D1 expression in neuroblastoma and breast cancer cells. Br J Cancer 2008; 27:145–154.

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.
Modern Pathology (2010) **23,** 662–672; doi:10.1038/modpathol.2010.47; published online 5 March 2010

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
Received 23 December 2009; revised and accepted 28 January 2010; published online 5 March 2010

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 (≤ 20, >20 to ≤ 50, >50 mm); (2) histological grade (1, 2, 3);[24] (3) tumor necrosis (absent, present);[25] (4) fibrotic focus (absent, fibrotic focus diameter ≤ 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

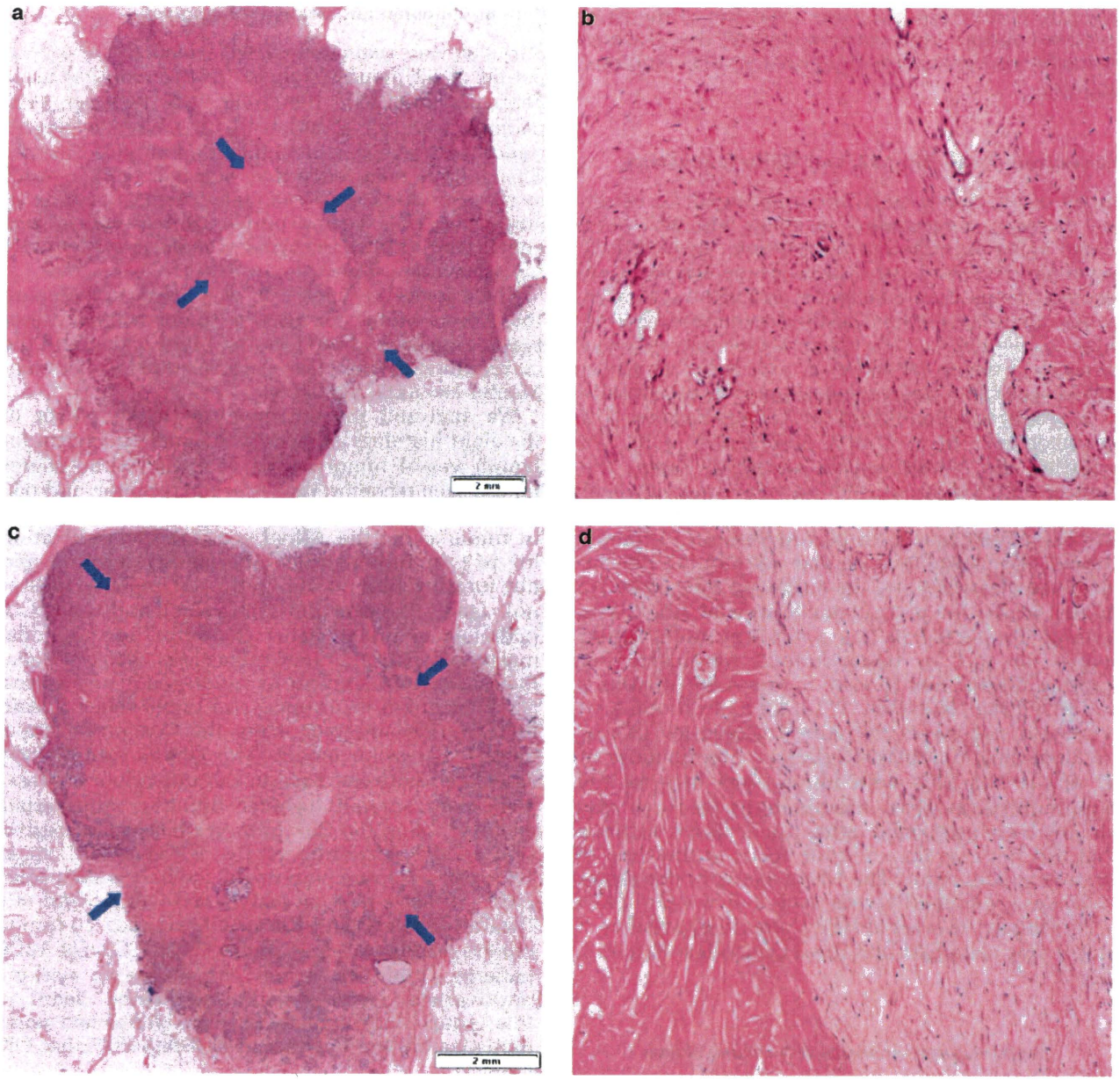

Figure 1 Invasive ductal carcinomas with fibrotic foci (**a–d**). (**a**) A fibrotic focus measuring 6.4 × 3.3 mm is visible within the tumor (panoramic view, arrows). The fibrotic focus shows a scar-like feature and is surrounded by invasive ductal carcinoma cells. (**b**) The fibrotic focus area consists 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.

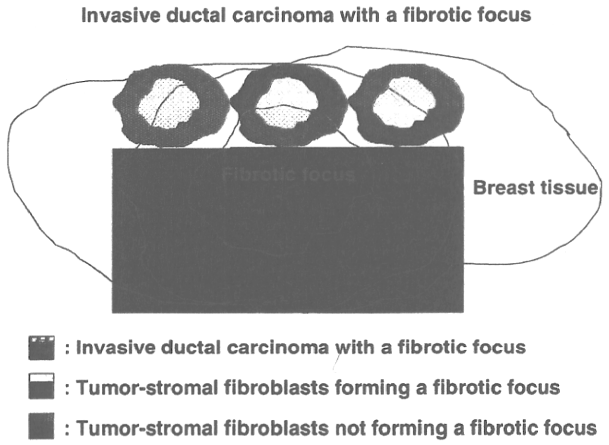

Figure 2 Schematic illustration of an invasive ductal carcinoma with a fibrotic focus.

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