9. Hayashi AH, Silver SF, van der Westhuizen NG. Treatment of invasive breast carcinoma with ultrasound-guided radiofrequency ablation. Am J Surg. 2003;185:429–35.
10. Izzo F, Thomas R, Delrio P. Radiofrequency ablation in patients with primary breast carcinoma: a pilot study in 26 patients. Cancer. 2001;92:2036–44.
11. Jeffrey SS, Birdwell RL, Ikeda DM. Radiofrequency ablation of breast cancer: first report of an emerging technology. Arch Surg. 1999;134:1064–8.
12. Noguchi M, Earashi M, Fujii H. Radiofrequency ablation of small breast cancer followed by surgical resection. J Surg Oncol. 2006;93:120–8.
13. Van den Bosch MAAJ, Daniel BL. MR-guided interventions of the breast. Magn Reson Imaging Clin N Am. 2005;13:505–17.
14. Curley SA. Radiofrequency ablation of malignant liver tumors. Oncologist. 2001;6:14–23.
15. Dickson JA, Calderwood SK. Temperature range and selective sensitivity of tumors to hyperthermia: a critical review. Ann NY Acad Sci. 1980;335:180–205.
16. Dickson JA, Calderwood SK. Thermosensitivity of neoplastic tissues in vivo. In: Storm F, editor. Hyperthermia in cancer therapy. Boston: GK Hall Medical; 1983. pp 63–140.
17. Susini T, Nori J, Olivieri S. Radiofrequency ablation for minimally invasive treatment of breast carcinoma. A pilot study in elderly inoperable patients. Gynecol Oncol. 2006;26:304–10.

Genes to Cells

Up-regulation of *PSF1* promotes the growth of breast cancer cells

Izumi Nakahara[1,2], Mamiko Miyamoto[1], Tatsuhiro Shibata[3,4], Sadako Akashi-Tanaka[5], Takayuki Kinoshita[5], Kaoru Mogushi[2], Kohtaro Oda[1,2], Masaya Ueno[6], Nobuyuki Takakura[6], Hiroshi Mizushima[2], Hiroshi Tanaka[2] and Tsutomu Ohta[1]*

[1]*Center for Medical Genomics, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[2]*Department of Computational Biology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*
[3]*Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[4]*Cancer Genomics Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[5]*Breast Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[6]*Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka Suita, Osaka 565-0871, Japan*

PSF1 is a subunit of the GINS complex that functions along with the MCM2-7 complex and Cdc45 in eukaryotic DNA replication. Although mammalian *PSF1* is predominantly expressed in highly proliferating cells and organs, little is known about the roles of *PSF1* in mature cells or cancer cells. We found that PSF1 was expressed at relatively high levels in breast tumor cells, but at low levels in normal breast cells. Knockdown of *PSF1* expression using small interfering RNA (siRNA) slowed the growth of breast cancer cell lines by delaying DNA replication but did not affect proliferation of normal human mammary epithelial cells. Reduced *PSF1* expression also inhibited anchorage-independent growth in breast cancer cell lines. These results suggest that *PSF1* over-expression is specifically involved in breast cancer cell growth. Therefore, PSF1 inhibition might provide new therapeutic approaches for breast cancer.

Introduction

Chromosomal DNA replication is tightly regulated in eukaryotic cells. Origin-recognition complexes (ORC) are believed to play a central role in the recognition of replication origins (Labib & Gambus 2007). In the late M and early G1 phases of the cell cycle, the mini-chromosome maintenance 2-7 (MCM2-7) complex and Cdc45 are localized to DNA replication origins along with ORC (Labib & Gambus 2007). The MCM2-7 complex and Cdc45 unwind the parental DNA duplex, allowing DNA polymerases to initiate DNA synthesis (Labib & Gambus 2007). The GINS complex was recently reported to participate in both the initiation and elongation phases of DNA replication through its ability to recruit Cdc45 and DNA polymerase (Pai *et al.* 2009). The GINS complex, which contains PSF1, PSF2, PSF3 and SLD5, was first identified as a component of prerecognition complexes by genetic analyses in *Saccharomyces cerevisiae* (Takayama *et al.* 2003). Genes encoding the GINS components are evolutionally conserved (Kubota *et al.* 2003). *PSF1* gene expression is essential for early embryogenesis, maintenance of immature hematopoietic cell pool size and acute bone marrow regeneration in mice (Ueno *et al.* 2005, 2009). PSF1 is predominantly expressed in highly proliferating cells but not in mature cells (Ueno *et al.* 2005) and is up-regulated in intrahepatic cholangiocarcinomas (Obama *et al.* 2005). Recently, it was shown that up-regulated PSF1 expression drove tumorigenesis and conferred metastatic properties (Nagahama *et al.* 2010). However, the role of *PSF1* in normal mature cells or mammalian cancer cells remains unclear.

In this study, we show that *PSF1* expression is up-regulated in breast cancer tissues and cell lines. Down-regulation of *PSF1* expression led to reduced growth of cancer cells, but not of normal mammary epithelial cells. Reduced *PSF1* expression also inhibited the anchorage-independent cell growth of breast

Communicated by: Masayuki Yamamoto (Tohoku University)
**Correspondence*: cota@ncc.go.jp

DOI: 10.1111/j.1365-2443.2010.01442.x

cancer cell lines. These findings indicate that PSF1 might have potential as a breast cancer biomarker and as a gene target for breast cancer treatment.

Results

PSF1 protein expression is enhanced in breast cancer cells

As *PSF1* promoter activity can be stimulated *in vitro* via 17β-estradiol (E2)-mediated estrogen receptor (ER) signaling (Hayashi *et al.* 2006), we speculated that *PSF1* expression might be up-regulated in breast cancer cells. To examine PSF1 expression in breast cancer tissues, we performed an immunostaining analysis of 34 tissue specimens. PSF1 immunohistochemical staining in normal breast tissues was very weak but was significantly enhanced in 41% (14 of 34) of cancer tissue specimens (Fig. 1A and Table 1). We also found that PSF1 was highly expressed in the invasive tumor area (Fig. 1B), suggesting that PSF1 might be predominantly expressed in advanced malignancy cells. The relationship between the level of PSF1 expression and clinicopathological parameters was also investigated, although no significant associations between the level of PSF1 expression and prognostic indicators could be established in the breast cancer specimens tested (Table 1). Next, to examine whether PSF1 expression correlated with hormone receptor expression and breast cancer biomarkers, we analyzed the expression of ER, progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2) and tumor suppressor gene product p53 by immunohistochemical staining of the same breast cancer samples used previously. No correlation between the expression of PSF1 and that of hormone receptors or breast cancer biomarkers was observed (Table 1), suggesting that PSF1 protein expression is not affected by hormone receptors (ER and PgR) or breast cancer biomarkers (HER2 and p53).

We analyzed the association between PSF1 expression and prognosis. The observation time (range: 0.6–3.4 years, median: 3.2 years) after surgery for the 34 patients did not allow for analysis of either the 5-year survival rate or 3-year disease-free survival rate. Therefore, we investigated *PSF1* expression levels and analyzed the survival rate using a publicly available microarray dataset of 295 patients with breast cancer (http://microarray-pubs.stanford.edu/wound_NKI/explore.html). Figure 1C shows the survival rates of the 127 and 168 patients who respectively had high and low *PSF1* expression levels. The 15-year survival rate of the low *PSF1* expression level group was higher ($P = 0.00466$), suggesting that *PSF1* expression might be a prognostic marker.

Promoter activity of *PSF1* is up-regulated in breast cancer cells

To examine *PSF1* expression in cell lines, we analyzed *PSF1* mRNA expression levels in breast cancer cell lines and normal breast cells using real-time RT-PCR. High *PSF1* expression levels were observed in breast cancer cell lines (Fig. 2A, lanes 3–5; upper panel), whereas only low levels were detected in normal human mammary epithelial cells (HMEC) or immortalized HMEC by expression of hTERT (catalytic component of human telomerase) (HMEC-tert) (Fig. 2A, lanes 1 and 2; upper panel). Next, we analyzed PSF1 protein levels in breast cancer cell lines and normal breast cells by Western blotting using anti-PSF1 antibody. PSF1 proteins were detected at high levels in breast cancer cell lines, but at low levels in HMEC and HMEC-tert cells (Fig. 2A; lower panel). These results suggested that both *PSF1* mRNA and PSF1 protein expressions were enhanced in breast cancer cell lines. We also analyzed the expression levels of the other GINS complex subunits (*PSF2*, *PSF3* and *SLD5*) in normal breast cells and breast cancer cell lines. Like *PSF1* expression, *SLD5* expression was up-regulated in all three breast cancer cell lines tested (Fig. 2B; lower panel), whereas expression levels of *PSF2* and *PSF3* were only up-regulated in specific breast cancer cell lines (Fig. 2B; upper and middle panels).

Because gene amplification of cancer-related genes has been observed in cancer cells, we investigated the possibility of *PSF1* gene amplification using a single-nucleotide polymorphism (SNP) chip. SNP IDs were rs2500406 and rs6083862. No amplification of the *PSF1* gene locus was detected in any of the breast cancer cell lines tested (data not shown), which suggested that *PSF1* up-regulation in breast cancer cell lines was not because of *PSF1* gene amplification. We then analyzed *PSF1* promoter activity using different promoter region lengths: 5, 1.6 and 0.5 kb upstream from the transcriptional start site. We found that when of each of the three regions was fused to the luciferase gene in T47D cells, the promoter activities were more than 10 times higher than those observed in HMEC-tert (Fig. 3A). This result indicated that the up-regulated *PSF1* expression was because of increased promoter activity of *PSF1* in breast cancer cells.

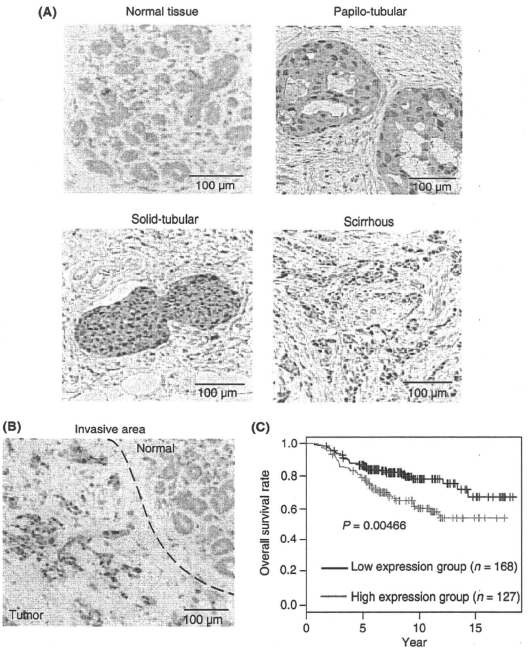

Figure 1 Increased PSF1 expression in human breast cancer tissues. Immunohistochemical staining of PSF1 in human breast cancer samples using anti-PSF1 antibody. Bars indicate 100 μm. (A) Nuclear PSF1 expression was detected in three types of breast cancer (papillo-tubular, solid-tubular and scirrhous). In rare cases, nuclear PSF1 was also detected in a few normal mammary epithelial cells located in the lobule where cell proliferation occurs physiologically. (B) Prominent and frequent nuclear accumulation of PSF1 was detected in invasive carcinoma cells (in tumor area), whereas no positive staining was observed in noncancerous mammary duct epithelium (in normal area). (C) The relationship between the level of *PSF1* expression and the survival rate in patients with breast cancer. The relationship between *PSF1* expression levels and the survival rate was analyzed by using publicly available microarray dataset of 295 patients with breast cancer (http://microarray-pubs.stanford.edu/wound_NKI/explore.html). The survival rates were determined using the Kaplan–Meier methods and were compared by means of the log rank test. The gray line shows a survival curve for 127 patients with higher *PSF1* expression levels and the black line for 168 patients with lower *PSF1* expression levels. The cutoff value of *PSF1* expression level was calculated by taking the mean value of the median expression levels of the good prognosis group (over 5-year survival) and the poor prognosis group (<5-year survival), respectively.

Down-regulation of *PSF1* led to reduced growth of breast cancer cells

To determine whether knockdown of *PSF1* expression impacted the growth of breast cancer cells, we measured the growth rate of breast cancer cell lines and normal cells treated with *PSF1*-specific siRNA. Knockdown of *PSF1* expression was detected by real-time RT-PCR in breast cancer cells (T47D, MDA-MB-231 and MDA-MB-361) and normal human mammary epithelial cells (HMEC and HMEC-tert) (Fig. 3B and Fig. S1 in Supporting

Table 1 Clinicopathologic features and immunohistochemical results of PSF1, ER, PgR, HER2 and p53

Patient number	PSF1	ER	PgR	HER2	p53	Stage	Histology
BC-1	0.5	0	0	3	2	2B	Papillo-tubular
BC-2	0.5	1	3	1	0	2A	Scirrhous
BC-3	1	2	3	2	0	1	Solid-tubular
BC-4	0.5	2	3	1	1	2A	Scirrhous
BC-5	1	2	3	0	1	2A	Scirrhous
BC-6	1	3	2	1	1	3B	Papillo-tubular
BC-7	2	1	1	0	2	1	Scirrhous
BC-8	2	3	3	1	0	1	Papillo-tubular
BC-9	2	3	1	1	2	2B	Scirrhous
BC-10	2	0	1	1	0	1	Papillo-tubular
BC-11	2	0	1	3	1	3A	Solid-tubular
BC-12	1	3	3	3	2	2B	Solid-tubular
BC-13	2	3	0	1	1	2A	Papillo-tubular
BC-14	2	1	2	3	2	3A	Solid-tubular
BC-15	1	0	0	1	2	1	Solid-tubular
BC-16	0.5	1	3	1	2	1	Scirrhous
BC-18	0.5	0	1	0	2	2B	Solid-tubular
BC-19	2	0	0	0	1	2A	Solid-tubular
BC-20	0.5	2	2	0	0	2A	Solid-tubular
BC-21	2	0	0	0	2	2A	Scirrhous
BC-22	0.5	1	3	0	0	2B	Solid-tubular
BC-23	2	0	3	1	2	2A	Scirrhous
BC-24	0.5	0	1	1	1	2A	Papillo-tubular
BC-25	1	2	2	0	2	2A	Solid-tubular
BC-26	0.5	1	2	0	0	1	Papillo-tubular
BC-28	2	3	3	0	1	1	Solid-tubular
BC-29	2	0	3	1	0	2A	Solid-tubular
BC-30	0.5	0	0	0	0	1	Scirrhous
BC-31	2	0	0	0	2	2A	Solid-tubular
BC-32	0.5	3	3	0	1	1	Papillo-tubular
BC-34	1	0	0	3	1	1	Papillo-tubular
BC-35	0.5	2	2	0	2	2B	Scirrhous
BC-36	0.5	2	3	0	1	2A	Papillo-tubular
BC-37	2	0	0	0	0	1	Solid-tubular

Staining extent was scored on a scale of 0–2 for PSF1, as follows: 0 = no staining, 0.5 = <5%, 1 = 5%–30% and 2 = >30% of tumor cells. Tumor cells with staining intensity 2 were considered as positive. Staining extent was scored on a scale of 0–3 for ER and PgR, as follows: 0 = no staining, 1 = <10%, 2 = 1%–10% and 3 = >10% of tumor cells. Tumor cells with staining intensity 3 were considered as positive. Staining extent was scored on a scale of 0–3 for HER2, as follows: 0 = no staining, 1 = <10%, 2 = 10%–30% and 3 = >30% of tumor cells. Tumor cells with staining intensity 2 and 3 were considered as positive. Staining extent was scored on a scale of 0–2 for p53, as follows: 0 = no staining, 1 = weak staining and 2 = strong staining in tumor cells. Tumor cells with staining intensity 2 were considered as positive.

ER, estrogen receptor; Pgr, progesterone receptor.

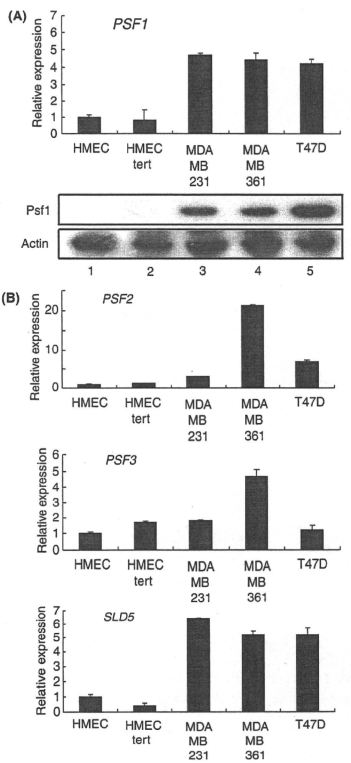

Figure 2 Expression levels of subunits of GINS in cell lines. (A) *PSF1* expression levels in cell lines. *PSF1* expressions in normal human mammary epithelial cells, HMEC and HMEC-tert (lanes 1 and 2) and in breast cancer cell lines, MDA-MB-231, MDA-MB-361 and T47D (lanes 3–5) were analyzed by real-time RT-PCR (upper panel) and by immunoblotting (lower panel). Level of *PSF1* expression in HMEC cells was set at 1. *CTBP1* and actin were internal controls. Data show the mean ± SEM (n = 3). (B) Expressions of *PSF2, PSF3* and *SLD5* in normal human mammary epithelial cells (HMEC and HMEC-tert) and in breast cancer cell lines (MDA-MB-231, MDA-MB-361 and T47D cells) were analyzed by real-time RT-PCR. Level of each gene expression in HMEC cells was set at 1. *CTBP1* was internal control. Data show the mean ± SEM (n = 3).

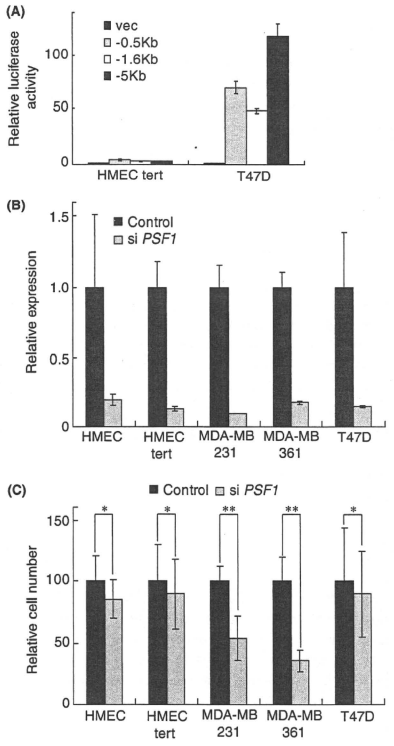

Figure 3 Up-regulation of *PSF1* promotes growth of breast cancer cell lines. (A) *PSF1* promoter (−0.5, −1.6 and −5 kb) activity using luciferase assay in normal human mammary epithelial cells (HMEC) and breast cancer cells. The pGL3-basic reporter plasmid (vec) containing the *PSF1* promoter (100 ng) was transfected into HMEC-tert and T47D cells. Luciferase activity in cell lysates was normalized to the *Renilla* luciferase activity of p RL-TK as an internal control. The activity in the absence of *PSF1* promoter was set at 1. Data show the mean ± SEM (n = 3). (B) Knockdown of *PSF1* expression by *PSF1* siRNA. The control siRNA or *PSF1* siRNA was transfected into HMEC, HMEC-tert, MDA-MB-231, MDA-MB-361 and T47D cells. After 2 days, the expression level of *PSF1* in the cells was analyzed by real-time RT-PCR. Level of *PSF1* expression in cells transfected with control siRNA was set at 1. *GAPDH* was an internal control. Data show the mean ± SEM (n = 3). (C) Growth rate of breast cancer cells by knockdown of *PSF1*. Six days after transfection of siRNA, cell numbers were counted. The number of cells transfected with control siRNA was set at 100. Data show the mean ± SEM, $*P > 0.05$, $**P < 0.01$ (n = 3).

Information). Six days after transfection, the numbers of HMEC, HMEC-tert and T47D cells transfected with either *PSF1*-specific or control siRNA were similar (Fig. 3C and Fig. S2 in Supporting Information). In contrast, MDA-MB-231 and MDA-MB-361 cell numbers after transfection with *PSF1*-specific siRNA were approximately 50% and 40%, respectively, of those transfected with control siRNA (Fig. 3C and Fig. S2 in Supporting Information). These results indicated that *PSF1* over-expression promoted growth in MDA-MB-231 and MDA-MB-361 cells, but not in normal HMEC and T47D cells.

To examine whether other components of the GINS complex were necessary for the growth of normal HMEC and breast cancer cells, we analyzed cell growth after knockdown of *PSF2, PSF3* and *SLD5* expression. Knockdown of these genes was confirmed by real-time RT-PCR (Fig. S3 in Supporting Information). Growth of normal human mammary epithelial cells (HMEC-tert) after knockdown of these three genes was not significantly influenced (Fig. 4A). In contrast, growth of breast cancer cells (MDA-MB-231) was reduced by knockdown of *PSF2* and *SLD5*, similar to that of *PSF1* (Fig. 4A; upper and lower panels) and was weakly reduced by knockdown of *PSF3* (Fig. 4A; middle panel). As the amount of PSF1 might be regulated by PSF2, PSF3 and SLD5, we analyzed the levels of *PSF1* mRNA and PSF1 protein after knockdown of GINS complex subunit expression. Reduced expression of *PSF2*, *PSF3* or *SLD5* had no effect on the level of *PSF1* mRNA (Fig. 4B; upper panel), but the level of PSF1 protein decreased (Fig. 4B; lower panel). This result could indicate that PSF1 protein is stabilized in the GINS complex in breast cancer cells.

Slow cell growth in response to reduced PSF1 expression due to delayed DNA replication

To examine whether *PSF1* knockdown induced apoptosis in breast cancer cells, we analyzed cell apoptosis using a fluorochrome inhibitor that covalently

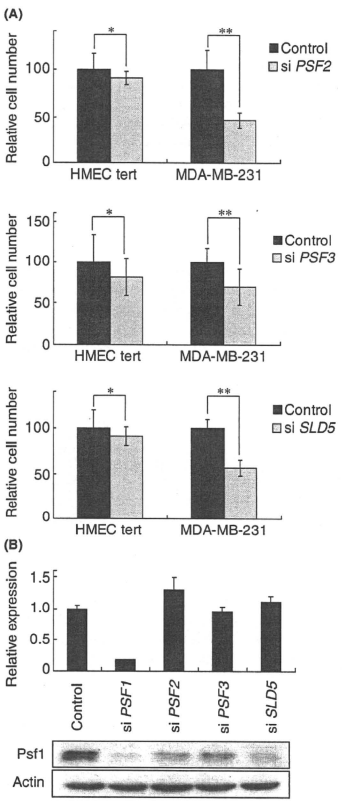

Figure 4 Knockdown of GINS complex subunits reduces growth of breast cancer cells. (A) Growth rate of normal cells and breast cancer cells by knockdown of *PSF2* (upper), *PSF3* (middle) and *SLD5* (lower). Control, *PSF2*, *PSF3* or *SLD5* siRNA was transfected into HMEC-tert or MDA-MB-231 cells. Six days after transfection of siRNA, cell numbers were counted. The number of cells transfected with control siRNA was set at 100. Data show the mean ± SEM, $*P > 0.05$, $**P < 0.01$ ($n = 3$). (B) Expression levels of *PSF1* mRNA and PSF1 protein in MDA-MB-231 cells transfected with siRNA of GINS complex subunits. Control, *PSF1*, *PSF2*, *PSF3* or *SLD5* siRNA was transfected into MDA-MB-231 cells. After 2 days, the expression level of *PSF1* was analyzed by real-time RT-PCR (upper panel). Level of *PSF1* expression in cells transfected with control siRNA was set at 1. *GAPDH* was an internal control. Data show the mean ± SEM ($n = 3$). Four days after transfection of siRNA, cells were collected and lysed by RIPA buffer. PSF1 protein was detected by anti-PSF1 antibody (lower panel). Actin was an internal control. HMEC, human mammary epithelial cells.

binds to active caspases (Bedner *et al.* 2000; Ishida *et al.* 2007). At 3 or 6 days after transfection with either control or *PSF1* siRNA, caspase-positive cells were not detected in the ~400 MDA-MB-231 cells examined (data not shown). Next, to determine whether *PSF1* knockdown affected the cell cycle, we analyzed DNA content using flow cytometry 5 days after transfection of breast cancer cells or normal cells with *PSF1* siRNA. FACS analysis showed that the number of cells in the cell cycle S phase increased after *PSF1* knockdown in MDA-MB-231 and MDA-MB-361 cells, but not in HMEC-tert cells (Fig. 5A). This result indicated that *PSF1* might participate in the S phase of the cell cycle in breast cancer cells, but not in normal HMEC. EdU incorporation assays were then performed in cells treated with *PSF1* siRNA. At 72 h after *PSF1* knockdown, EdU was incorporated for 75 min in cells. *PSF1* knockdown reduced cellular EdU incorporation in breast cancer cell lines (MDA-MB-231 and MDA-MB-361), but not normal human mammary epithelial cells (HMEC-tert) (Fig. 5B and C). These results supported the finding that reduction of *PSF1* levels slowed cell growth by delaying DNA replication in breast cancer cell lines.

Down-regulation of *PSF1* repressed anchorage-independent growth of breast cancer cells

To determine whether *PSF1* expression knockdown affected anchorage-independent breast cancer cell growth, we analyzed colony-formation activity of MDA-MB-231, MDA-MB-361 and T47D cells treated with *PSF1* siRNA on soft agar. Although MDA-MB-361 cells did not form colonies on soft agar (data not shown), 3 weeks after treatment, the number of colonies formed from T47D and MDA-MB-231 cells transfected with *PSF1*-specific siRNA was reduced approximately 40% and 10%, respectively, compared to those from cells transfected with control siRNA (Fig. 6). This result suggested that up-regulation of *PSF1* induced anchorage-independent growth of breast cancer cells.

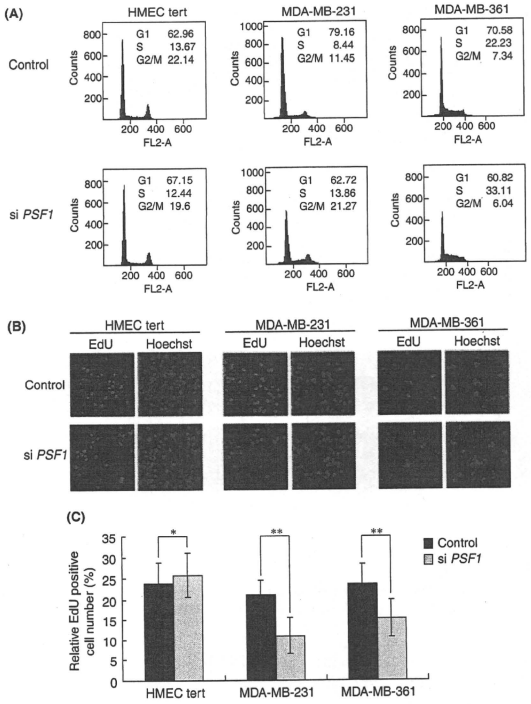

Figure 5 Knockdown of *PSF1* leads to delay in S phase of cell cycle in breast cancer cell lines. (A) Cell cycle analysis by flow cytometry. Five days after transfection of siRNA, HMEC-tert, MDA-MB-231 and MDA-MB-361 cells were collected and stained with PI. Cells were prepared using CycleTEST PLUS DNA REAGENT KIT (BD Biosciences). All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software. Counts and FL2-A indicate cell number and DNA content, respectively. (B) Incorporation of EdU. Control siRNA or *PSF1* siRNA was transfected into HMEC-tert, MDA-MB-231 and MDA-MB-361 cells. Three days after the transfection of siRNA, cells were labeled with EdU for 75 min and stained with anti-EdU antibody (green) and Hoechst (blue). DNA replication analysis was performed with Click-iT EdU Alexa Fluor 488 High-Throughput Imaging Assay Kit and confocal laser scanning microscope. (C) The bar graph indicates the relative EdU-positive cell number under certain fluorescence intensity condition in (B). Approximately 200 cells in each cell were counted. Data show the mean ± SEM, $*P > 0.05$, $**P < 0.01$ ($n = 3$). HMEC, human mammary epithelial cells.

Discussion

PSF1 immunohistochemical staining was significantly enhanced in 41% of breast cancer tissues tested but was very weak in normal breast tissues (Fig. 1A and Table 1). Although a strong correlation ($P < 0.05$) between PSF1 expression and that of gene markers (ER, PgR, HER2 and p53) was not observed in 34 breast cancer tissue specimens (Table 1), a weak correlation ($P = 0.116$) between expression of PSF1 and Her2 was observed. Therefore, the relationship between PSF1 and HER2 will be analyzed by

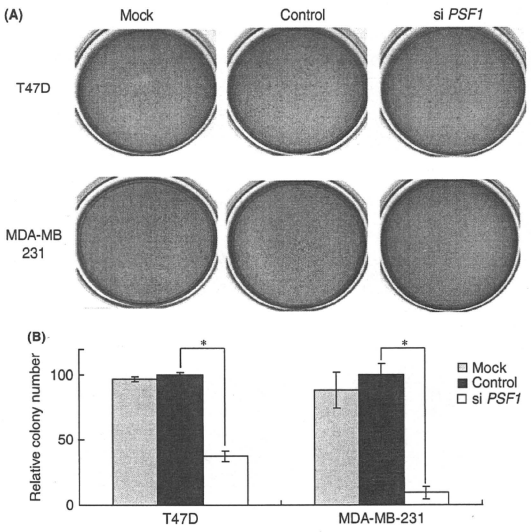

Figure 6 Knockdown of *PSF1* reduces anchorage-independent growth of breast cancer cell lines. (A) Colony-formation activity on soft agar. Mock, control siRNA or *PSF1* siRNA was transfected into T47D and MDA-MB-231 cells. Cells (5000 cells of T47D and 10 000 cells of MDA-MB-231) were cultured on soft agar for 3 weeks. (B) The bar graph indicates the relative colony number of cells in (A). The colony number of cells transfected with the control siRNA was set at 100. Data show the mean ± SEM, $*P < 0.01$ ($n = 3$).

increasing the number of specimens. We found that the 15-year survival rate of the group expressing low *PSF1* levels was higher than for patients expressing high *PSF1* levels (Fig. 1C). These results suggest that PSF1 might be useful as a new breast cancer biomarker or prognosis marker.

We determined that up-regulated *PSF1* expression in breast cancer cells was because of the increased activity of the *PSF1* promoter (Fig. 3A). Although stimulation of *PSF1* promoter activity by estrogen has been reported *in vitro* (Hayashi *et al.* 2006), the ER recognition sequences were not identified in the promoter regions (−5000b to +120b that contain the transcriptional start and upstream regions) of the *PSF1* gene. We also analyzed the expression levels of *PSF1* mRNA in breast cancer cell lines after treatment with the estrogen antagonist tamoxifen. Although tamoxifen significantly inhibited cell growth, it only weakly repressed the activity of *PSF1* expression in the ER-positive breast cancer cell line, T47D (data not shown). High levels of *PSF1* expression were also detected in the ER-negative cell line, MDA-MB-231 (Fig. 2A, lane 3). These results could indicate that ER is not a major factor for up-regulation of *PSF1* promoter activity in breast cancer cells. Therefore, to identify the factor(s) necessary for up-regulation of *PSF1* promoter activity, it will be important to understand the mechanisms of *PSF1* over-expression in breast cancer cells.

We found that knockdown of *PSF1* expression using siRNA slowed cell growth by delaying DNA replication (Figs 3,5). This result correlated with the finding that reduced *PSF1* expression using shRNA slowed cell growth in HeLa cells by increasing the number of cells in the G2/M phase (Nagahama *et al.* 2010). High-level expression of *PSF1* in LLC (lung carcinoma) and B16 (colon carcinoma) cells was also reportedly correlated with high proliferative activity (Nagahama *et al.* 2010). Our results, along with these reports, suggest that PSF1 over-expression might be

involved in cell growth of several cancers in addition to breast cancer by promoting changes in cell cycle progression. We found that down-regulation of *PSF1* led to reduced growth of MDA-MB-231 and MDA-MB-361 cells, but not of normal HMEC and T47D cells (Fig. 3C). This result suggested that breast cancer cells with specific genetic backgrounds might require large amounts of PSF1 for cell proliferation. Although there are reportedly many replication origins in the S phase of the cell cycle, only limited numbers of replication origins are activated in normal cells (Dominguez-Sola *et al.* 2007). The number of active replicons could be increased by c-Myc over-expression or oncogenic Ras expression in cancer cells (Di Micco *et al.* 2006; Dominguez-Sola *et al.* 2007). We did in fact detect c-Myc over-expression in MDA-MB-231 cells (data not shown). These reports together with our findings indicate that cancer cells having large numbers of active replication origins might require higher levels of GINS complex containing PSF1 when compared to normal mammary cells. We also found that down-regulation of *PSF1* reduced anchorage-independent cell growth in T47D cells (Fig. 6), but not cell proliferation (Fig. 3C). These results suggested that PSF1 over-expression could affect two types of cell growth, cell proliferation and anchorage-independent cell growth, of breast cancer cells. Although further studies will be needed to delineate the mechanism of PSF1 in increased breast cancer cell growth, PSF1 inhibition might be of therapeutic benefit for breast cancers with *PSF1* over-expression.

Experimental procedures

Tissue samples, cell lines and antibodies

Tumor tissues were obtained with informed consent from patients who received surgical treatment at National Cancer Center Hospital. Breast cancer cell lines (T47D, MDA-MB-231 and MDA-MB-361) were obtained from the American Type Culture Collection (ATCC). Normal HMEC was obtained from CAMBERX. HMEC-transfected human Tert (HMEC-tert) was obtained from Dr Kiyono (NCCRI, Japan). Anti-Psf1 antibody was used as described previously (Ueno *et al.* 2005).

Plasmid construction and reporter assay

The promoter DNAs of *PSF1* (−5000b to +120b, −1600b to +120b, −500b to +120b that contain transcriptional start and upstream regions) were isolated from human genomic DNA by PCR. These DNAs were sequenced and inserted in pGL3-basic (Promega) that contains a firefly luciferase gene. Reporter assay was performed as described previously (Ishida *et al.* 2007).

Immunohistochemical staining

Five-micrometer-thick sections of the formalin-fixed paraffin-embedded tumors were deparaffinized. After heat-induced epitope retrieval, the sections were incubated with mouse monoclonal anti-PSF1 antibody at a dilution of 1 : 50. The sections were incubated with a biotinylated secondary antibody against mouse IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 200 and then with the Vectastain ABC reagent (Vector Laboratories).

Real-time RT-PCR

Real-time RT-PCR were performed as described previously (Ishida *et al.* 2007) using the following primer sets: *PSF1*, 5′-TTCCCTGAGATTCAGATTGACTG-3′ (forward) and 5′-GGTCATAGACCA AAGTATAAAGC-3′ (reverse); *PSF2*, 5′-GACATTCTTCAATTCCACATCTG-3′ (forward) and 5′-GCCACCTCTGTGAGAGAGTC-3′ (reverse); *PSF3*, 5′-CCCTGACACCT CACAACTAGC-3′ (forward) and 5′-CAGAACATATTCATGTACAAAGC-3′ (reverse); and *SLD5*, 5′-GCCTCTCTCGCCGGAAGAGT-3′ (forward) and 5′-CCTGAC CTCATGATCCGC-3′ (reverse). *CTBP1* and *GAPDH* genes were used as internal controls.

Small interfering RNA and cell growth analysis

For the small interfering RNA (siRNA) experiments, 20 nM of siRNA for control (Qiagen), *PSF1* (SI00452501; Qiagen), *PSF2* (SI02653056; Qiagen), *PSF3* (SI00394478; Qiagen) and *SLD5* (SI04243323; Qiagen) was used. Transfection was performed as described previously (Ishida *et al.* 2007).

Flow cytometry and EdU incorporation assay

For DNA content analysis, cells were prepared using Cycle-TEST PLUS DNA REAGENT KIT (BD Biosciences). All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro software. DNA replication analysis was performed with Click-iT EdU Alexa Fluor 488 High-Throughput Imaging Assay Kit (Invitrogen) and confocal laser scanning microscope (Carl Zeiss).

Anchorage-independent colony assay

Anchorage-independent colony assay was performed as described previously (Ishida *et al.* 2007; Ohta *et al.* 2008). T47D (5000 cells) and MDA-MB-231 (10 000 cells) were plated on soft agar and incubated for 3 weeks.

Statistical analysis

Statistical analysis of clinicopathological characteristics was performed using the Fisher's exact test with a single degree of freedom. The survival rates were determined using the Kaplan–Meier methods and compared by means of the log rank test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using R software.

Acknowledgements

We thank Dr Tohru Kiyono (NCCRI, Japan) for HMEC-tert. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare.

References

Bedner, E., Smolewski, P., Amstad, P. & Darznkiewicz, Z. (2000) Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp. Cell Res.* **259**, 308–313.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Nuciforo, P.G., Bensimon, A., Maestro, R., Pelicci, P.G. & d'Adda di Fagagna, F. (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444**, 638–642.

Dominguez-Sola, D., Ying, C.Y., Grandori, C., Ruggiero, L., Chen, B., Li, M., Galloway, D.A., Gu, W., Gautier, J. & Dalla-Favera, R. (2007) Non-transcriptional control of DNA replication by c-Myc. *Nature* **448**, 445–451.

Hayashi, R., Arauchi, T., Tategu, M., Goto, Y. & Yoshida, K. (2006) A combined computational and experimental study on the structure-regulation relationships of putative mammalian DNA replication initiator GINS. *Genomics Proteomics Bioinformatics* **4**, 146–164.

Ishida, M., Miyamoto, M., Naitoh, S., Tatsuda, D., Hasegawa, T., Nemoto, T., Yokozeki, H., Nishioka, K., Matsukage, A., Ohki, M. & Ohta, T. (2007) The SYT-SSX fusion protein down-regulates the cell proliferation regulator COM1 in t(x;18) synovial sarcoma. *Mol. Cell. Biol.* **27**, 1348–1355.

Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H. & Takisawa, H. (2003) A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication. *Genes Dev.* **17**, 1141–1152.

Labib, K. & Gambus, A. (2007) A key role for the GINS complex at DNA replication forks. *Trends Cell Biol.* **17**, 271–278.

Nagahama, Y., Ueno, M., Miyamoto, S., Morii, E., Minami, T., Mochizuki, N., Saya, H. & Takakura, N. (2010) PSF1, a DNA replication factor expressed widely in stem and progenitor cells, drives tumorigenic and metastatic properties. *Cancer Res.* **70**, 1215–1224.

Obama, K., Ura, K., Satoh, S., Nakamura, Y. & Furukawa, Y. (2005) Up-regulation of PSF2, a member of the GINS multiprotein complex, in intrahepatic cholangiocarcinoma. *Oncol. Rep.* **15**, 701–706.

Ohta, T., Iijima, K., Miyamoto, M., Nakahara, I., Tanaka, H., Ohtsuji, M., Suzuki, T., Kobayashi, A., Yokota, J., Sakiyama, T., Shibata, T., Yamamoto, M. & Hirohashi, S. (2008) Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res.* **68**, 1303–1309.

Pai, C.C., Garcia, I., Wang, S.W., Cotterill, S., Macneill, S.A. & Kearsey, S.E. (2009) GINS inactivation phenotypes reveal two pathways for chromatin association replicative alpha and epsilon DNA polymerases in fission yeast. *Mol. Biol. Cell* **20**, 1213–1222.

Takayama, T., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A. & Araki, H. (2003) GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev.* **17**, 1153–1165.

Ueno, M., Itoh, M., Kong, L., Sugihara, K., Asano, M. & Takakura, N. (2005) PSF1 is essential for early embryogenesis in mice. *Mol. Cell. Biol.* **25**, 10528–10532.

Ueno, M., Itoh, M., Sugihara, K., Asano, M. & Takakura, N. (2009) Both alleles of PSF1 are required for maintenance of pool size of immature hematopoietic cells and acute bone marrow regeneration. *Blood* **113**, 555–562.

Received: 5 March 2010
Accepted: 16 June 2010

Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Knockdown of *PSF1* expression by using *PSF1* siRNA.

Figure S2 Growth rate of breast cancer cells by knockdown of *PSF1*.

Figure S3 Knockdown of GINS complex subunits by using siRNA in normal cells and breast cancer cells. Control, *PSF2*, *PSF3* or *SLD5* siRNA was transfected into HMEC-tert or MDA-MB-231 cells.

Additional Supporting Information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

British Journal of Cancer (2010) 103, 297 – 302

www.bjcancer.com

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

M Tanioka*[,1,2], C Shimizu[1], K Yonemori[1], K Yoshimura[3], K Tamura[1], T Kouno[1], M Ando[1], N Katsumata[1], H Tsuda[4], T Kinoshita[5] and Y Fujiwara[1]

[1]Breast and Medical Oncology Division, National Cancer Center Hospital, Tokyo, Japan; [2]Medical Oncology Division, Hyogo Cancer Center, Hyogo, Japan; [3]Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, Japan; [4]Division of Diagnostic Pathology, National Cancer Center Hospital, Tokyo, Japan; [5]Division of Breast Surgery, National Cancer Center Hospital, Tokyo, Japan

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

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

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

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

British Journal of Cancer (2010) **103,** 297–302. doi:10.1038/sj.bjc.6605769 www.bjcancer.com
Published online 6 July 2010

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

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

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

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

PATIENTS AND METHODS

Patients

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

*Correspondence: Dr M Tanioka; E-mail: tanioka@hp.pref.hyogo.jp
Received 17 March 2010; revised 1 June 2010; accepted 9 June 2010; published online 6 July 2010

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

Histopathology

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

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

Treatment

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

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

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

Clinical significance of locoregional recurrence after neoadjuvant chemotherapy

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

Statistical analysis

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

RESULTS

Characteristics of patients with relapse

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

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

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

Predictive factors for recurrence in all 88 patients with pCR

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

Table 1 Patient characteristics

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

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

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

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

DISCUSSION

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

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

Table 2 Characteristics of patients with recurrence

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

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

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

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

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

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

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

Table 3 Univariate analysis of predictive factors for recurrence

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

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

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

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

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

ACKNOWLEDGEMENTS

This study was supported by a grant for 'Validation study of pathological response criteria for neoadjuvant chemotherapy for primary breast cancer' from the Japanese Breast Cancer Society (2008).

REFERENCES

Anderson SJ, Wapnir I, Dignam JJ, Fisher B, Mamounas EP, Jeong JH, Geyer Jr CE, Wickerham DL, Costantino JP, Wolmark N (2009) Prognosis after ipsilateral breast tumor recurrence and locoregional recurrences in patients treated by breast-conserving therapy in five national surgical adjuvant breast and bowel project protocols of node-negative breast cancer. *J Clin Oncol* **27:** 2466–2473

Bear HD, Anderson S, Brown A, Smith R, Mamounas EP, Fisher B, Margolese R, Theoret H, Soran A, Wickerham DL, Wolmark N (2003) The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J Clin Oncol* **21:** 4165–4174

Bear HD, Anderson S, Smith RE, Geyer Jr CE, Mamounas EP, Fisher B, Brown AM, Robidoux A, Margolese R, Kahlenberg MS, Paik S, Soran A, Wickerham DL, Wolmark N (2006) Sequential preoperative or postoperative docetaxel added to preoperative doxorubicin plus cyclophosphamide for operable breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J Clin Oncol* **24:** 2019–2027

Bonadonna G, Valagussa P, Brambilla C, Ferrari L, Moliterni A, Terenziani M, Zambetti M (1998) Primary chemotherapy in operable breast cancer: eight-year experience at the Milan Cancer Institute. *J Clin Oncol* **16:** 93–100

Buzdar AU, Ibrahim NK, Francis D, Booser DJ, Thomas ES, Theriault RL, Pusztai L, Green MC, Arun BK, Giordano SH, Cristofanilli M, Frye DK, Smith TL, Hunt KK, Singletary SE, Sahin AA, Ewer MS, Buchholz TA, Berry D, Hortobagyi GN (2005) Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. *J Clin Oncol* **23:** 3676–3685

Buzdar AU, Valero V, Ibrahim NK, Francis D, Broglio KR, Theriault RL, Pusztai L, Green MC, Singletary SE, Hunt KK, Sahin AA, Esteva F, Symmans WF, Ewer MS, Buchholz TA, Hortobagyi GN (2007) Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human epidermal growth factor receptor 2-positive operable breast cancer: an update of the initial randomized study population and data of additional patients treated with the same regimen. *Clin Cancer Res* **13:** 228–233

Chollet P, Amat S, Cure H, de Latour M, Le Bouedec G, Mouret-Reynier MA, Ferriere JP, Achard JL, Dauplat J, Penault-Llorca F (2002) Prognostic significance of a complete pathological response after induction chemotherapy in operable breast cancer. *Br J Cancer* **86:** 1041–1046

Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* **19:** 403–410

Fisher B, Brown A, Mamounas E, Wieand S, Robidoux A, Margolese RG, Cruz Jr AB, Fisher ER, Wickerham DL, Wolmark N, DeCillis A, Hoehn JL, Lees AW, Dimitrov NV (1997) Effect of preoperative chemotherapy on local-regional disease in women with operable breast cancer: findings from National Surgical Adjuvant Breast and Bowel Project B-18. *J Clin Oncol* **15:** 2483–2493

Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A, Fisher ER, Wickerham DL, Begovic M, DeCillis A, Robidoux A, Margolese RG, Cruz Jr AB, Hoehn JL, Lees AW, Dimitrov NV, Bear HD (1998) Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* **16:** 2672–2685

Gianni L, Eiermann W, Semiglazov V, Manikhas GM, Lluch A, Tjulandin S, Feyereislova A, Valagussa P, Baselga J (2008) Neoadjuvant trastuzumab in patients with HER2-positive locally advanced breast cancer: primary efficacy analysis of the NOAH trial. *31st San Antonio Breast Cancer Symposium (abstract 31)*; 10–14 December

Gonzalez-Angulo AM, McGuire SE, Buchholz TA, Tucker SL, Kuerer HM, Rouzier R, Kau SW, Huang EH, Morandi P, Ocana A, Cristofanilli M, Valero V, Buzdar AU, Hortobagyi GN (2005) Factors predictive of distant metastases in patients with breast cancer who have a pathologic complete response after neoadjuvant chemotherapy. *J Clin Oncol* **23:** 7098–7104

Green MC, Buzdar AU, Smith T, Ibrahim NK, Valero V, Rosales MF, Cristofanilli M, Booser DJ, Pusztai L, Rivera E, Theriault RL, Carter C, Frye D, Hunt KK, Symmans WF, Strom EA, Sahin AA, Sikov W, Hortobagyi GN (2005) Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. *J Clin Oncol* **23:** 5983–5992

Guarneri V, Broglio K, Kau SW, Cristofanilli M, Buzdar AU, Valero V, Buchholz T, Meric F, Middleton L, Hortobagyi GN, Gonzalez-Angulo AM (2006) Prognostic value of pathologic complete response after primary

chemotherapy in relation to hormone receptor status and other factors. *J Clin Oncol* **24:** 1037–1044

Hayes DF, Thor AD, Dressler LG, Weaver D, Edgerton S, Cowan D, Broadwater G, Goldstein LJ, Martino S, Ingle JN, Henderson IC, Norton L, Winer EP, Hudis CA, Ellis MJ, Berry DA (2007) HER2 and response to paclitaxel in node-positive breast cancer. *N Engl J Med* **357:** 1496–1506

Kuerer HM, Newman LA, Smith TL, Ames FC, Hunt KK, Dhingra K, Theriault RL, Singh G, Binkley SM, Sneige N, Buchholz TA, Ross MI, McNeese MD, Buzdar AU, Hortobagyi GN, Singletary SE (1999) Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. *J Clin Oncol* **17:** 460–469

Kuerer HM, Singletary SE, Buzdar AU, Ames FC, Valero V, Buchholz TA, Ross MI, Pusztai L, Hortobagyi GN, Hunt KK (2001) Surgical conservation planning after neoadjuvant chemotherapy for stage II and operable stage III breast carcinoma. *Am J Surg* **182:** 601–608

Mauri D, Pavlidis N, Ioannidis JP (2005) Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J Natl Cancer Inst* **97:** 188–194

Mauriac L, MacGrogan G, Avril A, Durand M, Floquet A, Debled M, Dilhuydy JM, Bonichon F (1999) Neoadjuvant chemotherapy for operable breast carcinoma larger than 3 cm: a unicentre randomized trial with a 124-month median follow-up. Institut Bergonie Bordeaux Groupe Sein (IBBGS). *Ann Oncol* **10:** 47–52

Mazouni C, Peintinger F, Wan-Kau S, Andre F, Gonzalez-Angulo AM, Symmans WF, Meric-Bernstam F, Valero V, Hortobagyi GN, Pusztai L (2007) Residual ductal carcinoma *in situ* in patients with complete eradication of invasive breast cancer after neoadjuvant chemotherapy does not adversely affect patient outcome. *J Clin Oncol* **25:** 2650–2655

Morrell LE, Lee YJ, Hurley J, Arias M, Mies C, Richman SP, Fernandez H, Donofrio KA, Raub Jr WA, Cassileth PA (1998) A phase II trial of neoadjuvant methotrexate, vinblastine, doxorubicin, and cisplatin in the treatment of patients with locally advanced breast carcinoma. *Cancer* **82:** 503–511

Peintinger F, Symmans WF, Gonzalez-Angulo AM, Boughey JC, Buzdar AU, Yu TK, Hunt KK, Singletary SE, Babiera GV, Lucci A, Meric-Bernstam F, Kuerer HM (2006) The safety of breast-conserving surgery in patients who achieve a complete pathologic response after neoadjuvant chemotherapy. *Cancer* **107:** 1248–1254

Ring AE, Smith IE, Ashley S, Fulford LG, Lakhani SR (2004) Oestrogen receptor status, pathological complete response and prognosis in patients receiving neoadjuvant chemotherapy for early breast cancer. *Br J Cancer* **91:** 2012–2017

Rouzier R, Extra JM, Carton M, Falcou MC, Vincent-Salomon A, Fourquet A, Pouillart P, Bourstyn E (2001) Primary chemotherapy for operable breast cancer: incidence and prognostic significance of ipsilateral breast tumor recurrence after breast-conserving surgery. *J Clin Oncol* **19:** 3828–3835

Rowan K (2009) Trastuzumab before breast surgery? Large trial says yes but does not quell debate. *J Natl Cancer Inst* **101:** 448–449

Sataloff DM, Mason BA, Prestipino AJ, Seinige UL, Lieber CP, Baloch Z (1995) Pathologic response to induction chemotherapy in locally advanced carcinoma of the breast: a determinant of outcome. *J Am Coll Surg* **180:** 297–306

Smith IC, Heys SD, Hutcheon AW, Miller ID, Payne S, Gilbert FJ, Ah-See AK, Eremin O, Walker LG, Sarkar TK, Eggleton SP, Ogston KN (2002) Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. *J Clin Oncol* **20:** 1456–1466

van der Hage JA, van de Velde CJ, Julien JP, Tubiana-Hulin M, Vandervelden C, Duchateau L (2001) Preoperative chemotherapy in primary operable breast cancer: results from the European Organization for Research and Treatment of Cancer Trial 10902. *J Clin Oncol* **19:** 4224–4237

Wapnir IL, Anderson SJ, Mamounas EP, Geyer Jr CE, Jeong JH, Tan-Chiu E, Fisher B, Wolmark N (2006) Prognosis after ipsilateral breast tumor recurrence and locoregional recurrences in five National Surgical Adjuvant Breast and Bowel Project node-positive adjuvant breast cancer trials. *J Clin Oncol* **24:** 2028–2037

Wolmark N, Wang J, Mamounas E, Bryant J, Fisher B (2001) Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18. *J Natl Cancer Inst Monogr* **30:** 96–102

Human Pathology (2010) 41, 960–970

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Metaplastic carcinoma of the breast☆

Nao Okada MD[a,b,*], Takahiro Hasebe MD, PhD[a,*], Motoki Iwasaki MD, PhD[c], Nobuko Tamura MD[b], Sadako Akashi-Tanaka MD, PhD[b], Takashi Hojo MD[b], Tatsuhiro Shibata MD, PhD[d], Yuko Sasajima MD, PhD[e], Yae Kanai MD, PhD[f], Takayuki Kinoshita MD, PhD[b]

[a]*Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo 104-0045, Japan*
[b]*Department of Breast Surgery, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[c]*Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo 104-0045, Japan*
[d]*Cancer Genomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[e]*Clinical Laboratory Division, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[f]*Pathology Division, National Cancer Center Research Institute, Tokyo 104-0045, Japan*

Received 4 October 2009; revised 23 November 2009; accepted 25 November 2009

Keywords:
Metaplastic carcinoma;
Invasive ductal carcinoma;
Invasive lobular carcinoma;
Breast;
Prognosis

Summary The purposes of this study were to investigate whether the biological characteristics or outcomes of patients with metaplastic carcinoma, invasive ductal carcinoma, or invasive lobular carcinoma of the breast differ; to determine whether the metaplastic carcinoma subtypes have similar malignant potentials; and to identify accurate predictors of outcome in patients with metaplastic carcinoma. The subject comprised 6137 invasive ductal carcinoma patients, 301 invasive lobular carcinoma patients, and 46 metaplastic carcinoma patients of the breast. The metaplastic carcinomas were classified according to the World Health Organization classification. Multivariate analyses clearly demonstrated that the metaplastic carcinoma patients had a significantly poorer outcome than the invasive ductal carcinoma patients or the invasive lobular carcinoma patients independent of the nodal status or age not exceeding 39 years, whereas patients with triple-negative metaplastic carcinomas or triple-negative invasive lobular carcinomas had a poorer outcome than those with triple-negative invasive ductal carcinomas. Although no significant differences in clinical outcome were observed among the metaplastic carcinoma subtypes in multivariate analyses, an age not exceeding 39 years, the presence of skin invasion, and the presence of a squamous cell carcinoma component in nodal tumors were significant outcome predictors for metaplastic carcinoma patients. In conclusion, the results of this study clearly demonstrated that metaplastic carcinoma is more aggressive than invasive ductal carcinoma or invasive lobular carcinoma. Although the metaplastic carcinoma subtypes had no prognostic significance, an age not exceeding 39 years, the presence of skin invasion, and the presence

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

* Corresponding authors. Nao Okada is to be contacted at Department of Breast Surgery, National Cancer Center Hospital, Chuo-ku, Tokyo 104-0045, Japan. Takahiro Hasebe, Pathology Consultation Service, Clinical Trials and Practice Support Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo 104-0045, Japan.

E-mail addresses: naookada@ncc.go.jp (N. Okada), thasebe@ncc.go.jp (T. Hasebe).

doi:10.1016/j.humpath.2009.11.013

of a squamous cell carcinoma component in nodal tumors were significant predictors of outcome among metaplastic carcinoma patients.

1. Introduction

The World Heath Organization (WHO) classifies metaplastic carcinoma (MPC) into (1) epithelial type and (2) mixed type [1]. Epithelial-type MPC is in turn classified into (1) squamous cell carcinoma, (2) adenocarcinoma with spindle cell differentiation, and (3) adenosquamous carcinoma, whereas mixed type of MPC is classified into (1) carcinoma with chondroid metaplasia, (2) carcinoma with osseous metaplasia, and (3) carcinosarcoma [1]. Several studies have investigated whether the biological characteristics of these MPC subtypes differ [2-19] and whether outcome predictors exist for patients with these MPC subtypes [2-7,9,10,13-15,18]. However, almost all these studies involved survival analyses performed for a small number of MPC cases or that only used univariate analyses [2-7,9,10,13-15,18]. Thus, whether the presently used subtype classification reflects the malignant potential of these lesions remains uncertain; and which factors are the most important predictors of outcome in patients with MPC remains controversial.

Furthermore, although patients with MPC are though to exhibit a poorer outcome than patients with invasive ductal carcinoma (IDC) or invasive breast carcinoma [15,18], the survival periods of patients with MPC, IDC, or invasive lobular carcinoma (ILC) have not been compared using consecutive cases treated during the same period. Thus, no direct evidence indicating that MPC is more aggressive than IDC or ILC presently exists.

The purposes of this study were (1) to investigate whether the biological characteristics or outcomes of patients with MPC, IDC, or ILC of the breast differ; (2) to determine whether the MPC subtypes have similar malignant potentials; and (3) to identify accurate predictors of outcome in patients with MPC. The results of this study clearly demonstrated that patients with MPC have a significantly poorer outcome than patients with IDC or ILC; that the MPC subtype has no prognostic significance; and that an age not exceeding 39 years, the presence of skin invasion, and the presence of a squamous cell carcinoma component in nodal tumors were significant predictors of outcome among patients with MPC.

2. Materials and methods

2.1. Cases

The subject comprised 6137 consecutive cases of IDC, 301 consecutive cases of ILC, and 46 consecutive cases of MPC of the breast; all the subjects had undergone surgery at the National Cancer Center Hospital between January 1982 and March 2007. For the MPC cases, all the breast carcinomas diagnosed as squamous cell carcinoma, epidermoid carcinoma, MPC, carcinosarcoma, carcinoma with spindle cell metaplasia, carcinoma with chondroid metaplasia, or carcinoma with osteoid metaplasia at the National Cancer Center Hospital between January 1982 and March 2007 were reviewed; 46 cases of MPC were subsequently identified.

Clinical information was obtained from the patients' medical records. All the patients were Japanese women, ranging in age from 20 to 98 years (median, 53 years). Overall, 2094 patients were premenopausal and 3056 were postmenopausal. A partial mastectomy had been performed in 1208 patients, a modified radical mastectomy had been performed in 3340, and a standard radical mastectomy had been performed in 1139. A level I and II axillary lymph node dissection had been performed in all the patients, and some of the patients had been received a level III axillary lymph node dissection.

The protocol (20-112) for this study was approved by the Institutional Review Board of the National Cancer Center.

2.2. Neoadjuvant therapy and adjuvant therapy

Because standardized neoadjuvant therapy and adjuvant therapy for patients with breast cancer were started in the 1990s at the National Cancer Center Hospital, the effect of neoadjuvant therapy or adjuvant therapy was examined in patients with IDCs, ILCs, or MPCs that had been surgically treated since January 1990. Neoadjuvant therapy was performed in 467 out of 2039 patients with IDC, 28 out of 111 patients with ILC, and 4 out of 46 patients with MPC, whereas adjuvant therapy was performed in 1756 out of 2303 patients with IDC, 101 out of 122 patients with ILC, and 18 out of 46 patients with MPC. Among these patients, 378 received chemotherapy, 749 received endocrine therapy, and 693 received combined chemoendocrine therapy. In the 1980s, the main chemotherapy regimens in use were anthracycline based; but nonanthracycline-based regimens were used in some patient populations. In the 1990s, the chemotherapy regimens in use were anthracycline based and were combined with or without taxane. In the 1980s, the endocrine therapy regimens in use were tamoxifen combined with or without a gonadotropin-releasing hormone agonist, whereas an aromatase inhibitor was additionally used in the 1990s.

2.3. Histologic examination of IDCs and ILCs

The following IDC and ILC characteristics were obtained from the pathologic diagnostic records, which

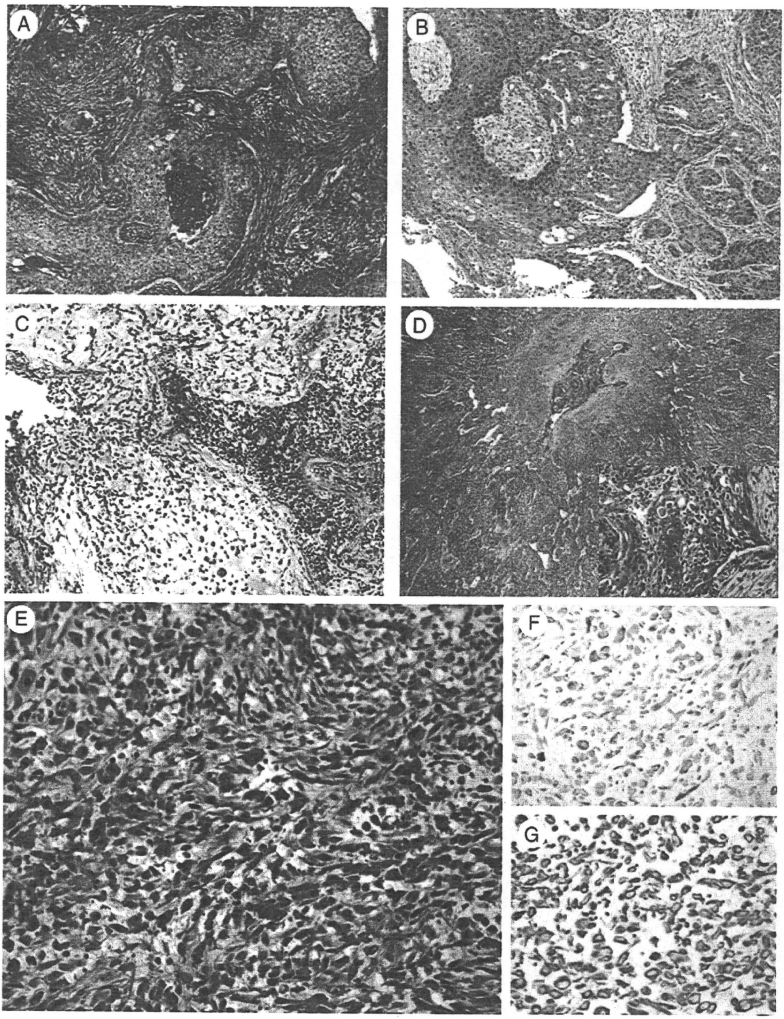

Fig. 1 Histologic features of MPC. A, Squamous cell carcinoma. The carcinoma cells invade as irregularly shaped solid nests showing squamous features with hyalinization. B, Adenosquamous carcinoma. The carcinoma cells invade as irregularly shaped solid nests and show squamous and tubular features. C, Carcinoma with chondroid metaplasia. The carcinoma cells invade as strands or solid nests with chondroid stroma. D, Squamous cell carcinoma in lymph node. Carcinoma cells metastasizing to the lymph node show squamous differentiation. E-G, Carcinosarcoma. Carcinosarcoma consists mainly of spindled tumor cells and epithelioid tumor cells admixed with pleomorphic tumor cells (E). Carcinosarcoma shows positive staining both for keratin (AE1/3) (F) and vimentin (G).