Frequent overexpression of epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation

T Shien, T Tashiro, M Omatsu, T Masuda, K Furuta, N Sato, S Akashi-Tanaka, M Uehara, E Iwamoto, T Kinoshita, T Fukutomi, H Tsuda, T Hasegawa

Aims: To evaluate the expression of common biological markers and the epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation (DCMDs).

Materials/methods: Thirty DCMDs were clinicopathologically and immunohistochemically analysed and compared with 36 control cases of high grade conventional invasive ductal carcinoma (IDC).

Results: EGFR, HER2/neu, oestrogen receptor, progesterone receptor, and p53 expression was seen in 21, one, three, four, and 20 of the 30 DCMDs, compared with eight, nine, 18, 17, and five of the 36 conventional IDCs (p < 0.05), respectively. In 16 of the 30 DCMDs, metastases were found in the brain, lung, bone, and liver, within a maximum of 47 months (mean, 13.9) after initial surgery, whereas only four of the 36 conventional IDCs metastasised to the lung and bone within a maximum of 27 months (mean, 18.0) after initial surgery (p = 0.0001). There was a significant difference in disease free survival between DCMD and conventional IDC (p = 0.001). EGFR was frequently overexpressed in DCMD compared with conventional IDC, whereas the expression of HER2/neu and hormone receptors was lower in DCMD. Fluorescent in situ hybridisation revealed that the mean EGFR to chromosome 7 centromere (CEP7) ratio of the 24 DCMD cases available for evaluation was 1.03, and EGFR gene amplification was not detected in the 21 IDC cases with EGFR overexpression.

Conclusion: Immunohistochemistry for myoepithelial markers and EGFR is useful for the accurate diagnosis and molecular target treatment of high grade DCMD.
as IDC with foci of tissue infarction in the central zone covering more than 30% of the tumour tissue area, accompanied by a varying degree of myxoid stromal matrix at the periphery (fig 1A). These carcinomas showed no features of CS, osseous or cartilaginous metaplasia, or MPC. MPCs showed infiltrating growth, mixed often with heterologous myoepithelial elements, from areas of bland chondroid and osseous differentiation to frank sarcomatous components (fig 1B). CSs had myoepithelial components composed of malignant, undifferentiated spindle cells that formed part of the tumour (fig 1C).

DCMDs were diagnosed based on the demonstration of specific cytoplasmic immunoreactivity for one or more myoepithelial marker. Immunopositivity for S-100 protein (fig 2A), α smooth muscle actin (fig 2B), and keratin 14 was seen in 23, 30, and 19 of the 30 cases, respectively. All three groups of tumours showed a similar immunohistochemical staining pattern for myoepithelial markers.

As a control group, samples from 36 patients with conventional IDC who were treated and followed in our hospital at the same time between 1982 and 2003 and matched for patient age, histological grade, tumour size, and lymph node status were selected from the pathology files, although it is not clear that this control group is large enough in view of the recognised heterogeneity between and within breast cancers. Clinical and follow up information was obtained by reviewing the medical records.

**Immunohistochemistry**

Immunostaining for HER2/neu, ER, progesterone receptor (PgR), and p53 was performed on 4 μm formalin fixed, paraffin wax embedded tissue sections using the labelled streptavidin biotin method (DakoCytomation, Glostrup, Denmark). Table 1 lists the specifications of the primary antibodies used in our study. After routine dewaxing and rehydration, the sections were moistened with phosphate buffered saline (PBS; pH 7.4). They were pretreated in an autoclave at 121°C for 10 minutes in 10 mMol/litre citrate buffer (pH 6.0), before being incubated with all antibodies, except for anti-S-100, in an automated immunostaining system (i6000™; BioGenex, San Ramon, California, USA) for 30 minutes. Immunohistochemical staining for EGFR was performed according to the instructions included with the EGFR detection system (EGFR pharmDx kit; DakoCytomation).

The immunohistochemical results for the HER2/neu, ER, PgR, p53, and EGFR specific antibodies were judged by two observers using a multihued microscope. A consensus judgment was adopted as the proper immunohistochemical score of the tumour based on strength: 0, negative; 1+, weak staining; 2+, moderate staining; 3+, strong staining. The distribution of positive cells was also graded in an effort to assess the diffuse or focal nature of the positive cells as sporadic (positive cells, ≤ 10%), focal (10–50% positive cells), or diffuse (positive cells, ≥ 50%). For ER, PgR, and p53, tumour nuclear staining in more than 10% of cells was evaluated as positive, regardless of intensity. For HER2/neu and EGFR, immunohistochemical scores of 2+ and 3+ with a focal to diffuse distribution were considered to be positive.

**Fluorescence in situ hybridisation**

FISH was carried out according to the manufacturer’s instructions. Briefly, after dewaxing, the slides were immersed in 0.2N HCl for 20 minutes and pretreatment solution (Vysis, Downers Grove, Illinois, USA) for 30 minutes at 37°C, then digested with protease for 60 minutes at room temperature, washed in 1× PBS for five minutes at room temperature, fixed in 10% formaldehyde for 10 minutes at room temperature, washed in 1× PBS for five minutes at room temperature, placed into prewarmed denaturation solution (Vysis) for five minutes at 72°C, and dehydrated by immersing in 70%, 85%, and 100% ethanol for one minute each at room temperature.

For interphase FISH, the slides were subjected to hybridisation with the LSI EGFR/chromosome 7 centromere (CEP7) dual colour probe (Vysis). A Spectrum Orange labelled EGFR specific probe and a Spectrum Green labelled centromeric
probe that hybridises to the centromeric region of the chromosome were used as controls to normalise copy numbers for chromosome 7. The FISH probe mix (10 μl) was added to the sample area of the slides at 45°C. The slides were coverslipped, sealed with rubber cement, and incubated at 37°C for 48 hours in a humidified chamber. They were then washed in post-hybridisation wash buffer at 72°C. Subsequently, 10 μl of 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Vysis) counterstain was placed on the slide and the slide was coverslipped. After hybridisation, all slides were maintained at –20°C in the dark. Hybridisation signals were visualised with an epifluorescence microscope, and images were captured on a CCD camera. For each specimen, the number of orange EGFR signals was counted for the 60 nuclei, and the mean EGFR copy number was calculated by dividing the total number of EGFR signals by 60. The mean green CEP7 copy number was also calculated by dividing the total number of CEP7 signals by 60. The EGFR : CEP7 ratio was also calculated for each specimen by dividing the total number of orange EGFR signals by the total number of green CEP7 signals. A ratio of > 2.0 was defined as EGFR DNA amplification.

### Statistical analysis

Statistical differences were calculated using the χ² test or Fisher’s exact test. Disease free survival (DFS) curves were plotted using the Kaplan–Meier method, and significant differences between the survival curves were calculated using the log rank test. Differences of p < 0.05 were considered significant.

### RESULTS

#### Patient characteristics

Table 2 shows the clinicopathological characteristics of the patients with DCMD (n = 30) and controls (n = 36). All patients with DCMD were female, with an average age at the time of initial diagnosis of 50.1 years (range, 26–77). All the control patients (with high grade conventional IDC) were female, with an average age at the time of initial diagnosis of 49.2 years (range, 28–77). None of the patients with DCMD or the control subjects had other cancers or endocrinological disorders. Each patient with DCMD presented with a palpable mass measuring between 1.2 and 5.2 cm (mean, 2.7) at the greatest diameter, whereas the control patients had a palpable mass measuring between 0.6 and 8.0 cm.

#### Table 2 Clinicopathological difference between the study and control patients

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>DCMD (n = 30)</th>
<th>cIDC (n = 36)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50</td>
<td>14</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;50</td>
<td>16</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>50.1</td>
<td>49.2</td>
<td></td>
</tr>
<tr>
<td>Histological grade 3</td>
<td>30</td>
<td>36</td>
<td>NS</td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 cm</td>
<td>10</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>&gt;3 cm</td>
<td>20</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Mean</td>
<td>2.7 cm</td>
<td>2.7 cm</td>
<td></td>
</tr>
<tr>
<td>LN metastases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (43.3%)</td>
<td>16 (44.4%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>17 (56.7%)</td>
<td>20 (55.6%)</td>
<td>NS</td>
</tr>
<tr>
<td>Distant metastases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>16 (53.3%)</td>
<td>4 (11.0%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>–</td>
<td>14 (46.7%)</td>
<td>32 (89.0%)</td>
<td></td>
</tr>
</tbody>
</table>

cIDC, conventional invasive ductal carcinoma; DCMD, ductal carcinoma with myoepithelial differentiation; LN, lymph node; NS, not significant.

### Table 1 Profiles of antibodies used in our study

<table>
<thead>
<tr>
<th>Antigen/antibody (dilution)</th>
<th>Source</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-100 protein (1/2000)*</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>None</td>
</tr>
<tr>
<td>SMA (1A4; 1/100)</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Keratin 14 (UU02; 1/50)</td>
<td>Ylem, Rome, Italy</td>
<td>Autoclave</td>
</tr>
<tr>
<td>EGFR (2-18C9; prediluted)</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>HER2/neu (1/100)*</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Autoclave</td>
</tr>
<tr>
<td>ER (1D5; 1/50)</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Autoclave</td>
</tr>
<tr>
<td>PgR (1A6; 1:50)</td>
<td>Novacastria, Newcastle upon Tyne, UK</td>
<td>Autoclave</td>
</tr>
<tr>
<td>p53 (DO7; 1/100)</td>
<td>DakoCytomation, Glostrup, Denmark</td>
<td>Autoclave</td>
</tr>
</tbody>
</table>

*Polyclonal antibody.

EGFR, epidermal growth factor receptor; ER, oestrogen receptor; PgR, progesterone receptor; SMA, smooth muscle actin.
(mean, 2.7) at the greatest diameter. Lymph node metastases were seen in 13 of the 30 patients with DCMD and in 16 of the 36 control subjects. There were no significant differences in age, histological grade, tumour size, or lymph node status between the two groups. Sixteen of the 30 patients with DCMD developed distant metastases within a maximum of 47 months (mean, 13.9) after surgery (p = 0.0001); 29 metastases were detected in the brain (nine), lungs (eight), bone (five), liver (three), skin (one), stomach (one), spleen (one), and soft tissue (one) during a follow up period ranging from two to 122 months (mean, 40.9). In contrast, only four of the 36 control patients developed metastases within 27 months (mean, 18.0) after surgery (two each in the bone and lung). Figure 3 shows the DFS curves. There was a significant difference in DFS between the DCMD and control groups (p = 0.001).

Immunohistochemical features

EGFR overexpression was detected in 21 of the 30 DCMD cases—12 of 20 patients with IDCs having a large central acellular zone, all five patients with MPC, and four of the five patients with CS (fig 2C)—and in eight of the 36 control subjects (table 3). HER2/neu overexpression was found in only one of the 30 patients with DCMD and in nine of the 36 controls. Positive reactions in the nuclei for ER and PgR were seen in three and four patients with DCMD, and in 18 and 17 of the controls, respectively. Immunoreactivity for p53 was seen in 20 of the 30 control patients developed metastases within 27 months (mean, 18.0) after surgery (two each in the bone and lung). Figure 3 shows the DFS curves. There was a significant difference in DFS between the DCMD and control groups (p = 0.001).

Of the 30 patients with DCMD, only two showed overexpression of hormone receptors without overexpression of EGFR. Coexpression of HER2/neu and EGFR was detected in only one patient. EGFR was overexpressed in 13 of 20 patients with immunoreactivity for p53 (table 4).

FISH analysis

FISH analysis revealed that the mean EGFR : CEP7 ratio of the 24 DCMD samples available for evaluation was 1.03, and EGFR gene amplification was not detected in the 21 patients with DCMD in whom EGFR was overexpressed (fig 4). The EGFR gene was not amplified in control cases with overexpression (data not shown).

DISCUSSION

Metaplastic carcinomas of the breast are rare neoplasms that account for <5% of mammary tumours and consist of different mixtures of neoplastic breast epithelial cells and metaplastic elements. Wargotz and Norris divided these...
EGFR in mammary high grade ductal carcinoma

Take home messages

- We evaluated the expression of common biological markers and the epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation (DCMDs).
- EGFR was frequently overexpressed in DCMD compared with high grade conventional invasive ductal carcinoma, whereas HER2/neu and hormone receptors were less frequently expressed in DCMD.
- p53 was also overexpressed in DCMD.
- Immunohistochemistry for myoepithelial markers and EGFR can be useful for the accurate diagnosis and molecular target treatment of DCMD.

We detected ER, PgR, and HER2/neu expression in 92 of 174 cases of high grade conventional IDC. Significant differences were observed between cases of metaplastic carcinoma with osteocartilaginous elements and controls for the expression of ER, PgR, and HER2/neu. EGFR expression was seen in 21 of 174 cases of IDC, whereas HER2/neu expression was infrequent, despite the high rate of EGFR overexpression in DCMD. Coexpression of HER2/neu and EGFR was detected in only one case, and EGFR and HER2/neu overexpression appear to be independent events in DCMD.

In our series, p53 was expressed in 20 of the 30 DCMD cases and only five of the 36 control cases. p53 expression was previously reported in 14 of 29 metaplastic carcinomas, and in our series, p53 expression was higher in DCMD than in controls. Tumours with both HER2/neu overexpression and p53 protein accumulation have been reported in several studies, and patients with such tumours were found to have a poor prognosis. In addition, earlier studies have shown that the presence of p53 protein in breast carcinoma is significantly correlated with the overexpression of EGFR; however, we did not find a similar correlation in our series, as described previously.

"EGFR and HER2/neu overexpression appear to be independent events in ductal carcinoma with myoepithelial differentiation"

In our series, EGFR gene amplification (assessed by FISH) was not detected in the 21 patients with DCMD and EGFR overexpression. Kersting et al recently reported that FISH revealed a low frequency of EGFR gene amplification, despite protein overexpression, in 222 invasive ductal carcinomas, including 29 high grade carcinomas. In their report, eight cases (4.7%) showed EGFR gene amplification by FISH and 31 cases (17%) showed overexpression of EGFR by means of immunohistochemistry. FISH showed EGFR gene amplification in only three of the 23 cases with immunohistochemical protein overexpression, whereas gene amplification was seen in two (1.6%) of the 124 cases without immunohistochemical protein overexpression. The findings of our series of patients with DCMD corroborate these previous results. Moreover, studies of EGFR gene amplification in other solid tumours have reported lower incidences of gene amplification relative to receptor overexpression, implying that in most solid tumours gene amplification is not the prevailing mechanism of EGFR overexpression, and that it is also unlikely to be of diagnostic value for most solid tumours.

The underlying mechanisms of EGFR protein overexpression are not understood completely. Gene amplification was proposed as a possible mechanism for many years, but recent
studies indicate that other mechanisms may play a role, such as post-translational changes or the length of polymorphisms in intron 1 of the EGFR gene.20 30

In summary, EGFR was frequently overexpressed in DCMD compared with high grade conventional IDC, whereas HER2/ neu and hormone receptors were less frequently expressed in DCMD. p53 was also overexpressed in DCMD. In suspected cases of DCMD, immunohistochemical examination for myoepithelial markers and EGFR can be useful for the accurate diagnosis and molecular target treatment of this special type of mammary ductal carcinoma with a different metastatic pattern and prognosis.

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Authors’ affiliations
T Shien, S Akashi-Tanaka, M Uehara, E Iwamoto, T Kinoshita, T Fukutomi, Division of Surgical Oncology, National Cancer Centre Hospital, Tokyo 104-0045, Japan
T Tashiro, M Omatsu, T Masuda, K Furuta, Division of Clinical Laboratory, National Cancer Centre Hospital
N Sato, Division of Diagnostic Radiology, National Cancer Centre Hospital
H Tsuda, Department of Clinical Pathology, National Defence Medical College, Saitama 359-8513, Japan
T Hasegawa, Department of Clinical Pathology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

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