Comparison of HER2 mRNA Amplification with Immunohistochemistry in Human Breast Cancer Using Laser Assisted Microdissection Technique

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In human breast cancer, overexpression of HER2 protein (score 3+) is a good indication for herceptin (trastuzumab) administration and can expect a favorable prognosis. In this study, we examined the correlation between the expression of HER2 protein and the status of mRNA amplification in human breast cancer tissues prepared using laser assisted microdissection (LAM) technique. Twenty breast cancers were selected for this study. The surgical specimens were obtained under informed consent. Fresh tumor tissues were cut into no more than 10x5x5 mm sections, and frozen immediately in hexane/dry-ice acetone. Eight-micrometer thick sections were cut and mounted on a membrane film-coated slide glass, fixed in methanol and stained with toluidine blue. Microdissection was carried out using a LAM instrument (LS 337 Laser Scissors™, Cell Robotics Inc. USA). RNA was extracted from the microdissected tumor tissues by means of guanidium thiocyanate method, and transcribed to cDNA. HER2 and GAPDH (internal control) mRNAs were analyzed by real time polymerase chain reaction (PCR) based on a fluorogenic TaqMan method. Amplification of HER2 mRNA was standardized with the copy number of GAPDH mRNA. Immunohistochemistry (IHC) for HER2 protein was carried out using LSAB (labeled streptavidine biotin) method. Regarding the status of HER2 mRNA amplification, we obtained 4 cases of invalid score and 4 cases of distinct and unequivocal amplification. The status of HER2 mRNA amplification of these 8 cases was well correlated with the expression of HER2 protein evaluated as score 0 and 3+. The cases evaluated as score 1+ and 2+ in the immunohistochemistry were quite heterogeneous and showed a relatively wide range of HER2 amplification.

A combination of LAM technique and real time PCR is a powerful and reliable method for the quantitative analysis of mRNA. Our results indicated that the groups of HER2 score 1+ and 2+ include candidates for herceptin therapy.

Key words: HER2, breast cancer, laser microdissection, immunohistochemistry, RT-PCR

I. Introduction

Human epidermal growth factor receptor-2 (HER2) has been shown as one of the prognostic and predictive factors in breast cancer [2, 5–6, 15, 20]. Information on the status of HER2 expression is critical to select patients for herceptin (trastuzumab) therapy in both primary and metastatic lesions [14, 16, 19]. Several methods are available for evaluating HER2 status, such as measurement of DNA, mRNA, or protein level [13]. The most common detection method for HER2 status is immunohistochemistry (IHC) [12]. IHC is particularly useful because it has some advantages such as lower cost, easier and simple technique and shorter time for evaluation. The expression of HER2 protein can be demon-
strated as membranous staining in individual cell. On the other hand, it has been shown that a variety of conditions influence greatly the results of IHC including tissue processing, storage term of the paraffin blocks, specificity of antibody and sensitivity of detection system used [13]. Fluorescence in situ hybridization (FISH) is a more recently developed method, which can visualize the number of gene copies present in the tumor cells and provide sensitive, accurate and reproducible results for the amplification of HER-2/neu gene, even in routine archival tissue [10]. Although FISH is reliable for HER2 gene amplification, this method requires a rather complex process, technical skills, much cost and suitable sampling [19]. Some of the HER2 assays are not practical for routine pathology laboratory use. Blottings (Western, Southern and Northern) can be used as a detection system for the various HER2 target molecules, but they are impractical for routine screening use because all these methods demand rather complicated techniques [13]. Recently, several studies demonstrated that HER2 gene amplification in tumor cells with a combination of LAM technique and quantitative gene expression analysis [4, 8, 9, 11, 17]. LAM is known to be quite useful for preparing target cells from the tissue specimen by means of microdissecting them out with a laser beam under microscopic observation and retrieving them for analysis. A variety of LAM instruments are now available. In this study, we describe an approach to detect the amplification of HER2 mRNA, in a small quantity of tissue sample using a combination of LAM and quantitative PCR in archival tissue specimens.

II. Material and Methods

Tissue samples
Stored fresh frozen tissue samples of 20 breast cancers were used under informed consent. For frozen tissue, tumor tissue without necrosis or hemorrhage was cut immediately from the surgical specimens. Tumor tissues no more than 10×5×5 mm in size were embedded in OCT compound with plastic containers, and then dipped gently into a refrigerant of hexane/dry-ice acetone. The frozen tissue blocks were stored at –80°C until use.

Immunohistochemistry
Formalin-fixed and paraffin embedded tissue sections of the same 20 breast cancers were examined immunohistochemically for the status of HER2 expression. The immunohistochemical visualization of HER2 protein was demonstrated by using a specific anti-c-erbB-2 (HER2-neu) protein polyclonal antibody (Zymed Laboratories Inc. Nichirei Co., Tokyo, Japan) and a labeled streptavidin biotin (LSAB) kit (Dako Japan, Kyoto, Japan).

Laser-assisted microdissection
Eight micrometer thick tissue sections were mounted on a membrane film-coated slide glass, fixed in 100% methanol and stained with toluidine blue. The slide glasses mounted with the tissue sections were air-dried at room temperature for up to 40 min. The target cells were then microdissected by laser beam under light microscope (Fig. 1). For microdissection in this study, we used CRI-337 Laser-Scissors™ (Cell Robotics Inc., Albuquerque, NM, USA), which is equipped with an ultraviolet (UV; 337 nm wave length) laser beam. The microdissected target tumor cells were retrieved into an Eppendorf™ lid with mineral oil (Fig. 2).

RNA extraction and reverse transcription
The cell mounted lids were carefully covered on the Eppendorf™ tubes containing 200 µl of denaturing buffer, guanidinium thiocyanate and 1.6 µl of β-mercaptoethanol. Total RNA was purified with 20 µl 2 M sodium acetate (pH 4.0), 220 µl citrate saturated phenol (pH 4.3), and 60 µl chloroform-isoamyl alcohol. These tubes were centrifuged for 15 min at 15,000 rpm and the upper aqueous layer was transferred into new tubes. Two hundred µl isopropanol, 2 µl glycogen was added as a carrier and the tubes were allowed to stand at –80°C for more than 30 min. They were recentrifuged for 30 min at 14,000 rpm, and the supernatant was decanted. The pellets were washed with 70% ethanol and air dried on ice. They were dissolved with 10 µl diethyl

![Fig. 1. Principle of laser assisted microdissection.](image-url)
pyrocarbonate (DEPC) treated water, and stored at −80°C until use. Total RNA was extracted from whole cryosection using the same protocol to check their quality. To assess the quality of the total RNA, 1 μl of them was directly analyzed on an RNA Labchip (Yokogawa Analytical System Ltd., Tokyo, Japan) and RNA6000 kit (Yokogawa Analytical System Ltd., Tokyo, Japan) using Agilent Bioanalyzer 2100 (Yokogawa Analytical System Ltd., Tokyo, Japan). The RNA extracted from microdissected tumor cells was reverse transcribed in a final volume of 20 μl using a Thermoscript RT-PCR system (Gibco-BRL, Invitrogen Japan K.K., Tokyo, Japan) according to manufacturer’s instructions.

**TaqMan quantitative real-time polymerase chain reaction**

In this study, the quantity of HER2 mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control were measured by TaqMan quantitative real-time PCR technique. In addition to the sense and antisense primers, a nonextendable oligonucleotide probe with a 5’ fluorescent reporter dye, either 6FAM or VIC, and a 3’ quencher dye, TAMRA, that hybridizes downstream of the sense primer to the target sequence were used. During the extension phase, the Taq polymerase hydrolyzes this probe thereby generating a fluorescent signal, which is directly proportional to the amount of PCR product synthesized. This fluorescent signal is monitored on-line using the laser detector of the ABI Prism 7700 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan).

The PCR amplification was performed using a 96-well tray and optical caps with a 25 μl final reaction mixture containing 900 nmol/L each primer, 250 nmol/L probe, 1×TaqMan® Universal PCR Master Mix (PE Biosystems, Foster City, CA). The sequences of each primer and probe are as follows: HER2 forward primer is 5’-TCACCTACAACACAGACACGTTTG, TaqMan probe is FAM5’-CCG-GTATACATTCGGCGCCAGCT-3’TAMRA, and reverse primer is 5’-ATCCACGTCCGTTAGAAGGTA. GAPDH forward primer is 5’-GAAGGTGAAGGTCGGAGTC, TaqMan probe is VIC5’-CCGACTCTTGCCCTTCGAAC-3’TAMRA, and reverse primer is 5’-GAAGATGGTGATGGGATTTC. The reaction mixture was preheated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

In this study, we used each of the RT-PCR products of GAPDH mRNA and HER2 mRNA as standard templates to measure the starting quantity, amplified with the same primers as described above. A total of 25 μl PCR reaction mixture contained 2 μl cDNA, 1×PCR Gold Buffer (PE

![Fig. 2. Microdissection and isolation of breast carcinoma cells (×400). A: Tumor cells were dissected by UV laser microbeam under a microscope. B: Section after removal of the target cells. C: Isolated tumor cells in the lid of a reaction tube.](image)

![Fig. 3. Colinearity of dilution and assay range of GAPDH as an internal control (A) and HER2 target mRNA (B). Threshold Ct values are plotted against relative copy number.](image)
Biosystems), 4 mmol/l MgCl$_2$, 0.2 µmol of each primer, 200 µmol/l of each dNTP, and 1.5 units AmpliTaq Gold (PE Biosystems). The PCR were carried out for 35 cycles at 95, 60 and 72°C, respectively. The products were purified with Microcon 100 (Takara Bio Inc., Shiga, Japan). To assess the concentration of the PCR products, 1 µl of the products was directly analyzed on a DNA 500 LabChip (Agilent Technologies Inc., Tokyo, Japan) according to manufacturer’s instructions. The concentrations (copies/µl) of GAPDH and HER2 PCR products were measured with the molecular weight of each target sequence. They were diluted with TE buffer from 1 to 1×10$^7$ copies/µl. Starting quantities of the samples were measured with each standard curve (Fig. 3A, B).

**Statistical analysis**

The results of the starting quantities of HER2 mRNA measured by real time RT-PCR were standardized with that of internal control GAPDH. To compare the status of HER2 between protein expression and the quantities of mRNA, student’s t-test was done among the groups evaluated as IHC score 0, 1+, 2+ and 3+.

### III. Results

#### Quality of total RNA from archival tissue sections

The quality of the total RNA extracted from the cryo-sections was confirmed by measurement of rRNA ratio (28S/18S). The gel images are shown in Figure 4A. The rRNA ratio (28S/18S) of the older (1995–1996) samples was close to 1.00, which means the quality of RNA was significantly poorer than that of recent (2000–2001) ones. However, both the bands (Fig. 4A) and peaks (Fig. 4B) of rRNA were clearly detected.

#### Table 1. Histology subtypes and immunohistochemical detection of HER2 protein expression

<table>
<thead>
<tr>
<th>No.</th>
<th>Year</th>
<th>Histology subtype*</th>
<th>HER2 protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1995</td>
<td>IDC, Solid.</td>
<td>2+</td>
</tr>
<tr>
<td>2</td>
<td>1996</td>
<td>IDC, Pap.</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1996</td>
<td>IDC, Sci.</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1996</td>
<td>IDC, Sci.</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1996</td>
<td>IDC, Solid.</td>
<td>1+</td>
</tr>
<tr>
<td>6</td>
<td>1996</td>
<td>IDC, Sci.</td>
<td>1+</td>
</tr>
<tr>
<td>7</td>
<td>2000</td>
<td>IDC, Sci.+Pap.</td>
<td>1+</td>
</tr>
<tr>
<td>8</td>
<td>2000</td>
<td>IDC, Sci.</td>
<td>1+</td>
</tr>
<tr>
<td>9</td>
<td>2000</td>
<td>IDC, Solid.</td>
<td>1+</td>
</tr>
<tr>
<td>10</td>
<td>2000</td>
<td>IDC, Sci.</td>
<td>1+</td>
</tr>
<tr>
<td>11</td>
<td>2000</td>
<td>IDC, Sci.+DCIS</td>
<td>1+</td>
</tr>
<tr>
<td>12</td>
<td>2000</td>
<td>IDC, Sci.</td>
<td>3+</td>
</tr>
<tr>
<td>13</td>
<td>2001</td>
<td>IDC, Sci.</td>
<td>1+</td>
</tr>
<tr>
<td>14</td>
<td>2001</td>
<td>IDC, Pap.</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>2001</td>
<td>IDC, Sci.+Solid.</td>
<td>3+</td>
</tr>
<tr>
<td>16</td>
<td>2001</td>
<td>IDC, Solid.</td>
<td>3+</td>
</tr>
<tr>
<td>17</td>
<td>2001</td>
<td>Med.</td>
<td>1+</td>
</tr>
<tr>
<td>18</td>
<td>2001</td>
<td>IDC, Sci.+Pap.+Solid.</td>
<td>1+</td>
</tr>
<tr>
<td>19</td>
<td>2001</td>
<td>IDC, Solid.</td>
<td>1+</td>
</tr>
<tr>
<td>20</td>
<td>2001</td>
<td>IDC, Sci.</td>
<td>3+</td>
</tr>
</tbody>
</table>


#### Histology subtypes and expression of HER2 protein

The summary of the 20 human breast cancers examined in this study is shown in Table 1. Nineteen samples were invasive ductal carcinomas and one was a special type. Their histology subtypes were 2 papillotubular, 5 solid tubular, 12 scirrhous, and 1 medullary carcinoma. Six samples were collected during the period of 1995–96 and 14 samples

![Fig. 4.](image)

Fig. 4. Gel images of total RNA by Agilent 2100 Bioanalyzer (A), a system of microcapillary electrophoresis. The quality of total RNA extracted from each whole frozen tissue sections were measured. This quality was expressed with the ratio of 28S and 18S rRNA concentrations measured as electrophologram (B). Lane M was RNA ladder. The samples in lanes 1 to 5 were obtained in 1995–1996, and these rRNA ratios were 28S/18S 2:1.39, 3:1.13, 3:1.22, 4:1.44, 5:1.27. The samples of lane 6 to 10 were obtained in 2000–2001, and these rRNA ratio were 6:1.10, 7:1.79, 8:1.74, 9:1.71, 10:1.33.
were during 2000–2001. The expression of HER2 protein was demonstrated immunohistochemically, and evaluated using a scoring system of score 0, 1+, 2+ and 3+ (Fig. 5). HER2 scores of 20 breast cancers are shown in Table 1.


IV. Discussion

HER2 mRNA amplification

In this study, we investigated the starting quantities of GAPDH mRNA as an internal control and Her2 mRNA by drawing a standard curve in Table 2. The starting quantity of HER2 mRNA was $10^{-3}$ to $10^{-4}$ times smaller than that of GAPDH mRNA. Although the starting quantity of HER2 mRNA was quite small, it was very different among the samples examined. The number of tumor cells microdissected for the assay was up to 1000 cells. However, the initial amount of RNA extracted was different from sample to sample. Therefore, we standardized the quantity of HER2 mRNA with that of GAPDH mRNA.

The result of comparison between the status of HER2 mRNA amplification and the protein expression is shown in Figure 6. The amplification of HER2 mRNA, standardized with GAPDH, was well correlated with the expression of HER2 protein in the cases showing the HER2 scores 0 and 3+. There was a significant difference between the two by Students’ t-test ($p<0.05$). The groups evaluating as HER2 score 1+ and 2+ (only one case) were quite heterogeneous regarding their amplification of HER2 mRNA.

IV. Discussion

The development and introduction of LAM technique brought about revolutionary advances in the molecular analysis of tissue samples, which are made up of heterogeneous cell population. Using this technique, pure cell populations can be obtained without any contamination from adjacent cells. Several studies for molecular analysis focused on pure cell population by LAM technique have been reported [3, 8, 9, 11, 17]. Even though using the pure cell population for molecular analysis, the results still depend greatly on the experimental design, and sufficient technical skill in including tissue processing.

In this study, we demonstrated that satisfactory results can be obtained in the analysis of HER2 mRNA amplification using a small number of tumor cells microdissected from the frozen tissues, which were stored for up to 7 years at $-80^\circ$C. Although the quality of the total RNA extracted from the older samples was poorer than that of the recent ones, the quality of RNA of both groups was sufficient enough to use for molecular analysis. Specht et al. reported the analysis of HER2 mRNA extracted from 50 cells [17], though the tissue samples used in their study were mostly tumors with strong expression of HER2 protein. We were also able to demonstrate the satisfactory amplification of β-actin mRNA, one of the housekeeping genes, in total RNA extracted from less than 50 cells of routine archival frozen tissue sections (data not shown). Regarding the initial copy number of HER2 mRNA in a single tumor cell, it is thought to be $10^{-3}$ to $10^{-4}$ times less than that of β-actin mRNA. In our study, the amplification of HER2 mRNA was consistently measurable in the samples made up of approximately 1000 cells microdissected from the frozen tissue, which was stored more than 5 years. It was surprising, because, to our knowledge, RNA is degraded easily by endogenous and exogenous RNase activities. For this reason, we paid great attention to tissue processing, such as tissue freezing, storing conditions of the frozen tissue blocks, cryosectioning, staining for the frozen section as well as the extraction of nucleic acid from the microdissected tissue samples. Regarding the LAM system, we used CRI-337 Laser Scissors, which is equipped with a UV laser beam module. Now the majority of the laser beams equipped to microdissection instruments is UV, which is non-thermal and thought to be better for RNA analysis.

We also investigated the correlation between the status of HER2 mRNA amplification and the expression of HER2 protein in breast cancer using a combination of LAM system and real time RT-PCR protocol. The HER2 status in breast cancer patients is one of the most important predictive and prognostic factors [2, 20], and known to be a key factor to select candidates for herceptin (trastuzumab) therapy [18]. In the surgical pathology laboratory, the immunohistochemical evaluation of HER2 status is most commonly used. It is well known that the immunohistochemical result is greatly dependent on tissue processing, staining conditions, and the properties of the immunohistochemical reagents including antibodies and visualization system.

A good correlation between the overexpression of HER2 protein and the amplification of HER2 DNA or mRNA has been shown in breast cancers with Herceptest score 3+. In addition, the usefulness of HER2 DNA analysis using FISH has been accepted in breast cancer patients with HER2 score 2+. However, it has been reported that overexpression of HER2 protein and amplification of HER2
mRNA were demonstrated without HER2 genetic abnormalities [1]. In this study, we examined the correlation between the immunohistochemical expression of HER2 protein and the amplification of HER2 mRNA by using real time RT-PCR protocol in breast cancers with various HER2 status, with scores of 0, 1+, 2+ and 3+.

In comparison with the FISH analysis, real time PCR and RT-PCR analysis provided more objective and quantitative data about HER2 status in breast cancer. In addition, FISH analysis may fail to demonstrate positive signals of lower gene amplification. We obtained a good correlation between the expression of HER2 protein and the starting quantity of HER2 mRNA in the groups of Herceptest score 3+ and score 0, respectively. On the other hand, the group of HER2 score 1+ was quite heterogeneous regarding the starting quantity of HER2 mRNA. It has been reported that the Herceptest score 1+ was quite heterogeneous between the immunohistochemical result and the status of HER2 mRNA amplification. This result suggests that the HER2 score 1+ group may include potential candidates for herceptin therapy.

In conclusion, LAM technique is a quite useful and reliable tool to obtain a pure cell population from routine archival tissue specimens. A combination of LAM and molecular analysis of HER2 mRNA provides quantitative and useful information, which is directly related to the choice of therapy. The quantitative analysis of HER2 mRNA seems to be a reliable method to pick out the candidates for herceptin therapy from the Herceptest score 2+ group and even from the 1+ group, which is a quite heterogeneous group for the status of HER2 mRNA amplification.

V. References