Analysis of the HER2/neu Gene Amplification in Microdissected Breast Cancer Tumour Samples

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Abstract. The HER2/neu oncogene has been reported to be amplified in >20% of invasive ductal carcinomas. In order to investigate the HER2/neu status in pure populations of breast cancer cells, a laser capture microdissection (LCM) system was used. Formalin-fixed paraffin-embedded breast tissue areas corresponding to normal ducts, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) were microdissected and genomic DNA was isolated by a modified proteinase K- phenol extraction method and subjected to PCR for HER2/neu analysis. One hundred % concordance for detection of the HER2/neu gene amplification was found between immunohistochemistry and PCR used in combination with LCM. Our results indicated that LCM is a powerful technique for isolating pure populations of cells from paraffin-embedded tissue sections and that these cells can be used to study genomic alterations at the DNA level.

Materials and Methods

Tissue samples. Formalin-fixed paraffin-embedded breast biopsies were obtained from the Department of Anatomic Pathology at Sunnybrook and Women’s College Health Sciences Centre, Canada. Five-micron sections were cut from the paraffin blocks and mounted on glass slides. The sections were prepared with a standard protocol for hematoxylin and eosin (H and E) staining. Areas of normal breast ducts, ductal carcinoma in situ, and invasive ductal carcinoma were identified from coverslipped slides, and matched against unstained/uncoverslipped serially-sectioned slides. The unstained slides were then used for LCM.

Laser capture microdissection. The Pixcell IIe Laser Capture Microdissection system was used for microdissection. A low-power infrared laser was used to melt 30 μm pulse sizes of special thermoplastic film over the cells of interest. The optimal settings for power and duration for capturing cells ranged between 21-33 mW and 6-9 milliseconds, respectively. In denser tissue, the cells were harder to lift and the same area was pulsed more than three times and the duration setting was increased to over 10 milliseconds. After microdissection, the cap was first placed on the special Capsure clean up pad to detach loosely attached cells that had been inadvertently removed from the tissue. Next, the cap containing the cells of interest was inserted into a 0.5 mL GeneAmp PCR reaction tube with an Arcturus cap insertion tool to retrieve cells for DNA isolation.

DNA extraction. Each GeneAmp reaction tube was filled with 100 μL of DNA digestion buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 1% Tween 20) and 10 μL of proteinase-K
Once the cap had been inserted into the reaction tube, the tube was inverted to facilitate contact between the buffer and the cells on the cap. The reaction tubes were then incubated at 65°C for 16 to 24 hours. After incubation, the tubes were centrifuged for 1 minute at 1,000 xg. The cap was removed from the tube and the solution was heated to 95°C for 10 minutes to inactivate proteinase-K. To ensure the purity of the extracted DNA, phenol extraction followed by ethanol precipitation was carried out.

**Genomic DNA analysis by PCR.** The purified genomic DNA was used to amplify the HER2/neu gene specific exon 3 fragment by 2 rounds of PCR. The first pair of HER2/neu primers had the following sequence: forward - ata tcc agg agg tgc agg g, reverse: ctt cga agc tgc agc tcc c. The second pair of primers (nested) had the following sequence: forward - ctc aca acc aag tga ggc ag, reverse - cag ggg tgg tat tct tca. The annealing temperatures were calculated based on the lowest melting temperature for the pair of primers. The purified genomic DNA was amplified by PCR with the following conditions: initial denaturing at 96°C for 10 minutes, 35 cycles of denaturing at 96°C, annealing at 58°C, and elongation at 72°C. The PCR product was then analyzed by gel electrophoresis. A nested PCR technique was utilized to increase the yield of amplified DNA. From the first PCR amplification, 4 µL of the product was used as a template for the second PCR reaction. During the first round of PCR, the annealing temperature was adjusted to 53°C and then to 58°C for the second PCR. Nested PCR enabled visualization of the DNA by gel electrophoresis. As expected, the first pair of primers amplified a 205-bp fragment, while the nested primers produced a fragment of 126 bp.

**DNA sequencing.** PCR products were separated on a 1% agarose gel, excised and cleaned with a Gel Purification Kit from Qiagen. Approximately 4 ng of purified HER2/neu product (~ 126 bp) was subject to sequencing using the Big Dye version 3.0 kit with 0.8 µM forward exon 3 primer, POP4 polymer and an ABI PRISM 3100 V 3.7 automated sequencer (ABI, Foster City, CA, USA). The data were analyzed with ABI SeqScape v 2.0 software.

**Immunohistochemistry.** Breast cancer tissue sections were dewaxed and antigen retrieval performed in citrate buffer pH 6.0 for 20 minutes in the microwave (9). The monoclonal anti-HER2/neu antibody (CB11) (Novacastra, Newcastle, UK) was diluted and used according to the manufacturer’s instructions. Positive reactions were developed using the Envision plus kit (DAKO) and diaminobenzidine as substrate, then counter-stained with hematoxylin, dehydrated and cleared (Figure 1). The results were evaluated microscopically. Strong complete membrane staining was considered positive for HER2/neu oncoprotein overexpression (9).

**Results and Discussion**

**Tumour samples and microdissection.** Serially sectioned breast tumors from 9 cases: 2 pure DCIS, 2 containing a mixture DCIS and IDC, 4 pure IDC, and 1 invasive lobular carcinoma (ILC) were studied (Table I). All 9 breast tumor samples were microdissected using the Pixcell IIe Laser Capture Microdissection system and the cells were captured onto a Capsure Macro LCM cap, as described in Material and Methods. Depending on the tissue quality, a selected area was pulsed 3 times for 6-10 milliseconds each time (Figure 2). When the tissue was completely dry, the cells of interest were easily lifted, and little or no contamination from neighbouring normal cells was evident.

**HER2/neu PCR amplification and characterization of microdissected tissue samples.** To investigate the lower limit of DNA that could be observed by our PCR protocol, initially we amplified the HER2/neu gene using a serially-diluted cancer cell line DNA. PCR amplification was examined by gel electrophoresis and it was determined that 1.725 ng of genomic DNA was sufficient to generate a clear HER2/neu band. To analyze gene amplification in primary breast tissues, DNA was isolated from pure cell populations of normal tissue, DCIS and IDC (Materials and Methods). It was estimated that each microdissected sample collected on the Capsure Macro LCM cap had approximately 150 cells. Previously, it has been estimated that 1 cell = ~ 10 pg of DNA (8). The HER2/neu gene was amplified by PCR using forward and reverse primers (Materials and Methods). Since it was not possible to visualize clear bands during the first PCR, a second round of PCR was preformed using nested primers to re-amplify the HER2/neu fragment. By performing 2
Figure 2. Ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) microdissected tissue samples. Both images, magnified at 200X, showed H- and E-stained sections of a high grade DCIS (a) and IDC (c): a) The DCIS was composed of a nest of tumour cells confined to the duct. b) The tissue area of interest was lifted by the LCM technique; gaps represent the foci of tumor cells that were removed. c) This IDC was composed of nests of tumour cells. d) The area of interest was lifted by the LCM technique; gaps represent the foci of tumor cells that were removed.

Figure 3. Detection of HER2/neu gene overexpression by nested PCR. Primers were derived from exon 3 of the HER2/neu gene. The arrow indicates the size of the HER2/neu fragment at 126 bp. Lane 1, the New England Biolabs 100 bp ladder. Lane 2, positive control from the cancer cell line, HT29. Lane 3, PCR product of high grade DCIS from DCIS/IDC sample #3 (Table I). Lane 4, PCR product of IDC from DCIS/IDC sample #3. Lane 5, PCR product of IDC sample #5 (Table I). Lanes 6 and 7, microdissected tissue of tumour samples # 7 and # 8 (Table I) with no HER2/neu amplification. Lane 8, negative control verifying no primer contamination.
rounds of PCR, a HER2/neu specific band was successfully produced. From the 9 cases microdissected, 2 showed amplification of HER2/neu by PCR (Figure 3). When the HER2/neu gene was amplified it corresponded to the 34.5 ng band in the serial dilution experiment. HER2/neu amplification was observed in both the DCIS and IDC portion of case number 3 and in case number 4 that was pure IDC (Table I). However, normal breast ducts were negative for HER2/neu amplification (data not shown). One of the HER2/neu bands from PCR analysis was purified and subjected to DNA sequencing. The BLAST analysis verified the identity of the amplified fragment (Figure 4).

Comparison of HER2/neu status by PCR and IHC. In this study, an attempt was made to eliminate the drawbacks of PCR detection of HER2/neu by utilizing the LCM technique. Of 9 cases studied, the 2 cases that showed overexpression of HER2/neu by IHC also showed amplification by PCR. In addition, all cases that were negative by IHC were also shown to be negative by PCR, giving a 100% correlation of results between the 2 techniques. Prior to this, the highest concordance between IHC and PCR had been 94% (10). Overexpression by IHC was detected in 24.3% of the cases studied, whereas PCR was only able to find amplification in 9.2% of the cases.

Table I. Pathological data on breast cancer cases for microdissection.

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumour type</th>
<th>Overexpression of HER2/neu by IHC</th>
<th>Amplification of HER2/neu by LCM/PCR</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>DCIS*</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>DCIS</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>IDC*/DCIS</td>
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<td>Positive</td>
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<tr>
<td>4</td>
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<tr>
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<td>8</td>
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</tr>
<tr>
<td>9</td>
<td>ILC***</td>
<td>Negative</td>
<td>Negative</td>
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*ductal carcinoma in situ
**invasive ductal carcinoma
***invasive lobular carcinoma

Figure 4. DNA sequence and BLAST analysis. (A) HER2/neu amplified DNA from IDC case # 5 was sequenced using the ABI DNA sequencing kit by the ABI 3100 Genetic Analyzer. (B) Blast analysis of the DNA sequenced in panel A.
studied (11). However, the poor results of the PCR method are usually attributed to the presence of tissue heterogeneity (11). Both PCR and IHC are reliable and rapid techniques used for the detection of amplification or the detection of overexpression of HER2/neu (12, 13). This illustrates that, by utilizing the LCM method, the PCR technique can be substantially improved. Also, this study suggested that analysis by PCR using microdissected tumour samples can provide the same level of accuracy and precision as IHC. In addition, analysis of HER2/neu by PCR has its benefits in being able to isolate the pure DNA for further analysis. Our study illustrated that the LCM technique can be successfully used to provide ample DNA for gene analysis by PCR. Our results indicated that 22% of the cases studied were positive for HER2/neu amplification, corresponding to the literature (14).

Future applications. The validity of genomic and proteomic techniques relies heavily on the preparation of homogeneous cell populations. Cells isolated from complex tissue by LCM have been successfully studied by various analytical techniques including cDNA microarrays and Q-PCR, to compare gene expression profiles between various cell types within a tissue (2). Complementary to these DNA and RNA analyses, mass spectrometric sequencing, peptide mass fingerprinting, or Western blot have also been used to identify proteins of interest. Newly developed protein chips may also prove useful for protein analysis of microdissected tissue and can provide important information not accessible through nucleic acid-based techniques, including post-translational modification, protein-protein interaction and protein-DNA interaction.

Previous studies utilizing LCM and PCR have not considered the importance of exploring the status of HER2/neu at specific stages in breast cancer. Using LCM in combination with sensitive molecular methods, we can further explore previously unanswerable questions about the development and progression of breast cancer.

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References


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