Detection of Breast Cancer Micrometastases in Axillary Lymph Nodes by Using Polymerase Chain Reaction

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ABSTRACT

Breast cancer micrometastases in axillary lymph nodes have been detected by serial sectioning and immunohistochemistry, and shown to have prognostic significance. We have used polymerase chain reaction (PCR) to see whether we could further improve the detection rate of micrometastases. Fifty-seven axillary lymph nodes from patients with breast cancer were examined histologically to assess the proportion of tumor involvement. Immunohistochemical staining with the use of an anti-keratin 19 antibody confirmed the histological findings. Reverse transcription PCR was then performed on extracted RNA by using K19 primers, and 18 histologically involved nodes yielded the expected 460-base pair product. Of 39 histologically negative nodes, 4 (10%) gave K19 bands detectable with ethidium staining and a further 10 (26%) gave K19 bands after Southern hybridization. To further increase the detection sensitivity a two stage amplification was performed by using nested primers, and K19 product was found in lymph nodes from patients without cancer, as well as in all the nodes from cancer patients. This was shown to be genuine low level expression from endogenous mRNA template, and not derived from amplification of a K19 pseudogene. Reducing the number of PCR cycles in the two amplification steps did not allow sufficient discrimination between normal nodes and those involved in which K19 expression was only detectable after Southern hybridization. The optimal "cut-off" point to distinguish involved nodes from normal nodes remained at the level of 40 cycles of PCR and Southern hybridization. PCR, using K19 as a tumor marker, has been demonstrated in this study to improve the detection of micrometastases in axillary lymph nodes in patients with breast cancer: sensitivity is limited by the specificity of the tumor marker.

INTRODUCTION

Following definitive local treatment and ipsilateral axillary lymph node dissection, more than 50% of patients with breast cancer have no evidence of residual or metastatic disease on conventional staging. However, approximately 30% of these women will relapse with distant metastatic disease within 5 years. Identifying micrometastatic disease may enable a better assessment of the prognosis of women with breast cancer, and define those women who will benefit from adjuvant chemotherapy.

Axillary node micrometastases in patients with breast cancer have been detected in several studies by serial sectioning (1—6) and immunohistochemistry (7—12). From an aggregate total of 2400 patients, approximately 13% converted from node negative to node positive, slightly more by using immunohistology (13). The presence of micrometastases correlates with conventional prognostic factors such as the size of the tumor and peritumoral vascular invasion (3, 7). When compared to node-negative disease, the presence of even a single micrometastasis in a lymph node is associated with a significant difference in recurrence and survival (12). Furthermore, perioperative chemotherapy for this group of patients gives an added disease-free survival advantage (7).

However, the number of patients converting from node negative to node positive is much less than the 25—30% who will recur within 5 years of surgery. Clearly, a more sensitive technique than either serial sectioning or immunohistochemistry is desirable.

Detection of specific gene sequences with the use of PCR2 would be a more sensitive technique than either serial sectioning or immunohistochemistry and may be expected to further increase the detection rate of micrometastases. Mattano et al., (14) showed that rare circulating neuroblastoma cells could be detected in peripheral blood to a level of 1 in 107 using PCR amplification, which was 100-fold more sensitive than immunocytoLOGY, while retaining specificity.

In this study, we show that an increased rate of detection of tumor involvement within an excised node can be achieved by measurement of a differentially expressed gene transcript. Using an RT-PCR amplification method, several target genes have been analyzed and K19 was found to be the most suitable to distinguish between involved and uninvolved lymph node tissue.

MATERIALS AND METHODS

Tissue. Lymph node specimens were obtained following axillary dissections of patients undergoing surgery for breast cancer at Charing Cross Hospital, London. Fifty-seven separate lymph nodes were collected from 18 patients. The mean age was 52.7 years (range, 24—74 years); 12 patients were postmenopausal and 6 were premenopausal. Fourteen patients had invasive ductal carcinoma, 3 had invasive lobular carcinoma, and 1 had tubular carcinoma. Special care was taken to exclude skin from all biopsies. Each lymph node was bisected and one-half was snap frozen in liquid nitrogen and stored at −70°C before extracting RNA, the rest being formalin fixed and paraffin embedded for histology and immunohistochemistry. For routine histology, a single 5-μm section was cut from each node, stained with hematoxylin and eosin, and carefully examined by one specialist breast histopathologist (S. S.). With small nodes (<0.5-cm diameter), up to 3 sections were examined. Similarly for immunohistochemistry, a single 5-μm section was cut from each node.

A further 11 lymph node specimens were collected from 11 patients who demonstrated no evidence of an epithelial malignancy, and these were used as controls and were referred to as normal.

Chemicals. MMLV reverse transcriptase was obtained from GIBCO BRL (Paisley, United Kingdom) and Taq polymerase was from Peninsular Laboratories (United Kingdom). Random hexamers and dNTPs were from Pharmacia (Uppsala, Sweden), and [32P]dCTP (3000 Ci/mmol) was from Amersham (United Kingdom). All other reagents were obtained from Sigma (Dorset, United Kingdom) unless indicated.

Oligonucleotide Primers. The primers for K19 were designed from previously published sequences (15) (Table 1), and were selected to maximize mismatches between K19 mRNA and the K19 pseudogene sequences (21). The primers were selected from two different exons with at least one intervening intron (22). To standardize samples and confirm the presence of cDNA template in each sample, a “housekeeping” gene, GAPDH, was also coamplified. The primers were synthesized by the Advanced Biotechnology Centre (Charing Cross and Westminster Medical School, London, United Kingdom) on an Applied Biosystems DNA Synthesizer.

RNA Isolation. Total cellular RNA was extracted from frozen lymph nodes by using the acid guanidium-phenol-chloroform technique (23) utilizing...
were added and the mixture was incubated at 37°C for 1 h. The enzyme was heat inactivated and the RNA was ethanol precipitated following phenol (Boehringer Mannheim). To a total volume of 100 μl containing 12 μg RNA, of the extracted RNA preparation was treated with RNase-free DNase 1 and samples were diluted to 0.3 μg/ml in water and stored at −70°C. RNA was checked electrophoretically and quantified spectrophotometrically, RNAzoI (Biogenesis, Bournemouth, United Kingdom). The integrity of the RNA was checked electrophoretically and quantified spectrophotometrically, and samples were diluted to 0.3 μg/ml in water and stored at −70°C.

DNase Treatment of RNA. To remove any contaminating DNA, a sample of the extracted RNA preparation was treated with RNase-free DNase 1 (Boehringer Mannheim). To a total volume of 100 μl containing 12 μg RNA, 10 mM MgCl₂, 0.1 mM dithiothreitol, 50 mM Tris-Cl, 10 units of enzyme were added and the mixture was incubated at 37°C for 1 h. The enzyme was heat inactivated and the RNA was ethanol precipitated following phenol chloroform extraction and was resuspended in water.

Reverse Transcription. First-strand cDNA was synthesized by using MMLV reverse transcriptase. RNA (4 μg in 12 μl water) was added to 1 μl of enzyme (200 units), 4 μl 5X reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KC1, 15 mM MgCl₂), 1 μl dNTP (20 mM concentrations each of dATP, dCTP, dGTP, and dTTP), 1 μl dithiothreitol, and 1 μl random hexamers (250 ng), to form a total reaction volume of 20 μl. Following incubation at 42°C for 1 h, the mixture was heated to 95°C, snap cooled, and stored at −20°C. With this experiment an additional tube which contained all the reagents except the enzyme was always included as a blank control.

Polymerase Chain Reaction. Specific cDNA sequences were amplified (24) in a reaction mix (100 μl) composed of 1–4 μl cDNA (equivalent to 100–400 ng RNA), 2 units of Taq polymerase, 2 mM MgCl₂, 200 μM dNTP, 200 ng of each of the 5‘ and 3‘ sequence-specific primers and buffer containing, in final concentrations, 67 mM Tris-HCl, pH 8.8 (at 25°C), 16.6 mM (NH₄)SO₄, 0.45% Triton X-100, 200 μM gelatin, and overlaid with 50 μl of mineral oil.

In the first round of PCR for K19, 40 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min with an extra 10-min extension for the last cycle. The second round of PCR using the nested K19 primers had identical reaction parameters except that the annealing temperature was 45°C.

Restriction Enzyme Analysis. To verify identity of PCR products obtained with the K19 primers, a sample (50 μl) was purified by using the Magic DNA clean-up system (Promega) and digested with 36 units HaeII (Boehringer Mannheim) in buffer containing Tris acetic (33 mM), potassium acetate (66 mM), magnesium acetate (10 mM), and dithiothreitol (0.5 mM, pH 7.9), and incubated at 37°C for 16–20 h. An aliquot was electrophoresed on 1.5% agarose alongside size marker HaeII-digested I X174 or the Cambio DNA ladder, and transferred onto HyBond N+ membrane (Amersham, United Kingdom) with a solution (60 μl/cm²) containing 50% (v/v) formamide, 0.1% SDS, 5X Denhardt’s solution (0.1% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 5 mM EDTA, 75 mM NaCl, 250 μg/ml denatured sonicated salmon sperm DNA, and incubated at 42°C for 4–6 h. After this time, the relevant probe (either plasmid or PCR product) labeled with [³²P]dCTP (to specific activities between 5 × 10⁶ and 5 × 10⁷ cpm/μg DNA), using the random primer method (25), was added and hybridization was continued for a further 16–20 h. Filters were subsequently washed in 2X standard saline citrate, 0.5% SDS for 15 min at 42°C with four changes of buffer, then in 0.1X standard saline citrate, 0.5% SDS for 15 min at 65°C with two changes of buffer, and exposed to Amersham hyperfilm at −70°C, with intensifying screens for periods between 1 h and several days.

Immunohistochemistry. Sections (5 μm) of formalin-fixed, paraffin-embedded lymph nodes were dewaxed in xylene and alcohol. They were placed in a 30% hydrogen peroxide-10% methanol solution to block endogenous peroxidase, and washed with TBS. Each slide was then covered with 10% normal horse serum in TBS which was removed before incubation with the primary mouse anti-keratin 19 antibody RCK108 (Dako, Ltd.) for 60 min at room temperature. After washing with TBS, a biotinylated second antibody (horse anti-mouse monoclonal) with added horse serum was applied and incubated for 60 min at room temperature. The avidin-biotin complex was added and then removed with TBS, before freshly prepared diaminobenzidine tetrahydrochloride solution was added for 10 min. The slides were then washed for 1 min in distilled water and sections were counterstained with Mayer's hematoxylin.

RESULTS

Of the 57 nodes examined from patients with breast cancer, 18 were found to have evidence of tumor involvement by histological criteria. The extent of tumor involvement in each node was estimated and ranged between 1 and 95% of the node. In the remaining 39 nodes there was no histological evidence of tumor. Cytokeratin 19 immunopositivity was present in epithelial tumor cells in the involved nodes (Fig. 1), but no reactivity was detectable in any of the histologically uninvolved nodes.

RNA was extracted from normal nodes and subjected to RT-PCR amplification using primers (Table 1) from several candidate genes which are known to be expressed in breast cells. These included heat shock protein 89, estrogen receptor, p52, epithelial membrane antigen, and K19. After 40 cycles of amplification, product was seen for all of the genes except K19 (data not shown). The rest of the study was therefore limited to analysis of K19 expression for the cancer samples.

RT-PCR, using K19 and GAPDH primers, was then performed on nodes from breast cancer patients. All 18 histologically involved nodes showed the expected 460-base pair product on gel electrophoresis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’-3’</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratin 19 (outer)</td>
<td>GAGGTGATACCCTGGTGGCTGAG</td>
<td>15</td>
</tr>
<tr>
<td>Keratin 19 (inner)</td>
<td>CTGCTCTGGGAAGGAAAGAT</td>
<td>15</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCCATCCAACACCTCTCCA</td>
<td>16</td>
</tr>
<tr>
<td>Epithelial membrane</td>
<td>GAGGTGGCCTGCTCACCTCTCAA</td>
<td>17</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>CCGAGCACTGATGGCTGCAA</td>
<td>19</td>
</tr>
<tr>
<td>p52</td>
<td>CAGGCAGATGTCGAAATGAC</td>
<td>19</td>
</tr>
<tr>
<td>Heat shock protein 89</td>
<td>CCTACACCTGTAAGAAGAC</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 1 Primer sequences

...
sis as well as the 379-base pair GAPDH product. Examples are shown in Fig. 2. In addition, of the 39 histologically negative nodes, 4 (10%) gave a K19 product detectable with ethidium staining after 40 cycles of PCR (Fig. 3). On Southern blotting and hybridization with 32P-labeled K19 cDNA, a further 10 (28%) gave positive signals. Examples are shown in Fig. 4. All 11 normal nodes, from patients without cancer, showed no evidence of K19-amplified products by ethidium staining (Fig. 5a) or Southern hybridization (Fig. 5b). Coamplification of the GAPDH gene did, however, give the expected product confirming the presence of amplifiable cDNA. These results are summarized in Table 2.

To further increase the sensitivity of this method, the PCR products were reamplified in a second stage PCR with the use of a pair of “nested” K19 primers. Under these conditions, all 11 normal lymph

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Total no. of lymph nodes</th>
<th>No. of nodes with K19 band by EB* staining</th>
<th>No. of nodes K19 positive by Southern hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologically positive</td>
<td>18</td>
<td>18</td>
<td>--</td>
</tr>
<tr>
<td>Histologically negative</td>
<td>39</td>
<td>4 (10%)</td>
<td>10 (28%)</td>
</tr>
</tbody>
</table>

* EB, ethidium bromide.

b Excludes those positive by ethidium staining.

Fig. 5. RNA was extracted from normal lymph nodes, resected from patients without cancer, was reverse transcribed and cDNA amplified by 40 cycles of PCR by using K19 and GAPDH primers. a, PCR products were electrophoresed in a 1% agarose gel and ethidium-stained bands corresponding to the 379-base pair GAPDH were seen in all eight samples, but K19 product was seen in only the histologically positive control (Lane 11). Lane 9 shows the blank control without RNA template, Lane M shows the Cambio DNA markers, and Lane 10 shows the PCR product, diluted 1:100, from a histologically involved node. b, autoradiograph of a Southern blot of the same PCR products hybridized with a mixture of 32P-labeled K19 and GAPDH cDNA. The lower band corresponds to GAPDH and the upper band to K19. The extra band in Lane 7 is probably derived from contaminating DNA in the RNA preparation.

Fig. 4. Autoradiograph of Southern blot showing hybridization of 32P-labeled K19 cDNA to 40-cycle PCR products obtained by amplification of RT product derived from nine histologically uninvolved nodes. A positive K19 signal (corresponding to a 462-base pair product) is seen in four samples (Lanes 1, 3, 4, and 7). Lane 10 is a blank control without RNA template and Lane 11 is PCR product from a histologically involved node diluted 1:100 to act as a positive control.
cycles for the normal node, a band was seen after 25 cycles for all the other nodes. At higher cycle numbers the difference was also quite marked. However, the difference in the number of cycles required before the K19 band appeared on ethidium staining was considered insufficient to assume a cut-off point between normal and involved nodes.

**DISCUSSION**

The axillary lymph nodes are routinely dissected in the surgical treatment of early breast cancer. Lymph nodes are the first site of arrest of tumor cells that have invaded the peritumoral lymphatics and histological involvement of the lymph nodes is the most important prognostic factor. The most accurate staging for early breast cancer therefore may be the assessment of axillary lymph nodes by using the most sensitive available technique.

The use of PCR relies on the amplification of unique or overexpressed genes in tumor cells, for example the bcr/abl fragment from the chromosomal translocation in chronic myeloid leukaemia (26), or prostate specific antigen in prostate cancer (27). No similarly specific tumor marker has been identified in breast cancer cells. We therefore initially examined several normal lymph nodes for a number of genes commonly expressed in breast tissue or which might be expected to display some specificity for epithelial cells (epithelial membrane antigen, estrogen receptor, pS2, and heat shock protein 89). However 40 cycles of PCR resulted in the appearance of product for all these genes from the RNA of normal nodes. This was interesting as immunohistochemistry using anti-EMA antibodies has been used in the detection of micrometastases in bone marrow aspirates (28, 29). Presumably the low level of mRNA expression in uninvolved host tissue is insufficient to produce enough protein to be detectable by immunohistochemical measurement, but invalidates measurements using PCR.

Cytokeratin 19 appeared to be a better discriminant: it is found in most benign and malignant breast tissue (30, 31) but has not been reported in normal lymph nodes, peripheral blood, or bone marrow with the use of PCR (32–34). In our experiments, there was no product for K19 from normal nodes after 40 cycles of amplification, but all histologically involved nodes displayed K19 expression. In addition, K19 mRNA was detected in a significant proportion of histologically uninvolved and immunohistochemically K19-negative nodes. Ethidium staining of electrophoresed PCR product was sufficient to detect 10% more positives in this group and the increased sensitivity afforded by Southern hybridization enabled detection of K19 in a further 28%. As none of the normal nodes from patients without cancer demonstrated K19 expression, and the negative control in each experiment, a sample without mRNA template, precluded extrinsic contamination, it is reasonable to suppose that those nodes which were considered histologically and immunohistochemically negative, and yet demonstrated a K19 product after 40 cycles of PCR, contained tumor cells.

The second-stage amplification using nested primers was designed to increase the detection sensitivity, but resulted in the appearance of K19 product in the normal nodes, as well as all the nodes from cancer patients. The presence of DNA contamination in some of the RNA samples was evident at this level of amplification. Contaminating DNA in the RNA preparations could be removed by DNase digestion but this treatment step also reduced the subsequent recovery of RNA. This step might therefore limit the initial sample size required for PCR analysis, and hamper analysis of very small lymph nodes or tissue sections. However, as the K19 primers extend across at least one intron, the DNA-derived product was easily distinguished from that expected from amplification of mRNA, and therefore DNA contamination does not pose a significant problem.

![Fig. 6. RNA was extracted from 11 normal nodes from patients without cancer, reverse transcribed, and amplified by 40 cycles of PCR with the use of K19 primers. One μl of this product was reamplified with nested K19 primers for a further 40 cycles and products were electrophoresed in a 1% agarose gel. Genomic DNA (Lane 12) was similarly amplified. Lane M shows Cambio DNA ladder markers. bp, base pairs.](image1)

![Fig. 7. PCR products obtained by two sequential rounds of 40-cycle amplifications with outer and nested K19 primers on RT product from two normal nodes (Lanes 1–6) and one histologically involved node (Lanes 7 and 8). Products were electrophoresed before (Lanes 1, 3, 5, and 7) and after (Lanes 2, 4, 6, and 8) digestion with HaeII. The sample in Lanes 5 and 6 was derived from the same node as for Lanes 3 and 4 except that the RNA had been treated with DNase. Lane M shows the Cambio DNA ladder marker. Fig. 7 shows that the expected band at 319 base pairs (bp) was a result of amplification of endogenous RNA, and not from contaminating DNA pseudogene by HaeII digestion of the 319-base pair product into two fragments of 237 and 81 base pairs in size. DNA contamination (600-base pair band) of the RNA sample can be removed by treatment with DNase 1 and this band is easily distinguished by size from the expected 319-base pair band.](image2)

![Fig. 8. RT products from a normal node (Lanes 1–5) and from three histologically uninvolved nodes (previously shown to be K19 positive only after Southern hybridization of 40-cycle first-round PCR products) (Lanes 6–20) were amplified by 20 cycles of PCR, using the outer K19 primers. One μl of these products was then reamplified by using the nested K19 primers for 20, 25, 30, 35, and 40 cycles, and aliquots of these products were electrophoresed in a 1% agarose gel. The lowest band (arrow) corresponds to the 319-base pair K19 nested product. The higher bands present in two of the samples are derived from contaminating DNA present in those RNA preparations. Lane 21 is a blank control with no RNA template and Lane M shows the Cambio DNA ladder markers.](image3)
There was also the expected K19 product appearing in normal nodes after two amplification rounds. It was necessary to determine whether the source of this PCR product was a K19 pseudogene present in contaminating DNA or due to low level endogenous mRNA expression. Although primers were chosen to maximize the mismatches between K19 and the pseudogene, they share a very high degree of sequence homology (82%) (16) and therefore it was possible that the pseudogene was amplified. That this was genuine K19 expression from an RNA template, albeit at a very low level, was confirmed by use of an endonuclease that is known to have a restriction site in the RNA sequence but not in the K19 pseudogene.

By reducing the number of cycles in the second-stage amplification, it was hoped that the nodes shown to be involved by PCR might be distinguished from normal nodes on gel electrophoresis alone, without the need for blotting and hybridization. However, there did not appear to be a sufficient difference in the number of cycles of PCR before normal nodes were shown to express the nested K19 product. This was not unexpected, as the exponential increase in PCR product with each cycle will obscure even considerable differences in the starting quantities of K19 mRNA. The cut-off point then at which K19 expression may be taken to indicate lymph node micrometastases, was achieved with 40 cycles of PCR and Southern hybridization.

Thus, the potential of PCR in the staging of breast cancer is limited by the specificity of the tumor marker. However, we have shown that PCR, using K19, is more sensitive than immunohistochemistry. These two techniques may complement each other: immunohistochemistry allows examination of the morphology of detected cells, thereby differentiating contaminating epithelial cells or heterotopic tissue in lymph nodes from metastatic breast cancer cells. Additionally, the number of micrometastatic cells has been suggested to be an independent predictor of early relapse in bone marrow (35).

PCR, using K19 as a tumor marker, has been demonstrated in this pilot study to improve the detection of micrometastases in axillary lymph nodes in patients with breast cancer. As K19 is a tissue-specific marker, there may be a broader application in the staging of other epithelial tumors. However, further studies are required to establish the prognostic significance of these micrometastases.

In this regard, we are currently extending our patient data base but it will be several years before the prognostic data become available. Clearly the method of analysis used in this study would, in its present form, be unsuitable for routine assessment, and we are therefore investigating two special techniques: reevaluation with longer follow up. Br. J. Cancer, 66: 523—527, 1992.


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