RESEARCH COMMUNICATION

# Combining Mammaglobin and Carcinoembryonic mRNA Markers for Early Detection of Micrometastases from Breast Cancers - a Molecular Study of 59 Patients

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## Abstract

**Introduction: As many as 30% of node-negative breast cancer patients relapse within five years, suggesting that current histological detection methods are inadequate for identifying metastatic disease. Detecting small number of cancer cells in the breast tissue or lymph node by reverse transcription-polymerase chain reaction (RT-PCR) assays using a combination of tissue and cancer specific markers might be very useful in the early detection or monitoring of the treatment. Mammaglobin is a member of the uteroglobin gene family and appears to be expressed only in breast tissue. Carcinoembryonic antigen has been the preferred molecular marker for detection of micro metastases in lymph nodes in almost all carcinomas. Materials and Methods: Samples were collected from randomly chosen breast cancer patients undergoing modified mastectomy or breast conserving surgery between September 2003 and July 2004. RT-PCR was applied to study the expression of MMG and CEA markers. Breast cancer micrometastases in axillary lymph nodes were also assessed. Results: The MMG marker was positive in 9/10 normal breast tissues, 3/3 breast fibroadenomas and 37/39 of breast carcinoma tissues, giving an overall sensitivity of 94%. The sensitivity was 80% for metastatic lymph node samples. On the other hand CEA showed 95% sensitivity for malignant breast tumors and 100% sensitivity for metastatic lymph nodes. Conclusions: RT-PCR using a combination of MMG and CEA markers is a powerful tool to complement current routine histopathology techniques for detection of breast cancer metastasis in axillary nodes.**

**Key Words**: Breast cancer - mammaglobin (MMG) - carcinoembryonic antigen (CEA) genes - RT-PCR

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## Introduction

Breast cancer is one of the most common cancers in women, and is rapidly increasing in incidence and death rate (Sloane et al., 1980; Branagan et al., 2002). Detecting small number of cancer cells in the breast tissue or lymph node might be very useful in the early detection or monitoring of the treatment (Sloane et al., 1980; Yeu-Tsu, 1994) As many as 30% of patients with breast cancer who have undergone curative surgery and show no evidence of locoregional or distant disease still have recurrent disease over 5-10 years (Hellman, 1994; International (Ludwig) Breast Cancer Study Group, 1990). Some of these treatment failures may be attributed to residual disease in the breast or axillary lymph nodes (International (Ludwig) Breast Cancer Study Group, 1990) that was not detected by routine histopathologic examination (Aihara et al., 1999).

Reverse transcription-polymerase chain reaction (RT-PCR) assays using tissue or cancer-specific gene transcripts (tumor markers) have been reported to constitute a sensitive method for detection of breast cancer dissemination in axillary lymph nodes (Duffy, 2001). Since most tumor markers in clinical practice lack organ specificity (Watson et al., 1994) combining several markers may be more promising for improving the incidence of cancer detection (Duffy, 2001). Mammaglobin is a member of the uteroglobin gene family, which is localized on human chromosome 11q13 and appears to be expressed only in breast tissue (Jnku et al, 2004; Marchetti et al., 2001). Mammoglobin messenger RNA expression has been reported in 70% to 95% of primary

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and metastatic breast tumor biopsies (Leygue et al., 1999; Masuda et al., 2000). Carcinoembryonic antigen has been the preferred molecular marker for detection of micrometastases in lymph nodes in almost all carcinomas. However, some evidence suggests that false positives may arise in some situations (Masuda et al., 2000). It has been shown that both of MMG and CEA are appropriate tumor markers, consistently expressed in tumor but not normal lymph nodes (Marchetti et al., 2001). In the present study, we applied a combination of mammaglobin (MMG) and carcinoembryonic antigen (CEA) genes for the detection of breast cancer micrometastases in axillary lymph nodes.

## Materials and Methods

*Patients and sample collection*

Samples were collected from 59 randomly chosen breast cancer patients undergone modified mastectomy or breast conserving surgery between September 2003 and July 2004. They included 52 breast tumor samples (3 fibroadenoma, 10 nonmalignant breast tissue, 39 breast carcinoma) and 13 axillary lymph node samples (5 metastatic, 8 non-metastatic). One half of each axillary lymph node was formalin fixed and embedded for routine histological examination by hematoxylin and eosin (H&E) staining. The other half of the lymph nodes and malignant and nonmalignant breast tissues were stored at 4˚C for a short period (usually between 12-24h) until RNA extraction.

*RNA extraction*

Total RNA was extracted with a single-step method (Greenberg et al., 2003) from surgical and pathological specimens using TriPure Isolation Reagent (Roche Applied) according to the manufacture's protocol. Fresh tissue specimens were cut into small pieces with clean surgical knives to avoid RNA degradation.

*Multiplex RT-PCR assay*

Complementary DNA (cDNA) was generated with moloney murine leukemia virus reverse transcriptase (Invitrogen life technologies) according to the manufacture's protocol. Briefly, 1µg of RNA was incubated at 65˚C for 5 min and then put on ice for at least 2 min before the addition of RT reaction reagents with Oligo-(dT) 18 (0.5¬µg/¬µl) priming. The RT reaction was performed at 37˚C for 5 min and 42˚C for 55 min, followed by heating at 70˚C for 10 min and 4˚C for 2 min.

Multiplex PCR was performed in a 50µl reaction mixture containing 5µl cDNA template, 1X Biotools PCR buffer, 1.5 mM $MgCl_2$, 1mM deoxynucleotide triphosphates,0.2 µM of each primer, and 2 units of Taq DNA polymerase 5u/µl (Biotools DNA polymerase).The PCR condition was initial denaturing at 94˚C for 5min, followed by 35 cycles at 95˚C for 0.5min, 60˚C for 0.5 min, 68˚C for 2.5 min, and for final extension 68˚C for 5 min. The primer sequences used for actin, mammaglobin and carcinomaembryionic antigen cDNA detection are shown in Table 1. The integrity of all RNA samples was verified by amplification of actin mRNA by RT-PCR.

**Table 1. Sequences of Actin, MMG, and CEA Primers**

| Primer | Sequence (5'-3') | Size (bp) |
|---|---|---|
| Actin MP1 | 5'-CTC-TTC-CAG-CCT-TCC-TTC-CT-3 | |
| Actin MP2 | 5'-AGC-ACT-GTG-TTG-GCG-AC-G-3 | 115 |
| | Hellman 1994 | |
| MMG D2 | 5'- GAC-ATA-AGA-AAG -AGA-AGG-TGT GG 3 | |
| MMG D2102 | 5'- CAG-CGG-CTT-CCT-TGA-TCC-TTG 3 | 430 |
| | Branagan et al., 2002 | |
| CEA forward | 5'- GAG-CGA-ACC-TCA-ACC-TCT-CCT-GCC-ACT-3 | |
| CEA reverse | 5'-TGT-AGC-TGT-TGC-AAA-TGC-TTT-AAG-GAA-GAA GC-3' | 366 |
| | Masuda et al., 2000 | |

## Results

*Validation of markers*

Eight breast tissue samples, 3 with malignant findings and 5 with normal histopathology, together with 2 pathologically malignant lymph nodes were used to establish the validity of MMG and CEA markers for the detection of breast tissue and breast cancer metastases. MMG was positive in all breast tissues as well as metastatic lymph nodes. On the other hand CEA was positive in all known malignant tissues and negative in normal ones. Actin which had been used as positive control was positive in all samples. These markers were selected on the basis of their previously reported specificity for normal breast tissues and breast cancer metastases in lymph nodes (International (Ludwig) Breast Cancer Study Group, 1990; Masuda N. et al., 2000).

*Detection of micrometastases: comparison between CEA RT-PCR and MMG RT-PCR*

A total of 1022 tissue samples from 59 breast cancer patients were analyzed using RT-PCR for expression of the validated markers. All patients were females, aged 19 to 88 (mean 47.5). The actin mRNA message was used as positive control in all experiments. Two samples which had negative results for actin were excluded from this study. The distribution of MMG and CEA positivity in nonmalignant breast tissues, fibroadenomas, primary breast cancers and metastatic and nonmetastatic lymph nodes is summarized in Table 2. Of 10 patients with non-malignant breast tissues, 9 had samples with MMG positive results. There was one CEA positive result among samples obtained from histologically non-malignant breast tissues samples. However 1 out of 9 axillary nodes in this patient was histologically positive. Of 39 patients affected with breast carcinoma, 37 were MMG positive. Among MMG positive patients, 2 were CEA negative. On the other hand, 37 patients were CEA positive among which 2 were MMG negative. Thus, all malignant breast tissue samples were either MMG or CEA positive. Five histologically positive and 8 histologically negative node patients were studied. All histologically positive nodes were CEA positive and 4 were MMG positive. In one histologically negative lymph node

**Table 2. MMG, CEA and B-ACTIN mRNAs in Non-malignant Breast Tissues, Fibroadenomas, Primary Breast Cancers and Axillary Lymph Nodes (LN)**

| Tissue/pathology | n | $MMG^+$ n (%) | $CEA^+$ n (%) | $MMG^+/CEA^+$ n (%) |
|---|---|---|---|---|
| Breast/normal | 10 | 9 (90) | 1 (10) | 9 (90) |
| Breast/Fibroadenoma | 3 | 3 (100) | 0 (0) | 3 (100) |
| Breast/carcinoma | 39 | 37 (95) | 37 (95) | 39 (100) |
| LN/metastatic | 5 | 4 (80) | 5 (100) | 5 (100) |
| LN/Non-metastatic | 8 | 2 (25) | 3 (38) | 3 (38) |

CEA was positive. Moreover, in 2 histologically negative lymph nodes, both MMG and CEA were positive. All these 3 patients had breast carcinomas.

## Discussion

To date, several studies have been performed to find appropriate tumor markers for breast cancer (Yeu-Tsu, 1994; Duffy, 2001). In the present study, we obtained a 94% sensitivity for the MMG marker overall and 80% for metastatic lymph nodes. CEA showed a 95% sensitivity for malignant breast tumors and 100% sensitivity for metastatic lymph nodes. Our finding of the absence of MMG marker expression in the 1 of 10 normal tissue samples is in accordance with similar earlier studies (Jnku et al., 2004; Watson et al., 1996). The fact that two out of 39 malignant samples showed no MMG expression may be explained by a lack of expression with advanced malignancies (Min et al., 1998; Masuda et al., 2000).

Our results are furthermore indicative of a higher sensitivity of these molecular markers for detection of micrometastases than standard histopathology. The 10-year disease-free and overall survival rates may be significantly better in patients without micrometastases (Chomczynski et al., 1987). However, the clinical and prognostic significance of our results need to be further investigated in a prospective study on a larger number of breast cancer patients. Obtaining lymph nodes for RT-PCR experiments is difficult due to the ethical concerns regarding use for a research only and therefore clinicians and pathologists sent samples for this part of the study only when they had sufficient tissue for their routine pathological studies.

The present study suggests that molecular detection of MMG and CEA markers by RT-PCR could be a powerful and sensitive complement to routine histopathological analysis. This study also suggests that the application of the two in combination would provide a higher detection rate. This approach needs good cooperation between surgeons, pathologists and molecular laboratories. Further evaluation in larger studies should help establish the clinical utility for breast cancer patient management.

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