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Application of a Multigene RT-PCR assay for the detection of Mammaglobin and complementary transcribed genes in breast cancer lymph nodes

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Abstract

Background: Mammaglobin mRNA expression is found in 70-80 % of primary and metastatic breast tumor biopsies. The potential breast tumor markers B305D, B726P and GABAπ complement the expression of mammaglobin. Collectively the expression profile of these four genes could be utilized as a diagnostic and prognostic indicator for breast cancer.

Methods: A multigene RT-PCR assay was established to detect the expression of mammaglobin, GABAπ, B305D and B726P simultaneously. Specific primers and Taqman® probes were used to analyze combined mRNA expression profiles in primary breast tumors and metastatic lymph node specimens.

Results: The multigene RT-PCR assay detected significant expression signals in 27/27 primary and in 50/50 lymph-node-metastatic breast tumor samples. Specificity studies demonstrated no significant expression signal in 27 non-breast cancer lymph nodes, in 22 various normal tissues or in 14 colon tumor samples.

Conclusion: The novel RT-PCR-based assay described here provides a sensitive detection system for disseminated breast tumor cells in lymph nodes. In addition, this multigene assay could also be used to test peripheral blood and bone marrow samples.

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Abbreviations:

RT-PCR reverse transcription-PCR;

GABAπ γ-aminobutyrate type A receptor π subunit;

CEA carcinoembryonic antigen;

MUC1 mucin1;

NDRI National Disease Research Interchange

FAM 6-carboxy-fluorescein.

ROC receiver operator characteristic

Mammaglobin, a highly glycosylated low molecular weight protein, is a homologue of the rabbit uteroglobin and the rat steroid binding protein subunit C3 (1;2). It is a member of the Uteroglobin family with yet unknown function. The known human family members (mammaglobin, lipophilin A and B, mammaglobin B (lipophilin C, lacryglobin), Clara cell 10kDA protein) are localized in a dense cluster on chromosome 11q12.2 (3). The mRNA expression of mammaglobin has been described in 70-80 % of primary and metastatic breast tumor biopsies (1;4-7). Furthermore, mammaglobin has been used as a marker to detect circulating mammary carcinoma cells in peripheral blood using RT-PCR (8).

Although mammaglobin is a promising tumor marker it is not universally expressed in breast cancers because these tumors are highly heterogeneous by gene expression profiling. Therefore, additional markers are needed for the development of a sensitive assay to detect malignant breast cancer cells. Three genes B305D, GABAπ and B726P have been identified by genetic subtraction, differential display, DNA microarray analysis and real-time PCR, as potential therapeutic and diagnostic targets in breast cancer. Recently we reported the complementary expression profile of these candidate genes in combination with mammaglobin in breast carcinomas (7). B305D is a novel gene that is located on chromosome 21q11.1, c1 region (AP001465.1). B305D cDNA has been isolated by using differential display RT-PCR technique and its sequence predicts a type II membrane protein.

The known gene γ-aminobutyrate type A receptor π subunit (GABAπ) was recovered by PCR substraction and found to be inversely expressed as compared to B305D. GABAπ (U95367) belongs to the $GABA_A$ receptor family. Unlike other $GABA_A$ receptors that are typically expressed in neuronal tissues, GABAπ shows low expression in lung, thymus and prostate tissues and higher levels in the uterus (9). The cDNA designated as B726P (AL357148) was derived from PCR subtraction. B726P is a novel gene located on chromosome 10 with several different putative open reading frames yielded by mRNA splicing. One of these splice forms has been recently identified (10) using reactivity with autologous breast cancer patient sera and referred to as NY-BR-1.

In the present study we established a real-time multigene RT-PCR assay to simultaneously monitor the expression level of these four breast cancer markers. The sensitivity and specificity of this assay have been investigated by using numerous primary breast tumors, metastatic lymph node and normal tissues.

Materials and Methods

TISSUE SAMPLES—Primary breast cancer, normal and tumor lymph node tissue samples were kindly provided by Dr. Elizabeth Repasky (Roswell Park Cancer Institute, Buffalo, NY) and Dr. Roberto Badaro (University of Bahia, Salvador, Brazil). Tissue samples were also obtained from National Disease Research Interchange (NDRI). Lymph node cDNA samples were kindly provided by Dr. Michael Mitas (Medical University of South Carolina, Department of Surgery, Charleston SC) and Dr. Timothy P. Fleming (Washington University School of Medicine, St. Louis, MO).

RNA ISOLATION AND cDNA PREPARATION—Total RNA was extracted from liquid nitrogen frozen tissue samples by homogenization in Trizol reagent (Gibco BRL, Bethesda, MD) and cDNA was prepared using Oligo dT (Boehringer Mannheim) primer with Superscript™ II reverse transcriptase (Gibco BRL, Bethesda, MD) for 60 min at 42 °C.

PCR ASSAY—A multigene real-time PCR assay was developed to detect the expression of mammaglobin, GABAπ, B305D and B726P simultaneously. The expression levels of single genes and their combined expression levels were measured by quantitative real-time PCR using

the ABI 7700 Prism™ sequence detection system (Applied Biosystems, Foster City, CA). Specific primers and 6-carboxy- fluorescein (FAM)-labeled Taqman® probes were used in combination. Primers were designed to span intron-exon junctions in order to exclude genomic DNA from amplification. Primer concentrations, limiting the reporter signal without decreasing the detected threshold cycle to avoid competition in the multigene PCR, were determined.

The forward primer for mammaglobin was 5′- tgccatagatgaattgaaggaatg-3′ and the reverse primer was 5′- tgtcatatattaattgcataaacacctca-3′, both used at 100 nM. The primer sequences for GABAπ were 5′- caattttggtggagaacccg-3′ forward and 5′- gctgtcggaggtatatggtg-3′ reverse, used at 300 nM and 50 nM respectively. For B305D amplification 300 nM of forward 5′- tctgataaaggccgtacaatg-3′ primer and 50 nM of reverse 5′- tcacgacttgctgtttttgctc-3′ primer were used.100 nM each of B726P forward 5′- gcaagtgccaatgatcagagg-3′ and reverse 5′- atatagactcaggtatacacact-3′ primer were added to the PCR mixture. Actin expression was measured in separate reactions and used to normalize expression levels. 300 nM each of forward primer 5′- actggaacggtgaaggtgaca-3′ and reverse primer 5′- cggccacattgtgaactttg-3′ were used for actin amplification. For real-time detection 4 pmol of each gene specific Taqman® probe with following sequences were used:

Mammaglobin FAM-5′- tcttaaccaaacggatgaaactctgagcaatg-3′-TAMRA,

GABAπ FAM-5′- catttcagagagtaacatggactacaca-3′-TAMRA,

B305D FAM-5′- atcaaaaaacaagcatggcctcacaccact-3′-TAMRA,

B726P FAM-5′- tcccatcagaatccaaacaagaggaagatg-3′-TAMRA,

Actin FAM-5′- cagtcggttggagcgagcatccc-3′TAMRA.

40 PCR cycles were performed with TaqMan® 1000 Rxn PCR Core Reagents (Part. No. 430 4439, Applied Biosystems, Foster City, CA) using 0.0375 U/μl TaqGold, 1× Buffer A, 5 mM $MgCl_2$, 0.2 mM dCTP, 0.2 mM dATP, 0.4 mM dUTP, 0.2 mM dGTP, 0.01 U/μl AmpErase UNG, 8 % (v/v) Glycerol, 0.05 % (v/v), Gelatin, 0.01 % (v/v) Tween 20. PCR conditions were one cycle at 50°C for 2 min, one cycle at 95°C for 10 min and for 40 cycles 95°C for 15″ followed by 60°C for 1′ and 68°C for 1′. For single gene PCR standard curves were established using serial dilutions of plasmid DNA containing target gene cDNA sequence. For the multigene assay combined copy numbers were determined by using serial dilutions containing four plasmids to establish a standard curve. TaqMan® SDS analysis software was used to determine standard curves and to calculate copy numbers. Final copy numbers were determined as average values of triplicate PCR reactions and normalized per 1000 pg Actin. The average variation within triplicates was 21 %, ranging from 3 to 35 %.

RECEIVER OPERATOR CHARACTERISTIC (ROC) CURVE ANALYSIS—Rockit 0.9B Beta Version software (Dr. Charles E. Metz, Department of Radiology, The University of Chicago, http://www-radiology.uchicago.edu/krl/toppage11.htm) was used to analyze expression data obtained from pathology positive breast cancer and normal lymph node specimens. Detected copy numbers in lymph node samples for the Mammaglobin, B305D, B726P, GABAπ or the Multigene RT-PCR assay respectively were processed for the 27 actually-negative and the 50 actually- positive cases. The generated output of True-Positive Fractions (TPF) and corresponding False-Positive Fractions (FPF) was used for the construction of ROC curves as sensitivity and 1-specificity.

Results

A panel of 27 primary breast tumor samples was used to compare the multigene real-time PCR assay with single gene PCR. The multigene assay, simultaneously detecting mammaglobin +

B305D + B726P + GABAπ, resulted in a significant expression signal from 27/27 tumor samples (Figure 1B) as compared to mammaglobin expression alone that detected 17 of these samples (Figure 1A). Table 1 further demonstrates the transcriptional complementation of the four target genes with B305D being highly expressed in 16, GABAπ in 10 and B726P in 14 tumor specimens. All samples tested were detected positive with a combined expression signal by the multigene assay.

The expression profile of B305D and GABAπ in the breast tumor samples showed an inverse relationship which is complementary at the level of quantitative detection. This results in a detection of 24/27 primary breast tumor samples with the expression of these two genes alone. B726P expression showed an independent but additive profile in comparison to the other markers.

We then tested the cDNA of 50 pathology positive metastatic breast cancer lymph node samples and 27 normal or non-relevant disease lymph node samples respectively. All 50 metastatic breast cancer samples were detected with positive expression signals (Figure 2) whereas non-breast cancer-containing lymph node specimens showed no significant signals. Table 2 illustrates the complementary expression profiles of the four candidate genes in the 77 lymph node specimens tested.

Significant mammaglobin expression alone is detected in 40 metastatic breast cancer lymph node samples, ranging from 19.7 to 4.1×10E6 copies/1000pg actin. High expression of B305D was found in 36 (5 - 5×10E3 copies/1000pg actin), of GABAπ in 15 (11.7 - 830 copies/1000pg actin) and of B726P in 22 (7 - 4.9×10E4 copies/1000pg actin) out of 50 pathology positive breast cancer lymph node samples. No significant expression signals were detected by mammaglobin, B305D, GABAπ, B726P or multigene RT-PCR in 24 normal lymph node specimens, in 2 nodes containing melanoma, or in one node with evidence of lymphoma.

The multigene real-time PCR assay detected a positive signal for all 50 metastatic breast cancer lymph node specimens, ranging from 17.2 to 1.9×10E4 copies/1000pg actin. Similar to primary tumor samples two genes in particular, B305D and GABAπ, added to the diagnostic sensitivity of mammaglobin detection in lymph node analysis. A combination of these three genes detected 50/50 lymph node breast metastases and 26/27 primary breast tumors evaluated. The inclusion of B726P enabled the detection of one primary tumor specimen. Moreover, the parallel detection of four genes enhanced the detection sensitivity for samples with low level single gene expression.

In order to assess the specificity of the multigene PCR assay described here a panel of 14 colon tumor and 22 normal tissue cDNAs was tested. Expression signals were detected in 0/14 colon tumors and in 4/22 normal tissues (Table 2). However, the low borderline signals detected in four normal tissues (esophagus, skin, trachea and salivary gland) were only ranging from 2 - 6 copies/1000pg actin, presumably due to low levels of mammaglobin expression in skin and of GABAπ and mammaglobin expression in salivary gland, trachea and esophagus. No background expression was detected in bone marrow and peripheral mononuclear cells (PBMC).

To compare the diagnostic performance of single markers and multimarker detection in lymph node analysis, we analyzed the expression data using Rockit 0.9B Beta Version software. Receiver operator characteristic (ROC) curves were established by plotting sensitivity versus 1-specificity to assess the accuracy of each assay (Figure 3). The software was used to calculate ROC curve sensitivities, specificities and the corresponding area under the curve. The area under the ROC curve (Az) indicates the performance of an assay to separate the group being tested into actually-negative and actually-positive cases. A perfect test is represented by an area of 1, an excellent test by 0.9 - 1, a good test by 0.8 - 0.9 and 0.7 - 0.9 indicates moderate

accuracy. Due to perfect decision performance of the multigene assay, no actual curve could be constructed. ROC analysis of the multigene data resulted in an area value under curve of 1. The best performance for a single gene expression assay was achieved by mammaglobin, with a ROC curve area value of 0.92. B305D expression data resulted in an area value of 0.84, which indicates good test performance. B726P RT-PCR with Az = 0.78 and GABAπ RT-PCR with Az = 0.77 can be categorized as assays with moderate accuracies.

Discussion

Lymph node staging provides the most important prognostic indicator in breast cancer (11). The presence of metastases in lymph nodes correlates directly to the risk of disease recurrence and patient survival. Conventionally, axillary dissection is used to collect lymph nodes for analysis. As an alternative, sentinel lymphadenectomy has been discussed as a less invasive strategy to identify lymphatic spread of metastatic cancer cells (5;12;13). Nodal metastases are identified by staining (haematoxylin or eosin) or immunohistochemical analysis for cytokeratin proteins (14). Inadequate sectioning in lymph node analysis can produce false-negative results by missing small metastatic foci. Approximately 30 % of the patients diagnosed with pathologically negative lymph nodes develop recurrent disease. Since undetected micrometastases can be considered as a potential source for relapse of disease, more sensitive techniques for lymph node analysis have been discussed (5;15-17). Molecular diagnostic approaches are potentially of higher sensitivity than immunohistochemistry. However, the application of RT-PCR for breast cancer cell detection is hampered by the lack of specific marker genes. Cytokeratins as epithelial cell marker genes have shown limited specificity for breast cancer cell detection (14;18). Other potential tumor markers, in particular CEA, MUC1 and mammaglobin, demonstrated higher specificity but only identify a subset of tumor specimens. (4;5;18;19). No specific universal tumor marker has been identified so far due to the biological heterogeneity of breast carcinomas (20).

In this study we demonstrate the use of complementary expressed breast cancer genes for the development of a sensitive and specific RT-PCR assay to detect metastatic breast cancer cells. The multimarker assay described here increases the probability of detecting disseminated tumor cells and also provides the potential to characterize micrometastases on a molecular level. This could lead to improvements in determination of prognosis, monitoring of disease and possibly to individualized therapeutic strategies in the future.

For the future, intraoperative analysis of sentinel lymph nodes based on RT-PCR might even be possible. Due to improved screening approaches, early detection of breast tumors with small diameters is increasing. Since the incidence of lymph node metastases is related to tumor size, for small tumors the value of routine axillary lymph node dissection, in consideration of the possibility of overtreatment and consequent morbidity, is a matter of much debate (21-23). The development of rapid quantitative RT-PCR protocols might provide the platform to realize highly sensitive intraoperative analysis of sentinel lymph nodes (24) and to gain prognostic information about the necessity of axillary dissection for disease control.

This study focused on lymph node analysis to evaluate specificity and sensitivity of the assay. However, the multigene assay has potential applications in various tissues as a sensitive tool to detect breast tumor cells. Recently, we reported initial studies for these four genes individually (7) and in combination (25) to detect circulating breast tumor cells in peripheral blood samples.

Metastatic cells in bone marrow are also discussed to provide a high predictive value (26) for relapse of disease, independent of lymph node status. In addition, cytokeratin positive cells detected in the bone marrow of early stage tumors are suggesting a potential for early diagnosis of tumor dissemination (27). RT-PCR for mammaglobin has been shown to successfully detect

bone marrow micrometastases (28). Mammaglobin gene expression is also a useful marker for detection of tumor cell contamination in leukaphereses, with potential application in autologous stem cell transplantation protocols for high-dose chemotherapy programs (29). Due to the increased sensitivity provided by the multigene assay compared to mammaglobin alone, we conclude that the method described here will be useful for the evaluation of peripheral blood, bone marrow and leukapheresis samples for disseminated breast cancer cells.

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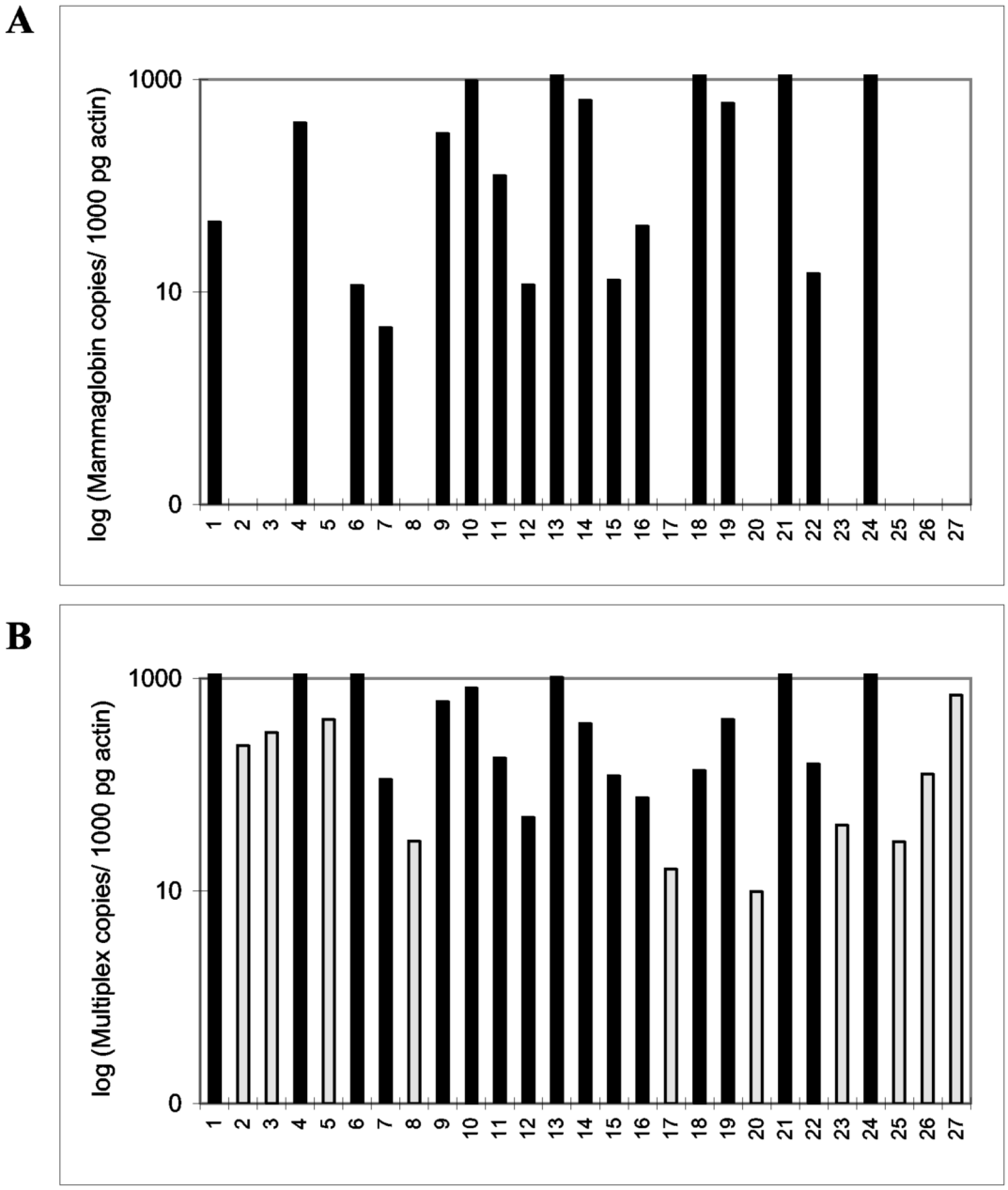

Fig. 1.

Comparison of mammaglobin expression and multigene signals detected in 27 primary breast cancer tumor cDNAs. Mammaglobin expression alone was detected in 17 out of 27 breast tumors (A). The multigene assay, co-detecting mammaglobin + B305D + Gabaπ + B726P, resulted in a significant expression signal from 27 out of 27 samples (B). Multigene copy numbers were determined by establishing a standard curve using a mixture of four plasmids containing inserts of either mammaglobin, B305D, Gabaπ or B726P cDNA. Average copy numbers were determined in triplicate PCR reactions. Actin copy numbers were detected in a separate PCR assay and used for normalization.

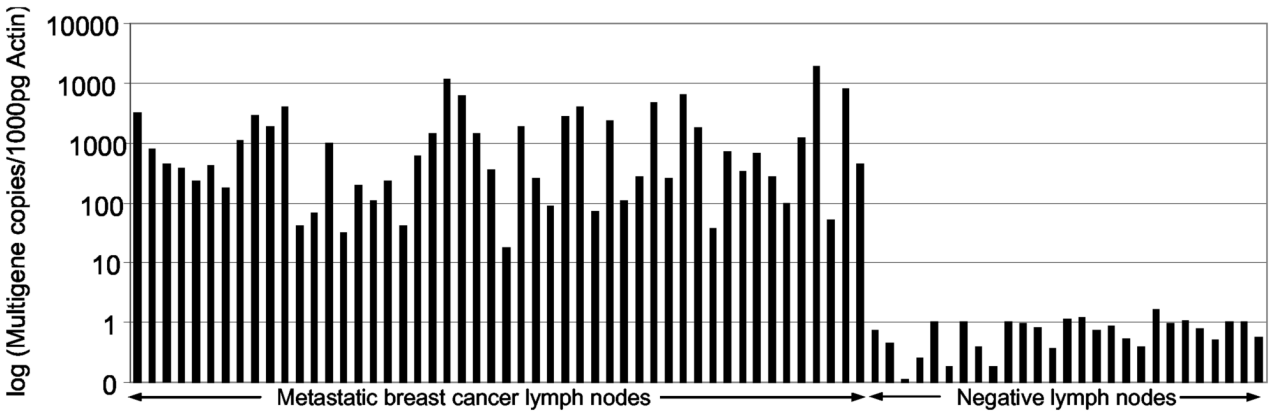

Fig. 2.

Breast cancer lymph node analysis using the multigene Real-Time RT- PCR assay. Combined copy numbers of mammaglobin, B305D, GABAπ and B726P normalized per 1000 pg actin were detected in 50 pathology positive metastatic breast cancer lymph nodes and in 27 negative lymph nodes (24 normal + 2 melanoma + 1 lymphoma lymph node samples). Average copy numbers were determined in triplicate PCR reactions and are shown in a logarithmic format. The range of copies/1000pg Actin in metastatic breast cancer lymph nodes is 17.2 to 18574.7; and 0 - 1.6 in negative lymph nodes.

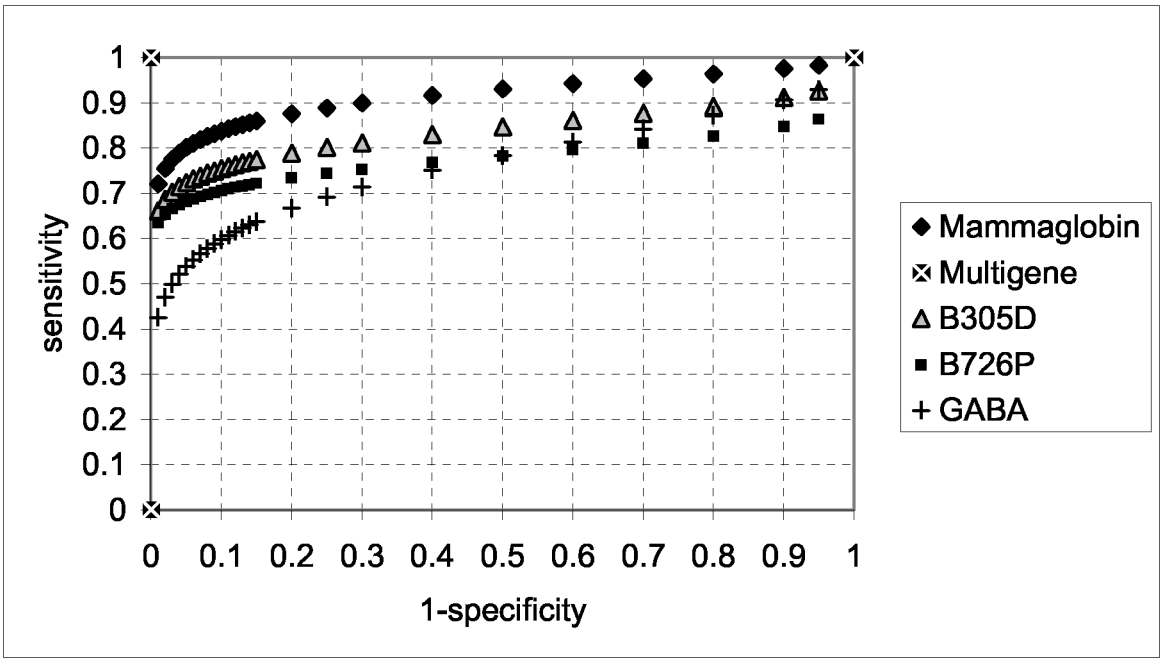

Fig. 3.

Receiver Operator Curve (ROC) analysis of Multigene and single gene Real-Time RT-PCR expression data. Expression data obtained from 27 true-negative and 50 true-positive lymph node specimens was analyzed using Rockit 0.9B Beta Version software (see Material and Methods). Sensitivity was plotted against 1-Specificity for single gene and multigene RT-PCRs. The area under each ROC curve is indicative for the diagnostic accuracy of the applied test. The maximal curve area is covered by multigene assay analysis, indicating a perfect decision performance in the sample population tested.

Table 1.

Expression pattern[a] of mammaglobin, B305D, GABAp and B726P in pimary breast tumor specimens.

breast primary tumor samples	Mammaglobin	B305D	GABAp	B726P	Multigene
1	+	++		+++	+++
2		+	+		++
3			++		++
4	++	+++		+++	+++
5		+		+++	++
6	+		+++		+++
7	+	++			++
8		++			+
9	++	+		+++	++
10	++	++		+++	++
11	++	+			++
12	+	++			++
13	+++	+			+++
14	++	+	++	+++	++
15	+		++		++
16	+	+		+	++
17			+		+
18	+++			+	++
19	++			+++	++
20		+			+
21	+++		+	+	+++
22			+	+	++
23				+	+
24	+++		+	+	+++
25			++	+	+
26		++			++
27		+		+	++
Total	17/27	16/27	10/27	14/27	27/27

[a] +, <10 copies/1000pg actin; ++, <100 copies/1000pg actin; +++, <1000 copies/1000pg actin.

Table 2.

Positive expression signals for single gene and/or multigene analyses in lymph node specimens, in colon tumor specimens and in normal tissues.

	Lymph node specimens		
	Metastatic breast cancer	**Melanoma /Lymphoma[a]**	**Normal**
Mammaglobin	40/50	0/3	0/24
B305D	36/50	0/3	0/24
GABAp	15/50	0/3	0/24
B726P	22/50	0/3	0/24
Multigene assay	50/50	0/3	0/24
	Colon tumor specimens		Normal tissues[b]
Multigene assay	0/14		4 /22[c]

[a] 2 melanoma and 1 lymphoma lymph node specimen.

[b] 2 normal colon, PBMC, bone marrow, heart, brain, pancreas, lung, liver, skin, kidney, spinal cord, salivary gland, small intestine, stomach, trachea, adrenal gland, aorta, skeletal muscle, bone, esophagus, bladder.

[c] a low expression signal was detected in esophagus, skin, trachea and salivary gland cDNA (ranging from 2-6 copies/1000pg actin)