Evaluation of Three mRNA Markers for the Detection of Lymph Node Metastases

JENNIFER BERGER[1,2], ELISABETH MUELLER-HOLZNER[1], HEIDI FIEGL[1], CHRISTIAN MARTH[1] and GUENTER DAXENBICHLER[1]

[1]*Department of Obstetrics and Gynecology, Innsbruck University Hospital, Innsbruck, Austria;*
[2]*Institute for Cell and Molecular Biology, The King's Buildings, University of Edinburgh, Edinburgh, U.K.*

Abstract. *The expressions of three mRNA markers were correlated with the results of extensive histopathological examination of a total of 290 axillary lymph nodes from 29 breast carcinoma patients. Included were two established markers for breast cancer (cytokeratin-19 and mammaglobin) and the novel marker DNA methyltransferase 3b (DNMT3b). DNMT3b was significantly overexpressed in breast cancer compared to normal breast tissue. The expression of the three markers in axillary lymph nodes was determined using quantitative real-time RT-PCR. DNMT3b expression showed a specificity of more than 99%, which was comparable to that of cytokeratin-19 and better than that of mammaglobin. The sensitivity of RT-PCR relative to histopathology was highest for cytokeratin-19 (96%), followed by DNMT3b (88%) and mammaglobin (68%). The overall agreement of histological and RT-PCR results was 96-99%. The results indicate that expression analysis of marker genes by quantitative RT-PCR can be a useful tool for lymph node diagnosis in breast cancer.*

Lymph node status represents one of the most important prognostic parameters for breast cancer. A large randomised clinical study showed that the proportion of disease-free patients and overall survival of lymph node-positive patients who received adjuvant chemotherapy was significantly higher than that of a control group (1). However, up to 30% of lymph node-negative patients ultimately develop recurrent disease (2). Histologically undetectable or dormant micrometastases in the lymphatic system can account for disease recurrence in these patients after variable disease-free intervals (3). In recent years, a number of more sensitive methods for the detection of micrometastases in lymph nodes have been developed. These involve serial sectioning of axillary nodes (4), immunohistochemistry (5) and molecular biology techniques, such as RT-PCR (6, 7). The RT-PCR detection of micrometastases in axillary lymph nodes was shown to be an independent predictor of clinical outcome in a retrospective series of patients (8). Real-time RT-PCR has the advantage of high sensitivity, high throughput capacity, quantitative readout system and minimal tissue requirements (9). Min *et al.* evaluated the expression of seven potential breast cancer markers and found that only *mammaglobin* was expressed in all breast cancer cell lines but not in normal lymph nodes (10). Mammaglobin expression was restricted to the mammary gland and was elevated in 25% of primary breast tumours (11, 12). We previously showed that *mammaglobin* expression is a useful marker for the detection of disseminated breast cancer cells in peripheral blood (13). Another marker, which was previously used with success is *cytokeratin-19* (*CK19*), an epithelial marker (14, 15). This gene is specifically transcribed by epithelial cells, but it is normally not expressed in lymphoid cells (16).

DNMT3b is one of the two *de novo* DNA methyltransferases, which are responsible for methylating previously unmethylated CpG dinucleotides (17). A role for DNA methylation in the differential regulation of gene expression was hypothesised many years ago (18) and since then, an inverse relationship between cytosine methylation and transcription has been observed for a large number of genes. Among the genes known to be methylated in breast cancer are *p16, BRCA1, RAR-β2, ER* and *E-cadherin* (19). A number of studies indicate that DNA methylation of multiple genes is a frequent event in cancer, (19, 20). Overexpression of *de novo* methyltransferase *DNMT3b* was observed in a variety of tumours (21, 22), including breast cancer (23) and may be involved in establishing aberrant methylation patterns during tumorigenesis. Recently, it became clear that a functional co-operation of DNMT3b and the maintenance DNA methyltransferase DNMT1 are essential for gene silencing in cancer cells (24), suggesting that, due to its ubiquitous overexpression in tumours, *DNMT3b* could be used as a general tumor marker.

Correspondence to: Guenter Daxenbichler, Department of Obstetrics and Gynecology, Anichstrasse 35, 6020 Innsbruck, Austria. Fax: ++43 512 504 23112, e-mail: Guenter.Daxenbichler@uibk.ac.at

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Table I. *Nucleotide sequences of primers and probes used for real-time RT-PCR and relevant GenBank accession numbers.*

Gene / GenBank accession No.	Oligonucleotide	Sequence
Mammaglobin	Forward primer	5'-CAA TGT TGA GGT GTT TAT GCA ATT AA -3'
AF015224	Reverse primer	5'- CCG TAG TTG GTT TCT CAC CAT ACC-3'
	Probe	5'FAM-CTG CAA GAC CTT TGG CTC ACA GAA CTG C-3'TAMRA
DNMT3b	Forward primer	5'-TACAATAGGATAGCCAAGTTAAAG -3'
AF176228	Reverse primer	5'-GCAGCTTCTGGCG- 3'
	Probe	5'FAM-CACAGACGTGTCCAACATGGGCCGTG-3'TAMRA
Cytokeratin-19	Forward primer	5'-TCGACAACGCCCGTCTG-3'
Y00503	Reverse primer	5'-CCACGCTCATGCGCAG-3'
	Probe	5' FAM-CCGAACCAAGTTTGAGACGGAACAGG-3'TAMRA

DNMT3b overexpression in neoplastic breast tissue compared to normal breast epithelium and a correlation with histopathological grading were verified (23). In this study the validity *DNMT3b* overexpression in detecting lymph node metastases in comparison to the two established tumour markers, *mammaglobin* and *CK19* was determined. Results of real-time RT-PCR and histopathological examination were correlated in a total of 290 lymph nodes from 29 breast cancer patients.

Materials and Methods

Patients and tissue samples. Twenty-nine samples of primary breast cancer tissue including 12 grade I samples, nine grade II samples and eight grade III samples were obtained immediately after resection of the breast or lumpectomy. A part of the tumour tissue was snap-frozen in liquid nitrogen and stored at –80˚C until lyophilisation. The rest of the specimen underwent routine histopathological examination. Seven non-cancerous breast tissue samples from patients who had undergone reductive plastic surgery were obtained in the same way.

Lymph node specimens were obtained from 29 unselected patients undergoing lumpectomy or tumorectomy only and axillary lymph node dissection for histologically proven carcinoma of the breast; 27 patients of this cohort were diagnosed with invasive ductal breast cancer, one patient had medullary and one lobular invasive breast cancer. In total, 290 lymph nodes were obtained immediately after resection. One half of each lymph node with a diameter ≥1 cm was subjected to fixation and staining according to a standard protocol and analysed by our pathologist (E. M-H.), and the other half was immediately put into 1 ml of lysis buffer (4 M guanidinium thiocyanate, 10 mM Tris.Cl, 10 mM EDTA pH 8.0, 1% sodium lauryl sarcosinate), snap-frozen in liquid nitrogen and stored at –70˚C until processed. For practical reasons, lymph nodes with a diameter ≤1 cm only underwent routine pathological analysis. This study was approved by the Institutional Review Board of the Department of Obstetrics and Gynecology of the University of Innsbruck, Austria. Informed consent was obtained from all patients prior to inclusion in the current investigation.

RNA extraction and reverse transcription reaction. Total cellular RNA was extracted from the tumour specimens using the acid guanidium thiocyanate-phenol-chloroform method (25). RNA concentration was quantified by measuring OD at 260 nm and checked for purity by evaluating the OD260 nm / OD280 nm ratio using a spectrophotometer (Perkin Elmer, Lambda EZ 201). Reverse transcription of RNA was performed in a final volume of 20 μl containing 1x RT-Buffer (50 mM Tris-HCl, pH8.3, 75 mM KCl, 5 mM $MgCl_2$), 40 units of rRNasin® RNase Inhibitor (Promega, Madison, WI, USA), 10 mM dithiothreitol, 200 units of M-MLV Reverse Transcriptase (Gibco BRL, Gaithersburg, MD, USA), 5 μM random hexamers (Applied Biosystems, Foster City, CA, USA) and 400 ng of denatured total RNA. Reverse transcription was performed at 37˚C for 50 min after an initial 10 min step at 25˚C, followed by 15 min incubation at 70˚C.

Primers and probes. CK19 primers and probe were previously described in Van Trappen *et al.* (14). Primer and probe sets for *DNMT3b* and *mammaglobin* PCR were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences of all probes and primers used and the GenBank accession numbers for the respective genes are provided in Table I. The *GAPDH* gene was used as an internal RNA control. Primers and probes were purchased from Applied Biosystems (human *GAPDH*, Cat. No. 4310859).

Real-time PCR amplification. Real-time PCR using TaqMan chemistry was performed using the ABI Prism 7700 Detection System (Applied Biosystems). Each reaction was carried out in a total volume of 25 μl reaction mixture containing 5 μl of cDNA, 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer and 250 nM of the TaqMan probe. Thermal cycling conditions comprised an initial step of 2 min at 50˚C, followed by a 10 min hold at 95˚C. The amplification protocol included 40 cycles, consisting of a 15 sec melt at 95˚C and a 1 min annealing/extension step at 60˚C. Gene expression was determined based on the threshold cycle number at which the increase in fluorescence signal associated with exponential amplification of PCR products began to be detected using Perkin-Elmer Applied Biosystems analysis software, all according to the manufacturer's instructions. Quantitative PCR reactions were performed in duplicates and repeated once for each sample-primer set. The mean of these four experiments was used as the relative quantification value.

Evaluation of data. For evaluation of relative *DNMT3b* expression in normal and malignant breast cancer tissues, gene expression was

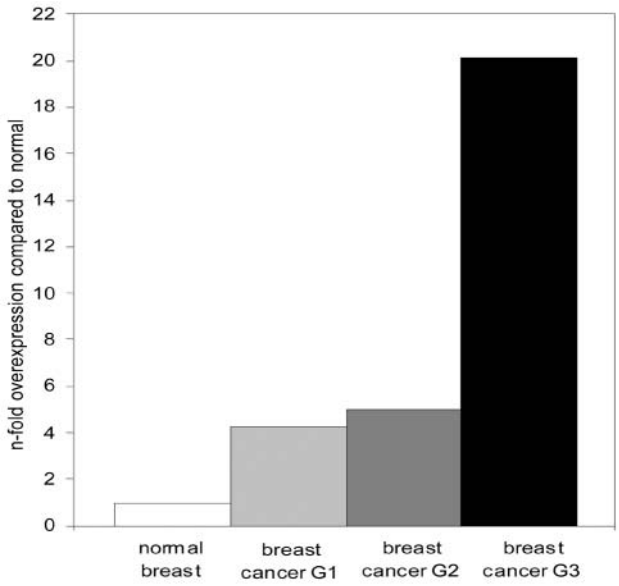

Figure 1. *Relative expression of DNMT3b in breast cancer tissue (grade 1-3) compared to normal breast tissue. DNMT3b expression was measured using real-time RT-PCR. Expression levels are shown as n-fold overexpression of breast cancer tissue compared to normal breast epithelium.*

normalised to the mean value for the seven normal breast tissue samples. N-fold overexpression of each category of tumours was calculated as: $2^{\text{(median Ct of normals – median Ct of each group of tumours)}}$. Student's *t*-test was performed to analyse the statistical significance in expression level differences between normal and malignant breast tissues (definition of significance: $p<0.05$).

Cut-off values for determining the presence of metastatic tumour cells in lymph nodes were chosen in order to gain a minimal number of discrepant results when compared to the histopathological diagnosis. Cases where only one micrometastasis with a diameter of <2 mm was detected in one half of the lymph node were evaluated separately, since, according to experience, it is very unlikely that a further micrometastasis is present in the same lymph node. RT-PCR-results for the genes investigated were related either to a reference gene (*GAPDH*) or total RNA (OD at 260 nm). In the latter case, no correction of results was necessary, as a constant amount of total RNA was always applied.

The normalisation of *mammaglobin* mRNA values to *GAPDH* was not possible, as most of the *mammaglobin* C_t values from negative lymph nodes were >40, *i.e.,* the signal was still negative after the maximum of 40 PCR cycles. Therefore, relation of *mammaglobin* to *GAPDH* would be artificial, *i.e.,* not true. Analysis of the validity of the *GAPDH*-normalised values for the other two mRNAs (*DNMT3b* and *CK-19*) revealed that these were slightly worse with respect to diagnostic power than the OD_{260}-related values. Furthermore, many genes commonly used as reference genes have been shown to vary in expression levels between different tissues (26), thereby influencing the reliability of normalisation. Therefore, in the final evaluation, the OD_{260}-related values were taken into consideration.

The diagnostic accuracy of each individual mRNA marker was evaluated by sensitivity (the probability that the mRNA marker is positive given that the histological examination reveals the presence of metastases) and specificity (the probability that the mRNA marker is negative given that the lymph node is histologically uninvolved).

Table II. *Distribution of the various gene expression levels in histologically-positive (histo +) and -negative (histo –) cases.*

	DNMT3b		CK19		Mammaglobin	
	Histo +	Histo –	Histo +	Histo –	Histo +	Histo –
Median	30.7	36.8	18.9	32.2	29.4	40.0
1st quartile	29.5	35.3	17.9	31.0	26.8	40.0
3rd quartile	31.6	38.9	22.1	33.1	39.4	40.0
Minimum	40.0	40.0	31.8	40.0	40.0	40.0
Maximum	26.4	31.9	16.2	19.9	18.0	23.7

Numbers of PCR cycles needed to generate a detectable signal in quantitative PCR (C_t). Equal amounts of total RNA were applied for all samples; no correction for a reference gene was performed.

Results

Overexpression of DNMT3b in breast cancer. DNMT3b mRNA levels were measured in 29 breast cancer samples and seven non-neoplastic breast tissues using quantitative real-time RT-PCR. As shown in Figure 1, average *DNMT3b* expression levels in breast cancer lesions were elevated compared to those in non-neoplastic breast tissue. *DNMT3b* expression correlated with increasing dedifferentiation, as determined by histopathological grading: mRNA levels of *DNMT3b* were elevated by about 6-fold in grade 1 lesions, 7-fold in grade 2 lesions and 13-fold in grade 3 lesions. The difference between mean *DNMT3b* expression levels in normal breast tissues and breast cancer lesions was statistically significant ($p=0.002$). When compared with the average *DNMT3b* expression level of normal breast samples, 9 out of 12 (75%) grade 1 samples, 8 out of 9 (89%) grade 2 samples and all 8 grade 3 samples showed *DNMT3b* overexpression.

Relative mRNA expression levels in lymph nodes containing metastatic breast cancer. The distribution of the expression levels for the three genes tested, indicated as C_t values, in histologically-positive and histologically-negative lymph nodes is provided in Table II. Equal amounts of total RNA were used for each PCR. No correction for a reference gene was performed. *CK19* showed the highest expression and accordingly, the lowest C_t values in positive lymph nodes among the three genes analysed. Most tumour-free lymph nodes also showed *CK19* expression, although mean expression levels were about 10,000-fold lower (corresponding to a mean difference in C_t values of 13.3) than in histologically-positive lymph nodes. Mammaglobin mRNA was undetectable in most of the histologically-negative lymph nodes (*i.e.,* no signal could be detected after 40 PCR cycles); positive lymph nodes showed a median C_t value of 29.4.

Table III. *Extreme values, i.e., highest C_t-values for histologically-positive (histo +) nodes and lowest C_t-values for histologically-negative (histo –) nodes are presented.*

DNMT3b				*CK19*				*Mammaglobin*			
cut-off = 32.0		cut-off = 13.0		cut-off = 24.0		cut-off = 2.0		cut-off = 35.0		cut-off = 11.0	
Histo + Ct	Histo – Ct	Histo + ΔC_t	Histo – ΔC_t	Histo + Ct	Histo – Ct	Histo + ΔC_t	Histo – ΔC_t	Histo + Ct	Histo – Ct	Histo + ΔC_t	Histo – ΔC_t
40.0[a]	**31.9**	**17.7**	**6.2**	**31.8**	**19.9**	**10.5**	**-2.5**	**40.0**	**23.7**	**20.6**	**3.5**
35.4	32.1	12.3	**6.6**	23.0	**20.1**	**3.0**	**0.6**	**40.0**	**28.8**	**18.3**	**6.8**
33.1	32.6	12.1	**6.8**	22.4	**21.2**	**2.8**	**0.7**	**40.0**	**29.2**	**16.5**	**7.8**
31.8	32.7	11.7	**7.1**	22.3	**23.7**	**2.3**	**1.0**	**40.0**	**30.6**	**16.5**	11.1
31.6	33.0	11.5	**7.2**	22.1	26.4	1.1	**1.1**	**40.0**	**30.7**	**16.3**	11.3
31.4	33.2	11.5	**7.2**	20.0	26.8	0.9	**1.1**	**39.1**	**32.4**	**16.0**	12.8
31.2	33.3	11.3	**7.4**	19.4	26.9	0.7	**1.7**	34.5	**33.9**	**15.7**	12.9
31.1	33.3	11.3	**7.5**	19.3	27.1	0.6	**1.9**	34.3	**34.9**	**13.3**	13.2
30.9	33.4	11.3	**7.8**	19.0	27.3	0.3	2.6	32.1	35.5	10.5	13.3
30.9	33.4	11.3	**10.5**	19.0	27.3	0.3	3.1	30.1	36.0	8.6	13.6

[a]Values in bold are disconcordant with histological findings at the chosen cut-off value. Results are either related to total RNA (C_t) or to GAPDH mRNA (ΔC_t).

Table IV. *Diagnostic validity of RT-PCR analysis of three different gene products in lymph nodes of mammary carcinoma patients.*

	DNMT3b		*CK19*		*Mammaglobin*	
	Total No.	%	Total No.	%	Total No.	%
Sensitivity	23/26	88	25/26	96	13/19	68
Specificity	262/263	99.6	257/261	98.5	228/236	96.6
Overall concordance	285/289	98.6	284/289	98.3	240/254	94.5
LN status pos. for RT-PCR and neg. for histology	1/28	4	2/28	7	1/25	4
LN status neg. for RT-PCR and pos. for histology	2/28	7	0/28	0	2/25	8

DNMT3b mRNA levels usually were also very low in negative lymph nodes; the median values for *DNMT3b* expression differed by about 70-fold between negative and positive histology. Results normalised against the reference gene *GAPDH* are not indicated, since these were not used for final evaluation.

Diagnostic validity of mRNA expression levels. Cut-off values were chosen in order to gain minimal discrepancy between histology and RT-PCR results. The highest C_t values for histologically-positive and the lowest C_t values for histologically-negative lymph nodes, highlighting the region where the discrepancy between RT-PCR results and histopathological examination occurred are provided in Table III. Disconcordant results were more frequent when normalising the data against the reference gene *GAPDH*. The three cases, where only one single metastasis of ≤2 mm was detected by histological examination, were negative for *DNMT3b* and *CK19*, but could not be analysed for *mammaglobin*. In one case, indicated in the first row of Table III, a metastasis with a diameter of 8 mm was detected in the histologically examined half, but all three RT-PCR tests gave a clearly negative result.

The diagnostic validity of the RT-PCR detection of the three gene products is given in Table IV. The best agreement for positivity between histology and RT-PCR (*i.e.,* the highest sensitivity relative to histopathology) was observed for *CK19*, reaching 96%. This correlation was slightly less pronounced for *DNMT3b* (88%) and clearly lower for *mamammaglobin* mRNA (68%). Specificity, *i.e.,* agreement in negative cases, was very high for all three mRNAs, *DNMT3b* (99.6%), *CK19* (98.5%) and *mammaglobin* (96.8%). The overall concordance of both methods, histology and RT-PCR, was very high, reaching more than 98% for *DNMT3b* and *CK19* and 95% for *mammaglobin*.

Misclassification of lymph node status of the patient by RT-PCR, when assuming histological classification as true, occurred in 3/28, 2/28 and 3/25 cases for *DNMT3b*, *CK19* and *mamammaglobin*, respectively. For further details see Table IV.

Discussion

Currently, routine pathological analysis of axillary lymph nodes in breast cancer patients consists of the preparation of one or two sections from the central area of the node followed by staining with hematoxylin and eosin and subsequent microscopic examination. This procedure, however, evaluates only a small portion of the node, thereby increasing the chance of false negative results. Immunohistochemistry has been used to assess the presence of micrometastases in nodes classified as uninvolved using conventional histology (27). RT-PCR analysis as a more sensitive method for the detection of tumour cells in lymph nodes was shown to be useful (6, 7), although this technique suffers from certain limitations, *e.g.* the fact that most molecular tumour markers are also expressed in uninvolved lymph nodes. Previous studies sought to overcome this problem by analysing a greater panel of markers and evaluating their data by taking into account the simultaneous expression of more than one marker (15). However, the analysis of multiple markers does not represent a practical approach for routine clinical screening. Analysis of expression of tumour-specific genes by quantitative RT-PCR methods could, therefore, lead to an improvement in accuracy and practicability of lymph node diagnosis.

Real-time RT-PCR allows for quantification and high through-put analysis and has become the technology of choice for the measurement of gene expression levels (28). Previous studies have used SYBR Green chemistry because of the somewhat lower costs (9). However, application of the more expensive TaqMan chemistry adds a higher level of specificity to the PCR reaction. In this study, we investigated the suitability of a potential new molecular marker, *DNMT3b*, in the detection of breast cancer metastases in axillary lymph nodes, in comparison to the already described mammary epithelial markers *CK-19* and *mammaglobin*. The first part of our study was designed to verify *DNMT3b* overexpression in mammary carcinomas and to optimise real-time RT-PCR conditions. In concordance with Girault *et al.*, we found evidence that *DNMT3b* expression was significantly elevated in breast cancer when compared to normal tissue. We also confirmed a correlation between *DNMT3b* expression levels and histopathological grading. The significantly higher mean of *DNMT3b* expression in grade 3 tumours compared to grade 1 or grade 2 tumours indicates that *DNMT3b* expression may increase with continuous dedifferentiation of tumour cells and, thus, breast cancer progression.

When using RT-PCR analysis of three gene products to detect lymph node metastases, cut-off values for RT-PCR results were fixed arbitrarily, in order to gain minimally discrepant results. Agreement of the RT-PCR and histological results was generally very high and better for *DNMT3b* and *CK19* than for *mammaglobin*. Our results do not provide a final conclusion whether *DNMT3b* or *CK19* is the most sensitive and specific marker, although there seems to be a tendency towards *CK19*. This marker has been used successfully in previous studies (14, 30).

In the cases where only a unifocal and small (≤2 mm) metastasis was detected, there was no agreement of both methods. This agrees with the experience that the presence in one half of the lymph node is associated with a high probability of absent tumour cells in the other half.

The lymph node status would change only in 2-3 of the 29 patients included in this study, when applying RT-PCR instead of extensive histopathological examination and when assuming that the situation in the two-halves of the lymph node examined by either method is the same with respect to metastases. Therefore, RT-PCR analysis of *DNMT3b* or *CK19* in lymph nodes would be equally sensitive as histological examination which, in our department, is performed in an extensive manner and is very time- and personnel-consuming. The clear advantage of molecular biological methods is their potential for automated analysis. For high through-put analysis, RT-PCR could be superior to histologic examination with respect to practicability and overall costs. Other studies have propagated the use of a panel of multiple expression markers in order to increase sensitivity (9, 15). This approach, however, is more cost- and time-consuming and, therefore, defeats the purpose of using molecular biology techniques. Another study employed the detection of aberrantly methylated tumour suppressor genes as a potential diagnostic tool for the identification of micrometastasis in cancer patients (29). However, each tumour type displayed a different methylation profile, and differences may even occur between individuals.

In summary, we were able to detect metastatic breast cancer cells in axillary lymph nodes using real-time RT-PCR determination of *CK19*, *mammaglobin* or *DNMT3b*. This molecular approach is comparable, at least for *CK19* and *DNMT3b,* to histopathological examination with respect to specificity and sensitivity, but allows higher through-put and more rapid analysis of samples. Since *DNMT3b* is overexpressed in various epithelial cancers, it could be used as a general tumour marker, thereby minimising the necessity to set-up specific diagnostic tools for each cancer type.

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