Quantitative Assessment of Promoter Hypermethylation during Breast Cancer Development

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The aberrant methylation of cytosine residues in the promoter region of growth regulatory genes is now widely recognized as an additional mechanism for gene inactivation in cancer cells. In this study we analyzed the methylation status of four growth regulatory genes (*p16*, *RASSF1A*, *cyclinD2*, *14-3-3 σ*) during breast cancer progression. For this purpose invasive and noninvasive tumor cell populations as well as hyperplastic cell proliferations were isolated from a series of archival breast tissue specimens ($n = 57$) using laser-assisted microdissection. A new real-time polymerase chain reaction-based assay was used for the sensitive and quantitative determination of the cell-specific methylation status. We found that aberrant promoter methylation was already prevalent in pure intraductal carcinoma with different frequencies and different methylation levels for the four genes analyzed. For *RASSF1A* and *14-3-3 σ* promoter methylation was also demonstrated in epithelial hyperplasia and intraductal papillomas. By contrast, aberrant methylation of *cyclinD2* and *p16* was restricted to cancerous epithelium. Increased methylation of the *cyclinD2* gene was significantly associated with a higher van Nuys grade. Furthermore, when intraductal and invasive tumor cells were compared, significant quantitative changes in the methylation level were detected primarily within the *cyclinD2* gene. These results demonstrate that promoter methylation is an early and frequent event in breast cancer development, but displays great quantitative and gene-specific differences, and changes in a gene-specific manner during tumor progression. (Am J Pathol 2002; 160:605–612)

Aberrant gene expression is the hallmark of cancer cells. As important as gain-of-function mutations is the loss of genetic information because of deletions or inactivating mutations.¹ In addition to these classical genetic mechanisms growth regulatory genes can be inactivated epi-

genetically via methylation of cytosine-residues in the promoter region of these genes.²

Through the recruitment of histone modifying enzymes to the DNA the cytosine methylation initiates the formation of a closed chromatin conformation thereby repressing transcription.^{3,4} This methylation-mediated inhibition of gene expression is now widely recognized as an important mechanism for the inactivation of growth regulatory genes in the process of malignant transformation.⁵

To answer the question whether the aberrant hypermethylation of regulatory sequences is an early event during cancer development, small and precancerous lesions have to be analyzed. These lesions are almost exclusively available as formalin-fixed, paraffin-embedded biopsy specimens. Isolation of homogeneous and morphologically defined cell populations from tissue sections has now become possible by laser-assisted microdissection.⁶

A very sensitive and widely used method for the detection of cytosine methylation in small tissue samples is the methylation-specific polymerase chain reaction (PCR).⁷ However this method provides only qualitative data regarding the methylation status of the regulatory region analyzed. This may mask potential quantitative differences between samples from different patients and also dynamic changes in the extent of methylation during tumor evolution. These differences might also be important for the classification of lesions and might have greater prognostic significance. For these reasons several groups including our own have developed independently from each other assays for the detection and quantification of CpG methylation,^{8–13} most of which are real-time PCR based. This technology combines the advantages of high through-put with a superior sensitivity and accuracy for quantification.¹⁴

In breast cancer promoter hypermethylation has now been described for several genes covering all aspects of cellular function.¹⁵ But almost all of these studies have analyzed advanced invasive mammary carcinomas in a purely qualitative manner. Therefore we started the anal-

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Table 1. Primer and Probe Sequences

Gene		Sequence
<i>p16</i>	Forward	5'-GGG GAG AGT AGA TAG CGG GC-3'
	Reverse	5'-AAC CAA TCA ACC GAA AAT TCC ATA-3'
	Probe	5'-FAM-TAC TCC CCG CCG CCG ACT CCA T-TAMRA
<i>RASSF1A</i>	Forward	5'-GCG TTG AAG TCG GGG TTC-3'
	Reverse	5'-CCC GTA CTT CGC TAA CTT TAA ACG-3'
	Probe	5'-FAM-ACA AAC GCG AAC CGA ACG AAA CCA-TAMRA
<i>cyclinD2</i>	Forward	5'-TTT GAT TTA AGG ATG CGT TAG AGT ACG-3'
	Reverse	5'-ACT TTC TCC CTA AAA ACC GAC TAC G-3'
	Probe	5'-FAM-AAT CCG CCA ACA CGA TCG ACC CTA-TAMRA
<i>actinβ</i>	Forward	5'-TGG TGA TGG AGG AGG TTT AGT AAG T-3'
	Reverse	5'-AAC CAA TAA AAC CTA CTC CTC CCT TAA-3'
	Probe	5'-FAM-ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA-TAMRA
<i>14-3-3σ</i>	Forward	5'-GAA GGT TAA GTT GGT AGA GTA GGT CGA AC-3'
	Reverse	5'-AAC TAC TAA AAA CAA ATT TCG CTC TTC G-3'
	Probe	5'-FAM-CTC GCC CTT CTC CAC GAC GCC-TAMRA

ysis of hyperplastic epithelia and intraductal as well as invasive breast cancer cells using a quantitative assay.

The aim of this study was to analyze the promoter hypermethylation of four key growth regulatory genes (*p16*, *RASSF1A*, *cyclinD2*, *14-3-3σ*) during breast cancer progression in a quantitative manner in morphologically defined laser-microdissected archival tissue specimens. The new real-time PCR-based methylation assay enables the detection of quantitative changes that may precede or accompany microscopically visible morphological alterations. To determine at which point of the morphological spectrum ranging from ductal hyperplasia to invasive carcinoma this epigenetic modification occurs, different hyperplastic and malignant lesions were analyzed.

Materials and Methods

Tissue Samples

A total of 40 cases of ductal carcinoma *in situ* (DCIS) were retrieved from the archive of the Institute of Pathology, Medizinische Hochschule Hannover, Hannover, Germany. In addition 7 specimens containing intraductal

papillomas and 10 specimens with intraductal epithelial hyperplasia were retrieved. Two pathologists (FL and HK) have reviewed each case independently and classified the DCIS specimens according to the van Nuys classification system.¹⁶ For control 10 blood samples were collected after informed consent from healthy volunteers without any history of neoplastic malignancy. Cell lines were purchased from the American Type Culture Collection (Rockville, MD) and cultivated according to the supplier's instructions given.

Laser Microdissection and DNA Isolation

All breast tissue samples used for methylation studies were prepared by laser-assisted microdissection from stained histological sections. Genomic DNA was isolated from fresh frozen biopsies, the peripheral blood mononuclear cell fraction, and from tissue culture cells using Proteinase K digestion and organic extractions according to standard procedures. Laser-microdissection of stained histological sections from breast tissue and subsequent isolation of DNA was performed essentially as described.¹⁷

Figure 1. A: Demonstration that the relative C_T values [C_T(target) – C_T(reference)] and thereby the calculated extent of methylation is constant in serial dilutions down to the threshold of detection. As an example a dilution for the *14-3-3σ* gene is shown. **B:** Validation of the quantitative methylation-specific PCR. Mixtures of DNA with a defined methylation status (completely methylated and completely unmethylated) were analyzed. Shown is the correlation between extent of methylation and ΔC_T value for the *RASSF1A* gene (mean of three independent experiments).

Figure 2. Two different morphological alterations from the same tissue section that display great differences concerning the methylation of the *RASSF1A* gene. **A:** Apocrine metaplasia of the breast (no methylation). **B:** Ductal hyperplasia (nearly complete methylation). H&E stain; original magnification, $\times 200$.

Quantitative Methylation Analysis

Bisulfite treatment of genomic DNA isolated from micro-dissected samples was performed as described.¹² All primer and hybridization probe sequences are listed in Table 1. For real-time PCR up to 5 μ l of the DNA samples were analyzed in a total volume of 25 μ l containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 200 μ mol/L dNTP, 240 nmol/L of each primer, 125 nmol/L hybridization probe, 1 μ mol/L ROX (Tibubiol, Bolin, Germany), 1.5 to 4.5 mmol/L $MgCl_2$, and 0.625 U Platinum-*Taq* (Gibco BRL, Life Technologies, Karlsruhe, Germany). After an initial denaturation step at 95°C for 5 minutes 45 cycles followed by 15 seconds at 95°C and 60 seconds at 62°C for annealing and extension.

For quantification of methylation a relative quantification algorithm was used. For standardization, samples with a known methylation status for a given gene were analyzed (cell line DNA and defined mixtures of cell line DNA) leading to calibration curves that allow the correlation between the measured ΔC_T (target – reference) values and the extent of methylation (Figure 1B). A region of the actin gene that contains no CpG dinucleotides and therefore is independent from the methylation status was used as a reference target.¹¹

Results

Establishment and Validation of the Quantitative Methylation Assay

Selection of the Primer/Probe Target Sequences

For the design of the primers and hybridization probes we took into account all published data (and also our unpublished observations) about the cytosine residues in the CpG island around the start site of transcription that are actually methylated. For the *p16* gene we have chosen the region identified by Huschtscha and colleagues¹⁸ and Wong and colleagues¹⁹ as the one that is early and most heavily methylated in human mammary epithelial cells (HMECs) during the process of cellular

transformation. For the *RASSF1A* gene we selected as a target for the methylation-specific real-time PCR the residues identified by Dammann and colleagues²⁰ by genomic sequencing as the critical residues methylated in lung and breast cancer samples. For the *cyclinD2* gene and the *14-3-3 σ* gene the regions identified by Evron and colleagues,²¹ Ferguson and colleagues,²² and Iwata and colleagues,²³ respectively, were selected for the development of the quantitative methylation-specific PCR. For all CpG sites analyzed in this study a close correlation between methylation and repression of transcription has been documented by reverse transcriptase-PCR or Western blotting analysis.^{19–23}

Evaluation of Reaction Efficiencies

The quantitative methylation-specific PCR assay used in this study is based on a relative quantification explained in detail elsewhere.²⁴ To use this relative quantification it has to be proven that the reaction efficiencies for all PCR systems that will be compared are equal. Figure 1A clearly demonstrates as an example for *14-3-3 σ* as the target gene and *ACT β* as the reference gene very similar reaction efficiencies over a wide range of template concentrations down to the threshold of detection indicated by a constant difference of the C_T values for these two primer/probe systems.

Validation of Quantification

To validate the reliability of the real-time PCR-based quantification we analyzed different mixtures of DNA isolated from cell lines for which the methylation status of the gene under study was known. Figure 1B demonstrates as an example for the *RASSF1A* gene a very good linear correlation ($r = 0.985$) between the extent of methylation and the measured differences of the C_T values [C_T (target gene) – C_T (reference gene)]. Similar calibration curves were obtained for all genes analyzed in this study (data not shown).

Promoter Methylation in Physiological and Hyperplastic Cell Proliferations of the Breast

To define whether promoter hypermethylation occurs already under luteinizing hormone (LH) stimulation and in hyperplastic proliferations such as epithelial hyperplasia or intraductal papilloma we analyzed two specimens of lactating breast epithelium, 7 intraductal papillomas, and 10 cases of epithelial hyperplasia. In addition the methylation status of the four genes under study was assessed in normal epithelium ($n = 6$), breast stromal tissue ($n = 2$), and one case of apocrine metaplasia (Figure 2A) a clearly benign alteration of the mammary epithelium. The results are summarized in Figure 3. Whereas for *p16* and *cyclin D2* no methylation or only very low levels of methylation could be detected, the *RASSF1A* and *14-3-3 σ* gene are quite frequently and also in part heavily methylated. *RASSF1A* was extensively methylated in cases of epithelial hyperplasia and papilloma but not in normal epithelium, apocrine metaplasia, and the highly proliferating lactating epithelium. By contrast, the *14-3-3 σ* gene was clearly methylated under physiological conditions in breast epithelium and also quite heavily in stromal tissue. Hyperplastic specimens exhibit *14-3-3 σ* gene methylation ranging from 5 to 85% (Figure 3).

Promoter Methylation in Lymphocytes and Normal Lymph Nodes

Because several groups have reported the sensitive detection of hypermethylated alleles in the blood of cancer patients by methylation-specific PCR as a marker of disease,^{25–27} we tested whether the four genes analyzed in this study could also be used for the early detection of breast cancer in blood samples. For this purpose we analyzed the methylation status of these genes in the peripheral blood mononuclear cell fraction of healthy individuals ($n = 10$) without any history of neoplastic malignancies to assess the normal level of methylation. As shown in Figure 3 for *RASSF1A* no methylation at all could be detected whereas for *p16* and *cyclinD2* only occasionally a very weak methylation signal ($<1\%$) could be seen. In contrast to this, for *14-3-3 σ* in all 10 samples a very high level of methylation ($>95\%$) was found. In addition to the real-time PCR-based assay we confirmed the almost complete methylation of the *14-3-3 σ* gene around the start point of transcription in the leukocytes of healthy individuals using a restriction enzyme-based methodology²⁸ (data not shown).

We also tested the DNA isolated from four resected lymph nodes without any morphological alteration and found very similar results to those observed with peripheral blood mononuclear cell fractions (data not shown). This excludes the use of the detection of *14-3-3 σ* hypermethylation in lymph nodes or peripheral blood for the screening for circulation tumor cells or micrometastasis that would otherwise be a promising approach because of the very high prevalence of *14-3-3 σ* hypermethylation in breast cancer cells (see Ferguson et al²² and our own results).

Figure 3. Methylation in lymphocytes from healthy donors and laser-micro-dissected, normal, and hyperplastic breast tissue and DCIS of the breast. Lymph, lymphocytes ($n = 10$); Stro, stromal tissue ($n = 2$); Norm, normal breast epithelium ($n = 6$); Lakt, lactating breast tissue ($n = 2$); ApoMe, apocrine metaplasia ($n = 1$); Hyp, epithelial hyperplasia ($n = 10$); Pap, papilloma ($n = 7$); DCIS, ductal carcinoma *in situ* ($n = 40$ for *p16* and *cyclinD2*, $n = 36$ for *RASSF1A*, and $n = 37$ for *14-3-3 σ*). In DCIS samples 1% or less methylation was found for *p16* in 39 cases (97.5%), for *RASSF1A* in 6 cases (17%), for *cyclinD2* in 16 cases (40%), and for *14-3-3 σ* in 12 cases (33%). Almost complete methylation was found for *p16* in 1 case (2.5%), for *RASSF1A* in 20 cases (56%), for *cyclinD2* in 11 cases (28%), and for *14-3-3 σ* in 2 cases (5%).

Figure 4. Methylation in pure intraductal carcinomas without accompanying invasive components (**left**: van Nuys grade 1 to 3, $n = 9$) and in low-grade DCIS (**right**: van Nuys grade 1 or 2, $n = 10$).

Promoter Methylation in DCIS of the Breast

Altogether 40 archival specimens of DCIS have been analyzed for methylation after laser-assisted microdissection of the intraductal tumor cells. The results are summarized in Figure 3. Marked differences between the four genes are clearly visible. Whereas the *p16* gene is only very rarely methylated, the *RASSF1A* gene is methylated in the vast majority of samples. In Figure 4 two subsets of samples are displayed in more detail: low-grade carcinomas (van Nuys grade 1 and 2, $n = 9$) and pure intraductal carcinomas without accompanying invasive components (van Nuys grade 1 to 3, $n = 10$).

In 9 out of 10 pure *in situ* carcinomas under study at least one of the four genes analyzed was hypermethylated. For the *cyclinD2* gene a statistically significant correlation between the histological grade according to van Nuys and the extent of methylation was found (Figure 5).

Figure 5. Extent of methylation within the *cyclinD2* gene in low- and high-grade DCIS (vN1–3, van Nuys grade 1 to 3): a statistically significant correlation was found between high-grade DCIS and a high level of methylation (chi-square test, $P < 0.001$).

Promoter Methylation during Progression to Invasive Growth

For 16 specimens intraductal and invasively growing tumor cells could be isolated from the same tissue section using laser-microdissection as shown in Figure 6. For the majority of cases the methylation of the *p16* gene, the *14-3-3σ* gene and the *RASSF1A* gene is very similar in the intraductal and in the invasive component indicating that in most cases the epigenetic inactivation takes place before invasive growth develops (Figure 7). Interestingly, the *cyclinD2* gene shows four different patterns of methylation during tumor progression: no methylation at all, high methylation already in intraductal tumor cells or a sharp increase as well as a sharp decrease in methylation during progression resulting in two types of invasive tumor cells (no methylation or nearly complete methylation). We could not find a correlation between the methylation level in the invasive components and the histological grade, the proliferation status (Ki-67 labeling) or the hormone receptor status (estrogen and progesterone receptor immunohistochemistry).

Discussion

The epigenetic inactivation of growth regulatory genes is well documented for advanced invasive breast cancer.^{15,29} Much less is known about intraductal carcinomas and hyperplastic alterations such as epithelial hyperplasia or intraductal papilloma. Therefore we started the systematic analysis of laser-microdissected DCIS as well as epithelial hyperplasia and intraductal papillomas. Whereas so far only a few cases of intraductal breast cancers have been analyzed^{21,30–32} this study investigates four genes in 40 histopathologically classified specimens of intraductal carcinomas.

For all CpG-rich regions analyzed in this study a close correlation between methylation and gene silencing has

Figure 6. Isolation of pure intraductal and pure invasive tumor cells using laser-assisted microdissection.^{6,17} **A:** Section of a ductal-invasive carcinoma of the breast. The reduced optical quality is because of the fact that the slides are dried and not coverslipped for microdissection. **B:** Section after removal of the intraductal and the invasive component, respectively. **C:** Isolated intraductal (**right**) and invasive (**left**) tumor cells in the lid of a reaction tube. Methylene blue-stained; original magnifications, $\times 100$ (**A–C**).

been shown.^{19–22} It has also been shown by bisulfite sequencing that the cytosine residues recognized by the primers and probes are actually methylated.

In comparison to conventional methylation-specific PCR,⁷ the real-time PCR-based assay for the detection of methylation has several advantages. First, the omission of all postamplification steps reduces the risk of contamination and increases the throughput of the system. Second, the assay is more stringent and more specific because in addition to the two PCR primers the fluorescent-labeled hybridization probe has to anneal correctly between the two primers. Third and most important, conventional methylation-specific PCR does not provide exact quantitative data.

Figure 7. Comparison of methylation levels for intraductal and invasive tumor cells isolated from the same tissue section using laser-assisted microdissection as illustrated in Figure 6.

The results concerning the nearly complete hypermethylation of the *14-3-3 σ* gene in normal lymphocytes (Figure 3) demonstrate the importance of microdissection that enables the isolation of pure morphologically defined cell populations. Laser microdissection also allows the direct comparison of neighboring intraductal and invasive tumor cells as illustrated in Figure 6. It cannot be excluded that the traces of *14-3-3 σ* gene methylation $<1\%$ found in a minor portion of the microdissected samples could be because of single lymphocytes occasionally present in otherwise homogenous tumor cell populations.

During the preparation of this manuscript, Umbricht and colleagues³³ published results concerning the methylation of one of the four genes analyzed in this study, namely *14-3-3 σ* , in epithelial hyperplasia of the mammary gland and intraductal breast cancer cells that principally confirm the results presented in this study for this particular gene.

Aberrant hypermethylation was found in benign hyperplastic lesions of the mammary gland and in low-grade intraductal carcinomas. The most frequently and most heavily methylated gene was the recently identified putative tumor-suppressor gene *RASSF1A*.³⁴ This gene was methylated to a similar extent in intraductal papillomas and epithelial hyperplasia, but never in normal breast

epithelium, lactating breast tissue, stromal cells, or lymphocytes (Figure 3). This finding suggests that methylation of *RASSF1A* might be a new marker for nonphysiological epithelial proliferation in the breast.

The observed dichotomy concerning the methylation of the *cyclinD2* gene in invasive carcinomas (Figure 7) may be explained by the opposing functions ascribed to the cyclin D2 protein during cell-cycle progression. The protein functions as activator of the cell cycle but also as inhibitor of proliferation and inducer of senescence depending on the cellular context.^{35,36} This has to be addressed in future studies.

The changes in methylation levels could be because of epigenetic variations in a given clone (eg, loss of methylation capacity). Alternatively, they could be caused by clonal selection during tumor progression.

During the immortalization of HMECs the *p16* gene is heavily methylated.^{18,19} Also several established breast carcinoma cell lines show clear hypermethylation of the *p16* gene.³⁷ In contrast to these findings in cell culture experiments, the methylation of this tumor suppressor gene in intraductal breast cancer cells, which are clearly malignant transformed, is only a rare event. Therefore the epigenetic pathways contributing to the immortalization and transformation *in vivo* seem to be different from those observed in cell culture using HMEC lines. These results demonstrate the importance of *in vivo* studies to elucidate the role of methylation in the process of malignant transformation and may indicate that the widely used HMEC lines are not in every respect a suitable model for the study of the malignant transformation of luminal breast epithelial cells that are the precursor cells of the vast majority of mammary carcinomas. In this context it should be mentioned that large scale cDNA array-based expression studies have revealed that HMECs are more similar to basal epithelial cells of the mammary gland than to luminal epithelial cells.³⁸ This might explain the difference concerning the epigenetic inactivation of *p16* in cultured HMEC lines *versus* DCIS seen in this study.

The frequency of *p16* gene hypermethylation in invasive growing tumor cells reported in this study is lower than in the majority of published studies.^{28,37,39–42} In contrast to previous studies performed predominantly on fairly large and advanced invasive tumors we have analyzed primarily intraductal lesions, part of them with accompanying small invasive lesions. Furthermore, it cannot be excluded that the comparably lower incidence of *p16* methylation reflects regional differences in patient cohorts because another study on breast cancer from this area applying a completely different method yielded also a low incidence of *p16* methylation not exceeding 5%.²⁸

We could find absolutely no indication for a reduced methylation of the *p16* gene in breast cancer cells during tumor progression (Figure 7) or in comparison to normal breast epithelium (Figure 3), as was reported by Van Zee and colleagues.⁴³ This discrepancy could be because of completely different methodology and to the fact that in the present study pure tumor cell populations isolated by laser-assisted microdissection were analyzed in contrast to the tumor homogenates analyzed in the earlier study.

In conclusion, our results demonstrate that the epigenetic modification of key regulatory genes is an early and frequent event in the development of breast cancer. A quantitative assessment of promoter methylation clearly shows huge gene-specific differences for the extent of methylation and also specific alterations of methylation levels during tumor progression.

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