Promoter Hypermethylation of the Death-associated Protein Kinase Gene in Breast Cancer Is Associated with the Invasive Lobular Subtype

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ABSTRACT

Expression of death-associated protein (DAP) kinase, a proapoptotic serine/threonine protein kinase, is frequently lost in human tumors. In a study of 134 primary breast cancer specimens hypermethylation of the DAP kinase gene was found in 15% of cases. A highly significant difference ($P < 0.001$) of DAP kinase inactivation was observed between invasive lobular cancer ($n = 19$) and invasive ductal cancer ($n = 85$; 53% versus 9%, respectively). Hypermethylation correlated with loss of RNA expression, estrogen receptor positivity ($P < 0.01$), and the absence of p53 overexpression ($P < 0.01$). In contrast to invasive lobular cancer, the in situ-growing precursor lesion lacked epigenetic modification of the DAP kinase promoter by aberrant methylation indicating a potential role in tumor progression. Unlike the DAP kinase gene, hypermethylation of the cyclin D1 and RASSF1A genes did not correlate with a particular histological subtype or to invasiveness. We conclude that different histological subtypes of breast cancer may not only differ concerning specific chromosomal abnormalities and DNA mutations but also with regard to epigenetic inactivation patterns.

INTRODUCTION

Inactivation of tumor suppressor genes is an important event contributing to the development of neoplastic malignancies. In addition to the classical genetic mechanisms of deletion or inactivating point mutations growth regulatory genes can be functionally inactivated without alterations of the primary sequence by methylation of cytosine residues in the promoter region of these genes (1).

Epigenetic inactivation is well established in invasive breast cancer (2) and already detectable in preinvasive intraductal carcinomas (3, 4). But thus far very little is known about the association of hypermethylation in breast cancer with particular clinical or histopathological features.

DAP$^3$ kinase is a positive mediator of IFN-γ-induced programmed cell death (5). It suppresses tumor growth and metastasis by increasing the occurrence of apoptosis in vivo (6). Recently we identified and characterized epigenetic alterations contributing to the development and progression of breast cancer (4). Extending this study to apoptosis regulating genes, we analyzed whether the DAP kinase gene is also methylated in mammary carcinoma. The hypermethylation of this important apoptosis regulating gene was demonstrated previously in a variety of B-cell lymphomas and epithelial tumors (Refs. 7, 8 and references therein), but not yet in breast cancer, one of the most frequent malignant neoplasias in women.

Therefore we, firstly, determined which cytosine residues in the DAP kinase gene are methylated in breast tumor biopsies. Secondly, based on these results, we developed a quantitative real-time PCR based assay for the rapid and sensitive quantitative detection of DAP kinase gene hypermethylation in a large series of histologically defined invasive breast cancer specimens, as well as in laser-microdissected premalignant and malignant preinvasive specimens. Thirdly, we analyzed the functional consequences of hypermethylation by measuring the DAP kinase mRNA transcript levels in primary tumor specimens using real-time PCR technology.

MATERIALS AND METHODS

Patient Samples and Cell Lines. A total of 106 invasive breast cancer specimens comprising 85 invasive ductal, 19 invasive lobular carcinomas, and 2 mucinous carcinomas were retrieved from the tumor bank of the Institute of Pathology, Medizinische Hochschule, Hannover, Germany. In addition 8 formalin-fixed, paraffin-embedded specimens containing LCIS and 4 resected lymph nodes without any morphological alteration were retrieved from the archive. Genomic DNA isolated from 34 laser-microdissected specimens (6 intraductal Paps, 8 epithelial Hyps, and 20 DCIS) from a previous study (4) were also analyzed.

For use as control, blood was collected after informed consent from 10 healthy volunteers.

Cell lines were purchased from American Type Culture Collection (Rockville, MD) and cultivated according to the supplier’s instructions.

DNA Extraction and Laser Microdissection. 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 was used for the study of in situ lesions. Subsequent isolation of DNA was performed essentially as described (9).

Bisulfite Sequencing and Methylation-specific PCR. Bisulfite treatment of genomic DNA was performed as described (10). Approximately 20 ng of converted DNA were amplified using the methylation-independent primer pairs 1H5'/3'1 and 2H5'/3'2 as described (10). All of the primers and probes used in this study are available at our website. For sequencing of individual clones the PCR products were inserted into an appropriate plasmid vector using the Topo cloning kit (Invitrogen, Karlsruhe, Germany), sequenced using the CycleReader Auto DNA sequencing kit from Fermentas (MBI Fermentas, St. Leon-Roth, Germany), and analyzed on a LI-COR 4200 automatic sequencer (LICOR, Inc., Lincoln, NE).

Quantitative Methylation Analysis. Quantitative methylation analysis was performed essentially as described (4).

RNA Extraction and Quantitative Real-Time PCR. Extraction of RNA from fresh-frozen biopsies and quantitative real-time PCR analysis was performed essentially as described (11).

Immunohistochemistry. The immunohistochemical stainings were performed after our routine diagnostic procedure using tyramine amplification (12).

RESULTS

DAP Kinase Gene Is Hypermethylated in Breast Cancer Cell Lines and Primary Breast Cancer Specimens. Analyzing a whole panel of breast cancer cell lines we found DAP kinase gene hypermethylation and concomitant loss of mRNA expression in 4 of 10 cell lines (data not shown).

After methylation-independent amplification and subsequent subcloning...
ing of PCR fragments, we used genomic bisulfite sequencing to determine the cytosine residues methylated in breast tumor cell lines and primary breast tumor biopsies (see Fig. 1). The DAP kinase mRNA-negative cell line MCF-7 shows nearly complete methylation of the 5' region of the DAP kinase gene, whereas the DAP kinase mRNA-positive cell line MDA-MB-361 contains nearly no methylated cytosine residues in this region of the DAP kinase gene (Fig. 1B). In contrast to other genes described in the literature (e.g., p15INK4b; Ref. 13), the DAP kinase gene is quite homogeneously methylated in tumor biopsies (Fig. 1C), which facilitates the design of appropriate primers and hybridization probes for quantitative methylation analysis.

Establishment and Validation of the Quantitative Methylation Assay. On the basis of the identification of cytosine residues methylated in primary tumor specimens an appropriate primer pair and the corresponding fluorescence-labeled probe were designed (see Fig. 1C for location). To use a relative quantification, it has to be proven that the reaction efficiencies for all of the PCR systems to be compared are equal. Fig. 2A clearly demonstrates for DAP kinase as the target gene and ACTβ as the reference gene very similar reaction efficiencies over many template concentrations. 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 DAP kinase gene was known. Fig. 2B demonstrates a very good linear correlation ($r^2 = 0.998$) between the extent of methylation and the...
measured differences of the C\textsubscript{T}-values [C\textsubscript{T}(target gene) – C\textsubscript{T}(reference gene)].

**DAP Kinase Methylation in Lymphocytes, Normal Lymph Nodes, and Normal Breast Tissue.** In the peripheral blood mononuclear cells of healthy volunteers (n = 10) and 4 resected lymph nodes without any morphological alteration, no DAP kinase gene methylation could be detected (data not shown).

Altogether 12 normal breast tissue specimens were tested for DAP kinase gene methylation. In one case a low level of methylation (\~{}1\%) could be detected (see Fig. 2C). This is in very good agreement with the results of Kang et al. (7) who also analyzed 13 specimens of normal breast tissue during their study of DAP kinase methylation in gastric carcinoma.

**Frequent DAP Kinase Gene Methylation in Invasive Lobular Breast Cancer.** A large series of invasive breast cancer specimens (n = 106) was analyzed for DAP kinase hypermethylation using the new quantitative real-time PCR assay. The overall frequency of hypermethylation was 16% (17 of 106). Fig. 2C demonstrates the clear distinction between samples with no or very low level methylation and clear-cut methylation. Varying the threshold between 5 and 20% does not change the results. Therefore, 10% was chosen as a threshold for classifying a sample as “hypermethylated.”

All of the cases underwent a morphological reassessment of the tumor specimens in a blinded fashion. Comparing methylation data with histopathological classification revealed a statistically highly significant association between DAP kinase gene methylation and the lobular subtype of invasive breast cancer: 53% hypermethylation in lobular invasive cancer versus 9% in ductal invasive cancer (Fig. 3A; \(\chi^2\) test, \(P < 0.001\)).

**Transcriptional Silencing of the DAP Kinase Gene in Invasive Lobular Breast Cancer.** To assess the functional consequences of promoter hypermethylation, the DAP kinase mRNA expression was quantified in samples with a high level of methylation and in a series of samples completely devoid of methylation. Only tumor samples were analyzed and compared with each other to avoid any distortion because of very different proliferation activity in normal and malignant transformed breast tissue. Fig. 4 clearly demonstrates that hypermethylation correlates very well with transcriptional silencing. Several samples without any signs of hypermethylation also showed a decrease of DAP kinase mRNA expression indicating that additional mechanisms may be responsible for loss of DAP kinase expression.

**DAP Kinase Gene Methylation in Hyperplastic Proliferations of the Breast and Intraductal Carcinoma.** To address the question whether hypermethylation is an early event in the development of lobular carcinoma preinvasive in situ lesions were analyzed. For this purpose pure tumor cell populations were isolated using laser-assisted microdissection. The methylation analysis of 8 cases of LCIS demonstrated that the hypermethylation of the DAP kinase gene is not an early event in the clonal evolution of lobular breast cancer. None of 8 intraductal specimens displayed hypermethylation (Fig. 3A, bar 4), whereas the cyclinD2 and the RASSF1A genes are frequently hypermethylated in LCIS (38% and 88%, respectively, Fig. 3B, bar 4; Fig. 3C, bar 4). In contrast to these findings in lobular cancer, the frequency of DAP kinase methylation in DCIS was very similar to that found in ductal-invasive carcinoma (compare Fig. 3A, bars 1 and 2).

To define whether DAP kinase gene methylation occurs already in premalignant proliferations of the ductal epithelium, laser-microdissected intraductal Paps (n = 6) and epithelial Hyps (n = 8) were analyzed for DAP kinase hypermethylation. No DAP kinase hypermethylation could be found in these premalignant lesions (Fig. 3A, bars 5 and 6). The methylation status of the cyclinD2 gene and the RASSF1A genes has been already assessed in a previous study (Ref. 4; Fig. 3B, bar 5 and 6; Fig. 3C, bar 5 and 6).

**Expression of DNMTs.** Because conflicting reports exist concerning the relationship between gene hypermethylation in tumor cells and mRNA expression of DNMTs (Ref. 14 and references therein), we determined in a subset of samples the expression of DNMT 1, DNMT 3A, and DNMT 3B using a real-time RT-PCR assay. No significant correlation between DNMT mRNA expression and DAP kinase gene hypermethylation could be shown (data not shown).

**Correlation of DAP Kinase Hypermethylation and Clinical Parameters.** No correlation between DAP kinase gene methylation and tumor size, lymph node involvement, and tumor grade could be found. For several genes an age-related increase in methylation levels has been described (15). Therefore, we analyzed the age-distribution of the whole patient group and the subset of samples displaying hypermethylation. No significant differences could be found (mean
age 59 and 60 years, respectively; median age: 59 and 58 years, respectively). But we found that epigenetic inactivation of the DAP kinase gene and p53 overexpression as determined by immunohistochemistry are mutually exclusive (χ² test with Yates-correction, \( P < 0.01 \)). In addition, a significant correlation exists between estrogen receptor positivity and DAP kinase gene methylation (χ² test, \( P < 0.01 \); data not shown).

**DISCUSSION**

The epigenetic inactivation of growth regulatory genes is well documented for invasive breast cancer (2). But nearly all of the studies published thus far do not analyze the potential correlation of hypermethylation with different histological subtypes of mammary carcinoma. In this comprehensive analysis of DAP kinase gene hypermethylation in breast cancer cell lines and primary breast cancer specimens, we determined by genomic sequencing after bisulfite methylation in breast cancer cell lines and primary breast cancer specimens, we found a highly significant association between promoter hypermethylation and invasive lobular breast cancer.

To the best of our knowledge this is the first study reporting an epigenetic alteration associated specifically with this histological subtype of breast cancer. Droufakou et al. (16) described in detail epigenetic inactivation of the E-cadherin gene in lobular breast cancer, but this event is not specific for this histological subtype (17).

To date only a few studies addressed the question whether different histological subtypes of cancer originating in the same organ differ in the epigenetic inactivation of crucial genes. Kim et al. (18) demonstrated for p16INK4a gene hypermethylation a significant correlation with the squamous cell type of lung cancer. Osada et al. (19) discovered a strong association between small lung cell cancer and 14--3--3σ hypermethylation, which was rarely found in non-small cell lung cancer. Graff et al. (20) found different methylation frequencies for the E-cadherin gene in different subtypes of thyroid carcinoma.

The finding that low-level methylation can be detected in normal breast tissue highlights the requirement for quantitative assessment of promoter hypermethylation in tumor specimens.

During the preparation of this manuscript Simpson et al. (21) reported also a good correlation between DAP kinase gene hypermethylation and loss of expression for pituitary tumors, which parallel our findings in breast tissue. By contrast, however, we found no indication for DAP kinase deletion in breast cancer. This may reflect tumor-specific inactivation mechanisms. Small or interstitial deletions or deletions affecting only a very small subfraction of tumor cells cannot be excluded.

The results described in the literature concerning the quantification of DNMT mRNA are not directly comparable because of different methodologies used. The data presented in this study support a model invoking post-transcriptional up-regulation or activation of these enzymes, as well as altered protein-protein interactions (14).

A morphological hallmark of invasive lobular carcinoma is a striking loss of cell-cell interaction not seen in most other cohesively growing epithelial malignancies. The functional loss of the DAP kinase gene may contribute to a resistance of the invasive tumor cells against apoptosis because of altered cell-cell and cell-matrix interactions.

The hypermethylation of the DAP kinase gene appears to be specific for malignant transformed cells, because the premalignant lesions ductal Hyp and intraducatal Pap are completely devoid of promoter hypermethylation (Fig. 3A, bar 5 and 6). The comparison with the cyclin D2 and the RASSF1A genes clearly shows that epigenetic inactivation in breast tissue is a gene and histological-type-specific phenomenon. In contrast to the age-related hypermethylation of several genes reported in the literature (see Ref. 15 and references therein), we did not find any correlation between DAP kinase gene methylation and the age of the patients, indicating also that the age-related increase in methylation seems to be a gene-specific phenomenon. That fact that the epigenetic inactivation of the DAP kinase gene and the overexpression of the p53 gene are mutually exclusive events in our series of primary breast cancer specimens supports results obtained previously with embryonic fibroblasts. Raveh et al. (22) could show that DAP kinase and p53 are acting in the same proapoptotic pathway counteracting oncogene-induced transformation.

The analysis of morphologically defined intraductal tumor cells after laser-assisted microdissection enabled the identification of DAP kinase hypermethylation as a rather late event in the clonal evolution of lobular breast cancer. Therefore, it may serve as a new progression marker in this histological subtype of mammary carcinoma.

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