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# Epigenetic alterations by methylation of *RASSF1A* and *DAPK1* promoter sequences in mammary carcinoma detected in extracellular tumor DNA

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**Abstract** Novel strategies for early detection of breast cancer, the most common and second most lethal cancer in women, are urgently needed. Silencing tumor suppressor genes via DNA methylation has established hypermethylation as one of the most frequent molecular alterations that may initiate and drive many types of human neoplasia including breast cancer. Detecting such epigenetic changes in DNA derived not only from tumor tissue, but also from bodily fluids, may be a promising target for the molecular analysis of cancer. In this study we examined serum, a readily accessible bodily fluid known to contain neoplastic DNA, from individuals with breast carcinoma. Using sensitive methylation-specific polymerase chain reaction, we searched for aberrant promoter hypermethylation of two normally nonmethylated genes: *RAS* association domain family member 1A (*RASSF1A*) and death-associated protein kinase 1 (*DAPK1*) in 26 patients with breast cancer, 16 patients with benign breast diseases, and 12 age-matched healthy controls. Hypermethylation of at least one gene was detected in 25/26 (96%) cancer patients, in 7/16 (43%) cases with benign breast diseases, and only 1/12 (8%) control subjects. Furthermore, methylation of both genes was found to be associated with ductal type of breast carcinoma. *RASSF1A* was hypermethylated in 18/26 cases (69%) and *DAPK1* in 23/26 (88%). However, *DAPK1* promoter methylation was more pronounced, as 12/23 *DAPK1* methylated cases (52%) were strongly methylated (>75%) compared to the weaker methylation of *RASSF1A* (none of the cases with methylation at the level of >75%). These findings, if confirmed in studies of extended cohorts, may lead to useful clinical application in early diagnosis of breast cancer and better management of the neoplastic disease. © 2010 Elsevier Inc. All rights reserved.

## 1. Introduction

Detection of malignant tumors at an early stage is the key to successful treatment and outcome. Because molecular alterations in neoplastic cells may precede clinically obvious cancer and can be detected at sensitive levels, they have emerged as a promising target for detection.

While original genetic investigations of neoplastic DNA changes were performed at the level of mutational analysis, large-scale deletions or amplifications, and chromosomal instability, newer evidence has shown that epigenetic modifications, such as DNA methylation, contribute to cancer development as well. DNA methylation refers to the addition of a methyl group to the cytosine ring of those cytosines that precede a guanosine (referred to as CpG dinucleotides) to form 5-methylcytosine. CpG dinucleotides are found at increased frequency in the promoter region of many genes, and methylation in the promoter region is frequently associated with "gene silencing" (i.e., the gene is expressed in the absence of methylation) [1]. In breast cancer, the second leading cause in cancer mortality, hypermethylation and hypomethylation of various genes was noted [2]. All these studies, however, require resected tumor samples. An alternative is offered by collecting the patients' peripheral blood. Next to detecting fetal DNA in the blood of pregnant females [3], testing for the presence of extracellular DNA (exDNA) from tumors in patients' serum and plasma has become feasible

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[4–6]. Since such tumor-specific exDNA has been noted to circulate freely and to be bound to blood cells [7], recovery of this DNA and analysis of its malignant modifications have been described for various carcinomas [8–11].

In addition to DNA sequence aberrations, epigenetic effects, including methylation at CpG sites, have proven to be an important parameter of neoplastic DNA, and their detection has become a powerful tool, not only for cancer diagnosis [12], but also for prognosis and response to treatment. One major reason is that both global and locus-specific methylation are linked to malignancy [13–15].

In this study, we examined the promoter methylation status of two normally nonmethylated, biologically significant cancer genes, *RAS* association domain family member 1A (*RASSF1A*) and death-associated protein kinase 1 (*DAPK1*), and their association with different clinicopathologic variables.

## 2. Materials and methods

### 2.1. Serum samples and DNA extraction

After approval of the study scheme by the Ethical Review Board of Tübingen University and obtaining informed consent from the included patients, peripheral blood samples were drawn before therapeutic intervention from 26 breast cancer patients and 16 patients with benign breast diseases who were treated at the Gynecology Department, Tübingen University Hospital. Twelve additional blood samples were drawn from age-matched healthy volunteers to be used as a control group. Blood samples were centrifuged at 2,000 × *g* for 10 minutes at room temperature, and 1-mL aliquots of serum samples were stored at −20°C.

Genomic DNA was extracted from sera using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

### 2.2. Bisulfite treatment and methylation-specific PCR (MSP)

Bisulfite treatment of genomic DNA was performed on the basis of the method developed by Herman et al. [16], with minor modifications described by Chan et al. [17].

The primer sequences for *RASSF1A* and *DAPK1* genes have been reported previously [18,19]. Polymerase chain reaction (PCR) amplification of modified DNA was hot-started using Hot Startaq DNA polymerase (Qiagen), the PCR started by initial denaturation at 95°C for 10 minutes, followed by 37 cycles of denaturation at 95°C for 30 seconds, annealing at 56–65°C for 40 seconds (depending on the respective primer set), and extension for 30 seconds at 72°C, with a final extension step of 5-minute annealing temperature to be used, each of which have been previously optimized for the PCR in our laboratories.

Human blood DNA from a healthy subject methylated by *Sss*I methylase (New England Biolabs, Beverly, MA) was used as a positive control for the methylated primer set in each PCR, and untreated bisulfite-modified genomic DNA from the same subject was used as a positive control for the unmethylated reaction. Water blank was used as a negative control for the PCR amplifications. The amplification products were separated on a 6% polyacrylamide gel and visualized by ethidium bromide staining and UV transillumination. For quantitation of the bands after gel electrophoresis, analysis software from INTAS UV Systems (Göttingen, Germany) was applied.

### 2.3. Statistical analysis

All the statistical analyses were performed with SPSS software 10.0 using chi-square test and correlation analysis. The results were considered statistically significant when $P \leqslant 0.05$.

## 3. Results

We examined the methylation status of the normally unmethylated *RASSF1A* tumor suppressor gene and the cancer gene *DAPK1*. In our study, promoter hypermethylation of both genes was found in patients of all ages, displaying different tumor types, grades, and stages (Table 1). Moreover, we found a statistically significant difference in promoter methylation of either of the genes in our comparison of breast cancer patients to control subjects and patients with benign breast diseases (Figures 1–3, Table 2).

On the basis of the data from the malignant sample group, *RASSF1A* methylation was shown to be associated with higher tumor grade (i.e. poorly differentiated tumors), while higher degrees of *DAPK1* methylation were associated with postmenopausal status. Moreover, promoter methylation of both genes was associated with the ductal tumor types (Table 3).

## 4. Discussion

The development of a palette of improved molecular and cellular techniques resulted in better understanding of genetic events leading to neoplasms. Next to alterations of DNA sequences in tumor cells, like point mutations, deletions, rearrangements, or copy number variations, epigenetic effects (i.e., methylation at CpG dinucleotides) were frequently found to be associated with malignancy. Hypermethylation has been reported in various dysplastic and neoplastic pathologies [20], albeit rarely in 100% of the cases observed [21,22]. Detection of DNA methylation was first assessed by restriction endonuclease pairs (isoschizomers, sensitive/nonsensitive for CpG methylation at the restriction site) and later by MSP using DNA isolated directly from tumor tissues. A more sophisticated approach

Table 1
Breast cancer patients characteristics

| Clinicopathological factors | Sample no. |
|---|---|
| Age | |
| Median, range | 54 years, 35–73 years |
| Menopausal status | |
| Premenopause | 6 (23.1%) |
| Perimenopause | 1 (3.8%) |
| Postmenopause | 19 (73.1%) |
| Family history of breast cancer | |
| Negative | 15 (57.7%) |
| Positive | 11 (42.3%) |
| Tumor type | |
| Ductal | 20 (76.9%) |
| Lobular | 4 (15.4%) |
| Others | 2 (7.7%) |
| Tumor grade | |
| Grade I | 3 (11.5%) |
| Grade II | 16 (61.6%) |
| Grade III | 7 (26.9%) |
| Tumor size | |
| Tis | 4 (15.4%) |
| T1a | 1 (3.8%) |
| T1b | 4 (15.4%) |
| T1c | 12 (46.3%) |
| T 2 | 3 (11.5%) |
| T4d | 2 (7.6%) |
| Lymph node metastasis | |
| pN0 | 21 (80.8%) |
| pN1 | 2 (7.7%) |
| pN2 | 2 (7.7%) |
| pN3 | 1 (3.8%) |
| Metastasis | |
| M0 | 25 (96.2%) |
| M1 | 1 (3.8%) |
| Estrogen receptor status | |
| Negative | 5 (19.2%) |
| Positive | 21 (80.8 %) |
| Progesterone receptor status | |
| Negative | 7 (26.9%) |
| Positive | 19 (73.1%) |
| *RASSF1* methylation | |
| No | 8 (30.8%) |
| < 25% | 5 (19.2%) |
| 25–50 % | 4 (15.4%0 |
| 50–75 % | 9 (34.6%) |
| >75% | 0 |
| *DPAK1* methylation | |
| No | 3 (11.5%) |
| < 25% | 6 (23.1%) |
| 25–50% | 2 (7.7%) |
| 50–75% | 3 (11.5%) |
| > 75% | 12 (46.2%) |
| Methylation of both genes | |
| Non-methylated | 1 (3.9%) |
| One methylated | 9 (34.6%) |
| Both methylated | 16 (61.5%) |

*Abbreviation*: Tis, tumor in situ.

was developed when tumor DNA became detectable in various fluids of cancer patients like urine, blood serum and plasma, and ductal lavage fluids [23–25].

Methylation of various genes was reported in breast cancer, the second leading cause in cancer mortality [26–28]. However, only a limited number of genetic

Table 2
Comparison of methylation of the *RASSF1A* and/or *DAPK1* genes between various studied groups

| | Breast cancer vs. control | Breast cancer vs. benign diseases[a] | Benign diseases vs. control |
|---|---|---|---|
| *RASSF1A* | | | |
| $\chi^2$ | 12.7 | 14.4 | 0.07 |
| *P* | <0.001 | <0.001 | NS |
| *DAPK1* | | | |
| $\chi^2$ | 26.9 | 20.6 | 4.6 |
| *P* | <0.001 | <0.001 | NS |
| Both genes | | | |
| $\chi^2$ | 29.6 | 21.5 | 2.7 |
| *P* | <0.001 | <0.001 | NS |

$P > 0.05$, nonsignificant; $P < 0.05$, significant.

*Abbreviation*: NS, nonsignificant.

[a] Benign breast disease is considered to be present when breast examination, mammogram, and breast sonogram coincide with the diagnosis.

markers were applied in extracellular DNA studies of breast cancer patients (*APC, GSTP1, RASSF1A, RARB, HIC1, ATM,* and *DAPK1*), with detection rates of methylation ranging from 30 to 80% (increasing numbers for more malignant or recurrent cases) [29,30]. Using the patients' peripheral blood, methylation microarray analysis revealed a list of breast cancer susceptibility gene candidates: out of those, *ATM* (ataxia telangiectasia mutated) was studied for methylation patterns at a very high resolution. A highly significant threefold increased risk of breast cancer was associated with decreased steady-state *ATM* mRNA levels, indicating the contribution of this regulatory mechanism and the feasibility of the technical approach [31]. Beyond using peripheral blood, pathologic nipple discharge was applied in a study of *RASSF1A, TWIST1*, and *HIN1* gene methylation, which showed to accurately distinguish between ducts with cancerous versus benign lesions [32]. However, a complete methylation of all CpG islands within a promoter can hardly be expected, as noted for the multiple methylation sites in of *TFF1* gene sequences in stomach cancer [33] or the *DAB2IP* gene in lung cancer [34].

Our study revealed that CpG promoter methylation of *RASSF1A* and/or *DAPK1* genes was found in all malignant

Table 3
Promoter methylation in breast cancer patients stratified by their clinicopathologic characteristics

| | *RASSF1A P* | *DAPK1 P* | Both genes *P* |
|---|---|---|---|
| Menopausal status | NS | <0.05 | NS |
| Family history | NS | NS | NS |
| Tumor histologic type | NS | NS | <0.05 |
| Tumor grade | <0.05 | NS | NS |
| Tumor size | NS | NS | NS |
| Invaded nodes | NS | NS | NS |
| Metastasis | NS | NS | NS |
| Estrogen receptor | NS | NS | NS |
| Progesterone receptor | NS | NS | NS |

*Abbreviation*: NS, nonsignificant.

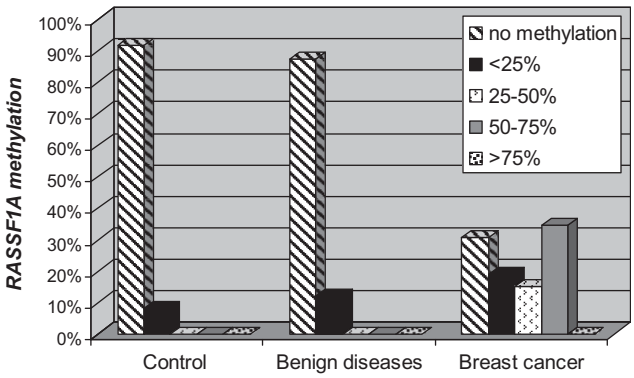


Fig. 1. *RASSF1A* methylation levels in the control, benign breast disease, and breast cancer groups compared to each other.

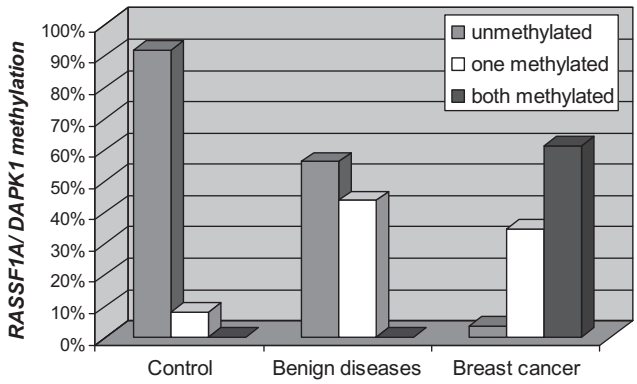


Fig. 3. Summary of methylation for both genes in the control, benign breast disease, and breast cancer groups compared to each other.

cases with the exception of only one case (Table I). All four cases with ductal carcinoma in situ displayed promoter hypermethylation of both studied genes. Furthermore, out of 17 cases with invasive carcinoma stage T1, 9 cases were showing hypermethylation of both genes, while 7 cases showed hypermethylation of only 1 gene, and only 1 case of invasive lobular carcinoma was noted to be nonmethylated. At least one gene was found to be methylated in patients with higher stages of neoplasia (stages T2 and T4). These findings are supported by a previous study that detected the presence of hypermethylated tumor suppressor genes in serum DNA and corresponding tumor DNA of pre-invasive and stage 1 breast carcinoma [30]. This indicates that hypermethylation can be a relatively early event in breast tumorigenesis, hence stressing its clinical importance.

Moreover, we detected hypermethylation of at least one gene in 7/16 cases with benign lesions (43%). Thus, our comparison of epigenetic information for the *RASSF1A* and *DAPK1* genes showed a highly significant difference in methylation of both genes in malignant cases versus patients with benign breast diseases or healthy controls (Table 2). The methylation status of both genes was not associated with age, presence of hormone (estrogen/progesterone) receptors, or TNM classification (T, tumor grading; N, lymph node status; M, metastatic status). Methylation of both promoters, however, was associated with the ductal tumor type (Table 3). A study performed with DNA extracted from tumor tissue and pyrosequencing for methylation status disclosed that out of 13 tumor suppressor genes, only 4 of them correlated with hormone receptor status [35]. In our collection, a more pronounced promotor methylation was apparent in the *DAPK1* gene, 12/26 cases (46%) were strongly methylated ($>75\%$), while *RASSF1A* showed none of such strong methylation (Table 1). Also, only 34% of cases (9/26) displayed a weak *DAPK1* methylation ($<25\%$ to none), and in *RASSF1A,* 50% of cases (13/26) were weakly methylated. The data from our set of patients suggest that *DAPK1* appears to be a more significant epigenetic marker than *RASSF1A*, but a larger sample number will be needed to substantiate this observation. Moreover, additional clinical data, such as the patients' status on hormone replacement therapy and intensified screen with additional markers, may not only improve analytical sensitivity, but also shed light on the biology of breast cancer development. Thus, it may have a useful clinical application as early diagnosis, providing new prognostic tools, identifying patients at higher risk of relapse, and finally allowing safe management and better outcome for breast cancer patients.

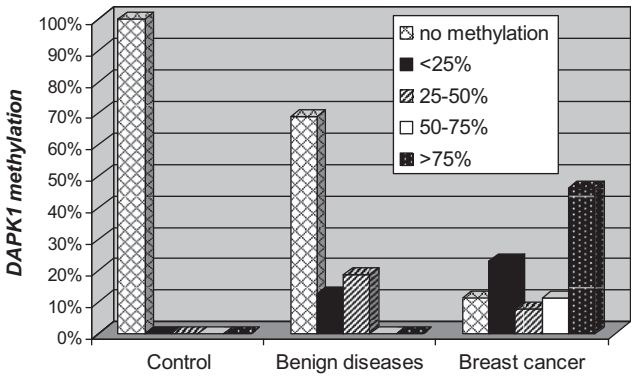


Fig. 2. *DAPK1* methylation levels in control, benign breast disease, and breast cancer groups compared to each other.

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