Hypermethylation of the CpG Island of Ras Association Domain Family 1A (RASSF1A), a Putative Tumor Suppressor Gene from the 3p21.3 Locus, Occurs in a Large Percentage of Human Breast Cancers

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ABSTRACT

The human Ras association domain family 1A gene (RASSF1A), recently cloned from the lung tumor suppressor locus 3p21.3, was shown to be hypermethylated in primary lung tumors, and reexpression of RASSF1A suppressed the growth of lung cancer cells (R. Dammann et al., Nat. Genet., 25: 315–319, 2000). In this study, we analyzed the expression and possible alterations of RASSF1A in breast cancer. In five breast cancer cell lines (MCF7, MDAMB157, MDAMB231, T47D, and ZR75–1), the CpG island and promoter of RASSF1A was completely methylated, and transcription was silenced. Treatment with the DNA methylation inhibitor 5-aza-2’-deoxycytidine reactivated the expression of RASSF1A. In 28 of 45 (62%) primary mammary carcinomas, the promoter of RASSF1A was highly methylated at its CpG sites. Coincident with methylation, the expression level of RASSF1A was lower in tumors compared with matching normal tissues. No somatic mutations were found in the samples that were unmethylated. The data suggest that hypermethylation of the CpG island promoter of RASSF1A may play an important role in breast cancer pathogenesis.

INTRODUCTION

Breast cancer is one of the most common malignancies in the world (1). Only about 7% of breast cancer cases in the United States are thought to be attributable to the presence of a autosomal dominant susceptibility allele (2). Two familial breast cancer genes (BRCA1 and BRCA2) have been identified (3). However, mutations in these genes are relatively rare in the general population (4), and mutations are extremely uncommon in sporadic breast cancers (5–8). Alterations or loss-of-function mutations in the coding region of a gene (9). It is very likely that additional genes contribute to inherited and sporadic breast cancer.

In general, both alleles of a tumor suppressor gene need to be inactivated by genetic alterations such as chromosomal deletions or loss-of-function mutations in the coding region of a gene (10). As an alternative mechanism, epigenetic alterations of tumor suppressor genes may occur in human cancers resulting in gene inactivation. Recent studies (11–13) have demonstrated that the CpG islands in the RB, p16, VHL, APC, and BRCA1 genes are frequently methylated in a variety of human cancers.

CpG islands that are hypermethylated in breast cancer are those of the genes coding for estrogen receptor (14–16), retinoic acid receptor (17), E-cadherin, and HER-2/neu (9). It is very likely that additional genes contribute to inherited and sporadic breast cancer.

Loss of genetic material from chromosome 3p21.3 is one of the most common and earliest identified events in the pathogenesis of lung cancer (28). LOH (39) at 3p21.3 is not limited to lung tumors, indicating that this region may harbor a broad-spectrum tumor suppressor gene (29). In breast cancer, LOH frequencies have been reported that are in the range of 25–35% using 3p21 markers (30–35). Frequent LOH and the presence of homozygous deletions suggest a critical role of the region 3p21.3 in tumorigenesis, and recently a region of common homozygous deletion in 3p21.3 was narrowed to 120 kb using several lung cancer cell lines and a breast cancer cell line (36).

In previous work (37), we have cloned and characterized the Ras association domain family 1A gene (RASSF1A) located within this minimal homozygous deletion region and found that this gene is epigenetically inactivated in 40% of primary lung cancers. Reexpression of RASSF1A in lung cancer cells reduced colony formation, suppressed anchorage-independent growth, and inhibited tumor formation in nude mice.

In this study, we investigated the methylation status, expression, and mutation of RASSF1A in primary breast tumor samples and in breast cancer cell lines.

MATERIALS AND METHODS

Cell Lines and Tissues. The breast cancer cell lines MCF7, MDAMB157, MDAMB231, T47D, and ZR75–1 were obtained from the American Type Culture Collection and were cultured in the recommended growth medium. All of the nonmicrodissected primary frozen breast tumors were classified and obtained from the Pathology Department of the City of Hope National Medical Center (Duarte, CA). Each tumor was scored based on formation of tubules, nuclear pleomorphism, and mitotic activity. Each of these was assigned a value of 1–3, and then the totals were added for a score and grade assignment (38).

Methylation Analysis. DNA was isolated from cells and tumors, and the methylation status of the RASSF1A promoter region was determined by a bisulfite genomic sequencing protocol (37, 39). Briefly, 1 µg of genomic DNA was denatured in 0.3 M sodium hydroxide for 15 min at 37°C. Cytosines were sulfonated in 3.12 M sodium bisulfite (Sigma Chemical Co., St. Louis, MO) and 5 mM hydroquinone (Sigma) in a thermocycler for 16 h at 55°C. The DNA samples were desalted through columns (Wizard DNA Clean-Up System; Promega), desulfonated in 0.3 M sodium hydroxide, and precipitated. PCR products were amplified by mixing 100 ng of bisulfite-treated DNA with primers MU379 (5’GGTTTGGTATGTAAATGTTAGTTGTGTTTTT) and ML730 (5’ACCCCTTCTCCCTAACAATAAAACTAACC) in 100 µl of reaction buffer containing 200 µM of each dNTP and Taq polymerase (Roche Diagnostics Corp., Indianapolis, IN) and incubated at 95°C for 15 s, 55°C for 15 s, and 74°C for 30 s for 20 cycles. A semi-nested PCR was performed using 1 of 50 of the initially amplified products and an internal primer ML561 (5’CCCCCAATCCCCCAACGCAACGAC) and primer MU379 with similar conditions as described for the preceding PCR amplification but for 30 cycles. The PCR products were purified using QIAquick PCR purification kits (Qia-gen, Valencia, CA). Products were sequenced directly to obtain average methylation levels. PCR products containing bisulfite-resistant cytosines were ligated into the pcCR2.1 vector (Invitrogen, Carlsbad, CA), and several clones were sequenced for confirmation. All of the described sequences were determined by cycle sequencing and run on an ABI 377 automated DNA sequencer.
The percentage of methylated alleles was estimated from the relative peak heights of the G and A peaks at methylated CpG sequences after normalizing the A peak for the average peak height along the entire sequence and subtracting the background for the G peak.

For the restriction enzyme analysis of PCR products from bisulfite-treated DNA (40), 50 ng of the PCR products were digested with 10 units of TaqI (New England Biolabs, Beverly, MA) according to conditions specified by the manufacturer of the enzyme and analyzed on a 2.2% Tris-borate EDTA agarose gel.

**RT-PCR Analysis.** Total RNA from cells or tissues was isolated by the guanidinium isothiocyanate method (RNAgents; Promega). RT-PCR was essentially performed as described (37). Briefly, 100 ng of RNA was preassociated with a lower primer from exon 4. After the reverse transcription reaction, half of the samples were pipetted into tubes containing PCR master mix and an upper primer from exon 2, and the remaining half were added into tubes containing PCR mix and an upper primer from exon 2β. These conditions selectively amplify transcripts RASSF1A and RASSF1C, respectively. PCR conditions were 95°C for 30 s, 60°C for 30 s, and 74°C for 1 min for 20 cycles for the RASSF1 gene and 15 cycles for the GAPD gene. These cycle numbers were chosen because they were in the exponential range of product amplification. PCR products were separated on 2% Tris-borate EDTA agarose gels, blotted, hybridized with a labeled probe from exon 3, and visualized by autoradiography.

**Reexpression of RASSF1A.** Breast cancer cell lines were treated with 5-Aza-CdR (Sigma). Cells (2 × 10⁶) each were grown for 4 days in the presence of different concentrations of 5-Aza-CdR. RNA was isolated, and RT-PCR was performed as described above.

**Mutation Screening.** Exon sequences were amplified by mixing 200 ng of genomic DNA with 10 pmol of each exon-specific primer in 100 μl of reaction buffer containing 200 μM of each dNTP and Taq polymerase (Roche Diagnostics Corp.) and incubated at 95°C for 30 s, 60°C for 30 s, and 74°C for 1 min for 35 cycles. PCR products were purified using QIAquick PCR purification kit (Qiagen), and both strands were sequenced directly without subcloning.

**LOH Analysis.** The microsatellite markers used were D3S4615/LUCA8.1 (28). For PCR amplification, one of the primers was end-labeled with γ[32P]ATP and T4 polynucleotide kinase (New England Biolabs). PCR was carried out in a 25-μl volume containing 50 ng of genomic DNA, 12.5 pmol of each primer, 200 μM of each dNTP, and Taq DNA polymerase (Roche Diagnostics Corp.) at 95°C for 20 s, 54°C for 20 s, and 74°C for 30 s for 35 cycles. PCR products were separated on an 8% denaturing polyacrylamide gel and visualized by autoradiography. LOH was defined as a more than 50% reduction of intensity in one of the two alleles as compared with that seen in the corresponding normal control.

**RESULTS**

In previous work, we have shown that the transcript of the RASSF1A gene was missing in lung cancer cell lines and that loss of expression correlated with hypermethylation of the CpG island promoter sequence of RASSF1A (37). To elucidate the status of RASSF1A during breast cancer pathogenesis, we analyzed the methylation pattern of five breast cancer cell lines (MCF7, MDAMB157, MDAMB231, T47D, and ZR75–1). We used bisulfite sequencing of genomic DNA (39) to determine the methylation status of the CpG island, in which RASSF1A transcription initiates. In this method, sodium bisulfite is used to convert all of the unmethylated cytosines to uracils and then to thymines during the subsequent PCR step. Because 5-methylcytosine remains nonreactive, all of the cytosines after sequence analysis represent only methylated cytosines. Therefore, all of the guanines present after sequencing in Fig. 1 are derived

![Fig. 1. Methylation analysis of the RASSF1A gene in breast cancer. Sequences of PCR products from bisulfite-treated DNA for the CpG island spanning the RASSF1A promoter were obtained from different cancer and normal samples. Methylated cytosines appear as a G signal in the complementary strand (bold Gs). The sequence is shown before and after bisulfite conversion at the top. The position of a TaqI restriction site is underlined. Two Sp1 consensus binding sites are boxed.](image-url)
Furthermore, we analyzed the methylation status of the RASSF1A promoter in 45 primary breast cancer samples obtained from the City of Hope Medical Center (Fig. 1). Of the 45 mammmary carcinomas analyzed, 28 (62%) were methylated in the promoter region. The methylation data are summarized in Table 1. Forty-one of these 45 cases were ductal carcinomas representing all of the grades of invasion. Of the two lobular carcinomas analyzed, one was methylated, and one colloid carcinoma was methylated. One phyllode cystosarcoma was not methylated. All of the three grade I carcinomas were methylated but at a lower degree than the corresponding tumor samples. In the 17 samples analyzed, no base changes were found. A second method to analyze the PCR fragments obtained from bisulfite-treated DNA is by further digestion with a restriction enzyme that has a CpG in its consensus sequence (40). TaqI (5’TCA-G’3’) will cut only previously methylated DNA after bisulfite treatment and PCR. The consensus sequence will be lost in unmethylated samples. The analyzed 205-bp fragment has two TaqI sites. Restriction digestion of methylated fragments results in three bands (90, 81, and 34 bp; the two larger ones migrating together). PCR fragments from the breast cancer cell lines MCF7 and T47D showed complete methylation at these TaqI sites (Fig. 2). The restriction digest of MDAMB231, MDAMB157, and ZR75–1 also showed 100% methylation (data not shown). In Fig. 2, we analyzed three primary ductal carcinomas by TaqI restriction (BC10, BC11, and BC12). The tumor samples showed approximately 50–60% methylation of the TaqI sites. The matching normal DNA samples were unmethylated. These data and several others (data not shown) confirm the results obtained by direct sequencing.

We analyzed the expression of RASSFIA in breast cancer cell lines by RT-PCR (Fig. 3). ZR75–1, T47D, MDAMB231, and MCF7 (Fig. 3), as well as MDAMB157 (data not shown), showed only traces of expression. At the same time, the alternative transcript RASSFIC (37) was present at high levels in all of the cell lines. Epigenetic inactivation of genes by DNA methylation can be reversed by treatment with the DNA methylation inhibitor 5-Aza-CdR (41). The four cell lines were treated with this compound for 4 days at various concentrations. 5-Aza-CdR reactivated expression of RASSFIA in all of the four cell lines (Fig. 3).

We also performed RT-PCR on primary tumors (Fig. 4). This analysis is more difficult because normal cells are present, and the RNA quality is not always very good. We were able to obtain good quality RNA from four matched samples of normal breast tissue and tumor in which the tumor DNA was methylated. In Fig. 4, we show by quantitative RT-PCR that expression of RASSFIA is three to ten times lower in the tumor tissues compared with the normal matching tissues.

Mutation analysis was carried out to search for changes in the coding sequence of RASSFIA in those samples that were unmethylated. In the 17 samples analyzed, no base changes were found. A larger series of sequencing data are shown in Fig. 1b. These nonmicrodissected breast tumors show an estimated frequency of methylated alleles of between 53 and 74%. With the exception of one CpG site in sample BC29, which showed only a low frequency of methylation, all of the other CpG sequences had a similarly uniform range of methylation levels of greater than 50% (Fig. 1b and data not shown).

Table 1. Summary of the methylation analysis

<table>
<thead>
<tr>
<th>Case</th>
<th>Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 breast cancer cell lines (MCF7, MDAMB157, MDAMB231, T47D, and ZR75–1)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>45 primary breast tumors</td>
<td>28 (62%)</td>
</tr>
<tr>
<td>41 ductal carcinomas</td>
<td>26 (63%)</td>
</tr>
<tr>
<td>2 lobular carcinomas</td>
<td>1 (30%)</td>
</tr>
<tr>
<td>1 colloid carcinoma</td>
<td>Methylated</td>
</tr>
<tr>
<td>1 phyllode cystosarcoma</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>3 grade I</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>15 grade II</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>15 grade III</td>
<td>9 (60%)</td>
</tr>
<tr>
<td>12 grade unknown</td>
<td>8 (67%)</td>
</tr>
<tr>
<td>40 matching normal tissues</td>
<td>3 (7.5%)</td>
</tr>
</tbody>
</table>

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DISCUSSION

In our previous work (37), we have cloned and characterized RASSF1A. RASSF1A was epigenetically inactivated in 40% of the analyzed primary non-small cell lung tumors and in several different cancer cell lines. In this study, we demonstrate that in primary breast tumors the RASSF1A promoter is methylated at an even higher frequency (62%). The degree of methylation at CpG sites in these nonmicrodissected carcinomas ranged from 50 to 75% and was nearly 100% for all of the five analyzed breast cancer cell lines. A degree of methylation of more than 50% would suggest either that both alleles are methylated or that one allele is methylated and the other one is lost. LOH of 3p21 for breast cancer was reported to be between 25 and 35% (30–35). Using the microsatellite marker D3S4615/LUCA8.1, which is approximately 140 kb proximal to the RASSF1 gene, we found LOH in 3 of 19 (16%) of informative cases. Two of the samples with LOH were methylated.

Methylation and LOH may be the major loss of function pathways for the RASSF1A gene because somatic mutations appear to be rare in this gene (37). Interestingly, we could detect a constant methylation frequency of RASSF1A in all of the different grades of the mammary carcinomas. RASSF1A inactivation was already very high in grade I tumors (Table 1). Thus, methylation of RASSF1A may be an early event during breast cancer pathogenesis. We could detect some methylation in 7.5% of the samples, which were classified as normal tissue removed with tumor surgery. It will be interesting to investigate whether this methylation occurs as part of the aging process, a phenomenon that has been described for other genes (42).

Hypermethylation of the RASSF1A promoter appears to be the main mechanism of inactivation. Our data support the revised Knudson two-hit theory (12). In this new hypothesis, epigenetic mechanisms of gene inactivation are included. Epigenetic silencing was shown to be a common mechanism for loss of function for several tumor suppressor genes including p16, VHL, MLH1, and also BRCA1. BRCA1 promoter methylation of sporadic breast carcinomas was at least four times less frequent compared with the hypermethylation of RASSF1A (20–23). The precise function of RASSF1A is still unclear, and more biochemical and genetic data are needed to understand its role in tumorigenesis. In a recent study, Vos et al. (43) have shown that RASSF1C binds RAS in a GTP-dependent manner, similar as its closest homologue, the mammalian Ras effector Nore1 (44). Overexpression of RASSF1C induced apoptosis. Because isoforms RASSF1A and RASSF1C share the identical Ras association domain, it is possible that RASSF1A will bind to RAS in the same manner as RASSF1C. Activated RAS proteins are usually associated with growth enhancement and transformation. However, RAS also can induce growth inhibitory effects manifested by senescence (45), terminal differentiation (46), or apoptosis (47). RASSF1 might be responsible for the RAS-dependent growth inhibition through its proapoptotic function (43). Loss of RASSF1 expression by methylation in human cancer may shift the balance of RAS activities toward a growth-promoting effect without the necessity of RAS-activating mutations.

None of the genes located within the 3p21.3 homozygous deletion region was found to be mutated in more than 5–10% of lung tumors (48). This supports the assumption that the putative 3p21.3 tumor suppressor gene is inactivated by mechanisms other than mutation of the coding sequence. The high frequency of epigenetic inactivation of the RASSF1A gene in breast cancer supports its role as a putative tumor suppressor gene.

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