Expression and promoter methylation of the *RASSF1A* gene in sporadic breast cancers in Chinese women

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Abstract. The novel tumor suppressor *RASSF1A* is frequently inactivated during human tumorigenesis by promoter methylation. In this study, we detected the *RASSF1A* promoter methylation by methylated-specific PCR and investigated *RASSF1A* gene expression by semi-quantitative RT-PCR and immunohistochemical staining in 36 cases of breast cancer and their adjacent normal tissues in Chinese women. The promoter methylation of the *RASSF1A* gene was found to be a frequent event in the breast cancers (61.1%). *RASSF1A* methylation was not found in the matched adjacent normal tissues. The loss frequency of *RASSF1A* mRNA was 33.3% and that of the RASSF1A protein was 44.4% in breast cancers. RASSF1A mRNA and protein were all expressed in adjacent normal tissues. The mRNA and protein expression level of RASSF1A was significantly lower in breast cancer than in adjacent normal tissue. However, the promoter methylation of the *RASSF1A* gene in breast cancers were not correlated with clinical parameters, such as ages, histological types, TNM stages and lymph node metastases. Thus, the promoter methylation of *RASSF1A* was one reason for the low level of RASSF1A mRNA and protein expression and was a frequent event in primary sporadic breast tumorigenesis in Chinese women.

Introduction

The Ras association domain family 1 (*RASSF1*) gene is located at 3p21.3 with a frequent loss of heterozygosity and homozygosity in a variety of human tumors. The *RASSF1* locus encodes two major transcripts and several tissue-specific splice variants, the most commonly detected being *RASSF1A* and *-C*. RASSF1A and -C proteins contain a Ras association domain. RASSF1A also contains a diacylglycerol-phorbolester binding domain near its NH_2 terminus (1-4). Loss of expression of the RASSF1A isoform is a frequent event in primary human tumors and the re-expression of RASSF1A in human tumor cell lines inhibits their tumorigenic phenotype (5). Moreover, knock-out mice, defective for RASSF1A, are prone to tumor development (6). It has been concluded that *RASSF1A* is a tumor suppressor that plays a key role in the development of human cancer. Inactivation of *RASSF1A* in human tumors typically occurs via promoter methylation (6-8). *RASSF1A* methylation occurs in a broad spectrum of carcinomas and other solid tumors. In several types of tumors, *RASSF1A* is inactivated at a high frequency, ranging from 40 to 50% in breast, prostate, and ovarian cancer to 30-80% in lung tumors and >90% in renal cell carcinomas (9). *RASSF1A* is probably the most frequently methylated gene described thus far in human cancer.

Different conclusions have been drawn about promoter methylation and the expression of *RASSF1A* in breast cancer (10-12) and some research has been done on the *RASSF1A* gene in breast tumors in Chinese women (13). We investigated 36 sporadic breast cancer cases and adjacent normal tissues in Chinese women. MSP (methylated-specific PCR), semi-quantitative RT-PCR and immunohistochemical staining were used to investigate the promoter methylation status and expression of *RASSF1A* gene in these tissues.

Materials and methods

Patient tissue samples and histological classification. The study group consisted of 36 female breast cancer patients aged 24-80 years. The patients were diagnosed at the First Affiliated Hospital of Nanjing Medical University (Jiangsu, China) during 2003-2004 and the patient samples were collected according to the ethics committee of the First Affiliated Hospital of Nanjing Medical University. Primary breast cancers and their nomal tissues (5 cm away from the adjacent tumor tissue) were obtained from each of the patients. None of them had received radiotherapy or chemotherapy. All the breast cancer patients were classified according to the World Health TNM criteria (4 of stage I, 17 of stage II, 12 of stage III and 3 of stage IV). Of these patients, 27 developed lymphatic metastasis. Data on tumor

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Abbreviations: RASSF1, Ras association domain family 1; MSP, methylated-specific PCR

Key words: methylation, breast cancer, *Ras association domain family 1 A*

Table I. The PCR primers and amplification size.

Primer	Primer sequence (5' → 3')	Size (bp)
RASSFIA (U)	F:5'-GGGGGTTTTGTGAGAGTGTGTTT-3' R:5'-CCCAATTAAACCCATACTTCACTAA-3'	204
RASSFIA (M)	F:5'-CGAGAGCGCGTTTAGTTTCGTT-3' R:5'-CGATTAAACCCGTACTTCGCTAA-3'	192
RASSF1A	F:5'-CAGATTGCAAGTTCACCTGCCACTA-3' R:5'-GATGAAGCCTGTGTAAGAACCGTCCT-3'	249
ß-actin	F:5'-AAAGACCTGTACGCCAACAC-3' R:5'-GTCATACTCCTGCTTGCTGAT-3'	219

U, unmethylated PCR primers; M, methylated PCR primers.

grade, histological type and lymphatic metastasis of the tumors were obtained from the Department of Pathology in the hospital. The tissues were first placed in liquid nitrogen and then frozen at -86˚C until the extraction of total RNA and DNA.

Bisulfite modification. DNA was extracted from breast cancer tissues by standard proteinase K digestion, phenol chloroform and ethanol precipitation proceeded. The method of bisulfite modification was performed according to previous methods (14,15), with some modifications. DNA (1 μg) in a volume of 45 μl was denatured by NaOH (final concentration of 0.3 mol/l) at 37˚C for 20 min and then treated with 3 mol/l sodium bisulfite 520 μl (pH 5.0) and 10 mmol/l hydroguinone 30 μl at 50˚C for 16 h. Tumor and control DNA were purified using the Wizard DNA clean-up system (Promega, USA) following the manufacturer's recommendations. The modified DNA was re-suspended in 20 μl TE (pH 8.0) and stored at -20˚C.

Methylation-specific polymerase chain reaction amplication. The primer sequences were determined according to previous reports (5) (Table I). The size of the PCR products for the methylated *RASSF1A* promoter was 204 bp and for the unmethylated *RASSF1A* promoter 196 bp. The solution (25 μl) contained 50 ng/μl of modified DNA, 10 pmol of each primer, 0.45 mM dNTP, 1.25 units HotStartTaq enzyme mixture supplied with 1X reaction buffer. The PCR was carried out in a thermocycler (PTC-100 Peltier thermal cycler, USA) under the following conditions: one cycle of 95˚C for 5 min, followed by 40 cycles of denaturing at 95˚C for 1 min, annealing at 56˚C for 1 min and extension at 72˚C for 1 min. This was followed by the final extension at 72˚C for 10 min. The PCR products were analysed by electrophoresis on 2% agarose gel and samples were evaluated. Methylation was considered positive only when there were obvious bands displayed on the agarose gel.

PCR product sequencing. PCR products were recovered by using a Quick gel extration kit. The ABI PRISM Big Dye Terminator kit was used to perform the direct sequencing. The product of sequencing PCR included 16 CpG sites.

RNA extraction and semi-quantitative RT-PCR. The primers of the *RASSF1A* gene were according to previous reports (1). Total RNAs were isolated from the sample tissues using a TRIzol procedure (Invitrogen, USA). An equal amount of RNA from each sample was added to 20 μl of reaction mixture and cDNA was synthesized by Rever Tra Ace-@ kit (Toyobo, Japan). PCR was performed using a PCR thermal cycler. The cycle was defined at 95˚C for 5 min, followed by 35 cycles of denaturing at 95˚C for 1 min, annealing at 60˚C for 1 min and extension at 72˚C for 2 min. This was followed by the final extension at 72˚C for 10 min. The size of the *RASSF1A* gene products was 249 bp. For ß-actin gene amplification, the thermal cycle was defined at 95˚C for 5 min, followed by 27 cycles of denaturing at 95˚C for 1 min, annealing at 60˚C for 1 min and extension at 72˚C for 2 min. This was followed by the final extension at 72˚C for 10 min. The size of the ß-actin gene products was 219 bp. The PCR products were analysed on 2% agarose gel.

Immunohistochemistry. A *RASSF1A* protein expression analysis was performed on formalin-fixed and paraffin-embedded malignant breast tissue and adjacent normal tissue (sliced in 5 μm sections), with RASSF1 (N-15) antibody (Santa Cruz, USA). Tissue sections were deparaffinized and stained according to the routine standard procedures. A routine streptavidin-biotin protocol using the SABC kit (SA1023-lgG, Bosten, China) was applied. The tissue sections mounted on glass slides were first incubated in 0.5% BSA in PBS to reduce non-specific protein binding and then sequentially incubated to react with monoclonal anti-RASSF1A primary antibody (1:200) overnight at 4˚C. For antibody detection all slides were incubated with StreptABComplex/biotin reagents following the manufacturer's recommendations. Biotin sites were visualized with 3,3'-diaminobenzidine (DAB) and counter-staining was performed with hematoxylin. The presence of RASSF1A was detected by light field microscopy as a dark brown reaction product in cytoplasm.

Statistical analysis. Proportions were compared by two-tailed Fisher's exact test. Associations with P-values <0.05 were considered to be significant.

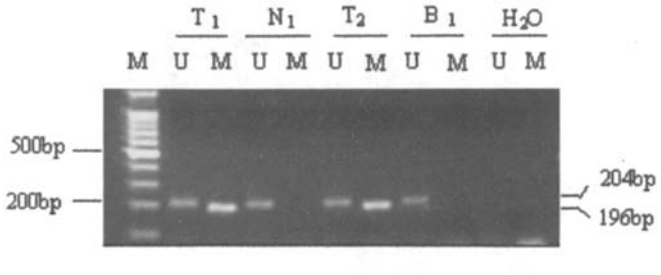

Figure 1. Methylated-specific PCR (MSP) analysis for methylation of the RASSF1A gene promoter in tumors. T1 and T2, breast cancer tissues; N, adjacent normal tissue, B1, normal tissue; H_2O, negative control; M, methylated and U, unmethylated.

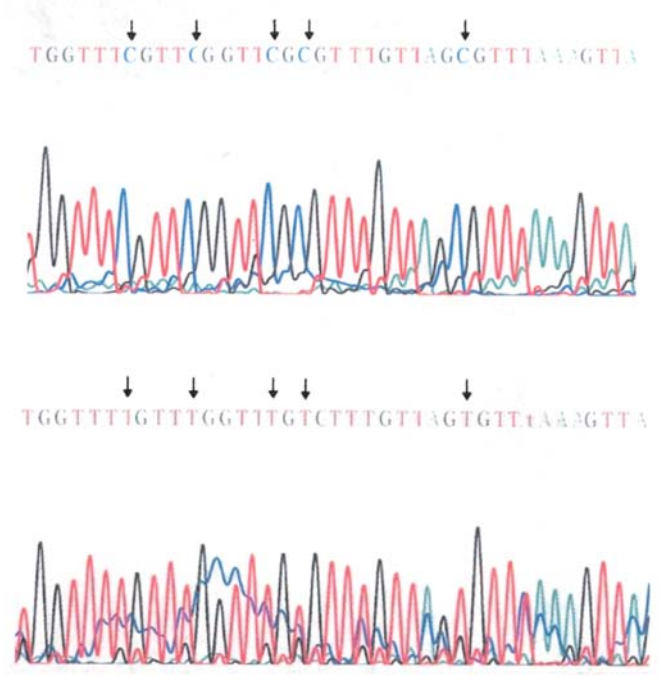

Figure 2. Methylation analysis of *RASSF1A* promoter by bisulfite sequencing. Two representative sequences showed the methylation status of cytosines at the target sites of MS-PCR primers. Methylated cytosines found in tumor cases remained unchanged as cytosines after bisulfite modification, whereas unmethylated cytosines were converted and sequenced as thymine.

Results

Methylation of RASSF1A. The *RASSF1A* promoter methylation was assayed using MSP. Genomic DNAs from breast cancer tissues and adjacent normal tissues were amplified by PCR with different primers for methylated and unmethylated DNA, respectively. Twenty-two (66.6%) of 36 breast cancer tissues showed promoter hypermethylation of *RASSF1A*. None of the adjacent normal tissues showed promoter methylation (Fig. 1). There was a significant difference in *RASSF1A* promoter hypermethylation between breast cancer tissues and adjacent normal tissues (P<0.05). This result suggested the gene promoter methylation was more common in breast cancer than in normal tissues.

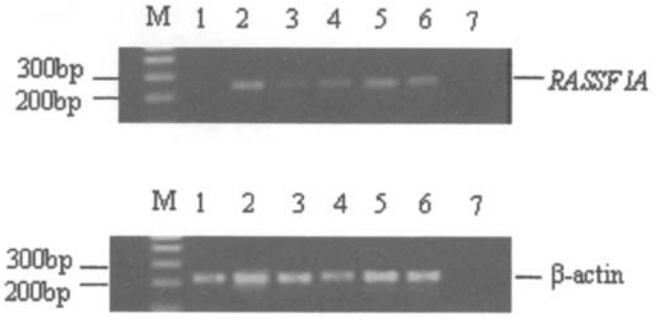

Figure 3. mRNA expression of *RASSF1A* gene. M, 100 bp DNA standard; 1 and 3, specimen of breast cancer; 2 and 4, matching adjacent normal tissues; 5 and 6, breast tumors; 7, negative control; upper panel, *RASSF1A* and lower panel, ß-actin.

RASSF1A CpG island sequencing analysis. Bisulfite sequencing analysis (Fig. 2) performed on the breast cancer tissues with fresh-frozen DNA and positive MS-PCR reaction, confirmed that these samples had methylated CpG islands in tumor tissues but not in the corresponding adjacent normal tissues of breast cancer tissues.

RASSF1A mRNA expression. Twelve cases of 36 breast cancer tissues did not show any detectable mRNA expression of the *RASSF1A* gene but the adjacent normal tissues all showed mRNA expression of *RASSF1A* gene. The mean relative intensity of the mRNA expression of the *RASSF1A* gene in breast cancer and adjacent normal tissues was 0.18264±0.1181 and 0.5561±0.2693, respectively (Fig. 3). The level of mRNA expression of the *RASSF1A* gene in breast cancer tissues was significantly lower than in adjacent normal tissues (P<0.05). Promoter hypermethylation was correlated with inactivation or a low mRNA expression of the *RASSF1A* gene.

RASSF1A protein expression. Out of 36 breast cancer tissues, 16 (44.4%) cases did not show a protein expression of the *RASSF1A* gene while 10 cases showed a reduced RASSF1A protein expression and the adjacent normal tissues showed RASSF1A protein expression. There was a significant difference in the RASSF1A protein expression between breast cancer and the adjacent normal tissues (P<0.05) (Fig. 4). Breast cancer tissues (20 out of 22) whose RASSF1A promoter was methylated had a reduced or no RASSF1A protein expression.

Tumor grade and histological type. The frequency of promoter hypermethylation in breast cancer tissues was not correlated with clinical parameters, such as age, histological type, TNM stage and lymph node metastases (P>0.05) (Table II).

Discussion

Biological features of breast cancer are determined by its underlying molecular alterations of the tumor, including the inactivation of tumor suppression genes, as well as mutations and deletions. It is now clear that the *de novo* promoter hypermethylation is a common mechanism to the inactive tumor suppressor. DNA methylation is one of epigenetic

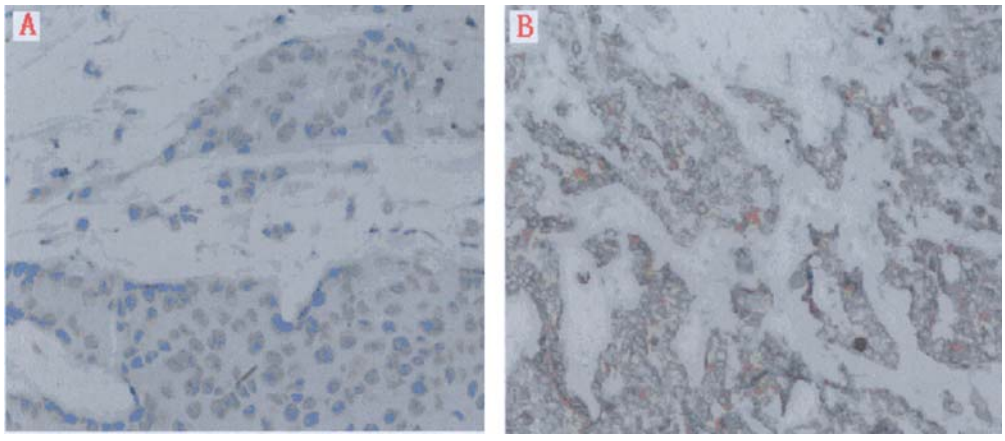

Figure 4. (A) Immunohistochemical expression of RASSF1A in breast cancer tissue, showing a markedly reduced expression of RASSF1A protein. (B) Immunohistochemical expression of RASSF1A in adjacent normal tissues, showing a wild-type expression of the RASSF1A protein.

Table II. The relationship between the promoter methylation of *RASSF1A* and clinicopathological features.

	Cases	Cases of methylation	Frequency of methylation (%)	P
Age				
≤50 years	17	9	52.9	>0.05
>50 years	19	13	73.5	>0.05
Clinical stage				
Stage I	4	2	50.0	>0.05
Stage II	17	12	70.6	
Stage III	12	6	50.0	
Stage IV	3	2	66.7	
Histological subtype				
Colloid carcinomas				
Lobular carcinomas	3	1	33.3	>0.05
Lymph node metastasis	33	21	63.6	
Yes	27	18	66.7	
No	9	4	44.4	>0.05

modifications (16). It occurs at cytosine bases located 5' to a guanosine and so-called CpG dinucleotide short regions of CpG dinucleotides known as CpG islands are found in the proximal promoter region of over half of human genes (17). The methylation of these gene promoters is generally not detected in normal tissues but in the hypermethylation of CpG islands resulting in a loss of gene function, which is a common feature in many tumor types, including breast cancer.

As an important tumor suppressor gene, *RASSF1A* inactivation has been detected in a variety of malignancies and it inactivated at a high frequency. For example, the methylation of *RASSF1A* is found in 80% of small cell lung cancers (7,8), in 90% of hepatocellular carcinomas (19-21) and >70% of prostate cancers (21,22). Although data on the methylation of the *RASSF1A* gene in breast cancer in some countries (5,11,12,23) exist, we reported here on the methylation status of 36 breast cancers and normal adjacent tissues in Chinese women. It was found that the frequency of the methylation of breast cancers was 61.1% but none was found in the adjacent normal tissues. The frequency of the promoter methylation in breast cancers was significantly higher than that in normal tissues. Clinical data showed that the gene methylation status of *RASSF1A* did not correlate with clinical parameters, such as patient age, histological type, clinical stage and lymph node metastases, indicating that the methylation of *RASSF1A* gene is an early molecular change in breast cancer tumorigenesis.

We analysed the mRNA level of *RASSF1A* gene in breast cancers using semi-quantitative RT-PCR. Breast cancer tissues had a loss of mRNA expression (33.3%), however, adjacent normal tissues all showed mRNA expression. Moreover, the level of mRNA expression in breast cancer was significantly lower than that in adjacent normal tissues. This result indicated that the low level or loss of *RASSF1A* expression played a role in breast cancer development. Dammann *et al* (8) found that mRNA expression in breast

cancers decreased significantly as compared to adjacent normal tissues and found that 5 breast cancer cell lines (MCF-7, MDAMB15, MDAMB23, T47 and ZR75-1) indicated a loss of mRNA expression of *RASSF1A*. The CpG islands and *RASSF1A* promoter were completely methylated. Treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine reactivated the expression of RASSF1A. The experiment further indicated that the promoter methylation may result in the loss or low expression of the *RASSF1A* gene.

In summary, our results showed the promoter hypermethylation of the *RASSF1A* gene in a considerable proportion of primary sporadic breast cancer in Chinese women. The majority of *RASSF1A* methylated cancers were found to have an absent or markedly reduced RASSF1A expression. The results are also consistent with other studies. Together, these findings suggest that *RASSF1A* promoter hypermethylation may also play an important role in breast tumor development in different nationalities.

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