Methylation of the *RASSF1A* promoter in breast cancer


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**ABSTRACT.** Tumor suppressor genes are the key targets of hypermethylation in breast cancer and may therefore lead to malignancy by deregulation of cell growth and division. Our previous pilot study with pairs of malignant and normal breast tissues identified a correlation between *RASSFIA* gene methylation and breast cancer. To determine the relationship between *RASSFIA* methylation and breast cancer, we conducted a larger study. We took samples from 108 patients with breast cancer, 28 patients with benign breast tumors, and 33 subjects with normal breast tissues at the Second Affiliated Hospital of Nanjing Medical University at Wuxi between July 2013 and September 2015. We used the samples to investigate methylation levels of the *RASSFIA* gene for associations with breast cancer. Quantitative real-time polymerase chain reaction (PCR) and methylation-specific PCR were used to investigate the levels of *RASSFIA* mRNA expression and *RASSFIA* methylation, respectively. *RASSFIA* was not expressed in 22 of the 108 breast cancer tissue samples (20.37%), and there was no statistically significant difference (P > 0.05); however, *RASSFIA* expression was significantly lower than that in the normal breast tissue samples (P < 0.05). Moreover,
the methylation rate of the RASSFIA gene promoter was significantly higher in the breast cancer tissues (64.81%) than in the normal breast tissues (18.18%) and benign breast tumors (17.86%) (P < 0.05). High methylation of the RASSFIA gene promoter was an important reason for its downregulation, and the gene played a critical regulated role in the incidence and development of breast cancer.

**Key words:** Breast cancer; RASSFIA gene; Gene promoter; DNA Methylation

**INTRODUCTION**

DNA methylation is an important biochemical process in the normal development of higher organisms. It involves the addition of a methyl group to the carbon at position 5 of the pyrimidine ring of cytosine or to the nitrogen at position 6 of the purine ring of adenine. This modification can be inherited through cell division.

Breast cancer is the leading cause of cancer-related death in women worldwide, but the exact etiology of breast cancer remains elusive. DNA methylation has been the focus of intensive investigation in recent years, and it is thought to play a significant role in the regulation of cancer-related genes (Das and Singal, 2004). Several tumor suppressor genes are silenced by promoter hypermethylation in breast cancer. They include *HIN1* (Krop et al., 2001), *RASSFIA* (Dammann et al., 2001), *RIL* (Boumber et al., 2007), and *CDH13* (Widschwendter et al., 2004). Aberrant DNA methylation of particular genes has been correlated with the clinical and pathological characteristics of breast cancer and its clinical outcomes (Feng et al., 2007).

DNA methylation markers have been applied as an alternative approach to the molecular profiling of breast cancer (Widschwendter et al., 2004; Fiegl et al., 2006; Feng et al., 2007). RASSFIA promoter methylation provides important prognostic information in early-stage breast cancer patients (Kioulafa et al., 2009). Together, these results suggest that DNA methylation profiling correlates with clinical status in breast cancer and could help predict response to hormonal and non-hormonal breast cancer therapy (Widschwendter et al., 2004). We investigated the association between RASSFIA gene promoter methylation and the incidence and development of breast cancer.

**MATERIAL AND METHODS**

**Samples**

Tumor samples from 108 patients aged 37-78 years with breast cancer, 28 patients with benign breast tumors, and 33 subjects with normal breast tissues were obtained by the collection of excess tissue during medically necessary excision procedures between July 2013 and September 2015 at the Second Affiliated Hospital of Nanjing Medical University, Wuxi. None of the patients who provided a tumor sample was given adjuvant therapy. All tissue samples were obtained after receiving informed consent according to institutional rules. None of the patients was recruited specifically for this study, which was approved by the Ethics Committee.
RASSF1A mRNA expression analysis

Total mRNA was extracted from patient samples using TRIzol (St. Louis, MO, USA), then reverse-transcribed into complementary DNA (cDNA) using a Reverse Transcription kit (Promega, USA). A polymerase chain reaction (PCR) was then conducted to determine the expression of RASSF1A mRNA, in which the cDNA was used as a template and the glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as an internal control. The primers for RASSF1A were: F, 5'-GGCGTCTGCGCAAAGGCC-3'; R, 5'-GGGTGGCTTCTTGCTGGAGGG-3'; and the primers for GAPDH were: F, 5'-GGACCTCATGGCCTACATGG-3'; R, 5'-TAGGGCCTCTTTGCTCAGT-3'. The amplification reaction program was as follows: initial denaturation for 5 min at 94°C; 32 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C; then extension for 2 min at 72°C. The PCR products were subjected to electrophoresis on a 1% agarose gel.

Methylation analysis

Total DNA was obtained from all samples using the TRIzol method, and was then modified using Na$_2$SO$_3$ and hydroquinone. Pyrosequencing methylation analysis was used to detect methylation of the RASSF1A gene. For each gene, we selected the CpG island region flanking the transcription start site at the 5'-untranslated region. The primers for pyrosequencing and PCR were: 1) no methylation primers: F, 5'-GGTTGTATTTGGTTGGAGTG-3'; R, 5'-CTACAAACCTTTACACACAACA-3'; and 2) methylation primers: F, 5'-AGCGAAGTACGGGTTTAATC-3'; R, 5'-GCACCACGTATACGTAACG-3'. The amplification reaction program was: initial denaturation for 5 min at 94°C; 36 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 58°C, and extension for 45 s at 72°C; then extension for 5 min at 72°C. The PCR products were subjected to electrophoresis on a 1% agarose gel.

Statistical analysis

Data are reported as median and range or mean standard deviation (SD). Data were analyzed using the $c^2$ test and the Fisher exact test, where appropriate. All tests were two-tailed with a 95% confidence interval. Statistical analysis was performed using the SPSS version 20.0 software. A P value of <0.05 was considered statistically significant.

RESULTS

RASSF1A mRNA expression

Of the 108 breast cancer tissue samples, RASSF1A mRNA was expressed in 86 cases, and not expressed in 22 cases; it was expressed in all of the 28 benign breast tumor tissue samples and the 33 normal breast tissue samples. There were no statistically significant difference in RASSF1A mRNA expression between normal breast tissues and breast cancer tissues (P > 0.05). The ratio of integrated optical density (RiOD) = (average optical density of RASSF1A x area) / (average optical density of GAPDH x area). Of the 108 breast cancer tissue samples, 86 cases expressed RASSF1A mRNA, but the expression levels were lower than in the benign breast tumors or the normal breast tissues, and the differences were statistically significant (P < 0.05). The results are shown in Table 1.
RiOD = ratio of integrated optical density. *P < 0.05 for comparison of normal breast tissues with benign breast tumor tissues.

### Results of methylation analysis of the RASSF1A promoter

Methylation-specific PCR was used to detect the methylation of CpG sites in the RASSF1A gene promoter in the normal breast, benign breast tumor, and breast cancer tissue samples. The results showed that the RASSF1A gene was partly methylated in six normal breast tissue samples (18.18%), methylated in five benign breast tumor tissue samples (17.86%), and methylated in 70 breast cancer tissue samples (64.81%). Eleven samples were partly methylated (10.19%), and the rest were not methylated. The methylation frequencies of CpG sites in the RASSF1A promoter were higher in the breast cancer tissues than in the normal breast and benign breast tumor tissues, and the differences were statistically significant (P < 0.05).

### DISCUSSION

It is evident that methylation plays an important role in breast cancer development and progression (Pu et al., 2003). Hypomethylation of DNA may lead to chromosomal instability. Tumor suppressor genes, such as RASSF1A and HIN1, are the key targets of hypermethylation in breast cancer, and may therefore lead to malignancy by the deregulation of cell and growth cycles (Tamura, 2002). In addition, there have been numerous studies on the association between the methylation of tumor suppressor genes and the clinical characteristics and outcomes of breast cancer. There has been a substantial limitation of these studies is that the data were highly variable, probably due to differences in the laboratory and statistical methods used, the sample sizes, and the clinical status of the subjects.

We found that expression of the RASSF1A gene was lower in samples from breast cancer patients than that from benign breast tumors or normal breast tissues, and the methylation frequency of the RASSF1A gene promoter was higher than in the benign breast tumor or normal breast tissue samples. The results suggest that the negative or downregulated expression of the RASSF1A gene in breast cancer was due to the hypermethylation of this gene promoter.

RASSF1A is a well-studied tumor suppressor gene, and plays an important role in many cell functions, such as apoptosis, cell cycle arrest, microtubule stabilization, and metaphase arrest (Agathanggelou et al., 2005; Dammann et al., 2005). Re-expression of RASSF1A in tumor cell lines decreases in *in vitro* colony formation and in *in vivo* tumorigenicity (Agathanggelou et al., 2005).

In conclusion, DNA methylation of RASSF1A would be expected to lead to loss of function and an increase in spontaneous or induced tumor formation. Our data, together with the data from other groups, suggest that RASSF1A methylation could be a potential molecular biomarker, but further replication analyses in larger cohorts and different populations are warranted.
Methylation of RASSFIA in breast cancer

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES


