

RhoBTB2 gene in breast cancer is silenced by promoter methylation

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Abstract. Mutation analysis in breast cancer has failed to explain the inactivation of RhoBTB2, a candidate breast cancer tumor suppressor gene on chromosome 8p. Some breast cancer-related genes in this region become inactivated by hypermethylation, and hypermethylation of RhoBTB2 abrogates its expression in bladder cancers. The aim of the present study was to determine whether RhoBTB2 was silenced by methylation in breast cancer. Nested methylation-specific PCR (nMSP) and quantitative reverse transcription PCR were used to analyze the methylation status and mRNA levels of RhoBTB2 in 50 paired breast cancer and normal tissues and the results were correlated with clinicopathological characteristics. Promoter methylation and the downregulation of RhoBTB2 mRNA was observed in tumor tissues ($P < 0.001$). mRNA levels were decreased in samples with methylation ($\chi^2 = 15.751$, $P < 0.001$). RhoBTB2 methylation was observed preferentially in progesterone receptor (PR)-negative samples ($P < 0.05$). The results demonstrated that aberrant methylation of RhoBTB2 may be responsible for the suppression of RhoBTB2 mRNA expression in breast cancer, a significant event during the genesis of breast cancer that correlated with PR status.

Introduction

Breast cancer is the leading diagnosed cancer and cause of cancer mortality in females worldwide. Breast cancer is responsible for ~23% of total new cancer cases and 14% of total cancer deaths in recent years in women (1).

DNA methylation in cancer has received attention as it has been shown to participate in the complex multistage process of malignant tumor emergence. Aberrant promoter methyla-

tion in CpG islands involves DNA methyltransferases (Dnmts) transfer of methyl groups from S-adenosyl-L-methionine to the fifth carbon position of the cytosines in 5'-CpG-3' dinucleotides (2). Hypermethylation may suppress gene transcription and reduce the stability of the genome by recruiting a complex containing transcriptional corepressors and histone deacetylases. This likely plays a crucial role in the inactivation of tumor suppressor genes, which is a step in tumorigenesis (3,4).

As a candidate tumor suppressor gene that is associated with 90% of sporadic breast cancers (5), RhoBTB2 was cloned by Hamaguchi *et al* using representational difference analysis (RDA) (6). RhoBTB2 is located on chromosome 8p, which is a hotspot region where many breast cancer tumor suppressor genes, including NGR1, FEZ1/LZTS1 and 14-3-3 σ (7-10), become inactivated by hypermethylation and their silencing contributes to breast tumor development. Aberrant methylation of RhoBTB2 was also found to reduce RhoBTB2 expression in bladder cancers (11). Results of the study by Fu *et al* revealed that mutations in the RhoBTB2 promoter and the seventh exon were seldom identified in Chinese patients, and are not associated with the risk of breast cancer as it is not a frequent mechanism of inactivation (12). Findings by those authors are consistent with results by Knowles *et al* suggesting other mechanisms may be involved, such as methylation, which are more common than mutations in RhoBTB2 (4), which are responsible for the loss of expression. CpGplot (<http://www.ebi.ac.uk/emboss/cpgplot>) was used to examine the RhoBTB2 promoter region and CpG islands were identified.

In this study, we determined the methylation status of RhoBTB2 and the mRNA expression in breast cancer tissues by nMSP and quantitative reverse transcription PCR (qRT)-PCR. We also correlated the methylation changes with the transcript expression and correlated results with clinicopathological characteristics to investigate CpG methylation and the role of RhoBTB2 in breast cancer.

Materials and methods

Tissues. Samples were collected after obtaining informed consent from 50 female patients, who were between 33 and 78 years of age (average age, 51.3 \pm 11.4 years), and who

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Table I. Summary of primer sequences for PCR.

Gene name	Primer sequence (5'→3')	Product size (bp)
β -actin (for both real-time PCR and semiquantitative PCR)	F: TCACCCACACTGTGCCCATCTACGA	295
	R: CAGCGGAACCGCTCATTGCCAATGG	295
RhoBTB2 (for real-time PCR)	F: ATGTGGTGGTTCTGTGCTTCT	209
	R: GGCAGGATTCATTAGGTTT	209
RhoBTB2 (for semiquantitative PCR)	F: TGTGGGCTCAGAGCTCAGGAGT	316
	R: CTGTAGAGGGCAGCATAACGCGT	316

F, forward; R, reverse.

underwent surgery due to breast cancer at the Union Hospital, Fuzhou, China, between September 2010 and April 2011. All of the cases were definitively diagnosed by pathology; including 46 infiltrating ductal carcinomas (IDC), two infiltrating lobular carcinoma (ILC), and two ductal carcinoma *in situ* (DCIS). None of the patients had received neoadjuvant therapy. Primary tumor samples and corresponding normal breast tissues, taken 5 cm from the cancer margin, were obtained. Samples were immediately snap-frozen in liquid nitrogen after resection and stored at -80°C overnight. The rest of the tumor was examined by routine histopathology and immunohistochemistry in the Pathology Department of Union Hospital, Fuzhou, China and the clinical data, including age of onset, tumor size, lymph node metastasis status of the patient, the histology grade, TNM staging, sex hormone and Her-2 levels, and the p53 gene status of the tumor were cataloged. The study was approved by the Ethics Committee of the Union Hospital, Fuzhou, China.

qRT-PCR and semiquantitative RT-PCR. Total RNA from tissues was extracted by TRIzol reagent (Takara, Shiga, Japan), and quantified with GeneQuant Pro (Amersham Biosciences, Pittsburg, PA, USA). Samples with a ratio of OD280/260 between 1.9 and 2.0 were processed further after total RNA was confirmed to be without degradation by agarose gel electrophoresis. The samples were stored at -80°C . Reverse transcription of RNA was performed in a total volume of 20 μl reaction mixture containing 2 μg RNA, in accordance with the protocol for reverse transcriptase reagent M-MLV (Biotek, Beijing, China). cDNA products were amplified using primers specific for RhoBTB2 and β -actin. β -actin was used for normalization of the quantity of cDNA.

RhoBTB2 primers for quantitative PCR were designed by Sangon Biotech (Shanghai, China), while the primers for PCR were obtained from previous studies (11,13); all primers were synthesized by Sangon. The primer sequences are presented in Table I, and quantitative PCR reaction systems are presented in Table II. The amplification conditions were as follows: 50°C for 2 min, 95°C for 2 min, then 30 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec.

qRT-PCR was performed using a Platinum[®] SYBR[®]-Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) and the ABI 7500 Sequence Detection System (Applied Biosystems, USA). Optimization of amplification conditions was carried out according to the manufacturer's instructions. Each experimental reaction was performed in triplicate and

Table II. Quantitative PCR reaction system.

Component	Volume
2X PCR mix	7.5 μl
Forward primer (10 pmol/ μl)	0.5 μl
Reverse primer (10 pmol/ μl)	0.5 μl
ROX	0.05 μl
cDNA template	0.5 μl
ddH ₂ O	up to 15 μl

the relative expression was calculated using the $\Delta\Delta\text{Ct}$ method (14). The relative levels of RhoBTB2 mRNA in the breast cancer tissues that were normalized to the internal control β -actin by subtraction were calculated as ΔCt (cancer tissue), and the levels in normal tissues as ΔCt (normal tissue). The RhoBTB2 mRNA score in each tissue pair was defined as follows: ΔCt of cancer tissue - the ΔCt of normal tissue yielded a $\Delta\Delta\text{Ct}$ value, which was used to calculate the result of $2^{(-\Delta\Delta\text{Ct})}$. RhoBTB2 mRNA was upregulated when the score was >1.0 and downregulated when the score was <1.0 .

Semiquantitative PCR was carried out in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA). Amplification was carried out in a 10 μl reaction mixture containing 0.5 μl of cDNA sample, 5 μl of 2X PCR mix (Biotek), and 0.5 μl of the primers (the final concentration of each pair was 10 pmol/ μl), with 3.5 μl of deionized water. The amplification program was: 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 64°C for 30 sec, 72°C for 45 sec, followed by a final extension at 72°C for 10 min. PCR products were examined with Marker (MBI Fermentas, Vilnius, Lithuania) on 2% agarose gel electrophoresis (100 V, 70 mA) and visualized under UV illumination (Syngene, Cambridge, UK). RhoBTB2 mRNA quantity in each sample was represented and analyzed in the form of Gray Intensity of RhoBTB2/ β -actin, then the relative level of RhoBTB2 mRNA in normal breast tissue was subtracted from the relative level in the tumor. RhoBTB2 mRNA was upregulated when the score was >0 and downregulated when the score was <0 .

DNA preparation and bisulfate modification. Genomic DNA was extracted from ~ 50 -100 mg of fresh tissue, using an

Table III. Summary of primer sequences for nMSP.

Gene name	Primer sequence (5'→3')	Product size (bp)
RhoBTB2 (outside primer)	F: GGTGGTTTATTTGGTGATATTG	439
	R: CCTACAACCTTACCTCCTAACAC	439
RhoBTB2 (M, inside primer)	F: GCGAGTTGGTATGTTATGTC	144
	R: TAATCTTACCCACGACGTTA	144
RhoBTB2 (U, inside primer)	F: GGTGAGTTGGTATGTTATGTT	144
	R: CTAATCTTACCCAC AACATTA	144

F, forward; R, reverse; M, methylated primer; U, unmethylated primer.

animal tissue genomic DNA isolation kit (Bioteke) according to the manufacturer's instructions. DNA was quantified with GeneQuant Pro (Amersham Biosciences). The samples with a ratio of OD280/260 between 1.7 and 1.9 were accepted, then bisulfate modification of 200 ng DNA was performed using protocols from the Methylamp™ One-Step DNA Modification kit (Epigentek, Brooklyn, NY, USA).

Nested methylation-specific PCR analysis. Nested primers for RhoBTB2 were synthesized by Invitrogen using primer sequences obtained from a previous study (11). The nMSP primer sequences are presented in Table III.

First round amplifications in 15 µl reactions included: 7.5 µl of 2X PCR mix (Bioteke, Beijing, China), 10 pmol of outsider primer and 0.5 µl of 200 ng modified DNA, with deionized water to make up the volume, using the following cycle parameters: 94°C for 5 min, then 35 cycles at 94°C for 30 sec, 56°C for 45 sec and 72°C for 45 sec, followed by a final extension at 72°C for 5 min. Aliquots of 0.5 µl PCR products were subjected to a second round of amplification.

Second round amplification was carried out using methylated and unmethylated primers in 15 µl reactions, with the following cycle parameters: 94°C for 5 min, then 30 cycles at 94°C for 30 sec, 50°C for 45 sec and 72°C for 45 sec, followed by a final extension at 72°C for 5 min.

Then, 15 µl of the final PCR products were confirmed by 2% agarose gel electrophoresis at 100 V for 30 min, and visualized under UV illumination. DNA from the peripheral blood of healthy adults, treated and untreated with DNA methyltransferase, was used as a positive control for methylated and unmethylated DNA. H₂O instead of DNA was used in the negative control in each set of PCR experiments.

Statistical analysis. Calculations were carried out using SPSS11.0 statistical software (SPSS, Inc., Chicago, IL, USA). The numerical data correlation among mRNA expression and epigenetic events was analyzed by the Chi-Square (χ^2) test. Fisher's exact test or continuity correction was used to test the statistical significance of the observed differences between the methylation status or mRNA expression and clinical parameters as appropriate. A comparison of mRNA expression in cancer and normal tissues was performed using the Paired-samples t-test. P-values presented were two-sided, and P<0.05 was considered statistically significant.

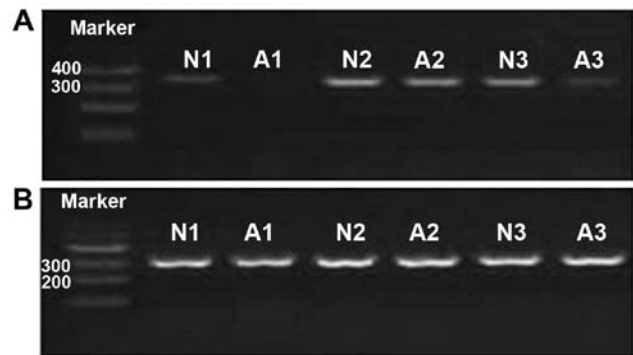


Figure 1. Semiquantitative PCR analysis of RhoBTB2 in breast cancer and corresponding normal tissue. Lane A, breast cancer tissues; lane N, corresponding normal tissues. The numbers following A and N are the sample code. Marker indicates the size marker lane. (A) RhoBTB2 amplification products indicate that the mRNA expression of RhoBTB2 (316 bp) in breast cancer tissues is reduced; (B) β -actin amplification products show that the β -actin bands (295 bp) were the same in all lanes, and was expressed at the same level in all the samples. RhoBTB2 mRNA quantity of each sample was represented and analyzed in the form of gray intensity of RhoBTB2/ β -actin.

Results

RhoBTB2 mRNA expression in breast cancer. In the 50 pairs of tumor and control tissues, RhoBTB2 mRNA in 29/50 breast cancer tumor samples (58%) was reduced, compared to the corresponding normal tissues. The average RhoBTB2 expression in breast carcinoma tissues was significantly lower than that in control tissues. The gray intensity value was 0.19 ± 0.01 in tumors vs. 0.25 ± 0.01 in normal tissues and the ΔCt value was 5.74 ± 0.45 in tumors vs. 3.07 ± 0.12 in normal tissues ($P < 0.05$). No significant relationship was observed between RhoBTB2 mRNA expression and the patient age of onset, tumor size, lymph node involvement, TNM staging, tumor grade, sex hormones or Her-2 levels, or p53 protein expression ($P > 0.05$) (Table IV).

All the samples were assayed in triplicate, including three assays for RhoBTB2 and three for β -actin. The analysis of solubility curves demonstrated that the curves of RhoBTB2 and β -actin presented a single peak, with the same DNA melting temperature (T_m) and a sharp peak. No abnormal reaction wave form was observed in other locations, which shows that the PCR products were specific. The DNA

Table IV. Correlation between clinicopathological factors and RhoBTB2 mRNA levels.

Variables	N	RhoBTB2 mRNA		P-value
		Down	Up	
Age (years)				
<50	24	13	11	0.598
≥50	26	16	10	
Tumor size (cm)				
≤2	27	16	11	0.845
>2	23	13	10	
Lymph node involvement				
No	26	15	11	0.441
Yes	24	14	10	
TNM staging				
I-II	39	24	15	0.543
III-IV	11	5	6	
Tumor grade ^a				
I-II	36	19	17	0.230
III	14	10	4	
ER status				
Negative	14	10	4	0.230
Positive	36	19	17	
PR status				
Negative	16	12	4	0.095
Positive	34	17	17	
Her-2 status				
Negative	33	18	15	0.479
Positive	11	8	3	
p53 status				
Negative	18	10	8	0.354
Positive	26	18	8	

^aThere are four non-infiltrating ductal carcinomas of the breast, with no mucinous carcinoma tumors that were included in tumor grade I.

Table V. Correlation between clinicopathological factors and methylation status of RhoBTB2 in breast cancer.

Variables	N	Methylation		P-value
		Yes	No	
Age (years)				
<50	24	12	12	0.786
≥50	26	14	12	
Tumor size (cm)				
≤2	27	16	11	0.266
>2	23	10	13	
Lymph node involvement				
No	26	15	11	0.402
Yes	24	11	13	
TNM staging				
I-II	39	22	17	0.240
III-IV	11	4	7	
Tumor grade ^a				
I-II	36	17	19	0.278
III	14	9	5	
ER status				
Negative	14	10	4	0.086
Positive	36	16	20	
PR status				
Negative	16	12	4	0.026
Positive	34	14	20	
Her-2 status				
Negative	33	16	17	0.162
Positive	11	8	3	
p53 status				
Negative	18	7	11	0.139
Positive	26	16	10	

^aThere are four non-infiltrating ductal carcinomas of the breast, with no mucinous carcinoma tumors that are included in tumor grade I.

amplification curves from the target gene of the same sample were smooth, full, and repeatable. In addition, 2% agarose gel electrophoresis verification (Fig. 1) showed the PCR products were pure and consistent with the results of quantitative PCR.

Methylation profile of RhoBTB2 in breast cancer. The determining standards for the methylated and unmethylated primers were: in one sample the amplification with the methylated and unmethylated primers was considered partial methylation, amplifications with the methylated primer yielding correct bands were considered full methylation, amplifications with the unmethylated primer yielding correct bands were considered methylation-negative. Both partial and full methylation were considered methylation-positive.

Fifty pairs of samples with the best quality of genomic DNA which had been previously examined for RhoBTB2 mRNA expression were selected for methylation analysis.

Methylation-specific bands were detected in 26 tumor samples (26/50, 52%). When we compared the methylation status in the tumors (52% positive methylation) and corresponding adjacent normal samples (0% positive methylation), the difference in the frequency of methylation between them was significant ($P<0.05$) (Fig. 2).

We analyzed the relationship between the RhoBTB2 gene methylation status and clinicopathological characteristics of patients (Table V). There was no notable association between RhoBTB2 methylation and patient age of onset, tumor size, lymph node involvement, TNM staging, tumor grade, ER status, Her-2 expression, or p53 gene status ($P>0.05$). There was a significant correlation of RhoBTB2 methylation with PR status ($P=0.026$) and the ratio of RhoBTB2 methylation was higher in PR⁻ than in PR⁺ tissues, 75.0 vs. 41.2%, indicating that the patients who were PR⁻ were prone to have RhoBTB2 methylation.

Table VI. Relationship between RhoBTB2 methylation and mRNA expression (n=50).

mRNA expression	Promoter methylation		Total
	Yes	No	
Low	22	7	29
Normal	4	17	21
Total	26	24	50

Compared with normal RhoBTB2 mRNA expression group, methylation was significantly higher in the low mRNA expression group, $\chi^2 = 15.751$, $P < 0.001$.

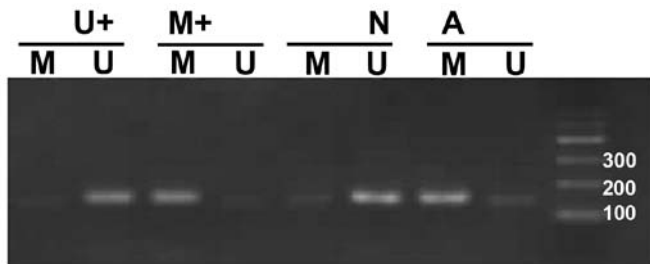


Figure 2. Nested-methylation analysis of the RhoBTB2 gene in breast cancer and corresponding normal tissues. Lane A, tumor sample; lane N, normal tissue; lane M, methylated-specific primers; lane U, unmethylated-specific primers. Lane U+, positive control of unmethylation (DNA from peripheral blood of healthy adult untreated with DNA methyltransferase was used as a positive control for unmethylation); lane M+, positive control of methylation (DNA from peripheral blood of healthy adult treated with DNA methyltransferase was used as a positive control for methylation).

Relationship between RhoBTB2 methylation and mRNA expression. The correlation of RhoBTB2 methylation with mRNA expression is shown in Table VI. There are 22 cases that were methylation-positive from the 29 cases which had suppressed RhoBTB2 mRNA expression (22/29, 75.9%), while only 4/21 cases were methylation-positive from the cases without suppressed mRNA expression (19.0%). Compared with the normal RhoBTB2 mRNA expression group, methylation was more frequent in the low RhoBTB2 mRNA expression group, and the difference was statistically significant ($\chi^2 = 15.751$, $P < 0.001$). Tumor tissue with hypermethylation of the gene tended to have lower RhoBTB2 mRNA expression, and the suppression of RhoBTB2 expression was observed in samples with gene methylation. This finding suggests that methylation of RhoBTB2 significantly contributes to the inhibition of transcription of the gene in breast cancer tumors.

Discussion

Aberrant DNA methylation plays a crucial role in the pathogenesis of human cancer since it may cause epigenetic inactivation in tumor suppressor genes in order to promote tumorigenesis. In this study, we detected mRNA expression of RhoBTB2 and the methylation status in breast cancer tissues and correlated the transcript expression and methylation with

clinicopathological characteristics of the patients. RhoBTB2 mRNA expression in breast carcinoma was significantly depressed compared to the corresponding normal tissues ($P < 0.001$), and the loss of mRNA was found in significantly more samples with methylation of the gene ($\chi^2 = 15.751$, $P < 0.001$). Additionally, there was a statistical correlation of the aberrant methylation changes with PR-negative cancer tissues ($P < 0.05$). To the best of our knowledge, this is the first study on CpG methylation-mediated transcriptional silencing of RhoBTB2 in breast cancer, which is a significant event during the genesis of breast cancer.

RhoBTB2 is located on chromosome 8p, which is a region of the chromosome with frequent abnormality in various types of cancer. As a member of the RhoGTPases family, RhoBTB2 plays a critical role in preventing the invasion and metastasis of malignant tumor cells through cytoskeleton remodeling in order to influence cell division, motility, contraction, and cytokinesis. It is also involved in nerve growth, mitogenesis, membrane trafficking, transcriptional activation, and cell growth control such as proliferation and apoptosis through upregulation of the E2F1 protein (15-18). Thus, inactivation of RhoBTB2 is important in the development of breast cancer, and the study of its methylation status is useful in the clarification of the causes of breast cancer.

In the analysis of RhoBTB2 mRNA expression from 50 cases, there were 29 breast tumor samples that had low or loss of expression, as the RhoBTB2 transcription in breast cancer was greatly suppressed compared with normal tissues ($P < 0.001$). The result of semiquantitative PCR was consistent with the quantitative PCR and the results were repeatable. According to the analysis of ISH and semiquantitative PCR reported by Mao *et al* (19), the mRNA and protein levels of RhoBTB2 in 60 breast cancer tissues was lower than that in the 30 benign breast lesions. In addition, follow-up observations of these cases revealed that the survival rate was significantly higher in RhoBTB2-positive patients than in RhoBTB2-negative patients, suggesting that the expression of RhoBTB2 may be regarded as an independent prognostic factor in breast carcinoma patients. As a breast cancer tumor suppressor gene, the downregulation of RhoBTB2 expression is a biological index of poor prognosis for breast cancer patients. In the RNA expression analysis in the study by Hamaguchi *et al*, absence of RhoBTB2 was found in 58% (11/19) of breast cancer tissues and 50% (7/14) of lung cancer tissues, but normal expression was found in the corresponding normal tissues (6). Knowles *et al* (4) found that, RhoBTB2 mRNA was decreased in 75% of bladder cancer cell lines. Compared with the corresponding normal tissues, bladder cancer tissues had significantly less RhoBTB2 mRNA, which was correlated with the clinical TNM stage and histological grade, and a low RhoBTB2 expression was regarded as a poor prognostic indicator for bladder cancer patients (11). However, the expression of RhoBTB2 was not decreased in colon tumors or other types of cancer (4), suggesting that RhoBTB2 is a tissue-specific tumor suppressor gene.

MSP results show that the total frequency of the RhoBTB2 promoter methylation in tumor tissues is 52%, which was significantly higher than that in the corresponding normal tissues ($P < 0.05$). In our breast cancer tissue samples, there were more methylation-positive samples with downregulation

of RhoBTB2 mRNA than there were cases without decreased RhoBTB2 mRNA (75.9 vs. 19.0%, $\chi^2 = 15.751$, $P < 0.001$). This suggests that RhoBTB2 mRNA expression is associated with its methylation status, and that the RhoBTB2 gene can be silenced by promoter methylation in breast cancer, which may affect the development of breast cancer. Jones and Takai (20) reported that the hypermethylation of CpG islands in the DNA promoter is the third mechanism of deactivation of an anti-oncogene along with mutation and deletion, and even a unique one in some cases. Aberrant methylation inhibits the transcription of genes and abrogates gene expression, but does not alter the DNA sequence or the gene product. This results in tumor suppressor gene silencing and the stability of the genome decreases (21,22), promoting tumorigenesis and tumor development. The RhoBTB2 gene is located on chromosome 8p21.3. In this same region of 8p, several breast cancer-related tumor suppressor genes, such as NGR1, FEZ1/LZTS1, and 14-3-3 σ , also exhibit expression abrogation by promoter methylation (7-10). Previous investigations into mutations in the RhoBTB2 gene have demonstrated that the occurrence of mutations was less than the occurrence of reduced mRNA expression (12). Knowles *et al* (4) hypothesized that there was another mechanism for RhoBTB2 silencing, for example, promoter methylation, that was noted more frequently than mutations in the gene. Recently, Shi *et al* (11) used MSP and RT-PCR and found that the frequency of methylation in CpG islands of the RhoBTB2 promoter is much higher in bladder cancer tissues than in normal tissues. The RhoBTB2 mRNA level in the tumor tissues with methylation is much lower than that in the tissues without methylation. Hypermethylation of the RhoBTB2 promoter is therefore a significant mechanism of RhoBTB2 deactivation in bladder cancer. Findings of Mao *et al* (19) demonstrated that the mRNA levels of RhoBTB2 were consistent with the protein levels, suggesting that RhoBTB2 expression was blocked at the transcription level. Downregulation of these transcripts and silencing of the promoter may be the primary mechanism of gene suppression, and our results are consistent with the abovementioned studies.

Both methylated and unmethylated genes were identified in several tumor samples, known as partly methylated, and the samples were considered methylation-positive. In the study by Herman *et al* on the sensitivity of MSP for detecting the methylated alleles in lung cancer samples (23), 0.1% of P16 DNA had methylated alleles, although the cells were always associated with normal cells that masked the presence of methylated sites. Tumor tissues consist of many normal cells, such as stromal, endothelial and inflammatory cells that do not have CpG methylation and their DNA may affect the results of the analysis. We suggest three reasons to explain partly methylated samples: first, we cannot avoid sampling normal interstitial cells within these tumor tissues. Second, DNA methylation may be important in early stages of tumor development (22) such as precancerous lesions, and the tumor tissues contain cells at various stages of development with methylation only occurring in the cells during the early stages of development. Third, since human genes are diploid, we cannot exclude the existence of different methylation status of the two alleles. Fan *et al* (24) suggested that, there is decreased and unstable aberrant methylation in part of the tumor, and

less methylation in an early tumor may represent a cancer cell subset with an unstable methylation status of tumor suppressor gene CpG islands. Methylation of CpG islands is a progressive process, with every pyrimidine base in CpG islands becoming gradually methylated. Some CpG islands are methylated early and then methylation expands to more CpG islands. Thus, key genes evolve into a silent steady state, and the gradual loss of function of these genes may be a mechanism that avoids early death of abnormal cells. This allows the evolution of an immortal tumor cell with its altered germplasm (25).

In the analysis of clinicopathological characteristics, RhoBTB2 methylation was observed preferentially in samples that were PR-negative ($P < 0.05$), but there was no notable association between RhoBTB2 methylation and other clinicopathological characteristics, such as age of onset, tumor size, lymph node involvement, TNM staging, tumor grade, ER status, Her-2 expression, or p53 gene status. This observation suggests that hypermethylation of the RhoBTB2 promoter may be involved in the initial stages of tumorigenesis. Moreover, the PR levels may be affected by hypermethylation of RhoBTB2 and aberrant methylation of RhoBTB2 is also important in the pathogenesis of certain types of breast cancer. These findings suggest that RhoBTB2 methylation may function as an early risk event for breast cancer in a phase-specific and tissue-specific manner. In addition, the clinicopathological characteristics analysis also revealed that there was no significant correlation between loss of RhoBTB2 expression and the clinicopathological characteristics. Mao *et al* (19) reported that the loss of RhoBTB2 mRNA expression was associated with age of onset, PR status and histopathological types but not with TNM staging, lymph node involvement, or ER and Her-2 status in breast cancer. Although our results are different from those of Mao *et al*, there were differences in the studies. The samples investigated by Mao *et al* were obtained from unpaired breast cancer tissues, whereas, we examined paired samples. Patients included in this study were from the Fujian region and there may be a difference in the genetic character of populations from different regions. Additionally, differences in laboratory and statistical methods may also have influenced the results. Moreover, Bi *et al* (5) compared differences in the age at onset of breast cancer, lymph node involvement, TNM staging, ER/PR, Her-2 status and survival time between groups, and showed no marked difference in RhoBTB2 expression. However, data from a larger sample may aid in obtaining better information on the distribution of RhoBTB2 expression in breast cancer patients.

In summary, we provide new evidence that hypermethylation of the RhoBTB2 promoter was an important mechanism associated with inactivation of RhoBTB2 transcription in breast cancer, which is important in the tumorigenesis and progression of breast cancer. There was also a correlation between RhoBTB2 methylation and PR downregulation, and a detailed explanation of the connection requires further investigation. At the present time, testing for DNA methylation is a feasible assay for determining the prognosis and for diagnoses by DNA-based biomarkers. Thus, our study revealed that the risk of breast cancer was connected with the expression of the RhoBTB2 gene, which is a new molecular target for breast cancer diagnosis, therapy and prognosis. As DNA methylation is reversible, the feasibility of a clinical application

of modifying methylation of the RhoBTB2 gene in order to restore its antitumor function merits further investigation.

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