STUDY ON METHYLATION SEQUENCE IN THE PROMOTER REGION OF THE ANTIGEN PRESENTING ELEMENT (APM) GENE IN TRIPLE NEGATIVE BREAST CANCER

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ABSTRACT

Objective: *The methylation levels of HLA-I and APM elements in TNBC tissues were analyzed by Sequenom MassARRAY® DNA methylation mass spectrometry to identify the leading epigenetic modification in the occurrence of triple-negative breast cancer (TNBC).*

Methods: *For TAP1 and ERp57 genes, the MassARRAY methylation detection technique was used to quantitatively analyze the methylation level of the CpG island in the promoter region of the candidate gene, to identify the differences in the overall and single CpG methylation rate between TNBC and non-TNBC patients, as well as breast cancer tissues and breast fibroma tissues.*

Results: *The total methylation level of the ERp57 gene in TNBC tissues was significantly lower than that in non-TNBC tissues ($P < 0.01$), and there was no statistically significant difference in the total methylation level of the TAP1 gene between TNBC tissues and non-TNBC tissues ($P > 0.05$). The total methylation level of the ERp57 gene in breast cancer tissues was markedly lower than that in breast fibroma tissues ($P < 0.05$), and the total methylation level of the TAP1 gene was dramatically higher than that in breast fibroma tissues ($P < 0.05$). The methylation level of ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-6.7, ER57-8-CpG-8, and ER57-8-CpG-9 sites in the promoter region of the ERp57 gene in TNBC tissues was remarkably lower than that in non-TNBC tissues ($P < 0.01$). The methylation level of ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-8, and ER57-8-CpG-9 sites in the promoter region of the ERp57 gene in breast cancer tissues was significantly lower than that in breast fibroma tissues ($P < 0.05$). The methylation level of the TAP1-14-CpG-29 site in the promoter region of the TAP1 gene in breast cancer tissues was significantly higher than that in breast fibroma tissues ($P < 0.05$).*

Conclusion: *The methylation of the promoter region of APM member TAP1 and ERp57 genes plays an important role in the occurrence and development of TNBC.*

Keywords: *triple-negative breast cancer (TNBC), antigen-presenting element, methylation.*

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Introduction

DNA methylation is a process whereby the methyl supplied by S-adenosine methionine is transferred to the 5th carbon atom of cytosine (via DNA methyltransferase catalysis) to form 5-methylcytosine. DNA methylation usually occurs at the 5'-cytosine, producing 5-methylcytosine, which regulates gene expression and protects DNA from degradation processes by specific restriction enzymes[(1)]. Numerous studies have shown that DNA methylation plays an important role in the occurrence and development of breast cancer[(2-3)]. During this process, DNA methylation can activate various oncogenes, leading to the loss of expression of multiple tumor suppressor genes, resulting in abnormal cell proliferation, and ultimately, the occurrence of breast cancer. Studies have shown that regional genomic hypermethylation and genome-wide hypomethylation can occur at different stages of breast cancer. The former may occur at the early stage of breast cancer, while the latter may be the mechanism of late breast cancer[(4)]. Given this, this study utilized the specimens of TNBC patients as the

research object. Based on our team's related research, the methylation localization and quantitative analysis of APM genes (TAP2 and ERp57) in breast cancer tissues were conducted using the Sequenom MassARRAY methylation quantitative analysis platform. Evaluating the effect of methylation of these genes on the occurrence of TNBC can provide a basis for quantitative testing of the methylation level of the TNBC gene.

Materials and methods

Experimental materials

Collection of clinical specimens

A total of 84 female breast cancer patients (including 59 TNBC patients and 25 non-TNBC patients) and 12 breast fibroadenoma patients in the cancer hospital affiliated with the third clinical medical college of Xinjiang Medical University were selected as the research subjects. The hospital ethics committee approved this study of our hospital.

Inclusion criteria and exclusion criteria for breast cancer

The inclusion criteria included patients who were 30 to 70 years of age, without a history of chemotherapy or radiotherapy, complicated with no other tumors or major organ disease (such as cardiopulmonary insufficiency and liver and kidney failure), primary breast cancer confirmed by clinicopathology, and complete data availability.

The exclusion criteria were complicated with organic lesions, unable to tolerate surgery, preoperative breast cancer patients, advanced breast cancer patients with distant metastasis, and incomplete clinical data.

Experimental methods

Preparation of genomic DNA

Purity and concentration detection of extracted DNA: 0.5 μL of DNA samples were absorbed, distilled water was added to 1 μL, thoroughly mixed, and transferred to a quartz cuvette. DNA purity was determined by measuring the optical density (OD) values at 260 and 280 nm. The OD260/OD280 ratio should be between 1.7 to 2.0, and the concentration of extracted DNA should be greater than 50 ng/μL. The quality control standard is the lack of significant degradation of the main electrophoresis band of genomic DNA, the main band is equal to or greater than 20 kb, a DNA concentration greater than 50 ng/μL, and the total DNA greater than 2 μg.

Quality identification of extracted DNA: The quality of DNA was identified by 0.8 % agarose gel electrophoresis. The qualified DNA concentration was adjusted to 75 ng/μL, then transferred to a 384-well plate and stored in a -20 oC refrigerator until further use.

Sulfite treatment of DNA samples

Preparation for the experiment: 24 mL of anhydrous ethanol was added to the wash buffer solution and stored at room temperature. To the CT Conversion Reagent was added 300 μl of M-Dilution Buffer, 900 μl of RNase-free water, and 50 μl of M-Dissolving Buffer. The solution was mixed well and stored at room temperature.

Primer design for gene methylation detection: The methylation primers were designed using Epidesigner software. At http://www.epidesigner.com, we input the entire sequence of the CpG island of the genes to be detected, including upstream and downstream addition sequences. The designed methylated primers displayed by the software system were selected. The 31-bp T7 promoter sequence cagtaatacgactcactatagggagaaggct was added at the 5' end of the reverse primer for subsequent in vitro transcription. The 10-bp sequence aggaagagag was added at 5' end of the forward primer to balance the PCR reaction. The bisulfite modified genome was amplified, and the length of the PCR product was 335 to 491 bp. PCR amplification products were identified by 2% agarose gel electrophoresis.

In vitro transcription and base-specific digestion: After fully mixing and sealing, the mixture was centrifuged at 4 °C (1000 rpm×1 min). The PCR protocol was 37 °C, 20 min; 85 °C, 5 min; 4 °C, hold.

Chip sampling and mass spectrometry: 15 nl was extracted from the pyrolysis products of 384 well plates and added to SpectroCHIP chips for detection using the MassARRAY Compact System. EpiTYPER software was used to analyze the mass spectrogram collected by the MassARRAY mass spectrometer, and then further quantitative analysis of methylation was performed.

Statistical analysis

All data were statistically analyzed using SPSS 22.0 statistical software. The Kruskal-Wallis method was used for the multi-group comparison, and the Wilcoxon test was used for the comparison between two groups. Statistical significance was assigned at $P < 0.05$.

Results

DNA purity and concentration

DNA purity and concentration of TNBC tissues

DNA from 59 cases of TNBC tissues was extracted. The detection standard was an OD260/OD280 ratio between 1.75 and 2.00. The total amount of DNA in samples was greater than 2 μg, the concentration was greater than 50 ng/μl, and there was no significant degradation of the main electrophoresis band. Among them, 18 cases of unqualified quality inspection were eliminated.

DNA purity and concentration of non-TNBC tissues

DNA from 25 cases of non-TNBC tissues was extracted. The detection standard was an OD260/OD280 ratio between 1.75 and 1.95. There was no significant degradation of the main electrophoresis band. The concentration of DNA in samples was greater than 50 ng/μl, and the total amount was greater than 2 μg. 14 cases of unqualified quality inspection were excluded due to the presence of RNA, contamination, degradation, and low total amounts of other impurities (labeled as "*").

Detection results of DNA purity and concentration of breast fibroma tissues

DNA from 12 cases of breast fibroma tissues was extracted. The detection standard was an OD260/OD280 ratio between 1.75 and 1.95. There was no significant degradation of the main electrophoresis band. The concentration of DNA in samples was greater than 50 ng/μl, and the total amount was greater than 2 μg. 14 cases of unqualified quality inspection were excluded due to the presence of RNA, contamination, degradation, and low total amounts of other impurities.

Detection results of MassARRAY mass spectrometry

The degree of methylation of each gene is shown in figures 1-2. The epigram shows the methylation profiles of the CpG island in the promoter region of the TAP1 and ERP57 genes in breast cancer tissues and breast fibroma tissues. The increase from 0% to 100% is incremented from pale yellow to dark blue.

Differential analysis of methylation levels of ERp57 and TAP1 genes in TNBC tissues and non-TNBC tissues

The total methylation level of the ERp57 gene in TNBC tissues was significantly lower than in non-TNBC tissues ($P < 0.01$), and there was no statistically significant difference in the total methylation level of the TAP1 gene between TNBC tissues and non-TNBC tissues ($P > 0.05$). The results are shown in Table 1.

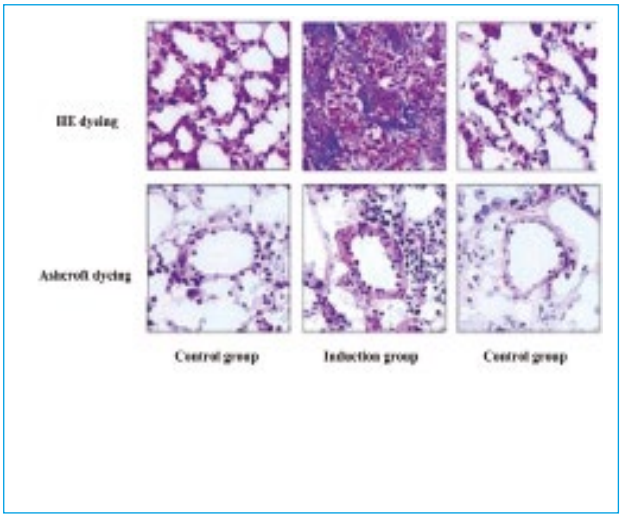

Fig. 1: Epigram of ERp57 gene methylation in breast cancer tissues.

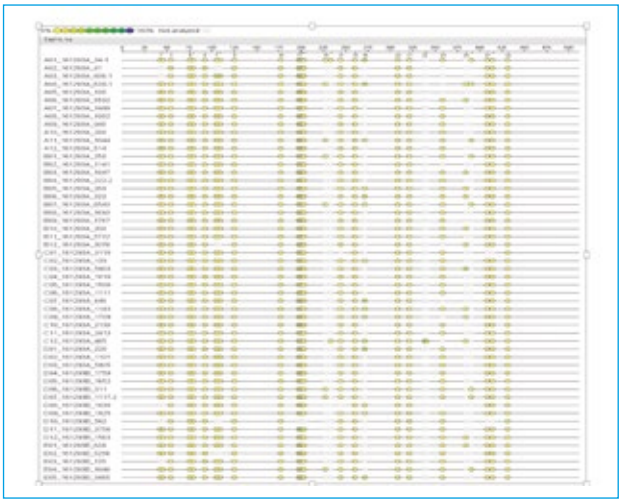

Fig. 2: Epigram of TAP1 gene methylation in breast cancer tissues.

Groups	ERp57	TAP1
TNBC tissues	0.4406 ± 0.2849	0.0331 ± 0.0108
Non-TNBC tissues	0.7727 ± 0.1678	0.0351 ± 0.0134
P value	0.000	0.671

Table 1: Differential analysis of methylation levels of ERp57 and TAP1 genes in TNBC tissues and non-TNBC tissues.

Differential analysis of methylation levels of ERp57 and TAP1 genes in breast cancer tissues and breast fibroma tissues

The total methylation level of the ERp57 gene in breast cancer tissues was markedly lower than

that in breast fibroma tissues ($P < 0.05$), and the total methylation level of the TAP1 gene was dramatically higher than that in breast fibroma tissues ($P < 0.05$). The results are shown in Table 2.

Groups	ERp57	TAP1
Breast cancer tissues	0.5183 ± 0.2968	0.0335 ± 0.0114
Breast fibroma tissues	0.7652 ± 0.1376	0.0287 ± 0.0056
P value	0.010	0.040

Table 2: Differential analysis of methylation levels of ERp57 and TAP1 genes in breast cancer tissues and breast fibroma tissues.

Differential analysis of methylation levels of the single CpG sites of ERp57 and TAP1 genes in TNBC tissues and non-TNBC tissues

The quantitative detection data of methylation at the single CpG site revealed by mass spectrometry showed that the methylation level of ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-6.7, ER57-8-CpG-8, and ER57-8-CpG-9 sites in the promoter region of the ER57 gene in TNBC tissues was remarkably lower than that in non-TNBC tissues ($P < 0.01$). The results are shown in Table 3.

CpG sites	TNBC tissues	Non-TNBC tissues	P value
ER57-8-CpG-1	0.4391 ± 0.3129	0.8127 ± 0.2176	0.000
ER57-8-CpG-2.3.4	0.5063 ± 0.3153	0.8740 ± 0.1737	0.000
ER57-8-CpG-5	0.4450 ± 0.2561	0.7500 ± 0.1531	0.000
ER57-8-CpG-6.7	0.3403 ± 0.2709	0.6610 ± 0.1404	0.000
ER57-8-CpG-8	0.4664 ± 0.2836	0.8036 ± 0.1605	0.000
ER57-8-CpG-9	0.4664 ± 0.2836	0.8036 ± 0.1605	0.000
TAP1-14-CpG-1.2	0.0364 ± 0.0355	0.0278 ± 0.0264	0.435
TAP1-14-CpG-3	0.0406 ± 0.0347	0.0455 ± 0.0448	0.744
TAP1-14-CpG-4.5	0.0342 ± 0.0235	0.0391 ± 0.0239	0.556
TAP1-14-CpG-6	0.0386 ± 0.0578	0.0409 ± 0.0653	0.918
TAP1-14-CpG-7.8	0.0345 ± 0.0535	0.0350 ± 0.0440	0.979
TAP1-14-CpG-9	0.0217 ± 0.0347	0.0136 ± 0.0273	0.434
TAP1-14-CpG-10	0.0283 ± 0.0481	0.0282 ± 0.0567	0.994
TAP1-4-CpG-11.12.13.14	0.0346 ± 0.0339	0.0436 ± 0.0375	0.485
TAP1-14-CpG-17	0.0436 ± 0.0449	0.0578 ± 0.0556	0.494
TAP1-14-CpG-18	0.0194 ± 0.0285	0.0245 ± 0.0262	0.587
TAP1-14-CpG-19	0.2011 ± 0.1438	0.1450 ± 0.1725	0.594
TAP1-14-CpG-20	0.0075 ± 0.0102	0.0064 ± 0.0092	0.732
TAP1-14-CpG-21	0.0181 ± 0.0326	0.0236 ± 0.0291	0.595
TAP1-14-CpG-24	0.0335 ± 0.0233	0.0329 ± 0.0229	0.944
TAP1-14-CpG-25	0.0167 ± 0.0255	0.0933 ± 0.1617	0.498
TAP1-14-CpG-27.28	0.0417 ± 0.0282	0.0340 ± 0.0222	0.375
TAP1-14-CpG-29	0.0242 ± 0.0187	0.0155 ± 0.0163	0.152

Table 3: Differential analysis of methylation levels of the single CpG sites of the ERp57 and TAP1 genes in TNBC tissues and non-TNBC tissues.

Differential analysis of methylation levels of the single CpG sites of ERp57 and TAP1 genes in breast cancer tissues and breast fibroma tissues

The quantitative detection data of methylation at the single CpG site provided by mass spectrometry showed that the methylation level of ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-8, and ER57-8-CpG-9 sites in the promoter region of the ERp57 gene in breast cancer tissues was significantly lower than that in breast fibroma tissues ($P < 0.05$). The methylation level of the TAP1-14-CpG-29 site in the promoter region of the TAP1 gene in breast cancer tissues was significantly higher than that in breast fibroma tissues ($P < 0.05$). The results are shown in Table 4.

CpG sites	Breast cancer tissues	Breast fibroma tissues	P value
ER57-8-CpG-1	0.5304 ± 0.3325	0.7840 ± 0.1664	0.021
ER57-8-CpG-2.3.4	0.5938 ± 0.3270	0.9100 ± 0.0258	0.000
ER57-8-CpG-5	0.5253 ± 0.2685	0.7520 ± 0.1486	0.021
ER57-8-CpG-6.7	0.4132 ± 0.2809	0.5920 ± 0.1698	0.080
ER57-8-CpG-8	0.5453 ± 0.2960	0.8140 ± 0.1060	0.001
ER57-8-CpG-9	0.5453 ± 0.2960	0.8140 ± 0.1060	0.001
TAP1-14-CpG-1.2	0.0345 ± 0.0337	0.0160 ± 0.0305	0.258
TAP1-14-CpG-3	0.0417 ± 0.0368	0.0420 ± 0.0268	0.983
TAP1-14-CpG-4.5	0.0353 ± 0.0234	0.0360 ± 0.0134	0.924
TAP1-14-CpG-6	0.0391 ± 0.0589	0.0320 ± 0.0444	0.753
TAP1-14-CpG-7.8	0.0347 ± 0.0510	0.0620 ± 0.0712	0.446
TAP1-14-CpG-9	0.0198 ± 0.0330	0.0220 ± 0.0492	0.926
TAP1-14-CpG-10	0.0283 ± 0.0496	0.0280 ± 0.0522	0.991
TAP1-14-CpG-11.12.13.14	0.0367 ± 0.0346	0.0240 ± 0.0230	0.309
TAP1-14-CpG-17	0.0464 ± 0.0469	0.0320 ± 0.0383	0.468
TAP1-14-CpG-18	0.0206 ± 0.0278	0.0440 ± 0.0666	0.479
TAP1-14-CpG-19	0.1838 ± 0.1482	0.0550 ± 0.0778	0.181
TAP1-14-CpG-20	0.0072 ± 0.0099	0.0020 ± 0.0045	0.063
TAP1-14-CpG-21	0.0194 ± 0.0316	0.0100 ± 0.0224	0.429
TAP1-14-CpG-24	0.0334 ± 0.0229	0.0225 ± 0.0222	0.407
TAP1-14-CpG-27.28	0.0400 ± 0.0270	0.0360 ± 0.0207	0.707
TAP1-14-CpG-29	0.0221 ± 0.0184	0.0100 ± 0.0071	0.013

Table 4: Differential analysis of methylation levels of the single CpG sites of ERp57 and TAP1 genes in breast cancer tissues and breast fibroma tissues.

Discussion

Relationship between DNA methylation and breast cancer

Previous studies have found that abnormal methylation of multiple genes occurs in breast cancer tissues. As a type D cyclin, Cyclin D2 is involved in the transition of the G1 phase to S phase in the cell cycle, which is mainly achieved by regulating cyclin-dependent protein kinases Cdk6 and Cdk4. Some researchers have used methylation-specific PCR (MSP) technology to analyze the Cyclin D2 methylation frequency in breast cancer cell lines and primary breast cancer and found that the gene promoter in primary breast cancer cells and breast cancer cell lines with no Cyclin D2 expression was

in a hypermethylated state[5-6]. Other studies have found that after 5-aza-dC treatment of breast cancer cell lines with Cyclin D2 gene hypermethylation, the gene can be demethylated, and the expression of the Cyclin D2 gene can be restored, suggesting that DNA methylation is one of the mechanisms causing the loss of Cyclin D2 expression in breast cancer[7].

Chromosome 17q21 has been shown to be strongly associated with early familial breast cancer, and the first gene associated with familial ovarian cancer and breast cancer to be successfully cloned was BRCAl. Studies have shown that more than half of patients with inherited breast cancer have heritable mutations in BRCAl, but the frequency of genetic mutations in the BRCAl gene is not high in patients with sporadic breast cancer[8-10].

Relationship between HLA-I and APM methylation and TNBC

In the process of TNBC occurrence and development, methylation modification is an important molecular event of epigenetic regulation of the genome, and the role of methylation epigenetic changes of tumor suppressor genes in the occurrence and development of TNBC is the most notable (11). In this study, MassARRAY methylation analysis was used to detect the methylation levels of TAP2 and ERp57 genes in breast cancer and breast fibroma tissues. The results showed that the total methylation level of the ERp57 gene in TNBC tissues was significantly lower than that in non-TNBC tissues ($P < 0.01$), and there was no statistically significant difference in the total methylation level of the TAP1 gene between TNBC tissues and non-TNBC tissues ($P > 0.05$). The quantitative detection data of methylation provided by mass spectrometry at the single CpG site showed that in the CpG island in each candidate gene's promoter region, not every site of abnormal methylation leads to abnormal gene expression. The methylation level of ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-6.7, ER57-8-CpG-8, and ER57-8-CpG-9 sites in the promoter region of ERp57 gene in TNBC tissues was remarkably lower than that in non-TNBC tissues ($P < 0.01$). TNBC is aggressive and has a poor prognosis. The results of this study showed that the six CpG sites (ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-6.7, ER57-8-CpG-8, ER57-8-CpG-9) of the ERp57 gene promoter were closely related to the occurrence of TNBC.

By comparing the methylation levels of TAP2 and ER57 genes in the breast cancer and breast fibroma groups, the results showed that the total methylation level of ERp57 gene in breast cancer tissues was markedly lower than that in breast fibroma tissues ($P < 0.05$), the total methylation level of the TAP1 gene was dramatically higher than that in breast fibroma tissues ($P < 0.05$), and there was no significant difference in the total methylation level of LMP7 gene between the two groups ($P > 0.05$). The quantitative detection data of methylation at the single CpG site provided by mass spectrometry showed that the methylation level of ER57-8-CpG-1, ER57-8-CpG-2.3.4, ER57-8-CpG-5, ER57-8-CpG-8, and ER57-8-CpG-9 sites in the promoter region of the ER57 gene in breast cancer tissues was significantly lower than that in breast fibroma tissues ($P < 0.05$). The methylation level of the TAP1-14-CpG-29 site in the promoter region of the TAP1 gene in breast cancer tissues was significantly higher than that in breast fibroma tissues ($P < 0.05$). Therefore, among the numerous CpG sites on the CpG island in the gene promoter region, not all can lead to gene expression silencing. Only partial methylation of the sites can lead to gene expression silencing, and these CpG sites are the key sites regulating gene function.

From the perspective of tumor immune surveillance theory, tumor cells exist in the body and constantly develop immune surveillance functions[12]. HLA-type molecules mediation of the TAP dependent antigen-presenting pathway plays an important role in the body's anti-tumor immune response. As a member of the ABC carrier protein superfamily, TAP is an HLA-I type antigen-presenting molecular chaperone in the endoplasmic reticulum lumen[13, 14]. TAP1 and TAP2 gene silencing is associated with the occurrence and development of various tumors and decreased expression of HLA-I type molecules on the cell surface, and TAP1 and TAP2 gene silencing are related to CpG island hypermethylation in the gene promoter region. Epigenetic changes play an important role in the occurrence and development of breast cancer. The occurrence of breast cancer is a multi-stage process of multi-gene changes. The occurrence of breast cancer proceeds through a series of stages involving the abnormal activation of several proto-oncogenes, the inactivation of tumor suppressor genes, and the stimulation of exogenous carcinogens. During breast cancer development, the pathogenic factors may continue to act on the body, in which DNA methylation plays a very important part.

In conclusion, methylation of the promoter regions of APM member TAP1 and ERp57 genes

play an important role in the occurrence and development of TNBC.

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