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Relation of promoter methylation of *mdr-1* gene and histone acetylation status with multidrug resistance in MCF-7/Adr cells

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Abstract: Objective To analyze the *mdr-1* gene promoter methylation and histone acetylation status in both MCF-7/Adr and MCF-7 cells and to preliminarily explore the epigenetic mechanism of multidrug resistance in breast cancer. **Methods** *mdr-1* gene promoter methylation status of the 2 cell lines was detected by methylation-sensitive PCR. mRNA expression of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) was detected by real-time quantitative PCR. Acetylation level of histone H3 and H4 was examined by optical density assay. **Results** Promoter hypermethylation of *mdr-1* gene was observed in MCF-7 cells whereas hypomethylation was found in MCF-7/Adr cells. Expression of *DNMT1*, *DNMT3a*, and *DNMT3b* mRNA in MCF-7/Adr cells significantly decreased compared with that of MCF-7 cells ($P<0.05$). H3 and H4 histone acetylation levels of MCF-7/Adr cells were obviously higher than those of the MCF-7 cells ($P<0.01$). Expression of *HDAC1*, *HDAC2*, *HDAC7*, and Sirtuin type 1 (*SIRT1*) mRNA in MCF-7/Adr cells was significantly reduced ($P<0.05$). **Conclusion** Hypomethylation of the promoter region of *mdr-1* gene, high H3 and H4 histone acetylation, and low mRNA expression of *DNMTs* and *HDACs* may be important epigenetic factors for the development of MDR in MCF-7/ Adr cells.

Key words: breast cancer; multidrug resistance; gene; methylation; histone; acetylation

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MCF-7/Adr 细胞 *mdr-1* 基因启动子甲基化和组蛋白乙酰化状态与多药耐药的关系

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[摘要] **目的:** 分析 MCF-7/Adr 及 MCF-7 细胞 *mdr-1* 基因启动子甲基化和组蛋白乙酰化状态,初步探讨乳腺癌多药耐药的表观遗传机制。**方法:** 用甲基化敏感 PCR 技术检测两个细胞系 *mdr-1* 基因启动子甲基化状态。实时定量 PCR 技术检测 DNA 甲基转移酶(DNA methyltransferases, *DNMTs*) mRNA 及组蛋白去乙酰化酶(histone deacetylases, *HDACs*) mRNA 的表达。光密度值法检测组蛋白 H3 和 H4 乙酰化水平。**结果:** MCF-7 细胞 *mdr-1* 基因启动子呈现高甲基化,MCF-7/Adr 细胞 *mdr-1* 基因启动子呈现低甲基

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Biography HUANG Chenghui, M. D., attending physician, mainly engaged in the research of mechanism and reversal of multidrug resistance in malignant tumor.

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化。与 MCF-7 细胞比较,MCF-7/Adr 细胞 *DNMT1*,*DNMT3a* 及 *DNMT3b* mRNA 表达显著下降($P<0.05$)。MCF-7/Adr 细胞组蛋白 H3 和 H4 乙酰化水平较 MCF-7 细胞明显升高($P<0.01$)。与 MCF-7 细胞比较,MCF-7/Adr 细胞 *HDAC1*,*HDAC2*,*HDAC7* 及 *SIRT1* mRNA 的表达显著下降($P<0.01$)。**结论**:*mdr-1* 基因启动子低甲基化、组蛋白 H3 和 H4 高乙酰化、*DNMTs* mRNA 及 *HDACs* mRNA 低表达可能是介导 MCF-7/Adr 细胞 MDR 形成的重要表观遗传学因素。

[关键词] 乳腺癌; 多药耐药; 基因; 甲基化; 组蛋白; 乙酰化
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The resistance of cancer cells to chemotherapeutic agents often leads to failure of chemotherapy. Multidrug resistance (MDR) reversal has become an urgent problem to be resolved. Many mechanisms are involved in tumor MDR formation, among which high expression of *mdr-1*/P-gp is the one that is most deeply and widely studied. Chemotherapy plays a major role in the treatment of patients with breast cancer, a kind of common malignant tumor. However, MDR often plagues a main obstacle to clinical efficacy after multi-chemotherapy. Epigenetics is a kind of inheritable gene expression mechanism with no change in DNA sequence, which involves in DNA methylation, histone acetylation, and chromatin remodeling. In recent years the epigenetic studies showed that DNA methylation and histone deacetylation could inhibit gene transcription[1]. To investigate the epigenetic mechanism of MDR in breast cancer, we analyzed the *mdr-1* gene promoter methylation and histone acetylation status in both MCF-7 breast cancer cell line and its variant MCF-7/Adr cell line to explore their roles in MDR.

1 MATERIALS AND METHODS

1.1 Cell culture

MCF-7 breast cancer cell line was provided by Cell Center of Xiangya School of Medicine, Central South University. MCF-7/Adr breast cancer cell line was purchased from the Shanghai Jintian Biological Technology Co. Ltd. The latter was cultured in doxorubicin (ADM, 1.0 mg/L) to maintain its drug resistance character and was used to do the experiment half a month later after ADM free culture. All these cells were cultured in RPMI 1640 medium containing 10% fetal calf serum at 37 ℃ in 5% CO_2 atmosphere with saturated humidity. The cells were subcultured every 3 to 4 days.

1.2 Main reagents

Trizol was purchased from Invitrogen Corporation in USA. *Msp*I/*Hpa*II restriction enzymes were purchased from Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. EpiQuiKTM histone extraction kit and histone H3 and H4 acetylation detection kits were purchased from Epigentek Group Inc. in USA. BCA protein assay kit was purchased from Pierce Chemical Company in USA. QuantiTest SYBR Green qPCR kit was purchased from Qiagen N.V. in Dutch. Real-time quantitative histone deacetylase (HDAC), DNA methyltransferase (DNMT), and β-actin gene PCR primers were synthesized by Shanghai Yinjun Biological Co. Ltd. with reference to the literatures[2-3]. Detailed primer sequences are shown in Tab. 1.

Tab. 1 Real-time PCR primers sequences of target genes (β-actin as internal control)

Target genes	Upstream primers	Downstream primers
HDAC1	5′-CAAGCTCCACATCAGTCCTTCC-3′	5′-TGCGGCAGCATTCTAAGGTT-3′
HDAC2	5′-AGTCAAGGAGGCGGCAAAA-3′	5′-TGCGGATTCTATGAGGCTTCA-3′
HDAC7	5′-CTTCTCCACAAGGACAAG-3′	5′-CTCCAGGGTTCTGTAGG-3′
SIRT1	5′-TACCGAGATAACCTTCTG-3′	5′-TCCAGTCACTAGAGCTTG-3′
DNMT1	5′-GAGCTACCACGCAGACATCA-3′	5′-CGAGGAAGTAGAAGCGGTTG-3′
DNMT3a	5′-CCGGAACATTGAGGACATCT-3′	5′-CAGCAGATGGTGCAGTAGGA-3′
DNMT3b	5′-CCCATTCGAGTCCTGTCATT-3′	5′-GGTTCCAACAGCAATGGACT-3′
β-actin	5′-GCACCACACCTTCTACAATGAGC-3′	5′-GGATAGCACAGCCTGGATGCAAC-3′

1.3 Detection of *mdr-1* gene promoter methylation status

DNA of MCF-7 cells and MCF-7/ADR cells were extracted. Genomic DNA was digested with *Hpa* II restriction endonuclease. *Msp* I was used to do another parallel control experiment. *Msp* I can cleave CCGG sequences regardless of CpG methylation status, but *Hpa* II can just cleave the CCGG sequences which were unmethylated. The sequence of *mdr-1* promoter region that can be identified by *Msp* I/*Hpa* II includes 2 segments. 2 μg DNA was digested with 20 U *Hpa* II restriction endonuclease at 37 ℃ for about 16 hours. Fragment I was about 121 bp. Sense PCR primer was 5′-TCTAGAGAGGTGCAACGGAAG-3′. Antisense primer was 5′-TCCGCCTCACCACAGATGAC-3′. Fragment Ⅱ was about 206 bp. Sense PCR primer was 5′-TGAAGTCCTCTGGCAG TCC-3′. Antisense primer was 5′-ATTCTCCCTCCCGGTTCC-3′. Each reaction system contained 0.25 μg *Msp* I or *Hpa* II digested DNA, 50 pmol sense and antisense primers, and 2 U TaqDNA polymerase. The amplification conditions consisted of an initial denaturation at 95 ℃ for 10 minutes, followed by 25 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 60 ℃ for 30 seconds, and polymerization at 72 ℃ for 30 seconds. PCR products were electrophoresised in 2% agarose gel.

1.4 Histone H3 and H4 acetylation detection

Histone of MCF-7 cells and MCF-7/ADR cells was extracted according to the EpiQuiK detection kit. Protein precipitation was dissolved by adding appropriate amount of double-distilled water. Protein solution was adjusted to the concentration of 200 μg/L. 5 mL protein solution was added to 8-well plate, incubated at 37 ℃ for 60 minutes. 50 μL H3 or H4 histone capture antibody (1 mg/L) was added to the solution for shaking incubation at room temperature for 60 minutes. The reactive plate was washed fully with washing liquid for 4 times and dried with filter paper. Then 50 μL H3 or H4 histone detection antibody (0.4 mg/L) was added for incubating at room temperature for 30 minutes. The plate was washed for another 5 times. 100 μL substrate solution was added to each hole, reacting at 37 ℃ in dark place for 10 minutes until the color of the holes turned blue. 50 μL suspension was added to the solution. Automatic microplate reader was used to detect every well's optical density values with 450 nm wavelength after mixing (EXL800UV, the United States Bio-Tek Inc). The mean value was acquired in each group. Each well was set for 4 duplicated holes. The experiment was repeated for 3 times. Formula as below was used for calculating the acetylation rate.

$$\text{Acetylation rate}(\%) = \frac{\text{OD (treated sample} - \text{blank)}}{\text{OD (untreated control} - \text{blank)}} \times 100\%.$$

1.5 Detection of DNMT and HDAC mRNA expression

Total RNA of 1×10^6 cells was extracted by using Trizol, and then cDNA was synthesized. The following components were added proportionally to each group: QuantiTest SYBR Green 10 μL, upstream primer (10 mg/L) 1.0 μL, downstream primer (10 mg/L) 1.0 μL, cDNA4.0 μL and then ribozyme-free water was added so that the end volume of reaction reached 20 μL. Reaction conditions: Initial denaturation at 95 ℃ for 15 minutes followed by 40 cycles of denaturation at 94 ℃ denaturation for 15 seconds, annealing at 55 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds. The fluorescence values were automatically recorded by real-time fluorescence quantitative PCR instrument, Rotor-Gene 3000 (Corbett Life Science in Australia). The relative values of target genes divided by β-actin could be drawn according to the target genes and β-actin cycle threshold values and standard curves. The relative quantitative results could be obtained by the value of targeted gene divided by those of β-actin gene.

1.6 Statistical analysis

Experimental results were the measurement data expressed as $\bar{x} \pm s$. Comparison between groups was performed by variance analysis and q test. $P < 0.05$ considered significantly different. Experimental data

were dealt with SPSS11.0 statistics.

2 RESULTS

2.1 *mdr-1* gene promoter methylation status of MCF-7/Adr and MCF-7 cells

Two pairs of primers used for the amplification containing the *Msp*I/*Hpa*II sites located in the promoter region of *mdr-1* gene. The amplification products of genomic DNA digested with *Hpa*II restriction endonuclease showed hypermethylation in *mdr-1* gene promoter region of MCF-7 cells but hypomethylation in MCF-7/Adr cells (Fig. 1 and 2).

2.2 *DNMT* mRNA expression of MCF-7/Adr and MCF-7 cells

DNMT1, *DNMT3a*, and *DNMT3b* mRNA were highly expressed in MCF-7 cells. Compared with MCF-7 cells, expression of *DNMT1*, *DNMT3a*, and *DNMT3b* mRNA in MCF-7/Adr cells significantly decreased ($P<0.05$, Tab.2).

2.3 H3 and H4 histone acetylation levels of MCF-7/Adr and MCF-7 cells

The results showed a low level of histone H3 and H4 acetylation in MCF-7 cells. Histone H3 and H4 acetylation levels were significantly higher in the MCF-7/Adr cells than those of MCF-7 cells ($P<0.01$, Tab.3).

2.4 *HDAC* mRNA expression of MCF-7/Adr and MCF-7 cells

HDAC1, *HDAC2*, *HDAC7*, and *SIRT1* mRNA expressions of MCF-7 cells were highly expressed. *HDAC1*, *HDAC2*, *HDAC7*, and *SIRT1* mRNA expressions of MCF-7/Adr cells were significantly lower than those of MCF-7 cells ($P<0.01$, Tab.4).

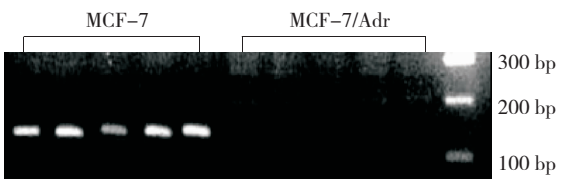

Fig. 1 ***mdr-1* gene promoter Fragment Ⅰ (121 bp) methylation status of MCF-7/Adr and MCF-7 cells.**

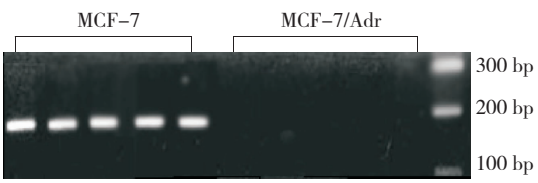

Fig. 2 ***mdr-1* gene promoter Fragment Ⅱ (206 bp) methylation status of MCF-7/Adr and MCF-7 cells.**

Tab. 2 Expression level of *DNMT* mRNA in MCF-7 and MCF-7/Adr cells ($\bar{x}\pm s, n=12$)

Groups	*DNMT1*	*DNMT3a*	*DNMT3b*
MCF-7 cells	2.56±0.14	2.89±0.15	3.16±0.21
MCF-7/Adr cells	1.14±0.06*	1.13±0.05*	1.69±0.26*

Compared with the MCF-7 cells, $*P<0.05$.

Tab. 3 Histone H3 and H4 acetylation levels of MCF-7 and MCF-7/Adr cells ($\bar{x}\pm s, n=12$)

Groups	H3 acetylation	H4 acetylation
MCF-7 cells	0.132±0.009	0.168±0.011
MCF-7/Adr cells	0.382±0.036**	0.461±0.013**

Compared with the MCF-7 cells, $**P<0.01$.

Tab. 4 *HDAC* mRNA expression of MCF-7 and MCF-7/Adr cells ($\bar{x}\pm s, n=12$)

Groups	HDAC1	HDAC2	HDAC7	SIRT1
MCF-7 cells	3.68±0.23	8.69±0.15	6.35±0.21	2.15±0.09
MCF-7/Adr cells	1.18±0.16**	3.65±0.25**	2.21±0.19**	0.98±0.03**

Compared with the MCF-7 cells, $**P<0.01$.

3 DISCUSSION

Induced by ADM in vitro, MCF-7/Adr cells, a breast cancer cell lines, which has become an important MDR cell research model, have the characteristics of high *mdr-1*/P-gp expression, resisting to many chemotherapeutic drugs such as adriamycin (ADM), daunorubicin (DNR), and vincristine (VCR)[4]. DNA methylation refers to transferring methyl to the fifth carbon atom of the cytosine and formation of 5-methyl-cytosine using S-adenosyl-L-methionine as methyl donor under the role of DNMTs. CpG islands (CGIs), 56% of coding genes related, are rich in CpG dinucleotide in the human genome sequences. Regulation of gene expression is their most critical function. DNA methylation is strictly regulated by DNMTs. The most widely studied methyltransferases are DNMT1, DNMT3a, and DNMT3b so far. DNMT1 is responsible for the maintenance methylation of de novo methylated DNA. DNMT3a and DNMT3b are responsible for maintaining the methylation of the unmethylated double-stranded DNA[5]. DNA methylation restrains certain genes' activation and hypomethylation upregulates expression of some genes[1]. Study on bladder cancer from Tada, et al.[6] showed that hypomethylation of *mdr-1* gene promoter is necessary for increasing gene expression and occurrence of drug-resistant phenotype. Our study showed that band didn't appear in the corresponding lane of Fragment Ⅰ and Fragment Ⅱ of *mdr-1* gene promoter region in MCF-7/Adr cells. All those cells showed a hypomethylation status, whereas the variant MCF-7 cells showed hypermethylation status. Further studies made clear that the *DNMT1*, *DNMT3a*, and *DNMT3b* mRNA expression levels in sensitive MCF-7 cells were significantly higher than those of drug-resistant MCF-7/Adr cells. These results suggest that hypomethylation of *mdr-1* gene promoter CGIs in MCF-7/Adr cells have apparent activation on gene expression and regulation which leads to MDR in tumor cells.

Histone is the core of chromatin. The covalent modification in its tail plays an important role in gene expression regulation. Histone acetylases together with HDACs regulate the state of histone acetylation under normal circumstances. Histone acetylation leads to gene activation and expression whereas histone deacetylation leads to gene silencing. HDACs can be divided into 3 types, among which Type I and Type II include HDAC1 ~ HDAC12. Type III HDACs also known as Sirtuins, of which longevity gene *SIRT1* is mostly studied[7]. Our study showed that the histone H3 and H4 acetylation levels were significantly lower in MCF-7 cells than those of the corresponding resistance MCF-7/Adr cells. The *HDAC1*, *HDAC2*, *HDAC7*, and *SIRT1* mRNA expression levels were significantly upregulated in MCF-7 cells compared with those of the drug-resistant MCF-7/Adr cells. These results demonstrate that low expression of HDACs and high histone H3 and H4 acetylation levels may play an important role in MDR formation by activating the *mdr-1* gene transcription in MCF-7/Adr cells.

At present, most studies consider that DNA methylation influencs histone acetylation and vice versa. Furthermore DNA methylation and histone acetylation can generate synergies. Participating in chromatin remodeling, DNMT1 can recruit many enzymes, including HDAC1, HDAC2, and the histone methyltransferases, which plays a crucial role in the maintenance of DNA methylation and the modification of histone H3[8-9]. El-Osta, et al.[10] studied the sensitive and drug resistant leukemia CEM cells and found an obvious demethylation treated by DNMT inhibitor 5-azacytidine, which induces a low expression of *mdr-1* gene alone. Single application of trichostatin (TSA), a kind of HDAC inhibitor, could not activate the hypermethylated *mdr-1* gene. However, 5-azacytidine combined with TSA could induce a high expression of *mdr-1* gene. Our study suggests that DNA hypomethylation in the *mdr-1* gene promoter region and histone acetylation are im-

portant factors leading to the gene activation, which may play an important role in the development of MDR. Histone deacetylation may contribute to inhibiting of drug resistant formation after chemotherapy on MCF-7 cell lines. However, further study should focus on whether there are deeper interactions between *mdr-1* gene promoter methylation and histone acetylation during the process of sensitive breast cancer MCF-7 cell lines becoming variant resistant strains. Clarifying the mechanism mentioned above would also provide a theoretical basis in exploring effective strategies in tumor MDR reversal.

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