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Original Research Article

Low Dose 5-Azacytidine Treatment Induce in KYSE450 Esophageal Carcinoma Cell Line

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Abstract: The objective is to discuss the treatment effects of low doses of 5 – Azacytidine to KYSE450 esophageal cancer cell line. The cells of KYSE450 esophageal cancer cell line were used different drug concentrations of 5-Azacytidine（5-Aza）, and used Western blot hybridization technique(Western blotting) detected ofγH2AX protein expression of cells, selected drug concentration of relative to the maximum and not produce cytotoxic for subsequent experiments. Methylation-specific PCR (Methylation Specific Polymerase Chain Reaction, MSP) detected methylation status changed circumstances of DNMT1 and DNMT3b gene promoter at different time points in pretherapy and post-treatment. 3. Western blotting analysis the protein expression changed of DNMT1 and DNMT3b genes at different time points in pretherapy and post-treatment. Real-time quantitative PCR (Quantitative Real time Polymerase Chain Reaction, qRT-PCR) technique detected mRNA expression of DNMT1 and DNMT3b genes at different time points in pretherapy and post-treatment. 5. Flow cytometry is used to analyze the cell cycle change of each cell. 1. Application of 5-Aza treatment KYSE450 esophageal cancer cells after 72h, measured 1000nM/L drug concentrations relative to the maximum dose of the drug does not produce toxicity. The cells of KYSE450 esophageal cancer cell line were treated with low doses of 5-Aza, DNMT1, DNMT3b gene methylation was decreases at first and increases late with time rendering. 3. Demethylation can increase silencing the expression of DNMT1, DNMT3b genes, so that is decreased expression of the mRNA and protein, there were the same trend with decreases at first and increases late, and can lead to cell cycle arrest, inhibition of cell proliferation. Low doses of 5-Aza does not produce toxic effects on cells, and can be reversed DNMT1, DNMT3b methylation status for clinical epigenetic therapy provides long-term basis. The expression of DNMT1 and DNMT3b gene decrease with mRNA and protein and cell cycle arrest, that is closely associated with decreased methylation status of the gene. DNMT1 and DNMT3b gene may be an important factor in the development of esophageal cancer, it offers a new way of molecular targeting treatment for esophageal cancer.
Keywords: esophageal cancer; DNA methyltransferases; epigenetic; 5-Azacytidine.

INTRODUCTION

Esophageal carcinoma (EC) is the one of the most common malignant tumor of world, its morbidity and mortality were malignant tumor of the world's sixth and fourth[1]. The occurrence of EC is a multi-stage, more genetic evolution under the influence of participation, its development present cumulative change, involving protocarcinogenic gene expression and tumor suppressor gene silence [2].

In recent years, epigenetics research is the most popular and fastest growing in the oncology research field, its main contain DNA methylation, noncoding RNA regulation, histone modification and chromatin remodeling, and so on. The occurrence and development of tumor is closely related to the abnormal changes of epigenetics [3, 4]. DNA methylation is one of the most important regulatory mechanisms of epigenetics [5]. In genetics, genetic mutations often cannot be reversed; but epigenetic changes under certain conditions can be reversed, the special clew of epigenetic repair may provide new opportunities for future anticancer treatment [6, 7].

Long and large doses of epigenetic therapy treatment can have serious side effects, what is more genetic mutations will happen to induce other tumors; its action mechanism, patient selection and dosage must be further research. Nucleoside class, now, is the more the epigenetic drugs, it can make the genome shows low methylation status, role and positively related to the

dose, but high drug concentration can affect the synthesis of DNA or RNA, cytotoxic effect.

Studies have shown [8], the DNA methylation inhibitors decitabine and 5-Aza are efficacious for hematological neoplasms at lower, less toxic, doses. Low concentration of drug dosage can reduce DNMTs in cells; reduce the degree of methylation gene.

DNA methylation is realized by DNMTs. DNMTs adjust DNA methylation status, not only in the growth of the organism, and plays a very important role in the aging process, but also in the development process of tumor plays a very important role [9-11]. Thus the DNMTs in epigenetic plays a very important role. In recent years, experimental studies are mainly concentrated in DNMTs tumor-suppressor gene methylation, but the express the change of the DNMTs itself whether be influenced by the promoter methylation of DNMTs gene and histone acetylation , the research is rarely. In EC, the change of the expression of DNMTs itself whether be affected by the promoter methylation of DNMTs gene, and the relationship with the effect of epigenetic treatment. These are not reported. This study intends to explore the change of the promoter methylation of DNMTs gene and DNMTs expression, and the relationship with the effect of epigenetic treatment, after EC cell lines by low dose of DNMTs inhibitor treatment. This provides the basis for epigenetic treatment of EC, in the future.

MATERIALS

Experimented cells & Drugs

KYSE450 esophageal carcinoma cell lines purchased from Cell Bank of Chinese Academy of Sciences (Shanghai). 5-Azacytidine dissolved in DMSO，preserved in minus 20℃.

5-Azacytidine treatment

Treatment group: Cells were treated with 5-azacytidine at a concentration of without cytotoxicity. Growth medium, conditioned with 5-azacytidine at a concentration of without cytotoxicity, was exchanged every 24 hours for a total of 72 hours of treatment. In the end, sample was extracted from the cells at 1 day, 4 day, 7 day, 14day and 21 day. Control group: Only add the same amount of DMSO in cells.

METHODS

Protein extractin and western blotting

Whole cell lysates were homogenized in RIPA buffer (PBS buffer with 1% NP40, 0.5% sodium deoxycholate, 0.1%SDS of final concentration and protease inhibitor cocktail [Roche]). Total proteins were extracted, and the concentration was measured. Then the total proteins were separated by 4%～12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA), transferred to PVDF (Millipore, Billerica, MA) and immunoblotted using anti-DNMT1, anti-DNMT3b, anti-GAPDH (Sigma- Aldrich), Donkey Anti-Rabbit and anti-Mouse IgG ECL Antibody, HRP Conjugated (GE Healthcare). The membrane was blocked with 5% nonfat milk. An enhanced chemiluminescence (Sangon, Shanghai, China) method was used to detect the protein bands and ECL plus western blotting detection system (GE Healthcare) was used for visualization.

Methylation specific PCR (MSP)

The application of DNA extraction kit to extract DNA and get through the sodium bisulfite modification of DNA.MSP primers were designed according to genomic sequences. MSP detect bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles. MSP of DNMT1 was carried out using primers 5'TTTTAGTAAATCGTGGAGT TTGGAC 3' (M-sense); 5' TACAAAAAAATAAAACGAAACC GAC 3' (M- antisense); 5' TTAGTAAATTGTGGAGTTTGGATGA 3' (U-sence); and 5' TACAAAAAAATAAAAC AAAACCAAC 3' (U-antisense). MSP of DNMT3b was carried out using primers 5'TTTATTTATTTTCGTTGT TTCGTTC 3' (M-sense); 5' CTTAACCACTTAACCCCAACG 3' (M-antisense); 5'TTATTTATTTTT GTTGTTTTGTTTG 3' (U-sence); and 5' CTTAAACCACTTA ACCCCAACACT 3' (U-antisense). Each MSP reaction included approximately 500 ng of bisulfite-treated DNA, 1ul of each primer, 2×HotMaste Taq PCR Master Mix 12.5ul, and ddH2O 7.5ul in a final reaction volume of 25ul. Cycle conditions of DNMT1were 94℃ 5 minutes, 35 cycles(94℃ 30 seconds, 58℃ 30 seconds, 72℃ 40 seconds);72℃ 5 minutes. Cycle conditions of DNMT3b were 94℃ 5 minutes, 37 cycles(94℃ 30 seconds, 57℃ 30 seconds, 72℃ 40 seconds);72℃ 5 minutes. MSP products were analyzed using 2% agarose gel electrophoresis.

RNA isolation and semi-quantitative reverse transcription PCR

Total RNA was isolated and extracted from cells with the TRIzol reagent Kit (Techonologies, Gaithersburg, MD). Agarose gel electrophoresis (1%) and spectrophotometric analysis (A260:280 nm ratio) were used to assess RNA quality and quantity. Amplify the produced cDNA in different microburettes, prepare reaction systems of 20μL based on the instruction of qRT-PCR kit with dye technique and make three accessory holes; Condition: pre degenerate them for 2 minutes in 50 ℃, 10 minutes in 95℃, 15 seconds in 95 ℃ and 1 minute in 60 ℃, for 40 cycles. The results, based on the standard curve, are automatically calculated from softwares.

The upstream primer of DNMT1: 5'AGGACTAGTTCTGCCCT CCC3', the downstream primer: 5'ACAGCTTCATGTCAGCCAAG3', the amplimer: 143bp. The upstream primer of DNMT3b:

5'GAATTACTCAC GCCCCAAGGA3', the downstream primer: 5'ACCG TGAGATGTCCCT CTTGTC3', the amplimer: 101bp. The upstream primer of GAPDH: 5'CAACTACATGGTTTACATGTTC3', the downstream primer: 5'GCC AGTGGACTCCACGAC3', the amplimer: 181bp. GAPDH was used to normalize all samples as an internal control.

Subcellular fractionation location

The separation of the nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

STATISTICAL METHODS

All the statistical analyses were performed using the statistical software package SPSS17.0. The results were compared in pairs. Associations with the research results were analyzed by t-test. A P-value of less than 0.05 was considered statistically significant.

RESULTS

The effects of the epigenetic treatment to KYSE450 Esophageal Carcinoma Cell Lines on the expression of γH2AX protein

KYSE450 cells were treated treating 72 hours with 5-azacytidine, drug concentration were respectively 0nM, 800nM, 900nM, 1000nM, 1100nM, 1200nM and 1300nM. The results show compare the expression of protein of control group with the experimental groups (respectively 800nM, 900nM and 1000nM) there were no significant differences($P>0.05$), control group with the experimental groups (respectively 1100nM, 1200 nM and 1300nM) have statistical significance ($P<0.05$). In consequence, 1000 nM/L drug concentrations relative to the maximum dose of the drug do not produce toxicity.

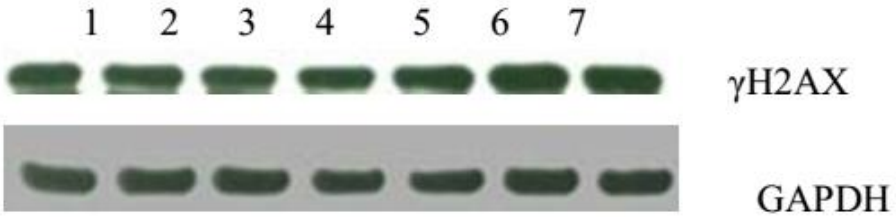

Fig 1: The expression of Western blotting for detection of γH2AX in KYSE450 esophageal carcinoma cells.
1、control group; 2:、The experimental group：800nM; 3、The experimental group：900nM; 4、The experimental group：1000nM; 5、The experimental group：1100nM; 6、The experimental group：1200nM; 7、The experimental group：1300nM;

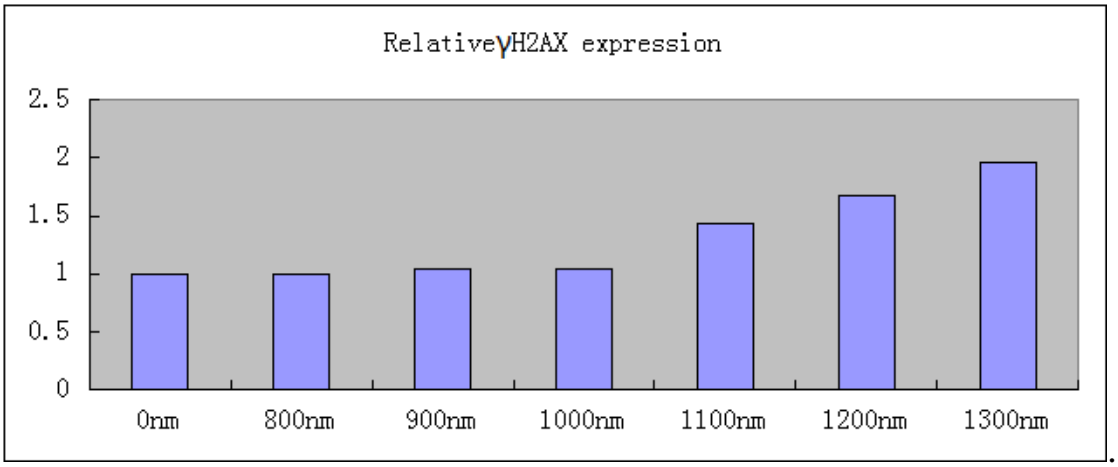

Fig 2: Relative expression ofγH2AX protein in cells of each group after treatment

Demethylation changes of DNMT1 and DNMT3b genes in KYSE450 cells.

KYSE450 esophageal cancer cells treated with low doses of 5-Aza, DNMT1, DNMT3b gene methylation was decreases at first and increases late with time rendering (Fig 3).

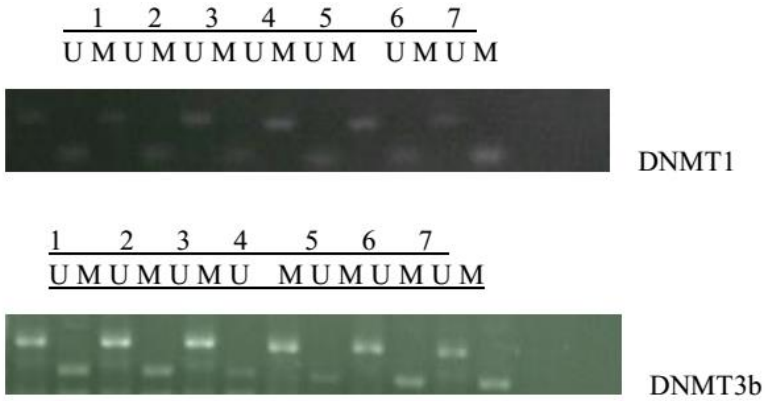

Fig 3: MSP for detection of methylation status of DNMT1、DNMT3b in KYSE450 cells after

1、control group; 2:、The 1 day after treatment; 3、The 4 day after treatment;4、The 7 day after treatment; 5、The 14 day after treatment; 6、The 21 day after treatment; 7、 represents water controls.

The not produce toxicity of the epigenetic treatment to KYSE450 Esophageal Carcinoma Cell Lines on the expression of DNMT1 and DNMT3b protein.

The expression of DNMT1, DNMT3b protein, there were decreases at first and increases late with time rendering. The results show compare the expression of protein of control group with the experimental groups of DNMT1 and DNMT3b have statistical significance ($P < 0.05$).

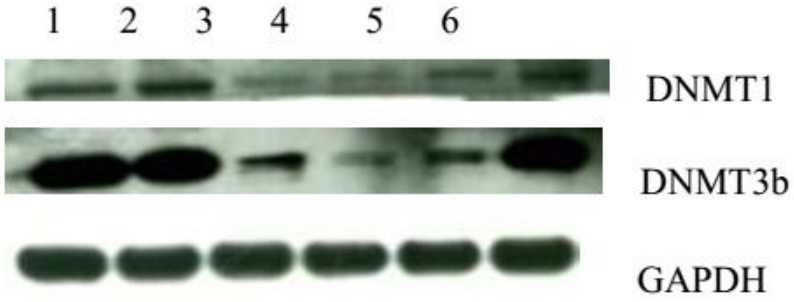

Fig 4: The expression of Western blotting for detection of DNMT1、DNMT3b in KYSE450 cells after treatment.

1、control group; 2:、The 1 day after treatment; 3、The 4 day after treatment; 4、The 7 day after treatment; 5、The 14 day after treatment; 6、The 21 day after treatment

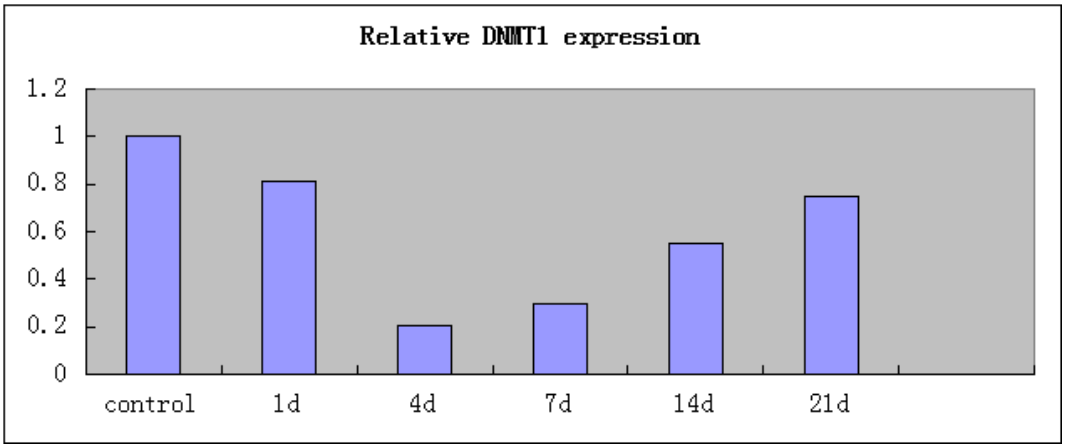

Fig 5: Relative expression of DNMT1 protein in cells of each group after treatment

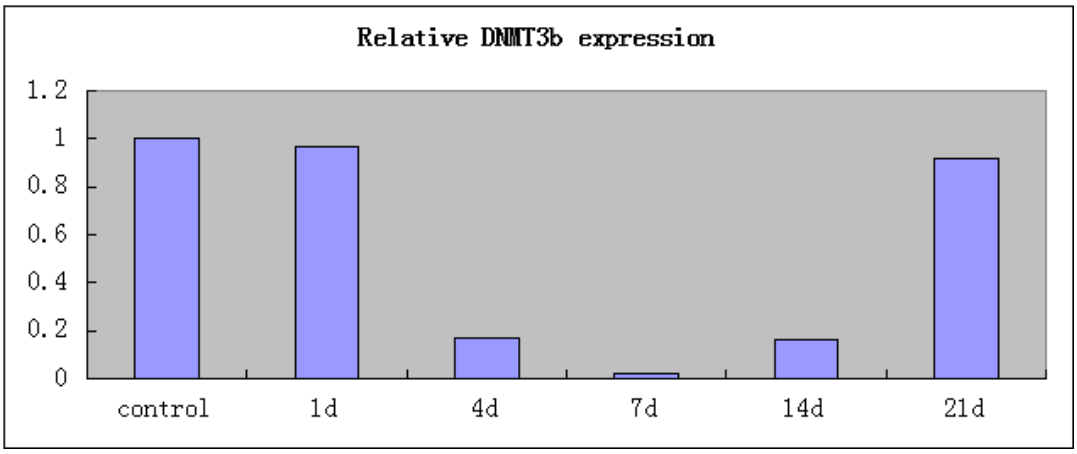

Fig 6: Relative expression of DNMT3b protein in cells of each group after treatment

Epigenetic treatment reduced the expression of DNMT1 gene and DMT3b gene in KYSE450 cell lines.

The mRNA of DNMT1 and DNMT3b reduced significantly in all the treatment groups compared to every control group. There were decreases at first and increases late with time rendering.

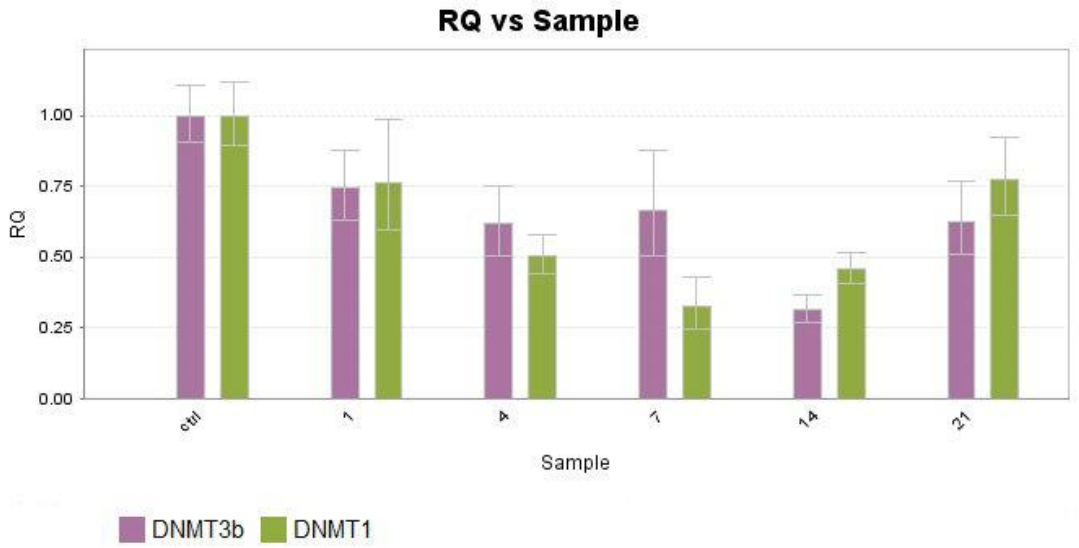

Fig 7: The expression of QRT-PCR for detection of DNMT1、DNMT3b gene in cells of each group.

Ctrl: control group; 2: The 1 day after treatment; 4: The 4 day after treatment; 7: The 7 day after treatment; 14: The 14 day after treatment; 21: The 21 day after treatment;

The cell cycle of the KYSE450 had changed after epigenetic treatment.

Epigenetic treatment that the maximum dose of the drug does not produce toxicity can lead to cell cycle arrest, inhibition of cell proliferation.

group	G1	S	G2
control group	45.88±5.16	39.62±2.00	14.36±3.19
The 1 day after treatment	41.50±2.21	49.54±4.05*	8.96±1.92*
The 4 day after treatment	56.56±1.19*	30.65±1.41*	12.80±0.44
The 7 day after treatment	36.30±0.72*	50.51±0.70*	12.98±0.61
The14 day after treatment	27.37±1.59*	46.59±0.47*	26.03±1.40*
The21 day after treatment	41.02±0.78	46.82±1.92*	12.16±1.21

Notes：comparisons to control group and treatment group,* $P < 0.05$,

DISCUSSION

Eukaryotic cell DNA and histone exists in the form of nucleosome in the nucleus, histone H2A as one of the five kinds of histones and highly conservative. H2AX is a subtype of the H2A histone family. Efficient repair of DNA double-strand breaks was observed in the γ-H2AX assay. Normally, with the passage of time (and over a predictable time in the presence of normal physiological repair mechanisms), the level of γ-H2AX diminishes as repair is completed; persistence of γ-H2AX indicates impaired DNA repair [12]. Due to the physical and chemical and biological factors, leading to cell DNA damage. In early response to DNA damage repair, γH2AX is the one of the earliest molecular reaction [13].

The results show compare the expression of protein of control group with the experimental groups (respectively 800nM, 900nM and 1000nM) there were no significant differences($P>0.05$), control group with the experimental groups (respectively 1100nM, 1200 nM and 1300nM) have statistical significance ($P<0.05$). In consequence, a 1000 nM/L drug concentration relative to the maximum dose of the drug does not produce toxicity. Use with this drug concentrations in the experimental group.

Epigenetics and genetics are two mechanisms in the development of tumor [14, 15]. Genetics research genetic structure, function, variation, and transfer and expression pattern of genes, to explore the gene sequence change caused by changes in the level of gene expression. Epigenetics research gene expression, its characteristic is the change of gene expression does not depend on the change of the DNA sequence, the change of DNA and histone modification. DNA methylation is one of the most important regulatory mechanisms of epigenetics [5]. DNA methylation of tumor suppressor gene silencing gene expression resulting in tumorigenesis, therefore, the use of demethylating drugs restore function of tumor suppressor genes, inhibition of tumor growth. It is a kind of new method for the treatment of malignancy in recent years.

DNMT consists of DNMTl, DNMT2, DNMT3a, DNMT3b and DNMT3L [16]. It plays an important role in the development of tumor that the increased expression of DNMTs leads to abnormal methylation of a variety of tumor suppressor gene in promoter region. In the process of canceration of gastric cancer, pancreatic cancer, liver cancer, lung cancer, breast cancer and cervical cancer, the increased expression of DNMTs is one of the early molecular changes [17-22]. Inhibit the expression of DNMTs can promote cell apoptosis in colon cancer and lung cancer [23, 24].

In recent years, experimental studies are mainly concentrated in DNMTs tumor-suppressor gene methylation, but the express the change of the DNMTs itself whether be influenced by the promoter methylation of DNMTs gene and histone acetylation , the research is rarely. In EC, the change of the expression of DNMTs itself whether be affected by the promoter methylation of DNMTs gene, and the relationship with the effect of epigenetic treatment, these are not reported. This study intends to explore the change of the promoter methylation of DNMTs gene and DNMTs expression, and the relationship with the effect of epigenetic treatment, after EC cell lines by low dose of DNMTs inhibitor treatment.

Based on the experiment results show that application of 5-Aza treatment KYSE450 esophageal cancer cells after 72h, measured 1000nM/L drug concentrations relative to the maximum dose of the drug does not produce toxicity. KYSE450 esophageal cancer cells treated with low doses of 5-Aza, DNMT1, DNMT3b gene methylation was decreases at first and increases late with time rendering. Demethylation can increase silencing the expression of DNMT1, DNMT3b genes, so that is decreased expression of the mRNA and protein, there were the same trend with decreases at first and increases late, and can lead to cell cycle arrest, inhibition of cell proliferation.

CONCLUSION

In conclusion, low doses of 5-Aza does not produce toxic effects on cells, and can be reversed DNMT1, DNMT3b methylation status for clinical epigenetic therapy provides long-term basis. The expression of DNMT1 and DNMT3b gene decrease with mRNA and protein and cell cycle arrest, that is closely associated with decreased methylation status of the gene. DNMT1, DNMT3b gene may be an important factor in the development of esophageal cancer, it offers a new way of molecular targeting treatment for esophageal cancer.

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Conflict of Interests: The authors declare that they have no conflict of interests.

Authors' Contribution:

Xingxing Peng wrote the paper. Xingxing Peng, Jiaqiang Luan, Kai Hu were in charge of experimental operation. Haiyong Wang, Zhenzong Du, Jianfei Song supervised the composition of the paper. All authors read and approved the final paper.

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