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Original Article

Role of Syk gene promoter demethylation in the biological behavior of medulloblastoma

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Abstract: Purpose: To investigate the demethylation status of the Syk gene promoter in human medulloblastoma cell lines and the interrelationship with biological behavior of medulloblastoma. Methods: D341 and Daoy cells were treated with 5-aza-2'-deoxycytidine (5-Aza-CdR) *in vitro*. RT-PCR, MSP, Western Blot and Transwell assay were used to detect Syk methylation, Syk mRNA, Syk protein, as well as the number of penetrating cells before and after treatment of 5-Aza-CdR. Results: Syk was expressed in D341 and Daoy cell lines. The expression of Sykm RNA and protein were up-regulated, the promoter methylation level of Syk gene was significantly decreased ($P<0.01$), and the invasion and metastasis ability were decreased ($P<0.01$) in D341 and Daoy cell lines after treatment with 5-Aza-CdR. Conclusion: Demethylation of Syk gene promoter can inhibit the invasion of the medulloblastoma.

Keywords: Medulloblastoma, Syk, demethylation, invasion

Introduction

Medulloblastoma is a common intracranial tumor in children and it is the second most common malignancy. The cause is unclear at present. The treatment effect is still poor and the prognosis is bad in a portion of patients [1]. In recent years, the study of the etiology and molecular genetics have been greatly promoted. However, the signal pathway and target genes still need to be studied in order to understand the molecular mechanism of the occurrence and development of tumor [2]. Spleen tyrosine kinase (Syk) is a type of non-receptor tyrosine kinase. Studies have shown that Syk is down-regulated in many malignant tumors. Syk plays an important role in tumor invasion and metastasis, suggesting that Syk is an important target in the gene therapy of malignancies [3]. Multiple studies [4, 5] showed that aberrant DNA methylation would lead to the loss of expression of Syk, and the loss of expression of Syk is related to the invasiveness of the tumor. At present, there is no report on the relationship between the invasive behavior and the expression of Syk in human medulloblastoma. The aim of our study is to investigate the relationship between changing the methylation status of Syk gene and the biological behavior of medulloblastoma cell lines, and the effect of Syk gene on the biological behavior of human medulloblastoma.

Materials and methods

Materials

D341 and Daoy cell lines were provided by the Beijing Union Medical College Hospital Cell Library (Beijing, China) and the Wenzhou Changfeng Biotechnology Co. (Zhejiang, China), respectively. The methylation transferase inhibitor 5-aza-2'-deoxycytidine (5-Aza-CdR) was purchased from the Sigma Co (USA). The RNA extraction reagent (Trizol) was purchased from the Invitrogen Co (Carlsbad, USA). The genomic DNA extraction kit and PVDF membrane were purchased from Kaiji Biotechnology (Shanghai, China). The Transwell Chamber was purchased from Corning Co. (USA). The rabbit anti human Syk monoclonal antibody was purchased from

Cell Signaling Co (USA). The rabbit anti human GAPDH antibody was purchased from Hangzhou Xiezi Biological Technology Co (Zhejiang, China). PCR Master Mix and real time fluorescence quantitative PCR kit and CDNA first chain synthesis kit and methylation specific PCR kit were purchased from Beijing Tiangen Biochemical Technology Co (Beijing, China). Other reagents and technical guidance were from BlueGene Biotech Co (Shanghai, China).

Cell culture

The D341 and Daoy cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO_2.

Experimental group

Cells were trypsinized to a single cell suspension with 0.25% trypsin adjusting the cell density to 5 × 10^4/mL. Culture medium had different concentrations of 5-Aza-CdR (0.1 mol/L, 1 mol/L, 10 mol/L). The experimental group was divided into three groups according to the concentration of the drug. The control group was without drug.

DNA extraction and methylation-specific PCR (MSP)

All procedures were done according to manufactures protocol. 20 μg DNA sample with 130 μl CT Conversion Reagent was heated at 98°C for 10 min and immediately lysed on ice. It was then incubated at 64°C for 2.5 h and was transferred to an IC Zymo-Spin tube. The tube was mixed with 600 μl M-Binding Buffer and centrifuged for 30 s. Then the tube was mixed with 100 μl M-Wash Buffer and centrifuged for 30 s. The tube was mixed with M-Desulphonation Buffer and was placed at room temperature for 15-20 min. Then was centrifuged for 30 s, was mixed with 200 μl M-Wash Buffer, and was centrifuged for 30 s. This was repeated once again. We added 10 μl M-Elution Buffer to the adsorption column matrix, and centrifuged at 4°C at 10,000 × g for 30 s. The DNA was washed with 70% ethanol after removing the supernatant. Finally, the DNA was diluted in 50 μl TE buffer and stored at -20°C. The methylated and unmethylated sequences were amplified with the nested PCR. The upstream and downstream primer sequences of methylated PCR were 5'-CGATTTCGCGGGTTTCGTTC-3' and 5'-AAA-ACGAACGCAACGCGAAAC-3', respectively. The upstream and downstream primer sequences of unmethylated PCR were 5'-ATTTTGTGGG-TTTTGTTTGGTG-3' and 5'-ACTTCCTTAACACACC-CAAAC-3'. PCR conditions consisted of 1 cycle of 95°C for 5 min, 30 cycles at 94°C for 45 s, at 50°C for 45 s, and at 72°C for 45 s, and 1 cycle of 72°C for 10 min.

Reverse transcriptase-PCR (RT-PCR)

RNA extraction was performed using the TRIzol approach with suspended cells and the purity was tested with the UV spectrophotometer A260/A280 ratio between 1.8 and 2.1. Reverse transcription was done according to the procedure of the first cDNA chain synthesis kit. For the cDNA synthesis reaction system, 2 μl RNA was mixed with 10 × RT mix, dNTP mix, 2 μl Oligo-dT15, 2 μl Super Pure dNTPs and RNase Free ddH_2O to a final volume of 14.5 μl. The mixture was incubated at 70°C for 5 min and immediately lysed on ice for 2 min, and was centrifuged for 5 min, then was added to 4 μl 5 × First-Strand Buffer and 0.5 μl Rnasin and 1 μl TIANScript M-MLV. The mixture was warmed at 42°C for 50 min, and at 95°C for 5 min, the final reaction was lysed on ice and diluted with ddH_2O RNase-Free to 50 uL. The SYBR-Green fluorescence dye was used during the real-time PCR. The 20-μl reaction system included 9 μl 2.5 × Real Master mix/20 × SYBR solution mixed reaction solution, 2 μl cDNA solution, 2 μl upstream and downstream primers (100 nM) and ddH_2O was added to make a final volume of 20 μl. The internal control was GAPDH gene. The forward and reverses primer for Syk were 5'-CATCAGCAATGCCTCCTGCAC-3', 5'-TGAGTCC-TTCCACGATACCAAAGTT-3', respectively. GAPDH gene was used as an internal control. The forward and reverse primers for GAPDH were 5'-CATGTCAAGGATAAGAA-3', 5'-AGTTCACCACG-TCATAGTAGTAATT-3', respectively. The Ct value was recorded in the three repeated experiments and averaged, and then calculated using the comparative ΔΔCt method.

Western blot

Each group of cells were lysed on ice with 250 μl of pre-cooled RIPA cell lysis buffer for 20 min and the total protein was isolated. The protein concentration was determined by BCA method. The protein samples were transferred to PVDF

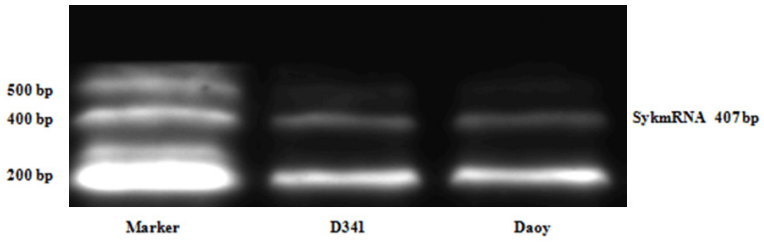

Figure 1. The expression of Syk in D341 and Daoy cell lines of human medulloblastoma.

membranes following SDS-PAGE. The blocking was performed with 5% non-fat milk powder and the primary antibody was incubated at 4°C overnight. The incubation with the secondary antibody was performed at room temperature for 1 h prior to washing and ECL treatment and finally the X-ray film was developed. GADPH was used as an internal control. The image analysis system was used to find the absorbance value ratio between Syk and GADPH. The value was recorded in the three repeated experiments and averaged.

Determination of cell invasion and migration abilities

Artificial basement membrane adhesive Matrigel was taken at -20°C, thawed at 4°C and lysed on ice. 50 μl of diluted Matrigel (2.5 μg) was added to the upper chamber of a 24-well transwell plate, which was then incubated at 37°C. The upper chamber was rinsed with serum-free RPMI-1640.D341 and Daoy Cells were collected using 0.25% trypsin after being treated for 72 h by the different concentrations of 5-aza-CdR and diluted to 3×10^5 cells/ml. Then, 200 μl of cell suspension was added to the upper chamber and 500 μl of culture medium to the lower chamber. Cells were grown at 37°C in an environment with 5% CO_2 for 24 h. Non-migratory cells were then removed from the upper chamber using a Q-tip. Transwell inserts were inverted, air-dried, and fixed with 90% ethanol fixed 15 min. Then, 500 μl of 0.1% crystal violet solution was added to a 24-well plate into which the transwell inserts were transferred. After incubation at room temperature for 10 min, inserts were removed and rinsed with PBS. Four fields were chosen and imaged. Cells were counted to determine the invasion ability of cancer cells. The migration ability of cells was performed similarly as the invasion assay, except that there was no Matrigel coating. Experiments were repeated three times.

Statistical analysis

SPSS 20.0 software was used for statistical analysis and the data are presented as mean ± standard deviation. A single factor analysis of variance was used to compare the number of multiple samples. $P<0.05$ was considered to indicate a statistically significant result.

Results

Expression of Syk in D341 and Daoy cell lines

The results of the western blot analysis of Syk protein expression were shown in (**Figure 1**). The result showed that D341 and Daoy cells all expressed Syk mRNA.

Effect of 5-Aza-CdR on the expression of Syk in D341 and Daoy cells

D341 and Daoy cells were treated with different concentrations of 5-Aza-CdR. The acting time was 24 h, 48 h, 72 h, respectively. The result (**Figure 2**) showed that the expression of Syk increased with raised concentrations and prolonged treatment.

Methylation status of the promoter region of Sky gene

The promoter methylation status of Syk gene were examined in D341 and Daoy cell lines of human medulloblastoma. Methylation of Syk gene was inhibited by 5-Aza-CdR (**Figure 3**).

Effect of 5-aza-CdR on the invasion and migration ability of D341 and Daoy cells

The number of cells through the artificial basement membrane and the polyester film was measured as the Transwell invasion and migration. The result (**Figures 4**, **5**) showed that the invasion and migration ability of the D341 and Daoy cell lines decreased with the treatment of the different concentrations of 5-Aza-CdR after 72 h. There was a concentration-effect relationship (**$P<0.01$).

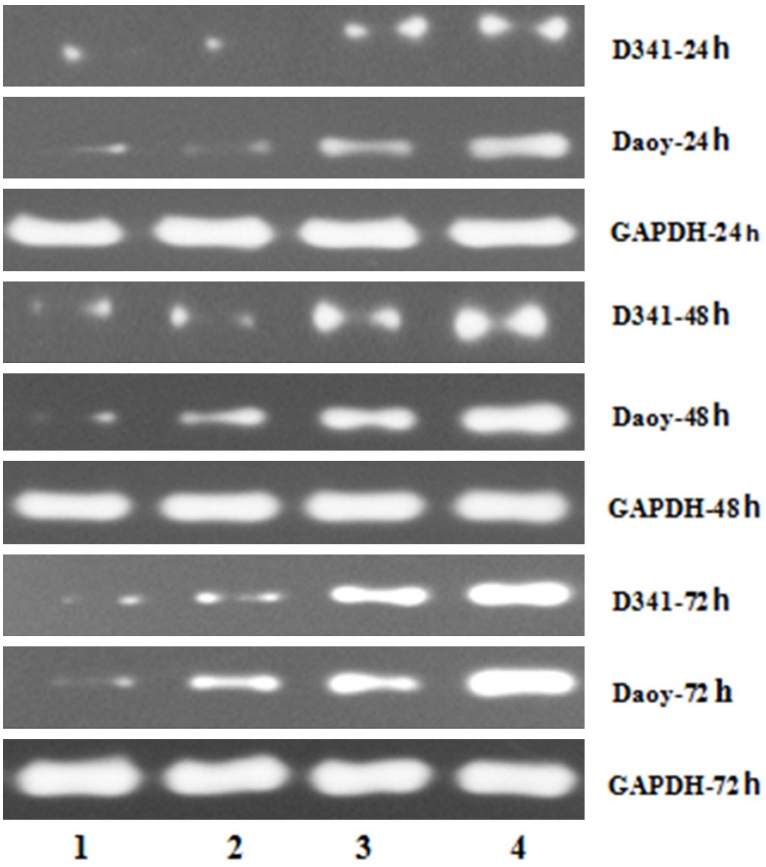

Figure 2. The expression of Syk after treatment with different concentration of 5-aza-2-CdR (1: control; 2: 0.1 μmol/L; 3: 1 μmol/L; 4: 10 μmol/L) for 24 h, 48 h and 72 h. GAPDH was used as internal control.

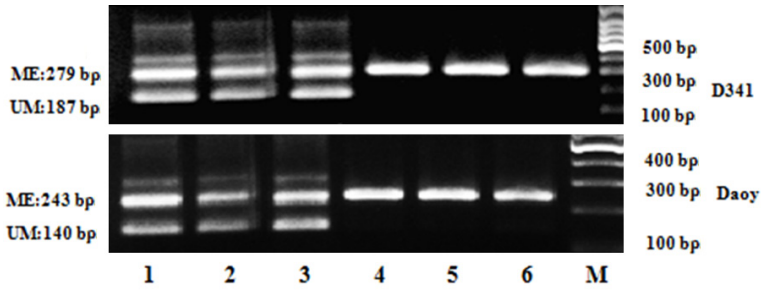

Figure 3. MSP was carried out to detect Syk gene promotor methylation status of D341, Daoy cell lines before (4-6) and after (1-3) treatment with 10 umol/L 5-aza-CdR for 72 h. Products of 279 bp and 187 bp were expected for methylated (ME) and unmethylated (UM) DNA in the D341 cell. 243 bp (ME) and 140 bp (UM) were in the Daoy cell. M: DL2000 Marker.

Discussion

Cancer is a disease caused by genetic material variation. The occurrences of cancer are closely related to the activation of oncogenes and the inactivation of tumor suppressor genes and abnormally modified genes [6]. It is the result of the mutual-function of many elements. Cancer is a severe health menace to the human being.

Syk [7, 8] is a significant molecule in the B cell antigen-receptor signaling pathway, and is considered to be a candidate tumor suppressor gene; the loss of which results in the retarded development and maturation of immune cells or even severe immunodeficiency. This may lead to the failure of monitoring cancer cell development and finally tumor growth. The mutation of Syk gene and the methylation of its promoter region are closely related to the formation and metastasis of tumors. Shakeel S [9] demonstrated that the polymorphic gene Syk could contribute to the development of breast cancer; the expression of Syk was normal in normal epithelial tissues and glandular and non-invasive breast cancer cells, while the expression was decreased or deleted in invasive breast cancer cells. Syk promoter methylation leading to gene silencing has been shown in breast cancer [10] and acute lymphoblastic leukemia [11]. The loss of Sky expression is thought to contribute to tumor progression by promoting tumor invasion, proliferation, and motility. At present, Syk plays the role of a tumor suppressor gene in a variety of malignant tumors, such as lung cancer [12], pancreatic cancer [5], and ovarian cancer [13].

Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer, including DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs, and microRNAs [14-16]. Aberrant DNA methylation, in the

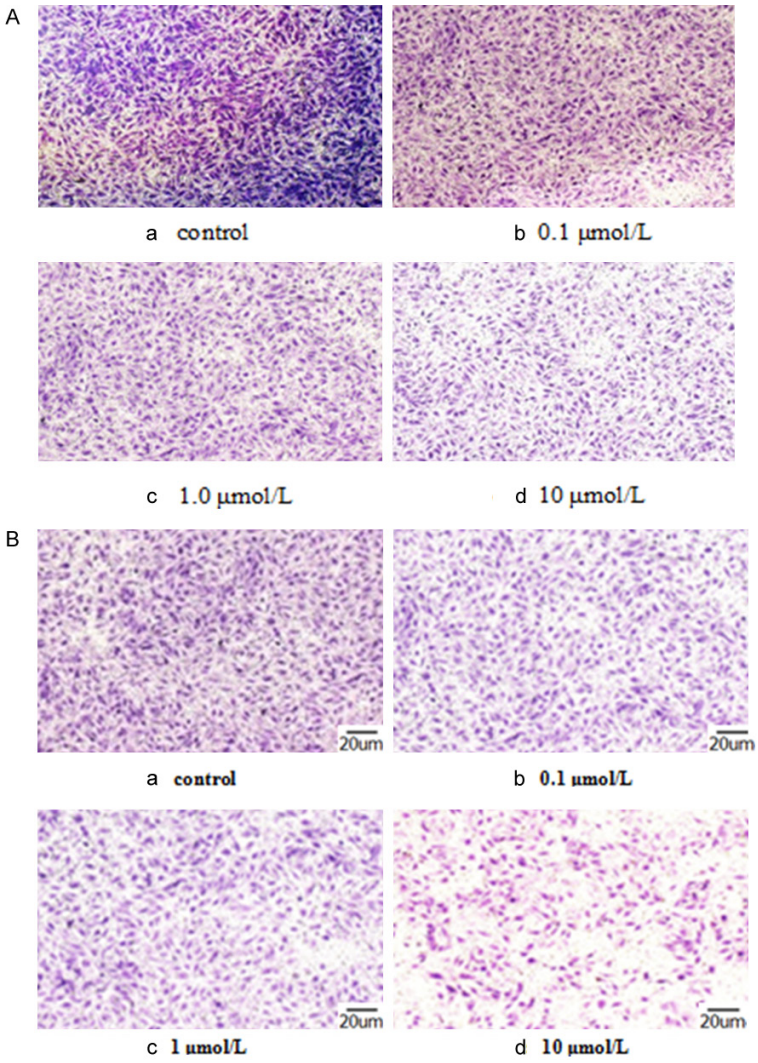

Figure 4. A. Experimental results of D341 cell migration in vitro; B. Experimental results of Daoy cell migration in vitro.

promoter regions of gene, leads to inactivation of tumor suppressors and other cancer-related genes in cancer cells. Liu Yet al. [17]showed that Syk hypermethylation was present in the HCCs and was associated with gene silencing. The tight correlation between Sky methylation and loss of Sky expression, together with the causal role of Sky methylation in gene silencing, indicates that epigenetic inactivation of Sky contributes to the progression of HCC. In this project, Shen B [18] explored the possibility of using Sky methylation as a prognostic marker in comparison to E-cadherin and TIMP-3 gene methylation.

The main focus of this study was to investigate the demethylation status of Syk gene promoter in human medulloblastoma D341 and Daoy cell lines and the interrelationship with biological behavior of medulloblastoma. We showed that the Syk gene was methylated in the promoter region and the expression level of Syk gene was clearly decreased, the expression of Syk mRNA and protein level were significantly increased after treatment with the methylation transferase inhibitor 5-aza-CdR ($P<0.01$), and the invasion and migration ability of cells were clearly decreased ($P<0.01$). This result was consistent with other relevant literature [19, 20]. Zmetakova I [21] has investigated the relationship between DNA methylation levels in invasiveness and metastasis associated genes with aberrant protein expression and also to evaluate whether a similar DNA methylation level is present in the tumor and circulating cell-free DNA for utilizing plasma DNA methylation as prognostic biomarker in breast cancer. They pointed out that the DNA methylation profiles manifested in our group of breast carcinomas are cancer specific, but they are not the only cause that affects the silencing of evaluated genes and the decrease of relevant protein products. The clinical utility of DNA methylation testing in peripheral blood cell DNA for cancer diagnosis and therapy needs further investigation.

Therefore, these experimental results show that the Syk gene had an inhibitory effect on the invasion and migration ability of medulloblastoma cell lines, and the promoter methylation of Syk gene led to the down-regulation of Syk expression. It plays a certain role in the molecular mechanism of invasion and metastasis of the medulloblastoma.

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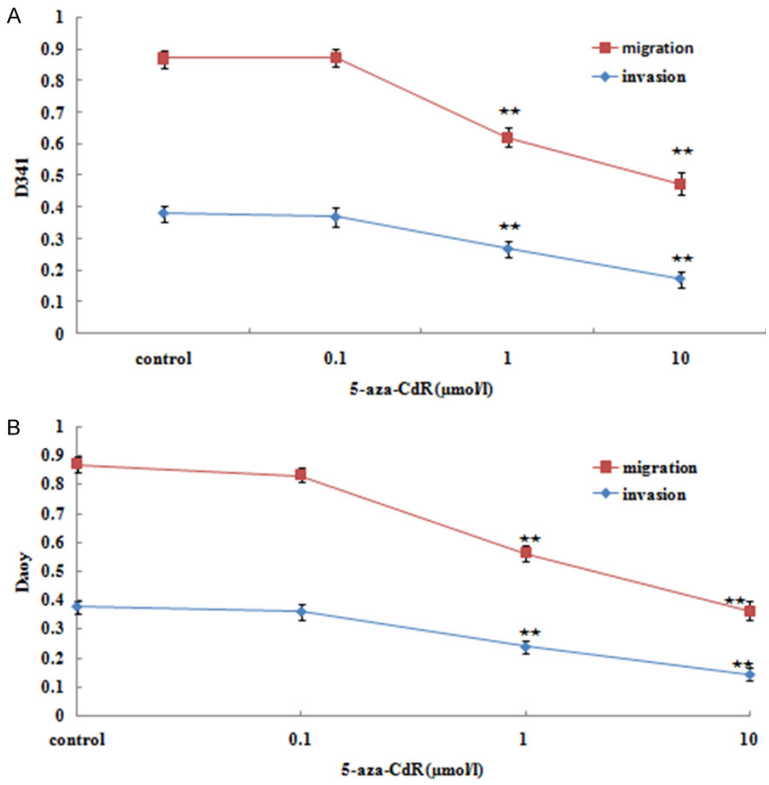

Figure 5. A. Effect of 5-aza-CdR on the invasion and migration ability of D341 cell. The invasion and migration ability of the D341 cell lines decreased with the treatment of the different concentrations of 5-Aza-CdR after 72 h. There was a concentration-effect relationship (**P< 0.01). B. Effect of 5-aza-CdR on the invasion and migration ability of Daoy cell. The invasion and migration ability of the Daoy cell lines decreased with the treatment of the different concentrations of 5-Aza-CdR after 72 h. There was a concentration-effect relationship (**P< 0.01).

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Disclosure of conflict of interest

None.

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