

The promoter methylation of the Syk gene in nasopharyngeal carcinoma cell lines

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Abstract. The aim of this study was to investigate the mRNA and protein expression levels of the Syk gene as well as its promoter methylation in nasopharyngeal carcinoma (NPC) cell lines. The CNE-1 (highly differentiated), CNE-2 (poorly differentiated) and NP69 (non-cancerous human immortalized nasopharyngeal epithelial cells) cell lines were used in the present study. The MS-PCR, Q-RT-PCR and western blotting methods were used to examine the Syk gene promoter methylation levels and mRNA and protein expression in the three cell lines. The promoter methylation levels in CNE-1, CNE-2 and NP69 cells were 36%, 62% and 0, respectively. The mRNA levels in CNE-1 and CNE-2 cells were 42 ± 3.5 and $28 \pm 2\%$ of that in NP69, respectively; the protein levels in CNE-1 and CNE-2 cells were 36 ± 4.5 and 16 ± 2.5 of that in NP69, respectively; the statistical differences between groups were significant. The lower differentiation levels of the NPC cell lines correlate with lower levels of mRNA and protein expression of the Syk gene, as well as higher promoter methylation levels.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common types of malignant cancer, with a high occurrence rate in the Chinese population compared with the western population (1). The genetic mechanisms of oncogenesis include the inactivation of tumor suppression genes, such as p53, Rb and p16, as well as the overexpression of oncogenes, such as bcl-2, ras and Cyclin D1. However, the mutations of these genes are rare in NPC patients. One possibility is that the regulation of gene expression through promoter methylation is changed

in NPC cases (2,3). The present study aimed to examine the differences in the mRNA and protein expression levels of the Syk gene as well as its promoter methylation in NPC cell lines with different differentiation levels, to understand the potential contribution of promoter methylation to NPC genesis.

Materials and methods

Materials. The NPC cell lines CNE-1, CNE-2 and NP69 were purchased from Yingrun Biotechnol Co. (Shanghai, China). The following reagents and instruments were used: Q-RT-PCR machine (ABI, Applied Biosystems, Carlsbad, CA, USA), Q-RT-PCR kit (Tiangen, Shanghai, China), keratinocyte-sfm culture medium (Invitrogen, Carlsbad, CA, USA), Genomic DNA extraction and purification kit, rabbit anti- β -actin, goat anti-rabbit secondary antibody, PVDF membrane, ECL kit (Kaiji Biotechnology, Shanghai, China).

Cell culture. The cell culture of the CNE-1, CNE-2 and NP69 cell lines was performed with complete RPMI-1640 medium at 37°C in a 5% CO₂ incubator. The cells were digested with 0.25% trypsin protease-EDTA.

MS-PCR. Following the DNA extraction, 8 μ g total DNA with ddH₂O in a total volume of 18 μ l was heated at 95°C and then immediately placed into an ice water bath. NaOH (3 M) was added for denaturation at 42°C for 20 min and the solution was then mixed with freshly prepared hydroquinone and sodium bisulfite. Liquid paraffin (200 μ l) was added and incubated at 50°C for 16 h. The liquid paraffin was then removed. The water phase was mixed with a purified solution of TBE buffer before de-salting with Wizard DNA purification system. An isopropanol wash was performed twice and DNA was eluted into 50 μ l ddH₂O, then centrifuged before being incubated with 3 M NaOH at 37°C for 15 min for denaturation. Then 33 μ l of 10 M ammonium acetate was added to buffer the NaOH before the ethanol was added to precipitate the DNA at -70°C for 1 h. Then the tube was centrifuged at 4°C at 10,000 x g for 20 min. The DNA was washed with 70% ethanol after removing the supernatant. Finally the DNA was diluted in 50 μ l TE buffer and stored at -20°C. For the MS-PCR detection, methylation-specific primers contained 9 CpG islands and non-methylation-specific primers contained 8 CpG

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Table I. Sequences of methylation and non-methylation primers for the Syk gene promoter.

Primer	Sequences	Length (bp)
Methylation	F: 5'-TTTCGGGTTTATGGGCGCGG-3'	202
	R: 5'-ACGAAAACGAACGCAACGCGAA-3'	
Non-methylation	F: 5'GTTTTAGTTGATTTTGTGTTAGTTTTG-3'	202
	R: 5'-ACCACCCACTCCTCCTCACT-3'	

F, upstream primer; R, downstream primer.

Table II. Sequences of Syk gene and GAPDH gene primers.

Gene	Sequences	Length (bp)
Syk	F: 5'-CAT GTC AAG GAT AAG AAC ATC ATA GA-3'	514
	R: 5'-AGT TCA CCA CGT CAT AGT AGT AAT T-3'	
GAPDH	F: 5'-ATG GCC TTC CGT GTC CCC ACT G-3'	398
	R: 5'-TGA GTG TGG CAG GGA CTC CCC A-3'	

F, upstream primer; R, downstream primer.

islands. The reaction was performed as follows: methylation system: denaturation at 95°C for 5 min, then 50 cycles of 95°C for 10 sec, annealing at 60°C for 15 sec and extension at 72°C for 25 sec; non-methylation system: denaturation at 95°C for 5 min, then 50 cycles of 95°C for 30 sec, annealing at 60°C for 15 sec and extension at 72°C for 25 sec; with a final step at 72°C for 10 min. PCR products (5 μ l) were used for 2% agarose gel electrophoresis and the image was analyzed for semi-quantitative absorbance values in order to calculate the methylation ratio [methylated primer band absorbance value/ (methylated primer extension increase in absorbance values + non-methylated primer band absorbance) x 100%]. The sequences of the primers are listed in Table I.

Q-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. The 18 S and 28 S integrity were analyzed with agarose gel electrophoresis. For the cDNA synthesis reaction system, 2 μ g RNA was mixed with 10X RT mix, dNTP mix, 2 μ l Oligo-dT15, 1 μ l Quant Reverse Transcriptase and RNase Free ddH₂O to a final volume of 20 μ l. The mixture was incubated at 37°C for 60 min. The SYBR-Green fluorescence dye was used during the real-time PCR. The 20- μ l reaction system included 9 μ l 2.5X Real Master mix/20X SYBR solution mixed reaction solution, 2 μ l cDNA solution, 2 μ l upstream and downstream primers (100 nM) and ddH₂O was added to make a final volume of 20 μ l. Each sample was repeated 3 times. The reaction conditions were: pre-denaturation at 95°C for 2 min, then 40 cycles of 95°C for 15 sec, 62°C for 30 sec, 68°C for 60 sec; final reaction at 95°C for 15 sec and 60°C for 1 min, then 95°C for 15 sec. The fluorescence signals were collected for the melting curve analysis. The Ct value was recorded in the three repeated experiments and averaged, then

calculated using the comparative $\Delta\Delta$ Ct method. The sequences of primers are listed in Table II.

Western blot analysis. The total protein samples were transferred to PVDF membranes (positively charged Heni dragon film) 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. GAPDH was used as an internal control. The image analysis system was used to find the absorbance value ratio between Syk and GAPDH.

Statistical analysis. The SPSS 17.0 software was used for statistical analysis and the data are presented as mean \pm standard deviation. The single factor analysis of variance was used to compared the Syk promoter methylation rate and mRNA and protein expression differences among the cell lines. P<0.05 was considered to indicate a statistically significant result.

Results

Promoter methylation in different cell lines. The electrophoresis results of the MS-PCR products suggest that CNE-1 and CNE-2 cells showed incomplete Syk promoter methylation (amplification with both groups of primers), while the NP69 cell line showed no promoter methylation (Fig. 1A). The methylation rates in CNE-1 and CNE-2 cells were statistically different at 36 and 62%, respectively (Fig. 1B; P<0.01).

Syk mRNA expression in different cell lines. The melting curves of the Syk and GAPDH genes had single peaks at 80.57°C and 88.73°C, respectively, suggesting high primer specificity. The

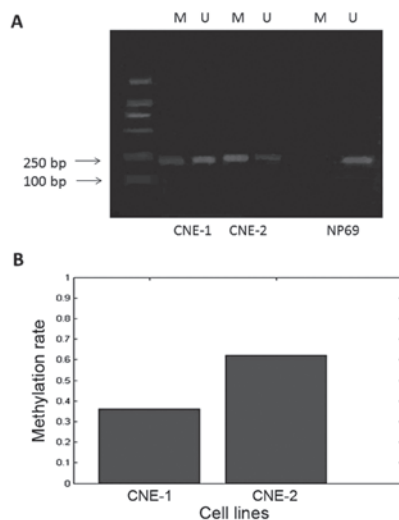


Figure 1. (A) MS-PCR electrophoresis image of the three cell lines. (B) Different methylation rate of the two NPC cell lines [(M/M+U) × 100%]. M, methylated primer band absorbance; U, non-methylated primer band absorbance.

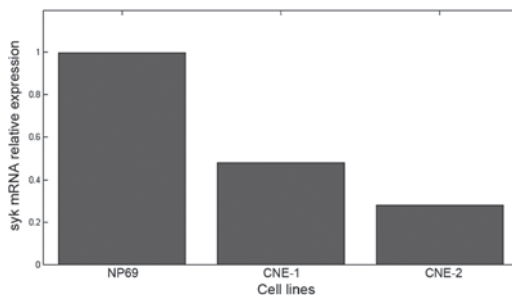


Figure 2. Syk mRNA expression levels in different cell lines (normalized to NP69).

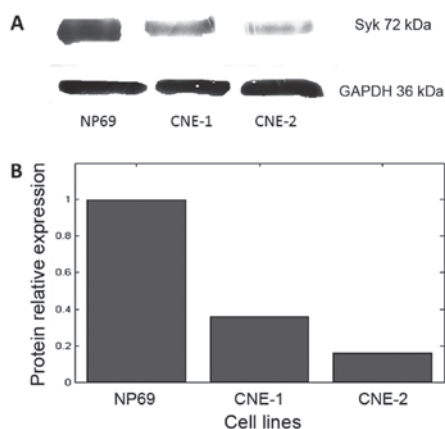


Figure 3. (A) Results of western blot analysis of the Syk protein. (B) Syk protein expression levels in different cell lines (normalized to NP69).

results of quantitative study of Syk gene expression are shown in Fig. 2. The mRNA was expressed in all three cell lines. The mRNA levels in CNE-1 and CNE-2 cells were lower, 42 ± 3.5 and $28 \pm 2\%$ of that in NP69, respectively; the differences between the groups were statistically significant ($P < 0.01$).

Syk protein expression in different cell lines. The results of the western blot analysis of Syk protein expression are shown in Fig. 3A. The protein was expressed in all three cell lines. The protein levels in CNE-1 and CNE-2 cells were lower, 36 ± 4.5 and $16 \pm 2.5\%$ of that in NP69 (Fig. 3B), respectively; the differences between the groups were statistically significant ($P < 0.01$).

Discussion

The epigenetic theory of cancer has emerged as a hot spot in recent years, especially for tumor suppressor genes (4,5). The present study used NPC as the model to investigate the epigenetic changes of tumor suppressor genes. Syk is a non-receptor tyrosine kinase which was first identified in 1991 (6) and locates to the q22 region of human chromosome 9. The protein contains 629 amino acids and has a molecular weight of 72 kDa. Syk 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 combined immunodeficiency (SCID) (7,8). This may lead to the failure of monitoring cancer cell development and finally tumor growth.

The present study used NPC cells lines with varied levels of differentiation with NP69 as the control to examine the changes in the expression of the Syk gene and correlated changes in epigenetic control. The results revealed that the mRNA expression level in NP69 cells was higher than that in the NPC cell lines ($P < 0.01$) and that in CNE-1 cells the mRNA expression level was higher than that in CNE-2 cells ($P < 0.01$), suggesting a positive correlation between mRNA expression and the differentiation level; similar results were also observed for Syk protein expression. Finally, the methylation level of the Syk gene promoter was negatively correlated with the differentiation level.

Previous studies have suggested that the tumor suppression function of Syk is due to its inhibition of cell proliferation and migration, with the downregulation of its expression in breast, gastric, colorectal and liver cancer, among others (8-10). The detailed roles of Syk in NPC growth and metastasis are not clear. Recent studies have highlighted the roles of epigenetic changes of tumor suppressor genes in oncogenesis (2,4,11,12). Our results are consistent with this theory, and the low differentiation level was associated with a low expression level of Syk and high methylation level of the promoter region. Further study is needed to elucidate the mechanism by which promoter methylation contributes to the altered expression of the Syk gene in NPC cell lines.

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