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Original article

RASSF10 is epigenetically inactivated and induces apoptosis in lung cancer cell lines

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ABSTRACT

Ras-association domain family 10 (RASSF10), the latest member of the RASSF family with Ras effector function, has been frequently inactivated by aberrant promoter hypermethylation in several human cancers. However, its role in lung cancer has remained unclear. In this study, we investigated the methylation status of RASSF10 by combined bisulfate restriction analysis (COBRA) and examined its preliminary function in lung cancer cell lines. RASSF10 was methylated in four out of six lung cancer cell lines, including NCI-H157, NCI-460, SPCA-1 and NCI-H446. Treatment with a DNA methylation inhibitor, 5-aza-2′-deoxycytiding (5-aza-DC), restored RASSF10 mRNA expression and the restoration of RASSF10 increased cell apoptosis in a dose dependent manner, whereas knockdown of RASSF10 improved cell proliferation ability and inhibited cell apoptosis rate significantly. Immunofluorescence revealed that RASSF10 protein was located in the cell membrane. Taken together, our data for the first time demonstrates the frequent epigenetic inactivation of RASSF10 in lung cancer cell lines. RASSF10 induces cell apoptosis and might function as a tumor suppressor gene in lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in males and the second most common cause in females worldwide [1]. It can be divided into two major types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Just like other tumor types, lung cancer arises out of a complex and poorly understood sequence of genetic/epigenetic alterations. Recently, epigenetic alterations including promoter DNA methylation and histone deacetylation have been established as one of the crucial mechanisms of carcinogenesis. Among them, transcriptional inactivation by promoter methylation in various tumor suppressor genes plays an important role in human malignancies including lung cancer. In this context, Ras effectors are of special interest.

The Ras-association domain family (RASSF) consists of 10 members (RASSF1 to RASSF10), and all of them are characterized by an RA-domain [2]. Several genes of this family, RASSF1A, RASSF2, RASSF4, RASSF5 and RASSF6, are epigenetically inactivated in cancer [3]. RASSF10 is a novel member of the N-terminal RASSFs, which is located at chromosome 11p15.2. RASSF10 has a CpG island which is vulnerable to be methylated leading to tumorigenesis [3]. Recently, several reports demonstrated that RASSF10 is frequently hypermethylated in childhood leukaemias, thyroid cancer, glioma, malignant melanoma of the skin and prostate cancer [4–8]. However, the epigenetic status of RASSF10 in lung cancer has not been investigated.

The purpose of this study is to illustrate the epigenetic deactivation of RASSF10 in lung cancer cell lines, and to evaluate its preliminary function in the lung tumorigenesis. To the best of our knowledge, this is the first report describing epigenetic inactivation of the RASSF10 candidate tumor suppressor gene in lung cancer.

2. Materials and methods

2.1. Cell line and cell culture conditions

Five NSCLC cell lines A549, NCI-H157, QG53, NCI-H460, and SPCA-1, and one SCLC cell line NCI-H446, were kindly provided by the Central Laboratory, First affiliated hospital of Dalian Medical University and cultured in RPMI-1640 (Hyclone, Logan, UT)

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supplemented with 10% fetal bovine serum, and maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. Methylation analysis

Genomic DNA isolation and bisulfite modify were operated using EZ DNA Methylation-Direct Kit (Zymo Research Corp., Orange, CA) according to the manufacturer's protocol. The methylation status of RASSF10 was assessed by combined bisulfate restriction analysis (COBRA) using specific primers in accordance with a previous report [6] (F: 5′-TTG TTT TTG TTG TTT TYG TYG TTT TAG TAG ATT-3′, R: 5′-CRA TTA AAC TTA ACC AAT TTA CRA AAA ACC TTA-3′; FN: 5′-GTG TGG ATT TGT TTT AGT AGA TT-3′, RN: 5′-CTA TTT CTC CTA AAT CAT AAC CAA ACT AA-3′) in a reaction buffer containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 10 pmol of each primer and 1.5 U Taq HotStart polymerase (TAKARA, Dalian, China). A nested PCR was carried out using with final annealing temperature 58 °C and standard PCR with annealing temperature 56 °C for secondary reaction. PCR products were digested with the restriction enzyme Taq I (NEB, Ipswich, MA) (TCGA) for 2 hours at 65 °C before visualization on a 3% agarose gel.

2.3. 5-aza-2′-deoxycytidine (5-aza-dC) treatment

Two lung cancer cell lines (SPCA-1 and NCI-H446) were treated with 5 μM and 10 μM of 5-aza-dC (Sigma, Taufkirchen, Germany) respectively to achieve genome demethylation. Treatment was carried out for 3 days with daily media changes and addition of fresh 5-aza-dC. Untreated cells were used as the negative control.

2.4. RASSF10 transfection and knockdown assay

The short hairpin RNA (shRNA) expression plasmids were purchased from GenePharma (Shanghai, China). The construction of shRNA expression vector for human RASSF10 was as follows: 5′-GGGACTGAGCTCTATGCATAG-3′. A random sequence of shRNA (shNC) was used as the negative control. 1×10^5 SPCA-1 cells were seeded into a 6-well culture plate without antibiotics and then were transfected with shRNA-RASSF10 plasmid and shNC plasmid respectively by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a final concentration of 0.2 nM according to the manufacturer's instructions. Transfection rates were monitored with fluorescence microscope. SPCA-1 cells without transfection were used as a blank control.

2.5. RT-PCR analysis of RASSF10 expression

Total cellular RNA was isolated from 5-aza-dC treated and untreated cells, shRNA transfected and shNC transfected cells using TRIzol reagent (TAKARA, Dalian, China). One microgram of DNase1-treated RNA was converted into cDNA by reverse transcription using random hexamer primers and RevertAid M-MuLV reverse transcriptase (Fermentas, Shenzhen, China). Expression of RASSF10 was confirmed by RT-PCR using following primers: forward 5′-GCG CCA TGG ATC CTT CGG AAAA-3′ and reverse 5′-GGC AGC GCC TCG TCG TCG TGGT-3′. β-actin expression was carried out concurrently using the following primers: forward 5′-CCG TAA AGA CCT CTA TGC CAA CA-3′ and reverse 5′-CGG ACT CAT CGT ACT CCT GCT-3′. A standard PCR was performed with an annealing temperature of 56 °C, total 40 cycles. RT-PCR products were analyzed on 2% agarose gels and stained with ethidium bromide.

2.6. Immunofluorescence analysis

At 48 h after transfection, cells were collected, gently washed by PBS and fixed with 4% paraformaldehyde for 1 h. After incubation with anti-RASSF10 rabbit polyclonal antibody (abcam, Cambridge, MA) for 1 h (1:50), DAPI (Beyotime, China) and TRITC anti-rabbit IgG (Zhongshan, Beijing, China) were added respectively. Images were observed under fluoresence microscope.

2.7. Western blot analysis

Cells were lysed in 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 50 mM sodium fluoride, 5 mM sodium pyrophosphate 1 mM sodium orth-ovanadate, 0.27 mM sucrose, 1% triton and protease inhibitor cocktail and protein was extracted. A total of 20 μg of protein extracts were resolved by SDS-PAGE gels, and then were transferred to PVDF membrane (Millipore, Billerica, MA). The primary antibody of RASSF10 (1:1000) was purchased from abcam (Cambridge, MA). β-actin (1:10000, Zhongshan, Beijing, China) was used as the internal control.

2.8. Flow cytometry analysis for cell apoptosis

SPCA-1 and NCI-H446 cells were treated with 5-aza-dC for 72 h and SPCA-1 cells were transfected with shRNA-RASSF10 plasmid or shNC plasmid respectively. Apoptosis was then assayed by Annexin V-FITC/PI detection kit (BD Biosciences, Pharmingen, USA) using flow cytometry. Briefly, cells were harvested and re-suspended in 1 × Annexin V binding buffer and stained with 5 uL of Annexin V and 5 uL of PI in the dark at room temperature. After additional 400 uL of binding buffer, cells were analyzed by flow cytometry.

2.9. Statistical analysis

Statistical analysis was performed using SPSS17 software (SPSS Inc., USA). Differences between groups were analyzed using Student t-test and Chi^2 test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. RASSF10 is hypermethylated in lung cancer cell lines

We first detected the methylation status of RASSF10 using COBRA in lung cancer cell lines. RASSF10 was methylated in 4/6 lung cancer cell lines including NCI-H157, NCI-H460, SPCA-1 and NCI-H446, whereas was unmethylated in A549 and QG53. One lung cancer cell line, SPCA-1, showed completely methylated, and the remaining 3 lung cancer cell lines (H157, H460 and H446) showed partially methylated (Fig. 1). Thus, RASSF10 is hypermethylated in lung cancer cell lines.

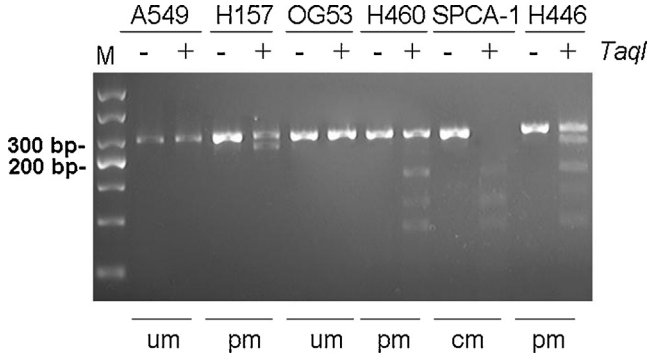

Fig. 1. Methylation status of Ras-association domain family 10 (RASSF10) in lung cancer cell lines. Combined bisulfate restriction analysis (COBRA) was performed to analyze the methylation of RASSF10. PCR products were digested by TaqI (+) or undigested (−) and resolved on 3% agarose gel. cm: completely methylated product; um: unmethylated product; pm: partially methylated product.