

Study on the relationship between the RASSF10 gene and the biological behavior of hepatocellular carcinoma cells

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Abstract. – **OBJECTIVE:** To investigate the relationship between Ras association domain family gene 10 (RASSF10 gene) and the biological behavior of hepatocellular carcinoma (HCC), including proliferation, invasion, and metastasis.

MATERIALS AND METHODS: HCC cell lines were generated with stable overexpression or low expression of RASSF10 protein. A cell line transfected with an empty vector was treated as control. At 12, 24, 48, and 72 h, the cell proliferation was determined by MTT assay, the invasion ability was determined by Transwell chambers, and the scratch assay was used to assess the migration ability. Additionally, cell lines were injected subcutaneously in the axillary fossa of nude mice aged 5-6 weeks. Tumors were measured weekly for 6 consecutive weeks to observe tumor volume, tumor growth rate, weight, and tumor metastasis in nude mice of the different groups.

RESULTS: In both the control group and low expression group, cell proliferation rates, cell invasion, and migration abilities, increased over time but decreased over time in the overexpression group. At each time point, data in the overexpression group were markedly lower than those in the control group, and highest in the low expression group. The differences were statistically significant ($p < 0.05$). In both control group and low expression group, tumor volume, tumor growth rate, weight, and tumor metastasis number were increased in nude mice over time, while they decreased in the overexpression group (except for tumor metastasis number). At each time point, data in the overexpression group were markedly lower than in the control group, and highest in the low expression group. The differences were statistically significant ($p < 0.05$).

CONCLUSIONS: Like a tumor suppressor gene, RASSF10 can inhibit the proliferation, invasion, and migration of HCC cells.

Key Words

RASSF10 gene, Hepatocellular carcinoma (HCC), Proliferation, Invasion, Migration.

Introduction

The distribution of hepatocellular carcinoma (HCC) has regional differences, with an annual increase of nearly 564000 new cases worldwide. The number of cases in China accounts for about half of total cases. HCC is inclined to metastasize at an early stage and spread through the circulation. Clinical symptoms are atypical, and there is a lack of simple diagnostic indicators. The opportunity for surgical removal of HCC is less than 30%, and the prognosis remains poor¹. Because of aflatoxin contamination and the high rate of hepatitis B virus infection in our country, HCC is an important disease that threatens the health of people^{2,3}. Modern molecular biology studies^{4,5} have shown that the initiation and progression of HCC is a complicated multistage process involving multiple genes and steps. The inactivation of tumor suppressor genes plays an important role in many of the biological behaviors of tumors, including tumorigenesis, proliferation, differentiation, invasion, and metastasis⁶. Ras association domain family gene 10 (RASSF10) is a newly discovered candidate tumor suppressor gene that is significantly downregulated in malignant glioma, prostate cancer, and stomach cancer^{7,8}. The methylation of CpG sites in the promoter region of RASSF10 can lead to the downregulation or loss of gene expression⁹. RASSF10 is also expressed in the normal liver tissue, but there are few studies on its relation with the occurrence of HCC. The aim of the present work was to analyze the relationship between RASSF10 expression and the biological behavior of HCC cells from the perspective of *in vitro* cellular experiments and an animal model. Previous researches¹⁰ used cell lines with transient overexpression or low expression of RASSF10 to study its function, while in this study its function was analyzed by generating stable HCC cell lines with overexpression or low expression of RASSF10.

Materials and Methods

The Construction of Cell Lines Expressing Different Levels of RASSF10

The HCC cell line, HepG2, was purchased from Shanghai Sangon cell experimental center (Shanghai, China). After routine recovery, culture, and passage, cells in the logarithmic growth phase were chosen for experiments.

RT-PCR was employed to clone the cDNA fragment (NM001080521) encoding the RASSF10 gene from normal liver tissue, and molecular cloning technology was applied to construct the eukaryotic expression vector, pcDNA3.1-RASSF10, before transfection in HepG2 cells with inactivated RASSF10 gene expression using liposome. G418 (1 mg/ml) was used for screening and RT-PCR and Western blot were employed to verify the expression of RASSF10. The constructed HCC cell lines that stably overexpressed RASSF10 were cultivated and passaged. Multiple siRNA fragments were designed and transfected into HepG2 cells that expressed RASSF10. Western blot was used to validate which siRNA fragment had the best effect on silencing RASSF10. The eukaryotic expression vector, pSUPER-RASSF10-siRNA, was then constructed and transfected into HCC cell lines with inactivated RASSF10 gene expression by the liposome. G418 (1 mg/ml) was used for screening, RT-PCR and Western blot were adopted to validate the expression of RASSF10, and constructed HCC cell lines with stable low expression of RASSF10 were cultivated and passaged.

Nude Mouse Model

Three different cell lines including cultivated HCC cell lines with stable overexpression and low expression of RASSF10 protein *in vitro*, and cell lines transfected with an empty vector as control (7×10^6) were injected subcutaneously in the axillary fossa of BALB/C nude mice aged 5-6 weeks. Each group contained 10 mice. After injection, tumor size (length $\times$ width $\times$ height $\times$ 0.5) was measured weekly for 6 consecutive weeks. Three mice from each group were sacrificed at 1, 3, and 6 weeks, respectively, and tumors were removed for comparison of tumor volume, tumor growth rate, weight, and tumor metastasis.

The Study of Cell Biological Behaviors

After 12, 24, 48, and 72 h of cell growth, the cell proliferation was determined by MTT assay, the invasion ability was determined with Transwell chambers, and the scratch assay was

used to test the migration ability. MTT assay: cells were trypsinized when they reached 85% confluent, and were then centrifuged at 2000 g for 15 min before discarding the supernatant. Phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) was used to resuspend cells to a concentration of 1×10^6 /ml. A total of 1 ml cell suspension was placed in each well of a 96-well plate (Sigma-Aldrich, St. Louis, MO, USA), and 40 μ l MTT solution was dispensed per well. The plate was cultured in the incubator set (ThermoFisher, Waltham, MA, USA) at 37°C, 5% CO₂ with saturated humidity for 4 h. The supernatant was then discarded, 150 μ l dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added, and shaken for 10 min. The plate was then placed in a microplate reader (ThermoFisher, Waltham, MA, USA), and the absorbance was measured at 490 nm (optical density, OD value), with 630 nm as the reference wavelength. The experiment was repeated three times and the values were averaged. Transwell chambers: the Matrigel matrix was placed at 4°C for 24 h to prepare the chamber, 50 μ l Matrigel (1:8) matrix and 400 μ l Opti-MEM culture medium were mixed in 1.5 ml EP tubes (Eppendorf, Hamburg, Germany) on ice and cultured in the incubator set at 37°C, 5% CO₂ with saturated humidity for 1 h. A volume of 50- μ l-cell suspension was added to the chamber, and it was allowed to continue to cultivate for 48 h. The chamber was then rinsed with PBS, fixed with ethanol for 5 min, dyed with 95% crystal violet solution for 7 min, rinsed with PBS, and air-dried at room temperature. The chamber membrane was placed on glass and sealed with neutral rubber. Cells were counted under an optical microscope (200 $\times$) (Olympus Optical Co., Ltd, Tokyo, Japan) in five randomly selected fields. GraphPad Prism 5 software (Version X; La Jolla, CA, USA) was used for analysis. Scratch assay: lines were drawn with a marker pen on the bottom of plates, cells were added, scratched in a straight line at the bottom of the well with the tip (200 μ l) of a sterile pipette, cultivated in Dulbecco's Modified Eagle Medium (DMEM) culture medium, and observed for cell migration in the scratched area.

Statistical Analysis

SPSS20.0 software (Version X; IBM, Armonk, NY, USA) was used for data analysis. Measurement data are presented as mean $\pm$ standard deviation, comparisons between groups were by one-way ANOVA, LSD-*t* test was applied for comparisons between two groups, and the

Table I. Comparison of cell proliferation rate.

Group	Low expression group	Control group	Overexpression group	F	p
12 h	1.3±0.2	1.1±0.3	0.9±0.2	5.632	0.026
24 h	1.7±0.3	1.2±0.3	0.7±0.2	6.124	0.020
48 h	2.0±0.4	1.3±0.4	0.5±0.1	8.527	0.007
72 h	2.1±0.5	1.4±0.4	0.4±0.1	12.302	0.000

variance analysis of repeated measurement data was used for intra-group comparisons. $p < 0.05$ was considered statistically significant.

Results

Comparisons of Cell Proliferation Rate

Cell proliferation rate in the control group and low expression group increased over time, while it decreased in the overexpression group. At each time point, the cell proliferation rate of the overexpression group was markedly lower than that of the control group, and it was highest in the low expression group. The differences were statistically significant ($p < 0.05$) (Table I).

Comparison of Invasion Ability

The invasion ability of cells in the control group and low expression group increased over time, while it decreased in the overexpression group. At each time point, the invasion ability of cells in the overexpression group was markedly lower than that of the control group, and the levels

were highest in the low expression group. The differences were statistically significant ($p < 0.05$) (Table II).

Comparison of Migration Ability

The migration ability of cells in the control group and low expression group increased over time, while it decreased in the overexpression group. At each time point, the migration ability of cells in the overexpression group was markedly lower than that of the control group, while it was highest in the low expression group. The differences were statistically significant ($p < 0.05$) (Table III).

Comparison of Tumor volume, Tumor Growth Rate, Weight, and Metastasis in Nude Mice

Tumor volume, tumor growth rate, weight, and metastasis in nude mice of the control group and low expression group increased over time, while they decreased (except for tumor metastasis number) in the overexpression group. At each time point, the tumor indexes in the overexpres-

Table II. Comparison of invasive ability (cell number/vision).

Group	Low expression group	Control group	Overexpression group	F	p
12 h	120±22	100±16	85±11	6.532	0.012
24 h	150±34	105±20	60±12	9.524	0.000
48 h	160±36	110±26	45±9	16.532	0.000
72 h	170±41	115±28	40±8	24.532	0.000

Table III. Comparison of migration ability (mm).

Group	Low expression group	Control group	Overexpression group	F	p
12 h	15.3±3.4	12.4±3.2	10.7±2.2	5.236	0.031
24 h	22.4±4.2	15.5±3.4	8.2±1.5	8.524	0.006
48 h	31.5±5.3	19.2±3.6	6.3±1.2	14.532	0.000
72 h	40.7±5.5	24.6±3.8	5.7±0.8	18.629	0.000

Table IV. Comparison of migration ability (mm).

Group		Low expression group	Control group	Overexpression group	F	p
Tumor volume (cm ³)	1 week	2.2±0.3	1.5±0.4	1.1±0.3	5.425	0.024
	3 weeks	4.3±0.6	1.8±0.5	0.8±0.2	12.324	0.000
	6 weeks	5.4±1.1	2.3±0.6	0.6±0.2	22.416	0.000
Growth rate (%)	1 week	65±12	48±13	32±7	6.635	0.011
	3 weeks	76±18	52±15	26±8	11.524	0.000
	6 weeks	83±23	63±17	22±6	17.629	0.000
Weight (g)	1week	22±3	16±2	12±2	8.632	0.009
	3 weeks	35±5	20±3	10±2	15.625	0.000
	6 weeks	41±7	23±3	9±2	23.265	0.000
Metastasis number (number)	1week	1.2±0.3	0.8±0.2	0.6±0.2	7.236	0.022
	3 weeks	2.1±0.4	1.0±0.3	0.5±0.2	9.213	0.005
	6 weeks	3.3±0.6	1.2±0.3	0.6±0.2	17.234	0.000

sion group were markedly lower than those in the control group, while the values were highest in the low expression group. The differences were statistically significant ($p<0.05$) (Table IV).

Discussion

At present, little is known about the biological function of the RASSF10 protein, although the amino acid sequence of the N-terminal of its polypeptide chain is highly homologous to the Ras effect gene, Nore 1/Haxp 1 in mice, and the chromosomal location of RASSF10 is adjacent to the Ras gene (RRAS2)¹¹. However, the relationship between Ras activation and silencing of RASSF10 has not yet been clarified. Through complete sequence analysis of RNAi¹⁰, downregulation of the homologous gene of RASSF10 in *Drosophila* can cause down-regulated expression of the Hedgehog signaling pathway, but this effect has not been observed in higher animals¹². The inhibitory effect of this gene in malignant glioma, prostate cancer, and stomach cancer cells has been confirmed by transient gene transfection *in vitro*, cell cloning, and agar cell culture¹³. Although the re-expression of RASSF10 can induce the apoptosis of prostate cancer cells, it has no effect on their cell cycle¹⁴. Hill et al¹⁵ found that the subcellular localization of RASSF10 in glioma cells is dependent on the cell cycle. It is located in the cytoplasm at the interphase stage of cell division and in the nucleus at the division stage.

The promotor region of RASSF10 contains typical duplex linked CpG sites. Abnormal methylation caused downregulated, absent, or reduced gene expression, which were verified in cell lines of leukemia, thyroid carcinoma, grade II-IV glioma,

prostate cancer, malignant melanoma, and gastric cancer¹⁶. Using methylation inhibitors, the expression of RASSF10 can be restored or upregulated¹⁷. The expression level of RASSF10 is closely related to clinical staging, therapeutic effect, and long-term prognosis of a variety of malignant tumors^{18,19}. RASSF10 is expressed in most human organs and tissues, including the liver. In 70% HCC tissue, RASSF10 expression is negative or down-regulated compared with the adjacent carcinoma tissue, and only 5% of the expression is higher than in adjacent carcinoma tissue²⁰. Methylation-specific PCR showed^{21,22} that the methylation level of the RASSF10 promoter increased significantly in the majority of HCC tissues, which was consistent with the trend of decreased RASSF10 mRNA expression in cancer tissue.

In the present study, tumor proliferation rate, invasion and migration abilities increased over time in the control group and the low expression group, while they decreased over time in the overexpression group. At each time point, the tumor indexes tested in the overexpression group were markedly lower than those in the control group, while they were highest in the low expression group. The differences were statistically significant. Similar results were obtained in nude mice regarding tumor volume, tumor growth rate, weight, and tumor metastasis numbers.

Conclusions

RASSF10, as a tumor suppressor gene, can inhibit the proliferation, invasion, and migration of HCC cells, and may play the role of a cancer suppressor gene by promoting the occurrence of cell apoptosis.

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Conflict of Interests:

The Authors declare that they have no conflict of interests.

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