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Expression profile of tumor suppressor gene *RASSF1* in lacrimal gland carcinoma

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ABSTRACT. We examined the expression pattern of the tumor suppressor gene RAS association domain family gene 1 (*RASSF1*) in lacrimal gland carcinoma and analyzed its relationship with the oncogenesis and progression of tumors. Sixty-two patients (30 males, 32 females, average age = 47 ± 3.5 years) admitted with lacrimal gland carcinoma to the Department of Ophthalmology of our hospital between January 2012 and January 2014 were enrolled in this study. Based on tumor malignancy, patients were classified into a malignant group (N = 25) and benign group (N = 37). Healthy lacrimal gland resections from trauma surgery (N = 35) were recruited as a healthy control group. Expression profiles of *RASSF1* in all groups were quantified using reverse transcription-polymerase chain reaction and western blotting. Recurrence of lacrimal gland carcinoma was surveyed through postoperative follow-up. Expression levels of *RASSF1* in samples from the malignant and benign groups were significantly lower than those in the healthy group ($P < 0.05$). Furthermore, the malignant group showed lower *RASSF1* expression than the benign group ($P < 0.05$). Postoperative follow-up identified 22 cases of recurrence in the malignant group, with a recurrence rate of 88%, while 15 cases in the benign group had a

recurrence rate of 40.5%. A direct relationship exists between *RASSF1* expression levels and the malignancy grade of lacrimal gland carcinoma. Patients with lower *RASSF1* expression showed a higher recurrence probability, indicating unfavorable prognosis. Therefore, measuring *RASSF1* expression can be used as a diagnostic method for lacrimal gland carcinoma.

Key words: Lacrimal gland carcinoma; *RASSF1*; Tumor suppressor gene

INTRODUCTION

Lacrimal gland carcinoma is a common orbital cancer in humans, showing relatively high recurrence and low survival rates. However, mortality is typically caused by metastasis into other substantive organs, rather than as a direct consequence of primary carcinoma (Singh et al., 2012; Stanford et al., 2014). Studies have revealed that the oncogenesis and progression of lacrimal gland carcinoma are regulated by multiple genetic functions, forming complicated mechanisms including activation of oncogenes and inactivation of tumor suppressor genes caused by gene loss, mutation, and replacement (Kanzaki et al., 2006). The tumor suppressor gene RAS association domain family gene 1 (*RASSF1*) was identified in human chromosome 3p21.3 in 1982 (Whang-peng et al., 1982). The *RASSF1* gene encodes RAS effective protein, which participates in RAS-related cellular signal pathways and regulates oncogenesis, cell differentiation, proliferation, and apoptosis of multiple malignant tumors (Zhou et al., 2014). Previous studies have revealed increased expression of *RASSF1* in normal human tissues, while lower levels were observed in some tumor cells (e.g., lymphoma, pulmonary carcinoma, and melanoma), indicating some relationships between decreasing *RASSF1* gene expression and tumor oncogenesis (Alkatan et al., 2014). Therefore, we analyzed the relationship between the expression level of *RASSF1* and tumor oncogenesis by determining the expression profile of *RASSF1* in lacrimal gland tissues from malignant patients, benign patients, and normal healthy subjects to understand the pathogenesis of lacrimal gland carcinoma.

MATERIAL AND METHODS

General information

Sixty-two patients (30 males, 32 females, average age = 47 ± 3.5 years) who were admitted with lacrimal gland carcinoma to the Department of Ophthalmology in our hospital with complete clinical data between January 2012 and January 2014 were enrolled in this study. All patients had undergone a single surgery. Based on histopathological examination of tumor tissues, patients were classified into the malignant group (N = 25, 13 males, 12 females, average age = 48 ± 4.2 years) or the benign group (N = 37, 17 males, 20 females, average age = 45 ± 2.5 years). We also obtained healthy lacrimal gland tissues removed from trauma surgery for use as the healthy control group (N = 35, 17 males, 18 females, average age = 47 ± 3.4 years). Statistical analysis revealed no significant differences in age or gender among the 3 groups. Lacrimal gland resections were performed by qualified surgeons, and tissues were stored in liquid nitrogen until further use.

Reagents

The total RNA extraction kit (Trizol-based) was purchased from Invitrogen (Carlsbad, CA, USA). DNA tags and polymerase chain reaction (PCR) reagents were provided by TaKaRa (Shiga, Japan). Other reagents, including absolute ethanol and propanol, were prepared in-house. PCR primer design and synthesis were performed by BGI (Shenzhen, China). Primer sequences had an expected amplification length of 310 base pairs: RASSF1-F: 5'-GCGTC ACGGC CAAGT GT-3'; RASSF1-R: 5'-TGCTG TCTGC CTTCC ACCTG-3'. β-Actin was selected as an internal reference gene (forward primer: 5'-TGACC CAGCT CATGT TT-3'; reverse primer: 5'-CTTTG CGGAT GTCCA CGT-3'; expected amplification length: 520 base pairs).

Total RNA extraction and cDNA synthesis

RNA extraction was performed in an RNase-free environment according to manufacturer instructions. Final RNA products were suspended in 25 μL diethylpyrocarbonate-treated H_2O and measured to determine the concentration. First-strand cDNA was synthesized by *in vitro* reverse transcription using oligo dT as the primer and extracted RNA (same concentration in all groups) as the template following manufacturer instructions (reverse transcription conditions: 25°C for 5 min, followed by incubation at 42°C for 1 h). cDNA products were stored at -20°C until further use.

Expression assay of the *RASSF1* gene

Using first-strand cDNA as a template, reverse transcription (RT)-PCR was performed as follows in a total volume of 50 μL: 25 μL 2X PCR mixture was added with 0.5 μL 10 μM of each *RASSF1* primer, 0.5 μL 10 μM of each β-actin primer, 2 μL first-strand cDNA, and 21 μL ddH_2O. PCR conditions were set as: 94°C for 5 min, followed by 35 cycles each of 94°C for 30 s, 60°C for 35 s, and 72°C for 1 min. PCR products were detected using electrophoresis in a 1% agarose gel. Using β-actin bands as the internal reference, *RASSF1* expression levels in all tissues were determined based on the optical density ratio of DNA bands (calculated as *RASSF1*/β-actin).

Protein assay of RASSF1

Following standard protocols, RASSF1 protein expression levels were semi-quantified using western blotting. Total protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were wet-transferred to the membrane at 30 V for 35 min. The membrane was blocked using 5% skim milk powder at 4°C overnight, and then incubated with 1:500 primary antibody in dilution buffer at 37°C for 2 h. After 3 washes with Tris-buffered saline containing Tween 20 for 5 min each, secondary antibody in dilution buffer was added for 1 h of incubation at 37°C. Following washing with Tris-buffered saline containing Tween 20, 3,3'-diaminobenzidine chromogenic substrate was added and the membrane was exposed for 15 s in automated western blot exposure equipment. Using β-actin bands for internal reference, positive cases of RASSF1 protein expression were counted in all groups. The relative expression level was determined based on the signal strength of blotting, and was further classified as high, normal, or low expression.

Data analysis

SPSS v11.0 (SPSS, Inc., Chicago, IL, USA) was employed to analyze all collected data. Relative expression levels of mRNA from all tissues are reported as means ± standard error of the mean and were compared using the Student *t*-test. The Fisher exact test was applied for between-group comparisons of protein expression levels. The significance level (α) was set as 0.05. $P < 0.05$ indicated a significant difference.

RESULTS

mRNA expressional profiling of the *RASSF1* gene

Optical density analysis of RT-PCR bands by the *RASSF1*/β-actin ratio revealed the *RASSF1* gene expression levels shown in Table 1. Normal groups showed significantly higher *RASSF1* mRNA levels compared to the other 2 groups. Further between-group comparisons showed that the benign group also had higher levels than samples from the malignant group ($P < 0.05$)

Table 1. Expressional profiling of the *RASSF1* gene.

Group	N	Optical density ratio	Comparison	t value	P
Normal (1)	15	0.5436 ± 0.1902	1 and 2	3.896	<0.05
Benign (2)	37	0.2216 ± 0.1302	2 and 3	13.324	<0.05
Malignant (3)	25	0.0435 ± 0.0402	1 and 3	19.367	<0.05

Expression levels of RASSF1 protein

Representative western blot results of the RASSF1 protein for all groups are shown in Figure 1. Protein levels were significantly higher in the normal group than in the other 2 groups. Statistical analysis among all groups showed significant differences, as shown in Table 2.

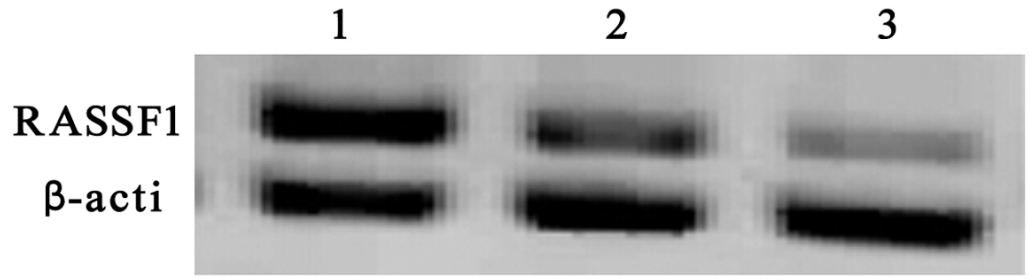

Figure 1. Representative results of western blot showing RASSF1 protein expression. *Lane 1*, normal group; *lane 2*, benign group; *lane 3*, malignant group.

Analysis of postoperative follow-up

All patients were followed-up after surgery and the results are listed in Table 3. In the malignant group, 88% of patients (22 of 25) developed recurrence, with an average time of 8

± 5 months. The recurrence rate was decreased to 40.5% (15 of 37) in the benign group, with an average time of 12 ± 4 months. Based on these results, expression profiling of *RASSF1* revealed a longer period of time between first surgery and recurrence in patients with higher *RASSF1* expression levels. In contrast, patients with lower *RASSF1* levels had a higher rate of recurrence over a shorter time. The normal group with no lacrimal gland carcinoma had the highest expression of *RASSF1*. Therefore, a direct relationship exists between *RASSF1* expression levels and postoperative recurrence rate; lower *RASSF1* expression indicates a higher recurrence rate and unfavorable prognosis.

Table 2. RASSF1 protein expressional levels.

Group	N	RASSF1 protein level			F value	P
		+++	++	+		
Normal (1)	15	15	0	0	0.013	<0.05
Benign (2)	37	24	12	1	0.000	<0.05
Malignant (3)	25	1	3	21	0.052	<0.05

Table 3. Postoperative follow-up results.

Group	N	Recurrence cases	Recurrence rate (%)	Time of recurrence (months)	RASSF1 level
Normal (1)	15	0	0	NA	+++*
Benign (2)	37	15	40.5	12 ± 4	+
Malignant (3)	25	22	88*	8 ± 5*	+

*Significant difference comparing to the normal and benign group, $P < 0.05$. NA = not applicable.

DISCUSSION

Lacrimal gland carcinoma is a common orbital cancer that affects human health and has a high recurrence rate. Most tumors develop from the lacrimal orbit. Pathological studies have shown that tumors are mostly cystic carcinoma without capsule, have infiltrative growth, and are classified into cribriform, tubular, and solid types. The oncogenesis of lacrimal gland carcinoma is not fully understood. However, most scholars agree that multiple genetic regulatory mechanisms, particularly activation of oncogenes and inactivation of tumor suppressor genes, underlie its occurrence and progression (Fang et al., 2013a; Alkatan et al., 2014; von Holstein et al., 2013, 2014).

Among various tumor-related genes, *RAS* has been well-established as an important cancer gene because it can both promote and inhibit cell growth, proliferation, and migration. In this study, we focused on the tumor suppressor gene *RASSF1*, which has a critical modulating function in cellular interactions in the normal human body. Previous studies have shown that the RASSF1 protein has a high level of homology with the RAS effective protein Norel. RASSF1 protein thus can modulate the balance of cellular growth via its competitive binding to RAS. The mutation or elimination of *RASSF1* will facilitate RAS-mediated cellular growth, further causing tumor occurrence (Tse et al., 2013; Zhou et al., 2014). This may be one of the mechanisms of RASSF1-mediated oncogenesis. Previous studies reported that abnormal expression of the *RASSF1* gene is a critical step in oncogenesis and that it is a potential factor controlling cell division (White, 2012; Zhou and Zhu, 2012; Chan and Katan, 2013; Fang et al., 2013b). We found that when *RASSF1* was expressed at high levels, the malignancy and re-

currence rate of lacrimal gland carcinoma were significantly lower than those in low *RASSF1* expression patients, further illustrating its importance during tumor occurrence.

To further investigate the relationship between the *RASSF1* tumor suppressor gene and lacrimal gland carcinoma, we compared the expression profiles of *RASSF1* and tumor occurrence by conducting expression assays in lacrimal gland tissues in malignant and benign patients as well as in healthy subjects. The results showed that the expression level of *RASSF1* decreased with the occurrence and progression of tumors, accompanied by higher postoperative recurrence rates. These results suggest that *RASSF1* gene expression alternations are closely related to the progression and recurrence of lacrimal gland carcinoma, and the malignancy and recurrence rate can be evaluated by assaying *RASSF1* expression levels. This provides a new diagnostic method for lacrimal gland carcinoma. However, the detailed function of *RASSF1* remains unclear, and additional studies are required to elucidate how its normal expression can suppress tumor occurrence.

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