SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 3: 1231-1236, 2012

Increased expression of Rab coupling protein in squamous cell carcinoma of the head and neck and its clinical significance

YAOZHANG DAI[1-3], YONG LIU[1,3], DONGHAI HUANG[1,3], CHANGYUN YU[1,3], GENGMING CAI[1,3], LEIMING PI[1,3], CAIPING REN[4], GEORGIA Z. CHEN[5], YONGQUAN TIAN[1,3] and XIN ZHANG[1,3]

[1]Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008; [2]Department of Otolaryngology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052; [3]Otolaryngology Major Disease Research Key Laboratory of Hunan Province; [4]Cancer Research Institute, Central South University, Changsha, Hunan 410008, P.R. China; [5]Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322, USA

Received November 14, 2011; Accepted January 17, 2012

DOI: 10.3892/ol.2012.652

Abstract. The role of Rab coupling protein (RCP) has not been previously investigated in squamous cell carcinoma of the head and neck (SCCHN). The aim of this study was to explore RCP protein expression and its clinicopathological significance in SCCHN. RCP protein expression in 95 SCCHN samples, 18 vocal nodule epithelia and 16 leukoplakia epithelia samples was analyzed by immunohistochemistry and correlated with clinicopathological parameters and patient outcome. Our data indicated that vocal nodule epithelia, leukoplakia epithelia and SCCHN showed a gradual increase in the expression of RCP protein. RCP overexpression was significantly associated with T classification, clinical staging, lymph node metastasis and recurrence. Survival analysis revealed that a high RCP expression was significantly correlated with shorter overall survival and disease-free survival. In conclusion, RCP protein may contribute to the malignant progression of SCCHN, and serves as a novel prognostic marker in patients with SCCHN.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common malignancy worldwide, and is a cancer with moderately low survival and high recurrence rates. Each year, approximately 50,000 new patients are confirmed as having SCCHN, and patients are usually diagnosed at around 60 years of age (1). Despite new therapeutic modalities, long-term outcomes of patients with SCCHN remain unsatisfactory (1). SCCHNs with similar histology and localization receiving identical therapies may have different clinical outcomes. Based on clinicopathological parameters, such as T and N classifications, it is not possible to reliably predict which patients are likely to respond successfully to treatment or may exprerience recurrence. Differential gene and protein expression in tumors may explain the variations in response to the same treatment modality (2).

The Rab proteins, Ras-related small G proteins (including the Rho-related proteins), serve a critical role in the regulation of intracellular transport events (3,4). A group of Rab11-interacting proteins has been described, which share several common domains and biological properties (5-7). A member of this family, termed Rab coupling protein (RCP), has been shown to be important in tumorigenesis and progression of breast cancer (8,9). However, there are few data reporting RCP expression and its clinicopathological significance in other types of cancer. The present study was carried out to investigate the expression status of RCP protein in SCCHN and analyze whether RCP expression was correlated with the clinical features and prognosis in patients with SCCHN.

Materials and methods

Patients and tissues. A total of 95 patients with SCCHN, who underwent partial or total laryngectomy at the Department of Otolaryngology of Xiangya Hospital in Central South University, China, between February 2003 and December 2005, were enrolled in this retrospective study. The patients had no history of previous malignancies, and no history of radiotherapy or chemotherapy. Recurrence and metastasis were diagnosed by physical examinations, imaging evaluation, surgical and postoperative pathological examinations. In addition, 18 vocal nodule epithelia (obtained from patients suffering from vocal nodules) and 16 leukoplakia epithelia of the larynx (precancerous lesions) samples were obtained between January 2008 and November 2010. Informed consent was obtained from all patients prior to surgery, and this investigation was

Correspondence to: Dr Xin Zhang, Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital, Central South University, 87 Xiangya Road, Changsha, Hunan 410008, P.R. China
E-mail: xinzh99@yahoo.com

Key words: squamous cell carcinoma of the head and neck, Rab coupling protein, prognosis, metastasis

approved by the Research Ethics Committee of Central South University, Changsha, China.

The main clinical and pathological variables of the patients are described in detail in Table I. There were 92 male and 3 female patients, with a median age of 57 years (range, 32-79). According to the TNM System of the International Union against Cancer (10), 21 cases were supraglottic, 65 were glottic, 1 was subglottic and 8 were hypopharyngeal carcinomas. There were 17 cases in stage I (T1N0M0), 21 cases in stage II (T2N0M0), 27 cases in stage III (T3N0M0 13 cases, T2N1M0 6 cases, T3N1M0 8 cases) and 30 cases in stage IV (T2N2M0 3 cases, T3N2M0 10 cases, T3N1M1 1 case, T4N0M0 4 cases, T4N1M0 7 cases and T4N2M0 5 cases). Considering pathological grading, 58 were staged as well-differentiated (G1), 29 as moderately differentiated (G2) and 8 as poorly differentiated (G3). A total of 40 patients with lymph node metastasis were validated by conventional postoperative pathological examinations and 46 patients experienced tumor recurrence following surgery.

Immunohistochemistry. Immunohistochemical staining was performed according to the manufacturer's instructions. Briefly, antigen retrieval was carried out in 10 mmol/l citrate buffer (pH 6.0) for 15 min at 100˚C in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. Slides were incubated with chicken IgY anti-RCP polyclonal antibody (Sigma, St. Louis, MO, USA) at 1:3000 dilution at 4˚C overnight, followed by the addition of HRP-labeled goat anti-chicken antibody (KPL, Gaithersburg, MD, USA) at 1:2000 dilution for 30 min. Immunoreactive proteins were visualized with 3,3'-diaminobenzidine (DAB) and counterstained with Mayer's haematoxylin. Negative control slides were probed with normal chicken serum under the same experimental conditions. Images of stained tissues were acquired with a Leica Qwin V3 image analysis system.

Evaluation of staining. Sections were independently evaluated and scored by two pathologists (Xiang Li and Xueping Feng) who were blind to all clinical data. Evaluation of staining was estimated by the pattern of staining quantity and intensity as described by Yuan *et al* (11): Quantity scores from 0 to 5 were respectively assigned if 0%, 1-10%, 11-30%, 31-50%, 51-80%, and 81-100% of the tumor cells were positive. The staining intensity was rated on a scale of 0 to 3: 0, negative; 1, weak; 2, moderate; and 3, strong). The multiplication of the intensity and extent scores was used as the final staining score for RCP. Theoretically, the scores ranged from 0 to 15. Scores above the median (≥7) were considered as high reactivity and 0-6 as weak reactivity.

Follow-up. A total of 95 patients with SCCHN were followed up after surgery. The follow-up period was defined as the interval between the date of tumor excision and that of the patient's mortality or the last follow-up. Recurrence and metastasis were diagnosed by clinical examination, imaging evaluation and pathological studies. Overall survival (OS) and disease-free survival (DFS) were calculated from the day of surgery to the date of mortality or that of tumor relapse. The lost follow-ups and mortality from other causes were treated as censored cases.

Table I. Clinicopathological characteristics of the 95 studied cases with SCCHN.

Variables	No. of patients	Percentage (%)
Age		
≤57	48	50.53
>57	47	49.47
Gender		
Female	3	3.16
Male	92	96.84
Alcohol intake		
Yes	52	54.74
No	43	45.26
Smoking		
Yes	66	69.47
No	29	30.53
Tumor site		
Hypopharyngeal	8	8.42
Supraglottic	21	21.11
Glottic	65	68.42
Subglottic	1	1.05
Tumor grade		
G1	58	61.05
G2	29	30.53
G3	8	8.42
T classification		
T1	17	17.89
T2	30	31.58
T3	32	33.68
T4	16	16.84
Clinical stage		
I	17	17.89
II	21	22.11
III	27	28.42
IV	30	31.58
Lymph node metastasis		
Negative	55	57.89
Positive	40	42.11

SCCHN, squamous cell carcinoma of the head and neck.

Statistical analyses. Statistical analyses were performed using the SPSS statistical software version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical significance between the expression of RCP protein and clinicopathological parameters was compared by the χ^2 test. Survival analyses were undertaken using the Kaplan-Meier method and curves were compared by the log-rank test. Identification of relevant prognostic factors was performed by the univariate and multivariate Cox regression analysis. Tests were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Table II. RCP expression in squamous epithelia from vocal nodules, leukoplakia tissues of larynx and SCCHN tissues.

	Staining score					
	0	1	2	3	4-7	8-15
Squamous epithelia from vocal cords (n=18)	15	1	2	-	-	-
Laryngeal leukoplakia (n=16)	1	2	11	2	-	-
SCCHN						
Low expression (n=30)	-	-	7	4	19	-
High expression (n=65)	-	-	-	-	-	65

SCCHN, squamous cell carcinoma of the head and neck; RCP, Rab coupling protein.

Table III. Clinicopathological characteristics of SCCHN and correlations with RCP expression.

Variables	Total	RCP expression		P-value
		Low (0-6)	High (7-15)	
Age				0.383
≤57	48	13	35	
>57	47	17	30	
Alcohol intake				0.658
Yes	52	15	28	
No	43	15	37	
Smoking				0.811
Yes	66	24	42	
No	29	17	12	
Tumor site				0.153
Glottic	65	24	41	
Others	30	6	24	
Hypopharyngeal	8	0	8	
Supraglottic	21	5	16	
Subglottic	1	1	0	
Tumor grade				1.004
G1	58	18	40	
G2+G3	37	12	25	
T classification				0.028
T1+T2	47	20	27	
T3+T4	48	10	38	
Clinical stage				0.012
Early stage (I+II)	38	18	20	
Late stage (III+IV)	57	12	45	
Lymph node metastasis				0.004
Negative	55	24	31	
Positive	40	6	34	
Recurrence rate[a]				0.034
No recurrence	44	17	27	
Recurrence	46	8	38	

[a]Five patients failed to follow-up. SCCHN, squamous cell carcinoma of the head and neck; RCP, Rab coupling protein.

Results

RCP expression in squamous epithelia from vocal nodules, leukoplakia tissues of larynx and SCCHN tissues. To investigate the protein expression profile of RCP in SCCHN, immunohistochemistry was initially performed in 95 paraffin-embedded, archival SCCHN primary tumor samples, 18 vocal nodules and 16 laryngeal leukoplakia specimens (precancerous lesions). Positive immunostaining was predominantly observed in the cytoplasm of carcinoma cells. Our data indicated that vocal nodule epithelia, leukoplakia epithelia and SCCHN revealed a gradually increased expression of RCP protein ($P<0.05$). As shown in Table II, only 3 (16.67%) of 18 vocal nodule epithelia showed a low RCP expression (scored 1-2) (Fig. 1A and B). Although 15 of the leukoplakia epithelia samples had a low expression of RCP (Fig. 1C and D), their scores all ranged from 1 to 3. While in SCCHN specimens, 65 (68.42%) cases demonstrated a high RCP expression (scored 8-15) (Fig. 1G and H), 19 cases showed a low RCP expression (scored 4-7) (Fig. 1E and F), and only 11 cases (11.58 %) were scored 2-3.

Correlation between RCP expression and clinicopathological variables. The association between RCP protein expression and clinicopathological characteristics of SCCHN was explored by the χ^2 test. As shown in Table III, RCP overexpression was significantly associated with tumor T classification ($P=0.028$), clinical staging ($P=0.012$), lymph node metastasis ($P=0.004$) and recurrence ($P=0.034$), respectively. However, no significant correlation was observed between RCP protein levels and variables such as age ($P=0.383$), alcohol intake ($P=0.658$), smoking status ($P=0.811$), tumor site ($P=0.153$) and tumor grade ($P=1.004$).

Patient follow-up and survival analysis. In total, 95 patients with SCCHN remained in follow-up after surgery. The median follow-up of the whole series was 58 months (range, 2-96), and the median follow-up of the patients alive at the last visit was 75 months (range, 51-96). During this follow-up period, 5 cases were lost due to change of address, 46 (48.42%) cases developed loco-regional recurrence (median recurrence time was 17 months), among which 2 cases (2.11%) had loco-regional recurrence twice and 1 case (1.05%) had local recurrence and distant lung metastasis. Forty-three (43/95; 43.16%) patients succumbed to the disease in this retrospective study, and the main cause of death was tumor recurrence.

In the survival analyses by the Kaplan-Meier method, the expression of RCP in SCCHN was significantly correlated with disease-free survival (Fig. 2A) and overall survival (Fig. 2B). The log-rank test further demonstrated that the survival time was significantly different between groups with a high and low expression of RCP protein, indicating that a high level of RCP was tightly correlated with a shorter survival time.

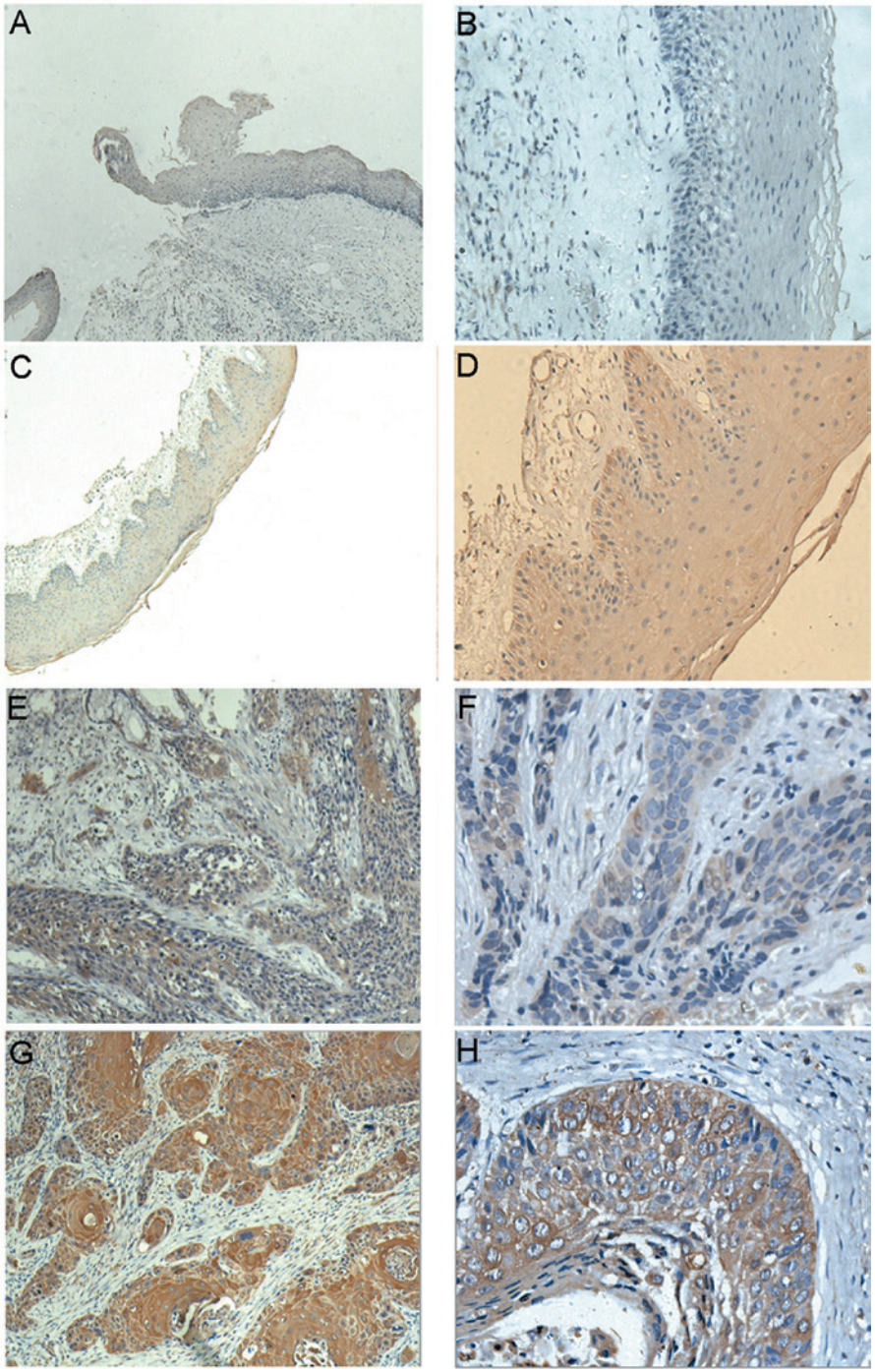

Figure 1. Immunohistochemical staining of RCP in the epithelial tissues of vocal nodules, leukoplakia of larynx and SCCHN. (A and B) Approximately negative RCP expression in vocal nodule epithelia. (C and D) Leukoplakia epithelia with a low RCP expression. (E and F) SCCHN tissue with a low RCP expression. (G and H) SCCHN tissue with a high RCP expression (left panel, magnification, x100; right panel, magnification, x400). RCP, Rab coupling protein; SCCHN, squamous cell carcinoma of the head and neck.

Univariate and multivariate Cox regression analyses were performed to identify relevant prognostic factors in overall survival using the Cox proportional hazards model (Table IV). Univariate analysis revealed that the following variables were significantly associated with a worse prognosis: Alcohol history (P=0.099); smoking (P=0.048); T classification (P=0.047); clinical staging (P=0.039); lymph node metastasis (P=0.007); recurrence (P=0.000); and RCP expression (P=0.015). However, the multivariate analysis showed that only recurrence had independent prognostic effects on the overall survival of patients with SCCHN (P=0.000).

Discussion

In the current study, we investigated the protein expression of RCP in a series of 95 clinical paraffin-embedded specimens with intact follow-up information. Immunostaining results revealed that RCP protein levels were markedly higher in

Table IV. Prognostic factors in overall survival by univariate and multivariate analyses (n=95).

Risk factors	Univariate analysis		Multivariate analysis	
	HR (95 CI)	P-value	HR (95 CI)	P-value
Age (≤57/>57)	0.865 (0.469-1.597)	0.643	0.991 (0.501-1.960)	0.098
Alcohol intake (Y/N)	1.696 (0.905-3.179)	0.099	1.093 (0.546-2.192)	0.801
Smoking (Y/N)	1.919 (1.005-3.663)	0.048	1.096 (0.454-2.648)	0.839
Tumor site (Glottic/others)	0.629 (0.329-1.202)	0.161	0.613 (0.252-1.494)	0.282
Tumor grade (G1/G2+G3)	0.719 (0.389-1.328)	0.292	1.134 (0.517-2.486)	0.754
T classification (T1+T2/T3+T4)	1.909 (1.010-3.609)	0.047	1.634 (0.452-5.901)	0.454
Clinical stage (I+II/III+IV)	2.037 (1.038-3.998)	0.039	0.442 (0.104-1.879)	0.269
Metastasis (Y/N)	2.350 (1.265-4.366)	0.007	1.804 (0.682-4.771)	0.235
Recurrence (Y/N)	38.221 (9.144-159.750)	0.000	37.458 (8.477-165.523)	0.000
RCP expression (High/low)	2.938 (1.234-6.996)	0.015	1.631 (0.648-4.107)	0.299

HR, hazard ratio; CI, confidence interval; Y/N, Yes/No; RCP, Rab coupling protein.

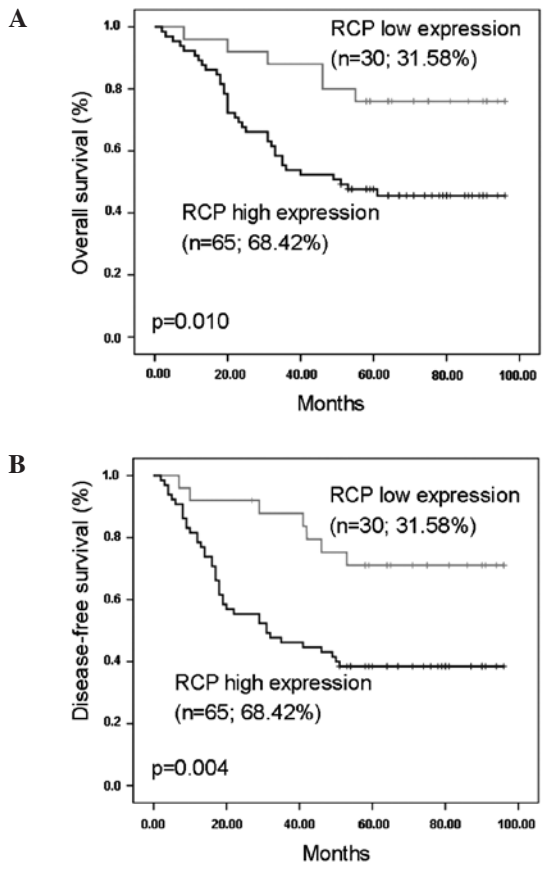

Figure 2. Kaplan-Meier survival curve analysis of (A) overall survival and (B) disease-free survival in the 95 patients with SCCHN. A high RCP expression is negatively correlated with overall survival and disease-free survival. The log-rank test was applied to calculate the P-value. SCCHN, squamous cell carcinoma of the head and neck.

SCCHN tissues compared with laryngeal leukoplakia (precancerous lesions), while RCP was hardly detected in squamous epithelia from vocal nodules. Our results were in agreement with previous studies. Zhang *et al* (8) showed that RCP had a higher expression in invasive breast ductal cancer than normal breast epithelium, ductal carcinoma *in situ* (pre-malignant), weakly aggressive mucinous and medullary histological types. In addition, overexpression of RCP in MCF10A normal human mammary epithelial cells resulted in the acquisition of tumorigenic properties. For example, RCP overexpression decreased growth factor-dependent cell growth; increased cell survival under anoikis conditions; induced cell motility, invasion and EMT *in vitro*; and increased tumor growth and progression *in vivo* (8). Thus, RCP is important in the malignant progression of SCCHN.

Subsequently, a detailed analysis for elucidating the correlation between RCP expression and clinicopathological variables was performed. RCP overexpression was found to be significantly associated with tumor T classification, clinical staging, particularly with lymph node metastasis, recurrence and a shorter survival time. The lymph node metastasis and postoperative tumor recurrence are important factors affecting prognosis (1,12-13). At present, there are few data concerning RCP expression and tumor metastasis in other types of cancer except for breast cancer. RCP overexpression induced cell motility, invasion and EMT *in vitro*; RCP knockdown weakened tumor progression and lung micrometastasis *in vivo* (8). The mechanism by which RCP promotes metastasis may be associated with certain downstream signals, such as EGFR and β1 integrin (9,14-15). Caswell *et al* (14) reported that activated RCP associates with β1 integrin and acts to link this integrin with RTKs at recycling endosomes. This consequently drives cell proliferation and cell migration in 2D and 3D matrices. Furthermore, when cells migrate in 3D matrices, the ability of RCP and its binding partner RAB25 to localize integrin and EGFR signaling to the cell front drives the extension of invasive pseudopods. This trafficking-dependent localization of signaling proteins such as β1 integrin and EGFR may contribute to the role of RCP in metastasis (9,14).

The prognostic value of RCP protein in patients with SCCHN remains to be determined. Currently, prognostic evaluation is mainly based on traditional methods including clinical stage, tumor site and histopathological grade. Previous

studies have suggested that other factors, such as molecular and cellular characteristics of the primary tumors, may improve our ability to prognosticate (16). In our present investigation, the multivariate analysis revealed that only tumor recurrence had independent prognostic effects on the overall survival rate. However, the expression level of RCP protein failed to be an independent prognostic factor in our Cox multivariate analysis, although its expression was inversely correlated with overall survival and disease-free survival. This failure may be explained by the fact that the proportion of the cases diagnosed as recurrence and lymph node metastasis in 95 patients with SCCHN was extremely large, which may reduce the RCP significance in multivariate analysis. Thus, the evaluation of the RCP protein may further provide new information for patient prognosis.

In conclusion, our current study indicated that RCP was upregulated in human SCCHN and that RCP overexpression was significantly correlated with tumor malignant progression and poor survival in patients with SCCHN, which suggested that RCP may serve as a specific and a novel prognostic marker in SCCHN. However, further studies are required to determine the molecular mechanism of RCP involved in SCCHN progression and prognosis, which may lead to further development of new approaches targeting RCP for effective tumor management.

Acknowledgements

The authors would like to thank Xiang Li (Department of Pathology, Xiangya Hospital) and Xueping Feng (Central Laboratory of Medical Research, Xiangya Hospital) for their evaluation of these clinical samples, and acknowledge Dr Vivek K. Rauniyar (Department of Neurology, Xiangya Hospital) for his critical review of this manuscript. This study was supported by grants from the National Natural Science Foundation of China (No. 81071757, 30872852, 30901664), the Key Program of Natural Science Foundation of Hunan Province (2010TP4012-1), the Research Fund for the Doctoral Program of Higher Education of China (20100162110036, 20090162110065) and the Graduate Degree Thesis Innovation Foundation of Hunan province (CX2011B059).

References

1. Jemal A, Siegel R, Ward E, *et al*: Cancer statistics, 2008. CA Cancer J Clin 58: 71-96, 2008.
2. Fischer CA, Jung M, Zlobec I, *et al*: Co-overexpression of p21 and Ki-67 in squamous cell carcinoma of the head and neck relative to a significantly poor prognosis. Head Neck 33: 267-273, 2010.
3. Hutagalung AH and Novick PJ: Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 91: 119-149, 2011.
4. Jordens I, Marsman M, Kuijl C and Neefjes J: Rab proteins, connecting transport and vesicle fusion. Traffic 6: 1070-1077, 2005.
5. Jing J and Prekeris R: Polarized endocytic transport: the roles of Rab11 and Rab11-FIPs in regulating cell polarity. Histol Histopathol 24: 1171-1180, 2009.
6. Hoekstra D, Tyteca D and van IJzendoorn SC: The subapical compartment: a traffic center in membrane polarity development. J Cell Sci 117: 2183-2192, 2004.
7. Somsel Rodman J and Wandinger-Ness A: Rab GTPases coordinate endocytosis. J Cell Sci 113: 183-192, 2000.
8. Zhang J, Liu X, Datta A, *et al*: RCP is a human breast cancer-promoting gene with Ras-activating function. J Clin Invest 119: 2171-2183, 2009.
9. Mills GB, Jurisica I, Yarden Y, *et al*: Genomic amplicons target vesicle recycling in breast cancer. J Clin Invest 119: 2123-2127, 2009.
10. Sobin LH and Wittekind CH (eds): TNM Classification of Malignant Tumors. Wiley-Liss, New York, 2002.
11. Yuan P, Temam S, El-Naggar A, *et al*: Over-expression of podoplanin in oral cancer and its association with poor clinical outcome. Cancer 107: 563-569, 2006.
12. Cerezo L, Millan I, Torre A, Aragón G and Otero J: Prognostic factors for survival and tumor control in cervical lymph node metastases from head and neck cancer. A multivariate study of 492 cases. Cancer 69: 1224-1234, 1992.
13. Liu Y, Xie C, Zhang X, *et al*: Elevated expression of HMGB1 in squamous-cell carcinoma of the head and neck and its clinical significance. Eur J Cancer 46: 3007-3015, 2010.
14. Caswell PT, Chan M, Lindsay AJ, McCaffrey MW, Boettiger D and Norman JC: Rab-coupling protein coordinates recycling of alpha5beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. J Cell Biol 183: 143-155, 2008.
15. Morello V, Cabodi S, Sigismund S, *et al*: β1 integrin controls EGFR signaling and tumorigenic properties of lung cancer cells. Oncogene 30: 4087-4096, 2011
16. Johann DJ Jr, McGuigan MD, Patel AR, *et al*: Clinical proteomics and biomarker discovery. Ann N Y Acad Sci 1022: 295-305, 2004.