Promoter methylation and expression of Raf kinase inhibitory protein in esophageal squamous cell carcinoma

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Abstract. Raf kinase inhibitory protein (RKIP) regulates multiple cellular processes, and its downregulation is associated with distinct human cancers. In the present study, the status of RKIP promoter methylation, as well as its expression and clinical significance in esophageal squamous cell carcinoma (ESCC), were examined. The promoter methylation status in the 5'-CpG island of the RKIP gene and the expression level of the RKIP protein were examined using a modified methylation-specific polymerase chain reaction (MSP) method and immunohistochemical staining, respectively, in 77 ESCC samples and matched paratumor normal tissues. The incidence of RKIP promoter methylation was significantly higher in tumor samples (75.3%) than in the matched normal tissues (27.3%; P<0.001). A higher incidence of promoter methylation was also detected in poorly differentiated cancers (93.5%) compared with well-differentiated cancers (50.0%; P<0.001), as well as in tumor samples with positive lymph node metastasis (86.7%) compared with those with negative lymph node metastasis (59.4%; P<0.001). Consistent with the promoter methylation status, the expression level of RKIP was significantly reduced in cancer tissues (36.4%) compared with matched normal tissues (76.6%; P<0.01), as well as in cancers with positive lymph node metastasis (24.4%) compared with those with negative lymph node metastasis (53.1%; P=0.01). Promoter methylation-induced gene silencing significantly correlated with the down regulation of RKIP and the development of ESCC. The results of the present study suggested that the methylation status of the RKIP promoter, when combined with its expression level, may serve as a biomarker for predicting the biological behaviors of ESCC.

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Introduction

Esophageal cancer is among the most prevalent cancers in China, ranking sixth in terms of incidence and fourth in terms of mortality, according to the 2011 Annual Report on the Status of Cancer in China (1). The highest morbidity rate of esophageal cancer in China reached 130 per 100,000 people in 2003 (2). Esophageal cancer can be further divided into two main pathological subtypes: Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), with the former being the predominant type and accounting for ~90% of esophageal cancer cases worldwide (3). Even though there have been significant advances in therapeutic approaches for ESCC, the prognosis of ESCC remains poor (the 5-year survival rate is <10%) (4). Therefore, it is essential to explore the pathogenesis of ESCC and to identify novel biomarkers for risk assessment, early detection and prognosis prediction.

Many genetic and epigenetic alterations have been implicated in the pathogenesis of ESCC. Among these alterations, DNA methylation-induced gene silencing of tumor suppressor genes plays significant roles in ESCC initiation, progression and metastasis; thus serving as important ESCC biomarkers (5). Although expression analysis alone is frequently used as the main approach to identify cancer biomarkers, Cheng *et al* recently proposed that integrating expression and epigenetic alteration analyses would provide a higher likelihood of identifying ESCC biomarkers (6).

Raf kinase inhibitory protein (RKIP) is a highly conserved, ubiquitously expressed and small cytoplasmic protein with various biological and pathological activities (7). RKIP inhibits Raf-1-mediated phosphorylation and activation of mitogen-activated protein kinase (MAPK) kinase (MEK)-1, as well as the subsequent MAPK and extracellular signal-regulated kinase activities (8). RKIP also negatively regulates nuclear factor (NF)-κB signaling and the signaling downstream of G protein-coupled receptor kinase (9,10). During cancer development, RKIP is characterized as a tumor suppressor gene because of its maintenance of chromosome stability, inhibition of cellular proliferation, promotion of cell differentiation and dynamic balancing of oncogene activities (11). Consistently, previous studies have reported RKIP absence or downregulation and its clinical significance in a variety of human cancers, such as prostate, breast and gastric cancers (12-14). These observations led to intensive studies on

the mechanisms and functions of RKIP in cancers; however, few previous studies have analyzed the RKIP methylation and expression status in ESCC. Using immunohistochemical analysis, Kim *et al* demonstrated that 71.4% of ESCC metastatic lymph nodes, 50.0% of primary ESCC and 28.9% of carcinoma *in situ* showed the loss of RKIP expression, suggesting that RKIP plays an inhibitory role in the invasion and metastasis of ESCC (15). In addition, Gao *et al* reported that positive expression of RKIP in ESCC occurred significantly less often than in paratumor normal tissues, and that reduced RKIP expression was significantly correlated with a higher risk of recurrence, implying its importance in prognosis prediction (16). Furthermore, the expression level of RKIP was decreased in the order of dysplastic Barrett's mucosa, low-grade dysplasia, high-grade dysplasia and EAC, suggesting its involvement in esophageal carcinogenesis (17).

Although these studies all support the significance of RKIP in ESCC, minimal information is known regarding the mechanisms leading to RKIP silencing in ESCC. Considering that promoter hypermethylation is an important mechanism for gene silencing, and that it plays an important role in downregulating RKIP in gastric carcinoma (18,19), the authors of the present study hypothesized that promoter methylation may be a potential mechanism for downregulating RKIP in ESCC and is thus responsible for the biological behaviors of ESCC. To test this hypothesis, the RKIP promoter methylation status and its expression level in ESCC tissues and matched paratumor normal tissues were examined using methylation-specific polymerase chain reaction (MSP) and immunohistochemical staining, respectively. Furthermore, their correlations with distinct clinicopathological features of the patients were analyzed.

Table I. Clinicopathological characteristics of esophageal squamous cell carcinoma cases in this study.

Characteristic	n	%
Gender		
Male	59	76.62
Female	18	23.38
TNM stage		
I + II	33	42.86
III+ IV	44	57.14
Pathological differentiation		
Well	20	25.97
Moderate	26	33.77
Poor	31	40.26
Age (years)		
<60	29	37.66
≥60	48	62.34
Lymph node metastasis		
Negative	32	42.86
Positive	45	57.14

TNM, tumor-node-metastasis.

Materials and methods

Patients and tissue samples. The present study was approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, China), where all data were collected, and written informed consent was obtained from all participants. A total of 77 patients (59 males and 18 females; mean age of 61.8±8.7 years) who underwent radical surgery of ESCC in the Fourth Hospital of Hebei Medical University between December 2008 and December 2010 were recruited to this study. No patients received pre-operative chemotherapy or radiotherapy. Among these patients, 29 were aged <60 years, while 49 were aged >60 years. According to the tumor, lymph node, metastasis (TNM) staging criteria from the Union for International Cancer Control and the American Joint Committee on Cancer (20), 33 cases were of stage I or II and 44 cases were of stage III or IV. According to the pathological grading system (21), 20 ESCC cases were well-differentiated, 26 were moderately-differentiated, and 31 were poorly-differentiated cancers. Among these ESCC cases, 45 showed positive lymph node metastasis, while 32 showed negative lymph node metastasis. The clinicopathological characteristics of the patients are summarized in Table I.

During surgery, the cancer tissues and the matched normal tissues were obtained from the primary tumors and at least 5-cm away from the tumor, respectively. All tissues were immediately stored at -80˚C, with some subsequently used for DNA extraction and some fixed in 10% formalin and embedded in paraffin for immunohistochemical staining. All matched normal tissues were confirmed by pathological examination to contain no invaded tumor cells.

Modified MSP method. Tissue samples were treated with proteinase K, and genomic DNA was extracted using phenol/chloroform. Using the TU-1800 PC UV-VIS spectrophotometer (Beijing, China) to measure the absorbance ratio at 260/280 nm, the total DNA purity was determined to be between 1.8 and 2.0. Bisulfite modification of 10 μg genomic DNA from each sample was performed as described previously (22). Briefly, genomic DNA was denatured using 2 M NaOH and then incubated with 10 M hydroquinone (Merck Millipore, Darmstadt, Germany) and 3 M sodium bisulfite at 50˚C for 16 h. Modified DNA was purified using the Wizard DNA purification resin (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The MSP was performed using primers (Table II) under the following conditions: Predenaturation at 95˚C for 12 min; 36 cycles at 95˚C for 60 sec, 52˚C for 45 sec and 72˚C for 60 sec; and a final extension at 72˚C for 10 min. The polymerase chain reaction (PCR) products were subsequently separated on 2% agarose gels by electrophoresis and analyzed using an ultraviolet light gel imaging system (FOTODYNE Incorporated, Hartland, WI, USA). The peripheral blood from a healthy individual without any diseases or tumors in the alimentary system was taken to isolate genomic DNA, which, following methylation treatment using DNA methyltransferase (Sss I; Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) was used as a positive control; that without Sss I treatment was used as a negative control. A water blank was also used as a negative control. The tissue samples showing positive PCR products only with methylated primers (full methylation) or with

Table II. Sequences, Tm and amplicon sizes of primers used for RKIP methylation-specific PCR.

	Primer sequences, 5'-3'	Tm, ˚C	PCR product size, bp
M	F TTTAGCGATATTTTTTGAGATACGA R GCTCCCTAACCTCTAATTAACCG	52.5	205
U	F TTTAGTGATATTTTTTGAGATATGA R CACTCCCTAACCTCTAATTAACCAA	52.5	205

M, methylated RKIP; U, unmethylated RKIP; Tm, melting temperature; F, forward; R, reverse; RKIP, Raf kinase inhibitory protein.

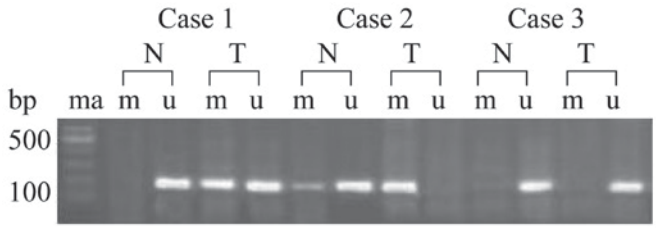

Figure 1. MSP analysis of RKIP promoter methylation in three representative ESCC tissues (T) and the matched paratumor normal tissues (N). The letters m and u indicate that the primers for methylated and unmethylated RKIP were used, respectively. RKIP, Raf kinase inhibitory protein.

both methylated and unmethylated primers (semi-methylation) were defined as methylated samples (23).

Immunohistochemical staining. Immunohistochemical staining for RKIP was performed on 4-μm formalin-fixed, paraffin-embedded tissue sections using the Immunohistochemical Streptavidin-Peroxidase kit (ZSGB-BIO, Beijing, China), according to the manufacturer's protocol. Briefly, the tissue sections were dewaxed, dehydrated and treated with 3% hydrogen peroxidase to block endogenous peroxidase activity. Following antigen retrieval in EDTA solution (pH 8.5) in a pressure cooker for 3 min, the sections were blocked with 10% normal goat serum at 37˚C for 40 min, followed by incubation with the primary rabbit anti-human RKIP antibody (catalog no., bs-1436R; dilution, 1:500; Bioss, Beijing, China) and then horseradish peroxidase-labeled streptavidin. Final color development was achieved using a 3,3'-diaminobenzidine solution, and the slides were counterstained with 1% Meyer's hematoxylin. The use of phosphate-buffered saline instead of the primary antibody served as a negative control.

Positive RKIP staining appeared as yellowish to brownish granules in the cytoplasm. The staining was scored and averaged by three independent clinical pathologists according to a scoring system modified from that proposed by Fromowitz *et al* (24). Briefly, five random fields were imaged from each slide under a BX41 light microscope (Olympus, Tokyo, Japan). A score was assigned based on the percentage of positive tumor cells averaged from all five fields: Score 0, ≤25%; score 1, 26-50%; score 2, 51-75%; and score 3, >75%. In addition, the staining was graded based on the intensity of the majority of the positively stained cells: 0, no staining; 1, weak yellowish staining; 2, moderate brownish staining; and 3, dark brownish staining. The final score was obtained by adding the percentage score and intensity grade, and stratified as: '-'=0; '+'=1-2; '++'=3-4; and '+++'=5-6, where '-' and '+' were classified as negative expression, while '++' and '+++' were classified as positive expression.

Statistical analysis. All statistical analyses were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Quantitative data are presented as a percentage or ratio of the total sample. The association between groups was assessed using the χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RKIP promoter methylation and protein expression in ESCC and paratumor normal tissues. To examine the status of RKIP promoter methylation in ESCC, MSP analysis on 77 ESCC tumor tissues and matched paratumor normal tissues was performed (Fig. 1). The incidence of RKIP promoter methylation in ESCC tissues was 75.3%, which was significantly higher compared with the matched normal tissues (27.3%; $P<0.001$; Table III). Given that methylation-induced gene silencing is an important mechanism for downregulating many tumor suppressor genes during cancer development (25,26), the expression level of RKIP in ESCC and normal tissues was also profiled by immunohistochemistry (Fig. 2). The incidence of positive RKIP expression in the ESCC tissues was 36.4%, which was significantly reduced compared with the matched normal tissues (76.6%; $P<0.001$; Table III).

Correlation between RKIP promoter methylation and the clinicopathological characteristics of ESCC. The correlation between RKIP promoter methylation in ESCC and various clinicopathological characteristics is shown in Table IV. The incidence of RKIP promoter methylation was not significantly correlated with the age ($P=0.528$), gender ($P=1.000$) or TNM stage ($P=0.321$), although it was significantly correlated with the differentiation status of the tumor (50.0% in well-differentiated tumors, 73.1% in moderately-differentiated tumors and 93.5% in poorly differentiated tumors; $P=0.002$), as well as the lymph node status (86.7 and 59.4% in ESCC patients with and without positive lymph node metastasis, respectively; $P=0.006$).

Correlation between RKIP protein expression and the clinicopathological characteristics of ESCC. The analysis of the correlation between the incidence of RKIP protein expression in ESCC and various clinicopathological characteristics showed that the expression of RKIP in the tumor tissues was

Table III. Methylation status and protein expression of RKIP in 77 ESCC tissues and the matched paratumor normal tissues.

	RKIP promoter methylation		RKIP protein expression	
Tissues	Methylation	No methylation	Positive	Negative
ESCC (n=77)	58 (75.32%)	19 (24.67%)	28 (36.36%)	49 (63.64%)
Normal (n=77)	21 (27.27%)	54 (72.73%)	59 (76.62%)	18 (23.38%)
χ^2	35.582		25.389	
P-value	P<0.001		P<0.001	

RKIP, Raf kinase inhibitory protein; ESCC, esophageal squamous cell carcinoma.

Table IV. Correlation between RKIP promoter methylation and the clinicopathological characteristics.

	RKIP promoter methylation				
Characteristic	n	M	U	χ^2	P-value
Age (years)					0.528
<60	29	23	6	0.398	
≥60	48	35	13		
Gender					1.000
Male	59	44	15	0.076	
Female	18	14	4		
Clinical stage					0.321
I+II	33	23	10	0.984	
III+IV	44	35	9		
Degree of differentiation					0.002
Well	20	10	10	12.511	
Moderate	26	19	7		
Poor	31	29	2		
Lymph node metastasis					0.006
Negative	32	19	13	7.494	
Positive	45	39	6		

M, methylated RKIP; U, unmethylated RKIP; RKIP, Raf kinase inhibitory protein.

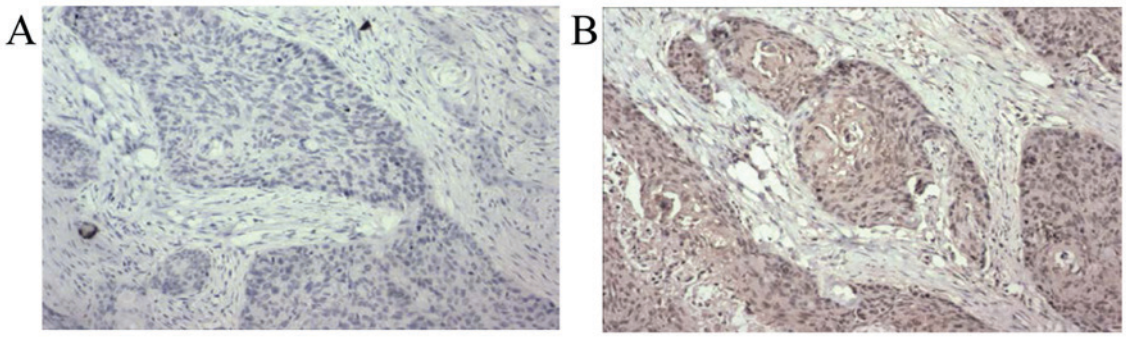

Figure 2. Immunohistochemical staining of Raf kinase inhibitory protein (brown signals) in (A) a representative ESCC tissue and (B) the matched paratumor normal tissue (magnification, x200).

only significantly correlated with lymph node metastasis (24.4 and 53.1% in ESCC patients with and without positive lymph node metastasis, respectively; P=0.01); it was not significantly associated with age (P=0.540), gender (P=0.760),

Table V. Correlation between RKIP protein expression and clinicopathological characteristics.

Characteristic	n	RKIP protein expression		χ^2	P-value
		Positive	Negative		
Age, years				0.571	0.450
<60	29	9	20		
≥60	48	19	29		
Gender				0.093	0.760
Male	59	22	37		
Female	18	6	12		
Clinical stage				3.667	0.056
I + II	33	16	17		
III + IV	44	12	32		
Degree of differentiation				2.535	0.282
Well	20	9	11		
Moderate	26	11	15		
Poor	31	8	23		
Lymph node metastasis				6.648	0.010
Negative	32	17	15		
Positive	45	11	34		

RKIP, Raf kinase inhibitory protein.

Table VI. Association between RKIP promoter methylation and protein expression in esophageal squamous cell carcinoma tissues.

RKIP methylation status	RKIP protein expression		Total	χ^2	P-value
	+	–			
Methylated	17	41	58	17.308	0.001
Unmethylated	11	8	19		
Total	28	49	77		

RKIP, Raf kinase inhibitory protein.

TNM stage (P=0.056) or the differentiation status of the tumor (P=0.282; Table V).

Association between RKIP promoter methylation and protein expression. Among the 58 ESCC tissues positive for RKIP promoter methylation, 29.3% were positive for RKIP protein expression. Of the 19 ESCC tissues negative for RKIP promoter methylation, 57.9% were positive for RKIP protein expression. There was a significant negative association between RKIP promoter methylation and protein expression (P=0.001; Table VI).

Discussion

The present study examined the status of RKIP promoter methylation and its expression in ESCC and matched paratumor normal tissues, and demonstrated that RKIP promoter methylation was significantly enhanced, while its protein expression level was significantly reduced, in the tumor tissues compared with the normal tissues. Functionally, RKIP promoter methylation was significantly correlated with the status of tumor differentiation and lymph node metastasis, while RKIP protein expression was associated with lymph node metastasis only. Consistent with these observations, RKIP promoter methylation was negatively associated with its protein expression in ESCC tissues.

Tumorigenesis is a complicated process involving numerous factors, multiple steps and genetic as well as epigenetic alterations in various genes. The pathogenesis of ESCC has been mainly attributed to environmental factors, including malnutrition, smoking, alcohol use and inflammation (27-29). However, recent studies have revealed that genetic and

epigenetic alterations also play significant roles in the development of multiple cancers, including ESCC (30-32). Among various epigenetic alterations, aberrant DNA methylation (including hypomethylation of oncogenes and hypermethylation of tumor suppressor genes) is the best characterized and most crucial mechanism modulating chromatin structure and the expression levels of oncogenes or tumor suppressor genes, contributing to tumor initiation and further development (33). Hypermethylation of the CpG islands within the promoter regions of tumor suppressor genes is a well-demonstrated molecular mechanism for transcriptional silencing and subsequent tumor progression (34). The presence of promoter CpG island hypermethylation in preneoplastic lesions of malignant cancers supports its significance in neoplastic transformation and tumor initiation (35-38).

Many studies have corroborated the nature of RKIP as a tumor suppressor gene. Li *et al* reported that promoter hypermethylation of the RKIP gene led to its silencing in colorectal cancer, which may underlie tumor metastasis (39). Al-Mulla *et al* demonstrated that chromosome loss in colorectal cancer was inversely proportional to RKIP expression levels, the silencing of which was mainly caused by methylation of the RKIP promoter (40). Consistently, other studies have supported the importance of RKIP promoter hypermethylation in the loss of its activity (41,42). In contrast, few studies have investigated the status or clinical significance of RKIP promoter methylation in ESCC. To address this issue, the present study compared promoter methylation of the RKIP gene in ESCC tissues and the matched normal tissues of 77 patients with ESCC, and showed that RKIP promoter hypermethylation was significantly enhanced in the tumor tissues compared with the normal tissues, supporting its potential involvement in tumor initiation. Furthermore, RKIP promoter hypermethylation was significantly correlated with tumor differentiation and lymph node metastasis, suggesting its value in predicting ESCC metastasis and prognosis.

In the paratumor normal tissues, 27.3% were positive for RKIP promoter methylation, and all these cases were semi-methylated (data not shown). One potential explanation for this was that we used the highly sensitive nested MSP for this study, which is capable of detecting alleles comprising <5% of the total genomic DNA (43). Therefore, minor invasion of the tumor tissue into the normal tissue (although negative in the pathological examinations) may have led to semi-methylation. Among the 58 ESCC samples positive for RKIP promoter methylation, 3 were semi-methylated (data not shown), which may be related to the degree of transcriptional silencing of RKIP; that is, an incomplete methylation corresponds to incomplete gene silencing (44).

In addition to promoter methylation, reduced or loss of RKIP expression has been associated with cancer development. Immunohistochemical analysis has demonstrated that the loss of RKIP expression is a key phenotype for many human cancers, including colorectal cancer, breast cancer, melanoma, prostate cancer and hepatocellular carcinoma, modulating distant metastasis, lymphatic metastasis, vascular infiltration and cancer mortality (45-49). Consistent with these studies, the present study showed that the incidence of positive RKIP expression in ESCC tissues was significantly less compared with the matched normal tissues, and that it was clinically correlated with lymph node metastasis. A further association analysis revealed that promoter methylation-induced RKIP silencing may be a major mechanism for downregulating RKIP and subsequent ESCC development.

The potential involvement of RKIP as a tumor suppressor gene in tumor invasiveness and metastasis has been well demonstrated in multiple cancers. Keller *et al* reported that RKIP expression was at its highest in normal prostate tissues, decreased in primary prostate cancers and was not detectable in metastatic tissues from prostate cancer (50). In addition, Schuierer *et al* demonstrated that RKIP expression was decreased concomitantly with the metastasis of melanoma (47). Mechanistic studies have suggested that RKIP is a metastasis suppressor gene that is responsible for blocking several signaling pathways in the metastatic cascade, including MEK, G proteins and NF-κB (51). Similarly, the present study showed that the percentage of RKIP expression in ESCC tumors with lymph node metastasis was significantly less compared with tumors without lymph node metastasis.

In summary, this study showed that promoter methylation may be responsible for RKIP downregulation and the oncogenesis of ESCC. Therefore, the incidence of RKIP promoter methylation may serve as a biomarker for the differentiation status and prognosis of ESCC. This study extends the molecular understanding of the pathogenesis of ESCC and provides a novel target for the early diagnosis and treatment of ESCC.

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