Int J Clin Exp Pathol 2017;10(2):1765-1773
www.ijcep.com /ISSN:1936-2625/IJCEP0046532

Original Article

Hypermethylation of RASSF1A CpG insland is associated with esophageal squamous cell carcinoma in Kazakh patients

Xiao-Bin Cui[1*], Ran-Ran Li[1*], Xiao-Li Diao[2*], Tao Ren[1], Hao Peng[1], Dan-Dan Wang[1], Bin Wang[1], Yun-Zhao Chen[1,2], Feng Li[1,2]

[1]Department of Pathology and Key Laboratory of Xinjiang Endemic and Ethnic Diseases (Ministry of Education), Shihezi University School of Medicine, Shihezi, China; [2]Department of Pathology, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China. [*]Equal contributors.

Received December 13, 2016; Accepted December 27, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Esophageal squamous cell carcinoma (ESCC) is a malignant tumor with poor prognosis and high mortality and incidence in Kazakh population. The decreased expression of RAS association domain family 1A (RASSF1A) by methylation in various cancers has been reported. This study aims to investigate the correlation of the RASSF1A promoter methylation and the esophageal squamous cell carcinoma (ESCC) in Kazakhs. To determine whether aberrant RASSF1A methylation occurs in esophageal cancer, the DNA methylation of 19 CpG units in the RASSF1A promoter was quantitatively analyzed by MALDI-TOF mass spectrometry in 100 ESCC tissues and 100 normal tissues from the Kazakh population. The results indicating that the overall methylation status of RASSF1A in Kazakh ESCC patients is higher than that in controls (χ^2=5.615, P=0.020). And the methylation levels of CpG_11.12, CpG_16.17 and CpG_24.25 were respectively 25.4%, 18.8%, 16.3%, which were significantly higher than the mean methylation rate (5%) in normal group (5%, CpG_11.12, P=0.00; CpG_16.17, P=0.002; CpG_24.25, P=0.001). However,no correlation was found between the RASSF1A overall methylation and clinicopathological characteristics (For age: t=1.716 P=0.089; gender: χ^2=1.487 P=0.475; differentiation: χ^2=0.770 P=0.680; HPV16 infection: χ^2=2.519, P=0.104. Similar conditions are to be found with CpG_11.12, CpG_16.17 and CpG_24.25 units (for age: t=1.706 P=0.079; gender: χ^2=1.847 P=0.415; differentiation: χ^2=0.670 P=0.7; metastasis: χ^2=3.765 P=0.463; HPV16 infection: χ^2=2.519, P=0.104). Our findings in this study suggest that the hypermethylation of RASSF1A play an important role in Kazakh esophageal squamous cell carcinoma (ESCC). Moreover, targeting RASSF1A methylation by demethylating agents may offer an approach for anticancer therapy of ESCC.

Keywords: RASSF1A, esophageal squamous cell carcinoma, Kazakh, methylation, CPG unit

Introduction

Esophageal carcinoma is the eighth most common cancer in the world and the seventh major cause of cancer death worldwide [1]. Esophageal carcinoma was divided into two primary histological subtypes: esophageal adenocarcinoma (EADC) and esophageal squamous cell carcinoma (ESCC). Esophageal squamous cell carcinoma (ESCC) still is the main predominant histological type of esophageal cancer and the leading cause of cancer-related deaths in China [2]. Compared with other ethnic populations in China, the Kazakh population in Xinjiang is characterized by higher incidence and mortality (90-150/100,000, age standardized) of ESCC than the general population of China [3-5]. Also, in this high-risk areasof ESCC there is a strong tendency toward familial aggregation [6], indicating that hereditary susceptibility with environmental risk factors both contributes to the high rates of ESCC. The gene methylation is an alternative mechanism which leads to gene inactivation that occur early tumor progression and thus changes gene expression without changing the DNA sequence [7-9]. Abnormal methylation on CpG island is one of the earliest molecular alternations occurring during carcinogenesis [10]. Recently, various kinds of ESCC-susceptible genes with preternatural DNA methylation gene expression have been identified, including RASSF1A genes.

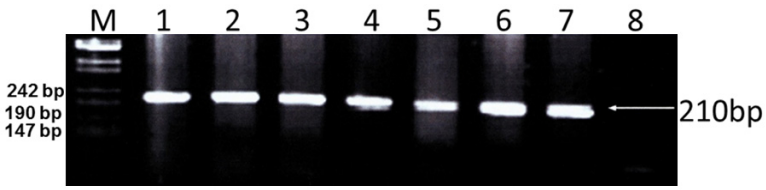

Figure 1. DNA quality evaluation. M: marker; 1: β-globin positive control; 2-7: β-globin target fragment samples, 8 blank control.

RAS association domain family 1A (RASSF1A) locates at 3p21.3 and participates in regulating cell cycle apoptosis, microtubule stability and other physiological activities [11]. RASSF1A inactivation by methylation is an extremely common event in many human cancers, including 80 to 100% of small-cell lung cancer cell lines and tumors, 30 to 40% of non-small-cell lung cancer cell lines and tumors, 49 to 62% of breast cancer cell lines and tumors, 67 to 70% of primary nasopharyngeal cancers, and 91% of primary renal cell carcinomas,indicating the RASSF1A promoter was a candidate tumor suppressor gene [11-13]. Some studies have also reported differences in the methylation frequencies of RASSF1A between ESCC cancer tissues and non-cancerous tissues. However, previous studies have indicated the possible crucial role of RASSF1A methylation on esophageal carcinogenesis in Chinese population, the methylation rates varied from 14.9% [14] in Beijing with a low incidence for ESCC, to 48.5% [15] in Hangzhou with a higher incidence for ESCC, indicating the inconformity of RASSF1A methylation with different environment background. Although the increased methylation of RASSF1A in ESCC has been founded [16-18], the critical CpG sites and its role on ESCC progression have not been clarified. Moreover, their study was conducted in Han population with traditional methylation-specific PCR (MSP) method, not the quantitative method [19]. So the effects of RASSF1A methylation on esophageal carcinogenesis have not been well characterized in the Kazakh population from this highest incidence area in China. Given these observations, revealing the molecular pathogenesis of Kazakh ESCC, especially the detection of abnormal CpG methylation, is therefore likely to provide new approaches to the prevention, diagnosis and treatment of ESCC.

In this study, we used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) approach to evaluate the methylation status of 19 CpG units of the RASSF1A promoter regions with a total 200 Kazakh subjects.We found that the promoter methylation level of the RASSF1A was increased in ESCCpatients than controls. Our results suggest that hypermethylation of RASSF1A is associated with esophageal squamous cancer. Therefore, status of RASSF1A promoter methylation has the potential to serve as a biomarkerfor ESCC patients and represents a new candidate therapeutic target by pharmacological compounds with DNA demethylating activity for ESCC treatment.

Materials and methods

Patients and tissue samples

One hundred esophageal tissues from Kazakh patients were confirmed ESCC by pathological examination, which were randomly collected by multistage cluster sampling. All patients were recruited from the First Affiliated Hospital of Shihezi University and the Peoples Hospitalof Xinjiang Uygur Autonomous Region between 1980 and 2004. No restrictions regarding age, sex, or disease stage were set. Patients were not pretreated with surgery other than diagnostic biopsies,radiation therapy, or chemotherapy, before gathered or any blood transfusion in the prior six months. The presentative tissue samples and matching normal tissue sampleswere surgically resected and fixed in 10% buffered formalin then embedded in paraffin, routinely processed. We also gathered clinical data on clinic-pathological variables, such as invasion depth, tumor site, and distant metastasis from the medical records of the patients. In addition, 100 samples of normal esophageal tissue were obtained from 100 healthy people without any primary esophageal tumor as controls. The healthy people were collected in Yili area, the high incidence area for ESCC.

In this study, various clinicopathological characteristics of Kazakh ESCC cases and controls were investigated. The age was 54.41 ± 9.14 (mean ± SD) years for the cancer samples and 52.81 ± 9.33 (mean ± SD) years for the normal sample (P=0.096). There were 60 (60%) males

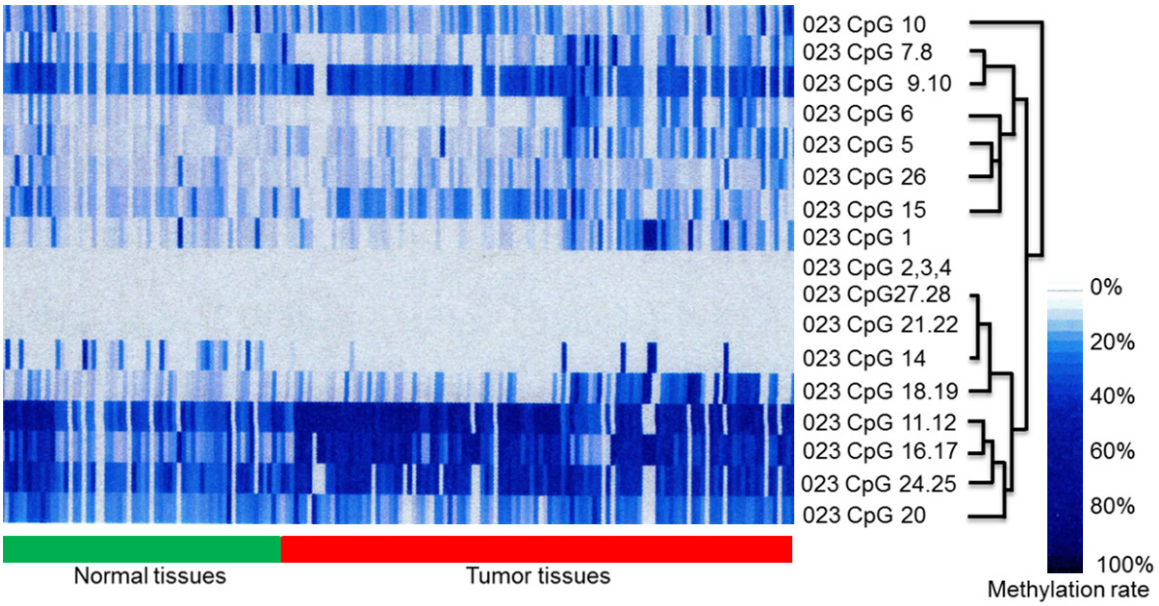

Figure 2. Two-way hierarchical cluster analysis of 100 Kazakh ESCC samples and 100 samples from Kazakh normal tissues (rows) and DNA-methylation of CpG Units in 17 promoter regions (columns). Each vertex indicates an individual CpG site. DNA-methylation levels are depicted in this false-color image on a continuous scale from white (nonmethylated) to blue (100% methylated). Poor quality data are in gray. The cancer samples show more-variable methylation patterns.

Table 1. Sequences of PCR primers used in this study

Primer	Sequence (5'-3')	Size (bp)
β-globin	5'-CAGACACCATGGTGCACCTGAC-3'	210
β-globin	5'-CAGACACCATGGTGCACCTGAC-3'	
RASSF1A	5'-aggaagagagtttagaataggttggaggtaggg-3'	311
RASSF1A	5'-cagtaatacgactcactatagggagaaggcttcccattaaaaaaataacactcaaa-3'	

and 40 (40%) females in the case group and 51 (51%) males and 49 (49%) females in the control group (P=0.087). All of the patients and controls were enrolled by written informed consent, and the study had obtained approval from Shihezi University's Research Ethics Committeeof Medicine, P. R. China. According to the ethical and legal standards, all subjects were handled and made anonymous in this research.

DNA isolation and bisulfite treatment

DNA was isolated from 10 tissue sections of 5 μm thickness by proteinase K digestion and a tissue DNA extraction kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's protocol. As an internal control, all purified genomic DNA samples were successfully tested by polymerase chain reaction (PCR) with humanβ-globin primers (Forward: 5'-CAGACACCATGGTGCACCTGAC-3' and Reverse: 5'-CCAATAGGCAGAGAGAGTCAGTG-3'), indicating that the suitable quality and quantity of DNA can be used to detect the profile of RASSF1A methylation (**Figure 1**). Genomic DNA was stored at -20°C until use as a template for each PCR reaction. The genomic DNA sodium bisulfite was performed with an EZ DNA Methylation KitTM according to the manufacturer's instructions (Zymo Research, Orange, CA, USA) (Catalog No. D5001). The transferred DNA was measured by an ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA).

RASSF1A CpG unit methylation analysis

The sequence of the RASSF1A CpG islands was determined by the UCSC genome browser. In view of that the genomic region site in the RASSF1A gene uncovering a prominent CpG island, we identified the area with proximal promoter activity in prior experiments [22]. The

Table 2. The comparison of the CPG sites number and rate of RASSF1A promoter methylation in Kazakh esophageal cancer and normal esophageal mucosa

	The number of cases		χ^2	*P* values
	Tumor cases (n)	Normal tissues (n)		
Number of RASSF1A sites methylation			5.445	0.000
≤3	50	25		
4-6	20	37		
≥7	30	38		
Methylation rate			4.035	0.001
≤10%	26	36		
11-50%	35	34		
≥51%	39	30		

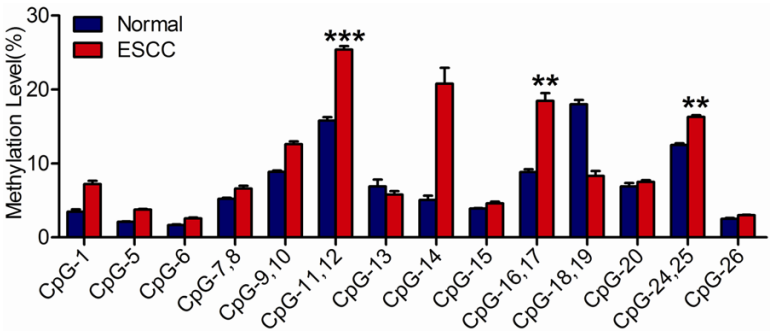

Figure 3. Evaluation of promoter methylation of RASSF1A CpG island. Median methylation levels of 14 informative CpG units in RASSF1A promoter between control and ESCC subjects. **P<0.05, ***P<0.001 (Two samples rank sum Wilcoxon tests).

analyzed region and the CpG sites of the RASSF1A promoter are shown in **Figure 2**. We designed primer sets for the methylation analysis of the RASSF1A promoter areas with EpiDesigner software (**Table 1**). For each reverse primer, we added an additional T7 promoter tag for in vivo transcription, and the forward primer was added a 10-mer tag to adjust for differences in melting temperature. The DNA methylation status of RASSF1A was quantitatively analyzed with the MassARRAY platform (SEQUENOM) as prior described.

The 5 μl PCR mixture contained 25 mM dNTP, 10 ng of bisulfite-treated DNA, 0.2 U of Hot Start TaqDNA polymerase (Sequenom Inc, San Diego, CA, USA), and a 1 μM mixture of reverse and forward primers. The cycles included preheating for 4 min at 94°C, followed by incubation for 45 cycles for 20 s at 94°C, 30 s at 62°C, and 60 s at 72°C and then by incubation at 72°C for 3 min. Two microliters of a shrimp alkaline phosphatase (SAP) mix containing 0.3 μl (1.7 U) and 1.7 μl of H_2O of SAP (Sequenom) was used for digesting redundant dNTPs with the following steps: 20 min at 37°C, 5 min at 85°C, and 4°C there. After the SAP treatment, 2 ml of premix including 0.3 U of SAP (Sequenom) was added for dephosphorylating of unincorporated dNTPs. The reaction mixture was incubated for 40 min at 37°C, and the SAP was heat-inactivated at 85°C for 5 min and was then maintained at 4°C. Five microliters of TCleavage Transcription/RNase Cocktail including 0.89 μl of 5× T7 polymerasebuffer, 0.24 μl of T cleavage mix, 3.14 mM dithiothreitol, 22 U of T7 RNA and DNA polymerase, 0.09 mg/ml of RNase A, and 2 μl of the PCR/SAP reactions production was mixed and incubated under the following steps: 37°C for 3 h of in vitro transcription and RNase A digestion. Fifteen nanoliters of cleavage reaction was then robotically executed (by a nanodispenser) with a matrix (SpectroCHIP; SEQUENOM, San Diego) onto siliconmchips preloaded. Mass spectra were collected by MassARRAY Compact MALDI-TOF (SEQUENOM), and the methylation rates of the spectra were generated by Epityper 1.0 software (SEQUENOM, San Diego). All the experiments were performed in triplicate. Inapplicable readings and their corresponding sites were eliminated from analysis. The methylation level was expressed as the proportions of methylated cytosines over the sum total of methylated and unmethylated cytosines.

Statistical analysis

Statistical analysis was carried out using SPSS 17.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 5.0. Two independent sample Wilcoxon

Table 3. Comparison of average methylation rate of RASSF1A CpG units in the Kazakh esophageal cancer and normal esophageal mucosa

CpG Units	Wilcoxon W	*P* Value
RASSF1A-CpG-1	279.500	0.675
RASSF1A-CpG-5	185.600	0.909
RASSF1A-CpG-6	165.700	0.674
RASSF1A-CpG-7.8	246.800	0.731
RASSF1A-CpG-9.10	289.300	0.080
RASSF1A-CpG-11.12	140.500	0.000*
RASSF1A-CpG-13	168.900	0.428
RASSF1A-CpG-14	332.500	0.714
RASSF1A-CpG-15	155.600	0.233
RASSF1A-CpG-16.17	264.000	0.002*
RASSF1A-CpG-18.19	180.900	0.969
RASSF1A-CpG-20	155.600	0.514
RASSF1A-CpG-24.25	178.900	0.001*
RASSF1A-CpG-26	125.600	0.0129

*P<0.05.

rank sum testswere performed to compare the RASSF1A methylation levels between normal control and tumor samples. A Mann-Whitney U-test was used to analyzethe RASSF1A methylation levels of every CpG site between the ESCC and control groups and between male and female samples. The correlation between each CpG site methylation of RASSF1A and the clinicopathological features was evaluated by a nonparametric test (the Mann-Whitney U-test between two groups and the Kruskal-Wallis H test for three or more groups). Hierarchical clustering analysis was used to describe the RASSF1A CPG units of 200 tissue samples. Each row represents the CpG unit of the amplicons and each column represents the CpG unit of a sample. All *P* value is two-sided, and *P* value of <0.05 was considered statistically significant.

Results

Hypermethylation of RASSF1A promoter in Kazakh patients with esophageal squamous cell carcinoma

The MassARRAY system is an instrument for quantitative analysis of a single CpG site's methylation at a target fragment (CpG island). We can obtain accurate data that represent the ratio or frequency of methylation incident on a CpG site by MALDI-TOFMS. This system was used to assess the methylation level of RASSF1A in all the samples recruited from Kazakh patients with ESCC (n=100) and from control subjects (n=100).

To examine relationships among ESCC and CpG sites, we made an unsupervised two-dimensional hierarchical clustering analysis, which provides an impartial view on these relationships (**Figure 2**). The patterns observed in the cluster analyses of the ESCC patients and normal controls show that the RASSF1A methylation profile of normal controls was remarkable different from that observed in tumor tissues. The sample clusters' result is separation into different terminal branches of the tree of all normal tissue samples from all ESCC samples. The normal samples are characterized by consistent low methylation levels. The ESCC samples show more-variable methylation patterns. What's more, the number of the CpG unit methylation of RASSF1A promoter in ESCC is higher than that in controls (χ^2=5.445, P=0.000, **Table 2**). Then, we summarized the methylation rate of RASSF1A promoter in all samples, the methylation level was increased in ESCC compared with normal samples (χ^2=4.035, P=0.001, **Table 2**). By using the two independent Wilcoxon test, we observe that the average methylation of CpG_11.12, CpG_16.17 and CpG_24.25 were respectively 25.4%, 18.8%, 16.3%, which were significantly higher than the mean methylation rate (5%) in normal group CpG_11.12, P=0.000; CpG_16.17, P=0.002; CpG_24.25, P=0.001, **Figure 3**; **Table 3**).

Correlation of RASSF1A methylation and clinicopathological features and HPV16 Einfection

Intriguingly, there was no relationship between the overall methylation of RASSF1A and gender, age and differentiation (For gender, χ^2=1.478, P=0.475; For age: t=1.716, P=0.089; For differentiation: χ^2=0.770, P=0.680, **Table 4**). What's more, the association between the patterns of the quantitative methylation of RASSF1A promoter CpG units and the clinicopathological features of the 100 patients with ESCC was further evaluated (**Table 5**). However, no correlation was found between the RASSF1A CpG units methylation and age, gender, tumor differentiation and metastasis (For age: χ^2= 1.706, P=0.079; gender: χ^2=1.847, P=0.415; differentiation: χ^2=0.670, P=0.780; metastasis: χ^2=3.765, P=0.463, **Table 5**).

Table 4. Relationship between promoter methylation status of RASSF1A and clinicopathological features

	RASSF1A Methylation Status			χ^2	*P* values
	<2 (n)	3-4 (n)	>5 (n)		
Gender				1.487	0.475
Male	14	31	20		
Female	9	27	10		
Age				t=1.716	0.089
Differentiation				0.770	0.680
Well	8	13	3		
Low and Moderate	10	27	5		
HPV infection					
Positive	8	31	15	2.519	0.104
Negative	12	19	6		

Our previous study evaluated the frequency of HPV16 infection in Kazakh EC patients (41.1%) was prominently higher than in the control group (15.2%) [20]. Therefore, we explore the relationship between HPV16 infection and RASSF1A methylation in ESCC. The overall methylation level was increased in HPV positive ESCC samples, but it was not statistically significant (χ^2=2.519, P=0.104, **Table 4**). Moreover, no correlation was found between the average methylation of CpG_11.12, CpG_16.17 and CpG_24.25 and HPV16 infection of the Kazakh ESCC (χ^2=3.765, P=0.463, **Table 5**).

Discussion

To our best knowledge, it is the first report to reveal the interactions of RASSF1A promoter methylation and the esophageal squamous cell carcinoma in Kazakh population. We have used the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) approach to conduct a comprehensive analysis on the RASSF1A gene methylation of Kazakh ESCC. The MassARRAY system is an instrument that capacitates high-through put detection and quantitative analysis of methylation at one individual CpG unit of a target Using MASSarray mass spectrometer to analysis the methylation level of RASSF1A promoter and the correlative between RASSF1A methylation and esophageal squamous cell carcinoma in Kazakh Ethnic, have yet to see clear reports at home and abroad. In consideration the obvious impact of environmental factors on DNA methylation, the advantage of our study is the large sample size with similar lifestyle background. All the ESCC patients and control subjects enrolled in this study were from the same high incidence area and they are all Kazakh national.

Our results demonstrated that the overall RASSF1A methylation statusand the average methylation level of CpG_11.12, CpG_16.17 and CpG_24.25 units in Kazakh patients were significantly higher than that in normal controls. The hypermethylation of the RASSF1A promoter in ESCC tissues was reported from 15% to 68%, indicating that RASSF1A is likely to be involved in the genesis of ESCC, and plays an important role in the progression of tumorigenesis [15, 18, 19, 21-24]. We successfully replicated the results of the study conducted on [19] Chinese Han population and Japanese [16, 25] population by the traditional method (methylation-specific PCR), although the subjects in both studies had different genetic and environmental backgrounds. Consequence, with non-quantitative MSP method in Chinese Han and Japanese population and the quantitative MassARRAY way in Kazakh population, the uniformity of the methylation of the RASSF1A promoter in both researches strengthens the association between such methylation and esophageal squamous cell carcinoma. Previous studies mostly focus on the overall methylation level in ESCC patients [1, 10, 11, 15, 21, 26, 27], but our result indicating that the overall methylation level cannot represent the application value,only the accurate information of CpG units methylation levels represents the clinical application value that could be used in in early diagnosis. The methylation of CpG_11.12, CpG_16.17 and CpG_24.25 were significant correlation with Kazakh ESCC, this result was not found in previous study. Perhaps it was the most remarkable founding of the present study. This result suggests that aberrant promoter methylation plays an important role in Kazakh patients with esophageal cancer and therefore represent promising targets for anticancer therapies.

In this study, we investigated whether the genetic and epigenetic alterations of RASSF1A could be used in addition to a prognostic biomarker as clinicopathological features in ESCC. We have described the CpG unitmethylation

Table 5. Association between RASSF1A promoter methylation and clinicopathological features in ESCC patients

Patients Characteristics	RASSF1A Methylation Units			X^2	*P* value
	CpG11-12	CpG16-17	CpG24-25		
Gender				1.847	0.415
Male	54	59	70		
Female	46	41	30		
Age				1.706*	0.079
Differentiation				0.670	0.780
Well	58	53	59		
Low+Moderate	42	47	41		
Metastasis				3.765	0.463
Yes	23	32	28		
No	12	16	13		
HPV infection					
Positive	23	32	28	3.765	0.463
Negative	12	16	20		

Note: *Students' t test, others were chi-square test.

status of the promoter region of RASSF1A in esophageal squamous cell carcinomas and its relationship with clinicopathological factors. Statistical analysis showed no significant correlation of RASSF1A hypermethylation with age, gender, histological differentiation, and metastasis. Similar findings were found in some previous studiesthat were determined by MSP method in ESCC patients [22]. However, the investigation conducted by Zhou et al displayed that age and histological differentiation were associated with RASSF1A promoter methylation [19]. What's more, Kuroki et al study showed significant relationship betweenRASSF1A methylation status and the TNM stage in ESCC, but histological differentiationfailed to support the existence of such a relationship [21]. Generally speaking, the relationship between RASSF1A methylation and clinicopathological features is still not exact and further research is needed.

Human papillomavirus (HPV) is a nonenveloped double-stranded DNA virus, which contains more than 90 genotypes play a crucial role in ESCC (especially HPV16) [28, 29]. Our previous study and Ru-xing Xi et al study common certified a significantly high prevalence of HPV16 E6-E7 infection in a case-control study [20, 30]. Besides, a similar observation was also found in previous studies in Xinjiang Kazakhs [31-33]. Recently, some previous studies have proved that virus infection increased the RASSF1A methylation level in cancer samples [34, 35]. However, the association between RASSF1A promoter methylation and virus infection is rarely studied and the findings are not the same. Toyooka et al [34] found that the frequency of RASSF1A abnormal methylation and the methylation index were significantly higher in SV40 sequence positive malignant mesotheliomas than in negative samples. Kang et al [35] detected the RASSF1A methylation in gastric cancer with or without EBV infection by MSP method, the results showed a higher prevalence of RASSF1A promoter methylation in EBV positive gastric cancer than EBV negative gastric cancer. Nevertheless, Kuzmin et al [36] verified that no synergistic relationship between HPV infection and RASSF1A methylation in cervical squamous cell carcinoma. In this study, our results showed no relationship between RASSF1A promoter methylation and HPV16 infection in Kazakh ESCC patients. Therefore, the relationship between RASSF1A methylation and virus infection is still little and controversial, needs our further investigation.

Conclusion

Our resultis not only the first time demonstrates that RASSF1A CpG island hypermethylation contributes to esophageal squamous cell carcinoma in Kazakh population but also shows that some particular CpG units methyaltion (CpG_11.12, CpG_16.17 and CpG_24.25) are associated with the esophageal squamous cell carcinoma. Most importantly, RASSF1A methyaltion may provide a mechanistic and molecular basis for the new therapeutic use of pharmacological compounds with DNA demethylating activity to treat Kazakh patients with esophageal carcinoma. Furthermore, once again we proved the prevalence of HPV16E6 in ESCC patients is significantly higher than that in control, but no correlation is found between RASSF1A methylation and HPV16E6 infection. Certainly, further work should be done to state

the mechanism of RASSF1A methylation in the progression of ESCC, and ultimately develop RASSF1A gene as one of the molecular biomarkers for high-risk subject screening and early detection for ESCC in the future.

Acknowledgements

This work was supported by Grants from the Major science and technology projects of Shihezi University (No. gxjs2014-zdgg06), the National Natural Science Foundation of China (No. 81360358, 81460362, 81560399), the Applied Basic Research Projects of Xinjiang Production and Construction Corps (No. 2016AG020), the high-level talent project of Shihezi University (No. RCZX201533), and the Foundation for Distinguished Young Scholars of Shihezi University (No. 2015ZRKXJQ02). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Feng Li and Yun-Zhao Chen, Department of Pathology and Key Laboratory for Xinjiang Endemic and Ethnic Diseases, Shihezi University School of Medicine, North 4th Road, Shihezi 832002, China. Tel: +86-137-0993-1299; +86-135-7945-7678; Fax: +86 99 32057-136; E-mail: lifeng7855@126.com (FL); cyz0515@sina.com (YZC)

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001; 94: 153-156.

[3] Cui XB, Chen YZ, Pang XL, Liu W, Hu JM, Li SG, Yang L, Zhang WJ, Liu CX, Cao YW, Jiang JF, Gu WY, Pang J, Yang L, Yuan XL, Yu SY, Li F. Multiple polymorphisms within the PLCE1 are associated with esophageal cancer via promoting the gene expression in a Chinese Kazakh population. Gene 2013; 530: 315-322.

[4] Cui XB, Pang XL, Li S, Jin J, Hu JM, Yang L, Liu CX, Li L, Wen SJ, Liang WH, Chen YZ, Li F. Elevated expression patterns and tight correlation of the PLCE1 and NF-kappaB signaling in Kazakh patients with esophageal carcinoma. Med Oncol 2014; 31: 791.

[5] Lu JB, Yang WX, Liu JM, Li YS, Qin YM. Trends in morbidity and mortality for oesophageal cancer in Linxian County, 1959-1983. Int J Cancer 1985; 36: 643-645.

[6] Guohong Z, Min S, Duenmei W, Songnian H, Min L, Jinsong L, Hongbin L, Feng Z, Dongping T, Heling Y, Zhicai L, Shiyong L, Quansheng G, Xiaoyun L, Yuxia G. Genetic heterogeneity of oesophageal cancer in high-incidence areas of southern and northern China. PLoS One 2010; 5: e9668.

[7] Deaton AM, Bird A. CpG islands and the regulation of transcription. Genes Dev 2011; 25: 1010-1022.

[8] Larsen F, Gundersen G, Lopez R, Prydz H. CpG islands as gene markers in the human genome. Genomics 1992; 13: 1095-1107.

[9] Zhu J, He F, Hu S, Yu J. On the nature of human housekeeping genes. Trends Genet 2008; 24: 481-484.

[10] Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. Nat Rev Genet 2012; 13: 679-692.

[11] Agathanggelou A, Honorio S, Macartney DP, Martinez A, Dallol A, Rader J, Fullwood P, Chauhan A, Walker R, Shaw JA, Hosoe S, Lerman MI, Minna JD, Maher ER, Latif F. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene 2001; 20: 1509-1518.

[12] Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet 2000; 25: 315-319.

[13] Dreijerink K, Braga E, Kuzmin I, Geil L, Duh FM, Angeloni D, Zbar B, Lerman MI, Stanbridge EJ, Minna JD, Protopopov A, Li J, Kashuba V, Klein G, Zabarovsky ER. The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci U S A 2001; 98: 7504-7509.

[14] Li B, Wang B, Niu LJ, Jiang L, Qiu CC. Hypermethylation of multiple tumor-related genes associated with DNMT3b up-regulation served as a biomarker for early diagnosis of esophageal squamous cell carcinoma. Epigenetics 2011; 6: 307-316.

[15] Cong DG, Wang SF. [Hypermethylation of promoter region of RAS association domain family gene1A in esophageal squamous cell carcinoma and significance thereof]. Zhonghua Yi Xue Za Zhi 2007; 87: 2932-2934.

[16] Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M, Croce CM. Promoter hypermethylation of RASSF1A in esophageal squamous cell carcinoma. Clin Cancer Res 2003; 9: 1441-1445.

[17] Lo PH, Xie D, Chan KC, Xu FP, Kuzmin I, Lerman MI, Law S, Chua D, Sham J, Lung ML. Reduced expression of RASSF1A in esophageal and nasopharyngeal carcinomas significantly correlates with tumor stage. Cancer Lett 2007; 257: 199-205.

[18] Mao WM, Li P, Zheng QQ, Wang CC, Ge MH, Hu FJ, Fang XH, Dong ZM, Ling ZQ. Hypermethylation-modulated downregulation of RASSF1A expression is associated with the progression of esophageal cancer. Arch Med Res 2011; 42: 182-188.

[19] Zhou SL, Cui J, Fan ZM, Li XM, Li JL, Liu BC, Zhang DY, Liu HY, Zhao XK, Song X, Wang R, Yan ZC, Yi HX, Wang LD. Polymorphism of A133S and promoter hypermethylation in Ras association domain family 1A gene (RASSF1A) is associated with risk of esophageal and gastric cardia cancers in Chinese population from high incidence area in northern China. BMC Cancer 2013; 13: 259.

[20] Zou N, Yang L, Chen L, Li T, Jin T, Peng H, Zhang S, Wang D, Li R, Liu C, Jiang J, Wang L, Liang W, Hu J, Li S, Wu C, Cui X, Chen Y, Li F. Heterozygote of TAP1 Codon637 decreases susceptibility to HPV infection but increases susceptibility to esophageal cancer among the Kazakh populations. J Exp Clin Cancer Res 2015; 34: 70.

[21] Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M, Croce CM. Allele loss and promoter hypermethylation of VHL, RAR-beta, RASSF1A, and FHIT tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. Cancer Res 2003; 63: 3724-3728.

[22] Wong ML, Tao Q, Fu L, Wong KY, Qiu GH, Law FB, Tin PC, Cheung WL, Lee PY, Tang JC, Tsao GS, Lam KY, Law S, Wong J, Srivastava G. Aberrant promoter hypermethylation and silencing of the critical 3p21 tumour suppressor gene, RASSF1A, in Chinese oesophageal squamous cell carcinoma. Int J Oncol 2006; 28: 767-773.

[23] Guo Q, Wang HB, Li YH, Li HF, Li TT, Zhang WX, Xiang SS, Sun ZQ. Correlations of promoter methylation in WIF-1, RASSF1A, and CDH13 genes with the risk and prognosis of esophageal cancer. Med Sci Monit 2016; 22: 2816-2824.

[24] Du Z, Ma K, Sun X, Li A, Wang H, Zhang L, Lin F, Feng X, Song J. Methylation of RASSF1A gene promoter and the correlation with DNMT1 expression that may contribute to esophageal squamous cell carcinoma. World J Surg Oncol 2015; 13: 141.

[25] Yamaguchi S, Kato H, Miyazaki T, Sohda M, Kimura H, Ide M, Asao T, Kuwano H. RASSF1A gene promoter methylation in esophageal cancer specimens. Dis Esophagus 2005; 18: 253-256.

[26] Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol1987; 196: 261-282.

[27] Laird PW, Jackson-Grusby L, Fazeli A, Dickinson SL, Jung WE, Li E, Weinberg RA, Jaenisch R. Suppression of intestinal neoplasia by DNA hypomethylation. Cell 1995; 81: 197-205.

[28] Syrjanen KJ. HPV infections and oesophageal cancer. J Clin Pathol 2002; 55: 721-728.

[29] Kamangar F, Qiao YL, Schiller JT, Dawsey SM, Fears T, Sun XD, Abnet CC, Zhao P, Taylor PR, Mark SD. Human papillomavirus serology and the risk of esophageal and gastric cancers: results from a cohort in a high-risk region in China. Int J Cancer 2006; 119: 579-584.

[30] Xi R, Pan S, Chen X, Hui B, Zhang L, Fu S, Li X, Zhang X, Gong T, Guo J, Zhang X, Che S. HPV16 E6-E7 induces cancer stem-like cells phenotypes in esophageal squamous cell carcinoma through the activation of PI3K/Akt signaling pathway in vitro and in vivo. Oncotarget 2016; [Epub ahead of print].

[31] Hu J, Li L, Pang L, Chen Y, Yang L, Liu C, Zhao J, Chang B, Qi Y, Liang W, Li F. HLA-DRB1*1501 and HLA-DQB1*0301 alleles are positively associated with HPV16 infection-related Kazakh esophageal squamous cell carcinoma in Xinjiang China. Cancer Immunol Immunother 2012; 61: 2135-2141.

[32] Hu JM, Li L, Chen YZ, Pang LJ, Yang L, Liu CX, Zhao J, Chang B, Zou H, Qi Y, Liang WH, Li F. Human papillomavirus type 16 infection may be involved in esophageal squamous cell carcinoma carcinogenesis in Chinese Kazakh patients. Dis Esophagus 2013; 26: 703-707.

[33] Lu XM, Monnier-Benoit S, Mo LZ, Xu SY, Pretet JL, Liu Z, Vuitton DA, Mougin C. Human papillomavirus in esophageal squamous cell carcinoma of the high-risk Kazakh ethnic group in Xinjiang, China. Eur J Surg Oncol 2008; 34: 765-770.

[34] Toyooka S, Pass HI, Shivapurkar N, Fukuyama Y, Maruyama R, Toyooka KO, Gilcrease M, Farinas A, Minna JD, Gazdar AF. Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. Cancer Res 2001; 61: 5727-5730.

[35] Kang GH, Lee HJ, Hwang KS, Lee S, Kim JH, Kim JS. Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. Am J Pathol 2003; 163: 1551-1556.

[36] Kuzmin I, Liu L, Dammann R, Geil L, Stanbridge EJ, Wilczynski SP, Lerman MI, Pfeifer GP. Inactivation of RAS association domain family 1A gene in cervical carcinomas and the role of human papillomavirus infection. Cancer Res 2003; 63: 1888-1893.