Original Article

Association of roasting meat intake with the risk of esophageal squamous cell carcinoma of Kazakh Chinese via affecting promoter methylation of *p16* gene

Weigang Chen MD[1], Chunmei Yang MD[1], Lan Yang MD[2,3], Cuihua Qi MD[1], Shuxin Tian MD[1], Yusheng Han MD[4], Yuqin Dou MD[5], Yungui Ma MD[6], Dean Tian MD[7,8], Yong Zheng MD[1]

[1]*Department of Gastroenterology, First Affiliated Hospital of the Medical College, Shihezi University, Xinjiang, China*
[2]*Department of Pathology, the Medical College of Shihezi University, Xinjiang, China*
[3]*The Local and National Key Laboratory of the Diseases of High Incidence in Xinjiang, Xinjiang, China*
[4]*Friendship Hospital of Yili State, Xinjiang, China*
[5]*The Hospital of No.4 Agricultural Division, the Xinjiang Production and Construction Corps, Xinjiang, China*
[6]*Hospital of Xinyuan County, Xinjiang, China*
[7]*Department of Gastroenterology, Tongji Hospital, Hubei, China*
[8]*Tongji Medical College, Huazhong University of Science and Technology, Hubei, China*

Background: Esophageal squamous cell carcinoma (ESCC) incidence is high in Kazak Autonomous Prefecture, Xinjiang, China. Roasting food has been reported to be related with the risk of various cancers and is very popular in the area, and may be related with the risk of ESCC. The promoter methylation inactivation of *p16* gene can increase the risk of ESCC. Thus, we want to know whether long-term roasting food is related with the risk of ESCC by effecting the promoter methylation of *p16* gene. Materials and Methods: Ninety ESCC patients and 60 healthy subjects were recruited from Kazak Autonomous Prefecture. MassARRAY was used to detect *p16* promoter methylation in ESCC tissues, as well as in normal esophageal tissues. The association between the *p16* promoter methylation and daily roasting meat intake was examined. Results: Daily roasting meat intake was related with the risk of ESCC (p<0.01) and the mean CpG methylation rates of *p16* promoter (p<0.01). In ESCC patients, the mean methylation rates of CpG 11-12 and CpG 33-34-35 were 29.4% and 37.4%, respectively, which was significantly higher than the rates in normal esophageal tissues (16.7% and 12.4%, respectively; p<0.01). The methylation of *p16* promoter is also related with daily roasting meat intake (p<0.01) in Kazakh Chinese with ESCC. For the CpG methylation of the *p16* promoter in the well, moderately and poorly differentiated ESCC, there are significant differences (p<0.05) for the 19 CpG units in the ESCC and controls. Conclusion: Roasting meat intake was associated with the risk of ESCC via effects on the methylation of *p16* promoter. These results suggest roasting food intake should be limited in the diet.

Key Words: esophageal squamous cell carcinoma, *p16* promoter methylation, Kazakh Chinese, MassARRAY, roasting meat

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a common malignancy and occurs with a high incidence among the Han Chinese in Linxian in Henan Province, and Kazakh Chinese in Xinjiang. Epidemiological studies reveal that the incidence of ESCC in north Xinjiang is the highest in the Xinjiang autonomous region. In the Yili Kazak Autonomous Prefecture, the incidence of ESCC is about 90/100,000 in Xinyuan County and in Tuoli County it is 155.9/100,000,[1,2] which is higher than the standardized mortality attributed to ESCC in China, and higher than that in Linxian Henan province (59.6/100,000).[3] Epidemiological studies show that numerous factors contribute to the high incidence of ESCC, including shorter dinner-to-bed time, hot beverage and high-temperature cooking, and a special living environment.[3-6]

ESCC is a highly invasive malignancy and severely threatens human health.[7] Investigators are focusing their

Corresponding Author: Dr Zheng Yong, Department of Gastroenterology, First Affiliated Hospital of the Medical College, Shihezi University, Shihezi, Xinjiang, 832002, China.
Tel: 0086-993-2858573; Fax: 0086-993-2858874
Email: zhengyongxj@163.com
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studies on identifying the biomarkers associated with ESCC. Apart from the frequent involvement of *p53* in ESCC among Kazakh Chinese in Xinjiang province,[8] multiple tumour suppressor genes, which become inactivated, are located on human chromosome.[9] It has been reported that *p16*, *p15* and *p14* in 9p21 all can lead to the silencing of target genes. The *p16* gene is an important target gene and is frequently observed in various cancers.[9-11]

In addition, there is evidence showing that aberrant *p16* promoter methylation is very common in ESCC,[11,12] and *p16* promoter methylation may serve as a valuable biomarker for early diagnosis.[13] However, little is known about the reason that causes *p16* promoter methylation and its relationship with the incidence of ESCC among Kazakh Chinese in the Xinjiang autonomous region. Deep-fried and burnt food and preserved (high salt) food are reported to be associated with increase in gastric cancer incidence.[14] Roasting meats are very popular among Kazakh Chinese in the Xinjiang autonomous region. Thus, we wanted to explore whether daily roasting meat intake was related with the risk of ESCC by affecting the methylation of the *p16* promoter. From the results, we hoped to provide evidence for early prevention of ESCC by controlling daily roasting meat intake.

METHODS

Participants

The study was approved by the Ethics Committee (IRB approval number: XJYY1328) of First Affiliated Hospital of the Medical College, Shihezi University, and informed consents were obtained from all participants. From March 10th, 2010 to March 16th, 2012, a total of 90 ESCC patients were recruited by the Clinical Research Centre at Friendship Hospital of Yili State (Xinjiang, China). At the same period, 60 healthy subjects were recruited. The ESCC subjects and healthy subjects were determined by the professional doctors. Daily roasting meat intake was documented according to self-description. Each person was well trained for cooperating with the test. The diagnostic requirements were included in the ESCC group. The diagnostic criteria for ESCC were used according to previous report.[15] Furthermore, all ESCC adults were reported to be diagnosed with ESCC within one year and not any other diseases, or cardiovascular disease, or inflammation disease. To exclude the interference of many other factors, all subjects were non-tobacco smokers, non-alcohol drinkers. All the subjects were with the similar economic and cultural background. All the other characters of the selected subjects were listed in Table 1. The body mass index (BMI) and daily calorie intake did not show the statistically difference between healthy subjects and ESCC patients (p>0.05). For all healthy subjects, were free of the ESCC symptoms.

We present here the cases from 50-year-old to 64-year-old males with histories of toasting meat intakes for the last 10 years. The reference recall period for diet was set at 10 years before diagnosis for ESCC cases and interview for controls. All participants reported food consumption frequency was at 3 times/day. The food consumption was adhered to a healthy Nordic food index as previously reported.[16] Calorie intake was assessed according to a previous report.[17] Information on daily dietary habits was solicited: (1) the main roasted food included kebob (roasted above a special iron oven about 1 meter long and 20 centimetres wide for 10 mins at from 200°C to 300°C), whole lamb (roasted in a fan assisted oven with a temperature of 200°C for 35 mins per kg) and baked meat (baked in a special oven, for 20 mins at 200°C); (2) vegetables and fruits; (3) grains; (4) varieties of olive oil, nuts and cocoa; (5) other meats, fish and eggs; (6) sweets and cake. Analytical studies showed normal arterial blood gases, immunoglobulins and alpha-1-antitryp-sin.

Endoscopic submucosal dissection (ESD) is accepted as a minimally invasive treatment for ESCC.[18] All patients with ESCC that was presumed to be confined to the superficial layers were referred to ESCC centre from First Affiliated Hospital of the Medical College, Shihezi University (Shihezi, Xinjiang, China) to undergo an endoscopic submucosal dissection (ESD). All patients were treated by esophagectomy and receive neither chemotherapy nor radiotherapy before surgery. All patients underwent that: Computed Tomography (CT) Scan for the neck, chest, and abdomen; chromoendoscopy after direct instillation of 1.5% iodine solution. ESD was usually performed according to a previous report.[19] The cases of ESCC were determined according to the Health Organization criteria (TNM system).[20] No injury was found after all surgical operation. For all healthy controls, they had normal endoscopic esophageal dissection and were free of the ESCC symptoms.

Sample collection

A total of 150 esophageal mucosa tissue specimens were collected from 90 patients with ESCC and 60 healthy adults. The tissues were collected by endoscopy and were stored in liquid nitrogen. Patients did not receive prior chemotherapy, radiotherapy, or surgery. ESCC was confirmed pathologically as squamous-cell carcinoma. Informed consent was obtained before the study, and complete pathological and clinical data on the patients were available.

Extraction of tissue DNA and its quality detection

The QIAamp DNA Mini Kit (QIAGEN Ltd, Valencia, USA) was used for DNA extraction. In brief, 50 μL of DNA solution were collected, and β-actin PCR amplification was used for the detection of DNA's existence. Following amplification, the negative samples were removed. DNA quality was determined using a spectrophotometer. The qualified samples were defined as having a DNA concentration >75 ng/μL, absence of degradation in the bands, and A260/A280 ratio of 1.7 to 2.1. The DNA samples were stored at -20°C until further use.

Sulfite treatment and p16 promoter methylation detection

Bisulfite PCR sequencing was performed with EpiTect Bisulfite Kit (Beijing Boao Biotech Co., Ltd, Beijing, China). The sequences of primers included 40 CpG sites. The primers are *p16* F 5'- aggaagagagGTTAGGAGGAG GTTTGTGATTAT-3' and *p16* R 5'- cagtaatacgactcactatagggagaaggctTCCCCTTACCTAAAAA AATACC-3'.

A MassCLEAVE Kit was used for alkaline phosphatase treatment, and a Spectro CHIP® Arrays and Clean Resin Kit (Tiangen Company, Beijing, China) was used for purification. A MassARRAY Nanodispenser RS1000 spotting (Sequenom, San Diego, CA, USA) was used to add samples into a SpectroCHIP microarray containing 384 spots. The microarray was loaded onto a MassARRAY Compact System (Sequenom, Inc., San Diego, CA, USA) for detection. The SpectroCHIP microarray assay was carried out using the MALDI-TOF (Shimadzu Biotech, Kyoto, Japan) and data were processed using EpiTYPER software (Sequenom, San Diego, CA, USA).

Statistical analysis

Differences between two groups were compared by one-way analysis of variance (ANOVA). We used Spearman's rank correlation coefficient to identify the association between daily roasting meat intake and the risk of ESCC. Meanwhile, Spearman's rank correlation coefficient was used to identify the association between daily roasting meat intake and the probabilities of *p16* promoter methylation. Spearman’s rank correlation was also used for eosinophils and lymphocytes counts. Data were analyzed using Statview 5.0 software (Abacus Systems, Berkeley CA, USA) with a *p* value of <0.05 accepted as significant.

RESULTS

p16 promoter methylation in ESCC and normal esophageal tissues

In this case-controlled study a total of 90 patients with ESCC and 60 healthy subjects were recruited from the same county. The amplicon in each sample encompasses 40 CpG sites, which can form 22 CpG units followed by T cleavage. Thus, each CpG unit has several CpG sites. Among these units, 19 were used for analysis, and CpG_2, CpG_26.27.28.29, and CpG_36.37 were undetectable. We studied 1,615 number of CpG units, and 1,534 CpG units could be analyzed (95%).

In the promoter region (DNA sequence up to 500 bp upstream of the transcription start site), the CpGs units are hypermethylated in ESCC. For confirmation of the sensitivity of our strategy, we analysed only specific CpGs that are generally observed to be aberrantly methylated in ESCC, and this may limit our methylation profile data since methylation patterns of CpGs have been observed to be heterogeneous in their occurrence. For example, CpG sites methylated in one patient need not be the same as in another one. A true methylation profile could be generated by MassArray techniques. A 500 bp region of the *p16* promoter containing 39 CpG sites, 31 of which could be examined by MassARRAY system. Among these units, only 8 CpG sites did not yield successful measurements. One hundred and fifty samples had good results for >90% of the samples. All the results show that MassARRY is more specific and sensitive than the traditional methylation-specific PCR.[21]

Our results showed that there were significant differences ($p<0.01$) in the mean methylation rates for CpG units in ESCC tissues (16.8%) and normal tissues (11.7%). Heatmap analysis was also performed to analyze the rate of CpG unit methylation, and this result is shown in Figure 1.

The methylation of 19 CpG units of the p16 promoter in ESCC and control group

The ANOVA test was employed to compare the methylation of the *p16* promoter between ESCC and normal tissues. The results showed that the methylation rates of CpG unit 11-12 (29.4%) and CpG unit 33-34-35 (37.4%) in ESCC tissues were significantly higher than rates the

Table 1. The characteristics of the patients with ESCC and healthy subjects

	Healthy	ESCC patients	*p* value
Male cases	33	55	
Age (y)	55.5±5.8	57.1±6.9	>0.05
BMI (kg/m^2)	25.7±6.1	26.8±6.7	>0.05
Daily calorie intake (kcal)	2228±366	2269±402	>0.05
Other meat and fish (g/d)	143±55	94±36	<0.05
Vegetables (g/d)	397±98	389±94	>0.05
Fruits (g/d)	213±176	201±154	>0.05
Grains (g/d)	103±53	110±44	>0.05
Oil, coca (g/d)	30±27	32±29	>0.05
Eggs (g/d)	15±10	16±11	>0.05
Sweets and cake (g/d)	40±31	43±34	>0.05
Roasting meat intake (g/d)	71±60	150±31	<0.01
Female cases	27	35	
Age (y)	57.9±6.1	58.1±5.6	>0.05
BMI (kg/m^2)	25.1±5.8	26.1±5.6	>0.05
Daily calorie intake (kcal)	1955±293	1907±309	>0.05
Other meat and fish (g/d)	130±50	101±31	<0.01
Vegetables (g/d)	354±74	361±80	>0.05
Fruits (g/d)	207±151	219±144	>0.05
Grains (g/d)	97±43	93±34	>0.05
Oil, coca (g/d)	26±19	23±17	>0.05
Eggs (g/d)	12±9	11±8	>0.05
Sweets and cake (g/d)	36±21	33±24	>0.05
Roasting meat intake (g/d)	69±52	142±41	<0.01

Data are shown as mean±SD.

corresponding tissues from the control group (16.7% and 12.4%, respectively) ($p<0.01$, Table 2).

Parametric and non-parametric statistical methods were used for comparing multiple groups. It can be seen from the average methylation rate histogram (Figure 2), there are total of 19 CpG units in the ESCC group and control group. In CpG3, CpG4 and CpG23.24.25, the average methylation rate of the control group is higher than that from the ESCC group, the other CpG units methylation rate in ESCC group are higher than that from control group. Among these data, the statistics of CpG_11.12 and CpG_33.34.35 are significant ($p<0.05$) (Table 2).

The methylation status of p16 promoter among the different degrees of ESCC differentiation

For the CpG methylation of the *p16* promoter in the well-differentiated, moderately differentiated and poorly differentiated ESCC, there are significant differences

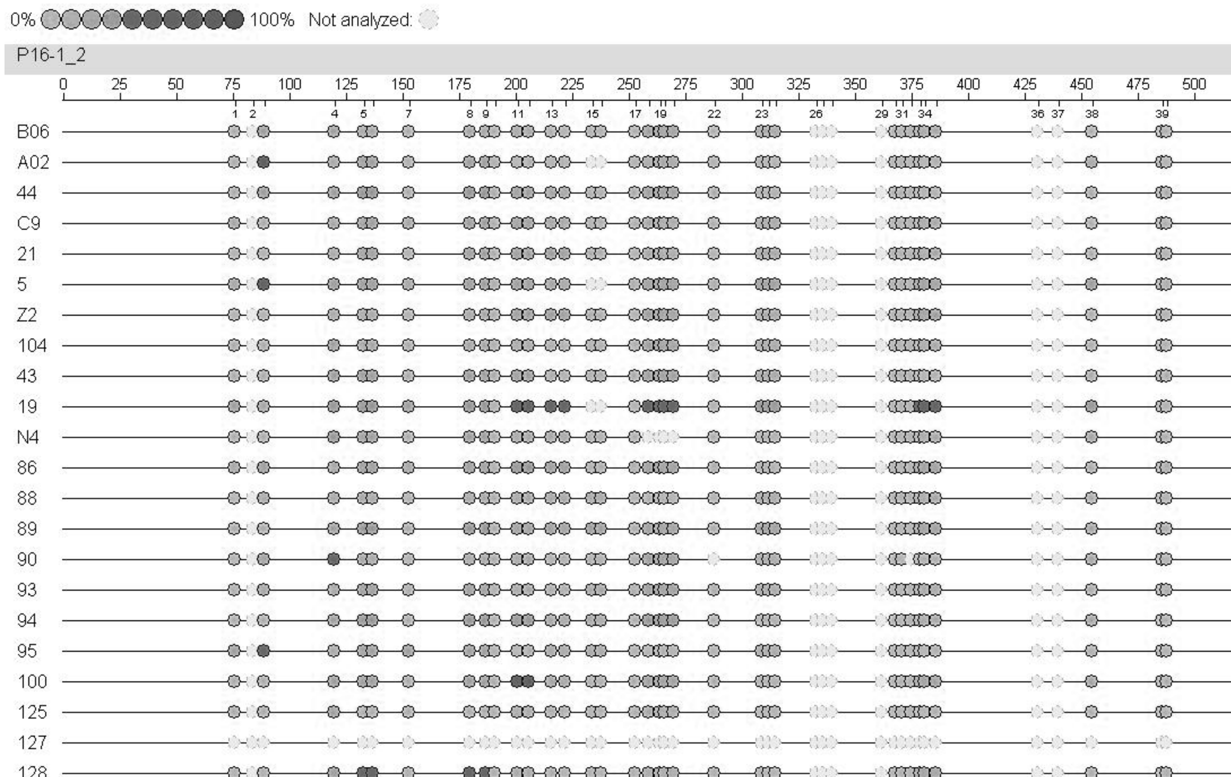

Figure 1. Heatmap analysis of CpG unit methymation of *p16* in ESCC and control group. Note: dark: 100%; medium: 0%; light: not analyzed. Each row represents the CpG unit of amplicon and each line represents the CpG unit in one sample.

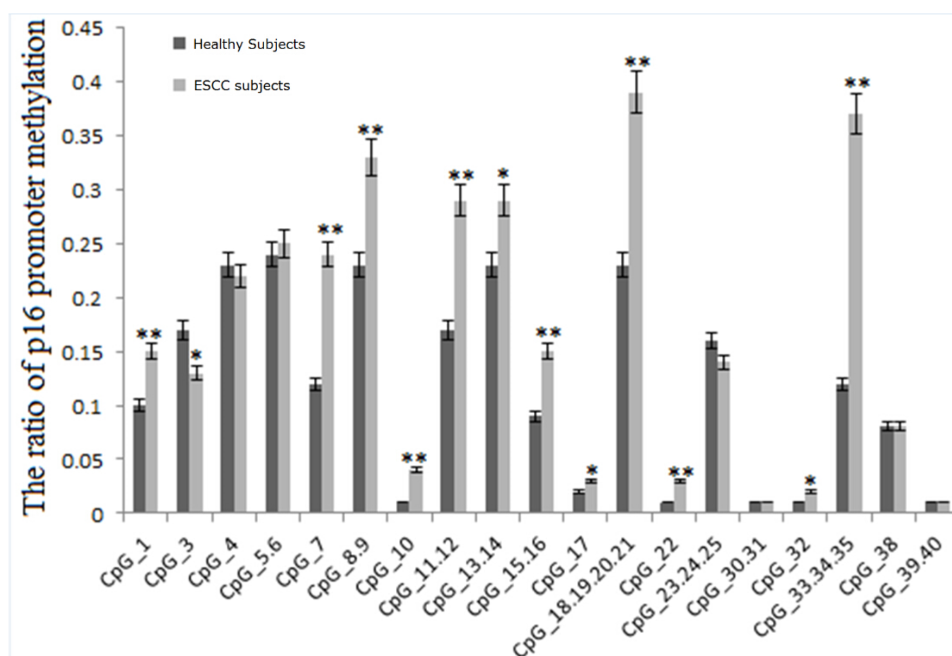

Figure 2. The average methylation of *p16* promoter CpG units in ESCC and normal group. There are total of 19 CpG units in the ESCC group and control group. Results are presented as means±SD. *$p<0.05$ and **$p<0.01$ via the controls.

($p<0.05$) for the 19 CpG units in the ESCC group and control group (Table 3). The results suggest that the different degrees of ESCC differentiation affect the methylation status of *p16* promoter. The mechanism is still not clear and requires further investigation. The ESCC tumours were well differentiated in 130 cases for *p16* promoter methylation (26%), moderately differentiated in 244 (49%), and poorly differentiated in 125 (25%). The carcinomas were staged according to the Tumour-Node-Metastasis classification.[22]

The methylation status of p16 promoter between both genders in ESCC

The results from Table 4 suggested the methylation levels of *p16* promoter were different between male and female. Among these data, the statistics of CpG_1, CpG_3, CpG_5.6, CpG_10, CpG13.14, CpG17 and CpG_22 were significant ($p<0.05$) (Table 4). The gender specific methylation of *p16* promoter implies the gender specific disease may be caused by the different levels of DNA methylation.

Daily roasting meat intake positively related with risk of the ESCC or the probabilities of p16 methylation

As Figure 3A showed, daily roasting meat intake was low in the healthy subjects, high in the ESCC patients and in between in the subjects with *p16* promoter methylation ($p<0.01$) (Figure 3A). The further investigated showed that daily roasting meat intake was positively related with the percent of *p16* promoter methylation in ESCC

Table 2. CpG methylation of the *p16* promoter in ESCC and normal tissues

No	Gene fragment	ESCC		Normal tissues		Wilcoxon W	p
		n	∑x/n±s	n	∑x/n±s		
1	CpG_1	26	0.10±0.10	53	0.15±0.06	1039	0.042*
2	CpG_3	27	0.13±0.16	55	0.17±0.20	995	0.045*
3	CpG_4	28	0.23±0.05	56	0.24±0.08	2369	0.917
4	CpG_5.6	55	0.25±0.10	27	0.24±0.09	2201	0.421
5	CpG_7	26	0.12±0.09	53	0.10±0.06	1039	0.992
6	CpG_8.9	27	0.33±0.10	55	0.22±0.09	2201	0.031*
7	CpG_10	27	0.04±0.05	55	0.01±0.04	2254	0.022*
8	CpG_11.12	27	0.29±0.11	54	0.17±0.16	2012	0.013*
9	CpG_13.14	26	0.29±0.10	54	0.22±0.14	2135	0.046*
10	CpG_15.16	22	0.15±0.05	50	0.09±0.08	1758	0.045*
11	CpG_17	27	0.02±0.05	55	0.03±0.04	2254	0.047*
12	CpG_18.19.20.21	25	0.39±0.12	50	0.23±0.08	1861	0.026*
13	CpG_22	27	0.02±0.10	54	0.01±0.04	2187	0.049*
14	CpG_23.24.25	23	0.16±0.07	44	0.14±0.08	775	0.043*
15	CpG_30.31	28	0.01±0.02	56	0.01±0.01	1126	0.524
16	CpG_32	27	0.02±0.10	54	0.01±0.04	2187	0.059
17	CpG_33.34.35	26	0.37±0.10	53	0.12±0.06	1911	0.009*
18	CpG_38	26	0.08±0.08	53	0.08±0.07	1021	0.842
19	CpG_39.40	28	0.01±0.02	55	0.01±0.01	1104	0.467

n represents the analysis of CpG number of units, said average methylation rate, s represents the standard deviation. * Indicates $p<0.05$.

Table 3. CpG methylation of the *p16* promoter in the three degrees of tumour differentiation

	Well-differentiated		Moderately differentiated		Poorly differentiated		p
	n	∑x/n±s	n	∑x/n±s	n	∑x/n±s	
CpG_1	7	0.17±0.22	13	0.14±0.12	6	0.11±0.50	0.037*
CpG_3	7	0.13±0.22	13	0.14±0.16	7	0.12±0.06	0.664
CpG_4	7	0.23±0.06	13	0.24±0.05	7	0.23±0.03	0.898
CpG_5.6	7	0.26±0.06	13	0.25±0.12	7	0.19±0.03	0.070
CpG_7	7	0.28±0.07	13	0.24±0.12	6	0.19±0.05	0.027*
CpG_8.9	7	0.36±0.06	13	0.25±0.12	7	0.19±0.04	0.017*
CpG_10	7	0.08±0.02	13	0.03±0.06	7	0.01±0.01	0.007*
CpG_11.12	7	0.39±0.15	13	0.22±0.09	7	0.13±0.08	0.010*
CpG_13.14	7	0.32±0.15	13	0.23±0.07	6	0.08±0.07	0.010*
CpG_15.16	5	0.23±0.04	11	0.08±0.06	6	0.05±0.03	0.028*
CpG_17	7	0.22±0.02	13	0.03±0.06	7	0.01±0.01	0.006*
CpG_18.19.20.21	7	0.33±0.10	13	0.26±0.15	5	0.17±0.03	0.014*
CpG_22	7	0.05±0.02	13	0.04±0.14	7	0.00±0.00	0.037*
CpG_23.24.25	6	0.17±0.09	12	0.13±0.06	5	0.08±0.03	0.041*
CpG_30.31	7	0.01±0.02	13	0.02±0.04	8	0.01±0.01	0.555
CpG_32	7	0.01±0.02	13	0.02±0.14	7	0.00±0.00	0.274
CpG_33.34.35	7	0.39±0.16	13	0.26±0.06	6	0.16±0.06	0.020*
CpG_38	7	0.09±0.11	13	0.09±0.09	6	0.07±0.03	0.940
CpG_39.40	7	0.01±0.02	13	0.02±0.04	8	0.01±0.01	0.555

n represents the analysis of CpG number of units, said average methylation rate, s represents the standard deviation. * Indicates $p<0.05$. ESCC is classified into Well-, Moderately- and Poorly-differentiated stages.

Table 4. CpG methylation of the *p16* promoter between genders in ESCC

Gene fragment	Male		Female		Wilcoxon W	p
	n	∑x/n±s	n	∑x/n±s		
CpG_1	14	0.18±0.12	12	0.13±0.05	161	0.008*
CpG_3	15	0.10±0.14	12	0.14±0.18	195	0.007*
CpG_4	15	0.25±0.05	13	0.22±0.04	154	0.118
CpG_5.6	15	0.25±0.12	12	0.19±0.04	167	0.028*
CpG_7	14	0.25±0.12	12	0.24±0.05	161	0.180
CpG_8.9	15	0.35±0.12	12	0.31±0.04	198	0.181
CpG_10	15	0.02±0.01	12	0.05±0.07	203	0.015*
CpG_11.12	15	0.30±0.13	12	0.27±0.09	207	0.505
CpG_13.14	15	0.32±0.12	11	0.19±0.07	195	0.021*
CpG_15.16	12	0.16±0.05	10	0.14±0.04	100	0.346
CpG_17	15	0.01±0.01	12	0.03±0.07	203	0.005*
CpG_18.19.20.21	14	0.39±0.15	11	0.38±0.04	130	0.721
CpG_22	15	0.00±0.01	12	0.05±0.01	191	0.003*
CpG_23.24.25	12	0.14±0.07	11	0.13±0.06	121	0.755
CpG_30.31	15	0.01±0.01	13	0.01±0.00	207	0.501
CpG_32	15	0.02±0.01	12	0.01±0.01	191	0.373
CpG_33.34.35	14	0.38±0.11	12	0.17±0.07	187	0.014*
CpG_38	14	0.10±0.11	12	0.07±0.06	155	0.742
CpG_39.40	15	0.01±0.01	13	0.01±0.01	207	0.650

Note: n represents the analysis of CpG number of units, said average methylation rate, s represents the standard deviation. * Indicates $p<0.05$.

($p<0.01$) (Figure 3B). For healthy subjects, daily roasting meat intake was not significantly related with the percent of *p16* promoter methylation in ESCC ($p>0.05$) (Figure 3C).

DISCUSSION

ESCC is a common malignancy in Kazakh Chinese from the Xinjiang autonomous region with a high incidence of ESCC. Although great progress has been achieved in the surgical intervention, chemotherapy, and radiotherapy of ESCC, the survival rate of ESCC patients is still lower than 10% after 5 years.[23] To date, the molecular mechanisms and pathogenesis of ESCC are still poorly understood.[24] In recent years, numerous studies have focused on epigenetic changes resulting from gene methylation. The *p16* gene is a newly identified tumour suppressor gene and *p16* promoter methylation has been a hot topic in tumour research.[25-27] The *p16* gene product can compete with cyclin D1 to bind to CDK4/6, which then inhibits the protein kinase activity of the cyclinD1/CDK4/6 complex. This blocks the transition from the G1 phase to the S phase and results in suppression of cell proliferation.[28] Promoter methylation of the *p16* gene is a chemical process, catalyzed by DNA methyltransferase, in which a methyl group is added chemically to the 5th carbon in the cytosine of CpG Island to form 5-methylcytosine.

We previously studied the product of the *p16* gene. Our results showed that *p16* expression reduces the malignancy and inhibits development of ESCC. Reduced *p16* expression may be attributed to the dysfunction of the *p16* gene. Related studies have revealed that gene methylation is a main cause of reduced *p16* expression. The methylation of *p16* usually occurs in the promoter. Therefore, the promoter has been a predominant site for the design of primers and detection of the methylation of the *p16* gene. To date, no studies have been conducted to investigate methylation of the *p16* gene among Kazakh Chinese in the Xinjiang autonomous region. In the present study we identified ESCC-associated *p16* gene methylation of CpG sites in tissues from Kazakh Chinese patients with ESCC and healthy control subjects.

MassARRAY was employed to detect the 22 CpG units in the promoter of the *p16* gene. Units CpG_2, CpG_26.27.28.29, and CpG_36.37 were undetectable, but 19 CpG units were detected. Our results showed that the CpG methylation rate in ESCC tissues was significantly higher than the rate in control tissues, suggesting hypermethylation of the *p16* gene may be related to the development of ESCC in this population. We found significant differences in the mean CpG methylation rates in ESCC and normal esophageal ($p<0.05$). In ESCC patients, the mean methylation rates of CpG 11-12 and CpG 33-34-35 were 19.4% and 17.4%, respectively, which was markedly higher than rates in normal esophageal tissues (16.7% and 12.4%, respectively; $p<0.05$). In the well-differentiated, moderately differentiated and poorly differentiated in ESCC there were no significant differences ($p>0.05$) in the 19 CpG units. There were also no significant differences in gender in ESCC ($p>0.05$).

However, Salam et al found that the methylation rate of *p16* was 72% in ESCC tissues in which the methylation-specific PCR method was used.[29] In addition, the methylation of the *p16* gene is often undetectable in some normal esophageal tissues. First, our results revealed a low, but detectable *p16* promoter methylation rate in normal esophageal tissues, which may be attributed to the association between a low rate of methylation and cell proliferation and apoptosis. Second, our findings revealed that the methylation rate of *p16* in ESCC tissues was lower than previously reported, which may be related to the different methods for the detection of DNA methylation.

On the other hand, methylation-specific PCR has been introduced for the detection of *p16* methylation. This method is sensitive and has wide application. However, the primers have limited CpG sites and can only be ap-

plied for qualitative measurement. In addition, the modification is incomplete and false positives are also observed. When compared with previous methods of detecting DNA methylation, MassARRAY offers high-throughput, rapid, and simple method. In addition, this method can be used to detect multiple CpG sites simultaneously.[30] When combined with EpiTYPER software, this allows the investigator to directly calculate the methylation rates at different methylation sites. Thus, MassARRAY is a quantitative method for the detection of *p16* promoter methylation.

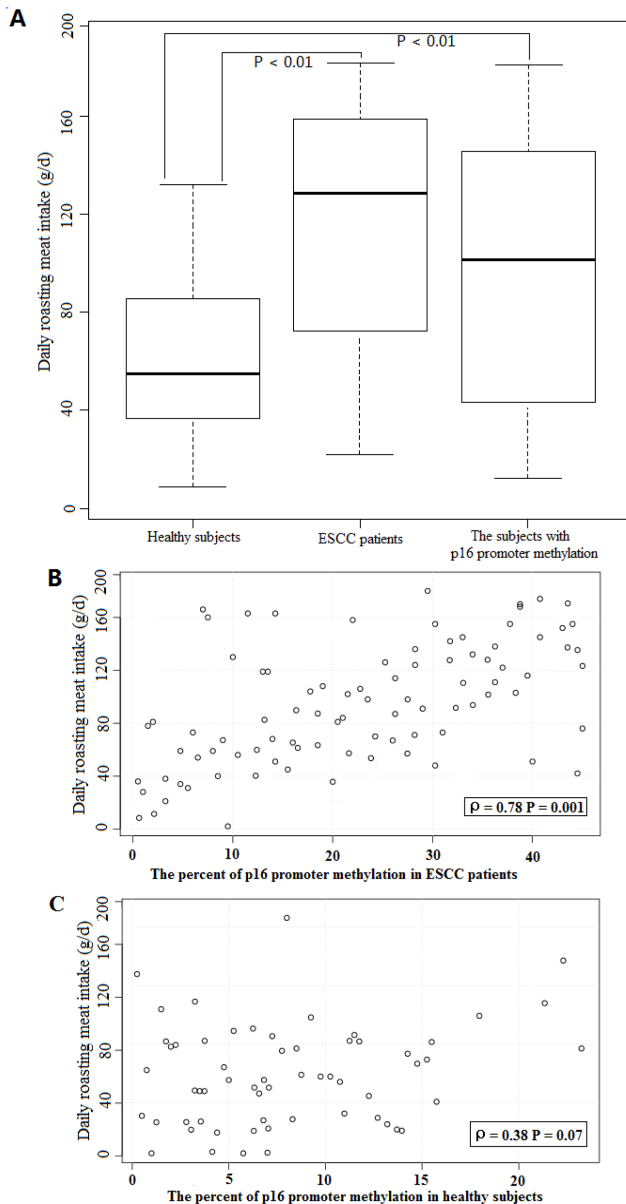

Figure 3. Daily roasting meat intake is positively related with the probabilities of *p16* promoter methylation. A, daily roasting meat intake in healthy subjects (n=60), ESCC patients (n=90) and the subjects (n=69) with *p16* promoter methylation. The bars in the boxes were average activities and the boxes represented 95% of the samples. The error bars were above or below the boxes. B, daily roasting meat intake was positively related with the probabilities of *p16* promoter methylation in ESCC subjects. C, daily roasting meat intake was not related with the probabilities of *p16* promoter methylation in healthy subjects. Statistical analysis was done by Spearman's rank correlation test. The value of rho falls between 0.5 and 1, there is a strong positive correlation. The value falls between 0 and 0.5, there is a weak positive correlation.

There is evidence to suggest that burning food or cooking food at high temperatures, can lead to the formation of minute quantities of potent carcinogens. There have been reports from the Food Standards Agency that have found the known animal carcinogen acrylamide is generated in carbohydrate foods that are fried or burnt.[31] The studies to discover any potential risk to humans are still underway at the FDA and European regulatory agencies.[31] People in Xinjiang enjoy roasting lamb chops or fish as a tradition. The lifestyle practice may increase the risk of ESCC.

Taken together, the methylation rate of the *p16* promoter in healthy Kazakh Chinese may be caused by the long-time roasting food intake. In the present study, MassARRAY is employed to detect the methylation of the *p16* promoter. The results showed that the hypermethylation of the *p16* promoter at CpG 11-12 and CpG 33-34-35 may be an early event in ESCC in Kazakh Chinese in the Xinjiang autonomous region.[11] Thus, *p16* promoter methylation may represent molecular biomarkers for the early diagnosis of ESCC. Although DNA methylation may result in aberrant gene expression, the DNA sequence and gene product remain unchanged, and the process is reversible. There is evidence showing that inhibitors of DNA methyltransferase can reactivate some genes, resulting in significant inhibition of cancer growth.[32] Therefore, the findings on *p16* promoter methylation may indicate that reversing methylation could be a viable strategy for treating ESCC in Kazakh Chinese. In addition, the roles of *p16* promoter methylation in the prognosis, genetic diagnosis, and gene therapy should be further studied.

The meat cooked at high temperature can increase the risk of various cancers. For example, high-temperature cooked meat contains heterocyclic amines, including 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP), and polycyclic aromatic hydrocarbons, such as benzo(a) pyrene. A high intake of PhIP can induce prostate tumours.[33] Another example, intake of fried meats can induce the risk of lung adenocarcinoma.[34] However, the association between ESCC and roasting food has never been reported. Here, we found the intake of roasting meats was related with the risk of ESCC. Dietary pattern and roasting meat intake should be controlled well and roasting food will be reduced. Furthermore, we found the association between two variables may be caused by affecting the methylation of *p16*. Actually, the expression of *p16* is affected by PhIP,[35] which is often produced in high-temperature cooked meat. Thus, the more clearly molecular mechanism for the association between the roasting meats intake and the risk of ESS can be explored in future.

There are still some limitations for the study. The large population are needed to be investigated for confirming our conclusion. We only investigated the persons in Kazakh Chinese and most Han Chinese was not studied, so we still did not know whether the same rule could be used for other Chinese. Mostly important, the roasting food can produce carcinogen, which was not considered here. Thus, the more precise molecular mechanisms need further work.

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AUTHOR DISCLOSURES

The authors declare no conflict of interest.

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Original Article

Association of roasting meat intake with the risk of esophageal squamous cell carcinoma of Kazakh Chinese via affecting promoter methylation of *p16* gene

Weigang Chen MD[1], Chunmei Yang MD[1], Lan Yang MD[2,3], Cuihua Qi MD[1], Shuxin Tian MD[1], Yusheng Han MD[4], Yuqin Dou MD[5], Yungui Ma MD[6], Dean Tian MD[7,8], Yong Zheng MD[1]

[1]*Department of Gastroenterology, First Affiliated Hospital of the Medical College, Shihezi University, Xinjiang, China*
[2]*Department of Pathology, the Medical College of Shihezi University, Xinjiang, China*
[3]*The Local and National Key Laboratory of the Diseases of High Incidence in Xinjiang, Xinjiang, China*
[4]*Friendship Hospital of Yili State, Xinjiang, China*
[5]*The Hospital of No.4 Agricultural Division, the Xinjiang Production and Construction Corps, Xinjiang, China*
[6]*Hospital of Xinyuan County, Xinjiang, China*
[7]*Department of Gastroenterology, Tongji Hospital, Hubei, China*
[8]*Tongji Medical College, Huazhong University of Science and Technology, Hubei, China*

烤肉摄入通过影响 *p16* 基因启动子甲基化增加中国哈萨克族人患食管鳞状细胞癌的风险

背景: 食管鳞状细胞癌（ESCC）在中国新疆维吾尔自治区伊犁哈萨克自治州有较高的发病率。据报道吃烧烤食物容易患癌，而烧烤食物在该地区非常流行或许增加患 ESCC 的风险。*p16* 基因的启动子甲基化会使该基因失活增加患 ESCC 的风险。因此，我们想知道长期食用烧烤食物是否会影响 *p16* 基因的启动子甲基化，进而增加患 ESCC 的风险。材料和方法：从哈萨克自治州召集 90 名 ESCC 患者和 60 名健康者，用 MassARRAY 技术检测 ESCC 组织和正常食管组织的 *p16* 基因启动子甲基化程度。同时调查了 *p16* 基因启动子甲基化与每日食用烤肉的关系。结果: 每日食用烧烤食物会增加患 ESCC 风险（$p<0.01$）和 *p16* 基因启动子甲基化程度（$p<0.01$）。在 ESCC 患者中, *p16* 基因启动子中 CpG 11-12 和 CpG 33-34-35 甲基化率分别是 29.4%和 37.4%, 高于正常组织中的 16.7%和 12.4%（$p<0.01$）。在哈萨克自治州的 ESCC 患者中, *p16* 基因启动子甲基化率与日常烧烤食物食用相关（$p<0.01$）。在健康人，良性、中性和恶性 ESCC 中，*p16* 基因启动子 19 CpG 甲基化也不同（$p<0.05$）。结论: 烤肉摄入可能影响 *p16* 基因启动子甲基化程度，从而增加患 ESCC 的风险。本结果警示在日常的饮食中应该限制食用烧烤食物。

关键词：食管鳞状细胞癌、*p16* 基因启动子的甲基化、中国哈萨克族、MassARRAY、烤肉