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Original Article

Tissue riboflavin levels and C20orf54 protein expression are regulated by C20orf54 hypermethylation in Kazak's esophageal cancer

Alimujiang Kelimu[1,3*], Juret Anwer[1*], Yanchao Deng[1], Ayshamgul Hasim[2], Desheng Li[1], Liwei Zhang[1], Ilyar Sheyhidin[1]

[1]Department of Thoracic Surgery, First Teaching Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China; [2]Department of Pathology, College of Basic Medicine of Xinjiang Medical University, Urumqi, Xinjiang, China; [3]Department of Neurosurgery, Shanghai East Hospital Affiliated to Tongji University, Shanghai, China. [*]Co-first authors.

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Abstract: C20orf54 is a riboflavin transporter gene that involved in the intestinal absorption of riboflavin, which is required for normal cellular functions in all aerobic forms of life. The posttranscriptional regulation of C20orf54 expression and its association with epigenetic modifications in cancer development remain unclear. We aimed to study C20orf54 hypermethylation and its regulation on C20orf54 protein expression in Kazak's esophageal squamous cell carcinoma (ESCC) patients. The methylation sequences of C20orf54 promoter regions were mapped in the esophageal cancer cell line, ECa109. MassARRAY analysis was used to quantitatively detect methylated DNA from Kazak's ESCC and normal adjacent tissues (NAT). C20orf54 protein expression and tissue riboflavin levels were assessed via immunohistochemistry and enzyme-linked immunosorbent assay, respectively. We identified 7 CpG sites that were methylated from 12 CpG sites of C20orf54 in ECa109 cells. The quantitative analysis of single CpG site methylation showed 5 CpG sites significant differences in methylation levels between ESCC and NAT. C20orf54 methylation was significantly higher in ESCC tissues (0.4758±0.0199) than in NAT (0.3574±0.0183). Riboflavin was also significantly decreased in ESCC tissues (17.32±3.54 μg/L) than in normal esophageal epithelia (22.12±4.01 μg/L). Increasing the methylation levels of C20orf54 promoter regions tended to decrease C20orf54 protein expression. An inverse association between tissue riboflavin levels and the methylation level of C20orf54 in ESCC tissues was also found. This suggests that C20orf54 may play a protective role in Kazak's ESCC by modulating riboflavin absorption.

Keywords: Esophageal squamous cell carcinoma, riboflavin transporter gene (C20orf54), DNA methylation, tissue riboflavin levels, bisulfite-sequencing PCR, MassARRAY

Introduction

Esophageal cancer (EC) is one of the most common malignancies in the world. Esophageal squamous cell carcinoma (ESCC) is the most frequent type of EC in Asia, with more than half of its cases occurring in China [1]. Recently, the Kazak population in Xinjiang has reported the highest incidence of ESCC in China, with age-standardized rates of 90-150/100,000 [2]. Epidemiological and etiological studies revealed that environmental and genetic factors have important roles in esophageal carcinogenesis [3-5]. These environmental factors include excessive smoking, excessive drinking, and nutritional deficiencies [6]. Because only a fraction of individuals who are exposed to these risk factors actually develop ESCC, the role played by genetic determinants in response to environmental exposures needs to be addressed.

Riboflavin is an essential component of the diet and circulates in the plasma after intake. It is intracellularly phosphorylated to form flavin mononucleotide, which is further metabolized to generate flavin adenine dinucleotide [7]. During metabolism, riboflavin functions as a cofactor for enzymes that are involved in oxidation-reduction reactions and plays a role in the

oxidative folding of proteins in the endoplasmic reticulum [8, 9]. Recent studies have indicated that the depletion of riboflavin in adult humans impairs the proliferative potential of intestinal cells, which may have implications for gastrointestinal function [10]. Epidemiology studies have suggested that riboflavin deficiency may play an important role in the etiology of esophageal cancer in high-incidence areas [11]. Although riboflavin is synthesized by plants and many microorganisms, it is not synthesized by animals [12]. The supplementation of diets with riboflavin has been shown to have an individual-specific effect on esophageal cancer, suggesting that an intra-individual mechanism is contributing to these results [13]. Our previous genome-wide association studies showed that genetic variations at 20p13 for the riboflavin transporter gene (C20orf54) significantly contribute to the risk of ESCC in Chinese Han and Uygur-Kazak populations [14]. We have confirmed that C20orf54 protein expression is significantly down-regulated in Kazak's ESCC tissues than in precancerous tissues [15]. Furthermore, the level of riboflavin is lower in Kazak's ESCC patients than in the normal population. This suggested that the down-regulation of C20orf54 protein expression is closely related to the lesion process of ESCC.

Epigenetic changes in DNA are common events in human cancer, and aberrant DNA methylation is an important mechanism for gene transcription and protein expression silencing. In the present study, we investigated whether epigenetic modifications can silence C20orf54 to indirectly modulate C20orf54 protein expression in Kazak's ESCC patients. We also examined whether methylation levels are associated with tissue riboflavin levels.

Materials and methods

Clinical characteristics and tissue samples

Thirty Kazak's ESCC patients were enrolled in the study. Sixty fresh and formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from the patients, and normal adjacent tissues (NAT) located in the esophageal mucosa and 5 cm away from the tumor were also collected from each patient as controls. All patients were treated at the Department of Thoracic Surgery of the First Affiliated Hospital in the Medical University of Xinjiang from March 2010 to June 2011. Informed written consent was obtained from all patients, and the study was approved by the Ethical Committee of the Medical University of Xinjiang. None of patients had received preoperative radiotherapy or chemotherapy. The mean age was 53.5 years; the youngest patient was 42 years old, and the oldest patient was 73 years old at the time of surgery. Each specimen was histologically examined, and tumors were graded by at least two experienced pathologists. The main characteristics of ESCC patients, including tumor grade, stage, and lymph node status of the tumor, were categorized according to the TNM (American Joint Committee on Cancer, 4th edition): (1) 10 cases as clinical stage I; (2) 12 cases as stage II; and (3) 8 cases as stage III. Of the 30 tumors, 12 were well differentiated, 7 were moderately differentiated, and 11 were poorly differentiated. Eighteen patients had lymph node metastases. Sixty biopsied tissue specimens were also collected within 30 min after resection and stored at -80°C for tissue riboflavin assays.

Cell lines

ECa109 cells were obtained from Wuhan University (Wuhan, China) and grown in RPMI 1640 medium (Invitrogen, USA), which was supplemented with 5% fetal bovine serum and penicillin/streptomycin, in a 5% CO_2 humidified incubator at 37°C. Cells were seeded in 6-well plates until they reached 60% confluence, and then transfections were performed.

DNA extraction, bisulfite treatment, and bisulfite-sequencing PCR (BSP)

DNA was extracted from ECa109 cells using a DNA extraction kit (QIAGEN, Valencia, CA, USA). Using the EZ Methylation Gold Kit (Zymo Research, Orange, CA, USA) and following the manufacturer's instructions, genomic DNA (500 ng) was modified with bisulfite. CpG island fragment-specific primers were designed by scanning gene promoter regions using the specialized Methyl Primer Express software (ABI Company), based on genetic information that was obtained from the Genbank database. Bisulfite-treated DNA from ECa109 cells was amplified by PCR. The following primers were used for subsequent amplifications: C20orf54 forward, AGGAGTGGTTTTTTTTAGAGAGGAA, and C20orf54 reverse, AACTCACCAATAATATA-

TACACCTTTT. Complete bisulfite modification was confirmed by sequence analysis. BSP amplifications were performed in 50 μl reaction mixtures containing 2 μl bisulfite-modified genomic DNA, 2 μl dNTPs, 1.2 μl primers, 2 μl $MgCl_2$, 20 nM ammonium sulfate, 75 nM Tris-HCl (pH 8.3), and 3 U Taq DNA polymerase. The touch-down PCR scheme was applied with the following cycling conditions: denaturation at 95°C for 15 min, 95°C for 20 s, annealing temperatures ranging from 62 to 56°C for 1 min, extension at 72°C for 1 min (45 cycles), and a final incubation at 72°C for 7 min. Annealing temperatures were as follows: 60°C. PCR products were then cloned into vectors and sequenced to identify CpG sites that were related to gene promoter methylation.

Quantitative DNA methylation analysis

For the quantitative detection of methylated DNA, MassARRAY (Sequenom, San Diego, CA, USA) was used to analyze esophageal tissue DNA for CpG content. Specific primer pairs against target genes were used to compare methylation levels of target fragments and CpG sites among different samples, according to the manufacturer's instructions and as described previously. The analyzed regions and CpG sites of candidate gene promoters were aggaagagagAGGAGTGGTTTTTTTTAGAGAGGAA (C20orf54 forward) and cagtaatacgactcactatagggagaaggctAAACTCACCAATAATATATACACCTTTT (C20orf54 reverse). The primers were designed according to the Sequenom Standard EpiPanel (Sequenom, November 2007 version). PCR amplification was performed with a mixture containing 10 ng bisulfite-treated DNA, 200 mM dNTPs, 0.2 U Hot Start Taq DNA polymerase (QIAGEN), and 0.2 mM primers in a total volume of 5 μl. PCR cycles included a hot start at 94°C for 15 min, followed by denaturation at 94°C for 20 s, annealing at 56°C for 30 s, extension at 72°C for 1 min (45 cycles), and a final incubation at 72°C for 3 min. Unincorporated dNTPs were dephosphorylated by adding 2 ml of premix, including 0.3 U shrimp alkaline phosphate (SAP; Sequenom). The reaction mixture was incubated at 37°C for 40 min, and SAP was then heat inactivated for 5 min at 85°C. After SAP treatment, 2 ml of the PCR product was used as a template for in vitro transcription, and RNase A cleavage was used for the reverse reaction, according to the manufacturer's instructions (Sequenom). The samples were conditioned and spotted on a 384-pad Spectro-CHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Irvine, CA, USA), followed by spectral acquisition on a MassARRAY analyzer compact MALDI-TOF mass spectrometer (Sequenom). The methylation analyses were carried out using the EpiTYPER application (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites.

Immunohistochemical studies

To detect C20orf54 protein expression in vivo, immunolocalization experiments were carried out on sections from representative blocks of 60 pairs of FFPE ESCC tissues and NAT. Tissue sections were deparaffinized and hydrated with phosphate-buffered saline (PBS, pH 7.4). Then, antigen retrieval was conducted by microwaving on high mode in Tris/EDTA buffer (pH 9.0) for 15 min. Sections were then pretreated with 3% hydrogen peroxide for 15 min to remove endogenous peroxidase. Subsequently, the slides were pretreated with 1% bovine serum albumin in PBS (pH 7.4) for 10 min, preincubated with a protein blocking solution for 15 min, and incubated with the anti-C20orf54 antibody (Abcam, Cambridge, USA; dilution 1:300) in a humid chamber at 4 μl overnight. Slides were then washed three times in PBS and incubated with a secondary biotinylated antibody for 15 min at room temperature. After 15 min, the sections were washed with PBS and treated with peroxidase-conjugated streptavidin for 15 min. Finally, the sections were stained lightly with hematoxylin, and PBS was used as a negative control. Immunostained slides were blindly evaluated by two pathologists under a light microscope. Protein expression was scored for each antibody separately and semi-quantitatively by assessing the stain intensity and the percentage of stained cells in the tumors. Staining intensity was scored as 0 (no staining), 1+ (weak), 2++ (medium), or 3+++ (strong). The percentage of stained cells was categorized as follows: 1, 0-10% stained cells; 2, 11-50% stained cells; 3, 50% stained cells or greater. The final score was obtained by multiplying the two scores. The sum of both scores was used to identify four categories of expression: strong expression (5-6), medium expression (3-4),

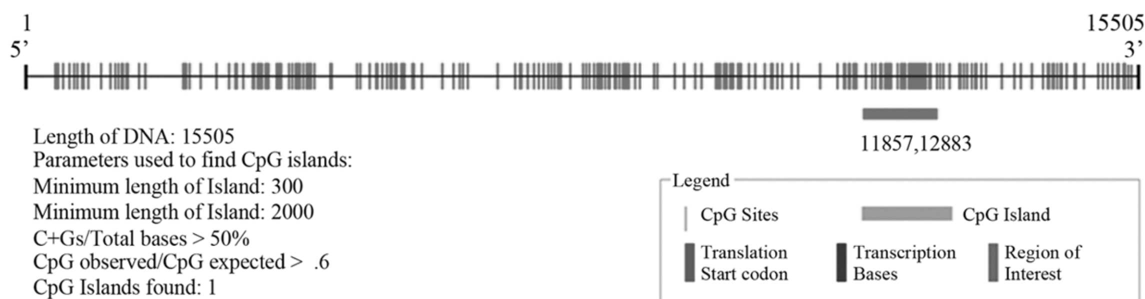

Figure 1. The CpG sites analyzed in promoters of C20orf54 gene. Each vertical indicate an individual CpG site. Solid line positions of CpG Island and the first solid line are the positions of BSP primers. All the BSP primers are designed to cover the transcriptional start site should be close to transcriptional start site.

Table 1. Quantitative analysis of c20orf54 genes single CpG site methylation by Sequenom MassARRAY

CpG site	Methylation levels ($\overline{x}$±s)		t	P
	Tumor tissues	Normal adjacent tissues		
c20orf54_CpG_1	0.2273±0.0139	0.1936±0.0268	0.965	0.345
c20orf54_CpG_2	0.1563±0.0079	0.1086±0.0088	3.763	0.001
c20orf54_CpG_3	0.1395±0.0108	0.0959±0.0087	3.441	0.002
c20orf54_CpG_4	0.5187±0.0275	0.4177±0.02741	2.257	0.035
c20orf54_CpG_5	NA	NA		
c20orf54_CpG_6	NA	NA		
c20orf54_CpG_7	0.5600±0.0279	0.4468±0.0285	2.617	0.016
c20orf54_CpG_8	0.6255±0.02781	0.5373±0.0297	1.916	0.069
c20orf54_CpG_9	0.6400±0.0271	0.5664±0.0262	1.906	0.070
c20orf54_CpG_10	0.6393±0.0287	0.5595±0.0259	2.237	0.036
c20orf54_CpG_11	NA	NA		
c20orf54_CpG_12	0.5600±0.0279	0.4466±0.02856	1.482	0.159

Table 2. Quantitative analysis of c20orf54 genes whole target CpG site methylation by Sequenom Mass ARRAY

	Methylation levels ($\overline{x}$±s)	t	P
Tumor tissues	0.4758±0.0199	9.293	0
Normal adjacent tissues	0.3574±0.0183		

weak expression (1-2), and total loss of expression (0).

Determination of tissue riboflavin levels

The enzyme-linked immunosorbent assay is a convenient method for measuring riboflavin levels in tissues [16]. Fresh tissues (100 mg) were collected from ESCC patients and weighed on an electronic balance. After weighing, tissues were added to l ml of 10 mM PBS and then homogenized. The homogenate was centrifuged for 20 min at 2,000 rpm, and the supernatant (200 μl) was removed for analysis. The optical density of the supernatant was measured at 450 nm within 15 min. A standard curve was used to determine the concentration of the sample.

Statistical analysis

All statistical analyses were performed with the SPSS Version 17 software package. All *P* values were two-sided, and the significance level was *P*<0.05. Fisher's exact test was used to evaluate associations with clinical pathological parameters. Quantitative DNA methylation data that were derived from MassARRAY were treated as continuous variables, and missing measurements were imputed into multivariable regression analyses using samples with replacements for non-missing values (single imputations). Linear associations between two continuous variables were quantified by the Pearson correlation coefficient.

Results

Methylation profiles of C20orf54 in Eca109 cells

Because of the theoretical relationship between gene promoter hypermethylation and the down-

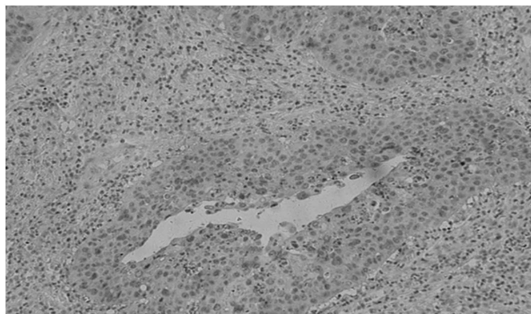

Figure 2. Expression of C20orf54 in ESCC tissues and normal adjacent tissues by IHC. The expression of C20orf54 protein was significantly lower in ESCC samples than in normal adjacent tissues.

Table 3. Inverse correlation between C20orf54 methylation and protein expression in ESCC

Protein expression	Methylation levels ($\overline{X}$±s)	F	P
-	0.4273±0.0139	7.69	0.041
+	0.2963±0.0079		
++	0.2195±0.0108		
+++	0.1187±0.0275		

regulation of gene transcription, we analyzed C20orf54, which is down-regulated in esophageal cancer. Computational analysis of the upstream region of C20orf54 revealed one CpG-rich island that was located between positions 11857 to 12883 relative to the transcriptional start site (**Figure 1**). By amplifying target CpG islands using genomic DNA as a template, followed by cloning and sequencing, we identified various extents of CpG site methylation at the promoter regions of C20orf54. The sequencing results show that C20orf54 contained 12 CpG sites, and 7 CpG sites were methylated.

C20orf54 DNA methylation in esophageal cancer from Kazak patients

The quantitative analysis of methylated DNA by the Sequenom MassARRAY platform is based on the detection of the methylation state of a single CpG site at a target fragment (CpG island). This analysis generates data representing the ratio or frequency of methylation events on a CpG site in the DNA of all samples. The results of single CpG site methylation show that the methylation level 5 CpG sites (CpG_2, CpG_3, CpG_4, CpG_7 and CpG_10) from CpG_1, CpG_2, CpG_3, CpG_4, CpG_5, CpG_6, CpG_7, CpG_8, CpG_9, CpG_10, CpG_12 significant differences between ESCC and NAT (**Table 1**). The total methylation level of target fragments of C20orf54 was statistically higher (0.4758±0.0199) in ESCC tissues than in NAT (0.3574±0.0183, $P<0.05$) (**Table 2**).

C20orf54 protein expression in ESCC tissues and its association with DNA methylation

The protein expression of C20orf54 was determined in ESCC tissues and NAT by immunohistochemistry. C20orf54 protein expression was significantly lower in ESCC samples than in NAT ($P=0.036$) (**Figure 2**). Furthermore, the relationship between C20orf54 expression and DNA methylation in ESCC tissues was analyzed. Alterations in the CpG island methylation of C20orf54 were associated with changes in C20orf54 protein expression (**Table 3**).

Riboflavin levels and their association with DNA methylation in ESCC tissues

The average riboflavin level in ESCC tissues was 17.32±3.54 μg/L, and the average level in matched NAT was 22.12±4.01 μg/L. There was an inverse association between tissue riboflavin levels and the methylation level of C20orf54 in ESCC (F=6.59, $P=0.044$).

Discussion

The purpose of this study was to elucidate whether epigenetic modifications can influence C20orf54 expression in esophageal cancer cells of Kazak's ESCC patients. We investigated the association of esophageal carcinogenesis with the aberrant regulation of C20orf54 expression at different levels, including gene promoter methylation and protein expression. The results show that C20orf54 protein expression tended to decrease as the methylation level of C20orf54 promoter regions increased. This indicates that C20orf54 promoter methylation contributes to the decrease in C20orf54 protein expression in Kazak's ESCC.

When analyzing ESCC specimens as solid tumor tissues, molecular analyses may be affected by tissue heterogeneity, due to the presence of necrotic areas and non-tumor cells, such as tumor-infiltrating leukocytes,

endothelial cells, and fibroblasts [17]. Furthermore, the heterogeneity of surgical tumor specimens can influence sequencing. To overcome the problem of tissue heterogeneity, it is necessary to have a viable and more homogeneous cell population that can retain the phenotypic and genomic profile of original tissues. Ideally, the cytological composition should be as homogeneous as possible to allow for the aberrant methylation of target CpG fragments at promoter regions to be identified. To attain this goal, we first used BSP to map the methylation status of CpG sites in C20orf54 promoters in an ESCC cell line (ECa109) and determine the target sequences of candidate gene methylation. Then, we quantitatively verified the methylation levels in tissue specimens from ESCC patients who had not received clinical treatment. MassARRAY analysis was used to examine the methylation status of every single CpG site in the promoter region of C20orf54. Because the target fragments are cleaved by enzymes prior to mass spectrometric detection, the methylation level (ratio) of a certain CpG site is independent from other CpG sites that are located at the same target fragment. Thus, to evaluate the methylation state of the whole-target fragment, the data provided by mass spectrometric detection must be further analyzed for the association of different CpG sites at the methylation level. Our results demonstrate that although the methylation level of whole-target CpG fragments of C20orf54 was significantly different between Kazak's ESCC tissues and corresponding non-cancerous esophageal tissues, hypermethylation was observed on certain CpG islands and sites. This suggests that there are multiple CpG sites. In addition, the methylation of some of these sites may not lead to the down-regulation of gene expression or loss of expression. Instead, the methylation of specific sites has been shown to influence genetic transcription [18]. Therefore, only the methylation level of individual CpG sites, and not the overall methylation level, has clinical value. Previous studies have reported that the methylation of CpG near the transcriptional start site is inversely correlated with mucin 1 gene (MUC1) expression [19]. Furthermore, MUC4 expression is regulated by the methylation of CpG sites near the transcriptional start site [20].

Our findings show that C20orf54 methylation played an important role in down-regulating protein expression and decreasing tissue riboflavin levels. We found an inverse association between tissue riboflavin levels and C20orf54 hypermethylation in ESCC tissues. Although the current study was limited by the small number of samples, our results confirmed the down-regulation of C20orf54 in ESCC tissues and suggest that aberrant promoter methylation is an important mechanism in the deregulation of C20orf54.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ilyar Sheyhidin, Department of Thoracic Surgery, First Teaching Hospital of Xinjiang Medical University, 1 Carp Mountain Road, Urumqi 830054, Xinjiang, China. Tel: +86 991 4366286; Fax: +86 991 4327997; E-mail: ilyarsha@163.com

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