RESEARCH ARTICLE

Epigenetic Regulation of Human Riboflavin Transporter 2(hRFT2) in Cervical Cancers from Uighur Women

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Abstract

In the present study, we studied the hypermethylation of the human riboflavin transporter 2 (hRFT2) gene and regulation of protein expression in biopsies from resected tissues from Uighur cervical squamous cell carcinoma (CSCC) patients and their neighboring normal tissues. hRFT2 gene promoter region methylation sequences were mapped in cervical cancer cell line SiHa by bisulfite-sequencing PCR and quantitative detection of methylated DNA from 30 pairs of Uighur's CSCCs and adjacent normal tissues by MassARRAY (Sequenom, San Diego, CA, USA) and hRFT2 protein expression was analyzed by immunohistochemistry. In SiHa, we identified 2 CG sites methylated from all of 12CpG sites of the hRFT2 gene. Analysis of the data from quantitative analysis of single CpG site methylation by Sequenom MassARRAY platform showed that the methylation level between two CpG sites (CpG 2 and CpG 3) from CpG 1~12 showed significant differences between CSCC and neighboring normal tissues. However, the methylation level of whole target CpG fragments demonstrated no significant variation between CSCC (0.476±0.020) and neighboring normal tissues (0.401±0.019, $p>0.05$). There was a tendency for translocation the hRFT2 proteins from cytoplasm/membrane to nucleus in CSCC with increase in methylation of CpG 2 and CpG 3 in hRFT2gene promoter regions, which may relate to the genesis of CSCC. Our results suggested that epigenetic modifications are responsible for aberrant expression of the hRFT2 gene, and may help to understand mechanisms of cervical carcinogenesis.

Keywords: Cervical squamous cell carcinoma - riboflavin transporter gene - DNA methylation

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Introduction

Cervical cancer (CC) is one of the most common cancers in women worldwide, which as a remarkable geographic distribution and differences in ethnic prevalence tumor has serious threat to women health in certain regions around the world (Binh et al., 2004; IARC, 1995). The prevalence and mortality of cervical squamous cell carcinoma (CSCC) in Uyghur ethnic group has been very high and beyond the average level in China, known as a high incident cancer of Xinjiang (Lalai et al., 2006). Epidemiological and etiological studies revealed that environmental and genetic factors have important roles in cervical carcinogenesis.The environmental factors include persisted infection of Human papillomavirus (HPV), and nutritional deficiencies (Brock et al., 1988; Zur Hausen, 2002). However, since only a fraction of individuals exposed to HPV infection actually develop CSCC, the role played by genetic determinants in response to environmental exposures needs to be addressed.

Some epidemiologic studies have reported a relationship between cervical cancer and diets low in riboflavin (Hernandez et al., 2003), and animal studies have shown that riboflavin deficiency can lead to disruption of the esophagus epithelium, in a similar manner to precancerous lesions in human's cervical cancer (Murphy et al., 2010). Riboflavin also participates in various metabolic redox reactions, and is involved in one carbon metabolism, which is a network of interrelated biochemical pathways that generate one carbon groups needed for physiologic processes (De Souza et al., 2006). Disruption of one carbon metabolism can interfere with DNA replication, DNA repair, and regulation of gene expression through methylation, each of which could promote carcinogenesis (Kim, 2004). A recent study showed that riboflavin transporter 2 (hRFT2) was found to transport riboflavin that is highly expressed in the small intestine and may be involved in such riboflavin absorption (Yonezawa et al., 2008), and it is also reported that hRFT2 is a transporter involved in the epithelial uptake of riboflavin in the small intestine for its nutritional utilization (Fujimura et al., 2010). Decreased protein expression of hRFT2 is related to malignant progression both esophageal squamous cell carcinoma and gastric cancer (Pangrekar et al., 1993), due to the hRFT2 is responsible for transporting riboflavin and riboflavin deficiency has been reported as a risk factor for cancer. Relatively speaking that alter expression of hRFT2 is the most important factors contributing to transporting and absorption of riboflavin in the intestinal (Maynur et al., 2012), whereas the Riboflavin deficiency can cause

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a variety of metabolic problems that lead to skin and mucosal disorders (Julaiti et al., 2012).

Epigenetic changes in DNA are now known as common events in human cancer. The aberrant DNA methylation is important mechanism for gene transcription and protein expression silencing. The posttranscriptional regulation of hRFT2 expression may associate with epigenetic modifications in cancer development which was not described so far. In the present study, we hypothesized whether epigenetic modifications modulate hRFT2 expression indirectly through silencing hRFT2 genes in Uyghur CSCC patients. To address this problem, we evaluated the aberrant methylation of CpG islands at gene promoter regions. CpG island fragment specific primers were designed by scanning gene promoter region using specialized software based on genetic information obtained from the Genbank database online, and bisulphate treated DNA of cervical cancer (SiHa) cell and to amplified with PCR followed by cloning into vector and sequencing to identify CpG sites related to gene promoter methylation, then quantitative detection of gene methylation in cervical tissue DNA by MassARRAY approach.

Materials and Methods

Clinical characteristics and tissue samples

A total of 60 fresh and formalin-fixed, paraffin-embedded (FFPE) tissues specimens were collected from Uyghur women with CSCC and matched nor¬mal mucous epithelia collected 5 cm away from the tumor. All patients were treated at the department of Gynecology of the First Affiliated Hospital in the Medical University of Xinjiang from July 2010 and May 2012. Of the 30 CSCC patients (none of whom had received pre-operative radiotherapy or chemotherapy). Each specimen was histologically examined, and the tumor was graded by at least two experienced pathologists. The main characteristics of CSCC patients, including tumor grade, stage, and lymph node status of the tumor, were categorized according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Frozen biopsies tissue specimens consisting of 30 cases CSCC and 30 cases matched normal mucous epithelia (5 cm away from the tumor) were collected within 30 min after resection and kept at -80ºC until used to determine gene promoter methylation. Written informed consent was obtained from all patients and controls participating in this study, and the study were approved by the ethics committee of first affiliated hospital of Xinjiang medical university.

Of patients with CSCC enrolled in this study, were 9 FIGO stage Ib, 11 FIGO stage IIa and 10 FIGO stage IIIb, Among them, 11 cases were pathologically characterized as well-differentiated, 10 moderately differentiated and 9 poorly differentiated tumors. Lymph node metastasis was documented for 21tumor patients. The mean age of cervical cancer women was 55.7 with extreme ages at 39 and 67.

Cell lines

The human cervical carcinoma cell line SiHa was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in RPMI 1640 media (Invitrogen) supplemented with 5% fetal bovine serum and penicillin/streptomycin in a 5% CO_2 humidified incubator at 37°C. Before transfection, SiHa cells were seeded in 6-well plates, the cells transfection were performed when the cells grew up at 60% confluence.

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

DNA was extracted from (SiHa) cells using a DNA extraction kit (QIAGEN, Valencia, CA, USA), and genomic DNA (500 ng) was bisulfite-modified using the EZ Methylation Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. CpG island fragment specific primers were designed by scanning gene promoter regions using specialized Methyl Primer Express software (ABI company) based on genetic information obtained from the Genbank database. Bisulfite treated DNA from cervical carcinoma cell line was PCR amplified. Primers used for subsequent amplifications are listed in Supplemental Table 1. 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 of Taq DNA polymerase. The touch-down PCR scheme was applied to amplify with the following cycling conditions: 95°C denaturation for 15 min, 95°C for 20 sec, annealing temperatures ranging from 62°C to 56°C for 1 min, extension at 72°C for 1 min for 45 cycles, and final incubation at 72°C for 7 min. Annealing temperatures were as follows: hRFT2: 60°C, PCR was followed by cloning into vectors and sequencing to identify CpG sites related to gene promoter methylation.

Quantitative DNA methylation analysis

For quantitative detection of methylated DNA, we used MassARRAY (Sequenom, San Diego, CA, USA) to analyze the cervical tissue DNA for CpG content. Target gene specific primer pairs 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 are shown in Supplemental Table 2. The primers used were designed according to Sequenom Standard EpiPanel (Sequenom, November 2007 version and Supplemental Table 3). PCR amplification was performed with the following parameters: the PCR mixture contained 10 ng bisulfite-treated DNA, 200 mM dNTPs, 0.2 U of Hot Start Taq DNA polymerase (QIAGEN), and 0.2 mM forward and reverse primers in a total volume of 5μl . The cycles included a hot start at 94°C for 15 min, followed by denaturation at 94°C for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min (45 cycles), with 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

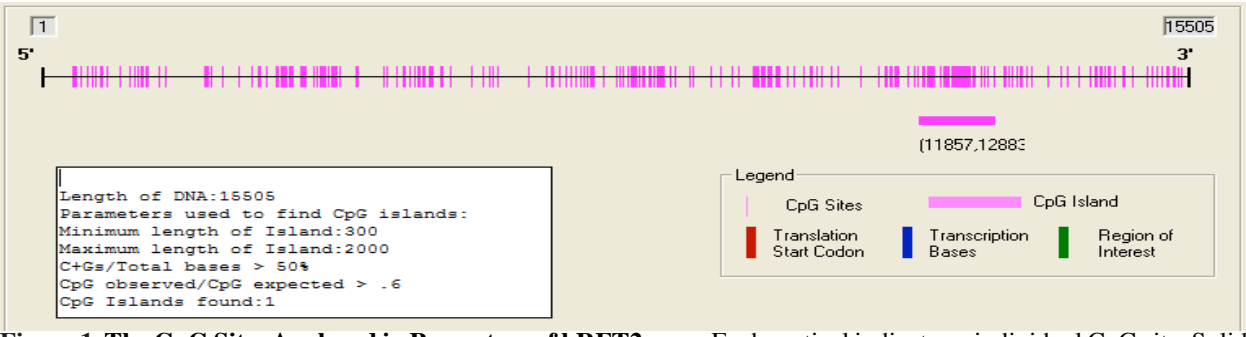

Figure 1. The CpG Sites Analyzed in Promoters of hRFT2 gene. Each vertical indicate an individual CpG site. Solid line positions of CpG Island and the first solid line are the positions of BSP primers. All the BSP primers are designed to cover the transcriptional start site should be close to transcriptional start site

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, per 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 hRFT2 protein *in vivo*, immunolocalization experiments were carried out on sections from representative blocks of 30 paired of FFPE CSCC and NAT. After deparaffinization and antigen retrieval, sections were incubated at 4°C overnight with rabbit polyclonal anti hRFT2 antibody (Santa Cruz, CA, USA). The slides were counterstained lightly with hematoxylin and mounted formicroscopic examination. Each slide was examined by an observer blinded to the diagnosis and clinicopathologic data, and reviewed and confirmed by a second blinded observer. Any intensity staining was considered to present a positive stain for hRFT2. The percentage of positive cells was scored as follows: 0 for ≤25%, 1 for 26-50%, 2 for 51-75%, and 3 for ≥76%. The intensity of staining was as follows: 0 indicated an absence of staining, 1 indicated weak staining, 2 indicated moderate staining, and 3 indicated intense staining. The sum of both scores was used to classify four categories of expression: strong expression (5-6), medium expression (3-4), weak expression (1-2), and total loss of expression (0).

Statistical analyses

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 for evaluation of associations with clinical pathological parameters. Quantitative DNA methylation data derived from MassARRAY were treated as continuous variables and missing measurements were imputed into multivariable regression analyses using samples with replacement for the non missing values (single imputations). Linear associations between 2 continuous variables were quantified by Pearson correlation coefficient.

Results

Methylation profiles of the hRFT2 gene in SiHa

Because of the theoretical relationship between gene promoter hypermethylation as an epigenetic modification with the down regulation of gene transcription, we analyzed hRFT2, due to the down regulation of these genes in ESCC and GC (Maynur et al., 2012; Julaiti et al., 2012). Map CpG-rich islands within the hRFT2 regulatory region by professional software with bisulfate sequencing primers, which used to amplify the gene promoter region sequences that are spanning 3 kb around the transcription start site. Computational analysis of the hRFT2 upstream region revealed one CpG-rich island located between positions 11857 to 12883 relative to the transcriptional start site (Figure 1). By amplification of target CpG islands using genomic DNA as template, and followed by cloning and sequencing, we identified various extents of CpG site methylation at promoter regions of hRFT2.The sequence result shown that hRFT2 gene contains 12 CpG sites and 2 CpG sites were methylated.

hRFT2 gene DNA methylation in cervical cancer from Uyghur patients

The quantitative analysis of methylated DNA by Sequenom MassARRAY platform, a mass spectrometry approach, is based on the detection of methylation state of a single CpG site at a target fragment (CpG island) and generates a data representing the ratio or frequency of the methylation events on a CpG site in all samples DNA. The data results of single CpG site methylation has shown that the methylation level between two groups CpG sites (CpG_2 and CpG_3) from CpG_1~12 significant differences between CSCC and nor¬mal mucous epithelia (5 cm away from the tumor) (Table 1). The global methylation level of target fragments of hRFT2 gene was higher in CSCC (0.452±0.020) than in neighboring normal tissues (0.401±0.019) but no significance ($p>0.05$) shown in Table 2.

hRFT2 proteins expression in CSCC tissue and its association with DNA methylation

Expression of hRFT2 was determined in CSCC tissues and normal adjacent tissues by IHC. The staining showed

Table 1. Quantitative Analysis of hRFT2 Genes Single CpG Site Methylation by Sequenom MassARRAY

CpG site	Methylation levels ($\bar{x}$±S)		*t*	p
	Tumor tissues	normal adjacent tissues		
hRFT2_CpG_1	0.227±0.014	0.194±0.027	0.965	0.345
hRFT2_CpG_2	0.156±0.007	0.109±0.009	3.763	0.001
hRFT2_CpG_3	0.139±0.011	0.096±0.009	3.441	0.002
hRFT2_CpG_4	0.519±0.028	0.498±0.027	1.057	0.155
hRFT2_CpG_5	NA	NA		
hRFT2_CpG_6	NA	NA		
hRFT2_CpG_7	0.560±0.028	0.547±0.029	1.007	0.116
hRFT2_CpG_8	0.626±0.0278	0.537±0.029	1.916	0.069
hRFT2_CpG_9	0.640±0.027	0.566±0.026	1.906	0.07
hRFT2_CpG_10	0.639±0.029	0.610±0.026	1.237	0.076
hRFT2_CpG_11	NA	NA		
hRFT2_CpG_12	0.560±0.028	0.447±0.0286	1.082	0.159

Table 2. Quantitative Analysis of hRFT2 Genes Whole Target CpG Site Methylation by Sequenom MassARRAY

	Methylation levels ($\bar{x}$±S)	*t*	*p*
Tumor tissues	0.452±0.020	1.021	0.105
normal adjacent tissues	0.401±0.019		

Table 3. Inverse Correlation Between hRFT2 CpG_2 and CpG_3 Methylation and Cytoplasm/Membrane Protein Expression in CSCC

Protein expression	methylation levels($\bar{x}$±S)	*F*	*P*
-	0.607±0.024	6.69	0.047
+	0.396±0.018		
++	0.219±0.011		
+++	0.118±0.028		

that there is a translocation expression tendency from cytoplasm/membrane to nucleus in hRFT2 from normal mucous epithelia to CSCC samples. Strong nucleus staining (3+) was detected in cervical cancer tissue. The nucleus positive rates (strong staining, medium staining, and weak staining) of hRFT2 expression in cervical cancer were 36.7%, 26.0%, and 20.0%, respectively. And the cytoplasm/membrane positive rates (strong staining, medium staining, and weak staining) of hRFT2 expression in cervical cancer were 6.7%, 13.3%, and 16.7%, respectively. But 21 (70.3%) medium cytoplasm/membrane staining (2+) of hRFT2 was detected in normal cervical epithelium adjacent to the tumor in 30 cases. Furthermore, the correlation between hRFT2 cytoplasm/membrane expression and DNA methylation in CSCC tissue was analyzed. The results demonstrated an inverse correlation of altered CpG_2 and CpG_3 methylation sites of hRFT2 with changes in cytoplasm/membrane protein expression (Table 3).

Table 3 Inverse correlation between hRFT2 CpG_2 and CpG_3 methylation and cytoplasm/membrane protein expression in CSCC

Discussion

The purpose of the present study was to elucidate whether epigenetic modifications influence expression of hRFT2 gene in cervical cancer cells of Uighur CSCC patients. In this study, the methylation pattern of the hRFT2 gene in patients with CSCC was detected by MassARRAY approach. However, CSCC specimens as solid tumor tissues, the 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 (Ingrid et al., 2011). Furthermore, heterogeneity of surgical tumor specimens can influence the sequencing. Thus, to overcome the problem of tissue heterogeneity, it is necessary to have a viable and more homogeneous cell material retaining the phenotypic and genomic profile of original tissue. Therefore, the cytological composition should be as homogeneous as possible to help identify the aberrant methylation target CpG fragments at promoter regions. To attain this goal, Human cervical cancer cell line SiHa were firstly used to screen specific methylated CpG fragments, cloning and sequencing of corresponding genes and then quantitative detection of gene methylation in clinical cervical tissue.

The results shown that hRFT2 contain multiple CpG islands, but hypermethylation occur only on 2 CpG sites, and it is inverse correlation with down-regulationof hRFT2 cytoplasm/membrane protein in CSCC, which indicates that there are multiple CpG sites, and not methylating of every site leads to down regulation of gene expression or translocation of expression. Only some of them produce genetic transcription. The sites which were methylated are the key CpG sites. It has been confirm that siRNAs targeted to CpG islands within the promoter of a specific gene can induce transcriptional gene silencing by means of DNA-methyltransferase-dependent methylation of DNA in human cells (Taira, 2006).

In our study, there is a translocation expression tendency from cytoplasm/membrane to nucleus in hRFT2 from normal mucous epithelia to CSCC samples, and the high methylation rate of the hRFT2 gene CpG_2 and CpG_3 sites was inverse correlation with down cytoplasm/membrane expression of hRFT2 protein in CSCC. It has been reported that riboflavin participates in various metabolic redox reactions, and is involved in one carbon metabolism, which is a network of interrelated biochemical pathways that generate one carbon groups needed for physiologic processes. Dysregulation of one-carbon metabolism and DNA methylation is believed to promote carcinogenesis (Kim, 2004). Riboflavin may also play a role in carcinogenesis, either by acting as cofactors in folate metabolism or by their independent roles in DNA synthesis, maintaining genomic stability, DNA repair, and regulation of cell division and apoptosis (Powers, 2003; Powers, 2005). However, whether there is correlation between genes methylation with changes localization of the protein expression was unknown. However, it has been reported that mutation in PDZK1 potentially changes transport property of various types of xenobiotic transporters by affecting their subcellular localization, possibly leading to change in disposition of various types of substrate drugs (Sugiura, 2011). Therefore protein localization changes may be associated with genetic polymorphisms, and it should further study.

Although our study has some limitations, the present

study provides little evidence about hRFT2 may be changing the protein activity or affect their fiboflavin transporter function by alter its express location, and promote the MTHFR, and other related factors just like genetic polymorphisms leads to the activation of cervical cancer.

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Competing Interests: The authors have declared that no competing interests exist.

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