RESEARCH ARTICLE

Relationships among MTHFR a1298c Gene Polymorphisms and Methylation Status of Dact1 Gene in Transitional Cell Carcinomas

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Abstract

Objectives: The purpose of this study was to determine the relationship between methylation status of the Dact1 gene and MTHFR a1298c polymorphic forms in transitional cell carcinoma tissues in a Chinese population. Methods: Polymorphisms of folate metabolism enzyme gene MTHFR were assessed by restrictive fragment length polymorphism (RFLP) methods and PCR-based DNA methylation analysis was used to determine the CpG island methylation status of the Dact1 gene. Associations between the methylation status of the Dact1 gene and clinical characteristics, as well as MTHFR a1298c polymorphisms, were analyzed. Results: aberrant methylation of the Dact1 gene was found in 68.3% of cancer tissues and 12.4% of normal tissues,. The methylation rate of the Dact1 gene in cancer tissues was significantly higher in patients with lymph node metastasis than in those without lymph node metastasis (46.3% vs. 17.2%, P = 0.018). No association was found between aberrant DNA methylation and selected factors including sex, age, tobacco smoking, alcohol consumption and green tea consumption. After adjusting for potential confounding variables, variant allele of MTHFR a1298c was found to be associated with methylation of the Dact1 gene. Compared with wild type CC, the odds ratio was 4.33 (95% CI: 1.06–10.59) for AC and 4.95 (95% CI: 1.18–12.74) for AA. The N stage in TNM staging and the occurrence of lymph node metastasis were associated with an MTHFR 1298 AA+AC genotype (P<0.05). Conclusion: MTHFR 1298 AC and AA genotypes might help maintain a normal methylation status of the Dact1 gene, aberrant CpG island methylation of which is closely related to the genesis and progression of transitional cell carcinoma.

Keywords: MTHFR - folate - epidemiology - methylation - transitional cell carcinoma - Dact1

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Introduction

Approximately 500,000 individuals suffered from bladder cancer in the U.S (Marsit et al., 2010) , transitional cell carcinoma (TCC) of the bladder cancer is one of the most common malignancies affecting the genitourinary tract and is characterized by multifocality and a high incidence of recurrence (Vikram et al., 2009). Although the carcinogenesis process is unclear so far, accumulation of multiple genetic and epigenetic alternations leading to the activation of proto-oncogenes and/or inactivation of tumor-suppressor genes (TSGs) is a common consensus (Hansen and Cavenee, 1988; Hunter, 1997; Jones and Baylin, 2007).

Aberrant DNA methylation is now recognized as an important epigenetic alteration occurring early in human cancers (Fang et al., 2006), involving bladder cancer (Tada et al., 2011). In general, DNA methylation alterations are probably the most widely studied epigenetic alterations in cancer (Brait and Sidransky, 2011), and plays a strong role in tumorigenesis (Eze et al., 2011), which increasingly becoming a hot research area. Carcinogenesis is associated with changes in this epigenetic phenomenon, including two distinct and seemingly opposing trends: global decrease in cytosine methylation (hypomethylation or unmethylation) and methylation of cytocine in CpG islands (hypermethylation) (Choi et al., 2007). One study done in Espana demonstrated that neoplasia is correlated with overall genomic hypomethylation (Gonzalo et al., 2008), failure to repress genes appropriately by abnormal demethylation of tissue-restricted genes or by hypomethylation of proto-oncogenes could result in the loss of tissue specificity and could promote cancer formation.

Thus, the exploration of its modulating mechanisms is expected to play important roles in early diagnosis, treatment and prognosis of tumors. As the monocarbon unit required for DNA methylation is provided by the folate metabolism pathway, Methylenetetrahydrofolate reductase (MTHFR) plays a critical role in folate metabolism, which is an important pathway of the methyl donor for DNA methylation. Impaired folate metabolism by these genetic variants (C667T and A1298C) of MTHFR could change the methylation pattern of DNA including

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promoter hypomethylation, which has been frequently observed in cancer (Oyama et al., 2004), however, little research has been done regarding this topic in TCC. So, we decided to investigate the correlation between the methylation pattern of the tumor suppressor gene Dact1 in TCC and the gene polymorphism of the folate metabolism enzyme, as well as clinical characteristics.

Materials and Methods

Samples Information

Frozen tissues and blood samples of 125 bladder carcinomas and 125 normal tissues were obtained from the Affliated Hospital of Xuzhou Medical College. The tumors type was classified in transitional cell carcinoma by two experienced pathologists according to the WHO standard. These patients had a well-documented clinical history and follow-up information. Included in the study were patients with a median age of 62-years-old among which 81 cases were male (accounting for 64.8%) and 44 cases were female (accounting for 35.2%). Twenty-three cases had pathological changes at 68 cases at bladder sidewall (accounting for 54.4%), 34 cases at bladder lateral wall (accounting for 27.2%) and bladder triangular area (accounting for 18.4%). According to TNM staging criteria, T1, T2, T3, T4 respectively accounted for 16.8%, 36.8%, 40.0% and 6.4%. Fifty-nine cases had regional Lymph node metastases, accounting for 47.2%, while remote metastasis only occurred in one case, accounting for 0.8%. In ten cases of normal controls, male and female took up 50% each, with a median age of 55 years old. The study was performed after approval by our institute Human Investigations Committee.

Isolation of genomic DNA from tissues

Genomic DNA was isolated from all tissues by using Promega's wizard DNA isolation kit according to the manufacturer's instructions. Bladder cancer tissues and normal kidney tissues were obtained after surgical resection and stored frozen at -70°C. The tissue were incubated at 55°C in homogenization buffer containing 50 mM Tris (pH 8.0),1 mM EDTA, 0.5% Tween 20, and 5 mg/mL proteinase K for 3 h, and the genomic DNA was isolated using Promega's DNA isolation kit.

PCR-based methods of DNA methylation analysis

Approximately DNA (500 ng) obtained was digested with 10 units of the methylation-specific restriction endonuclease HpaII (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's recommendations, which recognizes the methylated sequence 5'-CCGG -3', at 37°C for 14 h. The digested DNA was subjected to PCR amplification with the primer sets designed by using Primer 3 plus Software (SourceForge, Inc., USA) encompassing the CpG clusters in the DAct1 gene. The Forward Primer: 5'-ACTACTAATCAAAAACGCCCTACG -3' and Reverse Primer: 5'- AATAGTCGTGTTTTATITTCGGGTAC -3' were synthesized commercially at TIANGEN Biotechnologies (Beijing). Genomic PCR without HpaII digestion for each sample was used as internal control. Dilutions of DNA from the digestion reaction were then used for each PCR reaction. PCR conditions were 2 min at 94°C, followed by 27 cycles of 94°C for 30 s, 53°C for 30 s, and 68°C for 1 min for each primer sets .The PCR products were analyzed by 2% agarose gel electrophoresis, and amplified bands were analyzed in UV I Tech gel documentation system (UVI-Tech Ltd., Cambridge, United Kingdom). Undigested DNA of each sample was as an internal control. All other appropriate controls were set up with each batch of TCC samples processed.

Genotyping of the MTHFR gene by PCR-RFLP

Genomic DNA was extracted from fresh-frozen blood using a QIAamp DNA Mini Kit according to the manufacturer instructions (Qiagen, Hilden,Germany). The extracted DNA was stored at 4°C until analysis. Genotyping of the MTHFR a1298c and c677t were performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). respectively. Each amplification reaction was performed in a total volume of 25 μL, containing 10 × PCR buffer (1.8 mM MgCl2) 2.5 μL, 1 U Taq polymerase, 2.5 mmol/L of each dNTP (Tiangen, Beijing, People's Republic of China)2.0 μL, 5 pmol/L of each primer (0.5 μL) and 2 μL of genomic DNA, processing started with 94°C for 5 min and 34 cycles at 94°C for 45 s, 61.5°C for 40 s and 72°C for 50 s. This was followed by a final extension at 72°C for 7 min. Then 5 U of HinfI enzymes was added directly to the PCR products (5 μL) and digested at 37°C overnight. After restriction enzyme digestion of the amplified DNA, genotypes were identified by electrophoresis on 2 % agarose gels and visualized with ethidium-bromide staining ultraviolet illumination. Genotypes were scored by an experienced reader blinded to epidemiological data and serum lipid levels. The PCR products were purified by low melting point gel electrophoresis and phenol extraction, and then the DNA sequences were analyzed in Shanghai Sangon Biological Engineering Technology & Services Co, Ltd. People's Republic of China.

Data analysis and Statistics.

All statistical analyses were conducted with SPSS 11.0 (Chicago, Illinois USA) and STATA 9.2 (Texas USA) statistical software. Folate intake was calculated by monitoring the daily food intake of the subject, and then the sum of all folate taken from various foods was calculated as the total folate intake with reference to the nutrition values specified in the "food compositions". In addition, food folate might be affected by method of preparation, in that it degraded under high temperature or after oil frying; therefore, our study only investigated the fruit folate intake of subjects as a reference for overall folate level. Folate intake levels were divided into four groups according to the percentile interval P25, P50 and P75, i.e. Q1, Q2, Q3 and Q4 groups from low to high respectively. The correlation between Dact1 gene methylation frequency and age, sex, smoking history, history of alcohol consumption, history of tea consumption, sites of pathological changes and TNM staging etc, was done by Pearson χ^2 test. In the case of a sample size unsuitable for Pearson χ^2 test, a Fisher

Table 1. Association Between DACT1 Gene Methylation and Sex, Age and Selected Inducing Factors in TCC Patients

Variables Cases(n)		Frequency of methylation[n (%)]	
		Cancer tissues	Normal tissues
Sex			
Man	81	21(25.9)	8(9.9)
Woman	44	13(29.5)	6(13.6)
P value		0.664	0.561*
Age (years)			
< 60	49	12(24.5)	8(16.3)
≥ 60	76	22(28.9)	6(7.9)
P value		0.585	0.144
Tobacco smoking			
Never	68	20(29.4)	8(11.8)
Ever	57	14(24.6)	6(10.5)
P value		0.544	0.827
Alcohol drinking			
Never	71	21(29.6)	8(11.3)
Ever	54	13(24.1)	6(11.1)
P value		0.493	0.978
Green tea drinking			
Never	77	23(29.9)	10(13.0)
Ever	48	11(22.9)	4(8.3)
P value		0.395	0.422

*Fisher's exact test

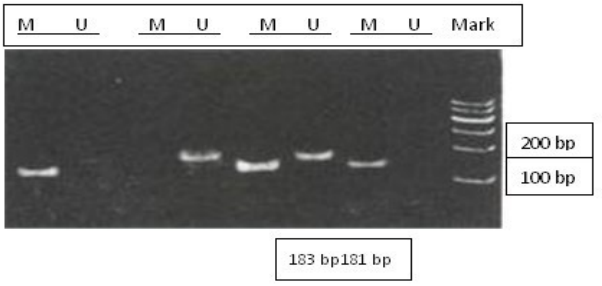

Figure 1. Methylation Status of the Promoter Region CpG Island Methylation of the DACT1 Gene as Assayed by Methylation-specific Polymerase (MSP) Chain Reaction. U : unmethylation, M : methylation

exact test would be used instead. Unconditional Logistic regression model was carried out to analyze for correlation between Dact1 gene methylation and MTHFR a1298c genetic polymorphism with age, gender and folate intake included as potential confounding variables.

Results

Methylation status of Dact1 and its correlation with clinical characteristics

According to PCR-based methods of methylation reaction electrophoresis result (Figure 1), methylation frequency of the Dact1 gene in TCC tissue was about 68.3% while methylation frequency of the Dact1 gene in paracancerous tissue was 12.4%. Among bladder specimens from 10 normal healthy controls, Dact1 gene promoter was all in the unmethylated state. The correlation between Dact1 gene methylation frequency and age, gender, smoking, alcohol drinking, tea intake etc., was not statistically significant (Table 1). The N stage in TNM staging, i.e., the occurrence of lymph node metastasis, was associated with Dact1 gene methylation frequency.

Table 2. Association Between Methylation of DACT1 and Clinical Characteristics in TCC Patients

Variables Cases(n)		Frequency of methylation[n (%)]	
		Cancer tissues	Normal tissues
Site			
Sidewall	23	8(34.8)	3(13.0)
Lateral wall	68	14(20.6)	7(10.3)
Triangular area	34	12(35.3)	4(11.8)
P value		0.193	0.929*
T stage			
T1/2	67	18(26.9)	8(11.9)
T3/4	58	16(27.6)	6(10.3)
P value		0.928	0.778
N stage			
N0	66	11(17.2)	7(10.6)
N1	59	27(46.3)	7(11.9)
P value		0.018	0.824
M stage			
M0	124	33(26.6)	14(11.3)
M1	1	1(100)	0
P value		0.272*	1.000*

*Fisher's exact test

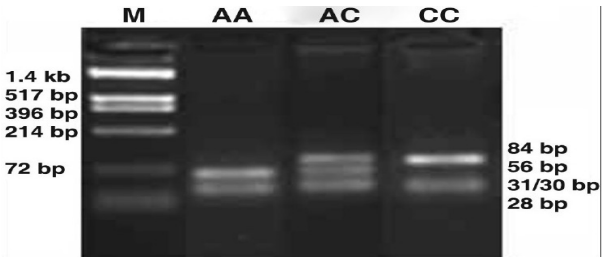

Figure 2. RFLP Analysis for the 1298(A→C) Mutation on a 163-bp PCR Fragment with MboII. The 1298(A→C) Abolishes an MboII Restriction Site. Digestion of the 163-bp fragment of the 1298AA genotype gives five fragments, of 56, 31, 30, 28 and 18 bp, whereas the 1298CC genotype results in four fragments, namely, 84, 31, 30, and 18 bp. The 18 bp fragment has been run off the gel

Specifically, the Dact1 gene in cancer patients with lymph node metastasis had significantly higher methylation frequency, up to 46.3%, which was significantly higher than that in patients without lymph node metastasis 17.2% ($P < 0.05$) (Table 2).

Relationship between Dact1 gene methylation status and polymorphism of MTHFR a1298c and c677t

We compared the correlation between Dact1 gene methylation status and MTHFR genotype in TCC tissue vs. normal tissue. After adjusting other potential confounding variables such as age, gender, folate intake and so on, we found that the Dact1 gene methylation frequency in the TCC tissue from carriers of 1298 AC + AA and 677 TT + CT genotypes was significantly increased, compared with MTHFR wild genotype CC (Figure 2 and Table 3).

Association between methylation of the MTHFR 1298 Genotype and clinical characteristics in TCC patients

The N stage in TNM staging, the occurrence of lymph node metastasis, was associated with MTHFR 1298 AA+AC genotype ($P<0.05$). However, there was no significant difference in other clinical characteristics in TCC.

Table 3. Association Between Methylation of DACT1 and MTHFR a1298c Polymorphisms in TCC Patients

Variables	Cancer tissues	Normal tissues
Age(years)	1.01(0.10-1.08)	0.91(0.83-1.00)
Sex		
Man	1	1
Woman	1.32(0.53-3.24)	2.37(0.65-8.65)
Folate intake†		
Q1	1	1
Q2	2.53(0.89-7.14)	2.65(0.58-11.99)
Q3	0.58(0.15-2.21)	0.34(0.03- 3.78)
Q4	5.09(1.02-25.36)	4.23(0.51-34.79)
MTHFR a1298c		
CC	1	1
AC	4.33 (1.06–10.59)	2.90(0.63-13.33)
AA	4.95 (1.18–12.74)	0.80(0.11-5.72)

*Adjusted by age, sex, folate intake and MTHFR a1298c genotypes; †The cut point of fruit folate intake is quartile (μg/day), Q1: 0–26.2; Q2: 26.3–99.4; Q3: 99.5–311.0; Q4: ≥311.1

Discussion

Genomic DNA methylation is the best-studied epigenetic modification currently discovered. The processs is promoted by DNA methyltransferases, which use S-adenosyl-methionine as the methyl donor (Batra and Mishra , 2007). 5-methyl-cytosine is formed through the addition of a methyl group to the fifth carbon position of the cytosinepyrimidine ring by DNA methyltransferases (Zhu et al., 2011). DNA methylation is an epigenetic mechanism which plays an important part in regulating gene expression at the transcription level (Huang and Vieira , 2006). Through methylation on cytosine of the CpG island in the gene promoter and the other CpG-riched region, some redundant genes in certain tissues or cells can be silenced, while demethylation can function as an activating some tissue-or stage-specific genes, which contributed to the temporal and spatial regulation of gene expression (Milutinovic et al., 2007; Nicholson et al., 2008). Previous studies have described global genomic hypomehtylation in several malignant cancers including carcinomas of bladder (Chalitchagorn et al., 2004) and other organic carcinomas, by evaluating genomic repetitive sequence. Therefore, the importance of these epigenetic mechanisms in the transcriptional regulation of genes plays critical roles in the process of cancer progression (Fraga et al., 2007).

The DACT1 (Dapper1/Dpr1) gene, located at chromosome 14q22.3, encodes a 836 amino acid protein with a putative leucine zipper (LZ) domain in the amino-terminal end and a consensus PDZ binding (PDZ-B) motif in the carboxy-terminal end that allows the DACT1protein to interact with the Dishevelled (Dvl) PDZ domain (Cheyette et al., 2002). Bioinformatic analyses have revealed that DACT1mRNA is expressed in the amnion, fetal brain, eye, heart, adult brain medulla, gastric cancer (signet ring cell features), RER+ colon tumor, acute lymphoblastic leukemia, germ cell tumor, chondrosarcoma, and parathyroid tumors (Katoh, 2003). Further-more, based on the evolutionary and functional conservation of Wnt signaling molecules, as well as the human chromosomal localization of DACT1, the DACT1gene is also predicted to be a potent cancer-associated gene (Katoh , 2003). DACT1 has been reported to be downregulated in hepatocellular carcinoma (Yau et al., 2005). A recent report identified a correlation between DACT1expression in lung cancer and poor histological grade, large tumor size, extent of tumor invasion, and lymph node metastasis (Yang et al., 2010). Although some studies have shown associations between DACT1 expression and cancer, the function of DACT1 in the WNT/ β-catenin signaling pathway remains unclear. The present study is the first to assess the methylation status of DACT1 promotor in samples from TCC and neighboring normal bladder tissue by PCR-based methylation assay. In our present study, the DACT1 gene methylation rate in TCC was 68.3 % compared with normal controls, The existence of regional lymph node metastasis was also associated with DACT1 gene methylation frequency, i.e., in the cancer tissues from patients with lymph node metastasis, the DACT1 gene methylation frequency (46.3%) was significantly higher than that of patients without lymph node metastasis (17.2%), suggesting that abnormal DACT1 gene methylation might serve as a valuable biomarker in the TCC diagnosis and potential indicator of TCC prognosis.

Unlike genetic changes, epigenetic changes are more dynamic and are often reversible, depending on the existence or removal of the inducing factors (Liu et al., 2008). Further exploration of the impact factors for epigenetic alterations was of great value for the early prevention and intervention of diseases, but knowledge in this field is still relatively superficial (Feinberg , 2004).Along with the research progress, it is found that environmental factors (for example dietary nutrients) associate with genomic DNA methylation, especially deficiency in methyl group donors such as vitamin A and methionine, etc. for example, Zhang et al. (2011) had proved that a dietary pattern characterized by a high intake of vegetables and fruits may protect against global DNA hypomethylation. Folate is an important dietary nutrient and accepts one-carbon units from one-carbon unit donors, DNA methylation status were determined as a functional endpoint, which suggesting that abnormal folate metabolism might affect on genomic methylation state negatively. MTHFR is a key enzyme regulating folate metabolism, which affects DNA methylation and synthesis (Friso et al., 2005). MTHFR converts 5, 10-methylentetrahydrofolate to 5-methyltetrahydrofolate, which is required for homocysteine methylation to methionine. Methionine is then activated to S-adenosylmethionine, a universal methyl donor in numerous transmethylation reactions, including methylation of DNA, RNA, proteins, and other molecules (Cellarier et al., 2003).moreover, enzyme activities with the MTHFR 677TT only met 30 % of that with wild 677CC genotypes, while enzyme activities with the MTHFR 677CT only met 60 % of that with wild 677CC genotypes (Weisberg et al., 1998). changing activity of MTHFR enzyme is associated with polymorphism in MTHFR gene would affect its abilities involving in DNA synthesis and the supply of methyl

group, therefore the association between MTHFR gene polymorphism and DNA methylation has gradually drawn interest from many researchers all over the world. Friso et al. (2005) reported that in human lymphocytes patients, the gene-nutrient interaction affecting DNA methylation in 1298AA is mainly due to the coexistence of the 677TT genotype. Axume et al. (2007) suggested that the MTHFR 677TT genotype and folate interact to lower global leukocyte DNA methylation patterns in young Mexican American women. Meanwhile, Supic et al. (2011) resulted in a significant association was found between TT genotype and methylation status of the RASSF1A gene in Oral Squamous Cell Carcinoma (OSCC) patients (P = 0.012). While A polymorphism which changes an A to C at position 1298 (a1298c) in MTHFR is a reasonably common variant of the gene. Neoplastic cells increase DNA-methyltransferase activity suggesting that methylation imbalance may contribute to tumor evolution (Lin et al., 2004; Jakubowska et al., 2007). Methylation status of the gene promoter shows different results in numerous investigations (Hilton et al., 2002; Paige, 2003). One factor influencing the results could be caused by contamination of specimens with cells from nearby tissues during analysis and isolation of tumor samples. Unmethylated DNA from the normal cells might compromise the results of the methylation levels of the tumor tissue (Hilton et al., 2002).

Because of the main role that MTHFR plays in the methylation status of genes, we designed assays to investigate that carriers of MTHFR 1298 mutation genotype had increased DACT1 gene methylation frequency in their TCC tissues, and this increase associated with the level of folate intake. Source of dietary folate, amount of folate intake, as well as the body metabolism all will affect the supply of methyl group required for DNA methylation. Ma et al. (1997) had also provided support for an important role of folate metabolism in colon carcinogenesis. In particular, these results suggest that the 677TT mutation in MTHFR reduces colon cancer risk, perhaps by increasing 5,10-methylenetetrahydrofolate levels for DNA synthesis, but that low folate intake or high alcohol consumption may negate some of the protective effect.It is generally believed that with sufficient folate intake, the risk of cancer in MTHFR 677CT or TT genotype carriers would be to reduced, since low activity of folate metabolic enzyme will not only help DNA synthesis reaction ,while adequate folate can but also provide sufficient methyl groups for DNA methylation process; oppositely, if the intake of folate is deficient, DNA synthesis, repair and the methylation process would all be affected. 5, 10-methylene THF supply deficiency would result in the difficulty of executing the protective function of MTHFR mutation genotype, so that the DNA methylation process would be disturbed (Wang et al., 2005; Yang et al., 2005). in this study, we found that the Dact1 gene methylation frequency in the TCC tissue from carriers of 1298 AC + AA and 677 TT + CT genotypes was significantly increased, which suggested that the effect of 1298AA polymorphism on DNA methylation is very likely due to the coexistence of the 677TT genotype (Friso et al., 2005). However, Impairment of the folate metabolism has been shown to be associated with global hypomethylation and promoter hypermethylation (Jones and Baylin , 2002). So the association between 1298AA and 1298AC and increased frequency of Dact1 promoter methylation is not a specific event or a consequence of a global increase of methylation of promoter CpG islands. In the process of carcinogenesis, the whole genome is in a hypomethylated state, accompanied by hypermethylation of other specific tumor suppressor genes, therefore, how the individual folate intake level and folate metabolism pathway disorder affect this co-existence of hyper- and hypo-methylation state, is still not clear. In the other hand, it has been shown that 1298AC and 1298CC is associated with increased risk of TCC (Safarinejad et al., 2011). while the N stage in TNM staging, the occurrence of lymph node metastasis, was associated with MTHFR 1298 AA+AC genotype (P<0.05). However, there was no significant difference in other clinical characteristics in TCC.

In summary, abnormal methylation in the promotor of DACT1 gene is associated with the origination and development of TCC. individuals with MTHFR 1298 AC or AA genotype maybe a negative factor in the process of carcinogenesis by methylating DACT1 gene in vivo. Therefore, exploration of abnormal DNA methylation distribution in other tumor suppressor genes is expected to play important role in the early individual diagnosis and prognosis monitor of TCC.

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