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Investigating the methylation status of *DACT2* gene and its association with *MTHFR* C677T polymorphism in patients with colorectal cancer

Ahmad Jalilvand[1], Mohammad Soleiman Soltanpour[2,*]

1) Department of Pathology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran
2) Department of Medical Laboratory Sciences, School of Paramedical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran

ABSTRACT

Colorectal cancer (CRC) is one of the common causes of cancer death in Iranian population. Both genetic and epigenetic changes have been implicated in CRC pathogenesis. *DACT2* gene as one of the WNT signaling pathway inhibitor was shown to display tumor suppressor activity in many cancers. The aim of present study was to investigate the methylation status of *DACT2* gene and its association with methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism in CRC patients. Fifty formalin-fixed paraffin-embedded cancerous and adjacent healthy tissues obtained from CRC patient were investigated. Genomic DNA was isolated using a FFPE commercial DNA extraction kit. The methylation status was evaluated by methylation specific PCR. Genotyping of *MTHFR* C677T polymorphism was performed using PCR-RFLP technique. Statistical analysis was done by GraphPad Prism 8. Results indicated that the frequency of methylated *DACT2* gene was significantly higher in cancerous tissue relative to adjacent healthy tissue ($P<0.001$). *DACT2* gene methylation was significantly more common among carriers of *MTHFR* 677CC genotype ($P=0.035$) and significantly less common among carriers of *MTHFR* 677T allele (P value =0.006). In conclusion the present study identified *DACT2* gene methylation as a significant risk factor for CRC development. Moreover, the low frequency of *DACT2* gene methylation among carriers of *MTHFR* 677T allele may confer a protective role for this common polymorphism against CRC risk.

Keywords: Methylation; Colorectal cancer; DACT2; Methylenetetrahydrofolate reductase

INTRODUCTION

Colorectal cancer (CRC) is a common malignancy in the worldwide and is one of the fifth common cancers in the Iranian population [1]. As the cause of 8.5% of all cancer deaths, CRC represents the fourth most common cause of cancer death in the world with approximately 694,000 deaths annually [2, 3]. A hallmark event in the CRC is the accumulation of multiple genetic and epigenetic changes leading to transformation of colon epithelial cells into cancerous

*Corresponding Author: Department of Medical Laboratory Sciences, School of Paramedical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran
Tel: +98-024-33772092; Fax: +98-024-33773153
E. mail: soltanpourm@zums.ac.ir

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cells. A growing body of evidence indicated that the frequency of aberrant epigenetic alterations are more common than the frequency of genetic mutations in the average CRC genome [4, 5].

One of the epigenetic alterations includes the DNA methylation of CpG island dinucleotide in the promoter region of genes that leads to down-regulation of gene without causing any alteration in the sequence of DNA [6]. The enhanced activity of WNT signaling pathways is a common finding in many cancers including CRC that results in extensive proliferation and disturbed differentiation. The Wnt signaling pathway consists of the canonical/β-catenin pathway as well as several non-canonical/β-catenin-independent pathways [7]. Numerous Wnt signaling pathway inhibitors including Secreted Frizzled Related Proteins (SFRP1-5), Dickkopf (DKK1-4), Wnt Inhibitory Factor-1 (WIF-1) and Dapper Dishevelled-associated antagonist of β-catenin (DACT2) have been described [7].

DACT2 is a key regulator of Wnt signaling pathway mapped to chromosome 6q27. DACT2 displayed tumor suppressor activity in many tumors including human breast cancer, gastric cancer and hepatocellular carcinoma and it's inactivation by DNA methylation may contribute to tumor pathogenesis [7-10]. 5-methyltetrahydrofolate (methyl-THF) acts as a methyl donor mediator in various biological reactions such as DNA methylation [11]. The methyl-THF is produced by the methylenetetrahydrofolate reductase (MTHFR) enzyme that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [11]. The C677T (rs1801133) polymorphism of *MTHFR* gene has been related to the reduced bio-availability of methyl-THF that may affect the level of DNA methylation [12]. So, the present study investigated the frequency of promoter DNA methylation of *DACT2* gene and its possible interaction with genetic C677T polymorphism of *MTHFR* gene in an Iranian population of CRC patients.

MATERIALS AND METHODS

Samples: The present study included 50 formalin-fixed paraffin-embedded (FFPE) cancerous and adjacent healthy tissues obtained from CRC patients referred to Mousavi hospital (Zanjan, Iran) between September 2015 and September 2017. The clinicopathological characteristics of CRC patients including tumor stages, tumor grades, tumor location, Lymph node metastasis and histologic type were obtained from medical records. The study was approved by ethical committee of Zanjan University of Medical Sciences (Ethical code: ZUMS.REC.1394.337), Zanjan, Iran.

DNA extraction and Methylation analysis: A 5-10 µm section of FFPE tissues was prepared and used for DNA extraction by a QIAamp DNA FFPE Tissue Kit (Qiagen, Germany). The purity and integrity of purified DNA was evaluated by nanodrop spectrophotometer. Next, 1-2 µg of extracted DNA was bisulfite-treated using the EpiTect Fast DNA Bisulfite kit (Qiagen, Germany), according to the manufacturer's protocol.

Methylation specific PCR (MSP) was used to analysis the methylation status of *DACT2* gene promoter, as previously described [9]. Briefly, two sets of primer specific for methylated and un-methylated status of gene were used for amplification. Each PCR reaction included 10 µL master mix 2x, 1 µL (0.5 µM) of each forward and reverse, 100 ng of bisulfite-converted DNA and appropriate volume of PCR grade water in total volume of 20 µL. Appropriate methylated and un-methylated controls was included in the PCR reaction (EpiTect PCR Control DNA Set, Qiagen, Germany). MSP products were visualized on 2% agarose gel and the presence of 161 bp and 152bp bands were indicative for unmethylated and methylated gene, respectively (Fig. 1).

***MTHFR* C677T genetic analysis:** The genotyping of C677T polymorphism of *MTHFR* gene was done using PCR-RFLP, as previously described [13]. Briefly, genomic DNA was

amplified by specific primers in a routine PCR condition. The size of PCR product was 198bp that following digestion with HinfI (Fermentas, Germany) restriction enzyme resulted in 175bp and 23bp in the presence of mutant T allele and an undigested 198 bp band in the presence of wild C allele.

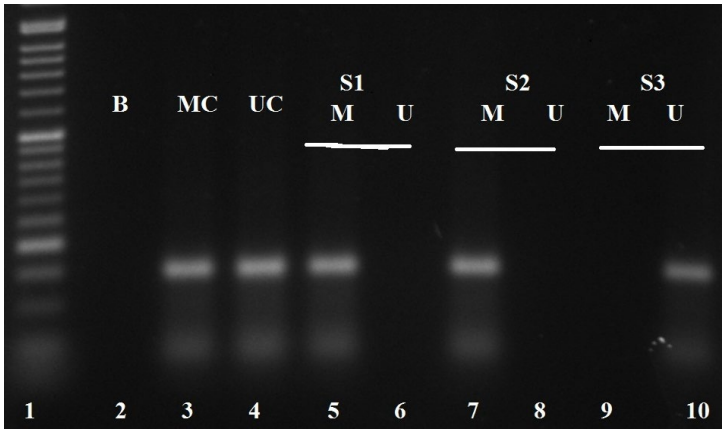

Figure 1: Electrophoresis of *DACT2* gene MSP products on 2% Agarose gel. 1: 50bp ladder; 2: blank; 3: methylated control; 4: un-methylated control; 5, 7, 9: methylated bands; 6,8,10: un-methylated bands; M: methylated; U:un-methylated; S:sample; C: control.

Statistical analysis: Methylation frequency between cancerous and adjacent healthy tissues was compared using χ^2 test. The association between methylation status of *DACT2* gene with clinical, pathological features and also *MTHFR* C677T genotypes was evaluated by χ^2 test, Fisher's exacts test or Pearson correlation coefficient test, as appropriate. Binary logistic regression analysis was done for investigating the independent association between *MTHFR* C677T genotypes and methylation status of *DACT2* gene. All statistical analysis was performed using GraphPad Prism 8 software.

RESULTS

The age of CRC patients ranged between 23-86 with the mean age of 59.5±11.1 years. The other clinical and pathological features of CRC patients were presented in Table 1. Methylation analysis of *DACT2* gene using MSP technique indicated that 23 out of 50 (46%) of CRC tissues were methylated while no methylation was found in the corresponding adjacent healthy tissues (P<0.001). The association between cliniciopathological characteristics of CRC patients with the methylation status of *DACT2* gene indicated no significant correlation between *DACT2* methylation and age, sex, tumor size, tumor stage and tumor histological type. However, a significant association was seen between *DACT2* methylation and tumor location (P<0.001), tumor grade (P=0.026) and tumor lymph node metastasis (P=0.022) (Table 1).

The investigation of *MTHFR* C677T polymorphism indicated that the genotype distribution of 677CC, 677CT and 677TT in the CRC patients were 28 (56%), 18 (36%) and 4 (08%), respectively. Also, the C allele and T allele frequency of *MTHFR* C677T polymorphism in the CRC patients was 74% and 26%, respectively. The association between *MTHFR* C677T polymorphism with methylation status of *DACT2* indicated that the 677CC genotype was more common among methylated samples relative to un-methylated samples (P=0.035). Also, the frequency of T allele was higher in un-methylated than methylated samples (P=0.006) (Table 2). Also, binary logistic regression analysis was used to investigate the independent association of *DACT2* methylation status with *MTHFR* C677T genotypes. Results indicated significant differences of *MTHFR* 677CC genotype (P=0.01) and *MTHFR* 677CT genotype (P<0.001) between methylated and un-methylated samples.

Table 1: the association between *DACT2* methylation status and clinical and pathological features of CRC patients

Clinical and pathological features	Number (n=50)	*DACT2* methylation status		P
		Methylated n=23	Unmethylated n=27	
Age				
<50	15	6	9	0.758
≥50	35	17	18	
Sex				
Female	24	14	10	0.155
Male	26	9	17	
Tumor size, cm				
<=5	23	11	12	0.998
≥5	27	12	15	
Tumor location				
Distal	27	19	8	<0.001
Proximal	23	4	19	
Grade				
I	8	2	6	0.026*
II	34	14	20	
III	8	7	1	
Stage				
I-II	17	9	8	0.556
III-IV	33	14	19	
Lymph node metastasis				
Positive	25	7	18	0.022
Negative	25	16	9	
Histological type				
Non-Mucinous	40	18	22	0.998
Mucinous	10	5	5	

* Calculated by Chi-square test.

Table 2: genotypic and allelic distribution of *MTHFR* C677T polymorphism according to methylation status of *DACT2* gene in CRC patient

MTHFR C677T	Methylated n=23 (%)	Un-methylated n=27 (%)	*P*	OR (95%CI)
CC	20 (86.96%)	08 (29.63%)	0.035	2.93 (1.13-8.17)
CT	02 (08.70%)	16 (59.26)	0.010	6.81 (1.46-31.7)
TT	01 (04.34%)	03 (11.11)	0.620	0.39 (0.02-2.82)
CT+TT	03 (13.04%)	19 (70.37%)	0.008	0.18 (0.05-0.65)
C allele	42 (91.30%)	32 (59.26%)	0.171	1.54 (0.83-2.75)
T allele	04 (08.70%)	22 (40.74%)	0.006	0.21 (0.07-0.61)

DISCUSSION

The main findings of present study were (I) the frequency of *DACT2* methylation was significantly higher in cancer tissues relative to healthy adjacent tissue (II) *DACT2* methylation was associated with tumor grades, lymph node metastasis and tumor location (III) the frequency of *DACT2* methylation was more common among the carriers of *MTHFR* 677CC genotype and the T allele frequency was higher in un-methylated relative to methylated state. In the present study the prevalence of *DACT2* methylation in cancer tissue was 46%. In another study by

Wang et al., the *DACT2* methylation was reported in 43.3% of CRC patients which was similar to our reported frequency [14]. Different rates of *DACT2* methylation was reported in other cancers including 55.7% in gastric cancer, 32.9% in breast cancers and 52.2% in esophageal squamous cell carcinoma [9, 10, 15]. So, *DACT2* methylation may be used as a diagnostic and prognostic biomarker for early management of numerous cancers.

According to the present study, tumors located in the distal portion of colon (19 out of 27; 70%) had significantly higher rate of *DACT2* DNA methylation relative to proximally located tumors (4 out of 23;27%) of colon (P value<0.001). This finding emphases a tumor location specific role of *DACT2* DNA methylation in the pathogenesis of CRC. Similarly, other studies have also reported a distinctive DNA methylation pattern of some specific genes in CRC located in proximal and distal portions of the colon [16]. Moreover, in current study a positive association was seen between *DACT2* gene methylation and tumor grade, so that the CRC patients with grade III had the highest rate of methylation (7 out of 8; 87.5%; *P* value=0.029). This observation suggests a role for *DACT2* gene methylation in disease severity and may have therapeutic and prognostic utility in CRC patients.

The association of *MTHFR* C677T polymorphism and occurrence of different cancers have been extensively studied [17, 18]. Zhao et al., reported *MTHFR* 677T allele as a protective factor against CRC risk [19]. Moreover, Teng et al., demonstrated a predisposing role for *MTHFR* 677CC genotype in the development of CRC among Caucasians [20]. Our study investigated the genotypic and allelic distribution of *MTHFR* C677T polymorphism between carriers of methylated and un-methylated *DACT2* gene and interestingly demonstrated that the frequency of *DACT2* gene methylation was significantly higher in CC carriers. This finding may explain to some extent the cause of increased risk of CRC among CC carriers. Previous studies have shown that the inheritance of *MTHFR* C677T polymorphism results in a thermolabile enzymatic variant associated with reduced bioactivity and decreased bioavailability of 5mTHF, a necessary precursor for methylation reactions [21]. So, it seems that the presence of *MTHFR* 677T allele restricts the bioavailability of 5mTHF and may impairs the methylation reaction, as our data indicated low methylation frequency in carriers of T allele.

Some limitations exists in present study including (I) the gene and protein expression levels of *DACT2* was not evaluated in the samples (II) common polymorphisms of *DACT2* gene and their role in CRC was not investigated.

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Conflict of Interest: The authors declare no conflicts of interest to be exist.

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