Original Article

TUSC3 Methylation in Peripheral Blood Cells as a Biomarker for Diagnosis of Colorectal Cancer

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Abstract

Background: Several case-control studies have suggested that global and loci-specific deoxyribonucleic acid (DNA) methylation in peripheral blood mononuclear cells (PBMCs) of DNA might be potential biomarkers of cancer diagnosis and prognosis. In this study, for the first time, we intended to assess the diagnostic power of the methylation level of tumor suppressor candidate 3 (*TUSC3*) gene promoter in patients with colorectal cancer (CRC).

Materials and Methods: In the current study, we quantitatively assessed the promoter methylation level of *TUSC3* in PBMCs of 70 CRC cases and 75 non-cancerous subjects via methylation quantification of endonuclease-resistant DNA (MethyQESD) method.

Results: The methylation level of the *TUSC3* was meaningfully higher in CRC cases than in non-CRC subjects (43.55 ± 21.80% vs. 16.07 ± 13.63%, respectively; $P < 0.001$). The sensitivity and specificity of this gene for the detection of CRC were 88.6% and 76.0%, respectively. The receiver operating characteristic (ROC) curve examination discovered an area under the curve (AUC) of 0.880, representing a very high accuracy of the *TUSC3* methylation marker in distinguishing CRC subjects from healthy individuals. However, there was no substantial diversity in methylation level between various CRC stages (*P*: 0.088).

Conclusion: For CRC screening, PBMCs are a reliable source for DNA methylation analysis and *TUSC3* promoter methylation can be utilized as a hopeful biomarker for early and non-invasive diagnosis of CRC.

Keywords: Biomarker, colorectal cancer, *TUSC3* gene, methylation

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Introduction

Evaluation of deoxyribonucleic acid (DNA) methylation biomarkers has been propounded as a promising tool for the accurate diagnosis and prognosis of various tumors.[1] Different sources can be recruited for the detection of DNA methylation biomarkers, including samples containing cell-free DNA such as plasma, serum, stool, and urine, and cell-based samples such as tumor and blood cells.[2] The significant challenge for the discovery of DNA methylation biomarkers is the high level of fragmentation and the low amount of circulating tumor DNA (ctDNA) (less than 0.01% of the total ctDNA), particularly in early-stage cancers, necessitating the development of efficient techniques for methylation analysis of ctDNA.[3,4]

Recently, some studies have introduced peripheral blood mononuclear cells (PBMCs) as a source of biomarkers for

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several cancers, particularly when taking a biopsy sample from the tumor cells is not easy.[5] Several studies have reported methylation alterations of LINE-1 sequence and promoters of *MMP9*, *IFNG*, *TFPI2*, *NDRG4*, *ITGA4*, and *BRCA1* genes in a variety of cancers.[6-9] The exact molecular mechanisms underlying DNA methylation changes in PBMCs have not yet been determined. However, one assumption is that PBMCs, as surrogate cells, can mimic the status of neighboring tissues such as cancer cells. Interplay between malignant cells and PBMCs is generally mediated by non-coding ribonucleic acids (ncRNAs) such as micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs) that mostly exist in exosomes.[5] This interaction may result in alterations in the epigenetic profile of PBMCs, especially methylation levels.

Regarding the critical impact of early diagnosis in the reduction of colorectal cancer (CRC) mortality and the limitations of traditional CRC screening tests comprising invasive nature, unpleasant feelings, and diet restriction, researchers have been looking for readily available and non-invasive sources of molecular biomarkers.[10-13] Also, considering the previously mentioned limitations of ctDNA which lead to low specificity and sensitivity and the advantages of PBMCs, in the present study, we chose PBMCs as a source of methylation biomarkers.

The hyper-methylation of *TUSC3*, as a tumor suppressor gene, has been described in some types of malignancies including CRC,[14] ovarian,[15,16] glioblastoma multi-form,[17] non-small-cell lung cancer (NSCLC),[18] and prostate cancer.[19] However, some studies have disclosed the oncogenic role of *TUSC3* in CRC.[20,21] On the other hand, most previous methylation studies employed methods in which bisulfite treatment was a required process.[22] This can cause a high level of ctDNA degradation; in contrast, methylation analysis with a combination of digestion by restriction enzyme and polymerase chain reaction (PCR) is devoid of this drawback. Therefore, given the role of *TUSC3* methylation in the progression of different cancers and the advantage of methylation analysis by digestion with restriction enzymes, for the first time, we quantitatively assessed the methylation level of *TUSC3* in PBMCs of patients with CRC and healthy subjects via the methylation quantification endonuclease-resistant DNA (MethyQESD), a bisulfite conversion-independent method.

Materials and Methods

Study population

We enrolled 70 sporadic CRC patients who were referred to Al-Zahra hospital of Isfahan University of Medical Sciences. The controls were 75 individuals with negative colonoscopy reports and with no personal and familial history of cancer. The features of individuals such as sex, age, body mass index (BMI), smoking status, and tumor stage were documented by employing a structured questionnaire and histological reports. The experimental design was confirmed by the AJA University of Medical Sciences with approval number IR.AJAUMS.REC.1401.016, and informed consent was received from each participant. For everyone, 2.5 ml of peripheral blood was obtained from 70 CRC patients and ethnicity-matched 75 healthy volunteers and stored in ethylenediaminetetraacetic acid anti-coagulant tubes at −20°C for further processing.

Quantitative methylation analysis

According to a previously described standard protocol,[23] the PBMCs were obtained by density gradient centrifugation over Ficoll-Hypaque (Ficoll-Hypaque, Sigma) from the peripheral blood samples of all individuals. DNA was extracted from the PBMCs using a Prime Prep Genomic DNA Isolation Kit (GeNetBio, Korea), and its quantity and quality were evaluated by gel electrophoresis and using a NanoDrop spectrophotometer, respectively.

Quantitative methylation analysis was accomplished using the MethyQESD technique.[24] This method is based on a combination of methylation-sensitive restriction enzymes and a real-time polymerase chain reaction (RT-PCR). According to the protocol, two different batches were considered, one a methylation-specific quantification digestion (MQD) with Hin6I and the other a methylation-sensitive endonuclease calibrator digestion (CalD) with the methylation-independent endonucleases, namely, XBaI and DraI. The sequence of primers was as follows: forward: 5'-TACCGCGCGTGGAGGAGACA-3'; reverse: 5'- GTGGGCAGGTACCGCAGCC-3 '. The enzyme digestion and RT-PCR protocol were described in detail by Duppel *et al*.[25]

Statistical analysis

The statistical tests to evaluate the methylation percentage of *TUSC3* promoter were performed via SPSS version 25 (Armonk, NY: IBM Corp). The diagnostic performance of the candidate biomarker was calculated by the receiver operating characteristic (ROC) curves. The sensitivity (true CRC/true CRC + false CRC-free), specificity (true CRC-free/true CRC-free + false CRC), and respective areas under the curve (AUCs) were measured to establish the best cutoff values for the percent of DNA methylation that can discriminate between CRC patients and healthy people. $P < 0.05$ was assumed to indicate a statistically significant difference.

Results

The characteristics of the 70 CRC cases and 75 sex- and age-matched controls are summarized in Table 1. There were not important differences between CRC group and healthy individuals in terms of BMI and smoking status (*P*: 0.624 and *P*: 0.196, respectively). According to the pathological reports, 20, 26, 15, and 9 of the CRC subjects were in stages I, II, III, and IV, respectively [Table 1].

TUSC3 methylation status

Our findings showed that the mean *TUSC3* promoter methylation levels in the CRC and control groups were 43.55 ± 21.80% and 16.07 ± 13.63%, respectively. The

difference in the mean methylation level of TUSC3 promoter between the CRC group and healthy individuals was substantial ($P < 0.001$) [Table 2 and Figure 1]. ROC curve analysis defined an optimal cutoff value of 18.68% TUSC3 methylation for specific and sensitive detection of sporadic CRC. Recruiting these methylation cutoff values, we obtained a specificity of 76.0% and a sensitivity of 88.6% for the classification of subjects into CRC and non-CRC groups. Besides, the ROC curve analysis revealed an AUC of 0.880, which represents a very high accuracy of the TUSC3 methylation marker in distinguishing CRC subjects from healthy volunteers [Table 2 and Figure 2]. However, there was not a significant difference in methylation level between various stages in CRC cases (*P*: 0.088). Besides, there was no significant difference between the smoking group (45.81%) and non-smokers (42.41%) in terms of the methylation level of the *TUSC3* gene (*P*: 0.158).

Discussion

PBMCs are defined as any blood cell with a round nucleus, including lymphocytes (B- and T-cells), monocytes, natural killer cells, and dendritic cells.[26] Recently, PBMCs have been proposed as one of the promising blood-circulating sources of biomarkers in several diseases, specifically malignancies.[27] In recent years, several DNA methylation-based biomarkers in leukocytes and PBMCs have been identified for the differentiation of cancer patients from healthy people.[6,7,28]

The first case-control studies in the field of DNA methylation alterations in cancer suggested that global DNA methylation of LINE-1 and Alu in leukocytes are potential susceptibility and diagnostic biomarkers. These studies confirmed that hypo- or hyper-methylation of these loci in blood leukocytes and PBMCs can be used as biomarkers for diagnosis and prognosis in different types of malignancies including head and neck, gastric, testicular, bladder, colon, liver, lung, nasopharynx, breast, and renal cell cancer.[28-35] On the other hand, subsequent studies focused on methylation assessment of specific promoter genes in blood cells, which led to the introduction of promising biomarkers such as *IGF2* in gastric and colorectal cancer;[36] *CDH1* in hereditary diffuse gastric cancer;[37] *MMP9*, *TFPI2*, *NDRG4*, *SDC2*, and *ITGA4* in CRC;[6,7,9,38] *NEUROD1*, *NUP155*, *SFRP1*, *TITF1*, and *ATM* in breast cancer;[39-41] *P53*, *CSF3R*, and *ERCC1* in lung cancer;[42,43] and *IL10*, *LCN2*, *AIM2*, *TAL1*, and *ZAP70* in pancreatic cancer.[44] Regarding this evidence as well as a hypothesis about possible connections between PBMCs and released exosomes from cancer cells, the reflection of the epigenetic status of tumor cells in PBMCs has made them a novel source of biomarkers.[5]

In the current study, for the first time, we quantitatively assessed the methylation status of *TUSC3* in PBMC samples of CRC patients and healthy individuals. Considering the disadvantage of bisulfite treatment for methylation analysis, MethyQESD was exploited for accurate measurement

Table 1: Characteristics of CRC patients and healthy controls in this study

Variable	Case (*n*: 70)	Control (*n*: 75)	*P*
Sex			
Male	39 (55.7%)	34 (45.3%)	0.246
Female	31 (44.3%)	41 (54.7%)	
Age (mean±SD)	56.0286±11.46004	54.7067±9.39490	0.447
BMI (mean±SD)	24.8000±3.89202	25.0933±3.25150	0.624
Smoker	23 (32.9%)	17 (22.7%)	
Non-smoker	47 (67.1%)	58 (77.3%)	0.196
Stage			
I	20 (28.57%)	–	
II	26 (37.14%)	–	
III	15 (21.43%)	–	
IV	9 (12.86%)	–	

BMI=Body mass index, SD=Standard deviation

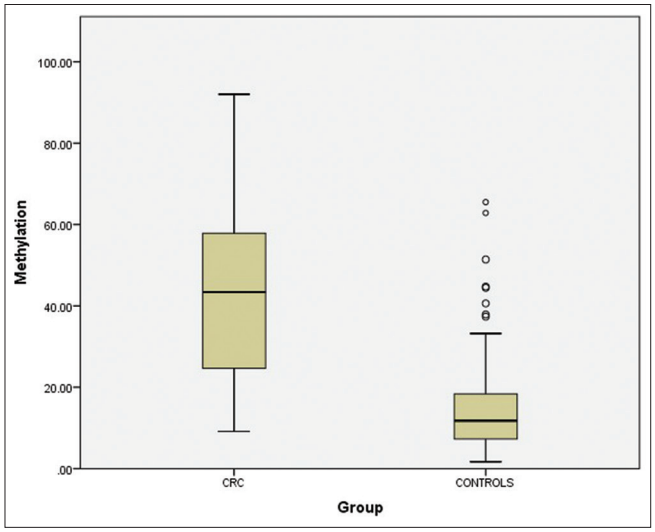

Figure 1: Comparison of *TUSC3* promoter methylation level between patients with CRC and healthy controls. $P < 0.001$*

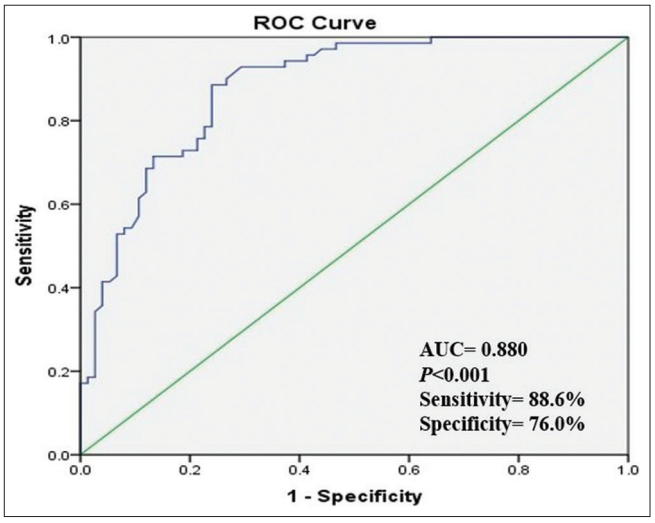

Figure 2: The receiver operating characteristic (ROC) curve of *TUSC3* promoter methylation level in patients with CRC compared with healthy controls

Table 2: The percentages of *TUSC3* promoter methylation in case and control groups and their diagnostic value

Group	AM%	*P*	Cutoff	Sensitivity	Specificity	AM% in I	AM% in II	AM% in III	AM% in IV	*P*
Case (*n*: 70)	43.55±21.80	<0.001*	18.68%	88.6%	76.0%	44.76±24.40	36.68±18.90	45.44±18.96	57.57±23.60	0.088
Control (*n*: 70)	16.07±13.63					_	_	_	_	_

AM=Average methylation, I=Stage I, II=Stage II, III=Stage III, IV=Stage IV * *P*<0.05

of DNA methylation levels because it is a bisulfite conversion-independent method.

Some studies have shown that *TUSC3* is upregulated in CRC and has an oncogenic function.[45,46] In this context, previous studies have suggested that in CRC, *TUSC3* could promote the formation of cellular stemness and induce drug resistance via the Hedgehog signaling pathway.[47] Besides, it has been revealed that this gene acts as an oncogene in the progression of CRC, leading to worse survival.[45] However, hyper-methylation of *TUSC3* has been reported in numerous studies and various types of tumors.[48] One study on clear cell renal cell carcinoma (ccRCC) revealed that higher expression of *TUSC3* is correlated with poor prognosis.[49] In CRC, hyper-methylation of *TUSC3* has been reported in cancer tissue, adenomatous colorectal polyps, and ulcerative colitis compared with normal colon, suggesting that *TUSC3* hyper-methylation might be an early event in CRC.[14,50,51] Moreover, partial methylation of this gene in normal cells progressively increases with age.[52] Previously, quantitative detection of *TUSC3* promoter methylation in tumors and serum samples demonstrated its robust diagnostic and prognostic power in different malignancies including ccRCC, lung, ovarian, and prostate cancers.[25,49,53,54]

Regarding the appropriate cutoff point, our findings indicated that 88.6% of patients with CRC and 76.0% of the disease-free samples were correctly detected (corresponding to sensitivity and specificity, respectively). The ROC curve analysis demonstrated that *TUSC3* promoter methylation poses a very high accuracy (AUC: 0.880, $P < 0.001$) for differentiating CRC individuals from controls [Figure 2].

In a similar study, Yuasa *et al*. discovered that the methylation level of *TUCS3* in the DNA of leukocytes was higher in cases with gastric cancer compared with normal subjects, although this difference was not drastic. Moreover, they unraveled that the methylation level of *TUSC3* is increased with smoking and age.[8] Along with this evidence, two other studies demonstrated that *TUSC3* in peripheral blood leukocytes is highly methylated during aging.[55,56] To prevent the putative effect of age on methylation level, we matched the age of the case and controls. Also, there was no significant difference between CRC patients and controls in terms of smoking status [Table 1]. In the other research, Zhang and colleagues showed that hyper-methylation of *TUSC3* in blood leukocytes was associated with a higher risk of gastric cancer in the Chinese population.[52]

Collectively, the results of our work and previous studies emphasize that PBMCs are a promising source for DNA methylation assessment for cancer diagnostic purposes. However, the influence of intervening factors such as age and smoking status should also be considered. Moreover, to the best of the authors' knowledge, this is the first study demonstrating the usage of *TUSC3* promoter methylation status in PBMCs as a novel promising biomarker that holds high power and accuracy in distinguishing CRC patients from healthy individuals in the early stages of the disease. Limitations of the present study were the relatively small sample size for detecting PBMC *TUSC3* methylation and the low number of the evaluated genes. Therefore, further comparative studies with larger sample sizes and various genes are required to corroborate the presented results.

Ethical approval

This study was approved by the ethics committee of AJA University of Medical Sciences.

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Conflicts of interest

There are no conflicts of interest.

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