# Epigenetic Inactivation of Protocadherin 10 by Methylation in Colorectal Cancer

**Sahar Tavakolian[1,2], Matineh Barati Bagherabad[1,3], Dor Mohammad Kordi-Tamandani[2,7], Mohammad Reza Abbaszadegan[1,4,5], Mohammad Amin Kerachian[1,4,6]**

[1] *Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*

[2] *Department of Biology, University of Sistan and Balouchestan, Zahedan, Iran*

[3] *Department of Biology, Ashkezar Branch, Islamic Azad University, Ashkezar, Yazd, Iran*

[4] *Department of Medical Genetics, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

[5] *Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*

[6] *Cancer Genetics Research Unit, Reza Radiotherapy and Oncology Center, Mashhad, Iran*

[7] *Genetics and Biotechnology Research Institute, University of Sistan and Baluchestan, Zahedan, Iran*

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**Abstract**- Aberrant promoter methylation of CpG islands of tumor-suppressor genes has been recognized as one of the important tumor markers for cancer detection. The aim of this study was to investigate the promoter methylation status of protocadherin 10 (*PCDH1*0), a tumor suppressor gene, in Iranian colorectal cancer (CRC) patients. Cancerous and the adjacent normal tissues obtained from 38 CRC patients were used to assess the methylation status of *PCDH10* with Methylation Specific PCR, in addition, to study the expression level of this gene by quantitative PCR. The relationship between hypermethylation and the demographic characteristics of these patients was analyzed. The promoter methylation level of *PCDH10* was statistically different between tumoral and normal tissues in CRC patients. Twenty-seven out of 38 patients showed hypermethylation with a sensitivity of 73% and a specificity of 97%. *PCDH1*0 expression decreased in 15 cases (46%) as 16 cases (50 %) showed overexpression and 1 case (4%) had no changes. Not a significant association was reported between *PCDH10* hypermethylation and the clinicopathological characteristics ($P>0.05$). Our results indicated that *PCDH10* methylation has a critical function in CRC, with a nearly elevated sensitivity and a high specificity in the Iranian population, qualify it as a potential candidate biomarker.



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**Keywords:** Protocadherin 10; PCDH10; Methylation; Gene expression; Colorectal cancer

## Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers, and it is the second cause of cancer death in the world (1,2). In Iran, it is the fifth most common type of cancer after skin, stomach, bladder, and prostate in men and third cause after skin and breast in women (3). Recent studies have shown that the incidence of CRC in the past three decades is increasing in Iran (4,5).

If cancer is diagnosed at its early clinical stages, i.e., stages I and II, it is curable in 90% and 75% of cases, respectively (6). The early detection of CRC through early detection and removal of adenomatous polyps prevents CRC formation and reduces the incidence and mortality rate of this cancer (7,8).

Although colonoscopy is the "gold standard" for CRC diagnosis, it is extremely costly, invasiveness and requires extensive preparation of the bowel (9,10). Currently, a non-invasive and low-priced procedure, guaiac fecal occult blood testing (gFOBT), is recommended for CRC detection, but its potential disadvantage is the low detection rate for early-stage tumors and precancerous lesions (11,12). Therefore, there is a requirement for sensitive and specific diagnostic methods for the early detection of CRC (13). In this regard, understanding the molecular pathogenesis of CRC could lead us to discover a better detection tool.

It is known that CRC develops through the

**Corresponding Author:** M.A. Kerachian
Department of Medical Genetics, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
Tel: +98 51 38002247, Fax: +98 51 38002247, E-mail addresses: amin.kerachian@mail.mcgill.ca, kerachianma@mums.ac.ir

accumulation of molecular alterations that lead to the conversion of normal colonic epithelial cells to adenocarcinomas (14). Among these alterations, epigenetic changes play a critical role in the development of cancer (15). Aberrant hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes has been recognized as one of the hallmarks of cancer that results in transcriptional silencing of corresponding tumor suppressor genes in CRC and other cancers (8). This event is considered as one of the most frequent alterations for gene inactivation in cancer (16). The feasibility of using DNA methylation changes as biomarkers for diagnosis and prognosis of cancer has been shown in several studies (17). Thus, promoter hypermethylation of CpG islands could be a promising candidate for early detection of cancer specifically CRC (16,17). Recent studies have demonstrated hypermethylation of protocadherin 10 (*PCDH10*, also termed *OL-PCDH* or *KIAA1400*) in several cancers such as lymphoid malignancies, prostate cancer, lung cancer, gastric cancer, bladder cancer, and CRC (18-23).

Cadherins are glycoproteins of transmembrane, which comprise a large superfamily that consists of classic cadherins, desmosomal cadherins, protocadherins, atypical cadherins, and cadherin-related neuronal receptors (24,25). The majority of them play important roles in the calcium-dependent homophilic cell-cell recognition and adhesion (26,27). Among cadherins, *PCDH10* belonging to the protocadherin gene subfamily is located at chromosome 4q22.2 (27). In 2013, Zhong and his colleagues indicated that *PCDH10* functions as an important tumor suppressor gene that inhibits cell proliferation and invasion of tumor cells (23). As a result, *PCDH10* methylation may be a novel diagnostic biomarker for CRC.

In the current study, we studied the methylation status and gene expression of *PCDH10* in tissue samples from CRC patients obtained from colorectal surgery, using Methylation Specific PCR (MSP) and quantitative PCR, respectively. In the case of tissue *PCDH10* hypermethylation, the *PCDH10* DNA methylation status of bodily fluids such as blood, stool, serum, and urine from cancer patients, maybe an attractive target for the future development of a detection assay for CRC among Iranian population.

## Materials and Methods

### Patients and collection of tissue DNA samples

Human tissue samples were collected from 76 CRC tumor and control samples (38 individuals including 38 fresh cancerous and 38 adjacent normal tissues) who had surgery in three Mashhad hospitals (Emam Reza, Ghaem and Omid Hospital Centers), Iran. Informed consent was obtained from every subject prior to the study. Inclusion criteria were primary CRC patients (all stages of cancer) and exclusion criteria were insufficient or unavailable tumor tissue sample, insufficient clinical information, and family history of CRC. Tumor characteristics such as location, size, and stage, as well as age, sex, and other necessary information were recorded in a questionnaire and presented in table 1. Approval of this study was obtained from our local ethics' committee, Mashhad University of Medical Sciences, Mashhad, Iran. Pathology slides from the surgical resection were recalled to confirm the diagnosis and tumor content of at least 70% of tumor cells in the tissue samples. The selection of CRC controls was based on the adjacent side of each sample with a minimum distance of 10 cm, which did not have any tumor. For DNA extraction from tumors, only the sections with confirmed CRC were used.

**Table 1. Clinical characteristics of studied subjects**

| | | |
|---|---|---|
| **Age (Mean±SD)** | | **57.73±15.02-year-old** |
| **Sex** | Female | 17 (40.5%) |
| | Male | 25 (59.5%) |
| **Grade** | 1 | 18 (42.8%) |
| | 2 | 23 (54.8%) |
| | 3 | 1 (2.4%) |
| **Location** | Proximal | 8 (19%) |
| | Distal | 34 (81%) |
| **Stage** | I | 4 (9.5%) |
| | IIA | 26 (61.9%) |
| | IIIA | 2 (4.1%) |
| | IIB | 1 (2.4%) |
| | IIIB | 6 (14.3%) |
| | IIIC | 3 (7.1%) |
| **T** | 1 | 1 (2.4%) |
| | 2 | 11 (26.2%) |
| | 3 | 25 (59%) |
| | 4 | 5 (11.9%) |
| **N** | 0 | 27 (64.3%) |
| | 1 | 12 (28.6%) |
| | 2 | 3 (7.1%) |

T: the size of the primary tumor and whether it has invaded nearby tissue, N: regional lymph nodes that are involved (TNM Classification of Malignant Tumors)

**DNA isolation from tissue samples**

DNA was isolated from colonic tissues (20 mg) by using the GeNet Bio tissue kit (Daejeon, Korea) according to the manufacturer's instructions and it was quantified by NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

**RNA isolation from tissue samples**

RNA from tissue samples was extracted with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and it was measured by NanoDrop1000 (Thermo Fisher Scientific, Waltham, MA, USA).

**cDNA synthesis and real time PCR**

Reverse transcription was performed on 4 µg total RNA using the RT kit (Pars Tous, Mashhad, Iran). The reverse transcription-PCR primers of *PCDH10* were as follows for forwarding strand 5'-TCCAACGAGACTAAACAC-3' and for reverse strand; 5'-GAGAAGAGATCCATACCAG-3'. GAPDH was used as an internal reference gene for normalizing the cDNA input.

The primers for *GAPDH* were 5'-CATGTTCGTCATGGGTGTGAAC-3' (forward) and 5'-CACAGTCTTCTGGGTGGCAG-3' (reverse). The product length of *PCDH10* and *GAPDH* was 191 and 178 bp, respectively. The mRNA expression ratio of *PCDH10* was defined as the ratio of the fluorescence emission intensity value of *PCDH10* reverse transcription-PCR products to that of *GAPDH* PCR products multiplied by 100. Real time PCR assays were done in a reaction volume of 10 µl SYBR Green (Ampliqon SYBR Green PCR Master Mix), 0.3 µl each primer (10 pmol/µl), 6.4 µl DEPC water, and 3 µl cDNA under the following conditions: 95º C for 15 minutes by 1 cycle followed by 95º C for 30 seconds and 57º C for 1 minute for 40 cycles. Amplification was performed in 96-well plates. Each plate consisted of cDNA samples and multiple water blanks, as well as positive and negative controls. Separate amplification assays were done for *PCDH10* and *GAPDH*, and each assay was done in triplicate following MIQE guidelines.

**Bisulfite modification**

DNA was chemically modified by sodium bisulfite to convert all unmethylated cytosines to uracils while leaving methyl cytosines unaltered. From each sample, 100 ng DNA was bisulfite-treated with EpiTect Bisulfite Kit (Qiagen, Germany) and eluted in 70 µL of elution buffer according to the manufacturer's protocol. CG Genome Universal Unmethylated DNA (S7822; Vial A; Millipore, Billerica, Massachusetts) and CG Genome Universal Methylated DNA (S7821; Millipore, Billerica, Massachusetts) were used as universal negative and positive controls, respectively in our study. One microliter bisulfite-treated DNA was used as a template for methylation quantification with fluorescence-based PCR (MSP).

**Methylation-specific PCR (MSP)**

The bisulfate-modified DNA was used as a template for MSP as described. Proper positive controls were included in each batch of PCR reactions. Methylated and unmethylated primer sequences used in this study, as well as the annealing temperature and product sizes, are given in table 2. For the MSP, 1 µL of bisulfite-converted DNA was used in each amplification reaction. PCR was performed in a reaction volume of 25 µL consisting of 18.3 µL $ddH_2O$, 2.5 µL 10X PCR buffer, 1.5 µL $MgC_{l2}$ (25 mM), 0.3 µL dNTP mixture, 200 nM of each forward and reverse primers, and 1 unit of Hot-Start Taq DNA Polymerase (2/5U/µL) (ParsTous, Mashhad, Iran). Thermal cycling profile performed as follow: a hot-start at 94° C for 10 min, followed by 35 cycles at 94° C for 30 sec, specific annealing temperature (56° C for methylated primer pairs and 51° C for unmethylated primer pairs) for 30 sec, and a final extension at 72° C for 10 min. To validate the results all PCR reactions have been performed triplicated. The MSP products were then analyzed by 2% agarose gel electrophoresis stained with ethidium bromide and visualized under ultraviolet illumination. Samples were scored as methylation-positive when methylated alleles were visualized as bands in the methylated DNA lane or both in methylated and unmethylated DNA lanes, and scored as methylation-negative when bands were seen only in the unmethylated DNA lane.

**Statistical analysis**

Statistical analyses were performed using statistical package using the SPSS for Windows™, version 15 software packages (SPSS Inc., Chicago, Illinois, USA). The data were analyzed systematically. Numerical data were expressed as Mean±SD. Pearson chi-square ($\chi 2$) or Fisher's exact test was used to assessing the relationship between PCDH10 methylation and clinical features. A two-sided $P<0.05$ was considered statistically significant.

**Table 2. *PCDH10* gene primers sequences, annealing temperature and product size for MSP assays**

| Primer | Sequences (5 ' -3 ' ) | Annealing temperature | Product size |
|---|---|---|---|
| **PCDH10 -MF** | TCGTTAAATAGATACGTTACGC | 56ºC | 152bp |
| **PCDH10 -MR** | TAAAAACTAAAAACTTTCCGCG | 56ºC | 152bp |
| **PCDH10 –UF** | GTTGT TAAATAGATATGTTATGT | 51ºC | 154bp |
| **PCDH10 -UR** | CTAAAAACTAAAAACTTTCCACA | 51ºC | 154bp |

## Results

**Detection of methylated *PCDH10* gene in colorectal tissue samples**

We examined the hypermethylation of the *PCDH10* gene in the DNA from 38 tumor tissues and 38 tumor-adjacent normal tissues by MSP. Patients were ranged from 27 to 86-year-old. In the aspect of CRC tumor grade, 30 and 12 samples were well and moderately differentiated, respectively. The other studied variables are present in table 1.

Our analysis showed that in 37 normal samples, *PCDH10* was unmethylated, and in only one sample, it was methylated, while in tumor tissues, there were 11 unmethylated and 27 methylated statues. Thus, *PCDH10* methylation showed a sensitivity of 73% (31 from 38 samples) and a specificity of 97% (27 from 38 samples).

Subsequently, we investigated the features association between *PCDH10* methylation and clinicopathological features in CRC patients, and the results are shown in table 3. This table shows that the relation of age and methylation of *PCDH10* in normal and tumor tissues is not significant ($P$>0.05) and the difference between methylation statuses between two genders was not significant, although most of the methylated and unmethylated tumors were in males (68%).

**Table 3. The main characteristics of patients with CRC and the relationship between *PCDH10* methylation and clinicopathologic parameters represented by the *P*-value (n=38).**

| | Sex | Location | Grade | T | N | Age | Size | Stage | Node involvement | Fold Change |
|---|---|---|---|---|---|---|---|---|---|---|
| **Normal tissue** | 0.71 | 0.48 | 0.68 | 0.21 | 0.83 | 0.39 | 0.19 | 0.570 | 0.92 | 0.44 |
| **Tumoral tissue** | 0.95 | 0.31 | 0.86 | 0.16 | 0.13 | 0.70 | 0.83 | 0.59 | 0.44 | 0.70 |

The difference between methylation statuses and three stages of the tumor was also not significant, although most of the methylated tumors were related to stage IIA. Besides, this difference was not significant in the normal group, as most of the specimens were unmethylated compared to methylated status. There was no significant difference between the methylation status and location, differentiation of tumors and number of involved lymph nodes in both groups of tumor and controls ($P$>0.05).

**Expression of *PCDH10* level**

We classified the expression to low expression (under -2 fold changes), normal expression (between -2 folds and +2 folds), and high expression (upper +2 fold changes). So our samples were in 15 (46%), 16 (50%) and 1 (4%) related to low, normal and high expressions, respectively. Our analysis indicated lower expression of *PCDH10* in methylated samples although the differences were not significant ($P$>0.05).

## Discussion

Recent studies in Iran have shown that the incidence of CRC is significantly increasing over the last three decades (28) and its age distribution is lower than those reported from Western countries (5,29). The detection of CpG island methylation in human DNA has been suggested as a hopeful way for noninvasive screening and early diagnosis of colorectal neoplasia (30). For the first time in Iran, we examined methylation status and expression of the *PCDH1*0 gene in the tumor and the coupled normal adjacent tissues in patients suffering from CRC. New investigations have revealed that *PCDH10* is down-regulated and hypermethylated in various human cancers (21). Moreover, obligatory

overexpression of *PCDH10* was capable of repressing the growth of several human cancers including nasopharyngeal, esophageal cancer, and colon cancer cells (31).

According to our findings, there is a significant difference between the methylation status of promoter *PCDH10* in tumor tissues and adjacent normal tissues in patients with CRC. Our study showed hypermethylation of the *PCDH10* promoter, causing gene silencing, occurs in 27 out of 38 in human CRC but hardly in their adjacent non-tumor tissues (1 out of 38). Our study presented that *PCDH10* methylation had a sensitivity of 73% (31 out of 38 samples) and specificity of 97% (27 out of 38 samples).

There are several studies regarding the methylation of *PCDH10* in CRC. In 2013, Zhong *et al.,* indicated that 100 % (8 out of 8) of CRC cell lines were silenced for *PCDH1*0, but not in normal colorectal epithelial cells. Demethylation treatment confirmed that the reduced expression is associated closely with its promoter methylation. Moreover, CpG methylation of *PCDH10* was also detected in 85 % of primary colorectal tumors, but not in adjacent normal colorectal tissues. They proposed that *PCDH10* as a tumor suppressor gene has critical roles of inhibiting cell proliferation, clonogenicity, and suppressing the invasion in the development of CRC (23). In 2013, Danese *et al.,* used a quantitative methylation-specific PCR to investigate a chosen CpG site in the *PCDH10* promoter of 67 tumor tissues, paired normal mucosae and plasma samples. The results demonstrated that *PCDH10* promoter methylation was detected in 63 out of 67 (94.0%) surgically resected colorectal tumors and in 42 out of 67 (62.7%) plasma samples. Additionally, in the current study, we compared *PCDH10* expression in paired colorectal tumors and their adjacent non-tumor tissues by qPCR. We found that *PCDH10* expression was reduced or silenced in 15 cases (49%), overexpressed in 16 cases (50%) and no changes in *PCDH10* expression in 1 case. The expression of *PCDH1*0 gene was silenced or markedly down-regulated in similar studies. Jao TM *et al.,* showed *PCDH1*0 down-regulation in 41 of 53 colorectal carcinomas compared with their matched normal mucosae (32).

To sum up, our study for the first time, reveals a higher methylation rate of *PCDH1*0 in CRC tumors in the Iranian population. We recommend further studies to be performed particularly prospective cohort studies which could help determine how this noninvasive diagnostic tool might improve colonoscopy yield and patient outcomes and potentially lower healthcare costs.

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