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Original Article

NDRG4 Methylation Change: A Promising Biomarker in Colorectal Cancer Diagnosis

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Abstract

Introduction: Colorectal cancer (CRC) is one of the most widespread Cancers across the world. Notwithstanding the diagnoses and treatments developed over the past four decades, patients' survival rate has improved moderately; and yet, it still has a 5-year survival rate of less than 50%.
In this study, hypermethylation of the NDRG4 gene was evaluated as a biomarker in CRC screening.

Materials and Methods: 70 samples were examined in this case-control study (45 CRC patients versus 25 healthy controls) totally, and Methylation-Specific PCR (Polymerase Chain Reaction) technique was used to investigate the NDRG4 methylation situation in plasma samples.

Results: The mean age in the control group and CRC patients was 58.4±3.4 years and 64.6±4.4 years, respectively. The male to female ratio in the control group and CRC patients was 1.5:1 and 1.1:1, respectively. Gastrointestinal disease history was positive in 12% and 33% of patients in the control group and CRC patients. In this study, NDRG4 gene hypermethylation was observed in 53.3% of patients, while it was shown in only 23.3% of healthy controls.

Conclusion: The results showed that NDRG4 could be a prognostic biomarker in CRC diagnosis and screen as a noninvasive blood-based biomarker.

Keywords: Colorectal Cancer, Methylation, Screening, NDRG4

1. Introduction

Colorectal cancer (CRC) is considered as the third of the most common and fatal cancers in the world [1]. This cancer originates from the internal mucosa of the colon [2]. Most colon and rectal cancer tumors grow slowly and may not develop symptoms until they reach an enormous size and eventually become dangerous [3]. CRC originates from an adenomatous polyp or flat tissue dysplasia with a background of inflammatory bowel disease. High-grade dysplasia in the backbone of adenomatosis most likely results in invasive adenocarcinoma. The invasion of the lamina propria and mucosa layer rarely results in metastasis to the lymph nodes. The factor that leads to invasive adenocarcinoma is the involvement of the submucosal layer [4, 5].
Colon cancer is currently the most common cancer recognized across the world.

Although significant improvement in diagnoses and treatments occured over the past four decades, and the survival rate of patients has improved to some extent, it still has a 5-year survival rate of less than 50%. The most important prognostic factor in colorectal cancer is the stage of the disease at diagnosis, and it is said to be the most effective way to improve the prognosis of the disease early on in its progression. Unfortunately, symptomatic colorectal cancer presents the symptoms when the disease has reached its advanced stages; hence, early detection and population screening for at-risk or early-stage patients is an effective way to reduce the incidence of the disease [6, 7].

The NDRG4 gene is a member of the NDRG gene family, including the other three NDRG1-3 genes, with approximately 60% homology between the products. NDRG4 is a candidate gene with a tumor suppressor role located in the 16q21-q22.3 chromosome region. Studies of mouse embryos have shown that under conditions of ventricular hypoplasia, NDRG4 gene expression is reduced, indicating the role of this gene in cell growth and proliferation. NDRG4 gene is hypermethylated in colorectal cancer. This methylation change suggests that this gene may be involved in the pathway of carcinogenesis and the development of colorectal cancer. A large number of studies have shown that the NDRG4 gene plays a role in tumor suppression in a variety of human cancers is increasing. Given the previously shown role of the NDRG2 gene in tumor suppression of colorectal cancer, and as NDGR4 has more than 60% similarity to the amino acid sequence of NGDR2, evidence can be drawn for the role of NDRG4 gene in tumor suppression of colorectal cancer [8, 9].

In the present study, considering the importance of early detection of colorectal cancer, the NDRG4 gene was selected as to investigate the promoter methylation changes. The promoter methylation of this gene was evaluated qualitatively by means of Methylation-Specific Polymerase Chain Reaction (MSPCR) in plasma samples of patients with colorectal adenocarcinoma compared to the control group.

2. Materials and Methods

2.1. Study Design and Patients:

In this study, 45 patients with colorectal cancer and 25 healthy controls (with no evidence of disease) were enrolled. After getting a written informed consent from the participants, a questionnaire was filled by every participant to obtain their demographic data, as well as their disease signs and conditions. Five milliliters of peripheral blood samples were collected from patients.

2.2. DNA Extraction:

DNA isolation performed in 500 µL plasma using the QIAamp DNA Blood Mini kit (Qiagen-Germany) and stored at -20°C.

2.3. Bisulfite Treatment:

As mentioned earlier, before performing MSP, DNA was treated with bisulfite to convert unmethylated cytosine to uracil [10].

2.4. MS-PCR:

Methylation-Specific PCR (MSP) was carried out to specify the methylation status of the gene. The NDRG4 gene primer sequences for the methylated and unmethylated templates were as follows:

Methylated (Forward):GGAGTTTAAATAAAGATTACGGTAGC,
methylated (Reverse): ATACGCTACGAAACCCTACC,
Unmethylated (forward): GGGAGTTTAAATAAAGATTATGGTAGT,
Unmethylated (Reverse): AATACACTACAAAACCCTACC.

NDRG4 PCR product size was 142 bp. Each PCR reaction mix included a total volume of 20 µl containing 10 µl hot start Taq Master Mix, 1 µM concentration of

each primer, 1 µl DMSO, 3 µl bisulfite-modified DNA and 5 µl RNase free water. The PCR temperature program is shown in table 1.

2.5. Gel Electrophoresis.

After PCR, a 3% agarose gel was used to manifest PCR products.

Table 1. PCR temperature program.

Stage	Temperature	Time	Cycles
Pre-incubation	94 °C	15 minutes	1
Amplification	94 °C	1 minutes	35
	59 °C	40 seconds	
	72 °C	40 seconds	
Final extension	72 °C	1 minutes	1

2.6. Statistical Analysis:

Two tests, including Fisher's exact test and $\chi 2$ test, were applied to investigate the statistical relationships between methylation situation of NDRG4 gene and pathological or demographical results and assess hypermethylation in patients. As the *P Value* was less than 0.5, it was considered significant.

3. Results

Demographic Data

Seventy individuals in two groups of CRC patients and healthy controls were enrolled in this study. Demographic data was as follows: The mean age in the control group and CRC patients was 58.4±3.4 years and 64.6±4.4 years, respectively. The male to female ratio in the control group and CRC patients was 1.5:1 and 1.1:1, respectively. Gastrointestinal disease history was positive in 12% and 33% of patients in the control group and CRC patients.

Status of Hypermethylation in Plasma Samples:

As shown in Table 2, 24 of 45 (53.3%) CRC patients and 6 of 25 (23.3%) healthy controls revealed hypermethylation in the NDRG4 gene. Gel electrophoresis result for the NDRG4 gene in 5 samples of CRC patients is shown in Figure 1. As displayed in Figure 1, there were 45 patient samples. Patients 1 and 5 have both methylated and unmethylated PCR amplification, which is interpreted as methylated heterozygotes. Only patient 2 can be interpreted as a methylated homozygote. Patients 3 and 4 can be interpreted as unmethylated homozygotes.

Table 2. Methylation status of NDRG4 in study population.

Group	Controls	CRC
Positive (Methylated)	6 (23.3%)	24 (53.3%)
Negative (Unmethylated)	19 (76.8%)	21 (43.2%)
Total	25 (100%)	45 (100%)

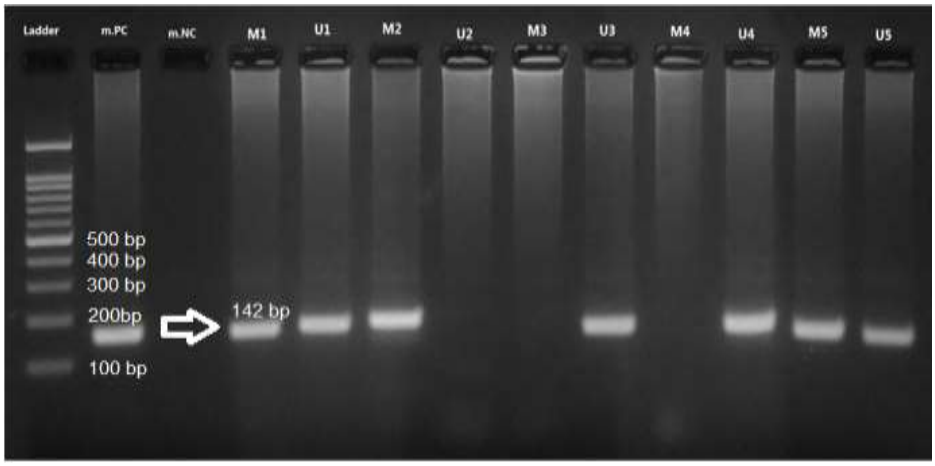

Figure 1. Gel electrophoresis result for NDRG4 gene. From the left, 100bp ladder, mP.C: positive methylated control, mN.C: M1-M5: patient samples with methylated primer. U1-U5: patient samples with unmethylated primer. PCR product: 142 bp.

4. Discussion

Among the different screening methods for CRC, markers that are examined using blood samples, given the ease of sampling, non-invasiveness, and relative patient satisfaction (compared to a procedure such as colonoscopy), seem to be the most suitable for biomarker presentation. Different serum and plasma markers have been studied in CRC research. One of the most well-known and proven of these markers is the SEPT9 marker, which has been discussed in several studies as a diagnostic biomarker [11, 12]. For the current research, the SEPT9 biomarker in the form of Epi proColon® commercial kits, ColoVantage®, has been used as CRC diagnostic kits by assessing methylated SEPT9 gene [13]. Imperial et al.[14] studied stool DNA testing in CRC screening. In this study, NDRG4 and BMP3 hypermethylation and fecal hemoglobin were examined. Based on the information obtained from this study, the rate of diagnosis of high-grade polyps and dysplasia using this panel was 62.9%. This study demonstrated that this method is suitable for population screening, given the non-invasive stool test [14].

There have been several studies that investigate the hypermethylation of the NDRG gene family; In the study of Liu et al.[15], the promoter methylation, mutations and genomic deletions of the NDRG2 gene were investigated in several cell lines from different cancers. The results of this study revealed that heterozygous deletion mutations, as well as promoter methylation of the NDRG2 gene, occur in a variety of cancers (including CRC) [15]. In the study of Melotte et al.[16], Methylation of the NDRG4 gene was evaluated in three CRC tissue samples, healthy colonic mucosal tissue and CRC cell lines using the MS-PCR technique. The results of this study indicated that 86% of the tissue cancers, and 70% of the cell lines showed hypermethylation in the NDRG4 gene. Furthermore, the expression of the NDRG4 gene was significantly decreased in cancer samples compared to healthy tissue [16].

The sample used in the present study was a blood plasma sample from patients; the most important reason for selecting a plasma sample was to find a non-invasive method for screening purposes. In recent years, tumor-associated cell-free DNA (cfDNA) in plasma and serum has been used as a non-invasive method for detecting cancers. This method is called liquid biopsy, which can detect genetic or epigenetic changes in cfDNA. In a study conducted by Ashoori et al.[17], epigenetic biomarker Bone Morphogenic Protein 3 for CRC screening was investigated. 75 percent of CRC patients showed hypermethylation

in the BMP3 gene in plasma samples. At the same time the percentage for the control group was only 30. In colonoscopy tissue biopsies, in 81.6% of CRC patients, BMP3 hypermethylation was showed. Specificity and sensitivity of this gene in CRC diagnosis were about 76% and 66%, respectively. [17].

In the present study, the mean age of CRC patients was 64.4 years, while in the control group, the mean age was 58.40 years. CRC has a direct relationship with age, and if age is considered as a risk factor, the risk increases with age. According to the Cancer Research Organization, the incidence of CRC is the most rapidly increasing age range of 50 to 54, reaching its highest level in people over the age of 75; 44% of the diagnosed CRC cases are patients aged 75 years and over; 54% of the patients in the present study were between the ages of 51 and 70, which is consistent with the Cancer Research Organization statistics.

5. Conclusion

In conclusion, the results of this study showed that NDRG4 can be a promising biomarker for the detection of adenocarcinomas of the colon and rectal and can be used as a noninvasive blood-based biomarker. Future studies can help achieve more promising results by investigating the relationship between the family record and environmental factors with epigenetic changes, especially gene methylation in colorectal cancer.

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Conflict of interest

The authors declare no conflict of interest.

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