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Evaluation of the Epigenetic Demethylation of *NRF2*, a Master Transcription Factor for Antioxidant Enzymes, in Colorectal Cancer

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Abstract

Background: Epigenetic changes in CpG islands of the promoter regions of homeostasis-related genes, including nuclear factor erythroid 2-related factor 2 (*NRF2*), have been shown to hold a significant role in the development of colorectal cancer. Therefore, we aimed to examine the DNA demethylation pattern of the *NRF2* promoter region in cancerous lesions from patients with colorectal cancer and the association of methylation status with clinicopathological features in the Iranian population.

Methods: In this cross-sectional study, 114 colorectal tissue samples were collected. These samples included: 34 tumour tissue samples, 60 precancerous polyps, and 20 normal tissue samples. The promoter methylation status of the *NRF2* gene was examined using methylation-specific PCR. Additionally, the relationship between the methylation status and the clinicopathological features was investigated.

Results: The frequency of *NRF2* demethylation in the tumour samples was significantly higher compared to the polyp tissues (p= 0.003) and normal tissue (p= 0.009), indicating that cancerous colorectal tissues exhibit increased demethylation of the *NRF2* promoter. After examining the demethylation status of tissue samples, the clinicopathological features were compared to the demethylation results. No significant association was found between *NRF2* promoter demethylation and the clinicopathological features of patient samples.

Conclusions: Our findings suggest that the epigenetic modifications leading to *NRF2* demethylation found in colorectal tumour samples may contribute to cancer progression from precancerous polyps to cancerous lesions.

Keywords: Colorectal Cancer, Epigenetic, Methylation-specific PCR, *NRF2*.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignant neoplasms, making it a major public health problem worldwide. Tumorigenesis begins as a polyp growing in the inner surface of the colon or rectum (1). These colorectal polyps are believed to be the main source of precancerous cells leading to the development of CRC (2). The mortality rate of CRC has reduced in developed countries as a result of early detection, polyp removal, and the treatment of precancerous polyps (3). However, the rates of CRC are increasing in developing countries, including Iran (4).

Recent research has explored the role of epigenetics in the development of CRC via genetic alterations of both oncogenes and tumour suppressor genes (5). Epigenetic modifications are defined as reversible heritable changes in gene expression that do not alter the primary DNA sequence (6). Various epigenetic mechanisms

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include DNA methylation/demethylation, chromatin remodelling, histone modifications, and non-coding RNAs (7).

The transcription factor, *NRF2* (nuclear factor erythroid-2-related factor 2), is a redox-sensitive master regulator that has been associated with antioxidant stress and drug metabolism. *NRF2* protects normal cells from oxidant stress and electrophilic attack generated by harmful exogenous agents (8). Additionally, NRF2 holds a significant role in cancer progression and dissemination by regulating the expression of numerous downstream genes involved in a wide range of biological processes, including cellular proliferation, survival, drug resistance, angiogenesis, and metastasis (9).

Current experimental evidence demonstrates that *NRF2* holds both protective and pathological roles in different human diseases and is associated with the development and progression of cancer, including breast (10) lung (11), and colorectal cancer (12). Therefore, regulation of *NRF2* expression is a critical target for cancer treatment and the prevention of cancer relapse. The regulation of *NRF2* activity has been shown to be influenced by epigenetic modifications (13).

Limited information exists on the epigenetic control mechanisms of colorectal polyps (14, 15).

Developing an understanding of the epigenetic mechanisms that underlie the pathological changes that occur in colorectal polyps would aid in creating effective screening tools for CRC.

The exact mechanisms involved in the epigenetic regulation of *NRF2* expression in the pathogenesis of CRC is essential for the development of safer and more effective strategies for CRC diagnosis and treatment.

In the present study, we hypothesized that the demethylation status of the *NRF2* promoter in colorectal polyps and cancerous lesions modifies *NRF2* expression and activity. This information can be used to aid in the development of a prognostic and diagnostic tools for CRC.

Materials and methods

Study Population and Sampling Procedure

This cross-sectional study was approved by the Ethics Committee of the Research Center for Gastroenterology and Liver diseases (RCGLD), Shahid Beheshti University of Medical Sciences (Ref No. IR.SBMU.RIGLD.REC.1395.925). Colorectal biopsies were obtained from individuals undergoing a colonoscopy at RCGLD of Shahid Beheshti University between 2015 and 2016. This study was performed on 114 fresh colorectal tissue samples, including 34 tumour tissue samples, 60 precancerous lesions and 20 normal tissue samples.

The demographic information of patients and clinical data was also retrieved (Table 1). All the tissues were histologically examined and classified by a pathologist as either polyp tissue, tumour tissue, or normal tissue. The grading and staging of tumour tissues were categorized according to the TNM (tumour, node, and metastases) staging system (16). Samples were snap-frozen and transferred to the laboratory in a liquid nitrogen dry shipper for further analysis.

Table 1. Frequencies of the demographic and clinicopathological characteristics of the studied Iranian patients with and without CRC.

Characteristic	Cases	Number (%)
Age (y) (mean ± SD)	61.20±10.61	
Gender	Male:58	50.9%
Age (y) (mean ± SD)	56.98±13.96	
Gender	Female: 56	49.1%
Age	<50: 23	20.2%
	>50: 91	79.8%
Smoking n (%)	NO: 96	84.2%
	YES: 18	15.8%
FH n (%)	NO: 94	82.5%
	YES: 20	17.5%
Diabets n (%)	NO: 97	85.1%
	YES: 17	14.9%
Location n (%)	Colon: 83	72.8%
	Rectum: 31	27.2%
IBD	NO: 101	88.6%
	YES: 13	11.4%
Stage	I: 8	23.5%
	II: 17	50%
	III: 9	26.5%

DNA Extraction and Methylation-Specific PCR (MSP)

Genomic DNA was extracted from frozen tissues using QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's protocol. Samples were then stored at −70 °C. The methylation status of the *NRF2* promoter region was determined using MSP technique. The primer sequences used are listed in Table 2.

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Table 2. Primer sequences for MSP analysis of *NRF2* promotor

Methylation status	Primer sequences	
	Forward	Reverse
Methylated	5′-AGGGAGGCGTAGTTTTTATATTAAC-3′	5′-AACTAAAATCCCAACAAACGAA-3′
Unmethylated	5′-GGAGGTGTAGTTTTTATATTAATGT-3′	5′-ACCAACTAAAATCCCAACAAACA-3′

Aliquots of the extracted DNA were bisulfite modified using Qiagen EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. MSP was performed on bisulfite-treated DNA of tumour, polyps and normal tissues. Briefly, PCR amplification with MSP primers was performed using 11 µl of Master Mix and 1.5 µl of bisulfite-converted DNA in a final reaction volume of 12.5 µL according to final volume detailed in Table 3. Human-control DNA sets, bisulfite-converted methylated and unmethylated DNA (Qiagen, Germany), were used for MSP reactions.

Table 3. Final concentration and volumes in the MSP master mix

Final Concentration	Volume/Reaction (µ L)	Reagent
10x MSP PCR buffer	1.25 µl	1x
Nuclease-free H2O	7.4 µl	-
Forward primer (M and U)	0.5 µl	0.1–0.5 µ M
Reverse primer (M and U)	0.5 µl	0.1–0.5 µ M
10mM dNTP mix	0.25 µl	200 µM of each dNTP
MgCl2	0.5 µl	1.5–5 mM
Q PCR buffer	2.5 µl	1x
HotStart Taq DNA Polymerase	0.1 µl	2.5 units/reaction
bisulfite-converted DNA	1.5 µl	1 µg/100 µL reaction
final reaction mixture	12.5 µl	-

The PCR was run for 34 cycles starting with initial denaturation at 95 °C for 5 min, ending with a final 10-min extension. Each PCR cycle was set as denaturation at 94 ºC for 40 sec, annealing at 57 °C for 35 sec, and extension at 72 ºC for 40 sec. The MSP products were analysed using 2% agarose gel electrophoresis, stained with a green viewer, and visualized with a UV transilluminator.

Statistical Analysis

Statistical analysis was performed using IBM SPSS statistical software, version 19 (SPSS Inc., Chicago, IL). Correlations were determined using Chi-square ($\chi 2$) test. Quantitative data was expressed as mean ± standard deviation (SD). Statistical significance was determined at $p< 0.05$.

Results

General Statistical Information

The mean age of patients with polyps, tumours, or normal tissue was determined to be 58.6±12.7, 61±13.7, and 57.3±9.2 years, respectively. The average BMI (kg/m) of patients with polyps, tumours, or normal tissues was 25.2±2.8, 24.9±2.9, and 26.3±3.8, respectively. Additional details of the demographic information of the patients and clinicopathological characteristics of tissue samples is summarized in Table 1.

Epigenetic analysis of NRF2 in the different types of colorectal specimens

The results reported in Table 4 reveal that the *NRF2* promoter was demethylated in most of the tumour samples (61.8%). In the colorectal polyps and normal colorectal tissue samples, the *NRF2* promoter was mostly methylated. Our findings indicate that the methylation status of *NRF2* was significantly dependent on type of the colorectal tissue sample examined ($p= 0.004$).

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Table 4. Correlations between *NRF2* gene methylation status and types of colorectal specimens.

Type of tissue	Methylation status		p value
	M	U	0.004*
Polyp	42 (70.0%)	18 (30.0%)	
Tumor	13 (38.2%)	21 (61.8%)	
Normal	15 (75.0%)	5 (25.0%)	

M: methylated U: unmethylated

*; According to $\chi 2$ test

Group comparison data on DNA methylation patterns in colorectal specimens

To further explore the differences in DNA methylation pattern of the *NRF2* promotor between tumour, polyp, and normal tissues, MSP results were pairwise compared (Table 5). A significant difference between the demethylation status of tumour and normal tissues was found (p= 0.009) and also between the polyp and tumour tissue samples (p= 0.003). These findings indicate that cancerous tissue had increased demethylation of the *NRF2* promoter region. However, no significant difference in methylation status was found between the polyp and normal groups (p= 0.66)

Correlations between the methylation of NRF2 promoter and Clinical Characteristics

As summarized in Table 6, no significant correlation was found between the methylation status of the *NRF2* promoter region and age, gender, BMI, FH, diabetes, HBP, or IBD.

Table 5. Group comparison data on DNA methylation patterns.

Comparison groups	Methylation Status		p value
	M	U	
Normal vs Tumor	15 (75.0%) vs 13 (38.2%)	5 (25.0%) vs 21 (61.8%)	0.009
Normal vs Polyp	15 (75.0%) vs 42 (70.0%)	5 (25.0%) vs 18 (30.0%)	0.66
Tumor vs Polyp	13 (38.2%) vs 42 (70.0%)	21 (61.8%) vs 18 (30.0%)	0.003

Table 6. Correlation of *NRF2* promoter methylation status and clinicopathological data.

	Methylation status		P-value
Variables	M	U	
Age (year)			0.13
Under 50	11 (15.7%)	12 (27.3%)	
Upper 50	59 (84.3%)	32 (72.7%)	
Sex (n%)			0.59
Female	33 (47.1%)	23 (52.3%)	
Male	37 (52.9%)	21 (47.7%)	
BMI (Kg/m2)			0.78
18 -23.9	19 (27.1%)	10 (22.7%)	
24 -29.9	40 (57.1%)	28 (63.6%)	
30 -35.9	11 (15.7%)	6 (13.6%)	
FH (n%)			0.71
No	57 (81.4%)	37 (84.1%)	
Yes	13 (18.6%)	7 (15.9%)	
Diabetes (n%)			0.43
No	61 (87.1%)	36 (81.8%)	
Yes	9 (12.9%)	8 (18.2%)	
HB (n%)			0.16
No	55 (78.6%)	39 (88.6%)	
Yes	15 (21.4%)	5 (11.4%)	
Smoking (n%)			0.12
No	56 (80.0%)	40 (90.9%)	
Yes	14 (20.0%)	4 (9.1%)	
Position (n%)			0.65
Colon	52 (74.3%)	31 (70.5%)	
Rectum	18 (25.7%)	13 (29.5%)	
IBD (n%)			0.53
No	61 (87.1%)	40 (90.9%)	
Yes	9 (12.9%)	4 (9.1%)	

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Discussion

Epigenetic modifications influence the activity of several genes involved in the pathogenesis of cancer. Therefore, the role of epigenetic mechanisms should be considered in cancer diagnosis, prognosis, and therapy (17). The methylation of CpG islands in the promoter region of a gene can lead to chromatin remodelling which interferes with the availability of the transcription apparatus, therefore altering gene expression (18). Research suggests that changes in the promoter region of genes, such as those involved in the oxidative stress homeostatic pathway, is related to an increase in mortality rates among patients with CRC (19, 20).

One of the major mechanisms involved in cellular defence against electrophilic and oxidative stress is the *NRF2-KEAP1* pathway. This pathway is necessary to maintain homeostasis through the activation of antioxidative enzymes (21). There are several cytoprotective and antioxidant genes controlled by *NRF2* activity, which is a basic leucine zipper (bZIP) transcription factor (22).

Growing evidence suggests that *NRF2* plays a fundamental role in both the prevention and pathogenesis of cancer. *NRF2* not only protects normal cells from being transformed into cancer cells, but also protects cancer cells from oxidative stress (23). This dual role of *NRF2* increases the survival rate of cancer cells and enhances cancer progression (24). However, the molecular mechanisms responsible for the role of *NRF2* have yet to be fully elucidated. *NRF2* over-activity has been detected in different types of malignancies (24-26). Considering the fact that *NRF2* has a potential role in CRC development and progression, effective diagnostic and therapeutic approaches depend on a better understanding of *NRF2* epigenetic regulation (27).

In the current study, we hypothesized that DNA demethylation of *NRF2* could lead to the development of CRC. The promoter region of *NRF2* was selected for MSP analysis. This region contains CpG islands near the transcription start site. Therefore, the demethylation of the *NRF2* gene could result in carcinogenesis.

Our findings show that demethylation of the *NRF2* promoter region promotes the development of CRC. Accumulating evidence indicates that epigenetic modifications to the promoter of *NRF2* contribute to the activation of *NRF2* in different cancers (28, 29). Although in certain types of human cancers such as prostate cancer, decreased *NRF2* expression and tumour aggressiveness could also be correlated with *NRF2* promoter hypermethylation (28). Nevertheless, previous research has reported increased activity of *NRF2* in colorectal tumours that leads to the overexpression of proteasome subunits and consequently increased proteasome activity (30). Recently, the overexpression of *NRF2* due to DNA demethylation of the *NRF2* promoter region was found in drug-resistant colon cancer cells (31).

According to our results, *NRF2* promoter demethylation is associated with the pathogenesis of CRC. Furthermore, the *NRF2* promoter exhibited increased demethylation in tumour tissues compared to the precancerous polyps and normal tissues.

In conclusion, our results provide further insight into the role of epigenetic changes underlying tumour progression from colorectal polyps to cancer. It is suggested targeting *NRF2* could be a potential strategy to prevent or treat CRC. Further investigation into the epigenetic regulation of the *NRF2* gene should consider disease clinical context such cancer stage. Additionally, future research should focus on developing fast and non-invasive diagnostic methods, such as those using peripheral blood or stool.

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