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The NRF2-KEAP1 pathway and epigenetic control in colorectal cancer: A promising prognostic and predictive biomarker

Zahra Taheri[1], Shiva Irani[1*], Hamid Asadzadeh Aghdaei[2]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Basic and Molecular Epidemiology of Gastroenterology Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
*Correspondence emails: shi_irani@yahoo.com; hamid.assadzadeh@gmail.com

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Abstract

In colorectal cancer (CRC), important genes in the *NRF2/KEAP1* pathway may undergo methylation, yet the exact genes that control this pathway in CRC are not entirely understood. Targeting the *NRF2/KEAP1* pathway could be a valuable therapeutic strategy for colorectal cancer. Our research examined the methylation and expression of the *KEAP1* gene in precancerous lesions and colorectal cancer, as well as investigated this pathway in the HT-29 cell line for potential CRC treatment. In this study, 208 frozen colorectal tissue samples were analysed, including 34 tumours, 60 precancerous lesions with adjacent normal tissues, and 20 normal tissue samples. The methylation-specific PCR and quantitative PCR were used to examine *KEAP1* gene promoter methylation, and *NRF2* and *KEAP1* gene expressions, respectively. Furthermore, the HT 29 cell line was treated with 5-Azacytidine, followed by quantitative real-time PCR to evaluate post-demethylation *KEAP1* and *NRF2* mRNA expression. Unlike *NRF2*, our research revealed a notable reduction in *KEAP1* gene expression in CRC tissues compared to adjacent normal tissues (p=0.02). Intriguingly, treating the HT-29 cell line with 5-Azacytidine led to a significant increase in *KEAP1* gene expression compared to untreated cells (P=0.03), with no significant alteration observed in NRF2 gene expression. Examination of CpG islands within the KEAP1 gene promoter indicated extensive hypermethylation in 58.8% of tumour tissues compared to adjacent normal samples. These findings indicate that decreased *KEAP1* gene expression resulting from promoter hypermethylation could interfere with the *NRF2/KEAP1* pathway, a crucial mechanism in colorectal carcinogenesis. The study proposes that *KEAP1* may play a significant role in the progression of CRC and could potentially serve as a biomarker for diagnosing and monitoring therapy in CRC.

Key words: *NRF2-KEAP1* Pathway, Epigenetic Control, Colorectal Cancer, Colonic Polyps

1. Introduction

Colorectal cancer (CRC) is the leading type of cancer worldwide that develops from malignant cells in different anatomic locations of the colon or rectum throughout the intestinal tract (Alzahrani et al., 2021).

Colonic polyps and chronic inflammatory conditions such as ulcerative colitis are said to be the primary causes of precursors for colorectal cancer generation (Altobelli et al., 2017; Grady & Markowitz. 2015). To date, limited data on epigenetic control mechanisms of colorectal polyps are available (Rezapour et al., 2019). Realizing the occurrence and underlying epigenetic procedure of pathological changes in colorectal polyps helps explain the effectiveness of a CRC screening program. Thus, renewing recent knowledge of colorectal polyps and CRC is essential. The mortality rate of colorectal cancer (CRC) has decreased in industrialized nations due to advancements in early detection, polyp removal, and treatment of precancerous lesions. However, this trend is on the rise in developing countries like Iran.

DNA methylation is a process whereby different tumour suppressor genes (TSGs) are inactivated in human tumours (Wang et al., 2019). Previous studies have shown that epigenetic modifications of essential genes like aberrant CpG methylation have important roles in CRC progression. It has been demonstrated that DNA methylation plays a critical function in suppressing the expression of nearby genes (Luo et al., 2014; Tokarz et al., 2017).

In mammalian cells, epigenetic regulation mechanisms, including DNA methylation, commonly occur in CpG islands, which are usually enriched in the promoters of genes (Zhang & Pradhan. 2014).

Many different genes are commonly methylated in various cancer types and could be used as epigenetic markers in the early detection of cancers. In 2010, several DNA methylation markers were reviewed as early detection markers for CRC (Azuara et al., 2010). Epigenetic modifications such as cytosine methylation in CpG-rich regions modulate gene expression without changing the linear sequence of DNA, which may also contribute to disease pathology and could be used as a diagnostic tool for early detection of CRC or be helpful in the assessment of clinical response to the CRC treatment. Early detection of precancerous polyps in the large bowel and their clinical management are the most essential purposes of colorectal cancer prevention and treatment of premalignant lesions. Finding novel molecular targets for earlier diagnosis of these lesions may be precursors for colorectal cancer. There is an urgent need to develop novel, safer, and more effective strategies.

The *NRF2/KEAP1* signalling pathway is crucial in protecting cells from oxidative damage and influences cancer progression and treatment resistance (Tossetta et al., 2023). Its modulation by natural and synthetic compounds affects cancer cell survival and therapy resistance (Tossetta et al., 2023).

Additionally, the pathway's role in various cancerous and non-cancerous diseases underscores its broad significance in cellular protection and disease modulation (Tossetta et al., 2023; Tossetta et al., 2023).

Nuclear factor-erythroid 2 (*NRF2*) is a transcription factor that regulates intracellular redox homeostasis by inducing transcription of various genes involved in protecting cells from oxidative stress and damage (He et al., 2020). Furthermore, *NRF2* plays a crucial role in cancer progression and metastasis by regulating the expression of numerous downstream genes involved in diverse biological processes, such as proliferation, survival, drug resistance, angiogenesis, and metastasis (Schmidlin et al., 2021). *KEAP1* contrarily controls *NRF2*. *KEAP1* is modified on its cysteine residues upon cell exposure to oxidative stress, such as reactive oxygen species (ROS). This alteration impedes the rapid degradation of *NRF2*, leading to the accumulation of *NRF2*, which then translocates into the nucleus, promoting the active transcription of downstream genes (Suzuki & Yamamoto. 2015).

Recently, Wang et al. reported that the expression of *KEAP1* is unexpectedly silenced due to CpG-island promoter hypermethylation in specific lung cancer cell lines and tissues (Wang et al., 2008). Besides, CpG methylation patterns of the *KEAP1* promoter region have been detected in some tumours, such as malignant glioma and breast cancer (Barbano et al., 2013; Muscarella et al., 2011).

Nonetheless, the methylation stage of *KEAP1* in colorectal polyps and colorectal cancer has not been interpreted.

According to the high morbidity and mortality rates of colorectal cancer, early detection of CRC is the most promising approach for reducing CRC-related death and improving the long-term survival of patients. Therefore, early detection of premalignant polyps in the large bowel and their clinical management are essential for colorectal cancer prevention and treatment of Precancerous lesions.

In the current findings, we hypothesized that epigenetic changes of *KEAP1* promoter in colorectal polyps and colorectal cancer could modify *KEAP1* expression and *NRF2* activity, which would be used as a prognostic and diagnostic tool in CRC.

2. Materials and methods

Study Population and Sampling Procedure

This study has received approval from the Ethics Committee of the Research Center for Gastroenterology and Liver Diseases (RCGLD), Shahid Beheshti University of Medical Sciences (Reference Number: IR.SBMU.RIGLD.REC.1395.925). Informed permission has been taken from all members before the research. Colorectal biopsies were taken from 114 individuals undertaking colonoscopies at the Research Center for Gastroenterology and Liver Diseases (RCGLD) of Shahid Beheshti University of Medical Sciences, Tehran, Iran, between 2015 and 2016. Samples have been frozen and transferred to the laboratory in a liquid nitrogen dry shipper and stored at -80 ºC till further use.

This research has been done on 208 frozen colorectal tissue samples, involving 120 precancerous lesions and adjacent normal tissues, 68 tumour tissue samples and adjacent normal tumour samples, and 20 normal tissue samples.

The clinical data involving high blood pressure (HBP), family history (FH), body mass index (BMI), diabetes, age, gender, smoking, and inflammatory bowel disease (IBD) have been gathered using a standard questionnaire finalized by a trained interviewer in a direct interview. A pathologist observed and defined all the tissues as adenomatous polyps, hyperplastic polyps, tumour tissues, and adjacent normal tissues. The marking and staging of tumour tissues have been labelled in line with the TNM system (Puppa et al., 2010).

DNA Extraction and MSP

The genomic DNA was isolated from frozen tissues using the QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's protocol, and subsequently stored at -70°C. The methylation status of the *KEAP1* promoter region was investigated .Primer sequences are listed in Table 1.

Table 1. Primer sequences for MSP analysis of *KEAP1* promoter.

Methylation status	Primer sequences (5′- 3′)	
	Forward	Reverse
Methylated	AGCGTTTGGGTAGTAGCGAC	GATAAAAACCGCTAAAAAACCG
Unmethylated	AGTGTTTGGGTAGTAGTGATG	AATAAAAACCACTAAAAAACCACT

Aliquots of the DNA extracted were subjected to modification using the Qiagen EpiTect Bisulfide kit following the manufacturer's guidelines. Methylation-specific PCR (MSP) was then conducted on the bisulfide-treated DNA samples sourced from polyps, tumours, and normal tissues. To outline the procedure, PCR amplification with MSP primers was performed using 11 µl of Master Mix and 1.5 µl of bisulfide-converted DNA, resulting in a final reaction volume of 12.5 µL. Additionally, a human-control DNA set containing bisulfide-converted methylated and unmethylated DNA (Qiagen, Germany) was employed for MSP reactions.

The PCR process comprised 36 cycles, starting with an initial denaturation at 94°C for 7 minutes and concluding with a final extension step lasting 10 minutes. In each cycle, denaturation occurred at 94°C for 40 seconds, followed by annealing at 58.5°C for 40 seconds, and extension at 72°C for 40 seconds. Subsequently, the MSP products underwent analysis using 2% agarose gel electrophoresis, staining with a green viewer, and observation under a UV transilluminator.

RNA Extraction and Real-time PCR Analysis

Entire RNA has been extracted from frozen tissues by RNeasy Mini Kit (Qiagen, Hilden, Germany), consistent with the producer's standard. NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE, USA) has determined RNA concentration and quality. The purified total RNA was snap-frozen and stored at -80°C for further analysis. cDNA was synthesized using a cDNA Synthesis kit (Thermo Fisher Scientific, United States) with random hexamer primer following the manufacturer's instructions. Subsequently, the cDNA fragments served as templates for amplifying the *NRF2* and *KEAP1* genes using SYBR Premix Ex Taq (Takara Bio Inc., Japan). Quantitative PCR (qPCR) was performed on a 7500 Real-Time PCR System (Applied Biosystems, USA) in a total reaction volume of 20 mL. Primer sequences are listed in Table 2.

Table 2. Reverse transcription-quantitative polymerase chain reaction primer sets

Primer sequences	Primer sequences (5′- 3′)	
	Forward	Reverse
NRF2	TTTCTCCCAATTCAGCCAGC	TACAAACGGGAATGTCTGCG
KEAP1	AACAGAGACGTGGACTTTCG	CGTGTGACCATCATAGCCT
BACTIN	GATTCCTATGTGGGCGACGA	GTACATGGCTGGGGTGTTGA

Cell Culture and Treatment

The HT 29 colorectal adenocarcinoma cell line has been cultured in RPMI 1640 medium (Biosera, France) enhanced with 10% fetal bovine serum (FBS) (Gibco, USA) and one percent penicillin-streptomycin (Biosera, France).

Cells have been maintained in a saturated incubator at 37 ° C and five percent CO2. The cells have been cultured in 6-well plates at equal concentrations of ~ $1x10^4$ CPW (cells per well). DNA demethylation was induced by adding ten µM 5-Azacytidine (Sigma-Aldrich) to the media. Control cells have remained without any use.

After 24 hours of exposure to 5-Azacytidine, the cells were trypsinized by trypsin-EDTA (Gibco). Total RNA has been extracted from the 5-Azacytidine-treated and untreated control cells by an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA has been produced by extracted RNA with a cDNA Synthesis kit (Thermo Fisher Scientific, United States).

Statistical Analysis

Quantitative data was expressed as mean ± standard deviation (SD). The statistical analyses and graphs were done using GraphPad Prism software version 7 (Graph Pad Inc., USA).

Group comparisons were made using the t-test. Correlations were analyzed by Fisher's exact test, one-way ANOVA, and Chi-square ($\chi 2$). The statistical significance level has been considered as $P < 0.05$. Relative quantification of the NRF2 and KEAP1 genes was performed using BACTIN as a housekeeping gene and results were expressed as 2-ΔΔCT method

3. Results

General Statistical Information

The mean age of patients with polyp and tumour and average individuals were 58.6±12.7, 61±13.7, and 57.3±9.2 years, respectively. The average BMI (kg/m) of patients with polyp and tumour and regular individuals was 25.2±2.8, 24.9±2.9 and 26.3±3.8, respectively.

QPCR and RT-qPCR Analyses

It was revealed that the *KEAP1* gene was significantly downregulated in the tumour group (n= 34) in comparison with corresponding adjacent normal tissues (n= 34) (p= 0.02, **Fig. 1. B**). However, a significant difference was neither found in the polyp group (n= 60) and corresponding adjacent normal tissues (n= 60) (p= 0.24, **Fig. 1. A**) nor in the polyp group and tumour group (p= 0.09, **Fig. 1. C**).

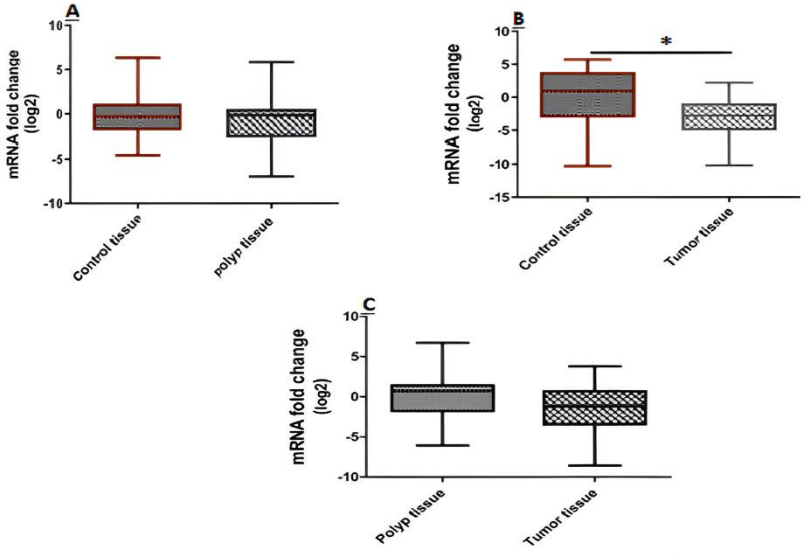

Figure1. Relative expression of *KEAP1* gene between polyp tissues and corresponding adjacent normal tissues (N=60 samples per group) (A), tumour tissues and corresponding adjacent normal tissues (N=34 samples per group) (B), tumour tissues (n= 34) and polyp tissues (n= 60) (C).

The *NRF2* expression level showed no statistically significant difference between the polyp group and adjacent normal tissues (P=0.82, see **Fig. 2. A**), as well as between the tumour group and adjacent normal tissues (P=0.51, see **Fig. 2. B**). Additionally, there was no significant variation in NRF2 expression level observed among the tumour and polyp groups (P=0.69, see **Fig. 2. C**).

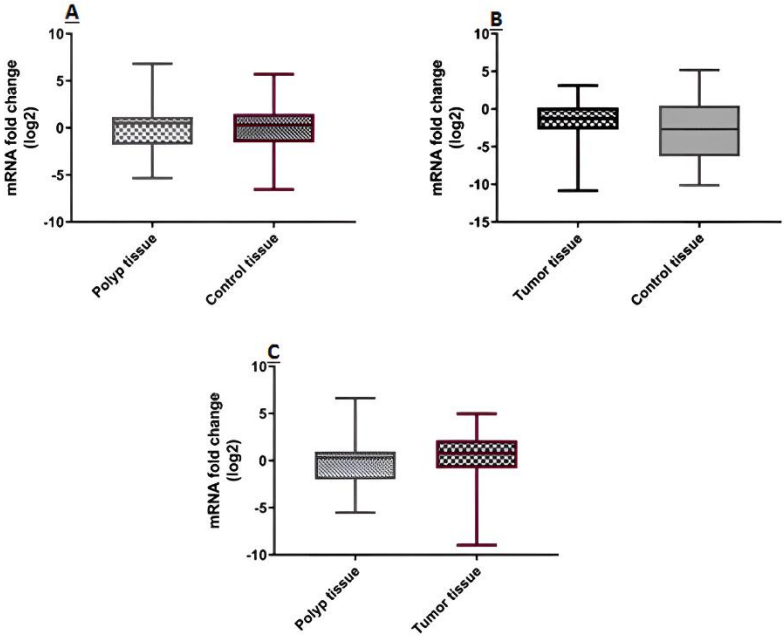

Figure 2. A) Relative expression of *NRF2* gene between polyp tissues and corresponding adjacent normal tissues (N=60 samples per group) (A), tumour tissues and corresponding adjacent normal tissues (N=34 samples per group) (B), tumour tissues (n= 34) and polyp tissues (n= 60) (C).

RT-qPCR analysis determined that *KEAP1* gene expression has been considerably upregulated in 5-Azacytidine-treated HT-29 cells in comparison with untreated HT-29 control cells (P=0.03, **Fig. 3. A**); however, there was no significant difference in *NRF2* gene expression between 5-Azacytidine-treated HT-29 cells and untreated HT-29 cells (p= 0.46, **Fig. 3. B**).

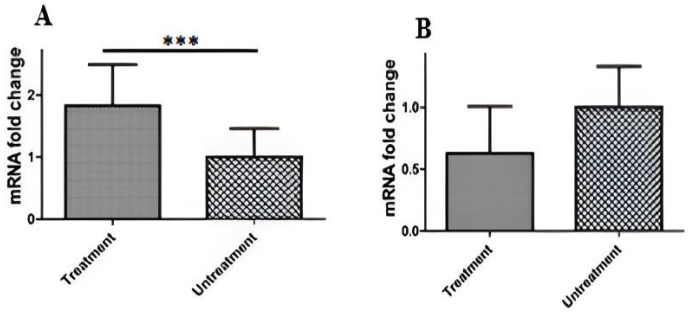

Figure 3. Expression level of *KEAP1* (A) and *NRF2* (B) in 5-Azacytidine-treated and untreated HT-29 cells.

MSP Analysis

MSP was done to determine the methylation status of the *KEAP1* gene promoter in all types of colorectal tissue samples. Results tabulated in Table 3 revealed that the *KEAP1* promoter was mainly unmethylated in the hyperplastic, adenomatous polyps and normal tissues. At the same time, 58.8% of the tumour (adenocarcinoma) samples were methylated, signifying that the methylation status of *KEAP1* is significantly reliant on the types of the colorectal tissue sample ($p = 0.001$).

Table 3. Correlations between *KEAP1* gene methylation state and kinds of colorectal samples

Type of tissue	Methylation status		P value
	M	U	0.001*
Adenoma	9(20.9%)	34(79.1%)	
Hyperplastic	3(17.6%)	14(82.4%)	
Tumour	20(58.8%)	14(41.2%)	
Normal	3(15.0%)	17(85.0%)	
M: methylated		U: unmethylated	

*; According to $\chi 2$ test

For further investigation into the differences in DNA methylation patterns of the *KEAP1* gene promoter among tumour, polyp, and normal groups, the MSP results were compared pairwise (Table 4). A significant alteration was observed not only between tumour (n=34) and normal (n=20) groups ($p = 0.002$) but also between polyp (n=60) and tumour (n=20) groups ($p < 0.001$). However, there was no significant difference between the polyp and normal groups ($p = 0.73$).

Table 4. Data comparing DNA methylation patterns

Comparison groups	Methylation Status		P value
	M	U	
Normal Vs. Tumour	3(15.0%) Vs. 20(58.8%)	17(85.0%) Vs. 14(41.2%)	0.002
Normal Vs. Polyp	3(15.0%) Vs. 11(18.3%)	17(85.0%) Vs. 49(81.7%)	0.73
Tumour Vs. Polyp	20(58.8%) Vs.11(18.3%)	14(41.2%) Vs. 49(81.7%)	< 0.001

In order to investigate potential connections between demographic characteristics and the methylation status of the *KEAP1* gene promoter, statistical analysis was conducted to assess the correlation between each demographic feature and MSP results. The analysis revealed that there is no significant association between the methylation status of BMI, HBP, FH, IBD, diabetes, and the *KEAP1* promoter (**Table 5**).

Table 5: The correlation between *KEAP1* promoter methylation status and clinicopathological features of participants.

	Methylation status		P-value
Variables	M	U	
Age (year)			0.10
Under 50	9(47.4%)	10(52.6%)	
Upper 50	21(28.0%)	54(72.0%)	
Sex (n %)			0.23
Female	18(37.5%)	30(62.5%)	
Male	12(26.1%)	34(73.9%)	
BMI(Kg/m2)			0.40
18 -23.9	5(21.7%)	18(78.3%)	
24 -29.9	22(36.7%)	38(63.3%)	
30 -35.9	3(27.3%)	8(72.7%)	
FH (n %)			0.55
No	25(33.3%)	50(66.7%)	
Yes	5(26.3%)	14(73.7%)	
Diabetes (n %)			0.39
No	28(33.3%)	56(66.7%)	
Yes	2(20.0%)	8(80.0%)	
HB (n %)			0.06
No	28(35.9%)	50(64.1%)	
Yes	2(12.5%)	14(87.5%)	
Smoking (n %)			0.21
No	29(33.7%)	57(66.3%)	
Yes	1(12.5%)	7(87.5%)	
Position (n %)			0.49
Colon	21(30.0%)	49(70.0%)	
Rectum	9(37.5%)	15(62.5%)	
IBD (n %)			0.51
No	28(32.9%)	57(67.1%)	
Yes	2(22.2%)	7(77.8%)	
Stage (n %)			
Stage I, II	14(56.0%)	11(44.0%)	0.57
Stage III	6(66.7%)	3(33.3%)	

The data is shown as number (%), and P value, with BMI representing Body Mass Index, FH indicating Family History, IBD standing for Inflammatory Bowel Disease, and HB representing High Blood Pressure.

4. Discussion

Methylation of CpG islands in the promoter region of a gene could be a trigger point for chromatin modification, which impedes the availability of transcription apparatus. As a consequence, gene expression alters. The effects of epigenetic changes on many genes in cancer suggest that epigenetic modifications and DNA methylation could play a role in cancer detection, prediction, and treatment.

DNA methylation has been recognized to be associated with cancer and has widely been used to screen various types of cancer, such as CRC. Prior research has demonstrated that gene methylation is a complicated process in colorectal lesions, and numerous hypermethylated genes are implicated in converting normal colon epithelium to aberrant crypt foci (Coppedè et al., 2014). Moreover, other methylated genes contribute to advancing an aberrant crypt to polyp/adenoma or CRC formation (Psofaki et al., 2010).

Therefore, epigenetic biomarkers often found in methylated status in tumour samples could be promising diagnostic or prognostic biomarkers in CRC.

In an attempt to diagnose CRC, methylation study of several hypermethylated genes has been introduced as valuable non-invasive biomarkers, comprising Septin9, adenomatous polyposis coli (APC), and MutL homolog 1 (MLH1), which were obtained from plasma or fecal samples (Liang et al., 2017; Ma et al., 2017; Molnár et al., 2015).

Some researchers have proposed that the modification of essential genes involved in the balance of oxidative stress is linked to higher death rates among people with colorectal cancer. A key mechanism for cellular defense against oxidative and electrophilic damage is the *NRF2-KEAP1* pathway which is essential for maintaining homeostasis by activating antioxidative enzymes (Panieri et al., 2020). *NRF2* activity regulates various antioxidants and cytoprotective genes, and it is a CNC-type essential leucine zipper transcription factor (Taguchi & Yamamoto. 2017).

NRF2 serves a dual function by safeguarding healthy cells against cancerous transformation and shielding cancer cells from cellular stress. This dual role of *NRF2* not only boosts the survival chances of cancer cells but also accelerates cancer advancement (Sajadimajd & Khazaei. 2018). *NRF2* over activity was seen in different kinds of cancer (Satoh et al., 2013; Yu et al., 2018; Yu et al., 2018). It was known that *NRF2* could be applied as an innovative target for CRC prevention (Li et al., 2022).

In the current study, we postulated that DNA methylation of a critical gene in the *NRF2/KEAP1* pathway can cause the development of CRC. Thus, the key regulator of the *NRF2/KEAP1* pathway with CpG islands in its promoter region was selected for MSP analysis. CpG islands of the *KEAP1* gene are situated near the transcription start site. Therefore, hypermethylation of the *KEAP1* gene could result in carcinogenesis. Based on our results, the evaluation of *KEAP1* gene expression exhibited a significant decrease in tumour samples compared with corresponding adjacent normal tissues.

We provided evidence that the downregulation of *KEAP1* in tumour samples might be associated with hypermethylation of its promoter region since our results showed aberrant methylation in *the KEAP1* gene promoter in CRC.

These results align with previous studies indicating that methylation of the *KEAP1* promoter correlates with the level of *KEAP1* expression in various colorectal cancer (CRC) cell lines. The possibility of considering the *NRF2-KEAP1* pathway as a progressive target for the treatment of colorectal diseases and prevention of its spreading towards malignancy is remarkable (Adinolfi et al., 2023). Many chemo preventive agents that target the *NRF2-*

KEAP1 pathway can avert cancer commencement and progression by inhibiting anomalous inflammation and decreasing oxidative stress; for instance, Isothiocyanate Sulforaphane has strong anti-cancer effects by increasing the production of antioxidant enzymes (Das et al., 2013).

Because the *NRF2-KEAP1* pathway is a plausible cause of CRC initiation and progression, a proper diagnostic and therapeutic approach depends on a better understanding of the control epigenetics of the *NRF2-KEAP1* pathway. To answer whether KEAP1 expression is under epigenetic control, an epigenetic drug was applied to the colorectal cell line.

Our research revealed that inhibiting DNA methylation in HT 29 CRC cells resulted in the upregulation of the KEAP1 gene, directly attributable to DNA demethylation.

Thus, it is proposed that the *KEAP1* promoter is under strong epigenetic regulation insofar as methylation of the promoter region of the *KEAP1* gene results in *KEAP1* downregulation, which, in turn, upregulates the *NRF2* gene. Therefore, *KEAP1* gene methylation is a promising biomarker for the early prognosis of colon tumorigenesis.

5. Conclusions

Our results provided extra perception into the role of epigenetic modifications causing tumour progression from colorectal polyps to cancer. It is recommended that targeting the *KEAP1-NRF2* pathway could prevent CRC progression. According to the present results, *KEAP1* promoter hypermethylation is consistent with *KEAP1* downregulation in CRC. Furthermore, the *KEAP1* promoter in tumour tissues has been more methylated than in normal adjacent tissues. Therefore, the methylation level of *the KEAP1* promoter could be an early detection biomarker in patients with CRC despite being an invasive and time-consuming method. More investigations are required to find and develop fast and non-invasive techniques, including peripheral blood and stool.

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Conflict of interests

The authors declare that they have no competing interests.

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