Iran Red Crescent Med J. 2021 April; 23(4):e80 doi: 10.32592/ircmj.2021.23.4.80
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Published online 2021 April 20 **Original Article**

Methylation Status of miR-200b Promoter in Colorectal Polyp and Adenocarcinoma Tissues

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Received 2021 January 10; **Revised** 2021 February 01; **Accepted** 2021 March 13.

Abstract

Background: Aberrant DNA methylation is a common molecular feature in colorectal cancer (CRC). Hypermethylation of miR-200b promoter, as an epigenetic factor, is involved in CRC tumorigenesis. The methylation status of miR-200b has been examined in CRC and adjacent normal tissues.

Objectives: This study aimed to investigate miR-200b methylation in a series of colorectal adenomatous polyps, hyperplastic polyps, and adenocarcinoma tissues as precursors of CRC in the Iranian population for the first time.

Methods: In this cross-sectional study (2017-2018), the methylation status of the miR-200b promoter was investigated on 131 fresh samples, including adenocarcinoma specimens (n=30), tumor-adjacent normal tissues (n=17), primary lesions (n=78; 55 adenomatous polyps and 23 hyperplastic polyps) and healthy individuals (n=6) using Methylation-specific polymerase chain reaction.

Results: Methylation of miR-200b was detected in adenocarcinoma samples (86%) and adenomatous polyps (85%); however, most of the hyperplastic polyps were unmethylated (69.6%). Neither control individuals nor tumor-adjacent normal tissues exhibited methylation in the miR-200b promoter. Aberrant methylation of miR-200b was significantly more common in tumor tissues and adenomatous polyps than in hyperplastic polyps (P<0.0001) and tumor-adjacent normal samples (P<0.0001).

Conclusion: The methylation status of the miR-200b promoter was significantly altered during CRC development and may be identified as an indicative biomarker for the early detection of the disease.

Keywords: Biomarkers, Colorectal cancer, Epigenetics, Methylation, MicroRNA200b, Polyps

1. Background

Colorectal cancer (CRC) is one of the most common cancers worldwide and the main cause of cancer-related deaths (1). It originates from the normal epithelial cells of the colon or rectum and the premalignant neoplasms may develop into invasive adenocarcinoma over time. Both genetic and epigenetic alterations may lead to this type of cancer, which converts normal cells to invasive and metastatic ones. DNA methylation is one of the most important epigenetic factors and plays a significant role in many cancers, including CRC. Aberrant methylation of some tumor suppressor genes and some oncogenes may occur in CRC (2,3).

Recently, identification of the biomarkers involved in the early detection and treatment of the disease has increased the survival rate of CRC patients (4). Treatment strategy for cancerous tissue depends mainly on tumor stage and histopathological changes of the tissue. Tumor markers can help the physician to select the best therapeutic regimen. Biomarkers have biological characteristics, which can be used as measurable indicators of normal biological or pathological processes, including cancer. A tumor marker may predict the risk for cancer development and progression in a specific tissue (5,6). MicroRNAs (miRNAs) are promising biomarkers for the diagnosis, prognosis, and treatment of cancers (7-9). They are single-stranded RNAs that regulate the expression of genes by binding to mRNAs. Several studies have reported that miRNAs may have important key roles in many cancers, including CRC (10-15). They play main roles in the diverse biological procedures, such as differentiation, proliferation, cell growth, migration, and survival (16). Activation of the epithelial-to-mesenchymal transition (EMT) program is implicated in the metastasis of colorectal tumors. Many genes are involved in this process, including miR-200b. MiR-200 family consists of five members (miR-200a, miR-200b, miR-200c, miR-429, and miR-141). MiR-200b is located on 1p36.33 and acts as a tumor

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suppressor and increases the expression of E-cadherin by directly targeting zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2 and inhibits EMT, cancer cell migration, and metastasis (17). The abnormal expression of miR-200b has been reported in various cancers (15,18-21). One of the most important mechanisms involved in miR-200 silencing is the hypermethylation of promoter CpG islands. A study conducted by Wen-rui et al. reported that miR-200b is partially silenced by DNA hypermethylation in human hepatocellular cancer (22). Some factors facilitate epigenetic regulation of miR-200b through demethylation of its promoter and loss of these factors leads to ectopic DNA methylation of CpG islands of the miR-200 family (23).

2. Objectives

The present study aimed to investigate the miR-200b methylation status in various colorectal polyps as precursors of CRC in the Iranian population for the first time.

3. Methods

3.1. Study population

In this study, a total of 131 colorectal specimens, including adenocarcinoma samples (n=30), tumor-adjacent normal tissues (n=17), primary lesions (n=78; 23 hyperplastic and 55 adenomatous polyps), and corresponding normal, and healthy control tissues (n=6) were collected from Research Center for Gastroenterology and Liver Disease of Taleghani Hospital, Tehran, Iran, from 2017 to 2018. This study was approved by the Research Institute for Gastroenterology and Liver Diseases (RIGLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran (89001357/ 15 August 2017).

The inclusion criteria included cases with pathological diagnosis of adenocarcinoma and polyps. On the other hand, the samples with low DNA quality for methylation-specific polymerase chain reaction (MSP) analysis were excluded from the study.

Written informed consent was obtained from the participants in the study. The control group consisted of healthy volunteers with no history of familial cancer and normal colonoscopy screening results. Demographic and clinicopathological characteristics of the participants were recorded which included such items as age, gender, smoking status, body mass index (BMI), family history, hypertension, inflammatory bowel disease (IBD), and location of samples. Data collection was conducted using a standard questionnaire, hospital health records, and laboratory data. The study variables were compared through Pearson's Chi-square test. A biopsy of each patient was examined histologically and the remaining biopsies were snap-frozen in liquid nitrogen. These samples were kept at −70 °C for DNA extraction. The stage of each colorectal cancer case was determined according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system. This study was approved by the Ethics Committee of the Gastroenterology and Liver Diseases Research Center in Shahid Beheshti University of Medical Sciences, Tehran, Iran (1392/704).

3.2. Genomic DNA extraction

Genomic DNA was extracted from the samples using the QIAamp DNA Mini kit (Qiagen, Germany), according to the manufacturer's protocol. The purity and concentration of all DNA samples were evaluated by the absorbance ratio at 260/280 nm and a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific).

3.3. Sodium Bisulfite Modification and MSP

The extracted DNA samples were modified by sodium-bisulfite using the EpiTect ® Bisulfite Kit (Qiagen, Germany). The methylation status of miR-200b in CRC tissues was identified using the MSP chain reaction method. The primer pairs for both the methylated and unmethylated sequences were reported in the previous study (22). The sequences for unmethylated primers were 5′-TGTTCTCT GTGGGGTGGGT3′ (forward) and 5′-GGCTTAATCA ATGGTGC-3′ (reverse). Moreover, the sequences for methylated primers were 5′-TGTTCTCTGTGGGG CGGGT-3′ (forward) and 5′-GGCTTAATCAATGGTGC-3′ (reverse). DNA samples were polymerase chain reaction (PCR)-amplified using either the methylated or the unmethylated primer pairs. The MSP reaction included 100 ng of template DNA per reaction template 10 pM of primer, 0.25 µL of 0.3 mM dNTP Mix 1.25 µL 10× PCR buffer, 2.5 µL Q PCR buffer, and 0.1 µL of HotStart Taq DNA Polymerase (Qiagen, Germany) in an ultimate reaction volume of 12.5 µL. The PCR amplification process included one cycle of 10 min at 95 °C for, 35 cycles of 94 °C for 45 s, annealing at 58 °C for 45 s, and finally a 45-s extension at 72 °C. The MSP products (5 µL) were loaded into 2% agarose gel and visualized by green viewer staining. Samples displaying unmethylated signals, methylated signals, and both unmethylated and methylated signals were considered as unmethylated, methylated, and hemimethylated, respectively. HeLa-methylated DNA and unmethylated DNA samples (Qiagen, Germany) were used as positive and negative controls, respectively.

3.4. Statistical Analyses

Data were statistically analyzed using SPSS software (version 21; SPSS Inc., Chicago, IL) and GraphPad Prism (version 6.01) for Windows. Methylation of miR-200b in the CRC, polyp tissues, corresponding normal, and healthy control tissues

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were analyzed using chi-square and Fisher's exact tests. The relation between miR-200b methylation, clinicopathologic, and demographic characteristics were also analyzed using chi-square and Fisher's exact tests. A p-value less than 0.05 was considered statistically significant.

4. Results

The present study was carried out on 131 colon tissues from 114 patients and healthy individuals. Demographic and clinicopathological characteristics such as age, gender, smoking status, BMI, family history, hypertension, IBD, and location of samples are presented in Table 1. In this study, four types of samples were used which included healthy control colon tissues (7%), adenocarcinoma (22.9%), tumor-adjacent normal tissues (13%), primary lesion tissues of hyperplastic polyps (17.6%), and adenomatous polyps (42%). The study participants included 53 males (46.5%) and 61 females (53.5%) in the age range of 26-83 years and the mean age of 60.33 years. It is worth mentioning that most of the samples were collected from the colon.

The association between miR-200b methylation and some demographic and clinicopathological characteristics is summarized in Table 2.

However, no significant association was observed between these characteristics and miR-200b methylation status (P>0.05). The methylation status of the miR-200b promoter was tested in the tissue specimen using the MSP technique. MiR-200b promoter was hemimethylated, completely methylated, and completely unmethylated in 65

Table 1. Demographic and clinicopathological characteristics of 114 individuals

Characteristics			Number (%)
Genger		Female	61 (53.5%)
		Male	53 (46.5%)
		Total	114 (100%)
Age		≤ 50	15 (13.2%)
		> 50	99 (86.8%)
BMI (Kg/m2)		18.5-24.9	59 (51.6%)
		25-29.9	48 (42.1%)
		30-34.9	7 (6.1%)
		35-39.9	0 (0%)
Smoking status		Yes	7 (6.1%)
		No	107 (93.9%)
		Total	114 (100%)
Family history		Yes	11 (9.6%)
		No	103 (90.4%)
		Total	114 (100%)
Sample location		Colon	105 (92.1%)
		Rectum	9 (7.9%)
		Total	114 (100%)
Type of sample	Tumor	Adenocarcinoma	30 (22.9%)
		Tumor-adjacent normal tissue	17 (13%)
	Polyp tissue	Adenomatous	55 (42%)
		Hyperplastic	23 (17.6%)
	Healthy control		8 (7%)
Underlying disease	Hypertension	Yes	11 (93%)
		No	106 (91.6%)
	IBD	Yes	10 (8.8%)
		No	104 (91.2%)

Table 2. The correlation between miR-200b methylation and demographic and clinicopathological characteristics.

Characteristics		HM	M	U	P-value
Gender	Female	31(50.8%)	8 (13.1%)	22 (36.1%)	0.273
	Male	34 (64.2%)	7 (13.2%)	12 (22.6%)	
Age	≤50	6 (40%)	3 (20%)	6 (40%)	0.22
	>50	59 (59.6%)	12 (12.1%)	28 (28.3%)	
BMI	18.5-24.9	30 (50.8%)	11 (18.6%)	18 (30.5%)	0.351
	25-29.9	31 (64.6%)	4 (8.3%)	13 (27.1%)	
	30-34.9	4 (%)	0 (0%)	3 (42.9%)	
	35-39.9	0 (0%)	0 (0%)	0 (0%)	
Smoking	Yes	4 (57.1%)	1 (14.3%)	2 (28.6%)	0.994
	No	61 (57%)	14 (13.1%)	32 (29.9%)	
Family history	YES	8 (72.7%)	1 (9.1%)	2 (18.2%)	0.540
	No	57 (55.3%)	14 (13.6%)	32 (31.1%)	
IBD	Yes	7 (70%)	1 (10%)	2 (20%)	0.683
	No	58 (55.8%)	14 (13.5%)	32 (30.8%)	
Sample location	Colon	57 (54.3%)	14 (13.3%)	34 (32.4%)	0.096
	Rectum	8 (88.9%)	1 (11.1%)	0 (0.0%)	

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Table 3. The relationship between methylation status and kinds of specimens.

Types of samples		HM	M	U	P-value
Adenocarcinoma		21 (70%)	5 (16.7%)	4 (13.3%)	<0.0001
Tumor-adjacent normal tissue		0 (0.0%)	0 (0.0%)	17 (100%)	
Polyp	Adenomatous	37 (67.3%)	10 (18.2%)	8 (14.5%)	<0.0001
	Hyperplastic	7 (30.4%)	0 (0.0%)	16 (69.6%)	

(49.6%) , 15 (11.5%), and 51 (39.9%) samples, respectively. Moreover, the miR-200b promoter was methylated in 86.7% of adenocarcinoma samples, while no tumor-adjacent normal tissue showed methylation.

These results demonstrated statistically significant differences between CRC and adjacent normal tissues in terms of the methylation status of miR-200b (P<0.0001).

Promoter methylation levels of miR-200b in the adenomatous and hyperplastic polyps were obtained at 85.5% and 30.4%, respectively. In addition, the miR-200b promoter was unmethylated in most hyperplastic polyps (69.6%), while it was hemimethylated in most adenocarcinoma tissue specimens (70.0%). In addition, a significant alteration was observed between hyperplastic and adenomatous polyps (P<0.0001). Neither control individuals nor tumor-adjacent normal tissues showed methylation in miR-200b promoter (Table 3). The increased level of miR-200b promoter methylation was higher in tumor and adenomatous tissues than the healthy controls and hyperplastic tissue; however, this difference was not related to the tumor stage. Moreover, demethylation level was higher in stage III samples, compared to stages I and II samples in tumor tissues (Table 4).

Table 4. The association between miR-200b methylation and tumor stage

Tumor stage		HM	M	U	P-value
Adenocarcinoma	Stage I	2 (50%)	2 (50%)	0 (0%)	0.0093
	Stage II	15 (88.2%)	1 (5.9%)	1 (5.9%)	
	Stage III	4 (36.4%)	2 (18.2%)	5 (45.5%)	

5. Discussion

Aberrant expression of miRNAs plays a major role in the development of CRC. Many studies have explored the mechanisms responsible for the dysregulation of miRNA expression (24). Recent findings have shown that DNA hypermethylation of CpG islands within the gene promoter can reduce the expression of tumor suppressor genes in many types of cancers (25, 26). The DNA methylation may have the main role in the regulation of miRNAs expression in CRC (27). Methylation of DNA is now recognized as a causal epigenetic event that occurs during colorectal cancer progression and impacts gene expression (2).

It has been indicated that miR-200b, as a member of the miR-200 family, plays a tumor suppressor role in CRC and is silenced by DNA hypermethylation (22). In this study, it was found that miR-200b was methylated in colorectal tumor tissues, however, this did not occur in adjacent normal tissues and healthy individuals.

The results of recent studies showed that the miR-200 family was silenced by DNA hypermethylation in various cancers (28-31). It seems that silencing of miR-200b through methylation of promoter CpG islands may play an important role in tumorigenesis. Some studies have indicated that the ectopic DNA methylation of CpG islands of miR-200s is related to their silencing during metastasis, whereas epigenetic drugs, such as 5-aza-2-deoxycytidine can revive their expressions. This indicates the important role of epigenetic mechanisms in regulating the expression of the miR200 family in cancer (32,33). Based on the results of a study conducted by Song et al., downregulation of demethylation factors leads to miR-200 hypermethylation (23). Another study performed by Wiklund et al. showed that the miR200 family is epigenetically repressed in invasive bladder cancer (34).

To the best of our knowledge, no study has assessed the methylation of miR-200b in hyperplastic and adenomatous polyps of the colon in the Iranian population. The results of the present study indicated that methylation of miR-200b promoters is a frequent event in adenomatous polyps since 85.5% of adenomatous polyp cases had at least one methylated miR-200b promoter. The results also showed that miR-200b was unmethylated in most hyperplastic polyps and no complete methylation occurred in these specimens. Moreover, most adenomatous polyps have the highest potential to become cancerous. These findings demonstrated that miR-200b methylation mostly occurs in these types of polyps, compared to hyperplastic ones (with no malignant potential).

In line with the findings of the present study, the study conducted by Vrba et al. evaluated the methylation and expression of miR-200b in human mammary epithelial cells and human mammary fibroblasts and showed cell-type-specific repression by DNA methylation (35). The results of another study

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have also confirmed that the CpG islands of miR-200b promoter were methylated differently (36). Based on these results, miR-200b methylation may be considered as an indicative biomarker for the early detection of CRC. The results of previous studies also suggested that miR-200s can be used as biomarkers for cancers (18,37). Therefore, regarding the findings of this study and those of the previous studies it can be concluded that miR-200b has the potential to be used as a prognostic marker in gastric cancers (38). The miR-200 family has been recognized as candidate biomarkers for the early detection of epithelial ovarian cancer (39).

The obtained results indicated that the demethylation level in tumor specimens was higher in stage III compared to stages I and II. Therefore, the progression of a tumor may not be dependent on miR-200b promoter methylation and possibly other genetic factors are involved in this process. Based on the obtained results, it seems that miR-200b plays a role in tumorigenicity. This is indicated by the fact that miR-200 expression decreased in metastatic samples compared to non-metastatic samples, and the downregulation of miR-200 may forestall the poor survival of patients (40).

Regarding the limitations of the present study, one can refer to the fact that the specimens from healthy people were very limited and the pyrosequencing method was not performed in this study.

6. Conclusion

Eventually, the current study proved the research hypothesis that miR-200b promoter may serve as a promising diagnostic biomarker in colorectal cancer.

Footnotes

Funding/Support: This work was financially supported by the RIGLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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