

Promoter Hypermethylation of the Eyes Absent 4 Gene Is a Tumor-Specific Epigenetic Biomarker in Iranian Colorectal Cancer Patients

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Abstract- The aim of this study was to investigate whether hypermethylation of Eyes Absent 4 (EYA4) is also implicated in Iranian Colorectal Cancer (CRC) patients or not. From fresh frozen tissues, samples from 38 paired (cancer and normal) CRC tissue specimens were used in this study, the DNA was isolated, sodium bisulfite treated and analyzed by methylation-specific polymerase (MSP) chain reaction using primers specific for unmethylated or methylated promoter sequences of the EYA4 gene. We also analyzed EYA4 mRNA expression using real time RT-PCR. Demographic characteristics of these patients including age, sex, tumor grade, location, stage, and TNM classification were evaluated and the relationship between methylation status of the gene and clinicopathological features was analyzed. Current study indicated that EYA4 promoter hypermethylation has a sensitivity of 81.57% and specificity of 78.94%. Findings showed lower expression of EYA-4 in methylated samples in comparison with its normal adjacent tissue, although it was not significant ($P>0.05$). No significant associations were observed between EYA4 hypermethylation and the clinicopathological characteristics. Although the clinical patient outcome of our 38 CRC patients was not associated with EYA4 promoter hypermethylation, the high frequency of this methylation and its high sensitivity and specificity to neoplastic cells may qualify EYA4 promoter methylation as a potential candidate screening marker in Iranian population and may help to improve early detection of CRC.

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Introduction

With over one-million new cases diagnosed every year in the world, colorectal cancer (CRC) is a major health concern globally (1). Each year more than 51,000 new cases of cancer are diagnosed in Iran, and 35,000 deaths are related to cancer. Based on Malekzadeh's report, the estimated number of new cases of CRC in Iran is 3641 each year, and 2262 Iranians die of CRC annually, accounting for about 6.3% of all cancer deaths in Iran (2). According to the statistics, it is the second and the fourth most common cancer in men and women, respectively worldwide. Roughly 70% of CRCs are sporadic, without any inherited predisposition (3). For

early diagnosed CRC patients, the 5-year survival rates are approximately 90% but this decrease to less than 10% in patients with vast metastases (4). The most effective approach to reducing CRC incidence and mortality is early detection of colonic lesions (5). This cancer is a suitable candidate for screening due to the underlying neoplastic processes. Early detection of CRC has been found to be cost-effective, and several guidelines and tests have been designed to reach this goal (6). While colonoscopy is still the most accurate diagnostic test for CRC; however, it is costly and is associated with poor patient compliance and procedure-related complications. In contrast, fecal occult blood testing (FOBT), another commonly used CRC detection

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method, is inexpensive and easy to perform but has a quite low specificity and sensitivity. Advances in understanding the underlying molecular alterations of CRC has caused the identification of encouraging early detection molecular biomarkers for use in non-invasive CRC screening assays (7). Stool or blood-based biomarkers have the potential to transform CRC diagnostics by providing patient-friendly, noninvasive, and cost-effective early detection of CRC.

In addition, cancer biomarkers may have substantial applications, ranging from disease risk analysis in otherwise healthy people, therapy response prediction, molecular classification to prognostics of the disease and surveillance in patients who have been treated for cancer.

Promoter methylation is a propitious CRC biomarker that has the potency to render into non-invasive CRC detection modalities. So far several DNA methylation markers have been suggested as useful biomarkers for early CRC detection.

The clinical application of DNA methylation biomarkers in CRC is rapidly growing into a competitive scientific field and has been noticed by the commercial industry as a marketable product (8). In Europe and the Middle East and now in the US, there is a blood-based assay that detects methylated SEPT9 called "Epi proColon" that is been in the market as a colon cancer screening assay (7,9).

Cologuard® is an FDA approved diagnostic tool, user-friendly and non-invasive and colon cancer screening test based on stool DNA science. In Cologuard® test, detection of methylated target DNA (*BMP3*, *NDRG4*), *KRAS* point mutations, and *ACTB* (a reference gene in order to estimate the total amount of human DNA in each sample) is carried out. According to the company claims, Cologuard® can detect 92% of cancers and 69% of the most advanced precancerous polyps in patients with average risk for CRC (6,10).

Eye Absent (EYA)4 is another proposed methylated gene that has been recently identified as an important tumor suppressor gene in different cancers. In *Drosophila*, EYA is known to mediate developmentally major protein-protein interactions (11). The EYA proteins were first described in the context of fly eye development. EYAs have been protected among commonly different animal species ranging from amphioxus to humans. Human EYA4 maps to 6q23. EYAs are an unusual class of proteins that combine tyrosine phosphatase, threonine phosphatase, and transactivation activities. EYAs are now involved in processes such as organ development, innate immunity,

repair of damaged DNA, photoperiodism, angiogenesis, and cancer metastasis (12).

A possible cancer role for EYA4 gene was suggested by a microarray-based methylation analysis indicated that EYA4 is normally methylated in CRC but not methylated in the normal mucosa (13). In 2015, Kim and his colleagues showed for the first time the function of the EYA4 protein as a tumor suppressor not only *in vitro* but also *in vivo* in xenograft study in CRC (14). EYA4 is often deleted, hyper- and hypomethylated in numerous independent lung tumor data sets, in both major non-small cell lung cancer subtypes and in the earliest stages of lung cancer (15). Recently a few reports about the abnormal hypermethylation of EYA4 promoters in pancreatic cancer, esophageal cancer, and lung cancer have been issued (14).

Four independent assays have shown EYA4 (methylation-specific polymerase chain reaction (MSP), methylation array, mass spectrometry, and pyrosequencing) to be hypermethylated in CRC. Other studies also showed that methylation levels of the EYA4 promoter in CRC were 13.6-fold higher than normal tissues (5,13,14,16,17).

In the present study, we studied the methylation status and gene expression of EYA4 in tissue samples from CRC patients obtained from colorectal surgery, using MSP and qPCR, respectively. In the case of tissue EYA4 hypermethylation, the EYA4 DNA methylation status of bodily fluids such as blood, stool, serum, and urine from cancer patients, may be an attractive target for future development of a detection assay for CRC among the Iranian population.

Materials and Methods

Clinical sample collection

Human tissue samples were collected from 38 individuals including 38 fresh frozen cancerous and 38 adjacent normal tissue dissected from the proximal tumor resection with distance of 10 cm at least, obtained from CRC patients who had surgery in three Mashhad hospitals (Emam Reza, Ghaem and Omid Hospital Centers), Iran. Informed consents were obtained from all individuals prior to the study. Inclusion criteria were primary CRC patients (all stages of the disease), and exclusion criteria were insufficient or absent tumor tissue sample, lacking clinical information and positive 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.

Table 1. Clinical characteristics of subjects studied

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%)
	IIIB	1 (2.4%)
	IIIB	6 (14.3%)
	IIIC	3 (7.1%)
T	1	1 (2.4%)
	2	11 (26.2%)
	3	25 (59%)
	14	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)

Approval of this study was obtained from our local ethics committee, Mashhad University of Medical Sciences, Mashhad, Iran.

DNA isolation from tissue samples

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

RNA isolation from tissue samples

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

cDNA synthesis and real time PCR

Reverse transcription was performed on 4 µg total RNA using RT kit (Pars Tous, Mashhad, Iran). The reverse transcription-PCR primers of EYA4 were as

follows for forwarding strand; GCACAGTATTATTCAGCATCA and for reverse strand; AACTCTCCTGGTTGGTTAG. GAPDH was used as a reference gene to normalize the cDNA input. The primers for GAPDH were CATGTTTCGTCATGGGTGTGAAC (forward) and CACAGTCTTCTGGGTGGCAG (reverse). The product length of EYA4 and GAPDH was 134 and 178 bp, respectively. Real time PCR assays were carried out in a reaction volume of 10µl consisting of 2 µl 5x HOT FIREPol EvaGreen qPCR Supermix (Solis Biodyne, Estonia), 0.3 µl each primer (10 pM), 4.4 µl DEPC water, and 3 µl cDNA under the following conditions: 95° C for 15 minutes by 1 cycle followed by 95° C for 30 seconds and 40 cycle and 57° C for 1 minute. Amplification was performed in a 96-well plate, including cDNA samples, water blanks, positive and negative controls. Amplification assays were done in triplicate for EYA4 and GAPDH.

Bisulfite modification

DNA was modified chemically by sodium bisulfite.

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Thus, all unmethylated cytosines were converted to uracils, but methylated cytosines were unaltered (EpiTect Bisulfite Kit, Qiagen, Germany) and eluted in 70 μ L 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 positive control in our study.

Methylation-specific PCR (MSP)

The bisulfite-modified DNA was used as a template for MSP as described previously. Proper positive controls were included in each batch of PCR reaction. Methylated and unmethylated primer sequences used in this study, as well as the annealing temperature and product sizes, are given in Table 1. MSP-PCR was performed in a reaction volume of 25 μ L consisting of 16.6 μ L ddH₂O, 2.5 μ L 10X PCR buffer, 2 μ L MgCl₂ (25 mM), 0.5 mM dNTP mixture, 200 nM of each forward and reverse primers, and 1 unit of Hot-Start Taq DNA Polymerase (2/5U/ μ L) (ParsTous, Mashhad, Iran). 2 μ L of bisulfite-converted DNA was used in each amplification reaction. Thermal cycling profile performed as follow: included hot-start at 94° C for 5 min, followed by 35 cycles at 94° C for 30 sec, each annealing temperature of 66° C for methylated primer pairs or 57° C for unmethylated primer pairs for 30 sec, 68° C for 30 sec, and a final extension at 72° C for 5 min. To validate the results all PCR reactions have been

performed triplicated. The MSP products were then analyzed by 2% agarose gel electrophoresis.

Statistical analysis

Pearson chi-squared and Fisher's exact tests were used to assessing the association between the methylation status of the EYA4 promoter in the DNA from all tissue samples, and to evaluate the association between methylated EYA4 promoter, tumor location, patient tissue (cancerous vs normal adjacent tissue), and demographic variables, including gender age and, the tumor node metastasis (TNM) classification. Numerical data were expressed as Mean \pm SD. A two-tailed *P* of *P*<0.05 was considered statistically significant for all calculations. The statistical analyses were performed with the SPSS 15 software package (SPSS Inc., Chicago, IL).

Results

Detection of methylated EYA4 gene in colorectal tissue samples

We examined hypermethylation of EYA4 gene in the DNA from 38 tumor tissues and 38 tumor-adjacent normal tissues by MSP. Patients were ranged between 27 to 86-year-old. In aspect of CRC tumor grade, 30 and 12 ones were widely and mild differentiated, respectively (Figure 1). The other variables are listed in Table 2.

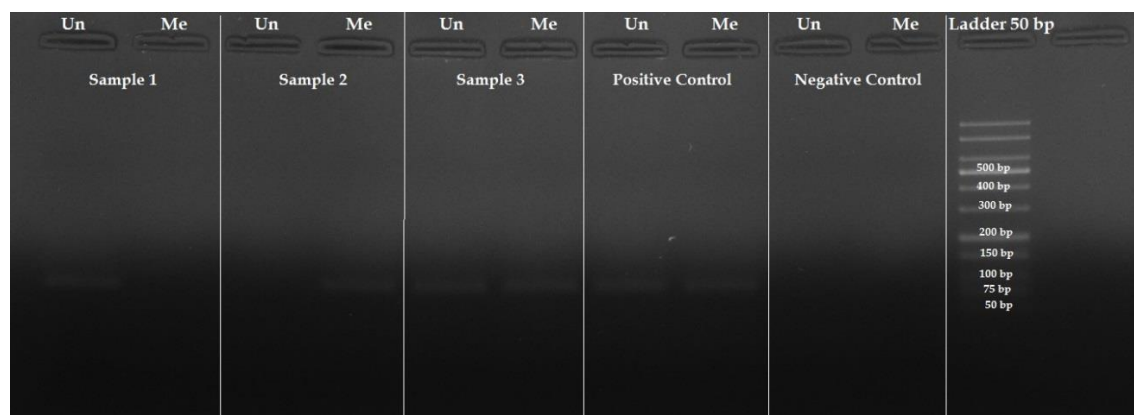


Figure 1. Detection of unmethylated (Un) and methylated (Me) EYA4 in tissue samples of three CRC patients (Sample 1-3) plus positive and negative controls. Product size for the methylated and unmethylated bands are 102 and 108 base pairs, respectively

Table 2. The main characteristics of patients with CRC, and the relationship between EYA4 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	0.48	0.19	0.17	0.84	0.54	0.27	0.42	0.38	0.71	0.52
Tumor tissue	0.27	0.19	0.80	0.87	0.90	0.68	0.95	0.56	0.64	0.52

Our analysis revealed that EYA4 was methylated and unmethylated in 32 out of 38 (84%) and 6 out of 38 (16%) tumor tissue samples, respectively while in their tumor-adjacent normal tissues only 8 out of 38 (27%) samples were methylated. So methylation percentages were significantly higher in cancer tissues in comparison to their adjacent normal tissues ($P=0.001$). Furthermore, EYA4 promoter hypermethylation showed a sensitivity of 81.57% (31 out of 38 samples), and specificity of 78.94% (30 out of 38 samples).

There was no significant association between EYA4 promoter hypermethylation and clinicopathological features. Relation of the methylation status of EYA4 in tumoral and normal adjacent tissues of CRC with some variables is presented in Table 3. This table shows that there was no association between age and methylation of EYA4 in the normal and tumoral tissues and the highest methylation was presented in age between 35 to 45 years old. Besides, the difference between methylation status in two genders was not significant ($P>0.05$).

Table 3. EYA4 gene primers sequences, annealing temperature and product size for MSP assays

Primer	Sequences (5' -3')	Annealing temperature	Product size
EYA4 -MF	CGCCACCGACTACTACGAACTCGTA	65 °C	102 bp
EYA4 -MR	ATAAAAACGGAGTGGGTTTTTCGCG	65° C	
EYA4 -UF	TCACCACCAACTACTACAAACTCATA	55° C	108 bp
EYA4 -UR	GTAAATAAAAATGGAGTGGGTTTTTGTG	55° C	

There was no remarkable relation between methylation status and the grade of the tumor, although most of the methylated tumors were related to grade I and grade III of cancer. Furthermore, this difference was not seen in the normal tissue, as most of the specimens were unmethylated in this group. Also, there was no correlation between the methylation status and location, tumor differentiation, number of nodes involved, tumor invasion and the stage of cancer ($P>0.05$).

Expression of EYA-4 level

The mean of EYA-4 was -1.88 (SD: 2.84) folds in all studied tissues. We divided 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 38.9% (-4.67 ± 1.96 folds), 55.6%, (-0.27 ± 1.03 folds) and 5.5% (2.98 ± 1.27 folds) related to low, normal and high expressions ($P=0.001$), respectively. Our analysis showed lower expression of EYA-4 in methylated samples in comparison with its normal adjacent tissues, but these differences were not significant ($P>0.05$).

Discussion

The low tolerance of present screening methods has fuelled the search for a non-invasive extremely sensitive screening test. There is substantial potential for the development of new DNA methylation markers or panels to enhance the sensitivity and specificity of current cancer detection tests (18). Recently, the

epigenetic alteration is highly attractive because of its fundamental role in gene regulation. DNA methylation as an epigenetic alteration has an effective role in tumor initiation and progression. Researchers have offered a large number of tumor-related methylated genes such as EYA4 as diagnostic biomarkers for CRC (14). There are numerous studies regarding the cellular mechanisms of the EYA4 gene, although there are only a few published papers related to its role in carcinogenesis. It is assumed that EYA genes might affect apoptosis in neoplastic cells during development (11,19-25) and interact with a network of other genes to induce apoptosis (16). They could be linked with the development and formation in certain human organs including eye, kidney, and inner ear (11,19-25). The participation of EYA4 methylation in CRC samples is nearly a new observation. In a study by Kisiel *et al.*, they found that methylated EYA4 could serve as a useful predictive marker of synchronous CRC in patients with ulcerative colitis (26). Since aberrant promoter methylation is the main feature of tumor suppressor genes (27,28), EYA4 functions probably as a tumor suppressor during the progression of CRC. Our study presented that EYA4 methylation had a sensitivity of 81.57% and specificity of 78.94%. Thus, EYA4 as a target of epigenetic inactivation, has high sensitivity and specificity for CRC, suggesting its usefulness as a molecular biomarker for cancer detection. Besides, in the current study, EYA4 expression was reduced in CRC samples although it was not significant ($P>0.05$). This result might be explained by the fact that methylation of EYA4 is associated with absent or decreased mRNA expression in CRC samples, which would be related to a

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possible tumor suppressor role. It has been proved that reduced gene expression is merely characteristic of a subset of methylated genes in CRC (7).

Besides, in this study methylation status did not show a significant statistical difference in different grades of tumor tissues while most of the methylated EYA-4 gene was related to grade I and III of CRC samples. The relation of tumor grade and methylation EYA-4 has been previously studied, but no studies were reported in CRC patients. Zou *et al.*, found that in esophageal adenocarcinoma patients, grade II had higher frequencies of methylation compared to the other different grades (13).

This study for the first time reveals higher methylation rate of EYA4 in CRC tumors in Iranian population. We recommend further studies to be performed particularly prospective cohort studies conducted in the CRC surveillance which would help determine how this noninvasive diagnostic tool could improve patient and colonoscopy outcomes, and potentially diminish healthcare costs.

The EYA4 gene, a tumor suppressor, is a high-frequency target of epigenetic inactivation in human CRC, distinctly suggested as a molecular biomarker for early detection of CRC.

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