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EVALUATION OF NEW EPIGENETIC MARKERS SPG20, ITGA4 AND ALX4 IN PLASMA OF COLORECTAL CANCER PATIENTS.

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ABSTRACT

Background: Colorectal cancer (CRC) has a high incidence and mortality. Remarkable increase in the incidence of CRC has been reported in different populations in the past decades. The risk is influenced by both environmental and genetic factors. DNA methylation is an effective molecular biomarker for diagnose of the cancer. **Materials and Methods:** In this study, blood samples were obtained from 73 individuals (41 cases vs. 32 controls) to test the performance of three gene markers, SPG20, ITGA4 and ALX4, using Methylation Specific PCR (MSP) method. **Results:** Results showed that the promoters of SPG20, ITGA4 and ALX4 genes are hypermethylated in 68.2%, 60.9% and 68.2% of patients respectively. **Conclusion:** Collectively it has concluded that the detection of simultaneous hypermethylation of SPG20/ITGA4/ALX4 promoter can specifically be applied as a biomarker in the diagnosis of patients with cancer risk and can be promising before colonoscopy.

KEYWORDS: CRC-methylation- ITGA4- SPG20- ALX4.

INTRODUCTION

Colorectal cancer (CRC), is a type of cancer that involves colon and rectum and is the fourth most common cause of cancer victims, accounting for 8% of all cancer deaths worldwide (Susanne et al., 2015; Jemal et al., 2011). Incidence, age at diagnosis and other statistics factors of CRC varies widely in different regions of the world. Although Epidemiological pattern of CRC has not been studied enough, The estimated number of new cases of CRC in Iran is increasing like other developing countries and is accounting for approximately 6.3% of all cancer deaths in Iran (Sajadi et al., 2005). The main theory of multi-stages cancer development suggests that CRC, is result of accumulation of mutations in oncogenes and tumor suppressor genes in colon mucosa cells as well as epigenetic modifications (Panzuto et al., 2003; Jasperson et al., 2012). Screening of CRC leads to early diagnosis that can reduce mortality and may also causes decrease in incidence (Jasperson et al., 2012). Traditional screening methods are based on colonoscopy and X-ray methods, which are somewhat invasive and are not effective enough (Muto et al., 2001). New screening methods are based on detecting genetic and epigenetic biomarkers in body fluids such as plasma, urine and stool specimens. These methods are non-invasive and sufficiently help to diagnosis of CRC and even precancerous lesions in early stages (Ahlquist et al., 2010; Tao et al., 2011). Recently, many of genes have discovered for early detection of CRC (Mitchell et al., 2014; Roperch et al., 2013; Kim et al., 2010). One of these gene alternations is aberrant DNA methylation, an epigenetic change that frequently occurs in cancer-associated genes in early stages of cancers (Lao et al., 2011; Ross et al., 2010). Studies has shown that ALX4, SPG20, ITGA4 genes have high rates of hypermethylation in CRC tumor tissues compared to normal tissues (Bethge et al., 2014; Kostin et al., 2010). ALX4 gene encodes Home box protein arista less-like 4 which found to be a transcription factor that binds to DNA (Wöhlke et al., 2006). Spastic paraplegia-20 (SPG20) encodes Spartin as a multifunctional protein that involved in intracellular epidermal growth factor receptor trafficking (Zhang et al., 2013). ITGA4 gene encodes Integrin alpha, an integral membrane protein (Holmes et al., 2011).

Methylation-specific PCR (MSP) is a qualitative technique used for detecting the presence of methylation in bisulfite-converted DNA (Antonia et al., 2009). It was

previously approved that MSP is a simple, rapid and inexpensive method to determine the methylation pattern of a gene (Hou et al., 2003). The aim of this study was to evaluate the hypermethylation of SPG20, ITGA and ALX4 promoters in plasma specimens for early detection of CRC using MSP method.

MATERIAL AND METHODS
Study population. A total of 73 patients who underwent colonoscopy procedure at the colonoscopy department of Baqiyatallah hospital, Tehran, Iran were enrolled in this study. Blood samples were collected from patients in K3EDTA vacationer tubes (Gold vac™-China) after obtaining written informed consent and questionnaire. Samples divided in to two groups, CRC patients and normal healthy individuals. Patient who had previous therapeutic interventions such as chemotherapy, other types of cancers and gastrointestinal disease were excluded from the study.

DNA extraction. The plasma was immediately separated from the cellular fraction by centrifugation at 2,500 rpm for 10 min and frozen at -80°C. DNA isolation from 400 µL plasma was conducted using QIAamp DNA Blood Mini kit (Qiagen-Germany), dissolved in 50 µL of sterile distilled water and stored at -20°C. Concentration of the isolated DNA was quantified using nanodrop spectrophotometer (Maestrogen-USA).

Methylation Specific PCR (MSP)
Bisulfite modification. 2 µg of extracted DNA was treated by sodium bisulphate to convert all unmethylated cytosins to uracils using the EZ DNA Methylation-Gold Kit (Zymo Research-USA). To confirm bisulfite DNA conversions, unmethylated and methylated human control DNA (Qiagen-Germany) was used as control.

MSP. The methylation status of the genes was determined using MSP. The bisulfite-modified DNA was used as the template for PCR. The primer sequences for the methylated and unmethylated templates are showed in Table 1. Each PCR reaction mix consisted of a total volume of 25 µl containing 12.5 µl hot startTaq Master Mix, 0.4 µM concentration of each primer, 1.5 µl DMSO and 3 µl bisulfite- modified DNA. Methylated human control DNA (bisulfate converted) was used as control for methylated primers and bisulfate DNA, unmethylated Human control DNA (bisulfite converted) for unmethylated primers and bisulfate DNA and unmethylated Human control DNA for unmethylated primers and unconverted DNA.

Table 1: primers designed for methylated and unmethylated genes

Gene		Primer Sequences
ALX4	Meth_F	5'ACGTTAAGAGTCGGGTTCGTTACGGC3'
	Meth_R	5'GACGACGACTAAAACTACGAATCGACGA 3'
	UnMeth_F	5'ATGTTAAGAGTTGGGTTTGTTATGGT3'
	UnMeth_R	5'ACAACAACAACTAAAACTACAAAATACAC3'
SPG20	Meth_F	5'ACGTGGTCGTAGGGGATC3'
	Meth_R	5'GCCGCCGCAACCTAAATC3'
	UnMeth_F	5'GTTGAGTAGTTGATGTGGTTG3'
	UnMeth_R	5'CACACTCTCAAACACTTCACA3'
ITGA4	Meth_F	5'TCGAAGGGTCGTCGTTCG3'
	Meth_R	5'ACGACCGAATAACCGAACAAC3'
	UnMeth_F	5'TTGTATTTGGAGAAGTAGTGTG3'
	UnMeth_R	5'ACCCACCACAAACAAAACATTC3'

Meth: Methylated. UnMeth: Unmethylated. F: Forward. R: Reverse.

Statistical Analysis. Statistical analysis was conducted using the SPSS20 software (version 17.0, SPSS Inc, Chicago, USA). The Fisher's exact test and $\chi 2$ test were applied to study the statistical relationships between either MSP status or pathological or demographical results as well as evaluation of hypermethylation in patients.

RESULTS
Sample collection. A total of 73 subjects were recruited in this study including 41 patients and 32 healthy controls. As shown in Table 2, there was no significant difference in age, gender and weight between study groups.

Methylation rate in PCR products
As shown in table 3, 28 of 41 (68.2%) CRC plasma samples contained hypermethylated ALX4 while only 8 of 32 (25%) normal samples showed hypermethylation. It indicates that the rate of hypermethylation of ALX4 promoter DNA was significantly higher in the cancer samples ($P < 0.008$). SPG20 evaluation illustrated that 28 hypermethylated cases of 41 patients (68.2%) ($P<0.000$) compared to 53.1% hypermethylation detected in normal samples. 25 of 41 cases showed hypermethylation of ITGA4 in patients (60.9%) with 30.3% healthy cases (Figure 1).

Statistical analysis of single methylation
Statistical analysis revealed that ALX4 gene was hypermethylated in 28 of 41 patients (68.2%), in which 24 individuals were homozygous and 4 were

heterozygous, while in case of healthy individuals, the hypermethylation was observed in 8 of 31 (25.8%) cases. These results indicated 74.9% sensitivity and 73.1% specificity for detecting CRC by using the plasma samples as a noninvasive method. Hypermethylation study in SPG20 gene showed 28 hypermethylated cases of 41 (68.2%) in which 27 individuals were homozygous and 1 was heterozygous, while 17 methylated SPG20 promoter DNA (53.1%) were detected from healthy individuals. These results indicated 46.9% sensitivity and 68.3% specificity of the test for detecting CRC by using the Plasma. In total of 41 patients, 25 cases showed hypermethylated ITGA4 (60.9%) containing 15 homozygous and 10 heterozygous. As shown (table. 4), 10 methylated ITGA4 promoter DNA (30.3%) were detected from healthy individuals. According to the results, ITGA4 promoter DNA has 69.7% sensitivity and 61.0% specificity of the test for detecting CRC by using the Plasma (figure 2).

The study of simultaneous methylation is shown in Table 4. It was revealed that the both ITGA4-SPG20 genes are methylated in 15 of 41 patients (49.2%) while it was reported in 6 of 30 (20%) in the control group (p< 0.007). Moreover, at least one gene was methylated in 48.6% of patients and no methylated SPG20 promoter DNA was observed in 8.6% of patient. Of 41 patients studied, 16 patients (48.5%) and 3 of 32 healthy individuals (13.6%) showed simultaneous methylation of SPG20 and ALX4 promoter DNA with at least one gene methylated in 68.2% of patients, and only 6% with no methylation (p< 0.022). The studied also 16 of 41 patients (44.4%) and 3 of 32 healthy individuals (11%) with simultaneous methylation of ITGA4 and ALX4 promoter DNA with at least one gene methylated in 44.4% of patients (p< 0.001). Simultaneous methylation of all three promoters DNA was also investigated in which 6 of 41 patients (18.8%) showed hypermethylation and no Methylation of three genes was observed in healthy group simultaneously. However, one or two genes were hypermethylated in 22 of 41 patients (53.6%) (P< 0.009).

Table 2: comparison of age and gender and weight between patients and normal individuals

Factor	Patients	Normal	P-value
Total number	41	32	
Mean Age (Year)	60.19 ± 15.5	54.71 ± 13.2	0.397
Male: Female	22:19 (53:47%)	15:17 (46:54%)	0.623
Mean Weight (Kg)	73.39 ± 3.15	70/50± 4/7	0.432

Table 3: Statistical analysis of the methylation of ITGA4, SPG20 and ALX4 promoters

Gene	Result	Case (CRC)	Control (Normal)	P value
ALX4	Positive	28 (68.2%)	8 (25)%	<0.008
	Negative	13 (31.8%)	24 (75%)	
ITGA4	Positive	25 (60.9%)	10 (32.2%)	<0.14
	Negative	16 (39.1%)	22 (67.8%)	
SPG20	Positive	28 (68.2%)	17 (54.8%)	<0.000
	Negative	13 (31.8%)	15 (45.2%)	

Table 4: Statistical analysis of the simultaneous methylation

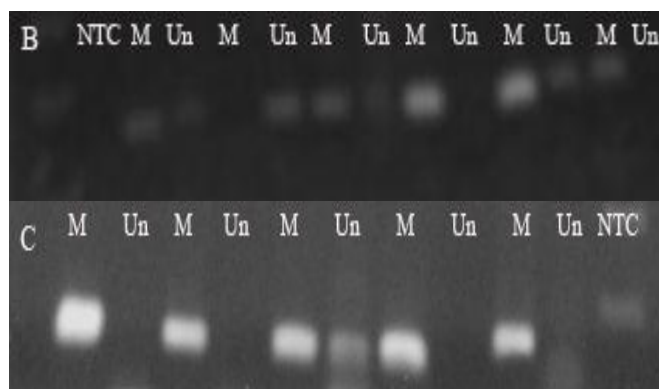| Genes		Simultaneous positive result	P value
ALX4 and ITGA4	Case	16 (39%)	p< 0.001
	Control	3 (9.3%)	
ITGA4 and SPG20	Case	15 (36.5%)	p< 0.007
	Control	6 (18.7%)	
SPG20 and ALX4	Case	16 (39%)	p< 0.022
	Control	3 (9.3%)	
SPG20 and ALX4 and ITGA4	Case	6 (14.6%)	p< 0.009
	Control	0	

Figure 1. MSP PCR of case and control samples. (A) Detection of methylated DNA of SPG20 gene in samples (M1, M2 and M3- NTC is negative control) (B) Detection of unmethylated (Un) and methylated (M) DNA of ITGA4 gene in samples (NTC is negative control). (C) Detection of unmethylated (Un) and methylated (M) DNA of ALX4 gene in samples (NTC is negative control).
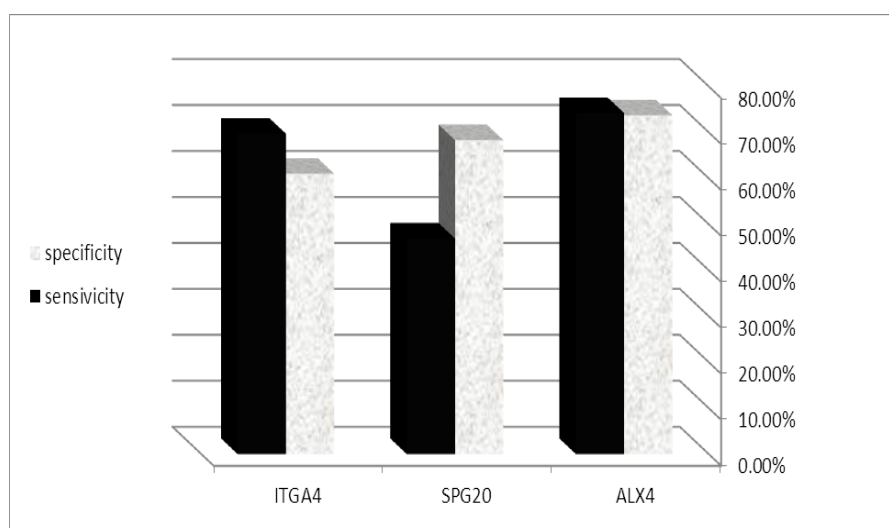

Figure 2: Specificity and Sensitivity of ITGA4, SPG20 and ALX4 based on Combine Cross tabulation in SPSS 17 software.

DISCUSSION

The most important prognostic factor in colorectal cancer is stage at diagnosis and one of most effective ways of improving prognosis is early diagnosis (Hardingham et al., 2015; Schreuders et al., 2015). CRC has some dedicated symptoms which rarely can be detected in the early stages by histological techniques since the removal of the tumor in the early stages of the disease is the only therapeutic approach (Kadiyska el al., 2015).

Besides genetic alterations, DNA hypermethylation of tumor suppressor genes is a frequently detected mechanism behind the inactivation of these genes leading to tumor initiation and is an efficient way to achieve cancer molecular markers (Kalmár el al., 2015). Using promoter hypermethylation as a diagnosis and prognosis marker involves several advantages. Hypermethylated region is an indicator of abnormality. For evaluation of methylation change, sensitive methods such as methylation specific PCR are well developed (Grützmann el al., 2008).

The aim of this study was Evaluation of three epigenetic markers SPG20, ITGA4 and ALX4 for early detection of CRC, since the hypermethylation of these genes is occurred at early stages of tumor formation and they are detectable in plasma and tissue samples. Within these years, several groups and research centers had reported a large amount of clinical results about the sensitivity and specificity of DNA tests by using different markers in colorectal cancer patients. ColoSure™ diagnostic test was presented in the United States in 2010 as a diagnostic test to detect colorectal cancer based on Vimentin methylation markers in stool samples of patients (Ned et al., 2011). Grutzmann et al identified SEPTIN9 marker as a methylation marker for early detection of colorectal cancer, which would detect colorectal cancer at an early stage in the plasma samples. In this study rate of methylation in CRC samples was 58% (Grützmann el al., 2008). In 2014, the cologuard company could receive its FDA approval for early detection of colorectal cancer using a panel of BMP3 and NDRG4 methylation markers along with KRAS mutations (Dhaliwal et al., 2015). In a study conducted by Eugene Chang et al, the specificity and sensitivity of

promoter methylation of ITGA4, SFRP2 and P16 was studied in fecal DNA with the aim of replacing occult blood test in adenoma and carcinoma of the colon. The results suggested that these genes can be used as a marker for early screening of colorectal cancer (Chang et al., 2010). Zhang et al., examined the possibility of detection of aberrant SPG20 promoter hypermethylation as a marker based on stool DNA for the non-invasive detection of colon cancer (Zhang et al., 2013). We detected hypermethylated SPG20 in plasma samples of CRC patients, as well as the healthy individuals. It indicated an 46.9% sensitivity and 68.3% specificity which was lower than what Lind et al's and Zhang et al's reported (about 90% an 88% specificity) (Zhang et al., 2013). Furthermore, we found that the hypermethylated ALX4 promoter DNA in blood samples had a high sensitivity and specificity (74.9% and 73.17%) for detecting CRC. In addition, we simultaneously detected hypermethylated SPG20/ITGA4, SPG20/ ALX4, ITGA4/ALX4 and SPG20/ITGA4/ALX4in samples from the patients with CRC. Accordingly, simultaneous study of all three genes hypermethylation as a panel of biomarkers in the plasma samples appears to suggest a powerful screening method for detecting CRC since no simultaneous methylation was observed in healthy group in case of SPG20/ITGA4/ALX4.

Moreover, based on the statistically significant results, the percentage of simultaneous methylation in patients was lower than single gene methylation which may be due to the fact that study population was not large enough and thus increase in the number of patients will lead to more accurate results. The study of hypermethylation of genes showed no statistical correlation with gender, age, weight, BMI, family history, meat and vegetables consumption, aspirin consumption and smoking. The only significant correlation as observed between the disease history and the hypermethylated ALX4 gene with a P value of 0.018. As the hypermethylation of ALX4 gene was increased in patients with a history of polyps or diabetes diseases.

CONCLUSION
Followed by careful statistical analysis of the MSP results of all three genes and pathology results from colonoscopy, it seems that the detection of simultaneous hypermethylation of SPG20/ITGA4/ALX4 promoter is a specific and sensitive biomarker panel in the diagnosis of patients with cancer risk and can be helpful before colonoscopy. Moreover, it is promising to achieve the relationships between environmental factors and family history with epigenetic modifications, especially gene methylation in colorectal cancer. Collectively, results of numerous studies on use of biomarkers, Specially by noninvasive methods, are expected to developed the landscape of CRC screening, and would resulted in reducing mortality of CRC through early detection of pre-cancerous lesions.

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