SEPT9: A PROMISING BLOOD-BASED BIOMARKER FOR EARLY DETECTION OF COLORECTAL CANCER.

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ABSTRACT
Background: Colorectal cancer (CRC) is the fourth most frequent cancer worldwide. CRC screening could reduce cancer-related mortality. The aim of this study was evaluation of seoptn9 methylated DNA for detection of CRC and precancerous colorectal lesions. Methods: Plasma samples were collected from 25 untreated CRC patients, 20 polyposis adenoma and 42 healthy control subjects before colonoscopy. We used methylation-specific PCR to assay the methylation status of seoptn9. Results: Septin9 was hypermethylated in 64% of CRC patients and 15% of polyposis adenoma. Sensitivity and specificity of seoptn9 methylated DNA test was 64% (95% CI: 43%-83) and 83% (95% CI: 68%-98) respectively. Conclusion: Results of this study suggest that seoptn9 is a valuable biomarker for CRC screening.

KEYWORD: seoptn9, colorectal cancer, methylation, screening test.

INTRODUCTION
Colorectal cancer (CRC) is the fourth common cancer worldwide with increasing rate of mortality and morbidity.[1] The estimated one million new CRC cases are diagnosed each year especially in developing countries.[2] A sufficient strategy for better management and increasing survival rate of patients is early diagnosis of CRC, such as other cancers.[3] Finding sensitive and specific biomarkers for early detection of CRC is required which in turn leads to decrease CRC-related mortality.[4] Different screening methods are used for detection of CRC in different populations.[5] These screening methods include fecal occult blood test, CT colonography, flexible sigmoidoscopy, colonoscopy and currently used methods such as molecular biomarkers.[6,7] Over the last decade, advances in genomics and molecular studies have demonstrated that different molecular pathways are involved in the development of CRC.[8] Epigenetic alternations in different oncogenes and tumor suppressor genes is an early changes in almost all types of cancer and is a promising field in screening and diagnosis of CRC.[9,10] One of these biomarkers, is plasma methylated Septin9 DNA (mSEPT9). SEPT9 is a member of septin gene family, and has been found to act as an oncogene and tumor suppressor gene in different types of cancer.[11,12] Several studies showed the role of SEPT9 in cancers, including leukemia, ovarian cancer, urologic cancer, brain tumors and CRC.[13,14] The molecular mechanism of SEPT9 in colon tumorigenesis is not well known yet.[14] Several studies have demonstrated that SEPT9 is hypermethylated in CRC patients.[12,15,17] Based on these studies, the mSEPT9 looks a powerful marker for non-invasive detection of CRC. In this study SEPT9 methylation in plasma samples in healthy controls, CRC cases and patients with polyposis adenoma was assayed by Methylation Specific PCR (MSP) to evaluate the hypermethylation of this gene as a non-invasive biomarker for early detection of CRC.

MATERIAL AND METHODS
Study design and patients: A total of 42 healthy controls (no evidence of disease), 20 patients with polyposis adenoma (more than 1 cm diameter) and 25 patients with CRC (approved by histopathologic results) were enrolled in this study in Baqiyatallah hospital, Tehran, Iran. After obtaining written informed consent and questionnaire, 6 ml peripheral blood samples were collected from patients using K3EDTA vacutainer tubes (Gold vac™-China).

DNA extraction. Plasma samples were obtained from 6 ml freshly collected blood samples by centrifugation at 3000 rpm for 10 minutes. Plasma samples were then stored at -80°C until further use. DNA isolation from 400 μL plasma was conducted using QIAamp DNA Blood
Mini kit (Qiagen-Germany), dissolved in 70 μL of sterile distilled water and stored at -20°C. Concentration of the isolated DNA was quantified using nanodrop spectrophotometer (Maestrogen-USA).

**Bisulfite Treatment.** 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 controls.

**Amplication of mSEPT9.** Methylation-Specific PCR (MSP) was performed to determine methylation status of SEPT9 gene. Primer sequences for the methylated and unmethylated templates are showed in Table 1. Each PCR reaction mix consisted of a total volume of 30 μl containing 15 μl hot start Taq Master Mix, 1 μM concentration of each primer, 1 μl DMSO and 3 μl bisulfite-modified DNA and 9 μl RNase free water.

**Table 1. primers designed for methylated and unmethylated SEPT9 gene.**

<table>
<thead>
<tr>
<th>SEPT9 Gene</th>
<th>sequence (5’&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M_Forward primer</td>
<td>TAGTTATTGGCGTTAGCGCG</td>
</tr>
<tr>
<td>M_Reverse Primer</td>
<td>TACGCTACGCTCACCATAC</td>
</tr>
<tr>
<td>U_Forward primer</td>
<td>TTGGGAAGTGTGGTGATTTTG</td>
</tr>
<tr>
<td>U_Reverse primer</td>
<td>ACCCCTACACTACACTAC</td>
</tr>
</tbody>
</table>


### Statistical Analysis

Statistical analysis was conducted using the SPSS20 software (version 17.0, SPSS Inc, Chicago, USA). The Fisher’s exact test and χ2 test were applied to study the statistical relationships between either MSP status and pathological or demographical results as well as evaluation of hypermethylation in patients. P value less than 0.05 was considered significant.

**RESULTS**

**Patients.** The study population consists of 87 individuals in three groups (CRC, polyposis adenoma and healthy controls). There was no significant difference in age, gender and weight between study groups (Table 2).

**Table 2. Demographic characteristics of the study population.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Polyposis</th>
<th>CRC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td>42</td>
<td>20</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Age (Mean)</td>
<td>55.6</td>
<td>63.5</td>
<td>64.5</td>
<td>0.5</td>
</tr>
<tr>
<td>BMI (Mean)</td>
<td>26.4</td>
<td>26.3</td>
<td>25.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Smoking</td>
<td>No</td>
<td>35(83.3%)</td>
<td>14(70%)</td>
<td>19(76%)</td>
</tr>
<tr>
<td>Yes</td>
<td>7 (16.7%)</td>
<td>6 (30%)</td>
<td>6 (24%)</td>
<td></td>
</tr>
</tbody>
</table>

**Methylation of SEPT9 in plasma samples**

As shown in table 3, 7 of 42 (16%) healthy controls contained hypermethylated SEP9. In CRC group the rate of hypermethylation is 16 of 25 (64%) and in polyposis adenoma group, 3 of 20 (15%) showed hypermethylation in SEPT9.

**Table 3. Methylation status of SEPT9 in study population**

<table>
<thead>
<tr>
<th>Group</th>
<th>Negative</th>
<th>Positive</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>35 (84%)</td>
<td>7 (16%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Polyposis</td>
<td>17 (85%)</td>
<td>3 (15%)</td>
<td>0.007</td>
</tr>
<tr>
<td>CRC</td>
<td>9 (36%)</td>
<td>16 (64%)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Specificity and Sensitivity of mSEPT9**

Evaluation of methylation status of SEPT9 in this study demonstrated that specificity and sensitivity of this gene is 64% and 83.3% respectively (figure 1).
Figure 2. SEPT9 Methylation Status in affected CRC patients using methylated and unmethylated primers on 1.5% agarose gel. M: methylated. U: unmethylated. P1-S: patients.

DISCUSSION

Colorectal cancer is still one of the most commonly diagnosed cancers worldwide,[18] and although survival after diagnosis has improved over the last decades the overall 5-year survival remains less than 50%.[19] According to importance of early diagnosis, it seems that screening is therefore the only sufficient method of early detection.[18] Based on several studies, DNA markers in stool, blood and other body specimens have been investigated and different DNA panels have been developed as a powerful screening approach.[19,20] For example, In our pervious study, it has been concluded that detection of simultaneous hypermethylation of SPG20/ITGA4/ALX4 gene promoters is a specific and sensitive biomarker panel in the diagnosis of patients with cancer risk and can be helpful before colonoscopy.[21] The aim of our present study was evaluation of mSEPT9 in plasma samples of CRC patients in comparison with healthy controls. The rate of hypermethylation in CRC patients was 64% while in polyposis adenoma and healthy controls 15% and 16% showed hypermethylation in SEPT9 respectively. Numerous studies have been evaluated SEPT9 as a biomarker in diagnosis and prognosis of CRC. Grutzmann et al conducted a study on methylation of SEPT9 in 354 plasma samples and concluded that detection of mSEPT in plasma is a minimally invasive screening method.[18] In a study conducted by Tänzer et al, 73% CRC patients showed hypermethylation in SEPT9 while in healthy control group was only 9%.[15] In another study, Warren et al concluded that SEPT9 could be a specific marker for CRC screening instead of colonoscopy.[22] Toth el al showed SEPT9 methylation in 8.3% of controls, 30.8% of adenoma and 88.2% of CRC in a study using Real-time PCR in plasma samples.[13] Overall, these studies demonstrated that mSEPT9 is a valuable biomarker in detection of CRC as a molecular screening method.

In this study, specificity and sensitivity of SEPT9 test was 64% and 83.3% respectively. In other studies, the rate of specificity and sensitivity is approximately similar. Sensitivity of SEPT9 test in plasma samples of 50 CRC patients in Warren et al study was 88%.[12] 73.3% in David A et al study.[24] and 68% in Potter et al study.[14]

CONCLUSION

Collectively, results of this study in comparison with other aligned researches demonstrated that evaluation of mSEPT9 seems to be a reliable biomarker for early detection of CRC and we hope future researches make it an efficient and powerful method for CRC screening.

ACKNOWLEDGMENT

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