Original Research Article

Septin-9: a novel biomarker for colorectal cancer screening

Alp Yildiz[1]*, Huseyin Gobut[1], Sezai Leventoglu[2], Aybala Yildiz[1], Mehmet Ali Ergun[2], Ayhan Kubar[3], Bulent Aytac[2]

[1]Department of General Surgery, Yildirim Beyazit University, Yenimahalle Training and Research Hospital, Ankara, Turkey
[2]Department of General Surgery, Gazi University, School of Medicine, Ankara, Turkey
[3]Department of Genetics, Gülhane Military Medical Academy, Ankara, Turkey

*Correspondence:
Dr. Alp Yildiz,
E-mail: alpyildiz2501@gmail.com

ABSTRACT

Background: The gold standard method for the diagnosis of colorectal cancer is colonoscopy. However, colonoscopy has several limitations and complications. Moreover, fecal occult blood test and serum based tumor markers do lack the desired specificity and sensitivity. Therefore, a novel biomarker is needed for early detection of colorectal cancer. With this prospective randomised controlled study author's aim was to evaluate the efficacy of Septin-9 on colorectal cancer screening.

Methods: Septin-9 is a protein which is encoded by the SEPT9 gene. SEPT9 has been detected in the blood of colorectal cancer patients. In this study, the results of fecal occult blood test, carcinoembryonic antigen and SEPT9 using fecal and blood samples of the patients obtained before colonoscopy were compared. DNA isolation and bisulfite transformation were made from the blood samples for SEPT9. The experimental and control groups were built up using the results of biopsy taken during colonoscopy.

Results: Fecal occult blood test was determined as positive in control group for 30% (6/20), in colorectal cancer group for 54.2% (13/24). FOBT specificity and sensitivity for colorectal cancer were 54.1%, 70%, respectively. Sensitivity and specificity of Septin9 at detecting colorectal cancers were found statistically significant according to either fecal occult blood test or carcinoembryonic antigen values.

Conclusions: When author evaluated their situation in the light of the data, it was seen that CRCs is an important public health problem. As being an easily applicable test by blood sample and having high sensitivity and specificity, SEPT9 should take its place in routine screening protocols.

Keywords: Cancer screening, Colorectal cancer, Septin-9

INTRODUCTION

Colorectal cancer (CRC) is a common type of cancer having high mortality and morbidity rates. The possibility of the early detection and intervention of CRC with the revelation of pathogenesis of the cancer with conducted researches along after molecular and biological developments show this cancer, which is constituted by genetic and environmental effects in a long time can be preventable.[1] Early detection in CRCs, beside decreasing mortality and morbidity rates, also decreases costs of treatment. The way to detect CRC in early stages is to catch illness in asymptomatic stage by screening programs.

In 2000, USA president Bill Clinton determined March as awareness month of CRC in order to raise awareness. Every year in March, CRC awareness events are

organized. Blue star become the symbol of this event. Since 2008, Europa colon has organized similar events. According to the studies published in American Cancer Society (ACS), screening rates of CRC would reach 80% by 2018 as a first goal and along with this, it is predicted that 277.000 new cases and 203.000 deaths would have been avoided for 20years period.[2] A 17% decrease in the incidences and a 19% decrease in mortality for CRC are expected by the end of 2020 and in 2030, 22% decrease in incidences and 33% decrease in mortality are expected.[2]

For that reason, importance and applicability of screening programs come into prominence. In screening programs, fecal occult blood test, sigmoidoscopy, colonoscopy and imaging methods are used. Tests based on stools are non-invasive, cheap but have low specificity and sensitivity, and have high false positiveness. However, colonoscopy is accepted as golden standard because of some limitations being invasive, simpler, more applicable and non-invasive screening methods are required.[2]

In present study, specificity and sensitivity comparison of methylated Septin9 as colorectal cancer biomarker in blood with fecal occult blood test (FOBT) and serum Carcinoembryonic Antigen (CEA) values was aimed.

METHODS

Septin9 is a kind of protein produced by SEPT9 gene. Septin9 gene (SEPT9) is a localized gene in 17q25 region. It codes 12 coded exons and 586 amino acids. Its molecular weight is 65369daltons. This gene is a member of Septin family, responsible for the control of cytokinesis and cell cycle. Septins, as GTPases, are interacting with microtubules and actins in cytoskeleton. It acts as tumor suppressor in uncontrolled and rapid cell division. It regulates cell growth or hold very rapid and uncontrolled dividing cells.[3] The tissues which they expressed are brain, colon, heart, kidney, leucocyte, liver, ovary, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen and testicles.[3]

The patients, who registered to Gazi University Medical Faculty General Surgery Department Endoscopy Unit for their routine screening of over the age of 50 with the complaints of rectal bleeding and having malignity suspicion were involved in the study.

Patients under the age of 18, pregnant patients and the ones having colon surgery history in their past were excluded from the study. Detailed medical story of 225 patients involved in the study were gathered and after physical inspection, detailed interviews were conducted and then informed consent forms were given to the patients and their consents were taken for the study. Demographic information and parameters for analysis were recorded into previously prepared standard forms. All patients were applied to colonoscopy procedure. Suspicious lesion localizations were recorded and

samples were taken by biopsy. Demographic data, SEPT-9 levels, FOBT results and CEA results of control group with normal colonoscopy and patients with malign results out of pathology were examined.

During the study, among 225 patients involved in the study, 38 patients were excluded because they did not bring their CEA and FOBT samples and 36 patients were excluded due to technical (sample with hemolysis etc.) reasons. Out of 151 patients, 105 patients with normal colonoscopy results were evaluated as control group and 46 patients with cancer detection were evaluated as colorectal cancer group.

The data analysis was done for 20 control group patients and 24 colorectal cancer group patients, whose DNAs were able to be isolated Table 1.

Table 1: Design of the study.
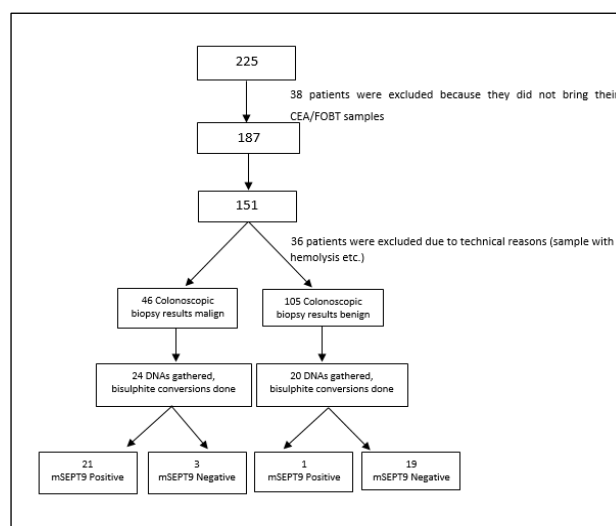
From every individual, either in patient group or control group, 2 pieces of 10ml complete blood count were gathered. These samples were kept waiting at room temperature and 3ml plasma were collected. Plasmas were separated to 1.7ml eppendorf tubes and they were kept under -8000°C until the time of study. For DNA isolation from samples, alkali phenol-chloroform method was used. For this purpose, each one sample was taken to 250ml 6-8 eppendorf tube and 12ml pronaseE (72mg/ml, SERVA, Germany) +250mm K buffer was added to, then they were incubated for 2hours at 470°C. 500mm alkali phenol-chloroform were added on the samples taken from there and they were vortexed. The mixture was centrifuged at 12.000rpm and +80°C for 10minutes. After centrifuge, transparent phase, which constituted upper part of the tubes, was taken to a new eppendorf tube and 700ml isopropyl alcohol (Merck, Germany) was added on them, they were centrifuged at 12.000rpm and +80°C for 10minutes. After centrifuge, supernatant was poured and 300mmm 75% ethyl alcohol was added to eppendorf tube and vortexed and were centrifuged at 12.000rpm and +80°C for 10minutes. After centrifuge, supernatant was

poured and eppendorf tubes were kept at 3700ºC for 10-15minutes and then were dried. Thereafter, 30mmm distilled water without DNAase and RNAase was added to the first eppendorf tube of 8 eppendorf tubes of the same sample and DNA was solubilized. By transferring the sample including 30mm DNA in this tube to other 7 eppendorf tubes respectively in the same way, 1.5-2.0ml plasma sample was concentrated to 30mmm. These samples were kept at -800ºC until PCR tests were done.
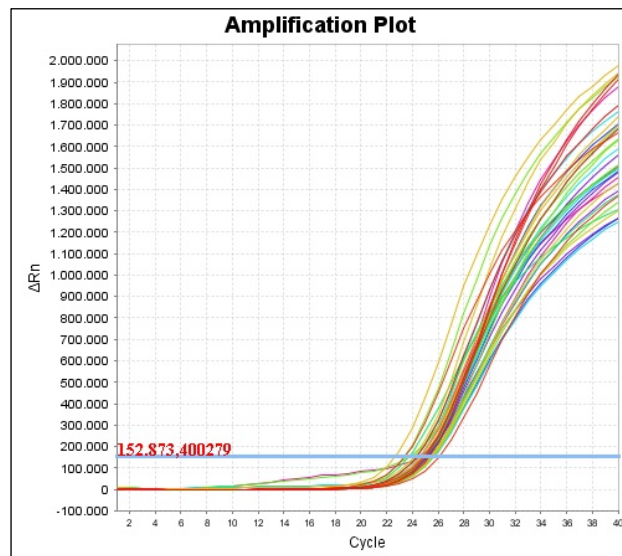
RESULTS

Among the patients included in the study the group which their colonoscopic determined as normal defined as control group (20 patients) and the cancer determined group defined as cancer group (24 patients).

Average age of the control group was determined as 57.05±2.56 (38-79). 13 of the patients (65%) were men. Average age of colorectal cancer group was determined as 57.91±1.45 (21-84). 14 of the patients were women (58.4%). Colonoscopy tumor localization was determined as rectum in 7 patients (29.1%), sigmoid colon in 7 patients (29.1%), left colon in 5 patients (20.8%), right colon in 3 patients (12.5%), caecum in 1 patient (4.1%) and transverse colon in 1 patient (4.1%). Invasion depth after surgery pathology report of 24 patients, who has determined to have colorectal cancer, was determined as pT1 in 2 patients (8.4%), pT2 in 6 patients (25%), pT3 in 10 patients (41.6%) and pT4 in 6 patients (25%).

Septin9 methylation was found 5% (1/20) in control group and 87.5% (21/24) in colorectal cancer group. The reason why Septin9 methylation could not detected at 3 patients in colorectal cancer group was due to technical reasons. The SEPT9 gene region PCR test results were found positive for these 3 patients before participating in bisulphite conversion reaction. However, SEPT9 gene region PCR test positiveness at plasma does not only come from colon epithelium cells but also comes from different tissues and organs in organism. Therefore, actually existing SEPT9 gene region PCR test positiveness reflects a general result and does not reflect specifically SEPT9 from colon epithelium. This situation causes that methylated SEPT9 gene region coming from colon epithelium was not analyzed as Methylation analysis being in test procedure instead, unmethylated SEPT9 gene region coming from other tissues was analyzed. For that reason, the DNA, which was needed to be examined in actual fact, could not be reached due to the low volume of patient plasma samples and patients were reported as normal as false negative (-). The most important problem encountered related with this test system was interpreted as this point. In other words, when starting real time PCR test processes, it was necessary to provide receival of colon epithelium DNA firstly. Author's suggestion for this was to bring, at least 6ml, patient's plasma to laboratory for DNA derivation. Linear amplification curves for showing patient and control sample's real time PCR tests conducted by using

Eva Green fluorescent paint. Ct values are predicted as >30 can be seen in Figure 1.

Ct values are predicted as >30.

Figure 1: Patient and control sample's real time PCR tests conducted by using Eva Green fluorescent paint.
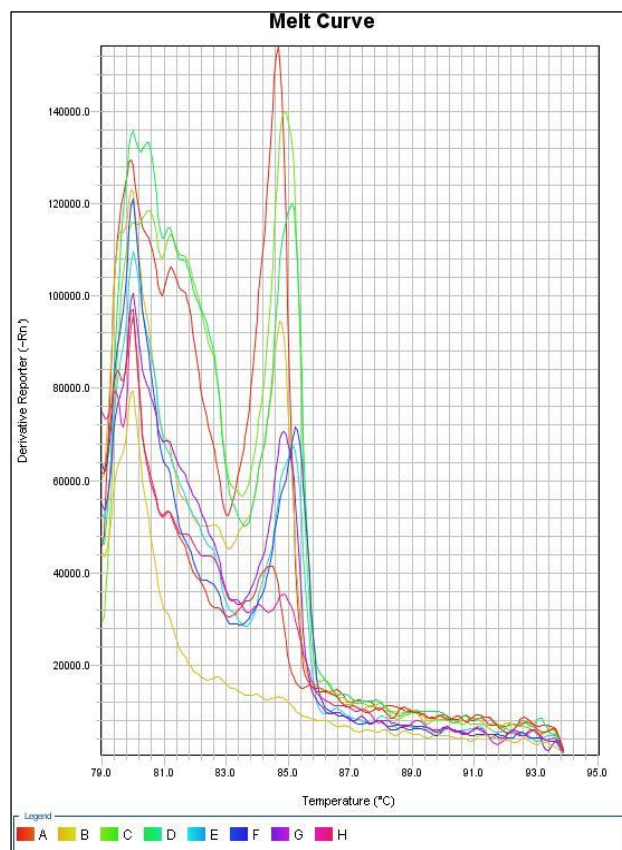

Figure 2: Melting curve peaks of DNA samples of colon cancer patients obtained as a result of methylation of C (cytosine) nucleotide out of bisulphite reaction and as a result of not able to be converted to U/T (Urasil/Timin) nucleotide.

Additionally, melting curve peaks of DNA samples of colon cancer patients obtained as a result of methylation of C (cytosine) nucleotide out of bisulphite reaction and as a result of not able to be converted to U/T (Urasil/Timin) nucleotide as shown in Figure 2.

Specificity and sensitivity of Septin9 for colorectal cancer were determined as 87.5% and 95% respectively.

Fecal occult blood test was determined as positive in control group for 30% (6/20) in colorectal cancer group for 54.2% (13/24). FOBT specificity and sensitivity for colorectal cancer were 54.1%, 70% respectively.

When CEA levels examined, 5% (1/20) of control group patients and 41.6% (10/24) of colorectal cancer patients have rates over 5ng/ml. CEA specificity and sensitivity for colorectal cancer were 41.7%, 95% respectively (Table 2).

Table 2: Patient results according to colonoscopic biopsy.

Patient	Colonoscopic biopsy malign	Colonoscopic biopsy benign	Total
Age	57.91±1.45 (21-84)	57.05±2.56 (38-79)	
Gender			
Male	10	13	23
Female	14	7	21
pT			
1	2	-	-
2	6	-	-
3	10	-	-
4	6	-	-
Localisation			
Caecum	1	-	-
Right colon	3	-	-
Transverse colon	1	-	-
Left colon	5	-	-
Sigmoid colon	7	-	-
Rectum	7	-	-
CEA			
Positive	10	1	11
Negative	14	19	33
FOBT			
Positive	13	6	19
Negative	11	14	25

When sensitivity and specificity of methods examined, sensitivity of CEA was determined as 41.7%, specificity of it was 95% (p=0.0006), sensitivity of FOBT was 54.1%, specificity of it was 70% (p=0.135), sensitivity of SEPT9 was 87.5%, specificity of it was 95% (p<0.0001) (Table 3). Sensitivity and specificity of Septin9 at detecting colorectal cancers were found statistically significant according to either fecal occult blood test or carcinoembryonic antigen values.

Table 3: Comparison of SEPT9 with CEA and FOBT.

Patient	Sensitivity (%)	Specificity (%)	p value*
mSEPT9	87.50 95 0.0001	87.50 95 0.0001	87.50 95 0.0001
CEA	41.70 95 0.0060	41.70 95 0.0060	41.70 95 0.0060
FOBT	54.16 70 0.1350	54.16 70 0.1350	54.16 70 0.1350

DISCUSSION

CRC is one of the most common cancer types in developed countries and its incidence increase gradually.[4] As it is ranked as the second most cause among cancer related deaths, it also constitutes 9% of cancer deaths, and constitutes 3% of all deaths.[5] Early detection and excision of precancerous polyps may avoid conversion to cancer. For that reason, many screening methods have been developed for the purpose of detecting precancerous polyps or colon and rectum cancers in early stages. The increase of these marker's clinical usage would make contribution to increase in CRCs lifespans and enhancements of their life qualities.[6] Whereas screening methods having very high sensitivity and specificity such as colonoscopy are invasive and have low patient compatibility, screening methods such as fecal occult blood test, carcinoembryonic antigen are less invasive and more tolerable by patients, they are the methods to be able to comply with. Handicap of these methods was their low specificity and sensitivity.[7]

Developments in molecular technology, mutation analyses and genome mapping cause discovery of a series of biomarkers (diagnostic, pharmacological, predictive and prognostic) for CRC.[8] CRC screening method proposed in England and at Council of Europe decision in 2006, as stool based studies have low compatibility, showed the need for new generation blood based biomarkers.[9] Starting from this point, detection of Septin9 methylation at plasma DNA of colon cancer patients was approved at New York State Health department in 2011 and made a great impact. Studies were conducted related with its usage as screening method by examining Septin9 methylation in blood.[10]

In this study, specificity and sensitivity comparison of methylated Septin9 as colorectal cancer biomarker in blood with fecal occult blood test and serum CEA values was aimed. Fecal occult blood test was examined both for control and cancer group.

The sensitivity of FOBT test was determined as 30-40% in previous studies but when the number repetition of test programs increase, its sensitivity increases up to 80-92%.[11]

In a systematic collection, it was reported that special diet application does not decrease false positive result rate but on the contrary, it decrease compatibility of patients to screening test.[12] Similarly, providing hydration before test, cutting iron supplements do not affect test negatively.[13] Author applied fecal occult blood test to patients at least two days before colonoscopy operation without applying special diet program. Author determined a proportion of 30% false positiveness. Author determined the sensitivity of FOBT as low for colorectal cancer in this study. In the literature, also, it was reported that although FOBT was used at colorectal cancer screening programs, it was not specific at gastrointestinal system bleeding and also its sensitivity was low. Besides this, thanks to this screening method, at a 15-33% rate decrease was shown in colon and rectum cancer incidences and mortality. At 6 patients in control group, who was determined to have false positive, neoplasia and polyp were determined. Hemorrhoidal illness, colonic inflammation or diet can be speculated as a reason for false positiveness. When compared with FOBT, Septin9 values gathered from blood test has statistically significantly higher specificity and sensitivity at detecting colorectal cancers (%87.5, %95, respectively). This was interpreted as an encouraging result with regards to suitability of Septin9 to be used as screening method.

CEA is known as a muscle test used for the purpose of examining existence of locoregional relapse or metastasis as a tumor marker after tumor surgery. For that reason, it was not used as screening test. At colorectal cancer detection, specificity of CEA highness was 85.2% whereas its sensitivity was reported as 51.8%.[7] Its low sensitivity values are also bringing out the importance of this test with regards to tumor monitorization. In colorectal cancer group, whereas CEA highness was found at 10/24 patients, Septin9 in this group was determined as positive at 1/20 of the group. Author determined specificity and sensitivity of CEA for colorectal cancers 41.7% and 95%, respectively. When compared with Septin9, both specificity and sensitivity of CEA were observed as significantly lower (p=0.006). This shows CEA was not a reliable test as a screening method for colon and rectum cancers.

Despite the differences due to changes in methylation of tumorigeneses of SEPT9 right and left colon cancers, it was very important clinically to develop a screening test, which has sensitivity independently from its localization, was based on blood, minimal invasive and CRC specific.[14]

In conducted studies, SEPT9 DNA methylation profile was examined at epithelial and stromal cells of healthy and ill individuals. SEPT9 DNA methylation change was examined only in SEPT9 CpG island and only heterogenous colorectal tissue samples were examined. In analyzed tissues, it was determined that SEPT9 is not methylated at normal tissues but in CpG island it is methylated and it is accepted as an early indicator at development of adenoma carcinoma.[15] Developing technology and PCR applications ease material augmentation and determination and sensitivity rate increase comparably to colonoscopy. Intestine preparation procedures, sedation, its requiring expertise and possible complications are limitations of colonoscopy. However, disadvantage of blood based SEPT9 methylated DNA method when compared with colonoscopy is it's not having curative property and its inability to demonstrate expected effectiveness at adenoma detection. It is evaluated as an easily applicable, non-invasive test which is suggestible to intermediate risk group, who do not want colonoscopy or colonoscopy is unattainable to.[10]

As PSA test is used in routine together with screening of prostate cancer, it was indicated that blood test usage is necessary with CRC screening method. Such kind of blood-based tests, which are conducted after colonoscopy, easily done, easily monitored and help to early detection of CRC have very effective potential to reduce mortality as a result of this illness.[16] In this research, author found compatible results with literature findings. With obtained findings, it was shown that SEPT9 gene methylation results have higher sensitivity and specificity values when compared with both CEA and FOBT. Obtained data in this study constitute an important background for SEPT9 gene methylation study to turn into be a commercial kit in this country and for extensive public scanning to be done.

On the other hand, this kit is predicted to be 25-30USD on the average when author make cost analysis. According to this statistical analysis results having high sensitivity and specificity values of SEPT9 kit showed also that it was more logical/rational to apply this test as screening test.

Author knew that early detection was important for colorectal cancer prevention and treatment and thus many studies showed that screening programs have positive contributions to colorectal cancer morbidity and mortality. Thus, asymptomatic patients should be screened for CRC regardless of their risk level. Society should be informed about early detection opportunity of screening tests and their role in reduction of CRC morbidity and mortality rates. Especially the ones having family members with CRC stories, all segments of society should be courage to make their screening tests done in symptomless periods.[2]

Screening methods are explained in detail for normal population and risk groups all around the world. When author evaluated the situation in the light of their data, it was seen that CRCs is an important public health problem. As being an easily applicable test by blood sample and having high sensitivity and specificity, SEPT9 should take its place in routine screening protocols.

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