RESEARCH ARTICLE

Editorial Process: Submission:11/02/2022 Acceptance:03/16/2023

The Clinical Significance of Septin 9 and Colon Cancer Specific Antigen-2 (CCSA-2) in Colorectal Cancer

Lamiaa Abd El Kader[1], Amira H Soliman[1], Salem E Salem[2], Samia Y Akel[1], Noha H Ibrahim[1]*

Abstract

Introduction: Colorectal cancer (CRC) is a major health problem Worldwide, Egypt shows a high rate of early CRC in the world as 35% of 1,600 Egyptian CRC patients were under 40 with threefold increased risk of death within 5 years. DNA methylation-based biomarkers as methylated Septin9 (mSEPT9) has a promising role for detecting CRC. As well as set of nuclear matrix proteins associated with changes in the nuclear structure/architecture. detection of these nuclear proteins resulted in identification of biomarkers that are specific for colon cancer. Particular interest has been placed on colon cancer specific antigen-2(CCSA-2). **Methods:** A total of 30 newly diagnosed CRC patients, 7 colonic adenoma patients, and 15 age- and sex-matched control subjects were recruited in this study. Plasma mSEPT9was assayed by Epi procolon kit, CCSA-2 by ELISA and, Occult blood in stool by Guaiac-based fecal occult blood test. The level of Colon Cancer mSEPT9 and CCSA-2 were carried on CRC patients both preoperatively and three months postoperatively. **Results:** mSEPT9 has 96.7% sensitivity and 95.5% specificity in differentiating colorectal cancer patients from non-malignant cases. Also, our study showed a highly statistically significant difference between the pre and three months postoperative expression of mSEPT9 in colorectal cancer as there was a dramatically decrease in the expression of mSEPT9 postoperatively (p value < 0.001). The CCSA-2 at the cutoff level of >1.43 would provide 93.3% sensitivity and 90.9% specificity in differentiation between malignant and non-malignant cases. Also, the study showed that there is a statistically significant difference between colorectal cancer patients preoperatively and postoperatively according to CCSA-2 with dramatic decrease in its level postoperatively (p value > 0.001). **Conclusion:** The plasma SEPT9 DNA methylation level and Serum CCSA-2 could be used as promising non-invasive methods for observing the CRC patients postsurgical response to predict the occurrence of complete remission or relapses.

Keywords: Methyl septin 9, CCS Ag2, CRC

Asian Pac J Cancer Prev, **24 (3)**, 1027-1036

Introduction

More than one million individuals develop CRC each year. It represents the third most commonly diagnosed cancer in males and the second in females, with 1.8 million new cases and almost 861,000 deaths in 2018 according to the WHO being the fourth cause of death worldwide (Ferlay et al., 2018). Egypt displays a high rate of early CRC in the world as 35% of 1,600 Egyptian CRC patients were under 40. It has been described that Egyptian patients who have CRC under the age of 30, are having threefold increased risk of death within 5 years compared to those who have CRC over age of 50. In the younger patients, the 5-year survival rate drops dramatically from 75% to 25% (Bader El Din et al., 2020).

However, it is one of the few preventable cancers, approximately 65 % of patients present with advanced disease with poor prognosis as cancer related survival is closely related to the clinical and pathological stage of the disease at the time of diagnosis (Luo et al., 2011). Five-year survival for cancer limited to the bowel wall at the time of diagnosis approaches 90 %, while it is 35 to 60 %when lymph nodes are involved and less than 10% if there are distant metastasis. Efforts to develop better screening strategies, to improve patient survival rates and to minimize chemotherapeutic toxicity are necessary (Levin et al., 2008).Unfortunately, currently available serological tumor markers for CRC such as CEA and CA19-9 have not proven to be ideal (Leilani et al., 2021).

Determination of epigenetic events is a strong candidate for early detection of disease since regulation of gene expression by aberrant DNA methylation is a well characterized event in tumor biology, and is extensively described for CRC (Béla et al., 2014). In recent years, SEPT9 gene methylation has been recognized as a hotspot and is considered to be a specific biomarker of the early

*[1]Department of Clinical Pathology, National Cancer Institute, Cairo University, Egypt. [2]Medical Oncology Department, National Cancer Institute, Cairo University, Egypt.*For Correspondence: Noha.hassan@nci.cu.edu.eg*

stages of colorectal cancer. It may be a reliable indicator for screening CRC among high-risk individuals (Yu et al., 2018).This test analyses the methylation status of the gamma promoter region of the SEPT9 gene V2 transcript, which is differentially methylated in CRC patients., (Leon Arellano, 2020).

Gene expression is highly dependent on the interaction between chromatin and the nuclear matrix. Changes in the framework of the nuclear matrix may alter gene expression by affecting DNA transcription or replication (Vatandoost et al., 2016). The nuclear framework consists of 10% of the nuclear proteins and is virtually devoid of lipids, DNA, and histones The detection of NMPs in the serum is possible because of the release of NMPs as tumor cells undergo lysis. Specific NMP fingerprint in colon cancer . Four proteins specific for colon cancer were identified that are not present in normal adjacent and normal donor colon tissue. Among the nuclear matrix proteins specific for cancer, particular interest has been placed on colon cancer specific antigen-2 (CCSA-2) (Degui Liu et al., 2014). The researches also revealed that the CCSA-2 had overall sensitivity of 97.3% and specificity of 78.4% in separating individuals with CRC and advanced adenomas from normal, non-advanced adenomas and hyperplastic populations (Warren et al., 2008). Degui Liu et al., (2014) also reported high expression of CCSA-2 in highly dysplastic adenomas and invasive cancers. So, CCSA-2 could be used as a potential marker for colon cancer detection with high sensitivity and specificity.

Up to our knowledge, this is the first study that inspects methyl septin expression with CCSA-2 levels in CRC preoperatively and postoperatively. This study aims to assess the clinical values of methylated septin 9 and, CCSA-g 2 in CRC patients and their relations to other factors such as the age, medical history, family history in addition to other known colorectal cancer tumor markers (CEA and CA19.9) in a trial to verify their ability as a non-invasive diagnostic and prognostic markers in colorectal cancer.

Materials and Methods

Patients

This study was done on 52 participants, including 30 newly diagnosed CRC Egyptian patients at different stages, 7 patients with potentially pre-malignant colorectal lesions (colonic adenomas and polyps), and 15 age- and sex-matched controls. Patients were recruited from surgical oncology outpatient clinics at National Cancer Institute (NCI) hospital, Cairo University, from September 2016 till March 2018. The study was permitted by the Institutional Review Board (IRB) of the NCI, Cairo University. It was permitted according to Helsinki guidelines of studies performed on human beings and written consent was obtained from all study subjects before their enrollment. All patients were subjected to; full history taking and clinical examination, ordinary biochemical and markers investigations, imaging techniques in the form of: CT and MRI, and endoscopic studies with biopsy taking and histopathological examination. Samples were obtained from all patients prior to any therapeutic or surgical intervention Participants' age showed as mean±SD of (45.40±8.23) years in CRC patients, (45.91±9.51) years in the adenoma patients, and (45.17±7.96) years in controls.

Methods

Specimen collection

Ten milliliters of venous blood were withdrawn under aseptic precautions, 7millimeters were put into sterile vaccutainer tube containing K2 EDTA and centrifuged at 3400 rpm for 15 min. Supernatant plasma was transferred to sterile, labeled eppendorf tube. These plasma samples were stored at -70°C until methyl septin9 DNA detection by Epi procolon kit (M5-02). The other 3 milliliters were allowed to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 3400 rpm. The supernatant serum was collected and stored at -70°C until ELISA assay of all samples for detection of Colon Cancer – Specific Antigen-2 (CCSA-2)by Enzyme – linked immunosorbent assay (ELISA), using sandwich ELISA Kit (Catalog No: E-EL-H0793) supplied by Elabscience Biotechnology Inc. according to manufacturer's instructions Figure1.

Determination of serum CEA, CA 19.9 using Cobas e411 Autoanalyzer, Roche. The analyser and Kits were purchased from Abott Architect diagnostics*(Jack et al., 2006) and, occult blood in stool was performed by Guaiac-based fecal occult blood test on stool samples according to manufacturer's instructions

Detection of methylated septin 9 DNA from the plasma

This was done using Epi proColon Plasma Quick Kit (M5-02-001) ,Epi proColon Sensitive PCR Kit (M5-02-002), Epi proColon Control Kit (M5-02-003).

The Epi proColon test principal involves two phases

First, DNA was extracted from plasma and treated with bisulfite using the Epi proColon Plasma Quick Kit (M5- 02-001) to produce bisulfite converted DNA (bisDNA). It was based on the nucleophilic addition of a bisulfite ion to a cytosine nucleotide and a subsequent deamination reaction to yield uracil sulfonate; methylated cytosine was protected from deamination by the methyl group. Thus, the sequence of bisDNA differed from the input DNA by the substitution of uracil nucleotides for unmethylatedcytosines, while retaining methyl cytosine bases.

Second, bisDNA was assayed by a duplex Real-Time PCR (M5-02-002). The control is designed to establish that sufficient input DNA was included in the reaction. The EpiproColon test detects a bisDNA sequence containing methylated CpG sites within the v2 region of the SEPT9 gene and a bisDNA sequence from the ACTB gene with no methylated CpG sites to discriminates methylated cytosines from cytosines using a combination of a blocker oligonucleotide and a methylation specific probe. The reaction comprises primers that are placed in regions lacking CpG dinucleotides, a blocker specific for bisulfite converted unmethylated sequences within the region was added to preferentially amplify methylated sequences, and a methylated Septin 9 specific fluorescent detection probe to exclusively identify methylated sequences amplified during the duplex PCR reaction (Figure 2).

B) Test procedure: Following the instructions of the manufacturer (Epigenomics AG, Berlin, Germany), 3.5 ml plasma samples and one bottle of EpiproColon Positive Control and EpiproColon Negative Control were thawed, then added to 3.5 mL EpiproColon Lysis Binding Buffer in 15 mL centrifuge tube and vortexed for 5 to 10 sec then, incubated at 25°C for 10 min then adding EpiproColon Magnetic Beads 90 µL (freshly suspended) and 2.5 mL of Absolute Ethanol and mixing them then, the tubes were placed into a rotator at room temperature for 45min at 10 to 20 rpm; adjusting rotator angle to 35 to 45 degrees. For DNA washing the tubes were put into the DynaMag™-15 magnetic racks for 5 to 10 min then the supernatant was aspirated carefully, then 1.5 mL EpiproColon Wash A Buffer was added and resuspend magnetic beads completely by vortexing for 5 to 10 sec, the magnetic beads then were transferred into 2.0 mL microtube completely and placed into the DynaMag-2 magnetic racks for 2 to 6 min. removing as much buffer as possible while microtubes are still in the DynaMag-2 magnetic rack, and spin down the microtubes and Put them into the DynaMag-2 magnetic racks for 2 to 6 min and remove the residual buffer. For elution, the microtubes were put into a non-magnetic rack adding 100 µL EpiproColon Elution Buffer to each microtube for 5 to 10 sec resuspend the magnetic beads by vortexing for 5 to 10 sec and put the microtubes into a thermoshaker set to 1,000 rpm and incubate at 80°C for 10 min then spin down the microtubes and place the microtubes into the DynaMag-2 magnetic racks for 2 to 6 min. Transfering the complete eluate, while microtubes are still in the magnetic rack, (~100 µL DNA solution) into fresh 2.0 mL microtubes. For bisulfite Conversion, 150µL EpiproColon Bisulfite Solution, 25 µLEpiproColon Protection Buffer were added to the tubes and mixed by vortexing for 5 to 10 sec then incubated for 45 min at 80 °C without shaking ,adding 1,000 µL EpiproColon Wash A Buffer,20µL EpiproColon Magnetic Beads (freshly suspended) mixing by vortexing for 5 to 10 sec. Placping them in the shaker at 1,000 rpm and incubate for 45 min at 23°C. Then the microtubes were spinned down and placed on the DynaMag™-2 magnetic racks for 2 to 6 min. removing as much liquid as possible while tubes are still in the magnetic rack. Three times wash steps using 800 µL EpiproColon Wash A Buffer then800 µL EpiproColon Wash A Buffer and finally 400 µl EpiproColon Wash B Buffer then allow the beads to dry and adding 60 µL EpiproColon Elution Buffer for 10 min at 23°C in a shaker at 1,000 rpm. spin down the microtubes, transfering the complete eluate (~ 60 µL DNA solution) into a 96-well plate and seal the plate with adhesive film using an adhesive film applicator

Preparation of PCR Master Mix using Epi proColon Sensitive PCR Kit (M5-02-002)

Put 15 µL PCR Master Mix into the selected wells of the MicroAmp® Fast Optical 96-Well Reaction Plate and adding 15 µl of each bisDNA solution to each well of the PCR plate, 15 µl of positive control as well as negative control were added to separate wells each run. Using Software version SDS v1.4 21 CFR Part 11 for plate loading and Thermal Cycler Program (Applied Biosystems 7500 Fast Dx with SDS v1.4)

Interpretation of Results

Positive control to be valid should determine septin 9 cycle threshold less than 41.1 and ACTB (house keeping gene) cycle threshold less than 37.2. While, negative control to be valid should determine no cycle threshold for septin 9 and determine ACTB cycle threshold less than 37.2. Single PCR is considered valid when determine ACTB threshold less than 32.1 which indicates sufficient input of bisDNA into the single PCR. The test result for a patient sample is "POSITIVE", if PCR of a Septin 9 had cycle threshold less than 45. The test result for a patient specimen is "NEGATIVE", if PCR of a Septin 9 had cycle threshold more than45.

Statistical analysis

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage. A one-way analysis of variance (ANOVA) when comparing between more than two means. A paired t-test is used when comparing the difference between two variables for the same subject. Chi-square (X^2) test of significance was used in order to compare proportions between two qualitative parameters. Pearson Correlation Coefficient was used to measure the linear correlation between two sets of data. Multivariate analysis logistic regression: was used to predict the outcome of categorical variable based on one or more predictor variables.The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the P-value <0.05 was considered significant.

Results

The study involved 3 groups; colorectal cancer group (30 patients; 13 female and 17 male), benign colorectal diseases group (7 patients; 2 female and 5 male) and normal healthy persons as control group (15 persons; 7 female and 8 male). Regarding sex and age distribution there was no significant differences between patients with colorectal cancer and both benign patients and controls suggesting that the frequency matching was adequate. There was a significant difference between the patients of colorectal cancer and both benign and control groups regarding the presence of medical history of diabetes, hypertension and smoking (p value = 0.012) and family history of colorectal carcinoma (p value = 0.049 as shown in Table 1.

Colorectal patients in this study mostly were of grade II constituting 67 %, while 6% were grade III and 4% were grade IV. There were a statistically significant differences between colorectal cancer patients and both benign patients and control regarding Hb, ALT, AST, and serum creatinine levels (Table 2). There was a highly statistically significant difference between the three studied groups regarding the preoperative presence of occult blood in the stool (Table 3). There was a high statistically significant difference between colorectal cancer patients and both

Table 1. Comparative Study of Sex, Age Distribution, Medical History and Family History in the Three Studied Groups

Demographic data	Colon Cancer Group (N=30)	Benign Cases (N=7)	Control (N=15)	F/x2#	p-value
Sex					
Male	17 (56.7%)	5 (71.4%)	8 (53.3%)	0.671#	0.715
Female	13 (43.3%)	2 (28.6%)	7 (46.7%)		
Age (years)					
Mean ± SD	45.40±8.23	45.91±9.51	45.17±7.96	1.742	0.289
Median (IQR)	45 (8.7)	46 (11.1)	44 (9.5)		
Range	34-56	37-55	32-55		
Medical History					
Diabetic	9 (30.0%)	0 (0.0%)a	0 (0.0%)a	8.219#	0.012*
Hypertensive	6 (20.0%)	0 (0.0%)a	0 (0.0%)a		
Smoker	9 (30.0%)	3 (42.9%)	0 (0.0%)ab		
Family History					
Negative	23 (76.7%)	7 (100.0%)	15 (100.0%)	5.932#	0.049*
Positive	7 (23.3%)	0 (0.0%)a	0 (0.0%)a		

benign patients and control regarding preoperative level of CCSAg2, CA19.9 and CEA (p value < 0.001) for all of them (Table 4).

ROC curve was drawn for CCSA-2 as malignant and non-malignant cases aiming to detect the reasonable cutoff level of CCS Ag2 regarding both sensitivity and specificity in our study. The area under the curve = 0.994 and this is evaluated statisically to be excellent one (Figure 3). Coordinates of the ROC curve of CCSA-2 indicated that the cutoff level of CCSA-2 at 1.43 would provide 93.3% sensitivity and 90.9% specificity in differentiation between malignant and non-malignant cases.

The post-operative follow up for CCSA-2 was carried on 28 out of 30 colorectal cancer patients showing a high statistically significant difference between colorectal cancer patients preoperatively and postoperatively

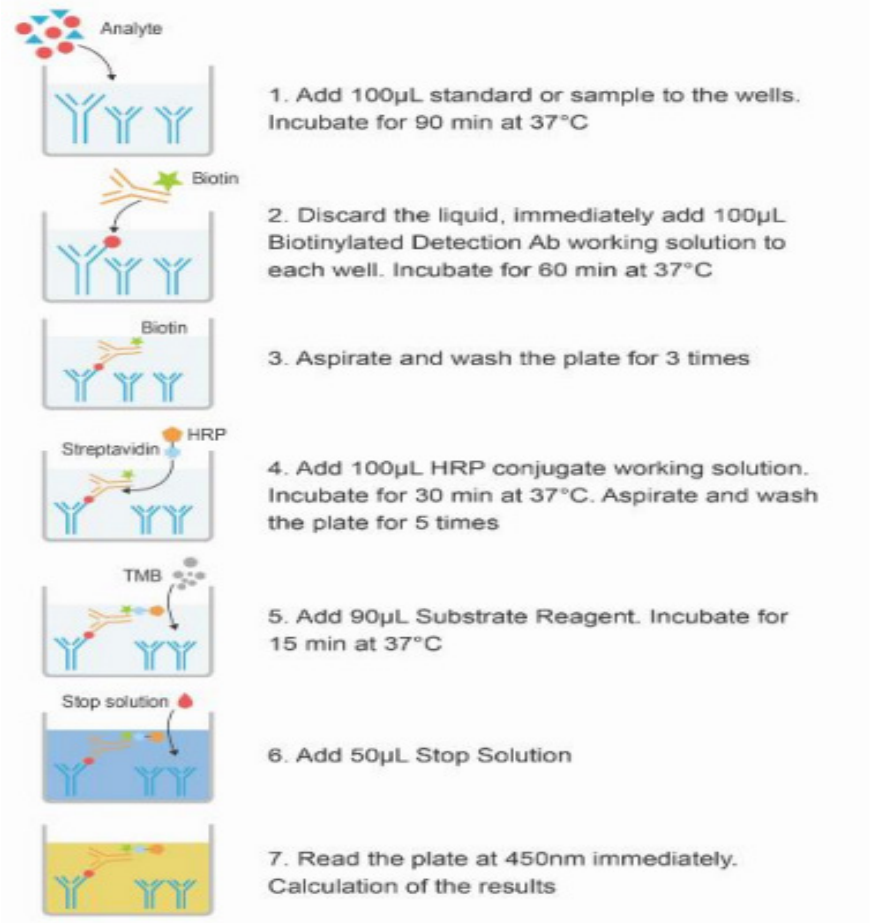

Figure 1. Cancer – Specific Antigen-2 (CCSA-2)by ELISA Catalog No : E-EL-H0793

Table 2. Comparison between the Three Groups Regarding the Laboratory Data

Laboratory	Colorectal cancer Group (N=30)	Benign Cases (N=7)	Control (N=15)	ANOVA	p-value
Hb (g/dl)					
Mean±SD	11.44±1.00	12.59±1.25a	12.99±0.78a	13.685	<0.001**
Median (IQR)	11.25 (1.5)	12.55 (12.3)	12.9 (1.4)		
Range	10.1-13.9	10.9-14.9	11.9-14.1		
Creat. (mg/dl)					
Mean±SD	0.93±0.20	0.90±0.16	0.77±0.14ab	3.908	0.027*
Median (IQR)	0.9 (0.30)	0.89 (9)	0.8 (0.1)		
Range	0.5-1.3	0.7-1.2	0.5-1		
ALT(u/l)					
Mean±SD	36.30±9.82	29.29±7.59a	17.13±4.17ab	26.602	<0.001**
Median (IQR)	36 (12.25)	29 (37)	17 (7)		
Range	18-56	19-38	26-Oct		
AST(u/l)					
Mean±SD	51.87±19.11	33.29±6.80a	21.73±5.32ab	8.65	<0.001**
Median (IQR)	44.5 (21)	33.37 (33)	20 (9)		
Range	19-164	23-42	13-30		

F-ANOVA test;*p-value <0.05 S;**p-value <0.001 HS; a, Significant between colorectal cancer group (p-value <0.05); b, Significant between benign cases (p-value <0.05).

with a high significant statistically decrease in its level postoperatively (p value = <0.001) (Table 5).

A high statistically significant difference between the three studied groups regarding the expression of methyl Sept. 9 preoperative (N=30), as All colorectal cancer patients except one were positive for methyl septin 9, While all control and benign patients except one were negative (p value =<0.001). This positive benign patient proved to have a precancerous polyp and developed colorectal cancer after one year during follow up. methyl Sept.9 has 96.7% sensitivity and 95.5% specificity in differentiating colorectal cancer patients from both bengin and control groups. There was a high statistically significant difference between pre and postoperative expression of methyl Sept.9 in colorectal cancer patients as there was a dramatically decrease in the expression of methyl septin 9 three months postoperatively (p value <0.001). There was no statistically significant correlation between CCS Ag2 and other parameters including; age, Hb, creat, Alt, Ast, CEA and CA19.9 levels (Table 6).

the medical history, family history, Hb (g/dl), ALT(u/l), CA19.9 U/ml, CCSA-2 had a high significant positive correlation on the colorectal cancer patient. While Creat. (mg/dl), AST (u/l), CEA (ng/ml), occult blood in stool

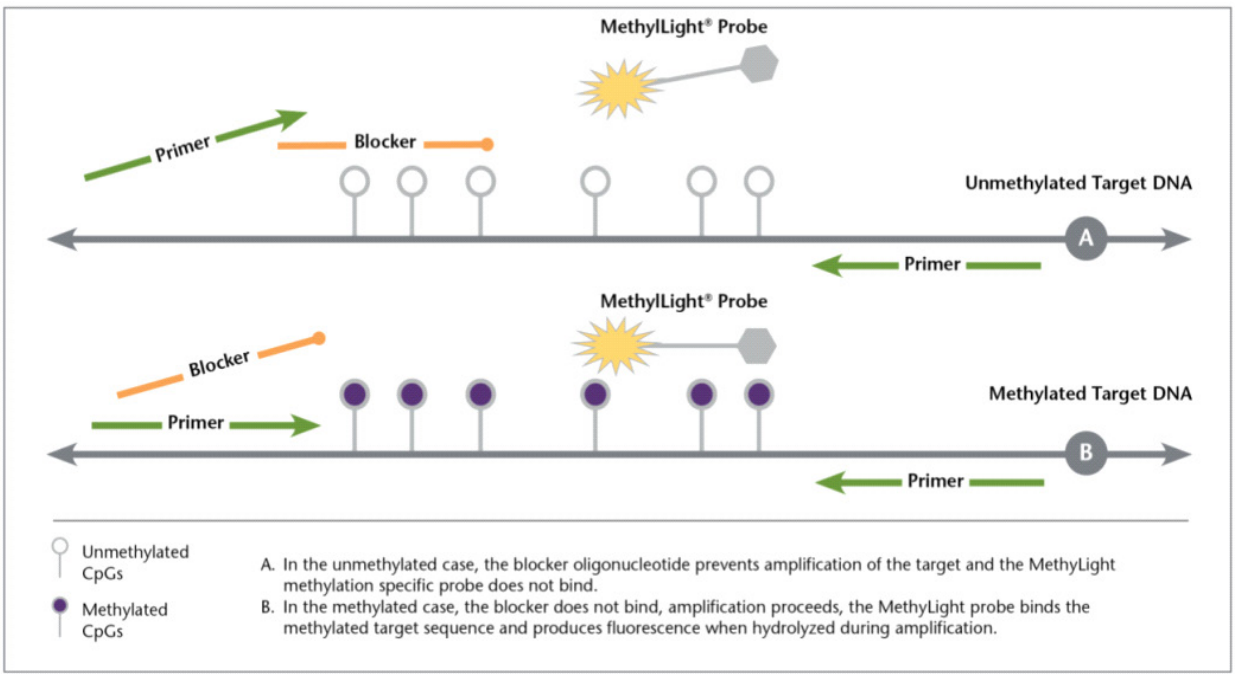

Figure 2. The Duplex PCR Reaction to Identify Methylated Septin 9 Gene

Table 3. Comparison between the Three Studied Groups According to the Presence of Occult Blood in the Stool

Occult blood in stool	Colorectal cancer Group (N=30)	Benign Cases (N=7)	Control (N=15)	p-value
Negative	0 (0.0%)	7 (100.0%)	15 (100.0%)	<0.001**
Positive	30 (100.0%)	0 (0.0%)a	0 (0.0%)a	

a, Significant difference with colorectal cancer group (p-value <0.05); b, Significant difference with benign cases (p-value <0.05)

Table 4. Comparison between the Three Studied Groups Regarding to CCs Ag2, CA19.9 and CEA levels

	Colorectal cancer Group (N=30)	Benign Cases (N=7)	Control (N=15)	ANOVA	p-value
CCS Ag2 (Preoperative					
Mean±SD	12.27±9.77	1.23±0.87a	0.49±0.33ab	14.977	<0.001**
Median (IQR)	8.77 (13.7)	1.02 (0.99)	0.48 (0.58)		
Range	1.2676-36.695	0.027-2.801	0.02-1.02		
CA19.9 U/ml					
Mean±SD	24.22±16.04	7.40±8.88a	2.74±1.41ab	15.935	<0.001**
Median (IQR)	25 (23)	11.7 (1.71)	2.8 (1.99)		
Range	0.8-60.70	1.4-25	0.32-5.86		
CEA (ng/ml)					
Mean±SD	11.17±7.99	1.34±0.40a	0.82±0.36ab	7.562	<0.001**
Median (IQR)	5 (21.4)	1.5 (0.72)	0.88 (0.43)		
Range	0.9-38	0.8-1.8	0.065-1.31		

F-ANOVA test;**p-value <0.001 HS; a, Significant between colorectal cancer group (p-value <0.05); b, Significant between benign cases (p-value <0.05)

had a lower significant positive correlation with colorectal cancer (Table 7).

The most predictor factor was septin 9 as it had a highly statistically significant correlation with colorectal cancer patients (p<0.001). Odds ratio multivariate analysis of relevant parameters presented in univariate analysis in colorectal cancer group showed that the most reliable is CCSAg2 with p value 0.022.

Discussion

Colorectal cancer (CRC) is a major health problem. Worldwide, In Egypt, the estimated rate of CRC is 6.5 % of all malignant tumors regarding the National Cancer Institute registry at Cairo University. Egypt shows a high rate of early CRC in the world as 35% of 1,600 Egyptian CRC patients were under 40 having threefold increased risk of death within 5 years compared to those who have CRC over age of 50. (Bader El Din et al., 2020).

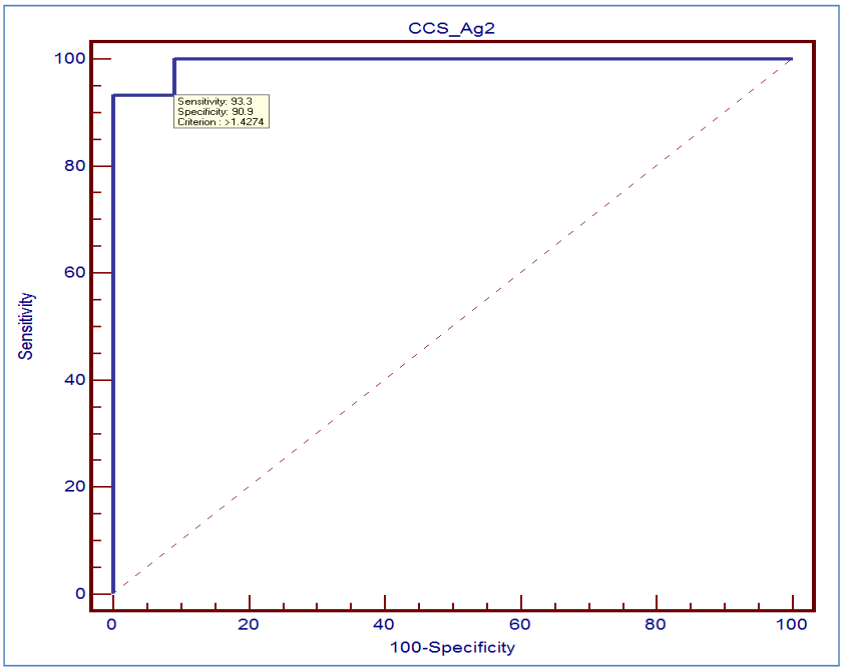

Figure 3. ROC Curve for CCSA-2 as Malignant and Non-Malignant Cases

Table 5. Comparison between Pre and Postoperative Level of CCSA-2 in Colorectal Cancer Patients (N 28 cases)

CCS Ag2	Preoperative (N=28)	Postoperative (N=28)	Mean Diff.	Paired Sample t-test
Mean±SD	12.27±9.77	5.97±3.55	6.29±4.76	5.099
Median (IQR)	8.77 (13.7)	3.04 (6.9)		
Range	1.2676-36.695	0.13-30.81		

t-Paired Sample t-test; **p-value <0.001 HS

Table 6. Correlation between CCS A2 with All Studied Parameters, Using Pearson Correlation Coefficient in Colorectal Cancer Group (N=30).

	CCS Ag2 (Preoperative)	
	r	p-value
Age (years)	0.016	0.935
Hb (g/dl)	-0.256	0.172
Creat (mg/dl)	0.035	0.855
ALT(u/l)	-0.256	0.172
AST(u/l)	-0.321	0.084
CEA (ng/ml)	0.197	0.298
CA19.9 U/ml	0.046	0.811

r, Pearson Correlation Coefficient

Unfortunately, currently available serological tumor markers for CRC such as CEA and CA19-9 have not proven to be ideal (Leilani et al., 2021). Determination of epigenetic events is a strong candidate for early detection of disease since regulation of gene expression by aberrant DNA methylation is a well characterized event in tumor biology, and is extensively described for CRC (Béla et al., 2014).

In recent years, SEPT9 gene methylation has been recognized as a hotspot and is considered to be a specific biomarker of the early stages of colorectal cancer. It may be a reliable indicator for screening CRC among high-risk individuals (Yu et al., 2018). As well as Nuclear matrix proteins (NMP) have been identified as an oncological "fingerprint" for some certain cancers, such as bladder, renal, and prostate cancers. Gang xue and his colleagues had identified colon cancer-specific nuclear matrix proteins that were present in cancer tissue, but not found in normal adjacent tissue or in the normal colon tissue (Gang xue et al., 2014).

The aim of this work was to assess the clinical values of methylated septin 9 and, CCSA-g 2 in CRC patients and their relations to other factors such as the age, medical history, family history in addition to other known colorectal cancer tumor markers (CEA and CA19.9) in a trial to verify their ability as a non-invasive tool to act as diagnostic and prognostic markers in colorectal cancer. Family history of colorectal cancer (CRC) is a known risk factor for CRC, and encompasses both genetic and shared environmental risk. There is evidence for higher prevalence of adenomas and of multiple adenomas in people with family history of CRC (Henrikson et al., 2015). In our study a positive family history is significantly higher in CRC group (23.3 %) when compared to control group (0%) with (p<0.001).

Several studies are in agreement with our result, Nine studies of populations size ranging from 30,353 to 7 million individuals were included. Five studies were conducted in the US, four others were from Britain, Sweden, Japan, and China. All of them considered the risk of developing colorectal cancer in those with family histories of CRC compared to those with no family history (Henrikson et al., 2015).

The preoperative expression of methyl Sept. 9 was significantly higher in CRC group (96.7 %) when compared to control group (0%) with (p<0.001). These results were in agreement with Patai et al., 2013, whose study included 92 patients with CRC and 92 healthy volunteers and found statistically significant difference between CRC group and the healthy control subjects

Table 7. Logistic Regression (Patients with Colon Cancer Group VS benign and Control Group for All Parameters

	B	S.E.	p-value	Odds Ratio	95% C.I.	
					Lower	Upper
Medical History	2.562	1.125	0.026*	4.618	1.521	6.829
Family History	3.381	1.475	0.024*	7.647	1.719	10.509
Hb (g/dl)	3.72	1.623	0.029*	8.412	1.891	11.56
Creat (mg/dl)	-0.038	0.015	0.112	2.154	1.146	3.622
ALT(u/l)	4.091	1.785	0.032*	9.253	2.08	12.716
AST(u/l)	-0.041	0.016	0.213	2.37	1.26	3.984
CEA (ng/ml)	-0.05	0.019	0.187	2.867	1.525	4.82
Occult blood in stool	-0.045	0.018	0.316	2.606	1.387	4.382
CA19.9 U/ml	4.951	2.16	0.043*	8.623	2.517	15.387
CCS Ag2 (Preoperative)	5.446	2.376	0.022*	9.485	2.769	16.925
Sept. 9 (Preoperative)	5.266	2.297	<0.001**	10.739	3.135	19.164

B, regression coefficient, SE: standard error; OR, odds ratio; CI, confidence interval

(p<0.001). Also, Xie et al., (2018) concluded in their research article that mSEPT9 demonstrated best diagnostic ability in CRC detection and may provide a new approach for future CRC screening. Another supportive study done by Sun et al., 2021 demonstrated that Patients with CRC had a significantly higher methyl septin 9 level than control group and that SEPT9 analysis might be popularized as a routine biomarker for CRC screening. Our resuts showed higher sensitivity (96.7) and specificity (95.5) as compared to Rohit et al., (2020) meta-analysis Based on 19 studies, the pooled estimates (and 95% CIs) for mSEPT9 to detect CRC were: sensitivity 69% (62–75); specificity 92% (89–95); this could be explained by ethnic difference as the meta analysis include CRC patients of different ethnicity not including Egyptian patients, as well 12 studies of the 19 studies were screening studies on patients prior to their diagnosis recruiting most patients of stage I while our patients were 67% grade II, 6% grade III, 4% were grade IV but no one was grade I.

Moreover our study showed higher sensitivity and specificity as compared to Gamal et al., (2020) done on Egyptian patients who establish that mSEPT9 achieved overall sensitivity of 84% and specificity of 78%, with an area under the curve value of 0.911 and this could be explained by different method of detection as Human serum mSEPT9 was assayed in this study using Sunred ELISA Kit (Shanghai Sunred Biological Technology Co. Ltd, Shanghai, China), whereas we used the Epi proColon 2.0 Plasma Quick Kit following the instructions of the manufacturer (Epigenomics AG, Berlin, Germany) with higher performance. Furthermore, the heterogeneity of CRC and patients number (sample size) might contribute to this discordance. That's why the need for standardization of assay technology, processing techniques, and target population size (Antonia et al., 2017).

In our study a statistically significant difference in the preoperative expression of methyl Sept. 9 was found between CRC group when compared to benign group (p<0.001) in agreement with Zaher et al., (2012). However, Kinga Tóth et al., 2014 found that Methylated SEPT9 was detected in all tissue samples of normal subjects, patients with benign lesions and CRC patients. In plasma samples, elevated mSEPT9 values were detected in CRC, but not in adenomas concluding that the tissue levels of mSEPT9 alone are not sufficient to predict mSEPT9 levels in plasma.

In our study there was no significant difference in the preoperative expression of methyl Sept. 9 between benign and control group (P=0.2), in accordance with Zaher et al., (2012). However this was opposed by Duku et al., (2011) who made a study on 50 patients with colorectal carcinoma, 36 patients with benign colorectal adenomas and 35 healthy subjects and showed a statistically significant difference between benign and healthy control group. This discrepancy between our findings and the other studies might be contributed to the relatively small number of the patients with benign bowel diseases; however, it was a finding which worth further studies on a larger sample size to support the results of the present study.

Our study showed that a highly statistically significant difference between pre and three months postoperative expression of methyl Sept.9 in colorectal cancer as there is a dramatically decrease in the expression of methyl septin 9 postoperatively (p value < 0.001), However this result is obtained from a 14 patients randomly chosen from our colorectal cancer group. Our data is in agreement with Arellano et al., (2020) who reported that the hyper methylation of SEPT9 could be useful as an epigenetic biomarker for total remission or after neo-adjuvant therapy in locally advanced rectal cancer. Similar finding was reported in the study performed by Yang et al., (2018), MSEPT9 analysis appears to offer promising novel prognostic markers and might be considered for monitoring CRC recurrence.

Leman et al., (2007) used indirect enzyme-linked immunosorbent assay (ELISA) to analyze the performance of CCSA-2 as a serum-based colon cancer marker in subjects with CRC, advanced adenoma, and other cancers and benign diseases of the colon. The sensitivity and specificity for advanced colorectal neoplasia were 97.3% and 78.4%, respectively. Interestingly, the authors found an increase in serum CCSA-2 mean values from hyperplastic polyps to non-advanced adenoma, advanced adenoma, and CRC. In another study, the same group reported similar statistical performance for serum CCSA-2 in a large series of patients with CRC (Elhossary et al., 2020). The authors concluded that CCSA-2 is a potential specific blood-based marker for colon cancer.The mechanisms by which CCSA-2 is found in the serum are currently under investigation. Leman et al., 2008 speculate that this protein is released into the blood by cellular breakdown or apoptosis and is quite stable once it gets there.

In our study CCSA-2 median level was significantly increased in CRC group (8.77 U/ml) when compared to both benign group (1.02 U/ml) and control group (0.48 U/ml) (p<0.001) which comes compatible with Knychalski et al., (2016). In preoperative colorectal cancer patients the cutoff level of CCS Ag2 At >1.43 would provide 93.3% sensitivity and 90.9% specificity in differentiation between malignant and non-malignant cases.

In agreement with our results, the study conducted by Elhossary et al. 2020 showed that CCSA-2 in serum had 88.8% sensitivity and 84.2% specificity for distinguishing cancer colon , and Xue gang et al., 2014 concluded that serum CCSA-2 concentration had a sensitivity of 98.10% and a specificity of 97.90% in distinguishing individuals with CRC from other contributor population

So, our study showed that CCSA-2 could serve as another potential serum-based marker for detection of colorectal cancer which is supported by Leman et al., (2008)'s study.

In contrast to our result, a study conducted by Knychalski (2012) showed that The diagnostic value of the novel marker CCSA-2 is slightly lower than previously understood and accepted in clinical practice – CEA (accuracy of tests: CCSA-2 - 52%, CEA - 60%). This discrepancy could be explained by his small sample size (40 CRC patients and 40 healthy controls in comparison to previous supported studies with large series of patients).

In our study, there is a statistically significant difference between colorectal cancer patients preoperatively and postoperatively according to CCSA-2 with decrease in

its level postoperatively (p value > 0.001) this was in agreement with Cristofaro et al., (2017) as well as Xue Gang et al., (2014) who showed that CCSA-2 could be used as a monitoring marker that expresses the response of the patient to therapy and predict the occurrence of relapses.

Pearson Correlation Coefficient was used showed that no statistically significant correlation between CCS Ag2 and other parameters including; age, Hb, creat, Alt, Ast, CEA and CA19.9 levels.

The fecal occult blood test (FOBT) has commonly been used for colorectal cancer screening, especially in developed nations. It has been widely practiced for more than 3 decades as an approach to colorectal cancer screening (Paul et al., 2004). The Brazilian Ministry of Health and National Cancer Institute recommend the screening of colorectal cancer for people over 50 years-old with Fecal Occult Blood Test. The FOBT, can be performed recently by immunological methods, and is aimed to detect hidden blood characterized by the presence of human hemoglobin, additional tests may be warranted to determine the source of the bleeding. In our study FOBT is significantly higher in CRC group (98%) when compared to control group (0%) with ($p<0.001$). This is in agreement with Almeida et al., (2018) whose studies showed that FOBT showed highly significant difference between CRC and control group.

The serum marker (CEA and CA19-9) are currently the tumor markers in use for colon cancer diagnosis and follow-up. Median level of CA19-9 was significantly increase in CRC group (25 U/ml) when compared to both benign group (11.7 U/ml) and control group (2.8 U/ml) ($p<0.001$). Also median level of CEA was significantly higher in CRC group (5 ng/ml) when compared to both benign group (1.5 ng/ml) and control group (0.88 ng/ml) ($P<0.001$). These results comes concurring with Dong et al., (2014) who studied 64 patients with CRC and 36 subjects with non-malignant colorectal disease (NMCD) and found significant difference between both groups ($p<0.05$). While, other studies have shown that significantly higher concentrations of CEA or CA 19-9 were found in adenoma tissue, as well as CRC tissue, in comparison to normal mucosa (Kim et al., 2017). In a retrospective study which assessed CEA and CA 19-9 levels in serum of 91 patients with histologically confirmed diagnosis of colorectal adenocarcinoma, Vukobrat-Bijedic et al., 2013 concluded that serum CEA and CA 19-9 are late tumor markers for the prognosis of CRC, and are significantly increased in many patients with locally advanced and distant metastatic, but not early CRC.

The various parameters studied in this study when analyzed by the multivariante analysis (logistic regression analysis), showed that family history, Hb (g/dl), ALT (u/l), CA19.9 U/ml, CCSA-2 had a high significant positive correlation with the colorectal cancer patient. While Creat (mg/dl), AST (u/l), CEA (ng/ml), Occult blood in stool had a lower significant positive correlation with colorectal cancer. The most predictor factor was septin 9 as it had a highly statistically significant correlation with colorectal cancer patients ($p<0.001$). Odds ratio multivariate analysis of relevant parameters presented in univariate analysis was done in colorectal cancer patients group and showed that the most reliable is CCSA-2 with p 0.022.

In conclusion, the plasma SEPT9 DNA methylation level as well as Serum CCSA-2 could be used as non-invasive methods for observing the CRC patients postsurgical response to predict the occurrence of complete remission or relapses. Therefore, it is recommended to extend the study on larger scale of colorectal cancer patients to confirm these results.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

The authors would like to thank chemists and technicians of the Clinical Chemistry Unit at NCI, Cairo University.

Funding Statement

This work was funded by NCI, Cairo University and was a part of Lamiaa Abd El Kader approved MD thesis.

Ethical Approval and Consent

The study was approved by the IRB of NCI, Cairo University. A written consent was obtained from all subjects before their enrollment.

Conflict of Interest

The authors declare no conflict of interet.

References

Almeida CMG, Azevedo R, Baraviera AC, et al (2019). Association of fecal occult blood tests results with colonoscopic findings in a general hospital and validation of the screening test. *J Coloproctol*, **39**, 121-6.

Bader El Din NG, Ibrahim MK, El-Shenawy R, et al (2020). MicroRNAs expression profiling in Egyptian colorectal cancer patients. *IUBMB Life*, **72**, 275- 84.

Béla M, Kinga T, Barták B, Tulassay Z (2015). Plasma methylated septin 9: A colorectal cancer screening marker. *Expert Rev Mol Diagn*, **15**, 1-14.

Di Cristofaro L, Scarpa M, Angriman I, et al (2012). Cost-effectiveness analysis of postoperative surveillance protocols following radical surgery for colorectal cancer. *Acta Chir Belg*, **112**, 24-32.

Dong H, Tang J, Li LH, et al (2013). Serum carbohydrate antigen 19-9 as an indicator of liver metastasis in colorectal carcinoma cases. Asian Pac J Cancer Prev, **14**, 909- 13.

Duku M Cree M, Duku M, Bhandari P, et al (2011). Circulating tumour markers can define patients with normal colons, benign polyps, and cancers. *Br J Cancer*, **105**, 239- 45.

Eldeeb GS, Fawzy AN, El-Hefnawy SM, et al (2020). Study of the role of serum methylated septin9 in early detection of colorectal cancers in comparison with colonoscopy. *Menoufia Med J*, **33**, 824.

Elhossary M, Hawash N, Badawi R, et al (2020). Comparative Evaluation of Colon Cancer Specific Antigen-2 Test and Chromocolonoscopy for Early Detection of Egyptian Patients with Colorectal Cancer. *Antiinflamm Antiallergy Agents Med Chem*, **19**, 302- 12.

Ferlay J, Bray F, Soerjomataram I, et al (2018). GLOBOCAN

estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Global cancer statistics.
Hariharan R , Jenkins M (2020). Utility of the methylated SEPT9 test for the early detection of colorectal cancer: a systematic review and meta-analysis of diagnostic test accuracy. *BMJ Open Gastroenterol*, **7**, e000355.
Kim NH, Lee MY, Park JH, et al (2017). Serum CEA and CA 19-9 levels are associated with the presence and severity of colorectal neoplasia. *Yonsei Med J*, **58**, 918- 24.
Knychalski B, Łukieńczuk T (2012). The evaluation of diagnostic value of the tumor markers: CCSA-2 and CEA in colorectal cancer. *Pol J Surg*, **84**, 86-92.
Knychalski B, Lukieńczuk T (2016). The evaluation of diagnostic value of the tumor markers: CCSA-2 and CEA in colorectal cancer. *Pol Przegl Chir*, **84**, 86–92.
Leilani L, Silvia S, Mathias W (2021). Diagnostic and prognostic value of CEA and CA19-9 in colorectal cancer. *Diseases*, **9**, 21.
Leman ES, Schoen RE, Magheli A, et al (2008). Evaluation of colon cancer–specific antigen 2 as a potential serum marker for colorectal cancer. *Clin Cancer Res*, **14**, 1349- 354.
Leman ES, Schoen RE, Weissfeld JL, et al (2007). Initial analyses of colon cancer–specific antigen (CCSA)-3 and CCSA-4 as colorectal cancer–associated serum markers. *Cancer Res*, **67**, 5600- 605.
Leon Arellano M, García-Arranz M, Ruiz R (2020). A first step to a biomarker of curative surgery in colorectal cancer by liquid biopsy of methylated septin 9 gene. *Dis Markers*, **2020**, 9761406.
Levin B, Lieberman DA, McFarland B, et al (2008). Screening and surveillance for the early detection of colorectal cancer and adenomatous Polyps,: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *CA Cancer J Clin*, **58**, 130- 60.
Li X, Xiyi J, Qian L, et al (2018). Diagnostic value of methylated Septin9 for colorectal cancer detection. *Front Oncol*, **8**, 247.
Liu D, Wang X, Yang Y, et al (2014). Colon cancer-specific antigen-2 may be used as a detecting and prognostic marker in colorectal cancer: a preliminary observation. *PLoS One*, **9**, e94252.
Nora BH, Elizabeth MW, Katrina AG, et al (2015). Family history and the natural history of colorectal cancer: systematic review. *Genet Med*, **17**, 702-12.
Paul JL, David A (2004). Ahlquist, in Colorectal Adenocarcinoma.
Sepulveda AR, Hamilton SR, Allegra CJ, et al (2017). Nowak Molecular Biomarkers for the Evaluation of Colorectal Cancer. **19**, 187– 25.
Sun J, Xu J , Sun C, et al (2021). Screening and prognostic value of methylated septin9 and its association with clinicopathological and molecular characteristics in colorectal cancer. *Front Mol Biosci*, **381**.
Tóth K, Sipos F (2013). Detection of Methylated SEPT9 in Plasma Is a Reliable Screening Method for Both Left- and Right-Sided Colon Cancers. *PLoS One*, **7**, e46000.
Tóth K, Wasserkort R, Sipos F, et al (2014). Detection of methylated septin 9 in tissue and plasma of colorectal patients with neoplasia and the relationship to the amount of circulating cell-free DNA. *PLoS One*, **9**, e115415
Vatandoost N, Ghanbari J, Mojaver M, et al (2016). Early detection of colorectal cancer: from conventional methods to novel biomarkers. *J Cancer Res Clin Oncol*, **142**, 341– 51.
Vukobrat-Bijedic Z, Husic-Selimovic A, Sofic A, et al (2013). Cancer antigens (CEA and CA 19-9) as markers of advanced stage of colorectal carcinoma. *Med Arc*, **67**, 397.
Wang Y, Pei-Min Chen, Rong-Bin Liu (2018). Advance in plasma SEPT9 gene methylation assay for colorectal cancer early detection. *World J Gastrointest Oncol*, **15**, 15–22.
Warren JD, Xiong W, Bunker AM, et al (2011). Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. *BMC Med*, **9**, 1-9.
Xue G, Wang X, Yang Y, et al (2014). Colon cancer-specific antigen-2 may be used as a detecting and prognostic marker in colorectal cancer: a preliminary observation. *PLoS One*, **9**, e94252.
Xue G, Wang X, Yang Y, et al (2014). Colon cancer-specific antigen-2 may be used as a detecting and prognostic marker in colorectal cancer: a preliminary observation. *PLoS One*, **9**, e94252.
Xue Y, Zhi-Jie X, Xi C, Shuang-Shuang Z (2018). Clinical value of preoperative methylated septin 9 in Chinese colorectal cancer patients. *World J Gastroenterol*, **25**, 2099- 109.
Zahir MN, Azhar EM, Rafiq S (2014). Clinical features and outcome of sporadic colorectal carcinoma in young patients: a cross-sectional analysis from a developing country. *ISRN Oncol*, **2014**, 461570.