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Circulating tumor DNA for monitoring colorectal cancer: A prospective observational study to assess the presence of methylated SEPT9 and VIM promoter genes and its role as a biomarker in colorectal cancer management

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ABSTRACT

Objective: Methylation status of Septin9 (SEPT9) and vimentin (VIM) genes in circulating tumor DNA of colorectal cancer (CRC) patients is a promising bio-marker for the early detection of CRC. The aim of the present study was to identify the methylation status in promoter regions of the SEPT9 and VIM genes in a cohort of Indian patients with biopsy proven colorectal cancer.

Material and Methods: Forty-five consecutive patients of colorectal cancer were recruited. 10 mL venous samples were collected from each patient and processed for isolation of cell-free DNA, bisulfite conversion of cell-free DNA, polymerase chain reaction (PCR) amplification and detection of SEPT9 and VIM genes.

Results: Partial methylation in vimentin was present in 42.22% of the patients and 57.78% showed no methylation and none of the tumors had complete methylation. Only three (6.66%) patients showed complete methylation patterns in SEPT9 and the remaining 42 (93.33%) tumors showed partial methylation. Considering the two genes together, only three (6.66%) out of 45 showed complete methylation. The association of methylation patterns in both genes (complete, partial, and no methylation) with sex, age, T stage, N stage, M stage, CEA, histology, and location (right or left colon) were explored and none of these parameters were statistically significant.

Conclusion: In our study, only 6.66% CRC patients showed hypermethylation and there was no association of methylation patterns in the both genes (complete, partial, and no methylation) with any of the parameters like age, sex, TNM stage, CEA, and histology.

Keywords: Colorectal neoplasms, cell-free nucleic acids, methylation

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide with 90 per 100.000 people affected by CRC. CRC accounts for 10% of all tumor types worldwide. Five-year survival for colon cancer in different geographical settings range from 28.5% to 57% in males and from 30.9% to 60% in females (1).

Several non-invasive tests like fecal occult blood test (FOBT), fecal immunochemical test (FIT) and carcinoembryonic antigen (CEA) are available for the early diagnosis of CRC but these tests suffer from poor sensitivity and specificity which limits their clinical application (2). The standard screening method of colonoscopy is an invasive test which requires bowel preparation and is uncomfortable leading to poor compliance among patients. Therefore, better tests are required to improve patient compliance (3).

DNA methylation of certain genes is related to the development of colorectal cancer (4). Aberrant methylation of the genome can lead to the silencing of certain tumor suppressor genes which can result in malignant transformation (5). Recently, SEPT9 gene methylation has been recognized as a specific biomarker for colorectal cancer which may be used to screen high risk populations (3). Vimentin is a member of the intermediate filament family and is responsible for maintaining cell shape and integrity of cytoplasm and stabilizing cytoskeletal interactions (6). Human vimentin gene is located on the short arm of chromosome 10 (10p13) and aberrant methylation of vimentin gene may lead to unexpressed vimentin protein which may contribute to CRC pathogenesis (7). Li et al. have shown in their meta-

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analysis of seven studies with 467 CRC patients that vimentin promoter methylation in cancer tissues was significantly higher than in normal or benign tissues (6).

Liquid biopsy based methylated ctDNA for SEPT9 and vimentin genes can be used as a biomarker for detection of cancer (8). Liquid biopsy screening tests are blood based screening tests for early detection of cancer which examine peripheral blood for circulating tumor DNA (ctDNA) which can be present in the earliest stages of tumorigenesis (8). Malignant cells release ctDNA fragments into the bloodstream during apoptosis and represent tumor DNA of cancer cells. These ctDNA are methylated at 5'-cytosine-phosphate-guanine-3' sites (9).

There are no data in the published literature from India on the methylation status of both SEPT9 and vimentin genes in tumors with colorectal cancer. Therefore, this study was taken up to understand the methylation status of these genes in cell-free DNA isolated from the plasma of patients with colorectal cancer in Indian patients.

MATERIAL and METHODS

A prospective observational study was conducted at a tertiary referral center in India from December 2018 to February 2020. The study was approved by the institute ethics committee (Ref. No. AIG/IEC32/10.2018-09). The study was HIPAA compliant and adhered to the tenets of Declaration of Helsinki. A written informed consent was obtained from each patient prior to enrolment.

Patients over 18 years with an established colorectal cancer diagnosis, post neoadjuvant chemo/radiotherapy colorectal cancer patients and metastatic and recurrent colorectal cancer were included in the study. Patients had to be willing to provide oral and written informed consent.

Patients with other associated cancers and patients not willing to provide written informed consent were excluded from the study. During the study period, 75 cases of CRC were diagnosed, 45 patients were included after applying inclusion and exclusion criteria.

The aim of the study was to study the presence of methylated circulating tumor DNA in genes SEPT9 and VIM in colorectal cancer patients in the Indian population and their association with histopathological characteristics, tumor location (left or right) and CEA levels.

Biochemical Analysis

Plasma from 10 mL venous blood was collected in EDTA Vacuette tubes. CEA levels were also measured in all patients.

Isolation of Cell-Free DNA

The samples were equilibrated to room temperature before processing. One mL of serum was taken in an Eppendorf tube to which 100 µL of proteinase K was added. The solution was mixed thoroughly by vortexing. To this solution, 800 µL of Buffer ACL was added and pulse vortexing was carried out for 30 seconds to ensure complete mixing of the sample. This lysate mix was then incubated at 60°C for 30 minutes. After the completion of incubation time, 1.8 mL of Buffer ACB was added to the lysate mix and was mixed thoroughly for 30 seconds.

The sample was incubated on ice for five minutes. About 700 µL of the mixture was loaded onto the QIA AMP Mini column and centrifuged at 10.000 rpm for one minute. This step was repeated until all of the solution had passed through the column. Next, to the column 600 µL of Buffer ACW1 was added and centrifuged. The flow-through was discarded. Later, 700 µL of Buffer ACW2 was added and centrifuged. After centrifugation, a new collection vial was placed and the column was centrifuged at full speed to remove any residual ethanol. The column was then incubated at 56°C to ensure complete drying of the membrane. The QIA AMP mini column was then placed into a new 1.5 mL Eppendorf tube and 100 µL of Buffer AVE was added to the center of the membrane. The column was finally centrifuged at 20.000 g for one minute to elute the cell-free DNA. The sample was stored at -20°C until further use.

Bisulfite Conversion of the Cell-Free DNA

A total concentration of 500 ng of cell-free DNA was taken into a 1.5 µL Eppendorf tube and made up to 20 µL using RNase/DNase free water. To this tube, 130 µL of CT conversion reagent was added and mixed thoroughly and centrifuged briefly to ensure complete mixing of the sample. The sample was then incubated in a thermal cycler with the conditions, 98°C for eight minutes, 64°C for 3.5 hours, and 4°C until further processing. After the completion of incubation, the samples were taken and added to the Zymo Spin IC Column containing 600 µL of M-Binding Buffer. The solution is mixed by inverting the column a few times and centrifuged at 10.000 g for 30 seconds. The flow-through was discarded. To the column, 100 µL of M-Wash Buffer was added and centrifuged at 10.000 g for 30 seconds. Later 200 µL of M-Desulphonation Buffer was added and the column was incubated at room temperature for 20 minutes.

After the completion of the incubation time, the sample was centrifuged and flow-through discarded. Again, 200 µL of M-Wash Buffer was added and centrifuged. This step was repeated twice. The column was placed into a fresh Eppendorf tube and 10 µL of M-Elution Buffer was added, centrifuged at 10.000 g for one minute to elute the DNA. The eluted DNA was stored at -20°C until further use.

PCR Amplification

The isolated cfDNA was evaluated for quantity and quality using the Nanodrop 2000 spectrophotometer. Primers specific

to bisulfite converted DNA were designed for each of the two genes namely SEPT9 and vimentin. The primers used for amplification of vimentin for bisulfite converted DNA was forward 5`-GGGTGAGTTTAGTTTAGATTATTAT3`, reverse 5`-AAAAAAAATCCCCTCCCACTAC3`. The primers used for amplification of SEPT9 for bisulfite converted DNA were, forward 5`-TTAATTAGTTTTATTTGGGGTGAGG and reverse 5`-AAATATAAAAAACTTATATACATAAAAAC3`.

Each PCR was carried out using 2.5 U of Epi Taq polymerase (Takara Bio), 10 pm/µL of both forward and reverse primer, 10 mM of dNTP's, 25 mM of MgCl2 and 5x Taq Buffer in a total of 25 µL. The sample was incubated in a thermal cycler with the PCR conditions: initial denaturation at 95°C for three minutes, denaturation at 95°C for 30 seconds, annealing temperature for vimentin gene 59.9°C and SEPT9 gene was 55.4°C, followed by an extension at 72°C for 30 seconds and a final extension at 72°C for five minutes. The amplicons were checked for their size by loading them on agarose gel. The amplicon sizes of vimentin and SEPT9 genes were 209 bp and 198 bp respectively.

DNA Sequencing

PCR Purification Procedure

Eighteen (18) µL of the amplified PCR product was taken into a 96-well sample plate and 20 µL of AMPure Magnetic beads (Beckman) were added. The PCR product and AMPurebeads were mixed until the color of the solution appeared homogenous. The mix was incubated for five min at room temperature. The sample plate was placed on a 96-well magnetic plate and incubated for two minutes to separate the beads from the solution. The clear solution from the sample plate was discarded. 200 µL of 70% ethanol was added to each well and incubated for 30 seconds. Ethanol was removed carefully without disturbing the magnetic beads. The step was repeated by adding 200 µL of 70% ethanol to each well and incubated for 30 seconds. Ethanol was removed carefully without disturbing the magnetic beads. The sample plate was removed from the magnetic plate and 40 µL of elution buffer was added to the wells. The samples were incubated for five min at room temperature. The reaction plate was then placed on the magnetic plate and incubated for two minutes to separate beads from the elution buffer. The sample was transferred to a new 0.2 mL PCR tube and stored at -20°C until further use.

Dilution of the Amplified PCR Product

The sample was diluted to a given concentration based on the product size of the gene of interest. A 300 bp product was diluted to a concentration of 33 ng/µL using the elution buffer. After dilution, the dye termination cycle sequencing was carried out for the samples.

Dye Termination Cycle Sequencing

PCR mix: About 0.5 µL of primer, 2 µL of DTCs (quick start master mix, Beckman), water 0.1-2.5 µL and DNA template 0.1- 2.5 µL were added to make a total volume of 5 µL. The sample was set up for the dye termination cycle sequencing step in the thermal cycler with the following conditions: 96°C for one minute, 50°C for 20 seconds, and 60°C for four minutes for a total of 40 cycles.

Ethanol Precipitation

The sequencing reaction was transferred to an appropriately labeled 0.6 mL microcentrifuge tube and was mixed thoroughly. Then 60 µL of 95% ethanol was added from -20°c freezer and mixed thoroughly. Immediately it was centrifuged at 14.000 rpm at 4°C for 15 minutes. The pellet was rinsed twice with 70% ethanol and centrifuged immediately at 14.000 rpm at 4°C for 2-3 minutes. After centrifugation, the supernatant was removed carefully with a micropipette. It was allowed to completely dry at room temperature for 10 minutes. The sample was re-suspended in 40 µL of sample loading solution. Sample preparation for loading: The re-suspended sample was transferred to the appropriate wells of a 96-well sequencing plate. One drop of light mineral oil was added. The sample was loaded into the instrument.

Statistical Analysis

Clinical data were collected using a separate study proforma (attached). All the data were entered into MS EXCEL for further analysis. Descriptive measures like median and interquartile range (IQR) were derived for continuous variables and categorical variables were expressed as % frequency distribution. The outcome measure by clinical parameters was compared by using Fisher's exact test and Chi-square test. Analysis was carried out by statistical package for social sciences (SPSS 21st version) and graphed quick calculator software. A p value <0.05 was considered as significant with two-sided.

RESULTS

Clinical and Demographic Characteristics

General demographic and clinical characteristics of the study group are as shown in Table 1. Median age of the study group was 53 years comprising predominantly males. The majority in the study group presented with tumors in the right colon 20 (44.4%) with a median CEA level of 4. Among the 45 patients, five had undergone emergency surgery. Based on pathological characteristics, 14 (31.2%) had T4 disease, 23 (41.1%) had lymph nodes (LNs) positive disease, 13 (28.6%) had lymphovascular invasion.

Table 1. Clinical and demographic characteristics of the study group

		Number (n)	Percentage (%)
Age (years)	Median	53 (18-46)	
Sex	Male	32	71.1
	Female	13	13
Diagnosis	Right colon	20	44.4
	Left colon	9	20
	Sigmoid	8	17.7
	Rectum	8	17.7
Histology	Lymphovascular invasion	13	28.9
Tumor (T)	T1	2	4.4
	T2	9	20
	T3	18	40
	T4	14	31.2
Node (N)	N1	15	33.3
	N2	4	8.9
	NO	22	48.9
Metastasis (M)	Yes	5	11.1
	No	40	88.9
Presentation	Emergency	5	11.1
	Elective	40	88.9
CEA	Median	4	
	IQR	2.2-7.1	
	Min	1.2	
	Max	128	

The Methylation Status-Vimentin Gene

A total of 12 methylation sites were identified in the study group and the details are as presented in Figure 1. Methylation status ranged from no methylation to partial methylation and complete methylation across these 12 sites. While tumors from twenty-five patients (26/45; 57.78%) showed no methylation, 19 (42.22%) samples showed partial methylation and none of the tumors exhibited complete methylation.

Association of Methylation Patterns in the Vimentin Gene with Clinical, Demographic Characteristics and Tumor Location

The association of methylation patterns in the vimentin gene (complete, partial, and no methylation) were explored for clinical and demographic characteristics. None of the parameters like sex, age, T stage, N stage, M stage, CEA-median, histology was statistically significant and are presented in Table 2. Vimentin methylation was present in 12 (60%) right sided tumors compared to eight (32%) left sided tumors (p= 0.060).

The Methylation Status-SEPT9 Gene

A total of four methylation sites were identified in the promoter region of SEPT9 gene and the details are as presented in Figure 2. Methylation status ranged from no methylation to partial methylation and complete methylation across these sites. Three patients (6.66%) showed complete methylation and 42 patients (93.33%) showed partial methylation.

Association of Methylation Patterns in the SEPT9 Gene with Clinical, Demographic Characteristics and Tumor Location

The association of methylation patterns in the SEPT9 gene (complete, partial) were explored for clinical and demographic characteristics. Of partially methylated tumors, 71.4% and 66.7% of completely methylated tumors respectively were advanced (T3 + T4) (2,30). Of partially methylated tumors 90.5%, and 100% of completely methylated tumors were node positive respectively (Table 3) (3,38). Complete methylation was present in three right sided tumors compared to none in left sided tumors (15% versus 0%). Significance (p values) between complete and partial methylation groups could not be calculated because of the very small sample in complete methylation group.

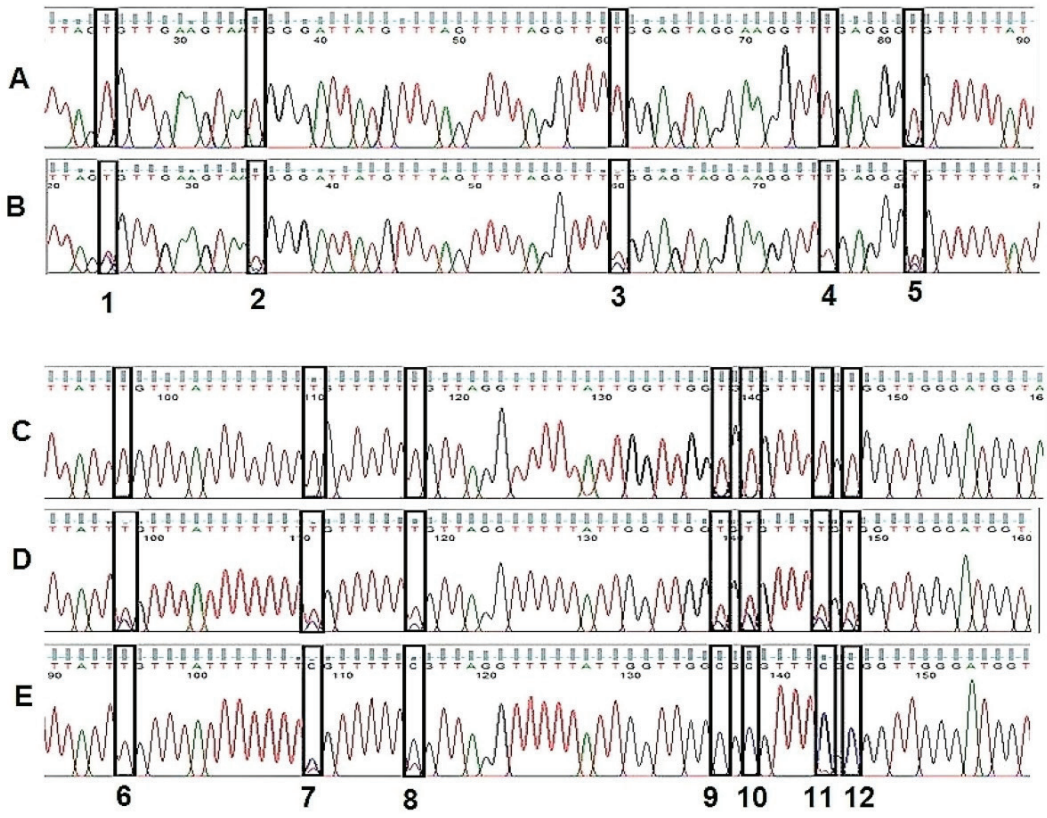

Figure 1. Representative electropherogram depicting different methylation sites (1-2) in the vimentin gene. Panels A-E shows the different methylation status in various sites of the gene. Site A1, A2, A3, A4, A5, B4, C6, C7, C8, C9, C10, C11, C12, E6.

Table 2. Comparison of demographic and clinical characteristics in the vimentin methylation group

		Methylation + (n= 19)		Methylation - (n= 26)		
		Number	Percentage (%)	Number	Percentage (%)	p
Sex	Female	5	26.3	8	30.7	1.000
	Male	14	73.7	18	69.3	
Age	<45	5	26.3	11	42.3	0.351
	>45	14	73.7	15	57.7	
T stage	T1 + T2	3	15.79	8	30.77	0.309
	T3 + T4	16	84.21	18	69.23	
N stage	N0	7	36.84	15	57.69	0.231
	N+	12	63.16	11	42.30	
M stage	M0	17	89.47	21	80.77	0.681
	M+	2	10.53	5	19.23	
Histology	LVI-	13	68.42	19	73.07	0.751
	LVI+	6	31.57	7	26.92	
Stage	1 + 2	7	36.84	14	53.85	0.366
	3 + 4	12	63.16	12	46.15	
CEA (median)	<4	11	63.16	10	38.46	0.349
	>4	7	36.84	14	58.34	

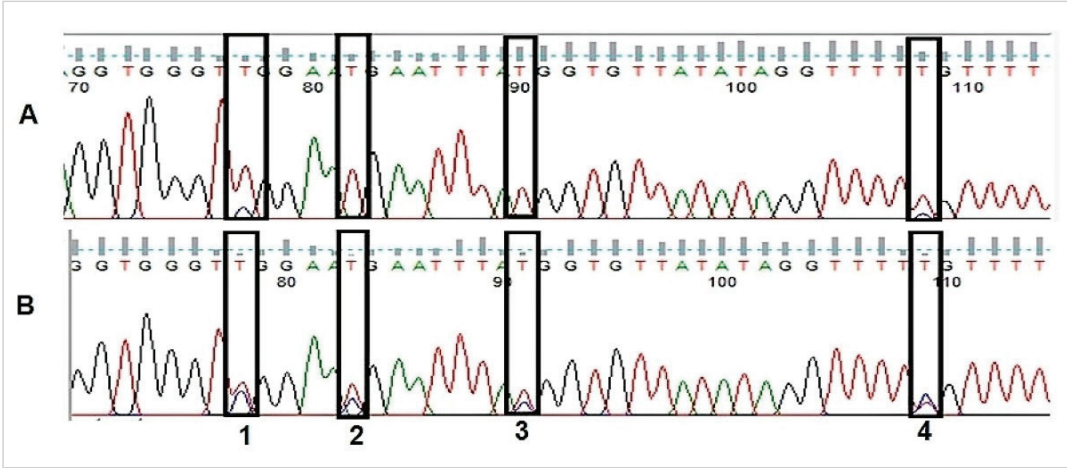

Figure 2. Representative electropherogram depicting different methylation sites (1-4) in the SEPT9 gene. Panels A-B shows the different methylation status at various sites of the gene. Site A2, A3 are the sites with no methylation. Site A1, A4, B1, B2.

Table 3. Comparison of demographic and clinical characteristics in SEPT9 complete and partial methylation group

		Complete methylation (n= 3)		Partial methylation (n= 42)	
		Number	Percentage	Number	Percentage
Age	<45	1	33.3	15	35.7
	>45	2	66.7	27	64.3
Sex	Female	2	66.7	11	26.2
	Male	1	33.3	31	73.8
T stage	T1 + T2	1	33.3	12	28.6
	T3 + T4	2	66.7	30	71.4
N stage	N1	3	100	38	90.5
	N2	0	0	4	9.5
Metastasis	M 0	3	100	37	88.1
	M +	0	0	5	11.9
Histology	LVI +	0	0	13	30.95
	LVI -	3	100	29	69.04
Stage	1 + 2	0	0	21	50
	3 + 4	3	100	21	50
CEA-median	<4	1	33.3	21	53.85
	>4	2	66.7	18	46.15

DISCUSSION

Several different methylation biomarkers have shown good sensitivity and specificity for the early detection of CRC (10). Some of the biomarkers currently under study are SEPT9, IKZF1, and BCAT1, ALX4, VIM. A combination of multiple biomarkers has significantly improved the ability of the plasma methylation test to detect CRC as compared with the measurement of a single marker (11).

SEPT9 gene is located on chromosome 17 at q25.3 (12). According to a systematic review by Nian et al., the sensitivity of methylated SEPT9 gene for CRC ranges from 37% to 88% (13). Another meta-analysis of 19 studies for mSEPT9 to detect CRC has demonstrated a moderate sensitivity of 69% and high specificity of 92% but poor diagnostic performance for precancerous lesions (8). Hu et al., in their meta-analysis of 22 studies with 2271 CRC patients, have shown that the diagnostic value was higher for Asian ethnicity compared to white populations, higher in advanced compared to early stage and higher in CRC compared to adenoma cases (2). Several studies have reported a sensitivity ranging between 30% and 75%, with

a specificity of approximately 90%, using the methylation status of the (SEPT9) gene promoter as a biomarker for detection of early CRC (14).

The PRECEPT clinical study has evaluated the utility of mSEPT9 in plasma for CRC screening and yielded a sensitivity of seventy-six percentage for stages I-IV cancers and 11.2% for advanced adenomas. Specificity was however as high as 91.5% (SAGE-tumor biology May 2018). It has promoter hypermethylation reaching sensitivities ranging from 51% to 90.0%, and the specificity from 73% to 96% in serum or plasma samples of CRC patients (15). In our study, only three patients showed complete methylation patterns in SEPT9 and the majority (43 tumors) showed partial methylation. There were no cases of unmethylated SEPT9 gene. None of the parameters like sex, age, T stage, N stage, M stage, CEA-median, histology was significantly different in complete methylation or partially methylated groups. Hu et al. have shown in their meta-analysis of twenty two studies with 2271 CRC patients that the rate of SEPT9 positivity was higher in advanced CRC cases compared to early stage CRC cases with no significant difference between left and right sided CRC (2). A single center study from Korea has recruited 111 patients of untreated CRC. mSEPT9 in plasma has been detected in only 44 (39.6%) patients unlike our study. The difference in the sensitivity of mSEPT9 among patients with adenomas and those with each stage of untreated CRC was statistically significant (Dukes' staging, p= 0.002 and TNM staging, p= 0.008). The sensitivity of mSEPT9 for each of the stages (I-IV) of untreated CRC patients were 20.7%, 54.1%, 36.6%, and 75.0%, respectively (16).

In our study, 44.5% of the patients showed partial vimentin methylation and 55.55% patients showed no methylation. Lu et al. have shown a vimentin promoter methylation rate of 41.1% in fecal DNA of 56 patients with CRC (17). Li et al. have shown in their meta-analysis of seven clinical cohort studies with a total of 467 CRC patients that the frequency of vimentin promoter methylation in cancer tissues was significantly higher than in normal and benign tissues (cancer tissues vs. normal tissues: OR= 32.41, 95% CI= 21.04 ~ 49.93, p< 0.001; cancer tissues vs. benign tissues: OR= 1.60, 95% CI= 1.05 ~ 2.42, p= 0.028). Ethnicity-stratified analysis has indicated that the frequency of aberrant vimentin promoter methylation was correlated with the pathogenesis of CRC in both Asians and Caucasians (6).

None of the parameters like sex, age, T stage, N stage, M stage, CEA-median, histology and tumor location were statistically significant between the methylated vimentin and non-methylated groups. In contrast, Shirahata et al. have shown in 44 colorectal cancer patients that four (9%) exhibited methylation of the vimentin gene in their serum DNA. Interestingly, methylation was significantly found in the serum of patients with liver metastasis, peritoneal dissemination, and distant metastasis (p= 0.026, p= 0.0029 and p= 0.0063, respectively), suggesting that vimentin methylation in serum might be detected more frequently in patients with advanced colorectal cancer (18).

Limitations of our study are the small sample size and lack of follow-up data.

CONCLUSION

In our study, only 6.66% of CRC patients showed hypermethylation and there was no association of methylation patterns in the both genes (complete, partial, and no methylation) with any of the parameters like age, sex, TNM stage, CEA, location, and histology.

Ethics Committee Approval: This study was approved by Asian Institute of Gastroenterology Ethics Committee (Study-Approval Number: AIG/IEC32/10.2018-09, Date: 06.11.2018).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - PG, ZA, PR; Design - PG, PR; Supervision - PR, GVR; Data Collection and/or Processing - PG, ZA; Analysis and/or Interpretation - PG, ZA, GVR; Literature Search - ZA, GVR; Writing Manuscript - ZA; Critical Reviews - PR, GVR, PG, ZA.

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Kolorektal kanseri izlemek için dolaşımdaki tümör DNA'sı: Metillenmiş SEPT9 ve VIM promotör genlerinin varlığını ve kolorektal kanser yönetiminde bir biyobelirteç olarak rolünü değerlendirmek için prospektif bir gözlemsel çalışma

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ÖZET

Giriş ve Amaç: Kolorektal kanser (CRC) hastalarının dolaşımdaki tümör DNA'sındaki Septin9 (SEPT9) ve vimentin (VIM) genlerinin metilasyon durumu, CRC'nin erken tespiti için umut verici bir biyobelirteçtir. Bu çalışmanın amacı, biyopsiyle kanıtlanmış kolorektal kanserli Hintli hastalardan oluşan bir kohortta SEPT9 ve VIM genlerinin promotör bölgelerindeki metilasyon durumunu belirlemekti.

Gereç ve Yöntem: Kırk beş ardışık kolorektal kanser hastası çalışmaya alındı. Her hastadan 10 mL venöz numune toplandı ve hücresiz DNA izolasyonu, hücresiz DNA'nın bisülfit dönüşümü, polimeraz zincir reaksiyonu (PCR) amplifikasyonu ve SEPT9 ve VIM genlerinin saptanması için işlendi.

Bulgular: Hastaların %42.22'si vimentinde sadece kısmi metilasyonla başvurdu ve %57,78'i hiç metilasyon göstermedi ve tümörlerin hiçbirinde tam metilasyon yoktu. Sadece üç (%6,66) hasta SEPT9'da tam metilasyon paternleri gösterdi ve geri kalan 42 (%93,33) tümörün tümü kısmi metilasyon gösterdi. İki gen birlikte ele alındığında, 45'ten sadece üçü (%6,66) tam metilasyon gösterdi. Her iki gendeki metilasyon modellerinin (tam, kısmi ve metilasyon yok) cinsiyet, yaş, T evresi, N evresi, M evresi, CEA, histolojiyle ilişkisi araştırıldı ve bu parametrelerin hiçbiri istatistiksel olarak anlamlı değildi.

Sonuç: Çalışmamızda sadece %6,66 CRC hastası hipermetilasyon gösterdi ve her iki gende (tam, kısmi ve metilasyon yok) metilasyon paternleriyle yaş, cinsiyet, TNM evresi, CEA ve histoloji gibi parametrelerin hiçbir ilişkisi yoktu.

Anahtar Kelimeler: Kolorektal neoplaziler, hücresiz nükleik asitler, metilasyon

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