RESEARCH ARTICLE

Identification of specific codon 201 mutation of the DCC Gene in the colonoscopic specimen of colorectal cancer

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Abstract

Objective: To identify the mutation in codon 201 of the deleted in colorectal cancer gene in colorectal cancer, and to correlate that mutation to the histopathological grading of colorectal cancer.

Method: The cross-sectional study was conducted from February 2019 to February 2021 after approval from the ethics review board of the Dow University of Health Sciences, Karachi, and comprised biopsy-proven colorectal cancer patients regardless of age and gender. After histopathological reporting, formalin-fixed paraffin-embedded tissue blocks of colorectal cancer were used for deoxyribonucleic acid extraction, followed by polymerase chain reaction optimisation and deoxyribonucleic acid Sanger sequencing for mutational analysis. Data was analysed using SPSS 25.

Results: Of the 100 biopsy specimens assessed, 45(45%) were selected. Of them, 13(29%) samples failed to show any band on gel electrophoresis. The remaining 32(71%) samples were used for Sanger sequencing. Of these, 1(3%) sample did not sequence, while 31(97%) showed sequencing. All the sequenced samples identified a mutation in codon 201 of exon 3 in the deleted in colorectal cancer gene; 30(97%) showed homozygosity, and 1(3%) showed heterozygosity. No significant association of point mutation was noted with various demographic and clinicopathological parameters ($p>0.05$).

Conclusion: The deleted in colorectal cancer gene's missense mutation in codon 201 was frequently observed in colorectal cancer patients.

Key Words: Colorectal cancer, Single nucleotide polymorphism, DCC gene, Codon 201 mutation.
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Introduction

Colorectal cancer (CRC) is now a global burden with 60% increase, likely resulting in >2.2 million cases and 1.1 million deaths by 2030[1]. It is the second leading reported cause of cancer deaths (9.4%) among both males and females throughout the world[2]. The incidence of young-onset CRC (<50 years of age) is also increasing in Asian countries[2]. An estimated frequency of CRC in 2010-19, as indicated by the Dow Cancer Registry, makes CRC 4 highest among both males and females (7%) and 3rd highest among children aged <18 years[3]. About 75% of young patients presented with advanced disease, frequently involving the rectum and rectosigmoid region[4] It is also estimated that the incidence will increase from 6.1 million in 2008 to 10.6 million in 2030 due to aging, growing population, changing lifestyles, increasing urbanisation, diet, obesity, tobacco use, alcohol consumption, chronic infections, and increasing lifespans[5].

CRC is a heterogeneous disease and constitutes different alterations in genetic and epigenetic levels that lead to tumour development and its progression to metastatic disease. It is important to identify the specific somatic mutation in CRC to understand its underlying molecular mechanism to help patient-specific medicine[6]. The development of an adenomatous lesion and its progression to the malignant tumour (cancer) manifest environmental as well as hereditary factors that contribute to the development and progression of CRC with the collection of mutations in the form of abnormal activation of proto-oncogenes to become oncogenes and inactivation of tumour suppressor genes and repair genes. The mutated genes exert different effects in terms of either gain or loss of functions due to the activation of different pathways that contribute to an abnormal and uncontrolled growth of normal colonic epithelium to cancer cells by histopathological transition from adenoma to carcinoma[7-9]. The deleted in colorectal cancer (DCC) is a tumour suppressor gene located on the long arm of chromosome 18, that blocks cell growth in the absence of its ligand netrin 1. It encodes cell surface

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protein in the colon which functions in cellular differentiation and apoptosis[9,10]. The mutation in the DCC gene progresses tumorigenesis and promotes metastasis[11]. The DCC gene is present in axons of the central nervous system (CNS) and differentiating cells of the intestine. In CRC, the mucous-secreting cells lose their ability to proliferate and differentiate. Approximately 70% of CRC showed loss of heterozygosity (LOH) in the DCC gene region[12]. A point mutation has been detected in the DCC gene as a single nucleotide polymorphism (SNP). Across all the ethnicities, 53 CRC risk loci for SNP mapping have been identified[13]. Deoxyribonucleic acid (DNA) sequencing identifies SNP by using a fluorescent dye in running sequence, and comparing it with the reference sequence. Codon 201 is the potential target for LOH with an allelic 71 deletions in the DCC gene. A point mutation at codon 201 in the DCC gene is an early premalignant event in CRC that promotes haematogenous and lymphatic dissemination[14].

There is a great need to investigate CRC at the molecular level for early diagnosis, followed by prompt and appropriate treatment. To the best of our knowledge, no work has been done previously to identify the mutation in codon 201 of the DCC gene in Pakistan. The current study was planned to fill the gap by identifying the mutation in codon 201 of the DCC gene in CRC cases, and to correlate that mutation to the histopathological grading of CRC.

Materials and Methods

The cross-sectional study was conducted from February 2019 to February 2021 after approval from the ethics review board of the Dow University of Health Sciences (DUHS), Karachi. The sample size was calculated by OpenEpi online sample size calculator by keeping the margin of error at a 5% confidence interval[15]. The mean CRC population size was taken as 60 from retrospective data of 2 years[16], and the frequency of codon 201 mutation was taken as 53%[17]. The sample was raised using non-probability convenience sampling technique from among patients who visited for colonoscopy at the Gastrointestinal (GI) Centre of Dowites78 Operation Theatre Welfare Society (DOTS) Complex, Dr Ruth Fau Civil Hospital, Karachi. Those included were biopsy-proven CRC patients regardless of age and gender with histopathological grade II with moderate differentiation in cellular pattern and grade III with poor differentiation. Those excluded were patients who had received neoadjuvant chemotherapy (NAC) or radiotherapy or with recurrent CRC.

Data was collected using a questionnaire that was filled after taking informed consent for biopsy and formalin-fixed paraffin-embedded (FFPE) usage for DNA extraction. This was followed by histopathological diagnosis of CRC with the grading of differentiation. All the diagnosed CRC samples were stored as FFPE tissue blocks for molecular analysis.

DNA extraction was done by using QIAamp DNA FFPE Tissue Kit (50) (Qiagen, Valencia, CA, United States, Catalogue No./ID: 56404) as per the manufacturer's guidelines. The quantity of extracted DNA was measured in ng/µl and purity by A260/280 absorbance ratio by NanoDrop spectrophotometer (Thermo Fisher Scientific Wilmington, DE 19810 U.S.A.). Ribonuclease (RNase) A 10mg/ml (Thermo Fisher Scientific cat no: EN0531) was used to degrade ribonucleic acid (RNA) from DNA samples to improve the quantity and purity of genomic DNA. All the concentrated DNA samples were stored at -20°C in a cryo box. Polymerase chain reaction (PCR) amplification was done by using specific forward and reverse primers for codon 201 of the DCC gene with a PCR product size of 296 at an annealing temperature of 60°C for 30 seconds at 35 cycles.

The forward primer used was:

5'GTAAAACGACGGCCAAGTCTTCATGGGAGACACAGTGCT 3'

The reverse primer used was:

5'AGGAAACAGCTATGACCAAAGAGCATTGCAATACCTGA 3'

The amplified PCR products were analysed for optimisation by gel electrophoresis. Lego agarose gel 1% (Thermo Fisher Scientific TopVision Agarose Catalogue No: RO491) was used to run 2µl of loaded DNA at 300V and 200A for 45 minutes by using DNA ladder 100bp (Thermo Fisher Scientific GeneRuler DNA Ladder Mix Catalogue No: SM0331) for determining the molecular weight of DNA fragments. All the DNA samples were processed for amplification, and the samples that did not amplify and showed PCR failure signals were not subjected to DNA sequencing.

The samples that showed PCR optimisation were sent for Sanger sequencing for the identification of a mutation exon 3 at codon 201 in the DCC gene at chromosome position of chr18: 50432602-50432602. The mutation at codon 201 was correlated with the histopathological grading of colorectal tumours. The results of sequencing were analysed by comparing them with the sequence of the DCC gene in the National Center for Biotechnology Information NCBI (NM_005215: c.601C>G)[18]. The variant analysis from sequencing data was performed by

DNASTAR Lasergene SeqMan7.0 software. Electropherograms were formed from each DNA sequence from where SNP was identified by using SnapGene Viewer 4.1.5 software.

Data was analysed using SPSS 25. Mean and standard deviations were calculated for quantitative variables, while frequencies and percentages were calculated for qualitative variables. Chi-square test was used to determine the association between dependent variables, such as CRC type, and mutation in codon 201 in DCC gene and tumour grading, with independent variables, such as age, gender, tumour site, CRC. P<0.05 was considered statistically significant.

Results

Of the 100 biopsy specimens assessed, 45(45%) were selected after histopathological diagnosis of colon or rectal cancers in grade II and grade III (Figure 1).

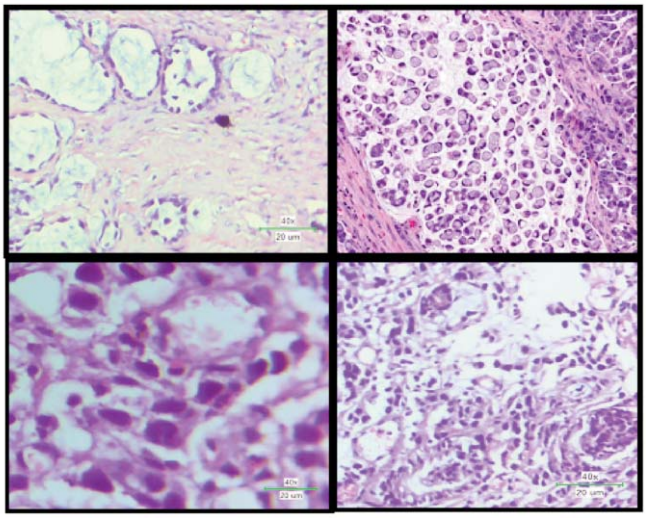

Figure-1: Photomicrographs of adenocarcinoma of rectum and colon with histopathological grading of cancer.

Of them, 13(29%) samples failed to show any band on gel electrophoresis and were subsequently discarded. The remaining 32(71%) samples were used for Sanger sequencing (Figure 2). Of these, 1(3%) did not sequence, while 31(97%) showed DNA sequencing. All the sequenced CRC samples identified a mutation in codon 201 of exon 3 in the DCC gene; 30(97%) showed

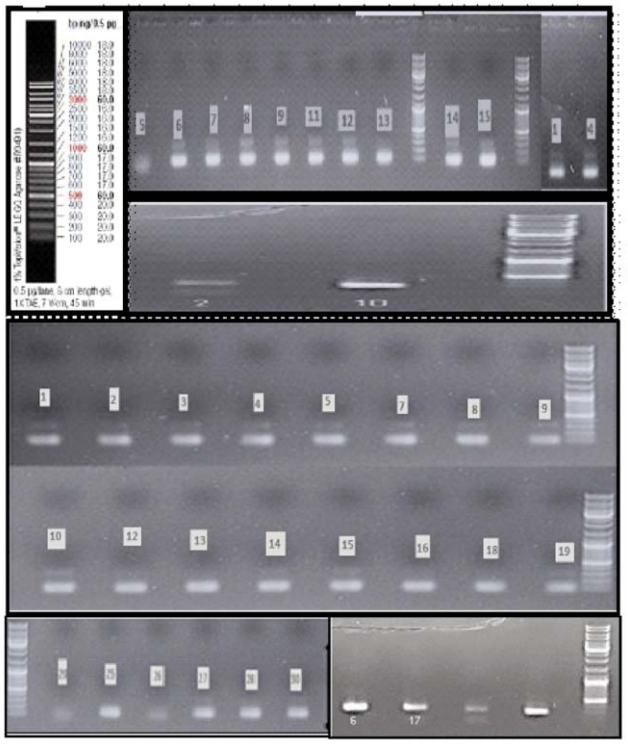

Figure-2: Qualitative analysis of amplified polymerase chain reaction (PCR) product on 1% Agarose gel. Both forward and reverse primers complementary to rs2229080 are seen amplifying c.601C>G region in exon 3.

homozygosity in SNP, and 1(3%) showed heterozygosity in transversion of the first base in codon 201 (Figure 3). A point mutation at the first base of codon 201 of the DCC gene was observed as transversion of guanine in place of cytosine in both forward and reverse sequences of DNA in the homozygous pattern of polymorphism. It was shown that despite genotype C/C, CRC patients showed G/G. A heterozygous variant of SNP showed a transversion of guanine in either forward or reverse sequence of DNA with genotype C/G. On electropherogram, the heterozygous variant showed a double peak of different colours, reflecting two nucleotides' substitution on one copy. The homozygous variants showed a single-coloured peak of a specific nucleotide. Out of 36(80%) participants of grade II, 25(69.4%) showed homozygosity, 1(2.7%) had a heterozygous pattern in the SNP variant, 9(25%) failed in PCR amplification and 1(2.7%) sample did not sequence.

Table 1: Association of Histological grading with SNP analysis of studied participants (n=45)

Variables		SNP analysis				
		Homozygosity SNP	Heterozygosity SNP	Sequencing Fail	PCRFail	*P-value*
Histologic Grading /WHO Grading	Grade II	25	1	1	9	0.648
	Grade III	5	0	0	4	

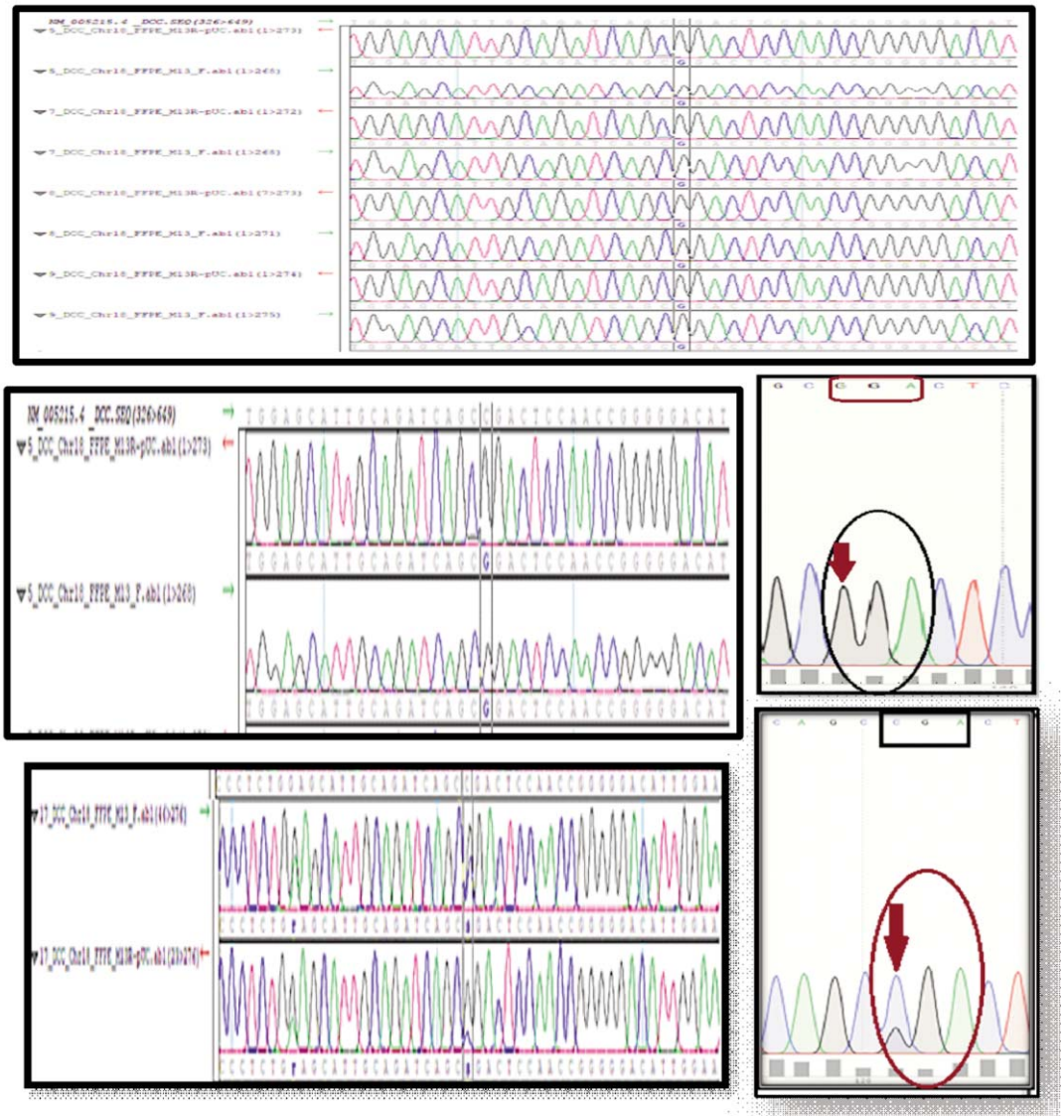

Figure-3: Sanger sequencing showing single nucleotide polymorphism (SNP) rs2229080 at c.601 C>G at codon 201 in exon 3 of deleted in colorectal cancer (DCC) gene.
A: Sanger sequencing showing SNP rs2229080 at c.601 C>G at codon 201 in exon 3 of DCC gene.
B: Homozygous variant (GG) by a single-coloured peak of a specific nucleotide. A transversion was seen of Guanine in both forward and reverse sequence of deoxyribonucleic acid (DNA) with genotype G/G (CGA>GGA).
C: Heterozygous variant (CG) shown by a double peak of different colours, reflecting two nucleotides' substitution on one copy. A transversion was seen of Guanine in either forward or reverse sequence of DNA with genotype C/G (CGA>GGA).

Out of 9(20%) grade III CRC patients, 5(55.5%) showed homozygosity in the SNP variant, and 4(44.4%) failed in PCR amplification.

As shown in Table 1and Table 2 the association between histopathological grades of CRC and independent variables to single nucleotide polymorphism (SNP) was statistically not significant as the p-value is > 0.05.

Discussion

The DCC is one of the tumour suppressor genes that inhibit the growth of tumour cells to avoid the progression from the adenoma stage to the carcinoma stage. The mutation in DCC gene in CRC promotes multiple effects on cancer cell progression. This exerts abnormalities in cellular metabolism, proliferation, differentiation and survival, ultimately promoting benign adenoma to carcinoma, and further progress to metastasis to distant organs. The current study showed a

Table-2: Association of independent variables with SNP analysis of studied Participants (n=45)

Variables	SNP analysis				
	Homozygosity SNP	Heterozygosity SNP	SequencingFail	PCR Fail	*P- Value*
Histopathological Diagnosis					
AC of Rectum	13	0	1	3	0.409
AC of Recto-sigmoid junction	2	0	0	5	
AC of Sigmoid colon	3	1	0	0	
AC of Descending colon	3	0	0	0	
AC of Transverse colon	2	0	0	1	
AC of junction of Transverse and Ascending colon	0	0	0	1	
AC of Ascending colon	5	0	0	3	
AC of junction of Ascending colon and Caecum	1	0	0	0	
AC of Caecum	1	0	0	0	
Gender					
Male	23	0	1	11	0.245
Female	7	1	0	2	
Age					
<20 years	2	0	0	1	0.823
21- 36 years	9	0	0	6	
37-52 years	8	1	1	2	
53- 68 years	6	0	0	3	
>68 years	5	0	0	1	
Site of Tumour					
Right	9	0	0	5	0.748
Left	21	1	1	8	
Type of Tumour					
Polypoidal mass	8	0	1	7	0.618
Fungating mass	1	0	0	1	
Exophytic & polypoidal mass	3	0	0	0	
Polypoidal & Ulcerative mass	1	0	0	0	
Fungating & Ulcerative mass	5	0	0	3	
Diffuse & infiltrative mass	4	0	0	0	
Annular & constricting mass	2	1	0	1	
Ulcerative mass	5	0	0	1	

comprehensive molecular analysis of FFPE tissue blocks of histopathologically-diagnosed CRC. Much work has been done on fresh tissues from biopsy and surgical intervention, and in order to create a novelty and promote valuable uses of FFPE tissue blocks, biopsy specimens were used for molecular analysis. It has been previously reported that FFPE tissue blocks of stained histological slides are frequently used worldwide in the diagnosis and in the assessment of disease prognosis during and after treatment of cancer[19] It has also been used in translational genomics for various complex diseases and in clinical trials for personalised medicine to improve the quality of healthcare facilities offered to the patients.[20]

DNA extraction from colonic and rectal biopsy tissue was performed. A biopsy tissue sample from any solid tumour has been used routinely for the diagnosis of the preoperative status of the tumour. It is also used for prognostic purposes and to assess the response of chemo-radiotherapy of tumours during and after treatment. However, this small biopsy specimen has also been used for molecular analysis to specify mutation and gene expression in preoperative cancer diagnosis to elaborate the strategy of appropriate therapy of cancer.[21]

A point mutation of missense type in the form of SNP in codon 201 was reported after DNA sequencing of all PCR optimised samples in the current study. This was shown by the transversion of a single nucleotide at the first position of codon 201. Most samples showed homozygosity with the transversion of CGA (arginine) to GGA (glycine) in both forward and reverse DNA sequences, as shown by a single-coloured peak of a specific nucleotide. A transversion of guanine in both forward and reverse sequence of DNA with genotype G/G (CGA>GGA) was noted. Only 1 sample showed transversion of CGA (arginine) to GGA (glycine) polymorphism either in forward sequence or in reverse sequence that showed heterozygote pattern of polymorphism as by a double peak of different colours, reflecting two nucleotides' substitution on one copy. A transversion of guanine in either forward or reverse sequence of DNA with genotype C/G (CGA>GGA) was noted.

It has been previously reported that codon 201 in the DCC gene is frequently mutated in CRC patients. Schmitt et al. analysed the mutation in codon 201 in cancer tissue as well as in adjacent normal colonic mucosa, and found a transversion in C to G of the first base in codon 201 that caused decreased gene expression. The current study showed that 100% cases had developed point mutation in codon 201 of the DCC gene in adenocarcinoma of the colon or rectum compared to a previous study in which 53% of CRC showed codon 201 mutations[17]. Kong X et al. revealed that a missense variation at codon 201 was strongly related to DCC gene mutation and loss of DCC expression[22]. Fearon et al. described a missense or nonsense mutation in a coding sequence of the DCC gene in somatic CRC[23, 24]. Honasko et al. identified a missense mutation in codon 201 resulted in the loss of wild type sequence due to transition of CGA (Arg) to GGA (Gly)[25]. Minami et al. reported that 54% of CRC patients showed codon 201 (glycine) DCC gene polymorphism after PCR and restriction fragment length polymorphism (RFLP) followed by DNA sequencing, and it was related to the advanced stage of CRC[26].

A total of 36 participants were diagnosed with grade II CRC in the current study. Of them, 25 participants showed homozygosity and only 1 showed heterozygous in the SNP variant, while 9 failed to be amplified, and 1 sample did not sequence. Among 9 grade III CRC participants, 5 showed homozygosity in the SNP variant, while 4 failed PCR amplification. The association between histopathological grades of CRC and independent variables to SNP was statistically not significant ($p>0.05$).

It was suggested by Limin M et al. that in CRC, the point mutation at codon 201 of the DCC gene acts as a premalignant event that progresses to deep invasion, and carcinomatous change from adenoma to carcinoma. Also, a homozygous mutation (codon 201 Gly G/G) in codon 201 was frequently observed compared to the heterozygous loss of wild-type codon 201 in CRC tissue[27]. The presence of this point mutation in tumour suppressor gene in CRC patients in Pakistan suggests the severity of disease.

The association between histopathological grades of CRC and independent variables to single nucleotide polymorphism (SNP) was statistically not significant as the p-value is > 0.05 as shown in Table 1and Table 2.

The current study has its limitations. There were PCR failure signals in some FFPE tissue blocks that might have been due to DNA fragmentation and formaldehyde cross-linkages. This PCR failure was not associated with the quantity of available biopsy tissue, but more likely related to the content of tumour tissue in biopsy and tissue fixation time in formalin. A small sample size and single-centre data also limit the generalisability of the findings. More CRC samples could not be included due to time and budget constraints.

In the light of the findings, it is recommended that the mutational analysis of codon 201 of the DCC gene can be included in the diagnosis of advanced stage CRC for treatment strategy as well as in the assessment of the prognosis of the disease.

Conclusion

The missense mutation in codon 201 of the DCC gene was frequently observed in grade II as well as in grade III CRC. The mutation analysis of codon 201 of the DCC gene can be included in routine practice for the detection of CRC prior to metastasis, and in taking a decision on treatment options for CRC.

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Author's Contributions

TS: Lab work and experiments, writing, final approval, and accountability for all aspects.

MW: Designed and supervised the study, lab work, data analysis, critical review, final approval, and accountability for all aspects.

FI: Designed and supervised the study, data interpretation, and final approval.

AS: Clinical supervisor, sampling, final approval and accountable for all aspects.