

## **Molecular Heterogeneity in Colorectal Cancer: Insights from Genetic Profiling**

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### **Abstract**

The high heterogeneity has become apparent in metastatic colorectal cancer (CRC) and the solitary leading causes of cancer mortality worldwide. Tumor from benign to malignancies derives by gradual and progressive genetic alterations develop into a collection of neoplastic diseases. The parameters are assessed to indicate the extent and prognosis of the disease such as tumor node metastasis stage, tumor grade, microsatellite status, lymphovascular invasion. It is investigated KRAS mutation status in a metastatic to predict the response to anti-epidermal growth factor. CRC has been described that have prognostic and therapeutic relevance at a distinct molecular level. In the present study, the researcher recruited 65 CRC patients with different stages of the tumor at the right-sided colon, left-sided colon and rectum. We have also recognized left-sided was more complex than right sided CRC patents. In CRC, it has revealed the major differences in the characterization of inflammatory infiltrations and cells location in tumor types. All the patients were stratified into different prognostic and therapeutic groups with the help of these parameters. Though, CRC show intra-tumor heterogeneity due to not clear-cut stratification. However, several CRC patients with single tumor mutations show differences in their mutational status, morphology, inflammatory infiltrate and gene expression. In conclusion, the primary focus on the concept of molecular heterogeneity their metastases and clinical implication in CRC.

**Keywords:** Colorectal Cancer, Genetic alternations, Heterogeneity, Tumor node metastasis

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## 1. Introduction

Colorectal cancer (CRC) is a most common and complex disease with an unpredictable clinical and imperative divergences in the response to treatment. Worldwide rate of incidence nearly 1.4 billion cases, new cases diagnosed more than 80% in above 50 years of age (Mohd et al., 2020). Ten fold higher incidence rate of CRC in western than in developing countries. In etiology of disease the environmental factor such as smoking habits, alcohol consumption and meat consumption play a central role in developing CRC (Younis et al 2018). Some other risk includes age, family history, personal history of cancer, chronic inflammatory bowel diseases and history of polyps in the colon. The healthiest prognostic parameters such as TNM classification system, designed by the American Joint Committee on Cancer and the Union for International Cancer Control are stratifying to CRC patients (Mohd et al., 2020). Mutational profile of KRAS metastases is play a central role in clinical practice in comparison to primary tumors and consistent KRAS mutations being an early event in CRC tumorigenesis. At the time of diagnosis therapeutic strategy is highly dependent on the stage of CRC (Hugen et al., 2016). The treatment for resectable metastases includes neoadjuvant chemotherapy, metastectomy and colectomy in stage IV tumors. For the symptom relief palliative chemotherapy can be considered at extensive unresectable metastases. The CRC is measured a highly heterogeneous and dynamic cancer categorized by multiple molecular pathways of tumor biology (Cuyle and Prenen, 2017). In CRC promising concept of tumor heterogeneity led to a change in the treatment concept towards the use of personalized medicine differences is the recent genetic insights.

In recent studies tumor heterogeneity has been remains a hot topic in cancer research. The CRC tumor heterogeneity can be divided into intertumor and intratumor heterogeneity. The intertumor heterogeneity comprises differences in colorectal tumors of the same histological type between patients and synchronic colorectal tumors occurring within a single patient. The intratumor heterogeneity can be sub-divided into spatial and temporal heterogeneity (Dagogo-Jack and Shaw, 2018). Spatial heterogeneity consigs to differences that can be observed within a single tumor and temporal heterogeneity consigs to the energetic nature of CRC with genetic alterations developing within individual tumors over time (Zellmer and Zhang, 2014). At different levels of genomics, transcriptomics, histopathologic and characterization of the inflammatory infiltration in tumor heterogeneity investigated. The study shows insight of genetic profiles regarding molecular heterogeneity in CRC and discusses the prognostic value of biomarkers and the influence of tumor heterogeneity.

## **2. Material and Methods**

### **2.1 Patient recruitment and sample collection.**

All the samples were collected after obtaining written informed consent from the patients. The patients were recruited based on the criteria i.e patients diagnosed with CRC. In present study, the researchers collected 65 samples from CRC patients. An equal number of normal and healthy individuals were selected as controls including those who have not exposed themselves to any kind of chemicals or radiation. The patients and the controls were divided into two groups based on age (Group I <50 years and group II > 50 years). Average patient age in group I was n=24 and in the group II was n=41 respectively. The anatomical distribution of the tumor was as follows: right bowel and left bowel. Tumor grades were separated into three categories; well-differentiated, moderately differentiated and poorly differentiated tumor.

### **2.2 DNA extraction from blood samples**

Whole genomic DNA was collected by following kit protocol (Bangalore Genei- blood DNA extraction kit). Whole blood was collected in EDTA coated collection tubes to avoid clotting. The first step in the extraction procedure was the lysis of the RBC using solution.

### **2.3 Agarose gel electrophoresis**

Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their molecular weight and is an intrinsic part of almost all routine experiments carried out in molecular biology.

### **2.4 Quantification of DNA**

Standard DNA was prepared using salmon sperm DNA at various concentrations (10, 25, 50 and 75µg/mL). The control DNA was serially diluted in distilled water. 50µL of the isolated genomic DNA was diluted in 1mL distilled water and OD was measured at 260nm using a spectrophotometer.

### **2.5 K-RAS gene polymorphism**

The PCR conditions were as follows. The reaction volume used was Template DNA (200ng) was 4.0µL, forward and reverse primers (1µM) was 1.0µL each, PCR master mix (2X) was 12.5µL, MilliQ water was 8.5µL and total volume was 25.0µL. The PCR products were electrophoresed on one percent agarose gels containing EtBr and viewed under ultraviolet light.

### **2.6 Restriction digestion**

The allelic variants were identified by the use of restriction enzymes that differentiate between alleles. For digestion of PCR product with HinfI restriction enzyme (Fermentas), the following protocol was used directly after amplification: PCR reaction product 10µL, Nuclease free water 18µL, 10X buffer R 2µL and HinfI enzyme 2µL.

### 3. Results

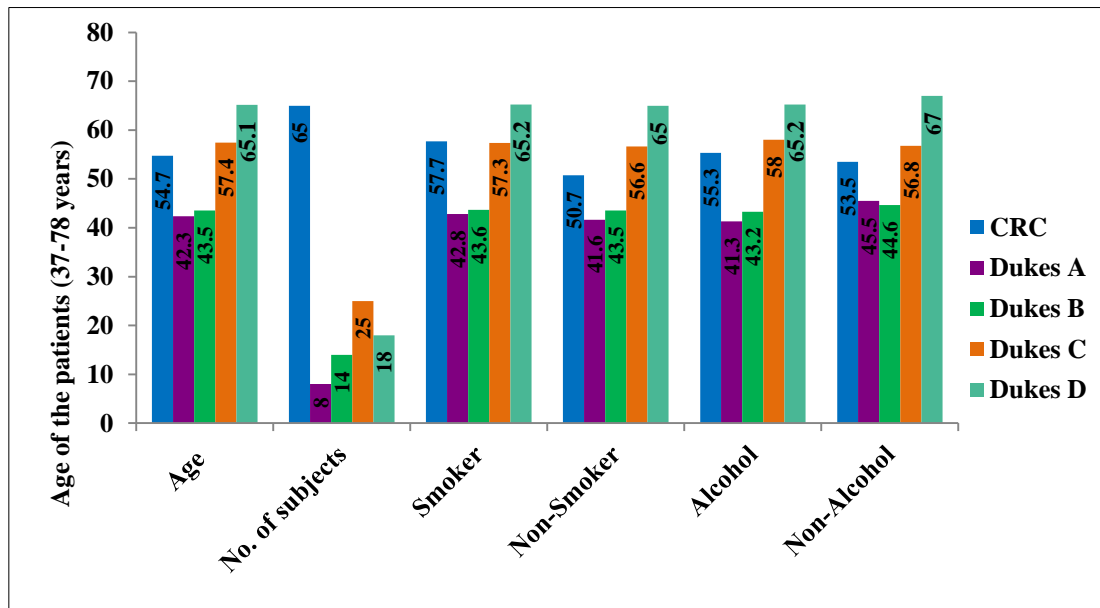
The clinical characterisation of the subjects recruited both experimental and control with age group between 37–78 (mean  $54.72 \pm 10.24$ ) and 36–77 (mean  $54.63 \pm 9.83$ ) was selected. The experimental and control subjects were characterised based on their age as the group I ( $\leq 50$  years; 24 subjects; 36.92%) and group II ( $> 50$  years; 41 subjects; 63.08%). Table 1 depicts the distribution of mean $\pm$ SD values of smoker and alcohol of experimental subjects along with their respective non-smoker and non-alcohol. The experimental subjects in Dukes A, Dukes B, Dukes C and D depicted increased mean age levels of smoker ( $42.80\pm 4.43$ ,  $43.66\pm 2.08$ ,  $57.38\pm 4.99$  and  $65.20\pm 6.29$ ) and alcohol ( $41.33\pm 3.26$ ,  $43.28\pm 4.07$ ,  $58.0\pm 5.32$  and  $65.21\pm 6.29$ ) when compared to control mean levels of non-smoker ( $41.66\pm 2.30$ ,  $43.54\pm 4.45$ ,  $56.63\pm 6.00$  and  $65.00\pm 3.60$ ) and non-alcohol ( $45.50\pm 3.53$ ,  $44.66\pm 3.98$ ,  $56.81\pm 5.82$  and  $67.00\pm 1.41$ ) respectively.

The mean level of smoker and alcohol frequency shows increased for CRC patients in Dukes A ( $42.80\pm 4.43$  and  $41.33\pm 3.26$ ), Dukes B ( $43.66\pm 2.08$  and  $43.28\pm 4.07$ ), Dukes C ( $57.38\pm 4.99$ ,  $58.00\pm 5.32$ ) and Dukes D ( $65.20\pm 6.29$  and  $65.20\pm 6.29$ ) respectively when compared to their respective non-smoker and non-alcohol Dukes A ( $41.66\pm 2.30$  and  $45.50\pm 3.53$ ), Dukes B ( $43.54\pm 4.45$  and  $44.66\pm 3.98$ ), Dukes C ( $56.63\pm 6.00$  and  $56.81\pm 5.82$ ), Dukes D ( $65.00\pm 3.60$  and  $67.00\pm 1.41$ ). The mean level of smoker and alcohol frequency was found to be significantly increased from Dukes A to Duke's D in CRC patients as compared to non-smoker and non-alcohol shown in Table 1. The CRC patients at Dukes D were found to be statistically higher ( $P<0.05$ ) mean level of smoker and alcohol compared to Dukes A, Dukes B and Dukes C shown in Table 1 (Figure 1). Smoker and alcohol CRC patients from Dukes A to Dukes D were increased when compared with their respective non-smoker and non-alcohol patients. It was indirect from the above data that the mean level of age in the all Dukes stage A, B, C and D was statistically significant ( $P<0.05$ ).

**Table 1. Mean  $\pm$  SD values of smoker, non-smoker, alcohol and non-alcohol of CRC patients based on stages of diseases**

S. No	Particulars	Age (years) (Mean $\pm$ SD)	No. of subjects	CRC			
				Smoker (Mean $\pm$ SD)	Non-Smoker (Mean $\pm$ SD)	Alcohol (Mean $\pm$ SD)	Non-Alcohol (Mean $\pm$ SD)
1	<b>CRC</b>	$54.72\pm 10.24$ (37-78)	<b>65</b>	$57.70\pm 9.93$	$50.78\pm 9.43$	$55.34\pm 11.01$	$53.50\pm 8.67$
2	<b>Dukes stage A (CRC)</b>	$42.37\pm 3.62$ (38-48)	<b>8</b>	$42.80\pm 4.43$	$41.66\pm 2.30$	$41.33\pm 3.26$	$45.50\pm 3.53$
3	<b>Dukes stage B (CRC)</b>	$43.57\pm 3.99$ (37-49)	<b>14</b>	$43.66\pm 2.08$	$43.54\pm 4.45$	$43.28\pm 4.07$	$44.66\pm 3.98$
4	<b>Dukes stage C (CRC)</b>	$57.40\pm 5.55$ (48-71)	<b>25</b>	$57.38\pm 4.99$	$56.63\pm 6.00$	$58.00\pm 5.32$	$56.81\pm 5.82$
5	<b>Dukes stage D (CRC)</b>	$65.16\pm 5.84$ (57-78)	<b>18</b>	$65.20\pm 6.29^*$	$65.00\pm 3.60^*$	$65.20\pm 6.29^*$	$67.00\pm 1.41^*$

CRC-Colorectal Cancer; Dukes stages: A, B, C and D; Duke A values are presented as Mean  $\pm$  SD.\*-Statistically significant compared Duke D ( $P<0.05$ ).



**Figure 1: Frequency of smoker, non-smoker, alcohol and non-alcohol of CRC patients based on their age**

The Table 2 depicts the distribution of mean±SD values of WBC of experimental subjects along with their particular control subjects. The experimental smoker and alcohol depicted increased mean levels of WBC (17419.16±9819.96 and 16847.38±9405.62) when compared to the non-smoker, non-alcohol and control mean levels of WBC (12906.54±3772.67, 13274.64±3802.47 and 7703.01±1263.12). Smoker and alcohol CRC patients showed increased levels of WBC when compared to non-smoker, non-alcohol and control with respect to controls, showed in Table 2 (Figure 2). It was indirect from the above data that the mean level of WBC values in the smoker and alcohol were statistically significant when compared to their respective non-smoker, non-alcohol and controls (P<0.05).

**Table 2. Mean ± SD values of WBC of CRC patients and controls based on their habits**

S. No	CRC						
	Category	CTL	CRC	Smoker (CRC)	Non-Smoker (CRC)	Alcohol (CRC)	Non-Alcohol (CRC)
1	No. of subjects	65	65	37	28	43	22
2	Age (years) (Mean±SD)	54.63±9.83 (36-77)	54.72±10.24 (37-78)	57.70±9.93 (38-77)	50.92±9.36 (37-67)	55.54±11.05 (37-78)	53.95±8.00 (37-68)
3	WBC/μL (Mean±SD)	7703.01 ± 1263.12	15475.42 ± 8081.86*	17419.16 ± 9819.96*	12906.54 ± 3772.67	16847.38 ± 9405.62*	13274.64 ± 3802.47

CTL-Controls and CRC-Colorectal Cancer; Smoker, Non-smoker, Alcohol and Non-alcohol, Values are presented as Mean±SD. \*.Statistically significant compared to controls (P<0.05).

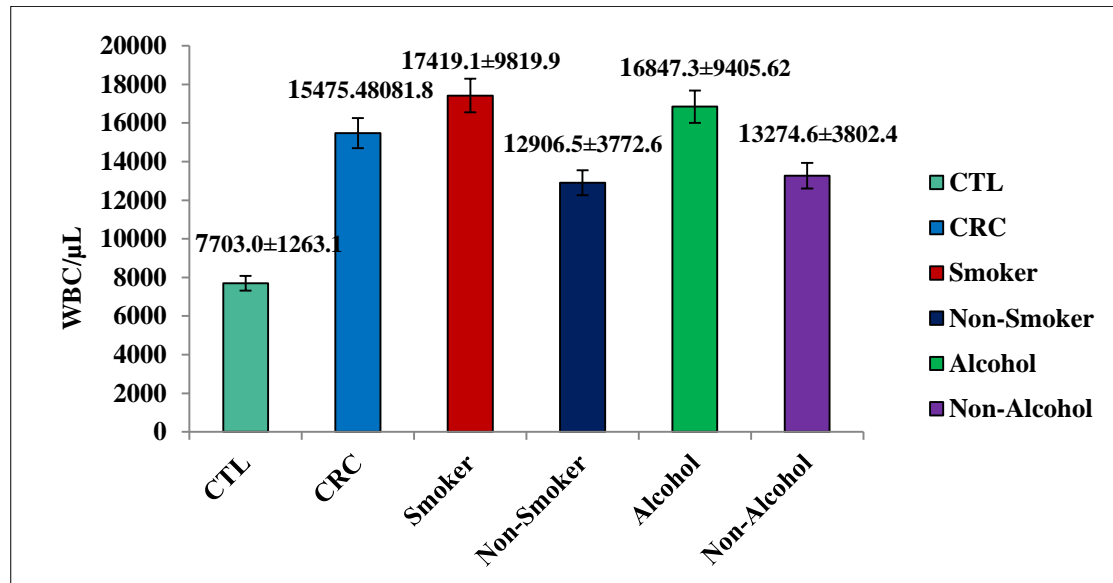


Figure 2: WBC frequency in CRC patients and controls based on their habits

The isolation of DNA was carried out from the peripheral blood samples and the genotype frequency was evaluated in CRC subjects and healthy controls. *K-RAS* genotypes were determined by using PCR-RFLP. Polymorphism representative RFLP analysis of the restriction digestion of the PCR products *K-RAS* gene was separate on 2 percent agarose gel. The *K-RAS* heterozygous genotype produced 3 bands of size 163bp, 222bp and 358bp. Among 65 experimental subjects, 26 subjects (40%) were found to have *K-RAS* gene mutation were found.

#### 4. Discussion

In the recent years, CRC has been increasing not only in India but also throughout the world and this increase is paralleled by the number of incidence. The mechanism of development of CRC involves a series of genes involved in effects on metabolism and potentially increased toxic/carcinogenic compounds (Alam *et al.*, 2018). A large number of molecular epidemiologic studies have been conducted with the purpose of identifying these genes and to assess their role in the etiology of the cancer. The majority of CRC expand from benign pre-neoplastic lesions, the adenomatous polyps or adenomas (Amersi *et al.*, 2005). The progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, which is referred to as the adenoma-carcinoma sequence (Fleming, 2012). For the uniformity and consistency in reporting, internationally accepted and used classification is that proposed by the WHO: adenocarcinoma, medullary carcinoma, colloid adenocarcinoma, “signet ring” squamous cell carcinoma, epidermoid carcinoma, adenosquamous, small cell carcinoma, undifferentiated carcinoma and

other types (Puppa *et al.*, 2010). The present study systematically showed the associations between polymorphisms in genes involved in the risk of colorectal adenomas and CRC. Although the etiology is not clear, CRC is considered a multifactorial disease, a significant role being attributed to the impact of environmental factors on a genetically prone area. The hereditary predisposition is considered a significant factor in colorectal carcinogenesis, although 80 percent of colorectal neoplasms occur in the absence of a family history of CRC (Lynch and Chapelle, 2003).

In the present study, 65 CRC patients with age range of 37-78 years have concluded the effect on age group incidence presented with 38.46 percent of Dukes stage C disease. Recent study patients with CRC included in the Department of Defense Automated Central Tumor Registry (January 1993 to December 2008) were stratified by age <40, 40 to 49, 50 to 79, and  $\geq 80$  years to determine the effect of age on incidence (Steele *et al.*, 2014). The young age at presentation (<50 years) was associated with advanced stage and higher recurrence of CRC, but with similar survival in comparison with older patients. About 79, 48 patients were identified; most (77%) patients were in the 50-79 year age, overall 25 percent presented with stage III disease (Steele *et al.*, 2014). Sudarshan *et al.* (2013) reported, 233 patients were diagnosed to have CRC. All the patients diagnosed below 40 years of age comprised 39.05 percent and those under age 20 comprised 4.29 percent. Among those under 40 years of age, the majority were males 63.73 percent, most occurred in the rectum 84.61 percent in Chhattisgarh, Raipur, India. The rising incidence of CRC with increasing age has been associated with the high risk of expansion of CRC (Wang *et al.*, 2017). This is similar to the observation from population-based data of developed countries. The age-specific rates in the older age group are much higher compared to that in pediatric, young and adult populations in the present study. Among 65 CRC patients, the group I were 24 subjects (36.92%) and group II were 41 subjects (63.07%). The number of patients belongs to group II and inferred that the above data showed that the age increases there will be a higher risk for the progression of CRC.

In the present study, the frequency of all four Dukes stage of CRC at the time of clinical presentation was Dukes A 12.30 percent, Dukes B 21.53 percent, Dukes C 38.46 percent, and Dukes D 27.29 percent, respectively. In an earlier report frequency were reported that in 481 cases (37.0%) of 1,299 cases of CRC, 36.0 percent in Dukes B and 37.5 percent in Dukes C disease respectively (Roth *et al.*, 2010). The results of the present study observed experimental Dukes A, Dukes B, Dukes C and Dukes D depicted increased mean levels of smoker and alcohol when compared to mean levels of non-smoker and non-alcohol. The present study observed the age wise distribution of Dukes stage A to D in experimental subjects. A study has been done by Walter *et al.* (2015), in Germany, where they reported smoking was

associated with decreased survival in Dukes stage A-C smokers with pack year's  $\geq 20$  in CRC cases. This was evident in the present finding also.

The experimental smoker and alcohol depicted increased mean levels of WBC when compared to the non-smoker, non-alcohol and control mean levels of WBC. Recently Al-Saeed *et al.* (2014) demonstrated pre-treatment WBC levels were found significantly high in right-sided CRC. Several studies depicted the WBC count has been predictive value in various cancers including CRC and increased WBC levels have been postulated as one of the mechanisms of hematogenous spread of metastases (Castillo-Perez, 2013). Earlier study findings demonstrate that elevated WBC is associated with an increase in both the mortality and incidence rates of colon cancer (Lee *et al.*, 2006). Malenica *et al.* (2017) reported that continuous cigarette smoking has severe adverse effects on haematological parameters (e.g., hemoglobin, white blood cells count, mean corpuscular volume, mean corpuscular hemoglobin concentration, RBC count, hematocrit) and these alterations might be associated with a greater risk for developing atherosclerosis, polycythemia vera, chronic obstructive pulmonary disease, and CRC. This was evident in the present finding also.

The *K-RAS* mutations play an important role in human tumorigenesis and are the most prevalent in pancreatic, thyroid, CRC and lung cancers (Tan and Du, 2012). Another earlier study suggested that hepatic metastases often exhibited deletions of chromosomes 2q, 5q, 8p, 9p, 10q and 21q21 as well as chromosomal gains of 1q, 11, 12qter, 17q12-21, 19 and 22q than their corresponding primary tumors. The study shows 12p12.1 copy number loss detected by array comparative genomic hybridisation in the tumor of five patients with a good response. A single tumor contained a loss of the whole chromosome, three tumors included a loss of the short arm of the chromosome and one tumor contained a loss of a 27.5 Mb region of the short arm of chromosome 12 including the *K-RAS* locus (Mekenkamp *et al.*, 2012). *K-RAS* oncogene is mutated in approximately 35-45 percent of CRC and *K-RAS* mutational status testing has been highlighted in the recent years (Kim *et al.*, 2015). The most frequent mutations were point substitutions in codons 12 and 13 validated as negative predictors of response to anti-epidermal growth factor receptor antibodies. Therefore, determining the *K-RAS* mutational status of tumor samples has become an essential tool for managing patients with CRC.

## 5. Conclusions

The heterogeneity is accepted information and heterogeneity seems predominantly evident in CRC. Heterogeneity is confined to the genetic level and with the tumor microenvironment. The considerable clinical interest in presence of tumor heterogeneity, as it directly impacts treatment decisions. The clinically method significant fall below certain detection thresholds makes the choice of testing. After treatment



initiation highly sensitive detection methods should be preferred, fractions of KRAS mutated cells have been shown to permit the development of secondary-resistance in tumor. Although the colorectal cancer, tumor heterogeneity must be considered in the clinical treatment concept.

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