# A STUDY OF NEWER PROGNOSTIC EPIGENETIC MARKERS SFRP1 & IGFBP3 IN COLORECTAL CARCINOMA

**THESIS**

SUBMITTED TO

**BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY**

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ESTABLISHED 1996

FOR THE DEGREE OF

**Doctor of Philosophy**

**IN**

**BIOTECHNOLOGY**


By

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**2019**

*Dedicated to my Parents and Teachers*

## DECLARATION

I, **Alok Kumar** hereby declare that the thesis entitled **"A STUDY OF NEWER PROGNOSTIC EPIGENETIC MARKER SFRP1 & IGFBP3 IN COLORECTAL CARCINOMA"** is an authentic research work carried out by me under the guidance of Dr. G. Sunil Babu, Assistant Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow and co-guidance of Dr. Dr. Pradyuman Singh, Professor, Department of Pathology, Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow. The research work is original, and no part of this work has been submitted for any other degree or diploma.

I also declare that the thesis is essentially free from all kinds of plagiarism.

All the above given information is true to the best of my knowledge.

Date: 20.09.2019

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# CERTIFICATE

This is to certify that the thesis entitled **"A STUDY OF NEWER PROGNOSTIC EPIGENETIC MARKERS SFRP1 & IGFBP3 IN COLORECTAL CARCINOMA"** submitted by **Alok Kumar** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other University.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow, satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulation-1999* as amended in *2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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## Ph.D. Course Work Certificate

This is to certify that **Mr. Alok Kumar**, Enrollment No. 231/13 Ph.D. Research Scholar, Department of Biotechnology of this University has successfully completed his Ph.D. Course work in the examination held during May, 2014.

**(A.K. Maurya)**
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# Institutional Ethics Committee
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EC Ref. No.: 01/IEC/BBAU/2015

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Subject: Approval of the research study – **"A study of newer prognostic epigenetic markers SFRP1 & IGFBP3 in Colorectal Carcinoma"** by the Ethics Committee

Dear **Mr. Alok Kumar**

With reference to the above mentioned proposal submitted by you for review; the comment and decision of Institutional Ethics Committee meeting on 20th October 2015 are given bellow for your information and necessary action.

Decision – **Approved**

Yours Sincerely,

Dr G. Sunil Babu
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**IEC No. 8/15**

Dear Mr. **Alok**

With reference to the above mentioned proposal submitted by you for review, the comments and decision at the Institutional Ethics Committee meeting on 03.03.2016 are given below for your information and necessary action:

**Decision:** Approved.

Thank you,

Yours sincerely,

(Deepak Malviya)
Member Secretary
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*Alok Kumar*

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## ABBREVIATIONS

| | | |
|---|---|---|
| APC | : | Adenomatous polyposis coli |
| EGFR | : | Epidermal growth factor receptor |
| 5-FU | : | 5-fluorouracil |
| HNPCC | : | hereditary nonpolyposis colorectal cancer |
| IBD | : | Inflammatory bowel disease |
| RAS | : | Ras-p21 protein coding gene |
| TNM | : | Tumor node metastasis |
| CRP | : | C-reactive protein |
| TNFα | : | Tumor necrosis factor α |
| IL | : | Interleukin |
| VEGFR2 | : | Vascular endothelial development factor receptor 2 |
| IGFBP3 | : | Insulin-like growth factor binding protein 3 |
| CRC | : | Colorectal carcinoma |
| MS-PCR | : | Methylation-specific PCR |
| CIMP | : | CpG island methylator phenotype |
| TP53 | : | Tumor protein p53 |
| TGFBR2 | : | Transforming growth factor β receptor type 2 gene |
| MGMT | : | O6-methylguanine DNA methyltransferase |
| VIM | : | Vimentin |
| SEPT9 | : | Septin 9 |
| SFRP1 | : | Secreted frizzled-related protein 1 |
| THBD | : | Thrombomodulin |

| | | |
|---|---|---|
| TYMS | : | Thymidylate synthetase |
| TFAP2E | : | Transcription factor AP-2 epsilon |
| SOCS1 | : | Suppressor of cytokine signaling 1 |
| CACNA1G | : | Calcium Voltage-Gated Channel Subunit Alpha1 G |
| RUNX3 | : | Runt-related transcription factor 3 |
| FFPE tissue | : | Formalin-Fixed Paraffin-Embedded tissue |

# INTRODUCTION

# CHAPTER-1

## INTRODUCTION

Colorectal cancer (CRC) is a cancer that begins from the colon or the rectum. CRC stands third most common cancer and second leading cause of cancer-associated mortality worldwide. In India, it is recorded as fourth most common cancer in males and fifth in female with 6.4%, 3.4% incidence rates (Bray F *et al.* 2018). Most of the CRC start as a growth on the inner lining of the colon or rectum and these growths are called polyps. These polyps are grouped into Two types, 1. Adenomatous polyps and 2. Hyperplastic polyps and inflammatory polyps. Most of the colorectal carcinomas are of adenocarcinomas, which constitute about 96% of CRC's. The major risk factors for the CRC are inflammatory bowel disease (IBD), diet, obesity, smoking, sedentary life style, alcohol intake, age, family history of colon cancer (Haggar FA *et al.* 2009). These risk factors may induce genetic and epigenetic alterations in colorectal epithelial cells that leads to tumorigenesis (Baylin SB *et al.* 2000). CRC can be treatable in early stages, but the its treatment becomes defiicult at advanced stages. Even with the development of several diagnostic, prognostic approaches for CRC, the existing treatments are not able to improve the chances of curability. CRC prognosis is still poor in India due to late presentation of CRC cases and its heterogeneous nature (Singh MP *et al.* 2017). Further, the CRC patients show a significant difference in prognosis, individual treatment responses, even in the same clinical stage of tumor due to heterogenicity of disease (Guastadisegni C *et al.* 2010). Heterogenicity of CRC plays a significant role in treatment response, patients outcome, survival after surgery. The major cause of heterogenicity of CRC is involvement of several genetics and epigenetic instability that attained during the lifetime, these may be inherited or non-inherited or lifestyle influenced, environment-related (Strambu V *et al.* 2014). These instabilities represent three major pathways that are characterised by three different types of genomic instability, Chromosomal instability (CIN) pathway, Microsatellite instability (MSI) pathway, Epigenetic instability pathway or CpG island methylator phenotype (CIMP) (Thorstensen L *et al.* 2005). Microsatellite instability (MSI) pathway develops as a result of defect in DNA mismatch repair genes that leads to microsatellite instability (Maestro ML *et al.* 2007). Chromosomal instability pathways develop as a result of mutations in *adenomatous polyposis coli (APC)* gene and some other genes that activate

Wnt signaling pathway (Fodde R *et al.* 2001). Epigenetic instability or CIMP pathway results in epigenetic silencing of several tumor suppressor genes by DNA methylation (Pancione M *et al.* 2012). Recently microRNAs are also reported to be involed in the generation of CRC (Pillai RS *et al.* 2005). All these pathways of CRC are characterized by specific pathological features, mechanisms of carcinogenesis and process of tumor development (Worthley DL *et al.* 2007). The molecular features of these pathways have been used clinically in the screening, diagnosis and management of patients with CRC (Gryfe R *et al.* 2000).

The Tumor Lymph-Node Metastasis (TNM) staging system is a most preferential method to forecast the survival and disease-associated risk of CRC patients but different patients with same TNM stage may have diverse long term prognosis and response to therapy (Marzouk O, *et al.* 2011). Prognostic biomarkers, which can predict the clinical outcome and distinguish high-risk initial stage CRC patients from low-risk initial stage patients, are needed for better management of CRC (Schmoll HJ *et al.* 2012). Therefore, validation based results about prognostic markers are desirable for better patients care. In last decade many epigenetic biomarkers have been reported and defined as capable cancer biomarkers in the literature (Mikeska T *et al.* 2014). However, only few biomarkers have been confirmed for clinical practice (Boland CR *et al.* 2010). In this thesis we focused on epigenetic instability pathway or CIMP, as it is one of the important and comparatively recent pathways that lead to colorectal cancer. Epigenetic regulation of gene expression is an important key mechanism that is working in normal tissues and has an essential role in the preservation of genomic stability, tissue differentiation and embryonic development. (Li E *et al.* 2002). In CRC gene promoter methylation mediate epigenetic silencing is very common event and play significant role in the carcinogenesis. It occurs at CpG (cytosine preceding guanine) island, these are short stretches of CpG rich regions (Gronbak K *et al.* 2007) and methylation of CpG islands within the promoter region cause transcriptional silencing of gene (Yoshiura K *et al.* 1995). In cancer cells, CpG islands may aberrantly hypermethylated, cause inappropriate silencing of gene expression (Jones PA et al. 2002). DNA methylation is an natural process in which enzyme adds a methyl group to the 5-position of cytosine by DNA methyltransferases (DNMT) to produce 5-methylcytosine (Valinluck V et al 2007). Usually, the favourite substrate for DNMT is a CG dinucleotide

sequence, which has therefore been termed CpG (Mojarad EN *et al.* 2013). Many tumor suppressor genes engaged in CRC formation and progression are often found epigenetically silenced through hypermethylation at their promoter regions and *IGFBP3, SFRP1, CACNA1G, IGF2, NUROG1, SOCS1, RUNX3* gene one of them (Tomii K, *et al.* 2007). Numerous tumor markers have been suggested and examined in CRC for the diagnosis, monitoring of treatment response in recent years (Van Cutsem E *et al.* 2014). Some methylation-based markers are translated as biomarkers for clinical practice in cancer (Costa Pinheiro *et al.* 2015). Many scientists and clinicians believe that epigenetic changes, such as promoter methylation of cancer-associated genes, can be promising diagnostic, prognostic and predictive biomarkers for cancer (Koch A *et al.* 2018). Hence, it is significant to focus research on an exploration for epigenetic changes that involved in cancer development, progression, and aggressiveness of disease.

We analyzed seven genes methylation status, they are *IGFBP3, SFRP1* and CIMP marker genes (*CACNA1G, IGF2, NUROG1, SOCS1, RUNX3*) and try to find the prognostic value of these genes in CRC. We have choosen these genes for study because they are reported as CIMP (CpG island methylator phenotype) marker genes. They represents a subtype of colorectal cancers that happen through an epigenetic instability pathway and are characterized by vast hypermethylation of promoter CpG island sites (Mojarad EN *et al.* 2013). However, the pathophysiology of hypermethylation CIMP related to CRC remains unclear needs studied further. Weisenberger DJ *et al.* 2006, reported CRC can be classified on the basis of their level of DNA methylation, and those cancers with high level of methylation known as CIMP high, represent a clinically and etiologically separate group that is characterized by epigenetic instability. CIMP associated cancers appear to have distinct epidemiology, histology, precursor lesions and different molecular features (Issa JP *et al.* 2008). The perception of CIMP led to the proposal of a tumorigenic pathway of CRC driven by promoter hypermethylation and hence epigenetic, rather than genetic, inactivation of tumor suppressor genes (Toyota M *et al.* 1999). Several studies defined that CRC can categorize two or more category on the basis of methylation status of CIMP gene marker. Barault *et al.* defined independent prognostic factor in MSS CRC (Gallois C *et al.* 2016). In this study we are looking methylation status of CIMP marker, IGFBP3 and SFRP1 genes. Tumor suppressor genes such as IGFBP3 and SFRP1 play a significant role

in the development of CRC, as both genes reported to be silenced in CRC by promoter hypermethylations (Ng J *et al.* 2015). IGFBP3 gene encodes Insulin-like Growth Factor Binding Protein 3- a multifunctional protein, facilitates the growth suppression and initiation of apoptosis by binding with insulin-like growth factors (IGFs) (Florini JR *et al.* 1996). It performs pivotal roles in cell survival, growth differentiation (Collett-Solberg PF *et al.* 1996). The promoter methylation mediated IGFBP-3 silencing was reported in many cancers, such as lung, hepatocellular, gastric, breast, and ovarian cancers including CRC (Zhu S *et al.* 2014).

Secreted Frizzled Related Protein 1 (SFRP1) gene is a antagosnist of Wnt signalling cascade and plays a significant role in the regulation of Wnt/βcatenin signalling pathway (Billiard J *et al.* 2005, R Prosperi J *et al.* 2010). β-catenin dependent canonical WNT signalling maintains crypt stem cell compartment in the bowel but hyperactivation of this pathway has been reported in colorectal carcinoma (Reya T *et al.* 2005, Spranger S *et al.* 2018). SFRP1 gene expression is found to be downregulated via aberrant methylation on its promoter region and this promoter methylation is a common epigenetic alteration found in human cancers including colorectal carcinoma (Suzuki H *et al.* 2002, Baylin SB *et al.* 2006). Reports pertaining to the SFRP1 as a prognostic marker asre getting accumulated in various cancers (Vatandoost N *et al.* 2016), we, explored for association of promoter methylations of SFRP1 with clinicopathological features of CRC and patients survival. The methylation status's of IGFBP3, SFRP1 and CIMP marker genes promoter region of stage II and III CRC in Indian patients and find its relationship with clinicopathological factors and determine the prognostic potential as biomarker. The relationships between gene promoter methylations and clinicopathological features may be used to more accurately to classify subgroups of CRC patients. To the best of our knowledge, no previous study has been reported in finding the relationship among promoter hypermethylations of *SFRP1, IGFBP3,CIMP* marker genes, their association with clinicopathological characteristics and survival of CRC patients in India.

# REVIEW OF LITERATURE

# CHAPTER-2

## REVIEW OF LITERATURE

### 2.1 Colorectal cancer (CRC)

When cancer develops from the large intestine (colon or rectum) it is called colorectal cancer or bowel cancer or colon cancer or rectal cancer. It is a heterogeneous disease and 4 major molecular pathways involve in CRC development. Major symptoms of CRC are constipation or diarrhoea, changes in bowel habits, a feeling that the bowel does not empty properly after a bowel movement, blood in stool or dark stools, bright red bleeding from the rectum, abdomen pain and bloating, a suffering of fullness in the abdomen, even after hunger situations, tiredness or fatigue, weight loss, a bump in the abdomen, unexplained iron deficiency in men.

### 2.2 Epidemiology of CRC

Incidence wise colorectal cancer is the third most common cancer worldwide and the second leading cause of cancer-associated deaths (9.2%). In India, Colorectal Cancer is the fourth, third most familiar type of malignancy in males, and female respectively (Ferlay J et al. 2013). ASR (age-standardized rates) of CRC has been estimated to be 4.2, 3.2 per lakh for males and females, respectively (NCRP ICMR Report, 2016). CRC is an elderly age cancer generally develops after the fifth decade of life (Kenneth R et al. 2004). The incidence rate of CRC is increasing in young age especially in developing countries, which is mainly due to changes in lifestyle related fact and food habits (Shahrudin MD et al. 1997; Pandey A et al.2008; Gupta S et al. 2010; Gupta R K et al. 2014).

### 2.3 Risk Factor Associated with CRC

**2.3.1** ***Alcohol*****:** Heavy alcohol drinking habit increases the risk of colon cancer several times (Roswall N et al. 2015). NSAID (Nonsteroidal anti-inflammatory drugs) and estrogen replacement therapy are also known to be protective factors (Cuzick J et al. 2009, Barone M et al. 2012).

**2.3.2** ***Smoking:*** Heavy smoking may increase the risk of colon and rectal cancer (Haggar FA et al. 2009).

**2.3.3** ***Family history of colon cancer:*** Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch Syndrome, familial adenomatous polyposis (FAP) and some type of colorectal cancer linked to family history of CRC (Winawer S et al. 2003).

**2.3.4** ***Diabetes:*** People suffering from diabetes or the ones who show resistant to Insulin show an increased risk of colon cancer (Giovannucci E et al. 2010).

**2.3.5** ***Older age:*** CRC can develop at any age of life but it is mostly seen between 50-60 years of age increasing age a big risk factor for CRC (Haleshappa RA et al. 2017).

**2.3.6** ***A sedentary lifestyle*****:** The people who are less active or inactive are more likely to suffer from colon cancer. Western-type of dietary habits with extreme caloric, red meat, and high animal fat diet, low fruits & vegetables diet, and low fibre in diet is a risk factor for Colorectum cancer (Adlercreutz H et al. 1990).

**2.3.7** ***Obesity*****:** Obesity increases the risk of CRC. The people who are obese have a greater risk of CRC (Calle EE et al. 2003).

**2.4 Bowel diseases**

Bowel diseases like Chronic inflammatory bowel disease or Inflammatory bowel disease (IBD) includes two chronic idiopathic inflammatory diseases such as ulcerative colitis as well as Crohn disease, particularly ulcerative colitis (UC) increases the risk of colorectal cancer development (Bernstein et al.2001).

**2.5 Diagnosis**

***2.5.1*** **Symptoms and sign:** Suffering from anaemia or haematochezia and different bowel habits such as diarrhoea or constipation. Particularly in rectal cancer, bloody stools mucous and urgency may happen. Weight loss, abdominal distension or pain, fatigue and bowel obstruction and perforation are symptoms of progressive disease, as are symptoms developed by cancerous diseases, such as hepatomegaly (Hamilton W et al. 2004).

***2.5.2*** **Medical check-up-** Up to 50% of rectum tumour patients may be diagnose by advanced rectum investigation (Lepisto et al. 2009). It is a vital way to check tumour

passion into pelvic structures and sign of bleeding in stools. Big size tumour in can be diagnose by abdominal palpation.

***2.5.3*** **Colonoscopy:** Colonoscopy is the furthermost common diagnostic technique for colorectal cancer. This helps to evaluate the size and location of the tumour. In rectal cancer, a rigid rectum-scopy may allow evaluation of the distance from the lowest part of the tumour to the anal canal, helps to decide the better technique for the surgical resection of CRC.

***2.5.4*** **Radiological Diagnosis of CRC:** Abdominal and thoracic CT scan (CT) before surgery allows diagnosis of malignant disease, and this technique can be helpful in examining tumour location and tumour size and adjacent organs metastasis, and tumour invasive deepness. The sensitivity of CT scan for lymph node metastasis (N stage) is 76%, and its specificity 55%; in the evaluation of distant metastasis (M stage), sensitivity is 85%and specificity 98% (Leufkens et al. 2011). In rectal cancer, CT is used for evaluating distant metastasis, whereas magnetic resonance imaging (MRI) is the examination method of choice for local staging. It enables evaluation of the distance of the tumour from the anal canal, depth of invasion, and enlargement of lymph nodes, all of which influence the choice of preoperative treatment and operative technique. MRI is highly sensitive in evaluating tumour invasion depth. The Mercury group study (2007) has been shown that MRI is equivalent to histopathological assessment of distant invasion of depth. In lymph node status assessment, accordance with 85% can be attained with histopathological evaluation of the inconsistency of the nodal margin and diverse intraocular signals but micro-metastasis cannot be ignored (Brown et al. 2003, Dworak et al. 1989). Intraluminal endoscopic ultrasound (EUS) can help for local rectum tumour staging, but lone for flat and distal cancer, The confirmation of the Tumour stage, by this technique the correctness is 90% as described by Massari et al. in 1998. EUS can discover lymph nodes bigger than 5 mm. EUS is, still, a very much operator-dependent investigation. Positron emission tomography or PET-CT scan visualizes metabolic changes in malignant cells, but its sensitivity is poor (Heriot et al. 2004). It can be used for sensing occult metastases, but it is unfit for before surgery local tumor staging. PET/CT along with MRI, a new alternative method for assessment of the local tumor staging, is under investigation (Lambrecht et al. 2010).

## 2.6 Screening of CRC

Colorectal cancer prognosis depends on cancer stage, so it become very important to diagnose cancer as soon as possible. Colonoscopy, endoscopy or colon computed tomographic (CT) scan can serve as better screening tool, but these methods are so costly and expensive resource- using. Positive faecal occult blood tests screening with full length colonoscopy in case of positive results is a means of finding asymptomatic tumours (Duffy et al. 2007). Trial testing of faecal occult blood screening testing begins start in Finland in 2007 for the age group of 60 to 69 years (Malila et al. 2011). The benefit of faecal occult blood test are non-invasiveness and less expensiveness, but the demerit of this test is low sensitivity and specificity. Better sensitive faecal testing than with occult blood is based on the examination of presence mutant DNA secreted from neoplastic tumour (Duffy et al. 2007). These screening tests are hopeful, but these tests are so costly not suitable for regular use.

**2.7 Treatment of CRC-** Treatment of CRC should be done as multidisciplinary teamwork to control tumour and improve patient chances of curability prognosis (Levine et al. 2012).

### *2.7.1* Surgical resection of tumour

**2.7.1.1 Surgery for Colon cancer:** Classic surgical resection of CRC tumour is based on the location of the tumour. Some of the traditional surgeries are: Right hemicolectomy used for caecum and ascending colon, and extended right hemicolectomy used for tumours present in the hepatic flexure or right side of transverse colon, extended left hemicolectomy surgery used for tumours present in left side of the transverse colon or for flexural lienal tumours, and left hemicolectomy used for descending colon or sigmoid tumours. minimum, 5-cm margin on both side of the specified tumour is suggested. The En-bloc excision is used for mesocolon with proximal ligation of vessels is done to improve the radicality of the surgery in locally advanced tumour and for staging. The tumours which attack nearby organs or the abdominal wall, needs en-block excision with non-tumours tissue margins. endoscopic resection is several time good options for surgical resection of small and local tumours (Manfredi et al. 2006). The total mesocolon extraction in right colon tumour growth may enhance the radicality (Eiholm et al. 2010). Now a days, the

laparoscopy-assisted colorectal cancer surgery having a major advantages for the patients over the conventional procedures i.e. helps patients to reduce hospitalize time, reduce post-surgery pain, and better improving results ( Hotta et al. 2011). The oncological results are testified to be similar (Patankar et al. 2003, Reza et al. 2006), and an even better result has been described for laparoscopically treated colorectal cancer (Lacy et al. 2002).

**2.7.1. 2 Surgery for Rectum tumour:** Surgical procedures of the treatment of rectum tumour is the removal of total mesorectal excision (TME), is the prime method (Heald et al. 1982) for low and mid rectum tumours, due to high risk of anastomotic leakage (Matthiessen et al. 2007). At least a 5 cm distal margin of partial removal of the mesorectum is generally considered acceptable. TME method reduces the risk of local recurrence of tumour (Martling et al. 2000, Heald et al. 1998). Before surgery radiation is suggested for T3 -4 size rectum tumours and for patient with suspected lymph-node metastases a short course for T3 or lymph-node positive tumours or both a long course for T4 size or fixed type tumours (Schmoll et al. 2012). Short-course before surgery radiation of 25-Gy in 5Gy fractions is delivered for 5 successive days. For long course of preoperative radiation therapy takes 5- 6 weeks with the total dose of 50.4Gy along with 5-Floro Uracile/capecitabine-based chemotherapy (CAPOX). After to generally it takes place after 42-64 days later the completion of radio therapy treatment (Glimeliuset al. 2008) however suggested time between radio therapy and surgical resection can become lengthier in the future ( Stockholm III trial NCT00904813 ). Before surgery radiation treatment testified to expand survival time and to reduce the chances local recurrence of cancer ( Kapitejin et al, 2002 ). In comparison of TME alone to, before surgery radiation treatment plus TME decreases chances of local recurrences up to 50%. The chemoradiotherapy might down-stage tumours in 60% of CRC cases and make them removable by surgery ( GarciaAguilar et al. 2003 ). Many hospitals use laparoscopic surgery in rectum tumour treatment; it has identical benefits to laparoscopic colon tumour treatment, with the oncological results similar to those of open surgery (Green et al. 2013 Arezzo et al. 2012,). Small rectum tumour may even be resectable by colonoscopy or by trans-anal endoscopic mucosectomy.

**2.8 Chemotherapy**

The aim of chemotherapy treatment is to reduce the risk for relapse and improve prognosis (Schmoll et al. 2012). Chemotherapy treatment is based on 5-fluorouracil (5-FU) or capecitabine, and together with oxaliplatin, it improves by 15 to 20% the prognosis in stage III CRC (Andre et al. 2004). Occurs in stage II tumours, but since here the survival is better, the final influence of 5-FU based treatment on survival is only 3 to 5%. Because of side-effects, Chemotherapy advised in stage II only for patients with risk factors like perforation, emergency surgery, T4 tumour, l vein or nerve invasion, or high-grade tumour less than 12 examined lymph nodes, (Schmoll et al. 2012). Capecitabine, / CAPOX the pre drug of 5-FU, has replaced 5-FU because of the greater acceptance of it and its resource advantages.

### 2.9 Medication of malignant CRC

Metastatic colorectal cancer can be surgically removable, depending on size and location tumour. Most common metastatic organs are liver, peritoneum, lungs, and other part of colon (AJCC Cancer Staging Handbook, 2018). Patients of CRC with presence of liver metastasis at the period of diagnosis, up to 10- 20% can be operated, and 10-15% of un-operate metastases can be operated after oncological treatment (Isoniemi et al. 2011 ). In Helsinki, primary colorectum tumour is removed t first, and if metastases respond to cancerous treatment, liver or pulmonary and other surgery is performed later. After whole removal of hepatic metastases, 5-year survival can be up to 50% ( Kopetz et al. 2009, Isoniemi et al. 2011). In stage IV Colorectal cancer, or recurrent tumour, FU/capecitabine, irinotecan, and oxaliplatin or CAPOX and FOLFOX are regularly used as chemotherapy, usually generally with combination of anti EGFR antibodies (cetuximab or panitumumab) for wild type KRAS tumours, or anti-VEGF antibody bevacizumab for patients with KRAS mutation ( Scmoll et al. 2012).

### 2.10 Pathological prognostic factor

#### 2.10.1 Stages

In colorectal cancer the best known prognostic factor is the cancer stage (de Leon et al. 1987, Chapuis et al. 2011).1 Dukes et al. 1932 described a staging system for rectum tumour which includes three stages: stage A, stage B, and stage C, and the stage D, added after more than thirty years by Turnbull et al.in 1967. In Dukes' staging system stage, A,

means the tumour invades the submucosa or at most the muscularis propria. In Duke stage B, tumour invades through the colon wall into the pericolic or perirectal fat and Duke stage C, the tumour attacks or penetrates the colon wall, and there occurs regional lymph-node metastasis. In Turnbull´s changes, Dukes' stage D represent that tumours have been resected non radically, that's mean there is distant metastasis, or local radicality is inadequate (Turnbull et al. 1967). The Australian Clinico-Pathological Staging (ACPS) classification system was described in 1982, (Davis et al. 1982). Dukes stage D was defined as clinical or microscopic sign of remaining cancer tissue. Dukes' stage is good, but a very old system prognostic factor for assessing the prognosis of colorectal cancer. 5-year survival rates were 90% for Dukes stage, 75% for stage B, 50% for Duke stage C, and below 10% for Dukes D in 1982-1998, at Helsinki University Central Hospital, (Carpelan-Holmström et al. 1995) The stage of cancer defines how far it has spread. Determining the stage helps chose the most appropriate treatment. Most commonly used system gives the stages a number from 0 to 4. The stages of colon cancer are: Stage 0: it is the primary stage of cancer, it starts when the tumour is still within the inner layer or mucosa, of the rectum and colon. It is also called carcinoma in situ.

### 2.8.2 Tumour node metastasis staging

Tumour lymph node metastasis (TNM) staging system was first published in 1950 by the Union of Internationale Contre le Cancer (UICC) ( Denoix 1950). For colorectal cancer, this describes tumour infiltration (T), and occurrence of lymph-node (N) and of distant metastasis (M). The American Joint Committee on Cancer (AJCC) included prognostic TNM subgroups in their staging system. Later the AJCC and UICC were joined, and the latest, the 7thedition, of the TNM classification was published in 2010 (Edge et al. 2010).

### 2.8.3 Histological differentiation

Tumor histology is classified according to glandular formation. WHO classification classify tumors according to histological differentiation into four groups that are well-differentiated tumors (grade 1), moderately differentiated (grade 2), poorly differentiated tumor (grade3), undifferentiated tumor (grade 4). High grade is an independent marker for poor prognosis of CRC (Compton et al. 2006).

### 2.8.4 Histological type of CRC

Around 90% of CRC are the dominant type of cancer, in which most of the tumours are adenocarcinomas type (Boyle et al. 2010), that are classified into different sub-types. Mucinous adenocarcinomas are tumors which have more than 50% of the lesion involving of extracellular mucin. Signet -ring -cell carcinomas, more than half of the cells shows intracytoplasmic mucin, and cells appearance like a signet ring structure. Few CRC tumour types are cribriform comedo-type adenocarcinoma, medullary carcinoma, small-cell carcinoma, adeno-squamous, micropapillary adenocarcinoma, and undifferentiated carcinoma, and spindle cell (Bosman et al. 2010). Undifferentiated carcinomas small-cell, and Signet-ring cells are graded as high.).

### 2.8.5 Lymphovascular invasion of tumour

tumour vascular invasion is an independent prognostic marker of adverse outcome (Compton et al. 2006). Malignant cell invasion into external veins which raises risk for liver metastasis and poor survival (Blenkinsopp et al. 1981). Lymph-node invasion (Maughan et al. 2007 Di F. et al. 2004,) and perineural invasion both are independent markers of worse outcome (Fujita et al. 2007 Ueno et al. 2001,).

### 2.8.6 Tumour site

In previous research shows, tumour located in the rectum was linked to worse outcome ( Park et al. 1999). Now, deviations have moderated because of availability of better adjuvant therapy and better surgical treatment for rectum cancer. The application of TME technique in rectum tumour decreases the chances of local recurrences and increase patient's survival (Heald et al. 1986). The macroscopic examination of the rectum tumour that is the completion of the mesorectum is what makes it feasible to estimate the survival outcome of patient ( Kapiteijn et al. 2002). The Microscopic examination permits circumferential in addition to proximal and distal marginals to be assessed, and these give prognostic indication about local recurrence of disease. If distal or proximal margin is not negative, the risk of recurrence of cancer increases 3.5-fold and the risk of mortality increses 2-fold. In rectam tumors, a distal margin of 2 cm is acceptable; in T1-2 tumors even 1 cm is adequate ( Washington et al. 2009). The lateral margins may be valuable in

forecast of local recurrence and, metastasis, and forecast of survival (Nagtegaal et al. 2008). In colon cancer similar results have been described for improving survival by the use of total mesocolon excision (Eiholm et al. 2010). The perforation caused by an obstructive tumour is linked to worse prognosis ( Anwar et al, 2006).

### 2.8.7. tumour immunity

The tumour-related immune response also a prognostic feature; intratumorally lymphocytes (TILs) associate with patient survival and inversely with tumour stage (Ropponen et al. 1997). It is also reported TILs is associate with the lack of tumour budding (Zlobec et al. 2007). It is also reported deficiency in peritumoral inflammatory reaction is associated with worse prognosis (Losi et al. 2006). The peritumoral CD68+ macrophages associate with improved patient survival ( Algars et al. 2011).

## 2.9 Molecular basis of Colorectal Cancer

Colorectal cancers pathogenicity depends on various epigenetic and genetic changes which are to some extent related to each other by following the multiple stage patterns has been reported by Fearon and Vogelstein (Fearon et al 1990). CRC is a heterogenous disease, influenced by many genetic and epigenetic alterations. CRC patients show a significant difference in prognosis and individual treatment responses, even when presenting at same clinical stage.

There are at least three major molecular pathways involved in CRC tumorigenesis (Colussi et al., 2013).

(1) Chromosomal instability pathway (CIN)

(2) CpG Island Methylator Phenotype (CIMP) Pathway

(3) Microsatellite instability (MSI) pathway

(4) Other pathway (miRNA mediated)

## 2.10 Genetic Basis of Colorectal Cancer.

The molecular characteristics of CRC initiation, and progression through the adenoma-carcinoma sequence has been reported by Eric R. Fearon and Bert Vogelstein in 1990. The adenoma-carcinoma sequence describes the stepwise progression from normal to dysplastic epithelium, along with the accumulation of several genetic alterations. This accumulation of gene mutations is non-random and initiates colorectal carcinogenesis

through the deregulation of pathways that modulate cellular differentiation, proliferation, and apoptosis. All CRCs harbour genetic alterations, including single base substitutions, and larger structural variations (e.g. aneuploidy). A detailed description of all the mutational events related to CRC is beyond the scope of this thesis; however, some of the most important genetic aberrations will be described below. The classical description of the multistep genetic model for CRC starts with the inactivation of *APC*. *APC* mutation occur in more than 80% of sporadic CRCs.78 *APC* germline mutation is also responsible for the autosomal dominant inherited FAP syndrome associated with multiple colorectal adenomas.79 *APC* inactivation is followed by two other frequent mutations, *KRAS* and *BRAF*. Mutation of *KRAS*/*BRAF* leads to an enhancement of the gene product, stimulating cellular growth through the MAP/ERK pathway. *KRAS* is mutated in 33% and *BRAF* in 10% of CRCs.80,81 The *KRAS*/*BRAF* protein is downstream of EGFR, making treatment with monoclonal antibodies against EGFR redundant in mutant tumors. Other additional mutations resides in the TGF-β (*SMAD4*), PIK3CA, and TP53 pathways along with loss of heterozygosity and aneuploidy.82 Indeed, these associations of chromosomal instability are seen in ~85% of invasive CRC.

**2.11 Biomarkers**

The development of colorectal cancer is a multi-step and complex process, which includes different phenomena in the normal cells leading to the cell become cancerous. Cancerous tumour has property to maintain proliferation signalling, protect cell death, evading growth suppressors, induce angiogenesis, supress apoptosis maintain replicative immortality, and permit tumour invasion and metastasis ( Hanahan et al. 2011). Cancer biomarkers/tumour markers are molecules which produced by cancer cell or by normal tissue in response to a malignancy they reflect these features of cancer. Cancer biomarkers can be detected in tissues or secreted body fluids, that may be helpful for treatment monitoring , cancer screening & diagnosis and can give information about treatment response, and prognosis of patient . In this study we studied epigenetic molecular markers for disease prognostication.

**2.11.1 Epigenetics** Aggregation of hereditary and epigenetic modifications transform typical colonic epithelial cells to adenocarcinoma cells. Genetic modifications including mutations in tumour silencer gene and oncogenes, while epigenetic components are characterized as heritable alteration in gene expression that is free of changes in the primary DNA sequence. Function of epigenetic mechanisms in the growth development and normal maintenance of organ & gene expression in specific tissue in now recognized. Malignant cellular makeover can happen due to any changes in epigenetic landscape, and maintenance of these heritable changes are achieved through different cycles of cell proliferation that cause the cells to be unique with similar genetic information, Epigenetic modifications in colorectal cancer growth (CRC) that alter colonic epithelial cells into adenocarcinoma cells include abnormal DNA methylation, chromatin alterations, and noncoding RNAs, particularly microRNA expression. CpG island DNA methylation and abnormal methylation of genes drive the beginning and progression of CRC. Histone changes have negative effect on chromatin structure and gene expression and its assume a significant function in gene silencing in CRC. Hypermethylation of DNA also common gene promoter its leads to epigenetic silencing of gene. Downregulation and abnormal expression of specific miRNAs having tumour suppression ability. Determination of main cause and function of epigenetic instability in the CRC pathogenesis will prompt successful prevention and therapeutic techniques for patients with CRC. Epigenetic drugs that underscore the reversible character of epigenetic events have led the plausibility of epigenetic treatment as a treatment choice in CRC (Khare S et al. 2012).

**2.11.2 DNA methylation**

DNA by natural process is known as DNA methylation. It can change the action of a DNA sequence without changing the original sequence. CpG islands are the small stretches of the DNA with high frequency of CpG sites.

**2.12 Methods for hypermethylation analysis**

DNA methylation can be detected by the following assays currently used in scientific research (Methylation-Specific PCR (MSP), which is based on a chemical reaction of sodium bisulfite with DNA that converts unmethylated cytosines of CpG dinucleotides to uracil or UpG, followed by traditional PCR (Hernández HG et al. 2013). However,

methylated cytosines will not be converted in this process, and primers are designed to overlap the CpG site of interest, which allows one to determine methylation status as methylated or unmethylated. Whole genome bisulfite sequencing**,** also known as BS-Seq, which is a high-throughput genome-wide analysis of DNA methylation but it is very expensive

**2.12.1 Methylated DNA immunoprecipitation** -Pyrosequencing of bisulfite treated DNA. This is sequencing of an amplicon made by a normal forward primer but a biotinylated reverse primer to PCR the gene of choice. The Pyrosequencer then analyses the sample by denaturing the DNA and adding one nucleotide at a time to the mix according to a sequence given by the user. If there is a mis-match, it is documented and the proportion of DNA for which the mis-match is present is distinguished. This gives the user a percentage methylation per CpG island.

**2.13.1 Methylation-specific PCR**- Methylation-specific PCR (MS-PCR or MSP) is one of the most common method used for DNA methylation analysis developed by Harman et al. 1994. In this method DNA undergoes bisulfite conversion of cytosine to uracil and then DNA fragments sequences are amplified with methylation specific primer and unmethylation specific primers. Bisulfite conversion of DNA converts unmethylated cytosine residues to uracil, methylated cytosine (5-methylcytosine) residues remain as unaffected. Therefore, DNA that has been converted with bisulfite retains only methylated cytosines remain as cytosines. Therefore, bisulfite conversion make known to specific changes in the DNA sequences that depend on the methylation status of cytosine, yielding single-nucleotide determination information about the methylation status of the fragment of DNA.

**1.13.2. Bisulfite sequencing** (**bisulphite sequencing**)- in this technique we use use of bisulfite conversion of DNA before routine sequencing to know the pattern of methylation. Bisulfite sequencing also use conventional sequencing methods on bisulfite-treated genomic DNA to analyse methylation status at CpG sites. All plans accept that bisulfite-induced treatment of unmethylated cytosines to uracil is complete, and this serves as the basis of all subsequent techniques. The methodologies can be generally

divided into strategies based on MSP and strategies employing polymerase chain reaction (PCR) performed under non-methylation-specific conditions. Methylation specific PCR is PCR type was first described by Herman *et al in 1992,* that helps to identify the methylated and unmethylated DNA in bisulfite converted DNA with the help of methylation and un-methylation specific primers.

**2.14 Molecular Pathways Involved in Colorectal Cancer:**

Colorectal cancers pathogenicity depends on various epigenetic and genetic changes which are to some extent related to each other by following the multiple stage pattern theorized by Fearon and Vogelstein (Fearon et al 1990). There are major 3 pathways involved in CRC development. In which most of the CRC follow Chromosomal instability pathway(CIN) which is characterized by chromosomal abnormalities and widespread loss of heterozygosity (LOH) (Lin, J.K. et al 2003, Leary et al 2008), second major pathway is MSI pathway approximately 15% of CRC follow a different pathway known as Mismatch Repair (MMR) system and consequential microsatellite instability (MSI). They are directly responsible for the production of polypeptides that recognize and repair single-nucleotide mismatches at microsatellite sequences that escape the proofreading mechanism of DNA polymerase. It was established in recent years that some other pathways also take part in the pathogenesis of CRC which includes inflammation, abnormal DNA methylation and miRNAs directly involved in the carcinogenicity of the CRC

**2.15 CIN Pathway**

CIN is one the most well understood and common pathway in CRC. This pathway involves various mitosis regulation of spindle checkpoints & proteins molecules capable of influencing chromosome stability in mitosis(Bardi, G et al 1993 ,1993).An initial "key" mutation is the early mutation of tumor-suppressor genes for adenomatous polyposis coli (APC) in both sporadic CIN and family adenomatous polyposis (FAP) when mutated by the gremlin(Shih, I.M et al 2001,Sieber et al 2002 ).A germline mutation of the APC gene was identified in FAP syndrome, an auto-dominant genetic disorder that has been identified in 60 to 80 percent in FAP families in the course of about in 16%-40% of patients with less than 100 polyps. AFAP and MAP are very comparable in phenotypical terms (Sieber et al 2003). The APC tumor silencing ability is associated with APC/β-catenin/Tcf

pathway. Its inactivation brings enhancement of WNT pathway, by its inability to degrade β-catenin. RAS Pathway- The previously mentioned early mutation of CIN pathway, are then trailed by events that advance new mutation and encourage the tumor's movement from benign to malignant stages. The p53 System**:** p53 loss of capacity is much of the time present in the later phases of colorectal tumorigenesis (Baker et al 1990).

**2.16 MSI Pathway (Microsatellite instability pathway):** MSI contributed approximately 15% of the colorectal cancer. This pathway activats due to defect in the MMR genes. The MSI pathway is the type of genomic instability associated with the origin of around 15% of sporadic colorectal disease and >95% of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) disorder.

**2.17 CIMP pathway:** This pathway develop due to epigenetic inactivation of several tumour suppressor gene and this pathway is characterized by special pathological features, initiates carcinogenesis and process of tumour development. CIMP and the "Serrated" Pathway: The third pathway by which CRC advances is the CpG island methylator phenotype (CIMP) (Samowitz et al 2005, Shen et al 2007). It is composed of various hypermethylation of CpG di-nucleotide sequence in the situated in the promoter region of genes involved in cell cycle control, apoptosis, angiogenesis, DNA repair, invasion and adhesion. Hypermethylation of the promoter leads to the loss of gene expression. Around 20-30% of CRC follows CIMP (Ogino et al 2006)

**2.18 DNA Methylation Biomarkers in Tissue**

**2.18.1 IGFBP3- Insulin-like growth factor binding protein 3 (IGFBP3)** is the main carrier of insulin-like growth factors (IGFs) in the circulation, where this complex regulates biologic function of IGFs (Kawasaki T et al. 2007). IGFBP3 has been shown to regulate cell growth and death, independently of its interaction with IGFs (Tonner E et al. 2002). *IGFBP3* promoter methylation and gene silencing are observed in human cancers including colorectal cancer (Hanafusa T et al. 2002), and have been associated with poor clinical outcome in lung and ovarian cancers (Merritt WM et al. 2008).

**2.18.2 SFRP1 gene-** The sFRP1 gene was proposed to lie at 8p11.2, within the region found to be deleted in our earlier tumour studies. Loss of expression has been shown

recently to correlate with lymph node metastases and increased mortality in breast tumours (Caldwell GM et al. 2004). uses of mortality worldwide. It is the third most common cancer in males and second in females globally and recorded as second major cause of cancer-related deaths worldwide (Bray et al. 2018). Secreted Frizzled Related Protein1 (SFRP1) gene is known for its ability to negatively modulate the Wnt signalling cascade (Mii and Taira. 2011). SFRP1 gene codes for SFRP1. The development of colorectal carcinoma (CRC) involves many genetic and epigenetic alterations and methylation being an important epigenetic event has been described as a diagnostic and prognostic biomarker. Secreted Frizzled- Related Protein 1 (SFRP1) gene regulates diverse physiological processes via the Wnt signalling. Promoter hypermethylation of SFRP1 gene is an epigenetic regulation mechanism that downregulates SFRP1 protein level in the tumour, and happens to be one of the significant events in colorectal carcinogenesis. We studied the clinicopathological relationship of CRC including survival outcomes with SFRP1 gene promoter methylation. Methods: We evaluated promoter methylation status of SFRP1 gene by methylation-specific PCR (MS-PCR) in the tumour tissue in 54 cases of stage II-III CRC patients in north India. The MS-PCR result was further validated by bisulfite sequencing.

### 2.18.3 CIMP marker

A third pathway by which CRC progresses is the CpG island methylator phenotype (CIMP) (Samowitz WS et al. 2005; Shen L et al. 2007). It consists of the aberrant hypermethylation of CpG dinucleotide sequences localized in the promoter regions of genes, which are involved in cell cycle regulation, apoptosis, angiogenesis, DNA repair, invasion and adhesion. The promoter hyper-methylations cause the loss of gene expression. CIMP is found in approximately 20%–30% of CRC and it was reported that clinical features of CIMP CRCs are similar to those associated with MSI (Ogino S et al. 2006). CIMP-positive CRCs are currently defined by a panel of CIMP markers, that are classified on basis of present or absent promoter methylation. We have selected five marker panel (*CACNA1G, IGF2, NEUROG1, RUNX3,* and *SOCS1)* and examined by a methylation specific PCR. Tumour with presence of 3 or more than 3 methylated markers are classified as CIMP High; if two or less than two marker found methylated case classified as CIMP Low.

# OBJECTIVES

# OBJECTIVES

1. To study CIMP status in all patients of histologically confirmed cases of CRC.

2. To look for hyper methylation of SFRP1, and IGFBP-3 genes in CRC tumor tissue.

3. To determine the potential of these genes as prognostic molecular marker vis-a-vis CIMP

# MATERIALS & METHODS

# CHAPTER-3

## MATERIALS AND METHODS

### 3.1 Chemicals, reagents & kits:

**3.1.1 Chemicals & reagents:** Agarose High EEO, Ethidium Bromide, Ethanol, Formaldehyde, Elution Buffer, Tris, Nuclease Free Water, Hematoxylin, Eosin, Xylene, Sodium Dodecyl Sulphate (Himedia), Ethylenediaminetetraacetic acid (EDTA), Tris-HCL, DNA Loading Dye (6X) Proteinase K were procured from Himedia and all the chemicals were molecular grade. Tris Acetate EDTA (TAE) Buffer (BioRad), DNA Ladders 50 bp (Invitrogen, USA), Methylation and un-methylation specific MS- PCR Primers (Integrated DNA Technology, USA).

### 3.1.2 Kits

QIAamp® FFPE DNA Extraction kit (Qiagen, Hilden, Germany), Epitect Bisulfite Conversion kit (Qiagen, Hilden, Germany), Ampli Taq Gold DNA Polymerase (Applied Biosystems, USA)

**3.2 Ethical approval-** The study was approved by the Institutional Ethical Committee of Dr. R. M. L. Institute of Medical Science, Lucknow (IEC no-8/15) and Institutional Ethical Committee of B. B. A. University, Lucknow. Written informed consent was taken from all the participants who participated in the study.

**3.3 Study Population**- Total of 56 CRC cases were included in this study. All these patients were admitted for curative surgery at Department of Surgical Gastroenterology and Department of Surgical Oncology, Ram Manohar Lohia Institute of Medical Science, Lucknow, Uttar Pradesh, India, from 2013 to 2017. The study was based on tissue samples of colorectal cancer. Tumour tissue and adjacent normal tissue samples were collected immediately after curative surgery. Patients were excluded if they had any distant metastasis or stage IV cases.

### 3.4 Inclusion and Exclusion Criteria:

**Inclusion criteria-** Histologically confirmed cases of CRC stage II and stage III.

**Exclusion criteria-** Stage IV CRC patient.

**3.5 Follow up**- Follow up of all these cases were recorded in regular intervals of 6 months up to five years after inclusion. Patients follow up and survival details was noted up to the finishing of study (2018).

**3.6 Genomic DNA extraction from FFPE Tissue**

Genomic DNA extraction from FFPE tissues was done by using commercially available QIAamp FFPE tissue Kit, (Qiagen GmbH, Hilden, Germany) by following the manufacturer's protocol. The selection of tumor and non-tumor regions were done by examining Hematoxylin-Eosin (H&E) stained sections. DNA quality and quantity checked by spectrophotometrically (Nano-drop) by using 260nm/280nm ratio (1.7-1.9). 0.8% agarose gel was used to determine the purity and integrity of the isolated DNA.

**3.6.1 DNA extraction protocol**

*3.6.1.1 Sample preparation*- Excess paraffin of FFPE tissue was removed by using a scalpel and trimmed excess paraffin from the sample block. 7-9 μm thick 6-8 sections cut from FFPE tissue block by using microtone and immediately place in 1.5 ml centrifuge tube**.**

*3.6.1.2 Paraffine removal*- 1 ml xylene was added to the sample (6-8 sections of FFPE tissue) mix by mild vortexing, centrifugation it at 15000 rpm for 2 min. The supernatant was discarded by gentle pipetting. Residual xylene removal were done by adding 1 ml ethanol (99%) to the pellet, Centrifuge at 15000 rpm for 2 min at room temperature (RT), then the supernatant was removed.

The residual ethanol was removed by putting the tube opened in room temperature for evaporation until all ethanol has completely evaporated (10 min.).

*3.6.1.3 Tissue lysis*- Tissue was lysed by adding 180 μl animal tissue lysis buffer (ATL) that contains SDS, EDTA, Tris with 20 μl proteinase K and samples were incubated at 56°C for 1h for complete lysis of samples. Then samples were incubated at 90°C for 1h for partially reverses formaldehyde modification of nucleic acids. Complete lysis was done by addition of 200μl buffer AL and 200μl ethanol (99%), mixed by pipetting for homogenize

the solution. After homogenization, the entire lysate was transferred to the elute column and then centrifuged the lysate.

*3.6.1.4 Washing*- Washing was done by adding 500 µl washing buffer AW1 (wash buffer 1) and AW2 (wash buffer 2) which remove the salts, impurities and precipitate the DNA on Qia-amp Elute column membrane.

*3.6.1.5 Elution*- DNA elution was done in 50 µl elution buffer (ATE). While adding of elution buffer, keep in view that the buffer should be added in dropwise manner and at the center of the membrane and incubated for 1 min then centrifuge it at 14,000 rpm for 1 min and purified eluted DNA stored at 4 °C for further use. All centrifugation steps were done at 15–25°C.

**3.7 Protocol of Bisulfite conversion**

*3.7.1 Reagent preparations*- 30 ml ethanol (99%) mixed to Buffer BW**.** 27 ml ethanol (99%) added to Buffer BD. Lyophilized carrier RNA (310 µg) dissolve in 310µl RNase-free water to obtain 1 µg/µl solution (Carrier RNA enhances binding of DNA to the spin-column membrane). Dissolved carrier RNA added into Buffer BL **(**100 µl carrier RNA solution added into 10 ml Buffer BL in the ratio of 1:100)

**3.7.2** *Protocol for Bisulfite DNA conversion*- 800 µl RNase-free water was added to dissolve the bisulfite mix. The bisulfite reactions prepared in PCR tubes (200µl) according to the following table.

**Table 3.1:** Bisulfite reaction components

| **Component** | **Volume per reaction (µl)** |
|---|---|
| DNA solution (1200ng–1600ng DNA) | Variable (maximum volume 20µl) |
| RNase-free water | Variable |
| Bisulfite Mix (dissolved), see step 1 | 85µg |
| DNA Protect Buffer | 35µg |
| **Total volume** | **140µg** |

DNA Protect buffer changes colour from green to blue after addition of the DNA. This indicates the proper mixing of bisulfite mix and the correct pH for the bisulfite conversion reaction. Bisulfite DNA conversion was performed by using thermal cycler. The thermal cycler was programmed according to the table given below.

**Table 3.2:** Bisulfite conversion thermal cycler conditions

| **Step** | **Time** | **Temperature** |
|---|---|---|
| Initial Denaturation | 5 min | 95°C |
| Incubation | 25 min | 60°C |
| Denaturation | 5 min | 95°C |
| Incubation | 85 min (1 h 25 min) | 60°C |
| Denaturation | 5 min | 95°C |
| Incubation | 175 min (2 h 55 min) | 60°C |
| Hold | Indefinite | 20°C |
| Start the thermal cycling incubation. | | |

*3.7.3 Clean-up of bisulfite converted DNA*

After completion bisulfite conversion, prepare a mix by adding 310µl freshly prepared Buffer BL(containing 10 µg/ml carrier RNA that enhance the binding of DNA to column membrane), 250µl ethanol (96–100%) added to sample, and mixed by vortexing. The entire mixture was taken out from the tube and added to EpiTect spin columns and centrifugation was done at 15000 RPM for 1 min. Followed by sample washed with 500 µl Buffer BW (wash buffer) centrifuge at 15000 RPM 1 min.

*3.7.4 De-sulphonation*

500 µl Buffer BD (de-sulfonation buffer) added to the spin columns and incubation was done for 15 mins at room temperature (15–25°C). Centrifuged the spin columns at 15000 RPM for 1 min. Discard the flow-through.

*3.7.5 Washing*- The samples were washed twice with 500 µl Buffer and BW added to spin column and centrifuged at 15000rpm for 1 min.

*3.7.6 Elution*- Elution of Bisulfite converted (BSC) DNA done by adding the in 20 µl Buffer EB (Elution buffer) at the center of the column's membrane. The column were centrifuged for 12000 rpm for 1 min for proper elution of the purified bisulfite converted DNA into the tube.

**3.8 Methylation-specific Polymerase Chain Reaction (MS-PCR)-**

Methylation-specific PCR was set up according to the method described by Herman *et al*. in 1996 (Herman *et al.,* 1996). 2.5 µl bisulfite converted DNA was amplified using methylation-specific primers i.e. one is methylation-specific and another one is un-methylation specific that recognizes either type of gene sequence after bisulfite conversion. For detection of the methylate and un methylate DNA samples, sequences of the primers for MS-PCR of the SFRP1 promoter region were commercially procured from the IDT (Integrated DNA Technology, USA). All PCR reactions were performed using AmpliTaq Gold PCR master mix (Applied Biosystems, USA). Positive controls for Methylated and un-methylated, bisulfite converted human control DNA were procured from Qiagen, Hilden, Germany.

*3.8.1 Sample Preparation*- MS-PCR analysis was done by using the bisulfite modified DNA samples that were extracted from FFPE tissue. The extracted nucleic acids were eluted in 20 µl elution buffer (EB). 2.5 µl Bisulfite converted DNA was further used for MS-PCR analysis.

*3.8.2 Optimization of primer sets for MSP analysis*-The primer sets were optimized in reference to annealing temperature and elongation time. This will ensure that the primers function optimally and amplify the correct PCR fragment. The unmethylated and the methylated fragments were optimized separately using bisulfite converted DNA.ols (provided in the kit).. Methylated and un-methylated, bisulfite converted human control DNA were procured from Qiagen, Hilden, Germany for positive controls.

*3.8.3 Reaction mixture preparation:*

The MS-PCR reaction mixture consisted of 50 ng/µl bisulfite converted template DNA, and AmpliTaq Gold fast PCR Master Mix which contains the DNA Polymerase (hot start Taq DNA polymerase) PCR Buffer, dNTPs, MgCl2 and Stabilizers. Milli-Q water was added to make up of reaction volume upto 25 µl. The DNA was amplified using a Proflex thermocycler (Invitrogen, California, USA).

**Table 3.3: Primer table for genes**

| Gene or Locus | Sequence (5'–3') | Size, bp | Annealing Temp (in $^0$C) | References |
|---|---|---|---|---|
| *CACNA1G*-mF | GGAGTCGGTCGGTTGGTTC | 104 | **58** | Lee *et al 2008* |
| *CACNA1G*-mR | AAAACATACTACCCGCGAAACG | | | |
| *CACNA1G*-uF | TTGGAGTTTGGGTGTGAAGTGA | 100 | **59** | |
| *CACNA1G*-uR | CACAAATCCCACTTCCCCTACA | | | |
| *IGF2*-mF | AGCGGTTTCGGTGTCGTTATC | 94 | **58** | |
| *IGF2*-mR | CGAACGCCCAACTCGATT | | | |
| *IGF2*-uF | GGATTGTGGGTGTTTAGTTTGGTT | 120 | **58** | |
| *IGF2*-uR | CCTTTCCACACTACATCCCAAAA | | | |
| *NEUROG1*-mF | AATTTATGTTCGCGGGAGGTC | 118 | **58** | |
| *NEUROG1*-mR | ACCAACTTAACCCGAACCGA | | | |
| *NEUROG1*-uF | TTGTTGGTTAATTGGTGGTGTTGT | 119 | **58** | |
| *NEUROG1*-uR | CATACCTCAACCACTAATCACCCA | | | |
| *RUNX3*-mF | TGTTTTCGTTTATTTTGTCG | 104 | **59** | |
| *RUNX3*-mR | CGCTATTATACGTATTCCCG | | | |
| *RUNX3*-uF | TTTGGGTTTATGGGAATATG | 120 | **59** | |
| *RUNX3*-uR | TTCTCACAACAACAACAACC | | | |
| *SOCS1*-mF | GTATTTTTTTGGTGCGCGATAGTC | 106 | **59** | |
| *SOCS1*-mR | CGACCGACCTAAAAATACACGC | | | |
| *SOCS1*-uF | GATGGTTGGAGTTAGAATTGGTTGTT | 116 | **59** | |
| *SOCS1*-uR | CTCTATACTCCACAAAACTCTTCCCA | | | |

**Table 3.4:** Primer for objective 2 (List of primer used for IGFBP3, SFRP1 gene)

| Gene Name | Sequence (5'–3') | Annealing Temp (in $^0$C) | Size of amplicon In bp | Reference |
|---|---|---|---|---|
| SFRP1-MSP-F | AGTTAGTGTCGCGCGTTC | **59** | **124** | Takada *et al*, 2004 |
| SFRP1-MSP-R | CCGATACCCATACCGACTC | | | |
| SFRP1-USP-F | GGAGTTGGGGTGTATTTAGTTTG | **58** | **136** | |
| SFRP1-USP-R | CCAATACCCATACCAACTCTACA | | | |
| IGFBP3-MSP-F | GACCCGAACGCGCCG | **62** | **219** | Tomii *et al*, 2007 |
| IGFBP3-MSP-R | AGGTGACGGGTTTCGGGC | | | |
| IGFBP3-USP-F | GATAAGGTGATGGGTTTTGGGT | **60** | **231** | |
| IGFBP3-USP-R | ATCTAAACAACCCAAACACACCA | | | |

*3.8.4 The PCR cycling conditions*- A initial denaturation step at 95°C for 10 minutes to activate the Taq polymerase enzyme, followed by 35 cycles of denaturation at 95°C for 30 seconds, 30 seconds of annealing at 59°-64°C, 30 seconds of elongation at 72°C, and a final extension step at 72°C for 7 minutes. The range of annealing temperatures used was adapted to the individual melting temperatures for each primer set (see table I). 3.5µl PCR products were mixed with 1.5µl gel loading dye (Bromo-Phenol-Blue) and separated on a 2.5%, (IGFBP3), 3.5% (CIMP, SFRP1 gene) agarose gel which contains ethidium bromide (EtBr) (a fluorescent intercalating dye) in 400 ml 1X TAE buffer in-tank BIO-RAD). The electrophoresis was performed for 55 minutes at 65 V and the PCR products were visualized on an UV trans-illuminator (Chemidoc XRS Gel Documentation System; BIO-RAD).

**The PCR condition for methylated allele**

10 min at 95 °C,
20 sec of 94 °C
30 sec of 59°C } 38 cycles
30 sec of 72 °C
Followed 7 min elongation on 72 °C

**3.9. Criteria for CIMP status:**

The 5 methylation markers were used (CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, SFRP1 and IGFBP3). CIMP-high was defined as ≥3 methylated markers, CIMP low as 1-2 methylate markers and CIMP-0 as the absence of methylated markers in accordance with the new CIMP panel. (Lee,. S et al, 2008)

**3.10 Bisulfite sequencing**

The sequencing of the amplicons were commercially done through outsourcing through Chromous Biotechnology, Bangalore, Karnataka (India).

**3.11 Methylation analysis by bisulfite sequencing -** Validation of MS-PCR results and methylation pattern of CpG in the promoter region was done by bisulfite sequencing using the method defined by Clark S J. *et.al* 1994. MS-PCR products from tumor and normal tissues were sequenced by using ABI sequencing platform Genetic analyzer 3500, and

sequence analysis and alignment were done by using Bioedit software and CLUSTALW online tool.

**3.12 Bioinformatics analysis-** The amplicon of the promoter region of SFRP1 & IGFBP3 were sequenced and the chromatogram retrieved from the sequencing was analyzed by the help of freely available software i.e. Chromas. Further, for the determination of the methylation status of CpG sites present in promoter region, we were used different sets of primers for the identification and methylation status of the given samples. For checking the authenticity of the primers and sequence matching, we used ClustalW (https://www.genome.jp/tools-bin/clustalw), which is freely available online. For the conversion of the sequence format, i.e. conversion of sequence in reverse complementary and C to U conversion, we were used the online available tool Bioinformatics.org (https://www.bioinformatics.org/sms/revcomp.html**)** website used. Further, the sequences found after sequencing were analysed manually for the identification methylation and un-methylated DNA.

**3.13 Statistical analysis –** Statistical analyses were done by using SPSS software (version 20). Chi-square test was used to analyze the statistical association between clinic-pathological data and methylation status of CIMP marker gene. Kaplan Meier survival curve and Log-rank test were used for survival analysis. To evaluate the prognostic impact, all clinicopathologic variables were evaluated along with methylation status by using univariate cox proportional hazard model analysis. P-value <0.05 was considered as significant. The Pearson Chi-square ($\chi 2$) and Fisher´s exact test are used to find the association of methylation with clinicopathological characteristics of patients.

# RESULTS

# CHAPTER-4

## RESULTS

**Clinicopathological characteristics of CRC:** In our study, we have included a total of 56 cases which includes 50% cases were of >50 years and 50% cases more than 50 years. Among the total cases, 60% of the cases were male and 40% are females. The study includes tumor stage T2, T3 and T4. 44.6% cases had T3, 39.2%, 16.2% patients diagnosed with T4 and T2 tumor size respectively. Presence of lymph node metastasis is shown by 51.7% of the total cases rest do not shows metastasis. Among all the histological grade of the tissue were 55.3% are well differentiated, 26.7% were moderately differentiated and 17.8% were poorly differentiated. (As shown in Table 4.1)

**Table 4.1 - Clinicopathological characteristics of patients**

| **Variable** | **Categories** | **No. of cases** | **percentage** |
|---|---|---|---|
| Age | <50 year | 28 | 50 |
| | ≥50 year | 28 | 50 |
| | | | |
| Gender | Male | 34 | 60.8 |
| | Female | 22 | 39.2 |
| | | | |
| Tumor Stage | T2 | 9 | 16.2 |
| | T3 | 25 | 44.6 |
| | T4 | 22 | 39.2 |
| | | | |
| Lymph-node Metastasis | Absent | 27 | 48.2 |
| | Present | 29 | 51.7 |
| | | | |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 83.9 |
| | Mucinous adenocarcinoma | 9 | 16.1 |
| | | | |
| Histological grade | Poorly differentiated | 10 | 17.8 |
| | Moderately differentiated | 15 | 26.7 |
| | Well differentiated | 31 | 55.3 |
| Tumor site | Colon | 41 | 73.2 |
| | Rectum | 15 | 26.7 |

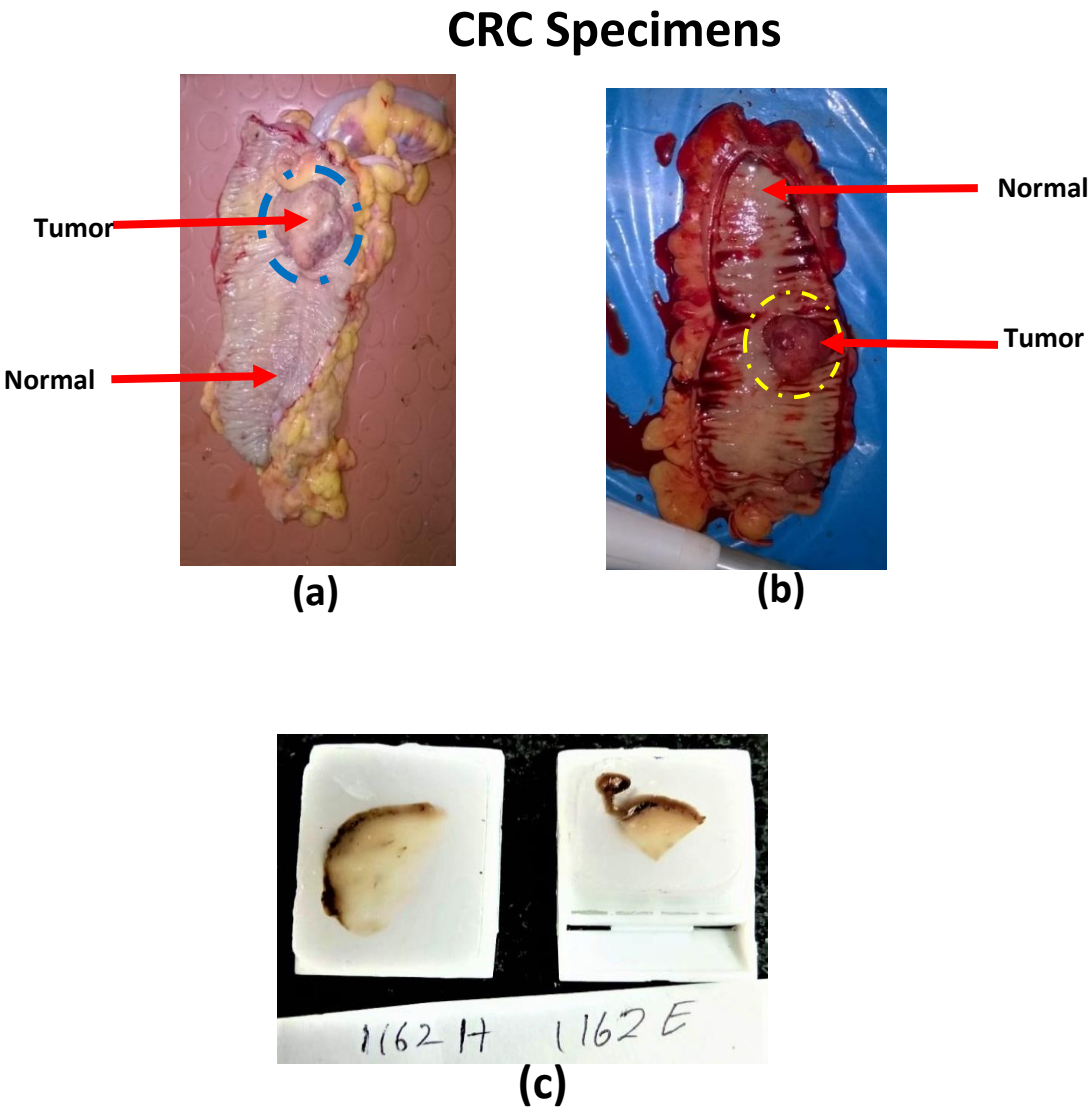



**Figure 4.1:- (a) & (b) CRC specimens-** Specimens of colorectal cancer collected after curative surgery for histopathological and molecular analysis. Tumor and non-tumor region marked by arrow. Tumor and adjacent non-tumor tissue used for Formalin-Fixed Paraffin-Embedded (FFPE) block formation for histopathological analysis and DNA extraction**. (c)** FFPE tissue block of colorectal tumor tissue, formed for histopathological analysis and DNA extraction for molecular analysis

## Histopathological analysis of CRC tumor

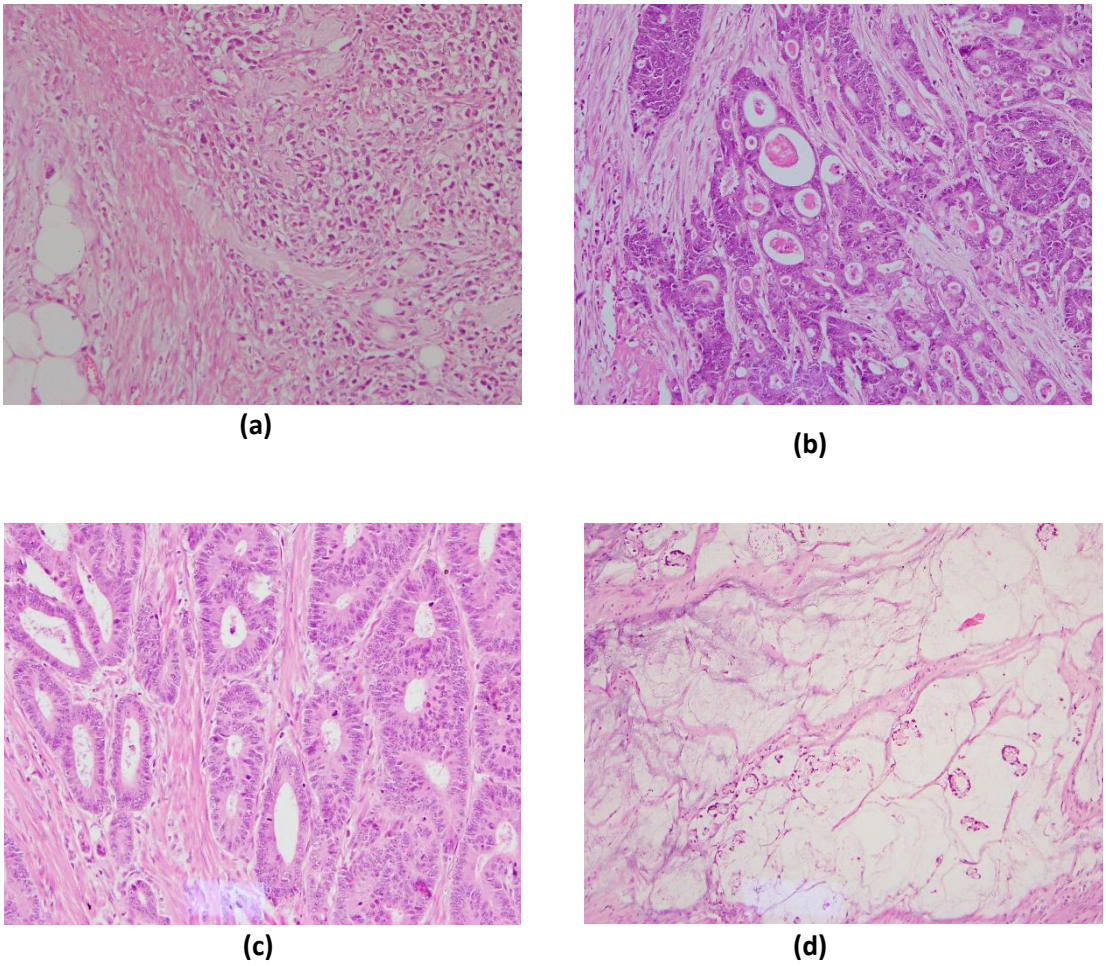



**Figure 4.2. Characterization of tumor by haematoxylin and eosin stain (HE staining)-** HE stained images of tumor tissue through histopathological investigation by pathologist **(a)** poorly differentiated tumor **(b)** moderately differentiated tumor **(c)** well differentiated tumor **(d)** is mucinous type well differentiated adenocarcinoma tumor.

**CIMP status analysis in CRC cases**

**Background-** Gene promoter hypermethylation is a common event found in CRC. CpG island methylator phenotype (CIMP) is a subtype of colorectal cancers that happen due to hypermethylation of multiple gene. The clinical significance of CpG island methylator phenotype is not well understood. Our aim is to analyse CIMP status of colorectal cancer cases using and new panel of CIMP marker gene described by Lee, et al 2008. To determine CIMP status of CRC cases we analysed methylation status of five marker genes panel i.e. CACNA1G, IGF2, NUROG1, SOCS1, RUNX3 using methylation specific PCR and phenotype of these cases verified by histopathological analysis. If three or more than three genes found methylated case catagerized as CIMP high if two or less than two gene found methylated case considered as CIMP LOW. We analysed all five marker genes in all 56 cases and individual findings of all gene described below.

On the basis of methylation status of marker genes 31(55.4%) cases were found CIMP-Low and 25 (44.6%) cases were found CIMP high. Clinicopathological characteristics of CRC cases described in table 4.2 images of histopathological investigation shown in figure 4.3. Sampling of tumor tissue and normal tissue shown in figure 4.2. DNA extraction and quantification picture shown in figure 4.3. MS-PCR results shown in figure 4.4 (a & b) and 4.5 and finding of marker gene methylation is listed in table 4.2 , characteristics of CRC cases listed in table 4.1. The results of individual CIMP marker gene methylation status and frequency was described in table 4.3. Trend of methylation frequency of CIMP marker shown in figure 4.6 (a) and (b).

**Association of CIMP status with clinicopathological characteristics-** To find the relationship between patients clinicopathological characters and CIMP status we perform chi square test. The results of chi square test shows the CIMP status was not associated with patients age, gender, The association of CpG island methylator phenotype was found with tumor site. 70% cases of poorly defrentited tumor found

**Molecular analysis**

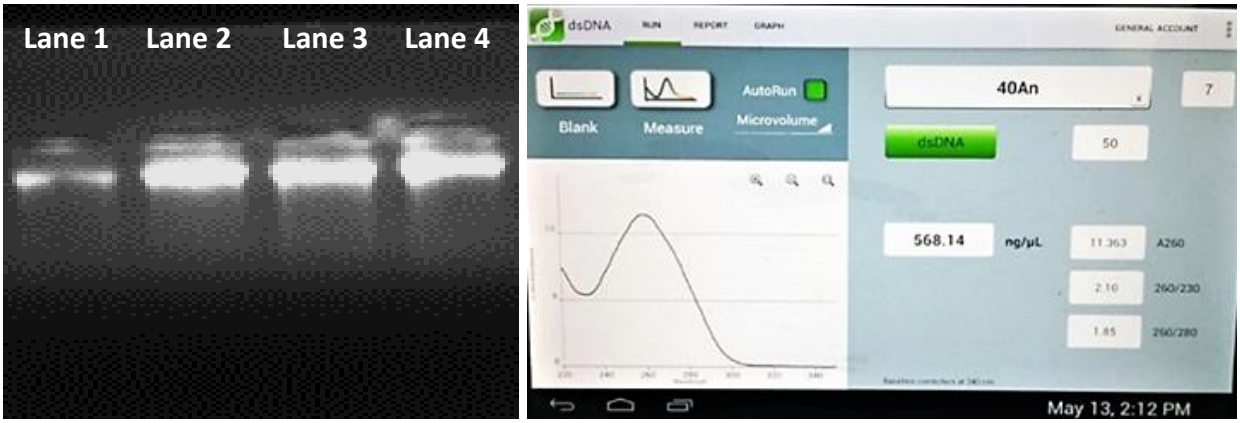
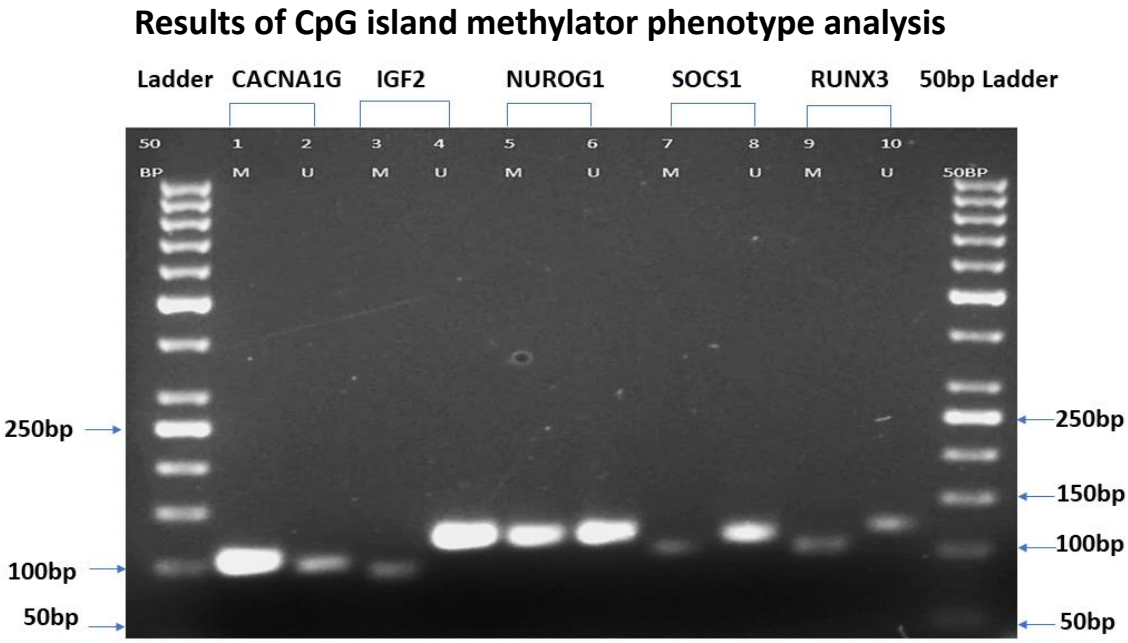


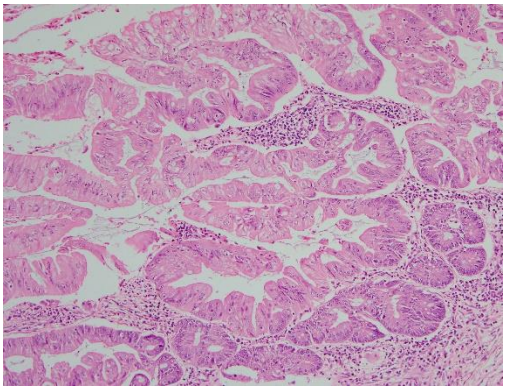

**Figure 4.3- Extracted DNA quantification-** (a) figure showing extracted DNA integrity in 0.8% agarose gel electrophoresis (b) showing lane 3rd DNA sample absorbance spectrum, quantity, and 260/280 ratio in spectrophotometer Lane 3 sample.

**CpG island methylator phenotype (CIMP) analysis-** CIMP analysis of CRC tumor done by Methylation specific PCR results are shown in figure 4.5, 4.6. We found 44.6 % CRC cases had CIMP high (fig 4.4) means 3 or more than 3 marker gene found methylated whereas 55.4% cases had CIMP low. CIMP marker IGF2 found methylated in 58.2% cases, NUROG1 gene methylation found in 57.1% cases, SOCS1 gene methylation found in 42.9% cases. CACNA1 gene and RUNX3 gene methylation frequency was low 37.5% and 33.9% respectively. CIMP analysis findings and markers genes methylation frequency listed in listed in table 4.3. Graphical representation of all marker methylation with trend shown in figure 4.6.

**Association of CIMP status and clinicopathological characteristics- We** found total 25 (44.6%) cases out of 56 cases had CIMP-High. In male 47.1% cases had CIMP-H and in female 40.9% females were had CIMP-H tumor. 41% patients who diagnosed with lymph-node metastasis had CIMP-H, 48% had CIMP-H tumor in patients who didn't having metastasis. Patients diagnosed with T2, T3, T4 having 33.3%, 40%, 50% cases CIMP-high tumor. 46.8% CRC cases of infiltrating adenocarcinoma NOS had CIMP high where as 33.3 % cases of mucinous adenocarcinoma found CIMP high. 70% cases of poorly differentiated CRC having CIMP high phenotype whereas only 33%, 41% cases of moderately and poorly differentiated CRC had CIMP high respectively. 66% cases of rectum cancer had CIMP high phenotype whereas only 36% cases of colon had CIMP high phenotype. Here we observe CIMP High phenotype was status was significantly associated with tumor site. High phenotype was more common in T4 tumor compare to T2 . (Table 4.4).




(a)



| S. No | Gene name | Size of the fragment in bp | |
|---|---|---|---|
| | | Methylated | Unmethylated |
| 1 | CACNA1G | 104 | 100 |
| 2 | IGF2 | 94 | 120 |
| 3 | NUROG1 | 118 | 119 |
| 4 | SOCS1 | 106 | 116 |
| 5 | RUNX3 | 104 | 120 |

(b)

**Figure 4.4**: **MS-PCR results- (a)** MS-PCR result of CIMP analysis. In this cases all CIMP marker found methylated band appears in lane 1, 3, 5, 7, 8, 9. 50bp DNA ladder run in first and last lane. PCR amplicon run 3% agarose gel. DNA bands also appears in unmethylation specific reaction this is due to presence some normal cells in tumor region This case is a CIMP high case due to more than 3 markers genes found methylated. **(b)** HE staining showing serrated type of morphology. Table showing the size of DNA amplicon. M= methylation specific PCR U= Unmethylation specific PCR

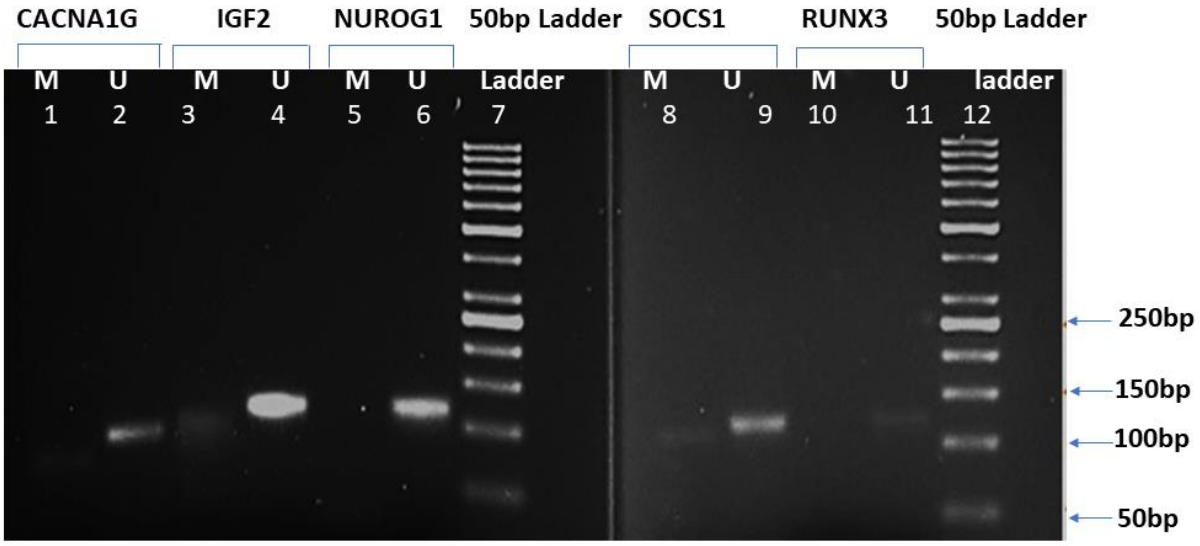



**Figure 4.5 CIMP analysis by MS-PCR-** Agarose gel picture of a CIMP low case. In this case no methylation specific amplicon appears.

**Table 4.2:** Table showing the size of methylated and unmethylated amplicon of all markers. M= methylation specific PCR U=unmethylation specific PCR

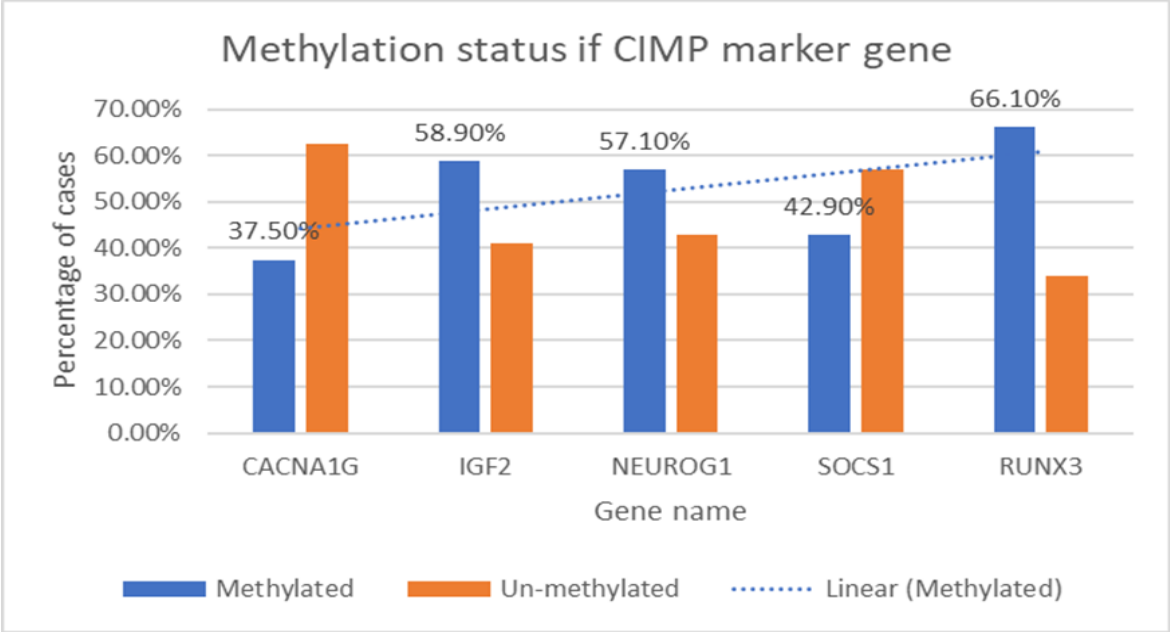
| S. No. | Gene Name | Size of Fragment (in bp) | |
|---|---|---|---|
| | | Methylated | Unmethylated |
| 1 | CACNA1G | 104 | 100 |
| 2 | IGF2 | 94 | 120 |
| 3 | NUROG1 | 118 | 119 |
| 4 | SOCS1 | 106 | 116 |
| 5 | RUNX3 | 104 | 120 |

**Table 4.3 –** Methylation pattern of CIMP marker gene

| | **Methylation analysis results** | | | |
|---|---|---|---|---|
| **S. NO** | **Gene name** | **Methylation status** | **No of cases** | **Percent** |
| 1 | SFRP1 gene | unmethylation | 14 | 25.0 |
| | | methylation | 42 | 75.0 |
| | | | | |
| 2 | IGFBP3 | unmethylation | 19 | 33.9 |
| | | methylation | 37 | 66.1 |
| | | | | |
| 3 | SOCS1 | Un-methylation | 32 | 57.1 |
| | | methylation | 24 | 42.9 |
| | | | | |
| 4 | CACNA1G | un-methylation | 35 | 62.5 |
| | | methylation | 21 | 37.5 |
| | | | | |
| 5 | IGF2 | unmethylation | 23 | 41.1 |
| | | methylation | 33 | 58.9 |
| | | | | |
| 6 | NUROG1 | Un-Methylation | 24 | 42.9 |
| | | methylation | 32 | 57.1 |
| | | | | |
| 7 | RUNX 3 | unmethylation | 37 | 66.1 |
| | | methylation | 19 | 33.9 |
| | | | | |
| 8 | CIMP STATUS | LOW | 31 | 55.4 |
| | | HIGH | 25 | 44.6 |
| | | | | **Total no of cases N = 56** |

**Table 4.4- Clinicopathological characteristics of CRC cases and their association with CIMP status**

| Variable | Categories | No. of cases | CIMP status CRC cases | | *p-value* |
|---|---|---|---|---|---|
| | | | Low 31 (55.4 %) | High 25 (44.6 %) | |
| Age | <50 year | 28 | 15 (53.6%) | 13 (46.4%) | 0.788 |
| | ≥50 year | 28 | 16 (57.1%) | 12 (42.9%) | |
| Gender | Male | 34 | 18 (52.9%) | 16 (47.1%) | 0.651 |
| | Female | 22 | 13 (59.1%) | 9 (40.9%) | |
| Tumor Stage | T2 | 9 | 6 (66.7%) | 3 (33.3%) | 0.459 |
| | T3 | 25 | 15 (60%) | 10(40%) | |
| | T4 | 22 | 10 (45.5%) | 12 (54.5%) | |
| | | | | | |
| Lymph-node metastasis | Absent | 27 | 14 (51.9%) | 13 (48.1%) | 0.611 |
| | Present | 29 | 17 (58.6%) | 12 (41.4%) | |
| | | | | | |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 25 (53.2%) | 22 (46.8%) | 0.456 |
| | Mucinous adenocarcinoma | 9 | 6 (66.7%) | 3 (33.3%) | |
| | | | | | |
| Histological grade | Poorly differentiated | 10 | 3 (30%) | 7 (70%) | 0.176 |
| | Moderately differentiated | 15 | 10 (66.7%) | 5 (33.3%) | |
| | Well differentiated | 31 | 18 (58.1%) | 13 (41.9%) | |
| | | | | | |
| Tumor site | Colon | 41 | 26 (63.4%) | 15 (36.6%) | **0.045** |
| | Rectum | 15 | 5 (33.3%) | 10 (66.7%) | |
| | | | | | |
| TNM Stage | II | 27 | 14 (51.9%) | 13 (48.1%) | 0.611 |
| | III | 29 | 17 (58.6%) | 12 (41.4%) | |
| | | | | | |
| IGFBP3 gene promoter methylation | Absent | 19 | 11 (57.9 %) | 8 (42.1 %) | 0.784 |
| | Present | 37 | 20 (54.1 %) | 17 (45.9 %) | |
| | | | | | |
| Alcohol Intake | Absent | 40 | 22 (55%) | 18 (45%) | 0.932 |
| | Present | 16 | 9 (56.2 %) | 7 (43.8%) | |
| | | | | | |
| Dietary Habit | Veg | 29 | 14 (48.3%) | 15 (51.7%) | 0.629 |
| | Veg + Non-Veg | 27 | 17 (63%) | 10 (37%) | |




(a)

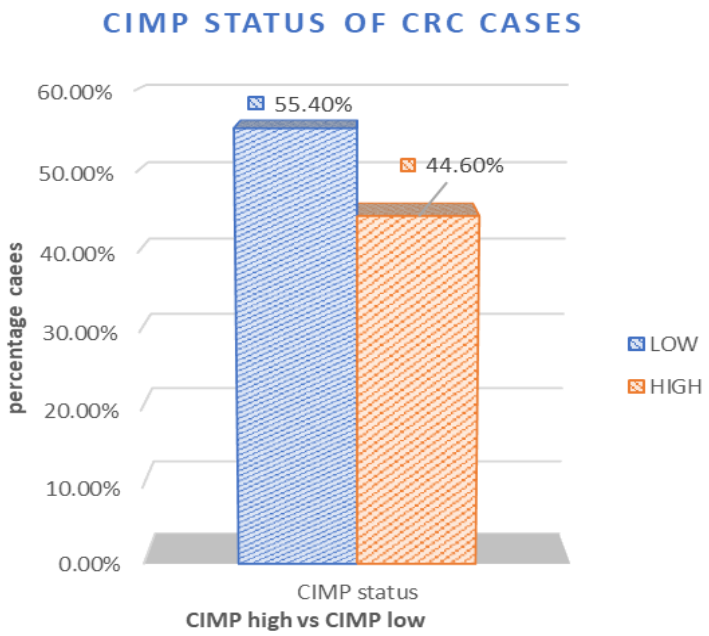



Figure 4.6 (a) Bar diagram showing percentage of cases found methylated vs unmethylated of CIMP markers gene (i.e. CACNA1G IGF2 NUROG1 SOCS1 RUNX3) percentage of methylated cases mentioned on top of bar. Dotted line shows the pattern of methylation frequency (b) Bar diagram of CIMP High (three or more than three CIMP marker found methylated) and CIMP low (two or less than two CIMP marker methylated)

**SFRP1 gene promoter methylation in tumor tissue-** We analyzed SFRP1 gene promoter methylation status in tumor tissue and adjacent normal tissue. In 42 out of 56 (75%) CRC cases SFRP1 gene was methylated while in 14/56 (25%) cases it was unmethylated. Whereas in only 2 out of 28 (7%) cases adjacent normal tissue showed methylated SFRP1 gene. Thus a significant difference in methylation status was present between tumor and non-tumor tissue. Methylated in relation to clinical stage was noted in 63% cases of stage II and 86.2 % cases of stage III tumor. To ascertain the methylation status of CpG sites present within promoter region of SFRP1 gene, we performed Bisulfite Sequencing of the 126 bp DNA fragment of SFRP1 gene amplified by MS-PCR in representative cases. Bisulphite sequencing showed methylated Cytosine nucleotide in the CpG sites. This 126 bp DNA sequence, in the cases showing methylated SFRP1 in MS-PCR, contained 22 CpG cites within which major no of the Cytosine were methylated (Figure 4.7).

**SFRP1 promoter methylation associated with lymph node metastasis-** Promoter methylation status of SFRP1 gene was compared with patient's clinicopathological characteristics such as age, tumor location, gender, lymph node involvement, tumor stage, and tumor grade (Table 4.5). Chi square test results showed that lymph node metastasis was significantly associated with methylation status of SFRP1 gene in the tumor. Lymph node involvement (pN1-3) was noted in 84.6% cases with methylated SFRP1. Location of tumor was not associated with methylation status. A higher incidence of methylation was detected in patients over 60 years age, however this was not statistically significant.

**Table 4.5-** Clinicopathological characteristics of CRC cases and their association with SFRP1 gene promoter methylation using Chi square test (significant p-value <0.05).

| **Variable** | **Categories** | **No. of cases** | **Methylation status of SFRP1 gene** | | *p-value* |
|---|---|---|---|---|---|
| | | | Un-methylated n =14 (25%) | Methylated n=42 (75%) | |
| Age | <50 year | 28 | 8(28.6%) | 20 (71.4%) | 0.537 |
| | ≥50 year | 28 | 6 (21.4%) | 22 (78.6%) | |
| | | | | | |
| Gender | Male | 34 | 7 (20.6%) | 27 (79.4%) | 0.343 |
| | Female | 22 | 7 (31.8%) | 15 (68.2%) | |
| | | | | | |
| Tumor Stage | T2 | 9 | 3 (33.3%) | 6 (66.7%) | 0.816 |
| | T3 | 25 | 6 (24%) | 19(76%) | |
| | T4 | 22 | 5 (22.7%) | 17(77.3%) | |
| | | | | | |
| Lymph-node metastasis | Absent | 27 | 10 (37%) | 17 (63%) | **0.045** |
| | Present | 29 | 4 (13.8%) | 25 (86.2%) | |
| | | | | | |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 12 (25.5%) | 35 (74.5%) | 0.834 |
| | Mucinous adenocarcinoma | 9 | 2(22.2%) | 7 (77.8%) | |
| | | | | | |
| Histological grade | Poorly differentiated | 10 | 3 (30%) | 7 (70%) | 0.881 |
| | Moderately differentiated | 15 | 4 (26.7%) | 15 (73.3%) | |
| | Well differentiated | 31 | 7 (22.6%) | 24 (77.4%) | |
| | | | | | |
| Tumor site | Colon | 41 | 10 (24.4%) | 31 (75.6%) | 0.862 |
| | Rectum | 15 | 4 (26.7%) | 11 (73.3%) | |
| | | | | | |
| CIMP Status | Low | 31 | 11 (35.5 %) | 20 (64.5%) | **0.044** |
| | High | 25 | 3 (12 %) | 12 (88 %) | |
| | | | | | |
| IGFBP3 gene promoter methylation | Absent | 19 | 10 (52.6 %) | 9 (47.4 %) | **0.01** |
| | Present | 37 | 4 (10.8 %) | 33 (89.2 %) | |

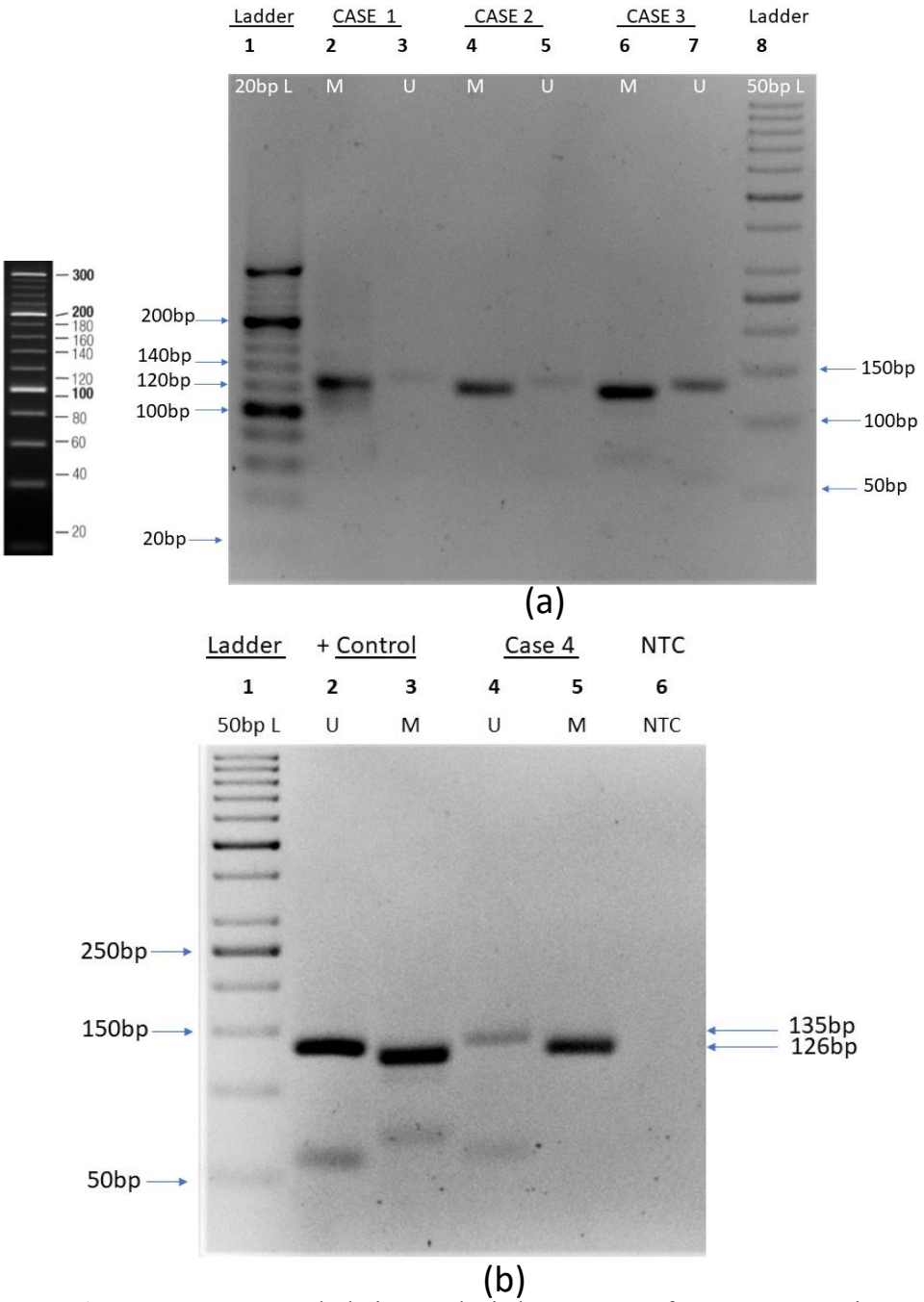



**Figure 4.7**- SFRP1 gene promoter methylation analysis by MS PCR for CRC tumor tissue. MS-PCR amplicon run on 4.0% agrose gel.

**(a)** Methylated gene band appear in lane 2,4,6 and their intensity higher than respective unmethylated band in lane 3,5,7 in 3 CRC tumour tissue. 20bp ladder in lane 1 and 50bp ladder in lane 8.

**(b)** Methylated DNA band in a CRC tumour tissue (lane 5) with control, positive control unmethylation (lane 2), positive control methyaltion (lane 3) and nigative control NTC ( lane 6) with 50 bp ladder (lane 1).

Size of Methylated and Un-methylated fragment is 126bp and 135bp respectively.

M (Methylation specific polymerase chain reaction), U (Un-methylation specific polymerase chain reaction), L (Ladder) NTC (Non Templete Control, water used instead of DNA template.

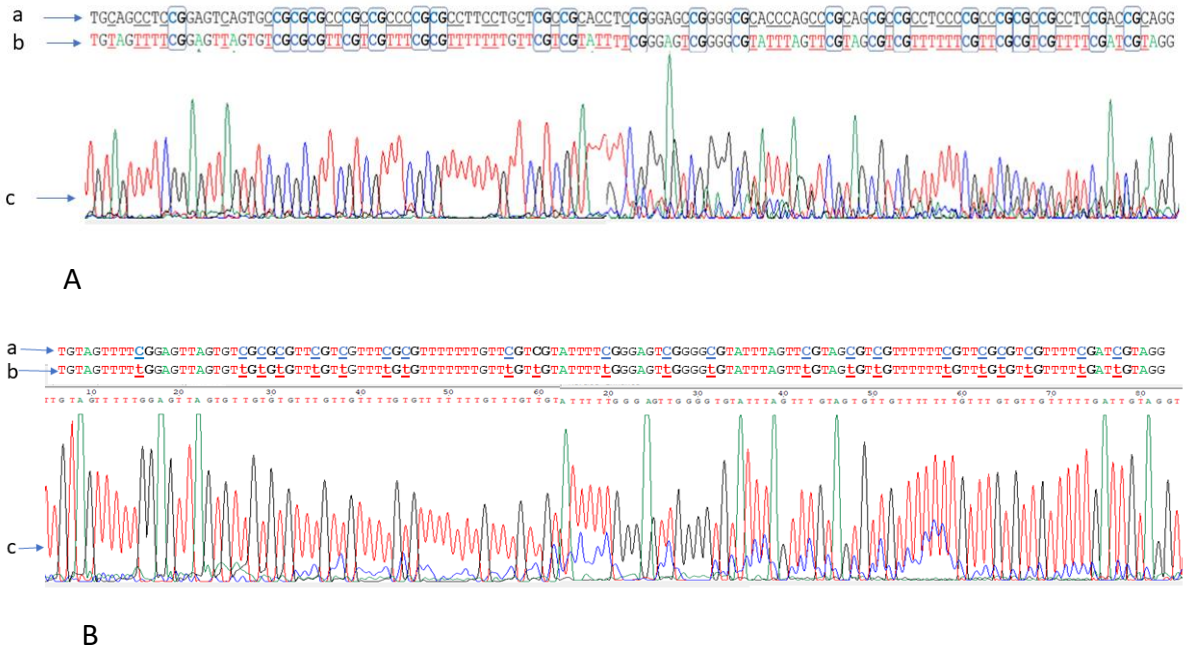



**Figure 4.8- Bisulfite sequencing of SFRP1 gene promoter for methylation analysis**

**(A)** Chromatogram of methylated SFRP1 gene promoter – Bold blue C represents methylated cytosine that's remained unchanged during bisulfite conversion due to its methylation and C represents un-methylated cytosine that converted to thymine represented T, and CG in round corner rectangle represents CpG sites.

**(B)** Chromatogram in a case of un-methylated SFRP1 gene t represented un-methylated cytosine that converted to thymine during bisulphite conversion.

(a) NCBI sequence of SFRP1 gene

(b) Sequence found in methylated and unmethylated gene in bisulfite converted DNA

(c) Chromatogram of sequencing

**IGFBP3 Promoter methylation analysis:**

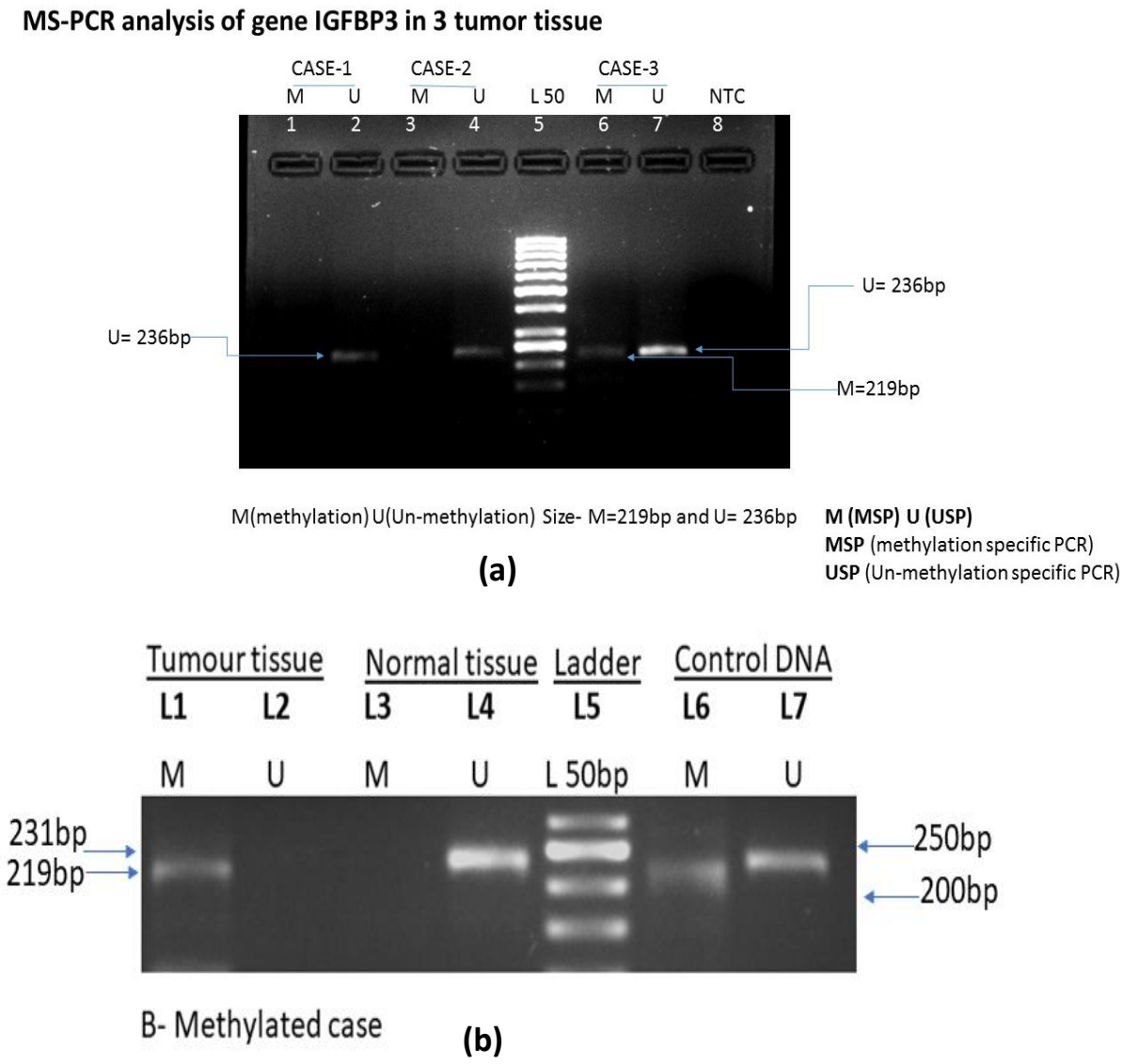



**Figure- 4.9 (a)** Methylation analysis of IGFBP3 gene in 3 CRC cases **(b)** methylation analysis in 1 normal and tumor tissue

M= Methylation specific PCR ; U= Un-Methylation specific PCR

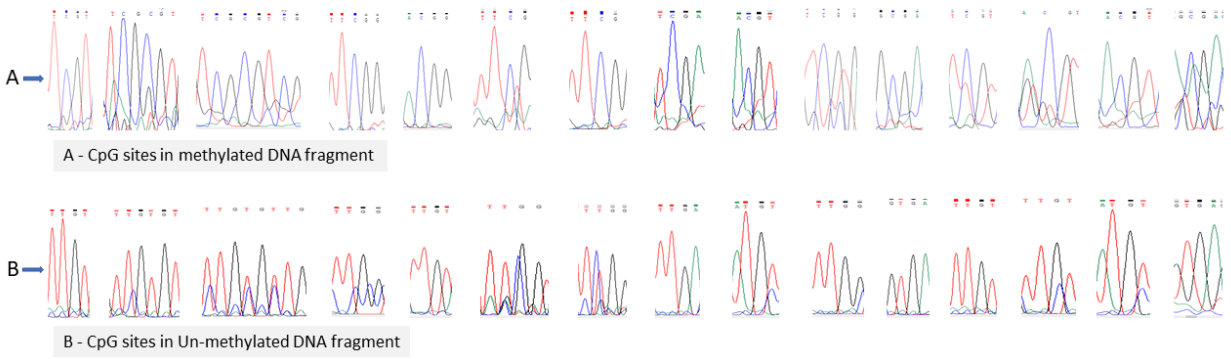



**Figure 4.10- IGFBP3 promoter methylation analysis using bisulfite sequencing-** figure shows the Methylated and unmethylated CpG sites present in IGFBP3 gene promoter

(a) Chromatogram showing cytosines of CpG sites remained as cytosines during bisulfite conversion due to methylated in methylated cases in IGFBP3 gene promoter region. CG remained unchanged during bisulfite conversion due to methylation.

(b) Chromatogram of CpG sites present in un-methylated gene here un-methylated cytosine converted to thymine during bisulphite conversion all un-methylated CG converted to TG.

**Methylation of status of *IGFBP-3* promoter**- Methylation status of *IGFBP-3* gene promoter was determined by MS-PCR results in tumor tissues results shown in figure 4.9 a & b. 37 cases out of 56 (66.1%) cases were methylated and 19 (33.9%) cases were unmethylated. *IGFBP-3* promoter methylation found in 13/27 (48.1%) cases of stage II and 24/29 (82.8 %) cases of stage III in tumor tissue. Patients of >50 year age group having slightly higher methylation frequency compare to <50 year age group the methylation frequency was 67.9% and 64.3% respectively. We found 61.8% male patients and 72.7% female patients had methylated *IGFBP-3* promoter. Methylation analysis also done in adjacent normal tissue but no methylation found in normal tissue which suggests that *IGFBP-3* promoter methylation could be a signature of malignancy figure 4.9.

**Association of Clinicopathological features with promoter methylation of *IGFBP-3*-** To determine whether promoter methylation status of *IGFBP-3* gene is associated with patient's clinical characters such as age, gender, tumor location, lymph node invasion, tumor stage, and histological grade, we performed Chi squire test and results are summarized in table 4.6**.** We found lymph node metastasis (p=0.006) was significantly associated with methylation of status of *IGFBP-3* gene promoter. Patients with positive lymph node (LN) metastasis (pN1-3) had 82.8% cases were methylated whereas only 48.1% cases were methylated with negative lymph node metastasis (pN0). On the basis of tumor site, 31 (75%) cases had methylated promoter out of 41 case of colon tumor and 6 (40%) cases of rectum tumor were methylated out of total 15 rectum cases. The association of promoter methylation with tumor site was not significant (P=0.013). In male 61.8 %, and female 72.7% tumor were having hypermethylated IGFBP-3 gene promoter. (Table 4.6)

**Table 4.6-** Clinicopathological characteristics of CRC cases and their association with IGFBP3 promoter methylation Showing association of clinicopathological parameters in relation to methylation status of IGFBP3 gene using Chi square test

| **Variable** | **Categories** | **No. of cases** | **Methylation status of IGFBP3 gene promoter** | | *p-value* |
|---|---|---|---|---|---|
| | | | **Un-methylated** n =19 (33.9%) | **Methylated** n=37 (66.1%) | |
| Age group | <50 year | 28 | 10 (35.7%) | 18 (64.3%) | 0.778 |
| | >50 year | 28 | 9 (32.1%) | 19 (67.9%) | |
| | | | | | |
| Gender | Male | 34 | 13 (38.2%) | 21 (61.8%) | 0.397 |
| | Female | 22 | 6 (27.3%) | 16 (72.7%) | |
| | | | | | |
| Tumour Stage | T2 | 9 | 3 (33.3%) | 6 (66.7%) | 0.955 |
| | T3 | 25 | 9 (36.0%) | 16 (66.4%) | |
| | T4 | 22 | 7 (31.8%) | 15 (68.2%) | |
| | | | | | |
| Lymph-node involvement | pN0 | 27 | 14 (51.9%) | 13 (48.1%) | **0.006** |
| | pN1-3 | 29 | 5 (17.2%) | 24 (82.8%) | |
| | | | | | |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 18 (38.3%) | 29 (61.7%) | 0.115 |
| | Mucinous adenocarcinoma | 9 | 1 (11.1%) | 8 (88.9%) | |
| | | | | | |
| Tumour grade | Poorly differentiated | 10 | 3 (30%) | 7 (70%) | 0.949 |
| | Moderately differentiated | 15 | 5 (33.3%) | 10 (66.7%) | |
| | Well differentiated | 31 | 11 (35.5%) | 20 (64.5%) | |
| | | | | | |
| Tumour location | Colon | 41 | 10 (24.4%) | 31 (75.6%) | **0.013** |
| | Rectum | 15 | 9 (60.0%) | 6 (40.0%) | |
| | | | | | |
| Diet | Veg | 29 | 11 (37.9%) | 18 (62.1%) | 0.512 |
| | Non-veg | 28 | 8 (29.6%) | 19 (70.4%) | |
| | | | | | |
| Alcohol habit | present | 40 | 15 (37.5%) | 25 (62.5%) | 0.372 |
| | absent | 16 | 4 (25.0%) | 12 (75.0%) | |

(significant p-value <0.05).

**Survival analysis with reference of *IGFBP-3* promoter methylation-** Based on the observed methylation status of *IGFBP-3* gene promoter in the tumor tissue, methylated and unmethylated groups were defined. Follow-up in 58 patients up to 60 months. During follow-up 25 (10 female 15 male) patients had died due to disease related event, advanced tumor stage, 18 patients were live and 15 patients lost during follow-up. The mean estimated overall survival (OS) of unmethylated group was 40.03 months and whereas methylated group OS was 21.58 months (in stage II and III both cases) Kaplan Meier survival curve shown in figure 4.13(h i). Unmethylated groups survival was significantly better as compare to methylated group (P=0.041 by Log rank test) and poor survival associated with methylation of *IGFBP-3* promoter. The combined estimated OS of all 56 cases were 34.69 months. To explore the effect of methylation in survival we analyzed survival in stage II and stage III CRC cases separately. We found stage II CRC patients survival was significantly differ in methylated and unmethylated group P= 0.04 the survival was 22.23 months, and 49.158 month respectively (fig 4.13 h (ii). Though in stage III CRC patient's the survival was 28.11 months, 22.91 months of unmethylated, methylated group respectively but difference in survival was not significant (P=0.73) (Fig 4.13 h (iii)). We also analyzed survival of CRC patients with reference of 8 deferent conventional pathological factors such as age group, gender, tumor stage, lymph node status, grade and tumor subtype but difference was not significant table 2. In univariate analysis, IGFBP-3 methylation status could be verified as an independent prognostic factor in stage II CRC cases. Univariate cox model suggesting, among these variables, IGFBP-3 methylation can serve as prognostic indicator of poor survival in stage II CRC.

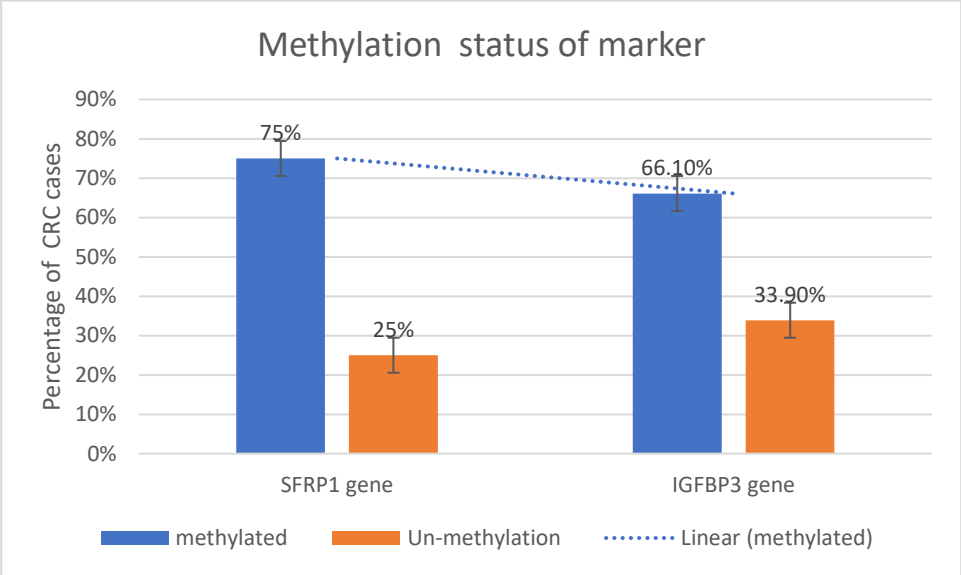



**Figure 4.11** Percentage of CRC cases that having methylated SFRP1, IGFBP3 gene promoter and un-methylated SFRP1, IGFBP3 gene promoter. Dotted line shows trend mrthylation

## Survival Analysis

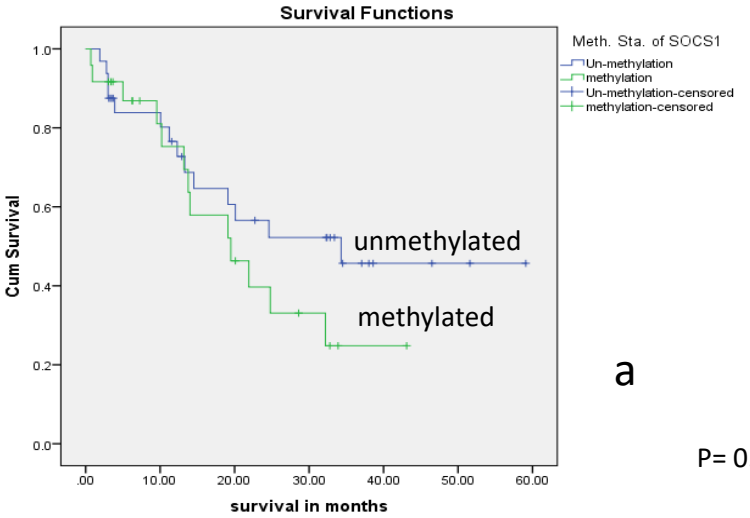
**Table 4.7- Survival analysis with of reference clinicopathological characters by using Kaplan-Meier survival analysis**

| Variables | Categories | Total No. | No. of Events (deaths) | Mean survival in months | P value |
|---|---|---|---|---|---|
| **Survival on the basis of clinicopathological factor** | | | | | |
| Clinical stage | II | 27 | 8 | 37.076 | **0.005** |
| | III | 29 | 19 | 22.99 | |
| | | | | | |
| Age group | <50 | 28 | 17 | 22.826 | 0.348 |
| | >50 | 28 | 10 | 36.877 | |
| | | | | | |
| Gender | Male | 34 | 18 | 26.80 | 0.113 |
| | Female | 22 | 9 | 33.607 | |
| | | | | | |
| Tumor stage | T2 | 9 | 3 | 31.19 | 0.583 |
| | T3 | 25 | 11 | 32.67 | |
| | T4 | 22 | 13 | 22.33 | |
| | | | | | |
| Histological type | Infiltrating adeno. NOS | 47 | 20 | 34.916 | **0.002** |
| | Mucinous adeno. | 9 | 7 | 12.496 | |
| | | | | | |
| Tumor grade | Poorly differentiated | 10 | 7 | 17.74 | 0.155 |
| | Mod. differentiated | 15 | 5 | 36.06 | |
| | Well. differentiated | 31 | 15 | 29.88 | |
| | | | | | |
| Tumor site | Colon | 41 | 18 | 29.96 | 0.565 |
| | Rectum | 15 | 9 | 27.309 | |
| | | | | | |
| CIMP Status | Low | 31 | 14 | 33.51 | 0.532 |
| | High | 25 | 13 | 22.24 | |
| | | | | | |
| Methylation status of SFRP1 | Un-Methylated | 14 | 4 | 45.34 | **0.033** |
| | Methylated | 42 | 23 | 22.435 | |
| | | | | | |
| Methylation status of IGFBP3 | Un-Methylated | 19 | 7 | 40.29 | **0.039** |
| | Methylated | 37 | 20 | 21.64 | |
| | | | | | |
| Methylation Status of SOCS1 | Un-Methylated | 32 | 14 | 34.746 | 0.255 |
| | Methylated | 24 | 13 | 22.50 | |
| | | | | | |
| | Un-Methylated | 35 | 13 | 37.048 | **0.033** |

| | | | | | |
|---|---|---|---|---|---|
| Methylation status of CACNA1G | Methylated | 21 | 14 | 18.319 | |
| | | | | | |
| Methylation status of IGF2 | Un-Methylated | 23 | 13 | 31.344 | 0.998 |
| | Methylated | 33 | 14 | 23.613 | |
| | | | | | |
| Methylation Status of NEUROG1 | Un-Methylated | 24 | 12 | 27.709 | 0.714 |
| | Methylated | 32 | 15 | 31.856 | |
| | | | | | |
| Methylation Status of RUNX3 | Un-Methylated | 37 | 17 | 33.40 | 0.46 |
| | Methylated | 19 | 10 | 21.073 | |
| | | | | | |
| Lymph Node | II | 27 | 8 | 37.076 | **0.005** |
| | III | 29 | 19 | 22.99 | |
| | | | | | |
| Alcohol Intake | Absent | 40 | 18 | 33.847 | 0.227 |
| | Present | 16 | 9 | 20.943 | |
| | | | | | |
| Dietary Habit | Veg | 29 | 14 | 24.28 | 0.225 |
| | Non Veg | 27 | 13 | 34.863 | |
| | | | | | |
| **Total** | | **58** | **25** | **42.081** | |

All P values by Log Rank test and significant P value showing in bold

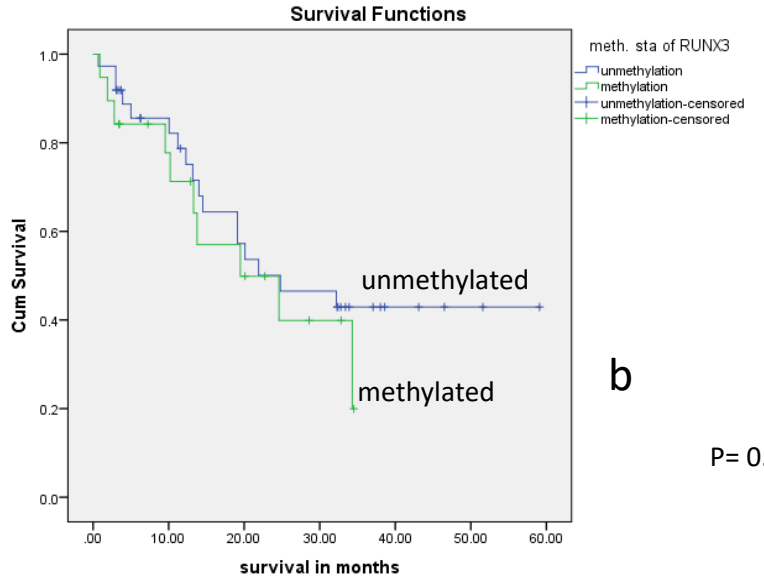
## Kaplan-Meier survival curve analysis




a

P= 0.255

**Figure 4.12a-** Survival curve on the basis of methylation of SOCS1 gene




b

P= 0.46

**Figure 4.12b**- Survival curve on the basis of methylation of RUNX3 gene

## Kaplan-Meier survival curve analysis

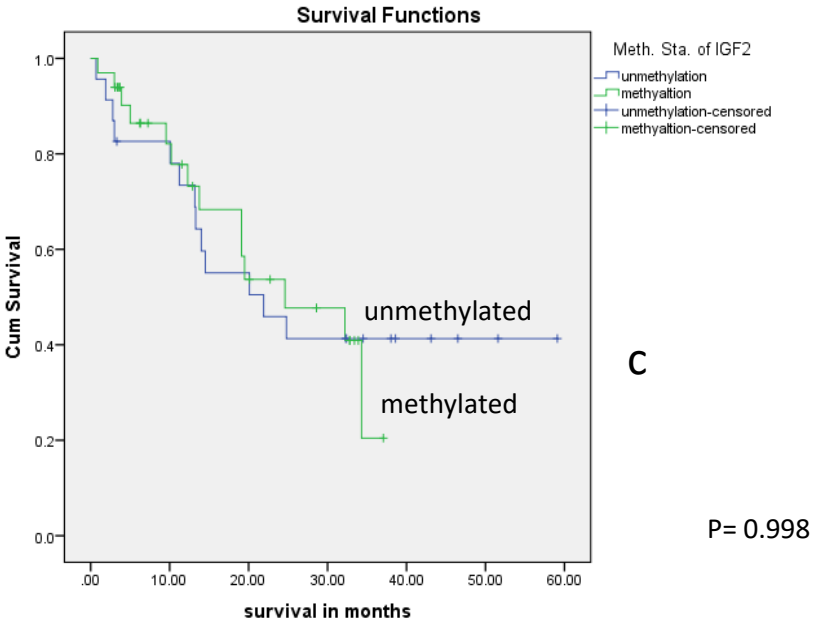



**Figure 4.12c**- Survival curve on the basis of methylation of IGF2 gene

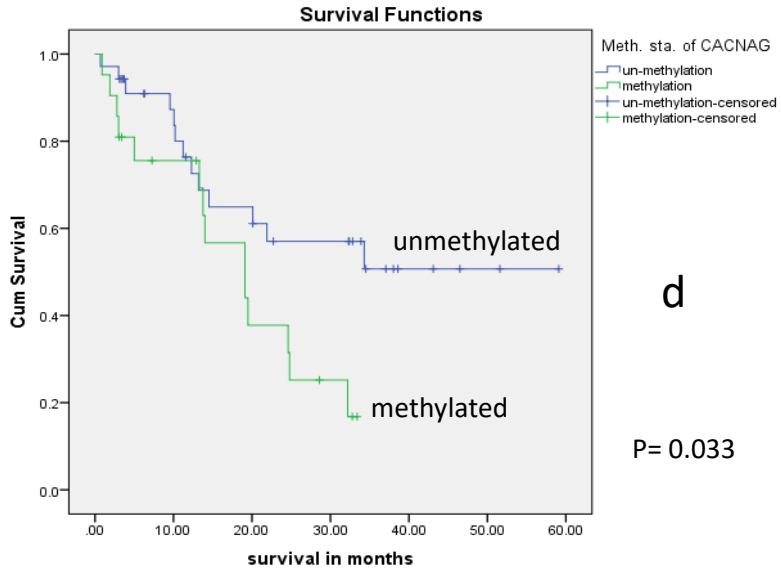
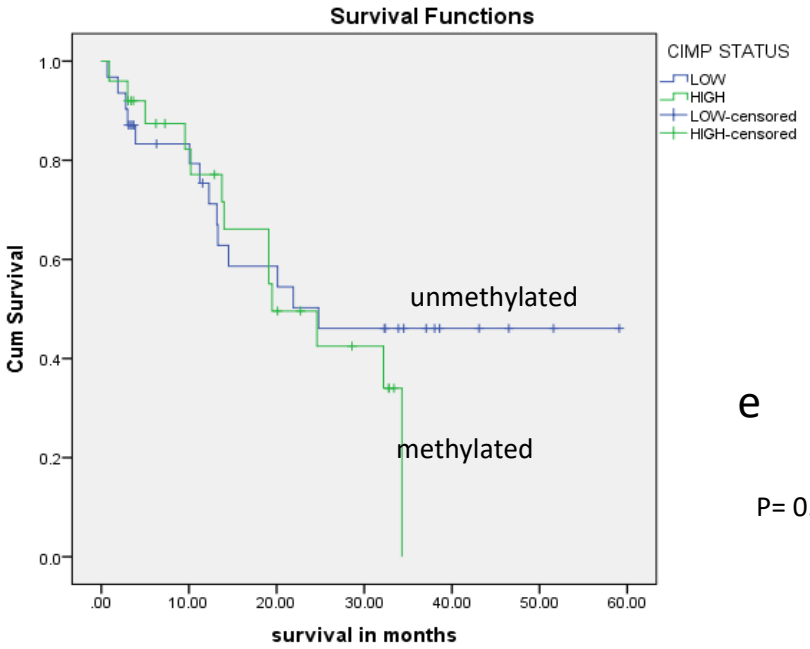



**Figure 4.12d** Survival curve on the basis of methylation of CACNA1G gene

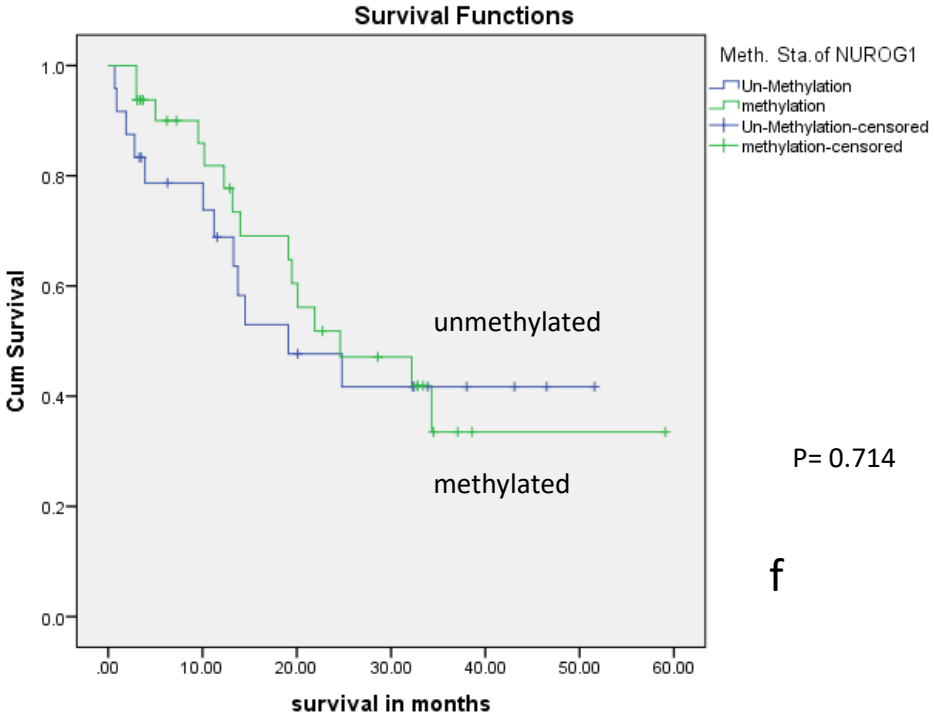
## Kaplan-Meier survival curve analysis




e

P= 0.532

**Figure 4.12e**- Survival curve on the basis of CIMP status gene




P= 0.714

f

**Figure 4.12f-** Survival curve on the basis of methylation of NUROG1 gene

## Kaplan-Meier survival curve analysis

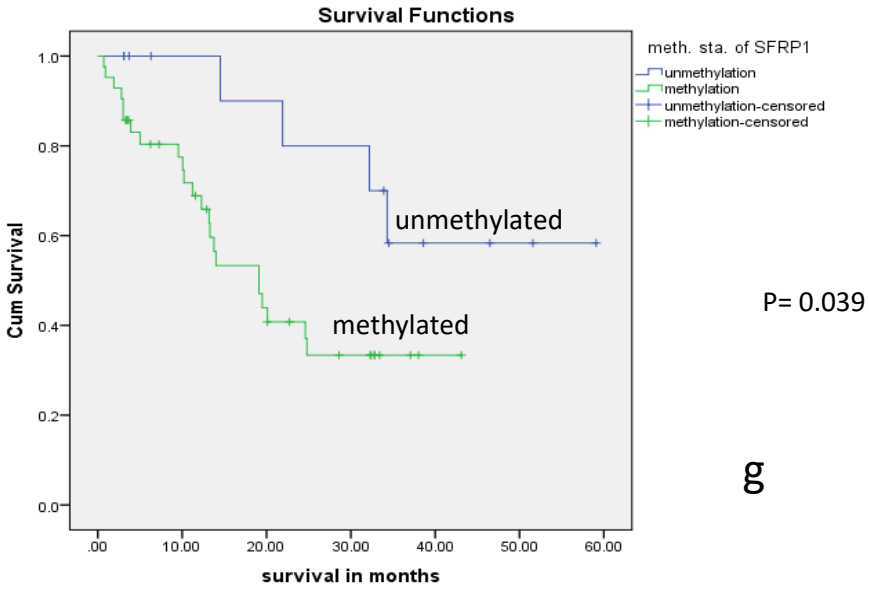



**Figure 4.12g**- Survival curve on the basis of methylation of SFRP1 gene

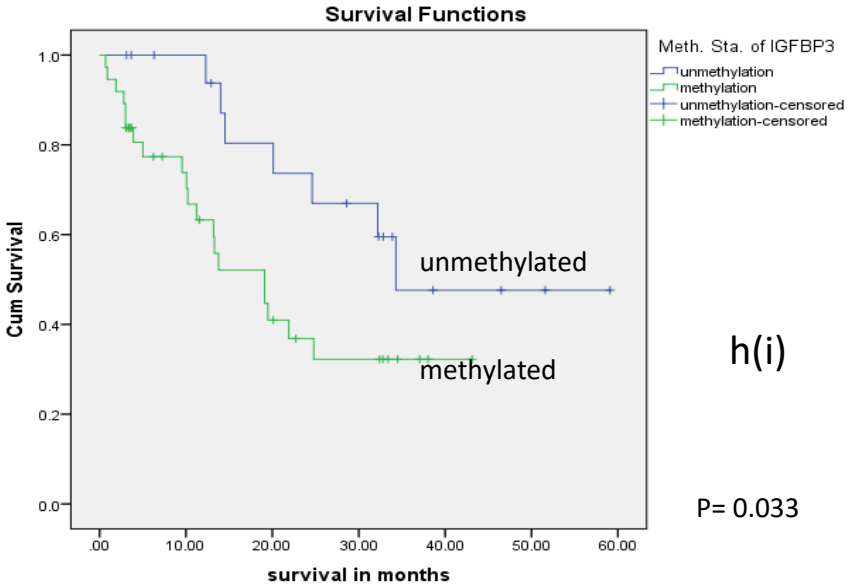
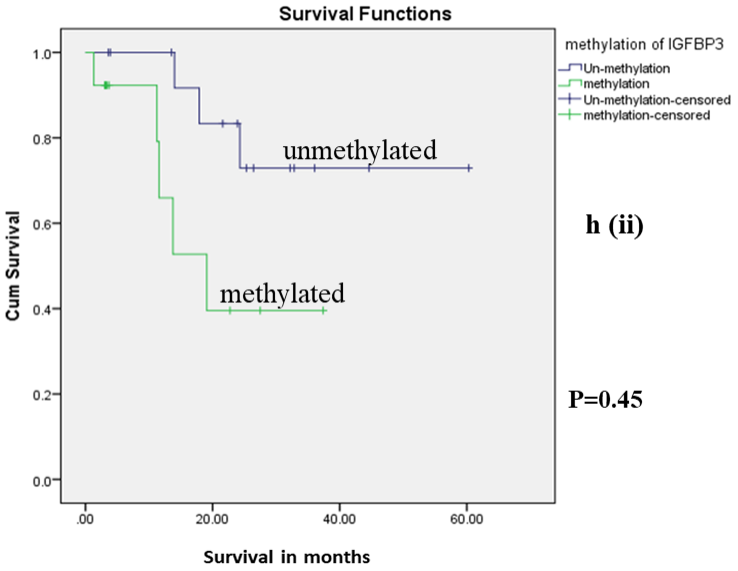


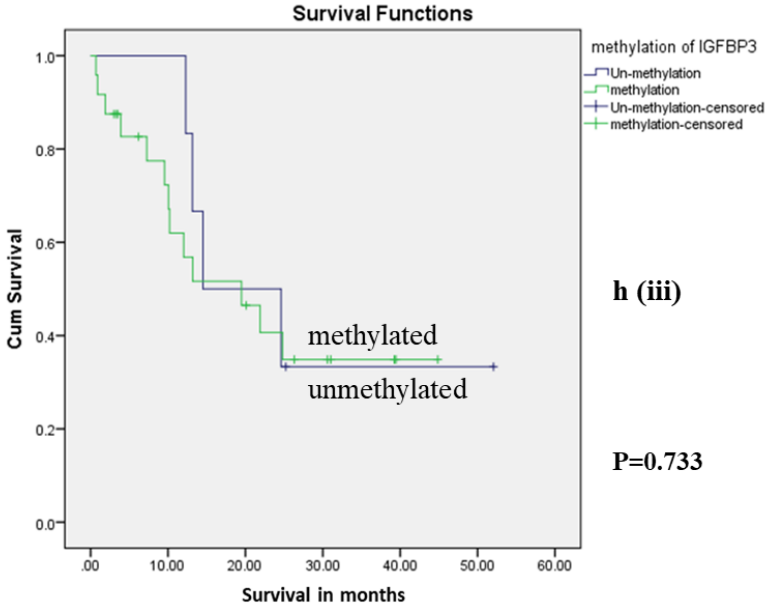

**Figure 4.12h-** Survival curve on the basis of methylation of IGFBP3 gene

## Kaplan-Meier survival curve analysis




**h (ii)**

**P=0.45**




**h (iii)**

**P=0.733**

**Figure 4.12h (ii & iii)-** Survival curve on the basis of methylation of IGFBP3 gene

**Promoter methylation of SFRP1 and pateints survival-** Based on the observed methylation status of SFRP1 gene in the tumor tissue, methylated and unmethylated groups were defined. Follow-up in 56 patients ranging from 12 month to 5 years (median follow-up 34 months) 27 CRC patients had died due to disease related event and advanced tumor stage and 12 patients lost to follow-up. The overall mean survival of unmethylated and methylated group was 45.34 months and 22.40 months respectively survival curve in (Figure 3a). The combined estimated OS of both the groups were 33.461 months. Unmethylated groups survival was significantly better as compare to methylated group (p= 0.033 by Log rank test) and poor survival associated with methylation of SFRP1. We also analysed survival of CRC patients with reference of 8 deferent conventional pathological factors such as Age group, Gender, Tumor stage, Clinical stage, Lymph node status, Differentiation of tumor and tumor subtype. *Kaplan-Meir* survival analysis results shows survival is dependent on many factors but it was majorly influenced by lymph node status and methylation of SFRP1. If we talk about survival on the basis of clinical stage II and III the mean survival was 37.76 and 22.99 months respectively(fig 3b). To explore the contribution of these variables and evaluation of their influence as potential prognostic marker, all these variables were analysed by univariate cox regression model analysis. In univariate analysis, only SFRP1 methylation status could be verified as an independent prognostic factor,. Univariate cox model suggesting, among these variables, SFRP1 methylation can serve as an independent prognostic indicator of poor survival in CRC.

**Table 4.8** Association between clinicopathological features of the CRC patients and prognosis.

| Univariate Cox regression analysis | | | | | |
|---|---|---|---|---|---|
| | | Univariate analysis | | | |
| Variables | Categorization | HR | 95.0% CI for HR | | Sig. |
| | | | Lower | Upper | |
| Age | >50 | .781 | .302 | 2.022 | .610 |
| | <50 | | | | |
| | | | | | |
| SFRP1 gene methylation | unmethylation | 3.795 | 1.058 | 13.609 | **.041** |
| | methylation | | | | |
| | | | | | |
| Sex | male | .578 | .230 | 1.451 | .243 |
| | female | | | | |
| | | | | | |
| CIMP Status | High | .638 | .221 | 1.840 | .405 |
| | Low | | | | |
| | | | | | |
| Differentiation of tumor | poor | 1 | | | .118 |
| | mod | .289 | .077 | 1.091 | .067 |
| | well | .357 | .118 | 1.077 | .067 |
| | | | | | |
| Lymph node involvement | present | 2.628 | 1.045 | 6.609 | **.040** |
| | absent | | | | |
| | | | | | |
| Tumor location | colon | 1.297 | .467 | 3.600 | .618 |
| | tumor | | | | |
| | | | | | |
| Tumor status | T2 | 1 | | | .784 |
| | T3 | 1.255 | .311 | 5.064 | .749 |
| | T4 | 1.590 | .379 | 6.681 | .526 |

**HR= Hazard Ratio; CI = Confidential Interval**

# DISCUSSION

# CHAPTER-5

## DISCUSSION

Colorectal cancer (CRC) is a common cancer occurring globally, representing the second cause of mortality related to cancer (Smith, *et al.* 2010). It is becoming a global burden that every year one million new cases are diagnosed around the world. Growing evidence suggests that CRC is heterogeneous disorder that can develop through different pathways involving distinct combinations of genetic and epigenetic alterations. These alterations effect the oncogenes, tumor supressor genes and DNA repair genes, which are all involved in critical pathways of CRC initiation and progression making CRC as a heterogeneous disorder (Harrison et. al. 2011). So, a better understanding of the molecular events involved in the progression of CRC could provide insights to develop the therapeutic targets and in assessing the risk assessment in CRC patients. The widely accepted pathways in the etiology of CRC is, 1. Chromosomal instability (CIN), Microsatellite instability (MSI) and CpG island Methylator phenotype (CIMP).

We collected a total of 56 CRC samples during our study which lies in both stage II and stage III of CRC and performed our studies. In this study we analysed the CIMP status, promoter hypermethylation of genes SFRP1 and IGFBP3. Follow-up has taken for all the 56 patients ranging from 12 months to 56 months (median follow-up period 28 months) after curative surgery. Eighteen patients had died due to disease related events and advanced tumour stage and Twelve patients miss the follow-up. The overall mean survival of unmethylated and methylated SFRP1 group was 45.34 months and 22.21 months respectively (fig 4.11g).

We started our study in identifying the CIMP status in these samples and try to find its association with the clinicopathological characters as well as the survival outcome of the patients. Hypermethylation of promoter of tumor-suppressor genes could leads transcriptional silencing of gene, CIMP is imagined to play important role in CRC progression (Herman JG *et al.* 2003, Nagasaka T, *et al.* 2008). Though, characterizations of CIMP diverse broadly between research paper with respect to methylation loci

considered and techniques or methods apply to conclude methylation. We used new panel of CIMP marker genes of CRC as reported by Lee at el 2008( CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1). The CIMP high and low distinction was done as mentioned in the materials and methods section. The results show that 44.6% of CRC cases show CIMP high status while 55.4% show CIMP low status. Till date the CIMP high status in CRC is reported was 20-30% and in our case it was 44.6%, this may be due to the fact that all the reports were from the countries other than India. Further, in our study we found CIMP status is associted with tumor location (P=0.045) whereas it doesnt show any association with other factors like patients age, gender, tumor stage (T2,T3,T4) Lymph node metastasis, histological grade (well difrentiated, modrately difrentiated, poorly defrentiated), histological type (Infiltrating adenocarcinoma NOS, Mucinous adenocarcinoma) and cancer TNM stage (II and III). Generally the CIMP status was reported in the tuour characterization such as its stage, grade and lymph node metastasis. However we got contrasting results, may be due to low number of patients in each type. The analysis of relationship between CIMP staus and patients lyfe style related factor such as alcohal intake, diatry habit (veg vs non-veg) gutka and tabacco chewing habits, we didn't found any sigficant association. The survival analysis studies also didn't show any significant associtionwith the CIMP status in our studies, however the CIMP high patients exhibits poor survival compared to CIMP low patients. The studies of Dahlin AM et. al. 2010, Weisenberger DJ *et al.* 2010, reported that CIMP is associated with transformed molecular and clinicopathological features. Juo *et al.* 2014, hypothesized that CIMP positivity is helpful in predicting the survival in CRC patients. The results obtained from our study indicate that in CIMP status is associated with the poor survival, tumor site and no other clinicopathological factors were associated.

We next analyzed the promoter methylation status of Secreted Frizzled Related Protein 1(SFRP1) and Insulin like Growth factor binding protein-3 (IGFBP3), both are reported to be tumour suppressor genes and reported to be silenced in CRC due to promoter methylations (Jogie-Brahim *et al.* 2009, Baxter RC *et al.* 2014, Hanafusa T *et al.* 2002, Chang YS *et al.* 2002). Methylated and unmethylated groups were defined based on the observed methylation status of SFRP1/IGFBP3 genes in the tumour and normal tissues.

The combined estimated overall survival periods (OS) of both the SFRP1 methylated and unmethylated groups were found to be 44.2 months. SFRP1 promoter methylation group show lesser survival period when compared to the unmethylated group (p= 0.033 by Log rank test). This result show survival period in inversely proportional to the promoter methylation of SFRP1. The analysis of association of promoter methylations of SFRP1 with the conventional pathological factors such as age, gender, tumour stage, clinical stage, lymph node status, differentiation of tumor and tumor subtype, we found lymph node metastasis ($p<0.05$) and survival period (*Kaplan-Meir* survival analysis ) is significantly associated with the promoter methylation. If we observe the survival on the basis of clinical stage II and III of CRC cases the mean survival period was 37 and 22 months respectively. To explore the possibility of promoter hypermethylation's as potential prognostic marker, all the variables mentioned above were analysed by univariate Cox hazard model analysis and the SFRP1 methylation status could be verified as an independent prognostic factor and poor survival.

In case of IGFBP-*3*, we found promoter methylations in 63.8% CRC cases. This was relatively higher with the gene we studied i.e., SFRP1. The different percentage of methylations were reported in earlier also, for example Kawasaki T *et al.* 2007 reported 29% Perez-Carbonell L *et al.* 2014 reported 83%. The findings of IGFBP3 promoter methylation association with clinicopathological characteristics show lymph node metastasis was significantly associated with both the stage II & III CRC cases (table 4.6). The data obtained by this study suggest that *IGFBP-3* promoter methylation is independent of patients age, sex, tumor location, tumor stage and differentiation. The survival analysis results show these more methylations less survival period. However, this statement was true to stage II CRC cases, but in the case of stage III CRC patients survival is better however it was not statistically significant ( fig 4.12 h iii) . The possible region of poor survival of patients who having methylated *IGFBP-3* promoter is might be its association with cancer progression, recurrence in CRC (Georges RB, *et al.* 2011, Hong J *et al.* 2002, Kansra S *et al.* 2000). Therefore, detection of *IGFBP-3* promoter methylation in stage II CRC may be helpful to identify those patients who were at high risk. This marker may be helpful improve the current strategy for CRC patients therapy management.

Through this study we concluded that the CIMP-high status can be used in predicting the survival period, i.e., CIMP high means less survival period. Further, the SFRP1 and IGFB3 promoter methylations lead to the more lymph node metastasis. This infers that transcriptional silencing of these genes increases the aggressiveness of the tumor in CRC cases. Further, the above conclusions are applicable to stage II CRC cases only not for the stage III CRC. SFRP1/IGFBP3 might have the ability in identifying the stage II CRC patients, who has high risk, poorer clinical outcome and therefore they may use adjunctive therapy besides the chemotherapy. In future these genes promoter methylations can be a potential target for the development of cancer drugs, like demethylating agents such as azacytidine and decitabine. Future research will be making a better understanding the fundamental mechanisms that explain the association between *SFRP1*/*IGFBP-3* promoter methylations and lymph-node metastasis, and to evaluate the role of demethylating agent alone or in combination with adjuvant therapy in patients with extreme risk stage II CRC. *IGFBP-3* gene promoter methylation as a prognostic biomarker for stage II CRC cases may be helpful for disease outcome prediction/finding of high-risk CRC patients. So, we admit the limitations in our study honestly that our sample size is small, and average follow up period (as these two factors are not in our control).

# SUMMARY

# CHAPTER-6

## SUMMARY

Colorectal carcinoma (CRC) is one of the leading causes of mortality worldwide. It remains a worrying human health issue, which contributes about 8.2% cancer associated deaths globally . It is the third most commonly diagnosed cancer around the globe, and in India it was recorded as fourth and fifth most common cancer in males and females with incidence rates of 6.4%, 3.4% respectively. The development of metastasis in distant organs, such as liver, lungs etc is the main cause of death, thus ~40-50% of patients relapse and die of metastatic disease even after curative surgery. However, CRC can be cured 9/10 times if we detect this in time. Many biomarkers as developed to detect the CRC, carcinoembryonic antigen (CEA) is the 1st one and widespread used. Epigenetic alterations such as DNA hypermethylation, can be used for the early detection of pre-malignant lesions, including adenomatous polyps in the colon. Promoter hypermethylation of tumor suppressor genes thereby transcriptional silencing gained significance as prognostic markers in the CRC diagnosis, prognosis and therapy. Generally, more than half of CRC cases were diagnosed at stage II and III , and the mainstay of treatment option is only surgery. After surgery, patients survival depends on disease relapse because in most of the cases recurrence will happen. So, it becomes important to identify the high risk patients who need additional adjuvant therapies besides the regular chemotherapy. Therefore, identifying the prognostic biomarkers are the need of the hour to identify high risk initial stage CRC cases. In this study we are looking for the epigenetic prognostic biomarkers SFRP1, IGFBP3, and CpG island methylator phenotype (CIMP) markers (CACNA1G, NEUROG1, SOCS1, IGF2, RUNX3) and analyzed their prognostic value in stage II and stage III CRC cases. Promoter methylated mediated silencing of IGFBP-3 and SFRP1 has been reported in many cancers, such as lung, hepatocellular, gastric, breast, ovarian cancers including Colorectal cancer

In this study we analyzed the CIMP status in the stage II and stage III CRC cases and found that CIMP high status is significantly associated with poor survival. We found 44.6% CRC cases had CIMP-high where as 55,4% had CIMP low. In CIMP marker genes analysis we found CACNA1 gene was methylated in 37.5% cases, IGF2 gene was found methylated in 58% cases, NUEROG1 was methylated in 57% cases RUNX3 was found methylated

33.9% cases and SOCS1 gene methylation was found in 42.9% cases. CACNA1G, SOCS1 are associated with poor survival but difference in survival was not significant. Methylation of a IGF2 gene doesn't show significant association with survival period however methylated NEUROG1 shows better survival. Though the survival period depends on many factors, a possible correlation was drawn by this study that CIMP high status means less survival in stage II CRC cases. There was no association observed in other clinicopathological factors such as age, gender, tumour stage, grading, histological type, dietary habits and alcohol intake.

Further, studies of promoter hypermethylation of the tumor suppressor genes such as SFRP1 and IGFBP3 also analysed and try to find associations on clinicopathological characters and the survival period of CRC patients. SFRP1 is an inhibitor of WNT signalling pathway, and transcriptional silencing leads to the WNT signalling activation promoting the tumorigenesis. In our study we found SFRP1 gene methylations in n 75% CRC cases, and is associated with lymph node metastasis. This clearly explains that suppression of this gene makes the tumour aggressive and evade the neighbouring tissue. The IGFBP3 promoter hypermethylation found in this study is 66% in the CRC. SFRP1/IGFBP3 promoter methylations were significantly associated with poor survival and lymph-node metastasis, but in the later case it was found to be stage II CRC.

To the best of our knowledge, no study has been examined the relationship between methylation of the IGFBP-3, SFRP1 and CIMP marker gene promoter with survival of patients in stage II and III of Colorectal cancer cases in India.

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# *PUBLICATIONS*

RESEARCH ARTICLE

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# Prognostic Relevance of *SFRP1* Gene Promoter Methylation in Colorectal Carcinoma

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## Abstract

**Background:** The development of colorectal carcinoma (CRC) involves many genetic and epigenetic alterations and methylation being an important epigenetic event has been described as a diagnostic and prognostic biomarker. Secreted Frizzled- Related Protein 1 (*SFRP1*) gene regulates diverse physiological processes via the Wnt signaling. Promoter hypermethylation of *SFRP1* gene is an epigenetic regulation mechanism that downregulates *SFRP1* protein level in the tumor, and happens to be one of the significant events in colorectal carcinogenesis. We studied the clinicopathological relationship of CRC including survival outcomes with *SFRP1* gene promoter methylation. **Methods:** We evaluated promoter methylation status of *SFRP1* gene by methylation-specific PCR (MS-PCR) in the tumor tissue in 54 cases of stage II-III CRC patients in north India. The MS-PCR result was further validated by bisulfite sequencing. **Results:** *SFRP1* gene was methylated in 72.2% cases and un-methylated in 27.8%. We found, that *SFRP1* gene methylation in tumor was associated with lymph node invasion (p=0.05). The mean overall survival was 22.318 months and 45.173 months respectively for patients with methylated and unmethylated *SFRP1* gene (p= 0.010, log rank test), (HR = 17.313, 95% CI: 2.021-148.290 P=0.009). **Conclusion:** Study indicates that promoter methylation of *SFRP1* gene is associated with lymph-node metastasis and poor mean overall survival and it can be a prognostic marker in CRC.

**Keywords:** Colorectal carcinoma- Methylation Specific PCR (MS-PCR)- Promoter hypermethylation,- *SFRP1* gene

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## Introduction

Colorectal carcinoma (CRC) is one of the leading causes of mortality worldwide. It is the third most common cancer in males and second in females globally and recorded as second major cause of cancer-related deaths worldwide (Bray et al., 2018). In India CRC is fifth most common cancer in females and forth in males with incidence rate of 3.1% and 5.8% respectively (Bray et al., 2018). There is an increasing incidence of CRC in India. CRC is a heterogenous disease, influenced by genetic and epigenetic alterations and the heterogenicity is due to several pathways involved in CRC tumorigenesis (Colussi et al., 2013). CRC patients show a significant difference in prognosis and individual treatment responses even when presenting at same clinical stage. Multiple factors deregulate the expression of cancer related genes (like APC, KRAS, BRAF, TP53, SFRPs MLH1, MSH1) and promoter methylation mediated silencing is one of them (Armaghany et al., 2012; Thiel et al., 2013; Fearon and Vogelstein, 1990; Wheeler et al., 2000). Secreted Frizzled Related Protein1 (*SFRP1*) gene is known for its ability to negatively modulate the Wnt signaling cascade (Mii and Taira, 2011). *SFRP1* gene codes for *SFRP1* protein that works as an antagonist of Wnt protein and plays a significant role in the regulation of Wnt/βcatenin signaling pathway. β-catenin dependent canonical WNT signaling maintains crypt stem cell compartment in the intestine but overactivation of this pathway by genetic or epigenetic changes has been seen in colorectal carcinoma (Novellasdemunt et al., 2015). This Wnt/βcatenin pathway also plays important role in tumorigenesis of several other types of cancers like breast, ovarian, gastrointestinal cancer (Clevers and Hans, 2006; Huang et al., 2006; Zhan et al.,2006). In CRC, *SFRP1* gene expression is found to be downregulated due to aberrant methylation in its promoter region and this promoter methylation is a common epigenetic alteration found in human cancers including colorectal carcinoma (Suzuki et al., 2004; Jones and Jomary, 2002). CpG islands are susceptible for methylation and since most of gene promoter regions are CpG island rich, it implies that promoter regions are most susceptible for hypermethylation and thus, promoter methylation leads transcriptional silencing of the gene (Nandakumar et al., 2011). If promoter hypermethylation occurs in tumor suppressor gene it may lead to tumorigenesis. Promoter hypermethylation mediated epigenetic silencing of *SFRP1* gene is a major

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cause of downregulation of *SFRP1* protein level and leads overactivation of Wnt signaling in CRC. Methylation based molecular makers are successfully being used in routine as prognostic/predictive marker for better patient management in various cancer Eg. MGMT in Gliomas (Weller et al., 2010). Some studies have also described value of *SFRP1* as prognostic/predictive biomarker in cancer (Leygo et al., 2017; Zheng et al., 2015). The aim of our study was to look for promoter hypermethylation of *SFRP1* gene in CRC, and find its prognostic significance. It explores for association of promoter methylation of *SFRP1* gene with clinicopathological features of CRC and patient survival.

## Materials and Methods

*Patients and tissue specimen*

We enrolled 54 histopathologically confirmed cases of CRC, who underwent curative surgery in Departments of Surgical Gastroenterology and Surgical Oncology, Dr. R M L Institute of Medical Sciences Lucknow, UP, India. Of these 54 cases 28 (51.85%) case were stage II and 26 (48.15%) cases were stage III at the time of diagnosis (Lippincott-Raven et al., 1998). After histopathological examination (HPE), the FFPE tissue blocks were taken for molecular analysis. HPE (staging and grading) were done by standard procedure. Patient demographic and histopathological details and follow up were recorded. This study was approved by Institutional Ethics Committee (IEC no-8/15) of Dr. RMLIMS, Lucknow, and written informed consent was taken for all cases included in this study. The selection of tumor and non-tumor regions was done by examining Hematoxylin - Eosin (H and E) stained sections. Patient follow up and mean survival was noted up to the close of study observations (July 2018) or the death of the patient which was earlier.

*Genomic DNA extraction*

DNA extraction from FFPE tissues were done by using QIAamp FFPE tissue Kit, REF no. 56404 (Qiagen, Hilden, Germany) by following manufacturer's protocol. DNA quality and quantity checked by spectrophotometrically. Purity and integrity checked by agarose gel electrophoresis in 0.8% agarose gel.

*Bisulfite modification of DNA*

The genomic DNA isolated from the CRC tumor and adjacent normal tissue were subjected to bisulfite methylation analysis. Bisulfite conversion of DNA was done by using Epitect Bisulfite kit (Cat No./ID: 59104 Qiagen, Hilden, Germany) by following manufacturer's protocol designed for processing DNA isolated from FFPE tissue samples. Briefly, the 20µl solution of DNA (500ng-2µg) mix with 35 µl of DNA protecting buffer and 85 µl bisulfite mix and incubated for conversion in thermo cycler at recommended temperature. After completion of bisulfite conversion reaction, 310 µl freshly prepared buffer BL containing 10 µg/ml carrier RNA (Carrier RNA increases binding of DNA to the spin-column membrane) added to sample then sample transferred to spin columns after that washing by wash buffer. Followed by desulphonation step performed by adding 500 µl de-sulfonation buffer BD to the spin columns and incubate for 15 min at room temperature. Then sample twice washed by wash buffer. Then final bisulfite converted DNA eluted in 20 µl elution buffer. Bisulfited converted DNA used for MS-PCR analysis within 24 hours.

*Methylation specific PCR (MS-PCR)*

Methylation specific PCR was set up according to the method described by Herman et al., (1996). 2.5 µl bisulfite converted DNA was amplified using methylation specific primers that specifically recognized either the unmethylated or methylated *SFRP1* gene sequence after bisulfate conversion (Takada et al., 2004) . Sequences of the primers for MS-PCR of the *SFRP1* promoter region were commercially procured. The sequences for Methylated primer were Forward: 5′-TGTAGTTTTCGGAGTTAGTGTCGCGC-3′, Reverse : 5′- CCTACGATCGAAAACGACGCGAACG-3′ (126bp); unmethylated primers, Forward-5′-GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT-3′, Reverse:5′CTCAACCTACAAATCAAAAACAACAC AAACA-3′ (135bp). All PCR reactions were performed using AmpliTaq Gold PCR master mix PCR cycling conditions were as following: initial denaturation at 95$^{o}$C for 10 min then 35 cycles consisting of three steps: 95°C for 10s, respective annealing temperature for 30s at 59$^{o}$C, extension at 68$^{o}$C for 10s followed by a final extension at 72$^{o}$C for 10 min. The annealing temperature for amplification of methylated and un-methylated *SFRP1* promoter region was 59$^{o}$C, and 58$^{o}$C respectively. Methylated and un-methylated bisulfite converted human control DNA procured from Qiagen, Hilden, Germany was used as positive control for methylation and unmethylation.

*SFRP1 Promoter methylation sequencing analysis*

Validation of MS-PCR results and methylation pattern of CpG in promoter region was done by bisulfite sequencing using the method defined by Susan et al., (1994). MS-PCR products from tumor and normal tissues were sequenced by using ABI sequencing platform Genetic analyzer 3500, and sequence analysis and alignment was done using Bioedit software and CLUSTALW online tool.

*Statistical analysis*

Statistical analyses was done by using SPSS software (version 20). Chi square test was used to analyze the statistical association between clinic-pathological data and methylation status of *SFRP1*. Kaplan Meier survival curve and Log-rank test were used for survival analysis. To evaluate the prognostic impact, all clinicopathologic variables were evaluated along with *SFRP1* methylation status by using univariate cox proportional hazard model analysis. P-value <0.05 was considered as significant.

## Results

*Clinicopathological characteristics*

The clinicopathological details are summarized in Table 1. The median age of patients at the time of

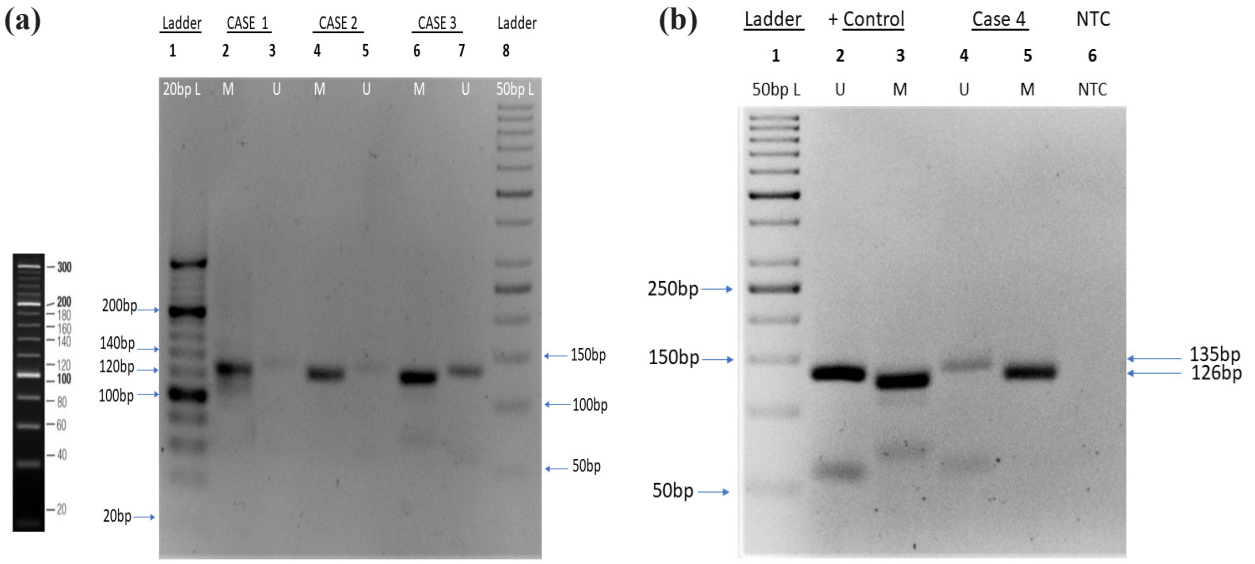


Figure 1. Promoter Methylation Analysis of *SFRP1* Gene in CRC Tumor Tissue by Methylation Specific Polymerase Chain Reaction (MS-PCR). MS-PCR amplified product run in 4.0% agrose gel. (a), Shows distinct band of methylated frgment in lane 2,4,6 and their intensity hingher than respective unmethylated band lane 3,5,7 in 3 Colorectal carcinoma tumour tissue. 20bp ladder in lane 1 and 50bp ladder in lane 8; (b), Shows the methylated DNA band in a CRC tumour tissue (lane 5) with control, positive control unmethylation (lane 2), positive control methyaltion (lane 3) and nigative control NTC ( lane 6) with 50 bp ladder (lane 1). Size of Methylated and Un-methylated fragment is 126bp and 135bp respectively. M (Methylation specific polymerase chain reaction), U (Un-methylation specific polymerase chain reaction), L (Ladder) NTC (Non Templete Control, water used instead of DNA template.

diagnosis was 49 years (range 18-76 years). Of these 33 (61.1%) were male and 21 (38.9%) were female patients (M:F = 1.57:1). 28 (51.85%) cases were of CRC stage II, and 26 (48.15%) stage III. The tumor site was right colon in 18 (33.3%), left colon (excluding rectum) in 20 (37%) and rectum in 16 (29.6%) cases. Histologically, 47 (87%) tumors were infiltrating adenocarcinoma NOS, 7 (13%) were mucinous adenocarcinoma. The tumor grade was well differentiated in 32 (59.3%) cases, moderate differentiation in 13 (24 %) and poor differentiation in 9 (16.6%) cases. Clinical follow up ranging from 12 to 56 months was available in these cases.

*SFRP1 gene promoter methylation in tumor tissue*

We analyzed *SFRP1* gene promoter methylation status in tumor tissue and adjacent normal tissue. In 39 out of 54 (72.2%) CRC cases *SFRP1* gene was methylated while in 15/54 (27.8%) cases it was unmethylated. Whereas in only

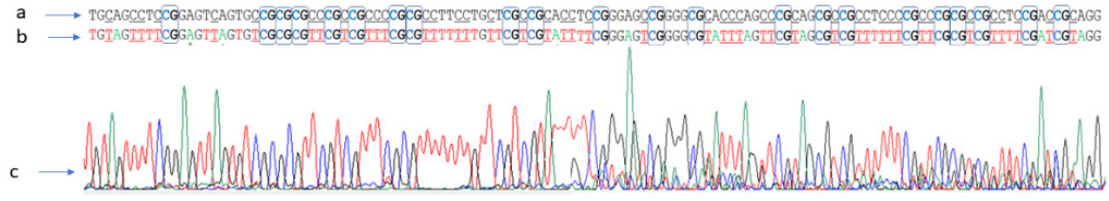


Figure 2a

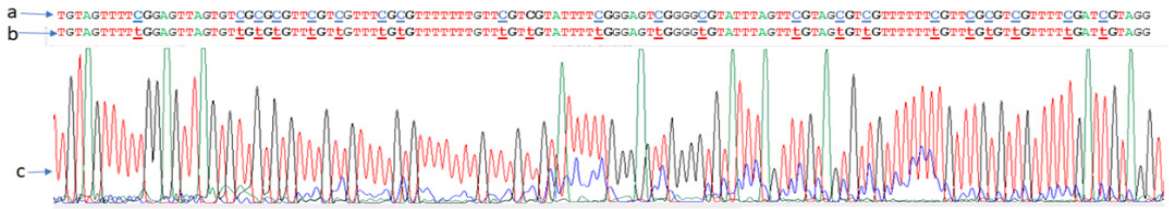


Figure 2b

Figure 2. *SFRP1* gene promoter methylation analysis by bisulphite sequencing. (a), Chromatogram of methylated SFRP1 gene promote – Bold blue C represents methylated cytosine that's remained unchanged during bisulfite conversion due to its methylation and C represents un-methylated cytosine that converted to thymine represented T, and CG in round corner rectangle represents CpG sites; (b), Chromatogram in a case of un-methylated SFRP1 gene t represented un-methylated cytosine that converted to thymine during bisulphite conversion. Arrow marked-"a" is sequence is normal 126bp of DNA sequence of SFRP1 gene of Homo sapiens *SFRP1* gene (NCBI Reference Sequence: NM_003012.4), "b" Bisulfite converted DNA sequence of methylated *SFRP1* gene of CRC case "c" chromatogram of bisulfite sequencing

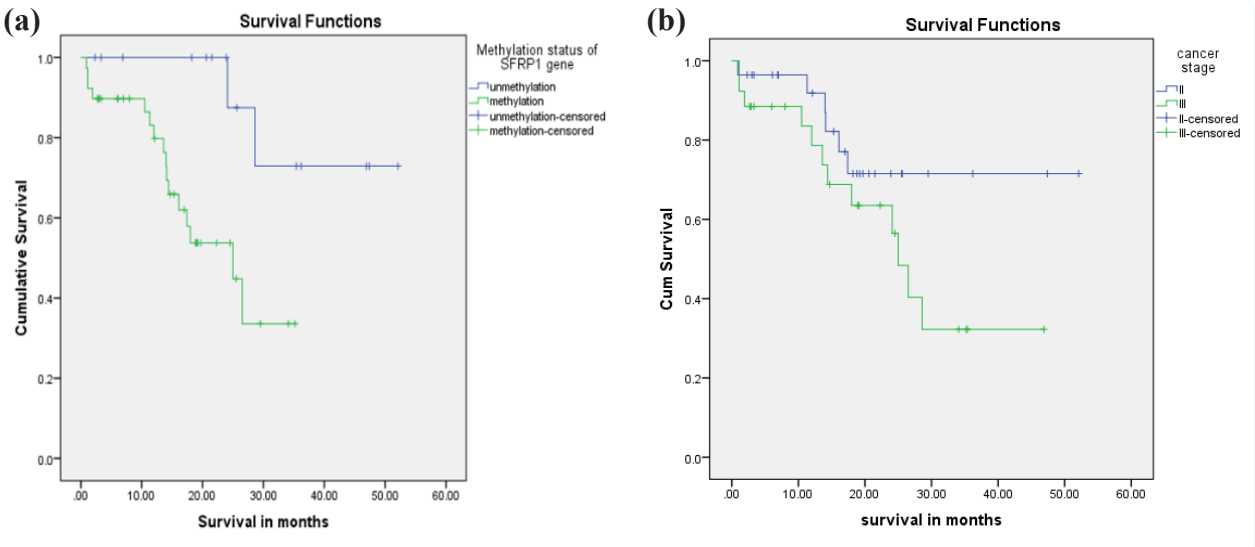


Figure 3. a, Kaplan-Meier survival analysis curve on the basis of methylation status of SFRP1 gene promoter region shows significance difference between methylated and un-methylated group p= 0.010 (Log rank test); b, Kaplan-Meier survival analysis curve on the basis of clinical stage II vs III P=0.135.

2 out of 28 (7%) cases adjacent non tumor tissue showed methylated *SFRP1* gene. Thus a significant difference in methylation status (P<0.0001) was present between tumor and non-tumor tissue (Figure 1). Methylated in relation to clinical stage was noted in 60.7% cases of stage II and 84.6 % cases of stage III tumor. To ascertain the methylation status of CpG sites present within promoter region of *SFRP1* gene, we performed Bisulfite Sequencing of the 126 bp DNA fragment of *SFRP1* gene amplified by MS-PCR in representative cases. Bisulphite sequencing showed methylated Cytosine nucleotide in the CpG sites. This 126 bp DNA sequence, in the cases showing methylated *SFRP1* in MS-PCR, contained 22 CpG cites within which most of the cytosine nucleotides were methylated (Figure 2).

*SFRP1 promoter methylation associated with lymph node invasion*

Promoter methylation status of *SFRP1* gene was compared with patient's clinicopathological characteristics such as age, gender, tumor location, lymph node involvement, tumor stage, and tumor grade (Table 1). Chi square test results show that lymph node metastasis was significantly associated with methylation status of *SFRP1* gene in the tumor. Lymph node involvement (pN1-3) was noted in 84.6% cases with methylated *SFRP1*. Location of tumor was not associated with methylation status. A higher frequency of methylation was observed in patients over 60 years age, however this was not statistically significant.

Table 1. Clinicopathological Characteristics of CRC Cases and Their Association with Methylation

| Variable | Categories | No. of cases | Methylation status of *SFRP1* gene promoter | | *p-value* |
|---|---|---|---|---|---|
| | | | Un-methylated n =15 (27.8%) | Methylated n=39 (72.2%) | |
| Age group | <50 year | 27 | 9 (33.3) | 18 (66.7) | 0.54 |
| | >50 year | 27 | 6 (22.2) | 21 (77.8) | |
| Gender | Male | 33 | 8 (24.2%) | 25 (75.8%) | 0.54 |
| | Female | 21 | 7 (33.3%) | 14 (66.7%) | |
| Tumour Stage | T2 | 9 | 3 (33.3%) | 6 (66.7%) | 0.77 |
| | T3 | 23 | 7 (30.4%) | 16 (69.9%) | |
| | T4 | 22 | 5 (22.7%) | 17 (77.3%) | |
| Lymph-node involvement | pN0 | 28 | 11 (39.3%) | 17 (60.7%) | 0.05 |
| | pN1-3 | 26 | 4 (15.4%) | 22 (84.6%) | |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 13 (27.7%) | 34 (72.) | 1 |
| | Mucinous adenocarcinoma | 7 | 2(28.6%) | 5 (71.4%) | |
| Tumour grade | Poorly differentiated | 9 | 3 (33.3%) | 6 (66.7%) | 0.83 |
| | Moderately differentiated | 13 | 4 (30.8%) | 9 (69.2%) | |
| | Well differentiated | 32 | 8 (25%) | 24 (75%) | |
| Tumour location | Colon | 38 | 10 (26.3) | 28 (73.7) | 0.747 |
| | Rectum | 16 | 5 (31.2%) | 11 (68.8%) | |

Table 1- Showing association of clinico-pathological parameters in relation to methylation status of SFRP1 gene using Chi square test (significant p-value <0.05).

Table 2. Association between Clinicopathological Characteristics and Prognosis of the Disease by Using Univariate Cox Regression Analysis

| Variables | Categories | No. of cases | No of Events (deaths) | HR | 95% CI# (Lower-Upper) |
|---|---|---|---|---|---|
| Age group | <50 year | 27 | 11 | 1 | Ref. |
| | >50 year | 27 | 7 | 0.672 | (0.183-2.475) |
| Gender | Male | 33 | 13 | 1 | Ref. |
| | Female | 21 | 5 | 0.308 | (0.089-1.063) |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 14 | 1 | Ref |
| | Mucinous adenocarcinoma | 7 | 4 | 2.404 | (0.664-8.701) |
| Tumor grade | Poorly differentiated | 9 | 4 | 1 | Ref. |
| | Moderately differentiated | 13 | 3 | 0.267 | (0.042-1.681) |
| | Well differentiated | 32 | 11 | 0.333 | (0.082 -1.356) |
| Tumor stage | pT2 | 9 | 3 | 1 | Ref. |
| | pT3 | 23 | 6 | 0.387 | (0.075-1.982) |
| | pT4 | 22 | 9 | 0.755 | (0.144-3.955) |
| Tumor location | Colon | 38 | 11 | 1 | Ref. |
| | Rectum | 16 | 7 | 1.15 | (0.391-3.385) |
| Lymph node involvement | pN0 | 28 | 6 | 1 | Ref. |
| | pN1-3 | 26 | 12 | 1.281 | (0.391-4.471) |
| Methylation status of SFRP1 gene promoter | Un-methylation | 15 | 2 | 1 | Ref. |
| | Methylation | 39 | 16 | 17.313 | (2.021-148.290) |

HR, (Hazard Ratio); #, Hazard Ratio (95% Confidence Interval); Ref., taken as reference

*Methylation of SFRP1 decreases the overall survival*

Based on the observed methylation status of *SFRP1* gene in the tumor tissue, methylated and unmethylated groups were defined. Follow-up in 54 patients ranging from 12 month to 56 month (median follow-up 28 months) 18 CRC patients had died due to disease related event and advanced tumor stage and 12 patients lost to follow-up. The overall mean survival of unmethylated and methylated group was 45.173 months and 22.318 months respectively survival curve in (Figure 3a). The combined estimated OS of both the groups were 33.461 months. Unmethylated groups survival was significantly better as compare to methylated group (p= 0.010 by Log rank test) and poor survival associated with methylation of *SFRP1*. We also analyzed survival of CRC patients with reference of 8 deferent conventional pathological factors such as Age group, Gender, Tumor stage, Clinical stage, Lymph node status, Differentiation of tumor and Tumor subtype (Table 3). Kaplan-Meir survival analysis results shows survival is dependent on many factors but it was majorly influenced by lymph node status and methylation of *SFRP1*. If we talk about survival on the basis of clinical

Table 3. Kaplan-Meier Survival Analysis with References to Clinicopathological Characteristics.

| Variables | Categories | No. of cases | No of Events (death) | Mean Survival in months | P value |
|---|---|---|---|---|---|
| Methylation status of *SFRP1* | Un-methylation | 15 | 2 | 45.17 | 0.01 |
| | methylation | 39 | 16 | 22.32 | |
| Age group | >50 year | 27 | 7 | 34.68 | 0.472 |
| | <50 year | 27 | 11 | 28.66 | |
| Lymph-node | pN0 | 28 | 6 | 40.96 | 0.135 |
| involvement | pN1-3 | 26 | 12 | 26.88 | |
| Gender | Male | 33 | 13 | 29.34 | 0.146 |
| | Female | 21 | 5 | 29.75 | |
| Tumour grade | Poorly differentiated | 7 | 3 | 27.10 | 0.68 |
| | Moderately differentiated | 14 | 4 | 29.33 | |
| | Well differentiated | 26 | 7 | 32.78 | |
| Tumour stage | pT2 | 9 | 3 | 26.99 | 0.284 |
| | pT3 | 23 | 6 | 37.81 | |
| | pT4 | 22 | 9 | 18.95 | |
| Histological type | Infiltrating adenocarcinoma NOS | 47 | 14 | 34.70 | 0.142 |
| | Mucinous adenocarcinoma | 7 | 4 | 24.85 | |
| Location of tumour | Colon | 38 | 11 | 26.17 | 0.931 |
| | Rectum | 16 | 7 | 34.06 | |

stage II and III the mean survival was 40.96 and 26.88 months respectively p=0.135 by log rank test (Figure 3b). To explore the contribution of these variables and evaluation of their influence as potential prognostic marker, all these variables were analyzed by univariate cox regression model analysis. In univariate analysis, only *SFRP1* methylation status could be verified as an independent prognostic factor, (HR = 17.313, 95% CI: 2.021-148.290, and *P-value* = 0.009, Table 2). Univariate cox model suggesting, among these variables, *SFRP1* methylation can serve as an independent prognostic indicator of poor survival in CRC.

## Discussion

Wnt signaling plays important role in embryonic development where it determines the cell fate, cell proliferation and cell migration (Clevers and Hans, 2006; Zhan et al. 2016). In life, Wnt signaling also controls tissue regeneration in adult bone marrow, skin and intestine (Goessling et al., 2009). Wnt signaling maintains intestinal stem cells by proliferation and differentiation. It is also involved in carcinogenesis of various tumors including CRC (Mii and Taira, 2011; Zhan et al., 2016; Zhou et al., 2015; Clevers and Hans, 2006; Huang et al., 2006). *SFRP1* gene is known for its ability to negatively modulate the Wnt/β-catenin signaling cascade. Promoter methylation downregulates the expression of *SFRP1* gene in CRC (Jones and Jomary, 2002; Suzuki et al., 2004; Shih et al., 2006, Fukui et al., 2005) Silencing of *SFRP1* gene, allows constitutive WNT signaling via binding to Wnt protein and inhibits its binding to Wnt-frizzled receptor, consequently altering the proliferation and differentiation of tumor cells. Limited studies have looked in to the association of *SFRP1* methylation with clinicopathological characters and survival in CRC. Studies done on other tumors suggest that methylation of *SFRP1* gene can serve as epigenetic diagnostic, prognostic and predictive marker in liver, gall bladder, upper gastrointestinal tract and lung cancers (Kim et al., 2016; Mo et al., 2018; Suzuki et al., 2002; Müller et al., 2004; Zou et al., 2005; Su et al., 2009) In the present study, we have studied promoter methylation status of *SFRP1* gene in CRC patients and its association with various clinicopathological characteristics. We found that *SFRP1* was frequently methylated in tumor tissue compared with adjacent non tumor tissues. The frequency of *SFRP1* gene promoter methylation in our patients was 72.2%. Previous studies, have shown a frequency ranging from 52-95% in colorectal cancer (Rawson et al., 2011; Barták, 2017; Zhou et al., 2015; Meng et al., 2011; Dallol et al., 2012; Salehi et al., 2012). In present study we noted a slightly higher incidence of hypermethylation of *SFRP1* in male patients than females (75.8% vs. 66.7%), however this difference was not statistically significant (p=0.54). The frequency of methylation was not influenced by the histological subtype of tumor. Infiltrating adenocarcinoma NOS and mucinous adenocarcinoma showed *SFRP1* gene promoter methylation frequency of 72.3% and 71.4% respectively. In 84% cases with lymph node metastasis, *SFRP1* gene methylation was noted which was significant (P=0.05). Other clinicopathological characters such as age, gender, tumor, location, tumor stage, tumor type, grade of tumor did not show any significant association with methylation status of *SFRP1* gene. Our data suggests that *SFRP1* promoter methylation is an epigenetic prognostic marker for poor survival in stage II and III CRC. The patients in the methylated group had shorter mean overall survival (22.318 months) as compared to the un-methylated group (45.173 months). A possible reason for shorter overall survival with methylated *SFRP1* gene in CRC could be that promoter methylation reduces expression of *SFRP1* gene allowing constitutive WNT signaling that may help tumor cell to proliferate. Epigenetic inactivation of SFRP genes allowing constitutive WNT signaling in colorectal cancer has been previously described by Suzuki et al., (2004). *SFRP1* gene has also been studied in other tumors such as Head and neck squamous Cell carcinoma, breast cancer and found to indicate poor patients survival (Alsofyani et al., 2016, Veeck et al., 2008, Kang et al., 2014) An implication of this findings can also be explored for targeted therapy in CRC using recombinant *SFRP1* (Cooper et al., 2012).

This study has some limitations such as smaller sample size and shorter duration of follow-up. However even then the findings obtained are important with prognostic significance. Similar studies with larger sample size and longer follow-up would be helpful to substantiate our findings.

In conclusion our study shows that promoter methylation of *SFRP1* gene occurs frequently in Colorectal Carcinoma. This *SFRP1* promoter methylation is significantly associated with lymph node invasion and poor survival outcome in stage II and III CRC patients and it appears to be a poor prognostic marker.

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*Date:15.09.2019*

To
**Dr. G. Sunil Babu,**
Assistant Professor,
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**Subject-**Acceptance letter for the manuscript entitled "Promoter methylation analysis of Suppressor of Cytokine Signaling-1 (SOCS1) gene in Colorectal cancer patients and its association with Clinicopathological characteristics"

Dear Sir,

Your manuscript has been peer reviewed by the reviewer's panel of IJSTS. As per the comments and review articulated by the reviewer's, we are pleased to inform you that your manuscript entitled **"Promoter methylation analysis of Suppressor of Cytokine Signaling-1 (SOCS1) gene in Colorectal cancer patients and its association with Clinicopathological characteristics" (Authors:** Alok Kumar, Pradyumn Singh, Anshuman Singh, Sunil Babu Gosipatala**)** has now been accepted and will appear in Volume 5, 2019 of IJSTS, which is scheduled on Nov 2019.

We appreciate your contribution and association with us & it is requested to you to motivate your colleagues and fellow researchers to publish their work in our esteemed journal.

Thank you for submitting your work in IJSTS.

(Prof. Shubhini A. Saraf)

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**Date:** 06 Aug 2020
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**From:** "Molecular Biology Reports (MOLE)" Abishek.Sundaram@springernature.com
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Dear Dr. Gosipatala:

I am pleased to inform you that your manuscript, "IGFBP3 gene promoter methylation analysis and its association with clinicopathological characteristics of Colorectal carcinoma" has been accepted for publication in Molecular Biology Reports.

Please remember to always include your manuscript number, #MOLE-D-19-03987R1, whenever inquiring about your manuscript.

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Rodrigo Guimarães
Editor-in-Chief
Molecular Biology Reports

COMMENTS TO THE AUTHOR:
Reviewer #1: The authors have sufficiently addressed my requests.

Note that IGFBP-3 and IGFBP3 are used interchangeably throughout the manuscript. Please correct. It might be clearer to use IGFBP3 as you're referring to both the locus and the protein throughout. The HGNC gene symbol of the locus is IGFBP3, while the common protein aliases can be IGFBP3 or IGFBP-3.

—

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Molecular Biology Reports

# IGFBP3 gene promoter methylation analysis and its association with clinicopathological characteristics of Colorectal carcinoma

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | MOLE-D-19-03987R1 |
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| **Abstract:** | Background- Promoter methylation mediated silencing of tumor suppressor genes plays an important role in the tumorigenesis of colorectal carcinoma (CRC). Tumor suppressor gene, Insulin-like Growth Factor Binding Protein-3 ( IGFBP-3 ) expression is frequently downregulated in CRC due to promoter methylations. The aim of this study was to analyze the methylation status of IGFBP-3 gene promoter in stage II and III of CRC cases; find its association with clinicopathological characteristics of CRC patients and the methylation patterns as a prognostic biomarker.<br>Materials and methods- 58 histopathologically confirmed cases of CRC were included in the study. Methylation status of IGFBP-3 gene promoter was determined by using methylation specific PCR (MS-PCR) and bisulfite sequencing. Kaplan-Meier survival curve and univariate cox regression analysis were used for survival analysis; Chi-square test used for association analysis.<br>Results- IGFBP3 promoter methylation was found in 37 (63.8%) out of 58 CRC cases. This promoter methylation status was significantly associated with lymph-node metastasis (p=0.013) and the survival period. In stage II CRC cases, unmethylated gene promoter status showed better survival than the methylated. Mean overall survival (OS) of methylated and unmethylated group was 22.23 months, and 49.15 months respectively (p=0.045), HR=6.432, 95% CI: 0.986-41.943.<br>Conclusion- The IGFBP-3 promoter methylations found in 63.8% CRC cases in this study. The methylations was found to be associated with lymph-node metastasis and overall survival of the patients particularly in stage II CRC patients. However, promoter methylation was not associated with other clinocopathological characteristics such as age, gender, tumor location etc. |

# APPENDIX

**RAM MANOHAR LOHIA INSTITUTE OF MEDICAL SCIENCES, LUCKNOW**

# INFORMED CONSENT FORM

Study Title- **A Study of newer prognostic epigenetic markers SFRP1 & IGFBP3 in Colorectal carcinoma**

Study Number_____
Subject's Full Name_____
Date of Birth/Age_____
Address_____
_____

1. I confirm that I have read and understood the information sheets dated_____ for the above study and have the opportunity to ask questions.

OR I have been explained the nature of the study by the Investigator and had the opportunity to ask questions.

2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason and without my medicine care or legal rights being affected.

3. I understand that the sponsor of the clinical trial/ project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and my further research that may be conduct in relation to it, ever if I withdraw from the trial. However, I understand that my Identity will not be revealed in any information released to third parties or published. I will not be entitled for any compensation.

4. I agree not to restrict the use of any data or result that arises from this study [provided such a use is only for scientific purpose(s)

5. I agree to take in the above study

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative
e:_____
Signatory's Name_____ Date_____
Signature of the Investigator_____ Date_____
Study Investigator's Name_____
Signature of Witness_____ Date_____
Name of Witness_____

## डॉ राम मनोहर लोहिया आयुर्विज्ञान संस्थान, लखनऊ

## <u>सूचित सहमति पत्र</u>

अध्ययन शीर्षक: **कोलोरेक्टल कार्सिनोमा में नए इपीजेनेटिक शकुन सूचकों (प्रोग्नोस्टिक मार्कर) SFRP1 & IGFBP3 का एक अध्ययन**

अध्ययन नंबर :

सहभागी का पूरा नाम :

जन्म तिथि/उम्र :

पता:

1. मुझे अध्ययन अनवेक्षक ने विस्तार से सब तथ्यो को समझा दिया है तथा मुझे प्रश्न पूछने का अवसर प्रदान किया ।

2. मैने समझ लिया है की इस अध्ययन मे मेरी प्रतिभागिता स्वाएचिक है, तथा यह की मैं बिना कोई कारण बताए किसी भी समय अपनी चिकित्सीय देखभाल या क़ानूनी आधिकारों पर भाव पड़े बिना हट जाने के लिए स्वतन्त्र हूँ ।

3. मैने समझ लिया है की इस चिकित्सीय सैयोजक की ओर से काम करने वाले अन्य, नैतिकता समिति तथा विनियामक अधिकारियों का चालू इससे संबंधित तथा हो सकने वाले किसी अनुसंधान से संबंधित मेरे स्वास्थ्य अभिलेखों को देखने के लिए मेरी अनुमति की आवश्यकता नही होगी, भले ही मैं इस परीक्षण से हट ही क्यों न जाऊं । तथापि मैंने समझ लिया है की तृतीय पक्ष को दी गई या प्रकाशित की गई किसी जानकारी में मेरी पहचान को उजागर नहीं किया जायेगा तथा मुझे किसी की प्रकार की श्रतिपूर्ति देय नही होगी ।

4. इस अध्ययन में प्राप्त किन्ही आकड़ो या परीक्षणों के प्रयोग पर पाबंदी न लगाने के लिए मैं सहमत हूँ बशर्ते कि ऐसे प्रयोग मात्र वैज्ञानिक प्रयोजन/नो के लिए ही हों ।

5. उपर्युक्त अध्ययन में भाग लेने के लिए मैं सहमत हूँ ।

सहभागी के हस्ताक्षर या अंगूठे का निशान /क़ानूनी रूप से स्वीकार्य प्रतिनिधि

हस्ताक्षर करने वाले का नाम दिनांक

अध्ययन अन्वेषक के हस्ताक्षर दिनांक

अध्ययन अन्वेषक का नाम दिनांक

गवाह के हस्ताक्षर दिनांक

गवाह के नाम

**DR. RAM MANOHAR LOHIA INSTITUTE OF MEDICAL SCIENCES, LUCKNOW- 226010**

**INFORMED CONSENT PROCESS**

# A Study of newer prognostic epigenetic markers SFRP1 & IGFBP3 in Colorectal Carcinoma

1. Nature and purpose of study stating it as research

The main purpose of study is development of good prognostic molecular marker for disease prognostication in colorectal cancer. Prognostic markers are factors that predict the likely outcome of disease such as risk of relapse or disease progression. In this study we are looking methylation of SFRP1 and IGFBP3 genes correlates its significances as a prognostic marker for CRC patients.

2. Duration of participation with number of participants
**Duration of Study 18 month and number of participants- 50 (approx.)**

3. Procedures to be followed –- **NA**

4. Investigations, if any, to be performed - **NA**

5. Foreseeable risks and discomforts adequately described and whether project involves more than minimal risk- **NO risk**

6. Benefits to participant, community or medical profession as may be applicable-
**The information obtained from this study would help us to treat patient with colorectal carcinoma in a better way in future ".**

7. Policy on compensation- **NA**

8. Availability of medical treatment for such injuries or risk management- **NA**

9. Alternative treatments if available- **NA**

10. Steps taken for ensuring confidentiality- **All information collected about you during the course of the research will be kept strictly confidential. Any information which leaves the hospital/clinic/laboratory will have the patient identity removed so that individual patients will not recognized from it."**

11. No loss of benefits on withdrawal- **NA**

12. Benefit sharing in the event of commercialization- **NA**

13. Contact details of PI or local PI/Co-PI in multicentric studies for asking more information related to the research or in case of injury-
Dr. Pradyumn Singh, Additional Professor, Dept. of Pathology Dr. Ram ManoharLohia Institute of Medical Sciences, Lucknow-226010**.**

14. Contact details of Chairman of the IEC for appeal against violation of rights-

15. Voluntary participation --- **NO**

16. If test for genetics and HIV is to be done, counselling for consent for testing must be given as per national guidelines- **NA**

17. Storage period of biological sample and related data with choice offered to participant regarding future use of sample, refusal for storage and receipt of its results – 3 year

# डॉ राम मनोहर लोहिया आयुर्विज्ञान संस्थान, लखनऊ

## सूचित सहमति प्रक्रिया (INFORMED CONSENT PROCESS)

### अध्ययन शीर्षक- कोलोरेक्टल कार्सिनोमा में नए इपीजेनेटिक शकुन सूचकों (प्रोग्नोस्टिक मार्कर) SFRP1 & IGFBP3 का एक अध्ययन

1. अनुसंधान के रूप में अध्ययन का उद्देश्य और प्रकृति

इस अध्ययन का मुख्य उद्देश्य कोलोरेक्टल कैंसर रोग के शकुन (पूर्वानुमान) के लिए अच्छे आणविक भविष्यसूचक (मॉलिक्युलर प्रोग्नॉस्टिक मार्कर) का विकास है। भविष्यसूचक ऐसे कारक हैं जो कि रोग प्रगति के जोखिम या पतन के रूप में रोग की संभावित परिणाम की भविष्यवाणी है । इस अध्ययन में हम देख रहे हैं SFRP1 और IGFBP3 जीन की मेथिलिकरण कोलोरेक्टल कार्सिनोमा रोगियों के लिए एक शकुन मार्कर के रूप में अपने महत्व संबद्ध।

2. प्रतिभागियों की संख्या और भागीदारी की अवधि।

प्रतिभागियों की संख्या 50 (लगभग) भागीदारी की अवधि अध्ययन 18 महीने (लगभग)

3. प्रक्रियाओं का पालन किया जाना है- **लागू नहीं**

4. जांच, यदि कोई हो, प्रदर्शन किया जाएगा- **लागू नहीं**

5. निकट जोखिम और पर्याप्त रूप से वर्णित असुविधाएँ और चाहे न्यूनतम जोखिम से अधिक परियोजना शामिल है- **कोई जोख़िम नहीं**

6. भागीदार को लाभ, समुदाय या चिकित्सा के पेशे के रूप में लागू हो सकता है ?-

जानकारी इस अध्ययन से प्राप्त हमें भविष्य में एक बेहतर तरीका में कोलोरेक्टल कार्सिनोमा के साथ मरीज का इलाज करने में मदद मिलेगी। '

7. मुआवजे पर नीति - **लागू नहीं**

8. इस तरह की चोटों या जोखिम प्रबंधन के लिए चिकित्सा उपचार की उपलब्धता- लागू नहीं

9. वैकल्पिक उपचार यदि उपलब्ध है- **लागू नहीं**

10. गोपनीयता सुनिश्चित करने के लिए उठाए गए कदम।

"सभी अनुसंधान / परीक्षण के दौरान आप के बारे में एकत्र जानकारी कड़ाई से गोपनीय रखी जाएगी | कोई भी जानकारी है जो अस्पताल / क्लिनिक और प्रयोगशाला से बाहर जाएगी, तो उसके उपर से आपका नाम और पता हटा दिया जायगा

11. वापसी पर लाभ का कोई नुकसान?- **लागू नहीं**

12. व्यावसायीकरण की स्थिति में साझा लाभ।- **लागू नहीं**

13. अनुसंधान करने के लिए या चोट के मामले में संबंधित अधिक जानकारी के लिए पूछ अध्ययन में गड़बड़ी या स्थानीय पीआई / सह पीआई के संपर्क विवरण-

**प्रमुख अन्वेषक: डॉक्टर प्रद्युमन सिंह**
अडीशनल प्रोफेसर, पैथोलॉजी विभाग
डॉ राम मनोहर लोहिया आयुर्विज्ञान संस्थान, लखनऊ-226010

14. अधिकारों के उल्लंघन के खिलाफ अपील के लिए आईईसी के अध्यक्ष के संपर्क का विवरण

15. स्वैच्छिक भागीदारी- **नहीं**

16. यदि आनुवंशिकी और एचआईवी के लिए परीक्षण किया जाना है, तो राष्ट्रीय दिशा निर्देशों के अनुसार परीक्षण सहमति के लिए परामर्श के लिए दी जानी चाहिए - **लागू नहीं**

17. नमूना, भंडारण के लिए इनकार के भविष्य में प्रयोग और उसके परिणामों की प्राप्ति के संबंध में भागीदार के लिए जैविक नमूने और पसंद के साथ संबंधित डेटा के भंडारण अवधि की पेशकश की- **3 साल**

# Study Proforma

**Patient Name:**

Study ref no

CR No.

Date of Birth: Age: **Sex:** Male ☐ Female ☐

**Father's name:**

Date of presentation

Date of Surgery

Address:

Consultant doctor / Dept. / unit

Contact number (mobile/ home):
Email:
**Relevant History:**
Occupation:

**Diet: –** Veg ☐ Non veg. ☐ **Habitat: -** Urban ☐ Rural ☐

**Smoker: Y / N** Bidi ☐ Cigarette ☐ How often and when? .....

**Tobacco: Y / N** Gutka ☐ Khaini ☐

**Alcohol –** Y/N Occasional ☐ habitual ☐
Any Previous disease or Co morbidity: DM / HTN / TB / Any other ……
Previous biopsy (if any) Ref No. ……

**Clinical findings:** (to be filled by the surgeon/clinician)

Endoscopic findings –
Biopsy-
Imaging -
CT scan-
MR
Endo-anal/ TRUS/MR any other –

**Clinical obstruction**: Absent ☐ Present ☐ **Perforation:** Absent ☐ Present ☐
**Nature of perforation:** Through tumour prior to surgery ☐ Away from tumour ☐
Through tumour during surgery mobilisation ☐

**Tumour location:**

| | | | | | |
|---|---|---|---|---|---|
| Caecum | ☐ | Ascending colon | ☐ | Hepatic flexure | ☐ |
| Transverse colon | ☐ | Splenic flexure | ☐ | Descending colon | ☐ |
| Sigmoid colon | ☐ | Rectosigmoid junction | ☐ | Rectum | ☐ |

**For synchronous tumours indicate each other site** (Note: Synchronous tumours should be reported separately – this identifies the presence of other synchronous tumours for which separate evaluation would be done.)

**Type of operation**

| | | | | | |
|---|---|---|---|---|---|
| Right hemicolectomy | ☐ | Extended right hemicolectomy | ☐ | Abdominoperineal resection | ☐ |
| Transverse colectomy | ☐ | Left hemicolectomy | ☐ | Anterior resection(High /Low /Ultralow) | ☐ |
| Proctocolectomy | ☐ | Hartmann's procedure | ☐ | Total colectomy with ileorectal anastomosis | ☐ |
| Other procedure(s): | ☐ | | | | |

**Pre-operative radiotherapy:** Y / N (if yes) Short course / Long course
**Surgeon's opinion on the existence of local residual cancer postsurgery:**

**Involvement of adjacent organs**

**New primary cancer or recurrence:** New primary ☐ Regional (local) recurrence ☐
Distant metastases (if yes) Details ☐

**Other relevant details**

Peritoneum:-
☐ Tumour invades to the peritoneal surface
☐ Tumour has formed nodule(s) discrete from the tumour mass along the serosal surface

**Histopathological Examination:**
**Macroscopic findings**

**Lymph nodes received (if yes)** ☐ **not received** ☐
No. of lymph nodes ☐
Above the tumour level ☐ at the tumour level ☐ below the tumour level ☐
**Polyps** Absent ☐ Present (if present) ☐
Polyp summary (numbers, diameter range and gross appearance)
Other macroscopic comments
Sessile ☐ pedunculated ☐
Length of stalk in cm ……..

**Specimen length** in mm –
**Tumour site**

| | | | | | |
|---|---|---|---|---|---|
| Caecum | ☐ | Ascending colon | ☐ | Hepatic flexure | ☐ |
| Transverse colon | ☐ | Splenic flexure | ☐ | Descending colon | ☐ |
| Sigmoid colon | ☐ | Rectosigmoid junction | ☐ | Rectum | ☐ |

**Maximum tumour diameter-** ……Mm
**Distance of tumour to the nearer proximal or distal 'cut end'** …….Mm
**Distance of tumour to the nonperitonealised circumferential margin……** mm
**Tumour perforation** Absent ☐ Present ☐

**Relationship to anterior peritoneal reflection (rectal tumours)**
Entirely above ☐ Astride ☐ Entirely below ☐
**Intactness of mesorectum (rectal resections)**
Incomplete (grade 1) ☐
Nearly complete (grade 2) ☐
Complete (grade 3) ☐

**Microscopic findings**

**Tumour type**
Adenocarcinoma, NOS ☐
Cribriform comedo-type adenocarcinoma ☐
Medullary carcinoma, NOS ☐
Micropapillary carcinoma ☐
Colloid carcinoma ☐
Serrated adenocarcinoma ☐
Signet ring cell carcinoma ☐
Adenosquamous carcinoma ☐
Spindle cell carcinoma, NOS ☐
Squamous cell carcinoma, NOS ☐
Undifferentiated carcinoma ☐
**Histological grade**

☐ Low grade
well and moderately differentiated

☐ High grade
poorly and undifferentiated

**Maximum degree of local invasion into or through the bowel wall**
☐ pT1-Tumour invades submucosa
☐ pT2-Tumour invades muscularis propria
☐ pT3-Tumour invades through muscularis propria into pericolorectal tissues
☐ pT4a-Tumour penetrates to the surface of the visceral peritoneum
☐ pT4b-Tumour directly invades or is adherent to other organs or structures

**Involvement of the proximal or distal resection ('Cut end') margins**
☐ **Involved** (distal ☐ /proximal ☐ )
☐ **Not involved (**Clearance if mrgin is less than 10 mm)

**Status of the nonperitonealised circumferential margin (rectal tumours)**
☐ Involved
☐ Not involved
Microscopic clearance…….mm

**Lymph node involvement**
☐ Absent
☐ Present (if present)

**SITE 1** Number of positive nodes /Total number of nodes from this site

/

**SITE 2** Number of positive nodes / Total number of nodes from this site

/

**SITE 3** Total number of nodes from this site /Number of positive Nodes

/

**Isolated extra-mural tumour deposits**:

Absent ☐

Present ☐

Apical node involvement

Not applicable ☐

Absent ☐

Present ☐

**Venous and small vessel invasion**

Intramural vein invasion- Not identified ☐ Present ☐

Extramural vein invasion-Not identified ☐ Present ☐

Small vessel invasion-

Not identified ☐ Present ☐ Present and extensive ☐

Perineural invasion -

Not identified ☐ Present ☐ Present and extensive ☐

**Histologically confirmed distant metastases**

☐ Absent

☐ Present (if present) Site(s)-

**Relevant coexistent pathological abnormalities**

☐ None noted

☐ Ulcerative colitis-

With dysplasia ☐

Without dysplasia ☐

☐ Crohn's disease-

With dysplasia ☐

Without dysplasia ☐

Other ☐

If other describe

Polyps ☐

Polyp details (type, number, polyposis syndrome criteria met etc.)

**Microscopic residual tumour status (completeness of resection)**

**Response to neoadjuvant therapy**

**No prior treatment** ☐

**Grade 0 (complete response)** ☐

No viable cancer cells ☐

**Grade 1 (moderate response)**

Single cells or small groups of cancer cells

**Grade 2 (minimal response)** ☐

Residual cancer outgrown by fibrosis

**Grade 3 (poor response)** ☐

Minimal or no tumour kill; extensive

Microscopic comments

**Ancillary test findings**

**CEA level Pre operation**

**Post operation**

**Day**

**Histopathology:**

**Microsatellite instability (MSI)**:

Unstable ☐ Stable ☐ Not tested ☐

Lab performing test and report number:

Comments

*BRAF* (V600E mutation):

Mutated ☐ Wild type ☐ Not tested ☐

Lab performing test and report no.& Comments

*KRAS* gene mutation (codons 12 and 13)*:*

Mutated ☐ Wild type ☐ Not tested ☐

Lab performing test and report no.&Comments

**Synthesis and overview**

**Tumour stage (AJCC 2010)**

T

N

M

**Stage group**

**Residual tumour status**

R0: Complete resection, margins histologically negative, no residual tumour left after resection (primary tumour, regional nodes)

R1: Incomplete resection, margins histologically involved, microscopic tumour remains after resection of gross disease (primary tumour, regional nodes)

R2: Incomplete resection, margins macroscopically involved or gross disease remains after subtotal resection (eg primary tumour, regional nodes, or liver metastasis).

**Follow up**

| Visit Date | Any new complaints | Clinical opinion | Investigation | Remarks |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |