

REVIEW ARTICLE

Molecular Signatures of Colorectal Cancer: A Prospective Study for Improvisation of Treatment Strategies

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ABSTRACT

Colorectal cancer (CRC) is the third most common cancer in males and second in females. Although there is an alarming increase in the number of cases in the last few decades, limited treatment strategies are available for these alterations. Chromosome Instability (CIN), which alone accounts for 85% of sporadic CRCs, is attributed by aberrant stem cell renewal process, chromatin remodeling, chromosome translocation, cell cycle checkpoints, genome maintenance and cell survival pathways; whereas the MSI pathway, hallmark of HNPCC/Lynch syndrome are associated with alterations of genes associated with genome maintenance. CIMP or methylator phenotype has been classified into "high" or "low", based on methylation of CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1. While familial CRC accounts for ~15 to 20% of global CRC, sporadic CRC accounts for 70–85% of CRC cases, involving CIN, MSI and CIMP pathways. Conventional treatment strategies of CRC with DNA intercalators alone or in combination with angiogenesis inhibitors can be further renovated with the approach of immunotherapy. In summary, a detailed analysis of genetic and epigenetic alterations would help in understanding of molecular subtypes and evolving multimodal treatment strategy for better prognosis and management of CRC.

KEYWORDS: CRC, CIN, MMR, CIMP

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in males and the second in females [1]. Based on the number of key pathways altered, underlying genetic and epigenetic changes, CRC has been classified into different subtypes with representative clinicopathological variations in CRC patients. However, limited treatment strategies are available for these alterations. Improved knowledge of alterations of different pathways in CRC led to the development of more innovative clinical trial design. Though the progression oriented treatment strategy include broad spectrum of mutational analysis prior to targeted therapy, a detailed analysis of genetic and epigenetic alterations would help in understanding of molecular subtypes and evolving multimodal treatment strategy for better prognosis and management of CRC.

It is known that approximately 20% of CRC cases are familial. Distinct genetic susceptibility to CRC has been reported to be associated with Lynch Syndrome (Hereditary Nonpolyposis Colorectal Cancer/HNPCC) and Familial Adenomatous Polyposis (FAP) and thus the risk assessment of patients are to be analyzed as per NCCN guidelines [2].

The HNPCC/Lynch syndrome is mostly defined by mutations of the MMR genes viz. (MLH1, MSH2, MSH6 and PMS2). They are primarily found in the right colon and are often associated with CpG island methylator phenotype [3]. Immunohistochemical analysis of reduced expression of MMR genes, microsatellite analysis of MSI to detect repeat length polymorphism are the two widely used methods in

predicting Lynch syndrome. Presence of BRAF gene mutation is predictive in identifying promoter methylation in MLH1 gene. According to the NCCN colon/Rectal cancer panel, MMR testing is highlighted for patients with stage II tumors [2].

Familial colorectal adenocarcinoma (CRC) accounts for ~15 to 20% of global CRC. Germline mutation of Adenomatous Polyposis Coli (*APC*) gene in chromosome 5 leads to FAP. It is an autosomal dominant hereditary syndrome and characterized by hundreds to thousands adenomatous polyps in the colon and rectum with progression to CRC if left untreated (4). According to a systemic classification model defined by Vogelstein *et al* (2015), FAP have been classified as D type/deterministic type tumors, owing to their high ERS (extra risk score), where not only the environmental hazards but also the *APC* mutation associated replication error as well as up regulated stem cell renewal pathways increases their susceptibility towards development of cancer [5].

On the other hand, sporadic CRC accounts for 70–85% of CRC cases and it can proceed through CIN, MSI and CIMP pathways [6].

Besides genetic predisposition, other risk factors of CRC include inflammatory bowel disease, smoking, consumption of red and processed meat, alcohol, diabetes, low level of physical activity, metabolic syndrome, and obesity [2]. It is also observed that number of mutations of self renewing tissues like colon is proportional to age and the propensity of tumor development is decided by a number of “driver” gene mutation that offer selective growth advantage Eg *APC*, *KRAS*, *PI3KCA*, *SMAD4*, *TP53* and several “passanger” mutations occurring in each round of self renewal process of colon [7].

CRC Carcinogenesis:

Vogelstein *et al* (2013) proposed a stepwise model for the development of CRC followed by an adenoma-carcinoma sequence [7]. A number of genetic and epigenetic changes lead to the inactivation of Adenomatous Polyposis Coli (*APC*) tumour suppressor gene. This is the most predominant mechanism for the development of CRC (2). In the recent years, three distinct pathways have been identified for the development of CRC. These include [1] Chromosomal Instability (CIN) [2] Microsatellite Instability (MSI) and [3] CpG Island Methylator Phenotype pathways (CIMP)(8). Recently, the role of aberrant microRNA (miRNA) expression in the development of CRC carcinogenesis has also been established [9].

Based on the available data, we envisage that CRC can be the outcome of four distinctly altered pathways (Figure 1), described as follows:

1. CIN Pathway:

CIN (chromosomal instability) alone accounts 85% of global sporadic CRCs (10). Genetic alterations of *APC* gene is the trademark of CIN and is observed in both sporadic and familial colorectal cancer [11, 12]. Apart from this, alterations of several other oncogenic pathways as defined by Vogelstein *et al.* [7] set the hallmark of CRC progression.

A) Factors determining Cell Fate in development of CIN:

Fate of the cells can be determined by the combination of different cellular processes like stem cell renewal pathway, chromatin modification, chromosomal translocation and transcriptional regulation [3,7].

i) Stem cell renewal pathway

Stem cell renewal pathway is necessary for the growth and metastasis of cancerous cells. Amongst the different pathways, only mitotic WNT signalling has been well characterized in development of CIN. Both the Wnt antagonist *APC* and agonist *Dvl* regulate the k-MT (Kinetochore-microtubule) attachment whereas *GSK3 β* , β -catenin, and *Axin2* localizes in the centrosome regulating a proper distribution of the chromosomes during division [13, 14, 15, 16] (Figure 2). *Conductin/Axin2* was also reported to localize in the mitotic spindle (17). In CRC, WNT signalling is often upregulated owing to inactivating mutation of *APC* and up regulation of β -catenin (18). *APC*'s k-MT association via a complex with *mDia* and *End-Binding protein 1 (EB1)* at microtubule plus ends [19] is altered in CRC through inactivating mutation of *APC*, resulting in aneuploidy.

Excessive WNT signalling leads to up regulation of *Dvl2*, that overrides Spindle Assembly Checkpoint (SAC) checkpoint signalling through *PIK1* ((Polo-like kinase 1) dependent phosphorylation, wherein phospho-*Dvl2* causes premature phosphorylation of SAC components viz. *Mps1*, *Bub1* and *BubR1*, leading to premature k-MT dissociation [20,21].

Conductin/AXIN2, another important target of WNT pathway, which is often up regulated in CRC, suppresses SAC through *Plk1* dependent microtubule disruption (22). Thus, it is evident that, excessive WNT signaling is unable to control k-MT attachment as well as centrosome segregation, resulting in CIN. From other study, it was also evident that WNT signaling activates *SMC2* (Structural maintenance of

chromosomes protein 2), a core subunit of the Condensin complex and thus retaining chromosome condensation, leading to nulliploidy [23].

According to a recent report of The Cancer Genome Atlas Network project, WNT signalling was reported to be altered in 93% of CRC cases, resulting from either biallelic silencing of *APC*, functional up regulation of *CTNNB1* through mutation, overexpression of WNT receptor, Frizzled (*FZD10*), deletion of negative regulators of WNT signalling viz. *DKK*, *AXIN2*, *FBXW7*, *ARID1A*, *FAM123B* genes [3]. Overexpression of *Dvl2* has been reported in CRC [24]. A cumulative effect of p53 deficiency coupled with WNT up regulation was also reported to contribute to aneuploidy [25].

No clear association of Hedgehog or Notch signaling, two other components of stem cell renewal pathway have been reported till date, in the development of CIN.

ii) Chromatin remodeling:

It is reported that *SMARCB1* (encoding SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B) gene product, that relieves repressive chromatin structures, is up regulated in CRC, resulting in worst prognosis in stage I/II patients [26]. In 2005, Vries *et al* [27] reported that the loss of *INI1/SMARCB1* results in polyploidy and chromosomal instability. It was also reported that a subset of histone acetyl transferase (HAT/Naa10) is significantly up regulated in CRC [28]. Components of the polycomb group (PcG) and H3K4 methyltransferases that recognizes H3K27Me3 rich nucleosomes of tumor suppressor genes (TSGs) are one of the important contributors for oncogenesis in CRC [29].

All these data establish that CRC progression is largely dependent on up regulated HAT mediated or SWI/SNF mediated decondensation of chromosome, with resultant up regulation of oncoproteins; while histone methyl transferase mediated silencing of TSGs could also be another important regulator of CRC progression (Figure 3).

iii) **Chromosomal Translocation:** Although rarely observed but recent studies of the Cancer Genome Atlas Network reported chromosomal translocation of the first two exons of *NAV2* gene of chromosome 11 to the 3' coding region of *TCF7L1*, wherein the resultant fusion protein lacked β -Catenin binding domain. Similarly translocation associated inactivation of *TTC8*, the downstream target of p53 could also account for evasion of apoptosis and tumor growth [3].

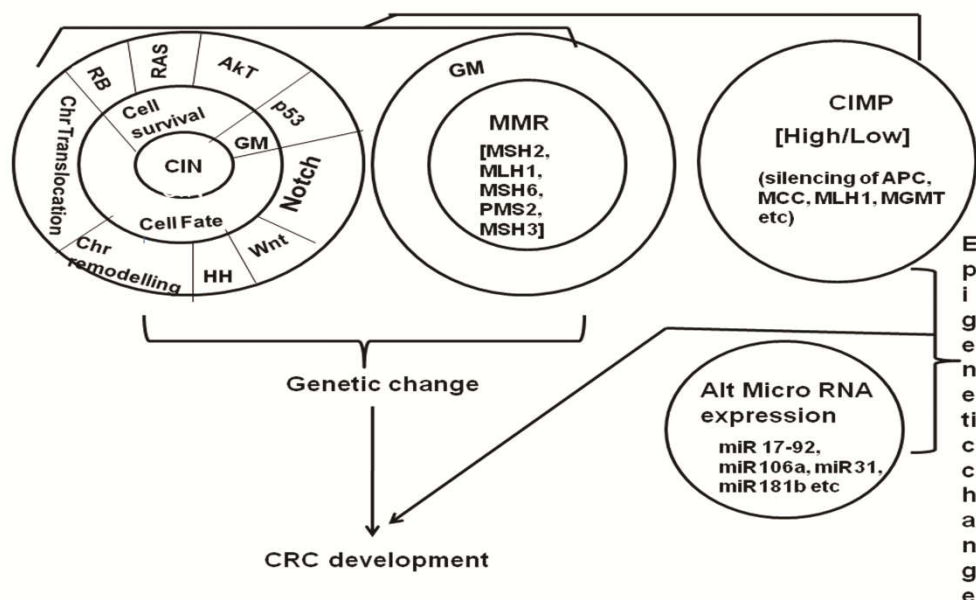


Figure 1: Schematic representation of the landscape of pathways altered during development of CRC [CIN, MMR, CIMP and miRNA expression]. GM: Genome Modification; HH: Hedgehog Signalling; CIMP: CpG Island Methylator Phenotype. CIN and MMR are attributed to both genetic (deletion, mutation) and epigenetic (methylation) defects; CIMP and altered miRNA expression.

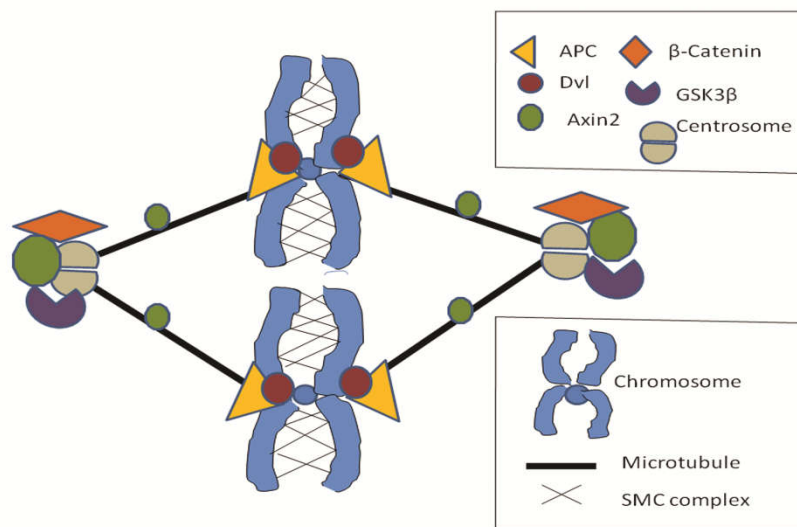


Figure 2: WNT pathway components in regulating kinetochore-microtubule attachment as well as centrosome-microtubule attachment. APC and Dvl stabilizes kinetochore-microtubule attachment whereas Axin2, β -Catenin and GSK3 β stabilizes the centrosome-microtubule adherence. Axin2 also stabilizes microtubule assembly. Aberrant Wnt signalling leads to improper SAC function, chromosome misalignment, early onset of anaphase and development of CIN. SMC complex; Structural Maintenance of Chromosome Complex. [Adaptation of report of Niehrs et al, 2012; Bernardo et al, 2013, Bahmanyar et al, 2008; Davalos et al, 2012]

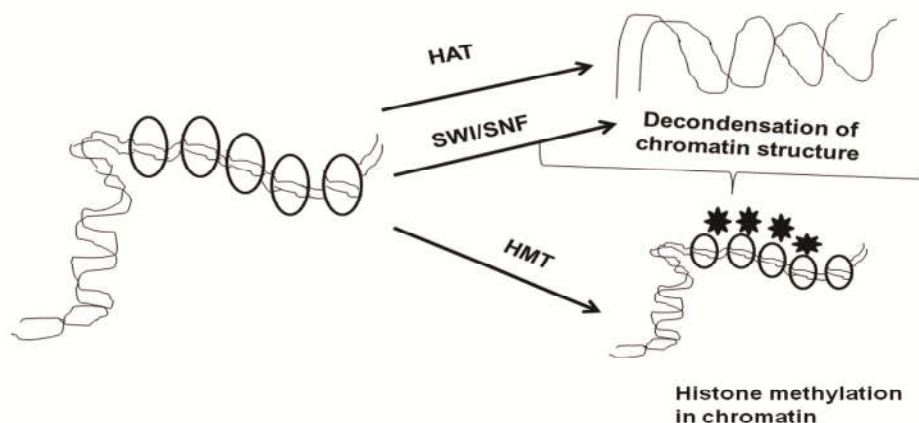


Figure 3: Role of Histone Acetyltransferase (HAT) and SWI/SNF (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B) mediated decondensation of chromatin and Histone Methyltransferase (HMT) (Indicated in black asterisk) mediated methylation of Histone, in chromatin remodelling and development of CIN.

B) Factors determining Cell Survival and development of CIN:

i) Cell -cycle check point and apoptosis:

Role of Rb protein:

The retinoblastoma tumor suppressor protein (pRb), an important regulator of G1/S checkpoint cycle [30] also has a role in regulation of CIN. Previous report showed that loss of pRb leads to over expression of Mad2, resulting in SAC (Spindle Assembly Checkpoint) dependent aneuploidy and CIN [31]. Other report showed overexpression of Mad2 leads to k-MT (kinetochore-microtubule) attachment errors that ultimately results in CIN, independent of SAC [32] (Figure 4). pRb also helps to maintain centromere integrity [33]. Loss of pRB function also alters the centromeric localization of CAP-D3/Condensin II, which leads to altered centromere geometry, errors in k-MT attachment and finally CIN [34, 35].

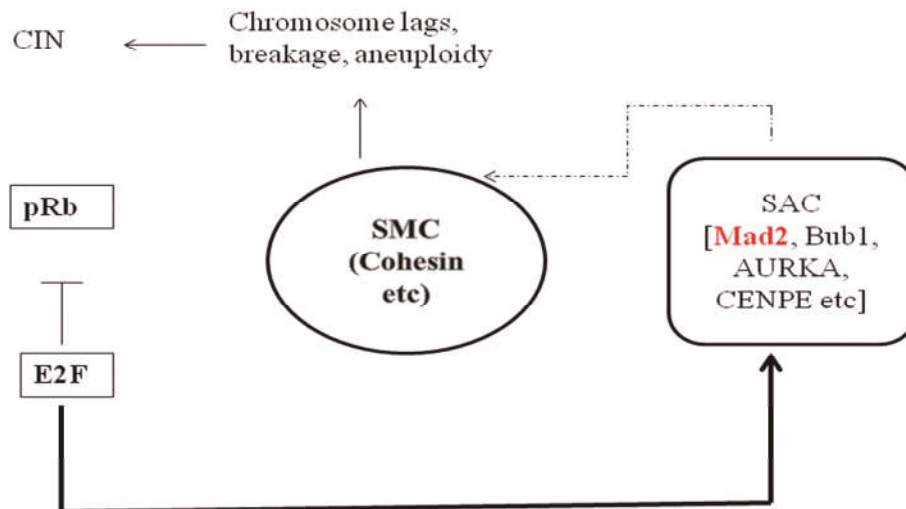


Figure 4: Role of pRb in development of CIN, through E2F mediated upregulation of SAC component Mad2 (shown in red font), leading to chromosomal instability.

ii) RAS Signalling Pathway:

Adenoma to carcinoma progression is followed by the subsequent mutations of Ras gene. These alterations affect cell cycle regulation, apoptosis, and cell survival (36). Apart from the known role in cell cycle progression, Ras signalling is also involved in maintaining genome integrity (37). Study revealed that at endogenous level, Ras expression is capable of mis-segregation of chromosome in cancerous cell with the help of Plk1 and the Anaphase-Promoting Complex/Cyclosome (APC/C) [38]. Raf kinase, the downstream target of Ras stabilizes Mps1 which hyper activates SAC and thus induce chromosome mis-segregation leading to CIN (39).

iii) The PI3K/AKT / mTOR Pathway:

The phosphoinositide 3-kinases (PI3K) are the lipid kinases that phosphorylate to activate AKT which in turn phosphorylate other proteins like mTOR. PIK3CA, catalytic subunit of PI3K is often mutated and induce oncogenic PI3K signalling. Previous report showed about 25% of PIK3CA somatic mutation in CRC (40). By dephosphorylating PIP3, PTEN negatively regulate PI3K/AKT signalling pathway and helps to maintain genomic stability (41). Frequent alterations of PI3K/AKT/mTOR signalling have also been reported in HNPCC [42].

iv) The MAPK Pathway:

Cell cycle arrest is mainly induced by the hyper activation of mitogen activated protein kinases (MAPK) / phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway. Study revealed that MAPK/PI3K/Akt pathway is also involved in genetic instability [43].

v) The JAK-STAT pathway:

JAK-STAT pathway is an important cytokine mediated immune response that involved in different cellular processes including proliferation, apoptosis and migration. Study revealed that in normal human cells, unphosphorylated STAT5A interacts with HP1 α (heterochromatin protein 1) and acts as a tumour suppressor (44). In CRC, nuclear JAK 2 displaces HP1 α from heterochromatin, thus altering heterochromatin stability (Figure 5) [44]. Therefore, JAK and STAT are involved in regulating heterochromatin integrity.

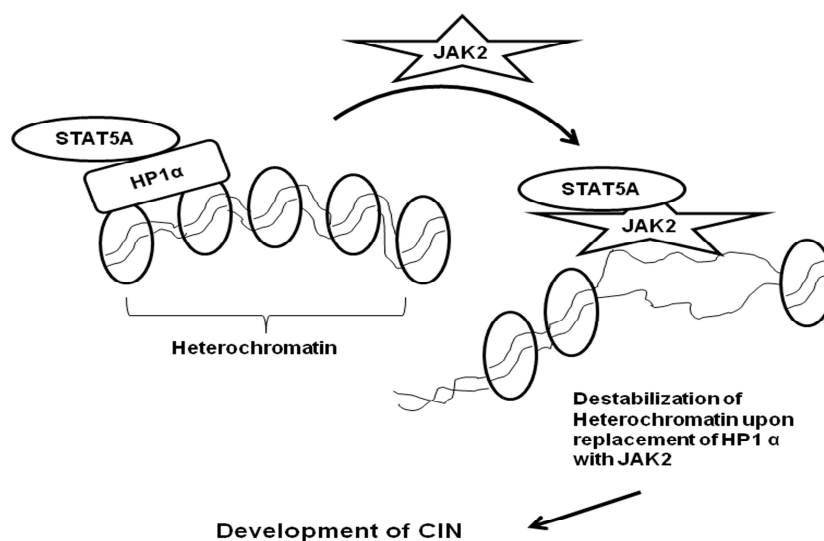


Figure 5: Role of JAK-STAT pathway in development of CIN. In normal cell, STAT5A interacts with nuclear HP1 α , thus maintaining heterochromatin stability. In CRC, nuclear JAK2 displaces HP1 α from heterochromatin region and thus chromatin stability is challenged.

C) Genome Maintenance and development of CIN:

i) DNA Damage Control:

Cellular response to DNA damage is mainly determined by the post-translational protein modification including, phosphorylation, ubiquitylation etc that transmit the DNA damage signal to elicit cell cycle arrest, DNA repair, apoptosis and senescence.

Role of p53 protein:

p53, the tumor suppressor gene that maintains cellular responses including DNA damage, oxidative stress, is mainly characterised by its loss of function in CRC [45]. The **p53** gene, located on the short arm of chromosome 17, encoding 393 amino acid residues is often mutated in CRC resulting in loss the cell cycle control and apoptosis. Study revealed that protective role of p53-activated fragment 1 (WAF-1) is absent when p53 is mutated, leading to growth of tumor cells [46]. MDM2, E3-ubiquitin ligase, and the related protein MDM4 (also known as MDMX), mainly regulate p53 under normal condition but the interaction between MDM2, MDM4, and p53 is lost when cells under high level of stress thus allowing p53 to exert its aberrant transcriptional function.

2. MSI Pathway:

Microsatellites are short repeat nucleotide sequences that leads to genomic instability and accounts for 15% of sporadic CRC cases [10] and >95% of Hereditary Non Polyposis Colorectal Cancer (HNPCC) syndrome. Microsatellite instability indicates inactivation of mismatch repair system (MMR), the factors responsible for genome maintenance. MMR defect leads to faulty replication error and increases 100 folds of mutation rate CRC cases (47). MMR system composed of multiple members like *MSH2*, *MLH1*, *MSH6*, *PMS2*, *MLH3*, *MSH3*, *PMS1*, and *Exo1* (48).

MSI-high, MSI-low, and microsatellite stable

According to the recommendation of "The International Workshop on Microsatellite Instability", a panel of five microsatellite loci (BAT25, BAT26, D5S346, D2S123, and D17S250) are used for detection of MSI(49). MSI classification system has been broadly redefined as:

- MSI-H- instability of at least two markers
- MSI-L- instability in one marker
- MSS -no apparent instability.

Other studies recommended BAT25, BAT26, NR21, NR24, and NR27 markers to identify MSI [50]. Importantly, in-vitro studies evident that MSI-high CRC does not respond to DNA – intercalating drugs like 5-Fluorouracil (5-FU) [51] and Cisplatin [52].

Lower rate of *KRAS* and *TP53* mutations have been documented in MSI-H tumors (53) but recently, germline deletions of the Epithelial Cell Adhesion Molecule (*EpcAM*) have been found in HNPCC that results in *hMSH2* gene silencing (54). *BRAF V600E* mutation is more common in sporadic MSI-H CRC, which helps to maintain cellular response to the growth signal through the RAS-RAF-MAP kinase pathway

(55). Transforming growth factor β type II receptor (TGF β RII) which inhibits cellular proliferation is found to be mutated in 90% of MSI-H tumors [56]. DNA repair genes (*RAD50*, *MSH3*, *MSH6*, *BLM*, *MBD4*, and *MLH3*), apoptosis (*APAF1*, *BAX*, *BCL-10*, and *Caspase 5*), signal transduction (*TGF β RII*, *ACTR11*, *IGF1IR*, and *WISP-3*), cell cycle (*PTEN* and *RIZ*), and the transcription factor (*TCF-4*) genes are mainly prone to mutations with defective MMR functions [57].

3. CpG Island Methylator Phenotype pathways (CIMP):

Genetic instability leads to molecular diversity of CRC. Epigenetic alterations (i.e. DNA methylation or histone modification) commonly in CpG dinucleotide promoter region are responsible for tumor suppressor gene silencing (58). Report showed approximately 20-30% of CRC cases are of CIMP and their clinical features are similar to those of MSI (59). Progression of histological grade of CRC is associated with the silencing of tumor suppressor gene *p16INK4a* that leads to uncontrolled cell proliferation (60, 61). DNA hypermethylation leads to silencing of several other genes in CRC including *APC*, *MCC*, *MLH1*, *MGMT* etc.

CIMP- High or Low:

Based on the methylated markers including *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*, CIMP can be classified into "high or low" [62].

Poorly differentiated, CIMP-High and MSI unstable tumors harbor BRAF mutation that leads to cell growth, progression of carcinogenesis, and high CRC specific mortality [63]. 90% CRC cases of sessile serrated adenoma (SSA) lesions are mainly characterized by BRAF V600E mutations. Study revealed that microsatellite unstable, CIMP-high sporadic CRC progression develop from serrated pathway [64]. Previous study showed 5FU- based adjuvant chemotherapy may not be beneficial for CRC patients with CIMP-H tumors [65] and cases with mutated KRAS (mostly belong to CIMP) showed poor response to Cetuximab chemotherapy [66].

4. Micro RNA:

Micro RNAs (miRNAs) are small (18-24 nucleotide), non-coding RNAs which regulate protein expression by translation inhibition in genes involved in cell differentiation, development and apoptosis. A large number of miRNAs involved in CRC pathogenesis, act as tumor suppressor or oncogene and are either up regulated or down regulated in CRC. Study showed that 13 CRC patients with altered miRNAs expression show either KRAS or BRAF mutations, in the RAS signaling pathway(67). Report showed other miRNAs including miR-17-92 cluster, miR-106a, miR-31, miR-181b, miR-183, miR-135a/b, the miR-200a/b/c family, miR-203 and miR-224 are altered in CRC 66. miR-31 up regulation is associated with stage IV CRC [68]. Lanza et al. demonstrated miR-17-92, miR-17-5p, miR-20, miR25, miR-92-1, miR-92-2, miR-93-1 and miR-106a up regulation in only microsatellite stable (MSS) CRC [69].

Apart from the classical model of function based classification, recent studies from the Cancer Genome Atlas Network classified CRC broadly into hypermutated and non-hypermutated groups. The hypermutable tumors with frequent mutations of *ACVR2A*, *TGFBR2*, *MSH3*, *MSH6*, *SLC9A9*, *BRAF* and *TCF7L2* genes show significantly lower mutation rate of *APC* and *TP53* than non-hypermutated tumors, indicating these two types of tumors develop through different pathways [3]. Also hypermutated tumors with MLH1 silencing have 50 fold higher chances of frameshift mutation than the non-hypermutated type [3]. For non-hypermutable type, the overall pattern of copy number variation, CIMP, mRNA, miRNA are similar in colon and rectum [3]. Oncogenic mutation of KRAS and inactivating mutations of tumor suppressor genes viz. *APC*, *TP53*, *FAM123B*, *SMAD4* are characteristic features of non-hypermutated tumors [3]. Besides the commonly observed chromosomal and sub-chromosomal changes, the study also reported 17 regions of significant focal amplification viz. 13q12.13 near the peptidase-coding gene *USP12*; *KLF5* at 13q22.1; at 20q13.12 near HNF4A, 8p12 encoding histone methyl-transferase-coding gene *WHSC1L1*, 17q21.1 encoding tyrosine kinase *ERBB2*, in CRC [3].

Conventional Treatment strategy: For resectable non-metastatic colon cancer, the preferred surgical procedure is colectomy with en bloc removal of the regional lymph nodes. Patients with stage II disease can be treated with DNA intercalators (viz 5FU/Capecitabine/Oxaloplatin). Patients with high risk stage II CRC can be considered for adjuvant chemotherapy with DNA intercalators (5FU/Capecitabine/Oxaloplatin) in combination with Folinic acid (FOLFOX). For stage III CRC, the recommended treatment strategy is a combination of DNA intercalator (viz FOLFOX, FLOX) and angiogenesis inhibitors (Bevacizumab, Cetuximab, Panitumab, Irinotecan etc) (4).

In metastatic setting, Cetuximab and Panitumumab, the EGFR inhibitors alone or in combination with Irinotecan, the topoisomerase 1 inhibitor, was reported to increase survival rates in CRC [6]. The caveat of conventional treatment strategy, which often leads to drug refractility and systemic toxicity is the

dearth of in depth molecular knowledge of the signature alterations of the tumor and thus warrants an extensive study at the molecular level.

Promising targets for CRC: It is reported that in CRC, CD133 (prominin-1) is widely expressed and can be a suitable target for therapy with Paclitaxal coated nanoparticles [70]. Studies by National Cancer Institutes to the FDA for Investigational New Drug suggested the use of Azacytidine as antineoplastic agent for treating various cancers including CRC [71]. A previous report by Flis *et al* [72] showed that DNMT (DNA methyltransferase) inhibitors Decitabine and Zebularine can improve the effect of chemotherapeutic agents on SW-480 and HT-29 CRC cell lines.

In majority of CRC patients, the drug induced systemic toxicity and bone marrow suppression has remained the major cause of mortality and thus immunotherapy has been considered a promising area of treating CRC. Immunotherapy presently is being considered for clinical trial under the following categories: [a] use of immunomodulators or anti-checkpoint inhibitors viz anti-PD-1 Antibody (Pembrolizumab) or anti-CTLA-4 Antibody (Ipilimumab), [b] monoclonal antibodies against surface antigens viz. RO5520985 (anti-VEGF-A antibody), [c] vaccination to elicit immune response (eg: dendritic cell vaccine), [d] adoptive cell therapy with tumor infiltrating lymphocytes, [e] oncolytic virus therapy eg Reolysin that can destroy cancer cells bearing an activated RAS pathway, [f] adjuvant chemotherapy with Epcadostat (INCB024360), an IDO inhibitor along with Nivolumab (PD-1 checkpoint inhibitor), [g] cytokine therapy with IL-10 74 etc.

As a conclusive remark, we must state that targeted therapy in CRC is still a challenging area, owing to the presence of molecular cross-talks amongst hitherto characterized pathways. Further research to understand the overlaps amongst different pathways and a patient stratified therapeutic regime would help us combat better against CRC.

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CONFLICT-OF-INTEREST

Authors have no conflict of interest.

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