Chapter 3

Canonical Wnt and MSI pathways may not govern tumorigenesis in a significant proportion of early-onset sporadic colorectal cancer patients in India.

3.0.1. Key words: EOCRC, Wnt-/MSI-, Wnt+/MSI-, canonical Wnt signaling, CRC progression model.


3.1. Introduction

As discussed in chapter 2, EOCRC patients constitute up to 47% of the total CRC patients in India. We endeavored to identify the underlying molecular pathways responsible for EOCRC. Pioneering studies conducted from the West in the past...
decades has identified two major genetic instability pathways Polakis (2007) viz. 1) Chromosomal Instability pathway (CIN) and 2) Microsatellite Instability (MSI); either of the two drive CRC initiation and proliferation in a majority of CRC (Figure 1). Among the two major cancer pathways, CIN is the predominant cause estimated to account for about 70-80 % of sporadic CRC and is primarily a result of protein truncating mutations in the Adenomatous polyposis coli (APC) tumor suppressor gene(Clevers 2006, Polakis 2007).

Figure 1: Contribution of major cancer pathways in CRC.

3.1.1. CIN pathway

The epithelial surface of the intestine arises from glandular structures called Crypts of Liberkuhn (Figure 2). The multipotent stem cells of the intestinal columnar epithelial cells reside at the base of the crypts where as differentiated cells originating from the stem cells make up the surface of the epithelial lining (Sancho, Batlle et al. 2004). The intestine maintains a highly controlled balance of cell division, migration and cell death
in the crypts in order to renew the epithelial layer every few days. This ensures that potentially mutated/worn out cells are disposed off from the epithelial lining of the intestinal lumen and are replenished by younger cells arising from the base of the crypt. This homeostasis is regulated by the controlled activation of Wnt signaling, a pro-proliferation signal (Vries, Huch et al. 2010). Wnt are a family of secreted glycoproteins. Though, there are different pathways involving Wnt signaling (discussed in detail in chapter-4), canonical Wnt signaling appears to be the major Wnt pathway in intestinal epithelial renewal. β-catenin is the central ‘effector’ protein in canonical Wnt signaling. There are two pools of β-catenin in epithelial cells, a membrane bound, and a cytoplasmic pool of free β-catenin (Murali D Bashyam 2013). In the absence of Wnt ligand (Figure 3) binding to the Frizled (Frz)/LRP receptor complex, cytoplasmic β-catenin is sequestered by a complex of APC, AXIN2, GSK3β, and CK1 (Pino and Chung 2010). This complex phosphorylates β-catenin at Thr41, Ser37 and Ser33. Phosphorylated β-catenin is ubiquitinilated by an F-box protein β-TrCP and is subsequently targeted for proteasomal degradation. In the absence of β-catenin in the nucleus, downstream targets of β-catenin (mostly belonging to pro-proliferation pathways) remain transcriptionally inactive. Upon binding of Wnt ligand to the Frz/LRP receptor complex, an alternate complex is formed with Frz/LRP, Dsh, CK1, GSK3β and AXIN2, circumscribing the prospect of formation of the β-catenin degradation complex that involves APC. This results in the stabilization of β-catenin in the cytoplasm and subsequent translocation to nucleus, where it recruits transcription factors such as Bcl9, Pygopus, TCF to form a β-catenin transcription complex which activates the transcription of target genes involved in proliferation.
The tight control of Wnt signaling is central to the homeostasis of replenishment in the intestinal epithelium, and is achieved through the gate keeper function of APC. Mutations in APC which affect its binding to β-catenin result in constitutively activated Wnt signaling which in turn results in uncontrolled and premature cell division, often with imperfect mitotic checkpoints. This leads to massive chromosomal aberrations (Geigl, Obenauf et al. 2008), including genomic deletions, duplications, translocations and chromosomal fusions and thus constitutive Wnt signaling (mostly via APC mutations) leads to CIN (Kaplan, Burds et al. 2001). In addition, a truncated APC protein devoid of the C-terminal domain (which ensures proper kinetochore-microtubule interaction) has been suggested to result in CIN. Because of the effect of aberrant Wnt signaling on genomic integrity, CIN and aberrantly activated Wnt signaling have been synonymously used in a few studies on CRC genesis ((Fernebro, Halvarsson et al. 2002), (Hadjihannas, Bruckner et al. 2006)). APC mutation has been proposed to be an early event in CRC genesis (Kinzler and Vogelstein 1996).
Figure 2: A representation of the intestinal epithelium with crypts of Liberkuhn (longitudinal section pictured). Modified and reproduced with permission: Elena Sancho, Eduard Batlle and Hans Clevers. Signaling pathways in intestinal development and cancer; Annual Review of Cell and Developmental Biology, Vol. 20: 695-72; Annual reviews© 2004.
Figure 3: Over view of the Wnt signaling pathway: Reprinted with permission; Maria S. Pino, Daniel C. Chung, The Chromosomal Instability Pathway in Colon Cancer; Gastroenterology, Volume 138, Issue 6, May 2010, Pages 2059-2072; Elsevier publications ©2010.
3.1.2. MSI pathway

The MSI pathway, caused primarily by the bi-allelic promoter CpG methylation induced inactivation of the mismatch repair (MMR) gene MLH1 drives tumors in about 15% of sporadic CRC patients (de la Chapelle and Hampel, Jass 2007). The constant renewal of cells in the intestinal epithelium necessitates an error free replication. MMR defects result in accelerated accretion of mutations in the genome, many of them potentially...
Cells with MMR defects are particularly prone to errors in repetitive regions of the genome and one of the easily discernable consequences can be identified in microsatellites (short tandem repeats or STRs). MMR defects often lead to an increase or decrease in the number of repeats in microsatellites (Figure 5) which is the cause of microsatellite instability. Though MSI is detected easily in microsatellites, due to the fact that they are more error prone, MMR defects actually result in enhanced rate of mutation all across the genome. Hence MSI tumors are in fact tumors with a ‘mutator phenotype’.

**Figure 5:** a) A depiction of microsatellite instability b) Major genes involved in MMR.
3.1.3. Additional events in CRC progression and the CRC progression model

Additional common genetic aberrations, including activating \textit{KRAS2} and inactivating \textit{TP53} mutations, may occur sequentially after \textit{APC} mutation during the adenoma to carcinoma transition (Vogelstein, Fearon et al. 1988), a cornerstone of the widely accepted CRC progression model (\textbf{Figure 6}).

\textbf{Figure 6}: A progression model of CRC was formulated by Vogelstein (Fearon and Vogelstein 1990) where in biallelic inactivation of \textit{APC} gene results in uncontrolled proliferation of intestinal crypt epithelial cells to form a mass of clonal cells called ‘aberrant cryptic foci (ACF)’. Subsequent genetic aberrations lead to expansion of the ACF to form early adenoma, late adenoma and adenomatous polyps. Additional aberrations lead to dysplasia and carcinoma which eventually results in metastatic carcinoma.
In the currently study, we have performed the first comprehensive molecular genetic analysis of CRC from India. We profiled status of Wnt, MSI, KRAS and TP53 on a panel of more than 320 sporadic CRC samples.

3.2. Review of literature

Colorectal cancer (CRC) is the second most common cancer with a worldwide incidence of 1.2 million (Ferlay, Shin et al. 2010). Two canonical genetic instability pathways appear to drive tumors in a majority of CRC patients (Kinzler and Vogelstein 1996). The chromosomal instability (CIN) pathway is estimated to account for about 70-80 % of sporadic CRC and is primarily a result of protein truncating mutations in the Adenomatous polyposis coli (APC) tumor suppressor gene [(Polakis 2007), (Clevers 2006). APC is the component of a multi protein complex (that targets cytoplasmic β-Catenin for degradation) which is inhibited by Wnt signaling allowing free β-Catenin to form a transcriptional activating complex with TCF/LEF transcription factors and activate transcription of pro-proliferative genes (Polakis 2000). In addition, a truncated APC protein devoid of the C-terminal domain (which ensures proper kinetochore-microtubule interaction) has been suggested to result in CIN(Kaplan, Burds et al. 2001); though this simple explanation has been challenged of late(Rusan and Peifer 2008). The microsatellite instability (MSI) pathway, caused primarily by the bi-allelic promoter CpG methylation induced inactivation of the mismatch repair (MMR) gene Mlh1, is the second major genetic instability pathway which drives tumors in about 15 % of sporadic CRC patients [reviewed (de la Chapelle and Hampel 2010), (Jass 2007)]. Colorectal
tumors originating due to either of the two canonical pathways (CIN and MSI) differ significantly with respect to location, aggressiveness, prognosis and response to therapy [(Gryfe, Kim et al. 2000), (Elsaleh, Joseph et al. 2000)]. Germ line mutations in either APC or in any one of several MMR genes, result in Familial Adenomatous Polyposis or Hereditary Non-Polyposis colorectal cancer, respectively and together account for about 6-8 % of all CRC cases in the west (Haggar and Boushey 2009). Taken together, either deregulated Wnt signaling or MSI appear to drive a majority of colorectal tumors.

APC mutation has been proposed to be an early event in CRC genesis [(Morin, Sparks et al. 1997). Additional common genetic aberrations include mutational inactivation of p53 and/or activating KRAS2 mutations; the three were suggested to act sequentially during the adenoma to carcinoma transition (Vogelstein, Fearon et al. 1988). Inactivating p53 mutations occur in 40-70 % of CRC cases (Fearon and Vogelstein 1990), (Luchtenborg, Weijenberg et al. 2005)], while mutually exclusive K-Ras [(Vogelstein, Fearon et al. 1988), (Polakis 2007)] and BRAF (Rajagopalan, Bardelli et al. 2002) mutations are detected in about 40 % and 10 % of CRC cases, respectively. Based on these findings, a model for CRC generation has been suggested (Vogelstein, Fearon et al. 1988). The advances in our understanding of CRC, in addition to implementation of a fecal occult blood test and sigmoidoscopy/colonoscopy-based screening program in adults [(Kahi, Imperiale et al. 2009), (Cunningham, Atkin et al. 2010)], have led to a significant improvement in survival rates of canonical age-related CRC in the West. In contrast, our understanding of sporadic early-onset CRC is meager
and their survival rates remain poor [(Fancher, Palesty et al. 2011), (Chan, Dassanayake et al. 2010)]. Several studies have indicated that CRC may arise through the CIN pathway involving aneuploidy, chromosomal changes and mutations in the APC and/or p53 genes [(Kapiteijn, Liefers et al. 2001), (Frattini, Balestra et al. 2004).

Ethnicity-specific variations in CRC incidence and etiology are well validated. The proportion of MSI positive (MSI+) CRC in African Americans has been reported to be significantly higher than in Caucasians (Brim, Mokarram et al. 2008 and Ashktorab, Smoot et al. 2003). More importantly, analysis of the ‘Cancer Incidence in Five Continents’ (CI5) database has revealed an increase in CRC incidence in developing countries including India (Center, Jemal et al. 2009). In addition, a high incidence of early-onset CRC among developing nations (Guraya and Eltinay 2006, Goh, Quek et al. 2005) including India (Mohandas and Desai 1999, Sarin 2001, Pal 2006) has been reported.

Identification of important cancer genes is a cardinal requisite for understanding the aggressive nature of young non-familial colorectal cancer patients as well as for predicting clinical outcome. The current study aimed at undertaking a systematic multi pronged molecular genetic analysis of CRC from the Indian population with specific focus on early (≤50 years of age) and classical late onset (≥60 years of age) CRC.
3.3. Materials and methods

3.3.1. Patient samples

The work was conducted as a collaborative study with four major hospitals in Hyderabad (detailed in chapter 2). The study was approved by independent ethics committees of each hospital. Patient samples were collected following informed consent as per the modified Helsinki declaration of 2004. A total of 608 patients including, 325 prospective (surgically resected) and 283 retrospective (archived formalin fixed paraffin embedded (FFPE) blocks) CRC samples corresponding to the period 2007-2011 were obtained. Several samples were omitted based on pathology (mucinous/signet ring cell carcinoma, undifferentiated carcinoma, gastro-intestinal stromal tumors (GIST) and lymphoma). Samples from patients with no known family history were used for the study. After omissions, 368 (149 archived and 219 resected) colorectal adenocarcinoma samples were included in the study. Patient clinical data as per a detailed questionnaire approved by hospital ethics committee was collected.

For each resected tumor, samples of corresponding normal tissue, located at least 5 cm away from the tumor, was also collected. Patient informed consent and clinico-pathological data were collected as approved by the local ethical committee. Individual samples were divided into three portions, one each for isolation of DNA and RNA and the third for histopathological analyses and archiving of the sample. The samples collected for nucleic acid isolation were snap frozen in liquid nitrogen and transferred to –80 ° Celsius for long-term storage. The samples for histopathology were collected in buffered formalin. The tumor samples that harbored at least 60 % of tumor epithelial
cells were only used for further processing. The resected normal mucosa was histopathologically analyzed to confirm zero tumor infiltration

3.3.2. Protocols

3.3.2.1. Nucleic acid isolation

3.3.2.1.1. Frozen tissue

For prospective samples, DNA was isolated from tumor (confirmed from multiple sections for >70% enrichment of tumor epithelia) and normal tissues using DNeasy Kit (Qiagen GmbH, Hildon, Germany) as per manufacturer’s protocol (detailed in chapter-4, section 4.1.3.2). The isolated DNA was quantified using spectrophotometric assays. The quantity and quality of the isolated DNA was determined using $A_{260}/280$ and $A_{260}/230$ values and by analyzing on a 0.8% agarose gel.

RNA was isolated from the tumor tissues using Trizol (Invitrogen, USA), as per manufacturers protocol (detailed protocol in chapter-4, transcriptome profiling section 4.2.2.2), and stored in 70% ethanol at –20°C till future use. Immediately prior to use, RNA was pelleted and dissolved in sterile water. RNA Quality was analyzed on Bioanalyzer (Agilent, Santa Clara, USA) and RNA Integrity number (RIN) was ascertained. Only samples with a minimum RIN value of 7 were used for further analysis.
3.3.2.1.2. Archived samples

Archived FFPE samples were collected from the collaborating hospitals. Serial 8µm sections were cut from the FFPE blocks and stained with hematoxylene. Tumor rich areas were marked by the collaborating pathologist. The tumor cells were manually microdissected from the marked regions under a microscope. DNA was isolated from the microdissected tumor using SDS/proteinase K digestion at 58°C overnight followed by standard phenol-chloroform extraction. DNA was isolated from matched normal samples of the same patient using the same method. Isolated DNA were quantitated using spectrophotometer and stored at -20 degrees.

3.3.2.2. Immunohistochemistry (IHC)

4-µm section from a paraffin block was deparaffinized in Xylene and hydrated in a graded series of alcohol solutions. Heat-induced antigen retrieval was performed by pressure cooker retrieval method in citrate buffer (1 mM, pH 6) before staining. Endogenous Peroxidase was blocked by pre-incubation with 2 % Hydrogen Peroxide in methanol. The sections were then incubated with either anti – β-catenin or anti - P53 antibody for 1 hour at room temperature followed by treatment with HRP-conjugated anti mouse secondary antibody (DAKO Envision Plus HRP kit, Dako Denmark A/S, Glostrup, Denmark) for 30 minutes. Sections were then subjected to chromogenic detection using DAB reagent from the Envision Plus HRP kit and were counter-stained with hematoxylene.
Samples with greater than 30% tumor cells exhibiting nuclear stain were considered as TP53 nuclear stabilization positive (NS+) ; lower than 20% as TP53 normal (NS-) and samples with a 20-30% nuclear stain were not included in the analysis. For β-Catenin, a sample was scored as Wnt positive if nuclear stain was observed in more than 35% tumor epithelial cells and Wnt negative if stain was detected in less than 25% cells; samples with intermediate stain (25-35% nuclear stain) were excluded from the analysis. Since accurate Wnt classification was important for the study, we employed multiple and reproducible procedures for β-Catenin IHC as follows. For each sample, two slides (each with two sections) treated with different primary antibody dilutions (1:3000 and 1:10000), were evaluated and the readings were normalized by averaging. Sections from a control FFPE block containing confirmed Wnt- samples (normal colonic mucosa) and Wnt+ sample (HepG2) were included in each batch of IHC experiment as control. In addition cytoplasmic membrane stain served as internal positive control for β-Catenin, where as absence of stain in infiltrating immune cells served as negative control for both the antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Threshold for negative</th>
<th>Threshold for positive</th>
</tr>
</thead>
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<tr>
<td>P53</td>
<td>DO-1 pantropic Calbiochem, USA</td>
<td>1:100</td>
<td>≤20% nuclear stain</td>
<td>≥30 % nuclear stain</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>Clone-12 BD, USA</td>
<td>1:3000 &amp; 1:10000</td>
<td>≤25% nuclear stain</td>
<td>≥35 % nuclear stain</td>
</tr>
</tbody>
</table>
At least two separate paraffin blocks were analyzed for each tumor sample, wherever possible. Intermediate, diffuse or varied staining in replicates were excluded from analyses of IHC.

3.3.2.3. Screening for Microsatellite Instability

In order to determine MSI status, genotyping was performed using Microsatellite markers. Screening was conducted as per guidelines set by the National Cancer Institute (NCI, USA) reference panel on microsatellite instability. The NCI panel of 5 microsatellites (BAT25, BAT26, D5S346, D17S250, D2S123)\(^{37}\) were amplified from DNA of tumor and matched normal samples. A tumor was reported as unstable for a Microsatellite marker if there was a detectable difference in the microsatellite alleles of the tumor and its corresponding normal, distinguished by the difference in size of the microsatellite repeats when analyzed by genotyping. Samples were classified as MSI-high (MSI-H) if two or more microsatellites exhibited instability, as MSI-low (MSI-L) or Microsatellite stable (MSS) respectively if one or none exhibited instability. MSI-H samples were designated as MSI positive (MSI+) and MSI-L and MSS samples were designated as MSI negative (MSI-). Only those samples were screened for MSI study where reproducible results for all five microsatellites were obtained.

Each of the five microsatellites were amplified (primer pairs are listed below) using Amplitaq Gold\(^{\text{TM}}\) (ABI, USA) as per manufacturer’s instructions with a 40 cycle amplification (95\(^{\circ}\)C 30s, 53\(^{\circ}\)C 40 s, 72\(^{\circ}\)C 60 s) followed by a single cycle of final extension (72\(^{\circ}\)C 20 min); the amplification cycle was preceded by a single cycle of initial
denaturation (95°C 10 min). The data analysis was performed on GeneMapper v4.1 (ABI Inc., USA).

Primer sequences and annealing conditions are listed in Table 1.

3.3.2.4. Mutation screening

Screening for mutations were performed by PCR-DNA sequencing on DNA isolated either from prospective or archived FFPE blocks on an ABI 3100 genetic analyzer (ABI Inc., USA) sequencing machine. All sequencing were carried out at National Genomics and Transcriptomics Facility (NGTF), CDFD.

3.3.2.4.1. Mutation analyses of Adenomatous Polyposis Coli (APC) - mutation cluster region (MCR).

The entire region spanning the MCR was amplified using Amplitaq Gold™ (ABI, USA). The PCR reactions included a 40 cycle amplification (94°C 30 s, 56°C 40 s, 72°C 60 s) followed by a single cycle of final extension (72°C 20 min); the amplification cycle was preceded by a single cycle of initial denaturation (95°C 10 min). Internal sequencing primers were utilized for detecting mutations.

MCR was sequenced from genomic DNA isolated from prospectively collected samples. Only MSI- samples were screened for APC mutations. Primer sequences and annealing conditions are listed in Table 1.
3.3.2.4.1.1. Identification of heterozygous frame-shift mutations

Heterozygous frame-shift mutations are difficult to identify by direct PCR-DNA sequencing. Suspected frame shift mutations were subjected to a PCR-clone-DNA sequence approach. Each amplicon harboring the frame shift mutation was cloned into the pUC18 using standard procedures to obtain clones harboring individual alleles. Several independent recombinant plasmids were then subjected to bi-directional DNA sequencing. Recombinant plasmids harbouring normal and the mutated sequence were detected in approximately equal proportion to confirm heterozygous mutations.

3.3.2.4.2. Mutation analysis of KRAS2

Mutations in KRAS2 hotspots results in constitutively active, GTP bound form of KRAS, which in turn results in constitutive activation of ERK/MAPKinase proliferation pathway. KRAS2 mutations are detected in 30-50% of CRC cases and are an important event in the CRC progression model. Mutations in KRAS2 tend to occur in three mutational hotspots viz. codons 12, 13 and 61. Among these codons 12 and 13 account for more than 90% of activating mutations. We screened exon 2 (harboring codon 12 and 13) of KRAS2 for mutation analysis by PCR-DNA sequencing. Amplicons were generated (primers listed in Table 1) using Amplitaq Gold™ (ABI, USA). The PCR reactions included a 40 cycle amplification (94°C 30 s, 56°C 40 s, 72°C 60 s) followed by a single cycle of final extension (72°C 20 min); the amplification cycle was preceded by a single cycle of denaturation (95°C 10 min).
As discussed previously, in order to avoid masking of mutations due to infiltrating normal cells, mutation screening was done on DNA isolated from microdissected tumor cells from paraffin sections (FFPE). Primer sequences and annealing conditions are listed in Table 1.

3.3.3. Analysis of Wnt target gene expression

1µg of total RNA was reverse transcribed using the Superscript-II reverse transcription system (Invitrogen, USA) in the presence of anchored oligo dT primers (Amersham, USA). The cDNA was diluted 1:10 with sterile water and then subjected to quantitative PCR in a total volume of 10 microlitre (Takara Bio Inc, Japan). Detailed protocols for cDNA synthesis and real time QPCR is given in chapter-4 section 4.2.2.5. Only MSI-samples were used for gene expression analysis. PCR condition for quantitation of AXIN2 and DKK1 cDNA included a 40 cycle amplification (95°C 15 s and 60°C 60s) preceded by a single cycle of initial denaturation (95°C 10 min). Melting curve analysis was carried out to confirm specific amplification. Δ-Δ-CT method was used to calculate relative AXIN2 RNA levels (with respect to GAPDH) following mean centering. AXIN2, DKK1 and GAPDH primer sequences are given in Table 1.
Table 1: Primer sequences and annealing conditions

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<th>Primer Name</th>
<th>Sequence</th>
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<td>BAT25_R</td>
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<tr>
<td>BAT26_F</td>
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<td>BAT26_R</td>
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<tr>
<td>D2S123_F</td>
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<td><strong>APC primers (for resected tissue DNA)</strong></td>
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<td>KRASshot1R</td>
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<tr>
<td>AXIN2-RT_R</td>
<td>CTTCATACATCGGGAGGCAC</td>
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</table>

*Annealing temperature. Primer modifications: ^TAMRA; 6-FAM; JOE.
*Sequencing primers.
3.4. Results

As discussed in chapter 2 the incidence of early onset (≤50 years) patients constituted a significant proportion of CRC and classical age associated CRC (late onset, ≥60 years of age), the predominant form of CRC in the West, was found to contribute to less than a third of all CRC incidences. Since deregulated Wnt activation and MSI are the two cardinal pathways that drive classical age associated CRC, we performed a comparative analysis of their respective status among early and late onset CRC patients from India to further decipher their molecular distinctions.

3.4.1. Wnt-/MSI- tumors constitute a major CRC subtype in India especially in EOCRC

3.4.1.1. Screening for CIN in Indian CRC patients

3.4.1.1.1. Screening for aberrant Wnt signaling in Indian CRC patients

CIN in CRC mostly results from aberrantly activated Wnt signaling. The first aim was to verify whether aberrant Wnt activation was the predominant CRC initiating event among Indian patients, as is well established in patients from Western nations. Among 303 samples only 159 (52.5%) exhibited β-Catenin nuclear localization (Figure 7A). This was substantially lower proportion than reported from the West [(44/52, 84.6% (Hao, Tomlinson et al. 1997); 100/100, 100% (Wong, Lo et al. 2004); 38/47, 80.6% (Hugh, Dillon et al. 1999)]. More importantly, only 43% (61/141) of early onset patients exhibited β-catenin nuclear positivity as against 67% (66/98) of late onset patients, a significant difference $P=0.002$ (Figure 7B).
Figure 7: A) Representative photomicrographs of β-catenin IHC. Left panel, Wnt-, cytoplasmic membrane staining of β-catenin is indicated by blue arrows. Right panel, Wnt+, nuclear staining of β-catenin is indicated by yellow arrow. Myofibroblasts and lymphocytes are negative for the stain and are visible with hematoxyline (blue) counter stain in the nucleus. B) Graphical representation of Wnt IHC results from early and late onset CRC patients. P value corresponds to Fisher exact text.
3.4.1.1.2. Wnt transcriptional target profiling confirms IHC results.

Since IHC based evaluation is qualitative, the next aim was to perform profiling of transcriptional targets of Wnt signaling to confirm the Wnt status determined using IHC. Two previously validated Wnt transcriptional targets, \textit{AXIN2} and \textit{DKK1} were selected based on literature [(Fodde and Tomlinson 2010), (Kocemba, Groen et al. 2012)]. In response to constitutively activated Wnt signaling (as observed in tumor tissue) expression level of \textit{AXIN2} is known to be upregulated (Fodde and Tomlinson. 2001) whereas \textit{DKK1}, a Wnt inhibitor, is known to be often down regulated (Kocemba, Groen et al. 2012). Our experiments revealed that \textit{AXIN2} transcript levels were significantly higher in Wnt+ (n=19) as compared to Wnt– (n=16) and normal (n=23) samples (\textbf{Figure 8A}). Similarly, \textit{DKK1} exhibited significantly reduced expression in Wnt+ as compared to Wnt- tumors (\textbf{Figure 8B}). The transcript profiling of the Wnt targets thereby confirmed our IHC based evaluation of Wnt status.
Figure 8: Transcript profiling of Wnt transcriptional targets by RT-Q-PCR; A) Axin2, B) Dkk1. Only MSI-samples (15 Wnt-, 18 Wnt+ and 17 normal) were selected for Axin2 transcript profiling. The ‘P’ value corresponds to Mann-Whitney U test.

3.4.1.1.3 APC mutation analysis further confirms IHC results.

APC mutations are known to be responsible for more than 80% of Wnt activated (Wnt+) CRC (Kinzler and Vogelstein 1996). Mutation Cluster Region (MCR) is a small region (including amino acids 1282-1513) in the APC gene spanning approximately 1000 bp, where 70% of the inactivating mutations of APC has been identified (Kinzler and Vogelstein 1996). MCR includes the coding region for β-catenin binding regulatory domain of APC. Mutations in MCR abrogate β-catenin binding by APC which subsequently results in constitutively activated Wnt signaling in tumors. We screened APC-MCR region from a subset of CRC samples. Further confirming our IHC based Wnt evaluation, screening of MCR region identified mutations (Table 2) in 11 of 20 Wnt+ (55%) samples but only 1 out of 27 (3.7%) Wnt- samples a statistically significant
result ($P=0.003$). Interestingly $APC$ mutations were detected at a significantly higher frequency in older patients (67%, 8/12) than in younger patients (11%, 3/27) ($P=0.001$) perhaps indicating different molecular mechanisms driving Wnt among young and older patients (Table 2). It is also possible that since our screening was limited to MCR, mutations in other regions of $APC$ could be causative event of aberrant Wnt activation. Our results therefore indicate a **significantly reduced occurrence of Wnt+ tumors among early-onset CRC patients in India.**

Clinico-pathological characteristics of samples screened for $APC$ mutations is given in (Table 1). $APC$ mutations detected in the screen are listed in Appendix-V, representative electropherogram of the mutation analysis is shown (Figure 9).
A)

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>OLD</th>
<th>YOUNG</th>
<th>Wnt+</th>
<th>Wnt-</th>
<th>MALE</th>
<th>FEMALE</th>
<th>COLON</th>
<th>RECTUM</th>
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</thead>
<tbody>
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<td>3*</td>
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<td>Wild type</td>
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<td>27*</td>
<td>24</td>
<td>23</td>
<td>9</td>
<td>33</td>
</tr>
</tbody>
</table>

*P=0.001
#P=0.003

B)

**Table 2:** analyses of APC mutations in CRC: panel A) Various clinico-pathological characteristics with respect to APC mutations, panel B) mutations detected in samples with respect to Wnt status.
Figure 9: Representative electropherogram of APC mutations panel A) electropherogram of a heterozygous APC mutation (p.R1450X) in a Wnt+ patient and panel B the same region without mutation and Validation of a heterozygous mutation nt4460(-CT) detected in a Wnt+ sample; panel C shows electropherogram of sequencing reaction carried using DNA derived from patient sample; panels D and E show electropherograms of sequencing reactions carried out on two separate plasmid constructs derived from the PCR product carrying the mutation as described materials and methods, indicating normal sequence (D) and mutated sequence with deletion (E), respectively, A bar in the panel D indicate the deleted bases. The sequencing reactions were performed using the reverse primer.
3.4.1.2. Screening for Microsatellite instability

We next sought to identify whether the second major CRC driver pathway, the MSI pathway, may account for tumorigenesis in Wnt- samples. We investigated the MSI status in a subset of early and late-onset CRC samples for which Wnt status was already determined (representative chromatogram showing MSI results are depicted (Figure 10). The proportion of MSI+ samples was marginally higher in early-onset (33%; 28/84) when compared to late-onset (29%; 14/48) samples; the difference however was not significant.
3.4.1.3. Combined analysis of contribution of the two pathways in CRC

In order to determine relative contribution of the two canonical pathways in early and late-onset CRC, we divided samples from the two age groups into four categories with respect to Wnt and MSI status namely Wnt+ MSI−, Wnt+ MSI+, Wnt− MSI+, and Wnt− MSI−. This enabled us to compare the frequency of Wnt activation in the two age groups exclusively in MSI− samples (a more appropriate comparison, since Wnt activation in MSI+ samples is expected to have occurred as a secondary event (ref) and therefore cannot be considered as a primary CRC initiating event). The proportion of MSI− CRC samples exhibiting Wnt activation was significantly lower in early (20/55; 36.3%) as against late-onset (25/32; 76%) samples (P= 0.0004; Figure 11). More importantly, the proportion of Wnt− MSI− samples was significantly higher in early (43.2%; 35/81) when compared to late-onset (18%; 8/45) CRC samples (P= 0.0056, Figure 11). Our results thereby reveal the existence of tumors not driven by either of the two canonical tumorigenesis pathways (Wnt or MSI) in a significant proportion of early-onset CRC patients.
Table 1: Combined analysis of Wnt and MSI status among Indian CRC.

<table>
<thead>
<tr>
<th>Criteria*</th>
<th>Early onset (numbers) n=81</th>
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<th>Late onset (numbers) n=44</th>
<th>% of late onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-M-</td>
<td>35*</td>
<td>43.2%</td>
<td>8*</td>
<td>18.2%</td>
</tr>
<tr>
<td>W-M+</td>
<td>15</td>
<td>18.5%</td>
<td>5</td>
<td>11.3%</td>
</tr>
<tr>
<td>W+M+</td>
<td>11</td>
<td>13.5%</td>
<td>6</td>
<td>13.6%</td>
</tr>
<tr>
<td>W+M-</td>
<td>20*</td>
<td>24.6%</td>
<td>25*</td>
<td>56.8%</td>
</tr>
</tbody>
</table>

* P= 0.0004

W-M- : Wnt-/MSI-
W-M+ : Wnt-/MSI+
W+M+ : Wnt+/MSI+
W+M- : Wnt+/MSI-

Figure 11: Combined analysis of Wnt and MSI status among Indian CRC.
3.4.2. Indian CRC tumors do not appear to follow the ‘adenoma-carcinoma sequential progression’ model

As discussed in the previous sections a progression model for CRC has been proposed where the initiating event (aberrant Wnt activation/ MMR inactivation) is followed sequentially by additional events such as activating KRAS and inactivating TP53 mutations. We sought to evaluate the sequential events in CRC samples.

3.4.2.1. Screening for P53 status

P53 status was evaluated using. Basal levels of P53 are undetectable whereas mutated P53 almost often results in its nuclear stabilization which is detectable by IHC. p53 status was evaluated in 208 samples (of the total 318 samples) and nuclear stabilization was detected in 143 (70%, Figure (12)) with no significant difference between samples from the two age groups (Table 2).
**Figure 12:** Analysis of TP53 status among Indian CRC samples. A and B) IHC based determination of p53 nuclear localization status in two representative CRC samples: In the absence of inactivating mutation, p53 is not directly detectable in cells by IHC (panel A); majority of mutations lead to stabilization of p53 which could be detected as nuclear staining by IHC (panel B). Nuclear staining is indicated with red arrow whereas absence of stain (counter stained with hematoxylene, blue colour) is indicated with green arrow (internal negative control). C) Graphical representation of the P53 IHC results.

NS+: Nuclear Stabilization Positive; NS-: Nuclear Stabilization Negative
3.4.2.2. Colorectal tumors occurring in young patients exhibit significantly lower frequency of KRAS2 mutations as against older patients

We screened the KRAS2 second exon for mutations; KRAS2 codons 12 and 13 located in the second exon account for 90% of all mutations detected in CRC and are known to render anti-EGFR therapy ineffective. Of the 174 samples tested, KRAS2 mutation was detected in 51 (29.5%; Figure 13 E), lower than the frequency identified from the West46. Representative sequencing results are depicted in Figure 13. More importantly, the frequency in early-onset CRC samples (21/87; 24%) was significantly lower than in late-onset samples (24/50; 48%) (p=0.0078; Figure 13). The mutation spectrum was however similar to what is reported for the western population (Appendix-V).

KRAS mutation did not exhibit significant association with tumor location, gender, stage or Wnt status but appeared to occur preferentially in samples exhibiting nuclear stabilization of TP53; though it was not a significant association. Interestingly, the frequency of KRAS2 mutation was lower (27.6%; 18/65) than p53 nuclear stabilization (66%; 43/65) when analyzed exclusively in Wnt+ samples indicating perhaps that KRAS2 may not sequentially follow Wnt deregulation during CRC progression among Indian patients, as against the classical CRC progression sequence. As reported earlier, KRAS2 mutations were preferentially detected in MSI-L/MSS samples rather than MSI-H samples. Previous studies from the West also indicated a low frequency of KRAS2 mutations in EOCRC patients.
E) KRAS2 mutation analysis

WT- wild type sequence; MUT – Mutation identified in exon-2

**Figure 13: Screening for hotspot mutations (exon 2) in KRAS2:** Exon 2 harbors ~90% of activating mutations (mainly at codons 12 and 13) in KRAS2. Representative electropherograms depicting normal sequence (Panel A) and mutations, p.G15D (panel B) p.G12V (panel C) p.G13 (panel D). Panel E: graphical representation of KRAS2 mutation screen. P value corresponds to Fisher exact test.
KRAS2 mutations identified in the study appendix –V.

3.5. Discussion

It is well established that deregulated Wnt signaling and MSI together account for 90-95% of CRC. Surprisingly, our analysis has revealed a significant proportion of Wnt-/MSI-tumors among Indian CRC patients, especially in early-onset CRC (fig 11). The Wnt status determined by β-Catenin IHC was confirmed by transcript profiling of Axin2 and DKK-1 and APC mutation screening. Though Wnt signaling results in β-Catenin induced transcriptional activation of several genes, many of these targets are also activated downstream of other pathways. However, AXIN2 appears to be exclusively activated by Wnt (Fodde and Tomlinson, Yan, Wiesmann et al. 2001). APC-MCR mutation screening identified mutations in 53% of Wnt+ samples; mutations in other regions of APC or in other components of the APC degradation complex such as Axin1/2 or β-Catenin, may account for Wnt activation in rest of the Wnt+ samples. Of note, APC mutation frequency in Wnt+ sporadic CRC samples has been proposed to vary between 30-70% [(Jass, Young et al. 2002, Luchtenborg, Weijenberg et al. 2005)].

It has been suggested that a majority of colorectal tumors (including MSI-H tumors) may exhibit active Wnt signaling either as a primary or a late event (Segditsas and Tomlinson 2006). Up-regulation of Wnt target genes in MSI-H tumors, due to mutations in APC/CTNNB1 (β-Catenin) or perturbation of a microsatellite located in the last exon
of TCF4, has been documented [(Shimizu, Ikeda et al. 2002), (Miyaki, Konishi et al. 1994)]. In our analysis, 41% of MSI-H tumors exhibited Wnt activation.

The TP53 stabilization frequency identified in this study is perhaps the highest reported so far. An earlier study from India however revealed a lower percentage (37%) but included only 48 samples (Bhatavdekar, Patel et al. 1997). A combined effect of APC inactivation and KRAS mutation in augmenting Wnt induced CRC initiation has been proposed (Janssen, Alberici et al. 2006). In the current study however, a significant majority of Wnt+ tumors did not harbor KRAS mutations, perhaps suggesting that the latter may not be essential for Wnt-dependent activation of CRC; a result similar to that obtained recently for FAP adenomas (Obrador-Hevia, Chin et al. 2010). KRAS and TP53 have been suggested to be involved in alternative pathways for generation of CRC [(Smith, Carey et al. 2002), (Calistrì, Rengucci et al. 2005). The combined presence of KRAS mutation and TP53 inactivation (although not a significant association) identified in the current study has however been reported from other developing nations as well (Soliman, Bondy et al. 2001). The significantly low KRAS mutation frequency in the younger CRC patients detected in this study (Figure 13) is similar to recent reports from the West [(Berg, Danielsen et al. 2010), (Yantiss, Goodarzi et al. 2009); EGFR targeted therapy therefore may yield better prognosis in these patients (Prenen, Tejpar et al. 2010). It should be noted that ERK/MAPK pathway, which is aberrantly activated by mutant KRAS2, could also be activated by mutations in other upstream and downstream effectors such as EGFR, BRAF or aberrations in any MAPKinase genes (Steelman, Chappell et al. 2011). Mutations in these genes occur at
a relatively lower frequency than KRAS2 and were not profiled in the current study. Additionally, we did not screen for mutations in PI3KCA and PTEN; activating mutations in PI3KCA (Steelman, Chappell et al. 2011) and inactivating mutations in PTEN have been implicated in CRC progression, albeit at lower frequencies.

As with other solid tumors, advanced age is an important risk factor for CRC (Soliman, Bondy et al. 2001). Nevertheless, a significant increase in CRC incidence among the young in the USA (Surveillance Epidemiology and End Result (SEER) data (Meyer, Narang et al. 2010)) as well as in developing nations (Siegel, Jemal et al. 2009) has been reported recently. Our results appear to indicate the occurrence of tumors among early-onset Indian CRC patients that do not appear to be driven by canonical Wnt signaling nor by microsatellite instability. It is therefore important to determine the tumorigenesis pathways in these tumors.

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3.7. References


