Chapter 5: Altered tumor biology in early onset patients; need to address the role of ethnicity or altered etiological factors.

5.0.1. **Key words:** EOCRC, ethnicity, canonical Wnt signaling, non-canonical Wnt signaling, Ca2+ signaling, NFATC2.

5.0.2. **Abbreviations:** EOCRC: early onset colorectal cancer, RC: rectal cancer, APC: *Adenomatous polyposis coli*, Wnt-/MSI-: Wnt signaling absent and MSI absent, Wnt+/MSI-: Wnt signaling present and MSI absent, *NFATC2*: *Nuclear factor of activated t-cells, cytoplasmic 2*, ROR2: *Receptor orphan receptor like 2*, *CCND1*: *Cyclin D1*, FFPE: Formalin fixed paraffin embedded.

5.1. **Summary**

Extensive studies on colorectal cancer (CRC) have provided crucial molecular and epidemiological insights into disease progression of late-onset colon cancer, the predominant CRC subtype in the West. Colon cancer is mainly driven by mutational inactivation of *Adenomatous Polyposis coli (APC)* tumor suppressor gene causing chromosomal instability due to constitutive activation of canonical Wnt signaling. A minor proportion is caused by inactivation of mismatch repair pathway resulting in microsatellite instability. Distinct etiological and molecular characteristics are however beginning to be unraveled in CRC occurring in developing nations. A preponderance of rectal (over colon) and early (over late-onset) cancers appears to be the hallmark of
CRC in developing nations. More importantly, the possible occurrence of unique non-Wnt driver tumorigenesis pathways in early-onset rectal cancer has been suggested in several populations. Though CRC research has focused on canonical Wnt signaling pathway for over two decades, it is imperative now to study alternative oncogenesis pathways to combat the ever increasing RC burden in developing countries.

With the aim to unravel the biology of early onset CRC in the Indian context, we performed a comprehensive analysis incorporating epidemiology with molecular biology, genomics and transcriptomics. The study revealed some interesting findings; the major ones are summarized below.

**5.2. Role of ethnicity in cancer incidence; high incidence of EOCRC**

The first major conclusion from this study (as discussed in chapter-2) is that EOCRC (≤50 years) contributes up to half of all the CRC incidences, a much higher proportion compared to their Western counterparts, where EOCRC contributes less than 20% of overall CRC incidence. Additionally, early onset rectal cancer constituted 22% of all cases in our study, thus becoming the single major subtype in the study. In fact, 51% of EOCRC patients harbored rectal cancer. This result is important since as per previous reports from the West, an early onset is commonly associated with Lynch syndrome, a major familial cancer syndrome. But Lynch syndrome is normally characterized by tumors in right colon and rarely in rectum. Classical late onset colon cancer (≥ 60
years), the predominant subtype of CRC in the West (contributing up to 70-80% of the CRC incidence), constituted a meager 14% of all cases.

Interestingly, the early onset patients did not exhibit significant association with family history of cancer, as could be expected on the basis of similar data from the West, nor did they show any significant difference from late onset patients for the etiological factors analyzed in the study. However, EOCRC was significantly more associated with prognostically important pathological variables, poor tumor grade and advanced pathological stage, perhaps indicating that EOCRC is an aggressive subtype. However, in a parallel study (not reported in thesis, Raman et al, 2013, personal communication) we identified that early onset patients, though harboring tumors with poor differentiation and advanced stage, had similar 5-year disease free survival rate as of late onset patients (when adjusted for stage and grade). Stage and grade could independently predict survival in that study.

Thus the early onset patients either gets diagnosed late or many of the tumors in early onset patients arise out of flat adenoma (or de novo cancer) which has a higher propensity to invade muscular layers of colorectum, and thus result in advanced stages at the time of diagnosis. Unlike the classical tumors in CRC which arise out of an adenomatous polyp (adenoma-carcinoma progression model), which can be detected in regular colonoscopy even among normal individuals, de novo tumors are thought to arise from flat adenoma which cannot be easily detected in colonoscopy. Adenomatous
polyps occur frequently among normal healthy individuals especially above age of 60, but previous reports indicate that Indians harbor significantly lower frequency of colorectal adenomatous polyps among normal population. Alternately, higher levels of incidence of flat adenomas were reported from Asia [Japan] among early onset patients and the colorectal tumors arising out of these flat adenomas were characterized by absence of APC mutations. It should be noted that mutations in APC is the tenet of the widely accepted ‘adenoma-carcinoma progression model’, which has been found to be correct model for a vast majority of classical (late-onset) CRC in the West.

Thus, the absence of APC mutations and above all absence of canonical Wnt signaling is pointing towards the need of alternate models to accommodate the diversity of tumorigenesis (perhaps linked to varied ethnicity or lifestyle) among different population. It is very likely that some of the early onset tumors in our study might have arisen out of flat adenomas, since the frequency of occurrence of benign polyps (detected during colonoscopy for diagnosing other gastro-intestinal diseases/ or during post mortem) among healthy Indians is much lower than in the West. Since polyps are thought to take decades to progress to fully malignant carcinoma, relative absence of polyps even among elderly Indians compared to Western populations perhaps points to alternate pathways of CRC progression. In any case, going by the hypothesis that tumors take decades to develop, it would be unlikely that an adenomatous polyp occurring in a young individual would have sufficient time required to become malignant and present itself with in short span of time, especially when the individuals are in the prime-time of
their health, immune system and tissue repair. Another possibility is that low penetrance familial syndromes exist in the Indian/Asian genetic background, but the increased incidence of EOCRC appears to be not limited to a few ethnicities and has been reported from populations with diverse genetic/ethnic background. A more likely explanation could be the adoption of un-healthy lifestyle including diet and lack of exercise, a phenomenal change due to rapid urbanization of a large number of countries, especially among younger population. A weak-point for such an explanation is that, in our study we observed no discernable difference in the frequency of urban and rural patients. An unhealthy lifestyle would be expected to be more among urban population and hence the frequency of younger patients would be expected to be more from urban population. Thus a more correct explanation would be that the current increase in incidence could be a mix of ‘nature and nurture’, i.e., an ethnic CRC risk factor is getting aggravated due to altered life style.

5.3. Molecular origins of CRC in india, do we need additional models?

The second major conclusion from this study (chapter-3) is that unlike their Western counterparts, a significant proportion of Indian CRC patients harbor tumors that do not exhibit deregulated Wnt signaling pathway. More importantly, 43% of early-onset CRC patients appear to harbor Wnt-/MSI- tumors suggesting thereby the existence of non-canonical pathways driving tumorigenesis. 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. The Wnt status determined by β-Catenin IHC was confirmed by transcript profiling of Axin2 and DKK-1 and APC mutation screening. Interestingly, early-onset patients harbored substantially lower levels of APC mutation compared to late-onset patients even in Wnt+ samples, perhaps indicating different biology. A combined effect of APC inactivation and KRAS mutation in augmenting Wnt induced CRC initiation has been proposed. 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. The significantly low KRAS mutation frequency in EOCRC patients detected in this study (Appendix V) is similar to recent reports from the West; EGFR targeted therapy therefore may yield better prognosis in these patients.

5.4. Understanding the biology of EOCRC in the absence of canonical CRC pathways

We sought to understand the molecular carcinogenesis of early onset Wnt-/MSI- CRC, using genome-wide approaches. Surprisingly, aCGH based copy number analysis revealed that even in the absence of β-catenin dependent (canonical) Wnt pathway activation, a sizeable proportion (70%, (21/30)) of early onset tumors exhibited CIN. In addition to recurrent gains observed in commonly affected oncogenes such as CCND1 and ERBB2, we detected recurrent gains at 11q11.3 and 20p13.1 more frequently in Wnt-/MSI- early-onset CRC. Interestingly, the recurrent CNA detected at 11q11.3 exhibited differential DNA copy number profile between the two classes of early onset
CRC. While 30% (6/20) Wnt-/MSI- tumors exhibited gain of copy number at the locus, 25% (5/20) Wnt+/MSI- tumors exhibited loss. Rest of the samples in both categories did not exhibit significant copy number changes. High-level recurrent CNA detected at 20p13.1 was loci known previously to be gained in various tumors, but definitive resident oncogene(s) has not yet been identified. The previously suggested oncogenes (ZNF216, AURKA) from this locus was well outside the minimum common region identified in the current study, indicating the role of another gene in the current context. **APCDD1L** was validated from the CNA due to the role of its homologue **APCDD1** in negative regulation of canonical Wnt signaling. Of not the gain was observed more frequently in Wnt-/MSI- subtype.

Comparative whole transcriptome profiling of Wnt-/MSI- and Wnt+/MSI- tumors identified the presence of non-canonical Wnt signaling pathway signature among the Wnt-/MSI- tumors. Unlike the canonical Wnt signaling pathway, which is almost always mediated through β-catenin, non-canonical Wnt signaling is a super-family of at least 5 sub-pathways. A majority of them are known to be involved in cell polarity, morphogenesis and cell movement (linked to aggressive nature of tumors) though not directly linked to oncogenesis. Recent evidences increasingly point to the potential role of non-canonical Wnt signaling in cancer, especially the Wnt/Ca2+ pathway. Among the several well characterized non-canonical Wnt signaling pathways, three viz. 1) Wnt/RAC and RHO pathway 2) Wnt/Ca2+ pathway and 3) Wnt5a/ROR2 pathway are known to be significantly associated with tumors. We performed initial validation of the existence of
non-canonical Wnt signaling pathway in EOCRC. A key non-canonical Wnt effector gene *NFATC2* (Nuclear Factor of Activated T-Cells Cytoplasmic 2, belonging to Wnt/Ca2+ signaling pathway) was validated using IHC on a tissue microarray (constructed from FFPE blocks representing samples used in transcriptome profiling). Our results confirmed that NFATC2 was not only over expressed in Wnt-/MSI- EOCRC at the transcript level, but also at the protein level. NFATC2 is a transcription factor and effector of Wnt/calcium signaling. In resting condition, the expression is detectable only in the cytoplasm, but upon activation translocates to nucleus and activates pro-invasive and pro-motility genes. Whether Wnt/Ca\(^2+\) signaling pathway is causatively associated with EOCRC, especially in the Indian context, could not be assessed in the current study.

5.5. Conclusion

Aberrant Wnt activation induced CIN and MMR inactivation induced MSI may account for a majority of aging related colon cancer; the predominant CRC subtype in the Western world. Several therapeutic approaches targeting the Wnt pathway are being tested and are expected to improve survival in metastatic late-onset colon cancer. However, as discussed above, it may not be accurate to assume a significant role of CIN and MMR inactivation in all forms of CRC. CRC in the developing world is predominantly a disease of the rectum occurring at a younger age. The current study revealed absence of the two main CRC pathways in a significant proportion of early onset CRC, especially in RC. In the absence of the two pathways, our study has
identified the presence of non-canonical Wnt pathway, yet unable to suggest whether the same is involved in the genesis of the tumor or as a genetic event occurring during the multistep progression of CRC. Further studies using Immunohistochemistry on a larger independent sample set and primary tumor tissue culture are required to identify the extent and role of non-canonical Wnt signaling in CRC in Indian genetic background. Our novel findings therefore include identification of a substantial proportion of early onset patients not harboring either CIN or MSI, which may have therapeutic implication. The role of non-canonical Wnt signaling in early onset CRC has not been investigated before. It is hoped that our study may lead to potential drug targets in early onset CRC in the Indian genetic background. Given that the worldwide CRC burden is becoming prominent in developing nations where the molecular and clinico-pathological CRC profile appears to be distinctly different, it is imperative to understand CRC biology in early-onset RC. Ongoing research in several laboratories is expected to hasten novel discoveries in the field of CRC molecular origin; which holds the promise for better patient management and treatment options for CRC in the developing world not driven by Wnt or MSI.