Identification and characterization of novel molecular markers in cancer prevalent among Indian patients

Thesis submitted to

Delhi Technological University

For the award of degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

Submitted by

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CERTIFICATE

This is to certify that the Ph.D. thesis entitled "**Identification and characterization of novel molecular markers in cancer prevalent among Indian patients**" is original and has been carried out by **Ms. Sanghamitra Mylavarapu** under our supervision at the Department of Biotechnology, Delhi Technological University, New Delhi and Invictus Oncology Pvt. Ltd., Delhi, for the award of the degree of Doctor Of Philosophy. It is further certified that the work embodied in this thesis has neither partially or fully submitted to any other university or institution for the award of any degree of diploma.

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DECLARATION

I, **Sanghamitra Mylavarapu**, certify that the work embodied in this Ph.D. thesis is my original work, carried out under the joint supervision of **Dr. Asmita Das**, Assistant Professor, Department of Biotechnology, Delhi Technological University (DTU), New Delhi and **Dr. Monideepa Roy**, Director, Research and Development, Invictus Oncology Pvt. Ltd., Delhi, for the duration from August 2012 to March 2019. The entire research work was carried out at Invictus Oncology Pvt. Ltd. Any part of the content of this thesis has not been submitted to any other university or institution for the award of any degree of diploma.

I declare that the work of other researchers, wherever cited throughout this thesis has been duly acknowledged and referenced. I further certify that I have not willfully plagiarized paragraphs, text, images and result reported in journals, books, reports, dissertation and thesis that are available in the public domain as part of this thesis.

Sanghamitra Mylavarapu
(Roll Number: 2K12/PhD/BT/01)
Date:

Dedicated to my parents

Gratitude

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CHAPTER 1

INTRODUCTION

CHAPTER 1

Introduction

1.1. Introduction to cancer

A normal mammalian cell grows and divides giving rise to progeny of its own kind. With the passage of time, older and damaged cells die, only to be replaced with new cells, a process that maintains tissue homeostasis (Figure 1). Cancer develops when this normal, highly regulated physiological process is disrupted. Renegade cells acquire the ability to grow and divide rapidly and bypass programmed death, thus accumulating more and more genetic and physiological anomalies with each cell division. Eventually, this abnormal cellular proliferation results in a mass of tissue called the tumor. Major advances in recent years have led to a detailed understanding of the pathophysiology of cancer where it is no longer considered to be a single malady but a pathological condition that encompasses a group of diseases exhibiting uncontrolled cell growth and division as a common feature.

1.1.2. Hallmarks of cancer

Transformation of a cell from normal to malignant form involves a series of molecular and physiological alterations that accumulate over time. Regulation of cell division (mitosis) is carried out by two main groups of molecules. One class – the activators, provide signals for stimulating cell growth and division, while the other, the suppressors, inhibit it. Some of these proteins trigger a separate signal for detecting damaged cells, allowing the aberrant cell to enter programmed cell death (apoptosis). A finely controlled balance between proliferative and inhibitory signals is therefore critical to maintain tissue homeostasis. Mutations or other changes in the activator/suppressor classes of genes disrupts this leading to the transformation of healthy cells into malignant ones. Cancer cells have evolved mechanisms that allow then to become unresponse to the signals that control cellular division like the way

normal cells do. Since cancer cells divide uncontrollably, they rapidly build up and grow into tumors.

The traits acquired by the normal cell as it gradually transforms into a precancerous stage and eventually into cancer are described as the "hallmarks of cancer" by Douglas Hanahan and Robert A. Weinberg (Hanahan D, Weinberg RA., 2000, Hanahan D, Weinberg RA., 2011). These acquired traits distinguish cancer cells from normal cells. Each hallmark contributes towards pathogenesis, but the presence of a complete set of characterisitics is a pre-requisite for cancer to fully develop (Figure 2). The framework that elucidates that development of cancer occurs through a set of discrete transformations or genetic events are described below.

1. *Self-sufficient growth*

Unlike normal cells, a malignant cell acquires the capability of generating endogenous growth stimulating signals, reducing their dependence on extraneous mitogenic stimulation in order to proliferate (Fedi P, Tronick S.R, Aaronson S.A., x1997). This is exemplified by the fact that cancer cells often overexpress epidermal growth factor receptor (EGFR), a cell surface receptor that feeds into the MAPK pathway. This renders the cell hypersensitive to stimulation with even normal levels of growth factors. Approximately 25% of human tumors contain structural mutation in the *Ras* oncogene that constitutively activate the MAPK pathway which sustains mitogenic signals promoting cellular proliferation (Medema RH, Bos JL., 1993).

2. *Insensitive to signals for suppression of cellular growth*

Concomitant with pro-proliferative signals, there are signals that halt cell growth and proliferation. Under normal physiology, the positively acting growth signals that stimulate proliferation are counterbalanced by negatively acting growth inhibitors to maintain tissue homeostasis. The growth inhibitory factors exert their effect by either forcing cells to enter G_0 state or into a post-mitotic differentiated state. In order to survive, cancer cells evolve mechanisms to bypass the effects of the anti-proliferative signals. Most anti-proliferative signals are transmitted via the retinoblastoma (Rb) protein. The tumor suppressor activity of Rb is via the sequestration of E2F transcription factors, thus preventing transcriptional activation of a host of target

genes that influence G1/S transition. Cancer related mutations in Rb can therefore release E2Fs resulting in cellular proliferation (Figure 1) (Weinberg RA., 1995).

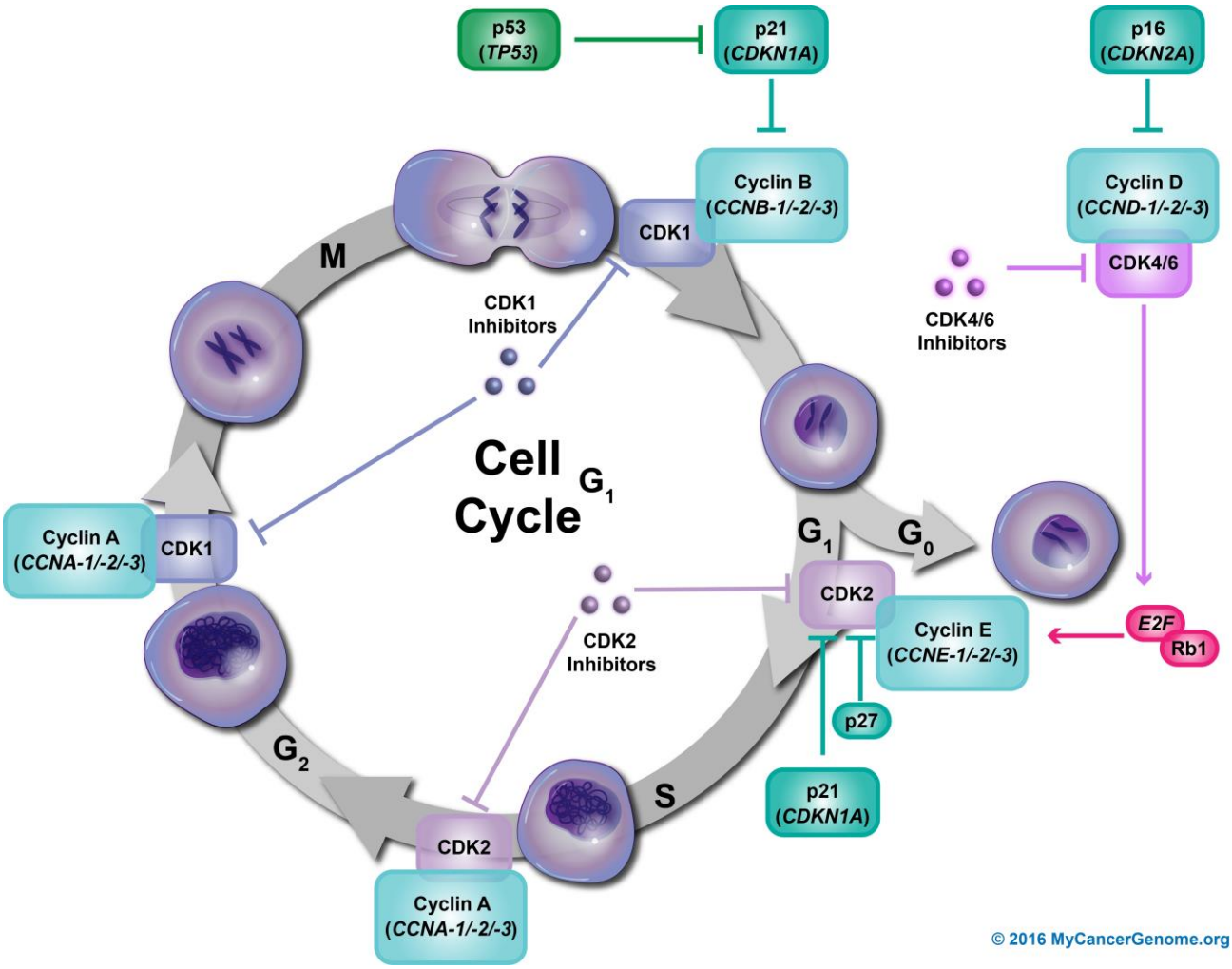

Figure 1: The cell cycle.

Cell cycle, consisting of four major stages G1 S, G2 and M, is the process through which cells divide to yield two identical daughter cells. Complex interactions between cyclin-dependent kinases (CDKs) and cyclins via formation of heterodimers control the progression through the cell cycle. The integrity of the daughter cells is maintained by activation and de-activation of checkpoints that function at various stages of the cell cycle – the G1/S checkpoint is activated when there is DNA damage, restricting the cell at the G1 phase. Genes belonging to E2F family control transcription of proliferative genes. CyclinD-CDK4/6 dependent phosphorylation inactivates Rb and released E2Fs to induce transcptional activation of proliferative genes, whereas sequestration of E2Fs by dephosphorylated Rb prevents the cell from progressing beyond the G1 checkpoint. The G2/M checkpoint, activated by DNA replication errors during S phase, prevents progression into the M phase via the activation of the tumor suppressor P53 (MyCancerGenome.org, 2016).

3. *Resisting cell death*

Old and damaged cells are eliminated via a process called apoptosis by which the body limits growth and discards cells containing damaged DNA, thus preventing propagation of DNA errors. Apoptosis is a key regulatory mechanism that keeps balance between cellular proliferation and cell death, thereby maintaining tissue integrity. The acquired ability to resist apoptotic cell death characterizes almost all malignant cells and is considered as one of the hallmarks of cancer. Two key components of the apoptotic machinery consist of sensors and effectors that together determine the fate of the cell. Sustained signaling by binding of IGF survival factors to the cell surface receptor IGF-R is a mechanism by which cancer cells evade apoptosis (Butt AJ, Firth SM, Baxter RC., 1999).

4. *Limitless replicative potential*

Tumors are formed by accumulation of billions of cells via unregulated cellular division, evasion of apoptosis and the ability to replicate infinitely. During mitosis, the terminal end of chromosomes called the telomere, are lost every time DNA is copied. Eventually, telomere loss reaches a critical point where the cell is unable to divide any longer. This is a process of self-limiting the replicative potential of healthy cells. However, the activation of telomerase, as enzyme that maintain telomeres, can allow cells to replicate indefinitely. Unlike normal cells, >90 percent of "immortalized" malignant cells have telomerase activation. Replicative immortality, or the potential for undergoing unlimited cellular division is an acquired feature that cancer cells display. The cell's intrinsic checkpoints that limit its replication capability are disrupted during malignant transformation and lead to tumor growth. Progressive loss of 50-100bp of DNA from the telomeres during each cell cycle leads to end-to-end joining of chromosomes and karyotypic disarray and crisis and cell death (Counter CM, Avilion AA, *et al*, 1992). Most cancer cells bypass this fate by upregulation of telomerase activity that maintain the telomeres via the addition of hexanucleotide repeats at the end of the chromosomes, thereby endowing the cells with unlimited division capabilities (Bryan TM, Cech TR., 1999).

5. ***Inducing angiogenesiss***

Growth of a tumor requires increased blood to provide nutrients and oxygen to the growing mass of cells. A process known as angiogenesis can stimulate the tumor to form new blood vessels that supply it with sufficient nutrients and thus promote its growth. Under normal physiological conditions, the generation of new blood supply is controlled by the balance between the production and secretion of pro- and anti-angiogenic factors such as VEGF, FGF1/2 and thrombospondin-1 or β-interferon respectively. Cancer cells acquire the ability to tip this balance towards the production of more VEGF and/or FGF1/2 that drive angiogenesis and concomitant suppression of thrombospondin-1 or β-interferon expression (Hanahan D, Folkman J., 1996). Once the tumor grows large enough, the cancer cells invade the surrounding tissues. During the course of its growth, the tumor mass starts producing pioneer cells that are endowed with the capability of breaking off and travel (metastasize) to distant tissues where they form new tumors. Most cancer related deaths are due to metastasis to distant sites (Sporn MB., 1996). In addition to the presence of all the above hallmarks of cancer, factors such as cell-cell interactions (via members of the cadherin family) and interaction of the tumor cells with the tumor microenvironment (via members of the integrin family) play critical role in determining the success of metastasis. Ubiquitously expressed E-cadherin on epithelial cells form homotypic cell-cell contacts and maintains tissue integrity. Additionally, E-cadherin mediated cell-cell contacts transmit growth suppressing signals into the cells by sequestering the growth promoting transcriptional activity of β-catenin via the Wnt/β-catenin pathway (Christofori G, Semb H., 1999). Functional E-cadherin is lost during malignant transformation of most epithelial cancers, whereas artificially induced E-cadherin expression have been shown to suppress invasion and metastasis both in cultured tumor cells and in mouse models (Christofori G, Semb H., 1999).

6. ***Ability to metastasize***

Unlike normal cells, cancer cells can possess the capability to metastasize – i.e. break through the basement membrane and invade surrounding tissue and spread to distant locations via the vascular system to form new tumors in secondary organs. It is the growth and spread of tumors that can interfere with normal functioning of vital organs which is the primary cause of death from cancer.

7. ***Ability to survive with low oxygen***

Inspite of induced angiogenesis, the core of the tumor may still be deprived of sufficient nutrients and oxygen. Non-malignant cells, that use oxygen to convert to glucose via aerobic metabolism would not be able to survive in such condition. The ability of cancer cells to change to anaerobic (oxygen-free) metabolism allows them to continue producing energy and thus survive in oxygen-deprived conditions.

8. ***Evading the immune system***

Under normal physiological conditions, foreign and abnormal cells are detected and destroyed by the body's immune system. Cancer cells are able to evade detection and destruction by hijacking the host immune system, allowing them to survive, proliferate and invade other tissues, although this process is not fully understood.

In addition to the above cancer hallmarks, there are two additional characteristics that enable the alterations necessary for carcinogenesis:

9. ***Genomic instability***

Maintenance of genomic integrity is the cornerstone that ensures health and survival of the organism. Constant surveillance and repair of DNA lesions by a host of DNA repair enzymes and mitotic fidelity that maintain karyotypic integrity are key cellular mechanisms that achieve genomic stability. Malfunction of these genomic caretakers are often the cause of cancer pathogenesis that may prove to be disastrous to the organism as a whole (Lengauer C, Kinzler KW, Vogelstein B., 1998). Accumulation of specific oncogenic mutations that promote cellular proliferation or disrupt control mechanisms such as mutation in the tumor suppressor genes can lead to transformation of mormal cells into cancer cells by acquiring hallmark characteristics. One of the most well studied members of the genomic caretaker is the p53 protein. Tumor suppressor function of p53 comprises of either DNA repair or arrest of the cell cycle in response to DNA damage. In the event of extensive DNA damage that cannot be repaired, p53 can also activate apoptotic cell death. In most cancers, p53 mutation abrogates its tumor suppression function resulting in compromised DNA damage repair that allow genomic instability to accumulate over multiple generations,

ultimately giving rise to mutant cells endowed with selective advantage (Levine AJ., 1997).

10. ***Inflammation***

The presence of chronic inflammatory conditions may result in molecular changes that can promote cellular proliferation, survival of the cancer cell and angiogenesis. DNA damaging free radicals may also be increased by inflammation.

The fact that there exists a multitude of traits that together are considered as hallmarks of cancer implicates that the human body has many layers of defenses that regulate mitosis and prevent DNA damage. Therefore, an agent that interferes with any one of the cancer-related hallmark processes is unlikely to cause cancer. But when multiple hallmarks are disrupted by a single or multiple agent(s), it can overwhelm the body's defenses, and can eventually lead to uncontrollable cellular growth, resulting in one renegade cell that rapidly divides itself to form the tumor. Thus, it requires multiple "hits" to cellular processes for carcinogenesis. This paradigm is known as the multi-hit model of carcinogenesis.

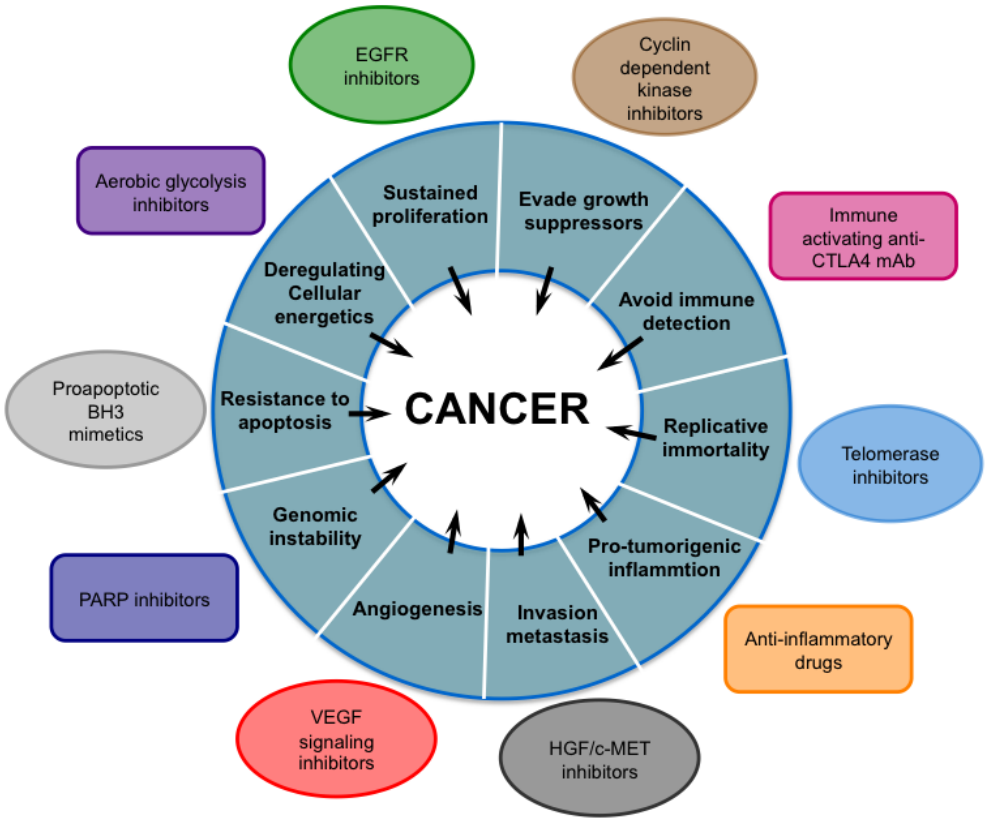

Figure 2: Hallmarks of cancer.

The six characteristics that differentiate malignant cells from cancer cells include automony of proliferative signals, ability to evade the effect of growth repressors, acquired resistance to apoptosis, almost infinite ability to undergo cellular divisions, generating new blood vessels for supply of oxygen and nutrients to sustain growth and ultimately, the invasion of nearby tissue and metastasis to remote location and recolonization (Adapted from Hanahan D, Weinberg RA., 2011).

1.2. Cancer incidence

As per WHO estimates, cancer has emerged as a second leading cause of mortality globally, claiming approximately 9.6 million lives in 2018. Middle and low-income countries bear nearly 70% of the burden of cancer related deaths. In addition, environmental factors and lifestyle choices significantly contribute to overall mortality rates. Use of tobacco poses a major risk factor and is responsible for nearly 22% of cancer related deaths in developing countries. Approximately 25% of cases in the middle and low-income countries are due to infections such as human papilloma virus (HPV) and hepatitis. Of all cancers, lung cancer and breast cancer have the highest incidence worldwide (2.09 million cases each), followed closely by colorectal cancer (1.8 million cases). 1.76 million deaths from lung cancer and 862000 deaths from colorectal cancer have been reported globally (WHO fact sheet, 2018).

In the Indian subcontinent, with a population of nearly 1.8 billion, there is an increasing trend of cancer incidence and is a major cause of healthcare concerns. There has been an increase in the number of cancer cases from 58400 in 1990 to 106900 in 2016. A similar trend is seen in cancer related mortality rates, whereby in 1990, 382000 people had died from cancer whereas in 2016, number of deaths had increased to 813000 (Dhillon PK, Mathur P, Nandakumar A,, *et. al*., 2018). Among men, the major sites of cancer are the oral cavity, lung, gastrointestinal tract including esophagus and stomach and colorectal, whereas in women, cervical and breast cancer are the most common followed by oral and ovarian cancer (Figure 3). Tobacco and alcohol use, diet and other lifestyle factors are responsible for the shifting trend. This is further compounded by viral infections, and exposure to environmental pollutants like pesticides and radiation (WHO fact sheet, 2018, Bray F, Farley, ME, *et al*., 2018).

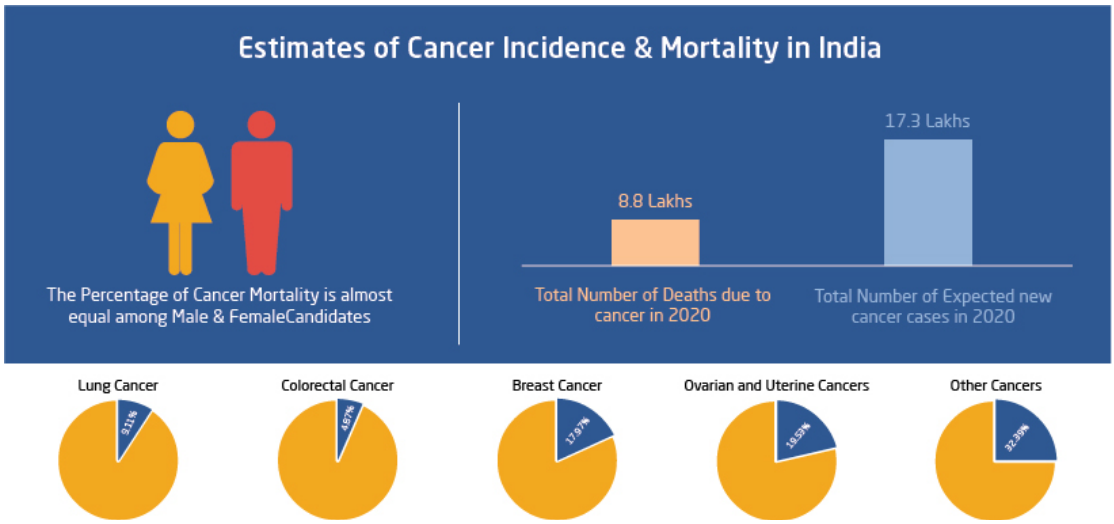

Figure 3: Estimates of Cancer Incidence and Mortality in India.
As per Indian Council of Medical Research (ICMR) estimates, by the year 2020, the number of new cancer cases in India is likely to be increase to approximately 17.3 lakhs, with 8.8 lakh cancer related deaths. Breast, ovarian and uterine cancers would have the highest incidence rates. Followed by lung and colorectal cancers (Source: Dsouza, N.D.R., Murthy, N.S. & Aras, R.Y., 2013).

1.3. Relevance of the research

Colorectal cancer (CRC) ranks third in incidence rates but is the second most common cause of death worldwide. The number of cases shows an upward trend in the developed countries although the rate of mortality is either stabilizing or declining. The rapidly developing countries however show an increasing trend both in incidence and mortality rates (Arnold M, Sierra MS, *et al*., 2017).

Rapid economic development and fast-changing lifestyle have seen an increase in the overall number of CRC cases while limited access to healthcare particularly among patients with poor socioeconomic background could account for the rapid increase in the number of CRC related deaths in India in the recent past. The estimated yearly incidence rates of colon and rectal cancers in India are approximately 4.4/100.000 and 4.1/100,000 respectively (Bhawna Sirohi, Shailesh V. Shrikhande, *et al*., 2014). Also, younger patients are being diagnosed with advanced disease (Patil PS, Saklani A, Gambhire P, Mehta S, *et al*., 2017). The rise in incidence and mortality rates in India, together with limited access to treatment facilities pose a massive burden on the management of CRC.

In the recent past, conceptualizing the mechanisms that determine onset and disease progression has been the focus area for research. Such studies have not only contributed to our present understanding of the factors that contribute to the pathogenesis and progression of CRC but also form the basis of current treatment modalities. However, most of the studies have been performed using patient samples or tumor derived cell lines from the Caucasian population, with almost no such study designed for cancers originating in India. Owing to the inherent genetic diversity of the Indian population (Xing J, Watkins WS, Hu Y, Huff CD, *et al*., 2010, Tamang R, Singh L, Thangaraj K. 2012, Metspalu M, Romero IG, Yunusbayev B, Chaubey G, *et al*., 2011, Soya SS, Padmaja N, Adithan C., 2005), coupled with a lack of mechanistic insight into the molecular events that lead up to disease onset, the current standard-of-care therapeutics are often rendered suboptimal for the Indian patients.

Therefore, there is a need to establish primary tumor derived cell lines from Indian patients that could be developed into models for studying the genetic basis of cancer in the Indian population. The present study was therefore designed to establish and characterize a tumor

derived primary CRC cell line from an Indian patient that may potentially be used as a tool for not only gaining insight into the fundamental mechanism of disease onset and progression, but also test therapy regimens that are currently in clinical practice for treatment of CRC. The unique molecular signatures present in the Indian cancers can be developed further either as population specific molecular markers or aid in development of new chemotherapeutic strategies with increased efficacy. With this background, we set forth to generate and characterize a novel primary tumor derived CRC cell line from a patient of Indian origin.

The current study enabled the isolation and establishment of a new cell line, CRC02, from an Indian CRC patient. In-depth morphological and molecular characterization of CRC02 provided an insight into the mechanism of pathogenic transformation that may be prevalent in the Indian population. In addition, it brought forth the subtle differences in the regulation of critical molecular machinery that may potentially translate into more drastic differences that eventually result in the standard therapies being rendered suboptimal in the Indian patients. Furthermore, these specific molecular signatures could be developed as markers to be used in the clinic as diagnostic tools and extended to develop tailored therapies for cancers that are specific to the Indian population.

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CHAPTER 2

RESEARCH OBJECTIVES

CHAPTER 2

Research objectives

2.1. Research objectives

There is a need to establish primary tumor derived cell lines from Indian patients that could be developed into models for studying the genetic basis of cancer in the Indian population. The present study was therefore designed to establish and characterize a tumor derived primary CRC cell line from an Indian patient that may potentially be used as a tool for not only gaining insight into the fundamental mechanism of disease onset and progression, but also test therapy regimens that are currently in clinical practice for treatment of CRC. The unique molecular signatures present in Indian cancers can be developed further either as population specific molecular markers or aid in development of new chemotherapeutic strategies with increased efficacy. With this background, we set forth to generate and characterize a novel cell line, CRC02.

Objective 1: Generation of primary tumor cell line, CRC02

Tumor tissue was surgically obtained from a female Indian CRC patient. Pathological staging of disease was done at $T_2N_1M_0$ (stage IIIA). The cancer had spread into the surrounding submucosa and into at least three lymph nodes, although distant metastasis had not occurred. Formalin fixed paraffin embedded blocks were prepared from a portion of the resected tumor tissue for histological analyisis by hematoxylin-eosin staining to examin the overall histopathology. Another portion of the tumor was used for establishing the primary CRC cell line, CRC02.

Objective 2: Characterization of CRC02 *in vitro*

Pathological transformation of CRC follows a series of well-defined molecular alterations in specific genes. Mutational status of these genes often form the basis for selecting therapeutic regimens such as those including cetuximab, an targeted therapeutic used to treat patients who have overexpression of EGFR. Preliminary characterization included examination of the morphology and growth characteristics of the cell line. Subsequently, the primary tumor and the derived cell line CRC02 were examined for the presence of mutations in the *BRAF* (V600E) and *KRAS* (codons 12, 13, 61 and 146) genes that are strongly associated with CRC pathogenesis.

In addition to gene mutations, the accumulation of gross chromosomal aberrations over time results in global genomic instability, a common feature and a major contributor towards the onset and progression of CRC. Karyotype analysis of CRC02 was performed to determine the chromosomal abnormalities present in CRC02. Further, DNA content analysis was performed to calculate the percentage of cells present at various stages of the cell cycle.

Objective 3: Molecular characterization of CRC02

CRC pathogenesis and progression involves an overall mis-regulation of multiple signaling pathways that often crosstalk with each other in complex networks. Within these networks are proteins that act as either oncogenes or tumor suppressors. Gain-of-function mutations in the oncogenes or loss-of-function mutations in tumor suppressors may result in these proteins' anomalous behavior, resulting in constitutive activation of signaling. This aberration is a prerequisite for cells to undergo malignant transformation. Therefore, we performed in-depth mRNA analysis using qRT-PCR to gain insight into the pathways that maybe mis-regulated in CRC02 and compared with established cell lines such as SW620, HT29 and HCT116. Further, functional effect of the altered gene expression profile was validated using wound healing and migration-invasion assays to measure the migratory and invasive potential of CRC02 cells. In addition, Wnt/β-catenin pathway, the most commonly mis-regulated signaling circuit in CRC pathogenesis, was analyzed in-depth. Subcellular localization, mutation, and turnover of β-catenin, the central molecule in this pathway, were studied in detail. The effect of aberrant β-catenin function was also correlated to observed phenotypes such as centrosomal and mitotic

defects in the form of supernumerary centrosomes, multipolar metaphase spindle and delayed cytokinesis.

The ability of a cell line to form tumors upon transplantation into animals is a prerequisite for it to be developed as a model for studying pathogenesis and a tool for screening drug candidates. *In vitro* tumorigenic potential of CRC02 was examined by growing these cells in an anchorage independent manner in soft-agar. Also, these cells were injected into SCID mice to establish xenograft to validate the same in-vivo.

Objective 4: Testing chemotherapeutic agents on CRC02

For a chemical compound to be developed as a drug candidate, it is crucial to measure its efficacy *in vitro* prior to injecting in animals. Therefore, *in vitro* cytotoxicity assay performed on tumor derived cell lines is an indispensable tool for screening vast libraries of compounds. The standard treatment for CRC involves a combination of drugs like 5-fluorouracil (5-FU), a nucleoside analog, oxaliplatin, a platinum drug, irinotecan, a topoisomerase inhibitor and cetuximab, the EGFR inhibitor.The therapeutic regimens that are in clinical practice for treating CRC comprises of combinations including 5-FU, leucovorin, oxaliplatin, irinotican and cetuximab. We tested these drug combinations on CRC02 to determine their efficacy by *in vitro* cytotoxicity assay.

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CHAPTER 3

GENERAL CHARACTERIZATION OF CRC02

CHAPTER 3

General characterization of CRC02 cell line

3.1. Background

Colorectal cancer (CRC) has emerged as the third major cause of cancer-related deaths worldwide and is continuing to rise at an alarming rate of 60%. By the year 2030, there would be ~2.2 million new cases and 1.1 million CRC related deaths (Arnold M, Sierra MS, Laversanne M, *et al*. 2017). Sedentary lifestyle, dietary habits and environmental factors are the major causes attributed to the ever-increasing risk of CRC. Early screenings and emergence of improved treatment strategies have resulted in gradual improvement in prognosis with the 5-year relative survival rate reaching almost 70% among CRC patients in Western countries (Arnold M, Sierra MS, Laversanne M, *et al*. 2017). However, it continues to impose as a major challenge in developing countries having a 5-year relative survival rate lower than 50% (Siegel RL, Miller KD, Fedewa SA, *et al*., 2017). In India, poor sensitivity to standard-of-care therapies and lack of early diagnostic parameters, coupled with limited access to healthcare facilities and poor socioeconomic backgrounds not only pose a massive challenge in the clinical management of CRC but also contribute to the increase in the incidence and death rates due to the disease burden.

In the past two decades, the overall survival (OS) has improved, particularly for those patients who have been diagnosed at an early stage, owing to the advances made in the therapeutic regimens for CRC (Chatenoud L, Bertuccio P, Bosetti C, 2014) (Heinemann V, von Weikersthal LF, Decker T, *et al*., 2014, Schwartzberg LS, Rivera F, Karthaus M, *et al*., 2014). Development of novel drugs have brough about this shift in the OS, perticularly for advanced stage CRC. This progress has been encouraging, although the current paradigm employs a “one-size-fits-all” approach that is beneficial to only a small group of patients.

In spite of the implementation of customary CRC screening at the population level resulting in diagnosis at a relatively early stage, a large number of CRC cases still diagnosed at the metastatic stage. Effective treatment of CRC relies on therapeutic agents that are developed

based on the mechanistic determinants of the disease. Recent years have seen rapid and significant advances being made in understanding the pathology and genetics of CRC. As a result, the field has experienced a significant progress in the development of novel therapies which have improved the overall patient survival. Fluoropyrimidine-based chemotherapy formed the backbone of CRC treatment, with irinotecan and oxaliplatin forming combination regimens that have significantly improved overall patient survival. The development of novel biologics in the past dacade, such as therapies directed against VEGF and EGFR have further transformed the therapeutic landscape of metastatic CRC. However, not all patients receiving these therapies respond to a similar extent. Individual patient- and tumor-related factors need to be considered before selection of the right patient groups for leveraging the optimal therapy.

In this context, it should be noted that most of the molecular and therapy response learnings have been based on tumor tissues and cell lines generated from samples derived from Caucasian patients, with almost no data available for Indian patients. Due to the inherent genetic diversity of the Indian subcontinent (Xing J, Watkins WS, Hu Y, *et al*, 2010), it is imperative to understand the genetic factors that govern pathogenesis in the Indian patients and is therefore crucial for the management of CRC in India. Although CRC cases are rising in India, there is a lack of tumor-derived cell lines generated from Indian patients that can be used to address this problem. We have therefore generated a new cell line, CRC02 and carried out molecular characterization that would unravel the mechanism that play crucial role in the pathogenesis of disease in Indian population. Understanding the panel of clinical- as well as tumor-associated characteristics in this primary tumor-derived cell line would set the foundation for establishing a more personalized treatment strategy among CRC patients in the Indian subcontinent. Based on patient characteristics, a tailored, biomarker driven approach may be adopted for selecting treatment regimen that can optimize therapy response and avoid unnecessary adverse effects.

3.2. Materials and methodology

3.2.1. Analysis of primary tumor section by immunohistochemistry

Paraffin embedded tissue blocks were used to perform hematoxylin-eosin and immunohistochemical staining of the primary tumor. ~3 μm sections of the tumor tissues were made and collected on positively charged slides. The collected sections were heated on a hot-plate at 60°C for 1 hour and deparaffinized. Rehydrated of the tissue sections was done by passing through an ethanol gradient and deionized water. Tissue sections were then placed in hot citrate buffer for 20 minutes for heat induced antigen retrieval. Blocking of endogenous peroxidase activity was performed by incubating the sections in 30% hydrogen peroxide solution for 20 minutes. Blocking of non-specific antibody binding was achieved by incubating the tissue sections in 10% normal goat serum, prior to adding primary antibody. After 1h, the tissue sections were washed with PBS and incubated in HRP labeled secondary antibody for 1h, followed by washing in PBS. Unbound antibody was washed off with PBS. Freshly prepared chromogenic substrate was added to aid visualization of the staining. Reaction was terminated by washing with PBS. All slides were counterstained using hematoxylin, dehydrated by passing through ethanol gradient and placed in xylene. The sections were then mounted and sealed. Leica's Aperio ImageScope software (V12.3.3.5048) was used for capturing representative images.

3.2.2. Establishment of CRC02 cell line

Surgically resected primary tumor tissue was obtained from a female Indian patient diagnosed with CRC. Clinical staging of the tumor at the time of resection was done at $T_2N_1M_0$. The resected tumor was washed extensively with PBS and incubated at room temperature for 10 mins with penicillin-streptomycin (1X). The primary tumor tissue was divided into small fragments of ~0.5-2.0 mm^3 size. The sectioned tumor pieces were then digested in DMEM containing 0.5X collagenase and antibiotics (penicillin-streptomycin) for 2-4h at 37°C. The digested tumor was passed through a fine-mesh cell strainer and centrifuges to collect the cells and seeded in flasks and allowed to attach. Tumor debis and unattached cells were washed off by PBS and replaced with the media. Fibroblasts were removed by differential trypsinization. The cells were also treated with media containing EDTA (0.01%) for 3 minutes and grown in

media containing only 5% FBS for 24h to inhibit fibroblast growth. This was repeated for at least for three passages to allow enrichment of the cultures for cancer cells and remove fibroblast contamination. Sequential passaging of the cells was performed in phenol red-free DMEM (containing 5.5mM glucose) supplemented with gentamicin (1X) to finally establish the primary tumor derived cell line CRC02.

3.2.3. Immunofluorescence staining of CRC02 cells

CRC02 Cells were seeded on pre-cleaned glass cover slips. Tthe cells were allowed to adhere overnight. Next day, the cells washed in PBS. Fixation was done in 3.7% formaldehyde 5 mins. Permeabilization solution (PBS + 1% BSA + 0.5% Triton X-100) was used to permeabilize the cells for 1-2 mins at room temperature. 1% BSA + 0.05% Triton Non-specific antibody binding was prevented by using blocking solution (PBS + 1% BSA + 0.05% Triton X-100) for 30 mins. This was followed by incubation with anti-α-tubulin primary antibody at a dilution of 1:1000 for 1h. The cells were washed with PBS prior to incubating with secondary antibody for 1h. The cells were again washed with PBS and stained with Hoechst 33258 at 1:5000 dilution from a 5mg/ml stock solution. The washed cells were then mounted in antifade mounting media (Prolong Gold, Invitrogen). The slides were dried overnight. Representative images were captured Nikon TiE Eclipse epifluorescence microscope.

3.2.4. Population doubling time

CRC02 cells were seeded in 12-well dish at an initial concentration of 15,000 cells/well. Next day, cells were trypsinized from a single well at regular time intervals, collected and counted. Generation time of CRC02 cells was calculated as per the following formula:

$$DT = \frac{T \, Log2}{Log\,(Xe/Xb)}$$

DT = Doubling time, T = the time interval between two cell collections, X_e = cell number at the end of the incubation, X_b = cell number at the start of the incubation.

3.2.5. Cytogenetic analysis

CRC02 cells were grown in 5ml of DMEM till approximately 70% confluency. Demecolcine solution was added to the culture at 100ng/ml. After 24 or 48h, the treated cells were harvested by centrifugation. The cells were resuspended in 0.075M KCl (hypotonic solution) followed by incubation at 37° for 20 mins. 500µl of cooled solution of methanlol and acetic acid solution (3:1) was added to fix the cells. The cells were collected by centrifugation and resuspended in fresh fixing solution. This was repeated two more times to ensure uniform and complete fixation, followed by storage overnight at -20°C. Post fixation, the cells added drop wise onto clean glass slides and air-dried. These were then baked for 8-12h at 65°C, followed by sequentially rinsing with trypsin solution, normal saline and finally deionized water. The washed slides were then stained with Giemsa solution for 5 mins. Excess stain was removed by washing with deionized water. Cytogenetic analysis was performed using Cytovision software (Leica Biosystems).

3.2.6. Cell cycle analysis of CRC02

For cell cycle analysis, the cells were trypsinized and collected by cenrifugation. The pelleted cells were washed with 1X PBS twice, and resuspended in 1ml PBS. The resuspended cells were mixed well to ensure a single-cell suspension. Ice cold ethanol (70%) was added to the cells for fixation and placed at 4°C for at least 4h. The ethanol solution was removed by centrifugation, the cells washed using PBS. Staining was done using 50µg/ml solution of propidium iodide (PI) containing RNAse A at a working concentration of 50µg/ml at 37°C for 20 minutes, followed by data acquisition by flow cytometry.

3.2.7. Mutation analysis of CRC02 cells

Purified genomic DNA from primary tumor tissue and CRC02 cells were used to perform PCR reactions. Primers were designed to amplify specific regions in the *BRAF* (codon 600) and *KRAS* (codons 12, 13, 61 and 146) genes. 50µl PCR reaction mix was prepared containing $MgCl_2$ (2.5 mM), dNTPs (0.2mM), forward and reverse primers (1µM each), and PhusionTaq polymerase (0.5 units). SW480 (having mutated *KRAS*) and Caco2 (having wildtype *KRAS*)

were controls for the PCR reaction. PCR was optimized for 25 cycles at the following cycling condition: 95 °C for 5 min, 25 cycles at 95 °C for 30 sec; 60 °C for 30 sec and 72 °C for 30 sec, followed by final extension for 5 min. The amplification products were resolved in a 1.5% agarose gel and the fragments were purified, followed by Sanger DNA sequencing to determine the presence of mutations.

3.3. Results:

3.3.1. Histopathological evaluation and analysis of colorectal cancer related mutations in the primary tumor and CRC02 cell line

The patient was diagnosed with stage IIIA colorectal carcinoma with TNM staging at $T_2N_1M_0$. Histopathological evaluation of the primary tumor sections stained with hematoxylin-eosin (H&E) showed moderate differentiation. Dark purple staining of the nuclei indicated the presence of cancer cells that formed irregular glandular structures (Figure 4A). We used anti-Ki67 antibody to perform IHC on the primary tumor sections in order to examine the presence of actively dividing cells. Tumor cells are endowed with the capacity to divide rapidly and therefore are expected to be positive for Ki67. We observed areas in the tumor sections that were strongly positive for Ki67 that marked the actively proliferating cancer cells within the tumor (Figure 4B). Since Wnt/β-catenin signaling pathway is often misregulated in CRC, we went on to examine its expression level using anti-β-catenin antibody. Immunostaining indicated abundant expression of the protein in the primary tumor sections (Figure 4C)

The original patient derived tumor and the cell line CRC02 were examined for the presence of common colorectal cancer related mutations in codons 12, 13, 61 and 146 of *KRAS* and codon 600 of *BRAF* genes. Our findings revealed the presence of wildtype sequence at these positions for both genes. This suggested that the clinically relevant molecular markers used for selecting therapeutic regimen that includes anti-EGFR therapy were unaltered in both the patient derived cell line CRC02 (Table 1).

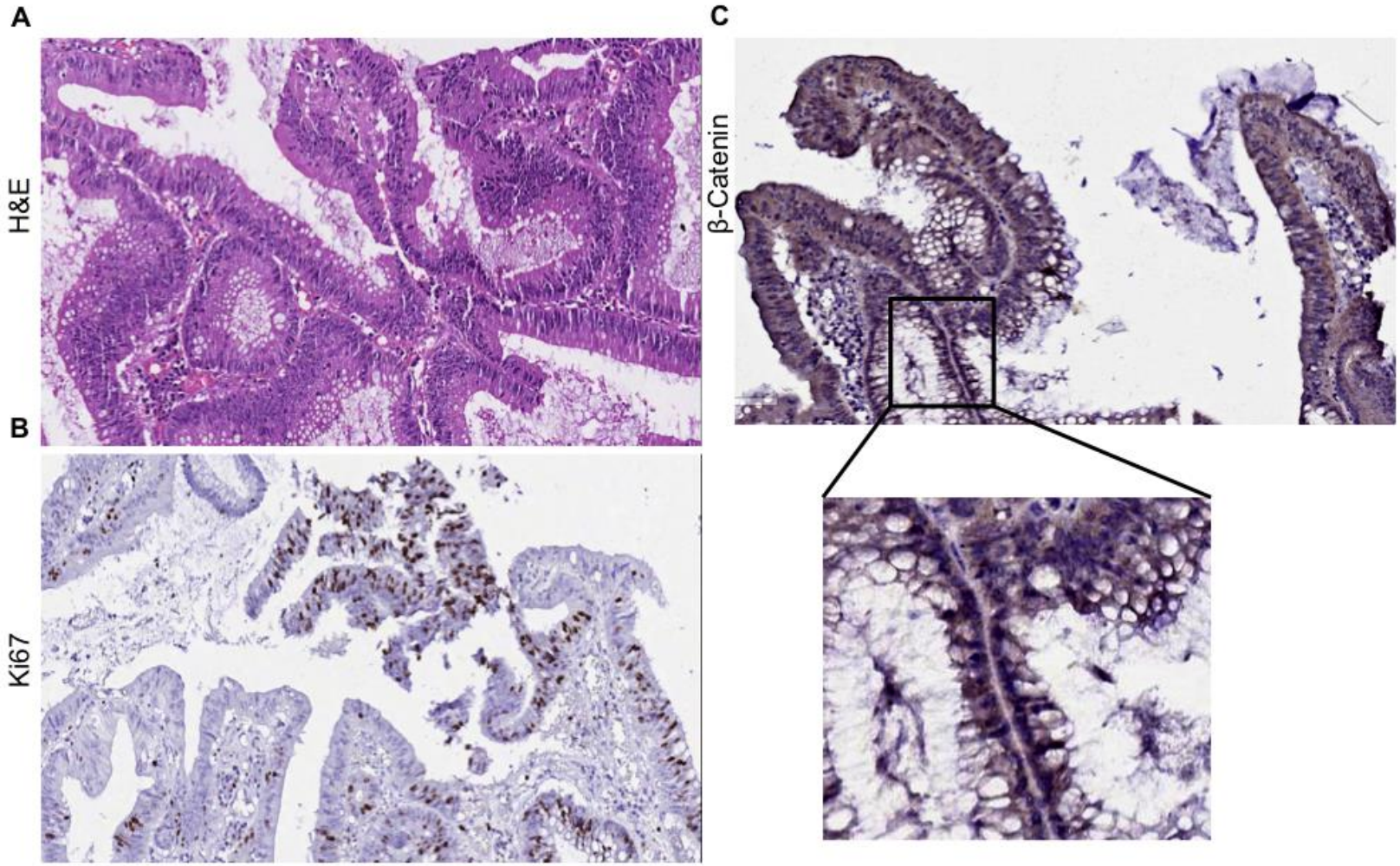

Figure 4: Histology of primary tumor. (A): *Moderate differentiation is indicated in the primary tumor as seen by H&E staining. Malignant cells form glandular structures of irregular shapes.* ***(B):*** *Actively dividing cancer cells are indicated by strong staining with anti-Ki67 antibody* ***(C:)*** *β-catenin is expression is abundant in the primary tumor.*

Table 1: Mutational analysis of CRC02

Clinically relevant mutations in KRAS and BRAF genes were absent in the patient derived tumor and the primary cell line CRC02.

CRC sample	**KRAS mutation**				**BRAF mutation (V600E)**
	Codon 12	Codon 13	Codon 61	Codon 146	
Original tumor	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype
CRC02	Wildtype	Wildtype	Wildtype	Wildtype	Wildtype

3.3.2. Morphological features and growth characteristics of CRC02

We have performed preliminary microscopic examination of CRC02 cells both at early and late passages (10 and 17), under low and high magnifications (10X, 40X). CRC02 cells formed a single layer and adhered strongly to the culture plate. These cells have a flattened morphology with polygonal shape that are typical of cells of epithelial origin and are polygonal in shape (Figure 5A, B). We have also performed immunofluorescent staining with anti- α-tubulin antibody that further highlighted the epitheloid nature of these cells (Figure 5C, D). CRC02 cell line was not derived from a single cell by clonal selection. Therefore, a heterogenous population of cells in the culture of RC02 was apparent. The generation time of an asynchronous culture of CRC02 was calculated to be ~24 hours (Figure 6).

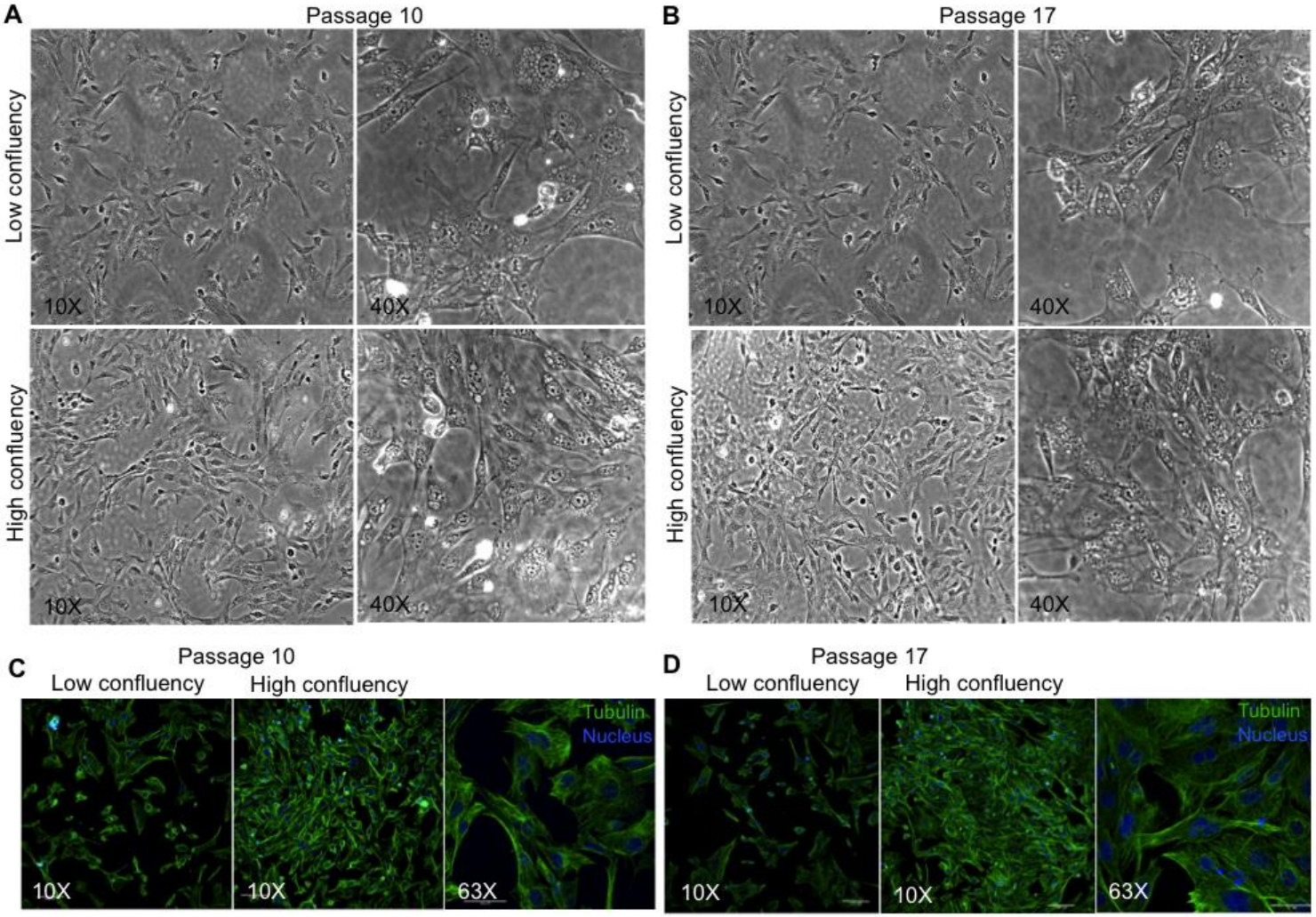

Figure 5: Morphological evaluation of CRC02. (A, B): *Low and high magnification phase contrast images of CRC02 cells reveal flattened cells with similar morphology as epithelial cells. These cells are adherent.* ***(C, D):*** *CRC02 cells have well formed cytoskeletal network as seen by immunostaining using anti-α-tubilin antibody.*

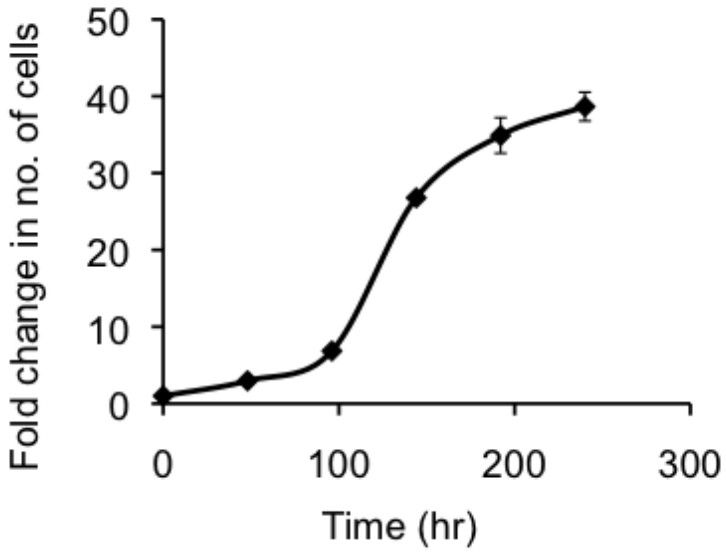

Figure 6: Population doubling time of CRC02.

Generation time of CRC02 cells was calculated to be ~24 hours.

3.3.3. Cytogenetic evaluation of CRC02

Chromosomal instability was observed in almost all CRC02 cells. The modal chromosomal number was found to be 89, indicating that these cells are near-tetraploid. Large number of structural and numerical aberrations was observed in more than one chromosome. Rearrangements of chromosomal segments were in the form of centric fission, as seen in multiple copies of duplicated chromosome 1. Loss of the short arm was observed in chromosomes 1, 2, 4, 5, 11 and 12. Loss of the long arm was seen in chromosomes 2, 9 and 19. In addition, there were derivative chromosomes present that could have resulted from addition of material, probably via translocation. These were present in the long arms of multiple chromosomes such as 18, 19, 20, 21 and 22. Also, the short arm of chromosome number 19 features additional translocated material (Figure 7). We have analysed cells from two different passages (passage 8 and 16) that gave identical results. This indicated that the cell line preserved its integrity across passages.

Figure 7: Karyotype of CRC02.

Karyotype of CRC02 shows abnormalities in the overall chromosome count and structural variations in the form of duplications and deletions in multiple chromosomes.

3.3.4. Mitotic defects in CRC02

3.3.4.1. CRC02 has high percentage of multinucleate and cytokinetically arrested cells

Mitosis is a tightly regulated cellular process preserving genomic integrity and ensuring overall survival of the organism. Dysregulation of cytokinesis, the final step in mitosis, results in aneuploid daughter cells with multiple nuclei (Rengstl B, Newrzela S, *et al.,* 2013). CRC02 had a 12-fold increase in the number of multinucleate cells as compared to HCT116 (Figure 8A-C). This elevation in the percentage of multinucleate cells suggested a high failure rate of cytokinesis in CRC02. In pathological conditions, it is known that controlled duplication of genomic material and division of cells are perturbed and defects in various stages of the cell cycle undermine mitotic fidelity resulting in aneuploid cells, a hallmark of cancer (Gordon DJ, Resio B, Pellman D., 2012). We therefore quantified the fraction of cells present in cytokinesis by observing the presence of characteristic, dense α-tubulin staining (signifying bundled microtubules) in elongated cytokinetic intercellular bridges. We found that approximately 15% cells were present in cytokinesis in CRC02 as compared to only about 6% in HCT116 (Figure 8D). The 2.5-fold elevation in the cytokinetic index in CRC02 cells indicated a pronounced delay/ arrest at the terminal stage of cell division.

3.3.4.2. CRC02 shows supernumerary centrosomes and spindle organization defects

Cancer cells often display high levels of aneuploidy due to the assembly of multipolar (as opposed to bipolar) mitotic spindles that increase the rate of chromosome mis-segregation at each cell cycle, resulting in extensive chromosome instability (Silkworth WT, Nardi IK., *et al*., 2009). Furthermore, in various cancer types, including colorectal and breast tumors, centrosome amplification has been implicated to have significant downstream effects on tumor progression (Mahathre MM, Rida PC, Aneja R., 2016, Levine MS, Bakker B, *et. al*., 2017). The observation of considerable defects in cytokinesis suggested that CRC02 might display other mitotic

defects in the preceding stages of mitosis as well, which has been commonly observed in several cancers (Dalton WB, Yang VW, 2007). We therefore evaluated the centrosomal status of the CRC02 cells in order to determine whether centrosome amplification could be observed in these cells. Immunofluorescence analysis using the centrosomal markers α-tubulin and γ-tubulin (Bahmanyar S, Guiney EL, *et al.*, 2010) revealed multiple centrosomes at interphase in CRC02 (Figure 9A). In addition, we examined metaphase spindles using anti-α-tubulin and anti-γ-tubulin antibodies and imaged them under the fluorescence microscope. Analysis revealed a 6-fold elevation in the fraction of multipolar mitotic spindles in CRC02 cells as compared to HCT116 (Figure 9B and C). Therefore, it is apparent that CRC02 cells exhibits extensive mitotic defects at various stages of the cell cycle in the form of supernumerary centrosomes at interphase, spindle organization defects at metaphase and cytokinetic defects that together contribute towards aneuploidy, as indicated by the presence of a higher fraction of multinucleate cells.

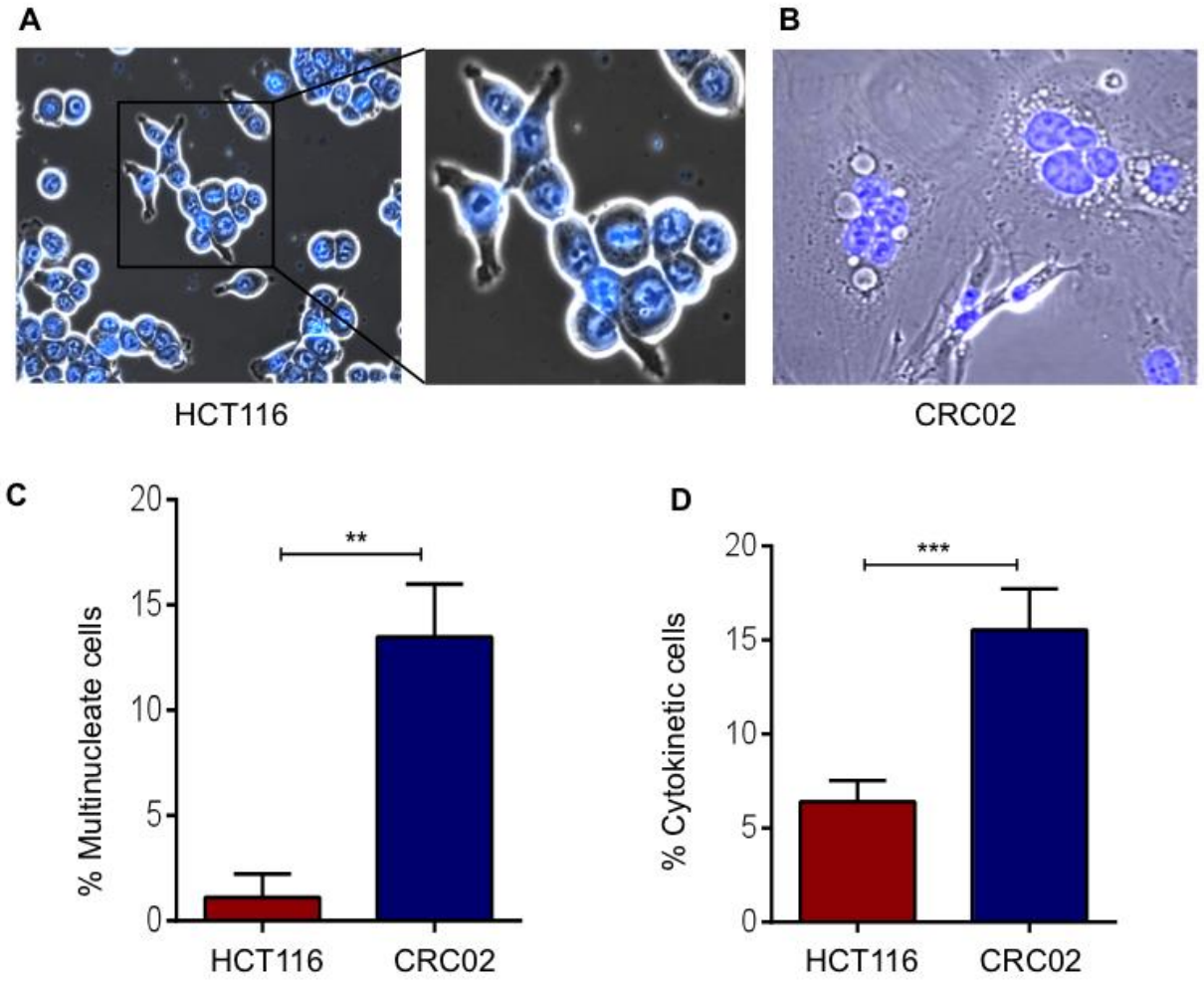

Figure 8: Increased number of multinucleate cells present in CRC02.

(A-C): *HCT116 cells are largely mononucleate (~3%), whereas there is an elevaton in the number of multinucleate cells in CRC02 (~15%).* ***(D):*** *There is a 2.5-fold increase in the number of cytokinetic cells inn CRC02 in comparison to HCT116 suggesting delay at cytokinesis in these cells.*

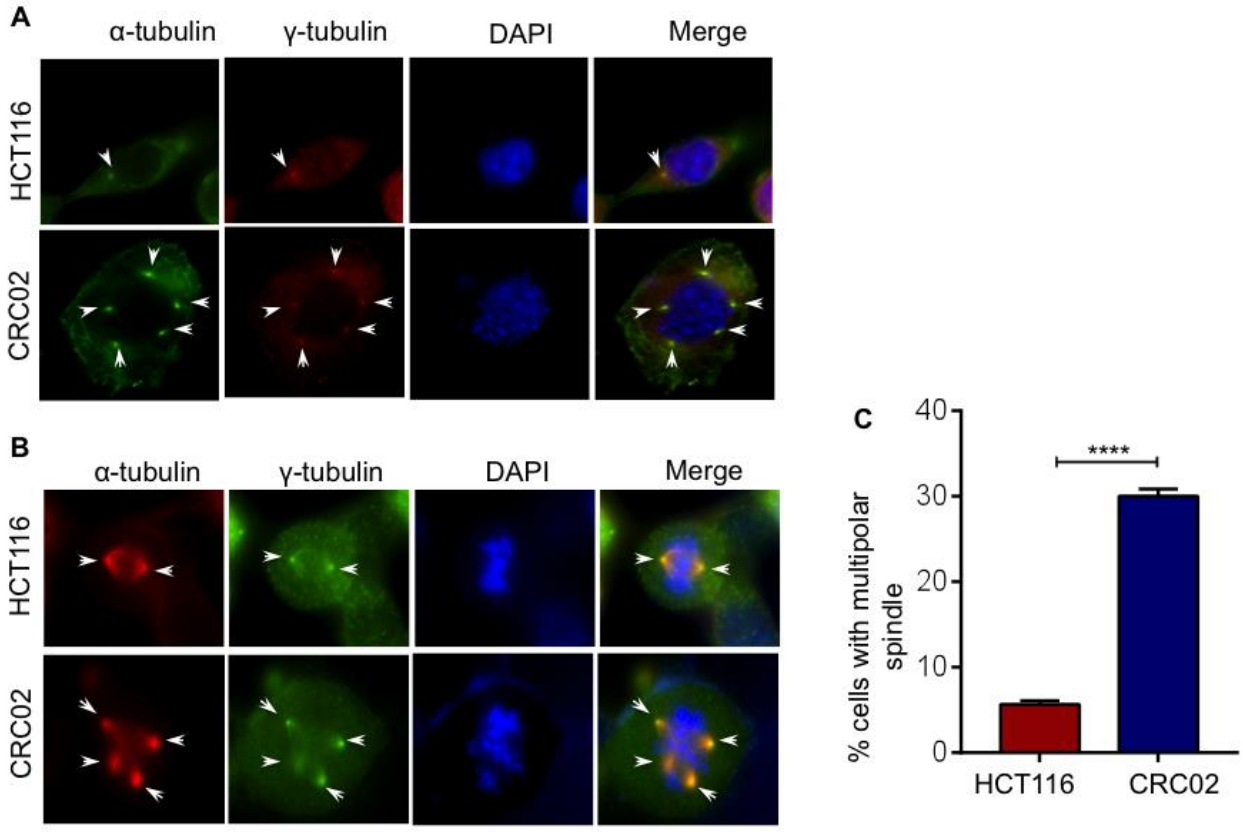

Figure 9: Supernumerary centrosomes and spindle apparatus defects in CRC02 cells. ***(A):*** *CRC02 cells have multiple centrosomes at interphase, whereas, HCT116 cells have one centrosome per cell.* ***(B):*** *Multiple centrosomes lead to formation of pultipolar spindle apparatus at metaphase in CRC02 cells as compared to bipolar spindle in HCT116.* ***(C):*** *Approximately 5% HCT116 cells show multipolar spindle whereas approximately 30% CRC02 cells shpw multipolar metaphase spindle (6-fold increase).*

3.3.5. Cell cycle analysis

We analysed the amount of DNA in asynchronous cultures of CRC02 cells by determining the relative fluorescence of propidium iodide (PI) incorporated at different stages of the cell cycle. ~46% of cells were at G1 phase indicating diploid (2n) DNA content. S phase contained ~24% cells (>2n) while ~15% cells were present at the G2/M phase representing tetraploidy (4n) (Figure 10). This corroborates elevated cytokinetic index in CRC02 as seen in Figure 8D and is indicative of a prolonged mitotic phase in this cell line.

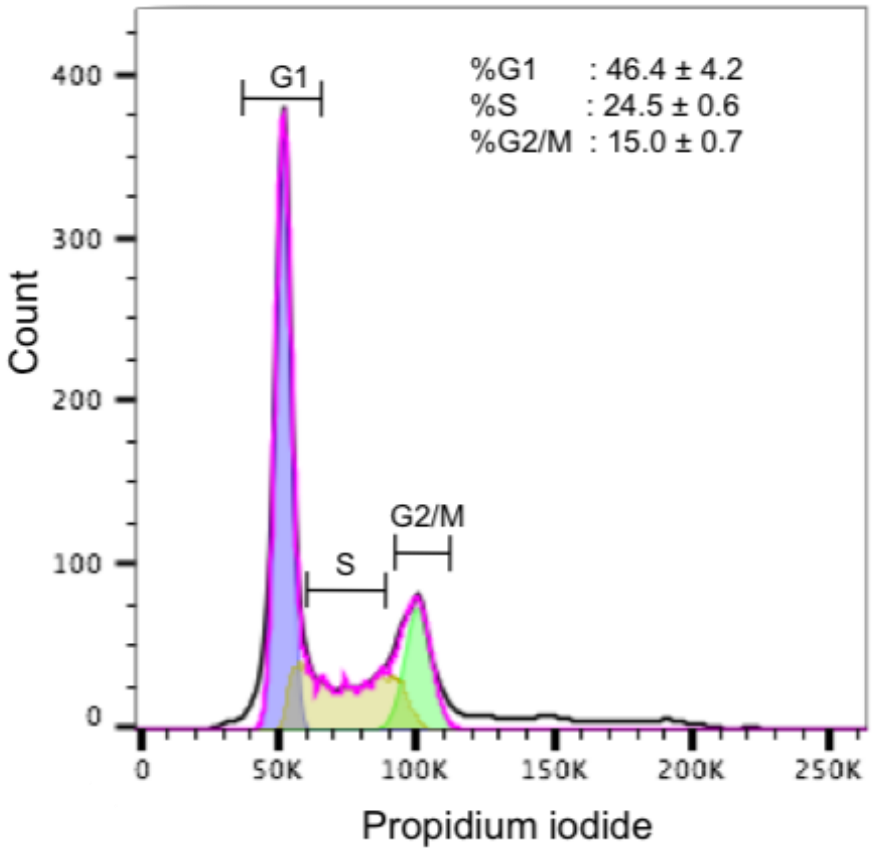

Figure 10: Cell cycle analysis.

DNA content analysis of CRC02 cells revealed ~46% cells in the G1 phase whereas, ~24% and ~15%were in S and G2/M phase of the cell cycle.

3.4. Discussion

CRC in India is fast becoming a major healthcare concern with 5-year relative survival rate lower than 50%. Major challenges in the clinical management of the disease burden are in the form of limited access to healthcare facilities due to poor socioeconomic conditions and suboptimal response to standard-of-care therapies. In order to develop effective treatment for CRC, it is imperative to understand the underlying mechanisms determining pathogenesis and progression of the disease. Great advances have been made in the recent past that have improved the overall survival among patients with CRC. However, the response to these therapies is not uniform among all CRC patients, perhaps due to their inherent genetic backgrounds that influence the treatment outcomes.

Thus far, tumor samples or cell lines derived from Caucasian patients have mostly been used for therapeutic developments for CRC, with almost no molecular information available on the endogenous population of India. Being a genetically diverse population (Xing J, Watkins WS, Hu Y, *et al*, 2010), it is therefore critical to understand the genetic basis of pathogenesis and progression of disease in the Indian population. Therefore, the newly developed cell line, CRC02, has the potential to provide the much-needed resource that could be a valuable tool to dissect the molecular basis of disease that may differ in the India population.

Histological examination of the primary tumor sections showed moderate differentiation along with high expression of the Wnt signaling molecule β-catenin. Genes that are strongly associated with CRC pathogenesis and form the basis for selection of treatment regiment containing anti-EGFR therapy were not mutated in the primary tumor. This was also preserved in the derived cell line CRC02. In contrast, the established CRC cel lines developed from Caucasian patients such as HCT116 and SW620 harbor mutated *KRAS*, whereas, HT29 contains mutation in the *BRAF* gene.

CRC02 had a polygonal shape that is typical of epithelial cells. Cytogenetic analysis revealed CRC02 to be near-tetraploid (n = 89) with large number of structural and

numerical chromosomal aberrations. Alongside, these cells also had abnormal centrosomal count that lead to significant defects during mitosis that led to a large number of cells being multinucleate. Overall, our preliminary examination of CRC02 cells revealed that CRC02 cells are different from the established and widely used CRC cell line HCT116.

3.5. References:

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CHAPTER 4

MOLECULAR CHARACTERIZATION OF CRC02

CHAPTER 4

Molecular characterization of CRC02

4.1. Background

4.1.1. Molecular alterations in colorectal cancer

CRC pathogenesis occurs from benign neoplasms called tubular adenomas and serrated polyps that are often present in the lining of the gastrointestinal tract, predominantly arising in the colon. The number of these small polyps in the lumen increases with age. Morphological changes and uncontrolled proliferation of the cells lining the colon often leads to these polyps to undergo malignant transformation to give rise to cancerous growth. Colonic polyps can be hyperplastic, adenomatous or malignant (Oving IM, Clevers 2002). An advanced adenoma or high-grade dysplasia is defined as a lesion of >10 mm with villous components showing mucosal invasion that does not extend below the muscularis mucosae (Benson AB, 2018), Whereas, a malignant polyp or early colorectal carcinoma consists of cancer cells invading the submucosal layer through the muscularis mucosaebut without distant metastasis (Kashida H, Kudo SE. 2006). Pedunculated polyps have stalks of variable lengths attached to the colonic mucosa and can range from invasive adenocarcinoma that are limited to the polyp head invading the muscularis mucosae to carcinoma cells infiltrating the submucosa potentially giving rise to metastasis (Haggitt RC, Glotzbach RE, Soffer EE, *et al.*, 1985).

Multiple factors contribute to the development and progression of CRC from the polyp to the carcinoma stage including accumulation of genetic mutations over time, epigenetic changes and inflammation.

The sequence of genetic and epigenetic alterations was first proposed in a model by Fearon and Vogelstein, three decades ago, that defined the initiating events and subsequent mutations that eventually led to the development of the final colorectal tumor (Figure 11) (Vogelstein B, Fearon ER, *et al.*, 1988). According to this

model, the cascade of events usually initiated with the loss-of-function mutation of the tumor suppressor *APC*, leading to increased Wnt mediated signaling (Shih IM, Zhou W, Goodman SN, *et al*., 2001). Thereafter, CRC progresses through either chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways (Figure 12, 13). 85% of CRC cases occur through the CIN pathway resulting in hypermutability via extensive loss of heterozygosity (LOH) along with gross abnormalities in chromosomes. The remaining 15% of CRC cases involve MSI, characterized mutations in the DNA mismatch repair (MMR) genes and CIMP pathways causing epigenetic silencing of *MLH1* via hypermethylation of the dinucleotide sequences (CpG islands) in the promoter region. This results in a defective MMR system. MMR pathway includes a multiprotein complex that proofreads and eliminates any mismatches in the nucleotide sequence which escape monitoring by DNA polymerase during DNA replication. There are numerous examples of such alterations at the chromosomal level in various malignancies – 3p21 (*CTNNB1*), 5q21 (*APC*), 9p (*p16, p15*), 13q (*Rb*), 17p (*Tp53*), 17q (*BRCA*), 18q (*DCC, SMAD4*), 16q (*CDH1*). Inefficient DNA mismatch repair during replication of long repeat regions or microsatellites (MSI) in genomic DNA often result in alterations in the length of these repeats that may introduce shift in the reading frame of the gene containing the MSI. This type of genomic instability is prevalent in numerous cancers including colorectal tumors (Aga Syed Sameer, 1990).

This model was further refined leading to our current understanding of CRC pathogenesis to be a result of discrete molecular pathways being altered. As per this model, colorectal tumors develop when there is activating mutations of oncogenes and/or loss-of-function mutation in tumor suppressors. Malignant transformation is possible when there are multiple mutated genes and these mutations accumulate over time. However, the sequence in which these genes mutate may not be of consequence. These salient features of the tumor progression model can also be applicable to other solid tumors (Vogelstein B, Fearon ER, et al., 1988).

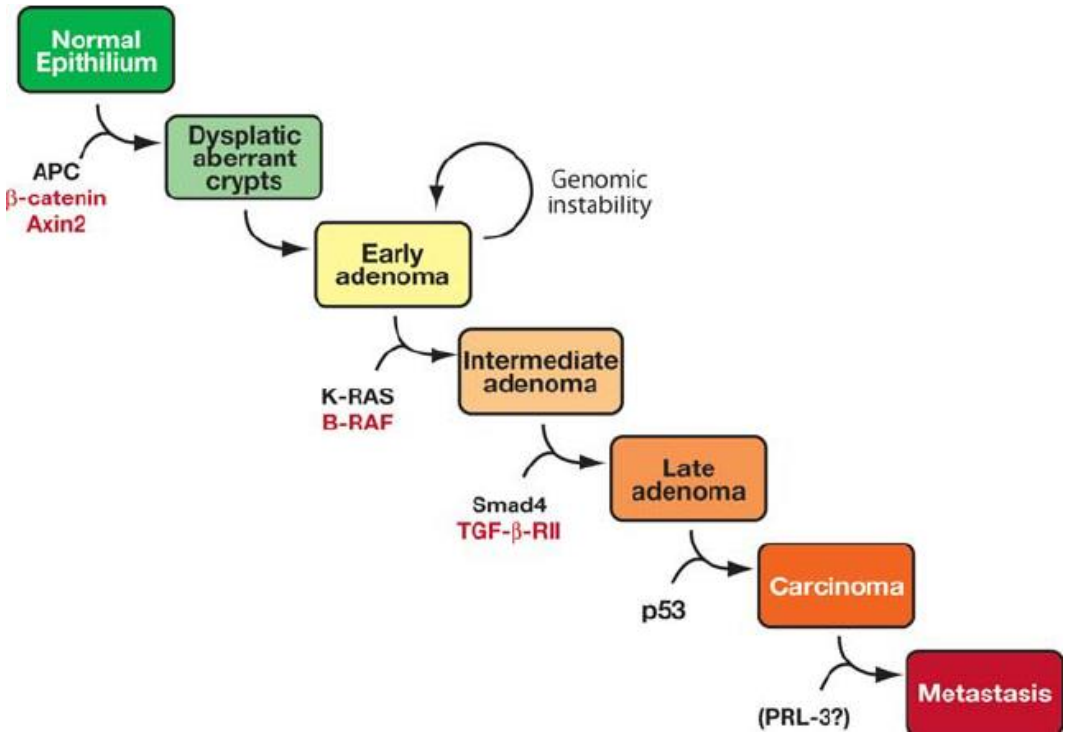


Figure 11: Vogelstein & Fearon model of progression of colorectal cancer.
There is a correlation between colorectal cancer progression and mutation accumulation, both epigenetic and igenetic. Hallmarks of colorectal carcinogenesis are genomic and epigenomic instability in the form of chromosomal instability (CIN), microsatellite instability (MSI), non-MSI hypermutability, aberration in DNA methylation and global DNA hypomethylation, together with alteration in specific genes such as loss of function mutation of tumor suppressor APC, gain of function of ongogene β-catenin, activating mutations in KRAS/BRAF (Sancho E, Batlle E, Clevers H., 2004).

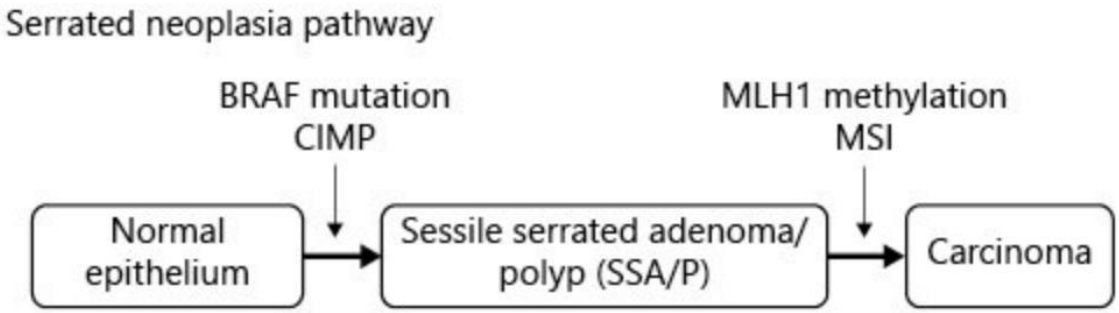


Figure 12. Alternate pathway for CRC pathogenesis: *The serrated neoplasia pathway is an alternative pathway toward CRC development. Serrated lesions are of different types, either hyperplastic polyps, sessile serrated adenoma or traditional serrated adenomas (IJspeert JE, Vermeulen L, et al., 2015).*

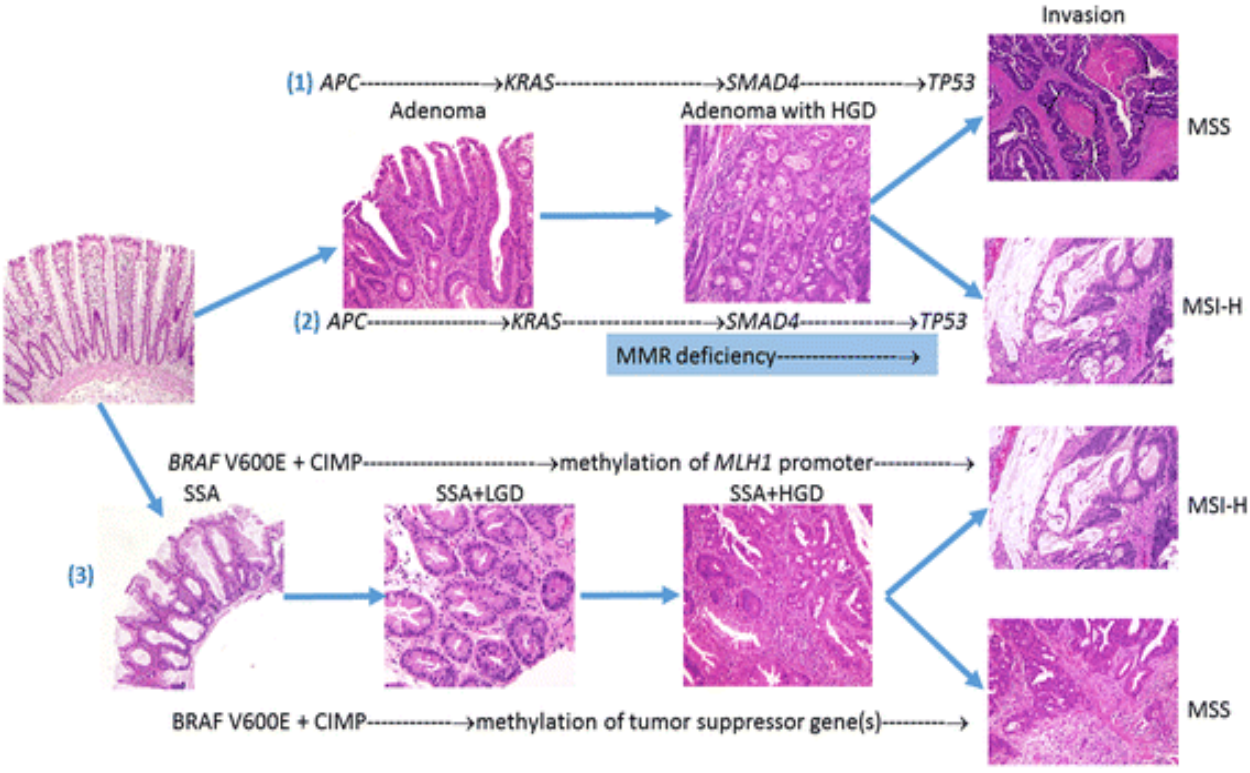


Figure 13. Three routes to CRC progression (1) *Microsatellite stable (MSS) cancers arise via the conventional adenoma-carcinoma sequence with activation of oncogenes such as KRAS and inactivation of tumor suppressors such as APC, SMAD4 and TR53;* ***(2)*** *MSI-high (MSI-H) cancers occur via MSI along with MMR* ***(3)*** *The serrated pathway with CIMP leads to either MSI-H cancers via methylation in MLH1 promoter or MSS cancers with methylation of tumor suppressors (Raul S. Gonzalez, Kay Washington and Chanjuan Shi, 2017).*

Further, population-based studies have revealed a few relevant genes that are correlated with onset and progression of CRC in current clinical settings. Some of these are described below:

***TP53*:** This gene encodes for the tumor suppressor p53 that regulates numerous cellular processes like apoptosis, DNA repair, cell cycle, and senescence. The most important function of p53 is to bring about cell cycle arrest and apoptotic cell death induced by DNA damage. Mutations in *TP53* often result in functional alterations that are critical in tumorigenesis. Nucleotide changes in exons 5-8 in *TP53* are observed in almost 75% of colon carcinomas and 6-25% colon adenomas. This region encodes for the structural domains of p53 that are highly conserved through evolution. Mutant p53 has higher stability along with diminished DNA binding ability that is in contrast to the wildtype protein. The expression level of p53 could be useful as predictive marker for patient survival with stage III CRC and/or rectal cancer (Tzeng ST, Tsai MH, Chen CL, *et al*., 2013).

***KRAS*:** Ras functions as small GTPase that control numerous pathways in a tissue specific manner including cell growth, differentiation and apoptosis (Khosravi-Far R, Der CJ., 1994). Oncogenic mutations give rise to constitutively activated Ras protein that affect cell proliferation and differentiation in the absence of extracellular signals (Fearon ER, Vogelstein B., 1990, Schubbert S, Shannon K, Bollag G., 2007). The RAS family comprises of *KRAS*, *HRAS* and *NRAS*. In CRC, *KRAS* is mutated in 25-60% tumors. Specifically, almost 90% of these mutations are present in codons 12, 13 and 61 (Vogelstein B, Fearon ER, *et al*., 1988, Fearon ER, Vogelstein B., 1990, Fearon ER, 1994, Bazan V, Migliavacca M, *et al*, 2002). 5% of CRC tumors have oncogenic *NRAS* mutations in the same exons as in *KRAS*. Among metastatic CRC, *KRAS* wild-type patients have favorable clinical outcome than patients who are *KRAS*-mutant. Among those with wildtype *KRAS*, treatment with cetuximab shows better response compared chemotherapy alone (Adelstein B-A, Dobbins TA, Harris CA, *et al*., 2011, Dahabreh IJ, Terasawa T, Castaldi PJ, *et al*., 2011, Loupakis F, Cremolini C, Salvatore L, *et al*., 2012, Vale CL, Tierney JF, Fisher D, *et al*., 2012). The combination treatment regimen containing anti-EGFR therapy and chemotheray has not been shown to improve clinical outcomes in patients that harbor *KRAS* mutation. In addition to not being beneficial to the patient, this can potentially be even

harmful. (Bokemeyer C, Bondarenko I, Makhson A, *et al*., 2009, Douillard JY, Siena S, Cassidy J, *et al*., 2010).

***BRAF*:** *BRAF* is a serine-threonine kinase belonging to the RAF subfamily of proteins acting downstream of *KRAS* in the MAPK pathway. *BRAF* is also a potential marker of prognostic value in addition to being a promising target for treatment interventions in CRC patients (Rizzo S, Bronte G, Fanale D, *et al*., 2010). Oncogenic mutations in codon 600 of *BRAF* are frequent during malignant transformation and is reported in various cancers including melanoma (50%), CRC (10%), and lung cancer (1% – 2%) (Berger MF, Hodis E, Heffernan TP *et al*., 2012, Kris MG, Johnson BE, Berry LD, *et al*., 2014). Approximately 80% of mutations in *BRAF* are represented by the transversion at T1799A position resulting in the amino acid substitution, V600E (Davies H, Bignell GR, *et al*., 2002). It has been reported that either *KRAS* or *BRAF* mutations is sufficient to activate the MAPK pathway during carcinogenesis and therefore are mutually exclusive events during oncogenesis (Fransén K, Klintenäs M, Osterström A, Dimberg J, *et al*., 2004).

***APC*:** Adenomatous polyposis coli or *APC* is a tumor suppressor that has been linked with familial adenomatous polyposis (FAP). A Wnt signal antagonist, its interaction with the transcription factor β-catenin is critical in maintaining cellular levels of the latter by modulating its degradation via the ubiquitin-dependent proteasomal pathway. In addition, binding of APC to β-catenin influences the transcription of genes that regulate cellular proliferation. Functional inactivation of APC is present in nearly 80% of colorectal cancer in humans making it one of the most frequently acquired mutations in CRC. Individuals that are heterozygous for such mutations have a higher predisposition to developing CRC (Lawrence N. Kwong and William F. Dove, 2009). The mutational cluster region (MCR) in *APC* is the hotspot for cancer related alterations and nearly 60% of colorectal tumors contain at least one mutation in this region (Samowitz WS, Slattery ML, *et al*., 2007).

***CTNNB1*:** This gene encodes for β-catenin and unlike APC mutations, *CTNNB1* mutations are comparatively rare in sporadic CRC comprising of only approximately 10% of all cases. Most frequently, the N-terminal regulatory region of β-catenin is

mutated in CRC resulting in unphosphorylated protein that escapes proteasome-mediated degradation and accumulates in the cytosol. The stabilized cytoplasmic β-catenin eventually is transported into the nucleus to activate target gene that regulate cellular proliferation (Polakis P., 2000). APC and β-catenin belong to the same signaling pathway (Wnt pathway), therefore, mutations in these two genes are mutually exclusive (Behrens J., 2005).

***PIK3CA*:** The catalytic subunit p110α of phosphatidylinositide-3-kinases (PI3K), is encoded by the gene *PIK3CA* and is mutated in many cancers including CRC. PI3K signaling regulates Ras-mediated cellular proliferation and tumor progression (Gray RT, Cantwell MM, *et al*. 2017, Karakas B, Bachman KE, Park BH., 2006). Mutations in this gene have been correlated with reduced survival in patients containing wildtype BRAF. In addition, concurrent nucleotide alterations in exons 9 and 20 of *PIK3CA* results in enhanced tumorigenicity and poorer survival in comparison to single mutaions either in exon 9 or 20 (Zhao L, Vogt PK., 2008, Rosty C, Young JP, Walsh MD, *et al*.., 2013). Amplification of *PIK3CA* has also been reported to be valuable diagnostic marker for CRC and an independent indicator for better survival for patients receiving adjuvant therapy (Jehan Z, Bavi P, Sultana M, *et al*., 2009).

***PTEN*:** A tumor suppressor that regulate cell-survival, is often hypermethylated in sporadic CRC with high grade MSI (Goel A, Arnold CN, Niedzwiecki D, *et al*., 2004, Tural D, Batur S, Erdamar S, *et al*., 2014). Poor prognosis is associated with PTEN loss in CRC patients where the cancer has metastasized to the liver (Laurent-Puig P, Cayre A, Manceau G, *et al*., 2009). It has been reported that *PTEN* expression could be a valuable predictive marker in patients carrying wildtype KRAS who are being treated with anti-EGFR therapy (Scalise JR, Poças RC, *et al*., 2016).

Table 2: Biomarkers for CRC used in diagnosis, prognosis, progression and treatment.

Established biomarkers for CRC			
Biomarker		**Potential prognostic value**	**Potential predictive value**
BRAF mutation	Metastasis, cetuximab resistance	√	√
KRAS mutation	CRC heterogeneity, cetuximab resistance	√	√
MSI	Resistance to 5-FU	√	
APC mutation	Poor overall survival	√	√
miRNA	Early detection, prognostic stratification, therapy-response prediction	√	√
PIK3CA mutation	Poor prognosis, resistance to anti-EGFR mAb	√	√
Loss of *PTEN*	Elevated metastatic potential, cetuximab resistance	-	√
NDST4 loss	Poor prognosis, metastasis prediction	√	√
Loss of *18qLOH*	Poor prognosis	√	-
IGFR-1R	Metastatic CRC, poor overall patient survival	√	√

Adapted from Vacante M, Borzì AM, Basile F, Biondi A, 2018.

4.1.2. Signaling pathways - misregulation and crosstalk in colorectal cancer

CRC pathogenesis and progression are results of activating oncogenic mutations or loss-of-function of tumor suppressors. Typically, these molecules are components of signaling circuits that are often dysregulated during malignant transformation. Few of these signaling pathways that are most often associated in CRC pathogenesis are the Wnt/β-catenin, Notch and TGF-β signaling (Wu WK, Wang XJ, 2013, Pellatt AJ, Mullany LE, 2018).

4.1.2.1. Wnt/β-catenin signaling pathway in colorectal cancer

A mutagenesis screen for eye phenotype in *Drosophila malanogaster* led to the discovery of a gene that affected body patterning during embryogenesis and was named *wingless*. Subsequently, other members of the Wnt family that played crucial roles during early embryonic development were discovered (Sharma R., 1973, Nüsslein-Volhard C, Wieschaus E., 1980). The involvement of Wnt signaling pathway in carcinogenesis was brought forth by the discovery that inactivation of Wnt1, an ortholog of the *Drosophila* gene *wingless*, resulted in tumors in mice (Tsukamoto AS, Grosschedl R, 1988, Nusse R, Varmus HE., 1982). During subsequent years, genetic tools and biochemical studies have enabled the identification of different components of the Wnt pathway such as the mechanism of Wnt ligand secretion, cell surface receptors for interacting with the secreted Wnt ligands, the regulatory role of β-catenin and the destruction complex. Profound analyses of the cancer genome in the recent past have provided conclusive evidence that mutations in the Wnt pathway occur at a high frequency in human cancers, thus establishing a strong correlation between the two (Clements WM, Wang J, et al., 2002, Satoh S, Daigo Y, et al., 2000, Segditsas S, Tomlinson I., 2006).

The signaling components of the Wnt pathway may be categorized as either positively acting (activating) or negatively acting (suppressing). Often, as in many oncogenic pathways, there is either alterations that lead to functional loss in the suppressors or a gain-of-function mutation in the activators. In either case, the overall effect is the activation of the signaling cascade. In CRC, mutated tumor suppressor APC accounts for approximately 49.8% of all cases (Zhan T, Rindtorff N, Boutros M., 2017). APC

mutation is common in FAP, the heritable genetic defect that leads to development of numerous polyps in the colon and in sporadic cases of CRC. The tumor suppressor function of APC is lost as a result of mutations in the MCR that produces truncated protein that may still retain the ability to bind β-catenin but may lose its regulatory function via the loss of the Axin binding regions (Figure 14) (Polakis P., 2007). Sporadic colorectal tumors often carry two mutations that inactivate APC – one that leads to protein truncation and the other that either has similar outcome or may result in loss of allele. The former is more common in FAP whereas adenomas may feature both truncation and allelic loss although it is rare to find tumors with homozygous deletion (A. J. Rowan, H. Lamlum, M. et al., 2000).

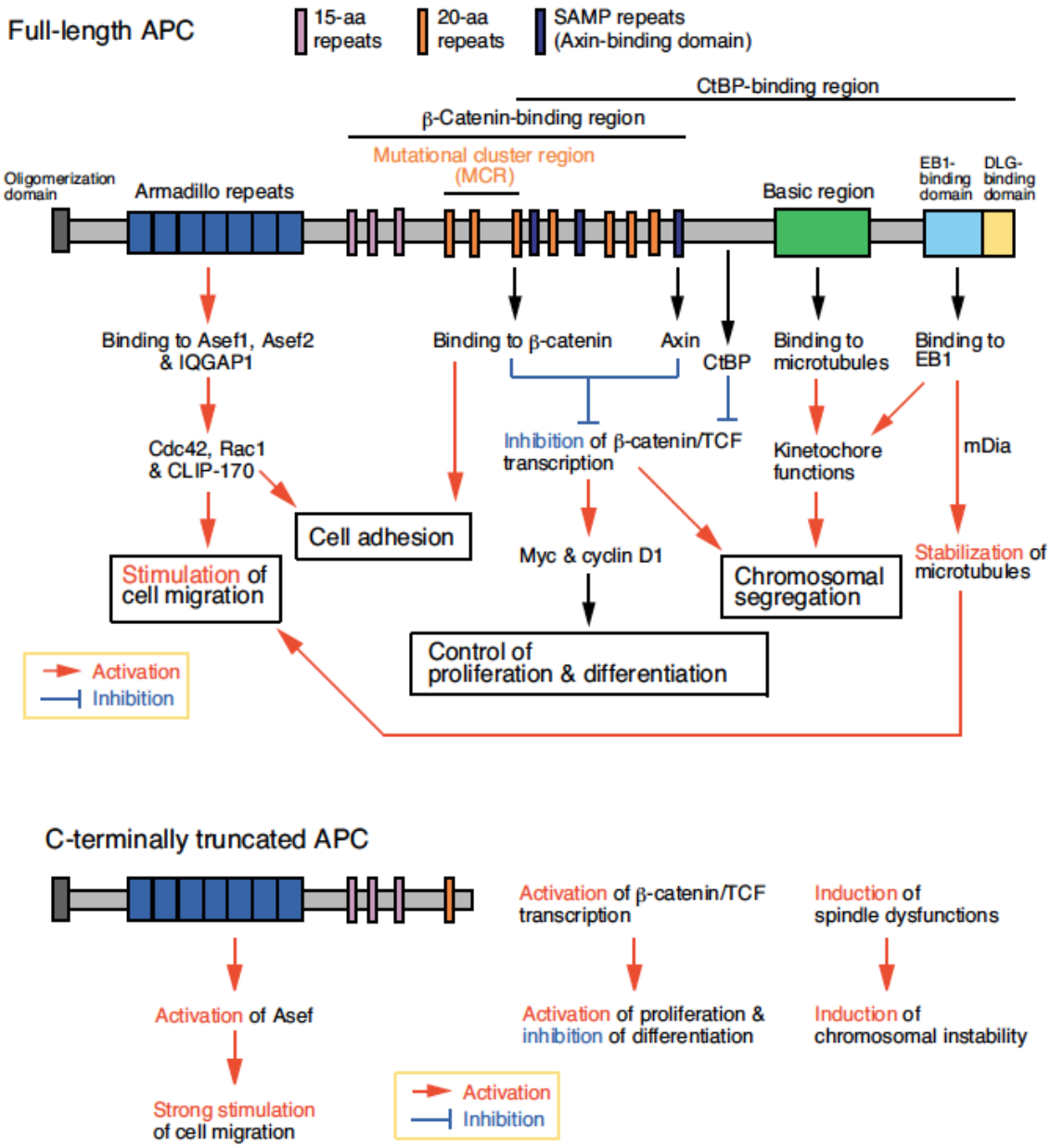


Figure 14: Functional domains of APC and cancer associated mutations.
APC participates in the canonical Wnt signaling via interaction with β-catenin. Since the binding region of β-catenin on APC and the MCR overlap, mutations in the MCR region directly affect β-catenin binding and turnover. The C-terminus of APC harbors the binding sites for components of the mitotic machinery such as the kinetochore and microtubules that play crucial role in cell division, Binding of IQGAP1 at the C-terminus is important for cell migration (Aoki K, Taketo MM., 2007).

Loss of APC is an event that occurs early in the development and progression of CRC. However, in nearly 4.7% of colorectal tumors, activating mutations in β-catenin drive carcinogenesis (Zhan T, Rindtorff N, Boutros M., 2017). Mutations in the N-terminal regulatory region leads to increased stability of β-catenin, altering its intracellular dynamics. Accumulation and enhanced transcriptional activity brings about activation of target genes, many of which are directly related to cellular proliferation. Additionally, mutation in APC compromises its ability to export β-catenin out of the nucleus abrogating its proteasomal degradation. Activation of the proto-oncogene *c-Myc* is modulated by β-catenin and APC. The physiological significance of this during carcinogenesis is evident by the finding that mutant APC induced cellular proliferation and migration is reversed by the loss of *c-Myc* in the intestine. In addition to activating target genes, β-catenin mediated gene suppression contributes towards malignant transformation – epithelial polarization gene ZO-1 is repressed by β-catenin resulting in loss of epithelial polarization (Wu WK, Wang XJ, Cheng AS, et al., 2013). Therefore, the central role of β-catenin in the pathogenesis and progression of CRC makes is a potential target suitable for development of anti-cancer therapeutics.

4.1.2.2. TGF-β/Smad signaling pathway in colorectal cancer

A key signaling cascade that regulates cellular proliferation and differentiation is the TGF-β pathway. Binding of the TGF-β ligand followed by receptor phosphorylation recruits Smad proteins, which act as the effectors of the signaling cascade. R-Smad, also known as Smad2/3, phosphorylates and forms heterodimer with Smad4 into a complex that once translocated to the nucleus, acts as a transcription factor (Figure 15) (Wu WK, Wang XJ, *et al.*, 2013). TGF-β signaling has been shown to play opposite roles depending on the cancer stage – early in carcinogenesis, the general function of the pathway is to promote cellular differentiation and apoptosis and suppress proliferation, thus acting as a tumor suppressor. However, in advance stages of cancer, TGF-β signaling has been shown to increase angiogenesis and metastasis (Wakefield LM, Roberts AB., 2002).

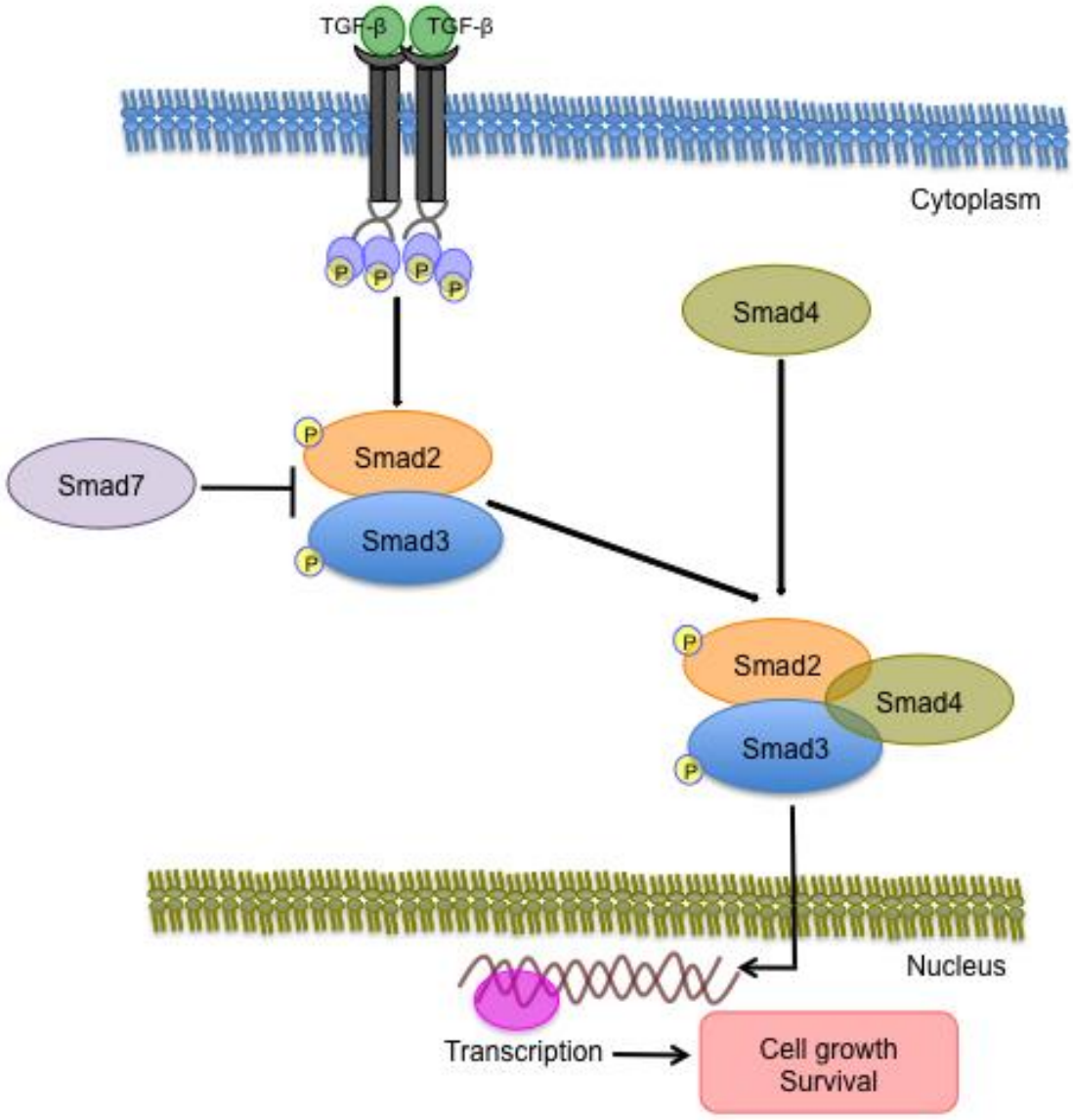


Figure 15: TGF-β signaling.

TGF-β ligand interacts with the cell surface receptors that leading to heterodimerization of the Type I and II receptors. This results in trans-phosphorylation and subsequent activation of the receptors. This in turn activates Smad2/3 via phosphorylation. The activated Smad2/3 interacts with Smad4 and translocates to the nucleus where it leads to transcriptional activation of pro-growth genes (Modified from www.mycancergenome.org).

Smad4 is the central mediator of signaling in the TGF-β signaling cascade. Smad4 deletion mutation has been reported in numerous cancers including CRC, gastric cancer, cholangiocarcinoma and prostate cancer. The frequency of Smad4 mutations vary between malignancies – 30% CRC cases have loss of Smad4, whereas 4.2% cholangiocarcinoma cases show Smad4 mutations. The loss of Smad4 in CRC has been reported to be positively correlated to disease progression and is believed to be an event that occurs late in pathogenesis. In addition, Smad4 loss has also been reported to make the cancer cells less responsive to therapy with 5-FU. In addition to cancer related mutations in Smad4, TGF-β pathway also interacts with other signaling circuits that may impact carcinogenesis. One such interaction is the crosstalk between TGF-β and the Wnt/β-catenin signaling pathways. Cytoplasmic interaction of Smad7 with the β-catenin destruction complex via binding to Axin results in destabilization of the destruction complex. This leads to decrease in the ubiquitin mediated β-catenin degradation and translocation into the nucleus. Smad2/3/4 complex has been shown to interact with β-catenin/LEF in the nucleus and synergistically regulate downstream gene expression (Zhao M, Mishra L, Deng CX., 2018).

4.1.2.3. Notch signaling pathway in colorectal cancer

The Notch signaling pathway regulates crucial physiological processes that are relevant during carcinogenesis such as cellular growth and proliferation, differentiation and apoptotic cell death. The Notch proteins are single-pass transmembrane proteins that are encoded by four Notch genes in humans, Notch1-4. Activation of the signaling pathway occurs when extracellular ligands Jagged and/or DLL bind to the Notch receptor leading to enzymatic cleavage of its extracellular domain. This renders the Notch receptor susceptible to a second cleavage by γ-secretase, releasing Notch Intracellular Domain (NICD). NICD translocation into the nucleus is followed by complexation with other transcription factor and activation of target genes *Hes* and *Hey* (Figure 16) (Wu WK, Wang XJ, Cheng AS, et al., 2013).

Aberrant Notch signaling is present during carcinogenesis and is maintained throughout the progression of the disease. Multiple factors may lead to the activation of the Notch pathway in cancer, including the Wnt/β-catenin signaling. It has been

reported that during colorectal carcinogenesis, Notch1 activity is enhanced via β-catenin mediated upregulation of Notch1 ligand Jagged-1. In addition, Wnt signaling has been shown to upregulate Notch2 expression. In the presence of mutated APC, expression of Notch target gene *Hes-1* is significantly increased in intestinal tumors (Wu WK, Wang XJ, Cheng AS, et al., 2013).

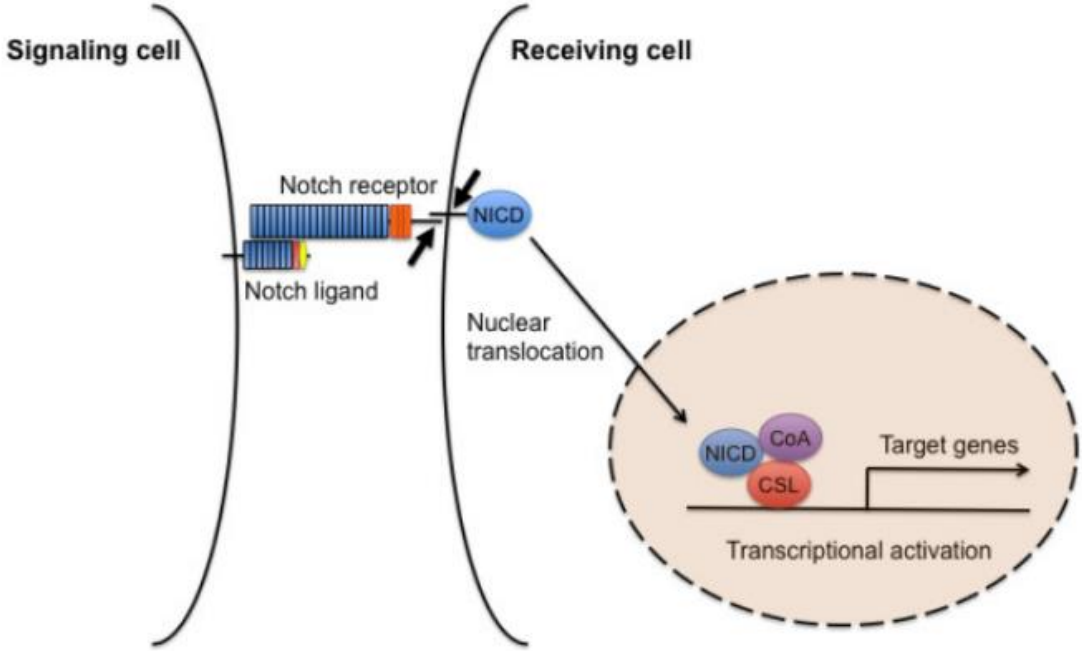


Figure 16: Canonical Notch signaling.
Notch signaling is activated when the ligands DLL and/or Jagged bind to the Notch receptor (Notch1-4) on the recipient cell. Upon receptor and ligand engagement, Notch receptor is cleaved releasing NICD into the cytoplasm, that trnslocates into the nucleus and complexes other transcriptional co-activators to increase the transcription of target genes (Kume T., 2009).

4.1.3. Epithelial to mesenchymal transition

Cells undergo differentiation to form various organs and tissues during embryonic development. Some cells however retain the plasticity that allows them to toggle between epithelial and mesenchymal forms. This is primarily driven by programmed molecular and biochemical changes that encompass the phenomena of epithelial to mesenchymal transition (EMT) and mesechymal to epithelial transition (MET). EMT involves transition of cells from epithelial nature to mesenchymal nature, wherein cells attain a spindle shaped morphology, lose polarity, cell-to-cell adhesion and become more migratory. MET is the reverse of EMT, wherein cells switch from mesenchymal to epithelial form, lose the enhanced migratory ability and gain stronger cell-to-cell adhesion (Lee JM, Dedhar S, Kalluri R, Thompson EW., 2006). EMT plays pivotal role during early embryonic development, facilitating cellular migration to enable key events such as gastrulation and development of the neural crest (Savagner P, 2015). This self-regulated EMT that is programmed to only generate new tissue, does not result in uncontrolled cellular proliferation and is classified as Type 1 EMT. In the event of tissue injury, Type 2 EMT is initiated wherein epithelial cells migrate to the site of damage to repair the wound. EMT Type 3 is associated with carcinogenesis and tumor progression resulting in pathological alterations in the tissue of origin. These alterations are characterized by uncontrolled cellular proliferation, eventually enabling the formation of secondary metastatic tumors (Kalluri R, Weinberg RA, 2009).

4.1.3.1. Molecular signatures of epithelial to mesenchymal transition

Biochemical changes that define EMT are orchestrated by an intricate signaling network that ultimately results in pathological changes culminating in uncontrolled cellular proliferation and eventually metastases to distant tissues (Kalluri R, Weinberg RA, 2009). Incoming extracellular cues originating in the tumor microenvironment are key drivers that bring about these molecular changes and result in the cancer cells acquiring a mesenchymal phenotype. Phenotypic expression of EMT is typically manifested in the form of alterations in morphology, modifications of the cytoskeletal architecture and acquired migratory and invasive capabilities (Figure 17) (Lamouille S, Xu J, Derynck R., 2014, Chaffer CL, San Juan BP, et al., 2016, Espinoza I, Miele

L., 2013, Wang Z, Li Y, Kong D, Sarkar FH., 2010). The underlying molecular changes in the form of either overexpression or suppression of certain molecules drive EMT and may be used as signatures defining the transition from an epithelial to a mesenchymal form. One of the most common changes during EMT is the loss of integrity of the cell-cell junctions that enable malignant cells to break off from the original tumor. Concomitant with this is the acquisition of enhanced motility and invasiveness that often govern the aggressiveness of the disease (Joëlle Roche, Robert M. Gemmill and Harry A. Drabkin, 2017).

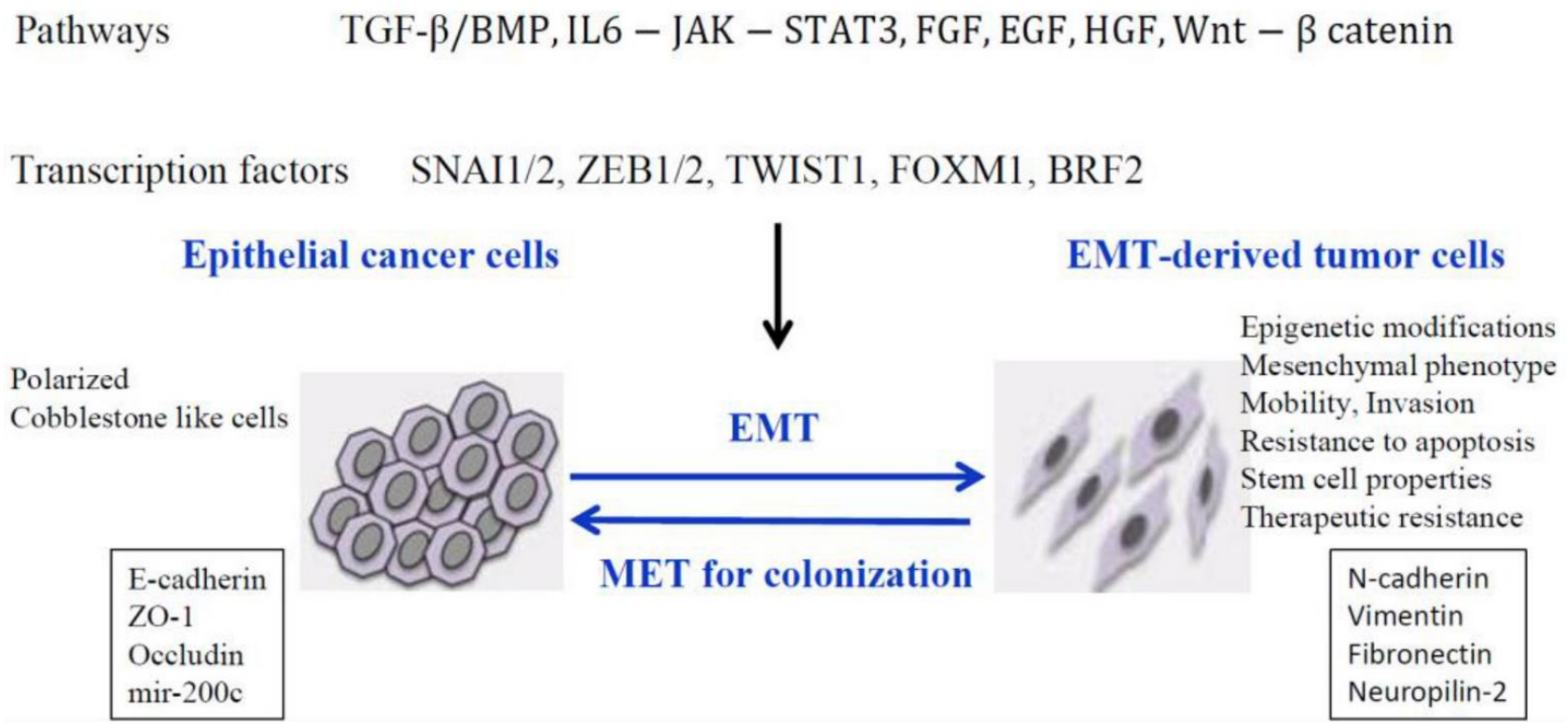


Figure 17: EMT program, in cancer.
Multiple signaling pathways lead to activation of the EMT program in cancer cells. The most important are the TGF-β/Smad, and Wnt/β-catenin signaling cascades. These networks activate transcription factors, eg., Snail, Twist, Zeb, which in turn transcriptionally activate target genes that cause increased cellular proliferation (Joëlle Roche, Robert M. Gemmill and Harry A. Drabkin, 2017).

Table 3: mRNA expression of various markers during EMT.

Multiple markers show differential expression pattern when cancer cells undergo EMT. In general, there is overexpression of mesenchymal markers, whereas epithelial markers are downregulated. Intercellular adhesion molecule such as E-cadherin is suppressed. Molecules that are implicated in cellular migration such as Vimentin are upregulated. Extrcellular matrix proteins too show differential expression. EMT related transcription factors like Snail, Slug and Twist show increase in expression and function (Modified from Zeisberg M, Neilson EG., 2009).

Markers	Expression during EMT
Cell Surface markers	
E cadherin	Downregulation
N cadherin	Upregulation
Integrin α5β1	Upregulation
Integrin αVβ6	Upregulation
Syndecan-1	Upregulation
ZO-1	Downregulation
Cytoskeletal markers	
Vimentin	Upregulation
β-Catenin	Upregulation
Cytokeratin	Downregulation
Extracellular matrix proteins	
Fibronectin	Upregulation
Laminin 1	Downregulation
Laminin 5	Upregulation
Collagen α1(I)	Upregulation
Collagen α1(III)	Upregulation
Collagen α1(IV)	Downregulation
Transcription factors	
Snail	Upregulation
Slug	Upregulation
Zeb1	Upregulation
Twist	Upregulation
LEF-1	Upregulation
FOXC2	Upregulation
Goosecoid	Upregulation

4.1.3.2. EMT induced loss of integrity of cell-cell adherens junctions

Cell-cell contacts are crucial in maintaining tissue integrity and are achieved via interaction of specialized proteins on the cell surface that forms the tight junctions, adherens junctions and desmosomes. Onset of EMT compromises these interactions leading to weakening of the intercellular contacts. These cells may eventually break off form the original tumor mass as cancer progresses. The proteins that are necessary for the formation and optimal functioning of the adherens junctions are either suppressed such as claudin and occludin, or mislocalized in cells that undergo EMT such as ZO1. E-cadherin, a transmembrane protein present at the plasma membrane is reported to undergo proteolytic cleavage and degradation as a consequence of the EMT program being turned on. In the absence of E-cadherin, β-catenin cannot bind precluding the formation of the cadherin-catenin complex. The free β-catenin accumulates in the nucleus and brings about transcriptional activation of target genes, thus activating the Wnt/β-catenin signaling pathway. Along with decrease in E-cadherin levels, cells undergoing EMT often express elevated levels of N-cadherin, a mesenchymal protein. This cadherin switch has been used to monitor progression of EMT in cancer (Lamouille S, Xu J, Derynck R., 2014, Zeisberg M, Neilson EG., 2009).

In addition to cadherin switch from E to N type during EMT, there is localized cytoskeleton remodeling that has implications on cellular morphology and migration. Microtubule network, actin cytoskeleton and intermediate filaments are the components of the cytoskeleton that not only determine cell shape but also provide the necessary mechanical strength to drive EMT induced migration and invasion. Activation of the EMT program leads to rearrangement of intermediate filaments that switch from being mainly composed of cytokeratins to primarily composed of vimentin networks that result in enhanced cellular motility. In cells undergoing EMT, enzymatically modified α-tubulin accumulate at microtubule-based membrane extensions called microtentacles. The polymerization and depolymerization cycle of tubulin is vital to multiple cellular functions like cell division, migration and invasion. Many anti-neoplastic agents are designed to take advantage of this dynamic process to arrest cells in certain stages of the cell cycle and thus trigger apoptotic cell death. Taxol, one of the first drug to be developed

as an anti-cancer therapeutic for treatment of multiple malignances including breast and ovarian cancers acts by stabilizing the tubulin network via prevention of depolymerization of the tubulin subunits (Whipple RA, Matrone MA, Cho EH, et al 2010, Sun BO, Fang Y, et al., 2015).

4.1.3.3. EMT related transcription factors

EMT related phenotypic changes such as cadherin switch, cytoskeletal rearrangement, morphological changes, and increased migratory ability is driven by enhanced activity of transcription factors. E-cadherin suppression is a hallmark for EMT – this is influenced by either direct or indirect interaction of transcription factors with the promoter region of *CDH1*. For instance, direct interaction of Snail and Zeb suppress E-cadherin while Twist indirectly influences downregulation of E-cadherin. Although these transctiption factors play pivotal roles during embryogenesis, their role in carcinoma pathogenesis and progression is of immense importance. Snail-1 has been associated with the transcriptional downregulation of E-cadherin and promotion of lymph node metastasis. Another EMT related transciption factor, Zeb1 represses E-cadherin expression but functions in the absence of Snail-1. In addition to being crucial during embryogenesis, Twist induces EMT in cancers and is reported to be associated with lymph node metastasis and poor survival. Along with suppressing E-cadherin, Twist also upregulates N-cadherin and vimentin, thus effecting cadherin switch during EMT. Clinical relevance of Twist is evident in the form of resistance to platinum and anthracyclin therapies (Figure 18) (Blanco MJ, Moreno-Bueno G, et al., 2002, Chou YS, Yang MH., 2015).

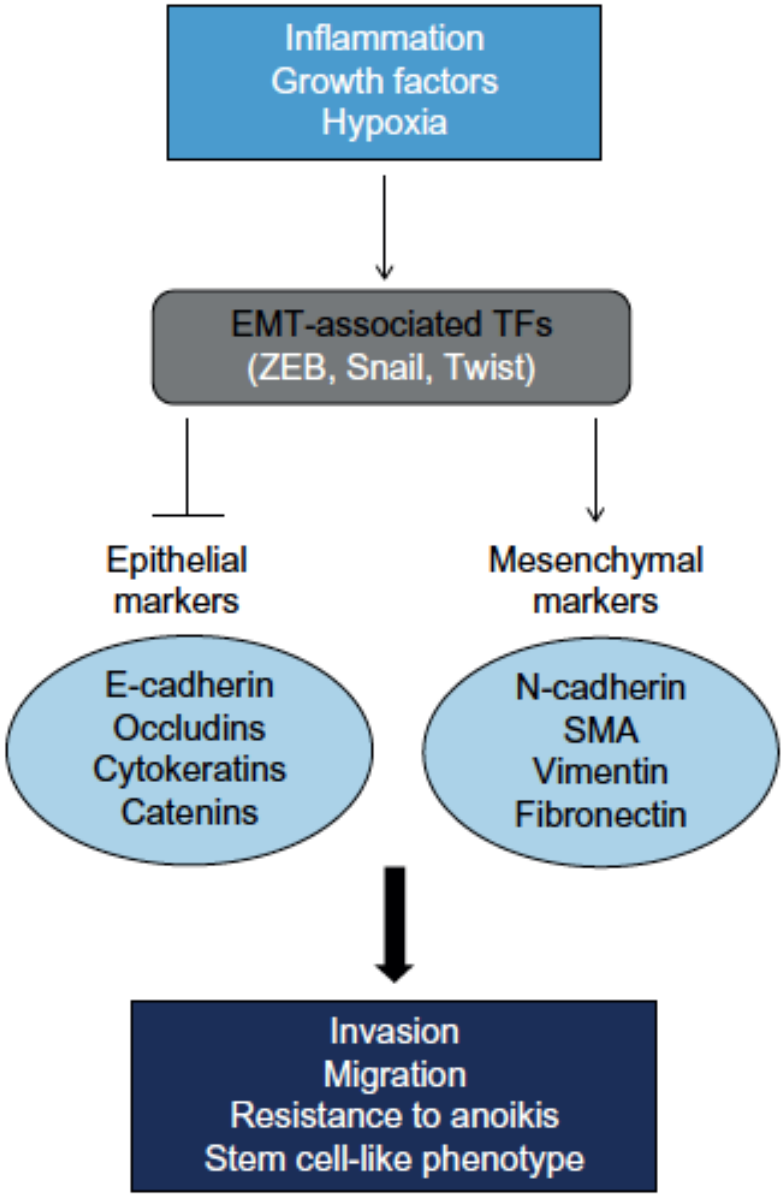


Figure 18: Regulation of EMT in cancer.
Multiple factors may induce EMT in cancer, including physiological stress like inflammation, hypoxia and increased growth signals. These conditions may lead to the enhanced activity of transcription factors that result in activation of mesenchymal markers and simultaneous repression of epithelial markers. EMT activation manifests in the form of increased cellular motility and invasiveness. In addition, these molecular changes also render the cells undergoing EMT less responsive to therapeutic agents (Guttilla Reed, I., 2015).

4.1.3.4. EMT and response to therapeutic agents

Often, tumors that were otherwise responsive to drugs evolve mechanisms to override their cytotoxic effects, resulting in recurrence and ultimately metastasis. The relation between EMT and response to therapy has gained prominence in the recent past. Multiple studies have correlated the expression of the EMT markers to treatment ourcome of standard therapy regimens. Although the mechanistic pathway of EMT induced chemoresistance is still unclear, multiple mechanisms such as DNA damage repair via homologous recombination driven by EMT related transcription factors or elevated drug efflux that result in resistance to apoptosis may be involved (Zhang P, Wei Y, Wang L, et al., 2014, Cortez MA, Valdecanas D, et al., 2014, Abdullah LN, Chow EK.,2013, Moitra K., 2015).

Among many different chemotherapeutic agents available, platinum drugs are the most frequently used to treat a wide range of cancers, either as a monotherapy or in combinatorial regimens with other drugs. Cisplatin, the first platinum-based drug to be used for treating cancers of the lung, head and neck, stomach, ovaries, etc., has been shown to be effective but comes at a cost in the form of severe side-effects, compounded by rapid development of drug resistance. Less toxic derivatives of cisplatin such as carboplatin and oxaliplatin have since been used for treating ovarian, lung, endometrial, bladder, breast, cervical and bladder cancers. Metastatic colorectal cancer is treated using therapy regimens such as FOLFOX, FOLFIRI that combine oxaliplatin, 5-FU, leucovorin and irinotecan The EMT-related transcription factors Snail and Twist have been implicated in development of resistance to platinum drugs. Cancer cells depleted of Snail and Twist become more sensitive to cisplatin via the activation of the JNK/mitochoncrial pathway. Resistance to 5-FU and oxaliplatin is reported to be partially induced by the activation of EMT mediated by transcription factor Twist, whereas, the downregulation of the EMT related miR-200c and miR-141 are partially responsible for rendering tumor cells resistant to irinotecan. Development of resistance to chemotherapeutic agents pose a major challenge in the treatment and clinical management of cancer, thus contributing to the overall disease burden in modern society (Wang C, Jiang K, Kang X, *et al*., 2012, Funahashi Y, Okamoto K, *et al.*, 2014).

4.2. Materials and methods

4.2.1. Anchorage-independent growth in soft agar

Tumorigenicity of CRC02 cells was measured using anchorage-independent growth is soft agar. 1ml of 5% agar was mixed with 9ml of complete media to prepare 0.5% base agar layer. The mixture was maintained at ~50°C at all times to prevent agar solidification prematurely. To each well of a 6 well dish, 1ml of the agar mix was added and set aside. After PBS wash, the cells were trypsinized and collected by centrifugation. The collected were resuspended in PBS at $5x10^3$ cells/ml. 0.3% top layer of agar containing cell suspension was made by adding 0.6ml of 5% agar to 9.4ml of media with cells. 1ml of this suspension was added to the already solidified base later and allowed to set. To prevent drying, 800µl of complete media was layered in each well. The cultures were incubated for 2 weeks at 37°C, 5% CO_2 atmosphere and 95% relative humidity. Images of the colonies were captured using Nikon TiE inverted microscope.

4.2.2. Establishment of xenograft model

In vivo tumorigenic potential of CRC02 cells was measured by implanting different number of cells in severe combined immunodeficient (SCID) mice. 4-6 weeks old female SCID mice were selected for the study. Three groups of 6 mice each were used for the study. $1x10^6$, $2x10^6$ and $4x10^6$ cells were mixed with matrigel (1:1 ratio) and implanted subcutaneously in the flank region of these mice. Post implantation, tumor volume was measured at regular intervals for upto 45 days.

4.2.3. Migration and invasion assay

Transwell migration assay using Boyden chambers was used to measure migratory property and invasiveness of CRC02 cells and compare with widely used CRC cell limes SW620, HT29 and HCT116. Transwells with PET membrane, 8µ pores (BD Falcon, Cat. No. 353097) were placed in each well of a 24-well cell culture plate.

Cells were suspended at a final concentration of $1x10^4$ in 200µl of media containing no serum and placed in the upper chamber of the transwell. 400µl of complete media was placed in the lower chamber of transwell. The cells were placed in an incubator at 37°C, containing an atmosphere of 5% CO_2 and 95% humidity. Non-migratory cells were scraped off from the upper part of the of the transwell membrane. After removing the non-migratory cells, the transwell membranes were fixed using 4% paraformaldehyde, followed by crystal violet staining. Cells that have migrated across the membrane were counted using a 10X objective. 100µg/ml of matrigel was used to coat the transwells for the invasion assay. $5x10^4$ cells per 200µl were seeded in each transwell chamber. The experiment was performed in a similar manner as that of the migration assay described above. For statistical significance, the experiments were repeated at least three times.

4.2.4. Drug sensitivity assay

Cytotoxicity of gemcitabine and camptothecin was tested on CRC02 and HCT116 cells. $3x10^3$ cells per well were seeded in 96-well plate in quadruplicate. Gemcitabine and camptothecin were added to the cells at different concentrations ranging from 0.3nM to 3µM. After 48 hours, 20µl of 5mg/ml stock of MTT solution was added to the cells and incubated for another 3 hours at 37°C. The precipitated formazab crystals were dissolved in 100µl of DMSO: methanol (1:1). Absorbance at 550nm was measured for each well with background corrected at 655nm. Three independent replicates were performed statistical significance. CRC02 cells were also treated with drug combinations that are clinically relevant for treatment of CRC. Combinations included 5-FU and leucovorin, FOLFOX, FOLFIRI and cetuximab. The concentrations of drugs used in the study were: 5-FU – 16.78µg/ml, leucovorin – 391ng/ml, oxaliplatin – 2.85µg/ml, irinotecan - 4µg/ml, cetuximab - 184µg/ml. All experiments were done in triplicate.

4.2.5. qRT-PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Cat # 74104). cDNA synthesis was done using 1µg of total RNA. qRT-PCR was done using SYBR Green reagent (SsoFast Eva Green supermix, Bio-Rad, cat # 1725202AP). GAPDH expression level was used as internal control. mRNA expression levels were normalized to HCT116 and represented as fold change with respect to HCT116.

Table 4: Primer sequences used for qRT-PCR evaluation of mRNA expression of signaling pathway components in CRC02 and HCT116 cells

Gene	Forward	Reverse
GAPDH	5' TCTGACTTCAACAGCGACAC 3'	5' TACTCCTTGGAGGCCATGT 3'
E-cadherin	5' CCAAAGACAGAGCGGAACTA 3'	5' GAGACTCCTCCATTCCTTCC 3'
N-cadherin	5' TTGTGGTGGGAGCAGTAAGT 3'	5' CCAATCTCATGGTCTCATCC 3'
Vimentin	5' ACTCCCTCTGGTTGATACCC 3'	5' ATTGCTGCACTGAGTGTGTG 3'
Twist	5' ATGCATTCTCAAGAGGTCGT 3'	5' GGCCAGTTTGATCCCAGTAT 3'
Snail	5' CGGGCAATTTAACAATGTCT 3'	5' AAATGTAAACATCTTCCTCCCA 3'
Slug	5' ATGCATTTCTTCACTCCGAA 3'	5' CATGAATTCCATGCTCTTGC 3'
Zeb1	5' GAAATCCTCTCGAATGAGCA 3'	5' TCCTGCAATTCTTCCATCTC 3'
Zeb2	5' GTTCGAGGAGGAAGAGGAAG 3'	5' TGGTCTGATTTGGTTTCCAT 3'
IQGAP1	5' CGCTGCTCTGAACTCTAAGG 3'	5' TCCTTTCATTTCCCTAGGCT 3'
Rac1	5' TCCTGTAGTCGCTTTGCCTA 3'	5' TGTTGTAGTGGCTGAAGGGT 3'
CDC42	5' GCATATGAGGAACCCTAGAGC 3'	5' AATTGGAAACTGCAACCAAA 3'
CLIP-170	5' TCTCCACGACACAGAGGATT 3'	5' CAGAAGGTTTCGTCGTCATT 3'
EB1	5' TTTCTGGTGGTAGCTTGTCC 3'	5' CTGTCATTTGAACAATGGCT 3'
Fzd	5' GCTGCAAGAGCTACGCTATC 3'	5' GGACCAGATCCAGAAGCC 3'
LRP6	5' TCTCTTCCAGGAATGTCTCG 3'	5' GGGAAGTAAGTGCCTTTGGT 3'
DVL2	5' GGGAAGTAAGTGCCTTTGGT 3'	5' CTTCCTCAGGCTGCTGTCTA 3'
GSK3b	5' TGCACTCTTCAACTTCACCA 3'	5' TGTCCACGGTCTCCAGTATT 3'
APC	5' GCTATTCAGGAAGGTGCAAA 3'	5' GATGAAATGGTGATCCCAGA 3'
Axin1	5' CGTGTGCTGGGATCTACTTT3'	5' AAGCTGTGTTGAAGGCACTC 3'
b Catenin	5' CAATGGCTTGGAATGAGACT 3'	5' CCCATCTCATGTTCCATCA 3'
SMAD2	5' AAACTGAGTGTCCCAAAGGTT 3'	5' GGTTTGCCTAGATCAAGAAGC 3'
SMAD3	5' AGTGCTGGTGACTGGATAGC 3'	5' AGACAAGGATCTGTGTCCCA 3'
SMAD4	5' GGTCAGGTGCCTTAGTGACCA 3'	5' CTGACGCAAATCAAAGACCT 3'
SMAD7	5' ACGCTGTTGGTACACAAGGT 3'	5' AGCTGATCTGCACGGTAAAG 3'
NOTCH1	5' AGTTTGGGAGGAGCAGATTT 3'	5' GCTGAGCCAAGTCTGACG 3'
NOTCH2	5' GGGTTTCACTGGATCCTTCT 3'	5' AGATTCACCAGGGTCTGACA 3'
NOTCH3	5' AGGACATGCAGGATAGCAAG 3'	5' AGTCTCTCCTGGGCTACGTC 3'
NOTCH4	5' CCAGAGCAGACATCTTCCAC 3'	5' TGCAATTCTTGGTTCCAACT 3'
DLL1	5' CAGGTTCTCCTCCTGAGGTC 3'	5' GCGTAATTCAGTTCACCCAT 3'
DLL3	5' TCCCTACCCTTCCTCGATT 3'	5' GAAGATGGCAGGTAGCTCAA 3'
DLL4	5' ACACCTTTGGGTGTCTGTCT 3'	5' GCTACTGCCACTCTCTCTGG 3'
JAG1	5' CAGAGCTTAAACCGAATGGA 3'	5' GGATTCTAAGTCAGCAACGG 3'
JAG2	5' CTTGTTCTTTCGGTGCTGTC 3'	5' GACTCAACAGAACCGTCTCG 3'
HES4	5' CACTCGAAGCTGGAGAAGG 3'	5' GCCAGACACTCGTGGAAG 3'
HEY1	5' TGCCTCCTATAGCAGAAAGG 3'	5' CCAACACTCCAAATGAGACC 3'

4.2.6. Analysis for statistical significance

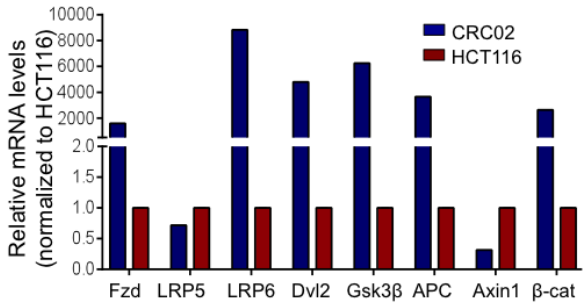
All graphs are plotted as mean ± SEM. Three independent biological replicates were used for calculating statistical significance using Student's t test. $P < 0.05$ was considered statistically significant.

4.3. Results

4.3.1. Differentially regulated signaling pathways in CRC02

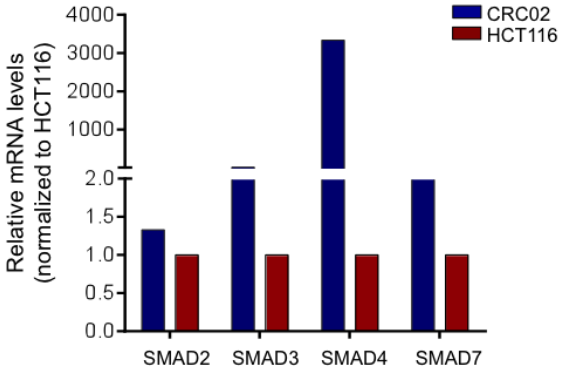
Pathogenesis and progression of CRC are driven by misregulation of Wnt, Notch and TGFβ signaling. Quantification of the relative transcript levels of various members of these pathways in CRC02 in comparison with HCT116. We observed several genes from these three pathways were upregulated in CRC02. In Wnt-β-catenin pathway, only Axin1 showed ~4-fold decreased expression in CRC02. In TGFβ pathway, Smad4 showed highest expression (~3000-fold). Varied mRNA expression levels were also seen in the Notch signaling pathway with Notch4 mRNA being ~23000-fold higher in CRC02 (Figure 19A-C). The above data present strong evidence that CRC02, the Indian CRC cell line, harbors differentially regulated signaling pathways as compared to the established Caucasian cell line HCT116 that may have clinical relevance in cancer pathogenesis in the Indian context.

A



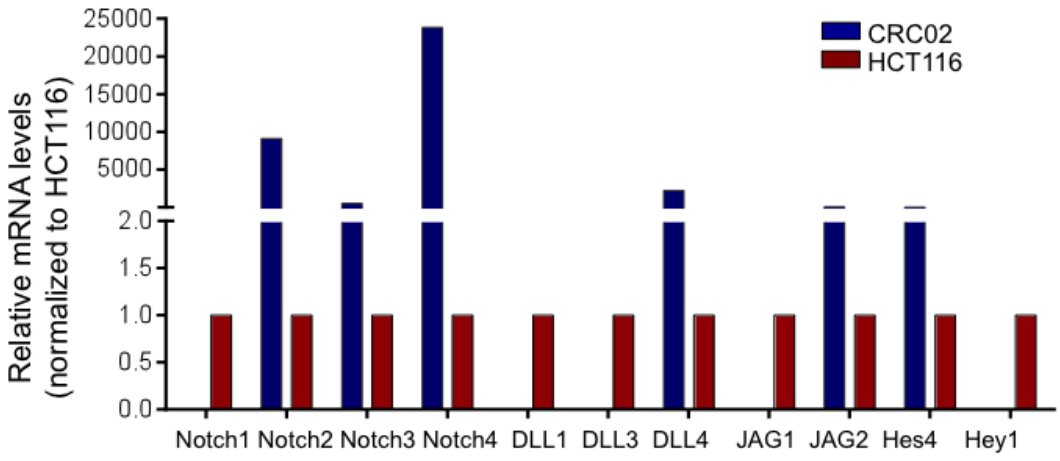
Gene	mRNA expression in CRC02	Fold change (over HCT116)
Frizzled	Upregulation	1500
LRP6	Upregulation	8000
Dishevelled	Upregulation	4700
GSK3b	Upregulation	6000
APC	Upregulation	3650
β-catenin	Upregulation	1800
Axin1	Downregulation	4

B



Gene	mRNA expression in CRC02	Fold change (over HCT116)
SMAD2	Upregulation	1.3
SMAD 3	Upregulation	3.6
SMAD 4	Upregulation	3000
SMAD 7	Upregulation	13

C



Gene	mRNA expression in CRC02	Fold change (over HCT116)
Notch1	Downregulation	Undetectable
Notch2	Upregulation	9000
Notch3	Upregulation	500
Notch4	Upregulation	23000
DLL1	Downregulation	Undetectable
DLL3	Downregulation	Undetectable
DLL4	Upregulation	2000
JAG1	Downregulation	Undetectable
JAG2	Upregulation	50
HES4	Upregulation	6
HEY1	Downregulation	Undetectable

Figure 19: Relative mRNA expression of important signaling pathways in CRC02
(A): *Most key genes of the Wnt/β-catenin pathway are overexpressed in CRC02. However, axin is the only gene showing ~4 folds downregulation*
(B): *All genes of the TGFβ are overexpressed in CRC02. SMAD4 shows ~3000 folds higher mRNA level in comparison to HCT116.* ***(C):*** *Notch2,3,4 are upregulated in CRC02. The Notch ligands DLL4 and JAG2 are also upregulated along with the downstream target gene Hes4.*

4.3.2. CRC02 cells exhibit molecular features of epithelial to mesenchymal transition

Majority of the cancer-related patient mortality is due to metastasis. The EMT program drives the molecular and physiological alterations enabling malignant cells to break away from the primary tumor mass, travel to distant tissues and find a new niche to re-colonize. Among the many molecules implicated to be involved in activating EMT, intercellular adhesion molecule E-cadherin and transcription factors such as Twist, Zeb, Snail and Slug and are considered to be markers of EMT activation. We evaluated the relative mRNA expression of these molecules in comparison to the established CRC cell line HT29, HCT116 and SW620. All mRNA expression levels were normalized to that of HCT116 cells. We observed highest expression of E-cadherin in HT29 (~3.5-fold higher). Diminished E-cadherin RNA levels were observed in SW620, whereas no detectable transcript was present in CRC02. As expected in EMT activation, we found a simultaneous increase of N-cadherin and vimentin transcript levels in CRC02 (~6-fold and ~4000-fold increase respectively). Between CRC02 and SW620, vimentin overexpression was more moderate in CRC02 than in SW620 (~8-fold increase) (Figure 20A). Further, mRNA expression analysis of EMT related transcription factor revealed a significantly higher level of Twist mRNA (~20-folds) and Zeb2 mRNA (~5000-folds) in CRC02. We could not detect transcripts for Snail, Slug, Zeb1 in CRC02. In SW620, all the transcription factors were upregulated. (Figure 20B). E-cadherin suppression coupled with increased N-cadherin, vimentin, twist, Zeb2 transcript level in CRC02 is an indicator of EMT program being activated in these cells.

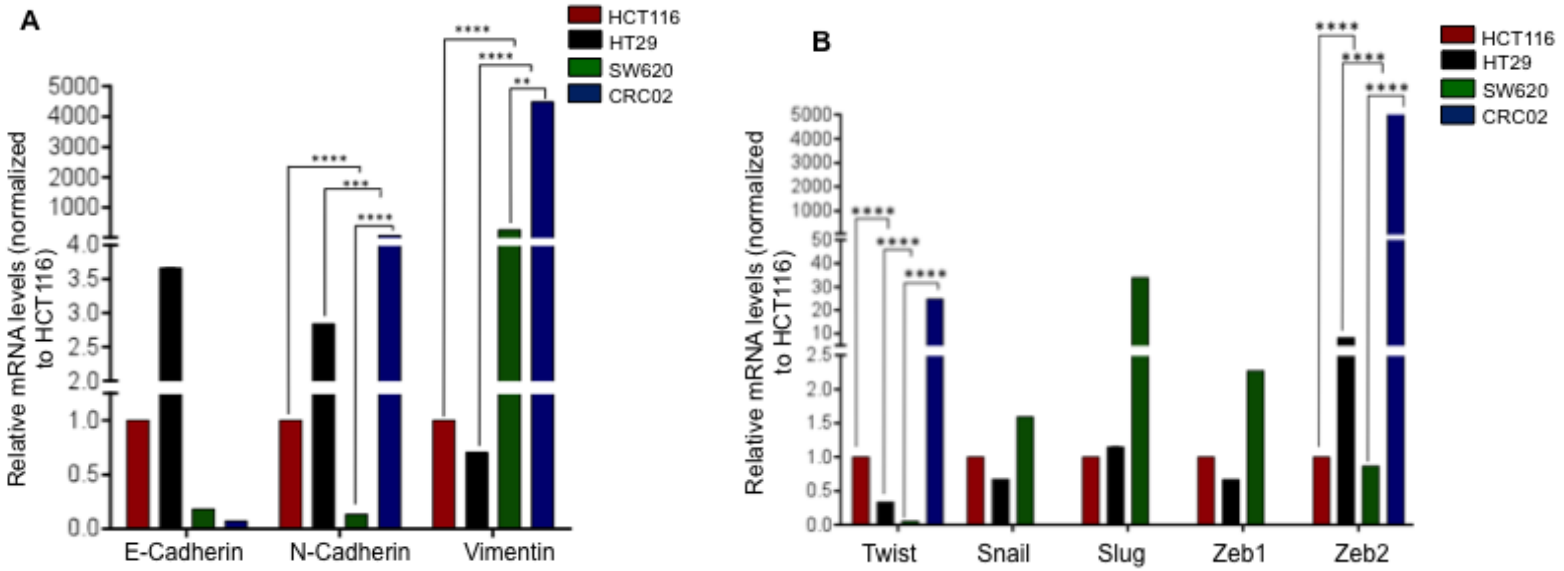


Figure 20: EMT markers in CRC02.
(A): *qRT-PCR analysis of mRNA expression shows suppression of E-cadherin and increase in N-cadherin indicating cadherin switch in CRC02.* ***(B):*** *EMT-TFs Twist and Zeb2 are overexpressed in CRC02.*

4.3.3. CRC02 cells are highly migratory and invasive

EMT endows tumor cells with enhanced migratory and invasive capabilities. We performed wound healing and invasion assays to assess whether CRC02 cells showed increased motility. A wound was created in a confluent monolayer of CRC02 and HCT116 cultures and monitored over a period of 48 hours. We observed that the rate at which CRC02 cells healed the wound was significantly faster than HCT116. CRC02 filled the wound within 48 hours whereas in HCT116 cultures, the wound was still visible at this time point (Figure 21A, B). The increased motility of CRC02 was validated by transwell migration assay where the cells were allowed to travel through the transwell pores from low serum towards high serum containing media. SW620 showed ~3.6-fold higher, whereas, CRC02 exhibited ~3.2-fold greater motility as compared to HCT116. HT29 cells however did not showe any significant migratory potential (Figure 21C, D). We obtained similar results for the invasion assay through matrigen coated transwell chambers. SW620 and CRC02 cells were showed increased invasive property (~9.8-fold and ~9-fold higher respectively, in comparison to HCT116) (Figure 21E, F). Therefore, CRC02 is similar to SW620, an established metastatic cell line in invasiveness and metastatic potential.

We also evaluated the mRNA levels of cytoskeletal binding protein IQGAP1 in all the cell lines by RT-PCR. Our data showed an enhancement by approximately 3500-fold in the transcript level of IQGAP1 in CRC02 as compared to HCT116. Moreover, there was a 2.5-fold elevation in the transcript levels of CLIP170, along with 41-fold enhancement in the mRNA levels of EB1. It has been reported earlier that IQGAP1 captures growing microtubule ends by binding CLIP-170, the plus end microtubule binding protein that promote and regulate microtubule dynamics (Fukata M, Watanabe T, Noritake J, 2002). In our study, Rac1 and CDC42 transcripts remained undetectable (Figure 22). Taken together, these observations suggest that CRC02 cells have undergone molecular changes that are strongly associated with EMT and are endowed with increased migratory property and are invasiveness.

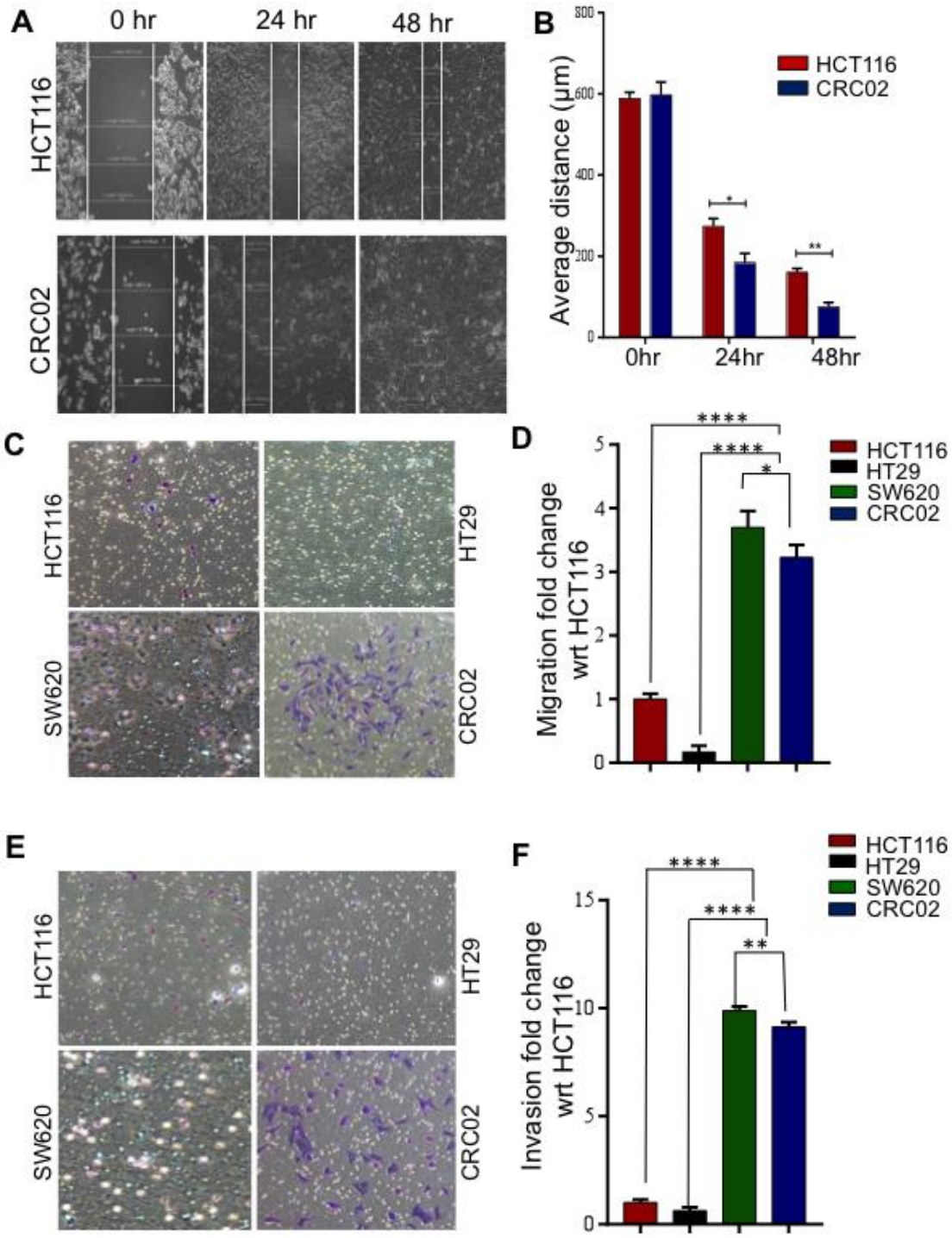


Figure 21: CRC02 is highly migratory and invasive.
(A, B): *CRC02 cells closed the wound in 48 hours, unlike HCT116 in which the wound persisted.* ***(C, D):*** *CRC02 and SW620 demonstrated greater migratory potential in a transwell migration assay (3.6 and 3.2-fold higher than HCT116).* ***(E, F):*** *Invasive potential of CRC02 and SW620 is greater compared to HCT116 and HT29.*

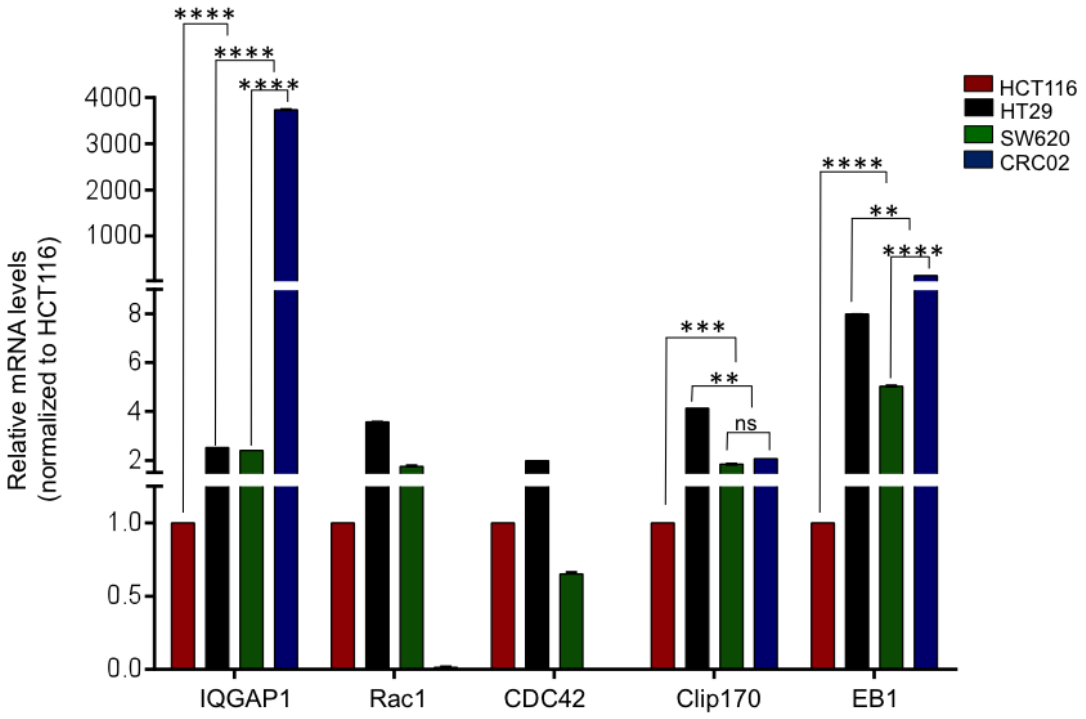


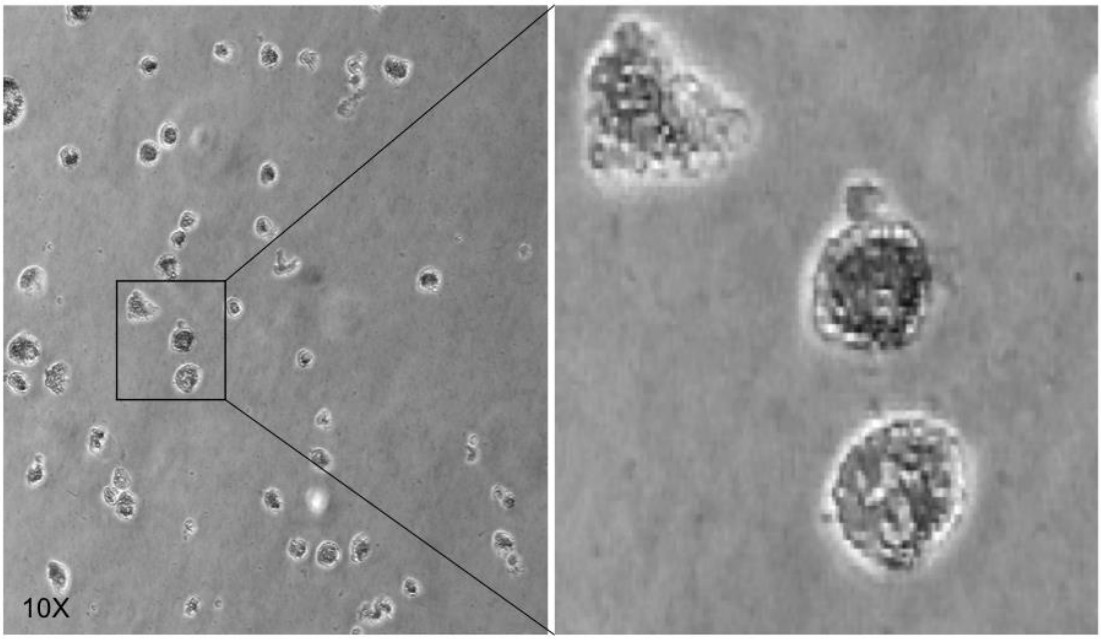
Figure 22: CRC02 exhibits increased expression of cytoskeletal modulating molecules.

Enhanced migratory ability is associated with localized cytoskeletal modulations. mRNA expression levels of actin and tubulin interacting molecules IQGAP1 and EB1 were elevated in CRC02

4.3.4. CRC02 cells exhibit *in vitro* anchorage-independent growth and tumorigenicity in mice

Anchorage independent growth assay was performed to evaluate the tumorigenic propensity of CRC02 cells. In two weeks after seeding, round colonies appeared (Figure 23A). These cells were also implanted in SCID mice to study their tumorigenic potential. Soon after cell implantaton, tumors started appearing in these mice and grew gradually until day 13 post injection. Thereafter, a spontaneous decrease in tumor volume was observed in all the groups, although complete regression of the tumors was not observed. Tumors started reappearing in the groups around day 29 and started growing rapidly. We continued monitoring the tumors until 45 days post-implantation. On day 45, the mean tumor volumes were recorded to be 232.53 mm^3, 347.48 mm^3 and 500 mm^3 respectively for the groups where $1x10^6$, 2 $x10^6$ and 4 $x10^6$ cells were injected (Figure 23B). No change in body weight was observed in any of the groups and the animals did not show any abnormal clinical signs. Therefore, we concluded that CRC02 showed a dose-dependent tumorigenic potential *in vivo* and can be a useful model for *in vitro* as well as *in vivo* mechanistic studies as well as a tool for screening novel therapeutic agents.

A



B

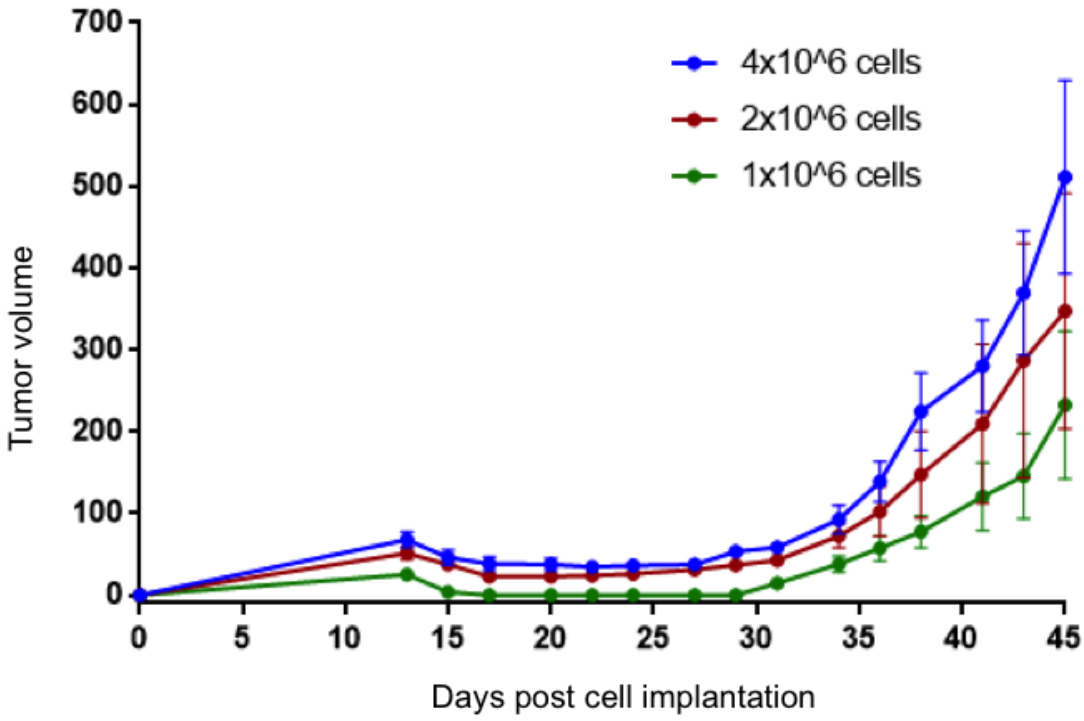


Figure 23: CRC02 exhibits anchorage-independent growth and are tumorigenic in mice.

(A): *CRC02 cells formed rounded colonies in soft agar indicating these cells have the capability of proliferating in an anchorage-independent manner.* ***(B):*** *CRC02 cells formed tumors upon implantation in SCID mice.*

4.3.5. CRC02 cells show lower sensitivity to standard-of-care therapeutics

Transition of cancer cells from epithelial to mesenchymal state often lead to diminished response to therapeutic agents. We tested the efficacy of clinically relevant drugs on CRC02. We used gemcitabine and camptothecin either as single agents or in combinations used to treat CRC in the clinic, with other drugs such as 5-FU, leucovorin and irinotecan. The EGFR inhibitor, cetuximab were also tested for efficacy. We found that there was no appreciable effect of any of these therapeutic regimens on CRC02 cells (Figure 24A, B). EMT activation has been reported to diminish the cytotoxic effect of therapeutic agents. Multiple mechanisms that are poorly understood bring about EMT-induced drug resistance. For instance, elevated expression of Twist has been linked to acquired resistance to oxaliplatin and 5-FU. Therefore, the increased migratory ability and invasiveness and the lowered response to therapeutic agents can be attributed to the ativation of EMT in CRC02 cells.

A

	CRC02	**HCT116**
DRUG	**IC_{50} (nM)**	**IC_{50} (nM)**
Gemcitabine	26.92 ± 1.8	18.43 ± 1.2
Camptothecin	14.89 ± 2.3	0.68 ± 1.3

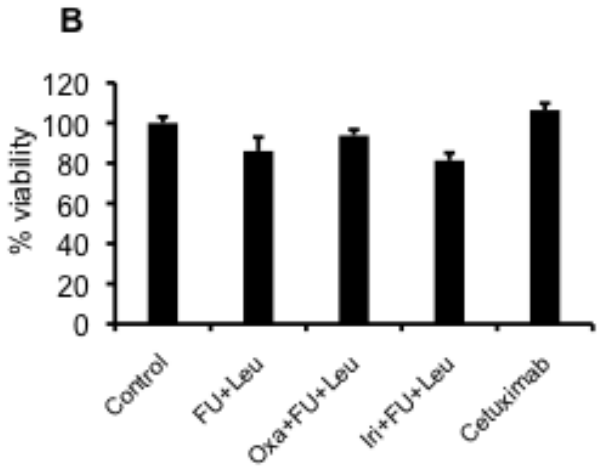


Figure 24: CRC02 has attenuated response to standard therapeutic regimen. ***(A):*** *CRC02 cells are less responsive to gemcitabine and camptothecin compared to HCT116* ***(B):*** *CRC02 did not show any appreciable response to drug combination used for treatment of CRC.*

4.4. Discussion

Misregulation of signaling cascades such as Wnt-β-catenin, TGFβ and Notch pathways are key to pathogenesis and progression of colorectal cancer. These are also implicated in EMT activation, metastasis and resistance to therapeutic agents, resulting in poor patient survival. Assessment of relative mRNA levels of these pathways revealed differential regulation of gene expression patterns in the novel cell line CRC02 in comparison to established cell lines HCT116. Repression of expression of E-cadherin and increase in N-cadherin and vimentin expression strongly suggest EMT program being activated in CRC02. Optimal E-cadherin expression and localization are critical in formation of adherens junctions and maintenance of tissue integrity under normal physiological conditions. However, malignant transformation results in suppression E-cadherin expression leading to disruption of cell-cell junction integrity. This is a pre-requisite for metastasis. In CRC02, cadherin switch from E to N type, together with elevated vimentin expression form the typical signature for a shift from epithelial to mesenchymal form.

In addition, we observed an elevated expression of IQGAP1 along with EB1 and CLIP170 in CRC02. All three proteins are directly involved with the localized remodeling of both actin and microtubule cytoskeleton during cellular migration (Watanabe T, Wang S, Kaibuchi K., 2015). Therefore, in CRC02, cadherin switch, together with misregulation of the cytoskeletal remodeling molecules confer an enhanced migratory and invasive capability resulting in at least a partial EMT event in this cell line as demonstrated by the transwell assays. Further, we observed upregulation of Twist and Zeb2. This corroborates with reported literature where Twist and Zeb are shown to bind to E-cadherin promoter region. This transcriptionally represses E-cadherin expression (Vesuna F, van Diest P, Chen JH, et. al, 2008). Additionally, a positive correlation exists between elevated expression of Twist, activation of the EMT program and poor clinical prognosis (Ansieau S, Morel AP, Hinkal G, et. al, 2010). Therefore, it may be reasonably assumed that overexpression of these EMT related transcription factors in CRC02 cells have led in the loss of E-cadherin expression. Our findings suggest that molecular alterations such as loss of inter-cellular junctions and localized modulation of actin and tubulin cytoskeleton may have contributed towards the enhanced migratory and invasive capability in CRC02 via the activation of the otherwise latent EMT program.

In recent years, a clear correlation has emerged between EMT activation and reduced efficacy of therapeutic agents (Voon DC, Huang RY, Jackson RA, 2017). Our analysis of the cytotoxic effects of nucleoside analogs and topoisomerase I inhibitor, gemcitabine and camptothecin, revealed that CRC02 is less responsive to these drugs as compared to HCT116. Also, the standard-of-care treatment regimens comprising of combinations of FOLFOX, FOLFIRI or the targeted therapy cetuximab had no appreciable cytotoxic effect on these cells. Several EMT activating molecules such as Twist and Zeb have been implicated to be important in induced drug resistance, although the mechanism is not well understood. These molecules bring about transcriptional repression of E-cadherin either directly (Zeb) or indirectly (Twist), which in turn modulate the cellular response to therapeutic drugs. Patients with low expression of Twist have been reported to have favorable treatment outcome, making the expression pattern of Twist in tumors a valuable indicator of predicting drug response (Brozovic A, Osmak M.,, 2007). Similar results were also reported with Zeb where silencing Zeb expression increased E-cadherin expression and drug sensitivity (Sánchez-Tilló E, Siles L, de Barrios O, *et al*., 2011). We observed a marked upregulation in the expression of Twist and Zeb2 in CRC02, implying that these could develop as indicators of disease prognosis among Indian patients.

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CHAPTER 5

β-catenin and its functional implications in CRC02

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5.1. Background

5.1.1. Wnt/β-catenin signaling

Wnt signaling is an evolutionarily conserved pathway that participates in tissue morphogenesis, neural patterning, cell polarity, cell migration and determining cell fate during embryonic development (Valenta T, Hausmann G, *et al.*, 2012, Yuko Komiya and Raymond Habas, 2008). The canonical Wnt signaling via the central regulatory molecule, β-catenin, is among the most studied pathways that are crucial during embryogenesis. β-catenin functions as a transcriptional co-activator, regulating the function of downstream target genes that are active during embryogenesis and tissue homeostasis (Bryan T. MacDonald, Keiko Tamai, Xi He, 2009). The other Wnt dependent signaling that are independent of β-catenin, the so-called non-canonical signaling, are the Wnt/Ca^{2+} signaling and the Planar Cell Polarity (PCP),,both of which are also crucial elements during embryonic development (Figure 25) (Yuko Komiya and Raymond Habas, 2008).

Involvement of Wnt/β-catenin signaling cascade in malignancies was first reported in 1991, with the discovery that the mutated β-catenin interacting protein, APC caused familial adenomatous poplyposis (FAP) (Kenneth W, Kinzler KW, Nilbert MC, Su LK, Vogelstein B, 1991, Nishisho I, Nakamura Y, Miyoshi Y, 1991). Loss-of-function mutations in APC was found to result in increased β-catenin signaling, thus establishing a direct link between Wnt/β-catenin pathway and colorectal cancer (Korinek V, Barker N, Morin PJ, et al, 1997). Rapid turnover of cellular β-catenin is critical in maintaining normal tissue homeostasis and APC is directly involved in maintaining low β-catenin levels in cells and therefore functioning as a tumor suppressor. This is achieved by the interaction of a large multiprotein complex called the destruction complex comprising of Axin1, APC, GSK3β, PP2A and CK1, that bind and rapidly degrade free β-catenin via the ubiquitination mediated proteasomal degradation pathway (Kimelman D, Xu W., 2006). Binding of Wnt ligand to the cell surface receptor

Frizzled (Fzd) and co-receptor LRP5/6 turns on the Wnt/β-catenin signaling via recruitment of the adaptor protein Dishevelled (Dvl). Interaction of Dvl with the ligand-receptor signalosome inhibits GSK3β mediated phosphoylation of β-catenin and destabilizes the destruction complex, resulting in release of β-catenin into the cytoplasm (Kim SE, Huang H, *et al.*, 2013). This cytoplasmic β-catenin is then transported inside the nucleus where it interacts with other co-transcription factors and activates transcription of target genes. Transport of β-catenin into the nuleus and activation of downstream target genes is the essence of β-catenin mediated Wnt signaling and therefore is a tighly relulated cellular process. However, pathological transformations that interfere with β-catenin turnover may lead to constitutively activated signaling and consequently over-activation of pro-growth genes resulting in abnormal cellular proliferation (Bilic J, Huang YL, Davidson G, *et al.*, 2007, Gerlach JP, Emmink BL, *et al*. 2014).

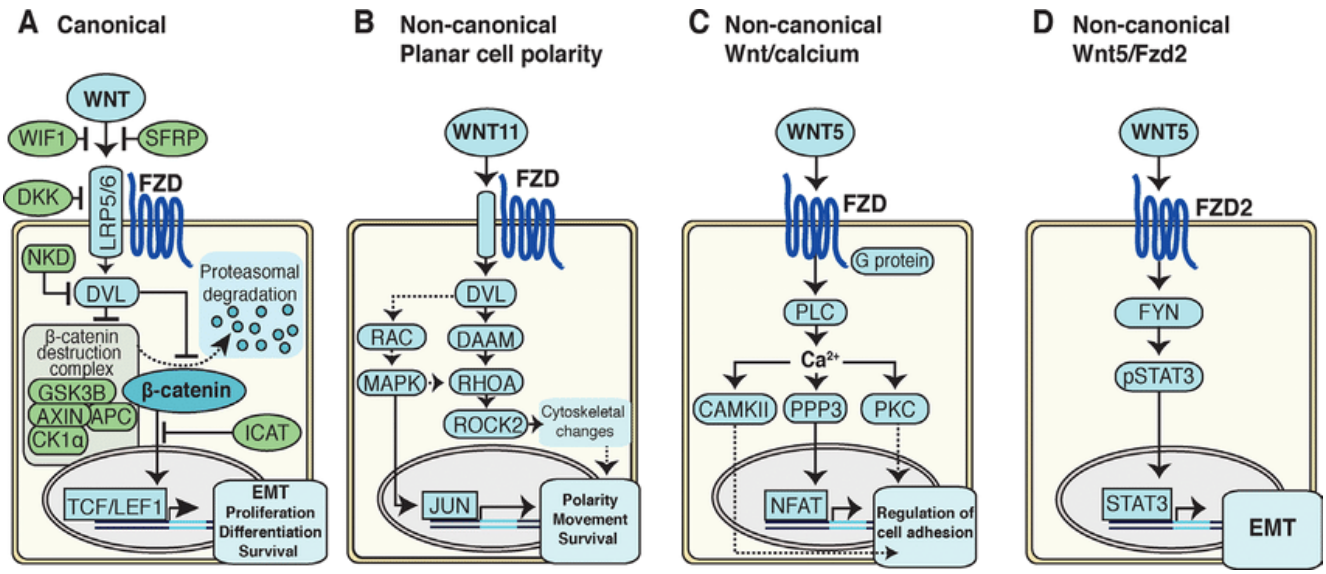

Figure 25: Canonical and Non-canonical Wnt signaling in cancer cells.
A: *Cellular β-catenin is degraded by the proteasomal degradation system in the absence of incoming Wnt signal. Binding of Wnt ligand to the cell surface receptor inhibits destruction complex and stabilizes β-catenin that eventually translocates to the nucleus. In the nucleus, β-catenin, along with other transcription co-factors brings about transcriptional upregulation of target genes.* ***B:*** *Planar cell polarity (PCP) signaling, a non-canonical Wnt signaling brings about cytoskeletal remodeling that affects cell polarity and migration during embryonic development.* ***C:*** *Wnt dependent calcium signaling, another non-canonical pathway, influences intracellular calcium dynamics that affect cell adhesion.* ***D:*** *Activation of the EMT program is brought about by Wnt5 signaling via FZD2 and FYN (Sandsmark E, Hansen AF, et al, 2017).*

5.1.2. β-catenin, the central regulatory molecule of Wnt/β-catenin signaling pathway

Human β-catenin is a 90 KDa protein, encoded by the *CTNNB1* gene. β-catenin is conserved through evolution and has high sequence homology among members of different zoological classes. For instance, there is more than 60% sequence homology between Cnidarian β-catenin and human β-catenin with identical structural architecture at the core armadillo repeats, differing only in the C terminus, indicating its preservation through evolution (Valenta T, Hausmann G, *et al.*, 2012). β-catenin was first identified as a component of the cell-cell adherens junction where it interacts with E-cadherin and APC (McCrea PD, Turck CW, Gumbiner B., 1991, Hülsken J, Birchmeier W, Behrens J., 1994). Crystal structure of the full-length Zebrafish β-catenin reveal a highly helical protein with 12 armadillo repeats forming the central region, flanked by an N-terminus helix and a flexible C-terminus helix called Helix C. Except armadillo repeat number 7, all other repeats are composed of three helices made of approximately 42 residues each. All 12 armadillo repeats together form a superhelix with a long groove that is positively charged (Figure 26) (Xing Y, Takemaru K, Liu J, et al., 2008).

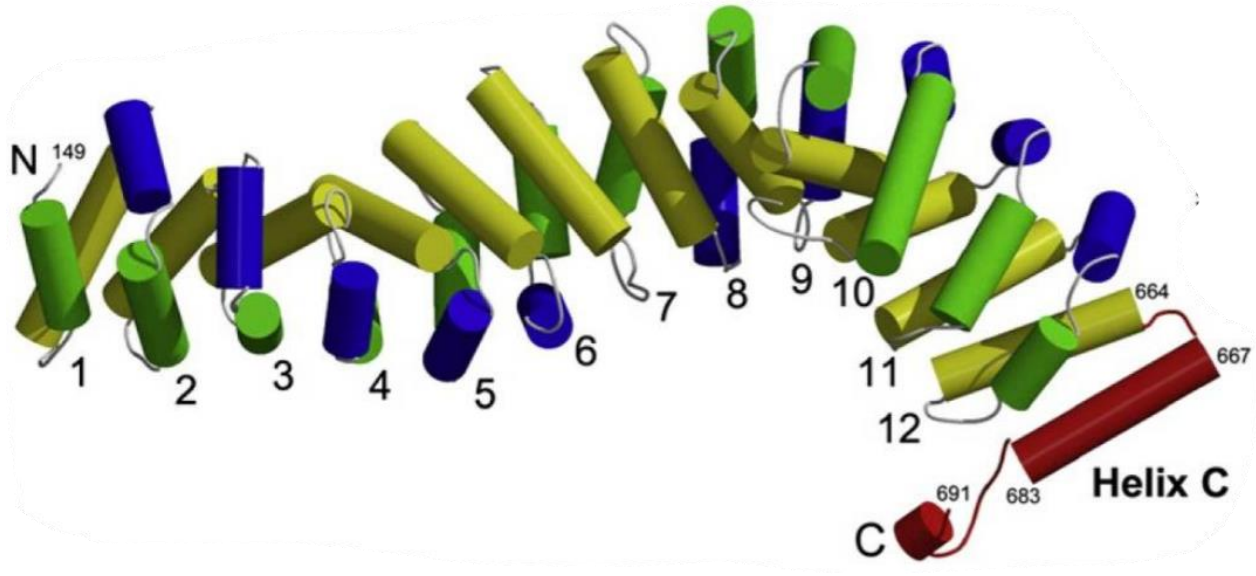

Figure 26: Crystal structure of human β-Catenin.
β-Catenin is a highly helical protein. The central region comprises of 12 armadillo repeats. Each repeat is made of three helices – blue -helix 1, green -helix 2 and yellow- helix 3 (yellow). The central core is flanked on either side by the N-terminal helix and the Helix C at the C terminal shown in red (Xing Y, Takemaru K, Liu J, et al, 2008).

The structure of β-catenin makes it conducive to support multiple protein-protein interactions that primarily govern its cellular functions. Its role as a component of the adherens junction is through its interaction with the plasma membrane anchored E-cadherin. β-catenin bridges the transmembrane E-cadherin and the cytosolic α-catenin to form a cadherin-catenin complex that modulates localized cytoskeletal remodeling (Valenta T, Hausmann G, *et al.*, 2012, Weis WI, Nelson WJ., 2006, Sayon Basu, Gal Haase, *et al.* 2016). Components of the destruction complex such as Axin1, APC, GSK3β interact with β-catenin to bring about its rapid turnover that maintains a low level of cytosolic free β-catenin (Kimelman D, Xu W., 2006). Binary interactions of transcription factors and transcription regulators bring about β-catenin dependent gene expression that is temporally separated during the life cycle of the cell (Valenta T, Hausmann G, *et al.*, 2012).

The gene *CTNNB1*, coding for β-catenin, comprises of 16 exons that span 23.2Kb on chromosome 3p21. (Machin P, Catasus L, Pons C, Muñoz J, et al, 2002). Mutations in *CTNNB1* that interfere with the turnover of β-catenin and its subcellular localization have been associated with numerous malignancies including colorectal cancer. These nucleotide alterations are often present within exon 3 of *CTNNB1* resulting in missense mutations at S33, S37, T41 and S45 and are clustered at the N-terminal regulatory domain of β-catenin. S33/S37/T41 and S45 are the binding sites for the kinases GSK3β and CK1 respectively, that sequentially phosphorylates β-catenin and tags it for proteasomal degradation. Therefore, exon 3 of β-catenin is considered a mutational hot spot that activate β-catenin mediated Wnt signaling that drives tumorigenesis (Gao C, Wang Y, Broaddus R et al, 2017). Colorectal cancer patient derived samples have revealed that ~ 58.1% tumors and 48.8% lymph nodes show cytoplasmic and nuclear staining for β-catenin. In addition, overexpression in cytoplasm and nucleus has been correlated with decrease in overall patient survival. Therefore, β-catenin expression, mutational status and subcellular localization could serve as a candidate prognostic predictor of disease progression (Rania Abdelmaksoud-Damak, Imen Miladi-Abdennadher, Mouna Triki, et al, 2015).

The interaction between β-catenin and the tumor suppressor APC is critical in maintaining the cellular homeostasis of β-catenin under normal conditions. This balance is however altered during malignant transformation. Loss of function mutations in APC that affect or abrogate its binding to β-catenin are common in colorectal cancer and have been mapped to exon 15 of

APC, also called the Mutation Cluster Region (MCR). It has been demonstrated using clinical samples that β-catenin and APC mutations are mutually exclusive in colorectal cancer pathogenesis. ~ 48% tumors with mutated β-catenin do not contain APC mutations. However, β-catenin mutations themselves are extremely rare in APC mutated colorectal tumors. This is because β-catenin and APC are both part of the same oncogenic pathway and either activation of β-catenin or inactivation of APC is sufficient to constitutively activate the signaling pathway (Rania Abdelmaksoud-Damak, Imen Miladi-Abdennadher, Mouna Triki, et al, 2015). Similar mutually exclusive mutations are present in other cancers such as BRAF and KRAS mutations in colorectal cancer and BRAF and NRAS in melanoma (Rajagopalan H, Bardelli A, et al, 2002, Colombino M, Capone M, et al., 2012).

5.1.3. Cellular functions of β-catenin

Cellular functions of β-catenin are largely governed by its subcellular localization and its interactions with its binding partners (Figure 27). As discussed earlier, β-catenin was first discovered as a member of the cell-cell adherens junction, bridging the transmembrane E-cadherin and the cytosolic α-catenin (McCrea PD, Turck CW, Gumbiner B., 1991, Hülsken J, Birchmeier W, Behrens J., 1994). The interaction between β-catenin and E-cadherin is independent of cell-cell adhesion and is constitutively active in cells in isolation (Stepniak E, Radice GL, Vasioukhin V., 2009). The formation of the cadherin-catenin complex helps both proteins escape proteasomal degradation – binding of β-catenin shields the PEST domain on E-cadherin from being ubiquitinated. Conversely, binding of E-cadherin to β-catenin prevents the destruction complex to gain access to β-catenin (Hinck L, Näthke IS, *et al*, 1994, Huber AH, Weis WI., 2001). Therefore, cadherin-catenin complex acts as a trap to sequester and stabilize β-catenin at the cell-cell contacts and function as a structural component of the inter-cellular junction. Loss of membrane bound E-cadherin releases β-catenin from the adherens junction, increasing the cytosolic pool of free β-catenin and thus promotes its function as a signaling molecule (Heuberger J, Birchmeier W., 2010).

The pool of free cytoplasmic β-catenin typically translocates to the nucleus where, together with other transcription co-activators, can initiate the activation of pro-growth genes. Under normal physiological conditions, free cytoplasmic β-catenin that is not bound to E-cadherin at

the plasma membrane is quickly degradation by the destruction complex (Kimelman D, Xu W., 2006). Association of β-catenin with either Axin or APC brings together kinases that phosphorylate a serine-threonine cluster at the N-terminal regulatory sequences of β-catenin. Casein kinase I (CK1α) mediated phosphorylation of S45 acts as a primer for sequential phosphorylation at T41/S37/S33 by GSK3β. The phosphorylated β-catenin is released from the destruction complex which then gets ubiquitinated by the Skp1/Cul1/F-box/β-TrCP E3 ubiquitin ligase complex and is degraded via the 26S proteasome (Hart M, Concordet JP, et al., 1999). Although GSK3β can phosphorylate a number of different substrates, β-catenin phosphorylation occurs only if the latter is associated with Axin and APC (Dajani R, Fraser E, Roe SM, et al., 2003). In the event that the N-terminally phosphorylated β-catenin is not associated with APC, rapid dephosphoryation of β-catenin occurs via PP2A (Su Y, Fu C, Ishikawa S, 2008).

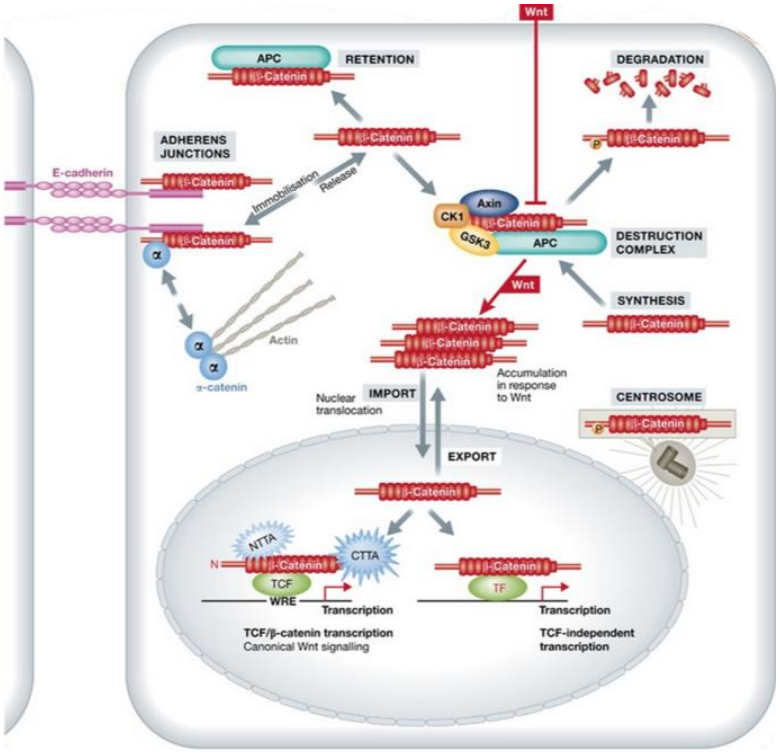

Figure 27: Cellular functions of β-catenin.
At adherens junctions, β-catenin forms cadherin-catenin complex with E-cadherin and α-catenin. Suppression of E-cadherin expression releases β-catenin from the adherens junctions, which rapidly gets degraded via phosphorylation dependent proteasomal degradation system. Wnt signaling abrogates phosphorylation of β-catenin leading to an increase in cytoplasmic free β-catenin that translocates to the nucleus and causes transcriptional upregulation of target genes. β-catenin is also present at the centrosomes and contributes towards centrosomal stability and integrity that is critical for maintaining mitotic fidelity (Valenta T, Hausmann G, Basler K., 2012).

Under normal physiological conditions, transcriptional activity of β-catenin is tightly regulated - β-catenin is exported from the nuclear compartment to the cytoplasm and rapidly degraded by the proteasome machinery. β-catenin lacks a Nuclear Export Signal (NES) and is therefore unable to translocate by itself out of the nucleus once its transcriptional functions are fulfilled (Beric R. Henderson, Francois Fagotto, 2002). Turnover of nuclear β-catenin is controlled by direct binding to APC (Henderson BR, 2000). A protein that has been implicated in regulating the movement of β-catenin between the cytoplasmic and the nuclear compartments is Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1). It has been reported that Pin1 stabilizes cellular β-catenin and that increased levels of Pin1 has direct correlation with the transactivation of β-catenin target genes in breast cancer tissue. Also, high Pin1 levels interfered with β-catenin-APC interaction thus preventing nuclear export of β-catenin. In addition, Pin1 influences intracellular β-catenin levels. Pin1 knockout mice have downregulated expression of β-catenin. Conversely, upregulation of Pin1 has been shown to increased β-catenin levels (Ryo A, Nakamura M, *et al.*, 2001). The mechanism of regulation of β-catenin trafficking by Pin1 depends on the phosphorylation status of the S246 on β-catenin. Once S246 gets phosphorylated, it forms a recognition motif for Pin1 to bind directly to β-catenin, which leads to cis-trans isomerization of the adjacent P247. This abolishes the binding of APC to β-catenin that adversely affects the export of the latter out of the nucleus resulting in its nuclear accumulation (Figure 28). This phenomenon has also been validated in multiple breast cancer tissue where elevated Pin1 levels are directly correlated with nuclear and cytoplasmic accumulation of β-catenin (Ryo A, Nakamura M, *et al.*, 2001).

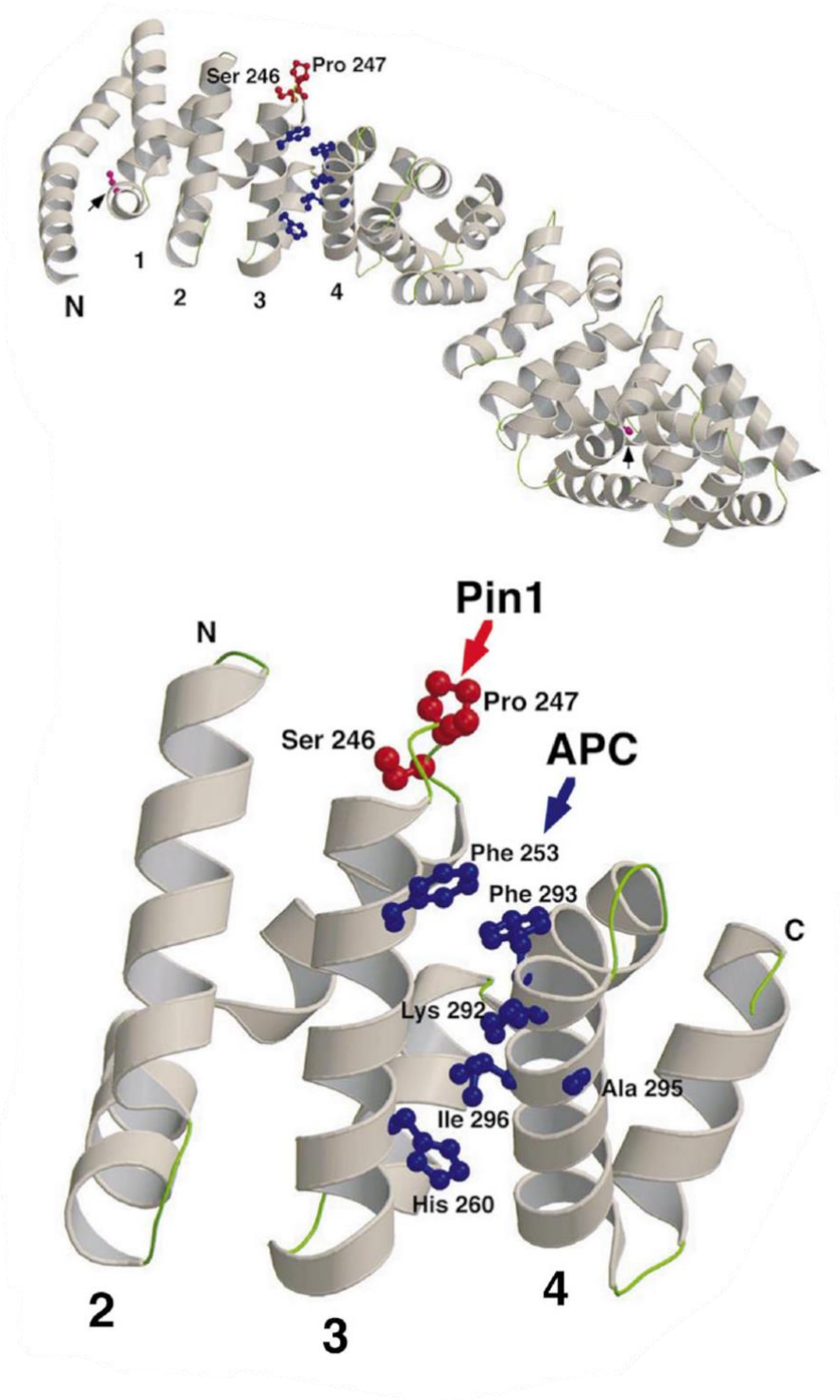

Figure 28: β-catenin binding to Pin1 and APC.
Pin1 binds to β-catenin at Ser246 (red) located in the loop region between the helices of the third armadillo repeat of β-catenin. This is very close to the six residues on the third and fourth armadillo repeats that are critical for binding APC (in blue). Binding of Pin1 at S246P of β-catenin induces conformational change (isomerization) that interferes with APC-β-catenin interaction, thus modulating the turnover and subcellular localization of β-catenin (Ryo A, Nakamura M, et al, 2001).

β-catenin as a structural component of the cell-cell adherens junction and transcriptional co-activator have been studied extensively. However, recent advances that has brought forth a novel role of β-catenin as a component of the centrosome and a regulator of cell division, have gained momentum (Figure 29) (Bahmanyar S, Guiney EL, *et al.*, 2010). Centrosomes are the primary microtubule organizing centers (MTOCs) of the cells and play important roles in cell motility, cell polarity, cell shape, cell division and vesicle transport (Schatten H, 2008). Centrosomes undergo cell-cycle dependent change in position and number inside the cell – interphase cells typically contain a single centrosome placed close to the nucleus. As the cell transitions through the cell cycle, significant reorganization within the centrosome occurs resulting in the duplication of the centrioles that go on to form the poles of the spindle apparatus during metaphase. This event is well synchronized with the duplication of DNA during the S phase. Formation of the bipolar spindle is a critical mitotic event and is of utmost importance in the proper segregation of chromosomes – anomalous spindle structures have been implicated in pathologies, including cancer (Schatten H, 2008).

The role of β-catenin as a centrosomal regulator was first brought to light by experiments where β-catenin overexpression not only resulted in centrosomal disorganization, but also, loss of cortical anchorage of microtubules in these cells. At adherens junctions, microtubules project towards the cell-cell contacts and are captured by cytoplasmic dynein at the junctions (Ligon LA, Karki S, et al, 2001). The level of cytoplasmic β-catenin varies with the cell cycle, steadily increasing at S phase and peaking at the G2/M boundary, thereafter, falling sharply once the cell enters G1. Any manipulation of β-catenin levels at the G2 phase affects centrosomal maturation and functions (Olmeda D, Castel S, et al., 2003). Cells depleted of β-catenin fail to nucleate microtubules and lack γ-tubulin containing centrosomes. These observations support the role of β-catenin in recruitment of some of the microtubule nucleation proteins to the centrosomes (Huang P, Senga T, Hamaguchi M., 2007). In addition, β-catenin localizes to centrosomes along with centriolar linker proteins Rootletin and C-Nap1 at interphase. As the cells enter mitosis, this pool of centrosomal β-catenin redistributes to the spindle poles and eventually to the mid-body at cytokinesis. It has been shown that depleting cells of β-catenin results in loss of centrosomal separation, a process that is crucial for forming the bipolar spindle apparatus during metaphase. These cells go on to form monopolar spindles that are unable to segregate chromosomes resulting in aneuploid cells (Kaplan DD, Meigs TE, Kelly P, Casey PJ., 2004). Conversely, stabilized β-catenin (via mutations that abrogates β-

catenin degradation) at the centrosomes increase the centriolar distance, thus affecting centrosome cohesion resulting in premature centrosomal splitting. Thus, β-catenin has been established as a negative regulator of centrosomal cohesion (Bahmanyar S, Kaplan DD, *et al.*, 2008).

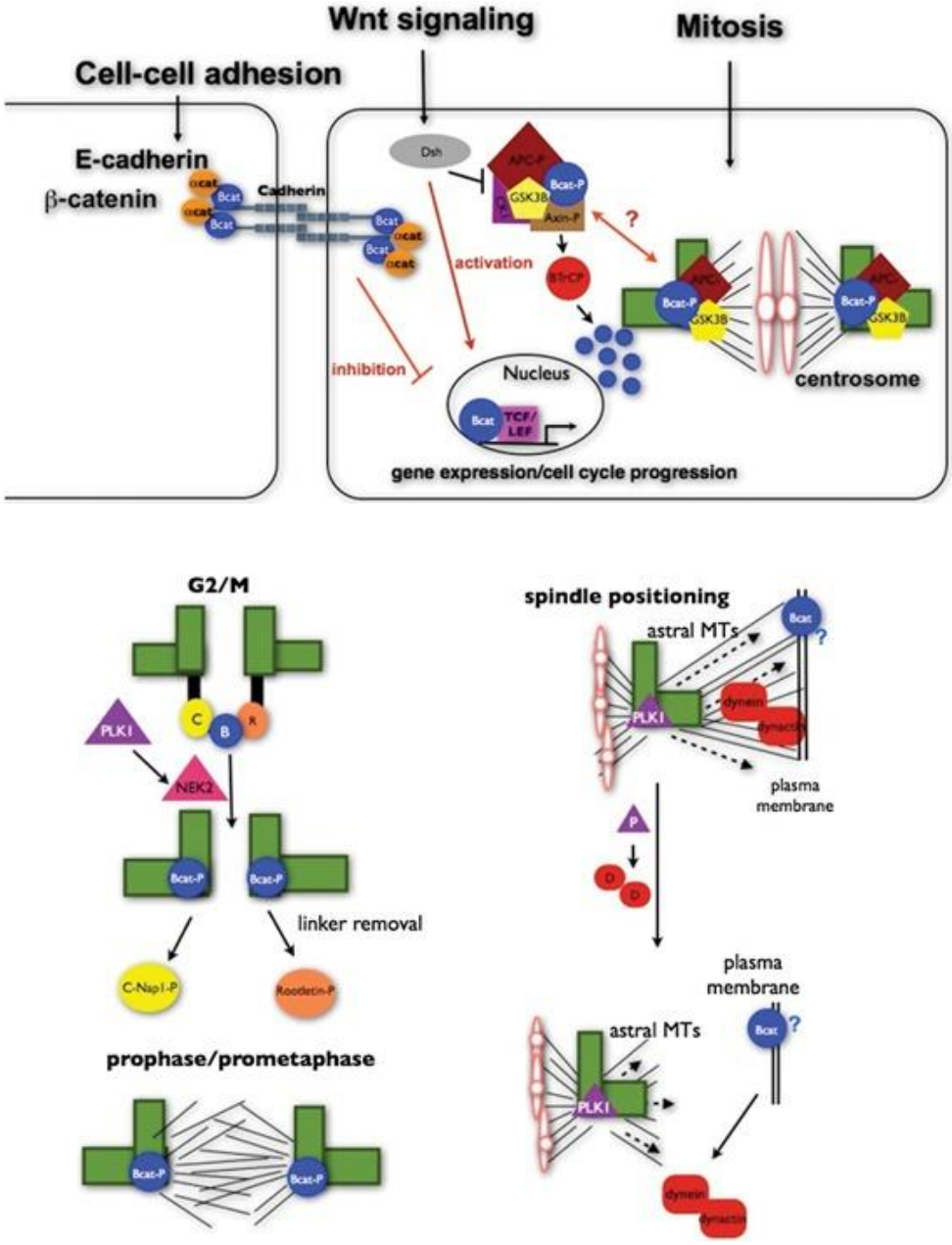

Figure 29: Centrosomal function of β-catenin.
In addition to being a structural component of the cell-cell junctions and localizing to the cytosol and nucleus, the Wnt signaling molecules β-catenin, APC, Axin and GSK3β have been shown to localize at the centrosome and function as regulators of cell cycle progression. Therefore, there may be cross talk between these distinct pools of Wnt signaling proteins that are spatially and temporally separated (Mbom BC, Nelson WJ, Barth A., 2013).

5.2. Materials and methodology

5.2.1. Real Time qPCR

Reat time qRT-PCR was performed as described in the materials and methodology section in Chapter 4.

The sequences of primers used for the analysis are as follows:
GAPDH: forward – TCTGACTTCAACAGCGACAC, reverse – TACTCCTTGGAGGCCATGT, β-catenin: forward -CAATGGCTTGGAATGAGACT, reverse – CCCATCTCATGTTCCATCA, E-cadherin: forward - CCAAAGACAGAGCGGAACTA, reverse – GAGACTCCTCCATTCCTTCC, c-Myc: forward –CAGCTACGGAACTCTTGTGC, reverse – CAAGACTCAGCCAAGGTTGT, Pin1: forward – TGCCTTCAGCAGAGGTCAGA, reverse – ACAATTCGGCCCTCGAGTCT.

5.2.2. Immunofluorescence staining

The method used for immunofluorescence analysis was as described in the materials and methodology section of Chapter 4.

Primary antibodies were used at the following dilutions:
α-tubulin – 1:1000
γ-tubulin – 1:500
β-catenin – 1:200
phospho-β-catenin – 1:200
E-cadherin – 1:200

5.2.3. Antibodies

Table 5: Antibodies used for the immunofluorescence and western blot analyses

Primary antibody	Company	Catalog number
β-catenin	Cell Signaling Technology	8480
phospho-β-catenin	Cell Signaling Technology	9561
APC	Thermo Scientific	MA1-26195
E-cadherin	Thermo Scientific	33-4000
α-tubulin	Sigma-Aldrich	T9026
β-actin	Sigma-Aldrich	A3853
γ-tubulin	Bethyl Labs	A302-631A
Pin1	Santa Cruz Biotechnology	SC-46660
Secondary antibody	**Company**	**Catalog number**
Anti-mouse Alexa-488	Molecular Probes, Invitrogen	A11008
Anti-rabbit Alexa-488	Molecular Probes, Invitrogen	A11001
Anti-mouse Cy3	Molecular Probes, Invitrogen	A10521
Anti-Rabbit Cy3	Molecular Probes, Invitrogen	A10520
Anti-mouse HRP	Jackson Immunoresearch	715-035-150
Anti-rabbit HRP	Jackson Immunoresearch	711—35-152

5.2.4. Western Blotting

1X Radioimmunoprecipiation Assay (RIPA) buffer with protease inhibitor (Roche, catalog no. 05892970001) and phosphatase inhibitor (sodium orthovanadate, New England Biolabs, catalog no. P0758S) were used to lyse the cells. The lysates were denatures at 95°C for 10 minutes in Laemmli buffer with β-mercaptoethanol. The denatured samples were electrophoresed in a 10% or a 12% SDS PAGE and the resolved proteins were transferred onto PVDF membrane. 5% BSA or 5% skimmed milk in TBST was used for blocking followed by addition of primary antibody. Incubation was done at 4°C overnight. The membranes were washed and HRP-labeled secondary antibody was added for 2 hours.

Membranes were washed and chromogenic substrate was added for chemiluminiscent imaging. Primary antibodies were used at the following dilutions: β-catenin – 1:1000, phospho-β-catenin – 1:500, Pin1 – 1:1000, β-Actin – 1:2000.

5.2.5. Drug sensitivity assay

MTT assay for evaluating the efficacy of bortezomib was performed as described in the materials and methodology section of Chapter 4. Different concentrations of bortezomib ranging from 0.03nM to 50nM were used for this assay.

5.2.6. Proteasome activity measurements

We used Proteasome 20S activity kit (Sigma-Aldrich, MAK172) for measuring the intrinsic activity of the proteasome degradation system in CRC02 and HCT116. Cells seeding was done at at a concentration of 80,000 cells per well in each well in 24-well plates. Next day, assay reagents (proteasome substrate and assay loading solution) were prepared as perthe manufacturer's instructions. The culture media was replaced with assay solution (1:1 ratio of assay loading solution and at 37°C, 5% CO_2 and 95% relative humidity. Fluorescence was measured post incubaton at λ_{ex} = 490nm and λ_{em} = 525nm.

200nM of bortezomib (ChemShuttle, Catalog no. 179324-69-7) was used for the proteasome inhibition assay. Cells were grown to approximately 85-90% confluency in 10 cm dishes. Bortezomib was added and the cells were incubated for differerent time points ranging from 30 minutes to 2 hours. Untreated cells were used as controls in the proteasome inhibition assay. The collected cells were lysed and western blot analysis was performed as described above.

5.3. Results

5.3.1. β-catenin is highly activated in CRC02

It has been reported earlier that differential accumulation of β-catenin at various subcellular compartments may orchestrate cell cycle progression by modulating centrosomal function (Mbom BC, Nelson WJ, Barth A., 2013). Moreover, mutations in β-catenin often lead to stabilization and accumulation of the protein in cells resulting in aberrant signaling in various malignancies (Polakis, 1999) and are often used as a marker for disease prognosis (Mårtensson A, Oberg A, *et al*., 2007, López-Knowles E, Zardawi SJ, *et al*., 2010). We tested the mRNA expression level of β-catenin in CRC02 using quantitative real time PCR analysis that revealed a dramatic increase (approximately 1800-fold) in relative expression of β-catenin mRNA in CRC02 as compared to HCT116 (Figure 30A).

We used anti-β-catenin antibody to study the localization of anti-β-catenin in CRC02 cells. Strong cytoplasmic staining and almost no cortical staining of β-catenin was observed in CRC02 cells. In addition, β-catenin also localized to the cytoplasmic microtubules but not to the cortical microtubules in CRC02 (Figure 30B). The cytosolic accumulation of β-catenin in CRC02 prompted us to investigate whether stabilizing mutations are present in β-catenin. It is known that cancer-associated mutations in β-catenin prevent its phosphorylation and subsequent degradation, resulting in its accumulation and eventual translocation into the nucleus leading to overexpression of its target genes (Bilic J, Huang YL, Davidson G, *et al.*, 2007, Gerlach JP, Emmink BL, *et al*. 2014). Upon DNA sequencing analysis we found that the sequence at S33, S37, T41 and S45 phosphorylation sites were not mutated in CRC02. In contrast, HCT116 contained the expected in-frame deletion at S45, as reported earlier (Ilyas M, Tomlinson IP, *et al*., 1997) (Figure 30C). The retention of the wildtype phosphorylation sites in CRC02-derived β-catenin indicated that normal phosphorylation might occur. To test this, we used a phospho-specific antibody that recognizes β-catenin phosphorylated at S33/S37/T41 to immunostain CRC02 and HCT116 cells. As expected, we observed the presence of a substantial amount of phospho-β-catenin in the cytoplasm and nucleus in CRC02 confirming that phosphorylation of β-catenin remains unimpaired (Figure 30D). Additionally, almost 75% of CRC02 cells showed accumulation of phosphorylated -β-catenin in the nucleus. This was in contrast to the ~20% of HCT116 cells that showed nuclear

accumulation of phospho-β-catenin.(Figure 30E). We next examined the mRNA expression of the downstream β-catenin target gene *c-Myc*. We observed a 1.5-fold increase in *c-Myc* expression in CRC02 as compared to HCT116 (Figure 30F). This observation indicated that the nuclear accumulation of β-catenin enhanced the expression of its target gene *c-Myc*. Therefore, in CRC02 cells, despite efficient phosphorylation in the N-terminal region, phosphorylated β-catenin not only accumulated in the cytosol and nucleus but also transcritionally upregulated the target gene *c-Myc*.

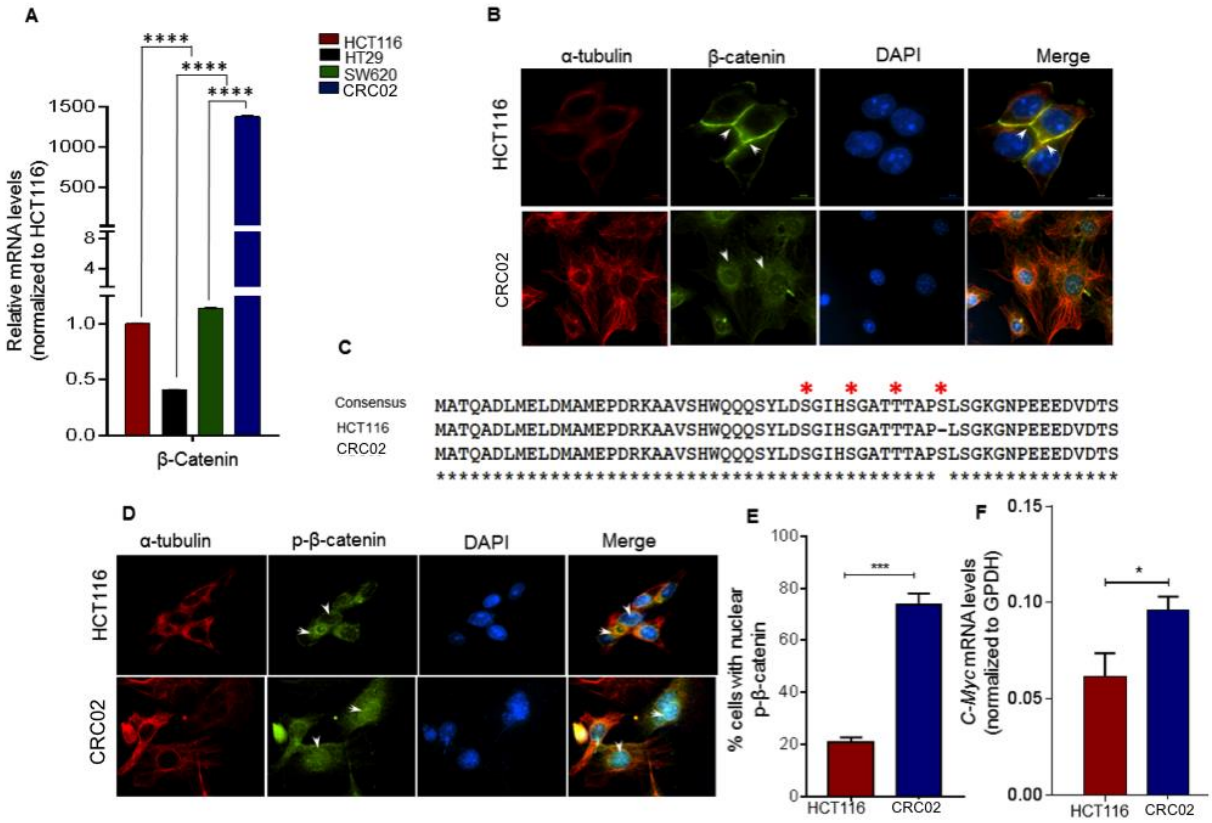

Figure 30: mRNA expression and subcellular localization of β-catenin in CRC02.
(A): *β-catenin is highly overexpressed in CRC02 in comparison to other CRC cell lines.* ***(B):*** *β-catenin (green) shows localization at the adherens junctions in HCT116. In contrast, β-catenin is primarily localized in the cytoplasm in CRC02. □□tubulin (red) and nucleus (blue) are also marked.* ***(C):*** *CRC02 derived β-catenin shows wildtype phosphorylation sites, S33/S37/T41 and S45 at the N-terminus (red asterix), whereas HCT116 contains an in-frame deletion at codon 45.* ***(D):*** *Presence of phosphorylated β-catenin (green) in the cytoplasm and nucleus (arrows) in CRC02 is confirmed by immunofluorescence imaging. However, only a small fraction of Phosphorylated β-catenin localizes to the nucleus in HCT116 (arrow).* ***(E):*** *~75% of CRC02 cells have nuclear phospho-β-catenin in comparison to only ~20% HCT116 cells.* ***(F):*** *c-Myc expression is elevated in CRC02. Error bars represent SEM calculated over three independent experiments.*

5.3.2. β-catenin localizes to supernumerary centrosomes and the inter-cellular bridge during cell division in CRC02

In earlier studies, it has been shown that in addition to cell-cell adhesion and Wnt signaling, β-catenin also plays an important role in establishing bipolar mitotic spindles (Kaplan and Casey, 2004) and regulates mitotic centrosome separation (Bahmanyar and Barth, 2008). Upon investigating the subcellular distribution of β-catenin in CRC02, we observed that during metaphase, β-catenin localizes at the spindle poles with distinct β-catenin foci that overlap with α-tubulin (Figure 31A) whereas at cytokinesis, prominent localization of β-catenin at the inter-cellular bridge was evident (Figure 31B). However, there was no discernible localization of β-catenin at either the spindle poles or at the inter-cellular bridge, although cortical β-catenin staining was prominent in HCT116. (Figure 31A, B). Therefore, the prominent localization of β-catenin at the intercellular bridge suggests that this protein may play a crucial role during cytokinesis in CRC02 cells.

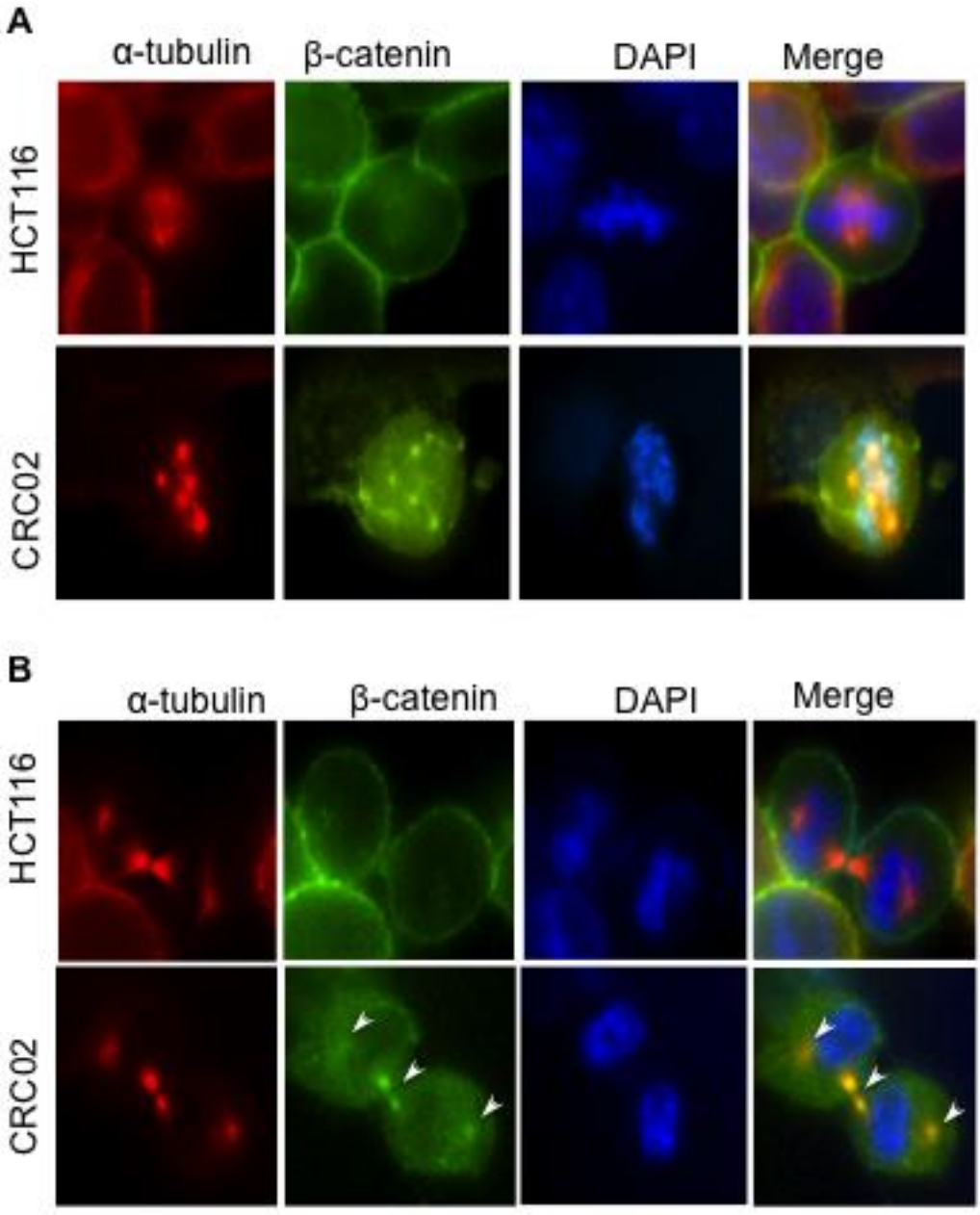

Figure 31: β-catenin localization during cell division.
(A): *Cortical β-catenin (green) is prominently visible in HCT116 during metaphse, however, at the spindle poles, no visible β-catenin foci can be seen. In CRC02, very prominent β-catenin foci are seen at the spindle poles at metaphase. (**B):** β-catenin localization at the intercellular bridge is marked by arrowheads in CRC02. In contrast, β-catenin is confined to the cell cortex in HCT116.*

5.3.3. CRC02 displays lack of cell-cell contacts due to loss of E-cadherin

A major fraction of cellular β-catenin localizes at the plasma membrane in complex with membrane bound E-cadherin, forming adherens junctions that is critical in maintaining tissue integrity (Valenta T, Hausmann G, *et al.*, 2012, Weis WI, Nelson WJ., 2006, Sayon Basu, Gal Haase, *et al.* 2016). As mentioned earlier, β-catenin is predominantly cytoplasmic in CRC02 cells, with almost no cortical localization as seen by immunofluorescence analysis. In HCT116 however, prominent cell-cell junction localization of β-catenin was observed. The absence of cortical β-catenin staining in CRC02 led us to examine the expression level and localization of E-cadherin in CRC02. We observed E-cadherin at the cell-cell junctions in HCT116 but not in CRC02 (Figure 32A). Also, mRNA transcript for E-cadherin was detectable on HCT116, whereas no detectable transcript was observed in CRC02 (Figure 32B). The lack of cortical β-catenin staining together with the absence of expression of E-cadherin in CRC02 is suggestive of a loss of intercellular contacts in CRC02.

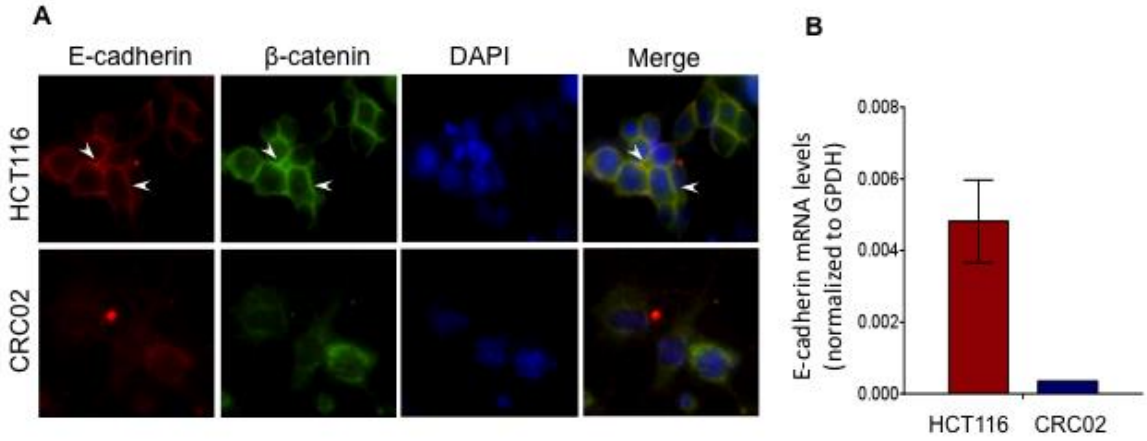

Figure 32: Loss of E-cadherin from adherens juctions in CRC02.
(A): E-cadherin and *-β-catenin localize at the cell-cell junctions in HCT116 (arrowheads), whereas, E_cadherin is lost from the cell-cell junctions in CRC02.* ***(B):*** *Measurement of relative mRNA levels show downregulation of mRNA expression in CRC02 as compared to HCT116. Error bars represent SEM calculated over three independent experiments.*

5.3.4. CRC02 shows elevated expression of Pin1 and APC truncation

β-catenin levels are tightly regulated in cells, prompting us to investigate whether alterations in its turnover process could result in the enhanced accumulation of phosphorylated β-catenin in CRC02. Pin1 is known to cause isomerization induced structural changes in β-catenin that can modulate its turnover (Ryo A, Nakamura M, *et al.,* 2001). In fact, changes in Pin1 expression affect levels of β-catenin expression in tumors including hepatocellular carcinoma, prostate cancer, salivary gland carcinoma and cervical carcinoma (Cheng CW, Leong KW, Tse E., 2016, Zhu Z, Zhang H, *et al.,* 2016, Schneider S, Thurnher D, *et al*., 2016). Apart from blocking the β-catenin and APC interaction, Pin1 also retains β-catenin in the nucleus thereby increasing the nuclear fraction of β-catenin (Ryo A, Nakamura M, *et al.,* 2001). To check whether Pin1 could account for the observed nuclear accumulation of β-catenin, we examined the expression of this protein in CRC02 cells. Our results revealed an increase of ~9000 fold in the mRNA level of Pin1 in CRC02 (Figure 33A). We also examined the level of pin1 protein in the whole cel lysates of CRC02 and HCT116. Our results demonstrated a significant increase (~ 3.5-fold) in Pin1 protein levels in CRC02 (Figure 33B, C). β-catenin lacks nuclear export signal and therefore gets exported out of the nucleus by binding to APC. Cancer associated truncation mutations in APC abrogate this interation resulting retention of β-catenin in the nucleus. We performed western blot analysis to determine whether CRC02 contains a truncated version of APC. We observed a band at ~150KDa for CRC02, whereas, HCT116 showed a band corresponding to the full-length APC (~300KDa), indicating that CRC02 does contain a truncated APC (Figure 33D). Therefore, we concluded that the elevated expression of Pin1 along with APC trunctation together are responsible for retaining β-catenin in the nucleus of CRC02 cells.

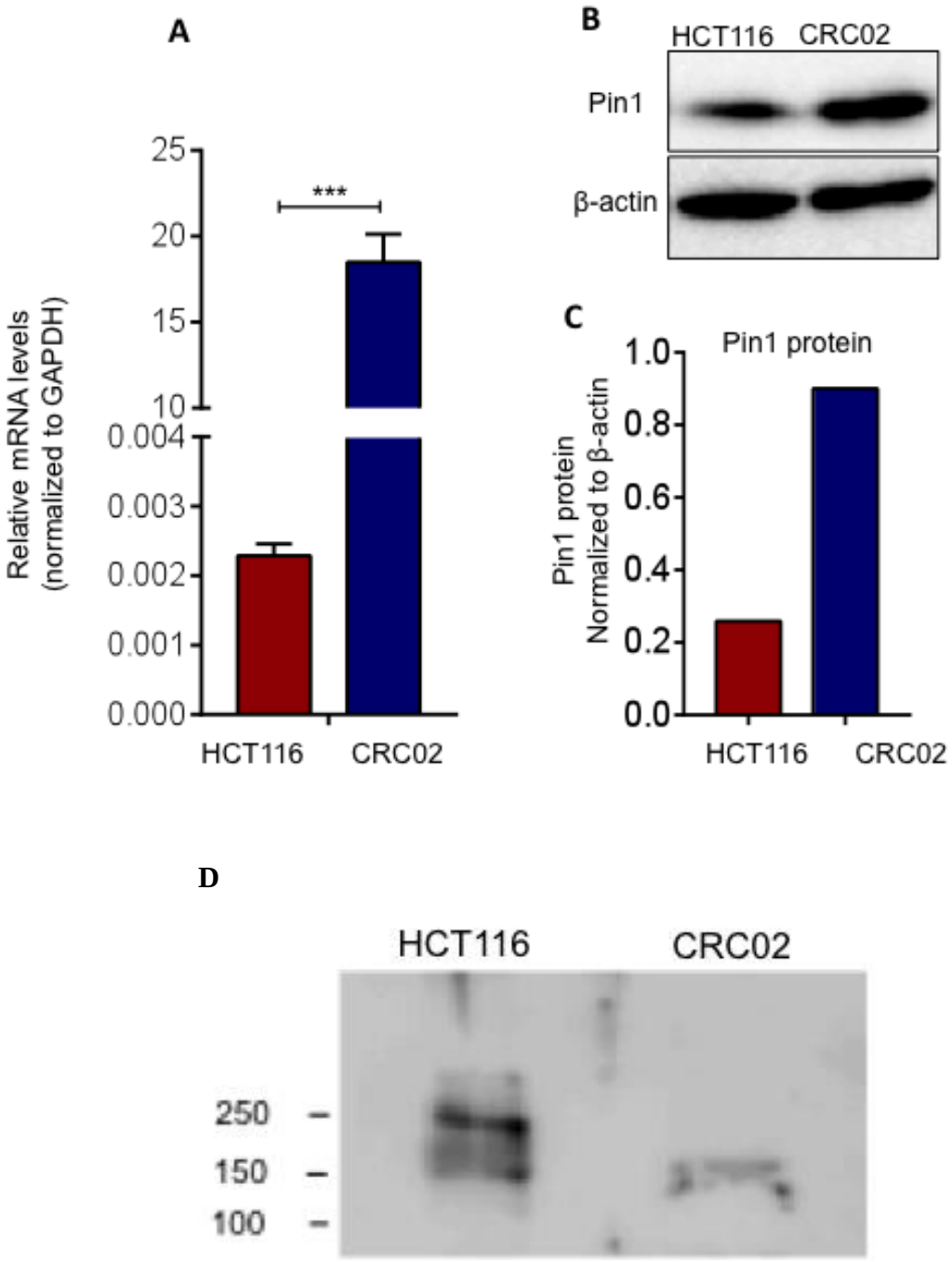

Figure 33: Increased Pin1 expression and APC truncation in CRC02.
(A): *Pin1 transcript levels are highly elevated in CRC02 as seen by qRT-PCR. (**B, C):** Immunoblot shows about 3.5-fold higher expression of Pin1 in CRC02 (**D):** Immunoblot analysis of APC shows a full-length protein in HCT116 whereas a truncated protein of ~150KDa in CRC02.*

5.3.5. CRC02 has high proteasomal activity

Ubiquitin mediated proteasomal degradation is a critical step in the turnover of β-catenin and for overall regulation of Wnt/β-catenin signaling cascade. This is effected by the multiprotein complex called the 26S proteasome (Hagen T, Vidal-Puig A, 2002). Elevated proteasomal activity has been reported in numerous malignancies, including colorectal cancer, to likely counter the numerous mis-regulated pathways that lead to abnormal cellular protein content (Datta K, Suman S, *et al.,* 2016). We postulated that increase in the proteasomal activity could be a possible compensatory mechanism for modulating β-catenin levels in CRC02. Therefore, we evaluated of basal proteasomal activity in CRC02. Our results revealed an increase of almost 6-folds in the instrinsic proteasomal activity in CRC02 in comparison to HCT116 (Figure 34A). Further, we used bortezomib, a clinically approved anti-cancer drug that blocks proteasomal activity that inhibits the chymotrypsin enzymatic activity of the 20S subunit of the proteasome (Richardson PG, Hideshima T, *et al.,* 2003, Roccaro AM, Vacca A, *et al.,* 2006, McBride A, Ryan PY., 2013, Wu WK, Cho CH, *et al.,* 2010), to test the comparative efficacies of the drug in the two cell lines. A higher IC_{50} value for bortezomib in CRC02 (~22nM) as compared to that in HCT116 (~5nM) meant that CRC02 cells were less sensitive to the proteasome inhibitor compared to HCT116 (Figure 34B). We also examined whether inactivation of the proteasomal degradation system could lead to an accumulation of cellular β-catenin. For this, we treated CRC02 cells with 200nM of bortezomib and retrieved cells at pre-determined time intervals after commencement of treatment. We used the whole cell lysates from the treated cells to measure the level of β-catenin protein using immunoblotting. Indeed, we found that there is an accumulation of β-catenin upon bortezomib treatment as early as 30 mins after treatment and continued thereafter upto 2 hours (Figure 34C). We further examined whether accumulation of β-catenin led to alterations in expression of *c-Myc* in cells with and without bortezomib treatment. We observed a 2-fold increase in *c-Myc* expression in the treated cells compared to the untreated control (Figure 34D). Our findings therefore suggest that the elevation in proteasomal activity in CRC02 not only partially compensates for the high β-catenin expression, but also makes these cells less responsive to the proteasome inhibitor, bortezomib. Further, inhibition of proteasome using bortezomib

leads to cellular accumulation of β-catenin that in turn leads to transcriptional upregulation of the target gene *c-Myc.*

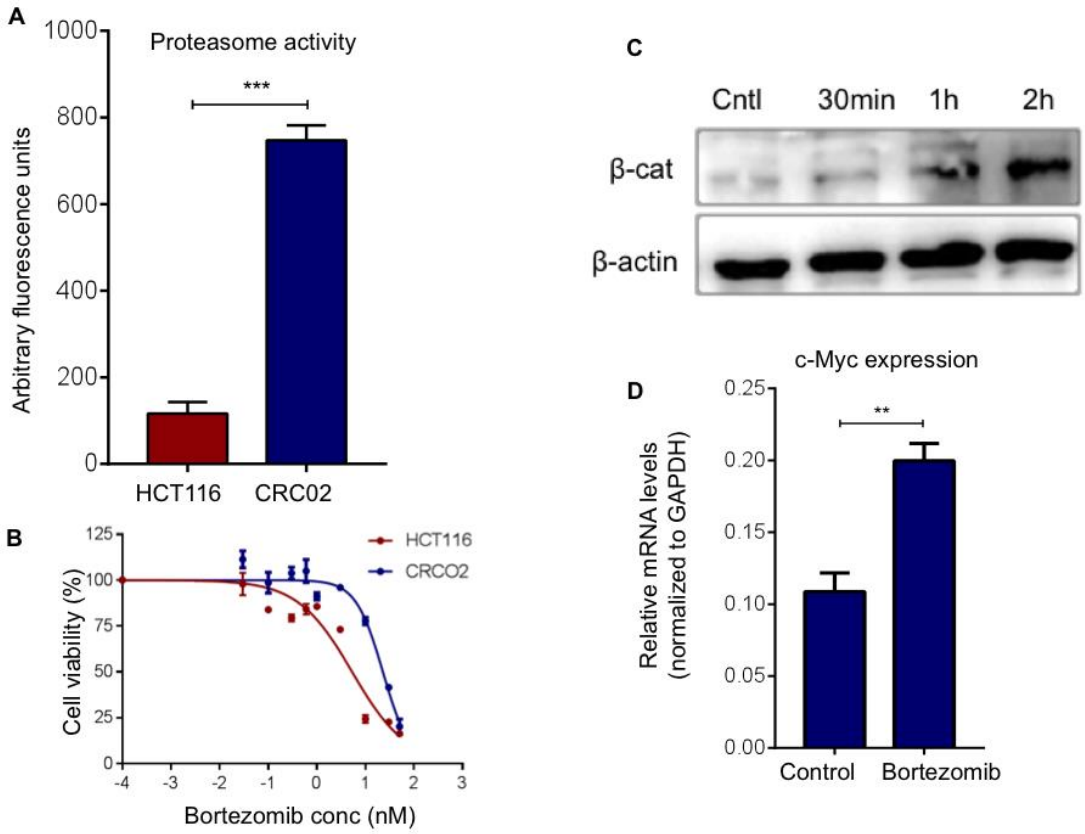

Figure 34: CRC02 has elevated proteasomal activity.
(A): *An ~ 6.5-fold higher endogenous proteasome activity is seen in CRC02 compared to HCT116.* ***(B):*** *CRC02 cells are less sensitive to proteasomal inhibitor bortezomib as compared to HCT116 as seen by the higher* IC_{50} *value.* ***(C):*** *β-catenin accumulation is seen as early as 30 mins upon proteasomal inhibition and increases thereafter upto 2 hours.* ***(D****): An ~2-fold increase in the relative mRNA level of c-Myc transcript is seen in bortezomib treated cells. The error bars represent SEM calculated from three independent experiments.*

5.4. Discussion

Wnt/β-catenin signaling has been closely associated with many malignancies, especially colorectal cancer (Fearon, E. R., 2011, Armaghany T, Wilson JD, *et al.*, 2012). Most of the cellular β-catenin localizes at the cortical region where it interacts with E-cadherin to form the intercellular junctions (Huber AH, Weis WI, 2001). In CRC02 cells, we observed not only a transcriptional upregulation of β-catenin, but also a predominant cytoplasmic localization of β-catenin protein. Concomitantly, there is a downregulation of expression and cortical localization of E-cadherin. Since the membrane bound E-cadherin anchors β-catenin to the cell membrane (Valenta T, Hausmann G, *et al.*, 2012, Weis WI, Nelson WJ., 2006, Sayon Basu, Gal Haase, *et al.* 2016), loss of membrane associated E-cadherin in CRC02 could result in the absence of the catenin-cadherin complex at the plasma membrane, releasing β-catenin into the cytoplasm. This pool of free cytosolic β-catenin would be available to translocate into the nucleus to bring about transcriptional activation of target genes as seen by the elevated expression of *c-Myc* in CRC02.

Cancer associated mutations of the N-terminus serine-threonine cluster abrogate phosphorylation dependent degradation of β-catenin resulting in its cytoplasmic accumulation and subsequent transcriptional activation of target genes (Bilic J, Huang YL, Davidson G, *et al.*, 2007, Gerlach JP, Emmink BL, *et al*. 2014). The presence of conserved, wild type residues at the regulatory site of CRC02-derived β-catenin as well as immunofluorescence analysis using phospho-β-catenin antibody demonstrate that β-catenin gets normally phosphorylated in CRC02 cells. Our data not only show the presence of cytoplasmic β-catenin in CRC02, but also phosphorylated β-catenin localized in the cytoplasm and nucleus in nearly 75% of the cells. This observation is supported by the work of Maher *et. al*., (2010), who showed that N-terminally phosphorylated β-catenin does not associate with cadherins and is mainly cytosolic and that T41/S45 phosphoryated β-catenin is largely nuclear (Maher MT, Mo R, *et. al*., 2010). Wild type S45 along with S33/S37/T41 ensures efficient phosphoryation that localizes phospho-β-catenin to the nucleus, as confirmed by immunofluorescence analysis of CRC02. HCT116 however, contains a

mutation at the S45 site (ΔS45), therefore cannot be phosphorylated at that site and is excluded from localizing to the nucleus (Maher MT, Mo R, *et. al*., 2010).

In addition to being an integral member of the destruction complex, APC is also important in β-catenin export out of the nucleus. Pin1 binding at the phosphorylated serine 247 and proline 248 (pS247P) causes β-catenin isomerization that interferes with the inteaction between APC and β-catenin. Increase in Pin1 has been reported in CRC and a positive relationship has been established netween Pin1 upregulation and β-catenin expression in CRC (Henderson BR, 2000, Ryo A, Nakamura M, *et al*., 2001, Kim CJ, Cho YG, et al., 2005). Our study revealed elevated transcript levels of Pin1 and correspondingly high Pin1 protein in CRC02. Therefore, the possibility of a compromised interaction between APC and β-catenin due to Pin1-mediated isomerization of β-catenin cannot be ruled out and the observed nuclear accumulation of phosphorylated β-catenin in CRC02 could be a result of this interaction.

The ubiquitin-proteasome degradation system is an important cellular machinery that regulates the flux of protein synthesis, degradation and re-synthesis, thus maintaining cellular homeostasis in the cellular milieu. Of the multiple mechanisms evolved by malignant cells to counter the unnatural protein load, change in proteasome activity is of primary importance (Chen L, Madura K, 2005). The intrinsic activity of the proteasome of CRC02 was found to be ~6-fold higher than HCT116. We used the clinically approved proteasome inhibitor bortezomib to inhibit the proteasome machinery in CRC02. This led to the accumulation of phosphorylated β-catenin over time. The transcriptional upregulation of *c-myc* in bortezomib treated cells indicate that the increase β-catenin level is perhaps leading to the upregulation of *c-Myc*. Whether the elevation in proteasomal activity evolved as a result of increased β-catenin expression or whether β-catenin expression increased in response to the elevated proteasomal activity is thus far unclear from the above results. Overexpression of β-catenin results in disorganized centrosomes and loss of cortical anchoring of microtubules. Conversely, depletion of β-catenin leads to to failure of microtubule nucleation, decrease in centrosomal cohesion and induction of extra centrosomal structures. These present compelling evidence towards the role of β-catenin in regulating centrosomal function (Ligon LA, Karki S, *et al.*, 2001, Huang P,

Senga T, *et al*, 2007, Bahmanyar S, Guiney EL, *et al.*, 2010, Bahmanyar S, Kaplan DD, *et al.*, 2008). Our data shows dramatic upregulation of β-catenin, presence of β-catenin at the multiple centrosomes. In addition, there is significant elevation of proteasomal activity in CRC02 that may partially neutralize the increased expression of β-catenin. However, the increased proteasomal activity appears to be insufficient to counter the dramatic increase in β-catenin expression that eventually may lead to the accumulation of β-catenin in the cytoplasm. Further, the possibility of NIMA-related protein kinase 2 (Nek2) mediated phosphorylation that excludes phospho-β-catenin from proteasomal degradation (Mbom BC, Siemers KA, 2014) could explain the inefficient degradation of β-catenin that result in the observed cytoplasmic accumulation in CRC02. Moreover, the absence of cortical E-cadherin in CRC02 leading to the loss of cortical sequestration of β-catenin could also contribute to the localized increase in concentration of the protein in the cytoplasm, which would drive the loss of centrosomal cohesion resulting in the observed supernumerary centrosomes (Bahmanyar S, Kaplan DD, *et al.*, 2008, Bahmanyar S, Guiney EL, *et al.*, 2010). Similarly, the increased local concentration of β-catenin could lead to its prominent localization at the inter-cellular bridge during cytokinesis in CRC02. β-catenin localization at the intercellular bridge at late telophase has been shown earlier (Kaplan DD, Meigs TE, *et. al.*, 2004). However, the functional significance of this localization or the role or has in cytokinesis has not yet been documented.

Our study revealed that the Wnt signaling pathway is misregulated in the form of transcriptional upregulation of β-catenin in CRC02. This cell line also has elevated activity of the proteasomal degradation machinery, which possibly helps to counter the increased transcriptional expression of β-catenin. The expression levels Pin1, of which β-catenin is a substrate, is also dramatically increased in CRC02. The loss of E-cadherin mediated adherens junctions led to β-catenin to be released into the cytosol and in spite of the high proteasomal activity, the cytoplasmically accumulated excess β-catenin led to a host of mitotic defects both at early and late stages of cell division. In addition, the excess β-catenin protein also accumulated in the nucleus and led to increased expression of the *c-myc* oncogene, resulting in uncontrolled cell proliferation.

5.5. References

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CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

CHAPTER 6

Conclusions and future perspectives

Decades of basic and clinical research have generated a wealth of information that today define cancer as a group of diseases that govern the transition of a normal cell into a malignant one. The lesions in the cancer genome may be as subtle as a point mutation that may either activate an oncogene or inactivate a tumor suppressor or may be more drastic such as a loss of an arm of a chromosome affecting multiple genes. In either case, the overall result manifests in the form of deregulated molecular machineries that control cellular proliferation, differentiation and cell death, generating a tumor through a multistep process that reflect the underlying discrete genetic alterations. The set of characters acquired during the malignant transformation that sets apart a cancer cell from its normal counterpart are described as the hallmarks of cancer. These include self-sufficiency in growth signals, insensitivity to growth suppressors, resistance to cell death, limitless replicative potential, induction of angiogenesis and the ability to invade distant organs. In addition, certain enabling conditions exist that promote the transition from normal to cancerous form such as the ability to adapt to low oxygen conditions, evading the host immune system and inducing inflammation.

Our current understanding of cancer as a disease is based on studies performed on patient derived primary tumor samples and cell lines. Not only have these been invaluable resources in understanding the underlying mechanism of pathogenesis and progression of cancer but have also proven to be a useful tool for designing and screening potential drug candidates. However, most of these studies have been performed using patient samples and cell lines originating in the Caucasian population. There is a dearth of cell lines derived from Indian patients that may be used to understand the genetic basis of cancer prevalent in the Indian population. Our goal for this study has been to generate a cell line derived from an Indian CRC patient and perform extensive molecular characterization of the same to highlight the

differences between the widely used and well characterized established CRC cell lines.

Preliminary characterization of the tumor derived primary cell line CRC02 showed characteristic morphology of cancer cells of epithelial origin with cells that are polygonal and adherent. Common CRC related gene mutations were absent in both the tumor as well as in CRC02. Cytogenetic analyses of CRC02 showed a gross overall genomic instability that is different from the well-established CRC cell lines. We conclude that this may have been the result of defects in the early as well as late stages of mitosis and delay in cytokinesis, leading to higher than normal percentage of cells becoming multinucleated, a signature for aneuploidy. In-depth molecular analysis of mRNA expression demonstrated that multiple CRC related signaling pathways are mis-regulated in CRC02, including Wnt/β-catenin, TGFβ and Notch. Although these cells were derived from a localized primary site of lesion, they had already aquired the characteristic signatures of having undergone EMT that empowers the aberrant cells with the potential to metastasize to distant organs. The enhanced ability of migration and invasion validated the elevated mRNA expression of the molecular markers for migratory potential and invasiveness. Moreover, CRC02 showed diminished response to standard therapeutic agents. This finding correlates with earlier reports that describe the mechanism of EMT induced drug resistance. Therefore, not only there is an overall differential regulation of key signaling pathways in CRC02, but also these cells are highly migratory and invasive in nature. Coupled with this, the attenuated response to therapeutics would make CRC02 a highly aggressive cancer. The ability to form tumors when injected into mice is critical if a cell line were to be developed as a tumor model, either for mechanistic studies or for screening drug candidates. CRC02 formed colonies in colony forming assay *in vitro* and also when injected into SCID mice *in vivo*, thus establishing a xenograft model. This has the potential to be developed further as a tool for drug screening.

In addition to playing a critical role during embryonic development, Wnt/β-catenin signaling is one of the most commonly mis-regulated pathways during CRC carcinogenesis. Our detailed molecular analysis of CRC02 cells demonstrated

transcriptional upregulation of β-catenin. Furthermore, this protein fails to associate with the cell-cell adherens junctions due to the loss of membrane associated E-cadherin expression following EMT driven cadherin switch in CRC02. Thus, free β-catenin accumulation in the cytoplasm and nucleus of CRC02 is party responsible for elevated expression of the downstream pro-proliferation gene *c-Myc*. Since, β-catenin lacks a nuclear export signal, its transport out of the nucleus is brought about by its binding with APC. This is regulated via Pin1 mediated phosphorylation dependent isomerization of β-catenin. Therefore, retention of β-catenin in the nucleus is affected by both a truncation mutation in APC as well as elevated levels of Pin1 in CRC02. β-catenin turnover is via proteasomal degradation – our data showed that CRC02 has elevated intrinsic proteasomal activity that may have evolved as a countermeasure to the increase in β-catenin expression.

There is a huge unmet need for developing more tumor derived primary cell lines from cancers that have originated in India with the aim to delineate the mechanisms of pathogenesis of cancer that could be different from the Caucasian population owing to the intrinsic genetic diversity among the Indian population. This study has brought forth the inherent molecular variations between cancer cell lines originating in different ethnic groups, pointing to the fact that underlying mechanistic differences exist in the way cancer developed in the two populations. Such differences are often the cause for suboptimal respose to standard therapy in certain patient groups. This is exemplified by the fact that the absence of the common CRC mutations in *KRAS* and *BRAF* genes (as seen in CRC02 cells), exclude the possibility of cetuximab containing therapeutic interventions being effective. Therefore, such mutational profiles may prove to be useful in selecting subsets of patients that are likely to respond well to a particular treatment regimen. This in turn would greatly impact the clinical management of CRC.

Similar studies on patient derived samples from India would not only help identify population specific novel molecular signatures that can potentially be developed further as diagnostic markers. Additionally, these markers could also serve as indicators of disease prognosis and predictors of response to therapy. This would in

turn open up the possibility of designing specific drugs that are highly effective in Indian patients and could alleviate the ever-increasing disease burden in India.

PUBLICATIONS

LIST OF PUBLICATIONS

1. **Mylavarapu S**, Kumar H, Kumari S, Sravanthi LS, Jain M, Basu A, Biswas M, Mylavarapu SVS, Das A, Roy M., Activation of Epithelial-Mesenchymal Transition and Altered β-Catenin Signaling in a Novel Indian Colorectal Carcinoma Cell Line, Front Oncol. 2019 Feb 15;9:54.

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Activation of Epithelial-Mesenchymal Transition and Altered β-Catenin Signaling in a Novel Indian Colorectal Carcinoma Cell Line

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Colorectal cancer is the third major cause of cancer-related mortality worldwide. The upward trend in incidence and mortality rates, poor sensitivity to conventional therapies and a dearth of early diagnostic parameters pose a huge challenge in the management of colorectal cancer in India. Due to the high level of genetic diversity present in the Indian population, unraveling the genetic contributions toward pathogenesis is key for understanding the etiology of colorectal cancer and in reversing this trend. We have established a novel cell line, MBC02, from an Indian colorectal cancer patient and have carried out extensive molecular characterization to unravel the pathological alterations in this cell line. In-depth molecular analysis of MBC02 revealed suppression of E-cadherin expression, concomitant with overexpression of EMT related molecules, which manifested in the form of highly migratory and invasive cells. Loss of membrane-tethered E-cadherin released β-catenin from the adherens junction resulting in its cytoplasmic and nuclear accumulation and consequently, upregulation of *c-Myc*. MBC02 also showed dramatic transcriptional upregulation of β-catenin. Remarkably, we observed significantly elevated proteasome activity that perhaps co-evolved to compensate for the unnaturally high mRNA level of β-catenin to regulate the increased protein load. In addition, there was substantial misregulation of other clinically relevant signaling pathways that have clinical relevance in the pathogenesis of colorectal cancer. Our findings pave the way toward understanding the molecular differences that could define pathogenesis in cancers originating in the Indian population.

Keywords: colorectal cancer, signaling, EMT, β-catenin, mitotic defects

INTRODUCTION

Colorectal cancer (CRC) ranks as the third most commonly diagnosed disease and a leading cause of cancer-related mortality worldwide. It is further complicated by the fact that about 50% of CRC patients develop liver metastases during their lifetime (1). Although there has been a significant advancement in the development of treatment regimens, there is no effective therapy in the clinic for advanced CRC presenting with metastasis.

Recently, shifting trends have emerged in the global incidence of CRC whereby rapidly developing countries present an increase in both CRC related incidence and mortality. In the developed nations however, there is an upward trend in incidence whereas mortality rates are either stabilizing or decreasing over time (1). CRC in India differs from the trends described in the developed nations mainly due to limited healthcare access coupled with poor socioeconomic backgrounds. In the Indian subcontinent, the annual incidence of colon and rectal cancers are 4.4 and 4.1 per 100,000 respectively (2). Additionally, there are reports of younger patients being usually diagnosed at an advanced stage (3). In recent years, understanding the underlying mechanisms that govern pathogenesis and progression of CRC have been the focus of research. However, majority of studies have been conducted on tumors and cell lines derived from Caucasian patients residing in developed countries. Consequently, most therapeutics used in the clinical management of CRC are based on data generated from the Caucasian population that often prove suboptimal for Indian patients. Since Indian population is genetically diverse (4), defining the contribution of genetic factors that lead to disease progression becomes even more critical for the management of Indian CRC patients.

Molecular alterations are the key contributors toward pathogenesis and progression of CRC, characterized by clearly defined stages starting from early adenoma, intermediate adenoma, late adenoma and carcinoma, leading into the final stage of cancer metastasis (5). Although germline mutations play a predominant role in many CRC patients, the vast majority of CRC cases are sporadic with no prior family history (6). Sporadic pre-cancerous polyps or lesions accumulate further genetic and epigenetic aberrations over time resulting in uncontrolled cellular growth, subsequently enabling the cells to acquire invasive and metastatic properties (7). An insight into the molecular events driving these cellular transitions, particularly toward enhanced invasiveness and metastasis, is crucial for the development of novel treatment regimens to combat CRC. One such critical event is the epithelial to mesenchymal transition (EMT), a series of programmed molecular and biochemical changes that result in cells losing their epithelial features while gaining mesenchymal features, such as loss of cell-cell contact, attaining an elongated spindle shaped morphology and increased motility. Complex networks of signaling pathways orchestrate this phenomenon. The inducers of EMT can downregulate E-cadherin while enhancing the N-cadherin and vimentin levels through modulating EMT-related signaling pathways, including Wnt/β-catenin, TGF-β, and EMT transcription factors, namely, zinc finger E-box binding homeobox (Zeb1/2) and Snail (8). This results in pathological changes in the tissue of origin, marked by uncontrolled cellular proliferation that eventually enables them to form secondary metastatic tumors (9). Activation of the EMT program is contextual whereby the incoming signals from the tumor microenvironment drive the molecular changes that ultimately set in motion the transition of cells from epithelial to mesenchymal states (10, 11). Among the myriad signaling molecules and pathways that are involved in the EMT program, crosstalk between these signaling pathways contributes toward pathogenesis and progression of CRC (12–14).

One of the key pathways involved in the EMT transition is the evolutionarily conserved Wnt/β-catenin signaling pathway that is involved in tissue morphogenesis during embryonic development (15, 16). β-catenin, the central effector in this pathway, participates in numerous cellular processes that are spatio-temporally separated. At cell-cell adherens junctions, β-catenin binds directly to the membrane anchored E-cadherin to form a catenin-cadherin complex—a critical mechanism that regulates localized cytoskeleton modulation (16–18). This interaction is thought to be a protective mechanism against proteasomal degradation, stabilizing β-catenin at the adherens junction (19, 20). Loss of E-cadherin from the membrane releases free β-catenin into the cytoplasm that eventually translocates into the nucleus and acts a transcriptional co-activator of pro-growth genes. Suppression of E-cadherin expression has been implicated in progression of cancer and is associated with poor prognosis and poor survival in many malignancies, including CRC (21). In the absence of an incoming Wnt signal, phosphorylation of a cluster of serine, and threonine residues at the N-terminus of β-catenin primes it for ubiquitination followed by proteasomal degradation, thus maintaining low levels of β-catenin in the cell. Mutations in these residues prevent phosphorylation resulting in stabilization and accumulation of β-catenin and subsequent increase in β-catenin mediated transcriptional activity (22–25). This is a tightly regulated step during normal cellular function—mis-regulation due to pathological transformation may lead to over-activation of pro-growth genes leading to abnormal cellular proliferation, a hallmark of cancer (26, 27).

In this article, we describe a novel CRC cell line, MBC02, derived from a patient of Indian origin that exhibited features of having undergone at least partial EMT. Significant overexpression of Wnt-β-catenin, TGFβ, and Notch pathways indicated an overall misregulation of clinically relevant signaling pathways that are also implicated in EMT. Transcriptional suppression of E-cadherin along with increased expression of N-cadherin, vimentin and EMT-related transcription factors, Twist and Zeb were corroborated by phenotypic changes such as enhanced migration and invasiveness and reduced response to standard-of-care therapeutics. MBC02 showed transcriptional upregulation along with cytoplasmic and nuclear accumulation of β-catenin, indicating activation of the Wnt/β-catenin signaling pathway in this cell line. In addition, increased nuclear accumulation of β-catenin could be linked to elevated levels of Pin1 expression in MBC02. These observations were further correlated with phenotypes that are typical of β-catenin driven mitotic defects such as supernumerary centrosomes at interphase and multipolar spindles at metaphase, resulting in cytokinetic failure and an enrichment of multinucleate cells that are aneuploid.

MATERIALS AND METHODS

Immunohistochemical Analysis of Primary Tumor Sections

Immunohistochemical staining was performed on sections prepared from paraffin-embedded tissue blocks. Sections of

~3 μm thickness were collected on positively charged slides (TOMO IHC adhesive glass slides, TOM-11). The slides containing the tissue sections were heated at 60°C for 1 h, followed by deparaffinization and rehydration by passing through gradient of ethanol solutions and finally placed in deionized water. Heat induced antigen retrieval was performed using citrate based antigen unmasking solution (Vector labs, Cat. No. H3300). Endogenous peroxidase was blocked by incubating the tissue sections in 6 ml of 30% hydrogen peroxidase solution for 20 min. Ten percent normal goat serum (Vector lab Cat. No. S-1000) was used for blocking non-specific proteins prior to incubation with primary antibody for 1 h, followed by washing with 1X PBS. The tissue sections were incubated with primary antibodies for 1 h, washed with 1X PBS and then incubated with HRP labeled secondary antibodies. The sections were again washed with 1X PBS. Freshly prepared chromogenic reagent (anti-rabbit HRP, Cell Signaling, Cat. No. 8114s or anti-mouse HRP, Cell Signaling, Cat. No. 8125s) was added to the sections for developing the color to aid visualization of staining. Dako Envision Kit (Cat. No. K5007) was used for Ki-67. All slides were counter-stained with hematoxylin (Merck, Cat. No. 6092530121730), dehydrated by passing through gradient of ethanol solutions, placed in xylene and finally mounted and sealed. Representative images were captured in 200X magnification using Leica's Aperio ImageScope software (V12.3.3.5048).

Establishment of MBC02

Surgically removed tumor tissue was obtained from the primary tumor site of a 37-year-old Indian female CRC patient with disease staging at $T_2N_1M_0$, after obtaining informed consent under Institutional Review Board (IRB) approved protocol of MITRARxDx India Pvt. Ltd., India (IRB# TS-04-2011). The tumor tissue was washed extensively with 1X PBS, followed by incubation with 1X penicillin/ streptomycin for 10 min at room temperature. The tumor tissue was then sectioned to obtain 0.5–2.0 mm^3 size sections. These were then digested with 0.5X collagenase in DMEM containing 1X penicillin/streptomycin at 37°C for 2–4 h. The disaggregated tumors were passed through a cell strainer and centrifuged to obtain a cell pellet. The cells were then counted by trypan blue exclusion method and seeded in flasks. Unattached cells and tumor debris were removed by changing the media, followed by differential trypsinization to remove fibroblasts. Further inhibition of fibroblast was achieved by growing the cultures in low serum and calcium containing media. The cultures were treated with 0.01% EDTA for 3 min and replaced with media containing 5% FBS for 24 h. Detached fibroblasts were removed by replacing with fresh media. This procedure was repeated for three passages to enrich the cultures for tumor cells. MBC02 cell line was established after sequential passaging and subsequent growth in phenol red free Dulbecco's Modified Eagle Medium (DMEM) containing 5.5 mM glucose supplemented with 10% FBS and 1X gentamicin. All assays reported in this article were carried out using cells between passages 6 to 20.

Cell Culture

MBC02 cells were grown and maintained in phenol red free DMEM—low glucose (HiMedia, Cat. No. AL183A) medium, containing 5.5 mM glucose, 10% fetal bovine serum (FBS) and supplemented with 1X gentamicin. HCT116, HT29, and SW620 cells were obtained from the American Type Culture Collection (ATCC), grown and maintained in DMEM—high glucose (HiMedia, Cat. No. AL007A) containing 25 mM glucose, supplemented with 10% FBS and 1X penicillin and streptomycin. All cell lines were grown at 37°C, 5% CO_2 atmosphere with relative humidity of 95%. Growth characteristics of MBC02 cells were measured by seeding cells at an initial concentration of 15,000 per well of a 12 well dish and allowed to grow. Cells were trypsinised from a single well and counted at designated time intervals. The population doubling time was calculated using the following formula: DT=T Log2/Log (X_e/X_b), where, T is the time period, X_e is the number of cells at the end of the incubation time and X_b is the number of cells at the beginning of the incubation time.

Karyotype Analysis

MBC02 cells from passage number 8 and 16 were cultured in 5 ml of recommended media supplemented with 10% FBS and incubated in a humidified CO_2 incubator at 37°C. Demecolcine solution (Sigma Aldrich, Cat. No. 7385) was added at a final concentration of 100 ng/ml and the cells were incubated for various time intervals (overnight, 24 and 48 h). Cells were harvested and centrifuged at 1,200–1,500 rpm for 10 min at room temperature. The cell pellets were resuspended in 8–10 ml of warm hypotonic solution (0.075 M KCl) and incubated at 37°C for 20 min, followed by addition of 500 μl of pre-cooled fixative (3:1 solution of methanol and acetic acid). The cells were gently mixed for uniform fixation. The cells were centrifuged and resuspended in fresh fixative. This process was repeated twice. The resuspended cells were placed in −20°C overnight. Next day, the fixed cells were dropped onto freshly washed glass slides and allowed to air dry. The dried slides were baked at 65°C for 8–12 h. The slides were then sequentially rinsed with trypsin solution (6.25 mg of trypsin in 50 ml Gurr buffer), followed by normal saline and deionized water. The washed slides were stained using Giemsa solution (2.0 ml of Giemsa stain added to 48 ml of Gurr buffer) for 5–6 min and washed with deionized water. Analyses of the stained slides were carried out using Cytovision software (Leica Biosystems).

Mutational Analysis

Genomic DNA was extracted from tumor tissues and MBC02 cell line using a QIAamp DNA Micro Kit (Qiagen) and subjected to PCR using region-specific primers to detect the mutational status of *KRAS* (codon numbers 12, 13, 61, and 146) and *BRAF* (codon 600). DNA fragment containing *KRAS* mutation hotspots were amplified with the intron-based primers (28). Reaction mix contained 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer set, and 0.5 units of PhusionTaq (ThermoFisher Scientific) in a total volume of 50 μl. SW480 bearing mutation in *KRAS* and Caco2 harboring wild type *KRAS* were used as controls for PCR and sequencing reactions. PCR was carried out at 95 °C for 5 min,

followed by 25 cycles at 95 °C for 30 s; 60 °C for 30 s and 72 °C for 30 s with a final extension for 5 min. PCR products were resolved on 1.5% agarose gel. The amplicons were excised and purified using a QIAquick gel extraction kit according to manufacturer's protocol (Qiagen) and processed for Sanger sequencing.

Anchorage Independent Growth Assay

Tumorigenic potential of MBC02 cells was asessed using the anchorage independent growth assay. The base layer of agar (0.5%) was prepared by mixing 9 ml of complete media to 1 ml of 5% agar. The temperature of the solution was maintained at 50°C to prevent premature solidification of the agar. 1 ml of the agar mix was added to each well of a 6 well plate and allowed to solidify completely. The cells were washed with 1X PBS and harvested by trypsinization. The cells were centrifuged and resuspended in 1X PBS and counted. The cell number was adjusted to 5×10^3cells/ml in complete media. The top agar layer (0.3%) was prepared by adding 0.6 ml of 5% agar to 9.4 ml of complete media containing cells. 1 ml of the top agar was layered over the base agar and allowed to solidify completely. 800 µl of complete media was layered on top to prevent drying of the agar. The plates were incubated at 37°C, 5% CO_2 atmosphere with relative humidity of 95% for 2 weeks. Colonies were imaged using Nikon TiE inverted microscope.

Cell Cycle Analysis

The culture media was removed and cells were washed with 1X PBS. Cells were harvested by trypsinization and collected by centrifugation at 2,000 rpm for 5 min. The cell pellets were washed twice with PBS and centrifuged at 2,000 rpm. The cells were resuspended in 1 ml PBS to obtain single cell suspension and fixed in ice cold 70% ethanol for at least 4 h at 4°C. After fixation, the ethanol was removed by centrifugation and the cells were washed twice with 1X PBS. Staining solution was prepared by adding propidium iodide at a final concentration of 50 µg/ml and RNAse A at a final concentration of 50 µg/ml. The samples were incubated at 37°C for 20 min and data acquired by flow cytometry (BD FACS Verse). Three biological replicates were performed to obtain statistically significant data.

Cell Migration and Invasion Assay

For would healing assay, MBC02 and HCT116 cells were seeded in 6 well plates and allowed to grow to confluency. After generating a wound in the monolayer, the media was removed and the cells were washed to remove detached cells. The cells were fed with fresh media and the wound was allowed to close. The gap between the invasion fronts was measured at regular interval to calculate the rate of wound closure. We used the transwell migration assay to evaluate the migratory and invasive potential of MBC02 in comparison to HCT116, HT29, and SW620. Boyden chambers with 8 µ pores (BD Falcon, Cat. No. 353097) were placed in 24-well cell culture plates. Cells were trypsinized, washed once in DMEM and counted using a hemocytometer. 1×10^4 cells were suspended in 200 µl of serum free media and added to the upper compartment of the Boyden chamber in each well of a 24 well plate. The lower compartment contained 400 µl of complete media with 10% FBS. After incubation for 24 h at 37°C, assays were terminated by scraping the top of the membrane to remove non-migratory cells. The membranes were fixed in 4% paraformaldehyde, stained with crystal violet and mounted on glass slides. Quantification of cells was carried out by counting at least three microscopic fields using a 10X objective. Matrigel coated Boyden chambers (100 µg/ml) were used for the invasion assay. 5×10^4 cells were suspended in 200 µl serum free media and seeded in each well. The lower chamber contained 400 µl of complete media with 10% FBS. After 24 h at 37°C, assays were terminated and cells were quantified as described above for the migration assay. The experiments were repeated at least thrice for obtaining data for statistical significance.

Gene Expression Analysis by qRT-PCR

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Cat. No. 74104). 1 µg of total RNA was used for cDNA synthesis using iScript cDNA Synthesis Kit (BioRad, Cat. No. 170-8891). Real time quantitative PCR was performed using SYBR Green reagent (SsoFast Eva Green supermix, BioRad, Cat. No. 1725202AP) in a CFX Connect Real Time PCR system (BioRad). Cycling conditions were optimized at initial denaturation for 5 min at 95°C followed by denaturation for 5 s at 95°C and annealing, extension for 45 s at 60°C for 40 cycles. GAPDH was used as the internal control and all CT values were normalized to either internal control or HCT116. Sequences of the primers are listed in **Table S1**. Three biological replicates were performed for each experiment.

Antibodies

Primary antibodies against Ki67 was procured from Dako (Cat. No. IR626, Clone MIB1) and caspase 3C from Cell Signaling Technology (Cat. No. 9661). Primary antibodies against β-catenin (Cat. No. 8480), phospho-β-catenin (Cat. No. 9561) were procured from Cell Signaling Technology; E-cadherin (Cat. No. 33-4000), APC (Cat. No. MA1-26185) from Thermo Scientific; α-tubulin (DM1A, Cat. No. T9026) and β-actin (Cat. No. A3853) from Sigma-Aldrich; γ-tubulin (Cat. No. A302-631A) from Bethyl Labs; Pin1 (Cat. No. SC-46660) from Santa Cruz Biotechnology. Anti-mouse and anti-rabbit Alexa-488 conjugated (Cat. No. A11008, A11001) and anti-mouse, anti-rabbit Cy3 conjugated (Cat. No. A10521, A10520) secondary antibodies for immunofluorescence were purchased from Molecular Probes, Invitrogen. Horse Radish Peroxidase (HRP) conjugated anti-mouse (Cat. No. 715-035-150) and anti-rabbit (Cat. No. 711-035-152) secondary antibodies from Jackson Immunoresearch were used for Western Blot analysis.

Immunofluorescence Staining

Cells were seeded on glass coverslips and allowed to adhere overnight. The media was removed and the cells washed with PBS and fixed in 3.7% paraformaldehyde at room temperature for 5 min. Permeabilization was achieved using PBS containing 1% BSA + 0.5% Triton X-100 for 1–2 min at room temperature. Blocking was performed using 1% BSA + 0.05% Triton X-100 in PBS for 30 min followed by incubation with primary antibody for 1 h. Primary antibodies were used at the following dilutions: α-tubulin – 1:1,000, γ-tubulin – 1:500, β-catenin –

1:200, phospho-β-catenin – 1:200, E-cadherin – 1:200. After washing with PBS, cells were incubated in secondary antibody for 1 h. The cells were washed with PBS and stained with Hoechst 33258 at a dilution of 1:5,000 from a 5 mg/ml stock solution. The cells were again washed with PBS and mounted in Prolong Gold mounting medium (Invitrogen). The slides were allowed to dry overnight before imaging using a Nikon TiE Eclipse epifluorescence microscope.

Western Blotting

Cells were lysed in 1X Radioimmunoprecipitation Assay (RIPA) buffer containing protease inhibitor (Roche, Cat. No. 05892970001) and phosphatase inhibitor (sodium orthovanadate, New England Biolabs, Cat. No. P0758S) at appropriate concentrations. Laemmli buffer containing β-mercaptoethanol was added and the samples were heated at 95°C for 10 min. Samples were loaded on a 10 or 12% denaturing gel and electrophoresis was performed before transferring the resolved proteins onto PVDF membrane (Millipore). Blocking was done in TBST containing either 5% skimmed milk or 5% BSA followed by incubation in primary antibody overnight at 4°C. Membranes were washed with tris-buffered saline containing 0.1% tween-20 (TBST) and incubated with HRP-conjugated secondary antibody for 2 h at room temperature. Blots were washed extensively with TBST and chromogenic substrate (Luminata Forte, Millipore) was added to develop the chemiluminescent signal. Images were captured in an Image Quant 4000 (GE) gel documentation system. Primary antibodies were used at the following dilutions: β-catenin – 1:1,000, Pin1 – 1:1,000, β-Actin – 1:2,000.

Drug Sensitivity Assay

Cytotoxicity of bortezomib (ChemShuttle, Cat. No. 179324-69-7) on MBC02 and HCT116 cells was measured using MTT assay. Cells were seeded in quadruplicate in the recommended media in 96 well plates at a density of 3,000 cells per well and allowed to attach overnight at 37°C, 5% CO_2 and 95% humidity. The cells were incubated for 48 h with different concentrations of bortezomib ranging from 0.03 to 50 nM. Untreated cells were used as control. Following incubation, 20 μl of 5 mg/ml MTT (HiMedia, Cat. No. RM1131-1G) solution was added to the cells and incubated for another 3 h. The media containing MTT was completely removed and the formazan precipitate was dissolved using 100 μl of 1:1 solution of DMSO: methanol. Absorbance was measured at 550 nm with background correction at 655 nm using a microplate reader (iMark, BioRad). The results were analyzed using GraphPad PRISM software (GraphPad, San Diego, CA). The IC_{50} values were calculated and result plotted as the mean ± SEM of the absorbance for each tested dose from three independent experiments. MBC02 cells were also treated with a combination of drugs that are the standard-of-care regimen for treatment of CRC. The therapeutic regimens included 5-fluorouracil (5-FU) + leucovorin (16.78 μg/ml + 391 ng/ml respectively), FOLFOX (oxaliplatin 2.58 μg/ml + 5-FU 16.78 μg/ml + leucovorin 391 ng/ml), FOLFIRI (irinotecan 4 μg/ml + 5-FU 16.78 μg/ml + leucovorin 391 ng/ml), and cetuximab (184 μg/ml) (29). DMSO was used as control for the drug sensitivity test. Experiments were performed in triplicate for statistical significance and results plotted as percent viability for each treatment group.

Proteasome Activity Measurements

Proteasome 20S activity kit (Sigma-Aldrich, Cat. No. MAK172) was used to measure the proteasome activity of MBC02 and HCT116 cells. 80,000 cells were seeded in each well of a 24 well plate and allowed to adhere overnight. Proteasome substrate stock and proteasome assay loading solution were prepared following the manufacturer's protocol. Media was removed and replaced with a 1:1 ratio of complete media and proteasome assay loading solution and incubated at 37°C, 5% CO_2 and 95% humidity for 4 h in the dark. Following incubation, the fluorescence intensity was measured at an excitation wavelength (λ_{ex}) of 490 nm and an emission wavelength (λ_{em}) of 525 nm. For the proteasome inhibition assay, cells were grown to ∼85–90% confluency in 10 cm dishes. Bortezomib was used at a concentration of 200 nM to treat cells for different time points ranging from 30 min to 2 h. Untreated cells were used as control in the proteasome inhibition assay.

Statistical Analysis

All quantitative data are represented as mean ± SEM, calculated from at least three independent biological replicates. Statistical significance was calculated using the Student's *t*-test. $P < 0.05$ was considered statistically significant. GraphPad PRISM (San Diego, CA) software was used for creating all graphs.

RESULTS

Primary Tumor Histology

Clinical diagnosis of the primary tumor revealed stage IIIA disease ($T_2N_1M_0$), indicating that the cancer may have progressed into the nearby submucosal tissue and into atleast 3 lymph nodes, although it had not metastasized to distant sites. Histopathological evaluation of hematoxylene-eosin (H&E) stained sections of the primary tumor demonstrated moderate differentiation with cancer cells forming irregular glandular structures. Cancer cells were marked by the dark purple staining of the nuclei (**Figure 1A**). Strong Ki67 staining in regions of the tumor showed the presence of actively dividing cancer cells (**Figure 1B**). Upon investigation we also observed an abundant β-catenin expression in the primary tumor (**Figure 1C**).

Preliminary Characterization and Cytogenetic Analysis of MBC02

We have developed a novel cell line, MBC02, from the primary tumor site of a 37-year-old female Indian CRC patient. As controls established colorectal cancer cell lines–HCT116, HT29, and SW620 were used. HCT116 and HT29 were obtained from primary carcinomas of an adult Caucasian male and female patient respectively, whereas, SW620 was derived from a metastatic tumor of a male Caucasian patient[1].

[1]https://www.atcc.org

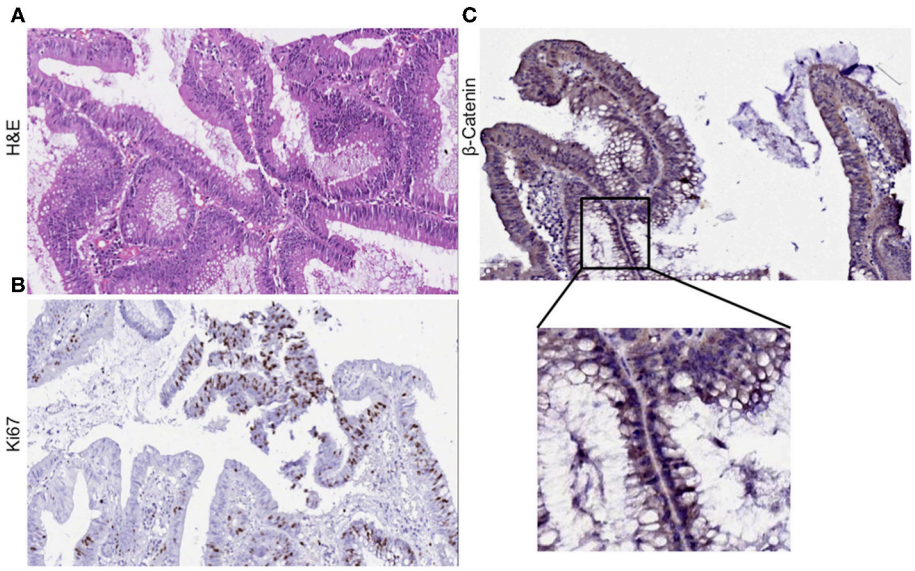

FIGURE 1 | Histological evaluation of primary tumor. **(A)** H&E staining of the primary tumor show moderate differentiation. Cancer cells form irregular glandular structures. **(B)** Ki67 positive staining mark the regions of actively dividing cancer cells within the tumor. **(C)** Primary tumor section was found to express β-catenin abundantly.

Microscopic examination of passage 10 and 17 under low and high magnification (10X and 40X) revealed that MBC02 cells grew as a monolayer that was strongly adherent to the substratum with a flattened morphology that is characteristic of epithelial cells (**Figures 2A,B**). Immunofluorescence staining using anti-tubulin antibody further highlighted the epithelial morphology of these cells (**Figures 2C,D**). Since MBC02 cell line was not derived by clonal selection, the presence of a heterogenous population is apparent with some cells being larger. The average population doubling time for MBC02 was calculated to be approximately 24 h (**Figure 3A**). In comparison, average population doubling time for HCT116, HT29, and SW620 were calculated to be 20, 25, and 28 h, respectively (**Figure S1**).

Karyotype analysis of MBC02 cells revealed chromosomal instability in almost all cells. These cells were near-tetraploid with a modal chromosome count of 89. Significant numerical and gross structural abnormalities were observed in chromosomes, some of which were present in multiple copies. A few of the structural rearrangements observed were centric fission detected in one or more copies of chromosome 1, deletion of the short arm of chromosome 1, 2, 4, 5, 11, and 12 and deletion of the long arm of chromosome 2, 9, and 19. In addition, derivative chromosomes as a result of addition of material of unknown origin, probably via translocation, were observed in one or more copies of the long arm of chromosomes 18, 19, 20, 21, and 22 as well as in the short arm of chromosome 19 (**Figure 3B**). Of note, HCT116 cells are reported to be near-diploid with modal chromosome number of 45 whereas HT29 and SW620 cells are hypertriploid (modal chromosome number of 71) and hyperdiploid (modal chromosomal number of 50), respectively[1]. Cytogenetic analysis of two different passages of MBC02 cells (passage 8 and 16) yielded identical results indicating that the integrity of the cell line is preserved between early and late passages. The abnormal karyotype could have resulted from gross defects in mitosis, both at early and late stages of cell division as well as during cytokinesis. This may lead to the presence of multinucleate cells. Indeed, we observed a ∼6-fold increase in the number of cells with multipolar metaphase spindle in MBC02 as compared to HCT116 (**Figure S2**). We also observed a ∼12-fold enhancement in the number of multinucleated cells in asynchronous cultures of MBC02 in comparison to HCT116. In addition, about 15% cells were present in cytokinesis in MBC02 whereas only about 6% of cells were observed to be undergoing cytokinesis in HCT116 (**Figure S3**). This 2.5-fold increase in the cytokinetic index in MBC02 could be the result of the presence of centrosomal and spindle defects in these cells (**Figure S2**). Based on the above results, it is apparent that mitotic defects at both early and late stages of cell division may have contributed toward MBC02 cells to have become aneuploid.

DNA content analysis of asynchronous culture of MBC02 cells was performed by measuring the fluorescence intensity of propidium iodide incorporated during the various stages of the cell cycle. We observed that ∼46% of cells were at G1 phase representing diploid DNA content (2n). ∼24% cells were at S phase (>2n) and ∼15% were at the G2/M phase (4n) (**Figure 3C**). To study the tumorigenic propensity of MBC02 cells, we performed anchorage independent growth assay. We observed the appearance of colonies after 2 weeks of plating the cells. The colonies appeared to be small and rounded (**Figure 3D**). This observation suggested that MBC02 cells have the potential to form tumors if injected into nude or SCID mice and may prove to be a valuable model system for not only studying cancer pathogenesis but also for screening novel therapeutics for clinical use.

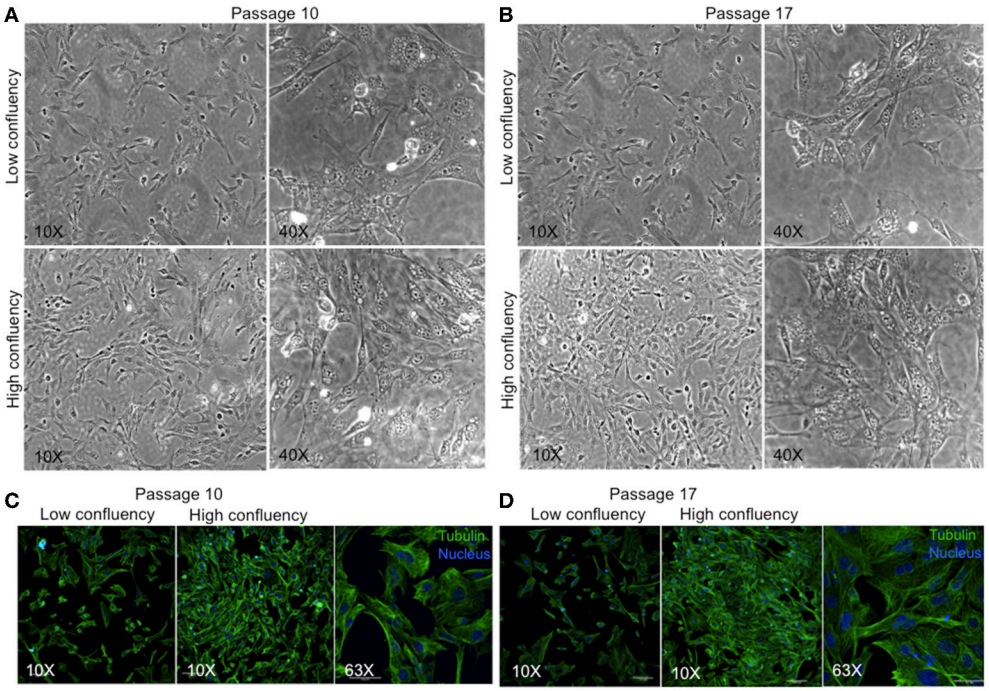

FIGURE 2 | Morphological evaluation of MBC02**. (A,B)** Phase contrast images of passage 10 and 17 of MBC02 at low (10X) and high (40X) magnification show that these cells are adherent to the substratum and have a flattened morphology similar to epithelial cells. **(C,D)** Immunofluorescent staining using anti-α-tubulin antibody show well-formed cytoskeletal network within these cells. The heterogenous nature of MBC02 cell line is apparent due to the presence of some cells that are larger than others.

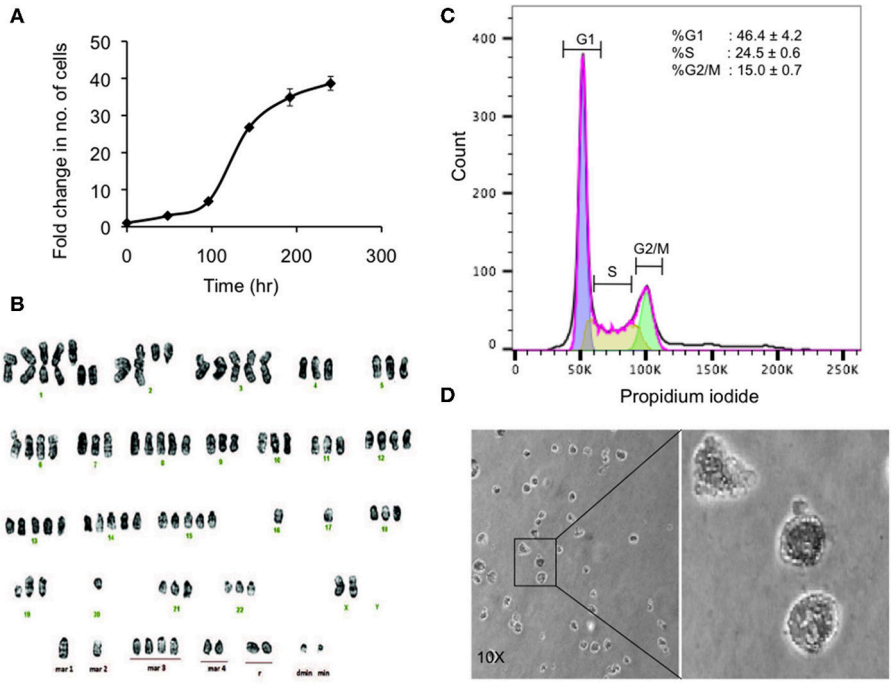

FIGURE 3 | Preliminary characterization of MBC02. **(A)** Population doubling time of MBC02 culture was calculated to be approximately 24 h. **(B)** MBC02 cells exhibit gross cytogenetic defects in the form of both structural and numerical aberrations ranging from centric fission, deletions of either long or short arms of more than one chromosome, duplications and complete loss of an arm of more than one chromosome. The modal chromosome number in MBC02 is 89. **(C)** DNA content analysis of MBC02 cells revealed ∼46% cells in the G1 phase whereas, ∼24 and ∼15%were in S and G2/M phase of the cell cycle **(D)** The ability of MBC02 cells to form colonies in soft agar indicate that these cells can proliferate in an anchorage independent manner and may form tumors if injected in mice.

TABLE 1 | Mutational analysis of MBC02.

CRC sample	KRAS mutation				BRAF mutation (V600E)
	Codon 12	Codon 13	Codon 61	Codon 146	
Original tumor	WT	WT	WT	WT	WT
MBC02	WT	WT	WT	WT	WT

MBC02 cells were evaluated for clinically relevant CRC related mutations in KRAS and BRAF. Codons 12, 13, 61, and 146 contained the wild type sequence. The V600E mutation in BRAF was also not present in MBC02 cells.

We next examined the original tumor and MBC02 cell line for the presence of common mutations in *KRAS* (codons 12, 13, 61, and 146) and *BRAF* (V600E) that are prevalent among CRC patients. Mutations in these genes were not present in either the tumor or the cell line (**Table 1**). These molecular markers used for the selection of anti-EGFR therapy were preserved in the patient tumor tissue as well as in the tumor derived primary cell line MBC02.

Key Signaling Pathways Are Differentially Regulated in MBC02

CRC pathogenesis is characterized by mis-regulation of a number of molecular pathways that often crosstalk in a complex network. Of note are the Wnt-β-catenin, TGFβ and Notch signaling pathways (30, 31). To investigate whether these pathways are differentially regulated in MBC02 cells in comparison to a standard CRC cell line, HCT116, we quantified the relative mRNA expression of key components of the Wnt-β-catenin, TGFβ and Notch signaling pathways. Our comparative findings revealed that several genes from each of the three pathways were overexpressed in MBC02. In the context of the Wnt-β-catenin pathway, all key signaling molecules showed highly upregulated expression, except for the scaffolding protein Axin1 that showed ∼4-fold downregulation as compared to HCT116. A similar trend was observed for the TGFβ pathway with the highest expression of SMAD4 (∼3,000-fold). The Notch signaling pathway also showed varied expression levels with Notch4 mRNA being ∼23,000-fold higher in MBC02 (**Table 2**). Our findings present strong evidence that clinically relevant signaling pathways are differentially regulated in MBC02, as compared to HCT116.

TABLE 2 | Key signaling pathways are mis-regulated in MBC02.

Signaling pathway	Gene	mRNA expression in MBC02	Fold change (over HCT116)
Wnt-β-catenin	Frizzled	Upregulation	1,500
	LRP6	Upregulation	8,000
	Disheveled	Upregulation	4,700
	GSK3b	Upregulation	6,000
	APC	Upregulation	3,650
	Axin1	Downregulation	4
	β-catenin	Upregulation	1,800
TGFβ pathway	SMAD2	Upregulation	1.3
	SMAD 3	Upregulation	3.6
	SMAD 4	Upregulation	3,000
	SMAD 7	Upregulation	13
Notch signaling	Notch1	Downregulation	Undetectable
	Notch2	Upregulation	9,000
	Notch3	Upregulation	500
	Notch4	Upregulation	23,000
	DLL1	Downregulation	Undetectable
	DLL3	Downregulation	Undetectable
	DLL4	Upregulation	2,000
	JAG1	Downregulation	Undetectable
	JAG2	Upregulation	50
	HES4	Upregulation	6
	HEY1	Downregulation	Undetectable

Differential gene expression in MBC02 cells was determined using qRT-PCR and fold change in mRNA levels was calculated with respect to HCT116. Important signaling pathways that are critical in CRC pathogenesis are mis-regulated in MBC02.

MBC02 Cells Lack Cell-Cell Contacts and Are Highly Migratory and Invasive

Altered molecular regulation in a cancer cell resulting in the acquired ability of enhanced cell motility is a key driver in cancer metastasis (9). We set forth to evaluate the relative mRNA expression levels of EMT markers in MBC02 and the established CRC cell lines HCT116, HT29, and SW620. E-cadherin was maximally expressed in HT29 (∼3.5-fold higher than HCT116). In SW620, we observed a decrease in E-cadherin expression and almost no detectable mRNA transcript in MBC02. There was a concomitant elevation of N-cadherin and vimentin expression in MBC02 (∼6-fold and ∼4,000-fold over HCT116). However, there was a more moderate increase in vimentin expression in MBC02 over SW620 (∼8-folds) (**Figure 4A**). The lowered expression of E-cadherin coupled with an enhancement in the levels of N-cadherin and vimentin constitute a typical molecular signature for EMT (32), suggesting that MBC02 may have undergone molecular alterations that could result in its transition from the epithelial to mesenchymal phenotype. Immunofluorescence analysis using an anti-E-cadherin antibody demonstrated localization of E-cadherin at cell-cell junctions in HCT116 as indicated by strong immunofluorescence staining. However, a similar pattern was not seen in MBC02 suggesting a loss of plasma membrane tethered E- cadherin that may have resulted in loss of adherens junction integrity in MBC02 (**Figure 4B**). Further, we examined the expression of EMT related transcription factors like Twist and Zeb that play critical roles in tumorigenesis and metastasis (33). There was a significant elevation in the levels of Twist (∼20-folds) and Zeb2 (∼5,000-folds) in MBC02 as compared to HCT116, while Snail, Slug, and Zeb1 transcripts were undetectable. However, all three transcription factors had elevated expression in SW620 as compared to HCT116 (**Figure 4C**). Together, these molecular alterations in MBC02 could be considered as indicators of these cells having undergone at least a partial EMT.

Transition of tumor cells from the epithelial to mesenchymal phenotype endows them with an enhanced capacity for migration. To explore whether there is an enhancement in the motility of MBC02 cells alongside the presence of the EMT signature, we performed a wound-healing assay. After

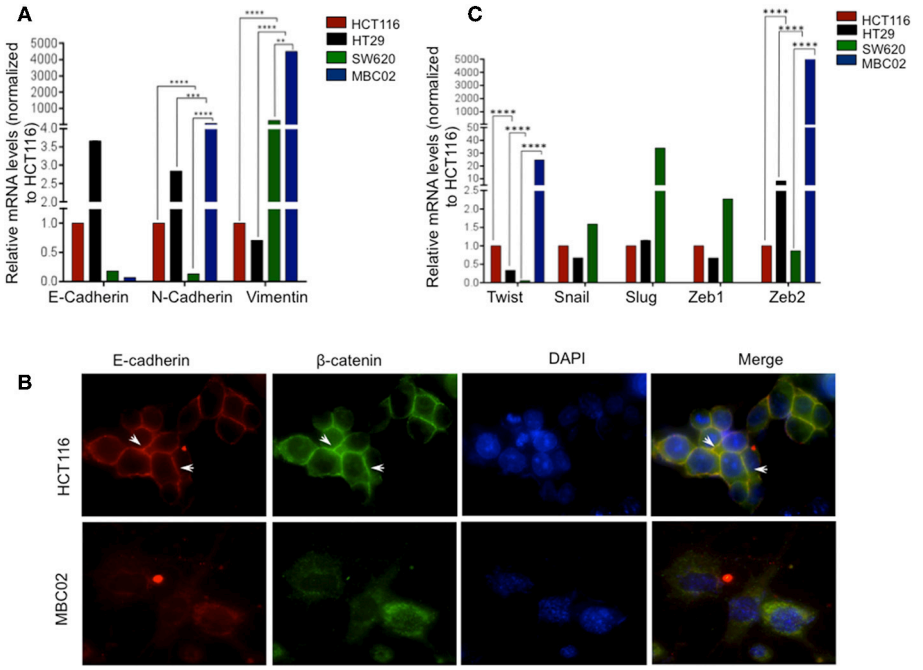

FIGURE 4 | MBC02 exhibits features of epithelial to mesenchymal transition. **(A)** mRNA expression analysis of CRC cell lines using qRT-PCR shows downregulation of E-cadherin and concomitant upregulation of N-cadherin and vimentin, indicating a cadherin switch in MBC02 and SW620 cell lines. **(B)** E-cadherin mediated intercellular junctions are lost in MBC02. Immunofluorescence analysis using anti-E-cadherin and anti-β-catenin antibodies reveal localization of both proteins at the cell-cell junctions in HCT116 (arrowheads), whereas no cortical staining is seen in MBC02. **(C)** EMT related transcription factors Twist and Zeb2 showed increased expression in MBC02 as compared to HCT116. Additionally, Snail, Slug, and Zeb1 are elevated in SW620. *p*-values are $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

wounding a confluent monolayer of cells, the cells along the wound edge were imaged by microscopy over a 48 h time period. As compared to HCT116, the rate of wound healing was significantly faster in MBC02, which led to more rapid healing of wounded monolayers. HCT116 cells reduced the width of the wound to 185 μm but MBC02 filled the wound in the same time interval (**Figures 5A,B**). The enhanced motility of MBC02 cells was confirmed by evaluating migration through transwell pores after subjecting the cells to a serum gradient for 24 h. SW620 showed ∼3.0-fold higher motility than HCT116, whereas, MBC02 exhibited ∼2.5-fold greater motility than HCT116. HT29 cells hardly showed any migratory potential (**Figures 5C,D**). Similar results were obtained in an invasion assay when these cells were added to matrigel coated Boyden chambers and allowed to migrate across the matrix along a serum gradient. SW620 and MBC02 cells were highly invasive compared to HCT116 (∼6.5-fold and ∼6-fold higher than HCT116). HT29 did not show any invasive property (**Figures 5E,F**). These observations indicated that the novel cell line MBC02 is similar to the metastatic cell line, SW620 in the expression pattern of EMT related molecular markers that has resulted in these cells being highly migratory and invasive in nature. Taken together, these results suggest that along with the loss of cell-cell junction integrity, MBC02 cells have gained enhanced migratory ability concomitant with increased invasiveness that present strong evidence of these cells having undergone EMT.

Response to Standard Therapy Is Attenuated in MBC02

Most often, the treatment regimens for CRC include the nucleoside analog 5-fluorouracil (5-FU) in combination with platinum drugs such as oxaliplatin or topoisomerase I inhibitor irinotecan (34). MBC02 cells were subjected to the various standard treatment regimens for CRC that are currently in clinical practice, namely a combination of 5-FU and leucovorin, FOLFOX (combination of oxaliplatin, 5-FU, and leucovorin), FOLFIRI (combination of oxaliplatin, 5-FU and irinotican) and the epidermal growth factor receptor (EGFR) inhibitor, cetuximab. We found that MBC02 did not show an appreciable response to any of these treatments (**Figure 5G**, **Table S2**). EMT is known to induce acquired drug resistance via multiple mechanisms that are not yet well-understood. Upregulation of Twist has been implicated in resistance to 5-FU and oxaliplatin (35, 36). Therefore, activation of EMT not only made MBC02 cells highly migratory and invasive, but also less responsive to standard therapeutic drugs for CRC.

Expression, Mislocalization, and Cytoplasmic Accumulation of β-Catenin in MBC02

Misregulation of Wnt/β-catenin pathway is a major cause of pathogenesis of CRC and mutations in β-catenin is often used as a marker for disease prognosis (24, 37, 38). We compared

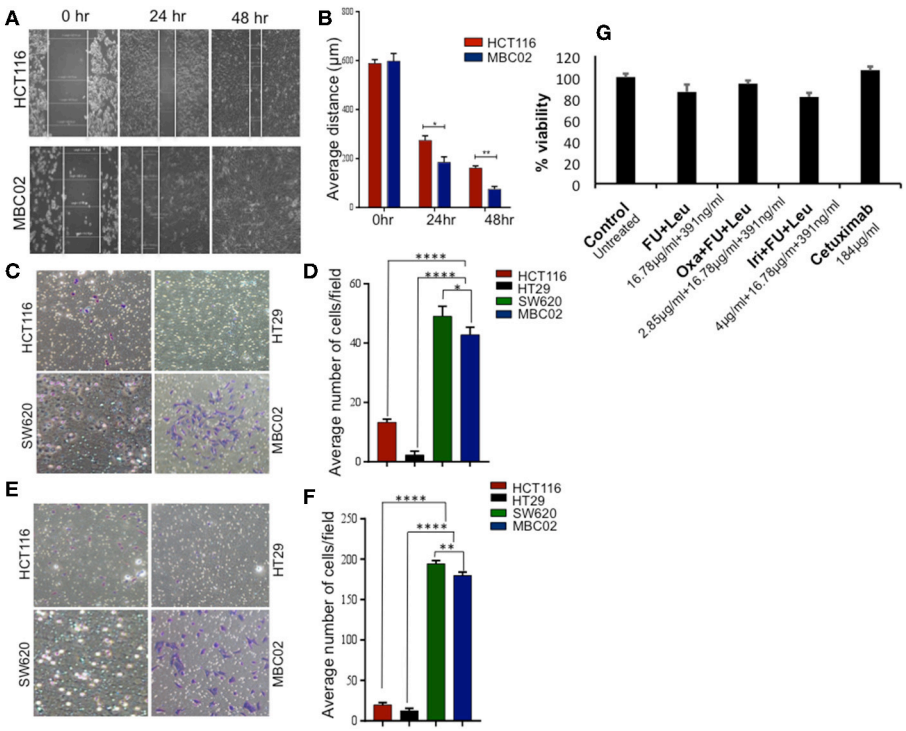

FIGURE 5 | MBC02 is highly migratory and invasive and has attenuated response to standard therapy. **(A,B)** Inherent migratory ability of MBC02 and HCT116 were measured using wound- healing assay. MBC02 cells were able to close the wound within 48 h whereas the wound still persisted in HCT116. **(C,D)** The ability of MBC02 and SW620 cells to migrate through a membrane barrier in a transwell migration assay suggests that MBC02 and SW620 cells have a higher migratory ability as compared to HCT116 and HT29. **(E,F)** Similar results were obtained in the invasion assay where more number of MBC02 and SW620 cells could cross the matrigel coated membrane barrier compared to HCT116 and HT29. **(G)** Clinically relevant drug combination including 5-FU, leucovorin, oxaliplatin, irinotecan and cetuximab were tested for efficacy on MBC02 cells. MBC02 did not show any appreciable response to any of the combinations and was similar to that of control. The data is represented as mean ± SEM calculated from three independent experiments. *p*-values are $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

the expression levels of β-catenin in MBC02, HCT116, HT29, and SW620 using qRT-PCR analysis. Our results showed a dramatic increase (∼1,500-fold) in relative expression of β-catenin mRNA in MBC02 in comparison to all the other cell lines. SW620 however showed similar β-catenin expression as HCT116 (**Figure 6A**). As the established cell lines have similar expression of β-catenin, we have used HCT116 as the control cell line for all further analyses. Immunofluorescence analysis using anti-β-catenin antibody revealed strong cytoplasmic staining and almost no cortical staining of β-catenin in MBC02 (**Figure 6B**). In addition, β-catenin also localized to the cytoplasmic microtubules but not to the cortical microtubules in MBC02. In contrast, β-catenin localization at the adherens junctions in HCT116 was confirmed by strong immunofluorescence at the cell-cell contacts (**Figure 6B**).

The cytosolic accumulation of β-catenin prompted us to investigate the presence of cancer associated stabilizing mutations in MBC02 derived β-catenin. Upon DNA sequence analysis we found that MBC02 contained the wild type sequence at the S33, S37, T41, and S45 phosphorylation sites. In contrast, HCT116 showed the expected in-frame deletion at S45, as reported earlier (34) (**Figure 6C**). The presence of the wild type phosphorylation sites in β-catenin of MBC02 is indicative of normal phosphorylation. To test this, we immunostained MBC02 and HCT116 cells using a phospho-specific antibody that recognizes β-catenin phosphorylated at S33/S37/T41. We observed significant accumulation of phospho-β-catenin in the cytoplasm and nucleus in MBC02 confirming that phosphorylation of β-catenin remained unimpaired (**Figure 6D**). Interestingly, a significantly higher fraction, ∼75% of MBC02 cells showed nuclear localization of phospho-β-catenin as compared to ∼20% for HCT116 (**Figure 6E**). Upon examination of the mRNA levels of *c-Myc*, a downstream target of β-catenin activation, we observed a ∼1.5-fold increase in *c-Myc* expression in MBC02 (**Figure 6F**). These results suggest that cytoplasmic and nuclear accumulation of β-catenin could partly be responsible for the increased expression of *c-Myc* in MBC02.

Upon investigating the subcellular localization of β-catenin in MBC02 during mitosis, we observed distinct foci of β-catenin at the spindle poles at metaphase that overlapped with α-tubulin (**Figure 6G**), while during cytokinesis, β-catenin localized prominently at the inter-cellular bridge (**Figure 6H**). In contrast, the presence of β-catenin at either the spindle poles or at the inter-cellular bridge was not discernible in HCT116 cells, although cortical β-catenin staining was prominent (**Figures 6G,H**). This differential localization of β-catenin during cell division suggested that the protein may be involved in

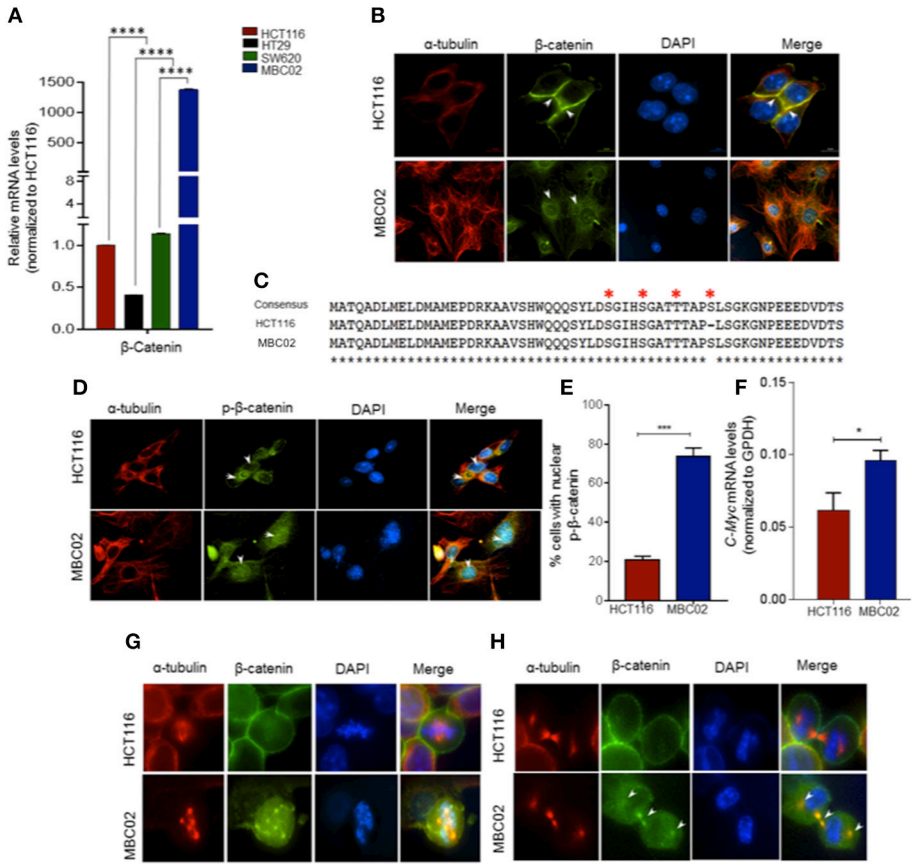

FIGURE 6 | β-catenin is highly activated in MBC02. **(A)** Relative mRNA expression of β-catenin in HCT116, HT29, SW620, and MBC02 cells was measured by RT-PCR and values were normalized to those of HCT116. β-catenin is highly overexpressed in MBC02 as compared to the other cell lines. **(B)** Immunofluorescence imaging of subcellular localization of β-catenin (green) show cortical localization in HCT116, whereas, in MBC02 it shows predominantly cytoplasmic and nuclear distribution. Arrowheads mark β-catenin localized to the cortex and at the inter-cellular junctions in HCT116 and cytoplasmic distribution in MBC02. α-tubulin (red) and nucleus (blue) are also marked. **(C)** Sequencing of MBC02 derived β-catenin shows the N-terminal phosphorylation sites, S33/S37/T41 and S45, contain wild type sequence (red asterisk) whereas in HCT116, there is an in-frame deletion of codon 45. **(D)** Immunofluorescence imaging confirms the presence of phosphorylated β-catenin (green) in MBC02 in the cytoplasm and nucleus (arrows). Phosphorylated β-catenin however, is excluded from the nucleus of HCT116 (arrow). **(E)** Nearly 75% of MBC02 cells show presence of phospho-β-catenin in the nucleus as compared to 20% HCT116 cells. **(F)** β-catenin target gene, *c-Myc* show elevated expression in MBC02 as compared to HCT116. **(G)** Prominent localization of β-catenin (green) at cell cortex is seen in HCT116 during metaphase. There is no visible staining of β-catenin at the spindle poles in HCT116. MBC02 however shows very prominent β-catenin localization at the poles of multipolar spindle during metaphase. **(H)** β-catenin localizes to the intercellular bridge during cytokinesis in MBC02 marked by arrowheads. In HCT116, β-catenin is confined to the cell cortex during early (metaphase) and late (cytokinesis) stages of mitosis, as marked by arrowheads. The data is represented as mean ± SEM calculated from approximately 200 cells per experiment, over three independent experiments. *p*-values are $^{*}p < 0.05$, $^{***}p < 0.001$, ****indicate statistical significance.

distinct roles during different phases of the cell division in MBC02.

Nuclear Retention of β-Catenin Could Have Resulted From Elevated Pin1 Expression in MBC02

The prominent presence of cytoplasmic and nuclear β-catenin in MBC02 prompted us to investigate whether alterations in its turnover could result in its enhanced cellular accumulation. Peptidylprolyl Cis/Trans Isomerase, NIMA-interacting 1 (Pin1) is known to cause isomerization induced structural changes in β-catenin that interferes with its interaction with adenomatous polyposis coli (APC) and thus modulate its turnover (39). We examined the level of expression of Pin1 in MBC02 to check whether Pin1 could account for the elevated nuclear accumulation of β-catenin in these cells. Our results demonstrated a dramatic increase of ∼9,000-fold in mRNA expression of Pin1 in MBC02 (**Figure 7A**). Western blot analysis of whole cell lysates of HCT116 and MBC02 showed a significant increase (∼3.5-fold) in Pin1 protein levels in MBC02 (**Figures 7B,C**). In addition, we performed western blot analysis of APC on MBC02 cell lysates to ascertain the presence of APC truncation, which is common in CRC. A band appeared at approximately ∼150 KDa size, indicating the presence of a truncated form of APC in MBC02 in comparison to the full-length protein observed in HCT116 (**Figure S4**). Taken together, the elevated levels of Pin1 and truncated APC in MBC02 could be responsible, at least in part, in retaining β-catenin in the nucleus of MBC02 cells.

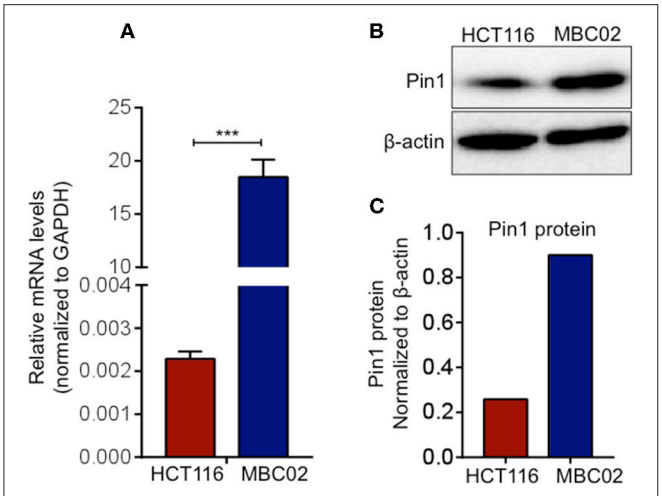

FIGURE 7 | Elevated Pin1 levels in MBC02. **(A)** qRT-PCR measurement of relative mRNA levels of Pin1 in MBC02 and HCT116 cells show that Pin1 transcript levels are highly elevated in MBC02. **(B,C)** Western blot analysis corroborates the RT-PCR data and shows about 3.5 fold higher Pin1 level in MBC02 as compared to HCT116. The data is represented as mean ± SEM calculated from three independent experiments. *p*-values are $^{***}p < 0.001$.

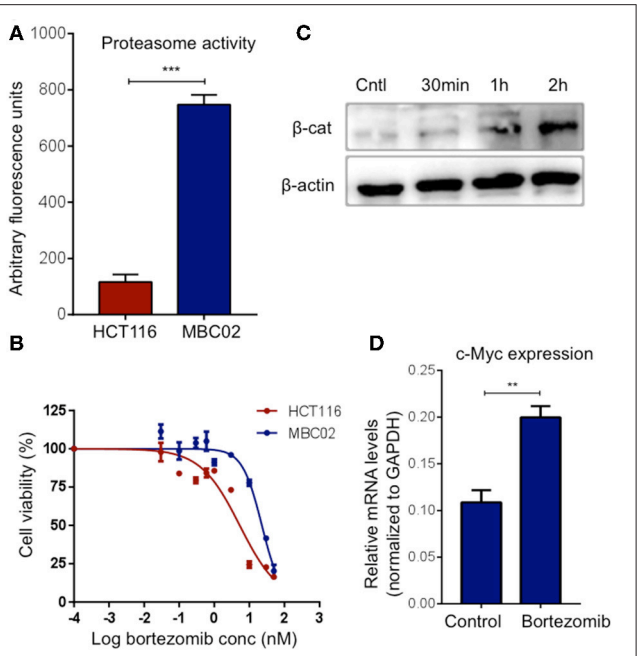

FIGURE 8 | High proteasome activity is observed in MBC02 cells. **(A)** MBC02 showed ∼6.5 fold higher endogenous proteasome activity as compared to HCT116. **(B)** MTT assay for checking the cytotoxicity of bortezomib in MBC02 and HCT116 show a higher IC_{50} value for MBC02 (∼22 nM) as compared to HCT116 (∼5 nM), indicating that MBC02 cells are less sensitive to bortezomib. **(C)** Western blot analysis of cell lysates from bortezomib (200 nM) treated cells shows β-catenin accumulation as early as 30 min post-treatment, thereafter not increasing appreciably up to 2 h. **(D)** Relative transcript levels of downstream target of β-catenin, *c-Myc*, were measured upon treatment with bortezomib (200 nM) and show ∼2 fold increase in mRNA levels as compared to untreated control cells. The data is represented as mean ± SEM calculated from three independent experiments. *p*-values are $^{**}p < 0.01$, $^{***}p < 0.001$.

MBC02 Has Elevated Intrinsic Proteasomal Degradation Activity

Ubiquitin mediated proteasomal degradation of β-catenin is a critical step in the overall regulation of the Wnt/β-catenin signaling cascade and is brought about by the multiprotein complex called the 26S proteasome (22). We postulated that increase in the proteasomal activity could possibly act as a compensatory mechanism for mitigating the high expression of β-catenin in MBC02. Evaluation of basal proteasomal activity in MBC02 revealed an almost 6-fold higher proteasome activity in MBC02 in comparison to HCT116 (**Figure 8A**). Further, we tested the comparative efficacy of bortezomib, a clinically approved anti-cancer drug that blocks the chymotrypsin activity of the 20S subunit of the proteasome, in MBC02 and HCT116 cells (40–43). A higher IC_{50} value in MBC02 (∼22 nM) as compared to that in HCT116 (∼5 nM) showed that MBC02 is less sensitive to the proteasome inhibitor compared to HCT116 (**Figure 8B**). To check whether the inactivation of the proteasome mediated protein degradation system could result in the accumulation of cellular β-catenin, we treated MBC02 cells with 200 nM of bortezomib, retrieved cells at regular time intervals after commencement of treatment and measured the level of β-catenin protein using immunoblotting. Bortezomib treated cells accumulated β-catenin as early as 30 min post treatment and continued thereafter up to 2 h (**Figure 8C**). We further examined whether the buildup of β-catenin led to alteration in expression of *c-Myc* in these cells. We observed that there was a 2-fold increase in *c-Myc* mRNA levels in treated cells as compared to control (**Figure 8D**). Our findings suggest that the enhanced proteasomal activity in MBC02 partially compensates for the unusually high expression of β-catenin mRNA. The higher proteasome activity in turn makes MBC02 cells less responsive to the proteasome inhibitor, bortezomib. Further, bortezomib-induced inhibition of proteasome activity allows β-catenin accumulation that brings about transcriptional activation of *c-Myc*.

DISCUSSION

Cancer cell lines have been the foundation for studies that allow not only elucidation of mechanisms of onset and progression of disease but also contribute toward screening of anticancer molecules for drug development. Patient derived cell lines from cancers originating in specific ethnic groups may potentially be of immense value owing to their inherent population specific genetic variations that ultimately influence disease progression. Although CRC cases are on the rise in India, there is a dearth of Indian patient derived cell lines that can facilitate studies to unravel the characteristics of CRC pathogenesis in the Indian population by serving as a relevant model to study various aspects of disease progression and its subsequent management in the clinic. Intra-tumor heterogeneity (ITH) or the coexistence of genetically distinct sub-clonal populations of cells within the same tumor, is the most defining aspect of all cancers as it

influences its response to a given therapy. Conventional cell line models fail to capture this heterogeneity of tumors as they are mostly clonal and highly homogenous in nature. In this study, we have established a primary cancer cell line, which is derived from a CRC patient addressing the above problem by propagating polyclonal tumor cells closely resembling the native patient tumor.

We observed morphological differences between MBC02 and the widely used established CRC cell lines (**Figure 2**). Gross numerical and structural anomalies in more than one chromosome were noted, indicating global chromosomal instability (CIN) in MBC02, a feature that is common in 80–85% of all CRC cases (44) (**Figure 3B**). The underlying reason could be the presence of multipolar metaphase spindles arising from the supernumerary centrosomes that ultimately result in defective mitosis and cytokinesis in these cells leading to the formation of large number of multinucleate cells (**Figures S2, S3**). Tumorigenicity of a cell line upon implantation in mice serves as an invaluable tool for testing and developing novel therapeutics. The ability of MBC02 cells to form colonies in soft agar in an anchorage independent manner (**Figure 3D**) alludes to its potential to be developed as a colorectal cancer model. The absence of the clinically relevant mutations in *KRAS* and *BRAF* (**Table 1**) in MBC02 suggests that cancer pathogenesis may have been driven by some other mechanism. Pathogenesis and progression of CRC have been associated with mis-regulation of inter-connected signaling pathways, of which Wnt-β-catenin, TGFβ, and Notch are prominent (12, 45, 46). These have also been implicated in the activation of the EMT program leading to metastasis, resistance to chemotherapy and poor survival (12). Our in-depth analysis of mRNA expression of Wnt-β-catenin, TGFβ, and Notch pathways highlight the presence of differentially regulated gene expression patterns in MBC02 (**Table 2**). Aoki et al. (47) demonstrated that activation of β-catenin/TCF contributed to chromosomal instability (CIN) in many cancers including gastrointestinal malignancies, which is independent of the p53 status. Therefore, taken together, these molecular features sets MBC02 apart from the widely used established CRC cell lines.

Under normal physiological conditions, an optimal level of E-cadherin expression is necessary to maintain structural integrity of the tissue via cell-cell adherens junction formation. In MBC02, there is a switch from E-cadherin to N-cadherin expression, accompanied by gain of expression of vimentin, along with upregulation of Twist and Zeb2 (**Figures 4A,C**). These transcription factors have been reported to bind to the E-cadherin promoter and bring about its transcriptional repression (48). Also, a positive correlation has been established between Twist expression, EMT and poor clinical prognosis (49). Therefore, it is reasonable to assume that high levels of these transcription factors may have resulted in reduction of E-cadherin mRNA expression in MBC02. Moreover, there is loss of E-cadherin from the cell-cell junctions in MBC02 (**Figure 2B**). In a tumor contexture, the functional outcome of this could be the acquired ability of the cancer cells to break off from the tumor mass and metastasize. MBC02 cells do exhibit an enhanced migratory and invasive nature that is similar to that of the established metastatic CRC cell line SW620 (**Figures 5A–F**) Therefore, our results demonstrate that the EMT program is likely to be activated in MBC02.

Recent years have seen EMT emerge as one of the major determinants of response to chemotherapy with clear correlation between the EMT phenotype and diminished efficacy of chemotherapeutic agents (50). The standard-of-care treatment regimen for CRC consisting either of a combination of 5-FU and leucovorin, FOLFOX, FOLFIRI or the targeted therapy cetuximab did not have any appreciable effect on these cells (**Figure 5G**). Although the mechanisms governing EMT induced drug resistance are not well-understood, several molecules are implicated to have key contributions, including EMT transcription factors Twist and Zeb (51). In addition to transcriptional repression of E-cadherin, these molecules also influence drug response (11, 52, 53). Low Twist and Zeb expression have shown favorable treatment outcome in patients and therefore, these could be valuable indicators for predicting drug response (54).

Membrane tethered E-cadherin anchors β-catenin at the plasma membrane forming a cadherin-catenin complex that not only is a component of functional adherens junctions but also shields β-catenin from proteasomal degradation (19, 20). Loss of E-cadherin in MBC02 (**Figure 4B**) may have resulted in compromised cell-cell junctions, releasing β-catenin into the cytoplasm, hence increasing the cytoplasmic pool of free β-catenin (**Figure 6B**). In MBC02, the presence of conserved, wild type residues at the regulatory site of β-catenin (**Figure 6C**), as well as immunofluorescence analysis using phospho-specific antibody demonstrate that phosphorylated β-catenin is present in abundance in both cytosolic and nuclear compartments (**Figures 6D,E**). This observation is supported by the work that showed that N-terminally phosphorylated β-catenin does not associate with cadherins and is mainly cytosolic and that T41/S45 phosphorylated β-catenin is largely nuclear (55). Wild type S45 along with S33/S37/T41 ensures efficient phosphorylation that localizes phospho-β-catenin to the nucleus, as confirmed by immunofluorescence analysis of MBC02. HCT116 however, contains a mutation at the S45 site (ΔS45), precluding phosphorylation at that site and excluding β-catenin from localizing to the nucleus (**Figures 6D,E**). The cytoplasmically accumulated β-catenin eventually translocates into the nucleus to bring about transcriptional activation of target genes as seen by the elevated expression of *c-Myc* in MBC02 (**Figure 6F**).

Our observation that β-catenin is present at the centrosomes in MBC02 (**Figures 6G,H**) is supported by earlier studies that have brought forth a relatively novel function of β-catenin in maintaining centrosomal integrity. Further, overexpression of β-catenin has been reported to result in disorganized centrosomes and loss of centrosomal cohesion leading to spindle organization defects (56, 57). The massive upregulation of β-catenin in MBC02 could be responsible for its prominent localization at the spindle poles and the cytokinetic bridge (**Figures 6G,H**). Although localization of β-catenin to the inter-cellular bridge

during late telophase has been reported earlier, its function in cytokinesis or its role in inducing cytokinetic defects has not been documented (57). It may be speculated that the increased amount of β-catenin within a localized area may perhaps play a role in inducing cytokinetic defects that may eventually lead to increase in the overall number of multinucleate cells in MBC02 (**Figure S3**).

Malignant transformations often lead to changes in the regulation of β-catenin turnover and result in abnormal accumulation in subcellular compartments (26, 27). Apart from being a core component of the destruction complex, the protein APC plays a crucial role in nuclear export of β-catenin. Phosphorylation dependent binding of Pin1 to β-catenin at the pS247P (phospho-Serine 247-Proline 248) motif brings about structural isomerization of β-catenin and prevents binding of APC to β-catenin. Pin1 upregulation has been reported in CRC and has been positively correlated with β-catenin expression (58–62). Most cancer-related mutations in APC occur within the Mutation Cluster Region (MCR) of APC that produces a N-terminal trunctated protein (∼150 KDa, instead of the full-length 310 KDa protein) which is unable to bind β-catenin and mark it for ubiquitination and subsequent proteasomal degradation (50). The elevated Pin1 levels in MBC02 (**Figures 7A–C**), together with truncated APC (**Figure S4**) could have a negative influence on the binding of β-catenin to APC, thereby inhibiting its nuclear export resulting in the accumulation of β-catenin in the nucleus (**Figure 6D**).

The ubiquitin-proteasome machinery plays an important role in maintaining cellular homeostasis by regulating the constant flux of synthesis, degradation and re-synthesis of proteins in the cellular milieu. Cancer cells have evolved various mechanisms to counter the abnormal production of proteins—altered proteasomal activity is one such mechanism (63, 64). MBC02 has approximately 6-fold higher basal proteasome activity over HCT116 (**Figure 8A**) and bortezomib mediated blocking of the proteasome led to a rapid accumulation of β-catenin (**Figure 8C**). However, it is unclear whether the high intrinsic proteasomal activity in MBC02 is a result of the dramatic transcriptional upregulation of β-catenin or the increased β-catenin mRNA level is a countermeasure for the elevated proteasomal activity in this cell line.

Proteasomal degradation of N-terminally phosphorylated β-catenin is well-documented (26, 27). Recently, it has been established that phosphorylation of β-catenin at S33/S37/T41 can also be achieved by NIMA-related protein kinase 2 (Nek2) and that binding of Nek2 to β-catenin prevents interaction of β-catenin with the E3 ligase β-TrCP resulting in inhibition of ubiquitination and proteasomal degradation (65). However, it is yet to be established whether Nek2 mediated phosphorylation of β-catenin is a mechanism that could shield β-catenin from GSK3β mediated phosphorylation and proteolytic degradation. It has been proposed that Nek2 mediated phosphorylation may be an alternate regulatory mechanism that could influence β-catenin stability that is independent of GSK3β (66). For our immunofluorescence analysis, we have used a phospho-S33/S37/T41 specific antibody that is unable to distinguish between the GSK3β or Nek2 mediated phosphorylated forms. We therefore speculate that in addition to the GSK3β mediated phospho-β-catenin, there may be a significant pool of Nek2 mediated phospho-β-catenin present that may escape proteasomal degradation and hence accumulate in the cytoplasm in MBC02.

Recent advances focusing on dissecting the molecular dynamics that govern cancer onset and progression are based on studies originating in the western countries with little or no data on Indian patients. India is known to be a genetically diverse population (4), therefore it may be reasonable to speculate that there may be differences in the underlying mechanisms of disease onset. Small molecular perturbations at the genetic level may translate to more drastic mechanistic differences, which could result in currently available therapies being rendered suboptimal in treating patients from various populations. Such molecular differences have the potential to be harnessed for developing either as molecular markers or targeted therapeutics that could be more effective in certain populations. Comparison of MBC02 cell line, originating from Indian patient, with the established Caucasian cell lines such as HCT116, HT29 and SW620, highlight such differences that may indicate underlying mechanistic differences in pathogenesis of CRC in these populations. However, detailed analyses from large patient cohorts would be needed in order to validate these findings that could be further utilized to design specific drugs tailored to targeted patient groups.

AUTHOR CONTRIBUTIONS

SM characterized MBC02, performed the cell-based assays, molecular characterization, high-resolution fluorescence imaging, and wrote the manuscript. HK performed the DNA sequencing and Western blot analysis. SK performed the MTT assays for determining the IC50 values. LS performed the anchorage independent growth assay. MJ performed the combinatorial drug sensitivity assay. AB developed the primary cell line along with initial characterization. MB performed immuno-histochemical analysis and reporting for the original colorectal tumor tissue. SVSM critically reviewed the manuscript and gave technical inputs on experimental design. MR supervised the design and execution of the experiments and data analysis. MR and AD supervised the overall study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00054/full#supplementary-material

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REVIEW
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Role of BRCA Mutations in the Modulation of Response to Platinum Therapy

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Recent years have seen cancer emerge as one of the leading cause of mortality worldwide with breast cancer being the second most common cause of death among women. Individuals harboring BRCA mutations are at a higher risk of developing breast and/or ovarian cancers. This risk is much greater in the presence of germline mutations. BRCA1 and BRCA2 play crucial role in the DNA damage response and repair pathway, a function that is critical in preserving the integrity of the genome. Mutations that interfere with normal cellular function of BRCA not only lead to onset and progression of cancer but also modulate therapy outcome of treatment with platinum drugs. In this review, we discuss the structural and functional impact of some of the prevalent BRCA mutations in breast and ovarian cancers and their role in platinum therapy response. Understanding the response of platinum drugs in the context of BRCA mutations may contribute toward developing better therapeutics that can improve survival and quality of life of patients.

Keywords: breast cancer, ovarian cancer, BRCA1/2 mutations, platinum drugs, response, resistance

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INTRODUCTION

Cancer is one of the leading causes of mortality worldwide. As per WHO estimates, 8.8 million cancer related deaths were reported in 2015, and this number is projected to rise to 13.1 million by the year 2030, with low- and middle-income countries bearing approximately 70% burden of all deaths worldwide. Of these, breast cancer has emerged as the second major cancer type comprising almost 25% of all cancers among women. Breast cancer is the most commonly occurring cancer among women with incidence rates varying widely across the world, having rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 92 in Northern America. But the mortality rate is lower in the developed countries as compared with low- and middle-income countries, because of higher survival of breast cancer patients in developed nations (1, 2).

Mutations in breast cancer susceptibility gene type 1 and type 2 (BRCA1 and BRCA2) put women at a higher risk of developing breast and/or ovarian cancer. In individuals harboring mutations in BRCA1, the probability of developing breast cancer over a lifetime is about 57–65% and that of ovarian cancer is about 39–40%. With BRCA2 mutations, the probabilities are at 45–49% for breast cancer and 11–18% for ovarian cancer (3, 4). Women with germline mutations are more prone to develop these cancers at a younger age with more aggressive disease and poorer prognosis as compared to those with somatic mutations. BRCA-mutated tumors exhibit both higher clinical grade and stage disease with greater metastatic potential (5). 70% of breast tumors containing germline mutations in BRCA1 fall in the category of triple-negative breast cancer (TNBC), a highly aggressive, highly metastatic subtype, comprising approximately 15% of all breast cancer cases, characterized by the absence of hormone receptors with no amplification of growth signal receptor (6).

Ovarian cancer patients with BRCA mutations exhibit a higher histological grade disease compared to those with sporadic disease and respond better to platinum therapy, having better prognosis (7, 8). In the absence of functional BRCA proteins, cells fail to repair intra-strand crosslinks formed by DNA cross-linking agents such as platinum drugs, leading to apoptotic cell death. Cisplatin is the most commonly used therapeutic agent for treating gynecological cancers either as a single agent before surgery or in combination with other drugs. Despite the favorable initial response, these cancers eventually develop tolerance to platinum leading to therapy failure. This review examines a few clinically relevant mutations that are common in breast and ovarian cancers. The structural and functional changes resulting from these mutations are explored further, focusing on their implications in modulating response to platinum therapy.

BRCA MUTATIONS IN BREAST AND OVARIAN CANCERS

BRCA1 and BRCA2 play a crucial role in maintaining genome integrity by repairing double-strand DNA breaks *via* the homologous recombination repair (HRR) pathway. Any mutations that cause functional disruption of these proteins may prove to be highly deleterious, leading to the development of cancer. In addition, BRCA1 and BRCA2 also play a critical role in cell division where they are transported to the cytosol to participate in regulating various molecular events during mitosis. Mutations impacting these important functions of BRCA1/2 can affect the delicate balance of the tightly regulated cellular processes that may lead to progression of disease.

BRCA mutations show huge diversity in various populations, many of which are functionally neutral or are of unknown pathological significance. However, there are some mutations that are more significant than others (**Table 1**). One of the most common cancer related mutations found in BRCA1 is the 5382insC, reported to have originated from a common European ancestor about 400–500 years ago. It was first described as the founder mutation in the Ashkenazi Jew population and could be present in other European populations as well. This mutation is also reported to be associated with a higher incidence of ovarian cancer (9.4%) but a lower incidence of breast cancer in Slavic countries (9). 185delAG located in exon 2 of BRCA1 is another common mutation reported in various ethnicities including Ashkenazi Jews and Indian population where it occurs at a high frequency of 16.4%. Missense mutation at the Cys61 (C61G) of BRCA1 is a founder mutation in Polish population and is included as a standard test for diagnosis and treatment of breast and ovarian cancer for Polish women (10, 11). 6174delT mutation is common in BRCA2 in the Ashkenazi Jewish population and other ethnic groups (12). In addition, BRCA1 and BRCA2 contain numerous other mutations that show a more population-specific distribution. This has been summarized in a review by Karami and Mehdipour (9). Screening for specific BRCA1/2 mutations that occur at high frequency in certain populations not only help in better clinical management of breast and ovarian cancers but can also be an invaluable tool in identifying healthy individuals who are currently disease-free but are at an increased risk of developing breast and/or ovarian cancer later in life (13). In addition to the presence of either BRCA1 or BRCA2 mutations in breast and/ovarian cancer patients, there are reports of patients being double heterozygous for both BRCA1 and BRCA2 mutations. These patients develop cancer at a much earlier age and with more severe disease (14, 15).

TABLE 1 | Clinically significant BRCA1/2 mutations in breast and ovarian cancers.

Type of mutation	Amino acid position	Amino acid change	Functional significance
BRCA1			
Missense	10	E → K	Familial breast and ovarian cancer
	23	E → K	Familial breast and ovarian cancer
	61	E → K	Breast and ovarian cancer. Interaction with BAP1 lost
	64	C → G	Loss of interaction with BAP1 in breast cancer
	67	D → Y	Breast cancer. Decreased ubiquitin function of BRCA1
	1685	T → I	Could be associated with susceptibility to cancer
	1699	R → Q	Reduced affinity for BRIP1 phosphopeptide in breast cancer
		R → W	Reduced protein stability breast and ovarian cancer
	1749	P → R	Reduced binding to BRIP1
	1775	M → K	Breast cancer. Interaction with BRIP1 and RBBP8 lost
Deletions	185delAG		Exon 2. Truncated protein. Functional null
	Δ369		Deleted in breast cancer
Insertion	5382insC		Breast and ovarian cancer. C-terminal truncated protein
BRCA2			
Missense	25	G → R	Breast cancer. PALB2 interaction lost
	31	W → C/R	Breast cancer. PALB2 interaction lost
	372	N → H	Common polymorphism that may elevate the risk of breast cancer
	Δ1286		Deleted in breast cancer
	Δ1302		Deleted in breast cancer
	2336	R → H	Decreased homologous recombination repair
	2722	T → R	Breast cancer. Exon skipping resulting in out of frame exons 17 and 19 fusion
	2723	D → H	Promotes RAD51 cytoplasmic localization in heterozygous state
Frameshift	6174delT		Truncated protein. BRCA1 C-terminus domain, NLS lost. Rad51 interaction lost

CANCER-ASSOCIATED MUTATIONS ALTER THE BIOLOGY OF BRCA PROTEINS

BRCA1 and BRCA2 are at the intersection of numerous key cellular pathways and perform multiple functions ranging from DNA

damage response and DNA repair activity, chromatin remodeling and transcription, and protein ubiquitination (16–19) (**Figure 1**). Single- or double-strand DNA breaks may arise from various sources like natural metabolic processes or from extraneous sources like chemical agents or irradiation. Left unrepaired, these DNA breaks may lead to the accumulation of deleterious mutations with a potential to cause genomic instability. Efficient repair of double-strand breaks *via* the HRR pathway requires functional involvement of BRCA1 and BRCA2 *via* their interaction with numerous other proteins (20, 21). Upon DNA damage, BRCA1 acts as a mediator bringing together components of the DNA repair pathway to the site of damage where it interacts with a large complex called the BRCA1-associated genome surveillance complex (BASC complex), along with the components of the DNA repair machinery (22, 23). Although BRCA1 and BRCA2 function in the DNA damage repair pathway, both have functionally distinct roles (23). BRCA1 functions as a DNA damage checkpoint activator and also in DNA repair, whereas BRCA2 is a core component of the HRR machinery. Homozygous BRCA1 knockout mice are embryonic lethal at age E7.5–E13.5, suggesting that functional loss of BRCA1 cannot be compensated by the presence of wild-type BRCA2 (24).

The BRCA proteins are organized into functional domains that enable numerous protein–protein interactions that are vital for their optimal function. BRCA1, a 220 kDa protein, contains an RING domain in the N-terminus that interacts with BRCA1-associated RING domain protein 1 (BARD1), the heterodimerization of which increases BRCA1 ubiquitin ligase activity by many folds. The BRCA1 C-terminus domain is conserved across many proteins that are involved in DNA repair and is the site for numerous phosphoprotein interactions. Limited structural data are available for the region of BRCA1 between exons 11 and 13, despite it being the binding site for a number of proteins that are involved in multiple cellular pathways. Some of the proteins that bind to this region of BRCA1 are retinoblastoma protein (RB), the transcription factor c-Myc, DNA repair proteins RAD50, RAD51, and PALB2 that forms a scaffold for BRCA1 and BRCA2 interaction (25). A common feature shared by many proteins that are at the hub of interconnecting pathways is the intrinsically disordered structure (26). Exons 11–13 of BRCA1 exhibit such disordered structure that perhaps provide a scaffold for multiple interactions and signal integration from various pathways (27). BRCA2 is a large 385 kDa protein with an N-terminus transactivation domain, a long exon 11 containing RAD51-specific binding site and a DNA binding domain toward the C-terminus (28). BRCA2, like BRCA1, contains disordered structure interspersed between more structured motifs, suggesting its participation in multiple cellular pathways (29, 30) (**Figure 2**). Here, we discuss

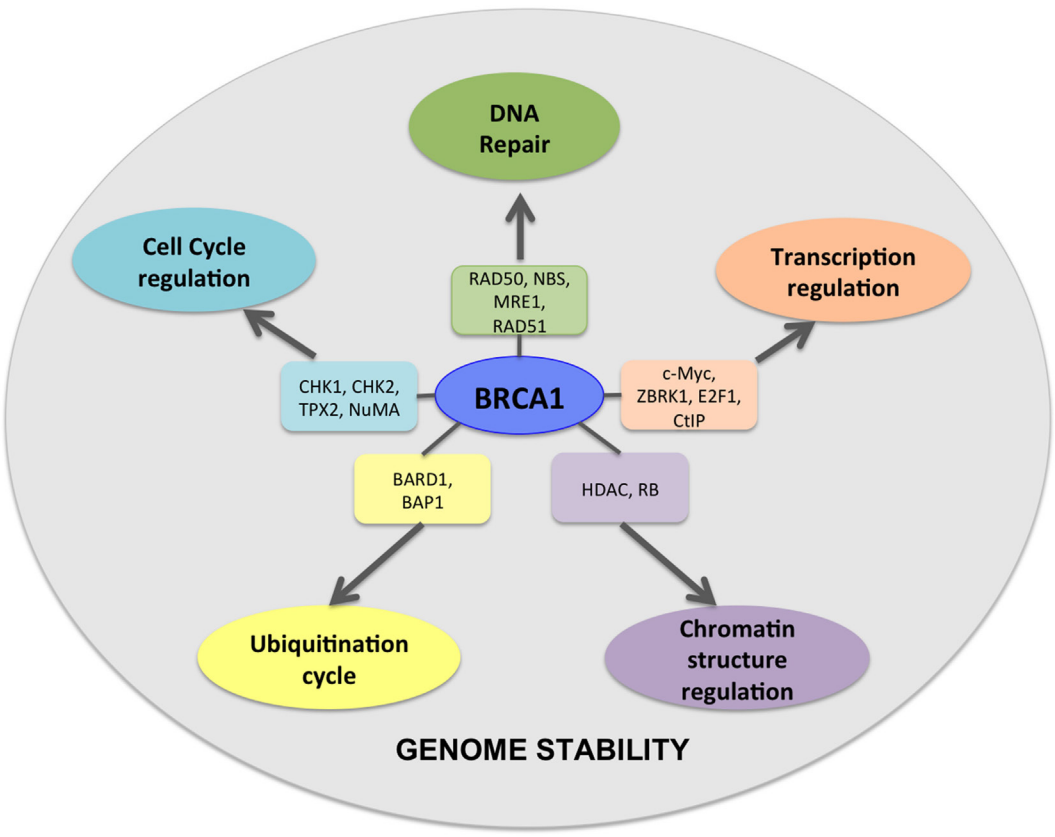

FIGURE 1 | BRCA1 is at the hub of numerous interconnecting cellular pathways. BRCA1 interacts with numerous intermediate proteins in these pathways that contribute toward genomic and cellular stability.

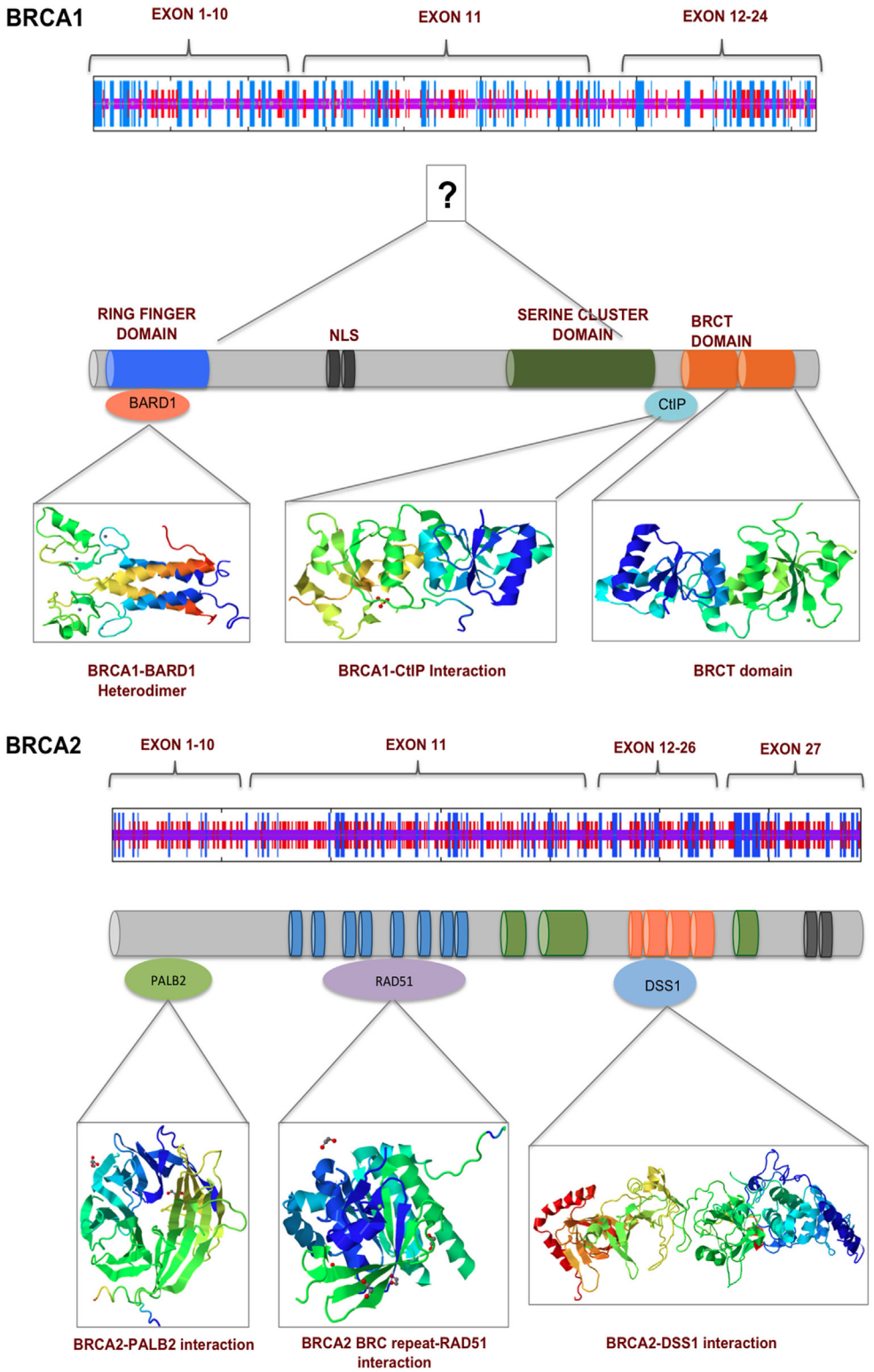

FIGURE 2 | BRCA1 and BRCA2 interact with numerous proteins *via* their multiple functional domains. The N and C termini of BRCA1 have structural motifs that allow multiple protein–protein interaction. Exons 11–13 in the middle of BRCA1 are more unstructured, which contains two nuclear localization signals. BRCA2 secondary structure prediction indicate a more helical middle region, which contains BRC repeats and binding sited for various proteins of the DNA damage response pathway.

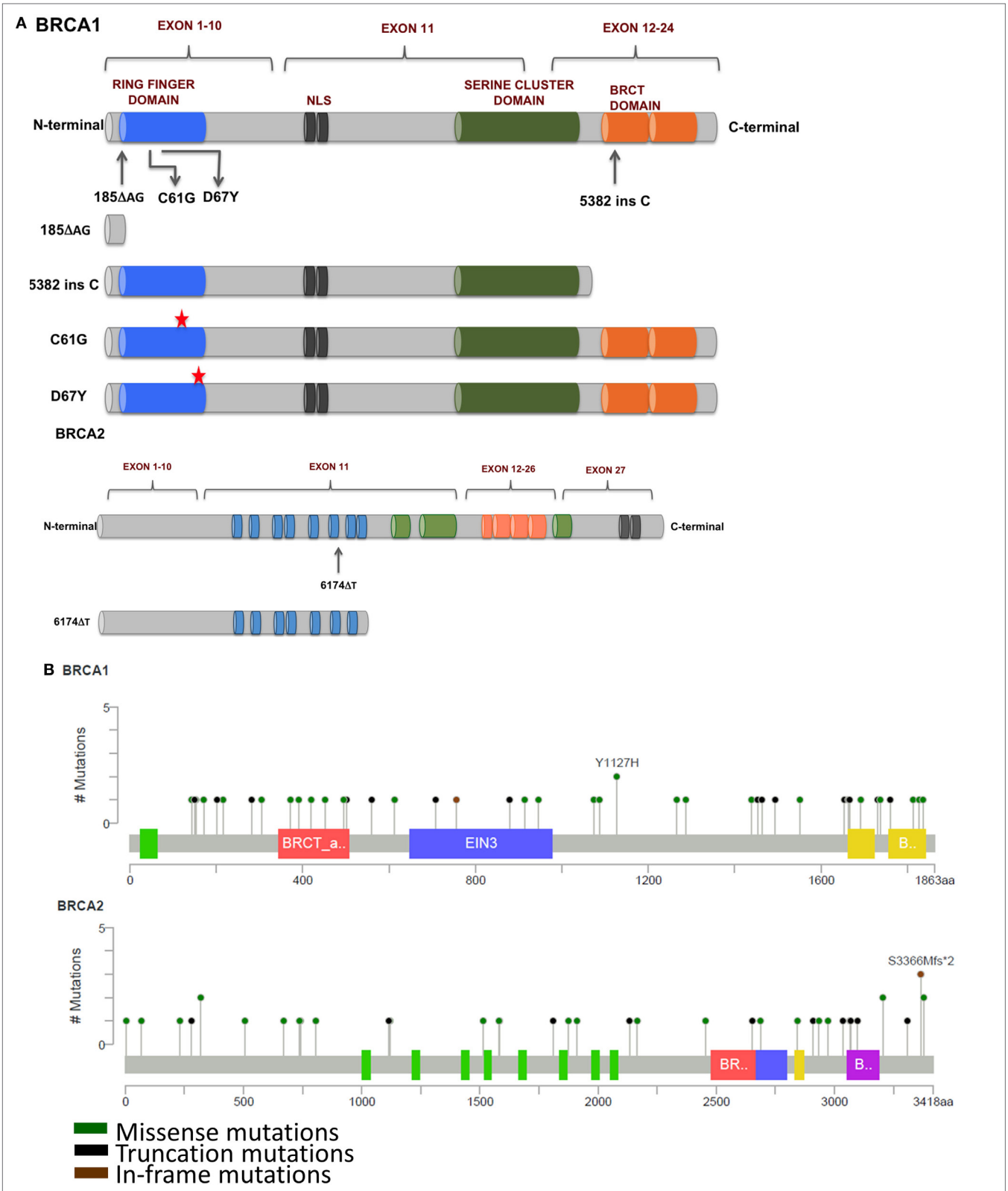

FIGURE 3 | **(A)** Mutations in BRCA1 and BRCA2 found in breast and ovarian cancers that affect response to platinum therapy. Deletion of multiple functional domains from BRCA1 result in truncated protein that is unable to repair DNA damage efficiently whereas missense mutations that result in amino acid substitutions result in altered response to platinum therapy. **(B)** Schematic representation of prevalent somatic mutations in BRCA1 and BRCA2 from breast cancer patients showing relative positions of various missense, truncations, and in-frame mutations (Adapted from www.cBioPortal.org) (31).

some key mutations in BRCA1 and BRCA2 that have strong correlation with breast and ovarian cancer and their functional consequences (**Figures 3A,B**).

185delAG—A two nucleotide deletion in exon 2 of BRCA1 produces a 39 amino acids long, functionally null truncated protein that has lost most of its functional domains as compared with a 220 kDa wild-type protein. The loss of the ring finger domain abrogates the binding of BARD1 and BAP1, which are important regulators of E3 ubiquitin ligase activity of BRCA1. Therefore, the loss of the ring finger domain of BRCA1 and consequently, loss of E3 ubiquitin ligase activity may be an important event in the development and progression of breast cancer (32, 33). BRCA1 interacts with both upstream and downstream effectors of cell cycle checkpoint kinases—deletion of binding domains result in the loss of these interactions and failure of the S and G2/M phase checkpoints allowing cell cycle progression even in the presence of DNA damage (34–37). Interaction of BRCA1 and BRCA2 is mediated by PALB2, and the complex is critical in RAD51-mediated HRR of damaged DNA. Disruption of this interaction by functional loss of BRCA1 protein results in defective DNA repair and consequently propagation of mutations that accumulate in daughter cells during cell division (38). However, nuclear expression of Maspin (mammary serine protease inhibitor), a member of the serpin superfamily and a target of truncated BRCA1, has been correlated with increased sensitivity to cisplatin with improved prognosis in ovarian cancer (39).

5382insC produces a C-terminal truncated BRCA1 commonly encountered in breast and ovarian tumors. The C-terminus of BRCA1 contains numerous domains for various protein–protein interactions including RNA-helicase binding, HDAC interaction, CtIP binding, and many others proteins (40–42). Thangaraju and colleagues have reported that the deletion of the C-terminus of BRCA1 through the 5382insC mutation resulted in loss of apoptosis in cell lines. It has been speculated that the deletion of the transactivation domains may be responsible for loss of transcriptional activation and/or repression of several genes, which may lead to apoptotic cell death (43).

6174delT—A frameshift mutation in the BRCA2 produces a 224 kDa truncated protein, about 2,002 amino acids long as compared with the 3,418 amino acids long wild-type protein (390 kDa). This truncation leads to the loss of two BRC repeat domains, loss of DNA binding, and the C-terminus RAD51 binding domain along with the nuclear localization signal, resulting in cells' defective DNA repair machinery that are unable to form RAD51 foci (44). In addition, cells carrying this mutation have a higher sensitivity to inhibitors of poly(ADP-ribose) polymerase (PARP). Edwards et al. reported that deletion of the 6174delT mutation could make the otherwise PARP inhibitor sensitive cells resistant to the drug. This acquired resistance to PARP inhibitors could be reversed upon restoration of the reading frame of BRCA2 (45), providing a window of opportunity for development of newer and effective strategies for clinical management of BRCA2-mutated cancers. C-terminal truncation of BRCA2 results in the loss of DSS1-mediated stabilization and rapid degradation of BRCA2, leaving cells vulnerable to DNA damage (46, 47). Other interacting partners of BRCA2 include EMSY and DMC1. Sporadic breast and ovarian tumors show amplification of EMSY, a binding partner of BRCA2, and have been associated with poor survival. Interaction of EMSY with exon 3 of wild-type BRCA2 (deleted in cancer) functionally inactivates BRCA2, suggesting a regulatory role for EMSY in the HRR pathway (48). The region between amino acids 2386 and 2411 of BRCA2 is highly conserved across species and is the binding site for DMC1, a germ cell counterpart of RAD51. Loss of this interaction due to the 6174delT mutation results in defective meiosis and propagation of chromosomal abnormalities in the germline (49).

SPORADIC CANCER AND BRCAness

BRCA1/2 mutations, seen most commonly in familial breast and ovarian tumors, impact the DNA repair pathway leading to genomic instability. However, some sporadic tumors that contain wild-type BRCA1 also have defective DNA repair pathway that may have resulted *via* other mechanisms. These characteristics of sporadic tumors that are similar to familial cancers are collectively called "BRCAness." Inactivation of BRCA1 in sporadic breast and ovarian tumors may be brought about by non-genetic mechanisms like promoter methylation that result in lowering of gene expression to undetectable levels and loss of heterozygosity. In contrast, BRCA2 inactivation does not occur by promoter hypermethylation—a significant number of sporadic breast and ovarian tumors show amplification of EMSY at the gene level. As discussed earlier, EMSY–BRCA2 interaction may regulate DNA repair *via* HRR pathway (50). A common feature that both BRCA-mutated cancers and those showing characteristics of BRCAness share is the elevated susceptibility to DNA cross-linking agents like platinum drugs and this has been the rationale for including these as therapeutic agents (8).

BRCA-MUTATED TUMORS ARE SENSITIVE TO PLATINUM THERAPY

Ever since its approval as an antineoplastic agent, cisplatin has been effective in BRCA-mutated breast cancer either as a single agent or in combination with other anticancer drugs (51, 52). The involvement of BRCA1 in efficient DNA repair mechanism has been highlighted by *in vitro* studies that showed that cells containing mutant BRCA1 showed increased sensitivity to platinum drugs as compared with those cells that have elevated BRCA1 levels. This heightened sensitivity could, however be, reversed upon restoration of the full-length functional BRCA1 (53, 54). BRCA1 has been identified as a key gene of the DNA repair machinery not only by siRNA screens but also using BRCA1, TP53 conditional knockout mice where animals after an initial favorable response to cisplatin, doxorubicin, and docetaxel, became resistant to doxorubicin and docetaxel but remained sensitive to cisplatin (55). Breast cancer xenografts using HCC1935 with mutated BRCA1 showed complete inhibition of tumor growth upon treatment with cisplatin whereas only partial response in xenografts that had the wild-type BRCA1 reconstituted. Moreover, significant cell cycle arrest at the S phase and G2/M transition was observed. This not only points to the role of BRCA1 in DNA repair pathway

and cell cycle checkpoint activation but also its involvement in modulating response to platinum drugs (56, 57).

Although BRCA-mutated breast tumors exhibit higher histological grade due to increased accumulation of chromosomal aberrations over time and are particularly sensitive to platinum drugs, their therapeutic use is limited to first-line treatment mainly due to development of resistance (58–62). This was demonstrated as early as 1988 when 47% partial response was achieved with a dose of 30 mg/m^2/day of cisplatin for 4 days, every 3 weeks, in six cycles as a first-line therapy for metastatic breast cancer (63). In another study, 83% pathologic complete response (pCR) was reported in breast cancer patients treated with cisplatin as a neoadjuvant treatment for TNBC. Also, higher response to platinum and pCR has been shown to be associated with low BRCA1 expression, promoter methylation, p53 frameshift or nonsense mutation, and E2F3 activation (64). Huang et al. reported more than 11 years of remission in a BRCA2-mutated metastatic breast cancer patient who received chemotherapy using high dose of cisplatin along with anthracyclin and alkylating agents (65). BRCA1-mutated metastatic breast cancer that was unresponsive to docetaxel, responded well to cisplatin and gemcitabine combination therapy for more than 6 months (66). In case of ovarian cancer, BRCA1 and BRCA2 patients exhibit a differential age of tumor development—BRCA1 carriers develop tumors earlier than those carrying BRCA2 mutations (48 vs. 57 years). Also, BRCA mutations seem to render the tumors more responsive to platinum drugs with a better survival (91 vs. 54 months) and longer disease-free interval (49 vs. 19 months) as compared with sporadic ovarian cancer (67, 68).

SECONDARY SOMATIC BRCA MUTATIONS CONTRIBUTE TO THE DEVELOPMENT OF RESISTANCE TO PLATINUM DRUGS

28.3% of recurrent ovarian cancer tumors contain secondary mutations as compared with only 3.1% in primary tumors, while 46.2% of platinum resistant tumors have secondary mutations that restored the function of BRCA1/2 as compared with 5.3% that are sensitive to platinum drugs (69). This implies that the secondary mutations in BRCA1/2 may be instrumental in the development of drug resistance to platinum therapeutics. The 39 amino acid long, non-functional BRCA1, produced due to the 185delAG mutation in the exon 2, severely impair its DNA repair function making the cells highly sensitive to platinum. This may get circumvented by the restoration of the reading frame that produces the full-length functional protein resulting in efficient DNA damage repair. Various studies indicate that platinum refractory BRCA1-mutated tumors carry two nucleotide insertions that are otherwise not present in BRCA1-mutated platinum-sensitive tumors. Similar results were demonstrated in case of BRCA2 mutations. In addition, new secondary mutations arise due to selection pressure exerted by prolonged exposure to platinum drugs resulting in resistance to both platinum drugs as well as PARP inhibitors (70). Such accumulation of secondary mutations could contribute to the development of resistance to platinum drugs over time. The presence of secondary mutations restoring the function of BRCA1/2 could be developed as a promising prognostic marker for either screening patients that are likely to respond favorably to platinum therapy or for predicting clinical outcome in patients already receiving platinum drugs or PARP inhibitor as single agent or in combination (71).

Apart from accumulated secondary mutations in BRCA, missense mutations in the ring finger domain affect response to platinum drugs. C61G missense mutation in exon 5 of BRCA1 abrogates BRCA1–BARD1 heterodimerization leading to loss of E3 ubiquitin ligase activity and cytoplasmic mislocalization of BRCA1. This results in diminished availability of BRCA1 at the site of DNA damage and impaired DNA repair, increasing vulnerability of these cells to platinum drugs. Homozygous C61G mice are embryonic lethal with severe developmental delays, mimicking phenotype of BRCA1 null mice. Interestingly, unlike BRCA1 null mice, these mice were reported to have developed resistance to both cisplatin and the PARP inhibitor olaparib, suggesting a hypomorphic activity of C61G mutation in BRCA1 (72). D67Y, another missense mutation that substitutes aspartic acid for a tyrosine residue at the 67th position result in decreased ubiquitin function of the mutated BRCA1 protein. In contrast to the C61G mutation, the D67Y mutation has been reported to increase cisplatin sensitivity *in vitro* (73).

In addition to mutational inactivation of BRCA function contributing to resistance to platinum drugs, several other mechanisms may exist that result in tumors not responding to platinum therapy (**Figure 4**). The dynamic balance between influx and efflux of platinum drugs influence its availability in the intracellular milieu to form DNA adducts and ultimately lead to apoptotic cell death. Copper transporters (CTR1 and CTR2) and copper-transporting p-type adenosine triphosphatase 1 and 2 (ATP7A and ATP7B) are key molecules that are responsible for intracellular transport of platinum drugs. In a meta-analysis study, Sun and colleagues reported positive correlation between CTR1 expression levels and response to cisplatin with favorable overall survival, progression, and disease-free survival. However, no such correlation could be made for CTR2, ATP7A, and ATP7B (74). Therefore, how a patient would response to platinum therapy is dependent not only on the occurrence of mutations that functionally inactivate BRCA1/2 but also to a large degree on other cellular mechanisms such as ion transport pathways that influence the dynamics of the drug within the tumor.

OVERCOMING PLATINUM RESISTANCE IN BRCA-MUTATED CANCERS

Treatment failure as a consequence of development of resistance to platinum drugs resulting in disease recurrence is a major roadblock in clinical management of cancers that carry BRCA mutations. In such cases, non-platinum-based drugs can achieve improved response and extended survival. PARP-1 and PARP-2, members of the PARP family, recruit the base excision repair (BER) machinery, a parallel, but less efficient mechanism for repairing damaged DNA, to the site of single-strand DNA breaks. In the absence of PARP, these lesions persist resulting in stalling

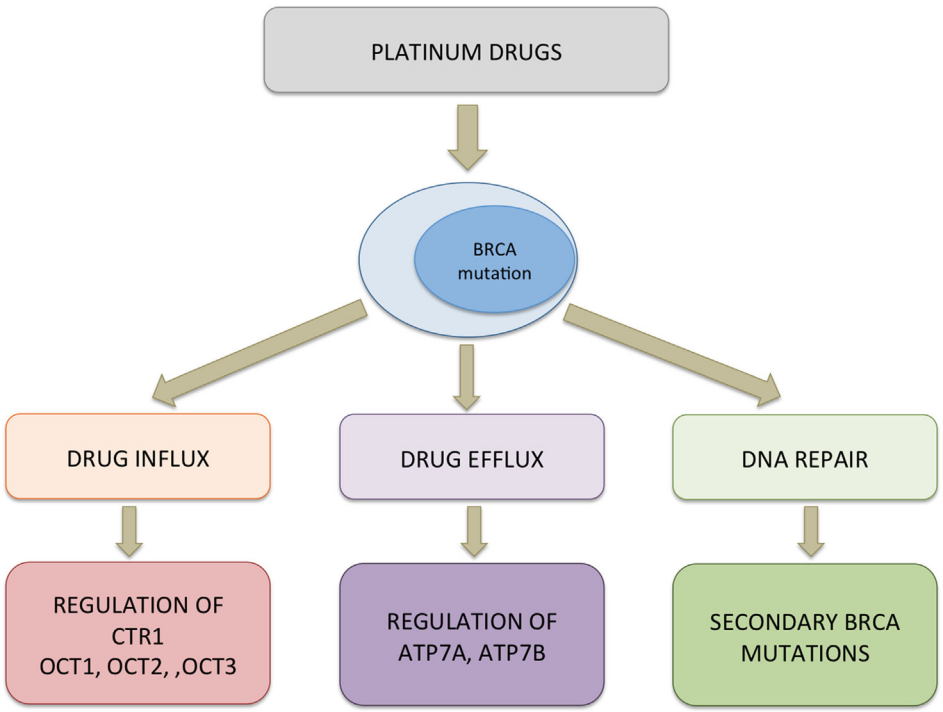

FIGURE 4 | Probable mechanism of resistance to platinum drugs in BRCA-mutated cells. Multiple pathways could be activated simultaneously conferring resistance to platinum drugs in cells. Downregulation of copper transporter (CTR1) and organic cation transporters (OCT1/2/3) can decrease the uptake of platinum drugs whereas upregulation of efflux pumps can bring about increased transport of the drug out of the cells. In the cytosol, platinum drugs can bind to a protein such as chaperons that interfere with the drug reaching the target. Functional restoration of BRCA1/2 and elevated expression of ERCC1 leading to effective removal of platinum–DNA adducts and efficient DNA repair contribute to decreased cytotoxicity of platinum drugs.

of the replication fork during DNA synthesis and formation of DSBs (75). The concept of synthetic lethality, the underlying premise of which is that functional depletion of two genes singly may not cause deleterious effect but together are lethal, was thus introduced to cancer therapeutics to develop better treatment strategies. This was mainly achieved by blocking the BER pathway by small molecule inhibitors against PARP in BRCA1/2-mutated cancers (76).

Poly(ADP-ribose) polymerase inhibitor olaparib has recently received FDA approval for treatment of ovarian cancer with mutated BRCA1/2 and EMEA approval for maintenance therapy for platinum-sensitive ovarian cancer (77). Randomized phase II clinical trial for evaluating the efficacy of olaparib, the first clinically approved PARP inhibitor, in combination with either paclitaxel or carboplatin, followed by olaparib monotherapy resulted in improved progression-free survival in BRCA-mutated high grade ovarian cancer patients compared with treatment with paclitaxel and carboplatin (78). PARP inhibitors have also been reported to be effective in other BRCA-mutated cancers. Clinical trials are underway to assess the efficacy of these (79, 80). Other PARP inhibitors such as niraparib, veliparib, and rucaparib are currently being tested in various clinical trials (81). Kim et al. reported that there is no clear correlation between BRCA1 expression and response to docetaxel (82), while Byrski et al. reported that non-BRCA1 mutation carriers showed higher complete or partial response to docetaxel as neoadjuvant therapy when compared with patients who were BRCA1 mutation carriers (83). *In vitro* studies using breast cancer cells indicate that BRCA1 mutations make the cells non-responsive to taxanes, an observation supported by *in vivo* experiments where targeted deletion of p53 and BRCA1 in mammary tissue rendered tumors resistant to docetaxel but not cisplatin (52). Clinical correlation between BRCA1 mutation and response to taxane is not clear with several studies reporting data that are largely inconclusive.

CONCLUSION

Structural organization of BRCA1 and BRCA2 into functionally distinct domains allow for multiple protein–protein interactions with numerous binding partners that facilitate participation in various cellular activities including DNA damage repair pathway. Specific protein–protein interaction such as BRCA1 and BARD1 that is crucial for the E3 ubiquitin ligase activity and the numerous phosphoproteins that bind to the C-terminus of BRCA1 make it an important node in the highly branched intracellular signaling network (29, 30, 84–88). Most common type of mutation observed among breast and ovarian cancer patients is deletion of multiple functional domains of BRCA1/2 that lead to not only impaired DNA repair but also abrogation of cell cycle checkpoints and transcriptional mis-regulation of genes, which eventually lead to global genomic instability. Suboptimal DNA repair mechanism arising from BRCA mutations render cells highly vulnerable to

DNA cross-linking agents, such as platinum drugs, making them useful therapeutics in many cancers. *In vitro* data have provided strong evidence toward better response to platinum drugs in the presence of BRCA mutations and is corroborated by clinical studies where BRCA mutation carriers exhibit better survival and longer disease-free intervals upon treatment with platinum drugs, suggesting beneficial therapy outcome. Along with targeting the suboptimal HRR pathway that results from non-functional BRCA1/2, the blocking of the BER pathway with PARP inhibitors significantly improves survival rates of cancer patients. Although BRCA1/2-mutated tumors are highly susceptible to platinum drugs and PARP inhibitors, development of resistance poses a major challenge in the clinical management of these cancers and secondary mutations significantly contribute toward this.

The inherent susceptibility of BRCA-mutated tumors to platinum drugs makes it an appropriate target for development of newer therapeutic agents. The ongoing efforts to design and develop novel inhibitors for the various components of the DNA repair pathway may yield encouraging results and in combination with platinum drugs could further improve the treatment options available for cancer patients. In addition, molecular signatures that can predict the outcome of a treatment regimen are being evaluated as biomarkers, which may help in identifying a target population that is more likely to respond to therapy. For instance, the C118T and C8092A polymorphisms in ERCC1 have been strongly correlated with objective response rate and overall survival (89). Similarly, the levels of annexin A3 in the peripheral blood may be a potential predictor for platinum resistance in ovarian cancer (90). Although molecular markers have the potential to predict therapy response, rigorous validation in large patient cohorts would truly bring out the benefits of such predictions. The formation of DNA–platinum adducts and subsequent progression toward apoptotic cell death was believed to be the mechanism of action for platinum drugs. Recent advances have brought forth other novel mechanisms by which platinum drugs exert their cytotoxic effect. One such mechanism is the ability of platinum drugs to modulate the host immune system. Treatment regimen that includes platinum drugs in combination with immune checkpoint blockers, such as anti-CTLA4 antibody and ipilimumab, has been approved for metastatic melanoma, whereas anti-PD-L1 antibody, atezolizumab, has been approved for metastatic lung cancer (91).

Platinum drugs, although highly efficacious in patients carrying BRCA1 and BRCA2 mutations, come at a cost in the form of severe side effects—nephrotoxicty and ototoxicity being two of the major unwanted effects of cisplatin treatment. The major challenge is to target the drug specifically to the tumor alone and minimize its accumulation in non-tumor tissue. Development of better drug-delivery vehicles that can ensure targeted delivery at the tumor site will not only maximize availability of the therapeutic agent at the site of the tumor but also reduce accumulation in healthier tissue, thereby minimizing toxicity. Thus, a therapeutic regimen that includes inhibitors for the DNA repair pathway, a tumor-specific platinum drug, together with radiotherapy may prove to be an effective way of treating and managing the cancer burden due to BRCA1 and BRCA2 mutations.

AUTHOR CONTRIBUTIONS

MR was involved in the conceptualization of the topic and supervised the writing of the manuscript. SM wrote the manuscript and drew the figures. AD critically reviewed the article.

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