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INVOLVEMENT OF ADENOMATOUS POLYPOSIS COLI IN COLORECTAL TUMORIGENESIS

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1. ABSTRACT

Colorectal cancer arises after a series of mutations in various tumor suppressor and proto-oncogenes, each of which is accompanied by specific alterations and pathological conditions. Recent advances have contributed a great deal of understanding of the molecular basis of events that lead to colorectal tumorigenesis. Mutation in the *adenomatous polyposis coli (APC)* gene is considered to be one of the earliest events in the colon cancer development. The familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) are the most commonly inherited colorectal cancers. FAP and HNPCC develop due to mutations in *APC* and DNA mismatch repair (MMR) genes, respectively. APC is known to regulate the levels of beta-catenin, an important mediator of cell-cell adhesion and transcriptional regulator. Mutations in *APC* gene are also linked with chromosomal instability in colon cancer cells. The role of APC is also implicated in cell migration, cell-cell adhesion, cell cycle control, and apoptosis. This article summarizes the structure-function studies and the role of *APC* mutations in colon cancer development.

2. INTRODUCTION

Colorectal cancer is one of the most predominant diseases in the Western world. It is the second leading cause of the world wide morbidity and mortality due to cancer. In the United States alone, approximately 146,940 new cases of colon and rectal cancers are expected in 2004. Of the new colon and rectal cancer cases, 106,370 will be colon cancer and 40,570 rectal cancer and its mortality may exceed 56,730 (1). The incidence rate of colon cancer varies up to 20-fold between high- and low-risk geographical areas through out the world (2). These results are mainly due to environmental and dietary factors. The history of colorectal cancer is well described but the mechanism of colorectal cancer initiation and progression is not well understood. The first step in the development of tumors from normal epithelium is usually the onset of dysplasia. Single dysplastic crypts (unicryptal adenomas) can be seen with the first histological manifestations of tumor development. It is now accepted that aberrant crypt foci (small areas of epithelium with irregular glandular architecture but no evidence of dysplasia) are precursor lesions of adenoma (3). Adenomas can gradually grow in size and change from a tubular to a villous architecture. The cells progressively show the mild, moderate, and then severe dysplasia followed by malignant changes resulting in local invasion with eventual metastasis to distant sites. A proposed model of colon cancer development through multiple histologically distinct stages is shown in Figure 1. Initially, Fearon and Vogelstein have suggested how the genes mutated during tumor progression relate in their order of occurrence to the histological stages of adenoma to carcinoma development (4). Genes which are mutated at different stages of colorectal cancer development include tumor suppressors, proto-oncogenes, DNA repair genes, growth factors and their receptor genes, cell cycle checkpoint genes, and apoptosis related genes (Figure 1). The multi-step colon cancer model describes an accumulation of genetic events, each conferring a selective growth advantage to an affected colonic epithelial cell

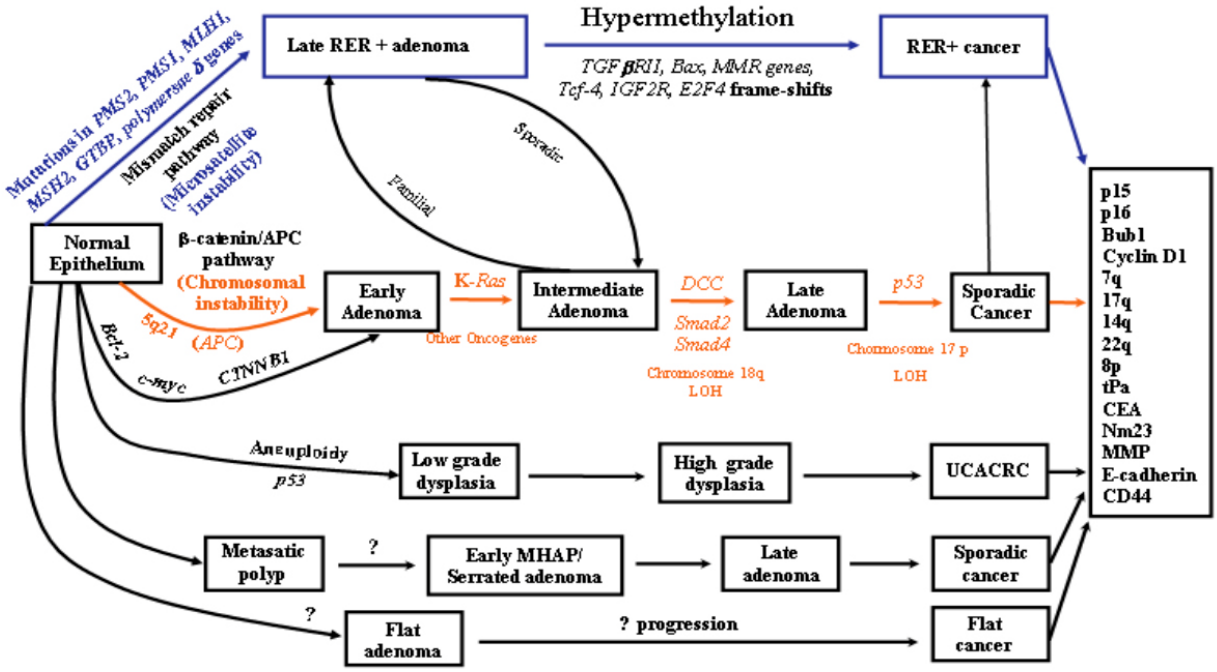

Figure 1. Model for genetic alterations in the development of colorectal cancer. Based on genetic analysis, at least two pathways are characterized in detail, which lead to colon cancer development. One pathway (indicated with red arrows) initiates with mutations in the *APC* gene followed by mutations in K-*ras*, deleted in colorectal cancer *(DCC)* and *p53* genes. The second pathway (indicated with blue arrows) is initiated by mutations in the MMR genes *(hMSH3, hMSH)* and other genes *(TGFbetaIIR, IGFIIR, PTEN, BLM, Tcf-4, Bax and E2F4)*. Beside these there are many other less characterized pathways with a high degree of overlapping among them. At least, seven gene mutations are needed to develop a normal epithelial cell into carcinoma. However, a cluster of genes and chromosome aberrations such as *p15, p16, Bub1, cyclin D1, tPa, CEA, Nm23, MMP*, E-cadherin (*CDH1*), *CD44*, 7q, 14q, 22q and 8p are observed in carcinoma and metastatic tumors. This illustration is adapted from (4).

These changes ultimately result in uninhibited cell growth, proliferation, and clonal development of tumor. The cumulative effect of these somatic mutations is the cause of sporadic colon cancer. Four main conclusions are drawn from the proposed model of sporadic colon cancer pathogenesis: 1) colorectal cancer is a consequence of the mutational activation of oncogenes and the inactivation of tumor suppressor genes; 2) at least four or five somatic mutations in genes of a normal colon epithelial cell are required for malignant transformation; 3) the accumulation of multiple genetic mutations rather than the sequence of mutations determine the biological behavior of the tumor, and 4) features of the tumorigenic process of colon cancer are applicable to other solid tumors, such as breast and pancreatic cancers (5).

The most commonly inherited colon cancer syndromes are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Each of these syndromes is the result of a specific germ-line mutation. In FAP, the germ-line mutation is always in the *adenomatous polyposis coli (APC)* gene (4, 5). In HNPCC, *hMLH1, hMSH2* and *hMSH6* are the most commonly mutated MMR genes (4, 5). Several hamartomatous polyp syndromes have recently been associated with germ-line mutations as well. One example is the Peutz-Jeghers syndrome, which results from mutations in the tumor suppressor gene *serine threonine kinase 11* (*STK11)* (6). Familial colon cancer in Ashkenazi Jewish population is probably the result of an *APC* germ-line mutation at I1307K residue; although the relative risk for tumor is much lower in a person with this mutation than germ-line mutations as noted in the cases of FAP (7, 8). Most germ-line mutations cause abnormalities in APC protein structure including I1307K germ-line mutation which causes a predisposition to sporadic mutations at distant sites of the gene, but the abnormalities in the APC protein structure occurs at later stages of the tumor development (7, 8). The main focus of this article is to provide an account of the role of APC in different signaling mechanisms, which are involved in the development of colorectal cancer.

Mutations in proto-oncogene *ras* are also detected in up to 50% of sporadic colorectal cancers and of large polyps. There are several pathways involved in the transduction of Ras-signaling. The best characterized once are the mitogen-activated protein kinases (MAPKs), including extracellular mitogen-regulated kinase (ERK), c-Jun amino-terminal kinases (JNK), and p38 (9). Activation of *ras* leads to the constitutive activity of the protein, which results in a continuous growth, inhibition of apoptosis, and or differentiation of cells that can be the basis of carcinogenesis. Recognition of *ras* mutations may be helpful in screening and early diagnosis of colorectal cancer

(10). The usefulness of a sensitive assay for the detection of *ras* mutations in the stool of patients with curable colorectal tumors has been studied (11). The farnesyl transferase inhibitors, which specifically inhibit *ras*-mediated signal transduction, have been used in patients with colorectal cancer exhibiting *ras* mutations. The *src* oncogene, first identified in Rous sarcoma virus, encodes for a transforming protein that directly modifies the cytoskeleton. Disruption of the cytoskeleton may be an early event in the process of malignant transformation and tumorigenesis (12, 13). Other oncogenes implicated in sporadic colon cancer include *c-myc* and *c-erbB2* (14, 15). The increased levels of c-Myc and c-Myb proto-oncogenes have been linked with the promotion of the development of colorectal tumors by suppressing normal apoptotic process (16). The oncogenic effect of c-Myc is also linked with the control of cell cycle progression by controlling the *Cdk4* gene expression (17). c-Myc rapidly increases *Cdk4* gene expression through four highly conserved c-Myc-binding sites within the *Cdk4* promoter (17). In proliferating human colon carcinoma cells (Caco-2), the epidermal growth factor (EGF) induces tyrosine phosphorylation of its receptor and several putative substrates of the receptor intrinsic kinase including c-erb B2. In addition, EGF induces stable association of the GTP-ase activating protein of p21ras to the p190 protein and p62 tyrosine-phosphorylated proteins which could coordinate progression through cell cycle, cell-cell interactions, and cell mobility (18). In another study in a large cohort of well-characterized colorectal tumors, and in a subset of lymph node metastases, no association was observed between either c-erbB-2 protein expression or the presence of the Val(655)Ile nucleotide polymorphism and patient survival (19). Although Val(655)Ile single nucleotide polymorphism is associated with an increased risk of breast cancer (20, 21). These studies suggest that c-erbB-2 is not a prognostic marker in colorectal cancer.

3. FAMILIAL ADENOMATOUS POLYPOSIS

FAP is an autosomal dominant condition which is characterized by multiple benign adenomatous polyps in the colon and rectum. Among hundreds and thousands of adenomatous polyps of the affected individuals, some progress into invasive tumor and metastasis. The polyps usually appear by adolescence or third decade of life. The risk of cancer is generally considered to be related to the polyp number (22). The incidence of FAP in the population is approximately 1 in 8000 (23). According to Knudsen's two-hit hypothesis, colorectal tumors from FAP patients harbor either additional somatic *APC* mutations or loss of heterozygosity at *APC* locus in addition to the germ-line mutation (24). If the germ-line mutation occurs between codons 1194 and 1392, then there is a strong selection of allelic loss of *APC* for the second hit resulting in the development of colorectal adenoma. If the germ-line mutation is outside of this region, then often a second hit in the mutation cluster region (MCR) results in tumorigenesis (25).

4. ATTENUATED FAMILIAL ADENOMATOUS POLYPOSIS

Attenuated FAP (AFAP) is characterized by the presence of less than 100 adenomatous polyps but still carrying a significantly increased risk of the development of colorectal cancer (26). Full colonoscopy is often required to establish the diagnosis because polyps may not be seen in the recto-signoid endoscopy as seen in classical FAP. A variant of FAP, known as Gardner syndrome, refers to the association of colonic polyps with recto-epidermoid skin cysts and benign-osteoid tumors of the mandible and long bones (27). Desmoid tumors, which usually arise in abdominal wall or bowel mesentery, are a cause of significant morbidity and mortality in FAP patients (26, 28). Hereditary desmoid disease, also attributed to mutations in the *APC* gene (28, 29), is characterized by autosomal dominant inheritance of multiple desmoid tumors in absence of colonic polyposis. The other manifestation of FAP is Turcot's syndrome, which refers to the association between multiple colorectal polyps and medulloblastoma, a primary brain tumor found in the cerebellum of children (30). Mutation in *APC* gene also leads to various other clinical conditions such as papillary carcinoma of the thyroid and adrenocortical adenoma (31, 32).

5. ADENOMATOUS POLYPOSIS COLI (APC)

5.1. Structure and transcriptional regulation of *APC* gene

A search for the genetic defect causing FAP led to the identification of the *APC* gene (33-35). *APC* gene encodes a large multi-domain protein that plays an integral role in cancer development. Analysis of *APC* gene and its gene product has revealed a broad spectrum of functions in normal and cancer cells (36). APC is known to associate with microtubules (37), beta-catenin (38), plakoglobin (39), EB1, and DLG (the human homologue of the drosophila discs large tumor suppressor protein) (40, 41). *APC* gene mutations results in a truncated protein product with abnormal function. In the sporadic colorectal tumors, besides the germ-line mutation, the somatic mutations in the *APC* gene are also found (41). The gene encoding human *APC* is localized on chromosome band 5q21-q22 and consists of 16 transcribed exons present on a 98-kb genomic fragment (40-43). The size of exons 1-16 of *APC* gene ranges from 85 to 398 bp, while the last exon, exon 15, is remarkably long consisting 6574 bp of the *APC* DNA. Most mutations occur in exon 16 of *APC* gene. With an 8538 nucleotide mRNA, *APC* encodes for a predicted 312 kDa protein consisting of 2843 amino acids (Figure 2). Mouse *APC*, localized on chromosome 18, has an mRNA of 8535 bp and a similar structure with 90% homology to human *APC,* was originally described as multiple intestinal neoplasia (Min) in mice. Min develops due to mutations in *APC* and it is pathologically similar to FAP (44). About 60% of the *APC* mutations in colorectal tumors are clustered in the central MCR region (amino acids 1284-1580; Figure 2) (45). Mutational analysis of the *APC* gene has shown that the majority of the germ-line mutations identified in FAP patients express a C-terminally truncated protein product by the introduction of a premature stop codon in the MCR region (46).

Recently we have shown that *APC* gene is inducible and is transcriptionally up-regulated by p53 in

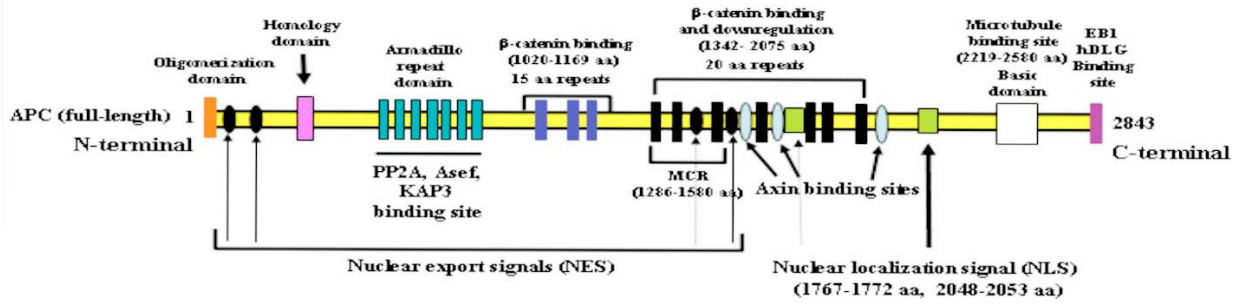

Figure 2. Structural features of the APC protein. Most of the mutations in APC occur in MCR region and create truncated proteins. The truncated proteins contain Asef and beta-catenin binding sites in the 15 amino acids armadillo-repeat domain but looses the beta-catenin regulatory activity which is located in the 20 amino acids repeat domain. Somatic mutations are selected more frequently in FAP patients with germ-line mutations outside of the MCR. Asef, APC-stimulated guanine nucleotide exchange factor; DLG, *Drosophila* discs large; EB1, end-binding protein 1, KAP3A, kinesin superfamily-associated protein 3A; MCR, mutator cluster region; NES, nuclear export signal; NLS, nuclear localization signal; PP2-B56alpha, protein phosphates 2A B56α subunit.

response to DNA damage (47). In order to understand the mechanism (s) by which *APC* gene is induced in response to DNA-damaging agents, we further characterized the *APC* promoter and identified its various regulatory elements. We have established that *APC* gene is transcriptionally regulated by upstream stimulating factors 1 and 2 (USF-1 and USF-2) (48). Our studies provide evidence that the phosphorylation status of p53 can critically up-regulate or down-regulate the *APC* gene expression in colon cancer cells (49). Currently, the consequence of transcriptional up-regulation of the *APC* gene by DNA-damaging agents is not clear. In various studies it has also been shown that the expression of many genes can be controlled through methylation in the promoter region. Hypomethylation could activate oncogenes while hypermethylation could inactivate tumor suppressor genes such as p14 ^{ARF} and p16^{ink4a}, *BRCA1* (50), and *APC* (51). The methylation at CpG sites in the promoter region of *APC* gene has been analyzed in colorectal tumors and cells (51, 52). The primary enzyme responsible for methylation of 5' CG is the enzyme DNA methyl transferase 1 (DNMT1). Interestingly, the *DNMT1* gene expression is indirectly regulated by the mutations in the *APC* gene (53). *DNMT1* induced methylation in the CpG region around the CCAAT-box in *APC* promoter is responsible for silencing *APC* gene expression by changing the chromatin confirmation and interfering with the binding of transcription factor CBF to the CCAAT-box (52). These studies suggest that the hypermethylation of the *APC* promoter provides an alternative mechanism of *APC* gene inactivation in early stages of colorectal tumorigenesis.

5.2. Structure and functions of APC protein

Our current understanding of APC function comes from studies of its protein structure, putative functional motifs, and from analysis of its interacting protein partners. The N-terminus of the APC protein, also termed the homodimerization domain, consists of several heptad repeats (apolar xx apolar xxx). It has been shown that 171 amino acids of APC are sufficient, and the first 55 amino acids are essential for homodimerization (54, 55). Some naturally occurring splice variants and some mutant APC are known in heterozygous cells that may dimerize to the wild-type APC and produce a dominant-negative effect on APC function (55). Whether dominant negative effect of APC is associated with colon cancer development was tested in mice. In these studies a forced expression of amino acids 1-716 or 1-1287 in the intestinal epithelium of mice did not lead to adenoma formation (56). Furthermore, it has also been shown that transgenic mice over-expressing truncated APC protein (Apc1638T) in the intestinal epithelium failed to develop intestinal tumors (57). These findings contradict the dominant negative role of *APC* gene mutations in colon cancer development.

Mice carrying one mutant *APC* allele display a dominant negative effect with a significant decrease in exterocyte migration in the intestinal villus (46). *In vitro* studies demonstrate that normal APC activity is severely abrogated on introduction of mutant APC (58). FAP patients carrying cytogenetic deletions, not just mutations, of *APC* gene have also been identified with a total loss of APC expression and function in these patients (59). The APC protein also contains seven Armadillo (Arm) repeats named for an amino acid motif repeated 13 times in the *Drosophila* homolog of beta-catenin. The Arm repeats are present in a number of other proteins, namely desmosomal proteins plakoglobin, plakophilin, p120-catenin (referred to as p120cas, cadherin-associated Src substrate, originally identified as a major target for this oncogenic tyrosine kinase) (60), the importin family of nuclear import receptors (61), and PF16 microtubule-associated protein (62). In beta-catenin, similar repeats are required for binding with APC, E-cadherin, and the architectural transcription factors belonging to Tcf/Lef family (63). The APC protein contains three 15 amino acid and seven 20 amino acid repeats in central third region of APC. The residues of the 20 amino acid repeats are highly conserved between the repeats.

The homology domain is localized just upstream of the Arm repeats and is highly conserved from fly to human. The Arm repeats are localized between amino acids 453 and 766 and consists seven copies of 42 amino acid motif (64). Varying numbers of Arm repeats have been identified in a variety of proteins with disparate activities

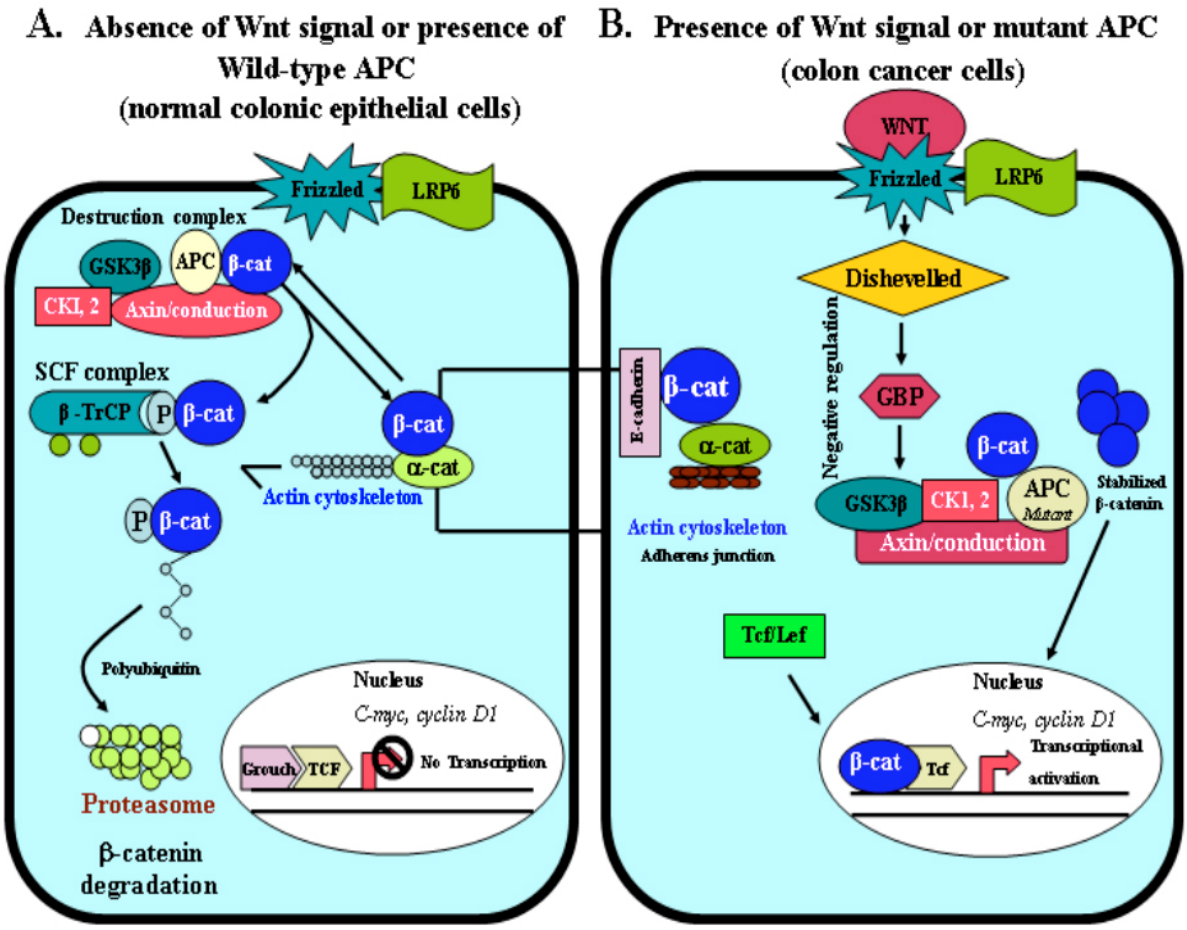

Figure 3. A model for the Wnt-signaling pathway. Panel A depicts the down-regulation of beta-catenin transactivation activity in normal colonic epithelial cells. Beta-catenin remains in a complex of Axin/Axil/conductin, APC, GSK3beta kinase and casein kinase 1 or 2 (CK1 or 2). In the absence of Wnt-signaling, GSK3beta and CK1 or 2 kinases become active and phosphorylate beta-catenin at serine and threonine residues in the N-terminal domain. Axin and APC promote phosphorylation of beta-catenin by acting as a scaffoled protein and bringing together enzyme(s) and substrate(s). The phosphorylated beta-catenin then binds with F-box protein beta-TrCP of the Skp1-Cullin-F-box (SCF) complex of ubiquitin ligases and undergoes proteoasomal degradation. Even though Tcf-Lef transcription factor without beta-catenin may bind to DNA in the absence of beta-catenin, the repressors and corepressors such as CtBP (carboxy-terminal binding protein), CBP (CREB-binding protein), Gro (Groucho), LRP (LDL-receptor-related protein) bind with Tcf-Lef and repress *c-myc* or *cyclin D1* gene expression to control cell cycle progression. Some other known genes which are regulated by beta-catenin/Tcf-Lef pathway are given here – *cyclin D1, CDH1, Tcf-1, c-jun, Fra-1, PPARd, Gastrin, uPAR, MMP7, Conductin, CD44, Id2, Siamois, Xbra, Twin and Ubx*. Panel B shows the role of mutations in the APC or beta-catenin protein in the regulation of beta-catenin level and its transactivation property in colon cancer cells. The mutant beta-catenin escapes its degradation through Wnt pathway and becomes stabilized in the cytoplasm. The stabilized level of beta-catenin then heterodimerizes with Tcf-Lef transcription factor and locates into the nucleus, where it actively transcribes cell cycle related genes causing cellular proliferation. The binding of beta-catenin with Tcf-Lef inhibits the binding of CtBP, CBP, Gro or LRP and potentiates its transcriptional activity.

including nuclear transport, cell adhesion, cell cycle control, and microtubule stability (55). This domain has been designated as a protein-protein interaction domain (Figure 2).

There are few proteins identified which bind to APC's Arm repeat region. These are B56 regulatory subunit of protein phosphatase 2A (PP2A), APC-stimulated Rac-specific guanine nucleotide exchange factor (Asef), and kinesin superfamily-associated protein 3A (KAP3A) (65-67). PP2A is one of the four major serine/threonine protein phosphatases whose regulatory domain interact with the Arm repeats of APC, while the catalytic subunit of this enzyme can also bind to Axin (68). It has been found that APC interacts with the kinesin superfamily (KIF) 3A and 3B proteins and microtubule plus-end-directed motor proteins through an association with the kinesin superfamily-associated protein 3 (KAP3) (66). The interaction of APC with KAP3 was required for its accumulation in clusters with Axin, while mutant APC derived from cancer cells were unable to interact efficiently (66). Axin and APC are both components of a tetrameric

destruction complex of the Wnt-signaling pathway. The Wnt-signaling pathway plays a key role in development, cellular proliferation, and differentiation. The disregulation of Wnt-signaling pathway results in multiple human malignancies, including the development of FAP (Figure 3). The co-localization and functional studies suggest that the APC-Asef complex may regulate the actin/cytoskeletal network, cell morphology, cell migration, and neuronal function.

Both three 15 amino acid repeats and seven 20 amino acid repeats of APC are involved in the binding with beta-catenin (69, 70). The binding of free cytoplasmic beta-catenin to the 20 amino acid repeat consensus region (TPxxxFSxxxSxSxL) of APC is modulated by phosphorylation through a serine-threonine kinase glycogen synthase kinase-3beta (GSK3beta) (70). The 15 and 20 amino acid repeats of APC are highly conserved from fly to human, which also interact with Axin/conductin. The Axin/conductin were originally identified as inhibitors of the Wnt-signaling pathway (71). They form a tetrameric destruction complex together with APC, beta-catenin and promote the phosphorylation of beta-catenin and subsequently mediate its ubiquitination and degradation in the proteasome (72-74), thereby controlling the Wnt-signaling pathway (Figure 3) (75). The C-terminal region of APC is known as the basic domain region which contains many arginine, lysine, and proline residues localized between amino acids 2200 and 2400. The basic domain of APC contains microtubule binding site (37). The C-terminal region of APC may play a role in cell cycle progression or growth control through binding to at least three different proteins, namely EB1, hDLG (human homologue of *Drosophila* disc large tumor suppressor gene), and protein tyrosine phosphatase (PTP)-BL (36, 40, 41, 75, 76). The yeast homologue of EB1 and Bim1p binds alpha-tubulin and localize to the mitotic spindle and to cytoplasmic microtubules (77, 78). The association of EB1 with microtubule cytoskeleton of the mitotic spindle is important for spindle assembly during cell cycle.

The hDLG is a member of the family of membrane-associated guanylate kinases, which localize at the sites of the cell-cell contacts of epithelial cells and in the presynaptic nerve termini of the central nervous system. These proteins are involved in the maintenance of cell polarity, migration, and blocking of cell proliferation. Nuclear localization of APC has been reported in few cell types (79). Since APC is a large protein to diffuse passively into nucleus, it is possible that APC is shuttled by an unconventional mechanism or else it is also possible that protein configuration tightly regulates its export and import into the nucleus. The structural analysis of APC showed two potential nuclear localization signals (NLSs) comprising amino acids 1767-1772 and 2048-2053 (80). Both APC NLSs, which are well conserved among human, rat, mouse, and fly, are necessary for optimal nuclear import. It has been demonstrated that phosphorylation of the NLS may inhibit nuclear import of wild type APC. This provides a regulatory mechanism for nuclear-cytoplasmic shuttling of APC. Since, APC is present in the nucleus it can directly interact with A/T-rich DNA and serve as a transcription factor (81). Recently, several groups identified the presence of nuclear export signals (NES) at the N-terminus of APC protein. There are at least five nuclear export signals, of which three NESs are located at amino acids 68-77, 165-174 and 1472-1481 (82). These NES's are located within the 20 amino acid repeats of beta-catenin binding domain of APC (80, 82). Only the first two nuclear export signals are functionally active. The highly conserved NES sequences are used to shuttle nuclear beta-catenin to the cytoplasmic destruction complex. The absence of the NES sequences leads to the accumulation of beta-catenin in the nucleus, which causes an inadvertent activation of Wnt target genes that may possibly be involved in tumor development. However, emerging evidence suggests that endogenous APC and beta-catenin can interact within nucleus, and subsequently APC may export beta-catenin from nucleus to cytoplasm and terminate Wnt-signaling, which may block the expression of cell cycle related genes. The control of APC's nuclear import is possibly regulated through phosphorylation near NLSs (80).

5.3. Mutations in *APC* gene

Most somatic mutations clustered between codons 1286 and 1513 are located in the central MCR region of the *APC* gene. APC mutations within the MCR region generate truncated APC proteins that lack most of the Axin-binding sites. The sequences upstream of the MCR might encode APC protein whose function is essential for cellular survival or tumor progression, while sequences downstream of the MCR might encode APC protein for tumor suppressor function of *APC* gene (83, 84). The following is the list of cell lines which have been characterized for their mutations in *APC* gene (Table 1) (84, 85).

Colorectal tumors from FAP proteins carry additional somatic mutations or loss of heterozygosity (LOH) in the *APC* gene locus in addition to the original mutation (86). The most common mutations in FAP occur at codon 1061 and 1309, which account for third of all mutations. If the germ-line mutation occurs between codons 1194 and 1392, then there is strong selection for allelic loss of APC. Somatic mutations are found in the majority of colorectal adenomas and carcinomas, including adenomas less than 5 mm in size (87). Somatic mutations results in the loss of both wild-type alleles of *APC* gene followed by DNA mismatch repair (MMR) genes in the majority of sporadic colorectal cancers (87).

6. BIOLOGICAL FUNCTIONS OF APC

6.1. Regulation of beta-catenin level

The functional clues of APC were assigned to its interaction with beta-catenin (88, 89). It has been demonstrated that APC and beta-catenin are important components of Wnt growth-factor-signaling pathway. The Wnt-signaling pathway has been well studied and characterized in the development of *Dictyostelium, Drosophila*, *Xenopus*, and animals (90-93). In *Drosophila*, the ubiquitously expressed APC localizes in the adherens junctions in epithelial cells of the embryo and in ovarian germ cells (92). This localization is actin-dependent and

Table 1. Mutation-site analysis in colon cancer cells

Cell line	First Mutation	Second Mutation
HCA 46	213	
C 32	776	-
LS 411	789	1556
C 75	811	1450
COLO 320 DM	811	-
HT 29	853	1555
LOVO	1114	1430
SW 948	1114	1429
HT 55	1131	1303
SW 403	1197	1264
C 106	1238	1490
SW 1222	1306	-
C 8	1307	1313
C 6	1309	1309
C 70	1309	-
LS 1034	1309	-
SKCO 1	1317	1443
C 14	1135	1340
SW 480	1338	-
SW 620	1338	-
VACO 4A	1354	-
VACO 4S	1354	-1373
C 99	1367	-
Ca CO 2	1367	-
C 23	1408	-
DLD 1	1416	1418
HCT 15	1417	-
GP 2D	1444	-
HRA 19	1450	-
SW 837	1450	-
SW 1417	1450	-
C8 4	1451	-
VACO 10 M	1454	1472
T 84	1488	-
COLO 205	1554	-
COLO 678	1554	-
VACO 5	1554	1499

The data presented in this table depict two hits on the *APC* gene in colorectal cancer cell lines derived from patients. These cell lines also show if the first mutation occurs out side of the MCR, the second mutation falls within the MCR (77). In these cell lines either the first or the second mutation introduces a nonsense codon in the *APC* gene, which transcribes a truncated APC protein.

seems to be mediated by the Arm-domain of APC, since a single amino acid deletion or exchange in this domain, APC(N175K), results in delocalization of APC from plasma membrane (88). Wnt-signaling is initiated by binding of Wnt to their receptors encoded by the gene frizzled (94-96). Binding of Wnt to the receptor leads to the phosphorylation of disheveled protein (Dsh) which through its association with Axin prevents GSK3beta, enabling GSK3beta to phosphorylate beta-catenin (Figure 3) (74, 97, 98). The phosphorylation of beta-catenin by GSK3beta leads to ubiquitination of beta-catenin by beta-transducin repeat-containing protein (beta-TRCP), an F-box component of the E3 ubiquitin ligase complex that recruits an E2 ubiquitin conjugating enzyme and promotes ubiquitination. The ubiquitinated protein is then targeted for subsequent degradation by the 26S proteasome system, while unphosphorylated beta-catenin escapes the recognition by beta-TRCP (73). Inhibition of both GSK3beta and proteasome could result in a rapid reduction of phosphorylated form of beta-catenin levels leading to the stabilization of the unphosphorylated form of beta-catenin in cytoplasm. The phosphorylated form of beta-catenin interacts with Tcf/Lef but cannot form ternary complex with DNA. Whether Dsh binds directly to Frizzled, the seven trans-membrane receptors for the Wnt ligands (98), or whether intermediary proteins are involved in the signal transmission between Frizzled and Dsh is unknown at present. It has been reported that Dsh interacts with a number of other molecules including the casein kinase 1 (CK1) and casein kinase 2 (CK2) and the inhibitor of Wnt-signaling protein GBP/Frat-1 (99-101). Activated Dsh subsequently inhibits the stabilization of the beta-catenin

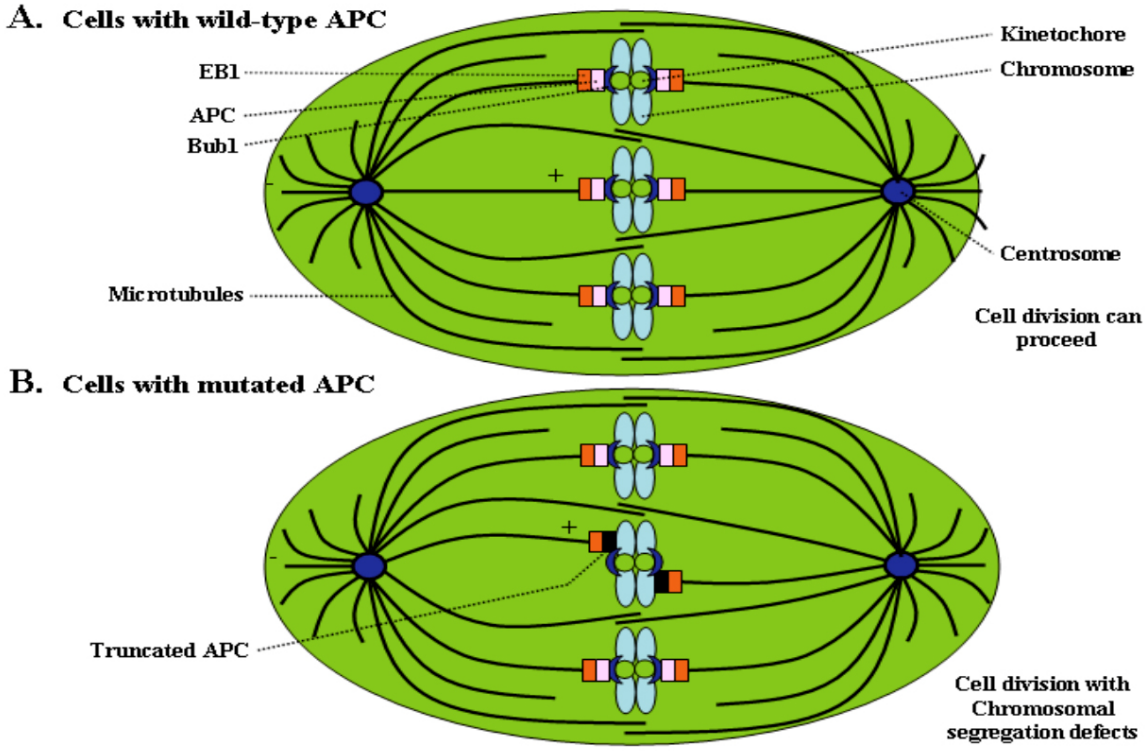

Figure 4. Chromosomal instability (CIN) in cells carrying mutations in *APC* gene. Panel A shows a model for the interaction of APC with plus-end of microtubules through EB1 and with kinetochore of chromosomes through Bub1 in normal colonic epithelial cells. APC can also bind microtubules directly *via* the C-terminal basic domain. Panel B shows a disruption in the interaction between spindle microtubules and kinetochores due to expression of truncated form of APC in colon cancer cells. Failure to interact efficiently with spindle microtubules and kinetochores results in defective chromosomal segregation during cell division.

destruction complex by directly binding to Axin (102). As a result there is an accumulation of free cytosolic beta-catenin. The unphosphorylated beta-catenin escapes recognition by beta-transducin repeat-containing protein (beta-TRCP), a component of an E3 ubiquitin ligase, and translocates to the nucleus where it engages with Tcf/Lef family transcription factors (76, 103). beta-catenin lacks a DNA-binding domain but contains an activation domain. Once beta-catenin binds to Tcf/Lef transcription factors, it stimulates the transcription of the Wnt target genes such as the cell cycle regulator *c-myc* (104), the G_1/S-regulator gene *cyclin D1* (105, 106), the gene encoding the matrix-degrading metalloproteinase *matrilysin* (107), the AP-1 transcription factors *c-jun* and *fra-1*, urokinase-type plasminogen activator receptor *WISP-1* (108), *PPAR-delta* (109), *AF17* (110), and the ectodermal-neural cortex 1 *(ENC1*) (111, 112). Mutations in APC, beta-catenin or axin/conductin can essentially influence the Wnt-signaling pathway. This can lead to continuous activation of target genes and initiate the process of tumorigenesis (113-115). Wnt-pathway is also critically regulated by the beta-catenin levels. In the normal or unstimulated cells beta-catenin levels are very low but both soluble and membrane bound protein accumulates during the Wnt-signaling pathway. The reduction of nuclear beta-catenin and beta-catenin-mediated transcription depends on a nuclear export function of APC (82, 116) which seems to be critical for its function as a tumor suppressor.

6.2. Chromosomal instability

Genetic instability is a central part of human cancer development (117). Two distinct chromosomal instability pathways for colorectal carcinogenesis have been identified. One is the microsatelite instability (MSI) and the other is chromosomal instability (CIN) (118). The MSI is caused due to a defect in mismatch repair machinery that results in a mutator phenotype at the nucleotide level in the microsatellite region of DNA (119, 120). DNA-damaging agents can further induce genetic instability in cells harboring defective DNA repair genes (121). The CIN and MSI are associated with two major inherited syndromes, FAP and HNPCC, respectively. In CIN, tumors exhibit a defect in chromosomal segregation, which results in variation of chromosome numbers among cells from individual clones (119, 120). Allelic imbalances have been observed in early colonic adenomas which are consistent with a potential role for CIN in tumor progression (121-124).

It has been shown that the C-terminus of APC is involved in maintaining chromosomal stability during mitosis. Recently, Fodde *et al*. (120) and Kaplan *et al.*

(125) suggested that the wild-type APC is involved in maintaining the proper orientation of chromosomes and serve as a platform between microtubules and chromosomes. APC binds at the plus ends of the microtubules through EB1, stretches it to the chromosomes, and inserts them into the kinetochores after binding with Bub1. It has been shown that APC co-localizes to kinetochores and forms complexes with Bub1 and Bub3, the two mitotic checkpoint proteins (Figure 4) (120, 125). It is also speculated that the phosphorylation of APC by Bub-kinases can negatively regulate kinetochore-microtubule attachment. In other words, the phosphorylation of APC could lead to decreased binding of microtubules with APC (126). The successful complex formation may facilitate proper growth of spindle formation and help in maintaining the ploidy of the cells. Once the *APC* gene is mutated, the truncated APC protein may loose its ability to bind with Bub1 and subsequently it may not properly align for the attachment of microtubules at kinetochores, resulting in defective segregation of chromosomes (125, 127). Accordingly, APC-mutant cells can have an abundance of spindle microtubules but cannot connect to kinetochores and develop CIN. Since the mitotic checkpoint gene *Bub-1* plays an important role in connecting APC with kinetochores, its loss may also induce CIN (123).

6.3. Cell migration and adhesion

Another important role for APC is assigned in cell migration. Colonic epithelial cells, derived from a committed stem cell, divide in the lower two-third of the crypts and migrate rapidly to the surface to form a single layer (128). During migration, they are differentiated into absorptive, secretory, paneth and endocrine cells. The function of a wild-type APC is necessary in maintaining the direction of upward movement of these cells along the crypt-villus axis. Loss of wild-type APC functions due to loss of expression or mutations affect cell migration. These cells, instead of migrating upwards towards the gut lumen, migrate aberrantly or less efficiently towards the crypt base where they accumulate and form polyps (129). In due time, these cells become aneuploid due to defects in chromosome segregation as well as acquiring beta-catenin stabilization and activation of genes for cell proliferation. The mechanisms by which APC might be involved in cell migration can be understood by its association with the kinesin superfamily-associated protein KAP3 that has been established in cell-cell adhesion and migration. It has been shown that APC, mediated by KAP3, interacts with kinesin motor proteins which transport it as well as beta-catenin along the microtubules to the growing ends of the cytoskeletal protruding into motile cell membranes (66, 130). At the tip of the microtubule, APC interacts with the end-binding protein EB1 and protein tyrosine phosphatase PTP-BL. PTP-BL modulates the steady state levels of tyrosine phosphorylations of APC associated proteins such as beta-catenin and GSK3 beta. In fact, GSK3 beta kinase activity has been implicated in microtubule dynamics (131, 132). In epithelial cells, since endogenous APC localizes at the tips of microtubules invading areas, microtubule depolymerizing agents have been found to inhibit the migration of epithelial cells (133). Recently, experimental evidence was presented describing the mechanisms by which the mutated APC might play a role in the migration of colorectal tumor cells (75). In these studies, an interaction of APC has been shown with Asef that may regulate the actin cytoskeletal network (67, 134). APC binds with Asef and controls its activity. Asef is activated in colorectal cancer cells containing truncated APC. Active Asef decreases E-cadherin-mediated cell-cell adhesion and promotes cell migration. Thus, the dynamic association of APC, EB1, Asef, catenins, EGFR or c-Met receptor, PTP-BL and E-cadherin proteins at cell-cell adherence junctions and microtubule ends play an important role in cell-cell communication, cell migration and carcinogenesis. Recently, the role of Rho GTPase and its effector, the formin mDia (Rho-mDia) has been implicated in cell migration (135). In these studies it is shown that lysophosphatidic acid (LPA) stimulates Rho-mDia to capture and stabilize microtubules in fibroblasts in association with APC and EB1. Expression of either full-length EB1 or APC, but not an APC-binding mutant of EB1, is sufficient to stabilize microtubules. These studies concluded that an evolutionarily conserved pathway for microtubule capture exists in which mDia functions as a scaffold protein for EB1 and APC to stabilize microtubules and promote cell migration. In a recent study using a novel inducible Ahcre transgenic line in conjunction with a loxP-flanked Apc allele, it has been further shown that loss of Apc acutely activates Wnt-signaling through the nuclear accumulation of beta-catenin and perturbs differentiation, migration, proliferation, and apoptosis (136).

Actin cytoskeletal integrity is necessary to maintain the shape and adherence junctions of cells. The imbalance in actin cytoskeletal integrity can cause disturbance in cell-cell adhesion and cell migration. The role of APC in actin cytoskeletal maintenance is predicted through its interaction with beta-catenin. Beta-catenin establishes a link between APC and actin by providing a bridge to alpha-catenin (137). In *Drosophila*, mutations in APC have been shown to affect the organization of adherence junctions (41, 138, 139). Another link of APC with actin is shown through its interaction with PDZ domain of DLG protein. Since APC co-localizes with DLG in the cytoplasm in rat colon epithelial cells, the APC-DLG complex may participate in regulation of cell cycle progression (41). Mutant APC lacking the S/TXV motif for DLG binding exhibits weaker cell cycle blocking activity at G_o/G_1 phase than the intact APC (140).

Interaction of APC with beta-catenin and the members of the cadherin family of proteins have been implicated in cell-cell adhesion (113, 133, 141). The C-terminal domain of E-cadherin interacts with beta- and gamma-catenin, which associate with alpha-catenin and form an E-cadherin complex with actin cytoskeleton. This complex maintains the stable cell-cell adhesion (141). APC becomes a part of the cell-cell adhesion complex linked with E-cadherin, since it directly binds with beta-catenin, gamma-catenin, and actin filament (113, 114). The tyrosine phosphorylation of beta-catenin by epidermal growth factor (EGF), hepatocyte growth factor (HGF) and c-Met receptors is important in modulating cadherin-catenin complexes from membrane bound form to free cytosolic

form (142). The phosphorylation of beta-catenin at tyrosine residue, which is blocked by tyrosine phosphatase Pez, is involved in epithelial cell migration (143). If the Wnt-pathway and the EGFR or c-Met receptors pathway are activated at the same time, then the degradation of beta-catenin can be inhibited and it may translocate to the nucleus, bind to the Lef-Tcf transcription factor, and down-regulate the transcription of E-cadherin gene, *CDH1*, expression (144). These complex interactions may finally result in the reduction of E-cadherin-mediated cell-cell adhesion and proliferation of cells (137, 145, 146). Recently, it has been reported that histologically normal enterocytes in a Min (Apc/+) mice migrated more slowly in vivo than enterocytes with either wild-type (Apc+/+) or with heterozygous loss of Apc protein (Apc1638N) (147). Further the effect of the Apc(Min) mutation was observed upon cell-cell adhesion by examining the components of the adherens junction (AJ), in which a reduced association was observed between E-cadherin and beta-catenin in Apc(Min/+) enterocytes (148).

6.4. Cell cycle regulation

Consistent with its role as a tumor suppressor, an increased expression of APC has been found to arrest the cell cycle in the G_o/G_1-S phase (149-151) and G_2/M phase (152). Overexpression of APC blocks cell cycle progression from G_o/G_1 to S phase by negatively modulating the activity of cyclin-Cdk complex (149). Overexpression of hDLG, which interacts with APC, suppresses cell proliferation by blocking cell cycle progression from the G_o/G_1 to the S phase, suggesting that this complex plays an important role in transducing the APC cell cycle-blocking signal (140). Mutant APC lacking the interaction sites with hDLG fails to effectively block cell cycle progression, further indicating that the APC-hDLG complex formation is necessary for cell cycle arrest (153). Mutant APCs recovered from FAP and/or sporadic colorectal tumors are less effective in arresting cell cycle progression than the normal APC. The cell cycle-blocking activity of APC was alleviated by the overexpression of cyclin E/Cdk2 or cyclin D1/Cdk4 (149).

The role of APC in cell cycle control with damaged DNA is yet not clear. There are two levels of cell cycle arrest in cells challenged with DNA-damaging agents, one at the G_o/G_1 phase and other at the G_2 phase. Cells with damaged DNA have an opportunity to repair the damaged DNA during arrest in G_o/G_1 or G_2 phases before proceeding to the next phase of cell cycle. If the damaged DNA is not repaired on time during these two phases, then cells may encounter either the programmed cell death pathway or become carcinogenic. In our studies, we found that treatment of HCT-116 colon cancer cells with lower concentrations of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) increased APC levels which was associated with increased G_2/M phase arrest of these cells (154). However, at higher concentrations of MNNG treatment the APC levels were decreased and cells were arrested senescence-like growth arrest which was also correlated with the loss of microtubule organization and telomeric DNA (154). In a recent study we have shown that the treatment of colon cancer cells with zinc chloride increases the APC protein level in colon cancer cells. We further showed that the increased levels of APC were linked with G_2/M phase arrest of these cells (152). A role for APC at the G_2/M arrest has also been explored with the observation that APC is hyper-phosphorylated during M phase and is a target of the M phase kinase Cdc2 (155, 156). In a recent study using MCA3D (mouse immortalized epidermal keratinocytes) and HaCa4 (mouse squamous cell carcinoma) cell lines, the role of wild-type beta-catenin and APC has been shown in cell cycle arrest and apoptosis (157). These studies showed an increased cytoplasmic and nuclear localization of beta-catenin and APC during S and G_2/M phase arrest of the cell cycle. From these studies it is clear that APC is involved in cell cycle control in various cell types including colon cancer cells.

6.5. Apoptosis

Biological processes are governed with a homeostasis mechanism that regulates cell birth and cell death. Dysfunction of the physiological pathways of programmed cell death promotes the proliferation of malignant cells. One of the earliest manifestations of this imbalance in the homeostasis of cells is the formation of polyps in colon cancer development. The role of APC in apoptosis is controversial and its precise mechanism remains to be understood. In some studies it is reported that the increased level of APC induces apoptosis, and in other studies the decreased or mutant protein level of APC is linked with apoptosis. Recently it had been shown that overexpression of APC in human colorectal cancer cell lines containing an endogenous inactive *APC* allele results in a substantial diminution of cell growth due to the induction of cell death through apoptosis (158). Contrary to the overexpression, the down-regulation of APC in mice has been found to increase apoptosis. In these studies, mutant mice harboring *APC* disrupted specifically in the neural crest by using the Cre-loxP recombination system developed severe craniofacial and cardiac defects due to massive apoptosis in the neural crest development (159). These studies suggest that, apart from its role in colon carcinogenesis, APC also plays a role in neural crest development (159). In vitro antisense inhibition of APC has been shown to increase beta-catenin protein expression leading to an incomplete myotube formation due to increased apoptosis. This suggests a role for the APC/beta-catenin pathway in myotube development.

A dynamic process is continuously maintained in colonic mucosa where migrating cells achieve apoptosis and newly generated cells move upward (160). In this process, the disruption of APC impairs the equilibrium between new cell formation at the base of the crypt and cell death at the top of villus, leading to the relative expansion of the mutant cells (161). There are several other studies in which the reduced level of APC is found to be linked with apoptosis. It has been observed that proteolytic cleavage of beta-catenin, gamma-catenin, APC, E-cadherin, and Rb are involved in drug-induced apoptosis of cancer cells (162-168). Our studies support the hypothesis that a reduced level of APC is associated with apoptosis in colon cancer cells. We have shown that C_2-ceramide induced apoptosis in colon cancer cells and the reduced levels of APC are

responsible for apoptosis (169). We have also described that the decreased levels of APC along with beta-catenin and E-cadherin are involved in curcumin-induced apoptosis in colon cancer cells (170). The stress-induced decrease in the APC protein level can be due to activation of caspase proteases, stress-activated protein kinases, and mitochondrial pro- and anti-apoptotic factors (169-173). Recently, it has also been shown that the APC levels are necessary to regulate the caspase activity in Min (APC/+) mice harboring multiple polyps, indicating that mutant forms of APC can induce repression of selected terminal caspases as a potential means of attenuating responses to apoptotic stimuli (174). Results also described that a reduction in caspase protein levels resulted in resistance to apoptotic-inducing agents and restoration of caspase levels reinstated apoptotic capacities (174). Taken together, it appears that the both decreased and increased levels of APC play a role in apoptosis in which the APC may not be playing a direct role but may be involved through other factors to execute its action. Also the type of signal induced in the cell may also determine whether and how increased or decreased levels of APC are involved in apoptosis. These ideas need to be tested in future studies.

7. SUMMARY AND PROSPECTIVE

Colorectal cancer is a consequence of mutations in several genes in the complex pathway of carcinogenesis. It has been established that mutations in *APC* gene are one of the earliest events in the process of colorectal carcinogenesis. Most frequent mutations in *APC* gene are localized in the MCR region of the *APC* gene. However, it is still unclear how the mutations in MCR region could initiate the process of colorectal carcinogenesis. Inactivation or malfunction of APC's tumor suppression function results in the onset of colorectal carcinogenesis. There are several known functions of APC, among which are its involvement with Wnt-signaling pathway, cell migration, cell-cell adhesion, CIN, cell cycle regulation, and apoptosis. Loss of APC function is a key step in the oncogenic activation of beta-catenin and promotion of malignant transformation of normal colorectal epithelial cells. The APC exists in various pools of cytoplsmic and nuclear fractions; however, their exact function within the cell is not clear. It has been shown that APC can shuttle between the nucleus and cytoplasm. Future studies should focus to decipher the role of cytoplasmic and nuclear fractions of APC and beta-catenin in colon cancer development. Among the many functions described for APC, it is not clear whether one or combination of them is necessary to induce colorectal tumorigenesis. These issue need to be more clearly understood so that an appropriate chemo- or gene therapy approach can be developed for colon cancer treatment. Additionally, future studies should focus on the use of potential chemopreventive agents extracted from medicinal plants to determine their mechanisms for the prevention of colorectal cancer. The plant products along with surgery and radiation may prove a safe and useful strategy for dealing with this deadly disease.

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Abbreviations: APC, adenomatous polyposis coli; AFAP, attenuated familial adenomatous polyposis; Arm, armadillo; Asef, APC-stimulated Rac-specific guanine nucleotide exchange factor; beta-TRCP, beta-transducin repeat-containing protein; CK, casein kinase; CIN, chromosomal instability; DLG, drosophila discs large; FAP, familial adenomatous polyposis; GSK3beta, glycogen synthase kinase-3beta; HNPCC, hereditary nonpolyposis colorectal cancer; KAP3A, kinesin superfamily-associated protein 3A; MCR, mutation cluster region; MMR, mismatch repair; MSI, microsatelite instability; NES, nuclear export signal; NLS, nuclear localization signal; PP2A, protein phosphatase 2A; PTP, protein tyrosine phosphatase; Tcf/Lef, T-cell factor/lymphoid enhancer factor

Key Words: Adenomatous polyposis coli, Apoptosis, Beta-catenin levels, Cell cycle regulation, Cell migration and adhesion, Colorectal cancer, Chromosomal instability, Familial adenomatous polyposis, Mutator cluster region, Review

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