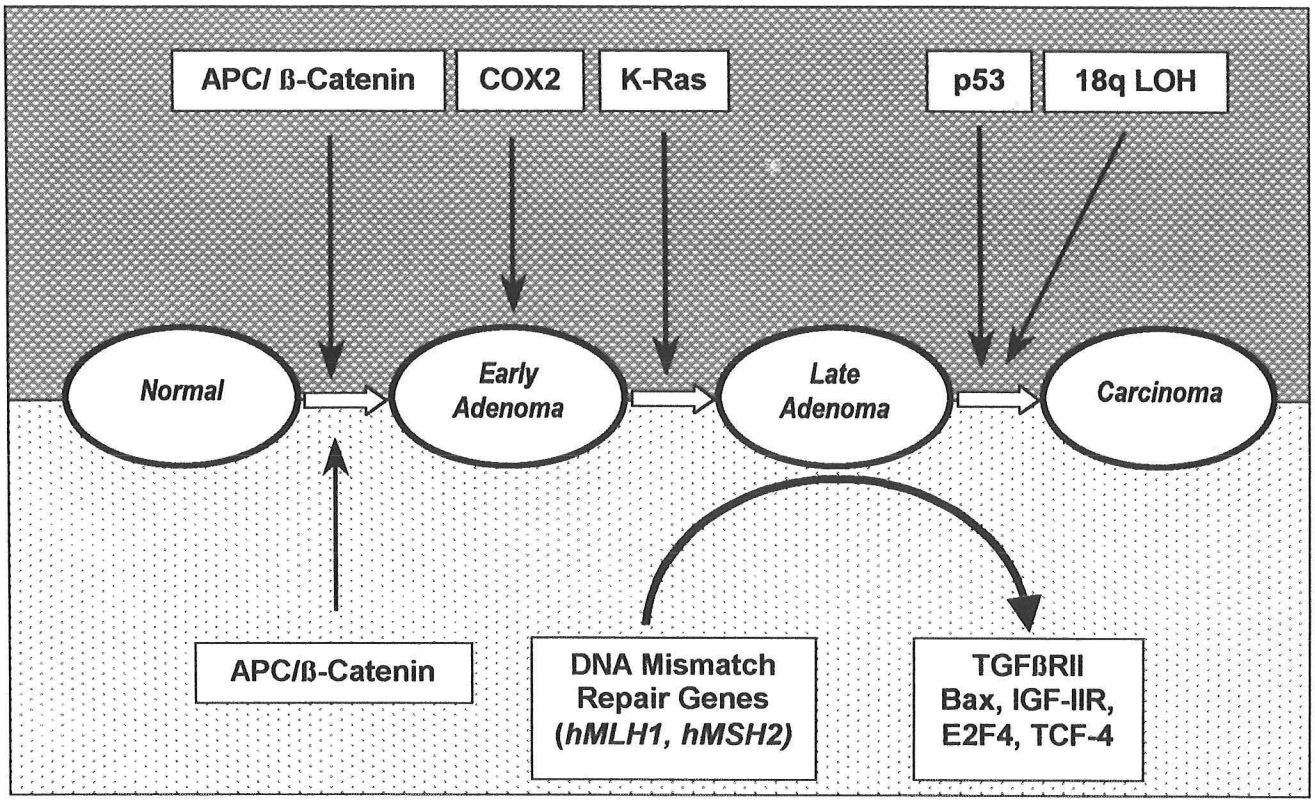

An Update in the Genetics of Colorectal Cancer

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Interests: Colorectal cancer screening
Telomerase/telomere biology
Epithelial cell cancer progression

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INTRODUCTION

Colorectal carcinoma (CRC) is the 3rd most common internal malignancy in the United States. On average, Americans have a 6% lifetime risk of developing the disease. Approximately 135,400 new cases were diagnosed and 55,000 deaths were attributed to CRC in 2001 (data from www.cancer.org). Over the past 15 years, major advances have been made in our understanding of the natural history of colon cancer. One consequence of this knowledge has been the implementation of population-based screening for all American men and women over the age of 50 (reviewed extensively elsewhere[1]). Our understanding of the molecular mechanisms underlying colon cancer progression has also increased tremendously, and this has translated into improved risk assessment by genetic testing in targeted individuals.

Approximately 80% of all colorectal cancers are considered to be "sporadic", with no evidence that the disease was inherited. In the remainder of cases, there appears to be a significant genetic component. Although the etiology of CRC is heterogeneous, several common themes have emerged involving both sporadic and inherited CRCs. The two best characterized genetic syndromes, familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC), together account for only 10% of all CRCs. Yet, these disorders continue to play an important role in the elucidation of additional genetic events, which will ultimately translate into improvements in the clinical management of all individuals at risk for colorectal cancer.

ADENOMA-CARCINOMA SEQUENCE

The natural history of CRC is well described, and strongly suggests that the majority of colorectal cancers arise from adenomatous polyps with a transition time of approximately 10 years. In 1988, Fearon and Vogelstein published a novel paradigm for colorectal tumorigenesis[2]. They proposed that colorectal cancer development is a multi-step process, resulting from the accumulation of genetic alterations in adenomatous polyps

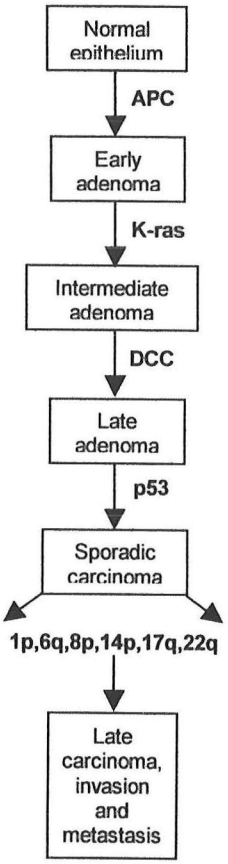

Figure 1. *Putative genetic pathway in sporadic colorectal carcinogenesis.*

(Figure 1). The number of genetic events and the order in which they occur appear to be somewhat variable, but our best understanding is that virtually all cancers must acquire six functional capabilities[3] (Table 1). The mechanism by which this is accomplished probably varies from one cancer type to another. This protocol will

review the major genetic alterations associated with the development of sporadic and heritable colorectal cancer, utilizing as a framework the six cancer hallmarks.

Table 1. *The Six Hallmarks of Cancer*

- Self sufficiency in growth signals
- Insensitivity to anti-growth signals
- Evasion of apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

THE SIX HALLMARKS OF CANCER

Self-Sufficiency in Growth Signals: the Ras Oncogene

All normal cells require growth signals in order to move from a quiescent state into a state of active proliferation. Growth signaling molecules such as extracellular hormones, growth factors, and cytokines bind to transmembrane receptors, which in turn communicate with the nucleus of a cell through a network of intracellular signaling pathways[3,4]. Normal cells will proliferate only as long as there is an abundant supply of appropriate diffusible growth factors and a proper substratum. In contrast, tumor cells universally demonstrate a markedly reduced dependence on exogenous growth factors. This independence is accomplished in a variety of ways including: 1) production of their own growth factors, 2) alteration of the transcellular transducers of growth signals, or 3) alteration of the intracellular circuitry that converts the growth signals into action.

The *ras* genes (K-ras, H-ras, and N-ras) produce a small family of G proteins with a potent transforming potential. The activity of Ras proteins is controlled by a cycle between a GDP-bound inactive state and a GTP-bound active state (Figure 2). Ras proteins situated at the inner face of the plasma membrane are transiently activated in response to a variety of extracellular growth signaling molecules. Activated Ras, in turn, stimulates a cascade of serine/threonine kinases that activate multiple signaling pathways. Thus, Ras acts as a molecular switch, converting the signals from the cell membrane to the nucleus[5]. A number of recent reports suggest that different Ras homologues preferentially mediate distinct

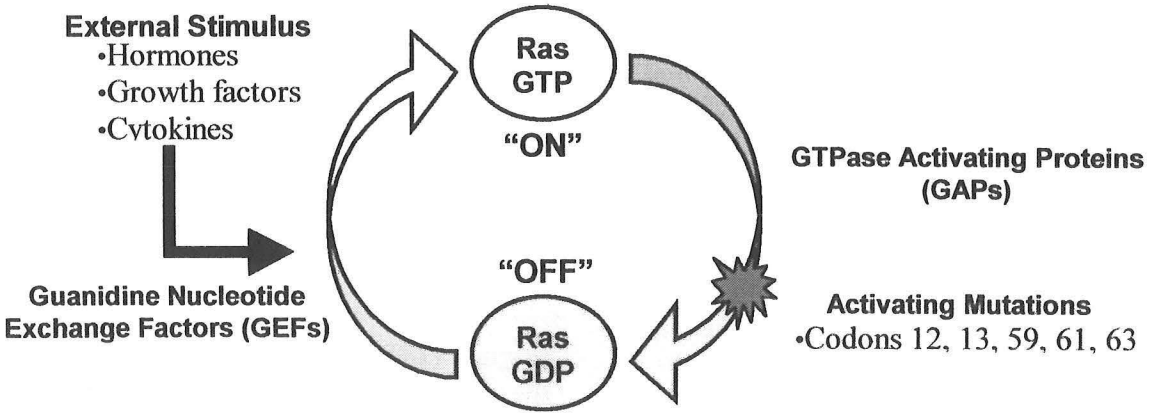

Figure 2. *The Ras molecular "on/off" switch. Ras localizes to inner plasma membrane. In response to external signals, GEFs are recruited and promote GDP/GTP exchange, leading to transient activation of Ras. Activating Ras mutations render it insensitive to the hydrolysis by GAPS. From*[5].

cellular processes, including proliferation, differentiation and apoptosis[6,7].

Ras gene mutations are the most frequently mutated oncogenes detected in human cancer, having been identified in approximately 30% of all cancers and 50% of sporadic colon carcinomas. Oncogenic mutations in *ras* encode constitutively activated proteins that enable them to release mitogenic signals into cells without stimulation by their normal upstream regulators, thereby stimulating cell proliferation and inhibiting apoptosis. The majority of *k-ras* mutations, gain-of-function missense point mutations in codons 12,13 and 61, occur early during the pathogenesis of colorectal cancer. In the largest series, Andreyev et al. evaluated over 2,721 colorectal cancer cases for *k-ras* mutations[8]. They found that the presence of a codon 12 glycine to valine (G12V) mutation (8.6% of all cases) independently increased the risk of CRC recurrence ($p = 0.007$) and death ($p= 0.004$). Thus, for CRCs, a G12V mutation of *K-ras* appears to predispose to more aggressive biological behavior in patients with advanced cancer.

One mechanism by which mutant *K-ras* may confer a more aggressive phenotype is by conferring resistance to the growth inhibitory properties of Transforming Growth Factor-β (TGF-β)[9]. Although *K-ras* expressing cancer cells become much more sensitive to TGF-β, the nature of their response is altered such that added TGF-β results in more rapid growth. This paradoxical growth-inducing effect of TGF-β is consistent with studies showing elevated levels of TGF-β in the serum of CRC patients with progressive disease. The importance of TGF-β in colorectal carcinogenesis is now being fully realized as a result of recent studies demonstrating an important link between the mismatch DNA repair system and the TGF- β receptor. This is discussed in further detail below.

Insensitivity to Anti-growth Signals: the Retinoblastoma Protein (pRb)

The second hallmark of cancer involves alterations in the negative control of normal cellular proliferation. The retinoblastoma (Rb) gene was first identified in association with an inherited eye tumor. Its role as a tumor suppressor was suggested when it was demonstrated that the disease was caused by a loss of function of the Rb protein product, pRb. Although mutations in the Rb gene *per se* are uncommon in colorectal cancer, a variety of genetic alterations in its downstream effectors have been identified, most of which achieve a similar endpoint.

When a cell leaves the quiescent phase, G0, to enter the metabolically active phase, G1, the decision to undergo division is made at the restriction point (R point). The cell cycle is controlled by the orderly activation and inactivation of protein kinases known as cyclin-dependent kinases (CDKs). Each CDK associates with a cyclin subunit that is essential for catalytic activity. The D cyclins serve to drive the cell cycle forward by binding to CDKs and forming a catalytically active complex that phosphorylates the pRb protein. Phosphorylation of pRb results in the release of E2F, a transcription factor for other critical cell cycle control genes (Figure 3). Due to the critical nature of the G1–S transition in the cell cycle, it is not surprising that perturbations of several G1 specific protein regulators have been demonstrated in most human cancers. Alterations in Rb, cyclin D1, the potent CDK inhibitor, p16, and CDK4 all contribute to disruption of the cell cycle, rendering it insensitive to anti-growth signals.

In light of the pivotal role of Rb/E2F in the control of cellular proliferation, it might be expected that genetic alterations in the pathway would be central to all colorectal cancers. However, analyses of colonic neoplasms have revealed surprisingly few

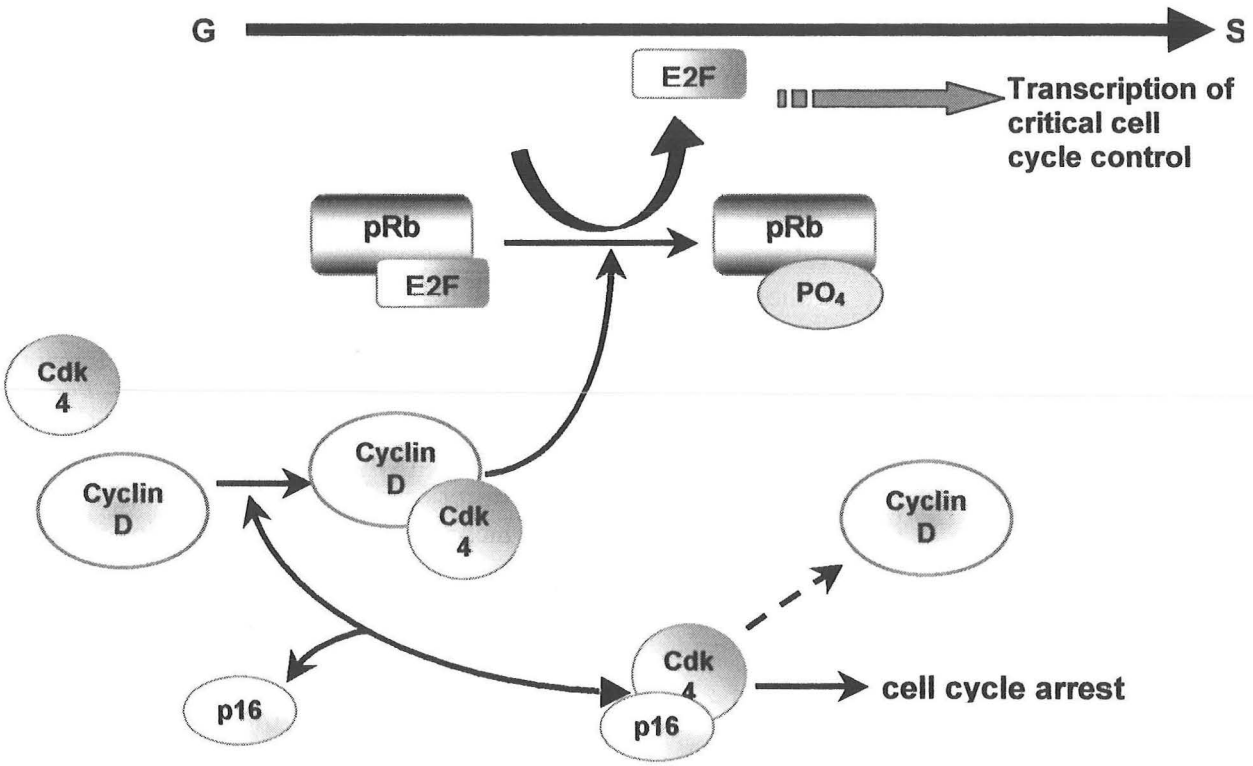

Figure 3. *Phosphorylation of pRB by the cyclin D/CDK4 complex releases the transcription factor E2F, resulting in transcription of cell cycle control genes and G1/S progression. See text for additional details. Modified from*[10].

mutations within genes encoding for Rb/E2F pathway proteins[11-19]. Although it is possible that other gene product directly involved in the Rb/E2F pathway will be identified, an alternative would be the development of mutations outside the pathway that impact the cell cycle in a less direct fashion. For example, in both sporadic and heritable CRCs, the role of β-catenin/Tcf4 in the activation of cyclin D1 has an important impact on disruption of the cell cycle[20]. Since β-catenin accumulation is regulated by the adenomatous polyposis coli (APC) gene, and mutations in APC have been detected in most colorectal cancers, activation of the Rb/E2F pathway would be one consequence of the loss of APC function. The role of APC in CRC development is discussed in detail below.

Evasion of Apoptosis: the p53 Tumor Suppressor Pathway

The growth of tumor cell populations depends not only on the rate of cell proliferation, but also on the rate of cell loss. The extent of cell loss, in turn, is determined primarily by programmed cell death, or apoptosis[21]. Current evidence suggests that most, if not all, human cancers are characterized by a resistance to apoptosis; alterations in the p53 tumor suppressor gene have been detected in at least 50% of all human cancers.

In normal human cells, both the intra- and extracellular environments are constantly monitored for conditions unfavorable to cell survival. These "sensors" regulate a second set of regulators that serve as effectors of programmed cell death. Specifically, the intracellular environment is monitored for

abnormalities including DNA damage, hypoxia, insufficiency of survival factors, and signal imbalances due to oncogene activation[21]. The extracellular environment is monitored for cell-matrix and cell-cell adherence-based survival signals; their absence triggers activation of the apoptotic machinery. The apoptotic cascade begins with disruption of cellular membranes, and proceeds through the breakdown of the cytoskeleton, extrusion of the cytosol, chromosomal degradation, fragmentation of the nucleus, and ends with the cellular corpse being engulfed by neighboring cells.

Mutations in p53 appear to mark the transition from adenoma to carcinoma, and are detected in approximately 70% of sporadic CRCs[2,3,67]. Inactivation of this pathway results in the elimination of a critical DNA damage sensor that can induce apoptosis. The p53 protein is important in maintaining chromosomal integrity, since DNA damage results in a p53-mediated G1 cell cycle arrest, followed by either DNA repair or apoptosis, depending on the extent of damage. Loss of p53 function, regardless of the mechanism, leads to karyotypic instability, impaired G1 cell cycle arrest, and reduced apoptosis, ultimately allowing cells to survive with an increased genetic burden. Mutations in the p53 gene occur only rarely in small adenomas, suggesting a late role for p53 in tumor progression (Figure 1).

Limitless Replicative Potential: telomere maintenance and telomerase

Self sufficient growth, insensitivity to anti-growth signals, and resistance to apoptosis each work to disengage the cell from growth controls imposed by the cell cycle regulators. For the successful propagation and continued growth of malignant cells, however, intrinsic mechanisms limiting proliferative capacity must be also subverted. Current evidence indicates that in almost all human cancers this is achieved by stabilization of telomeric DNA repeats via reactivation of ribonucleoprotein, telomerase. Telomeres are long stretches of non-coding DNA repeats of the six base pair sequence, TTAGGG, located at the ends of all eukaryotic chromosomes. Evidence suggests that as chromosome "caps", telomeres have at least three critical functions: 1) to protect chromosome ends from enzymatic degradation and abnormal fusion reactions; 2) to serve as a buffer zone to protect against the "end-replication" problem; and 3) to serve as a gauge for mitotic age (the divisional clock).

The existence of an intrinsic divisional clock was first suggested in 1965 by Leonard Hayflick, who demonstrated that cells maintained in culture have a finite capacity to proliferate[22]. During each round of cell division, 50-200 base pairs are lost from the ends of linear human chromosomes[23,24]. This "end-replication" problem occurs because conventional DNA replication machinery is unable to completely replicate the 3' ends of chromosomal DNA during the S phase of each cell cycle. In 1972, Olovnikov suggested that erosion of the chromosome ends could lead to the loss of essential genes and an exit from the cell cycle. Harley et al. introduced a modification of the Olovnikov theory, proposing a telomere-based mechanism to account for the process of "cellular aging"[25]. Specifically, it postulated that after a certain number of divisions, telomeres are no longer sufficient to protect chromosome ends from degradation and aberrant fusion reactions. Through signaling mechanisms that are not entirely understood, a few short telomeres may trigger exit from the cell cycle at G1 and entry into a permanent growth arrested state (senescence). In 1996, Wright et al.[26] constructed cell hybrids with artificially elongated telomeres and observed that these cells had a longer lifespan than that of cell

hybrids in which telomeres had not been elongated, providing the first direct evidence that telomere length is the counting mechanism that limits the proliferative capacity of human cells.

The ability to alter cellular proliferative capacity by manipulating telomere length provides a mechanistic basis for earlier observations of cellular lifespan *in vitro* (Figure 4). Normal human fibroblasts maintained in culture undergo a finite number of divisions determined by their initial telomere length, after which they become senescent, also known as the first mortality stage (M1). Cells nearing M1 can be forced to proliferate beyond this point by the introduction of certain viruses or oncogenes that abrogate the function of the tumor suppressor genes, p53 and pRb. These observations suggest that p53 and pRb mediate cell cycle exit at G1 in response to telomere shortening. Bypass of M1 allows additional rounds of cell division until further, critical telomere shortening occurs, resulting in a state of "crisis", characterized by widespread cell death. This second stage is known as the second mortality stage (M2). As a low frequency ($\sim 10^{-7}$) event in human cells, a subpopulation of cells escapes from crisis, giving rise to cells that have a limitless replicative capacity (i.e. are immortal). The characteristic feature of such immortal cells is the ability to maintain their telomeres.

The dual role of telomeric DNA as protector of chromosomal integrity and mitotic clock implicates cellular senescence as a natural and effective initial protection mechanism against the development of cancer. It is generally believed that tumors are initiated by multiple genetic events in cells that result in the inappropriate activation of growth stimulatory signals, an insensitivity to anti-growth signals, and a resistance to apoptosis. However, transformation to fully malignant derivatives does not occur in most cases because the majority of these aberrant

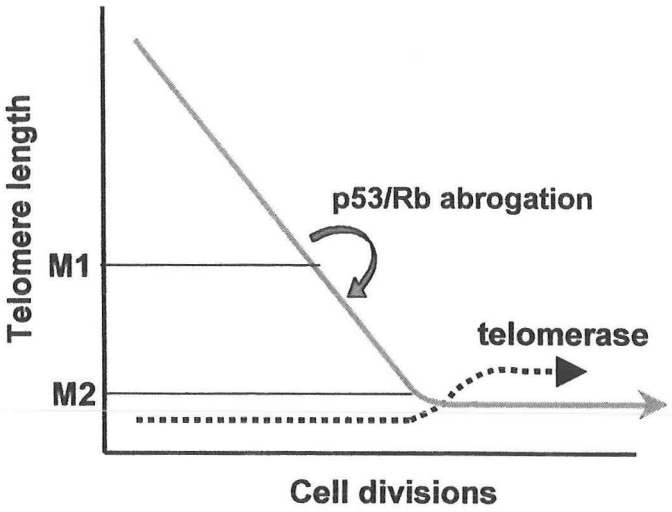

Figure 4. *The telomere hypothesis. Telomere loss due to cell division leads to senescence (M1). This can be bypassed by abrogation of the p53/Rb pathways. Widespread cell death (M2 crisis) results from critically short telomeres unless telomerase is reactivated.*

cells will have exhausted their endowment of allowed divisions. Consistent with this hypothesis, most cancers have a significantly shorter telomere shorter telomere length than non-cancerous tissue from the same patient[22]. In culture, cancer cells generally have short but stable telomeres, suggesting that human cancers have developed strategies for the maintenance of telomeric DNA at a length above the critical threshold. In 85%-95% of human cancers, this telomere stabilization is achieved by reactivation or upregulation of the ribonucleoprotein telomerase[27].

Telomerase is an RNA-protein complex that utilizes its RNA as a template for the addition of TTAGGG repeats to the 3' ends of chromosomes, thereby compensating for losses due to the end-replication problem. Most human adult somatic cells lack telomerase activity, however, high levels are found in germ cells. Telomerase activity is detectable at low levels in the basal cells of renewal tissues (skin and intestine), activated T and B cells and germinal centers of lymphoid organs. In these tissues and cells, however, the presence of detectable levels of

telomerase activity is not sufficient to prevent eventual telomere attrition.

The demonstration that telomere maintenance by exogenous telomerase immortalized human mammary epithelial cells, foreskin fibroblasts, and retinal pigmented epithelial cells, dermal keratinocytes and umbilical vein endothelial cells provided direct evidence that short telomere length regulates entry into senescence[28-30]. To date, there are no reports of naturally occurring tumors that have engaged telomerase-independent mechanisms of telomere stabilization, such as are telomerase negative[32-36]. Reactivation of telomerase appears to occur rather late in the adenoma-carcinoma sequence, consistent with observations that the average telomere length in colorectal tissues is shorter than adjacent normal mucosa[37]. Taken together, the existing evidence strongly suggests that circumvention of senescence *in vivo* represents an essential step in tumor progression that is required for the approach to and bypass of the M2 mortality stage.

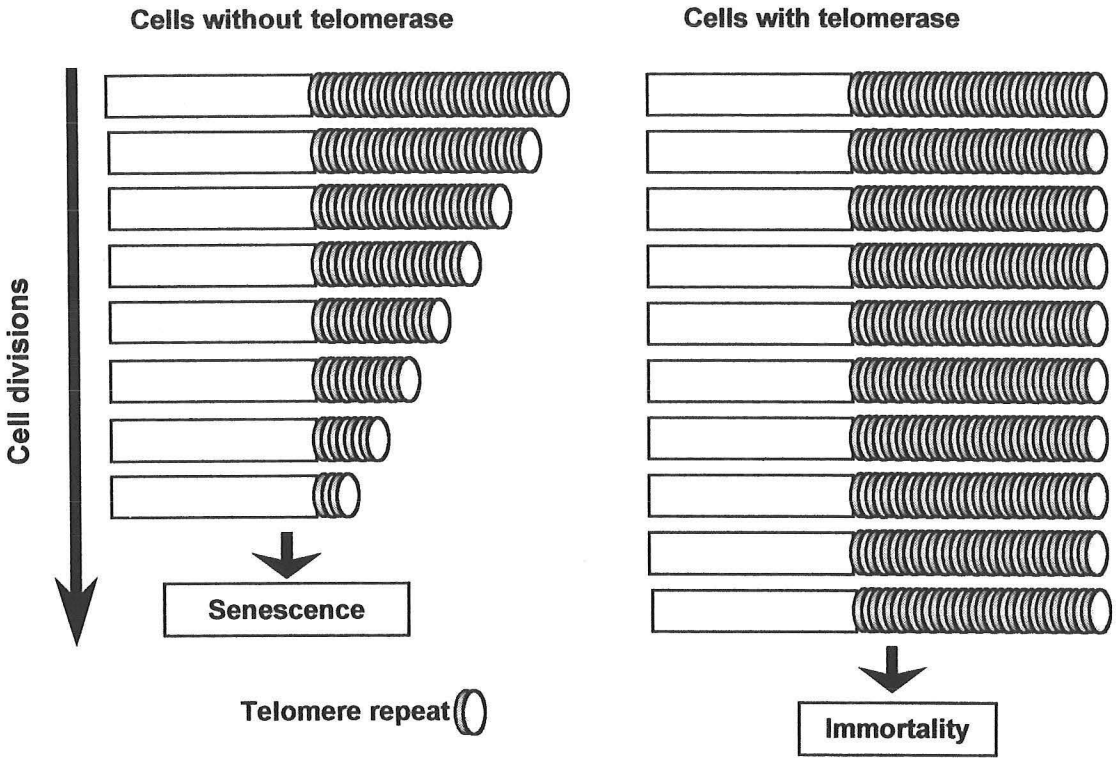

Figure 5. *In the absence of telomerase, telomeric DNA repeats are lost with each round of cell division. One or more short telomeres triggers a permanent growth arrest (senescence). The introduction of telomerase results in maintenance of telomeres and a limitless replicative potential.*

the so-called ALT pathway, which maintains telomeres through recombination-based-interchromosomal exchanges of sequence information[31].

The majority of colorectal carcinomas express high levels of telomerase, whereas normal colonic mucosa and small adenomas

Sustained Angiogenesis: Vascular endothelial growth factor (VEGF)

Normal tissue growth is tightly coordinated with the development of new blood vessels; cells within aberrant proliferative lesions initially lack angiogenic ability. However, angiogenesis is an essential

requirement for the development, progression and metastasis of malignant tumors. Two of the most well studied angiogenic signals are vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2). The VEGFs are a family of potent angiogenic growth factors that stimulate endothelial cell proliferation and migration *in vivo* and *in vitro*[38,39]. Relatively little is known about the physiological role of VEGF in the adult. However, the gene encoding one family member, VEGF-D, is under the control of the *c-fos* protooncogene, which is known to be essential for malignant progression[40] {403}. In the human, VEGF-D is a soluble factor that binds and activates transmembrane tyrosine kinase receptors (VEGFR-2 and VEGFR-3) located on the surface of endothelial cells. VEGFR-2 is upregulated in tumor angiogenesis and VEGFR-3 is upregulated in breast cancer and other tumor types. Furthermore, current evidence suggests that VEGFR-3 may be important in the maintenance of endothelial integrity during tumor angiogenesis. There is now substantial evidence that many, if not all, human tumors are capable of inducing and sustaining angiogenesis by the increasing the expression of VEGF and/or FGFs. The ability to induce angiogenesis appears to be acquired as an early to midstage event during multi-step tumorigenesis[41-43]. Two important studies have demonstrated the importance of sustained angiogenesis for the continued growth of tumors. Kim et al. used anti-VEGF antibodies to impair neovascularization and growth of subcutaneous tumors in nude mice[44]. Shortly thereafter, Millauer et al. obtained similar results in glioblastoma cells using a dominant-interfering version of the VEGF receptor 2[45].

Angiogenesis has been studied in colorectal cancer. Several studies have demonstrated an increase in microvascular density during the progression from normal colon to adenoma and frank carcinoma[46-51]. Recently, Wong et al. studied the relationship between VEGF expression and colorectal tumor progression using RT-PCR and *in situ* hybridization for VEGF[52]. Although 72% of normal colonic mucosa expressed VEGF compared to 100% of colorectal cancers, the CRCs demonstrated statistically significantly higher levels of VEGF expression compared to normal tissues ($p<0.0001$). Adenomas also showed a statistically significant upregulation of VEGF expression over normal colonic mucosa, with a further increase during the development of carcinomas. No additional signal was detected during the progression from *in situ* to invasive cancer, suggesting that the capacity for angiogenesis is acquired prior to the invasive phenotype. In each case, tumor cells formed the major source of VEGF expression, with a minor contribution from mononuclear cells.

Angiogenesis has been shown to have prognostic significance in colorectal cancer[49]. Using immunohistochemistry, White et al. analyzed archival specimens of colorectal cancers as well as normal colonic mucosa and adenomatous polyps for expression of VEGF-D and VEGFR-3[53]. VEGF-D was recently shown to be present in approximately 75% of colorectal cancers compared to 0% of adenomatous polyps and 22% of normal mucosa. By multivariate analysis, VEGF-D, but not VEGFR-3, was shown to be an independent negative prognostic indicator for both disease-free and overall survival and lymphatic involvement.

Tissue Invasion and Metastasis: E-cadherin and β-catenin

Although metastases account for over 90% of deaths attributable to cancer[54], the mechanisms underlying tissue invasion are perhaps the least understood of the cancer hallmarks. Once class of proteins, however, appears to play an integral role in the acquisition of metastatic or invasive capabilities. Cell-cell adhesion molecules (CAMs) such as the cadherin glycoproteins,

mediate cell-cell interactions. The most well studies member of this family is E-cadherin, which is present on the surface of all epithelial cells (reviewed extensively in[55-57]). In the cytoplasm, E-cadherin interacts with the catenins (α, β and γ-catenin), which link E-cadherin to the actin cytoskeleton. Adjacent cells are coupled by E-cadherin bridges, which serve to maintain the tight interconnections characteristic of normal epithelia. E-cadherin function is lost in most, if not all, epithelial cell cancers by a variety of mechanisms including mutation of the E-cadherin gene[55] (Figure 6).

Moreover, changes in the expression of proteins that are part of the E-cadherin complex, such as beta-catenin (β-catenin), can also impair cell adhesion. Introduction of E-cadherin to cultured cancer cells results in a reversion of the invasive phenotype, suggesting that loss of this pathway is a key step in the acquisition of invasive and/or metastatic properties[55]. Finally, evidence is beginning to emerge suggesting that the E-cadherin complex-mediated adhesion might transduce anti-growth signals to the nucleus, modulating gene expression and ultimately, cell behavior. The E-cadherin-β-catenin complex has been studied extensively in colorectal cancers and is discussed further below.

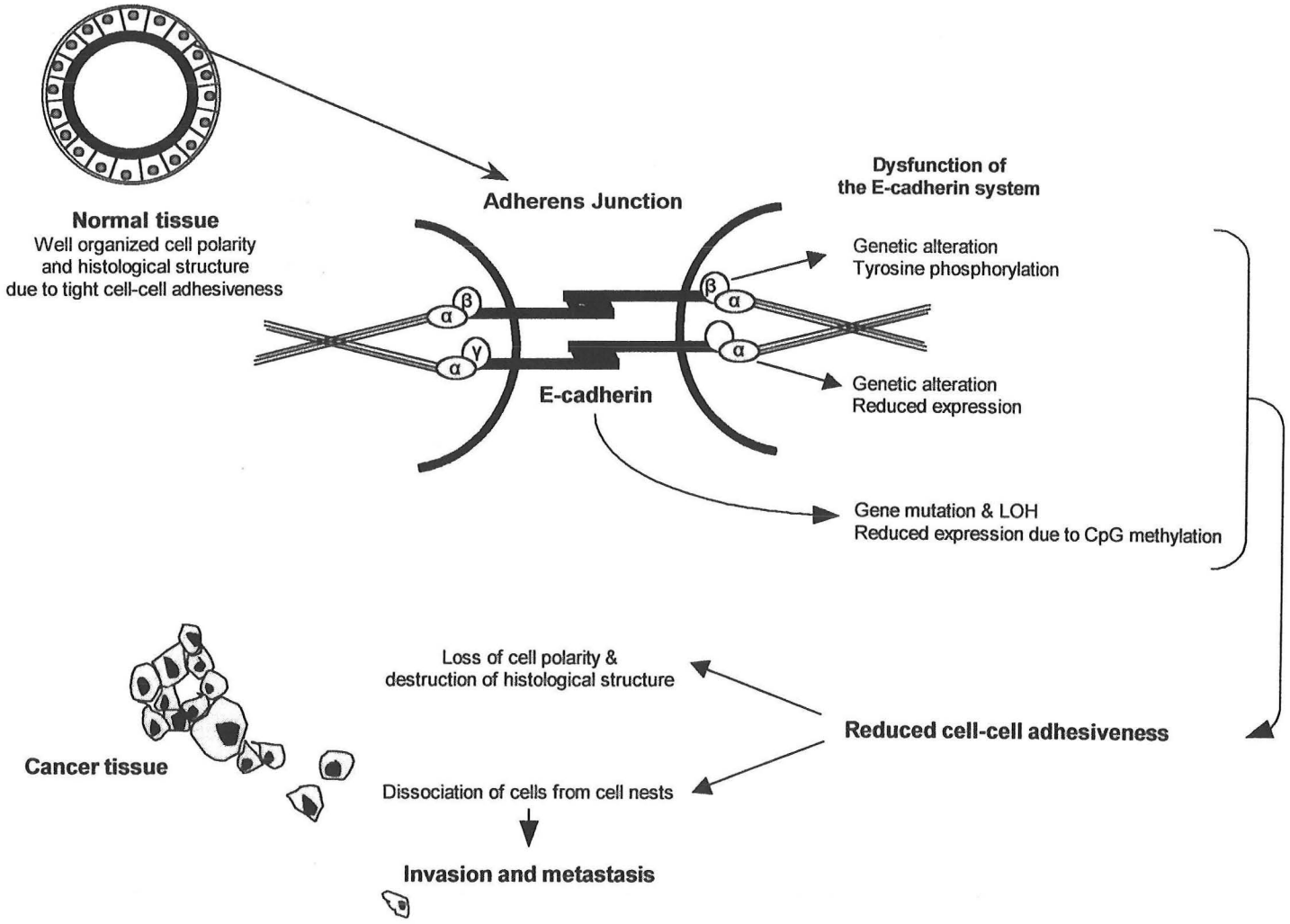

Figure 6. *Mechanisms of inactivation of the E-cadherin-mediated cell adhesion system in cancer. From*[57].

HEREDITARY CRC SYNDROMES

The hereditary colorectal cancer syndromes can be broadly divided into 2 categories: (1) tumors with chromosomal instability, which tend to be left-sided, display characteristic mutations, and behave aggressively (familial adenomatous polyposis), and (2) tumors with microsatellite instability (MSI), which occur more-often on the right side, harbor diploid DNA and characteristic mutations, and behave indolently (hereditary non-polyposis colorectal cancer). Together FAP and HNPCC account for approximately 6% to 9% of all colorectal cancer cases in the United States.

Familial Adenomatous Polyposis (FAP)

Germline mutations in the adenomatous polyposis coli (APC) gene are responsible for the autosomal dominantly inherited syndrome, familial adenomatous polyposis (FAP). Somatic mutations in APC are also present in ~80% of sporadic colorectal cancers[58]. The APC gene is located on chromosome 5q, and mutations almost always result in a truncated protein with abnormal function. Mutations in the APC gene follow the classical 2-hit model of tumor suppressor activation in that patients with FAP inherit one germline mutation; with tumors developing only after the second APC allele sustains a somatic mutation.

FAP is a rare disorder, affecting approximately 1 in 10,000 individuals and accounting for 1% of all colorectal cancers. The syndrome is characterized in most cases by the development of hundreds to thousands of colorectal adenomatous polyposis beginning at an early age (before 20 years). Due to the large number of polyps, the risk of developing colorectal cancer approaches 100%, with an average age at the time of cancer diagnosis ranging from 35 to 43 years[59,60]. Thus, prophylactic colon resection is recommended to minimize the risk for developing colorectal cancer.

In addition to colorectal cancer, FAP is also associated with a variety of extracolonic manifestations (reviewed in[61]). These include gastric and small intestinal polyps (adenomas and fundic gland retention polyps of the stomach), pancreatic, periampullary and thyroid adenomas and adenocarcinomas, osteomas, desmoid tumors, epidermoid cysts, dental abnormalities, hepatoblastomas, CNS tumors, and congenital hypertrophy of the retinal pigmented epithelium (CHRPE). Periampullary carcinoma is the most common cause of cancer death in FAP patients who have undergone a prophylactic colectomy[62,63]. The clinical association of FAP with desmoid tumors and osteomas is referred to as Gardner syndrome, while Crails syndrome is characterized by the association of FAP with CNS tumors, primarily meduloblastomas.

The APC gene is a tumor suppressor gene, with 15 exons that encode for a 2,843 amino acid protein. Over 300 mutations in the APC gene causing FAP have been identified, and the risk of developing a specific manifestation of FAP is often correlated with the position of the inherited APC mutation[64]. For example, severe polyposis (more than 5000 colorectal polyps) is usually seen in patients with mutations between codons 1250 and 1464. In contrast, APC mutations at the extreme 3' and 5' ends of the gene lead to an attenuated form of FAP characterized by the presence of dozens to hundreds of polyps. Classic FAP results from mutations between codons 169-1393 (Figure 7). CHRPE is present only in patients with mutations between codons 457 and 1444. The correlation between genotype and phenotype will become increasingly important in the future for targeted genetic testing, however, there is considerable inter- and intrafamilial phenotypic variability even in persons with identical genetic mutations, suggesting the

presence of important modifier genes that may influence the severity of the disease.

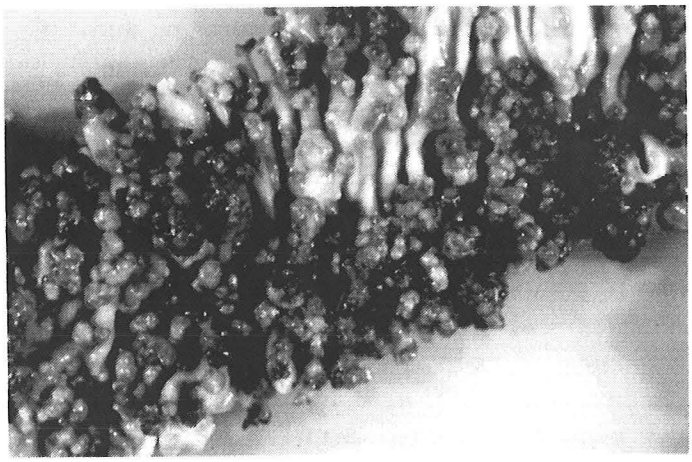

***Figure** 7. Fixed resection specimen from total colectomy in a patient with FAP. Courtesy of Ed L. Lee, Dallas VA Medical Center.*

In addition to the above-mentioned mutations, a specific APC gene mutation (I1307K) has been found in subjects of Ashkenazi Jewish descent that may explain a portion of the familial colorectal cancer occurring in this population[108]. Unlike other mutations in the APC gene, which result in an alteration in the protein length, the I1307K mutation is termed a missense mutation. It is hypothesized that the APC I1307K mutation itself does not cause colon cancer; rather this particular mutation appears to create a weak spot in the gene that makes it more susceptible to additional genetic changes that may in turn lead to colon cancer. The presence of a specific mutation in a well-defined population creates the possibility of genetic screening of Ashkenazi Jewish individuals with or without a family history of colon cancer.

Overall, APC functions as a tumor suppressor gene, affecting diverse physiological processes, from cell growth to apoptosis in a number of cell types and organisms. Characterization of FAP has provided new and important insights not only into hereditary and sporadic colorectal tumorigenesis, but also to cancer in general. The complex architecture of the normal colonic epithelium is strictly maintained by a variety of homeostatic controls. Sophisticated growth mechanisms and cell-cell and cell-extracellular matrix relationships tightly regulate cell turnover in order ensure that the number of new epithelial cells is approximately equal to the number of cells undergoing apoptosis. The system must be flexible enough, however, to allow for transient increases in proliferation under appropriate circumstances, such as after tissue damage. The APC gene product is an important regulator of epithelial homeostasis, directly and indirectly modulating a variety of processes such as cell adhesion, signal transduction and transcriptional activation.

Role of APC in the Wnt Signaling Pathway

Largely through its interaction with ß-catenin and the kinase, GSK3β, both of which are essential components of a signaling pathway known as Wnt, APC serves as an important regulator of transcription in colonic epithelial cells. Wnt signaling plays a critical and highly conserved role in cell differentiation and cell proliferation. The APC protein targets gene transcription through its modulation of intracellular levels of beta-catenin (β-catenin)[65,66,20,67]. APC forms a complex with Axin, which recruits β-catenin, facilitating its phosphorylation by GSK3ß, which targets β-catenin for degradation via ubiquitination. Upon binding of Wnt to its transmembrane receptor, the disheveled (Dsh) protein is activated and inhibits GSK3β. If not phosphorylated, β-catenin is not degraded, and accumulates within the cell cytoplasm and nucleus. Inside the nucleus, β-catenin associates with members of the T cell factor (Tcf)/lymphoid enhancer factor family of transcriptional activators, forming a complex that activates transcription of target genes, including the protooncogenes c-myc and

cyclin D1 (Figure 8). Another recently identified APC target gene is the peroxisome proliferator-activated receptor (PPAR) δ

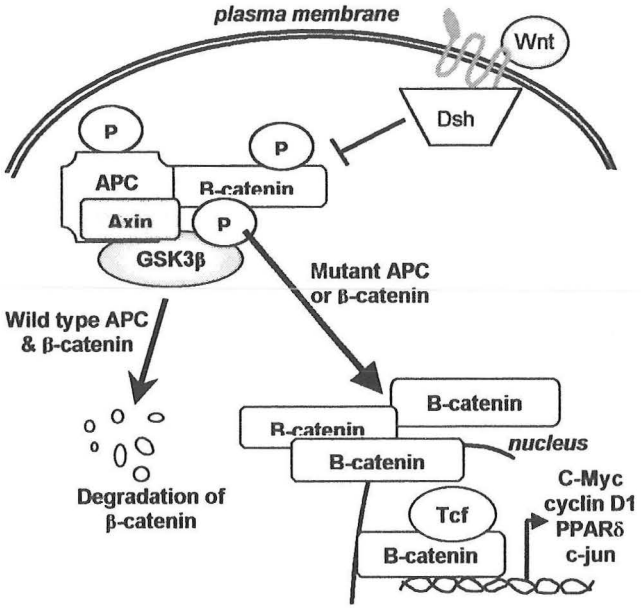

Figure 8. The APC/β-catenin pathway. In the presence of wild type APC, GSK3β phosphorylates β-catenin & targets it for destruction. Mutations in APC lead to the accumulation of β-catenin, which enters the nucleus and up-regulates gene transcription. Wnt signaling activates the disheveled (Dsh) protein and inhibits GSK3β, leading to the accumulation of β-catenin. From[68] {410}.

gene, which is also regulated by non-steroidal anti-inflammatory drugs (NSAIDs)[69]. Therefore, by preventing β-catenin accumulation, APC acts to control the upregulation of genes involved in cell cycle entry and progression.In addition to its role in Wnt signaling, APC may also be involved in cell adhesion by competing with E-cadherin for β-catenin binding. Through binding of β-catenin with APC, no association with E-cadherin can occur, leading to disruption of adherens junction integrity and disorganization of epithelial cells.

Genetic Testing for Familial Adenomatous Polyposis (FAP)

Guidelines for genetic testing to screen for FAP have been developed by the American Gastroenterological Association (AGA) as well as other organizations and are reviewed extensively elsewhere[61,70-76]. The importance of pretest genetic counseling and written informed consent prior to testing cannot be overemphasized. APC gene testing is indicated in patients clinically affected with FAP, for first-degree relatives of FAP patients, for patients in whom the attenuated form of FAP is suspected due to the presence of ≥20 cumulative adenomas, and first degree relatives of attenuated FAP patients beginning at age 10 years. Under ideal circumstances, APC gene testing begins with the clinically affected member. A positive test for FAP mutations may prompt consideration of a prophylactic colectomy and establish a basis for testing unaffected family members. Once a deleterious APC mutation is detected, testing can be

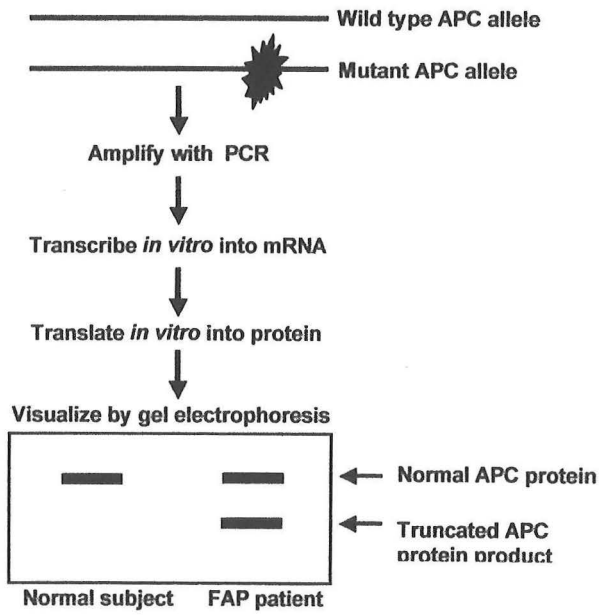

Figure 9. The APC protein truncation assay. Genomic DNA is isolated from leukocytes and fragments of the APC gene are amplified by PCR. The PCR products are transcribed and translated into protein and resolved by gel elexctrophoresis. A mutated (truncated) APC protein is detected in addition to wild type protein in patients with 1 mutant APC allele.

performed in at-risk members with a high degree of accuracy. A negative test in the clinically affected individual, however, is generally considered uninformative since the patient may have a mutation undetected with current methodology. Consequently, testing of at-risk family members should not be conducted. If a clinically affected family member is not available for testing, then at-risk family members should be tested. APC gene testing under these circumstances, however, can only provide positive results (i.e. the patient is affected with the disease) or inconclusive results.

Although there are a variety of available methods for APC gene testing, the APC protein truncation assay is currently the preferred method[76] (Figure 9). The sensitivity of this assay ranges from 79% to 90%. In contrast to sequencing, which can detect variants of unknown significance, the protein truncation assay usually detects disease-causing mutations, is less costly, and is the results are generally obtained faster. The combination of full sequence analysis and protein truncation, however, is the most sensitive, and has the added advantage of detecting attenuated FAP mutations, which are usually located at the 3' and 5' ends (personal communication, Gail Tomlinson). Genetic testing for the APC I1307K mutation is available for persons of Ashkenazi Jewish descent. Testing can begin in either affected patients or at-risk individuals, who receive either a true positive or a true negative result.

A recent adaptation of the protein truncation assay for germline APC mutations was reported by Traverso et al.[109] Stool was collected from colonic effluent of 28 patients with known CRC, 18 patients with large adenomas, 28 controls. Genomic DNA was purified from cells in each fecal sample and amplified by the PCR reaction. The PCR products were then subjected to an *in vitro* transcription-translation assay and visualized using gel electrophoresis (Figure 10). APC

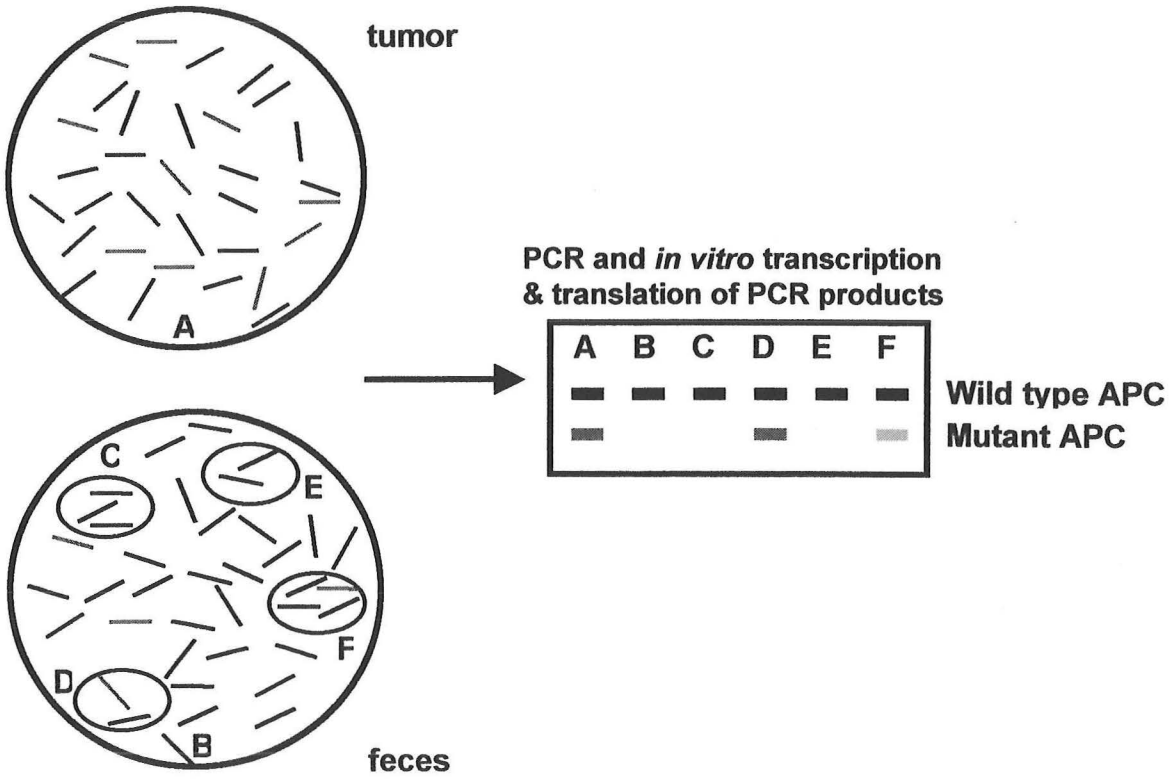

Figure 10. APC gene testing in the feces of persons with FAP. From[109].

mutations were identified in 26 of the 46 patients with large adenomas or CRCs (57%, 95% CI, 41 to 71%) and in none of the controls.

Hereditary Non-Polyposis Colon Cancer (Lynch syndrome)

Hereditary non-polyposis colorectal cancer (HNPCC) is the most commonly encountered form of hereditary colorectal cancer, accounting for 5%-8% of all colon cancer cases[77]. HNPCC is caused by a germline mutation in one of the 5 DNA mismatch repair genes (hMSH2, hMSH6, hMLH1, hPMS1, and hPMS2), with mutations in hMLH1 and hMSH2 occurring most often. The incidence of this condition is 1:1000 in the general population and approximately 1-6:100 among colorectal cancer patients. HNPCC is characterized by an 80% lifetime risk for colorectal cancer. Individuals with HNPCC develop colon cancer at an earlier age (mean of 44 years) compared to sporadic CRC. Furthermore, HNPCC-associated CRCs are predominantly right-sided (60%-80%), and 45% of affected individuals will develop synchronous and metachronous tumors within 10 years of resection. The histopathology of HNPCC colorectal cancers is also distinct, with poor differentiation, abundant extracellular mucin, and a robust lymphoid infiltrate.

A variety of extracolonic malignancies have been associated with HNPCC. For example, women who are HNPCC gene carriers carry a 60% lifetime risk for developing endometrial cancer. Additionally, there is an increased relative risk of ovarian cancer, transitional cell carcinoma of the ureter and renal pelvis, adenocarcinoma of the stomach, small bowel, and biliary tract in HNPCC pedigrees. Less commonly, there are reports of excess pancreatic, laryngeal, brain, breast and hematopoietic malignancies. Phenotypic signs of HNPCC are uncommon and include café au lait spots, sebaceous gland tumors, and keratoacanthomas[78].

The International Collaborative Group (ICG) on hereditary nonpolyposis colorectal cancer initially established research criteria for the identification of likely gene carriers since the genetic mutation was not known[79] (Amsterdam I criteria, Table 2). In response to concerns that the criteria were to stringent, the criteria were expanded to include extracolonic malignancies[80] (Amsterdam II criteria, Table 2). Epidemiologic studies suggest that approximately 1%-5% of all colorectal malignancies satisfy these criteria for the definition of HNPCC. Other diagnostic guidelines, such as the Bethesda criteria, were subsequently developed to address limitations imposed by strict requirements in the Amsterdam criteria. The Bethesda criteria aim to identify individuals with CRC in whom additional testing should be considered based on clinical and pathological features[81] (Table 3).

Table 2. *Amsterdam Criteria I and II (International Collaborative Group) for HNPCC Diagnosis*

Amsterdam criteria I
• ≥ 3 relatives with CRC, 1 of whom is a 1st degree relative of the other 2; FAP should be excluded
• ≥ 2 generations affected with CRC
• ≥ 1 CRC cases diagnosed before age 50
Amsterdam criteria II
• ≥ 3 relatives w/HNPCC associated cancer, 1 of whom is a 1st degree relative of other 2
• ≥ 2 generations affected with CRC
• ≥ 1 cancer cases diagnosed before age 50

Table 3. *Bethesda Criteria for Testing Colorectal Tumors for MSI* {431}.*

Individuals with cancer in families that meet Amsterdam criteria.
Individuals with 2 HNPCC-related cancers, including: - synchronous and metachronous CRC, or - associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small intestinal, renal pelvis and ureteral).
Individuals with CRC and a first-degree relative with CRC and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; 1 of the cancers was diagnosed at age <45 yrs, and the adenoma diagnosed at age < 40.
Individuals with CRC or endometrial cancer diagnosed at age < 45 yrs.
Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform, defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces; medullary carcinoma) on histopathology diagnosed at age < 45 yrs.
Individuals with signet ring cell-type CRC (composed of > 50% signet ring cells) diagnosed at age < 45 yrs.
Individuals with adenomas diagnosed at age < 40 yrs.

Abbreviations: HNPCC-hereditary non-polyposis colorectal cancer; CRC-colorectal cancer

*Meeting all features listed under any of the 7 criteria is sufficient.

Screening for HNPCC

Guidelines for colorectal cancer screening in HNPCC have been published by several groups on the basis of expert and consensus opinion, and are reviewed extensively elsewhere. In patients in whom genetic testing is not available, first-degree relatives (at 50% risk for HNPCC) of affected individuals should undergo colonoscopy every 1-2 years, beginning between 20 and 30 years of age, and annually after 40 years or alternating every 1-2 years, beginning at age 25 years. Colorectal screening has been shown to decrease morbidity and mortality from CRC for children of HNPCC patients[82]. Annual screening for endometrial cancer is also recommended beginning at age 25-35[61]. Although there is no consensus as to the optimal method, transvaginal ultrasound and endometrial aspiration are 2 reasonable options.

Testing for Mismatch Repair (MMR) and Microsatellite Instability (MSI)

HNPCC is caused by a germline mutation in one of several mismatch repair genes. The role of these genes is to maintain fidelity of DNA during replication. MMR is accomplished by the MMR proteins, which recognize and correct nucleotide base mispairs and small insertions or deletions generated by misincorporation or slippage of DNA polymerases during replication. Studies in yeast and *E. coli* first demonstrated that failure of the MMR system results in instability of DNA repeat sequences[83,84]. Studies in mammalian cells suggest that the MMR system is analogous to that in lower organisms. Thus, the associated phenotype of instability of microsatellite DNA sequences has become the molecular hallmark of MMR deficiency.

Microsatellites are short DNA sequences (1-6 nucleotides, repeated 10 to 60 times) that are distributed throughout the human genome. Since they are not known to code for proteins, their exact function is not known. Although microsatellite sequences are highly polymorphic within a population, they are conserved and inherited from one generation to the next. For a single individual, therefore, a given microsatellite demonstrates the same fixed length in all of that individual's different tissues.

Microsatellite sequences can be easily detected in normal and tumor tissue samples. In HNPCC, the pattern of microsatellite lengths in a colon cancer differs from that in somatic or germline tissue from the same individual. This phenomenon, known as microsatellite instability (MSI), reflects the existence of unrepaired DNA due to a deficiency in MMR. Both alleles of a MMR gene need to be inactivated to cause MMR deficiency. Intact MMR function is present in the lymphocytes of most individuals with HNPCC despite mutations in one of the MMR genes, suggesting that the mutations are recessive at the cellular level, and inheritance of the MMR phenotype as a dominant trait occurs due to the high rate of a second hit.

MSI has been found in > 90% of HNPCC cases that fulfill the Amsterdam criteria, as well as 15% of sporadic colorectal cancers[61]. The strong association between MSI and MMR deficiencies in HNPCC, as well as the avaibility of methodology for the detection of MSI in tissue led to the development of the Bethesda criteria (Table 3). In 1997, The National Cancer Institute proposed 5 specific markers for MSI testing and created a new classification for MSI[81,85]. Tumors are classified as MSI-high (≥ 2 markers unstable), MSI-low (1 marker unstable) and MSS (microsatellite stable). MSI is considered to be present in the specimen only if the result is MSI-high. The occurrence of MSI in various populations with CRC is shown in Table 4. Overall, the presence of MSI-high substantially increases the likelihood of detecting a germline MMR mutation. Using multivariate analysis, Wijnen et al.[110] found that a young age at CRC diagnosis, fulfillment of the Amsterdam criteria, and the presence of endometrial carcinoma in the family were independent predictors of mutations of *hMLH1* and *hMSH2*.

There are 5 human mismatch repair genes, *hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6*. Although germline mutations can occur on any of the MMR genes, mutations in *hMLH1* and *hMSH2* account for at least 95% of all HNPCC kindreds. Reports of *hMSH6* mutations have increased steadily and appear to be associated with a later cancer onset, a higher frequency of endometrial cancer, and a low degree of MSI

Table 4. *Summary of MSI Test Results*[61].

Population	Mean % MSI positive (range)
HNPCC; meets Amsterdam criteria I	81 (44-100)
HNPCC suspected; positive family history; does not meet Amsterdam criteria I	36 (9-62)
Multiple CRC (with or without family history)	35 (26-50)
CRC < 30 yrs	32
CRC < 35 yrs	35
CRC < 45 yrs	21
CRC < 50 yrs	17
Unselected CRC	21 (7-38)
	MSI-L 25 (22-27)
	MSI-H 13 (9-16)
Sporadic right-sided CRC	44 (31-57)
Sporadic left-sided CRC	0
Sporadic CRC (all)	15 (5-20)

CRC, Colorectal cancer

in tumor tissue[86-90]. Of the 15% of sporadic CRCs with MSI, only 6%-10% harbor germline mutations of *hMSH2* and *hMLH1*. Many of these cases are also due to inactivation of *hMLH1* by promoter hypermethylation, which is often biallelic[91]. Paradoxically, Amsterdam-positive families tested for MMR mutations of *hMSH2* and *hMLH1* show a mutation in only between 45% and 86% of cases. The MMR intact cases may be due to subtle mutations in the known MMR genes, or perhaps to novel genes yet to be identified.

Tumor development in HNPCC is thought to occur as a result of the accumulation of widespread mutations within

repetitive sequences. Mutation rates in tumor cells with MMR deficiency are 100-1000 fold higher than in normal cells[92,93]. In addition to MSI, mutations also occur in genes with exons that have repetitive sequences. One example is the type II TGF-β receptor gene[94]. Since TGF-β is a potent inhibitor of colonic epithelial cell growth, this finding validated the importance of TGF-β signaling in colorectal tumorigenesis. Other target genes have been identified including the insulin-like growth factor, BAX, E2F-4, TCF-4, the intestinal homeobox factor CDX2 and even *hMSH3* and *hMSH6*[94-100].

Genetic Testing for HNPCC

The identification of HNPCC gene carriers using clinical criteria is difficult due to the lack of an identifiable phenotype. The importance of accurate documentation of the family history of malignancy combined with clinical observation remains the first (and therefore most important) step in identifying at-risk individuals.

MSI. MSI testing is indicated for patients who meet the Amsterdam or Bethesda criteria (Table 5); MSI evaluation of the adenocarcinoma or adenoma serves as the initial screening test for HNPCC. MSI testing is performed on tumor tissue of patients with potential HNPCC; the detection of MSI-high provides evidence for a germline mutation in a MMR gene, and such patients should undergo specific genetic analysis of the *hMSH2* and *hMLH1* genes (information on commercial testing available through **www.myriad.com**). In persons with MSI-low or MSS tumors, additional testing can be suspended since these persons are unlikely to harbor germline MMR mutations.

A recent study showed that large-scale molecular screening could be feasible through MSI evaluation followed by mutational analysis. Ninety four percent of patients exhibiting *hMLH1/hMSH2* mutations had one of the following three criteria: young age, previous tumors or positive family history[101]. This subset of patients may have an inherited predisposition to develop CRCs, emphasizing the need for accurate review of their family histories.

MMR. Mismatch repair gene testing is indicated for confirmational or presymptomatic testing in adults affected with or at risk for HNPCC (Table 5). Gene testing is offered to individuals with possible HNPCC due to the presence of an MSI-high tumor result. If a mutation is found in an affected family member, then genetic testing of at-risk relatives will provide a true positive or true negative result. If a pedigree mutation is not identified, then further testing of other at-risk relatives should not be performed, because a negative result will be inconclusive. If tumor tissue is not available, consideration can be given to germline testing if any of the first 3 of the Bethesda criteria are met. When an affected family member is not available for testing, testing of at-risk family members can only provide positive or inconclusive results[61].

Table 5. *Indications for Gene Testing*

Gene test	Indications
MSI testing	Affected individuals in families meeting Amsterdam criteria
	Affected individuals meeting Bethesda criteria
MMR gene testing (*hMSH2, hMLH1*)	Patients with MSI-high tumor test
	Affected individuals in families meeting any of the first 3 criteria of the Bethesda criteria, or tumor tissue not available
	First-degree adult relatives of those with known mutation

Recently, immunohistochemistry (IHC) for *hMLH1* and *hMSH2* expression has been reported as a clinically useful, inexpensive adjunct to MSI testing of colorectal cancers[102-105] {476, 475, 474, 473}. Lack of expression of either *hMLH1* or *hMSH2* in tumors is correlated with MSI in the tumor. Additionally, absent *hMSH2* staining by IHC has been observed to correlate with a

germline mutation in the gene[105]. Due to its technical ease of use, immunohistochemistry for MMR is likely to become routine clinical practice in the future.

The TGF-β / SMAD Pathway & MSI

Transforming growth factor beta (TGF-β) belongs to a family of molecules that mediate a wide variety of biological effects, most notably inhibition of cell proliferation. After TGF-β binds to its receptor, type II TGF-β receptor (TGFβRII), the type I receptor is recruited and activated via phosphorylation. This initiates a signaling cascade, whereby the type I receptor sequentially phosphorylates a series of so-called SMAD proteins (SMAD2, SMAD3, and SMAD4), which then form a complex. This hetero-oligomeric complex translocates to the nucleus, and modulates transcription of specific genes through cis-regulatory SMAD binding sequences. Genes regulated by the SMAD pathway include plasminogen activator inhibitor I, the CDK inhibitors p15 and p21, cyclin D1, and TGF-β itself.

The potential role of TGF-β/SMAD pathway in colorectal carcinogenesis was realized with the demonstration that TGFβRII gene mutations were common in colorectal tumors with MSI. Functional studies confirmed that these mutants were resistant to the effects of TGF-β. Mutations have now been detected in genes that function at various points along the pathway, including *SMAD4*, *SMAD2*, and germline TGFβIIR alterations[106,107]. Interestingly, both SMAD2 and SMAD4 lie on chromosome 18q21, the same location of the "deleted in colon cancer" (DCC) locus; loss of heterozygosity on 18q is present in about 70% of colorectal cancers[2].

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