

GENETICS OF COLORECTAL CANCER

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Summary. *Advances in the molecular biology of colorectal cancer (CRC) broadened our understanding of this disease, but also provided insight into the pathogenesis of sporadic and inherited CRC. Mutations of APC gene are responsible for familial adenomatous polyposis (FAP), but mutations in six mismatch repair (MMR) genes: MSH2, MLH1, PMS1, PMS2, MSH6 and MSH3 for hereditary non-polyposis colorectal cancer (HNPCC). Development of genetic tests for detection of reported mutations of genes would make it possible to diagnose gene carriers and to discover CRC in early stage.*

Key words: *Mutations, APC gene, FAP, Lynch syndrome (HNPCC)*

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths and the third leading cause of cancer in the United States (1) and most Western countries, with 130 000 new cases per year and 56 600 deaths per year. The overall lifetime risk of CRC in the general population of United States is 6% (2), with lifetime risk of 6.14% in men and 5.92% in women (3). The crude incidence of CRC in the European Union is 53/100 000 cases; the death rate is 30 cases/100 000/year. Much has been learned over the past ten years about the molecular genetic alterations that give rise to CRC (4).

Molecular biologists and geneticists have detected at least two completely different pathogenetic pathways for CRC. Both are thought to begin with inactivation or loss of the adenomatous polyposis coli (APC) gene, followed by the sequential loss of other tumor-suppressor genes and activation of cellular oncogenes (5).

Adenocarcinomas of the large bowel arise "de novo" from normal colonic epithelium in a small percentage of patients. In vast majority CRCs arise through a series of genetic mutations that activate proto-oncogenes and disable tumor suppressor genes, normal colonic epithelium gives way to precancerous adenoma development and eventually frank adenocarcinoma (6). CRC screening programs tend to detect CRC in an early and curable stage, but CRC prevention programs tend to diagnose and remove precancerous lesions (adenomas-accumulation of benign neoplastic cells), thereby preventing their progression to CRC (7,8). In recent years it has become clear that there is an increased risk of developing CRC in hamartomatous polyps, which are accumulations of non-neoplastic cells (9).

CRCs have been classified as sporadic or hereditary (10).

Sporadic CRC

Sporadic CRCs comprise about 60% of all colorectal malignancies (6). In last decades it has been confirmed that these sporadic forms originate from colorectal adenoma, through adenoma-carcinoma sequence (11). This classic multi-step model process occurs over a period of approximately 10 years (12).

The single cell acquired genetic alteration that provides a growth and survival advantage to that cell and its progenitors is the first event in the molecular progression pathway to CRC. The somatic mutation of a gene critical to the control of cell growth or death would be a typical genetic alteration (13). Somatic gene mutations are the consequence of DNA point mutations, DNA rearrangements, amplifications or deletions. Mutations of any form may result in the inappropriate activation or deactivation of the gene (14). Inappropriate gene promoter methylation is a mechanism other than mutation influenced in critical gene functions. This epigenetic gene regulation is essential to normal cell growth and differentiation. Abnormal DNA methylation, both hypomethylation and hypermethylation, is common in CRC (15,16). Hypomethylation of a gene promoter will lead to inappropriate overtranscription of the gene or elevated mutation rates (17). Aberrant hypermethylation of the promoter will lead to transcriptional silencing (18). The abnormal methylation status of gene can play a critical role in the neoplastic transformation (19).

The earliest genetic alteration observed in sporadic CRC is inactivation of the adenomatous polyposis coli (APC) gene located in the long arm of chromosome 5 in position 5q21-22 (12,20,21). Mutations of APC gene lead to abnormal cell growth. Recent literature data link tumorigenesis initiated by mutations of APC gene to the

activation of β -catenin signaling, which in turn induces growth-promoting genes (22,23). Inactivation of APC gene occurring by gene deletion or gene mutation permits a hyperproliferative epithelium to develop from normal colonic epithelium. Deletion of APC gene has been detected in 30% of adenoma (24), but mutation of APC gene in approximately 63% of adenoma (25). Deletions of APC have been detected mainly in adenomas less than 1 cm in size (24), but mutations of APC were found in adenomas as small as 0.5 cm, suggesting that inactivation of APC gene is found an early event in the development of CRC (25). "Hotspot" for somatic mutation in APC gene in patients with sporadic CRC was found at codon 1554. APC mutation in the "mutation cluster regions", especially those in codon 1300 are associated with allelic loss, whereas tumors with mutations outside this region tend to harbor truncating mutations (26). Genetic alterations in sporadic CRC are characterized by losses of large chunks of chromosomal DNA (24). Loss of large chunks of DNA containing multiple gene loci occurs by mitotic recombination or aberrant chromosomal segregation. This process is called loss of heterozygosity (24,27). Gene mutations in sporadic CRC are acquired by the effect of environmental factors (somatic mutations). In sporadic CRC both copies of the gene must become somatically mutated (6).

The next early, but key genetic event appears to be the alteration of the ras gene, which transforms the hyperproliferative colonic epithelium into benign adenoma (11). H-ras and K-ras proteins have been identified as human oncogenes (28,29). Oncogenes are mutated forms of cellular proteins, which, when activated, can cause unregulated cellular proliferation. Mutated K-ras activate intracellular signaling cascades that promote neoplastic change (30). Mutations of K-ras oncogene are observed in a half of adenomas greater than 1 cm (24). Deletion of tumor suppressor gene deleted in colon cancer (DCC) located on chromosome 18q occurs when colorectal adenoma becomes more dysplastic (31). Deletion of DCC occurs mostly in adenomas with foci of carcinoma, suggesting DCC role in the later stages of the adenoma-carcinoma sequence (24,32). It is believed that the final progression to carcinoma is associated with the loss of chromosome 17p (the locus for the tumor suppressor gene p53). The p53 gene product is a multifunctional protein essential for control of cell growth (33). Deletion of p53 is rare in adenomas, but occurs in 75% of carcinomas, so it is believed to promote the transition from adenoma to carcinoma (24). The time course of adenoma-carcinoma sequence by way of sporadic CRC pathway is approximately 10 years (12).

Hereditary CRC

Molecular genetic findings have enabled hereditary CRC to be divided into two groups: (1) tumors which show microsatellite instability, occur more frequently in

the right colon, have diploid DNA, harbor characteristic mutations [e.g. transforming growth factor β type II receptor and BAX (HNPCC)]; and (2) tumors with chromosomal instability, which tend to be left-sided, show aneuploid DNA, harbor characteristic mutations such as K-ras, APC and p53 (familial adenomatous polyposis – FAP) (34). Hereditary CRCs are detected in several hamartomatous polyposis syndromes as juvenile polyposis.

FAP

CRCs in patients with FAP constitute about 1% of total CRC (35). FAP is autosomal dominant disorder affecting approximately 1 in every 8000 births (worldwide), with penetration rate over 90% (36). In the period between 1961-1990, incidence of FAP was 0.62-2.38/1,000,000 inhabitants, but prevalence was 0.88-26.3/1,000,000 (37). Affected patients develop 100 to 5000 adenomas (mean number more than 1000) of tubular structure, mostly less than 1 cm in diameter, located in all segments of the large bowel (38). One or more adenomas inevitably progress to CRC unless they undergo prophylactic colectomy. Most patients with FAP will develop hundreds colorectal adenomas by the age of 16, and if untreated, will acquire CRC at mean age of 39 (in some patients between 25 and 30 years), and die by the age of 42 years (39). Over 90% of CRC in FAP patients are diagnosed by the age of 40 (38).

FAP is characterized by numerous extracolonic manifestations: intraabdominal desmoids, epidermoid cysts, multiple osteomas, skin fibromas, CNS tumors, dental abnormalities, osteodystrophy, congenital hypertrophy of retinal pigment epithelium – CHRPE (40-42). There is a high frequency of stomach and duodenal polyps (43). In some patients with FAP duodenal and periampullar carcinoma as well as gallbladder and biliary tract malignancies are observed (44-46).

FAP is caused by a germ line mutation of the tumor suppressor APC gene located on the long arm of chromosome 5q21 (38,47-49). APC gene is a multifunctional protein included in processes of transduction, apoptosis, regulation of cell cycle and cell adhesion (42). In most identified APCs, mutation is located in 5' segment of gene. Mutations toward 3' APC gene segment are rare (50). APC is a large gene containing 15 exons, 2844 codons and 8532 nucleotides (47-50). Sequencing the entire gene, including introns, untranslated, and promoter regions is impractical. All confirmed FAP-causing mutations detected to date result in truncations of the protein (51,52). Over 200 germline APC mutations have been described, with 20% confined to two mutational hot spots in exon 15 (53,54). One of the major consequences of these mutations is the disruption of APC gene ability to inhibit the function of β -catenin (55,56). It is considered that APC gene normally binds to β -catenin (57,58) promoting its degradation (59), thereby preventing activation of growth-promoting genes, such as c-myc (60), by a β -catenin/Tcf-4 tran-

scription complex (61,62). It is believed that there may be at least one additional gene, besides APC, that can give rise to FAP (63).

The specific location of a mutation may determine in part the disease phenotype (49,64). Nagase et al (65) made the initial observations that the site of mutation correlated with the degree of polyposis. Mutations of APC between codons 450 and 1600 are associated with more than 100 existing colorectal adenomas (66), especially mutations between codons 1250 and 1330 (49). Mutations at either end of the gene (for example proximal to codon 158 or distal to codon 1900) are associated with an attenuated variant of the FAP (attenuated adenomatous polyposis coli-AAPC) characterized by sparse polyposis (50,67). A late onset of the disease and phenotype of AAPC characterized mutations in exons 3 and 4 (68) in the splice sites of intron 3 in the extreme 5' region of the gene (69-71). Others have recorded an association of AAPC with mutations in exon 6 (72), at the splice site of (73) or within (69,74) the alternatively spliced exon 9, in the 3' end of the gene (75,76), and with deletion of the whole gene, cytogenetically detectable (77). With respect to the extreme 5' APC mutation, localized within exons 3 and 4, it was originally proposed that there existed a specific "phenotypic-boundary" between AAPC and FAP residing in codons 157-168 of exon 4 (68). Marshall et al (78) further reported "phenotypic-boundary" to be between codons 159 and 168 and according to Walon et al (79) to codons 159-163. Nasioulas et al. (80) reported "phenotypic boundary" between codon 159 and 163. Deletion of 5 pair of base at codon 1309 (exon 15) cause development of colorectal adenoma in young age, but patients died from CRC 10 years earlier than patients with the other mutations (81). APC mutations in gene region between codons 1445 and 1578 are associated with severe desmoids, osteomas, epidermoid cysts and polyps of the proximal segments of the digestive tract (82). CHRPE is present in patients in whom the mutation lies downstream to exon 9, but it is not seen in individuals with mutations upstream of this exon (83,84). Mutations beyond codon 1600 are associated with AAPC and different number of extracolonic manifestations (85). In 20-50% of patients it is impossible to detect APC mutation (APC negative FAP). In these patients FAP is presented with significant mild phenotype in correlation with APC positive patients, suggesting influence of different genetic factors (86).

Genotype-phenotype correlation is useful in increasing the accuracy and effectiveness of screening, surveillance and treatment (87).

Patients with FAP have virtually a 100% risk of developing CRC from their adenomas in contrast to patients with sporadic CRC who develop cancer infrequently. It is a question why a mutation in the same APC gene should cause a 5% risk of CRC in patients with sporadic adenomas and a 100% risk in patients with FAP (6). Possible explanation could lead to the understanding of the mechanisms of tumorigenesis and

the "abnormal gatekeeper hypothesis" of CRC development (88).

Gatekeepers are genes that normally play a central role in the inhibition of normal cellular growth (89). Gatekeeper genes play a very important role in controlling the cellular growth machinery. The inactivation of these genes alone is enough to initiate tumor formation. These genes are tumor specific. It is known that for colonic epithelial cells APC gene has been proposed as the primary gatekeeper gene (22). Patients with FAP inherit a germline mutation in the APC gene. This mutated form of APC gene is expressed in every cell in the body, including all colonocytes. Only one additional acquired (somatic) mutation is needed to inactivate both APC alleles initiating tumorigenesis in patients with FAP. Second mutation can occur in any of the colonocytes because all of them contain one mutant copy of APC. Sporadic CRC patients have to acquire two somatic mutations in APC in the same colonocyte in order to initiate tumorigenesis, the likelihood of which is much less than for FAP. After initiation of tumorigenesis and adenomas form in either sporadic disease or FAP, the accumulation of mutations by "adenoma-carcinoma sequence" occurs over a 10-year period in both groups. The early age of CRC onset seen in FAP patients is a result of the early onset of adenoma development because of mutation in the APC gatekeeper gene (22).

The rate of mutation identification in the APC gene in FAP families is about 80% (90,91). If an APC mutation is detected in the index patient, the accuracy of the test in the family members approaches 100% because all affected family members inherit the same APC mutation (92). Genetic testing should not be performed without genetic counseling being provided (92).

The mutation status determines the choice of surgical procedure. In FAP patients with mutations proximal to codon 1250 a subtotal colectomy with ileo-rectal anastomosis is primary therapeutic choice, but in patients with mutations distal to codon 1250 a total colectomy with ileal pouch should be done (93).

Lynch syndrome (HNPCC)

Lynch syndrome or HNPCC (hereditary non polyposis colorectal cancer) is an autosomal dominant inherited cancer syndrome characterized by the development of (CRC) at an early age of onset, a preponderance of cancers in the proximal segments of colon (predominantly on the right side), an excess of multiple CRC, synchronous and metachronous CRC (94,95), and poorly differentiated and mucinous CRC (38,96). Lynch syndrome is associated with a small number of adenomas that are located predominantly on the right side of the colon (97). The mean age of cancer diagnosis is approximately 42 years, with a range of 20-80 years (98). To facilitate the clinical diagnosis of HNPCC, the International Collaborative Group on hereditary non-polyposis colorectal cancer (ICG-HNPCC) proposed the "Amsterdam criteria" (Amsterdam minimal criteria) in 1990.

Amsterdam criteria include: the presence of three or more relatives with CRC, one as the first degree relative of the other two; that the relatives affected by CRC span two or more generations; and at least one relative is affected by CRC before the age of 50 (99). Patients with HNPCC are at risk, in addition to CRC, of developing extracolonic tumors including endometrial, ovarian, stomach, small bowel, renal pelvis, ureteral, hepatobiliary, skin, etc. These extracolonic tumors are included in modified Amsterdam criteria (100). Bethesda criteria developed in 1997 with the aim to better identify HNPCC families (101). These criteria take into account the molecular and pathologic features of CRC and adenomas in addition to family history.

New clinical criteria have been proposed by the ICG-HNPCC, which encompass both the Amsterdam and Bethesda criteria (102). These criteria include:

1. Familial CRC meeting the Amsterdam criteria,
2. Familial clustering of colorectal and/or endometrial cancer,
3. Development of associated cancers: stomach, ovary, ureter/renal pelvis, brain, small bowel, hepatobiliary tract, and skin,
4. Development of multiple cancers, including synchronous or metachronous CRC or extra-colonic cancers,
5. Development of CRC with: (1) predilection for proximal colon; (2) improved survival; (3) increased proportion of mucinous tumors, poorly differentiated tumors, and tumors with marked host-lymphatic infiltration and lymphoid aggregation at the tumor margin,
6. Development of colorectal adenomas with: (1) the number varying from one to a few; (2) an increased proportion of adenomas with villous growth pattern; (3) a high degree of dysplasia; and (4) the age of diagnosis <40 years,
7. High frequency of microsatellite instability in the tumor,
8. Immunohistochemistry: loss of MLH1, MSH2, or MSH6 protein expression,
9. Germline mutation in mismatch repair (MMR) genes (MSH2, MLH1, MSH6, PMS1, and PMS2).

The genetic defect underlying HNPCC is a defect in DNA MMR genes. In the presence of normal DNA MMR, abnormally paired nucleotides are excised and replaced with the proper nucleotide base pair. When inactivation of the mismatch repair system is present (characteristic of HNPCC), errors in base pairing will accumulate, predisposing the cell to malignant progression. This persistent error reported in base pairing found in DNA of tumors from patients with HNPCC has been referred to as the "mutator" phenotype (103,104), "the replication error" (RER) or microsatellite instability (6).

In human genome there are about 100 000 microsatellites, short repeated sequences of mono-, di- or three nucleotides. The best known is CA (n), where the value of n is 10-30 (105). During carcinogenesis sequences take or lose "short or repeated sequence" demonstrating microsatellite instability (105-107). These observations,

along with the results of genetic linkage analysis in HNPCC (108,109) and the elucidation of DNA MMR mechanisms in bacteria and yeast (110,111), led to the identification of two major genetic susceptibility loci for HNPCC, hMSH2 and hMLH1.

Clues to the mechanism underlying the molecular basis of tumor microsatellite instability emerged from the study of the effects of mutations within the mismatch repair genes in bacteria and yeast (112). MMR system of the *Escherichia coli*, MutHLS, is responsible for the repair of most nucleotide mispairs in *E. coli* (113,114). The *E. coli* repair system requires several mut gene products: MutH, MutL, MutS and MutU, along with single stranded DNA binding protein, exonuclease I, exonuclease VII, RecJ exonuclease, DNA polymerase III holoenzyme and DNA ligase (113). The MutHLS MMR system has been identified in both prokaryotes and eukaryotes. Eukaryotic MMR system is more complex than the prokaryotic MutHLS system. Six yeast homologues of the *E. coli* MutS (MSH1-6) as well as two MutL homologues (MLH1 and PMS1) have been identified in *Saccharomyces cerevisiae* (115). Frequency of tract instability is elevated in *E. coli* strains with mutS and mutL mutations (111). Similarly, tracts of simple repetitive DNA in yeast were destabilized in cells bearing mutations in any of three (MSH2, MLH1, and PMS1) genes involved in MMR (116). It is suggested that the microsatellite instability in some colorectal tumors reflected mutations in genes involved in DNA MMR (112). It became apparent that DNA MMR genes were ideal candidates for the HNPCC susceptibility loci. The human homologues of the bacterial and yeast mismatch repair proteins were localized and cloned, and their role in HNPCC was examined. There are multiple homologues of the prokaryotic MutL (hMLSH1, hPMS1, hPMS2 and hPMS3-8) and MutS genes (hMSH2, hMSH3 and hMSH6) on chromosomes 3p21.3, 2q31-33, 7p22, 7q21/7q22, 2p22-p21, 5q11-q12 and 2p16, respectively (115). hMSH2 gene was the first cloned in some HNPCC kindreds (117,118), but later germline mutations of hMLH1 gene showed within other HNPCC kindreds (119,120).

Six human genes have been identified as participating in the function of MMR. These genes include MSH2, MLH1, PMS1, PMS2, MSH6 and MSH3 (117-119, 121-123).

In HNPCC, a vast majority of investigations have focused on the mutation analysis of hMSH2 and hMLH1 (124-127). Approximately 30% of families with HNPCC have germline mutations in hMSH2, while another 30% have mutations in hMLH1 (126-128). Most of the hMSH2 mutations consist of frameshifts or nonsense changes (127,128). On the other hand, hMLH1 mutations include both frameshift and missense changes. Splice site alterations are common in hMLH1, but less common in hMSH2. hMSH2 mutations are randomly distributed throughout the coding sequence (127), but clustering of hMLH1 mutations in exons 15-16 has been reported (129).

In patients with HNPCC, adenomas form at the same rate as in patients with sporadic CRC, because there is no germline defect in gatekeeper gene function. Rate progression from adenoma to carcinoma is accelerated due to an abnormality in the caretaker genes (22,88).

Caretaker genes maintain the integrity of the genome (89). Inactivation of caretakers does not directly initiate tumor formation, but enhances the rates of mutation in other genes, including gatekeepers (89). Patients with HNPCC inherit a single mutant allele (copy), caretaker (MMR) gene. A subsequent somatic mutation of the normal allele is required to inactivate the MMR system, resulting in the "mutator phenotype". MMR inactivation allows any mutations occurring during replication to be incorporated permanently into the cells' DNA. Inactivation of the MMR system promotes tumor formation, but does not initiate it. Thus, inactivation of MMR must be followed by other mutations (for example, APC gene), which are directly involved in malignant transformation (89). In patients with HNPCC, at least three mutations (one caretaker and two gatekeeper genes) are essential for tumor formation. So, the familial risk for HNPCC is increased only 5- to 50-fold above the general population (89).

In a recent study in Finland, 2.7% of CRC cases were found to be carriers of HNPCC-associated mutations, leading to an estimate that the population prevalence of the mutations was 1/740 (130). Male mutation carriers have a lifetime CRC risk of 74% or more; female mutation carriers have a lower risk, which is still many times higher than the risk in the general population. Lifetime endometrial cancer risk is 42% or more (131).

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Juvenile polyposis

Juvenile polyposis is an autosomal dominantly inherited polyposis syndrome that only recently has been shown to carry an increased risk of cancer (6). The relative risk of cancer compared to the general population has not been well-defined (132). The polyps in juvenile polyposis have hamartomatous origin, primarily composed of stromal components, with lack of dysplastic changes (133). Epithelial cells in hamartomatous polyps are still at an increased risk of malignant alteration. The molecular pathway by which hamartomatous polyps undergo malignant transformation has been termed the "abnormal landscaper hypothesis" (88).

Abnormal landscaper hypothesis suggests that the abnormal stromal environment surrounding the epithelial cell is the inducer of neoplastic changes (88, 133). In some juvenile polyposis patients, germline alterations have been localized to the PTEN (phosphate mutated in endometrial cancer) and the SMAD4 genes (134-136). The germline mutations in juvenile polyposis predispose the stromal cells rather than the epithelial cells to proliferate and form a polyp (124). Once hamartoma forms, the overlying epithelial component becomes susceptible to mutations in gatekeeper genes such as APC, leading to adenomatous changes and subsequent progression to carcinoma (137). It is suggested that mutations in the lamina propria cells within the polyp may alter interactions with the overlying epithelial cells and initiate adenomatous transformation of the hamartomatous polyp (137).

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GENETIKA KOLOREKTALNOG KARCINOMA

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Kratak sadržaj: *Napredak u molekularnoj biologiji kolorektalnog karcinoma je proširilo naše razumevanje bolesti i omogućilo shvatanje sporadičnog i naslednog kolorektalnog karcinoma. Mutacije APC gena su odgovorne sa porodičnu adenomatoznu polipozu, a mutacije 6 gena (odgovornih za popravku rasparenih baza): MSH2, MLH1, PMS1, PMS2, MSH6 i MSH3 za nasledni ne-polipozni kolorektalni karcinom. Razvoj genetskih testova za otkrivanje saopštenih mutacija gene omogućice otkrivanje nosioca gena i dijagnozu kolorektalnog karcinoma u ranoj fazi.*

Ključne reči: *Mutacije, APC gen, FAP, Lynch sindrom (HNPCC)*