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Novel strategies for comprehensive mutation screening of the APC gene

Minireview

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Colorectal cancer is the 4th most common cause of cancer related deaths worldwide and new possibilities in accurate diagnosis and targeted treatment are highly required. Mutations in adenomatous polyposis coli (APC) gene play a pivotal role in adenoma-carcinoma pathway of colorectal tumorigenesis. The quarter century from its´ first cloning, APC became one of the most frequently mutated, known driver genes in colorectal cancer. Intensive routine molecular testing of APC has brought the benefits for patients with family history of polyposis or colorectal cancer. Nevertheless, multiple mutational disease-causing mechanisms make the genetic testing still challenging. This minireview is focused on implementation of novel APC mutation screening diagnostic strategies for polyposis families according to the current findings. A further understanding and improved algorithms may help to increase the mutation detection rate. APC germline mutations achieve close to 100% penetrance, so more comprehensive approach followed by preventive and therapeutic strategies might reflect in decrease in burden of colorectal cancer.

Key words: colorectal cancer, familial adenomatous polyposis, large deletions, noncoding mutations, molecular diagnostic algorithms

Familial adenomatous polyposis and *APC* gene

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women [1]. About 70% of all CRCs represent sporadic cases, developed due to the somatic mutations. Familial predisposition account for 10–30%, whereas hereditary diseases present about 5–7% of all CRCs [2]. Well-described form of hereditary CRC is familial adenomatous polyposis (FAP; [MIM 175100]) characterized by hundreds to thousands (classic FAP) or less than one hundred colorectal adenomas (attenuated FAP, AFAP). Apart from colorectal adenomas, extracolonic manifestations such as desmoids tumors, osteomas, dental abnormalities, congenital hypertrophy of the retinal pigment epithelium (CHRPE), lipomas, epidermoid cysts and uppergastrointestinal polyps may also develop in patients with FAP [3]. In 2003, a genetically distinct type of AFAP has been identified, called MAP for *MUTYH*-associated polyposis (MIM 608456) [4]. The phenotype is similar to the AFAP, but the mode of inheritance is more complicated and the patients with MAP have fewer adenomatous polyposis (generally >100), later age of onset of adenomatous polyposis and colorectal cancer than classic FAP [5].

FAP is caused by dominant inheritance of germline mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene localized on chromosome 5q21-22. It encodes several tissue specific transcript in which the major transcript has an open reading frame of 8543 bp [6]. The *APC* gene has two promoters, 1A and 1B, which generate different mRNA products and appear to undergo tissue-specific regulation [7,8]. Promoter 1B is located approximately 30 kb upstream of promoter 1A. *APC* promoter 1A is down-regulated through hypermethylation in healthy gastric mucosa and gastric cancers [9]. Rohlin et al. showed that significant levels of transcript

are generated from promoter 1B in blood and in colorectal mucosa, as well as in a number of additional normal tissues. They estimated absolute levels of expression from promoter 1B to be 25- and 250-fold higher compared with two different transcript generated from 1A in normal colorectal mucosa and 100- and 1000-fold higher for each transcript in blood. It is thus established that promoter 1B has an important role in the regulation of *APC* in a variety of normal tissues [7].

APC is a large scaffold protein with multiple binding partners and function, ubiquitiously expressed in a variety of tissues, including the brain and gastrointestinal tract. It is a key regulator of the oncogenic protein β-catenin in the Wnt signaling pathway. Failure to control cytosolic levels leads to an increase in nuclear β-catenin levels, where it binds to transcription factors and facilitates tumorigenesis. In addition to maintaining β-catenin concentration, APC is also a regulator of mictotubules (MTs) and APC mutations influence MT stability, growth [10], cell migration, adhesion, apoptosis and DNA repair [11]. Deletion of APC is also linked with intellectual and autistic disorders. Mohn and colleagues elucidate the importance of APC in the mammalian brain by showing that it is an essential regulator of both synaptic adhesion complexes and signal transduction networks [12]. While it is known that both mutant and full-lenght forms of APC locate at centrosomes, there are major gaps in our understanding of the targeting, dynamics and regulation of APC in this structure. The dynamic profile of APC at the centrosome was surprisingly unaffected by loss of the C-terminal half (~1534 amino acid) of the protein, indicating that all the key sequences that mediate transient and strong protein associations reside within the N-terminal region of APC [13]. C-terminus is predicted to assemble additional protein complexes required to regulate MT nucleation [14] and other activities.

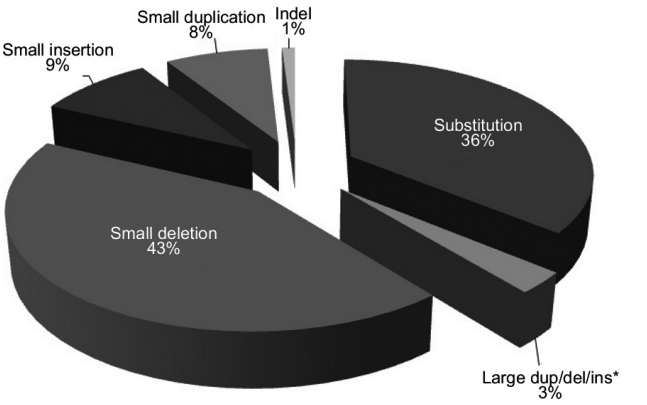

Figure 1. Frequency of APC mutations according to the InSiGHT Colon Cancer Gene Variant Databases (http://chromium.lovd.nl/LOVD2/colon_cancer/home.php?select_db=APC), last updated February 04, 2016.
*** large dup/del/ins – large duplications/deletions/insertions**

Mutation spectrum of the *APC* gene

Over 1500 mutations have already been identified [15], with some genotype-phenotype correlations published in the literature [16,17]. Novel mutations are still being reported showing approximately 15% to 20% of FAP patients have de novo germline mutations [18]. The most common *APC* mutations in FAP patients are base substitutions and small deletions representing 36% and 43%, respectively (Figure 1). About 60% of the *APC* mutations in colorectal tumors are clustered in the central domain of *APC* (amino Acids 1284-1580), also called the mutation cluster region (MCR) [19]. *APC* mutation within the MCR results in a truncated APC protein that lacks all of the axin binding sites and all but one or two of its 20-amino acid β-catenin binding sites. There are two hotspots for germline mutations at codons 1061 and 1309, and another two hotspots for somatic mutations at codons 1309 and 1450 [20]. In accordance, our previous results showed the most frequent mutations in tested Slovak FAP families were located within codons 1309 and 1061 represented 15% and 7%, respectively. Moreover, the expressive phenotype, large amount of colorectal polyps and congenital hypertrophy of the retinal pigment epithelium (CHRPE) were associated to all mutations within both these codons [21]. The vast majority of *APC* gene variations result in the expression of truncated protein that might have a dominant-negative, or gain-of-function, effect at least in the colon. Nontruncating single-base substitutions in the coding *APC* sequence or unique variants in less conserved intronic regions close to the splice sites have rarely been reported in FAP. Most of these *APC* variants are pathogenic due to aberrant splicing [22]. Recent data suggest that gross alterations in the *APC* gene may have been underreported initially, with up to 20% of FAP families potentially carrying a gross alteration [23]. However, the frequency of pathogenic *APC* mutations is very high compare to variants with non-significant relevance (Figure 2).

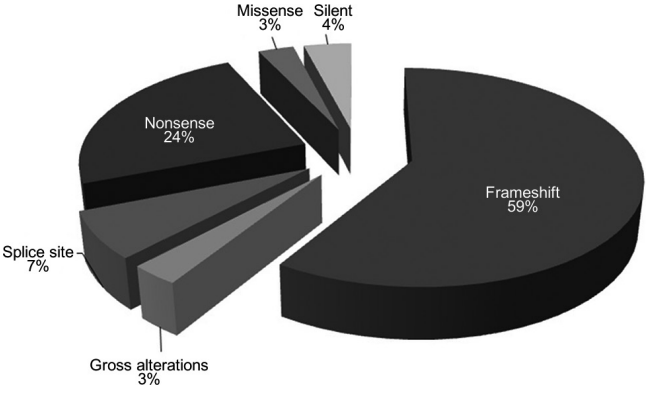

Figure 2. Molecular consequences of APC variants according to the InSiGHT Colon Cancer Gene Variant Databases (http://chromium.lovd.nl/LOVD2/colon_cancer/home.php?select_db=APC), last updated February 04, 2016

Large deletions in noncoding regions of the *APC* gene

APC gene was cloned in 1991 and since then worldwide establishment of molecular-genetic screening in patients at risk took place. However, 20% of classical FAP and 70% of AFAP cases remain mutation-negative after routine testing [16]. Mutations in *APC* noncoding regions, such as intronic mutations or promoter regions and in other causative genes like *MUTYH*, combined with older testing technologies account for some of the undetected mutations. Introduction of multiplex ligation-dependent probe amplification (MLPA), large but submicroscopic genomic *APC* deletions were more readily detected and are recognized to constitute around 12% of FAP cases [24]. The introduction of next generation sequencing using whole-genome sequencing, whole-exome sequencing and multigene panels have made it possible to identify a spectrum of new mutations and also new causative genes in hereditary CRC. Zhang et al. identified by next generation sequencing a novel heterozygous large deletion (c.423_8532del) of the *APC* gene. This is the first reported large deletion in the Chinese population associated with FAP [25]. Large deletions could be detected using microarray-based comparative genomic hybridization (array CGH). In this method patient and control DNA samples should be labeled with different florescent dyes [26].

Charames et al. described a single family with a large deletion containing promotor 1A, associated with complete silencing of the deletion-containing allele. Owing to the lack of promoter 1B-specific probes, it remains unsettled whether or not the deletion also affected promoter 1B. The mechanism of allelic silencing could not be determined at the time [27]. Rohlin et al. described a ~61 kb heterozygous deletion which resulted in reduction in expresion (approximatelly 90%), but not silencing, of the *APC* allele [7]. This deletion affects an open reading frame within it. Lin et al. found ~11 kb deletion, encompasses exon 1B and promoter 1B, which silenced one *APC* allele in affected individuals [28]. Pavicic et al. found that a large deletion of 132kb resulted in a reduction of the deleterious allele expression to 40-60% of the wild-type allele [29]. Yamaguchi et al. identified a deletion of ~10 kb encompassing the promoter 1B resulted in a marked decrease of *APC* transcripts in spite of the remaining *APC-1A* transcript. This deletion caused 87% reduction of mutant allele expression. Although deletions of promoter 1B reduced the expression of mutant *APC* allele, the degree of reduction caused by the deletions is different. Interestingly, the residual transcription was maintained by promoter 1A although the activity of promoter 1B was completely lost [30]. This compensatory mechanism against the impaired activity of promoter 1B has been reported by Rohlin et al. and Yamaguchi et al. [7,30].

In colorectal carcinomas, methylation of promoter 1A is presented in 20-45% of tumors [31,32] whereas promoter 1B is not prone to aberrant methylation [33]. The phenotype of classical FAP with no extracolonic manifestations appears common to all three families with promoter 1B deletions [7,29,34]. Due to the small sample size it would not be appropriate to speculate about genotype-phenotype correlation. Snow et al. speculate that the loss of expression from the *APC* promoter 1B deletion allele combined with low levels of promoter 1A expression may modify the presence of gastric and duodenal polyps [35]. Point mutation in the promoter 1B of *APC* [36] and description of gastric polyposis and gastric cancer in some individuals with large deletions around the promoter 1B [7,28,29,34,35], suggest that families with alternations in the promoter 1B are at risk of gastric adenocarcinoma. Li et al. study show that specific point mutations in the Yin Yang 1 (YY1) binding site in the promoter 1B of *APC* are the cause of gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), a new and potentially severe variant of FAP [36]. YY1 is a ubiquitously expressed transcription factor that has been shown to have multiple roles in oncogenesis and can act as both an activator and repressor of transcription [37].

Molecular diagnostic algorithms for polyposis families

Imbalance in allele-specific expression (ASE) of the two *APC* alleles as a cause of FAP has been recognized in several families [38]. Reduced ASE of the *APC* gene has been shown to be associated with a predisposition to FAP. The degree of expression reduction for one allele has been variable, ranging from around 50% to complete silencing [38,39]. Castellsagué et al. propose a molecular diagnostic algorithm for polyposis families that undergo *APC* mutation screening beginning with analysis of gross rearrangements. In samples with ASE imbalance, sequencing of exons 1-14 at the cDNA or gDNA level would be followed by analysis of the promoter region and, possibly, the 3´UTR region. This strategy would have made it unnecessary to sequence the largest exon of the gene (exon 15) [38]. In contrast Hegde et al. recommended that FAP testing be performed using full sequencing of the *APC* gene. If no mutation is detected, then testing for large gene rearrangements should be performed [23]. It is difficult to say, which algorithm is better. Comprehensive analysis of the entire *APC* gene is necessary for diagnostic testing of FAP. A mutation is detected in ~80% of patients with a clinical diagnosis of FAP, with DNA sequencing detecting 87% of point mutations and small insertions or deletions [40]. The remaining 10-15% of mutations represents gross deletions and duplications, which can be detected by MLPA, Southern blot, or real-time quantitative PCR analysis. Recently, New Generation Sequencing (NGS) techniques afford the opportunity to screen patients for a comprehensive panel of colorectal cancer susceptibility genes in a cost-effective fashion [41].

Targeted therapy and prognostic role of the *APC* gene

APC was presumed to be an important „initiator“ gene for the majority of CRCs [42,43]. The Cancer Genome Atlas (TCGA) project has profiled and characterized the landscape

of CRC mutations. In 94% of analysed samples, a mutation in one or more members of the WNT signaling pathway occurred, mainly the *APC* gene [44]. Yu et al. successfully affirmed *APC* as one of the most frequently mutated genes by validation of 187 recurrent and pathway-related genes in analyzed colon cancer series [45]. Classical tumor progression model *APC→KRAS→TP53* is a widely perceived sequential pathway of the key driver mutation events commonly occurring in CRC development [43,46]. The analysis performed on 468 colorectal tumor samples across 1321 genes associated with human cancer revealed that *APC* mutation usually co-occurs with either *KRAS* or *TP53* mutations or both [47]. This suggesting that *APC* mutations need to make a partnership with one or more additional driver mutations to advance to CRCs. Our results showed the presence of truncating *APC* mutation in codon 1060 together with p53 mutation in codon 210 in FAP patient with extraordinary expressive phenotype [48]. While *APC* mutations did not affect survival in a cohort of 107 CRC patients [49], a novel prognostic, five-class multigene mutation classification system comprising *APC* to play a central role in the context of its partnering mutations (with *KRAS* and *TP53*) and its bi-allelic mutation status has been developed [47].

Despite the prevalence of APC truncations in CRC, there are currently no therapeutics directly targeting them. Zhang et al. identified a candidate small molecule, TASIN-1 (truncated APC selective inhibitor-1), which specifically kills CRC cell lines with truncated APC through induction of apoptotic cell death. TASIN-1 inhibits cancer cell growth in human tumor xenografts and in a genetically engineered mouse model of CRC [50]. Considering the high prevalence of *APC* mutations in patients with CRC, targeting truncated APC should be an efefctive therapeutic strategy for prevention and intervention of CRC and a potential marker for stratifying patients in future personalized medicine clinical trials.

Conclusion

APC gene size, allelic and locus heterogenity, and multiple mutational disease-causing mechanisms continue to make the detection of disease-causing mutations in patients with colorectal adenomatous polyposis challenging. Technological advances as whole-genome sequencing in combination with ASE analysis by deep sequencing may be a useful strategy to identify deleterious genetic alterations in the regulatory regions undetected by routine genetic screening. Although genetic risk of FAP can be evaluated through mutation testing, refined correlations between specific mutations and clinical phenotypes remain limited and still do not provide the guidance for the clinical management of patients with FAP disease. Moreover, recent studies point to possible prognostic role of *APC* in tumor evolution. Comprehensive approach and development of multigene CRC classification systems might help to identify appropriate therapy for subpopulations of cancer patients.

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