Gonzalez et al. Applied Cancer Research (2017) 37:13
DOI 10.1186/s41241-017-0020-1

Applied Cancer Research

REVIEW Open Access

Current applications of molecular pathology in colorectal carcinoma

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Abstract

Molecular pathology is playing an increasingly important role in the treatment and overall management of patients with colorectal carcinoma. Three distinct genetic pathways have been identified that play a role in carcinogenesis: the chromosomal instability pathway, the microsatellite instability pathway, and the CpG island methylator phenotype pathway. Certain genetic mutations, some of which overlap with the aforementioned pathways, can also indicate that a carcinoma patient has a genetic predisposition syndrome, such as familial adenomatous polyposis, Lynch syndrome, and hamartomatous polyposis syndromes. A variety of advanced methods, including next-generation sequencing, are available to test for these and other mutations, such as targetable mutations that may allow tailoring of a treatment regimen to a patient's specific cancer (e.g., *KRAS* and *BRAF* mutations). The possible future role of testing circulating tumor cells is also addressed. New mutations and syndromes continue to be discovered, ensuring that our knowledge of colorectal carcinoma and our ability to treat it will advance in the future.

Keywords: Colorectal carcinoma, Hereditary syndromes, Molecular pathology, Therapeutic targets, Next generation sequencing

Background

Understanding of the molecular pathogenesis of colorectal carcinoma (CRC) began with the adenoma-carcinoma sequence, wherein tubular adenomas that arise within the colon accumulate additional molecular mutations over time, with oncogene activation and tumor suppressor inactivation, leading the adenoma to develop into adenocarcinoma (Fig. 1) [1]. A number of genes have been implicated in this sequence, including *KRAS* [2], *TP53* [3], *APC* [4], and less commonly *BRAF* [5].

While this pathway of molecular oncogenesis, termed the chromosomal instability pathway (CIN), accounts for roughly 60% of CRCs, a variety of other insults can accumulate to cause the remaining proportion of the disease [6]. The most well-studied of these is the microsatellite instability (MSI) pathway (Fig. 1) [7]. Certain genes (most notably *MLH1*, *MSH2*, *MSH6*, and *PMS2*) encode proteins that repair mismatched DNA bases, preventing the formation of deleterious microsatellite sequences. If this functionality is lost due to gene mutation, an adenoma can form and may, within a few years, progress to malignancy as well; hypermethylation of *MLH1* (see below) accounts for perhaps 12% of CRCs, with germline mutation of a mismatch repair gene accounting for about 3% [7].

A third pathway, the CpG island methylator phenotype (CIMP) or serrated pathway, causes 35% of CRCs and involves epigenetic hypermethylation of CpG islands in promoter regions of tumor suppressor genes, preventing them from undergoing transcription and therefore effectively inactivating them (Fig. 1). Such tumors typically arise from a sessile serrated adenoma, rather than a tubular adenoma (Fig. 1) [8]. This shares some overlap with the MSI pathway, as *MLH1* undergoes hypermethylation in a large proportion of cases. Other potentially targeted genes include *CDKN2A* (which encodes p16) and *THBS1* [9]. CIMP tumors are often proximally located and also harbor a *BRAF* mutation [10], which may have been present in the precursor sessile serrated adenoma.

These three pathways provide a basic but incomplete outline regarding the overall pathogenesis of CRC, as the molecular profile of each individual tumor is highly complex and variable. CRCs collectively have a median

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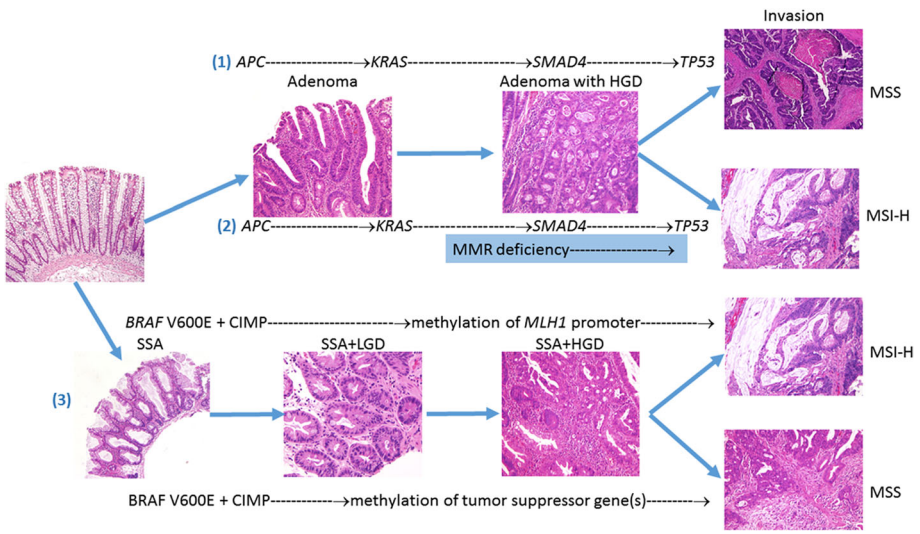

Fig. 1 Three major pathways leading to colorectal cancers. (1) Conventional adenoma-carcinoma sequence with oncogene (e.g. *KRAS*) activation and tumor suppressor (e.g. *APC*, *SMAD4* and *TP53*) inactivation, resulting in microsatellite stable (MSS) cancers; (2) Microsatellite instability (MSI) pathway with mismatch repair (MMR) protein deficiency in patients with Lynch syndrome, resulting in MSI-high (MSI-H) cancers; (3) Serrated pathway with CpG island methylation phenotype, resulting in either MSI-H cancers if methylation occurs in MLH1 promoter or MSS cancers if methylation occurs in tumor suppressor genes. HGD: high-grade dysplasia; LGD: low-grade dysplasia; SSA: sessile serrated adenoma

of 76 identifiable mutations, though only some truly contribute to oncogenesis [11]. Furthermore, the Cancer Genome Atlas Network recently reported that 16% of CRCs are hypermutated, with three-fourths of these demonstrating hypermethylation and the remaining one-fourth harboring mutations in *POLE* or in mismatch repair genes [12]. Their report also noted that *ARID1A*, *SOX9*, and *FAM123B* mutations are often identified in CRC. As additional molecular findings are cemented in the literature, classifications of colorectal carcinoma may become based primarily on molecular properties, rather than histologic subtype; indeed, such classifications are already being proposed [13, 14].

Identification of a CRC's molecular underpinnings can impact a patient in several ways. Targeted therapy against the EGFR signaling pathway has become a mainstay in the treatment of metastatic disease [15]. Furthermore, several inborn syndromes exist that predispose patients to develop CRC more frequently and/or at a younger age than non-syndromic patients in the general population. The most famous of these are familial adenomatous polyposis (FAP) and Lynch syndrome (formerly termed hereditary non-polyposis colorectal cancer, or HNPCC), which respectively embody the CIN and MSI pathways of oncogenesis. There are other, less common syndromes that share some similarities with these two. Additionally, several hamartomatous polyposis syndromes exist as a side note. These syndromes will be discussed in the next section of this article, followed by an overview of the role of molecular testing in patient diagnosis and treatment. Finally, the future of molecular evaluation of CRC will be covered, with a look at promising upcoming targets.

Colorectal carcinoma syndromes

Although fewer than 10% of CRCs can currently be attributed to hereditary cancer syndromes, they are important for several reasons [16]. For one, they afford an opportunity to observe the key molecular factors leading to carcinogenesis. Second, their identification can have a lasting impact on the entire family of a cancer patient. Finally, as family history is a strong risk factor for CRC even in the absence of a known syndrome, there exists potential for discovering new syndromes and novel molecular associations [17]. For these and other reasons, the American Society of Clinical Oncology recently released clinical practice guidelines aimed at identifying syndrome-associated CRCs [18]. A brief review of important syndromes follows.

Familial adenomatous polyposis

Patients with FAP develop hundreds or thousands of adenomatous polyps throughout their colon. While each individual polyp has a very low risk of progressing to adenocarcinoma, the sheer number of polyps leads to a lifetime risk of CRC of effectively 100%, which occurs on average around 34 years of age [19]. Therefore, patients are generally offered a prophylactic colectomy, as early as in their teenage years. However, despite the fact that FAP is the most common gastrointestinal polyposis syndrome, it only accounts for 0.25% of CRC [20]. Extra-colonic manifestations are somewhat common but

comparatively benign. They include gastric fundic gland polyps (which may harbor dysplasia), gastric adenomas, duodenal adenomas, and congenital hypertrophy of the retinal pigment epithelium [21]. Additionally, a rare form of papillary thyroid carcinoma known as the cribriform-morular subtype generally arises only in FAP patients [22].

FAP has a few associated syndromes, which effectively consist of FAP plus an additional lesion or lesions [21]. In Gardner syndrome, patients develop skeletal osteomas, epidermoid cysts, and desmoid fibromatosis, usually of the abdomen. Turcot syndrome has been described as FAP combined with medulloblastoma. (Turcot syndrome is also considered a variant of Lynch syndrome; see next section.)

FAP, Gardner, and Turcot syndromes all result from a germline mutation in *APC* (adenomatous polyposis coli), a tumor suppressor gene located at 5q21 that encodes a protein responsible for degrading β-catenin within the Wnt signaling pathway; it also stabilizes microtubules [23]. The syndromes are inherited in an autosomal dominant fashion, though roughly one-fourth of cases arise from a *de novo* mutation [20]. Mutations are typically toward the 3′ end of exon 15. Mutations at the 5′ end of the gene have less severe manifestations than mutations at the 3′ end, leading to attenuated FAP (AFAP), wherein patients develop fewer than 100 adenomatous colon polyps and have a lifetime risk of CRC of approximately 70% [21].

CRC in FAP develops via the conventional CIN pathway. This sequence is initiated by activation of the Wnt signaling pathway, typically by mutation of one copy of the *APC* tumor suppressor gene in non-syndromic patients, or by the germline mutation in FAP patients. The second *APC* allele is then inactivated by deletion or additional mutation. These *APC* alterations lead to the development of dysplasia, first in aberrant crypt foci and then in true adenomatous polyps. As additional genes become mutated (including *KRAS* and *TP53*), malignancy develops. Overall, the CIN pathway causes gains or losses of large stretches of chromosome material, hence its name [24]. In general, CRC caused by CIN has an unfavorable prognosis [25].

In a side note, the overall molecular alterations in colitis-associated CRC (in patients with ulcerative colitis or Crohn's disease) are similar but occur in a different order; for example, *TP53* is affected early in the pathogenetic sequence, and *APC* is one of the final genes mutated [26, 27].

Lynch syndrome

Formerly known as hereditary non-polyposis colorectal cancer [28], Lynch syndrome accounts for roughly 3% of all CRC and therefore is the most common cause of syndrome-associated CRC [7]. Much as FAP embodies the CIN pathway of carcinogenesis, Lynch syndrome exemplifies the MSI pathway. Patients have a germline mutation in *MLH1*, *MSH2*, *MSH6*, or *PMS2*, and secondary loss of the functional allele is the first insult on the pathway to CRC [7]. Loss of one or more of these mismatch repair proteins allows errors in microsatellites to accumulate during DNA replication, ultimately leading to genome-wide microsatellite instability [29]. In rare cases, Lynch syndrome may occur in a patient with a germline mutation in *EPCAM* (which is adjacent to *MSH2* on chromosome 2) [30].

Lynch syndrome is inherited in an autosomal dominant fashion. The lifetime risk of developing CRC is up to 53%, with a mean age at diagnosis of about 45–50 years [31]. CRC in Lynch syndrome patients develops from a tubular adenoma (unlike in non-syndromic patients with a sporadic MSI-high cancer, where it develops from a sessile serrated adenoma). These tumors have several identifiable characteristics; they are typically right-sided, they harbor a multitude of tumor-infiltrating lymphocytes, and they are more likely to be medullary, mucinous or signet ring cell in microscopic appearance (Fig. 2). They also have an improved prognosis compared to CRC arising via CIN, but they are less responsive to 5-fluorouracil [32, 33]. Lynch syndrome patients are also at increased risk for malignancy in a wide variety of other organs, including uterus, stomach, ovary, and brain [34]. While approximately 15% of all CRC are MSI-high [7], the majority of these are due to sporadic hypermethylation of *MLH1* (i.e., the CIMP pathway), which has not been linked to a heritable cancer syndrome at this time [35], though certain methylation patterns have been linked to family history of CRC in some patients [36].

As with FAP, there are several syndromes related to Lynch syndrome. Muir–Torre syndrome is also caused by germline mutation in a mismatch repair (MMR) gene (generally *MLH1* or *MSH2*); patients are at increased risk of developing cutaneous sebaceous neoplasms, in addition to the sequelae of Lynch [37]. Roughly 28% of families with known Lynch syndrome have a member with Muir–Torre [38].

Patients with a biallelic germline loss in an MMR gene have constitutional mismatch repair-deficiency syndrome [39]. In addition to Lynch syndrome-related tumors, patients develop hematologic malignancies and brain tumors; often, a patient will develop a tumor from more than one of these categories. Café au lait spots are also common. Some patients with Lynch syndrome-related tumors and glioblastomas have been said to have a form of Turcot syndrome [40], in addition to those Turcot patients with FAP and medulloblastomas, as discussed above.

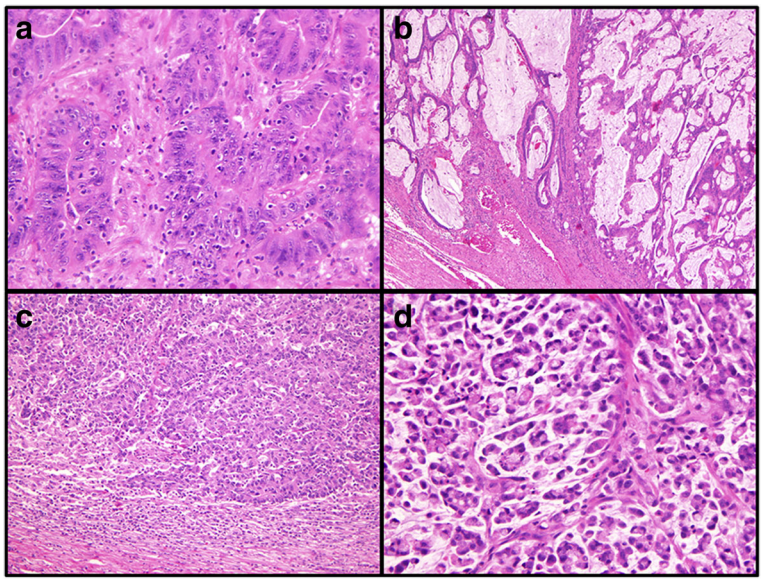

Fig. 2 Representative microsatellite instability-high colorectal carcinomas. **a** Moderately differentiated adenocarcinoma with prominent tumor-infiltrating lymphocytes (original magnification 200X); **b**. Mucinous adenocarcinoma (original magnification 100X); **c**. Medullary carcinoma with a pushing border and prominent tumor-infiltrating lymphocytes f2:3 (original magnification 100X); **d**. Signet ring cell carcinoma (original magnification 200X)

MUTYH-associated polyposis

Attenuated FAP is not the only syndrome characterized by the development of a modest number of colorectal adenomas. In *MUTYH*-associated polyposis (MAP), patients with biallelic *MUTYH* mutations develop colonic adenomas (usually more than 10, but varying from 1 to several hundred [41]) and are at increased risk for CRC [42, 43]. MAP appears to be responsible for fewer than 1% of CRC [44], but it may be underdiagnosed. By age 70, patients have an 80% risk of developing CRC [45], and testing for possible *MUTYH* mutation is therefore recommended in patients with more than 10 adenomatous polyps, a family history of CRC, and/or a known lack of *APC* mutations [46].

While two mutated alleles must be present in order for MAP to manifest, single-allele *MUTYH* mutations may be present in up to 1% of the people in certain populations [47]. Patients by definition lack a germline *APC* mutation. In additional to colorectal polyps and carcinoma, some patients may develop duodenal and gastric adenomas; extra-intestinal manifestations are rare [43].

MUTYH, located on 1p34, encodes for a base excision protein that repairs oxidative DNA damage [48]. MAP therefore shares some conceptual similarity with Lynch syndrome, as both involve malfunctioning repair of damaged DNA. While most patients harbor a c.536A > G or c.1187G > A mutation [47], more than 200 different gene mutations have been reported [49].

POLE/POLD1-associated syndrome

The role of *POLE* and *POLD1* in the development of colorectal polyposis and carcinogenesis has only recently been elucidated. These genes, which encode protein subunits of DNA polymerase complexes, may play a role in the development of hypermutated, microsatellite-stable CRC [50]. Patients with a germline *POLE* p.L424V mutation or one of a handful of *POLD1* mutations appear to be at risk of developing CRC, as well as tumors of the brain, breast, and endometrium [51, 52]. Patients may develop an attenuated adenomatous polyposis, or may develop only a few polyps. To this end, the term "polymerase proofreading-associated polyposis" (PPAP) has been suggested [52]. *POLE* and *POLD1* mutations may be inherited or arise *de novo* [53]. Germline mutations in these two genes appear to be fairly rare, and relatively few syndromic patients have been identified to date. Future reports on additional patients will likely help cement the clinicopathologic features associated with this syndrome.

Hamartomatous polyposis syndromes

There exist several syndromes that cause patients to develop multiple hamartomatous polyps throughout the colon (and often the rest of the gastrointestinal tract).

Peutz–Jeghers syndrome (PJS) is caused by germline mutation or deletion of *STK11*. This autosomal dominant syndrome leads to Peutz–Jeghers polyps in the stomach, small intestine, and colon, as well as characteristic mucocutaneous pigmentation [54]. These polyps have a characteristic "arborizing" architecture, with bundles of smooth muscle creating a framework that gives the polyps a tree-like appearance. These polyps are often harmless, though dysplasia and even carcinoma can

occur within (Fig. 3a). Roughly half of patients develop a gastrointestinal cancer by age 70 [55]; they also are at increased risk for a variety of other neoplasms. Experts have argued that Peutz–Jeghers polyps only arise in syndromic patients, meaning that a patient diagnosed with one likely also has PJS [56].

Germline mutations in *SMAD4* and *BMPR1A* can lead to juvenile polyposis syndrome (JPS), an autosomal dominant condition characterized by juvenile polyps growing through the gastrointestinal tract [54]. Microscopically, these polyps appear expanded and edematous, with dilated glands. As with Peutz–Jeghers polyps, they can develop dysplasia and give rise to malignancy (Fig. 3b); however, a lone juvenile polyp does not suggest that the patient is syndromic [57]. Up to 39% of patients with JPS will develop CRC in their lifetime, at a mean age of 44 years [58]. In at least some patients, a *BMPR1A* mutation instead leads to hereditary mixed polyposis syndrome, an autosomal dominant disorder causing a wide variety of colonic polyps to develop [59]. In other patients, this syndrome arises due to a *SCG5* mutation [60].

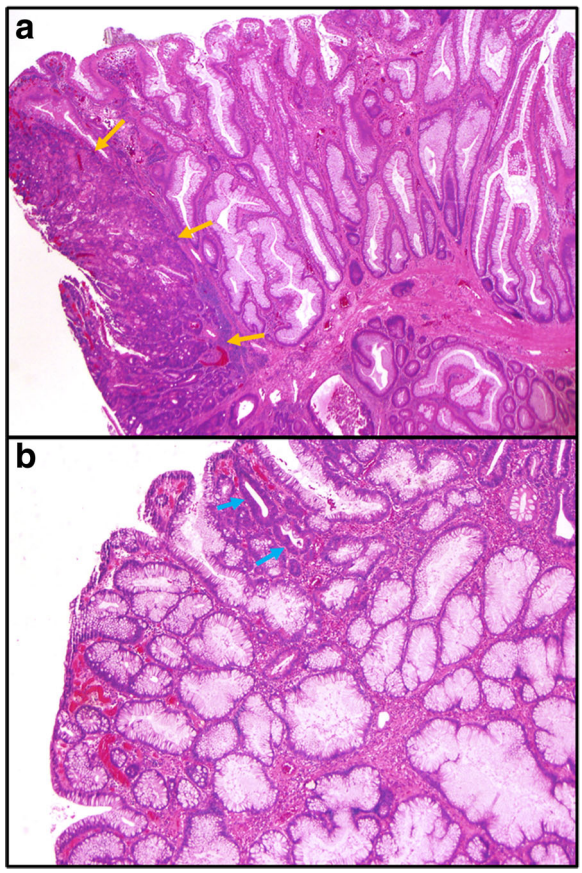

Fig. 3 Neoplastic changes in hamartomatous polyps. **a** Intramucosal carcinoma (yellow arrows) arising in a Peutz-Jegher's polyp showing arborizing smooth muscle proliferation covered by hyperplastic colonic epithelium (original magnification 20X); **b** Low-grade dysplasia (blue arrows) arising in a juvenile polyp showing inflamed stroma and dilated glands (original magnification 20X)

Patients with a germline *PTEN* mutation may develop one of a number of syndromes. The most common of these is Cowden syndrome, which causes a wide variety of gastrointestinal hamartomas (including juvenile polyps and ganglioneuromas), along with characteristic skin lesions and a variety of visceral lesions [54]. In the majority of cases, more than 50 gastrointestinal polyps are found [61]. Patients have a 9% chance of developing CRC; the risk of breast cancer is much higher, at 85% [62]. The other syndromes caused by germline *PTEN* mutation are Bannayan–Riley–Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome, all of which manifest in childhood. Only the first is particularly associated with gastrointestinal polyposis, and none appear to confer an increased risk of malignancy [63].

Finally, Cronkhite–Canada syndrome is a rare, acquired hamartomatous polyposis syndrome characterized by unusual, juvenile-like polyps developing throughout the gastrointestinal tract [64]. Both these polyps and the background flat mucosa show stromal edema, gland dilation, and congestion. Patients also demonstrate ectodermal abnormalities. Up to 25% of patients develop CRC. While the pathophysiologic and genetic underpinnings of Cronkhite–Canada syndrome remain unclear, the disease may be autoimmune in origin [65].

Serrated polyposis

In serrated polyposis, patients develop a multitude of sessile serrated adenomas throughout their colon. This uncommon syndrome was originally termed "hyperplastic polyposis" prior to the discovery of sessile serrated adenomas, which bear histologic similarity to hyperplastic polyps [66]. Mean age at diagnosis is 55 years, and up to half of patients report a family history of CRC. Some patients develop numerous small left-sided polyps with *KRAS* mutations, while others develop fewer, larger right-sided polyps with *BRAF* mutations; a phenotype intermediate between these two has also been described [67]. One large cohort study detected CRC in 35% of patients with serrated polyposis, with an increased number of polyps conferring higher risk [68]. Despite these known phenotypes and molecular alterations, underlying germline mutations and a mode of inheritance have not yet been established for serrated polyposis, which may prove to be a rather heterogeneous disease or category of related diseases. In support of this is the finding that multiple serrated polyps have been described in some patients with MAP [69].

Base excision repair polyposis

This term has been suggested for patients with a germline mutation in the base excision repair gene *NTHL1*, which predisposes them to develop adenomatous polyposis and CRC. Only one report on this entity appears to exist so far [70].

Current molecular testing

Molecular testing can be used to identify patients with hereditary CRC syndromes or individuals genetically susceptible to developing CRCs. In addition, molecular testing of CRCs helps identify molecular biomarkers, which may improve patient care by individualizing cancer treatment.

Molecular testing in hereditary colorectal cancer syndromes

Lynch syndrome

Patients with Lynch syndrome may be identified clinically via use of the Amsterdam criteria and the revised Bethesda guidelines [71]. The revised Bethesda guidelines are more sensitive than the Amsterdam criteria in identification of these patients. However, it is now known that a significant portion of Lynch syndrome patients could still be missed by using the revised Bethesda guidelines [72, 73]. Therefore, the recently updated National Comprehensive Cancer Network (NCCN) guidelines recommend screening for Lynch syndrome in all individuals with CRC or individuals with CRC diagnosed at <70 years old and those ≥70 years old who meet the revised Bethesda guidelines.

Immunohistochemistry for the four MMR proteins, PCR-based MSI testing, or both can be used as an initial screening test for Lynch syndrome. Immunohistochemical stains for the proteins MLH1, MSH2, MSH6, and PMS2 are a sensitive and specific way to detect MMR deficiency [72, 74]. Approximately 90% of MMR-deficient CRCs show loss of nuclear staining for one or more of the MMR proteins (Fig. 4a-b). The four proteins play a critical role in mismatch recognition and initiation of repair [29]. MLH1 and PMS2 form the hMutLα heterodimer, and loss of MLH1 invariably results in the degradation of PMS2. Similarly, MSH2 and MSH6 form the hMutSα heterodimer, and loss of MSH2 is consistently accompanied by loss of MSH6. However, the converse is not true, because loss of PMS2 or MSH6 does not always cause the degradation of MLH1 or MSH2, respectively. Therefore, when a tumor shows loss of MLH1 and PMS2 or loss of MSH2 and MSH6, it is likely due to defective MLH1 or MSH2, whereas isolated loss of PMS2 or MSH6 indicates a defect in PMS2 or MSH6, respectively.

PCR-based MSI testing can detect the genetic changes associated with an MMR defect, namely microsatellite instability. Approximately 90% of MSI-high tumors can be detected by MSI testing. Currently, most laboratories in the US use a fluorescence-based PCR assay from Promega (Madison, WI). The assay includes five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D). Both normal tissue and tumor tissue are analyzed. The mononucleotide markers are used for detection of microsatellite instability, and the pentanucleotide markers are used to ensure that both normal tissue and tumor are from the same patient. The results are interpreted as MSI-high when 2 or more mononucleotide markers show instability, MSI-low when only 1 marker is unstable, and microsatellite stable (MSS) when all markers are stable (Fig. 5).

Both immunohistochemistry and MSI testing have their advantages and disadvantages. Technical failure is not uncommon in MMR immunohistochemistry. In addition, the stains can be falsely negative in treated rectal cancers [74]. The major advantage of the immunohistochemical assay is specific identification of possible defective MMR protein(s). On the other hand, the PCR-

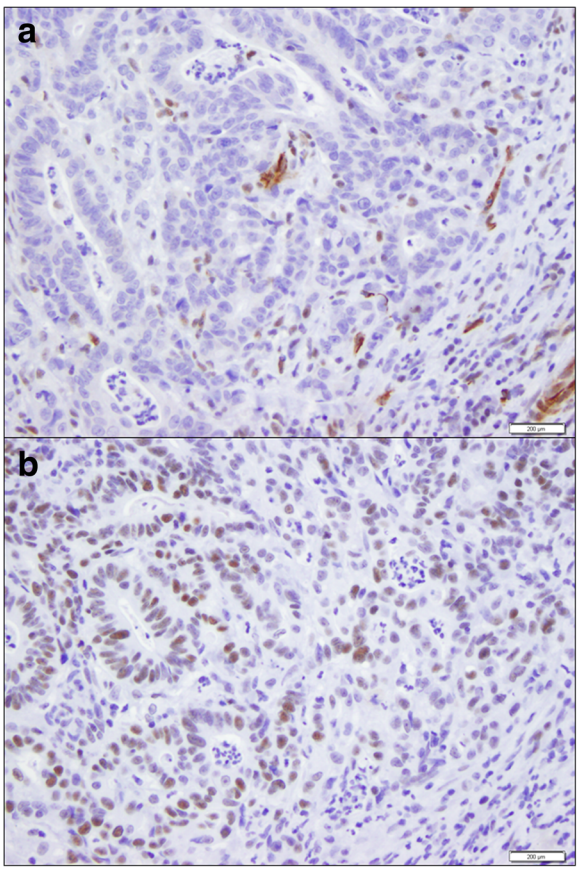

Fig. 4 Immunohistochemical stains for MLH1 and MSH2. **a** Loss of nuclear staining for MLH1 in the cells of a colorectal adenocarcinoma. The tumor also showed PMS2 loss. **b** Intact nuclear expression of MSH2 in the same tumor. It also showed intact MSH6 expression

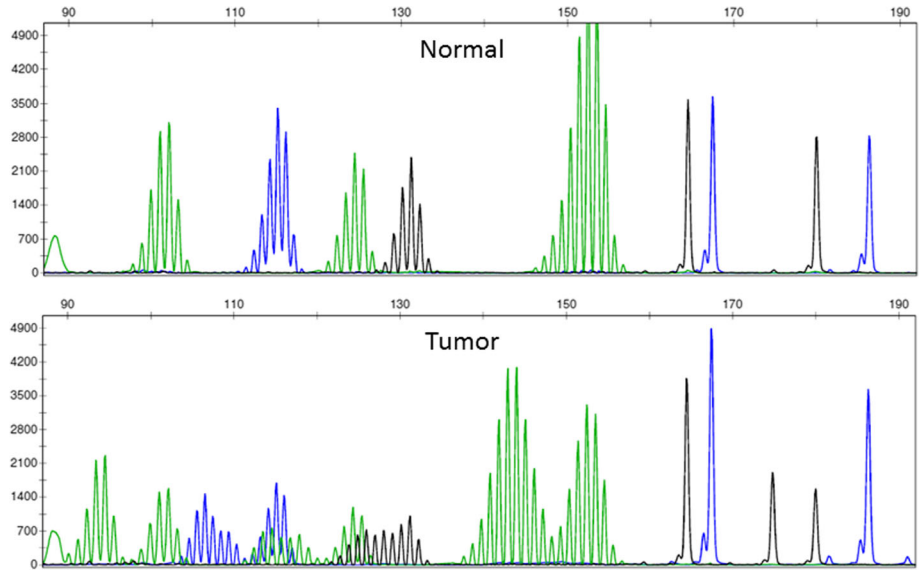

Fig. 5 PCR-based MSI testing showing microsatellite instability in 5 of 5 mononucleotide markers from a colorectal carcinoma. Normal tissue from the same patient is shown for comparison

based MSI testing requires sufficient tumor tissue (at least 10–30% nucleated tumor cells in a sample). However, the results from the PCR-based assay are easy to interpret. In addition, it can be used in treated CRCs. Either MMR immunohistochemistry or PCR-based MSI testing can be used as an initial screening test. If one test indicates MSS in patients with a high suspicion for Lynch syndrome, the tumor should be tested using the second method to increase the sensitivity.

MSI-high CRCs can be sporadic or hereditary. In fact, most MSI-H CRCs are sporadic, caused by *MLH1* promoter hypermethylation, and display loss of MLH1 and PMS2 by immunohistochemistry. In addition, sporadic MSI-high CRCs frequently harbor *BRAF* V600E mutation, which is extremely rare in Lynch syndrome-associated CRCs. If a tumor is MSI-high by PCR-based MSI testing or shows loss of MLH1 and PMS2 by immunohistochemistry, testing for the *BRAF* mutation and/or *MLH1* promoter methylation assay should be followed. Such an algorithmic approach can be used to identify Lynch syndrome patients (Fig. 6).

As mentioned above, MMR immunohistochemistry is able to provide information regarding the relevant gene(s). When there is loss of MLH1/PMS2 with no *BRAF* mutation and no *MLH1* promoter hypermethylation or loss of any other proteins (MSH2, MSH6, or PMS2), germline evaluation should be carried out for the genes corresponding to the absent proteins. For CRCs with loss of MSH2, a test for *EPCAM* deletions need to be included, since a deletion in the 3′ region causes somatic hypermethylation of *MSH2* [75]. Mutations in the MMR genes include missense, nonsense, and splice site mutations as well as regulatory mutations. Large deletions are seen in 5–10% of *MLH1* and 17–50% *MSH2* gene mutations. DNA extracted from a blood sample is required for germline genetic testing. Sanger sequencing can be used to sequence all coding exons and intron/exon boundaries of the relevant MMR gene(s). Next-generation sequencing can also be used for that purpose. However, *PMS2* cannot be sequenced by next-generation sequencing, due to high homology between the *PMS2* functional gene and pseudogenes. Sanger sequencing on long-range PCR using functional gene-specific primers has been used to detect mutations in the *PMS2* gene [76].

If MMR gene mutations are not detected by sequencing, analysis for large rearrangements or deletions of the MMR genes should be performed. Multiplex ligation-dependent probe amplification (MLPA) is commonly used to detect large deletions/rearrangements. Other methods used for large gene rearrangements include Southern blot hybridization, multiplex amplifiable probe hybridization, quantitative PCR analysis, and gene-targeted array-based comparative genomic hybridization [77].

FAP and attenuated FAP

The majority of germline mutations in *APC* are point mutations, short deletions, and short insertions, most of which introduce a stop codon, consequently resulting in a truncating APC protein. Two recurrent mutations at codons 1061 and 1309 are detected in approximately 30% of FAP cases. In addition, approximately 20% of the mutations are gross deletions, insertions, or complex rearrangements. Germline genetic testing of *APC* should be carried out for

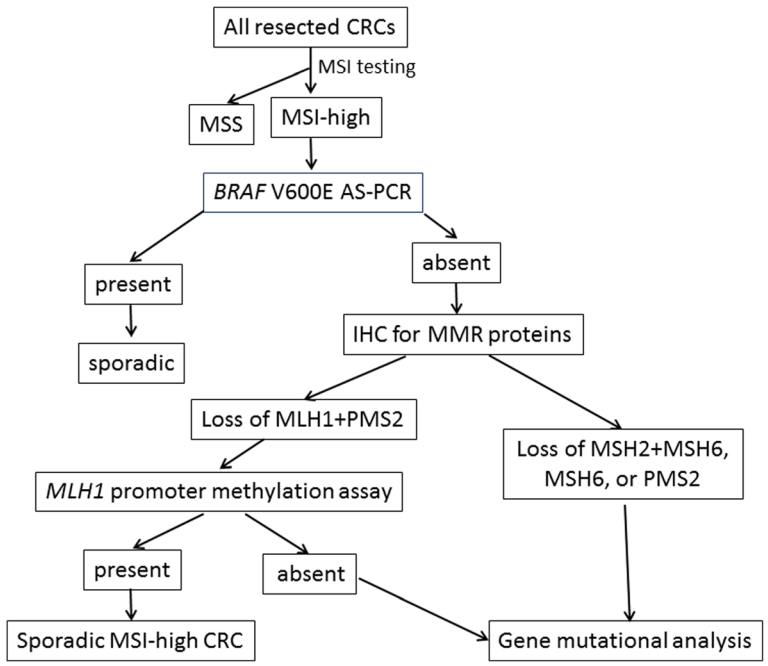

Fig. 6 An algorithmic approach used to identify Lynch syndrome patients at our institution. CRCs: colorectal cancers; MSI: microsatellite instability; MSS: microsatellite stable; ASaPCR: allele-specific PCR; IHC: immunohistochemistry; MMR: mismatch repair

all patients suspicious for FAP or AFAP. Full sequencing of *APC* using Sanger sequencing or next-generation sequencing can be employed to detect the mutations. If no mutations are detected by sequencing, large rearrangement analysis of the gene should be performed [77].

MUTYH-associated polyposis

The presentation of MAP is similar to that of attenuated FAP. In individuals with ≥ 10 colonic adenomas but no germline alterations in *APC*, mutational analysis of *MUTYH* is recommended. Most mutations associated with MAP are missense mutations in the *MUTYH* gene. Two in particular, p.Y165C and p.G382D, are seen in 70–80% of the southern European Caucasian population [78]. Deletions, frameshift, and nonsense mutations have also been reported. Germline testing of *MUTYH* can be initiated by screening for the two most common mutations in the white population by PCR/restriction enzyme digestion-based techniques, or other technologies such as denaturing high-pressure liquid chromatography, pyrosequencing, Sanger sequencing, or allele-specific PCR [77]. If it is heterozygous for the mutations, full sequencing of the gene should be performed. Full sequencing should also be considered for nonwhite individuals suspected of having MAP [77].

Polymerase proofreading-associated polyposis

Missense mutations affecting the exonuclease domains of the polymerase genes *POLE* and *POLD1* have recently been identified to be responsible for PPAP, which can present as a polyposis and/or a Lynch syndrome-like phenotype [51]. Individuals presenting with familial or early onset MMR-proficient CRC and/or *APC*-negative and *MUTYH*-negative polyposis should be screened for germline *POLE* or *POLD1* exonuclease mutations. Sanger sequencing or next-generation sequencing can be used to detect the mutations.

Hamartomatous polyposis syndromes

Mutations in *STK11* are responsible for approximately 90% of PJS cases. Most patients inherit the disease in an autosomal dominant manner; however, up to 45% of cases may be caused by a de novo *STK11* mutation. Most of the genetic alterations are missense/nonsense and small deletion/insertion mutations. Others include large deletions/insertions and rearrangements [79]. Gene sequence analysis of the entire coding regions and the splicing sites by Sanger sequencing and gross deletion/duplication analysis of the gene by MLPA are used to characterize the mutations.

Up to 50% of JPS patients have a mutation in *SMAD4* or *BMPR1A*. Single nucleotide variants explain about 40–45% of cases, and the remaining 10–15% of detectable cases are caused by gross deletions of either gene [80]. The initial test is concurrent sequence analysis of the *BMPR1A* and *SMAD4* genes. If no causative mutations are identified by sequencing, gross deletion/duplication analysis of both genes should be carried out.

PTEN mutations are identified in a small number of JPS patients. In addition, patients with a germline *PTEN* mutation may develop one of a number of other syndromes, such as Cowden syndrome. When no alterations are identified in the *BMPR1A* and *SMAD4 genes,* mutational analysis of *PTEN* may be considered.

Role of next-generation sequencing in hereditary colorectal cancer syndromes

Genetic testing for CRC susceptibility can either focus on well-characterized mutations based on a clinical suspicion or use panel testing to screen for multiple mutations in multiple genes simultaneously. Genetic panel testing can be achieved by next-general sequencing or Sanger sequencing. However, next-generation sequencing has demonstrated benefits on both costs and time required compared to Sanger sequencing [81]. Ambry Genetics (Aliso Viejo, CA) and GeneDx (Gaithersburg, MD) offer next-generation sequencing panels for hereditary CRCs. Similar testing is provided by the Mayo Clinic (Rochester, MN) and the University of Washington (Seattle, WA).

Molecular biomarker testing in colorectal cancers

MSI testing

MSI status may influence therapy decisions in stage II colorectal cancers. MSI is a good prognostic factor, though MSI-high cancers may not benefit from 5-FU-based adjunctive chemotherapy. Therefore, post-operative chemotherapy is not recommended for patients with stage II MSI-high CRC. The 2015 NCCN guidelines recommend that MSI testing or MMR immunohistochemistry should be performed in all patients with stage II CRC. In addition, MSI tumors contain significant more mutations then MSS tumors. They may be more likely to respond to immunotherapy, such as anti-PD1 agents [82]. Therefore, MSI or MMR testing should be performed in all patients with metastatic colorectal cancer if not previously done.

Extended RAS and BRAF mutational analysis for metastatic CRC patients

Mutations of *KRAS* and *NRAS* in exons 2, 3, and 4 result in constitutive activation of the EGFR signaling pathway. Clinical trials have provided evidence that these mutations are negative predictors of response to anti-EGFR therapy [83–88]. BRAF is an immediate downstream molecule of RAS. *BRAF* mutations also constitutively activate the enzyme activity, consequently persistently stimulating the EGFR signaling pathway. Although the results regarding the predictive role of *BRAF* mutations have been controversial, *BRAF* mutations in MSS cancers confer a poorer prognosis [83]. Therefore, the 2015 NCCN guidelines recommend that all patients with metastatic CRC should be tested for *KRAS*, *NRAS* and *BRAF* mutations. The NCCN guidelines also state that extended RAS mutations should be tested whenever possible.

Mutations in *RAS* and *BRAF* are missense single nucleotide substitutions (point mutations). Sanger sequencing, allelic-specific PCR, and pyrosequencing can be used to detect point mutations. However, these assays cannot be multiplexed, and Sanger sequencing requires high tumor cellularity. Many laboratories have developed multigene assays, which are more sensitive than Sanger sequencing and more efficient than the above assays. One example of a multigene assay is the SNapShot platform [89], which is a combination of multiplex PCR amplification of tumor DNA with single base extension of the PCR product following by capillary electrophoresis (Fig. 7). Recently, next-generation sequencing is receiving more widespread used for the same purpose.

Multiple gene mutation analysis by next-generation sequencing

In addition to *KRAS* and *BRAF*, some CRCs harbor mutations in other genes encoding for key intracellular molecular transducers of EGFR activation, such as *PIK3CA* and *PTEN*. Although their predictive and prognostic role is uncertain in metastatic CRC, they are potentially actionable genetic alterations. In fact, the PI3K/AKt/mTOR signaling pathway has been utilized as the therapeutic target for metastatic CRC in several clinical trials. Therefore, identification of mutational status of these genes, in addition to *KRAS* and *BRAF*, could help select

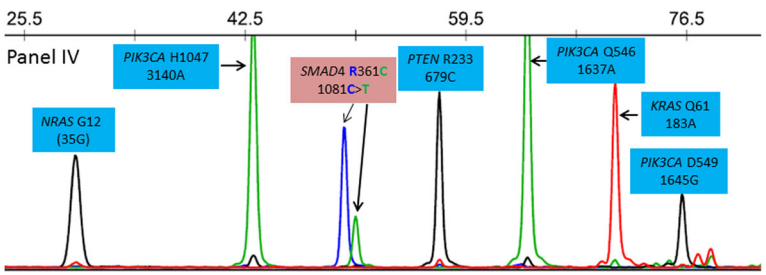

Fig. 7 A panel from the SNapShot assay showing a mutation in *SMAD4* (p. R361C; 1081C > T)

better treatment for patients with metastatic CRC. Analysis of multiple mutations in multiple genes requires implementation of multi-target testing methodologies. Next-generation sequencing has been proven to be a robust and cost-effective tool to analyze a large number of gene alterations simultaneously [90, 91]. In addition, next-generation sequencing may identify uncommon genetic alterations, which could be potential actionable targets.

Although next-generation sequencing has been used for detection of gene mutations in malignancies for clinical implications in some centers, specific guidelines have not been developed. There is always a question regarding which sample should be used for analysis of gene mutations (metastasis versus primary tumor). Theoretically, it is more appropriate to test the metastatic lesions, because they are the cause of clinical morbidity and mortality. Multiple studies have compared *KRAS* alterations and have demonstrated rare discordance between primary cancer and metastasis [83]. However, significant discordance could occur when analyzing a larger panel. One study demonstrated a very different genotype in metastases from the primary tumor in about half of patients with synchronous metastatic CRCs [92]. Therefore, metastases may be a preferred specimen for next-generation sequencing of cancer genes, especially for patients with synchronous metastatic colorectal cancer. Nucleated tumor cellularity should be estimated by examination of an adjacent hematoxylin- and eosin-stained slide. Next-generation sequencing platforms have a minimum sensitivity of approximately 5%. Therefore, tumor cellularity of ≥ 10% is necessary for detection. During the data analysis, mutant-allele frequency (mutant/wild-type ratio) should be calculated. Both tumor cellularity and mutant-allele frequency help determine tumor heterogeneity and mutant allele-specific imbalance.

Future molecular testing in colorectal cancer

Characterization of biomarkers in circulating tumor DNA may be the future of personalized medicine in oncology. It is a non-invasive approach analyzing tumor genotypes in real time. A recent study demonstrated identification of tumor-associated mutations including *KRAS*, *BRAF,* and *PIK3CA* mutations in plasma DNA from the majority of 503 patients with metastatic CRC enrolled in the CORRECT trial [93]. Detection of genetic mutations using circulating tumor DNA may allow clinicians to follow the genetic evolution of the tumor noninvasively and may aid in predicting treatment response. However, to see widespread use in clinical practice, the technologies detecting circulating tumor cells need to be validated in large-scale studies.

Conclusions

Molecular testing has become of critical importance in the management of patients with CRC. It can identify the existence of a tumor predisposition syndrome, and it can help specify tumor-specific targets for patient therapy. Therefore, the genetic makeup of a particular CRC simply cannot be disregarded if the standard of care is to be met. As our knowledge of the genetics of CRC advances, new molecular targets and new genetic syndromes will almost certainly be discovered.

Abbreviations

CIMP: CpG island methylator phenotype; CIN: Chromosomal instability pathway; CRC: Colorectal cancer; FAP: Familial adenomatous polyposis; HNPCC: Hereditary non-polyposis colorectal cancer; JPS: Juvenile polyposis syndrome; MAP: *MUTYH*-associated polyposis; MLPA: Multiplex ligation-dependent probe amplification; MMR: Mismatch repair; MSI: Microsatellite instability; NCCN: National Comprehensive Cancer Network; PJS: Peutz–Jeghers syndrome

Acknowledgements

None.

Funding

None.

Availability of data and material

Not applicable.

Authors' contributions

All authors have contributed to the writing and editing. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Publisher's Note

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Received: 14 October 2016 Accepted: 5 May 2017
Published online: 11 May 2017

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