Review paper

# Molecular pathology of colorectal cancer

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Colorectal cancer (CRC) is one of the most intensively studied cancer types, partly because of its high prevalence but also because of the existence of its precursor lesions, tubular or villous adenomas, and more recently (sessile) serrated adenomas, which can be detected endoscopically and removed. The morphological steps in the adenoma-carcinoma sequence have been elucidated at a molecular level, which has been facilitated by identification of the genes responsible for familial intestinal cancer. However, apart from early detection of familial forms of CRC and its use in genetic counseling, until recently such detailed molecular knowledge has had little impact on clinical management of the disease.

This has dramatically changed in the last decade. With drugs specifically targeting the epidermal growth factor receptor (EGFR) having been shown effective in CRC, mechanisms responsible for resistance have been explored. The finding that *KRAS* mutated cancers do not respond to anti-EGFR treatment has had a profound impact on clinical management and on molecular diagnostics of CRC. Additional genetic tests for mutations in *NRAS*, *BRAF* and *PIK3CA* contribute to determining who to treat, and others will follow. New therapies effective in patients with advanced CRC are under investigation.

Remaining burning questions for optimal management are which patients will relapse after resection of the primary tumor and which patients will respond to the standard 5FU-oxaliplatin adjuvant treatment regimen. Predictive tests to address these issues are eagerly awaited. New classifications of CRC, based on molecular parameters, are emerging, and we will be confronted with new subtypes of CRC, for which the definition is based on combinations of gene expression patterns, chromosomal alterations, gene mutations and epigenetic characteristics. This will be instrumental in designing new approaches for therapy but will also be translated into molecular diagnostics. Both will contribute to improved clinical management of CRC.

**Key words:** colorectal carcinoma, molecular pathology, chromosomal instability, microsatellite instability, CpG island methylator phenotype (CIMP), KRAS.

## Introduction

The face of 'cutting edge' medicine is rapidly changing. In a recent paper in which they reflect on the changes taking place, Stephen Friend and Leroy Hood [1] refer to this as P4 medicine: predictive, personalized, preventive and participatory. Much of this has been brought about by the molecular genetic revolution. It is now possible to sequence a whole genome in a matter of days and at an affordable cost. In the near future we might all have our personal genomic information with us on a smart card, containing the relevant elements of our personal medical history. We will focus here on colorectal cancer (CRC), one of the most frequently occurring cancers, globally responsible for a high number of cancer-associated deaths annually [2]. Colorectal cancer is one of the most intensively studied cancer types, partly

because of its high prevalence but also because of the existence of its precursor lesions, tubular or villous adenomas and more recently (sessile) serrated adenomas, which can be detected endoscopically and removed. Theoretically, removal of these adenomas would prevent most cases of CRC from developing. Characteristic morphological steps in the evolution of these precursor lesions have been elucidated at a molecular level, and the adenoma-carcinoma sequence, as this has become known, is one of the classical examples of stepwise progression of cancer. Gaining this knowledge has been facilitated by the occurrence of a variety of forms of familial intestinal cancer, the molecular genetic background of which has been largely clarified [3]. Apart from early detection of familial forms of CRC and its use in genetic counseling, until recently detailed molecular knowledge has had little impact on the clinical management of CRC. Classical clinico-pathological parameters have remained essential factors in determining how a CRC patient will be treated. It is the purpose of this paper to review how advancing knowledge of the molecular events involved in the development of CRC and its precursor lesions up to the metastatic stage have influenced our approach to (early) detection and treatment of this globally widespread disease, which still kills half of those who develop it [2].

## Pathways in the development of colorectal cancer

Molecular studies of CRC have elucidated that several signaling pathways are involved in its development. We now have a fairly detailed insight into the main (epi)genetic events involved in colorectal carcinogenesis. What has become clear is that CRC is not a single disease [3]. The morphological heterogeneity, in terms of site, grade and type of the tumor, has been expanded by several added layers of molecular complexity [4-6]. We now realize that CRC is a very heterogeneous disease, in terms of clinical presentation, likelihood of cure, pattern of extension and response to treatment, to name but a few determinants of this heterogeneity. A structured approach towards understanding at least parts of this heterogeneity follows the molecular pathways involved in its genesis. Three main pathway concepts have now been developed [7-9], and a fourth has been recently added [10].

### The chromosomal instability pathway

The first elucidated is the **classical pathway**, of which chromosomal instability (CIN) is a hallmark. These carcinomas are usually highly aneuploid and show striking variation in gene copy number. The concept of this pathway was developed by Vogelstein in a landmark paper [7], and it is involved in roughly 80% of colorectal carcinomas. The events in this pathway are illustrated in Fig. 1. Elucidation of the pathway was facilitated through the discovery of the adenomatous polyposis coli (*APC*) gene, the gene responsible for familial adenomatous polyposis (FAP) [11, 12]. *APC* mutations activate the Wnt signaling pathway, which plays a central role in the development of CRC, as in about 90% of all CRC cases gene abnormalities occur which activate this pathway [13]. Most of these are loss of function mutations of *APC*, but rare β-catenin mutations have been reported. The second allele of *APC* is subsequently silenced through mutation, allelic loss or gene promoter methylation. Loss of function of the APC protein interferes with phosphorylation of β-catenin, which is then no longer ubiquitinated and fails to be eliminated in the proteasome. When β-catenin accumulates, it is translocated to the nucleus, where it assumes a transcription factor function in interaction with LEF/TCF and upregulates the expression of genes that promote cell growth (Fig. 2). Wnt pathway activation is already present in adenomatous polyps, precursor lesions of CRC, and hence is considered as an early event in CRC carcinogenesis.

That colon adenomas are precursor lesions of carcinomas was known, long before molecular knowledge started to elucidate the mechanisms involved. Over time, the progression of an adenomatous polyp into a malignant carcinoma became known as the ad-

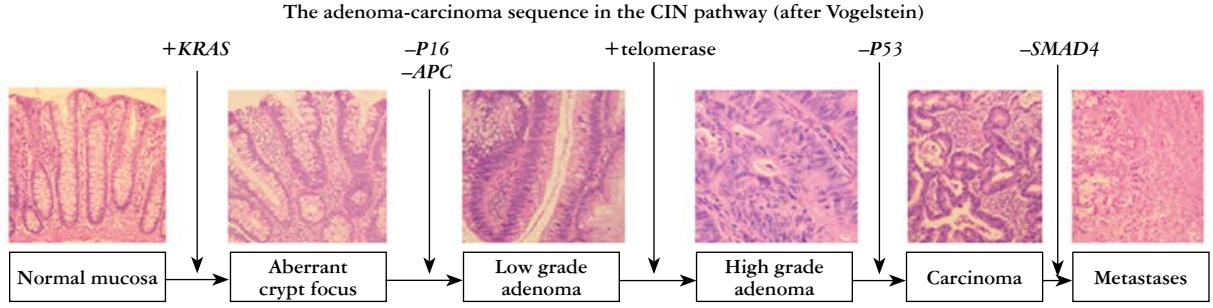


**Fig. 1.** The model of stepwise progression of colorectal cancer as originally proposed by Vogelstein [7]. The histology panels show morphological characteristics of the lesion at each step; purported molecular lesions associated with each transition are indicated above the arrows. + activating mutation; – inactivating mutation

*Source: World Cancer Report 2014. IARC, WHO press 2014, p. 395. ISBN 978-92-832-0429-9. Published with permission.*

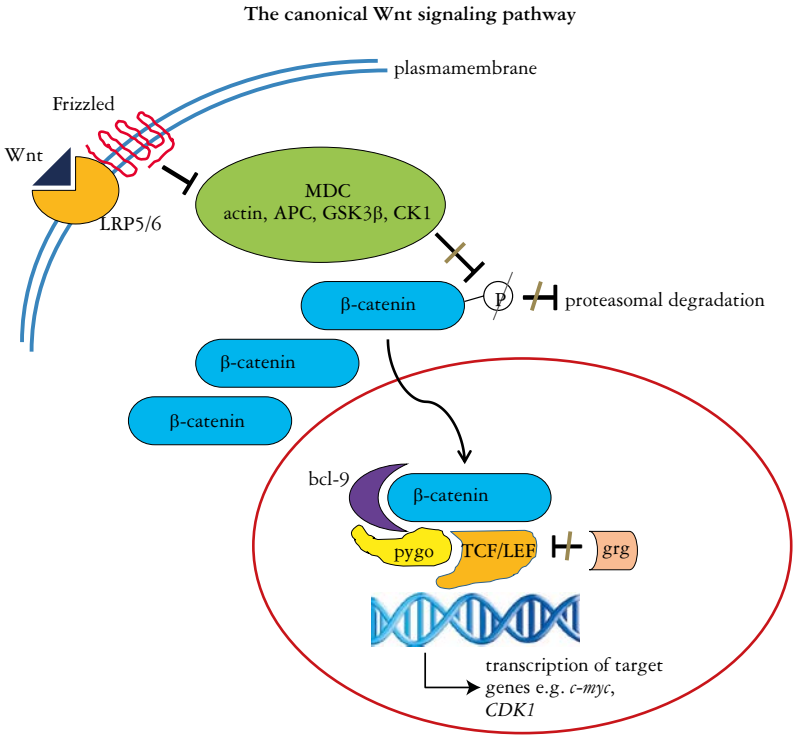


**Fig. 2.** The canonical Wnt pathway. In the absence of Wnt β-catenin is phosphorylated by GSK3β and CK1 in the multiprotein destruction complex (MDC), which contains axin, APC, GSK3β and CK1, and subsequently degraded in the proteasome. Binding of Wnt to its receptor Frizzled and the co-receptors LRP5/6 creates a receptor complex. This destabilizes the MDC, as a result of which it no longer phosphorylates β-catenin, the key mediator of the pathway. β-catenin is then no longer degraded in the proteasome, accumulates in the cytoplasm, and migrates to the nucleus. Here it forms a transcription complex with pygopus (pygo), Bcl-9 and TCF/LEF, which is no longer inhibited by the transcriptional repressor groucho (grg). Transcription of proliferation-stimulating target genes, including c-myc and CDK1, ensues

enoma-carcinoma sequence. With the elucidation of molecular mechanisms involved, Vogelstein [7] supplemented this concept with molecular data, which with the equally well established stepwise progression concept resulted in the molecular model which has become almost a standard approach to address molecular carcinogenesis (Fig. 1).

FAP patients with a germline mutation of *APC* develop hundreds to thousands of adenomatous polyps already at a very young age and have a 100% lifetime risk of the development of CRC. Development of neoplastic lesions is not limited to the colorectum: neoplasms occur also in the upper intestinal tract (stomach, duodenum) and in connective tissue (desmoid tumors). There is a striking genotype-phenotype relationship regarding the clinical characteristics of FAP patients. Mutations before codon 157, after codon 1595, and in the alternatively spliced region of exon 9 are associated with a much lower adenoma frequency (attenuated FAP, for a review see 14) and also with the occurrence of lesions in the upper gastrointestinal tract (duodenum and stomach). In contrast, mutations between codons 1250 and 1464 are associated with severe polyposis. Mutations after codon 1444 are associated with occurrence of desmoid tumors [14]. Also in sporadic CRC, which usually develop at advanced age (mostly from the seventh decade on), *APC* mutations occur but these are somatic, not germline [15].

Additional molecular events are activating mutations of *KRAS* and *BRAF* in the MAPK pathway. Mutations of *KRAS* are found in around 45% of CRC and constitutively activate the MAPK signaling pathway (Fig. 3). *BRAF*, downstream of *KRAS* in the MAPK pathway, is mutated in less than 10% of the cases. Mutations in the MAPK pathway also occur in *NRAS* and in phosphoinositide-3-kinase (*PIK3*) [16]. We will come back to this issue in the context of molecular predictors of response to targeted treatment in CRC.

In the classical pathway, loss of function mutations of the *TP53* gene occur in about 70% of the cases with accumulation of the mutated protein in the nucleus (Fig. 4), but as a rule in the progression from high-grade adenoma to carcinoma, when also telomerase is activated [17], which confers an unlimited lifespan on the transformed cells. Activation of the TGF-β pathway, which is reflected in the loss of its downstream effector SMAD4, is probably involved in metastatic progression [18].

The classical pathway is associated with FAP and germline mutations in the *APC* gene, but not all pa-

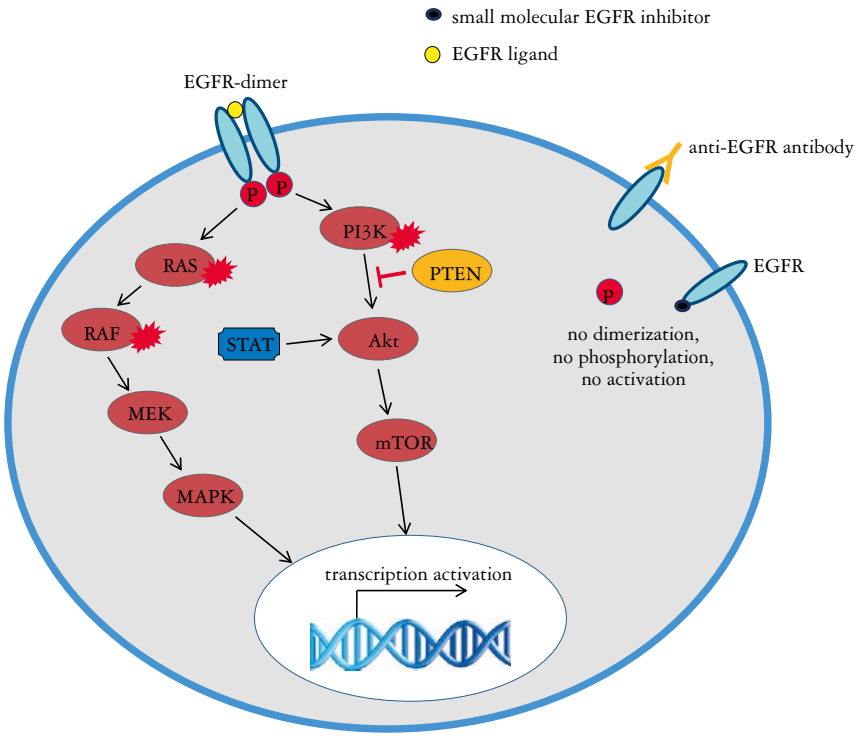


**Fig. 3.** The MAPK pathway. Normally the pathway is activated by receptor-ligand interaction, upon which the EGF receptor dimerizes and phosphorylates a downstream signaling molecule. This finally leads to transcription of genes involved in a variety of cellular activities, including proliferation and invasion. Antibodies or small molecules that bind to EGFR can inactivate this process. Targeting EGFR is only an effective approach to silence the pathway if there are no downstream proteins constitutively activated by mutation (such as KRAS, BRAF or PIK3CA)

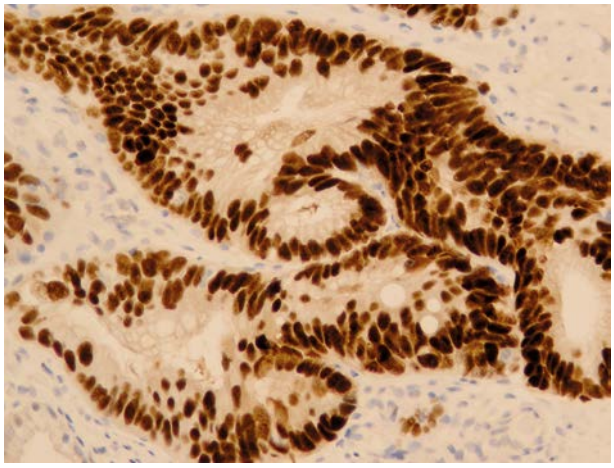

**Fig. 4.** Immunohistochemical staining of p53 protein in a colon carcinoma. Note diffuse strong staining of all nuclei, indicative of mutated p53

tients with polyposis have an *APC* mutation. Detailed analysis of the genome of such patients has led to the discovery of the involvement of the *MUTYH* gene, which is involved in base excision repair following oxidative damage to DNA [19]. *MUTYH* polyposis (or MAP) to a large extent follows the same sequence of events as FAP patients, but MAP patients often have fewer polyps, carcinomas appear between the fourth and seventh decade, and the lifetime risk for the development of CRC is lower than for FAP patients [20].

## The microsatellite instability (MIN) pathway

This pathway is responsible for the development of hypermutating carcinomas, which in contrast to the CIN carcinomas usually show little aneuploidy. The prototype of these pathways was discovered in the early 1990s while searching for the molecular genetic origins of hereditary non-polyposis colon cancer (HNPCC), which is now known as Lynch syndrome [21]. A landmark discovery was that Lynch syndrome is caused by a mutation in one of the genes encoding the proteins involved in mismatch repair (MMR) [22]. During DNA replication errors occur, such as single base mismatches or deletions and short insertions, which are corrected by MMR. A protein complex is formed by the proteins involved in MMR which binds the mismatch and uses the information from the (correct) complementary strand to excise the error and repair it. When MMR does not function, the cells accumulate errors, which occur also in microsatellite sequences. These are repeating sequences of DNA, mostly 1-6 base pairs in length, which occur throughout the genome. MMR deficiency generates novel microsatellite sequences, which can be detected by a PCR-based assay. In this assay a panel of microsatellite marker loci, including BAT25, BAT26, D2S123, D5346, and D17S250, is used to detect microsatellite instability (MSI) [23]. When two or more markers are unstable, it is called MSI-H(igh); when

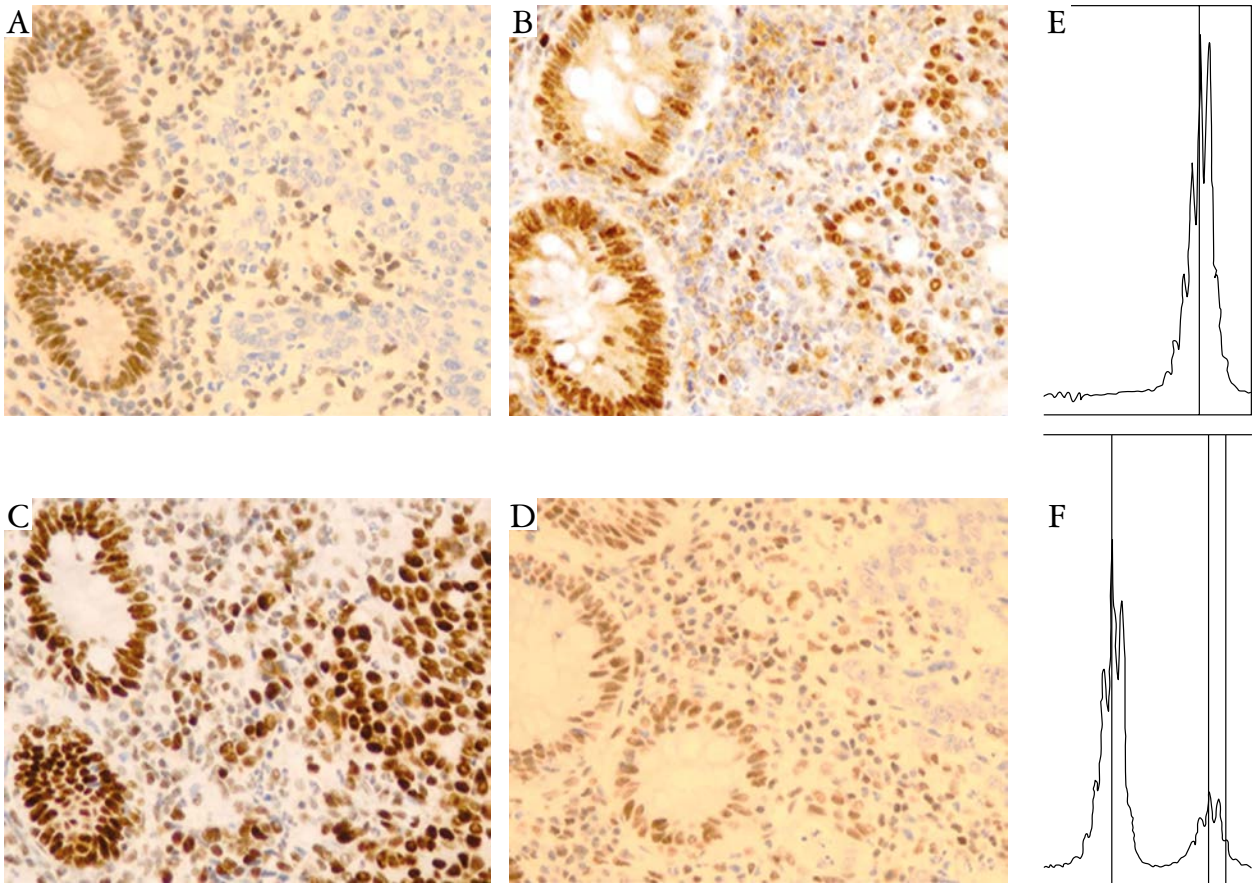


**Fig. 5.** A typical example of mismatch repair deficiency, as reflected in loss of immunohistochemical staining of MLH1. A) MLH1 staining. Note absence of staining of tumor cell nuclei whereas normal crypt cells and stromal cells are positive. This as a rule goes along with absence of PMS2 staining (panel D), which is absent as it cannot complex with mutated MLH1. B) MSH2 staining. Tumor cell nuclei are stained, as for MSH6 (panel C). Panels E and F confirm microsatellite instability as reflected in a shift in the position of the BAT26 microsatellite marker, due to an aberrant number of repetitions

only one marker is instable, it is called MSI-L(ow); and when all markers are stable, it is called microsatellite stable (MSS) (Fig. 5). As mismatch repair proteins are easily detectable in routinely processed tissue sections, immunohistochemical detection of loss of expression of one of the relevant proteins (MLH1, MSH2, MSH6 and PMS2) has become mainstream in the detection of mismatch repair and a screening tool for the detection of Lynch syndrome [24] (Fig. 5). In pathway terms, this MSI-H is equivalent to the microsatellite instability (MIN) pathway. Lynch syndrome is responsible for about 3% of CRC, but MSI occurs in around 12% of sporadic CRC cases. This is due to promoter methylation of *MLH1*, which shuts down *MLH1* transcription, resulting in deficient MMR and MSI-H [25].

MSI carcinomas display a relatively high frequency of *BRAF* gene mutations. There are other genes preferentially mutated, often characterized by mononucleotide tracts in their coding regions, as is the case for the TGF-β receptor II gene and the pro-apoptotic gene *BAX*, which are as a consequence frequently inactivated in MMR-deficient cancer. The MS status has gained quite a bit of clinical interest for sporadic CRC lately as mismatch repair deficient carcinomas have a better prognosis than mismatch repair competent carcinomas. Microsatellite instability status is gradually entering into clinical decision making [26].

In the MIN pathway, the development of neoplastic lesions is quite similar to that in the CIN pathway: an adenoma-carcinoma sequence. However, cancers that arose through this pathway behave differently from those in the CIN pathway: they have a better prognosis, respond differently to standard chemotherapy, have fairly characteristic morphology as they are situated in the right colon, are of mucinous or medullary histology (Fig. 6), and display a characteristic lymphocytic infiltrate [27].

It had been noted in the The Cancer Genome Atlas (TCGA) whole genome sequencing effort for CRC that a subgroup of hypermutating carcinomas are not microsatellite instable. In addition, families were identified with oligopolyposis and microsatellite stable CRC at young age but without *APC* or *MYH* mutations. This stimulated the search for additional mechanisms responsible for a hypermutating state. Recently it was found that germline mutations in the proofreading domain of two DNA polymerases (*POLE* and *POLD1*)

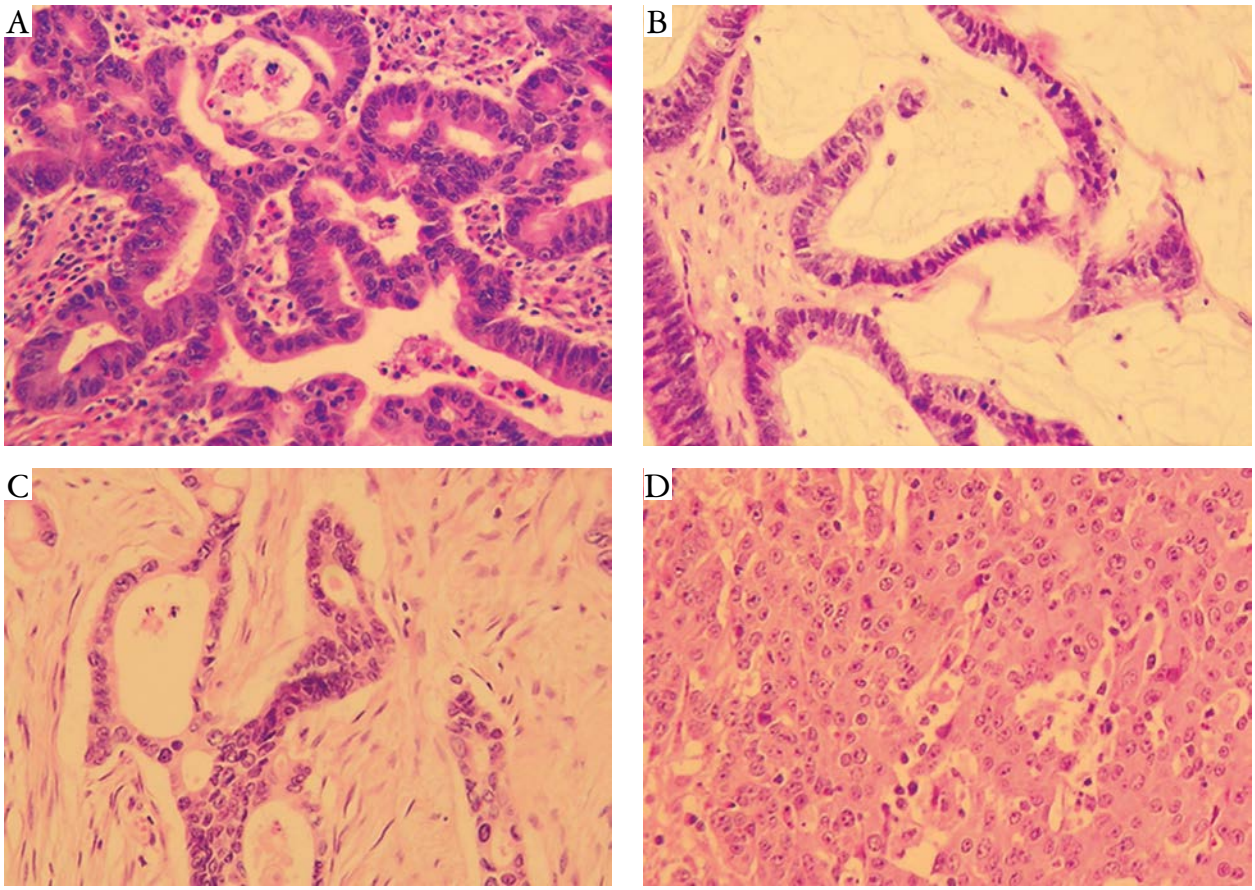


**Fig. 6.** Morphological heterogeneity of colorectal cancer. Panel A shows complex tubular and serrated architecture with moderate cellular atypia, as the cell polarity is still maintained. Panel B shows a mucinous pattern. If this pattern dominates (> 50%), the tumor is called mucinous carcinoma. Panel C shows a pattern characterized by strong stromal reaction (for which the term desmoplastic is used) in the presence of moderate numbers of tumor cells). Panel D shows a pattern of poorly differentiated cells growing in syncytial sheets, characteristically with diffuse infiltration of tumor infiltrating lymphocytes (TIL), known as medullary carcinoma

are associated with this syndrome [10]. Lack of correction of mistakes in DNA replication apparently generates mutations, with a mutator phenotype as a result [28]. This syndrome has been called polymerase proofreading-associated polyposis (PPAP).

### The CpG island hypermethylation phenotype pathway

Hypermethylation of gene promoter associated CpG islands, involved in the regulation of transcription, is a frequently encountered mechanism responsible for silencing of tumor suppressor genes. If this phenomenon occurs genome-wide, it is called CpG island hypermethylation phenotype (CIMP) [29]. What causes the CIMP phenotype and what exactly its consequences are remains elusive.

Around the turn of the century, it was noted that flat non-adenomatous mucosal lesions, notably in the right colon, often have a CIMP. These lesions occur as a result of abnormal retention of surface epithelium due to hyperproliferation and inhibition of apoptotic cell loss. They are composed of glands with a saw-tooth like appearance (hence the name serrated) and were long considered as largely innocuous hyperplastic polyps. More recently it was shown that a subtype of them with particular morphology has significant malignant potential, and hence these are called sessile serrated adenomas or polyps (SSA/P) [30]. They resemble (benign) hyperplastic polyps but show a more irregular crypt architecture (Fig. 7) and occasionally features of dysplasia. SSA/P have been associated with increased risk for the development of CRC. The extent of the risk has not yet been adequately established, but current evidence suggests that it is lower than the risk of progression of traditional adenomas [31]. Carcinomas with CIMP were noted to be associated with serrated precursor lesions, and in addition CIMP carcinomas often harbor *BRAF* mutations. Putting CIMP and serrated morphology together led to the development of the CIMP pathway concept [32].

The molecular events in the pathway include in addition to CIMP mutation of the *BRAF* gene, characteristically the V600E mutation, methylation of the promoter of a variety of genes (as a re-

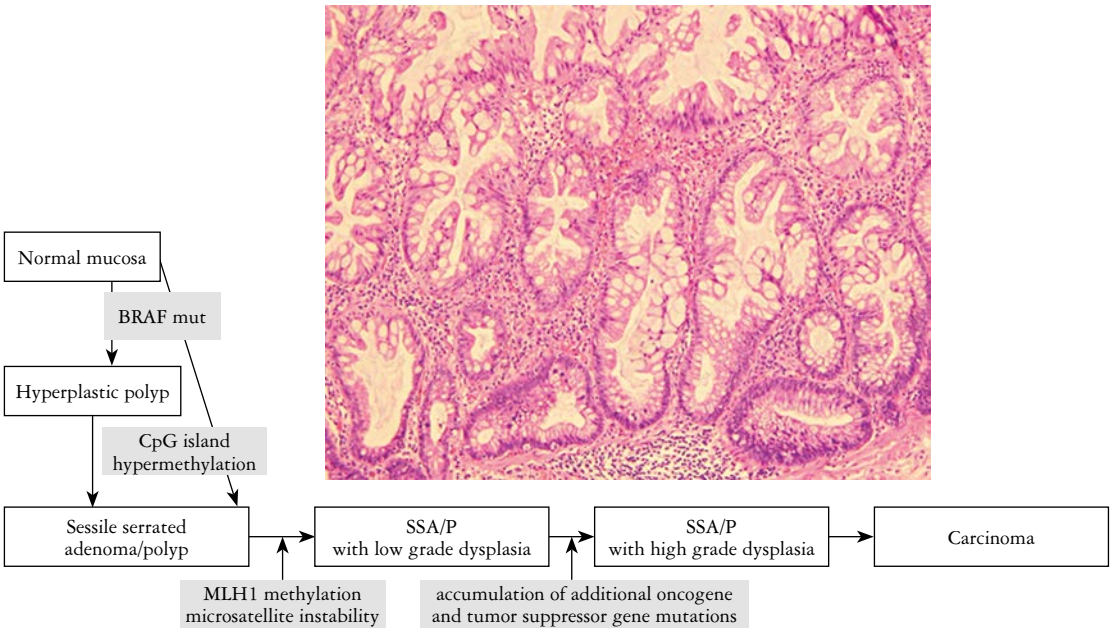


**Fig. 7.** The CpG island methylator phenotype (CIMP) pathway (also known as the serrated pathway) of colorectal carcinogenesis. In the pathway, early lesions are characterized by BRAF mutation (typically the V600E mutation) and CIMP. Later lesions show mismatch repair deficiency due to hypermethylation of the MLH1 promoter, morphologically characterized by serrated architecture. These lesions are called sessile serrated adenoma. Further progression is a result of hypermutability of the genome due to mismatch repair insufficiency and comprises genome alterations also found in the classical pathway (Fig. 1)

*Source: Molecular Surgical Pathology. Cheng L, Eble JN (eds.). Springer Verlag 2013; p. 13 (fig. 1.7). Published with permission*

sult of CIMP), and subsequent methylation of the *MLH1* gene promoter with silencing of the expression of MLH1 as a result. This is then responsible for MMR insufficiency, MSI, and a hypermutating state.

It is important to emphasize here that these pathway notions constitute a model framework which is useful in further defining molecular events in colorectal cancer in association with clinical and pathological parameters. In reality, a variety of subgroups of colorectal cancer exist with molecular characteristics which overlap to a significant degree [30]. Recently, several approaches to the development of molecular classifications of CRC have been published. These not surprisingly show significant similarities between each other and they also align with some of the pathway characteristics [4–6]. These molecular classifications add a layer of complexity to the heterogeneity of CRC. Currently, efforts are underway to develop a consensus molecular classification in the context of open source collaborative genomic data analysis of which the first results have already been published [33]. It remains to be seen how exactly these molecular classifications will impact on clinical management of CRC, which is still largely determined by classical TNM parameters, even though the MS status recently has gained importance as a molecular parameter to be taken into account in the choice of adjuvant treatment of stage II/III CRC and potentially also the choice of treatment of metastatic CRC. This brings us to the question of whether, and if so how, these pathway concepts have impacted on the clinical management of CRC.

## Molecular solutions to problems in the management of colorectal cancer?

Understanding disease is what pathology ultimately strives for, but this understanding should serve patients through the development of applications from which they profit. The issue is therefore which burning questions in the present modalities of care for CRC patients might profit from a molecular solution. In this context the following questions are relevant:

- Can molecular pathology provide more efficient approaches towards population screening for CRC?
- Can molecular tests predict which patients are at high risk for development of recurrent disease after primary surgery?
- Can molecular tests predict which individuals of the cohort of patients at high risk for recurrent disease most likely will respond to currently used adjuvant (chemo) therapy?
- Can molecular tests predict which patients most likely will respond to the available modalities of targeted treatment?

### Molecular screening for colorectal cancer

Colorectal cancer screening programs have risen to global prominence, given the high prevalence of this disease and the possibility to identify and adequately treat precursor lesions by colonoscopy. The standard approach for these screening programs is fecal occult blood testing (FOBT), in order to identify a more limited population qualifying for colonoscopy in case of a positive FOBT. Fecal occult blood testing is relatively sensitive but lacks specificity [34]. A variety of molecular approaches are under investigation to develop more specific tests. Most focus on the detection of tumor DNA in fecal matter, looking for tumor-specific gene alterations such as hypermethylation or specific mutations. In a laboratory setting such tests have shown promising results, but as yet sensitive and specific molecular tests to detect cancer are not available [35].

### Molecular prognostic tests for colorectal cancer

A key problem in the clinical management of most cancers is the question of which patients will present recurrent disease after initial therapy, which for most cancers is surgical removal of the primary tumor, occasionally after neoadjuvant (chemo- and/or radio-) therapy. The issue is clear: those patients with a high likelihood of recurrent disease, which for now means stage III and high-risk stage II cases, might be treated with adjuvant chemotherapy. Those with a low likelihood of recurrent disease, stage I and low-risk stage II patients, should be spared this therapy, which is associated with significant side effects. Presently used criteria used for patient stratification lack accuracy, as even among stage I cancers a small proportion will relapse and about 30% of stage III patients will not relapse [36]. Several prognostic molecular signatures have been developed, which potentially would allow stratification of patients according to the risk of recurrence [37-39]. A recent study has independently assessed the performance of these signatures in an independent patient population and concluded that all were prognostically significant [40]. Strikingly, however, they did not use similar gene sets, nor did they identify the same patients as high risk. A combination of these signatures, not surprisingly, performed better than any single signature. These data indicate that even though finding effective (molecular) approaches towards identification of high-risk CRC patients remains urgent, as yet the efficacy of attempts to attain this goal remains questionable.

### Molecular tests predictive for response to chemotherapy

Of the CRC patients who qualify for adjuvant chemotherapy (the 'high-risk' – based on clinical and histopathological criteria – stage II and the stage III patients), only about 50% respond [41]. Those that do not respond are exposed to the usual side-effects of chemotherapy without having any benefit. It would therefore be valuable to have (clinical, molecular) parameters predicting response to this regimen, which as a rule consists of a combination of 5-fluorouracil, leucovorin and oxaliplatin (known as FOLFOX). Some attempts have been made to develop molecular tests to this effect, but as yet none are used in daily practice due to lack of sensitivity or specificity [42, 43]. The search therefore remains on for more effective approaches towards adjuvant treatment as well as for tools to predict treatment response.

### Molecular markers predictive of a response to targeted therapy in colorectal cancer

As stated before, the standard therapy for advanced CRC is based on the combination of 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX). In the last decade, evidence was obtained that for a subpopulation of patients a therapeutic approach addressing the epidermal growth factor receptor (EGFR) might be advantageous. Monoclonal antibodies were developed against EGFR with some efficacy in CRC. In prospective clinical trials, initial observations were validated, initially showing a response to cetuximab [44] and subsequently also panitumumab [45] but only in patients with a *KRAS* wild type tumor. This finding rapidly promoted *KRAS* mutation testing in CRC to one of the most frequently performed tests in molecular pathology laboratories. An important limitation of the *KRAS* mutation status is, however, that it identifies (*KRAS*-mutated) patients who will not respond to anti-EGFR therapy, which eliminates about 40% of the non-responders, but of the *KRAS*-wild type patients only about 40% respond. The capacity to effectively predict who will respond rather than those patients who will not would be an improvement. Assessing the mutation status of other genes in the MAPK pathway has been investigated [46], and an approach combining testing of the mutation status of several genes involved in the MAPK pathway effectively increases the predictive value. The accuracy of the presently available test approaches is, however, still far from ideal, and the search is on for molecular tests that more accurately predict targeted therapy response [47].

An important issue in this context is therapy resistance. The beneficial effect of anti-EGFR is as a rule of short duration because of the development of resistance [48]. Ongoing studies explore the mechanisms responsible for this treatment failure [49]. Others search for new molecular targets to treat advanced colorectal cancer [50].

## Concluding remarks

We are not at the end of what has been a fascinating adventure in terms of discovering the details

of colorectal carcinogenesis. We have come from classical histopathology (including key gross pathology parameters) through a continuously more refined TNM classification approach to molecular parameters to stratify patients for therapy, befitting the specific characteristics of their cancer. We might not understand colorectal carcinogenesis completely, but our knowledge is much better than it was two decades ago. We are in the middle of the development of parameters predicting response to available targeted therapies, of the development of new therapies targeting elements of the pathways involved in colorectal carcinogenesis, and of molecular tests that will predict the response to them. Global attempts are being made through TCGA project to gain more insight into the molecular biology of colorectal (and many other) cancers. Pathology plays a key role in this development, as much of the knowledge that has been gained has been (and still is) based on molecular studies on tissue samples that pathologists use to make a diagnosis. Pathology and pathologists should remain in that central position as a partner in a multidisciplinary effort, along with molecular (cancer) biologists, bioinformaticians and (molecular) oncologists. Eventually, this will improve early detection and provide more accurate prediction of (adjuvant) treatment need, more efficient drugs, and accurate predictors of drug response, from which our patients will profit.

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