Histopathology 2007, **50**, 113–130. DOI: 10.1111/j.1365-2559.2006.02549.x

REVIEW

Classification of colorectal cancer based on correlation of clinical, morphological and molecular features

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(2007) *Histopathology*, **50**, 113–130

Classification of colorectal cancer based on correlation of clinical, morphological and molecular features

Over the last 20 years it has become clear that colorectal cancer (CRC) evolves through multiple pathways. These pathways may be defined on the basis of two molecular features: (i) DNA microsatellite instability (MSI) status stratified as MSI-high (MSI-H), MSI-low (MSI-L) and MS stable (MSS), and (ii) CpG island methylator phenotype (CIMP) stratified as CIMP-high, CIMP-low and CIMP-negative (CIMP-neg). In this review the morphological correlates of five molecular subtypes are outlined: Type 1 (CIMP-high/MSI-H/*BRAF* mutation), Type 2 (CIMP-high/MSI-L or MSS/*BRAF* mutation), Type 3 (CIMP-low/MSS or MSI-L/*KRAS* mutation), Type 4 (CIMP-neg/MSS) and Type 5 or Lynch syndrome (CIMP-neg/MSI-H). The molecular pathways are determined at an early evolutionary stage and are fully established within precancerous lesions. Serrated polyps are the precursors of Types 1 and 2 CRC, whereas Types 4 and 5 evolve through the adenoma–carcinoma sequence. Type 3 CRC may arise within either type of polyp. Types 1 and 4 are conceived as having few, if any, molecular overlaps with each other, whereas Types 2, 3 and 5 combine the molecular features of Types 1 and 4 in different ways. This approach to the classification of CRC should accelerate understanding of causation and will impact on clinical management in the areas of both prevention and treatment.

Keywords: cancer, classification, colorectal, DNA methylation, microsatellite instability, pathways

Abbreviations: CIMP-high, -low or -neg, CpG island methylator phenotype-high, -low, or negative; CIN, chromosomal instabilty; CRC, colorectal cancer; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stable

Introduction

The role of the histopathologist is no longer limited to issuing an accurate tissue diagnosis but is increasingly directed towards the provision of prognostic information and additional findings directly relevant to patient management. This ongoing refinement of reporting practice should not obscure the more fundamental role of the pathologist in the classification of disease. Classification is more than the mere naming of disease entities or even the collation of their particular diagnostic features. It includes the elucidation of clinicopathological correlation, which is the starting point for the investigation of the causation, evolution and natural history of a disease. It is necessary for a disease to be properly classified in order to achieve effective clinical management and meaningful laboratory investigation of the underlying mechanisms. The classification of cancer has traditionally been based mainly on microscopic morphology supplemented, in more complex forms of malignancy, by immunophenotyping and, more rarely, molecular approaches. Molecular technology has been mainly limited to subtle

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refinements of classification, particularly when markers are shown to contribute prognostic information or predict chemoresponsiveness.

In the case of colorectal cancer (CRC), both clinical management and research have proceeded for many decades on the basis that CRC is a homogeneous entity. Nevertheless, particular morphological subtypes, such as mucinous carcinoma, have long been recognized and clinical features have been shown to differ according to anatomical subsite.[1] The evolution of CRC was also understood to proceed on the basis of a relatively uniform and linear sequence of steps, with *APC* inactivation initiating adenomas and additional genetic changes, notably *KRAS* mutation, and *TP53* inactivation promoting the emergence of increasingly aggressive subclones.[2] The condition familial adenomatous polyposis (FAP), caused by germ-line mutation of *APC*, was perceived as the hereditary counterpart of the 'vast majority' of sporadic CRCs.[3] While the mutational events driving tumorigenesis were deemed to be selected on the basis that each would confer a biological advantage,[4] an additional factor was required to explain how the accumulation of multiple genetic changes could occur within the limited lifespan of a cell. This additional factor, known as genetic instability, implicates the loss of a mechanism (or mechanisms) not only critical for the maintenance of genomic fidelity during cell division but also capable of triggering apoptosis in the setting of accumulating genetic damage.[5]

Types of genetic instability

The condition FAP illustrates the requirement for genetic instability. Without this ingredient many thousands of adenomas may be initiated by inactivation of *APC*, but the fate of the vast majority is merely to grow harmlessly over several decades. In the context of sporadic CRC an individual adenoma would appear (on the basis of the relative frequency of adenoma versus carcinoma) to have a much higher risk of malignant transformation.[6] The concept that lesions of similar appearance could have markedly different biological properties was highlighted by a second form of familial CRC: Lynch syndrome, known also as hereditary non-polyposis colorectal cancer. In this condition it is evident that a high proportion of adenomas will, if left untreated, progress to CRC and do so within a short timeframe.[7] Most adenomas in subjects with Lynch syndrome show loss of expression of a DNA mismatch repair protein (usually MLH1 or MSH2) and display a form of genetic instability characterized by the accumulation of numerous mutations which specifically target repetitive sequences of DNA.[8] These sequences occur most frequently in non-encoding microsatellite regions, hence the term microsatellite instability (MSI). Following inactivation of a DNA mismatch repair gene one may detect such mutations at a high frequency throughout the genome, hence the term MSI-high (MSI-H). MSI-low (MSI-L) will be discussed below. Because short repetitive sequences also occur within the encoding regions of certain tumour suppressor genes such as *TGFβRII*, *IGF2R* and *BAX*, these may be mutated and inactivated.[9–11] CRCs with MSI have a diploid DNA content with few losses or gains of chromosomal regions.[12] Genetic instability was therefore conceived as operating on two levels, a more subtle level affecting DNA sequences (MSI-H), and chromosomal instability (CIN) affecting whole chromosomes or parts of chromosomes.[13] These forms of instabilty are mutually exclusive, so that CRCs with CIN will be MS stable (MSS).

Notwithstanding the mutual exclusivity of these two forms of genetic instability, all CRCs were considered to evolve through a similar linear sequence of genetic alterations. Indeed, *APC*, *KRAS* and *TP53* were all shown to be mutated in CRCs with MSI-H from patients with Lynch syndrome and in malignant cell lines with MSI-H.[14–20]

Need for an alternative pathway to explain sporadic CRCs with MSI-H

Support for the existence of two largely independent pathways to sporadic CRC was slow to develop. Acceptance of such a notion had to supplant an attractive and elegant paradigm, in which *APC* inactivation and loss of DNA mismatch repair were envisaged to initiate and promote (respectively) an essentially similar evolutionary pathway in both sporadic and familial settings.[13] There was no obvious imperative for an alternative pathway to sporadic MSI-H CRC on the basis of the literature amassed within basic science journals. Notably, sporadic colorectal adenomas could show MSI-H,[21] whereas a similar spectrum of somatic mutations occurred in CRC cell lines regardless of microsatellite status.[20] Why complicate the picture by introducing hyperplastic polyps (or closely related lesions) when these lesions had been dismissed as harmless for decades?[22] Why suggest that sporadic and Lynch syndrome-associated MSI-H CRCs are not in fact direct counterparts?[23]

ABSENCE OF EXPECTED GENETIC SIGNATURES

The reluctance to counter the status quo meant that reports providing contrary data were either ignored or

rebutted with tendentious arguments. For example, with the single exception noted above,[21] MSI-H consistent with DNA mismatch repair deficiency was rarely observed in sporadic adenomas[24] and the few such examples found turned out to be mainly derived from patients with Lynch syndrome.[25] These findings gave rise to the suggestion that, unlike the adenomas in Lynch syndrome, MSI-H must occur as a relatively late event in sporadic adenomas.[21] Why, it may then be asked, were the genetic alterations associated with initiation and early progression of sporadic adenomas not found in sporadic MSI-H CRC? For example, in studies that carefully distinguished sporadic MSI-H CRC and Lynch syndrome, the sporadic MSI-H subset showed infrequent *APC* mutation or loss of the *APC* locus on chromosome 5q, while *KRAS* mutation was also rare.[26–28] These observations were countered by the argument that alterations in other components of the Wnt signalling pathway could substitute for *APC* inactivation, notably an activating mutation of *CTNNB1* (encodes β-catenin). While it is certainly correct that *CTNNB1* is sometimes mutated in CRC with MSI-H,[29] this mutation is mainly limited to Lynch syndrome cancers,[30,31] whereas it is absent or very rarely detected in sporadic MSI-H CRC.[26,31,32] The non-involvement of *APC* and *CTNNB1* in sporadic MSI-H CRC is fully supported by the immunoexpression pattern for β-catenin, in which the normal distribution along lateral cell membranes is maintained while aberrant translocation to the nucleus is infrequent.[27,33] A single study from Japan linking mutation of *CTNNB1* with sporadic MSI-H CRC has not been confirmed in Western populations.[34] In fact, a subsequent study from the same group in Japan showed that mutation of *CTNNB1* was negatively associated with both *BRAF* mutation and methylation of *MLH1*, which are the hallmark genetic alterations in sporadic MSI-H CRC.[35] The finding of *CTNNB1* mutation in early-onset cases of MSI-H CRC[29] could be due to either Lynch syndrome or germ-line hemi-allelic methylation of *MLH1*.[36] The over-representation of *CTNNB1* mutations in MSI-H CRC cell lines[37] is probably due to the fact that very few such cell lines are derived from sporadic MSI-H CRCs.

Methylation of the *APC* promoter could fill the mutational gap in theory, but this epigenetic change occurs in only 18% of CRCs, may affect the wild-type allele when there is already an *APC* mutation, and is not associated with either MSI-H or with methylation of other genes.[38] Furthermore, it has been shown that at least one *APC* allele must be retained in a truncated form to drive proliferation and tumorigenesis.[39] This indicates that bi-allelic methylation of *APC* (leading to complete silencing) may not provide an important growth advantage. Invoking other components of the Wnt signalling pathway such as *AXIN2*[40] or *TCF4*[41] in the initiation of sporadic MSI-H neoplasia does not provide a surrogate directly equivalent to *APC* inactivation, since these genes are mutated at a relatively late stage (after the acquisition of MSI-H status). The widely accepted notion that other components of the 'canonical' Wnt pathway can be invoked in the initiation of the subset of CRCs without *APC* mutation is unproven.

PRESENCE OF UNEXPECTED GENETIC SIGNATURES

In addition to the absence of adenoma-specific mutations, sporadic MSI-H CRCs are characterized by alterations, specifically extensive DNA methylation and *BRAF* mutation, that are not only rare in sporadic adenomas[42–45] but are also not observed in Lynch syndrome CRC.[46,47] The association between *BRAF* mutation and CIMP has been shown to be extremely strong in CRC with an odds ratio of over 200.[48] While DNA methylation may occur in sporadic adenomas,[49] it is seldom marked in small tubular adenomas,[50] although it may implicate more loci in adenomas with high-grade dysplasia and/or villous change.[51] By contrast, very extensive DNA methylation is the usual finding in serrated polyps occurring in the proximal colon[52,53] that also show frequent *BRAF* mutation.[44] The most convincing evidence for the existence of a serrated pathway to MSI-H CRC is the direct observation of a serrated polyp–dysplasia–carcinoma transition supported by immunohistochemical and molecular correlation. This has been achieved by the demonstration of MLH1 loss in dysplastic or malignant subclones and the presence of MSI-H in the DNA extracted from such subclones.[54–57] Methylation of the *MLH1* promoter is the principal mechanism underlying the silencing of *MLH1* and loss of mismatch repair proficiency in sporadic MSI-H CRC.[58] However, based on the spectrum of genetic alterations in serrated polyps, these lesions must also serve as the principal source of sporadic MSI-H CRC, whereas the conventional adenoma–carcinoma sequence initiated by *APC* or *CTNNB1* mutation and subsequently driven by *KRAS* mutation is more likely to be associated with the early evolution of CRC in Lynch syndrome.

Heterogeneity of sporadic MSS CRC: stratification based on DNA methylation and low-level MSI

Removal of the two forms of MSI-H CRC (familial and sporadic) leaves the large MSS subset comprising

around 85% of CRC. It might be supposed that this group is homogeneous at the molecular level and comprises CRC with mutation of *APC*, *KRAS* and *TP53*. In practice, however, only around 10% of CRCs are characterized by this 'classic' genotype.[59,60] Not only is the MSS group highly heterogeneous, but it includes some CRCs with molecular features that characterize the sporadic MSI-H subset, notably *BRAF* mutation,[44,61] extensive DNA methylation or the CpG island methylator phenotype (CIMP),[62–66] and diploid status or chromosomal stability.[67–70] It is notable that MSS CRCs with high-level CIMP and/or *BRAF* mutation also share certain clinical and pathological features with the sporadic MSI-H subset with CIMP. These features include: (i) a predilection for females,[61,63,66] (ii) increased age at onset,[66] (iii) a predilection for proximal colon,[61–63,65,66] (iv) poor differentiation,[61,63,65,66] (v) mucinous differentiation[61–63,65,66,71] and (vi) round and vesicular nuclei with a prominent nucleolus.[62] However, there are also differences from the sporadic MSI-H subset with CIMP, including: (i) a higher incidence of presentation at an advanced pathological stage,[61,63,65,66] (ii) infiltrative growth pattern with discohesive tumour cells,[65] (iii) lack of tumour-infiltrating lymphocytes (TILs),[62,63] (iv) poor prognosis[64,72] and (v) responsiveness to adjuvant treatment with 5-fluorouracil.[64] MSS CRCs with *BRAF* mutation and/or DNA methylation are likely to show a degree of overlap with MSS CRC with diploid DNA status or infrequent loss of heterozygosity. For example, MSS CRCs with diploid DNA content and/or little evidence of CIN have been shown to be more frequent in the proximal colon,[68,69] to present at an advanced stage[68] and to be mucinous and/or poorly differentiated.[70] Furthermore, concordant silencing of multiple tumour suppressor genes through promoter region methylation would explain how neoplasia may develop without a background of either MSI or CIN.

SIGNIFICANCE OF LOW-LEVEL MSI

While the MSS group lacks MSI-H by definition, a subset of non-MSI-H CRC shows MSI-L. The concept of MSI-L has been controversial and CRCs with MSI-L do not represent a clearly defined group. Nevertheless, there is now good evidence that MSI-L status occurs as a non-random and biologically based phenomenon and is not merely a polymerase chain reaction-based artefact.[73,74] MSI-L CRCs were distinguished from both MSI-H and MSS CRCs on the basis of gene expression profiles[75] and also differ from MSS CRCs in showing frequent instability in the trinucleotide repeat region of *RAS-induced senescence 1* (*RIS1*).[76] MSI-L status has been shown to be an independent adverse prognostic feature in stage III CRC from patients not treated with adjuvant chemotherapy[77,78] and particularly when occurring in association with mutation of *RIS1*.[76] MSI-L CRCs were found to be over-represented among CIMP-high CRCs that were not MSI-H.[79] Additionally, CRCs with both *KRAS* mutation and MSI-L showed more extensive DNA methylation than MSS CRCs with *KRAS* mutation or non-MSI-H CRCs without either *KRAS* or *BRAF* mutation.[80] While the preceding points might link MSI-L with both DNA methylation and diploid DNA status, one study has shown that MSI-L CRC in fact had higher rates of loss of heterozygosity than the MSS group.[69] Two mechanisms for MSI-L status have been advanced: (i) increased generation of methylG:T mismatches due to loss of expression of *O-6-Methylguanine DNA Methyltransferase* (*MGMT*) that would stress the DNA mismatch repair machinery,[81] and (ii) partial methylation and loss of expression of the DNA mismatch repair gene *MLH1*.[82,83] These mechanisms might also synergise and account for the high end of the range of MSI-L or 'super-low' status.[73] Involvement of *MLH1* (partial methylation) alone might result in MSI-L without chromosomal instability or *KRAS* mutation. Involvement of *MGMT* (with or without *MLH1*) would be associated with *KRAS* mutation[84] and chromosomal instability on the basis that methylG:T mismatches give rise to futile cycles of DNA excision and attempted repair that may culminate in chromosomal damage.[85,86] Methylation of *MGMT* was found to be most frequent in the subset of CRC with both MSI-L status and *KRAS* mutation.[80]

HETEROGENEITY WITHIN CIMP

Differences between CIMP-high and CIMP-low may not be merely quantitative. CIMP-high CRCs have frequent *BRAF* mutation and show methylation of many markers, consistent with a generalized increase in *de novo* methylation (described as CIMP1).[87,88] By contrast, CIMP-low CRCs have very frequent *KRAS* mutation (92%) and show a denser pattern of methylation affecting a smaller number of genes, suggesting an epigenetic defect influencing the spread of methylation from methylation centres (described as CIMP2).[87,88] It is likely that synergy between *BRAF* or *KRAS* mutations and particular patterns of DNA methylation is necessary to bring about early tumorigenic events. Activated ras and raf have been linked to cell senescence characterized by irreversible cell cycle arrest.[89,90] Interestingly, hyperplastic and closely related polyps initiated by either *KRAS* or *BRAF* mutation

(see below) have been traditionally linked with cell senescence.[91,92] A tumorigenic effect requires the additional inactivation of tumour suppressor genes normally associated with cell cycle arrest, such as *CDKN2A* (encodes p16), $p14^{ARF}$ and *TP53*.[89,90] This could explain the association between *KRAS* mutation and methylation of *CDKN2A* and/or $p14^{ARF}$ in subsets of CRC.[80] In the case of *BRAF*, it has been suggested that more widespread methylation of pro-apoptotic genes such as *RASSF1*, *RASSF2*, *NORE1* (*RASSF5*) and *MST1* is required to bring about a tumorigenic effect.[93] Genes that happen to be methylated in colon and other cell lines not only share distinct functional properties (cell signalling, cell adhesion, cell–cell communication and ion transport) but have common sequence motifs in their promoters.[94] This suggests that *de novo* methylation is not a random process but occurs through a specific instructive mechanism.[94] The evidence for a genetic basis for CIMP is outlined in the following sections.

MECHANISMS FOR CIMP

As well as the strong association with *BRAF* mutation, subjects with CIMP-high or CIMP1 CRC are more likely to have a positive family history of CRC. In a large population-based study in which subjects were selected on the basis of having MSS CRC with *BRAF* mutation, the odds ratio for a positive family history compared with patients with MSS/*BRAF*-negative CRC was 4.23 (95% confidence interval 1.65, 10.84).[61] Among subjects with MSI-H CRC, *BRAF* mutation was a negative predictor for a positive family history.[61] However, subjects with MSI-H/*BRAF*-negative CRC were relatively young and many would be expected to be from Lynch syndrome families. When the same population-based set of cases was studied with respect to CIMP and family history, the link was less strong.[66] However, this analysis employed a cut-off for CIMP in which only about one-third of 'CIMP-positive' CRCs had *BRAF* mutation. The hereditary link appears to be with CIMP-high and/or *BRAF* mutation. Two high-risk family clinic-based studies have suggested that patients with CIMP CRC or *BRAF*-positive CRC may represent a new cancer family syndrome with an increased risk of extracolonic as well as colorectal malignancy.[95,96] A third clinic-based study identified Lynch syndrome-like families in which CRCs showed variable MSI status with combinations of MSS, MSI-L and MSI-H CRC.[97] In Lynch syndrome all tested CRCs would be expected to be MSI-H, whereas in the MSI-variable families most of the CRCs were either MSS or MSI-L. In these families, about half of which met the Amsterdam criteria, a high proportion of both polyps and CRC showed mutation of *BRAF* and/or methylation of the CIMP marker MINT31. Many of the polyps were advanced serrated polyps (serrated adenomas or mixed polyps) and two family members had hyperplastic polyposis.[97] One hospital-based study found no increased family history of cancer in subjects with CIMP CRC. However, this study excluded families meeting unspecified criteria for Lynch syndrome and used a loose definition of CIMP.[98]

The preceding studies suggest that there is likely to be a genetic predisposition to DNA methylation which results in polyps and CRC with CIMP-high (CIMP1). This is supported by the finding of extensive DNA methylation in the normal colorectal mucosa in three unrelated subjects with hyperplastic polyposis.[93] Some patients with hyperplastic polyposis develop multiple CRCs that may be MSS, MSI-L and MSI-H within the same subject.[55] Conceivably, hyperplastic polyposis is inherited as an autosomal recessive disorder associated with multiple polyps and cancers. Subjects with a single copy of the altered gene may develop small numbers of serrated polyps and be at increased risk of developing CIMP CRC. The early evolution of CIMP CRC may be the same regardless of MSI status. Modifying genetic factors may then affect the likelihood of methylation and inactivation of *MGMT* or *MLH1*, which will in turn determine whether the pathway diverges to give CRCs that are MSS, MSI-L or MSI-H.[93]

CIMP-high or *BRAF*-positive CRCs may share an underlying genetic predisposition and constitutional factors, as indicated by the association with female gender. In addition, particular environmental factors may be important in the pathogenesis of these CRCs. The increased risk of CRC associated with smoking is largely explained by the subset with *BRAF* mutation and/or CIMP.[99] Smoking is also associated with hyperplastic polyps, suggesting that the increased risk is related to the earliest evolutionary steps.[100] Interestingly, a polymorphism in the promoter region of *MLH1* (93G→A) modifies the risk of hyperplastic polyps (mainly left-sided) in smokers and raises the possibility of a gene–environment interaction that could predispose to partial methylation of *MLH1* and an MSI-L/CIMP-low pathway (see above).[101] Chronic inflammation in the context of ulcerative colitis has also been linked with DNA methylation.[102]

LINK BETWEEN LOSS OF IMPRINTING AND CIMP

Genomic imprinting occurs through methylation of one allele so that a gene is expressed only through the

non-imprinted (usually paternal) allele. *IGF2* is one of the more well-known imprinted genes. Loss of imprinting (LOI) of *IGF2* has been asociated with MSI-H CRC.[103] A study from Japan failed to show this link but found that CRCs with LOI had the morphological features of CRCs with CIMP, notably poor differentiation, mucinous differentiation and proximal location.[104] The link between LOI and CIMP may be explained by the fact that LOI depends upon the methylation of a controlling element known as the *H19* differential methylated region.[105] The fact that LOI of *IGF2* may be found in normal colonic epithelium and even normal leucocytes as well as CRC[106] suggests that the *H19* differential methylated region is exceptionally sensitive to methylation pressures. The observation that *IGF2* LOI in normal leucocytes is associated with a personal and family history of CRC[106] provides additional evidence for an inherited basis for CIMP.

Molecular classification of colorectal cancer

It would undoubtedly be more convenient for cancer researchers if CRC could be viewed as a homogeneous disorder, because an individual CRC or cell line could then be considered representive of all CRC. At one level this may still be true, insofar as the acquisition of the full malignant phenotype probably depends upon the combined disruption of all the major signalling pathways. Indeed, while it has been argued above that familial and sporadic MSI-H CRC evolves through different pathways, there is very considerable overlap in the altered gene expression signatures of these two types of CRC, as shown by microchip array-based analysis.[107] However, this does not refute the concept that the pathways differ at a fundamental level. Rather, it highlights major limitations of present-day biotechnology insofar as it is incapable of either explaining the evolutionary history of a malignancy or resolving subtle differences in levels of gene expression existing at the key control points of signalling pathways. Based primarily on: (i) the underlying types of genetic instability, and (ii) the presence of DNA methylation, the following five molecular subtypes of CRC (with approximate frequencies) are suggested:

1 CIMP-high, methylation of *MLH1*, *BRAF* mutation, chromosomally stable, MSI-H, origin in serrated polyps, known generally as sporadic MSI-H (12%).

2 CIMP-high, partial methylation of *MLH1*, *BRAF* mutation, chromosomally stable, MSS or MSI-L, origin in serrated polyps (8%).

3 CIMP-low, *KRAS* mutation, *MGMT* methylation, chromosomal instability, MSS or MSI-L, origin in adenomas or serrated polyps (20%).

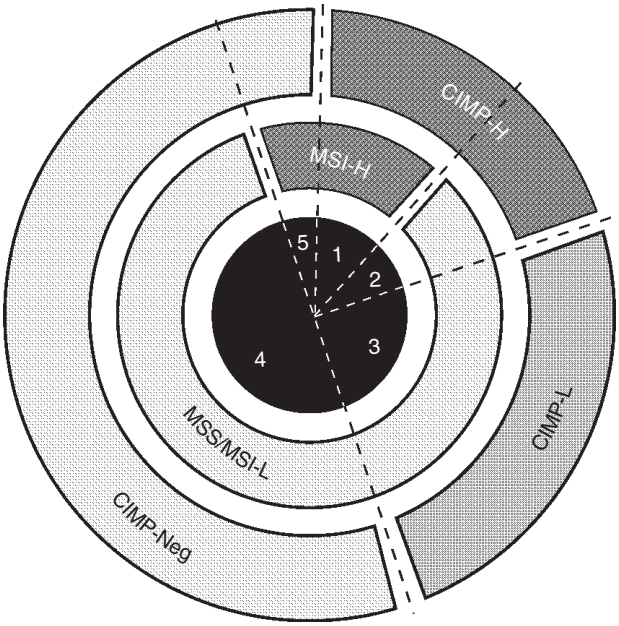

Figure 1. Derivation of molecular colorectal cancer groups 1–5 based on CpG island methylator phenotype (CIMP) status (H, high; L, low; Neg, negative) and DNA microsatellite instability (MSI) status (H, high; L, low; S, stable).

4 CIMP-negative, chromosomal instability, mainly MSS, origin in adenomas (may be sporadic, FAP-associated or *MUTYH* (formerly *MYH*) polyposis associated[108]) (57%).

5 Lynch syndrome, CIMP-negative, *BRAF* mutation negative, chromosomally stable, MSI-H, origin in adenomas (3%) (described also as familial MSI-H CRC in this review).

Sporadic MSI-H CRCs are deliberately termed as group 1 because they are the most obviously homogeneous group with respect to their clinical, morphological and molecular features. However, group 5 CRCs share features with group 1 CRCs and these groups may be conceived as completing a circle rather than representing the ends of a spectrum (Figure 1). Overlaps between the groups are not excluded. For example, *KRAS* rather than *BRAF* mutation may occasionally occur in association with CIMP-high.[48]

Morphological correlations

While particular morphological correlates have been demonstrated for each of the preceding subtypes, it is not necessarily possible to recognize each group on the basis of morphological features alone. In particular, there are no studies of the morphological distinction of groups 3 and 4. It is often the case that a particular feature will characterize two or more groups. The

Table 1. Molecular, clinical and morphological features of colorectal cancer groups 1–5

Feature	Group 1	Group 2	Group 3	Group 4	Group 5
MSI status	H	S/L	S/L	S	H
Methylation	+++	+++	++	+/−	+/−
Ploidy	Dip > An	Dip > An	An > Dip	An > Dip	Dip > An
APC	+/−	+/−	+	+++	++
KRAS	−	+	+++	++	++
BRAF	+++	++	−	−	−
TP53	−	+	++	+++	+
Location	R > L	R > L	L > R	L > R	R > L
Gender	F > M	F > M	M > F	M > F	M > F
Precursor	SP	SP	SP/AD	AD	AD
Serration	+++	+++	+	+/−	+/−
Mucinous	+++	+++	+	+	++
Dirty necrosis	+	+	?	+++	+
Poor differentiation	+++	+++	+	+	++
Circumscribed	+++	+	?	++	++
Tumour budding	+/−	+	?	+++	+
Lymphocytes	+++	+	?	+	+++

MSI, microsatellite instability; H, high; S, stable; L, low; Dip, diploid; An, aneuploid; Serration, serrated morphology; SP, serrated polyp; AD, adenoma; Circumscribed, circumscribed invasive margin.

primary basis for the classification of CRC is therefore molecular. In this section the focus will be on the various discriminating morphological features and the extent to which they cut across the molecular subtypes. An overview of the morphological findings in CRC groups 1–5 is shown in Table 1.

SERRATED MORPHOLOGY

The term 'serrated adenocarcinoma' was introduced to describe CRC with such a close structural and functional (histochemical) resemblance to the hyperplastic polyp that it was difficult to dismiss a direct histogenetic relationship between the two (Figure 2a).[109] Serrated adenocarcinomas were subsequently described in association with multiple serrated adenomas and hyperplastic polyps[110] and finally in considerable detail when observed either with or without a contiguous serrated adenoma (Figure 2b).[56,82] It should be strongly stressed that glandular serration in isolation is a non-specific feature that may be produced by branching and folding of proliferating epithelium that occurs in CRC regardless of early histogenesis. Serrated adenocarcinomas are recognized by the presence of additional features which include: (i) cribriform, lace-like and trabecular structures, (ii) secretion of intracellular and often abundant extracellular mucin, (iii) a low nuclear:cytoplasmic ratio, (iv) round or ovoid nuclei that are vesicular with a prominent nuclear membrane (chromatin condensation at the nuclear membrane) and large nucleolus, (v) well-preserved nuclear polarity, and (vi) an overall 'pink' appearance due to relatively abundant eosinophilic cytoplasm and lack of nuclear hyperchromatism.[82] Serrated morphology has been linked with MSI, but the association was significant for MSI-L CRC, with only a trend for MSI-H CRC.[82] Many of the structural and cytological features accompanying serrated morphology are linked with DNA methylation. However, glandular serration in isolation was not shown to be associated with CIMP,[65] emphasizing the importance of a more global appraisal. Nevertheless, glandular serration was more frequent

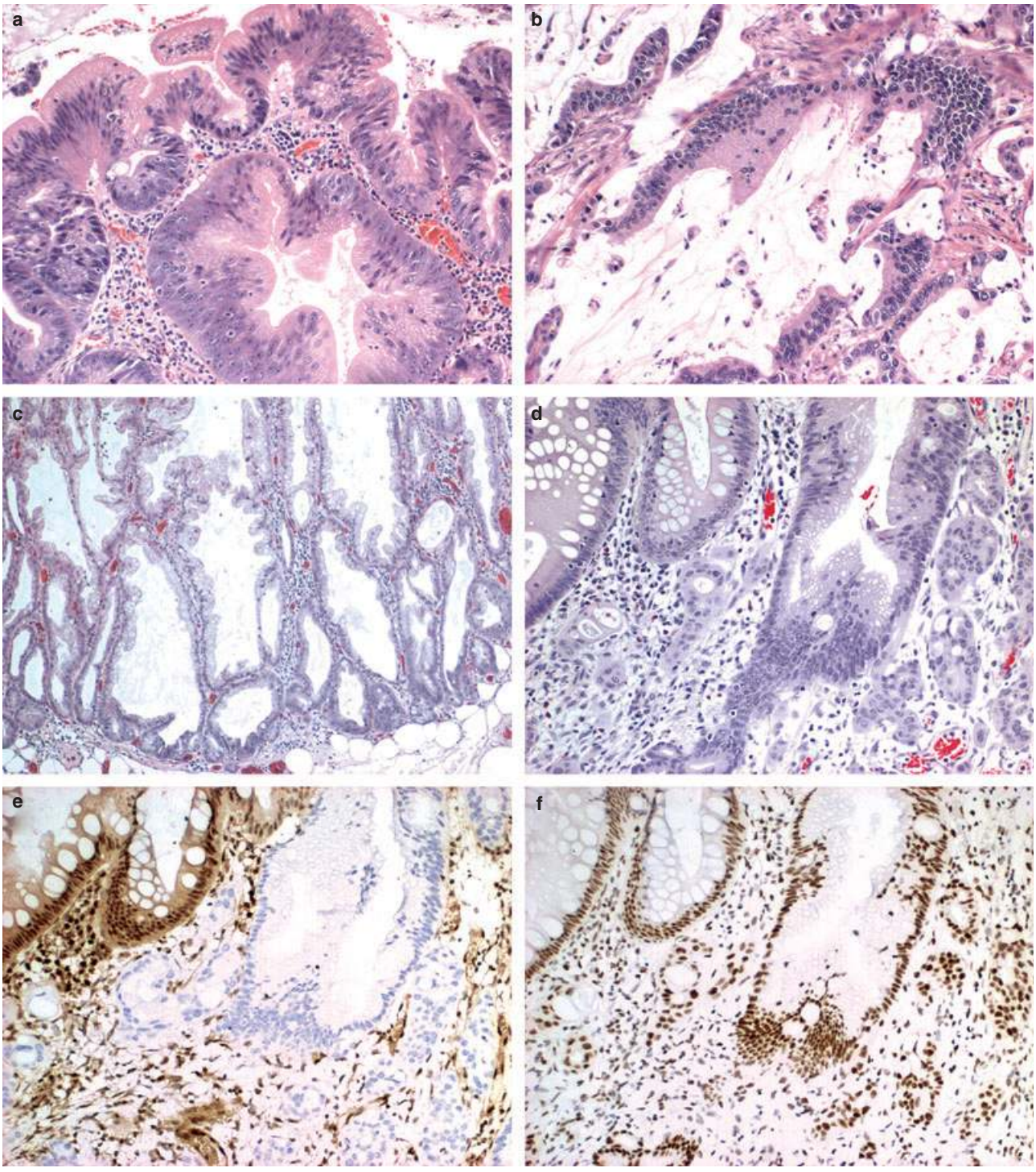

Figure 2. Colorectal cancers with serrated morphology and serrated precursor lesions. Serrated adenoma (**a**) that was contiguous with a microsatellite instability-high serrated adenocarcinoma (group 1) (**b**). Serration is not as obvious in the carcinoma (**b**) as the serrated adenoma (**a**) but the carcinoma shows other features of serrated morphology, including abundant eosinophilic cytoplasm, ovoid nuclei and extracellular mucin. Sessile serrated adenoma showing branched, dilated and back-to-back glands but no cytological atypia (**c**). Poorly differentiated group 2 carcinoma [**d,** H&E; **e,** immunohistochemistry for Methylguanine DNA Methyltransferase (MGMT); **f,** immunohistochemistry for MLH1]. The glands to the left are normal and show nuclear expression of both MGMT and MLH1. The gland with features of serrated adenoma and the poorly differentiated adenocarcinoma infiltrating the lamina propria show loss of nuclear expression of MGMT but not MLH1 (ABC technique).

in CRC from members of MSI-variable families in which the CRCs showed frequent *BRAF* mutation and/or methylation of the CIMP marker MINT31.[97] A serrated morphology will therefore be over-represented among group 1 and 2 CRCs and may help to distinguish sporadic from familial (Lynch syndrome) MSI-H CRC.[111]

PRECURSOR LESIONS

Lesions similar to hyperplastic polyps but characterized by large size, aberrant architecture, increased proliferation and a predilection for the proximal colon have recently been linked with sporadic MSI-H CRC and termed 'sessile serrated adenoma (SSA)',[112,113] 'sessile serrated polyp'[22] or 'sessile polyp with atypical proliferation' (Figure 2c).[53,114] The finding of either this lesion or traditional serrated adenoma in contiguity with a CRC would serve as evidence of molecular groups 1 or 2.[82,115,116] Most of the polyps contiguous with CRC in Lynch syndrome are conventional adenomas,[115,117] but serrated adenoma has been observed on rare occasions.[118] The transition from SSA to CRC will usually be through an intermediate stage of dysplasia or intraepithelial neoplasia (giving a mixed polyp), even if this step is transient. Importantly, dysplasia in the serrated pathway may not resemble adenomatous dysplasia. Instead of being elongated, pseudo-stratified and hyperchromatic, nuclei are round and vesicular with a coarse nuclear membrane and a prominent nucleolus and nuclear polarity is well maintained.[119,120] In other words, the cytological atypia resembles (not surprisingly) the aberrant cytology associated with group 1 and 2 CRC as described above.

Molecular alterations occurring at the key transition from hyperplasia to dysplasia include loss of expression of MLH1 in group 1 CRC and loss of expression of MGMT and/or aberrant expression of p53 in group 2 CRC (Figure 2d–f). Mixed polyps, in which the dysplastic component shows normal expression of MLH1 but aberrant expression of p53, have been termed 'fusion' polyps since they combine molecular features of the serrated pathway (e.g. *BRAF* mutation and DNA methylation) with an abnormality characteristic of the adenoma–carcinoma sequence.[120] Therefore, group 2 CRC could be regarded as a group 1/group 4 hybrid. Group 3 CRC is associated with *KRAS* mutation and CIMP-low (CIMP2) (see above). The principal precursors of group 3 CRC are likely to be adenomas with *KRAS* mutation. DNA methylation occurs in adenomas but becomes more evident with increasing size, dysplasia or villosity.[49–51] However, there is less extensive marker methylation in adenomas compared with serrated polyps.[116] *KRAS* mutation is closely linked to villous change and dysplasia (but not size) and is found in some mixed polyps and serrated adenomas as well as conventional adenomas.[120,121] Therefore, group 3 CRC may arise within mixed polyps or serrated adenomas as well as conventional adenomas with villous change.[120]

MUCINOUS DIFFERENTIATION AND DIRTY NECROSIS

Mucinous carcinoma is diagnosed when at least 50% of the tumour comprises secretory mucin. The mucin is intraluminal in the case of well or moderately differentiated CRC and forms interstitial pools surrounding the irregular trabeculae in poorly differentiated CRC (Figure 3a,b).[122] Mucinous carcinoma is over-represented among group 1 and 2 CRC[61–63,65,66,123] and the latter may secrete appreciable amounts of mucin without meeting the strict quantitative definition. In the case of group 1 (sporadic MSI-H) CRC there is often a zoning pattern with mucin secretion confined to the deeper tumour compartment only,[124] or there may be marked tumour heterogeneity with areas of mucinous carcinoma alternating with other patterns.[125] The strict definition of mucinous carcinoma may therefore lack sensitivity when it is used as a marker of group 1 and 2 CRC.

Secretory mucin associated with group 1 (sporadic MSI-H) CRC comprises both intestinal (MUC2) and gastric (MUC5AC) mucin.[123,126] Secretory mucin associated with serrated adenocarcinomas comprises non-O-acetylated sialic acid substituents, as in small intestine.[109] A mixed gastric and small intestinal mucinous phenotype is also associated with hyperplastic polyps, mixed polyps and serrated adenomas of the colorectum.[109,127] By contrast, secretory mucin production in conventional colorectal adenomas is decreased, leaving expression of the transmembrane glycoprotein MUC1 only in areas of high-grade dysplasia.[128] Expression of non-O-acetylated sialic acid is also restricted to foci of high-grade dysplasia in adenomas.[129] A shared secretory mucinous profile across serrated polyps, group 1 (sporadic MSI-H) CRC and serrated adenocarcinoma (occurring among group 1 and 2 CRC) provides strong evidence for the existence of a serrated pathway of colorectal tumorigenesis which parallels the molecular arguments presented above.

Some sporadic mucinous carcinomas arise in villous adenomas (Figure 3b).[130] The increased frequency of villous adenomas in Lynch syndrome[7] may account for the higher incidence of mucinous carcinoma in this condition.[131,132] The link between mucinous differentiation and Lynch syndrome was observed in CRC

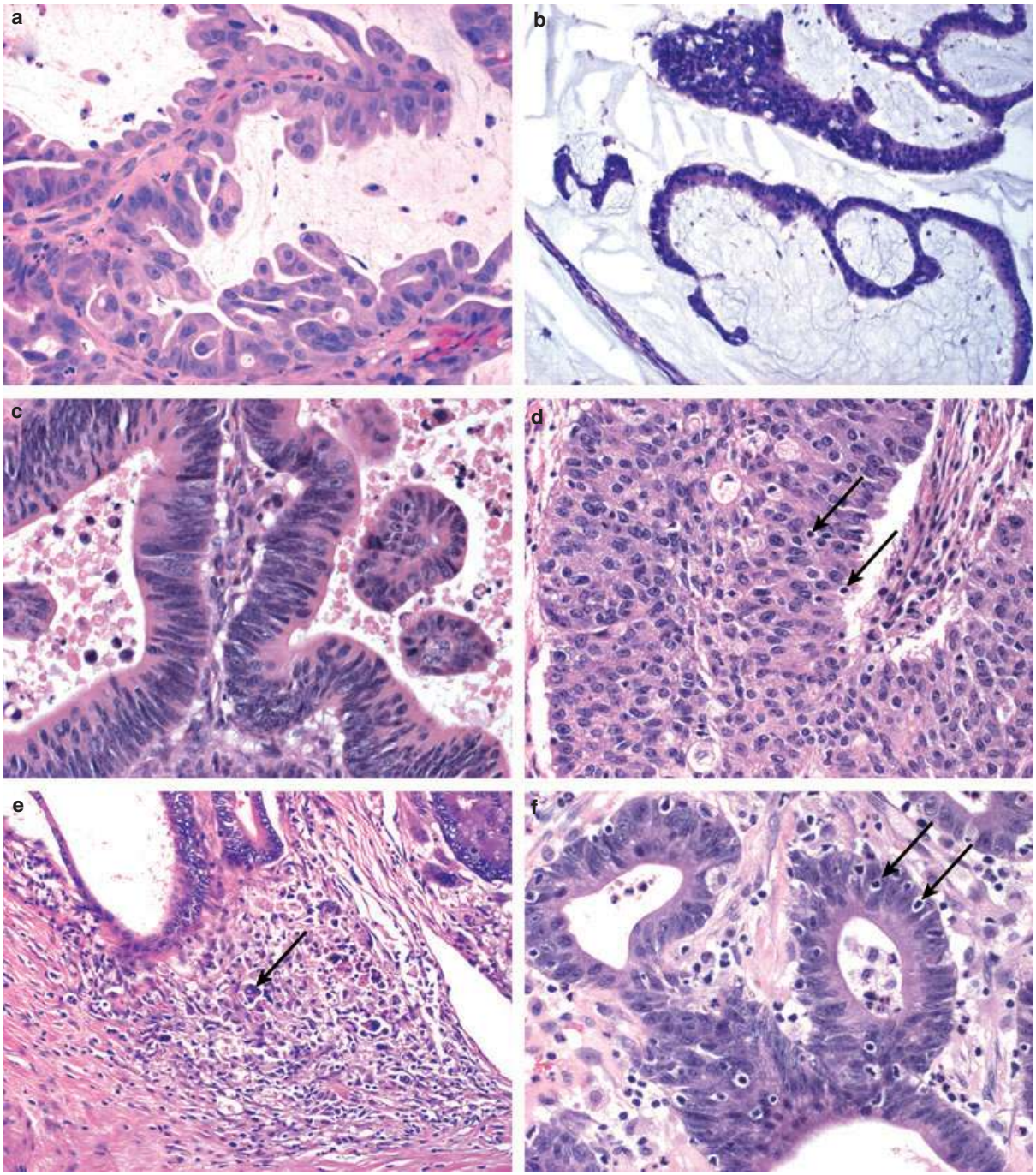

Figure 3. Differentiation, tumour budding and lymphocytic infiltration. Mucinous carcinoma with serrated morphology (group 1) (**a**) is compared with mucinous carcinoma (group 3 or 4) that developed within a villous adenoma (**b**). In addition to the serrated contour of epithelium, the mucinous carcinoma arising within a serrated precursor lesion (not shown) is characterized by an abundant eosinophilic cytoplasm and vesicular ovoid nuclei that are ovoid and vesicular with a prominent nucleolus (**a**). The cytology of the mucinous carcinoma arising in a villous adenoma (not shown) is characterized by a dark and amphophilic cytoplasm and nuclei which are hyperchromatic rather than vesicular (**b**). Moderately differentiated adenocarcinoma (group 4), in which lumen contains necrotic cellular debris ('dirty necrosis') and epithelium shows elongated and stratified nuclei which are hyperchromatic and lack distinct nucleoli (**c**). The cytology is consistent with an origin in a conventional adenoma and not a serrated polyp. Medullary carcinoma (group 5, Lynch syndrome) composed of solid sheets of cells and infiltrated by lymphocytes (arrows) (**d**). Tumour budding characterized by small clusters of de-differentiated cells (arrow) at the invasive margin (**e**). Moderately differentiated adenocarcinoma (group 5, Lynch syndrome) with intraepithelial lymphocytes (arrows) (**f**). The cytology is consistent with origin within a conventional adenoma.

obtained from subjects meeting clinical criteria for Lynch syndrome and before its molecular basis had been uncovered. Following the demonstration of DNA mismatch repair deficiency and the associated MSI phenotype, sporadic and familial CRCs with MSI-H were initially grouped together on the assumption that they were equivalent tumours.[124,133] However, a marked mucinous component has been described in 35%,[133] 43%,[125] 36%[134] and 31%[135] of MSI-H CRCs that were mainly sporadic. When the mucinous phenotype was defined on the basis of any amount of secretory mucin, this feature was found in as many as 67% of mainly sporadic MSI-H CRC.[136] In contrast, mucinous differentiation was observed in only 19%[132] and 22%[125] of likely Lynch syndrome CRC, whereas, among 64 CRCs from subjects with a proven germ-line mutation in a DNA mismatch repair gene, the frequency of mucinous carcinoma was not significantly greater than in CRC from in general population.[137] It is likely that there is a slight over-representation of mucinous carcinoma in Lynch syndrome, but with interfamily differences. Mucinous carcinoma has been reported in five members of a single family[138] and has also been associated specifically with *MSH2* germ-line mutation.[137]

Mucinous carcinoma has traditionally been regarded as relatively aggressive, although this impression derives mainly from the study of rectal cancer.[130,139] It is clear that mucinous differentiation occurs in multiple molecular subtypes, each with differing site predilections and prognosis. Since mucinous differentiation is not specific to a single clinicopathological entity, the lack of a clear prognostic effect is not surprising.

When not filled with secretory mucin, malignant lumina in haematoxylin and eosin (H&E) sections may either appear empty or contain deeply eosinophilic material that is frequently admixed with necrotic cell debris (Figure 3c). This eosinophilic material ('dirty necrosis') is strongly positive with period acid–Schiff and expresses the transmembrane glycoprotein MUC1.[140] The presence of dirty necrosis is negatively associated with CRC showing MSI-H.[136]

POOR DIFFERENTIATION, MEDULLARY AND SIGNET RING CELL SUBTYPES

Poor differentiation, as in other tumour types, indicates a marked loss of morphological resemblance to the parent tissue and, in the case of adenocarcinoma, a loss of glandular development. Like mucinous differentiation, this feature has been associated with poor prognosis. However, and serving as a parallel with mucinous differentiation, poorly differentiated adenocarcinoma is over-represented among group 1[141] and Lynch syndrome CRC[131,132] that are associated with a relatively good prognosis. A series of eight largely undifferentiated CRCs with a pushing tumour margin was found to be associated with an unexpectedly favourable clinical outcome.[142] Two of the subjects were very young (a female aged 31 years and a male aged 39 years) and may well have had Lynch syndrome. The term medullary carcinoma has subsequently been applied to poorly differentiated large cell carcinoma in which the epithelium is arranged in closely packed trabeculae or solid aggregates.[143] Medullary carcinoma is distinguished from undifferentiated carcinoma by its good overall circumscription, lack of nuclear pleomorphism, presence of lymphocytic infiltration, which may be intraepithelial, peritumoral or within Crohn-like nodules, and presence of focal glandular differentiation (Figure 3d). Medullary carcinoma occurs in both group 1 and Lynch syndrome CRC but is uncommon in both. However, Group 1 CRCs frequently show morphological heterogeneity with the medullary pattern being represented in subclones.[125] The homeobox gene *CDX2* is mutated in CRC with MSI-H[144] but (and consistent with the rarity of medullary carcinoma) mutation of this gene was observed in only 3.2% of Lynch syndrome CRC.[145] Loss of expression of CDX2 was strongly associated with medullary carcinoma (described as large cell minimally differentiated carcinoma).[146] In grading the biological aggressiveness of CRC it is clear that a single feature, such as glandular differentiation, provides limited prognostic information when assessed in isolation from other features, whether morphological or molecular.

Although associated with group 1 and Lynch syndrome CRC, signet ring cell carcinoma is, like medullary carcinoma, an uncommon malignancy and is probably less specific than medullary carcinoma with respect to an association with MSI. Due to its relative rarity it is not known if the highly aggressive nature of signet ring cell carcinoma is modified in CRC with MSI-H status. This possibility is, however, suggested by the description of well-circumscribed signet ring cell carcinomas with an exophytic growth pattern and limited aggression.[147] CRCs with both CIMP and MSI-L status (group 2) are more likely to include subclones comprising signet ring cells.[148]

INVASIVE MARGIN AND TUMOUR BUDDING

Morphological findings at the invasive tumour margin provide, after stage, the most important prognostic information in CRC.[149–151] A diffusely infiltrative margin is characterized by widespread invasion and dissection of normal tissue structures (smooth muscle or

adipose tissue) so that there is no clear boundary between tumour and host tissue. This pattern of spread is closely correlated with lymphovascular and perineural invasion and the presence of discontinuous mesenteric deposits.[152] A diffuse growth pattern is also associated with a feature described as tumour budding or de-differentiation, in which there is a transition from glandular structures to single cells or clusters of up to four cells at the invasive margin (Figure 3e).[151] Tumour budding may and should be distinguished from a subclone showing poor differentiation by its presence along the entire invasive interface. Furthermore, budding cells have the properties of malignant stem cells, including the potential for re-differentiation both locally and at sites of metastasis.[153] In other words, the morphological and immunophenotypic features associated with budding cells are reversible and therefore likely to be under epigenetic control. Expression patterns associated with budding cells include up-regulation of β-catenin,[154,155] laminin5-γ2,[156] matrix metalloproteinase-7 (MMP-7 or matrilysin),[157] membrane type-1 MMP,[157] p16,[28] cyclin D1,[33] urokinase-like plasminogen activator receptor,[158] CD44,[158] COX-2[158] and tenascin-C[159] and down-regulation of E-cadherin[155,160] and Cdx-2.[161] Budding cells also show evidence of autonomous movement characterized by the presence of podia[162] that express P-glycoprotein at points of attachment to mesenchymal elements.[163] Mesenchymal markers including fibronectin are also expressed[155] and tumour budding is synonymous with the epithelial–mesenchymal transition described in other cancer model systems.[159]

While tumour budding is likely to be triggered through an increased sensitivity to mesenchymally derived growth signals,[161] the change will occur only in cancer cells primed by particular genetic alterations. Tumour budding is uncommon in group 1 CRC[28,164] and when it does occur the full immunophenotype is not apparent.[165] For example, budding cells in group 1 CRC do not show increased expression of β-catenin or laminin5-γ2 and lack the development of podia.[165] It is possible that the low frequency of mutation of the Wnt pathway genes *APC* and *CTNNB1* accounts for the lack of tumour budding in group 1 CRC. While the full budding phenotype may not be an absolute requirement for metastasis, the relative absence of budding among group 1 CRCs could be at least one explanation for their good prognosis.

LYMPHOCYTIC INFILTRATION

A marked peritumoral lymphocytic infiltrate was initially described in CRC from subjects meeting clinical criteria for Lynch syndrome.[132] Intraepithelial lymphocytes, known also as TILs, were initially associated with undifferentiated or medullary carcinoma with MSI-H,[166] but were subsequently also shown to be a useful biomarker for all group 1 (sporadic MSI-H) CRC.[141] TILs serve as the most important marker for sporadic and familial MSI-H CRC and diagnostic cut-offs based on cell counts in either H&E sections[136,167] or utilizing CD3/CD8 immunohistochemistry[124,168] have been established. The finding of at least five intraepithelial lymphocytes in at least one of 10 high-power (× 40) fields provides a sensitive cut-off (Figure 3f).

Intraepithelial cytotoxic (CD8) T cells are observed under normal physiological conditions. Accordingly, one might postulate a mechanism leading to active destruction of these cells in non-MSI-H CRC, for example by the 'Fas ligand counter-attack'.[169] However, intraepithelial T cells in MSI-H CRC are: (i) generally more numerous than under normal physiological conditions, (ii) associated with a peritumoral lymphocytic reaction including Crohn-like nodules of B cells[170] and (iii) associated with an improved prognosis within the MSI-H subset.[171] These findings indicate the existence of a clinically beneficial specific immune reaction against mutator-generated tumour antigens and not merely the passive retention of T cells within the intraepithelial compartment.

There is a negative correlation between lymphocytic infiltration and mucinous differentiation[139] and this explains why these features are independent markers of MSI-H status. Lymphocytic infiltration (peritumoral and Crohn-like) was more marked in Lynch syndrome than group 1 (sporadic MSI-H) CRC,[115] a finding which could be related to the increased frequency of mucinous differentiation in the latter (see above). It is also possible that the adverse prognosis associated with TIL-depleted MSI-H CRC is explained by the deleterious effects of increased mucin production. TILs are not restricted to the MSI-H subset of CRC. Increased TIL counts have been associated with MSI-L status[168] and particularly in CRC with both CIMP-high and MSI-L (group 2).[148] Since this subset is not associated with a good prognosis, the link between TILs and survival remains unclear.

Conclusion

A classification of CRC that incorporates an understanding of the earliest evolutionary steps is necessary in order to dissect out the various risk factors that explain causation or pathogenesis or identify early targets for chemoprevention.

The notion that adenomas give rise to CRC was developed by pathologists. It was subsequently re-worked by basic scientists, who promulgated the view that adenomas are initiated through bi-allelic inactivation of *APC* and progress to CRC through a predictable linear sequence of molecular alterations. The dogmatic linking of the 'vast majority' of CRC to this mono-directional model implied that if a minority subset outside the model existed it was too minuscule to warrant further consideration. Until comparatively recently there has been a failure to recognize that CRC is in fact a multipathway disease comprising disparate subgroups with particular clinical, pathological and molecular features. The unfortunate consequence has been a delay in the progress of research that depends absolutely on such an understanding. In particular, the oversimplification of the evolutionary pathway has confounded the identification of risk factors for CRC, whether genetic, constitutional or lifestyle related.

While it has been usual to establish the molecular correlates of existing morphological classifications, the decades-long tendency of considering CRC as a single entity means that the circle has had to be completed in the reverse order. It should be stressed, however, that the correlation of morphological and immunohistochemical features with molecular subtypes has been an iterative process, in which genetic instability and CIMP have been shown to be fundamental classification criteria through a process of trial and error. This correlative process incorporates clinical, morphological and biological components. Furthermore, since genetic instability and CIMP are acquired at the precancerous stage, the suggested typing of CRC has a strong basis in pathogenesis. While the proposed classification remains speculative, it has the advantage of conveying powerful meaning through the synthesis of clinical, pathological and molecular features. An exclusively molecular classification carries little meaning. John Constable said that we see nothing until we truly understand, but we can also say that we understand nothing until we truly see. The recognition of the heterogeneous nature of CRC means that pathology has now become integral to CRC research.

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