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Genetic Pathways in Colorectal and other Cancers*

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Cells from cancers show aberrant behaviour such as unrestrained growth, invasion into adjacent tissue and metastasis. All these features of cancer cell behaviour can be explained in terms of genetic changes and the functional impact of these changes. In this review, colorectal cancer (CRC) is examined as a classical example of multistep carcinogenesis. First there is an overview which shows that cancers develop by a process of somatic evolution. This gives rise to preferred genetic pathways of tumorigenesis. The factors which may influence the development and ultimate choice of genetic pathways are then examined. Next, CRC is studied as a specific disease and the putative genetic pathways are described. The mutations that comprise these pathways and the possible functional sequelae of these are explored. The review concludes with a look at those avenues which may further elucidate the natural history of CRC and lead to improved therapy.

Key words: colorectal cancer, genetics, genetic pathways, review
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INTRODUCTION

It is generally accepted that carcinogenesis is a multistep process [1–3]. These steps represent gene mutations or epigenetic changes such as methylation resulting in either activation of a dominantly acting oncogene or inactivation of a tumour suppressor. Each mutation alters the behavioural responses of the cells giving some growth advantage. As the mutations accumulate, there is a progressive change from normal growth to malignant behaviour, characterised by autonomous and unrestrained growth, invasion into the surrounding tissue and metastasis. It is most unlikely that a single mutation will be sufficient to cause a malignant phenotype to develop and, given that the mutation rate in human tissues is low [4, 5], it is a statistical near-impossibility that all the requisite mutations will occur simultaneously. It follows from this that each mutation will occur at a different time and, thus, that all the steps in a particular multi-step process will occur in a sequence. The mutations which occur and the order in which they occur form the *genetic pathway* and it should, in principle, be possible to construct a genetic pathway for each cancer and identify those steps in common to particular types of cancers.

The genetic pathway is reflected histologically by precursor lesions showing a progressive increase in atypia leading to features of malignancy. Precursor lesions can be seen in most cancers, although some tumours do occur in which they are not described. In these cases, either the precursor lesion is histologically not distinct from normal tissue or, more probably, it has been obliterated by the tumour.

All tissues are subject to growth controls and, as a general principle, the mutations (or epigenetic changes) lead to escape from these growth controls. As different tissues have different growth control mechanisms, many of the actual mutations involved will vary from tissue to tissue. Conversely, tumours arising from any particular tissue will have to escape the same growth controls and, therefore, may be expected to develop along similar genetic pathways. A number of factors influence mutation selection and these are considered next.

THE SOMATIC EVOLUTION OF CANCER: FACTORS WHICH INFLUENCE THE GENETIC PATHWAYS

Escape from environmental growth constraints

Normal tissues have a highly complex architecture which is maintained through strict regulation of tissue growth and cell-cell and cell-extracellular matrix relationships. This necessitates sophisticated and effective growth control mechanisms to ensure that the number of new cells produced is approximately equal to the number which undergo apoptosis (programmed cell death) and that tissue architecture is maintained with cellular turnover. During repair of tissue damage, these mechanisms must allow a sufficient increase in proliferation to restore the normal architecture and then bring the tissue back to the normal equilibrium. Signals for controlling cell growth may arise endogenously as part of the differentiation process or may be received from the immediate environment. The latter may arise from direct interaction of membrane receptors (such as adhesion molecules) with other cells, from molecules present in the extra-cellular matrix (such as basement membrane components), or from secreted molecules (such as growth factors and cytokines) [6–13]. Cells which escape these growth constraints will have a greater 'fitness' than other cells and will, according to the principles of Darwinian evolution, grow in preference.

All cells are prone to mutations which may occur spontaneously or may arise as a result of environmental factors. Mutations occur at random throughout the genome but only those which give some sort of survival advantage and thereby make the cell 'fitter' will be selected. The limitations to tumour growth, or selection pressures, will change as the tumour evolves and, therefore, each stage will provide a 'window of opportunity' for selection of mutations appropriate to that stage. For example, in the early stages of development, escape from growth control exerted by stromal adhesion molecules may give the greatest selective advantage and mutations affecting the function of membrane receptors may be selected in preference to those causing increased proliferation. In the latter case, the growth controls may match an increase in cell number with an increase in the rate of apoptosis, thus giving no growth advantage. At a later stage, tumour hypoxia may be the growth limiting factor and mutations causing secretion of angiogenic factors or enhanced anaerobic metabolism will be selected over those causing increased cell proliferation as these will aggravate the state of hypoxia. In a well vascularised tumour, however, the relative selective advantages of these mutations may be reversed and mutations causing an increase in the rate of proliferation will be selected over the others. Although the main evolutionary drive may come from environmental constraints, competition between cells within a tumour may also

occur. In this situation, secretion of growth inhibitory or cytotoxic factors by cells which are resistant to those factors may give a selective advantage and allow sub-clonal evolution [14, 15]. In this way, although mutations will occur randomly, the mutations which are selected and the order of selection, i.e. the genetic pathway, will be non-random [16, 17].

Occasionally a tumour will contain a mutation which has neither selective advantage nor disadvantage. If such a 'neutral mutation' occurs coincidentally with a selected mutation it may be carried as a 'bystander' through the selective advantage given by the latter [18]: a phenomenon that has been called 'hitch-hiking' in a general evolutionary context. *Such bystander mutations would be expected to be rare at any one locus and will not form part of the genetic pathway*. The exception to this is if a tumour has an intrinsic or acquired high mutation rate such as cancers with defective DNA mismatch repair. These cases generate numerous bystander mutations and are discussed in more detail later.

Interaction of mutations: the inverted pyramid versus nexus hypothesis

Although environmental constraints will predominantly determine which mutations are selected, mutations which have already occurred may influence selection pressures associated with subsequent mutations. Thus, mutations alter the normal activities within cells and two mutations may interact to enhance or neutralise the effects of each other. This 'oncogene collaboration' has been shown *in vitro* where co-transfection of different combinations of activated oncogenes into fibroblasts has resulted in different phenotypic effects. Some pairs of oncogenes appear to result in more efficient transformation of cells than others and this is thought to be due to synergistic activities of the oncogenes [19–22]. Thus, the environment will determine which change is necessary to overcome the constraints present at that time. If a number of mutations can produce this change, and they are truly redundant i.e. they give an equal selective advantage, then which happens first, and so is actually selected for, will be down to chance. If, however, these mutations have some impact on the activity of earlier mutations, then the one which best complements the pre-existing mutations or interferes least with their activity, will be selected.

It is not generally known whether tumours at different stages of development become 'resistant' to the effects of mutations which occurred at earlier stages of development. For example, is the functional change induced by the mutation which occurred at the very beginning of tumour development still essential for tumour survival at the very late stages of metastasis? At first glance the answer to this question is 'no', as the metastatic cells are now in a completely different tissue and environment, with a new set of selection pressures. Carcinogenesis is, however, a very complex process in which the interactions of the mutations may be extremely important. Thus, it may be the case that the tumorigenic process is like an inverted pyramid (Figure 1). In this model, the first mutation predicts the selection of the second mutation and they interact to ensure optimal activity of both. These together form the basis for selection of the third mutation and so on until a number of mutations has accrued. Although the environment will still give the major evolutionary drive, the internal selection pressures may result in a number of inter-dependent mutations. Reversal of the first mutation will affect the activity of the second mutation which may then have a knock-on effect up to the top of the pyramid. These changes may then affect the viability of the tumour

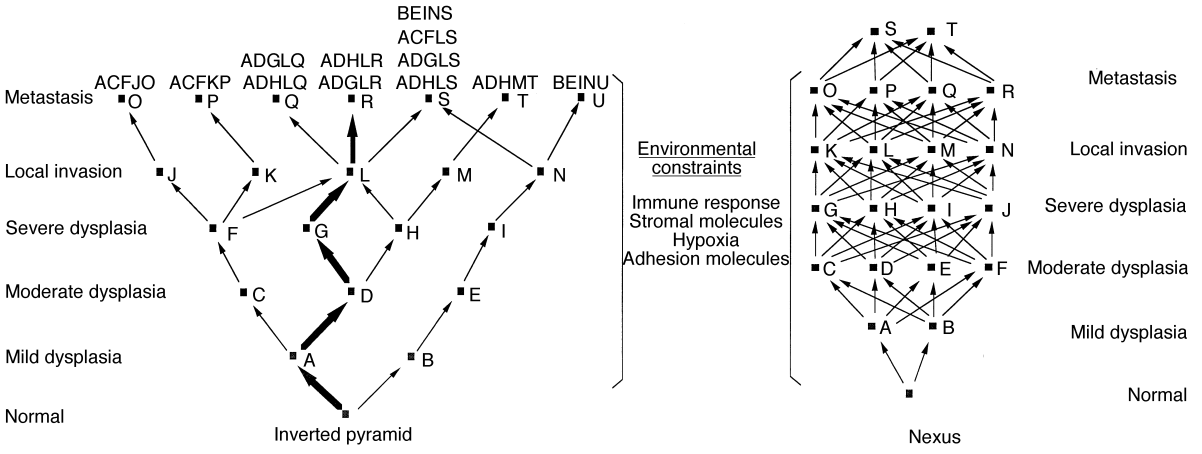

Figure 1. Two models of tumour evolution. Each point represents a specific gene which is mutated and the evolution is from normal through to metastasis. In both models the selection pressures are mainly from the environment and tumour progression is reflected by histological changes. At each step there are a number of alternative genes which may be mutated. In the 'inverted pyramid' model there is a large degree of interdependence of the mutations and early mutations provide internal selection pressures for later mutations. Thus, a cell with mutation 'A' may select 'C' or 'D' but, since 'A' and 'D' interact with each other better than 'A' and 'C', mutation 'D' will usually be selected. Mutations 'A' and 'E' interact to provide a selective disadvantage and thus, are never seen together. Mutations 'AD' can interact equally well with mutation 'H' and this provides a point of true redundancy, whereas 'AC' can only interact with 'F'. Mutation of 'H' with 'A' alone will not give a selective disadvantage since the environmental constraints at that stage will not be conducive to selection of 'H'. In this way, the selection of mutations will occur in sequence. Mutation of 'L' may be a promiscuous event seen in many pathways because it gives a bigger selective advantage than the other possibilities at that stage. The selection of mutations at metastasis will be determined by the site in which the metastasis occurs and several different genetic pathways may share just one final mutation. The optimal choice in this scenario is 'ADGLR'. If the interdependence of mutations is great, then reversal of the first mutation may affect the activity of the second mutation and all subsequent mutations and, in effect, the 'pyramid' will collapse. In the nexus model, the mutations are not interdependent and there are no internal selection pressures. In this case, once each stage has been passed, the mutation leading to that stage are no longer required and reversal of these does not affect the tumour.

cells. If this hypothesis is correct, it will have important therapeutic consequences, because targeting of therapy should be towards reversing either the first mutation or the changes induced by it. Targeting of later mutations may just take the tumour back to an earlier stage and allow it to evolve in another direction.

Alternatively, it is possible that, although there is interaction of mutations, there is no strict interdependence. In this case tumour development could be viewed as a nexus of interconnecting points (or mutations) in which selection pressures apply at each point, but previous mutations may no longer be essential for survival. The truth probably lies somewhere in between.

Modifier genes and epistatic effects

Another factor which may affect the genetic pathway of a tumour will be the host complement of modifier genes which may also be potential susceptibility factors. The definition of modifiers is limited here to polymorphic genes which have alleles of differing levels of activity and which do not usually form part of the genetic pathway. The relative activity of the alleles present will have an impact on the genetic pathway, either in terms of speed of progression along the pathway or on the mutations which actually comprise the pathway. Modifier genes may act at both the cell-autonomous and non cell-autonomous level and, as more is learnt about them, tumour modifiers may be sub-categorised in accordance with how they affect tumour development (e.g. mutation rate, vascularisation, proliferation, adhesion etc.) [23]. Some of the possible activities of modifiers are exemplified in the following three hypothetical scenarios.

(1) At a very simple level, modifiers may affect the mutation rate and thereby influence the chance of tumour initiation and speed of progression. DNA can be damaged by mutagens both from the environment and from within the cell (e.g. oxygen free radicals). A number of metabolic enzymes (such as the glutathione-S-transferases) can detoxify these substances [24] and it follows that a host with high enzyme activity levels may, for example, be relatively protected compared to a host with low levels of enzyme activity. Similarly, host-to-host variation in the multitude of activities involved in DNA repair or immune response will give variation in the risk of tumour development.

(2) At another level, modifiers may code for a secreted factor which may even affect selective values of certain mutations. For example, a growth factor gene may have alleles which code for different levels of secretion of that factor. If a host A has alleles for high secretory levels whilst host B has alleles for low secretory levels, then all things being equal, host A will have more rapid tumour growth than host B. In this way speed of progression may be altered without actually altering the genetic pathway. It is also possible that in host B there may be selection of a gain-of-function mutation of the growth factor receptor whilst in host A, this step is obviated by the relatively high secretion of the growth factor. In this way, the genetic pathway itself may be affected by the modifier.

(3) At yet another level, modifier genes may interact directly with genes on a genetic pathway or may even become part of the genetic pathway themselves. No molecule acts in isolation and the activities of most molecules are tightly regulated. Mutations result in a change in activity and the way the compensatory mechanisms cope with these changes will influence the impact of the mutation on the cell and selectability of that mutation. Modifier genes may form part of these compensatory mechanisms. Consider a tumour suppressor gene X which converts substrate (a) into substrate (b). If accumulation of (a) beyond a threshold gives a selective advantage (through, for example, increased transcriptional or enzymatic activity) then loss-of-function mutations of X will be expected. A modifier gene, M, may also have a small degree of activity in the processing of substrate (a) either as a non-essential co-factor for X or through independent activity. Normally mutations of M will not be selected because in the presence of wild type X, substrate (a) will still be degraded. If however, X is mutated, then the activity of the particular alleles of M which a host possesses may determine how quickly substrate (a) builds up beyond the threshold level. Low activity of M may mean that a single mutation of X is sufficient whilst high activity may either reduce the value of the mutation to a non-selected level or may drive a second mutation of X for full selective advantage. In this scenario, high activity alleles may themselves become targets for mutation (and thereby form part of the genetic pathway) but only in the context of a mutation of X.

The activity of modifier genes can be complicated and context dependent. In mice it has been possible to quantify the contribution various alleles of particular susceptibility loci make to tumour development [25, 26]. This may also eventually be possible in humans and understanding modifiers could make an important contribution to understanding carcinogenesis in humans.

Mutation rates in the development of cancer

Recently the notion that an increase in mutation rate is a necessary component of the genetic pathway has gained currency in the world of cancer genetics [27, 28]. This idea is appealing because many malignant tumours eventually become grossly aneuploid. A sub-group of near-diploid tumours exists, but these usually have loss of DNA mismatch repair (discussed in more detail later) resulting in an increase in the mutation rate. The observation that most tumours have some deregulation of mechanisms for genome integrity dovetails with claims that an increase in mutation rate is necessary if a tumour is to develop within the lifetime of an individual [29, 30].

However, there are mathematical models which show that an increase in the mutation rate is not essential for tumour development and that clonal expansion through mutation followed by selection is sufficient [18, 31]. Thus, it appears odd that a mutation for increasing the mutation rate would be selected at all since the advantage of this would not be felt for several generations and another mutation giving a more immediate advantage would be selected before this. One possibility is that mutations giving an increase in the mutation rate are not selected for but are usually bystander events which are present in the dominant clone because they confer a long-term selective advantage.

It is more likely that genomic instability arises as a consequence of mutations which are selected primarily for reasons other than increased mutation rate. For example, mutations of the *TP53* gene confer resistance to apoptosis and this is the most likely reason for their selection [32–34]. Mutations in *TP53* are also associated with the development of aneuploidy [35, 36]. Other genes monitor the fidelity of chromosomal segregation at mitosis and activate apoptotic pathways in those cells in which chromosomal non-disjunction occurs [37]. When *TP53* is mutated, however, cells which become aneuploid may be refractory to apoptosis and aneuploidy will persist with no direct selective advantage. It would then be expected that chromosomes which can be lost or gained without cost to the cell will be lost and gained randomly. In fact the authors of this review have interpreted one study, in which karyotypic changes were studied in cell lines *in vitro* over several generations, as showing just this [38]. Aneuploidy does not alter gene function but only changes gene dosage. Many cellular functions can certainly be radically altered simply by changes in gene dosage but dominant oncogenes and many tumour suppressor gene effects depend on function changing mutations so that chromosomal imbalance itself cannot be the primary mechanism of genetic change in tumour evolution.

Loss of mismatch repair function has been linked to resistance to apoptosis induced by alkylating agents and to alterations of cell cycle control [39]. Mutations of mismatch repair genes are probably, therefore, selected for this or other similar reasons with increase in mutation rate occurring as an associated, although possibly important phenomenon.

Further evidence for the power of selection comes from the recent description of a novel polymorphism of codon 1307 of the *APC* gene [40, 41]. This T to A substitution creates an A8 tract which is hypermutable and approximately 50% of tumours arising in patients with this polymorphism have insertion/deletion mutations in this run of 8 nucleotides. Repeat tracts are usually the targets in cells which have a mutator phenotype with loss of mismatch repair function (see later) and are rarely mutated in mismatch repair competent cells, though such tracts may nevertheless have a relatively high spontaneous mutation rate. None of the patients in the above studies had evidence of loss of mismatch repair function, showing that where the selective advantage is sufficient, mutations in repeat tracts (which will naturally occur more often than elsewhere) will be selected for and will be the most commonly observed mutations; an increase in mutation rate in not necessary for mutations at these sites if there are sufficient selection pressures. Conversely, the type II TGF-b receptor gene contains an A10 tract in exon 3. This is mutated in most RER+ CRCs but is infrequently mutated in RER+ endometrial cancers showing that, despite being hypermutable, selection pressures apparently keep this gene intact in endometrial cancers [42].

Changes in mutation rates will affect the rate of progression along a genetic pathway but should not themselves alter that genetic pathway. Clonal selection and expansion are mostly sufficient to account for the somatic evolution of cancers and so an increase in the mutation rate is usually an epiphenomenon which is not essential for tumour development, though it does accelerate the process when it occurs.

Is tumour progression inevitable?

Once a cell has acquired a first mutation which gives it a growth advantage over other cells then, normally, one might expect its continued evolution into a cancer. Nevertheless, many tumours remain benign and never progress to malignancy. It is unlikely that large multicellular organisms would have developed without some means of dealing with 'rogue' elements and the fact that there are so few tumours given the large numbers of cells in the human body attests to this. Natural tumour prevention strategies probably exist at multiple levels and no doubt include limiting the incidence of mutations by repair or apoptosis and induction of apoptosis when cell behaviour becomes abnormal.

Alternative pathways

Tumours arising in any particular tissue may develop along several different genetic pathways. Genetic differences between individuals will affect this choice and even within the same tissue there may be subtle regional differences which will ultimately affect mutant selection. The stochastic nature of mutation will also be an important factor influencing the probability of which particular selectively advantageous mutation happens to occur at a given stage of tumour evolution. Different magnitudes of the selective advantage of different mutations will ensure that some pathways will be preferred over others.

The concept of genetic pathways can be used to explain apparent discrepancies between genotype and phenotype. For example, mutations of the *TP53* gene are found in a high proportion of colorectal and pulmonary carcinomas. Patients with the Li-Fraumeni syndrome who have germline *TP53* mutations, nevertheless do not have these tumours as part of the clinical syndrome [43, 44]. This is probably because early *TP53* mutations do not initiate tumour development in the lung and colon but only give a selective advantage when combined with earlier mutations, such as in *APC*, which characterise these tumours at early stages. Conversely, germline mutations of some genes give rise to a family cancer syndrome whereas mutations of these genes are not found in sporadic tumours. Mutations of the *BRCA1* and *BRCA2* genes, for example, give rise to familial breast and ovarian cancers and somatic mutations of the wild type alleles are found in these tumours [45, 46]. However, mutations of these genes have not been found in sporadic breast or ovarian cancers. This could be explained by a weak selective advantage arising from mutations of these genes such that, in sporadic tumours, an alternative is usually chosen. In the familial cases, the mutation is already present in all cells and so a weak selective effective is more likely to be effective.

Tumour evolution is a highly complex process which is affected by a variety of factors. In the remainder of this review the putative genetic pathways leading specifically to the development of colorectal cancer are considered.

THE MOLECULAR PATHOLOGY OF COLORECTAL CANCER

Epidemiology of CRC

Colorectal cancer (CRC) is predominantly a disease of the Western world and, after lung cancer in men and breast cancer in women, is the most common cause of cancer related death. The peak incidence of the disease is in the seventh decade and it is fairly equally distributed between men and women. There are approximately 30 000 new cases of CRC diagnosed each year in the U.K. and it accounts for approximately 17 000 deaths per annum [47, 48]. In patients who develop the disease, prognosis depends on the stage of the

tumour at presentation. Surgery is the main and most effective current form of treatment.

Most cases of CRC arise sporadically (namely with no background of a family history of the disease), although inherited cancer syndromes may account for up to 5% of all cases. In addition, there are a number of cases in which a family history can be elucidated which does not follow a classical Mendelian pattern presumably because of incomplete penetrance and multifactorial effects. Even in cases of sporadic colorectal cancer, there is a definite increase in risk in their relatives. There is also an increased risk of tumour development in patients with chronic inflammatory diseases of the large bowel such as Ulcerative Colitis and colonic Crohn's Disease [49–51].

There is wide variation in the incidence of CRC around the world but migrating populations tend to assume the relative risk of the region into which they move [52]. This shows that factors in the environment play a major role in the development of CRC, with diet as one of the most obvious candidates. The way in which diet acts is, however, uncertain. There is speculation that the Western diet results in increased exposure to carcinogens thereby inducing mutations. Chemical carcinogens often cause transversion mutations and frequently have signature mutations [53]. These are not found in CRC and it is more likely that mutations arise spontaneously and the diet affects tumorigenesis through other mechanisms, such as altered proliferation (i.e. promotion) due to the effects of dietary breakdown products.

NATURAL HISTORY OF CRC

The natural history of CRC is well described, if not well understood. The first step in the development of tumours from normal epithelium is usually taken to be onset of dysplasia. Single dysplastic crypts (unicryptal adenomas) can be seen and are thought to be the first histological manifestations of tumour development. Recently, it has been proposed that aberrant crypt foci (small areas of epithelium with irregular glandular architecture but no evidence of dysplasia) are precursor lesions which give rise to adenomas. The data regarding mutations found in aberrant crypt foci are inconclusive and their relationship to adenomas remains unproven [54–58]. Adenomas can gradually grow in size and change from a tubular to a villous architecture. The cells show first mild, then moderate, and then severe dysplasia followed by malignant change resulting in local invasion with eventual metastasis to distant sites.

Tumours can arise anywhere in the large bowel, although the majority of sporadic cancers are left-sided (i.e. distal to the splenic flexure). The process of tumour development probably takes up to 20 years or more, and there are several lines of evidence which suggest that not all adenomas will progress to carcinomas. The frequency of adenomas in the general population is higher than the frequency of carcinomas, taking into account the fact that some patients will die before the adenomas have had sufficient time to progress to carcinomas [59]. Other studies have shown that the presence of a tumour on the left side of the large bowel indicates a high risk of synchronous or metachronous tumour development elsewhere in the bowel [60]. The simple presence of a tumour is associated with approximately a 30% chance of occurrence of a second tumour. However, risk of a malignant second tumour seems to be associated with advanced features in the first (i.e. tumour size > 1 cm, villous architecture, high grade dysplasia [61]. Thus, in patients who have a tumour with low-grade features, the risk of a second tumour is high but the chances of that tumour progressing are low.

Patients with the autosomal dominant condition Familial Adenomatous Polyposis (FAP) are born with germline mutations in the *APC* gene. They can develop thousands of adenomatous polyps throughout the large bowel and are usually treated with early total colectomy. It is a well recorded fact that after surgery, the polyps which occur in the residual rectum may undergo significant regression [62]. Further evidence for regression comes from another study in which sporadic tumours < 1 cm in size were not removed but were instead followed endoscopically. Many of these tumours also showed regression with time, again demonstrating that progression is not inevitable [63].

Thus, although the path taken by a colorectal tumour as it develops is histologically well described, it is not always certain whether a tumour will complete the 'journey' to cancer. The factors which limit or inhibit tumour progression are unknown and identification of the factors, other than chance, which control tumour progression remains one of the challenges of the future.

PUTATIVE GENETIC PATHWAYS IN COLORECTAL CANCER

Sporadic CRC

Colorectal cancers develop via a number of histologically distinct steps (Figure 2a) and the term "adenoma to carcinoma sequence" was coined by Morson and colleagues at St Mark's Hospital in London to describe this histological progression [64]. Fearon and Vogelstein [65] and Bodmer [3] have suggested how the genes mutated during tumour progression relate in their order of occurrence to the histological stages of the adenoma to carcinoma sequence (Figure 2b).

Adenomatous polyposis coli (APC) gene mutations. The earliest gene mutation detected in colorectal cancer is the tumour suppressor gene *Adenomatous Polyposis Coli (APC)*. This is located on chromosome 5q21 and is found to be mutated in 60–80% of sporadic colorectal cancers [66–70]. Germline *APC* mutations are found in the autosomal dominant condition Familial Adenomatous Polyposis (FAP) [71]. Patients with this condition are prone to developing hundreds to thousands of colorectal adenomas and consequent early-onset carcinoma. They are also prone to the development of small intestinal adenomas (and carcinomas), intra-abdominal desmoid tumours, congenital hypertrophy of the retinal pigment epithelium (CHRPE), fundic gland polyps in the stomach, osteomas and bone cysts [62]. It is apparent from this, that APC is involved in the regulation of a number of colonic and extra-colonic tissues and analysis of the protein is beginning to give some insight into APC activity and the functional sequelae of *APC* mutations.

The *APC* gene product is a 312 kD protein consisting of 2843 amino acids. It is a multifunctional protein which contains several amino acid motifs and domains that allow it to interact with numerous other molecules [72]. Studies of patients with FAP have shown a very interesting genoptype-phenotype correlation with regard to the location of the germline mutations. In general, mutations in the central region of the molecule give a profuse polyposis phenotype with large numbers of polyps, whilst mutations at the 5′ or 3′ ends gives an attenuated phenotype characterised by relatively few adenomas and a later onset of cancer [73, 74].

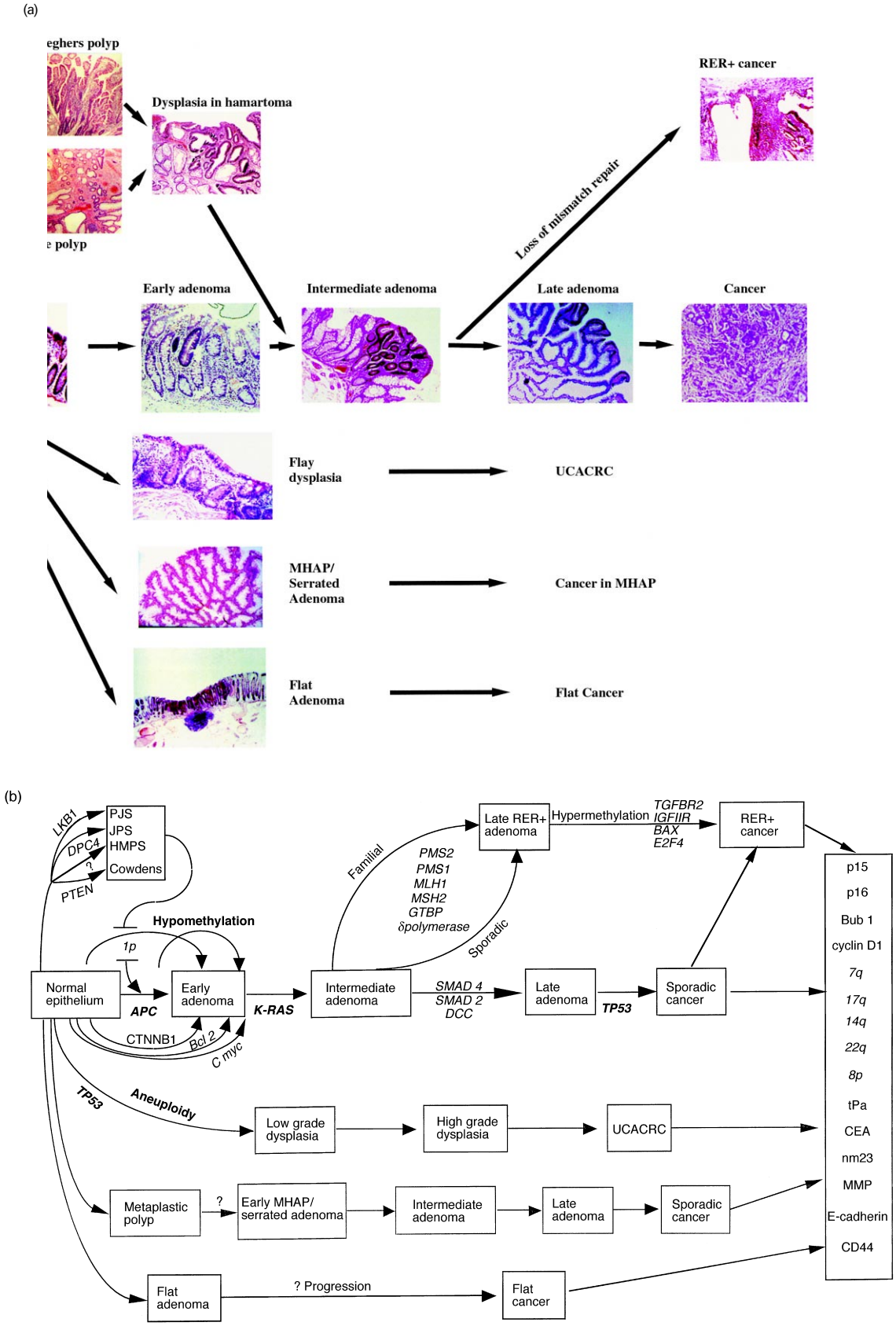

Figure 2. (a) The histologically distinct subtypes of cancer which serve as a basis for and which are a 'reflection' of the genetic pathways. As well as distinct histological features, the subtypes of cancer also have distinct clinical histories. (b) Putative genetic pathways in colorectal cancer. The Fearon and Vogelstein model is shown in bold and the majority of tumours may develop along this pathway. Little is known about the other pathways but there is probably a high degree of overlap between the various pathways.

Patients with the attenuated disease usually have a later age of cancer development. Mutations between codons 450 and 1444 are also associated with onset of CHRPE and this shows that disturbance of different functional domains of the same molecule can result in different phenotypic effects [72].

A relatively high proportion of the somatic *APC* mutations which are described in colorectal tumours occur in the same region that gives the profuse polyposis phenotype and are clustered between codons 1286 and 1513 which comprises just 10% of the *APC* coding sequence and is termed the mutation cluster region (MCR) [69]. The somatic mutations are almost all truncating mutations which result in the loss of the functional domains downstream from the MCR. Such a tight clustering of mutations implies that of all the functions downstream to the MCR which are lost, the loss of the functions which are immediately adjacent to the 3′ limit of the MCR may be more important to tumour development. One of these functions is the down-regulation of the levels and activity of β-catenin [75–77]. It is thus possible that the main selective advantage of *APC* truncation mutations is the loss of regulation of β-catenin activity. This is supported by the fact that, in those colorectal tumours which have only wild type APC, up to 50% contain gain-of-function mutations of the β-catenin gene [78–80]. One of the effects of increased β-catenin activity is increased nuclear signalling in association with Tcf-4, a transcription factor [81]. One of the proposed targets for this signalling is the *C-MYC* gene which is often up-regulated in colorectal tumours [82].

The presence of a 5′ limit to the MCR also suggests that retention of the functions upstream of this region is necessary for tumour development and this is supported by the fact that complete loss of APC protein is almost never seen in CRC cell lines. Some of these gain-of-function effects of truncated APC may lie in the ability of this protein to complex with β-catenin. This may then sequester β-catenin from the cell adhesion molecule E-cadherin (which functions through β-catenin) and so allow a subtle inhibition of E-cadherin function and complement the increased signalling activity of deregulated β-catenin [83]. In general, APC protein truncated in the MCR may be particularly effective in disrupting or sequestering components of the complex of proteins that binds APC, and so mediate relatively strong dominant negative or other gain of function effects.

Since *APC* is a tumour suppressor gene, then according to Knudson's two hit hypothesis, mutation of the first allele should be followed by inactivation of the second allele either through a second mutation or through its loss, as detected for example by loss of heterozygosity [84]. Patients with FAP, who have a germline *APC* mutation, do not develop adenomas until puberty and not every crypt is dysplastic even in older patients. The second event in *FAP* is probably another *APC* mutation and somatic *APC* mutations have been described in FAP derived adenomas consisting of just two crypts [85]. Mutations in other genes will, of course, determine the subsequent pattern of tumour development, just as in the case of sporadic tumours.

Given the fact that it has a large number of functions, the activities of the APC protein are probably influenced by a large number of 'modifier' genes. This is demonstrated by variable expression of identical germline mutations both within and between FAP kindreds. The impact of modifiers of APC on tumour development is best illustrated in animal models. The *Min* (Multiple Intestinal Neoplasia) mouse contains a truncating mutation of the murine *APC* gene and is prone to the development of mainly small bowel and a few large bowel polyps when in a C57Black 6 (B6) genetic background [86]. When, however, the *Min* allele is introduced into the germline of AKR mice, these are almost completely resistant to tumour development [87]. The AKR mice thus carry one or more modifier genes which confer a large amount of resistance even in the presence of a germline APC mutant. One suggested modifier is Pla2g2a on chromosome 4 [88], which is the region where a major modifier effect has been mapped. The B6 mice contain null alleles for this gene, and when wild type alleles are introduced into B6 $APC^{min/+}$ mice, there is a dose dependent decrease in polyp number [89]. The B6 mice with two copies of wild type Pla2g2a do not appear to be as resistant as the AKR mice suggesting that the latter also possess other resistance alleles. However, though the association between the modifier effect and lack of Pla2g2a activity is strong, it is likely that Pla2g2a is simply acting as a marker for an effect of another yet to be identified closely linked gene.

As well as the impact of modifiers, variation in APC function and tumour development may arise as a result of missense variants or polymorphisms. Classical FAP is transmitted as an autosomal dominant disease with a variably expressed phenotype. Recently, however, variants of the *APC* gene have been found which show sequence polymorphisms causing amino acid changes in the crucial β-catenin binding region [40, 90]. These variants are associated with an increased risk of tumour development, but this risk is not transmitted in the classical Mendelian pattern of inheritance. These variants may, thus, be low-penetrance alleles which explain some of those cases with a family history of colorectal cancer which does not follow a strictly Mendelian pattern.

Methylation status. It has been suggested that general changes in patterns of DNA methylation may follow *APC* mutations or other early changes in gene expression in colorectal cancer [65, 91]. The basis for this demethylation is unknown and its role in tumour development is uncertain since it often co-exists with focal (i.e. gene specific) hypermethylation. Changes in methylation can impact on tumour development in a number of ways, including induction of mitotic non-disjunction and chromosomal irregularities [92]. The expression of many genes can be controlled through methylation in the promoter region and thus hypomethylation could activate an oncogene whilst hypermethylation could inactivate a tumour suppressor gene. Mice in which the DNA methyltransferase enzyme has been knocked out or inhibited, and so are hypomethylated, are relatively resistant to tumour development [93]. This may be due to a reduction in the number of 5-methylcytosine residues which are prone to demethylation resulting in point mutations.

K-RAS gene mutations. Up to 50% of sporadic colorectal tumours are found to contain mutations of the *K-RAS* oncogene. The Ras family of proteins is involved in signal transduction and is a part of the signalling pathways of a large number of functionally diverse molecules. The majority of the *K-RAS* mutations seem to be gain-of-function missense mutations limited to codons 12 and 13 [94]. When combined with *APC* mutations these presumably give an added selective advantage resulting in sub-clonal evolution. This may be seen histologically by, for example, the emergence of a focus of moderate dysplasia in an otherwise mildly dysplastic adenoma. Ras family proteins are involved in signalling by

surface receptors and forced over-expression can affect both cell adhesion and the cell cycle [95–98]. It is difficult to dissect out the precise effect of *K-RAS* mutations which may be multifarious.

There have been several reports of *K-RAS* mutation in histologically normal colonic mucosa [99–101] and it has been suggested that dysplasia is seen only when *K-RAS* mutations occur in association with other mutations such as *APC* [102]. This implies that *K-RAS* mutations only give a selective advantage to cells with *APC* mutations and, in isolation, may be neutral in normal mucosa.

Mutation of the deleted in CRC (DCC) *gene/allelic loss on 18q.* Fearon and Vogelstein, following Dutrillaux's demonstration of chromosome 18 abnormalities in colorectal cancers, showed a relatively high frequency of LOH around 18q22 and suggested that the gene *DCC*, which they had identified in this region, was the target for this. The *DCC* gene codes for a large membrane protein which is a member of the immunoglobulin gene superfamily and which appears to be a receptor for netrin, a nerve growth factor [103]. Few mutations have been described in *DCC* although loss of the 5′ part of the mRNA has been reported [104]. This, together with the failure of *DCC* knockout mice to have a significantly altered phenotype (crossing DCC knockout mice with Min mice produced no effect other than that seen in Min mice), and the fact that 18q contains a number of other potential tumour suppressor genes has cast doubt on the importance of *DCC* in tumorigenesis [105].

Loss of activity of one or more genes on 18q does appear to be an important step in tumour development, but identification of the target has so far remained elusive. While the whole chromosome is often lost, loss of the region 18q21-18tel appears to be the main target [106]. Candidates in this region include Smad (also known as Deleted in Pancreatic Carcinoma (*DPC*) 4) and Smad 2 (also known as JV18-1). Both of these are involved in the TGF-β signalling pathway and mutations have been found in CRC, albeit at a lower frequency than the allelic loss [107, 108]. It is possible that these are redundant for tumour development, so that loss of either will give an equivalent selective advantage.

TP53 mutation. Mutation of the *TP53* gene has been shown to occur in the adenoma to carcinoma sequence and most probably occur before metastasis. *TP53* mutations thus appear to mark the transition from a benign adenoma to a malignant carcinoma, and are found in up to 70% of sporadic colorectal cancers. The gene maps to chromosome 17p and, although mutant p53 does exert a dominant negative effect, allelic loss at the *TP53* locus is a common event [32], suggesting a strong disadvantage of residual wild-type activity.

The physiological role of p53 is described more fully in another review [33]. Suffice it to say that it has a number of functions and is central to many apoptotic pathways. For example, DNA damage in cycling cells induces post-translational stabilisation of p53 which then activates genes such as *p21* and *GADD45* to cause cell cycle arrest in G1 [33]. This allows for repair of the DNA before proceeding to S-phase although, if the damage is too great for effective repair, p53 mediated activation of genes such as *BAX* results in apoptosis [109, 110]. The key selective advantage of *TP53* mutation is most probably resistance to apoptosis, although the nature of the apoptotic stresses are not yet defined. Relaxation of cell-cell and cell-ECM adhesion due to early changes is likely to render a cell susceptible to apoptosis, as it is known that this happens when epithelial cells are detached from their substrate. This places a premium on selection for 'anti-apoptotic' mutations, such as *TP53*. It is also possible that when tumour cells invade into the stroma they encounter a hostile environment causing pro-apoptotic signals either directly (e.g. through stromal cells or molecules) or indirectly (e.g. through hypoxia). Survival may thus depend on escaping from these signals. *TP53* mutations will also allow tumour cells to survive with an increased genetic burden although, as previously stated, this is more likely a consequence than a cause for mutation selection.

Colorectal cancers with defective mismatch repair

The Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome is an autosomal dominant condition which probably accounts for up to 5% of all colorectal cancers in Western countries [111–113]. Patients with HNPCC develop colorectal cancers and may also develop cancers in the small intestine, stomach, ovary, bladder and endometrium [114]. HNPCC kindreds are defined clinically by the relatively strict 'Amsterdam criteria' which include onset of cancer in at least two first degree relatives of the proband, cancer in family members spanning at least two generations and cancer in at least one case occurring below the age of 50 years [115]. The syndrome arises as a result of germline mutation in one of several DNA mismatch repair genes described so far (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH3*, *hMSH6* (*GTBP*) [116–121]. These genes control the repair of DNA base pair mismatches and are responsible for ensuring correct DNA synthesis during replication [122]. DNA polymerases are error-prone enzymes especially in areas of DNA containing nucleotide repeat sequences. Even in normal cells these sites are inherently unstable and inappropriate base insertion or DNA replication slippage at these sites results in insertion or deletion loops consisting of multiples of the nucleotide repeat sequence. These are normally repaired, but in the absence of efficient mismatch repair function, these loops often become 'fixed' and, at the next round of replication, result in alleles of different sizes. Microsatellites are usually intronic nucleotide repeat sequences and are thus prone to insertion/deletion mutations. Over several generations multiple different sized alleles may accumulate within a tumour which has one or more defective mismatch repair genes and this is called 'microsatellite instability'. Because these errors arise during replication, these tumours are termed Replication Error positive (RER+).

In HNPCC cancers, somatic mutation or promoter methylation of the wild type allele results in complete loss of mismatch repair function and, consequently, the cancers which develop are characterised by microsatellite instability [123–125]. Loss of mismatch repair results in an increase in the mutation rate and RER+ tumours are said to have a 'mutator phenotype' [126, 127]. Microsatellite mutations occur as a result of the physical structure of DNA, not selection and, because they are usually selectively neutral (unless they occur in the expressed region of a functionally critical gene), they represent true 'bystander' mutations. The development of a mutator phenotype will increase the chances of bystander mutations occurring, a fact which must be remembered when examining the genetic pathways of these tumours.

In addition to the tumours in HNPCC patients approximately 10–15% of sporadic colorectal cancers are found to be

RER + although there is some disagreement as to the exact definition of an RER + phenotype [128]. Criteria have ranged from instability at any one microsatellite locus, to a minimum of two unstable loci, to instability at 30% of the loci studied [113, 129, 130]. A distinction has also been drawn between those tumours with large numbers of new alleles and those with few new alleles at these loci [131]. A consensus has been brought nearer by the identification of BAT 26 [132], a marker which appears to have high sensitivity and specificity in identifying RER + tumours. Thus far few studies have drawn a distinction between sporadic and familial RER + cancers although some genes have been found mutated in sporadic cancers (e.g. δ-polymerase [133]) but not yet shown in the germline of HNPCC kindreds. As well as microsatellite instability, RER + cancers have many phenotypic features which make them clinico-pathologically distinct from the more usual sporadic colorectal cancers. RER + cancers in HNPCC occur at an earlier age and stage at presentation and are predominantly right-sided. RER + cancers in general tend to have a mucinous histology; poor differentiation; peri and intratumoural lymphocytic infiltrate may show more global hypermethylation and better a prognosis [114, 130].

Phenotype is ultimately a reflection of genotype and from these features RER + colorectal cancers appear, at first glance, to develop along a different genetic pathway to sporadic colorectal cancers. In addition, in contrast to sporadic colorectal tumours, RER + cancers have a near diploid karyotype [124] and probably, as a result, show a relatively low frequency of allelic loss [113]. It has been shown that the incidence, number and distribution of adenomas in at-risk members of HNPCC kindreds tends to be no different from that seen in the general population, although the size of the adenomas may be larger with a more frequent mucinous histology [135]. This suggests that initial development of tumours is no different. However, tumour progression appears to be accelerated in a proportion of HNPCC cancers, probably those in which the loss of the remaining wild-type allele occurred early, leading unusually to an early increase in the mutation rate. In addition, microsatellite instability is not seen in normal gut epithelium in patients with HNPCC, and is present in only 50% of HNPCC associated adenomas as opposed to over 90% of HNPCC colorectal cancers [112, 136]. Thus, loss of mismatch repair function does not give a selective advantage in normal epithelium or in early adenomas. This suggests that tumour initiation and early development involve the usual steps of mutation in *APC* and *K-RAS*. This appears to be the case, since the frequency of *APC* and *K-RAS* mutations appears to be similar in both sporadic and HNPCC associated colorectal tumour [112]. The pattern of mutation in *APC* may, however, be different and one study has found significantly higher frequencies of mutation at short mononucleotide repeat sequences in the *APC* gene in HNPCC associated cancers [137]. This suggests that, in these cases, somatic mutation or loss or down regulation by methylation of the wild type allele of the mismatch repair gene, may have occurred before *APC* mutation. A study of sporadic RER + CRCs did not show a similar bias in types of *APC* mutation suggesting that in these cases, the first effects of loss of mismatch repair did not occur until after *APC* mutation [138].

Irrespective of when exactly the loss of mismatch repair occurs, as has been discussed, selection for the mismatch repair mutations is probably for some feature such as resistance to apoptosis rather than an increased mutation rate. The mode of inactivation of the wild type allele may be variable and few somatic mutations have so far been identified.

As stated, RER + cancers tend to be of diploid or near-diploid karyotype [134]. There appears to be some mechanism which maintains chromosome number, the nature of which and whose relationship to mismatch repair function is as yet unknown. Alternatively, selection of mismatch repair mutations may preclude selection for mutations that normally disrupt the karyotype. Intriguingly, RER + cell lines which have *TP53* mutations, an event generally associated with aneuploidy, are also usually near diploid [139]. It may be that normal cellular mechanisms which prevent aneuploidy through the induction of apoptosis are not mutated in these tumours, or that aneuploidy together with the mutator phenotype gives too much of a genetic burden for survival.

Once loss of mismatch repair has occurred, not only microsatellites but all nucleotide repeat sequences become targets for insertion/deletion type mutations. A number of genes have been identified which have mono- or dinucleotide repeat sequences in exonic coding regions. Loss of function mutations in RER + cancers but not RER − cancers have been found in four such genes. These include genes coding for Type II TGF-β receptor (*TGFBR2*) [140], the IGF II receptor (*IFGIIR*) [141], Bax protein (*BAX*) [142] and the E2F4 cell cycle regulator (*E2F4*) [143]. The frequency of mutation in these genes is variable and their relationship to each other is not completely known although mutations of *TGFBR2* and *IGFIIR* seem to be mutually exclusive [141]. It has been shown that where there is a selective advantage, mutations at repeat sequences will naturally also occur in RER − cancers since even in the absence of a mismatch repair defect, such mutations will be relatively more frequent. Thus the fact that these mutations are rare in RER − cancers is suggestive, but not proof, of an alternative pathway; as has been discussed, the mutations may be bystander events occurring as a consequence of instability at nucleotide repeat sequences. However, both RER + and RER − CRCs have to overcome the same environmental constraints and it is likely that, at points of redundancy in the genetic pathway, the choice of mutation for a given selection pressure, will be for those genes that have the higher spontaneous mutation rate i.e. genes with repeat sequences. For example, inactivation of TGF-β signalling may be important in the development of CRCs. In RER + tumours this may be achieved optimally by inactivation of the type II TGF-β receptor due to the high frequency of mutations of the A10 tract. However, in RER − tumours, mutations of Smad 2 and 4 (which lie further downstream in the same pathway) may be selected in preference because they give the same selective advantage. Similarly, although *TP53* mutations do occur in RER + cancers, they are reported in a much lower frequency than in RER − cancers. Bax is induced by p53 as an effector of apoptosis and thus *BAX* mutations in RER + cancer may be an alternative to *TP53* mutations in RER − tumours.

The phenotypic and genotypic evidence suggests that tumours with loss of mismatch repair probably develop along a genetic pathway different to sporadic RER − tumours. The early part of the pathways may be the same, but once there is loss of mismatch repair, the RER + cancer probably develop along a different pathway which is accelerated as a consequence of the mutator phenotype. Whether sporadic

RER+ cancers are the same as familial RER+ cancers remains to be clarified.

Ulcerative colitis associated colorectal carcinomas

Patients with ulcerative colitis have an increased risk of colorectal carcinoma [49]. The risk increases with duration of the disease, early age of onset of disease (especially under the age of 15 years) and pancolitis [50]. Clinico-pathologically, ulcerative colitis associated colorectal carcinomas (UCACRCs) are distinct from sporadic colorectal cancers. UCACRCs present at a younger age [144] than sporadic tumours and, although they have a predominantly left-sided distribution [145], they tend frequently to be multiple and often appear mucinous on histology [146]. The precursor lesions of UCACRCs, in contrast to the well circumscribed polypoid sporadic adenomas, are usually diffuse flat lesions which show progression from low-grade to high-grade dysplasia [147]. In addition, the immunophenotype of UCACRCs may be different with a lower frequency of Bcl-2 expression than that seen in sporadic cancers [148]. Although the UCACRCs are mainly left-sided, the precursor lesions are equally distributed on the left and right sides of the colon suggesting a complex evolutionary process [145].

Additional differences between conventional sporadic colorectal cancers and UCACRCs lie in the relationship of the dysplastic epithelium with the pericryptal smooth muscle cells. These cells form a sheath around the crypts, probably contribute to the formation of basement membrane and appear to migrate towards the surface along with the epithelial cells [149]. In sporadic tumours, the pericryptal cells are closely apposed to the epithelium until the late stages of an adenoma or early carcinoma when they become dispersed [150]. In ulcerative colitis-associated dysplasia, the relationship between the pericryptal cells and the epithelium breaks down at an early stage. These changes in the distribution of pericryptal smooth muscle cells may play a role in UCACRC tumour development. There is some direct genetic evidence to support the assertion of a different pathway for UCACRC tumours. Several studies have shown that *TP53* mutation is probably an early event in the development of these tumours and may even occur before the onset of the features of dysplasia [101, 151–153]. The reason for early selection of *TP53* mutations is unknown, but one possibility is that it represents a means of escaping from apoptotic stresses imposed by the inflammatory cytokines and cells during the periods of inflammation. In addition several studies have shown early development of aneuploidy in UCACRCs which may or may not be a consequence of *TP53* mutation [154–157].

The frequency of truncating *APC* mutations is lower in UCACRCs than in other sporadic cancers. Two studies using *in vitro* synthesis of protein to detect truncating mutations found *APC* mutations in only l2/43 (30%) of UCACRCs, in contrast to 60–80% expected in sporadic colorectal cancers [158, 159]. Similarly, the reported frequency of *K-RAS* mutations is much lower in UCACRCs suggesting that this also does not form part of the pathway in most of these tumours [101, 160–162]

The role of the mismatch repair genes in the UCACRC genetic pathway is uncertain. Mutations of these genes have not been reported in UCACRCs, but tumours have been shown to be RER+. However, microsatellite instability has also been described in histologically non-neoplastic inflammatory lesions, often at a higher frequency than in the neoplastic lesions [163, 164]. It is claimed that this arises because the mismatch repair process is saturable [165]. Thus once the rate of proliferation in epithelium (and presumably inflammatory cells) crosses a certain threshold the mismatch repair proteins cannot function efficiently and microsatellite instability can arise. If this is truly the case, microsatellite instability cannot be used as a surrogate marker for the involvement of mismatch repair genes in the genetic pathway of UCACRCs which will instead depend on direct analysis of the genes themselves.

Flat carcinomas

In recent years flat adenomas and carcinomas have drawn more attention from both clinicians and pathologists. They may have escaped scrutiny before because they are easily missed on endoscopy and often only become visible after painting the mucosa with a dye [166]. Flat adenomas are usually small tumours which, histologically, rise little above the surrounding normal epithelium [167]. In contrast to the polypoid sporadic adenomas, in which severity of dysplasia and risk of tumour invasion generally increase with the size of the tumour, flat adenomas (and a variant called depressed adenoma) show high grade dysplasia in relatively small tumours [168]. The absence of a polypoid precursor lesion and the presence of high grade dysplasia have resulted in these tumours also being called *de novo* carcinomas (which is probably an inappropriate name).

Little is known about the biology of flat adenomas. They have a mainly right-sided distribution and tend to show general dissipation of the pericryptal smooth muscle cells [169]. Some studies have shown that *K-RAS* mutations occur at a lower frequency than in sporadic polypoid adenomas [170–172]. Even the natural history of flat adenomas and carcinomas is not clear and the possibility has been raised that flat adenomas are not the precursor lesions of flat carcinomas [170–174].

Hamartomatous polyposis syndromes

Hamartomas consist of normal looking elements of a tissue which are, however, arranged so that the normal architecture of the tissue is lost and replaced by another pattern of tissue organisation. Several different types of hamartomatous polyps have been described in the gastrointestinal tract. They may occur as single sporadic tumours or they may be present in large numbers as part of an inherited polyposis syndrome. The latter include the Peutz-Jeghers Syndrome (PJS) [175], juvenile polyposis syndrome (JPS) [176], Hereditary Mixed Polyposis Syndrome (HMPS) [177] and Cowden's Syndrome [178]. Patients with all these conditions have an increased risk of colorectal cancer. A small subset of JPS is due to germline *DPC4* [179] mutations, whilst Cowden's syndrome and PJS are single gene disorders arising from loss of function mutations of the *PTEN* and LKB1 [180, 181] genes, respectively. *DPC4* has been described as a 'landscaper' gene which results primarily in a change in the stroma surrounding a tumour. It has even been suggested that the somatic mutation of *DPC4* occurs in the stromal cells [28].

The intestinal polyps in these syndromes develop as a result of somatic mutation of the wild type allele and polyps from all four syndromes may show epithelial dysplasia with eventual progression to carcinoma. Whether there is a distinct 'hamartoma-carcinoma' sequence is as yet uncertain. Few mutations of the *LKB1* and *PTEN* genes have been found in sporadic colorectal cancers and dysplastic juvenile

polyps have been shown to harbour *APC* mutations [182]. It is possible that loss of function mutations of these genes results in a weak modifier effect on *APC* which is expedient to tumour development which otherwise progresses along the same genetic pathway as sporadic tumours. In this case mutations would only be of benefit if they occurred before the *APC* mutations and would not be expected outside the context of the inherited polyposis syndromes.

Mixed hyperplastic adenomatous polyps/serrated adenomas

Hyperplastic polyps are usually small, benign epithelial lesions which have a unique architecture that gives the glands a serrated appearance [146]. When features of dysplasia are present they are called mixed hyperplastic adenomatous polyps (MHAPs) or serrated adenomas [183]. These are thought to have the potential to progress to malignancy, and foci of severe dysplasia or carcinoma *in situ* have been reported in up to 10% of MHAPs. Whether hyperplastic polyps actually give rise to MHAPs, or whether the latter represent tumours which have a similar histological appearance to hyperplastic polyps is uncertain. The association of hyperplastic polyps with colorectal carcinoma is well described. They are frequently found clustered around tumours, but are not thought to confer any risk for dysplastic change beyond that of the normal background mucosa [62]. This has led some to suggest that they may be surrogate markers for high-risk of cancer development while posing no risk in themselves. A syndrome of giant hyperplastic polyposis has, however, been described which is associated with an increased risk of cancer development and so suggests that hyperplastic polyps can at least sometimes develop into MHAPs [184]. Little is known about the natural history of these polyps and even less is known about the genetic basis of tumour development. Results are mixed, with some reporting low frequencies of *APC* and *K-RAS* mutations, whilst others have shown no differences in these, but a high frequency of *TP53* mutations [185–187]. Further studies will reveal whether there is indeed a novel MHAP-carcinoma pathway and whether this proposed pathway shares features in common with that of the more usual sporadic colorectal carcinomas.

Expanding the genetic pathways

New mutations. We have used clinical, pathological and genetic data to describe a number of different pathways which may lead to the development of colorectal cancer. These pathways are far from complete and are complicated by the fact that there may be varying degrees of overlap between the pathways and that there may be redundancy at various points along the pathways. In addition, several genetic changes have been described in colorectal cancers which need to be added to the appropriate genetic pathways. These changes are considered together, as many of the studies were performed on tumours before different pathways were described.

Loss of heterozygosity (LOH) can be used to identify chromosomal regions containing tumour suppressor genes. Frequencies of LOH of around 50% have been reported on chromosomes 1p, 8p, 7q, 14q, 10q, 17q and 22q [188–197]. Allelic loss at 1p35-36 occurs relatively early in the development of adenomas and potential target genes which map to this area include *p73* (a homologue of *TP53* and *Rad 54* (a recombination repair gene).

Non mutated genes with altered function. As well as those genes whose function is altered as a result of mutation, many proteins show altered expression in tumours without evidence of mutation in the relevant gene. For example, Bcl-2 protein is over-expressed in adenomas, while there appears to be loss of expression in carcinomas [148, 198, 199]. Other proteins such as vascular endothelial growth factor, matrix metalloproteinases [200], urokinase plasminogen activator [201], and NM23 [202] show increased expression in tumours; in the case of CD44 [203, 204], there is also expression of aberrant splice variants of the protein. In contrast, some proteins, such as E-cadherin [205], show reduced or complete loss of expression without evidence of mutation. These changes must all have a genetic or epigenetic basis although this may be indirect and possibly occur through mutation of another apparently unrelated gene. Many of these changes will form part of the selective advantage of a particular gene mutation and it is important to distinguish them from other selectively neutral changes. The oncogene *C-MYC*, for example, is over-expressed in colorectal tumours without evidence of gene amplification or mutation. It has now been shown that *APC* mutation results in increased β-catenin levels and that β-catenin then complexes with Tcf 4 to activate directly the *C-MYC* promoter. Thus, *C-MYC* activation may be an indirect consequence of *APC* mutation and not an epiphenomenal change arising as a consequence of increased proliferative drive. Many of the proteins with altered expression have been postulated to have a role in tumour progression. For example, CD44 splice variants and NM23 are thought to promote tumour metastasis, whilst loss of E-cadherin expression has been suggested as an important step in local tumour invasion.

Future perspectives

We have reviewed the evidence which shows that normal cells become malignant tumours as a result of somatic evolution. Tumours undergo continuous evolution, and metastasis of tumour cells to a new environment may drive evolution of the metastatic deposits in a different direction to the primary tumour. For a full understanding of the process, all the genetic pathways need to be identified. A complete genetic pathway would include all the genes involved, whether mutated or functionally altered without mutation, and the order in which they become involved from the first change through to metastasis in different tissues. In addition, a full description of all the modifier genes and the impact of their alleles (individually and in combination) on speed of progression along the genetic pathways is desirable. These will form the basis of eventually achieving a complete understanding of all the functional effects and interactions of these genes to reveal exactly how a tumour evolves, potential points of intervention to disrupt this process and prognostic implications of the changes that have already occurred.

Achievement of these objectives may appear to be very demanding. However, automation and major improvements in technology such as the light cycler (which allows PCR reactions to be completed in 10 min), nucleotide arrays on silicon chips (to allow mutation detection and quantitation of gene expression) together with rapid capillary based sequencers will allow high throughput genomic analysis. The rate limiting step will probably be getting access to appropriate material, since microdissected material from different areas of a large number of tumours at different stages will be required.

Animal models will play an increasingly important role in cancer investigation, both in terms of identifying new genes

and for testing theories. The Min mouse, as already described, is the murine homologue of FAP [86]. When Min mice are crossed with *DPC4* (Smad 4) knockout mice [206] or *Cdx-2* knockout mice [207], there appears to be some acceleration of tumour development, whereas crossing Min mice with *Cox 2* knockouts appears to inhibit tumour development [208]. *SMAD3* knockout mice develop metastatic colorectal tumours which appear to be wild type for *APC* [209]. These examples may be models for alternative pathways to the general CRC pathway we have discussed. With Cre-lox technology, genes of interest can be conditionally knocked out, or knocked in, to study their effects at specific times in specific tissues. Since activation or inhibition is conditional on exposure to Cre-recombinase, multiple genes can be studied in one mouse without imposing a large genetic burden during embryogenesis. It may even be possible to create a mammalian artificial chromosome which contains all the relevant genes that can be conditionally activated to induce a cancer and this would serve as an excellent model for further experimentation.

A thorough understanding of the functional development of colorectal cancers will identify new potential target areas for therapy. It may be possible, for example, to alter intracellular molecules to make the cell refractory to the effects of a given mutation, for example, farnesyl transferase inhibitors in tumours with *K-RAS* mutations. Targeting of gene therapy will also depend on understanding the interactions of the mutations. As has been previously mentioned, if tumours are dependent on all mutations for survival, this will target gene therapy to early mutations such as *APC*, since attacking later mutations may just take the tumour back to that stage and force it to evolve along a different pathway. Alternatively, if tumours become resistant to the effects of early mutations then targeting the early mutations will be pointless and gene therapy should be directed elsewhere.

In the future it may be possible to do a full genotypic analysis of a person to identify quite precisely the likelihood and timing of tumour development and to allow intense screening during a dangerous period. This possibility does raise important ethical questions which will have to be addressed, given the need to maintain strict confidentiality of data relating to the genotype of an individual. If a tumour does develop, then biopsies could be taken from different parts of the tumour, either when it is resected or by needle biopsy under X-ray guidance. The biopsies could be rapidly genotyped and the mutation profile of the tumour used to identify the prognostic factors and to create a chemotherapy and gene therapy strategy tailored to the individual tumour. If cell lines were also made from the biopsies, these therapies could be tested on the tumour cells *in vitro* or in nude mice.

Our knowledge and understanding of the genetic pathways in cancer development, though advancing rapidly, is still very limited. The potential is there for an exponential increase in our understanding of tumour biology, which will improve the still relatively poor prognosis of colorectal and other cancers.

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