Genetic prognostic markers in colorectal cancer

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Abstract
The contribution of molecular genetics to colorectal cancer has been restricted largely to relatively rare inherited tumours and to the detection of germline mutations predisposing to these cancers. However, much is now also known about somatic events leading to colorectal cancer. A number of studies has been undertaken examining possible relations between genetic features and prognostic indices. While many of these studies are small and inconclusive, it is clear that a number of different pathways exist for the development of this cancer and some molecular characteristics correlate with clinicopathological features. With the advent of methods for the rapid genotyping of large numbers of colorectal cancers, it should be possible to evaluate fully the clinical usefulness of colorectal cancer genotypes through multivariate analyses.

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Colorectal cancer accounts for over 90% of the malignant tumours of the large bowel. After lung and breast cancer, it is the most common cause of death from malignant disease in western countries. The incidence of the disease in England and Wales is about 30 000 cases per year, resulting in around 17 000 deaths per annum and it has been estimated that at least half a million cases of colorectal cancer occur each year worldwide. Incidence rates of the tumour are increasing in many countries. This, together with a reduction in smoking, may result in colorectal cancer becoming the most common cause of death from malignant disease in the near future. Unfortunately, despite improvements in medical and surgical provision, there has been little change in mortality from colorectal cancer during the past 40 years.

Natural history of colorectal cancer
In western countries, approximately 60% of primary colorectal cancers are situated in the rectum and sigmoid. Of the remainder, one half are located within the caecum. The tumours are staged according to Dukes's system into categories A, B, C, and D. Grade can either be expressed simply as degree of differentiation (well, medium, or poorly differentiated) or according to the more complex Jass grouping. Prognosis correlates well with both stage and grade.

Patients fall into two broad groups at time of presentation. The first group either have non-resectable cancers or disseminated disease. These patients have a very poor prognosis, with a median survival of seven months. The other two thirds of patients will undergo a resection of their primary tumour. Despite an apparently "curative" resection, around 50% of patients will die within five years, and of these around 80% will have had a detectable recurrence within two years. The majority of these patients die as a result of liver secondaries, but there is evidence that they also have widespread extrahepatic disease. Studies of occult hepatic metastases based on postoperative computed tomography have estimated that the mean age of deposits at time of surgery is 18 months. This suggests that many colorectal cancers metastasise early, but that some tumours cannot or do not metastasise, and that the factors that determine the propensity to metastasise have acted before presentation. There are many reports of the five year survival rates for colorectal cancer. Figures vary from around 20% to over 70%. Whether these differences can be ascribed entirely to patient selection and diligent surgical resection or whether some cancers have a more inherently indolent biological behaviour remains unclear.

Clearly, improved success in the treatment of this disease requires a better understanding of its development and behaviour. Molecular studies have shown that the natural history of all colorectal cancers is not the same. In some cases, the molecular basis for the clinicopathological features of the tumour has been partially determined. If genotypic markers can be identified that correlate with tumour behaviour and patient prognosis, then this should lead to a more accurate prediction of prognosis and tailoring of treatment.

Molecular genetics of colorectal cancer
THE ADENOMA–CARCINOMA SEQUENCE
Histological observations led to the concept that the majority of colorectal cancers develop from normal epithelium through sequentially worsening degrees of adenomatous dysplasia.
The genetic pathway model for the pathogenesis of sporadic disease proposed by Fearon and Vogelstein is based on this concept of an adenoma–carcinoma sequence (fig 1). While the total accumulation of mutations is the principal factor in development of the disease, the model proposed that in most colorectal cancers the causative mutations in tumour suppressor and oncogenes occur in a particular order (specifically, adenomatous polyposis coli (APC) gene mutations, global hypomethylation, K ras mutations, deleted in colonic cancer (DCC) gene mutations, and finally mutations in the p53 gene). The Fearon and Vogelstein model was proposed seven years ago. Since then, other mutations that occur at a high frequency in colorectal cancer have been identified and, therefore, the original model needs to be modified. Specifically, it needs to be adapted to take account of the alternative pathways for the development of colorectal cancer that are now known to exist.

INITIATION OF COLORECTAL CARCINOGENESIS

There is good evidence that only two mutations are required for the initiation of colorectal carcinogenesis. In most cases, these mutations occur at the APC tumour suppressor locus (5q21 q22). APC mutations, which generally lead to a truncated APC protein, or take the form of allele loss, are detected in about 75% of sporadic colorectal cancers, and are seen in the earliest adenomas from which these cancers develop. In addition to the role of the APC gene in the aetiology of sporadic colorectal cancer, germline mutations in this gene cause familial adenomatous polyposis coli (FAP), which is characterised by florid gastrointestinal adenomas and many extra colonic features.

The APC protein is probably a dimer. It is likely that the gene product exerts its tumour suppressor actions through intracellular signalling, interactions with the cytoskeleton, and controlling cellular proliferation, possibly affecting the rate of cell division or apoptosis. Whether APC mutations are always the first events in colorectal carcinogenesis or whether germline defects in one of the mismatch repair (MMR) genes could provide an alternative initiating step has been questioned. Mutations in four MMR genes cause the dominantly inherited syndrome hereditary non-polyposis colorectal cancer (HNPCC): hMSH2 on chromosome 2p, hMLH1 on chromosome 3p, hPMS1 on chromosome 2q, and hPMS2 on chromosome 7q. Colorectal cancers from patients with mutations in these MMR genes consistently show microsatellite instability, a form of replication error. Although, in general, it is assumed that mutations in the MMR genes in HNPCC families act only to increase the mutation rate (including mutations at APC), it is also possible that the MMR mutations themselves may have a direct role in initiation of tumorigenesis. MMR mutations also occur in sporadic colorectal cancers; however, when these mutations arise somatically they occur after APC mutations and, therefore, are involved in the progression of tumours, rather than initiation.

Colorectal cancers associated with ulcerative colitis do not usually develop from adenomas, suggesting a different genetic pathway from sporadic cancers. The low frequency of APC mutations in colorectal cancers associated with inflammatory bowel disease suggests that mutations in this gene are not the initiating event in these types of tumours.

The possibility that mutations in other genes can initiate colorectal tumorigenesis is suggested by reports of adenomas from families not linked with APC. In addition, evidence is emerging that a hamartoma–adenoma–carcinoma sequence exists and genes for several hamartoma syndromes have been localised or cloned (such as Peutz Jeghers syndrome on chromosome 19q (PJS1), some forms of juvenile polyposis, and Cowden's syndrome/PTEN on chromosome 10q). Furthermore, there is some support for a hyperplastic polyadenoma–carcinoma sequence (fig 1).

COLORECTAL CANCER PROGRESSION: EARLY ADENOMA TO CARCINOMA

While mutations in the APC gene initiate events in colorectal tumorigenesis, it is most likely that these mutations alone are insufficient for adenomas to progress and without mutations at other loci, regression might occur. Probably, several genes are involved in the progression of early adenomas to early carcinomas.

Early candidates for adenoma progression were the ras oncogenes. The K ras gene is one of a family of three human genes (K ras, H ras, and N ras). These encode small GTP binding proteins localised on the inner leaflet of the cell.
membrane that are involved in transducing signals from receptor tyrosine kinases such as epidermal growth factor receptor (EGFR). The receptors are coupled to the ras proteins through an intermediate complex of GRB and SOS2 proteins. Downstream elements of this transduction pathway include the cytoplasmic RAF serine threonine kinase and mitogen activated protein (MAP) kinase cascades. The ras proteins are activated on binding GTP and deactivated by intrinsic GTPase activity from two GTPase activating (GAP) proteins. One of these is ras-GAP-p120 and the other is neurofibromin, the product of the NF1 gene. The ras oncogenes are activated by point mutations that prevent the activation of GTPase. More than 50% of colorectal cancers display specific mutations in the K ras gene, with an increasing frequency of mutation in larger and more advanced lesions. The consequence of K ras mutations during tumour development may be a growth advantage of those cells with both APC and K ras mutations over cells with APC mutations alone. Although K ras mutations are seen within histologically normal mucosa, they appear to be present with coexisting APC mutations only in dysplastic mucosa. This supports the notion that K ras mutations confer no growth advantage in the absence of a mutation in the APC gene.

Originally, the MCC (mutated in colonic cancer) and DCC tumour suppressor genes were thought to play a role in colorectal carcinogenesis. In the model of Fearon and Vogelstein, mutation at the DCC locus represented the third step in the genetic pathway. The MCC and DCC genes were identified as a result of the frequent allele loss close to their locations on 5q21–q22 and 18q21.3, respectively, in colorectal cancers. A small number of mutations at MCC were described originally in these tumours, but subsequent studies have found very few mutations, suggesting that APC is the primary target for allele loss on 5q21–q22. DCC is a neural cell adhesion molecule homologue and, therefore, its mutation may have a role in colorectal tumour progression, invasion, and metastasis (although, in general, allelic loss at DCC occurs before malignancy). However, there is now evidence suggesting that another gene, DPC4, may be the target of allele loss on chromosome 18q in some tumours.

There is no doubt about the role of p53 mutations in the progression of colorectal tumours. p53 protein is important in maintaining DNA integrity. DNA damage results in p53 mediated arrest in the G1 phase of the cell cycle, followed by repair or, if the damage is too great, p53 induced apoptosis. Therefore, loss of function of p53 by mutation or deletion allows cells to accumulate mutations throughout the genome and results in karyotypic instability, impaired G1 cell cycle arrest, and reduced apoptosis. Mutations in the p53 gene occur in approximately 75% of colorectal cancers, but the frequency is lower in carcinomas that are mucinous and those that arise in the proximal colon, as is seen in HNPCC. p53 mutations are rare in adenomas, suggesting that p53 plays a role in tumour progression, but it is not an absolute requirement for malignant transformation because a significant proportion of cases have no demonstrable abnormality. These mutations also tend to occur at the late adenoma stage (although they occur earlier in colorectal cancers associated with inflammatory bowel disease, which do not develop from adenomas). Dominant, gain of function mutations in p53 are common in colorectal cancers and these can be detected reliably using immunohistochemistry for p53 protein. Allele loss close to p53 (17p13.1) also occurs frequently, either because of loss of the wild-type allele or possibly because another gene nearby is targeted.

The sites of other candidate tumour suppressor genes that may be involved in colorectal tumour progression have been identified by allele loss studies. Mutations of the FHIT gene and at the p16 (MTS1) locus may be important in colorectal tumours, the latter through failure of cell cycle arrest. Locations of other tumour suppressor loci include chromosomes 1p36–q42 (close to the putative human Mom-1 homologue), 6q25, 8p12, 14q32, and 22q. Typically, allele loss occurs at these locations at frequencies between 30% and 60%.

The roles of MMR mutations in colorectal tumorigenesis have been mentioned briefly. In HNPCC tumours, one mutation is inherited and the other occurs somatically. In about 15% of sporadic colon cancers, two MMR mutations (or two mutations at a related locus) occur in the somatic genome. Normal mucosa from HNPCC patients does not display microsatellite instability, only 50% of HNPCC adenomas (compared with 90% of cancers) exhibit microsatellite instability, and the frequency of early lesions such as APC mutations is similar in replication error positive and negative tumours. Therefore, in sporadic cancers, defective MMR function may be an alternative route to allele loss or the acquisition of mutations, and loss of MMR may simply "catalyse" the progress of a tumour down the same pathway as microsatellite instability negative cases. MMR mutations may act as alternatives to p53 mutations in colorectal tumours, albeit through a different mechanism. Although genomic instability in p53 mutant cancers tends to take the form of karyotypic abnormalities, instability resulting from MMR mutations can lead to near diploid lesions. It has been found that MMR mutations are associated negatively with mutant p53 and that, like p53 mutations, MMR mutations often occur in late colorectal adenomas. Genomic instability may also be caused by somatic or germline mutations in other genes involved in DNA replication and repair. Support for this idea comes from a study of two apparently sporadic colorectal cancer cases that were both shown to carry DNA polymerase γ variants.

MALIGNANCY

Few genetic changes specific to mature colorectal cancers have been identified. Several regions of allele loss have been detected but
their roles in tumour progression are unclear. The types of mutations that would be important in the mature colorectal cancer are those that cause sustained replication, decreased apoptosis, or angiogenesis. Malignancy requires colorectal tumour cells to exhibit several features, namely the ability to: (a) erode the basement membrane, (b) disrupt normal cell junctions, and (c) survive in the blood or lymphatic systems and in a new tissue environment.

Cell adhesion molecules are candidates for involvement in the process of invasion and metastasis of colorectal cancers. In addition to effects on adhesion, mutations at these loci may also have effects on growth. For example, E cadherin forms part of the adherens junction complex of epithelial cells. Loss of the E cadherin protein occurs in several cancers, including colorectal cancer, and is associated with the development of invasive properties. Other proteins that may be associated with invasion by colorectal cancers include those involved in tissue degradation, such as urokinase plasminogen activators and matrix metalloproteinases/collagenases. The cells of metastases may have genotypes and/or patterns of gene expression distinct from those of primary tumours. Variation at a number of gene loci may alter the behavioural pattern of the mature colorectal cancer. These include the NM23 gene, which has a possible role in the metastasis of several cancers, and CD44.

**Clinicopathological correlations: prediction of prognosis**

The recognition that there are probably several different genetic pathways for the development of colorectal cancer suggests that correlations exist between the molecular and clinicopathological features of tumours that are not apparent using routine methods such as histology. These correlations may serve as prognostic determinants and/or enable the partitioning of patients into groups for different therapies. A number of studies have sought to examine the relation between genotypic variation in these tumours and clinicopathological features, especially patient prognosis. Most work has been in the form of case control studies, using a comparison of the frequency of genotypes in primary and secondary tumours as a surrogate for survival. However, a small number of studies have examined the relation between genotype and prognosis by classical survival analysis. In this section, we review the evidence for genetic variation in colorectal cancers as markers of prognosis, including some studies of protein or mRNA expression that have been assumed to be indicators of underlying mutations.

**Allele loss studies**

In an early allelotyping study based upon 56 patients, Vogelstein et al showed that patients with more than the median percentage of allelic deletions had a considerably worse prognosis than other patients; based on a mean follow up period of 38 months, the tumour recurrence and death rates were 30% and 68%, and 26% and 64%, respectively. Later studies have examined the relation between specific chromosome abnormalities and tumour behaviour. Chromosome 18q allele loss has a well-established association with colorectal cancer progression and has been evaluated as a prognostic marker in a number of studies. Overall, its value may not be great. However, based on more than 100 patients with stage II and III cancers it appears that allele loss at this region represents a significant prognostic marker. After adjustment for tumour differentiation, vein invasion, and stage it has been shown to be a strong predictor for survival with a hazard ratio for death of 2.5 (95% confidence interval (CI), 1.1 to 5.7, p = 0.036).

Cytogenetic and allele loss studies of chromosome 17 have been based on fewer patients than the analyses of chromosome 18. Most have found that both 17p and 17q abnormalities are associated with invasion and angiogenesis. Allele loss at 17q has also been shown to provide independent prognostic information. Mutations that are likely to be important prognostically are those that involve genes involved in tumour progression rather than initiation. Therefore, it is not surprising that allele loss at chromosome 5q, the site of the APC gene, has not been shown to be a prognostic marker. The high rate of allele loss at other chromosomes such as 8p, 1p, and 11q during tumour progression suggests that they may be sites of other tumour suppressor genes that are important for the progression of colonic tumours. A correlation between 8p allele loss and microinvasion (a prognostic marker independent of Duke's stage) has been reported in one small study of 14 colorectal cancers. A relation between tumour progression and chromosome 17p deletion has also been shown. However, analysis of 126 sporadic colorectal cancers for allele loss at 11q22 has failed to show an association with Duke's grade or degree of differentiation.

**K ras**

Along with p53, K ras mutations are one of the most common genetic lesions in human cancer. Since the discovery of the human ras gene family, there has been much debate concerning the potential for differential tumour behaviour as a result of different ras mutations. Point mutations in codons 12, 13, and 61 of the K ras gene are early events in the pathogenesis of colorectal cancer. However, the impact of the number, type, and position of such mutations on the progression of adenomas, as well as the clinical behaviour of colorectal carcinomas, is not fully established. To date, most studies have indicated that the second base of codon 12 is mutated more heavily in colorectal cancers than the first or third bases. Therefore, it is conceivable that the type of K ras mutation determines tumour behaviour directly.

In a relatively large study by Finkelstein et al of 194 consecutive primary, recurrent, and metastatic colorectal adenocarcinomas, a significantly higher mutation rate in K ras was
seen in lymphogenous, haematogenous metastases. When colorectal carcinomas were analysed by specific K ras mutation type, it was found that codon 13 mutated tumours did not progress to local or distant metastasis. Tumours having a codon 12 valine substitution did not metastasise beyond pericolonic, perirectal lymph nodes. In contrast, colorectal cancers with codon 12 aspartic acid substitutions accounted for most of the distant haematogenous metastases. Tumours with normal K ras accounted for most intraperitoneal deposits. On the basis of these data, Finkelstein et al. proposed that genotyping of colorectal adenocarcinoma by K ras status would identify subsets of patients likely to pursue indolent or aggressive forms of disease.

Some, but not all, reports have supported the proposal that the possession of a K ras mutation is associated independently with shorter survival rates. However, the relation between specific mutation and prognosis is unclear. Early in vitro observations suggested that codon 12 K ras mutations elicited stronger transformation responses than codon 13 K ras changes in NIH/3T3 cell assays. However, this notion has not been supported by some biochemical analyses of different ras proteins. On the basis of the available clinico-pathological data in colorectal cancer, it is likely that patient survival is related to the occurrence of K ras mutations, but is not necessarily related to the specific type of mutation.

DELETED IN COLON CANCER
If allelic loss of chromosome 18q predicts a poor outcome in colorectal cancer then the DCC gene must be a prime candidate for the cause of this association. In a single study of 132 patients with curatively resected stage II and III colorectal carcinomas, the expression of DCC protein was a strong positive predictive factor for survival. Patients with stage II disease whose tumours expressed DCC, had a five year survival rate of 94.3% compared with a survival rate of 62% for patients with DCC negative tumours. In patients with stage III disease, the survival rates were 59% and 33% for patients with DCC positive and negative tumours, respectively.

MISMATCH REPAIR GENES AND MICROSATELLITE INSTABILITY
Evidence directly correlating molecular and clinico pathological data for colorectal cancer has come from HNPCC. Colorectal cancers developing in carriers of HNPCC mutations are poorly differentiated and frequently multiple. Paradoxically, despite multiplicity and poor differentiation of cancers, early observations suggested that colorectal cancers in HNPCC carried a more favourable prognosis that in sporadic cases. Convincing evidence for improved survival in HNPCC is provided by a large study from Finland. The survival rates of 175 colorectal cancer patients with HNPCC were compared with those of 14,000 patients with sporadic colorectal cancers diagnosed before the age of 65 years. One hundred and twenty of the patients with HNPCC came from families segregating a germline mutation in the hMLH1 gene. The overall survival rate was 65% for patients with HNPCC and 44% for patients with sporadic cancer. Furthermore, the survival rates of HNPCC patients were better in all strata analysed. A similar study has been undertaken in Japan. The five year survival rate for colorectal cancer in patients from families conforming to the Amsterdam criteria for HNPCC (n = 14) was 92%, in putative HNPCC families (n = 131) the survival rate was 70%, while in sporadic colorectal cancer (n = 1604) the survival rate was 60%.

To determine whether microsatellite instability characterises a subset of sporadic colorectal cancers as well as patients with HNPCC, Ishimari et al. examined its incidence in 80 primary colorectal cancers and 36 liver metastases. The replication error phenotype did not show correlation with any clinicopathological parameters of tumour aggressiveness, such as Duke's staging, histological grade, or survival suggesting that the microsatellite instability phenotype is not associated with aggressiveness.

A systematic analysis of 215 sporadic colorectal cancer patients showed that those with microsatellite instability had a survival advantage over patients without it, independent of other prognostic factors. Microsatellite instability was found in 16.4% of colorectal cancers. The hazard ratio of patients with tumours showing microsatellite instability to those without was estimated to be 0.39 (95% CI 0.19 to 0.82). At any point in time after diagnosis, a patient whose tumour displayed microsatellite instability had a risk of dying, which was approximately 59% of the risk of a patient with a tumour without microsatellite instability, even after allowing for the effect of other predictive factors. Only one of the 24 patients exhibiting microsatellite instability in their colorectal cancers possessed a detectable germline defect in hMSH2. These findings suggest that although the genetic basis of HNPCC and sporadic cancers with microsatellite instability is different, tumours in the two groups share some biological characteristics in terms of prognosis.

P53 AND P27
An increased intracellular concentration of p53, which is frequently but not always related to p53 mutation, has been proposed as being associated with poor prognosis in some tumour types. In immunohistochemical studies of colorectal cancer, although p53 overexpression correlates with chromosome 17p loss, hyperdiploid DNA content, and tumour site there have been conflicting findings about its role as a prognostic indicator. This reflects the fact that the degree of association between p53 mutations and protein expression depends, in part, on the specific antibody used. In studies of the relation between p53 mutations and prognosis, the situation is much clearer. Studies suggest strongly that colorectal cancers harbouring p53 mutations are more aggressive, are associated with a higher propensity for
lymphatic and haematogenous spread, and have a worse prognosis.126 132

p27 is a member of the cip/kip family of cyclin-dependent kinase inhibitors, which bind to cyclin/cyclin dependent kinase (cdk) complexes and block progression through the cell cycle. p27 regulates progression from G, into S phase by binding to and inhibiting the cyclin E/Cdk2 complex, which is required for cells to enter S phase. In contrast to the p53 gene, mutations in p27 are rare. However, cell cycle regulation of p27 concentrations occurs at the post-transcriptional level through proteasome mediated degradation.133 Recently, reduced expression of p27 has been shown to correlate with poor survival in a study of 149 patients with primary colorectal cancer.134 Patients whose tumours expressed p27 had a median survival of 151 months, whereas those who lacked p27 (10%) had a median survival of only 69 months. In this study, p27 expression was shown to be an independent prognostic marker and the risk of death associated with reduced expression was increased 2.9-fold.

Conclusions

The incidence of colorectal cancer is increasing and, unfortunately, the prognosis remains poor for the majority of patients. Identification of patients who are at a high risk of recurrent local and metastatic disease is important for the selection of appropriate treatment. Prognostic variables that have been found to be statistically significant include pathological stage and grade, type of tumour growth, chromosomal aneuploidy, and the presence of microinvasion. Until recently, there has been little understanding of the molecular basis of these indices. Despite the continuing interest in understanding the field of biology, in the future, the genetic features of colorectal tumours will almost certainly become useful indicators of prognosis and of the most appropriate therapy. To date, one of the problems with clinicopathological associations has been that most studies have, for entirely understandable reasons, analysed only small numbers of tumours. However, there is some evidence that colorectal cancers harbouring defects in the MMR genes are associated with a better prognosis and those with chromosome anomalies, such as deletion of 18q or 17q and mutations in K ras and p53, a worse prognosis. With the advent of methods for rapid genotyping it should be possible to construct mutation profiles of tumours and use multivariate analysis to determine which molecular features correlate with the clinicopathological data.


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