Oncogene mutations in cancer and dysplasia associated with inflammatory bowel disease

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ABSTRACT: Chronic inflammatory bowel disease (IBD) predisposes affected individuals to the development of invasive colon cancer. Increased cancer risk has been seen in ulcerative colitis and Crohn’s disease in Western societies as well as in schistosomal colitis in China. In this study it was found that the frequency of the 12th codon of c-Ki-ras, as well as the specific amino acid substitutions, were similar in sporadic colon cancers, cancers associated with ulcerative colitis in the United States, and colon cancers found in patients with schistosomal colitis in Jiangsu province in China. Further, activating mutations of codon 12 of e-Ki-ras were found in high grade dysplastic lesions in chronic ulcerative colitis. The latter finding indicates that some dysplasias are clonal proliferative lesions with genetic characteristics associated with invasive cancer.

Key Words: Carcinogenesis, Dysplasia, Oncogene, Ulcerative colitis

IT HAS BEEN RECOGNIZED FOR MANY years that patients with longstanding ulcerative colitis are at increased risk for developing invasive colon carcinoma. This association is most evident in patients with extensive disease of more than 10 years' duration (1-4). Population-based studies indicate that cancer risk is approximately 0.5 to 1% per year beginning 10 years after diagnosis. Epithelial dysplasia is considered a precancerous lesion in ulcerative colitis and is used as a histologic predictor of cancer development (5). The relationship between dysplasia and cancer in ulcerative colitis has been inferred from the observation that colons resected for cancer in ulcerative colitis almost always have regions of associated dysplasia (6). Also, specimens resected because of dysplasia frequently harbour occult carcinomas (7). Because dysplasia may be difficult to distinguish from reparative changes in ulcerative colitis, a standardized classification has been developed and used to grade the severity of the dysplastic changes (8). It is presumed, but not unequivocally proven, that neoplastic transformation involves the progression of a colonic epithelial cell from normal morphology through increasing grades of dysplasia to invasive carcinoma, and that this process is associated with the accumulation of specific
genetic lesions. Colectomy specimens from patients with chronic ulcerative colitis are an excellent resource to study the biology and genetics of the precancerous phase of colon cancer, since specimens often have carcinoma, varying grades of dysplasia and relatively normal or regenerative epithelium.

Cancer associated with inflammatory bowel disease (IBD) is of considerable importance in other regions of the world as well, particularly in southeastern China. Schistosoma japonicum infection is endemic in Jiangsu province and leads to a chronic schistosomal colitis. The colon cancer incidence rates in Jiangsu province, 44 cases per 100,000, are the highest in Asia. Dr. Chen Ming- Chai and his colleagues (9-12) have described the epidemiology and clinical aspects of schistosomal colitis in this region and presented the histologic evolution of inflammatory lesions progressing to invasive cancer. The histopathology of chronic schistosomal colitis is remarkably similar to that found in chronic ulcerative colitis and, in both disorders, flat dysplastic changes appear to be the precancerous lesion.

Invasive colon cancer is a genetically complex disease, evidenced by the finding of multiple karyotypic abnormalities and a high frequency of allelic loss at multiple chromosomal sites in colon cancer cells (13-16). Nevertheless, a series of nonrandom generic changes have been identified which occur frequently and appear to play a significant role in the molecular pathogenesis of this disease. These include alterations of dominantly acting oncogenes (ras, c-myc and c-src) and of presumed 'suppressor' genes located on chromosomes 5q, 17p and 18q.

Of the dominantly acting oncogenes, the ras genes have been studied in greatest detail, and approximately 50% of colon cancers have been found to have an oncogenic ras mutation (17,18). The majority of mutations of ras genes in colon cancer occur at codon 12 or 13 of c-Ki-ras (approximately 90%), the remainder occurring at codon 61 of c-Ki-ras and at similar positions in N-ras. These mutations are associated with diminished GTPase activity and enhanced transforming properties of the p21 molecule in vitro. 

pp60c-src tyrosyl kinase activity has been found to be increased, compared to normal colonic mucosa, in the majority of colon cancers, with some tumours demonstrating disproportionately high kinase activity compared to pp60c-src protein levels (19-22). This has given rise to the notion that pp60c-src is 'activated' in colon cancer, as can be seen with the neural form of pp60c-src, oncogenic mutations of c-src (eg, at positions 338, 378, 441, 527 of chicken c-src, and 3) when a high proportion of pp60c-src molecules are phosphorylated at Tyr 416 relative to Tyr 527. In previous studies the author was unable to detect activating mutations of c-src in colon tumours expressing high levels of pp60c-src kinase activity (23). The author's interpretation of these data is that colon cancer may represent the clonal expansion of a colonic epithelial cell at a specific stage in differentiation at which the cell normally expresses high levels of pp60c-src tyrosyl kinase, and this activity is not necessarily linked to cell transformation.

Both cytogenetic and molecular studies have shown that a portion or all of chromosomes 5, 17 and 18 are frequently lost in advanced colon carcinoma. This suggests that recessive genes on these chromosomes may be activated by loss of a normal balancing allele. A locus on 5q (APC) has been linked to familial adenomatous polyposis of the colon and has been mapped to 5q21-22 (24,25). Nineteen to 36% of sporadic colon carcinomas have been reported to have hemizygous loss of 5q (14,26-29). If genetic markers closely flanking the APC locus are used, approximately 50% of sporadic colon cancers will demonstrate loss of a portion of 5q, indicating the importance of this locus in the molecular pathogenesis of sporadic colon cancer (30).

A high incidence of allelic loss at chromosome 17p has also been found in a number of studies of colon cancers (13-15). In a number of instances this has been associated with mutations of the p53 gene in the remaining allele (30-32). The described mutations occur in two evolutionary conserved regions of p53 and suggest that these mutations alter or inactivate the normal function of the gene product. Allelic loss at chromosome 18q occurs in approximately 75% of colon cancers, determined by karyotypic analysis and molecular studies (11,27). A candidate 'suppressor' gene involved in the allelic deletion on 18q has recently been described (33). The gene on 18q shows sequence similarity to neural cell adhesion molecules and has been shown to have diminished expression as well as being mutated in a number of colon carcinomas.

The high frequency of specific genetic alterations in colon cancer allowed Vogelstein and his colleagues (27) to define the sequence of these genetic changes in the polyp to cancer sequence. They found that ras mutations and 5q deletions occurred with equal frequency in polyps and cancers but that 17p and 18q deletions occurred more frequently in invasive carcinomas. Their data suggest that the ras mutation and 5q deletion occur early in this sequence (but after the polyp has developed) and that the 17p and 18q deletions occur at the transition to, or after carcinoma has developed.

In this work, the author addresses a number of issues related to the role of oncogene mutations in cancers and dysplasia associated with IBD. First, the frequency and type of ras mutations in sporadic colon cancers (evolved from polyps) are determined and contrasted with colon cancers associated with IBD both ulcerative colitis in the United States and schistosomal colitis in China. Second, evidence is presented that the ras mutation may occur in the dysplastic phase of IBD.

PATIENTS AND METHODS

Tissue procurement: Resected colon specimens from American patients with sporadic colorectal cancer and ulcerative colitis were obtained at University Hospital and the Massachusetts General Hospital, in Boston, Massachusetts. Colectomy specimens from schistosomiasis patients with colon cancer were obtained from Kun San Hospital, Jiangsu province,
People's Republic of China. Tumour specimens and samples of normal colonic mucosa were stored in liquid nitrogen until nucleic acids were extracted.

**ras gene mutation analysis:** Mutations at codons 12 and 13 of c-Ki-ras and N-ras were scored by restriction fragment length polymorphisms of ras genes amplified in a polymerase chain reaction (PCR). The 5' end oligonucleotide primer used in the PCR was immediately adjacent to codon 12 or 13 and contained base mismatches compared to the wild-type sequence, which generated an artificial restriction site at codon 12 or 13 (34). Mutations were scored as a loss of the restriction site.

**DNA extraction:** Heavy molecular weight DNA was extracted from colon tumours or normal mucosa by homogenization in a solution containing sodium dodecyl sulphate (0.1%) and proteinase K (200 mg/mL) followed by multiple extractions with phenol/ chloroform as described previously (35).

**Oligonucleotide primers and PCR:** Primers were synthesized using a Cyclone DNA synthesizer (Millipore Corp) and purified by polyacrylamide gel electrophoresis. The primers used to score restriction fragment length polymorphism (RFLP) at codons 12 and 13 of c-K-ras and N-ras are noted below. The bases modified, with respect to the wild-type sequence, to create artificial restriction sites are underlined.

**c-Ki-ras (codon 12):** The 5' end primer used generates a Bst N1 site at codon 12. The 3' end primer contains an internal Bst N1 site (CCTGG).

**5' end primer:**
5'-ACTGAAGTACAACGTGTTGTTG
GAGCT 3'

**3' end primer:**
5'-TCAAAGATGGTCCTGACAC 3'

**c-Ki-ras (codon 13):** The modification of the 5' end primer creates a BglII site at codon 13 to score mutations at that site. The 3' end primer used is that shown for the assay of codon 12.

**5' primer:**
5'-ACTGAATATAAACCCTTGTGTTG
GAGCT 3'

**3' primer:**
5'-GCGGCTACACCTGACAC 3'

**N-ras codon 12 and 13:** The 5' end primer introduces a Bst N1 site to score mutations at codon 12. The aspartic acid mutation of codon 13 (G to A at position 2) creates an Hph I recognition site. Therefore, this mutation which is the most common mutation of codon 13, was scored by using primers as shown below for codon 12 followed by digestion with Hph I.

**5' end primers:**
5'-ACTGAAGTACAACGTGTTGTTG
GAGCT 3'

**3' end primers:**
5'-GGGGCTACACCTGACAC 3'

**DNA sequencing:** Amplified DNA products were extracted with chloroform, diluted with 2.5 mL water and subjected to centrifuge-driven dialysis using a Centricon C-30 filter (Amicon, Massachussetts), to remove excess primers. The double-stranded DNA was sequenced by the dideoxy-terminator method (36) using the 3' end amplification primer and the modified T7 DNA polymerase, Sequenase (US Biochemicals, Ohio) as previously described (37).

**RESULTS**

Restriction fragment polymorphism assays were performed to detect mutations of codon 12 and 13 of c-Ki-ras and N-ras in 42 sporadic colon cancers, 15 colon cancers associated with schistosomal colitis and three tumours associated with ulcerative colitis. The majority of mutations detected in the cancers in this study was at codon 12 of c-Ki-ras (Figure 1). These were scored using an artificially generated Bst N1 site at codon 12 using the PCR as described in the 'Patients and Methods' section. Twenty-three of 47 sporadic cancers, 12 of 15 colon cancers associated with schistosomiasis, and two of three cancers associated with ulcerative colitis were positive for ras mutations (Table 1). Eight of the mutations in cancers associated with schisto-
Mutations In 180

Table 1: c-Ki-ras mutations in sporadic inflammatory bowel disease-associated colon cancers

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Positive/tested (%)</th>
<th>Codon 12</th>
<th>Codon 13</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic colon cancer</td>
<td>23/47 (49)</td>
<td>23</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Colon cancer (schistosomiasis)</td>
<td>12/15 (80)</td>
<td>11</td>
<td>1</td>
<td>6-aspartate</td>
</tr>
<tr>
<td>Colon cancer (ulcerative colitis)</td>
<td>2/3 (67)</td>
<td>2</td>
<td>-</td>
<td>1-aspartate</td>
</tr>
</tbody>
</table>

Table 2: c-Ki-ras mutations in ulcerative colitis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of patients</th>
<th>Positive/tested</th>
<th>Mutation of codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colonic mucosa</td>
<td>14</td>
<td>0/14</td>
<td>-</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>9</td>
<td>0/37</td>
<td>-</td>
</tr>
<tr>
<td>High grade dysplasia</td>
<td>1</td>
<td>2/5</td>
<td>Aspartate</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2/2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>1/1</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND Not done

Discussion

In this study the frequency of ras mutations in sporadic cancers (approximately 50%) was similar to that found in a number of previous studies (17,18). The authors were unable to obtain a sufficient number of samples of sporadic colon cancer from China to compare the mutation frequencies in sporadic cancers from China and the United States. The finding of 12 of 15 (80%) schistosomiasis-associated colon dysplasia and regions of inflammatory mucosa devoid of dysplasia. Multiple histologic sections were obtained to ensure that dysplastic areas were devoid of invasive carcinoma. In the study of the first specimen, an RNase protection assay was used on five contiguous samples of high grade dysplasia; those results were previously reported. Subsequent to that manuscript a carcinoma was found on the same specimen. RFLP analysis and DNA sequencing were performed on both cancer and dysplasia. Multiple RFLP assays were performed on the second specimen. In case 1 the invasive cancer and two contiguous regions of high grade dysplasia had a glycine to aspartate mutation of the c-Ki-ras codon 12 (Figure 2). In case 2, RFLP analysis demonstrated c-Ki-ras codon 12 mutations of the cancer and two regions of high grade dysplasia, but a normal c-Ki-ras gene was found in DNA from normal appearing mucosa distant from the tumour (Figure 2).

**Figure 2** Results of c-Ki-ras of codon 12 mutation assays in colectomy specimens from patients with ulcerative colitis. In case 2 (right) (+) signifies the presence of a codon 12 mutation and (−) indicates a wild type codon 12. Asp Aspartate; Gly Glycine
cancers and two of three cancers in ulcerative colitis patients positive for ras mutations may represent a true higher incidence of mutation in this disease or more likely a selection bias in a small series. The assay of additional cancers associated with IBD will clarify this issue. The c-Ki-ras mutations were primarily of the 12th codon and were either aspartate or valine substitutions for glycine. These are two frequent substitutions reported in sporadic cancers in the United States although serine and cystine mutations, which are also detected in these tumours.

Of interest was that four carcinomas had faint mutation bands in the RFLP assay on repeated analysis using ethidium staining or radioisotopically labelled oligonucleotide primers. The authors have examined the histologic sections immediately adjacent to the tissue assayed for mutations, and more than 80% of the cells were identified as tumour cells in all four tumours (data not shown). The lower limit of sensitivity of the RFLP assay is the detection of a mutation in approximately 5% of cells. It appears that the ras mutation was present in only a subpopulation of tumour cells, probably 5 to 10% of the cells. Therefore, it seems likely that the ras mutation occurred late in the evolution of these cancers or, less likely, that the mutated allele was lost from a major population of tumour cells in the progression of the tumor. Other work has suggested that the ras mutation occurs early in most sporadic cancers, ie, in the adenoma stage (27).

The finding of activating c-Ki-ras mutations in high grade dysplasia is consistent with the finding of ras mutations in some polyps (27) and supports the notion that this mutation can frequently occur prior to the invasive stage of colon cancer development. The assay of additional specimens with low and intermediate grades of dysplasia will elucidate what the earliest stage in the transformation process is in which the ras mutation can be detected. Further, these studies will indicate whether activating mutations of ras will be a useful adjunct to histopathology in the assessment of cancer risk in chronic IBD.

In two instances the authors were able to score ras mutations in multiple samples throughout coloectomy specimens from patients with ulcerative colitis with both cancer and dysplasia. It was found that dysplastic cells containing the identical mutation covered a wide area of mucosa, suggesting that dysplasia is not a focal lesion but rather it is a clonal, proliferative abnormality which spreads horizontally but is not vertically invasive. These data are supported by recent studies of cellular DNA content using flow cytometry and cytophotometric techniques demonstrating aneuploid clones of dysplastic epithelial cells in ulcerative colitis (34,35). Further, it was found that there were regions of high grade dysplasia which lacked ras mutations even though they were contiguous to morphologically identical epithelium which scored positive in ras mutation assays. These data suggest that either the mutation occurs late in the evolution of the dysplastic clone or that there are multiple dysplastic clones in longstanding IBD. In two instances carcinoma contiguous to a region of dysplasia has been detected in which both cancer and dysplasia had the identical activating c-Ki-ras mutation. In these instances it is likely that the cancer evolved from the area of dysplasia.

In summary, these studies have shown that activating mutations of c-Ki-ras represent a common genetic pathway for transformation of colonic epithelial cells irrespective of etiology or ethnic background. Further, this genetic lesion can be found in the non-invasive stage in the evolution of colon carcinoma associated with IBD.

REFERENCES


