

Frequency and Spectrum of c-Ki-ras Mutations in Human Sporadic Colon Carcinoma, Carcinomas Arising in Ulcerative Colitis, and Pancreatic Adenocarcinoma

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Sporadic colon carcinomas, carcinomas arising in chronic ulcerative colitis, and pancreatic adenocarcinomas have been analyzed for the presence of c-Ki-ras mutations by a combination of histological enrichment, cell sorting, polymerase chain reaction, and direct sequencing. Although 60% (37/61) of sporadic colon carcinomas contained mutations in codon 12, only 1 of 17 specimens of dysplasia or carcinoma from ulcerative colitis patients contained c-Ki-ras mutations, despite a high frequency of aneuploid tumors. In contrast, a higher percentage (16/20 = 80%) of pancreatic adenocarcinomas contained mutations in c-Ki-ras 2, despite a lower frequency of DNA aneuploidy in these neoplasms. Moreover, the spectrum of mutations differed between sporadic colon carcinoma, where the predominant mutation was a G to A transition, and pancreatic carcinomas, which predominantly contained G to C or T transversions. These results suggest that the etiology of *ras* mutations is different in these three human neoplasms.

Introduction

Neoplastic progression is hypothesized to occur by a series of sequential genetic and epigenetic alterations within a clonal population of cells that culminate in a phenotype characterized by loss of proliferative control (1,2). In the human gastrointestinal tract, genetic alterations such as mutational activation of *ras* proto-oncogenes have been implicated in tumor progression. Mutations in c-Ki-ras 2 have been reported in 40 to 60% of sporadic colon carcinomas (3-7) and in 75 to 90% of pancreatic carcinomas (8-11). Their occurrence in precursor lesions such as colonic adenomas suggests that *ras* mutations may be an early, initiating event in some human tumors.

In animal model systems, activation of *ras* oncogenes by specific base pair mutations appears to be an important mechanism during the experimental induction of tumors by chemical carcinogens (12,13). Moreover, in

many cases, the spectrum of mutations produced within *ras* oncogenes by a particular direct-acting chemical carcinogen correlates with the proposed mechanism of action by that class of carcinogen (14-21). In human tumors, however, the role of specific carcinogens in the activation of *ras* oncogenes has not been defined. Although carcinogens have been implicated in the genesis of sporadic colon cancer (22), much less is known about the role of specific carcinogens in the development of pancreatic carcinoma. Clearly, studies using human tumor model systems are needed to clarify the relationship between carcinogenic exposure and *ras* mutations in the development of human cancer.

We have undertaken a retrospective analysis of the frequency and spectrum of *ras* mutations in sporadic human colon carcinomas, carcinomas arising in patients with long-standing ulcerative colitis, and pancreatic carcinomas using a combination of histologic enrichment, cell sorting, the polymerase-catalyzed chain reaction (PCR), and direct sequencing. In contrast to sporadic colon carcinomas, mutations in c-Ki-ras were infrequently observed in carcinomas or areas of high-grade dysplasia in patients with chronic ulcerative colitis. Moreover, differences in the frequency and spectrum of mutations observed in sporadic colon carcinoma and pancreatic carcinoma suggest that a different class of

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carcinogens may be involved in the initiation of these two tumors.

Methodology

Case Selection

Cases of sporadic colon carcinoma were selected from patients without a prior history of malignancy, inflammatory bowel disease, familial colorectal carcinoma, or prior chemotherapy. Cases from patients with chronic ulcerative colitis were chosen with the following criteria: at least 10 years of ulcerative colitis with evidence of dysplasia or cancer, the absence of small intestinal disease, the absence of pathologic changes suggestive of Crohn's disease, and colonic involvement extending proximal to the rectosigmoid. Twenty paraffin-embedded cases of pancreatic adenocarcinoma were analyzed for flow cytometric alterations in DNA content; cases exhibiting aneuploid subpopulations were sorted prior to DNA extraction and gene amplification, and diploid carcinomas were enriched histologically to ensure that at least 75% of the section contained tumor cells prior to DNA extraction.

Flow Cytometry, Cell Sorting, PCR, and DNA Sequencing

The detailed methodologies for our histologic enrichment procedure, flow cytometric and cell sorting techniques, PCR, and DNA sequencing methods for the c-Ki-ras 2 first exon have been previously described (6,7,23).

Results

Sporadic Colon Carcinoma

Mutations in the c-Ki-ras proto-oncogene have been demonstrated in 40 to 60% of sporadic colon carcinomas by a number of different techniques, including oligonucleotide hybridization, RNase A mismatch cleavage analysis, and PCR followed by direct sequencing (3-7). Mutations have also been found in adenomas adjacent to regions of carcinoma and in adenomas not associated with cancer, suggesting that these mutations may be an early event in neoplastic progression (3-5,7).

We have analyzed 61 sporadic colon carcinomas for

flow cytometric abnormalities and c-Ki-ras mutations (6,7). Forty-six (75% \pm 6%) contained aneuploid DNA populations, and 37 (60 \pm 6%) contained mutations in codon 12 of c-Ki-ras 2 (Table 1). Twenty-seven of the 46 (59% \pm 7%) aneuploid carcinomas and 10 of the 15 (67% \pm 12%) diploid carcinomas contained mutations within codon 12, but this difference was not statistically significant.

Twenty-eight carcinomas were sorted into aneuploid and diploid subpopulations and analyzed for c-Ki-ras mutations. Six cases (21% \pm 8%) exhibited mutations only within the aneuploid population, 10 cases (36% \pm 9%) contained mutations within both aneuploid and diploid subpopulations, and 12 (43% \pm 9%) cases did not contain ras mutations in either sorted cell population. In two cases, mutations were detected in regions of histologically benign mucosa adjacent to carcinoma (7).

The predominant mutation observed was a G to A transition at the first base pair site of codon 12 (32/37); G to T transversions were observed less frequently (4/37), and only one tumor with a G to C transversion was observed. Although G to A and G to T transversions have been previously reported to be the most frequent mutations in colorectal carcinomas (28/30 = 93%) (4), our results differ by the predominance of first base pair site mutations in these tumors.

Statistically significant correlations were not observed between the patient's age, sex, stage of disease and the presence of c-Ki-ras mutations (7). Our results demonstrate that ras mutations may precede the development of flow cytometrically detectable alterations in ploidy and even morphologically identifiable changes that are usually associated with histologic progression in the development of colorectal carcinomas.

Carcinomas and Dysplasia Arising in Ulcerative Colitis

Ulcerative colitis is a chronic inflammatory disease that predisposes patients to the development of colorectal carcinoma. In contrast to sporadic colon carcinoma, in which the development of aneuploidy correlates spatially with the histopathologic location of the adenoma or carcinoma, the regions of DNA aneuploidy in ulcerative colitis patients may cover large areas of colonic mucosa and may involve areas without recognizable dysplasia (24-28). Moreover, dysplasia within

Table 1. DNA content and Ki-ras codon 12 mutations in sporadic colon carcinoma and ulcerative colitis-associated dysplasia and carcinoma.*

Disease	Number of patients	Percent aneuploid	Percent with Ki-ras codon 12 mutations
Sporadic colon carcinoma	61	75 (46/61)	61 (37/61)
Ulcerative colitis carcinoma and dysplasia	17	88 (15/17)	6 (1/17)

*Shown are the tumor type, the number of patients analyzed, the percentage and fraction of aneuploid tumors as assessed by flow cytometry, and the percentage and fraction of tumors containing mutations in codon 12 of c-Ki-ras as assessed by polymerase chain reaction and direct sequencing of the first exon of c-Ki-ras 2.

ulcerative colitis may produce no grossly recognizable lesion, whereas the dysplastic epithelium in sporadic colon carcinoma characteristically produces an exophytic mass. These clinical and flow cytometric differences suggest that alternative pathways for tumorigenesis may be involved in these two diseases.

Twenty-eight samples of carcinoma and dysplasia from seventeen patients with chronic ulcerative colitis were analyzed for flow cytometric alterations and mutations in *c-Ki-ras* 2 (Table 1) (23). Aneuploid cell populations were identified in 15/17 (88% \pm 8%) of the patients, within areas of high-grade dysplasia, indefinite for dysplasia, and negative for dysplasia.

In contrast to our results with sporadic colon carcinomas, however, *c-Ki-ras* codon 12 mutations were only found in one case of ulcerative colitis-associated carcinoma. The mutation (a G to T transversion at the first base pair site of codon 12), which was present in the aneuploid cell population of a carcinoma, was absent in the diploid carcinoma subpopulation, two adjacent regions of mucosa with high-grade dysplasia, and a region of nearby mucosa with low-grade dysplasia (23). The difference in frequency of *c-Ki-ras* mutations in sporadic colon tumors compared to carcinoma and high-grade dysplasia in ulcerative colitis patients is statistically significant.

Although inflammatory cells may constitute a high proportion of total cells in ulcerative colitis specimens, it is unlikely that this would have prevented our detection of mutated *ras* sequences. In the 88% of cases containing aneuploid populations, cell sorting allowed separation of aneuploid tumor cells from diploid stromal and inflammatory cells. In the remaining diploid cases, regions were selected for analysis that contained minimal inflammation and in which tumor cells composed greater than 75% of the section as assessed microscopically.

Despite our tumor cell enrichment procedure, it is possible that *c-Ki-ras* mutations may be present in ulcerative colitis-associated carcinomas and dysplasias below the sensitivity of detection by direct sequencing. In mixing experiments, we have been able to detect mutations that were present in 20% of the cells within the mixed sample (10% of total alleles, assuming the *c-Ki-ras* gene in the tumor is heterozygous for the mutation); we cannot reliably identify mutations that are present in lower frequency. Thus, if mutations are present in ulcerative colitis, the cells that contain them must be a minority population within the tumor, which is in marked contrast to sporadic colon carcinomas, where mutations are readily detected by PCR and direct sequencing. These results suggest that the *ras* mutations may play significantly different roles in the genesis of sporadic colon carcinoma and carcinoma and dysplasia arising in ulcerative colitis.

Pancreatic Adenocarcinomas

A number of investigators have demonstrated that a high frequency (75–90%) of human pancreatic adeno-

carcinomas contain mutations within the *c-Ki-ras* proto-oncogene (8–11). The role of *ras* mutations in the etiology of pancreatic carcinoma is uncertain, since the presence of mutations has not been found to correlate with the extent of disease or the degree of differentiation of the tumor, and more than one type of mutation has been detected within individual carcinomas or only within a small subset of cells within a carcinoma. However, investigators have suggested that the spectrum of mutations in these carcinomas differs from those in colorectal carcinomas and may more closely resemble the spectrum of mutations seen in human lung adenocarcinomas (29,30). This observation has led to the hypothesis that carcinogens within cigarette smoke may serve as risk factors in the etiology of both of these diseases (29,30).

Fifty pancreatic adenocarcinomas have been analyzed for DNA content abnormalities by flow cytometry and to date, 20 of these cases have been analyzed for *ras* mutations by PCR and direct sequencing (Table 2). In contrast to the high frequency of aneuploid sporadic colon carcinomas and ulcerative colitis-associated carcinomas, only 31 of the 50 (62% \pm 7%) of the pancreatic carcinoma specimens contained aneuploid cells. Moreover, the percentage of aneuploid cells within the pancreatic carcinoma specimens was often lower than in aneuploid colon carcinomas, and only six tumors contained greater than 50% aneuploid cells.

Despite the lower percentage of aneuploid pancreatic neoplasms, 16 of the 20 tumors analyzed (80% \pm 9%) contained mutations in codon 12 of *c-Ki-ras* 2. Of tumors with *ras* mutations, 11 (69% \pm 12%) contained aneuploid cells, and 5 (31% \pm 12%) were diploid neoplasms. Mutations were not observed in codon 13 or within the remaining codons of the first exon of *c-Ki-ras* 2. There

Table 2. DNA content and *c-Ki-ras* codon 12 sequence of pancreatic adenocarcinomas.^a

Case	DNA content	K-ras codon 12 sequence	Amino acid codon 12
P-1	2.37	GTT	Val
P-2	2.2	GCT	Ala
P-3	2.3	CGT	Arg
P-4	3.3	GTT	Val
P-5	2.0	GTT	Val
P-6	2.0	GGT	Gly
P-7	2.0	GAT	Asp
P-8	2.0	GGT	Gly
P-9	2.2	GGT	Gly
P-10	4.0	GTT	Val
P-11	2.0	GGT	Gly
P-12	3.0	TGT	Cys
P-13	3.4	CGT	Arg
P-14	2.0	CGT	Arg
P-15	4.6	CGT	Arg
P-16	2.0	CGT	Arg
P-17	2.7	CGT	Arg
P-18	2.5	CGT	Arg
P-19	2.0	CGT	Arg
P-20	3.9	TGT	Cys

^aShown are the case number of the pancreatic carcinoma, the DNA content (2.0 = diploid), the sequence of *c-Ki-ras* codon 12 within the tumor, and the corresponding amino acid for the sequence.

Table 3. Spectrum of c-Ki-ras codon 12 mutations in pancreatic adenocarcinomas.*

Number analyzed	Percentage of tumors with mutation						Reference
	AGT (ser)	CGT (arg)	TGT (cys)	GAT (asp)	GCT (ala)	GTT (val)	
28	0	4	36	32	1	28	(8)
12	0	17	0	67	0	17	(11)
49	0	31	0	37	2	31	(10)
16	0	50	13	6	6	25	This report

*Shown are the number of neoplasms containing c-Ki-ras mutations, the percentage of these tumors with the indicated codon 12 sequence, and the published reference from which the data was obtained.

was no statistically significant correlation between the age or sex of the patient, the degree of differentiation, or the stage of the tumor.

The aneuploid pancreatic carcinomas containing *ras* mutations were further sorted into aneuploid and diploid subpopulations prior to PCR and sequencing. The same mutations were observed in the tumors before sorting and in the aneuploid populations analyzed after sorting.

In contrast to our results with sporadic colon carcinoma, where the predominant mutation consisted of a G to A transition at the first base pair site, a wider spectrum of mutations was observed within our pancreatic carcinomas (Table 3). The predominant mutations observed were G to C or G to T transversions. Our results are in agreement with investigators who suggest that the spectrum of mutations in sporadic colon carcinomas differs from that in pancreatic neoplasms; however, we do not see the predominance of G to T transversions that have been reported in lung carcinomas which harbor a c-Ki-ras codon 12 mutation (29,30).

Conclusion

This preliminary survey of three different human tumors indicates that even in adenocarcinomas of the gastrointestinal tract, there is diversity in both the frequency and spectrum of c-Ki-ras codon 12 mutations. Our data provide support for the suggestion that different carcinogens may be involved in producing *ras* mutations in sporadic colon carcinoma and pancreatic carcinoma and provide evidence for a different role for c-Ki-ras mutations in the genesis of neoplasms associated with sporadic colon carcinoma and carcinomas associated with ulcerative colitis.

The finding of G:C to A:T transitions as the predominant alteration in colon cancer suggests the possibility that these mutations result from errors in DNA replication or repair during the course of tumor progression. The finding of G to T transversions in pancreatic carcinomas could be indicative of a chemical alteration in DNA resulting in the formation of an apurinic site (31). The heterogeneity found in the spectrum of mutations in pancreatic carcinomas reported from different laboratories, however, underlines the need for studies with a large series of tumors before generalizations can be

made regarding the role of any specific carcinogen in the development of a human tumor.

This work was supported by the NIH NIDDK grant DK32971 to Glenna Burmer and Peter Rabinovitch, by the National Institute on Aging grant AG07359 to Glenna Burmer, and by the NCI grant R35CA39903 to Lawrence Loeb.

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