

Activation of c-Ki-ras in Human Gastrointestinal Dysplasias Determined by Direct Sequencing of Polymerase Chain Reaction Products¹

Stephen J. Meltzer,² Shrikant M. Mane, Patrick K. Wood, James H. Resau, Carnell Newkirk, John A. Terzakis, Burton I. Korelitz, Wilfred M. Weinstein, and Samuel W. Needleman

Gastroenterology [S. J. M., P. K. W.] and Hematology [S. W. N.] Divisions, Department of Medicine, and Department of Pathology [S. J. M., S. M. M., J. H. R., C. N., S. W. N.], University of Maryland School of Medicine, and University of Maryland Cancer Center [S. J. M., S. M. M.], Baltimore, Maryland 21201; Department of Pathology [J. A. T.] and Gastroenterology Division, Department of Medicine [B. I. K.], Lenox Hill Hospital, New York, New York 10021; Gastroenterology Division, Department of Medicine, University of California Health Sciences Center, Los Angeles, California 90024 [W. M. W.]; and Department of Medicine, Veterans Administration Medical Center, Baltimore, Maryland 21218 [S. J. M., S. W. N.]

ABSTRACT

Activation of c-Ki-ras by point mutation within exon 1 was studied in 33 specimens of dysplastic gastrointestinal lesions or of cancers presumed to arise from dysplasia. Samples were obtained from patients with underlying ulcerative colitis or Barrett's esophagus, two diseases associated with dysplasia and increased rates of colonic or esophageal adenocarcinoma, respectively. Genomic DNA was amplified using primers bounding this exon in the polymerase chain reaction. Polymerase chain reaction products were analyzed by direct dideoxy sequencing. Three point mutations in codon 13 of c-Ki-ras were found, all in colonic specimens (two high-grade dysplasias and one adenocarcinoma arising in ulcerative colitis). No point mutations were observed in the second exon of c-Ki-ras or in and around codons 12, 13, and 61 of c-N-ras and C-Ha-ras in a partial sampling of the specimens. These data indicate that ras family protooncogene activation is an uncommon event at this level of malignant progression in these disease states. Carcinogenesis in ulcerative colitis and Barrett's esophagus may proceed via different pathways than in sporadic colon cancer, perhaps involving loss or inactivation of suppressor genes.

INTRODUCTION

Ulcerative colitis and Barrett's esophagus are two diseases of the gastrointestinal tract characterized by increased incidence rates of colonic and esophageal adenocarcinoma, respectively (1-3). It is also typical, in these disease states, for mucosal epithelial dysplasia to develop prior to the occurrence of cancer (2-5). Dysplasia is believed to constitute a premalignant state with a high propensity to progress to carcinoma (4). It may thus represent the early stages of neoplastic progression in these illnesses (6).

The molecular basis of dysplasia is unknown. Increased rates of DNA synthesis (7) and of ras p21 expression (8) have been reported in dysplastic ulcerative colitis tissue, and elevated thymidylate synthesis rates have been noted in Barrett's esophagus (9). These disease states are therefore characterized by increased rates of DNA synthesis, perhaps as a result of rapid cell turnover induced by inflammation, which occurs in both illnesses. Rapid DNA synthesis may lead to an increased incidence of replication errors, including point mutations.

Point mutations in ras family protooncogenes, particularly c-Ki-ras, have been observed in 40% of colon cancers and in 10 to 50% of premalignant adenomatous colon polyps (10-13). When present in a colon cancer, they are usually also present

in benign adenomatous tissue adjacent to the cancer. These mutations thus appear to represent an early step in the evolution of sporadic colon cancer (14). We therefore undertook to determine how often they occur in flat dysplasia, an alternative premalignant phase in the evolution of certain cancers of the colon and esophagus.

MATERIALS AND METHODS

Frozen Tissues. Eight of the 12 esophageal specimens were obtained as endoscopic biopsies or from surgical resections, frozen within one-half h, and stored in liquid nitrogen until DNA extraction was performed. Four of the five esophageal adenocarcinomas and two of the seven dysplasia samples were obtained in this way. None of the ulcerative colitis specimens was obtained in frozen form. Parallel fixed and stained portions of all frozen esophageal tissues were read by a single morphologist (W. M. W.) and classified according to the method of Reid *et al.* (5).

Paraffin-embedded Tissues. Blocks of tissue from endoscopic or colonoscopic biopsies or from surgical resections were obtained from pathology archives at the UCLA School of Medicine, Lenox Hill Hospital, and the University of Maryland Hospital. Five of seven dysplastic Barrett's esophagus specimens and one of five esophageal adenocarcinomas were obtained in embedded form. All 21 dysplastic or cancerous ulcerative colitis specimens were read and graded by the same pathologist (J. A. T.) according to the system of Riddell *et al.* (4). These specimens had been fixed in varying fixative solutions and ranged in age from 3 mo to 5 yr.

Microdissection. Microdissection was performed on all 21 ulcerative colitis specimens and on the paraffin-embedded esophageal specimens. All microdissections were performed by the same operator (C. N.). Each block was sectioned completely into 10- μ m slices, and one of every 10 slices was stained with hematoxylin and eosin. All stained slides were reviewed by one of the authors (S. J. M.) to determine the areas to be selected. Only dysplastic (in samples containing only dysplasia) or cancerous (in samples containing cancer) regions were chosen. These regions comprised as little as 5% of the entire block and, thus, were extremely important in improving the sensitivity of the point mutation assay. The stained sections were used as templates on which the unstained neighboring sections were overlaid. A scalpel was then used to cut out and remove the desired regions of the slides. The architecture and morphology of the unstained sections were quite easily visible when a stained section was used as template. Tissue selected in this way was scraped off the slide and collected into a 500- μ l Eppendorf tube. Image analysis revealed that 90% of the nuclear area within the microdissections consisted of malignant epithelial nuclei, while only 10% was contributed by benign cells (mostly small lymphocytes).

DNA Extraction. DNA was extracted from both frozen and embedded tissues by the method of Goelz *et al.* (15). The average length of DNA isolated from frozen tissues was greater than 20 kilobases, while DNA isolated from paraffin-embedded tissues averaged 500 kilobases in length. Yields ranged from 10 to 1500 μ g of DNA from frozen tissues and from 0.5 to 50 μ g from paraffin blocks.

Oligonucleotide Primers. Twenty-nucleotide sequences near both ends of exons 1 and 2 of c-Ki-ras, c-N-ras, and c-Ha-ras were synthesized on an Applied Biosystems Model 380 DNA synthesizer. The

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² To whom requests for reprints should be addressed.

sequences used were selected in such a way as to minimize 3' primer complementarity and keep GC/AT percentages in the 40 to 60% range. Primers were also checked against the University of Wisconsin Genbank database to ensure that they were only homologous to the desired *ras* sequences. Second sets of primers not overlapping the first set were used when mutations were seen. The second sets of primers did not recognize any sequences within the PCR³ products generated from the original primer sets. In this way, cross-contamination of genomic DNAs with PCR product was nullified.

Polymerase Chain Reaction. Five tenths to 2 μg of genomic DNA were subjected to 35 cycles of amplification using standard protocols (16, 17). In some cases, a modification of these protocols allowing direct sequencing without purification of PCR products was used (18).

Sequencing. A modification of the method of Engelke *et al.* (19) was used. Briefly, 20 ng (3 pmol) of PCR primer were end labeled with T4 polynucleotide kinase and [γ-³²P]ATP. Nested primers were not used, since perfectly adequate results were obtained with the primers used for the PCR. The labeled primer was annealed on ice with 100 ng (3 pmol) of heat-denatured PCR product. The annealing mixture was aliquoted into 4 separate termination reactions, one each for dideoxy G, A, T, and C. Terminations were performed for 3 min at room temperature followed by 2 min at 37°C. Reactions were stopped by the addition of 95% formamide/20 mM EDTA. Eight μl of each reaction were heated to 90°C for 4 min and then loaded onto 8% acrylamide/50% urea gels. Gels were run at a temperature of 55°C until being taken off for exposure. Exposures were performed at -70°C with Dupont Cronex intensifying screens from 15 min to 4 days, depending on band intensities.

Analysis of Data. Point mutations were defined as the visible presence of two bands at a given locus. All mutations were verified by sequencing at least twice in the same direction as well as by sequencing in the reverse direction using the downstream primer. In addition, mutations were verified by reamplification and resequencing utilizing a second, nonoverlapping set of primers. All mutations reported in "Results" below exhibited perfect concordance in these studies. The presence of more than two bands at a given locus was considered artifact. Wild-type bands were assumed to be contributed by the remaining (heterozygous) normal allele and/or by admixture of homozygous wild-type cells in the sample.

RESULTS

An example of point mutation in codon 13 of *K-ras* is depicted in Fig. 1. Both of these sequences represent dysplastic ulcerative colitis specimens; one contains the point mutation, while the other does not.

Results of direct sequencing of PCR products are summarized in Table 1. Activation of c-Ki-*ras* by G→A transitions in position 1 of codon 13 was observed in 3 of 33 dysplasias or cancers presumed to arise from dysplasia. All three mutations occurred in high-grade dysplasia or in frank adenocarcinoma. This finding implies that *ras* activation, when it occurs, may be a late event in ulcerative colitis. In addition, all mutations were seen in ulcerative colitis, with none being observed in Barrett's esophagus. It is possible that the source of the DNA samples contributed to this tendency; the ulcerative colitis specimens were microdissected, whereas the esophageal samples were not. Thus, the sensitivity of detection of mutations in the esophageal samples may have been compromised as a consequence of this fact. However, it is also possible that anatomic predilections to acquired genetic lesions in *ras* protooncogenes exist, or that these lesions occur in a disease-specific manner.

DISCUSSION

Activation of *K-ras* at codons 12 and 13 has been observed frequently in colon cancers and polyps (10-13). It is of note

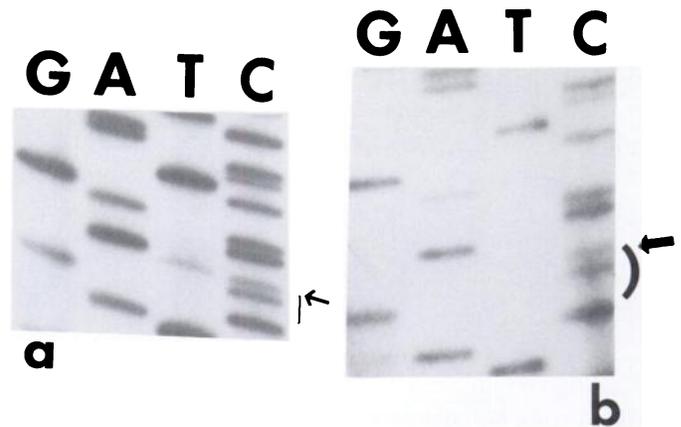


Fig. 1. DNA sequences of PCR products from two dysplastic ulcerative colitis specimens (Lanes a and b). The area amplified surrounds *K-ras* codons 12 and 13, with codon 13 indicated in brackets. The normal sequence of codon 13 (noncoding strand) is GCC, read from bottom to top. The sample on the left (Lane a) contains two bands at position 1 of *K-ras* codon 13: one in the normal "C" lane; and one in the "T" lane (arrow). The change from a C to a T results in substitution of serine for glycine at codon 13. The normal signal in the "C" lane is contributed by either the unmutated *ras* allele (if still present in the dysplastic cells), or by contaminating normal cells. The sample on the right (Lane b) contains only a wild-type band in the "C" lane (arrow). There is no mutation in this sample

that all 3 mutations reported here comprise G to A transitions. G to A transition mutations that cause *ras* activation in rodent mammary cancer have been shown to arise from exposure to alkylating carcinogens, through *O*⁶-alkyl-guanosine adduct formation (20). Dietary nitrosamines implicated as gastrointestinal carcinogens may act analogously on colonic epithelium, contributing to the mutations observed in these ulcerative colitis specimens. The present data show for the first time that these lesions do occur in flat dysplasias and in cancers presumed to arise from flat dysplasias. However, the frequency of *ras* point mutations in this study (10%) is much lower in flat dysplasias than that reported in polypoid colonic lesions (up to 50%). This finding suggests that neoplastic progression in flat colonic mucosa usually occurs by a different, as yet unknown, mechanism.

Numerous clinical characteristics of the dysplasias and cancers occurring in ulcerative colitis and Barrett's esophagus are in fact consistent with a unique tumor biology. Both of these diseases are distinguished by underlying chronic inflammation, as opposed to sporadic colonic polyps and carcinomas, which are not associated with inflammation. In the case of Barrett's esophagus, inflammation is caused by chronic gastroesophageal acid reflux, while the cause of inflammation in ulcerative colitis is unknown. True adenomatous polyps are uncommon in ulcerative colitis. Most cancers that occur in ulcerative colitis are associated with foci of flat microscopic dysplasia elsewhere in the colon at the time of resection. Dysplasia is often found at the margins of adenocarcinomas in this disease. Thus, circumstantial evidence supports the hypothesis that adenocarcinoma occurring in ulcerative colitis arises as a clone from a cell within a dysplastic lesion.

Esophageal adenocarcinomas share similar characteristics. At resection, they are almost invariably accompanied by adjacent dysplastic mucosa and also by benign Barrett's metaplasia. Thus, they appear to require two phases of evolution prior to becoming cancerous: (a) a metaplastic Barrett's phase, probably as a reparative response to inflammation and ulceration; (b) a dysplastic phase, usually occurring after years of prolonged reflux esophagitis. This biological behavior is also quite distinct from that of sporadic colon polyps and carcinomas, which do not pass through metaplastic or flat dysplastic phases.

³ The abbreviation used is: PCR, polymerase chain reaction.

Table 1 Tissue samples and ras codons analyzed

Genomic DNA was extracted from tissues, and ras codons were amplified using the polymerase chain reaction. PCR products were directly sequenced to determine the presence or absence of point mutations. Tissue classification was performed according to the methods of Reid *et al.* (5) for esophageal specimens and Riddell *et al.* (4) for colonic specimens.

Sample	Tissue classification	K12/13	K59/61	N12/13	N59/61	H12/13	H59/61
<i>Barrett's esophagus</i>							
E	High-grade dysplasia	WT*		WT	WT		
E10	High-grade dysplasia	WT	WT	WT	WT		WT
35	Adenocarcinoma	WT			WT		
36	Adenocarcinoma	WT	WT				
L	Indefinite, probably negative	WT					
205	Indefinite, probably negative	WT					
303	Intramucosal carcinoma	WT	WT	WT		WT	
354	Intramucosal carcinoma	WT	WT	WT	WT	WT	WT
351	Adenocarcinoma	WT		WT	WT		WT
393	Indefinite, probably positive	WT	WT		WT	WT	
398	Adenocarcinoma	WT	WT		WT		
CC	Adenocarcinoma	WT					
<i>Ulcerative colitis</i>							
84	Low-grade dysplasia	WT			WT		
88	High-grade dysplasia	Mutant (G → A, position 1)					
318	High-grade dysplasia	WT					
320	High-grade dysplasia	WT		WT	WT		WT
1	High-grade dysplasia	WT		WT		WT	
2	Indefinite, probably negative	WT					
3	Indefinite, probably positive	WT				WT	
4	Indefinite, probably negative	WT				WT	
1-1	Indefinite, probably positive	WT					
1-2	Adenocarcinoma	WT					
1-4	Low-grade dysplasia	WT					
1-10	Adenocarcinoma	WT					
1-11	Adenocarcinoma	WT					
1-12	Adenocarcinoma	Mutant (G → A, position 1)					
A	High-grade dysplasia	WT					
B	Low-grade dysplasia	WT				WT	
C	Low-grade dysplasia	WT				WT	
D	Low-grade dysplasia	WT				WT	
E	Indefinite, probably negative	WT				WT	
AA	High-grade dysplasia	Mutant (G → A, position 1)			WT		
BB	Low-grade dysplasia	WT			WT		

* WT, wild type.

Therefore, the current study suggests that unique events are involved in the process of neoplastic transformation in ulcerative colitis and Barrett's esophagus. Two recent studies have shown a low incidence of ras activation in esophageal cancer (21, 22). However, at least one of these studies (21) looked at squamous carcinomas, while the other study (22) did not specify any histological diagnosis. Squamous cancers do not usually arise from flat dysplasia occurring in Barrett's esophagus, but rather occur *de novo* in the setting of chronic tobacco and ethanol consumption. Thus, the low incidence of mutations in these studies is probably not related to the low incidence in esophageal adenocarcinoma and dysplasia.

Several mechanisms should be considered in the design of future studies. Loss of heterozygosity has been noted at several genetic loci in colonic adenomas and adenocarcinomas, most notably on chromosomes 5q, 17p, and 18q. These loci probably harbor suppressor genes whose loss allows cancer to develop or progress (23, 24). Full development of the neoplastic phenotype probably requires several steps, some of which involve activation of oncogenic sequences and some of which involve inactivation or loss of antioncogenic sequences (12). Since genetic losses are known to occur in premalignant colonic adenomas, they are also likely to be found in other premalignant tissues, such as dysplastic ulcerative colitis and Barrett's esophagus. It is quite possible that genetic loss(es) occur(s) early in the process of malignant transformation in these diseases as well.

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