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High Incidence of C-K-RAS Oncogenes in Human Colon Cancer Detected by the RNAse A Mismatch Cleavage Method

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ABSTRACT

*We have recently developed a new method to detect and characterize single base substitutions in transcribed genes which is based on the ability of RNAse A to recognize and cleave single base mismatches in RNA:RNA heteroduplexes. The RNAse A mismatch cleavage assay was applied to screen human colon carcinoma cell lines and primary tumors for the presence of mutant c-K-***ras** *oncogenes. We have determined that the mutant c-K-***ras** *allele is overexpressed and amplified relative to the normal in the SK-CO-1 human colon carcinoma cell line. The oncogene mutation has been characterized by this method as a glycine to valine substitution at codon 12 of the c-K-***ras** *gene. This result was confirmed by cloning and sequencing. We have previously reported that about 40% of primary human colon tumors contain c-K-ras genes mutant at codon 12 (Forrester et al, Nature 327: 298, 1987). We report here the characterization by molecular cloning and sequencing of the mutation in the c-K-***ras** *oncogene from two of these tumors (tumors 3 and 28). We also describe the histopathological characterization of these two tumors and demonstrate, by Southern blot hybridization of NIH3T3 transformants, the simultaneous presence of mutant c-K-***ras** *and N-***ras** *oncogenes in villous adenoma 28. Our results provide evidence for the frequent association of ras somatic mutational activation in the early stages of tumor development in this common type of human cancer.*

INTRODUCTION

Proto-oncogenes are a class of cellular genes which possess oncogenic potential. Genetic alterations of a diverse nature can lead to the activation of the malignant potential of cellular proto-oncogenes. The activated proto-oncogenes are known as oncogenes. Increasing evidence supports the concept that combinations of multiple genetic abnormalities, some of which result in the activation of cellular oncogenes, are intrinsic to the tumorigenesis process (see ref 1 for a review).

Three characterized members of the *ras* gene family (c-H-*ras*, c-K-*ras*, and N-*ras*) acquire direct oncogenic activity in certain cultured cells by point mutations in their coding regions (reviewed in 2). These mutations result in the synthesis of proteins containing single amino acid substitutions at preferential locations: codons 12, 13, and 61. Mutant *ras* oncogenes are frequently and reproducibly involved in the generation of defined tumors in some animal model systems (see ref 3 for a recent review). In addition to these qualitative changes, other genetic alterations resulting in increased gene expression are associated with the manifestation of the *ras* gene malignant potential (reviewed in 4). These findings support the hypothesis that *ras* oncogenes contribute in a dominant but dose-dependent manner to the multistage process of tumor development and progression.

Although *ras* oncogenes have been identified in a significant proportion of human tumors, their incidence and distribution according to tumor type have not been accurately determined. A reason for the lack of accurate information on the incidence of *ras* oncogenes in primary human tumors lies in the technical difficulties intrinsic to the methods used for the detection of single base substitutions in genes from organisms with large genomes.

We have previously developed a novel method to detect and characterize single base substitutions in transcribed genes (5) which offers several advantages over other methods currently available. The method is based on the ability of RNAse A to recognize and cleave single base mismatches in RNA:RNA duplexes. A homogeneously labeled antisense RNA probe is hybridized to total cellular RNA. The RNA:RNA duplexes are then digested with RNAse A and the resulting RNA fragments are analyzed on a denaturing polyacrylamide gel. Because the size of these fragments is dependent on the position of the mismatch in the RNA duplex, the method permits for the localization of the point mutations in the gene transcripts. A schematic representation of the method is outlined in Figure 1. We have applied this method to the analysis of the involvement of *ras* mutational activation in human cancer. We describe here our findings on the incidence of mutant c-K-*ras* oncogenes in human colo-rectal tumors.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection Experiments

The origins of the human tumor cell lines SK-CO-1, SW480, PR371, and normal foreskin fibroblasts (HNF) have been previously described (5). Human cells were grown in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum. NIH3T3 mouse fibroblasts were grown in DMEM containing 10% bovine calf serum. Transfection assays were performed using the calcium phosphate coprecipitation method (6,7) as previously described (8). Foci of morphologically transformed NIH3T3 cells were scored 3 weeks after transfection and individual transformants were isolated and grown in mass culture for further analysis.

RNA and DNA Extractions and Southern Blot Hybridizations

The sources of tumor tissue and diagnostic characterization have been previously described (9). RNA was prepared from cultured cells or from frozen tumors by sequential guanidine HCl-ethanol and phenol extractions as described (10). The quality of the RNA was examined by formaldehyde-agarose gels and staining with ethidium bromide. Only samples of tumor RNA showing the presence of ribosomal 28S and 18S RNA stained bands at approximate ratios of 2:1 were used for the RNAse A mismatch cleavage method. High molecular weight DNA was prepared from frozen tumors and cultured cells as described (8,11). Southern blot experiments were performed by transfer of restriction endonuclease-digested DNA to nitrocellulose membranes and hybridization with ^{32}P-labeled c-K-*ras* or N-*ras* specific DNA probes as described (10).

RESULTS AND DISCUSSION

Characterization and Expression of Mutant c-K-*ras* Genes in Human Colon Carcinoma Cell Lines

Using the RNAse A mismatch cleavage method (Figure 1), we have previously described the presence of single point mutations at codon 12 of the c-K-*ras* gene of SK-LU-1 lung carcinoma and SK-CO-1 colon carcinoma human tumor-derived cell lines, and we predicted that these mutations resided at the second position of the triplet (5). We have now characterized the mutation of SK-CO-1 tumor cells (Figure 2), using an antisense RNA probe corresponding to the first coding exon of the c-K-*ras* oncogene from SW480 colon carcinoma cells, which contains a G-T transversion at the second position of codon 12 resulting in the replacement by valine of the glycine encoded by the normal triplet (GGT) (17).

When total cellular RNA from SW480 and SK-CO-1 was hybridized to the antisense RNA probe corresponding to the first normal exon of the c-K-*ras* gene (anti GLY), digestion with RNAse A yielded, together with a 114 nucleotides protected band, RNA fragments of 67 and 46 nucleotides diagnostic of the presence of a point mutation at codon 12 of the c-K-*ras* transcript. These bands were also present after hybridization and digestion of RNA from PR371 lung carcinoma cell line, which contains a c-K-*ras* gene with a cysteine mutation (TGT) at codon 12 (11). However, RNA from normal fibroblasts only yielded a major 114 nucleotides band representing the full-length protected RNA fragment.

When hybridized to the antivaline mutant probe (anti VAL), RNA from normal fibroblasts was cleaved by the enzyme, yielding RNA fragments of 88 and 67 nucleotides diagnostic of a mismatch at codon 12. The difference in size of one of the mismatch-specific RNA fragments (46 or 88 nucleotides with the anti GLY or anti VAL probes, respectively) is due to the presence of 5' untranslated exon sequences in the valine mutant probe. The c-K-*ras* transcripts present in PR371 RNA were cleaved to almost completion by the enzyme generating the diagnostic 88 and 67 nucleotides subbands. However, these bands were absent when RNA from SW480 cells was hybridized and digested under the same conditions, and only a major band of 154 nucleotides, corresponding to the full-length protected RNA fragment, was apparent. These results indicate the absence of normal c-K-*ras* transcripts in SW480 colon carcinoma cells, in agreement with the results obtained by cDNA cloning and sequencing (17). A minor band of about 114 nucleotides corresponding to the unspliced c-K-*ras* transcript and other faint nonspecific bands were also

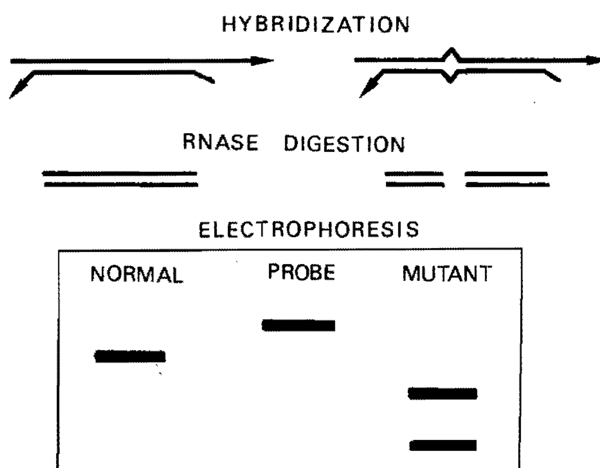


FIGURE 1. Schematic Representation of the RNAse A Mismatch Cleavage Method

apparent. SK-CO-1 RNA was mostly protected from the nuclease attack although a minor proportion of the hybrids were also cleaved, generating the 88 and 67 nucleotides codon 12 mismatch-specific subbands.

These results indicate that the normal c-K-*ras* allele is present in SK-CO-1 cells. These results also predict that the mutation in the c-K-*ras* gene of the SK-CO-1 cell line is the same valine mutation as that present in the SW480 cell line. This prediction was confirmed by cloning and sequencing the first coding exon of the c-K-*ras* gene from SK-CO-1 cells (Figure 3), demonstrating the applicability of the RNAse A cleavage method to characterize single point mutations in transcribed genes.

Comparison of the extent of cleavage by RNAse A of heteroduplexes generated by hybridization with the normal or mutant RNA probes indicated that, similar to the situation in Calu-1 and PR371 lung carcinoma cell lines (5), the mutant c-K-*ras* allele is overexpressed (2 to 3 fold) relative to the normal allele in SK-CO-1 colon carcinoma cells (Figure 2). Analysis of the c-K-*ras* alleles using restriction endonuclease polymorphisms in the c-K-*ras* locus (5), revealed that the mutant allele is amplified 2-4 fold relative to its normal counterpart in SK-CO-1 tumor cells (data not shown).

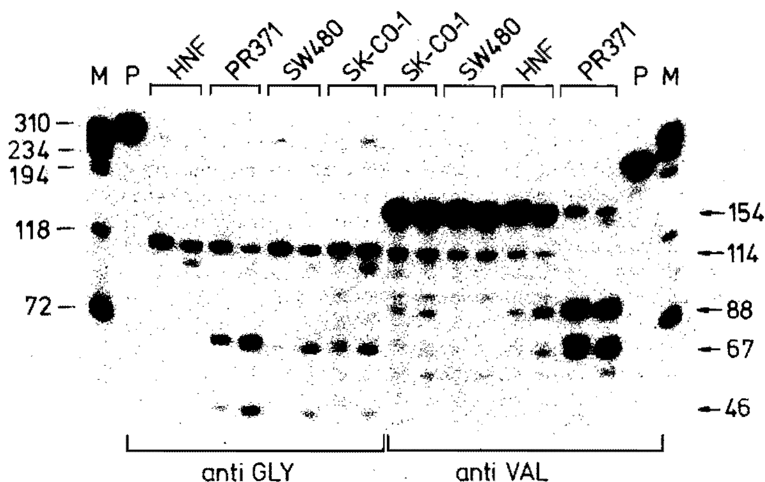


FIGURE 2. <u>RNAse A Mismatch Cleavage Analysis of c-K-*ras* Transcripts from SK-CO-1 Colon Carcinoma Cell Line.</u>

Thirty micrograms of total cellular RNA from the cell lines indicated at the top were hybridized with ^{32}P labeled antisense RNA (10^5 cpm) directed by pAK1Ngly and pAK1Mval vectors as described in Experimental Procedures. RNA hybrids were digested with RNAse A for 30 and 90 minutes (shown from left to right) at 30°C and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. HNF: human normal foreskin fibroblasts. P: undigested RNA probes. Numbers at the left indicate the position and size (in nucleotides) of OX1794 DNA *Hae*III restriction fragments used as molecular weight markers (M). Arrows at the right indicate the size of the RNA fragments generated by RNAse A digestion of the hybridized transcripts.

Gene Cloning and Sequencing

DNA fragments containing the first coding exon of the c-K-*ras* gene from tumors 3 and 28 and from the tumor cell line SK-CO-1 were isolated by constructing partial genomic libraries of *Hind*III digested DNA cloned into the lambda 47.1 phage vector and screening with c-K-*ras*-specific DNA probes as described (11). The 15Kb *Hind*III fragment of the c-K-*ras* gene from tumor 3 was isolated from DNA of a NIH3T3 primary transformant, while the gene fragments from tumor 28 and cell line SK-CO-1 were isolated directly from the human tumor cell

DNA. Three recombinant phages were isolated from the NIH3T3 transformant library, and one recombinant clone was isolated from each of the human DNA libraries. The 280 bp *Sau*3A-*Pst*I fragments containing sequences of the first coding exon of the c-K-*ras* gene from tumors 3 and 28 and the 1.4Kb *Stu*I-*Xba*I fragment containing the first coding exon of the gene from the SK-CO-1 cell line were subcloned into the *Bam*HI-*Pst*I and the *Sma*I-*Xba*I sites of the pGEM-4 vector (Promega Biotec.), respectively. The first coding exons of the c-K-*ras* gene from these human tumor cells were sequenced by the Maxam-Gilbert chemical method as described (11).

Plasmid Constructions and *in vitro* Transcriptions

A plasmid directing the synthesis of antisense RNA containing a valine mutation at codon 12 of the c-K-*ras* gene (pAK1Mval) was constructed by subcloning the 154 bp *Sau*3A-*Pst*I fragment of p3035 (obtained from Oncor) into the *Bam*HI-*Pst*I sites of the pSP65 vector (Promega Biotec.). p3035 is a full length cDNA clone of the c-K-*ras* oncogene from the SW480 colon carcinoma cell line (12). Construction of plasmids pAK1Ngly and pAK1Mser has been previously described (5,9). The 200 bp *Sau*3A-*Hind*III fragment corresponding to the first coding exon of the human N-*ras* gene (13) was similarly cloned into the *Bam*HI-*Hind*III sites of pGEM4, generating pAN1Ngly. The N-*ras* fragment was subcloned from pNR9.2, a plasmid containing the 5' 9.2Kb *Eco*RI fragment of the human N-*ras* gene cloned into the *Eco*RI site of pAT153 (13). Plasmid pKRSm2.6 contains the 2.6kb *Eco*RI-*Sma*I fragment corresponding to the 5' distal region of the c-K-*ras* gene (5).

Radioactive RNA probes were synthesized with the SP6 Polymerase *in vitro* transcription system (14) as previously described (5). One microgram of the appropriate plasmid was linearized with *Hind*III and incubated with SP6 RNA Polymerase (Promega Biotec.) in the presence of 0.5mM each of ATP, GTP, and UTP and 12uM of CTP and ^{32}P-CTP (Amersham, 10mCi/ml, >400Ci/mmol) for 60 minutes at 40°C. After removal of unincorporated nucleotides by ethanol precipitation, the RNA probes were purified by denaturing polyacrylamide gel electrophoresis (15). We found that this step eliminated some unspecific background bands originating from the presence of foldback products generated during the *in vitro* transcription reaction (14,16). Gel slices containing the synthesized RNA were transferred to sterile microfuge tubes and shaken at 37°C for three hours in 375ul of 2M NH_4OAc and 0.1% SDS. The samples were centrifuged and the supernatants were precipitated with ethanol in the presence of carrier tRNA. The pellets were washed with ethanol, lyophilyzed and dissolved in hybridization buffer (5). The RNA probes were stored at -70°C and used within 4 days.

Hybridizations and RNAse A Digestions

Total cellular RNA was hybridized to the appropriate antisense ^{32}-P-labeled RNA probe (10^{5} cpm) for 12-18 hours at 50°C and digested with RNAse A (Type IIIA, Sigma or PL Biochemicals) essentially as previously described (5). For RNAse A treatment, the enzyme concentration, length of digestion and incubation temperature were adjusted depending on the nature of the mismatches present in the RNA hybrids. Generally, digestions using RNAse A at concentrations of 20-40 ug/ml at 25-30°C for 30-60 minutes were sufficient. RNAse treatment was terminated by digestion with Proteinase K (Sigma) in the presence of SDS and the protein was removed by phenol extraction. The RNA was ethanol precipitated, and analyzed by denaturing polyacrylamide gel electrophoresis as described (5).

Detection of Mutant c-K-*ras* Genes in Human Primary Colon Tumors

We have screened a large panel of primary human colon tumors for the presence of c-K-*ras* genes with mutations in its first coding exon using the RNAse A mismatch cleavage method. Total cellular RNA prepared from frozen tumors was hybridized to an antisense RNA probe. This probe spans most of the c-K-*ras* first coding exon, from the *Sau*3A site to the 5' splice acceptor site and approximately 180 additional nucleotides of upstream intron sequences (5) an encodes either a glycine or serine at position 12 (5,9). The hybrids were digested with RNAse A and analyzed by denaturing polyacrylamide gel electrophoresis.

Hybridization of this antisense RNA probe to mature normal c-K-*ras* mRNA

TABLE 1.

Incidence of c-K-*ras* Oncogenes in Primary Human Colon Tumors

a: Type of primary colon tumor. b: Position of the mutated nucleotide in the normal triplet (GGT) at position 12 of the c-K-ras gene. N: A, T, or C. c: Number and total percentage of mutations detected at either the first of second position of codon 12 by the RNAse A mismatch cleavage method. d: number and total percentage of c-K-ras oncogenes as determined by the NIH3T3 transfection assay. e: Grade of invasiveness of the tumors following Duke's classification (18). The numbers are positive tumors/total tumors analyzed in each group. 0, non-invasive tumor. f: Metastases present (+) or absent (-) at time of surgery. The numbers are positive cases/total cases analyzed.

TYPE [a]	NUMBER	MUTATION [b]	RNAseA [c]	NIH3T3 [d]	GRADE [e] 0	A	B	C	D	METASTASES [f] -	+
ADENOCA.	68	CODON 12	27/68 40%	7/35 20%	1/1	5/13	9/23	10/21	2/4	15/37	12/27
		NGT	9	2	1	3	2	3	0	6	3
		GNT	18	4	0	2	7	7	2	9	9
(VILLOUS ADENOMAS)	(8)		(7/8) (87%)	(3/5) (60%)	(1/1	4/5	1/1	1/1	0/0	6/7	1/1)
		NGT	5	3	1	3	0	1	0	4	1
		GNT	2	0	0	1	1	0	0	2	0
ADENOMAT. POLYPS	6		1/6 16%	ND	NA	NA	NA	NA	NA	1/6	NA

followed by digestion with RNAse A generates a protected RNA duplex of 114 bp. When the probe is annealed to c-K-*ras* transcripts containing single base substitutions at codon 12, digestion with the enzyme generates fragments of 67 and 46 nucleotides indicating the presence of a mutant c-K-*ras* gene at this position (see Figure 2). The results obtained in our studies with a series of 68 primary colon tumors has recently been reported (9) and are summarized in Table 1.

Fragments of the c-K-*ras* genes from two tumors which scored positive in our assay (tumors 3 and 28) were molecularly cloned and their first coding exons sequenced. The result of these experiments confirmed the presence of point mutations at codon 12 of the c-K-*ras* gene in these tumors. The mutations were characterized as cysteine (TGT) and serine (AGT) substitutions, respectively (Figure 3). The activation of the transforming potential of the c-K-*ras* gene by

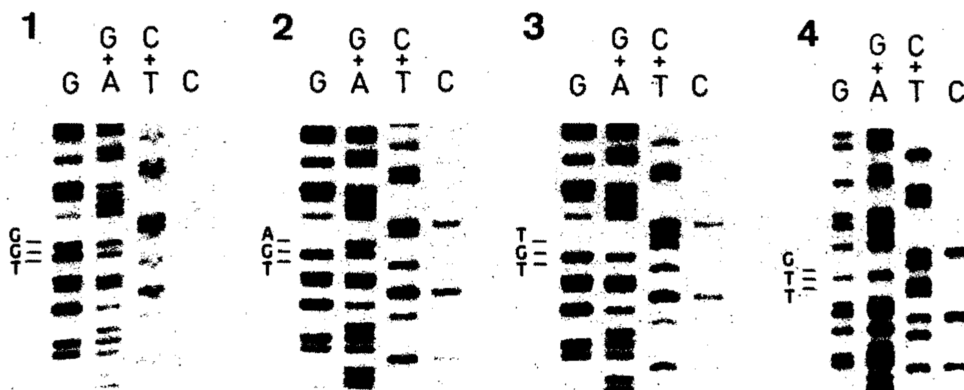


FIGURE 3. Sequence Analysis of the Mutant c-K-*ras* Genes from Colon Tumors 3 and 28 and from Colon Carcinoma Cell Line SK-CO-1.

Autoradiography of sequencing ladders of the c-K-*ras* first coding exon from colon tumors 28 (2), 3 (3) and colon carcinoma cell line SK-CO-1 (4). Parallel sequence analysis of the first coding exon of PR310 lung carcinoma cell line, which contains a c-K-*ras* gene normal at position 12 (11) is also shown (1). The nucleotide sequence of codon 12 is indicated at the left.

these point mutations was demonstrated by transfection of chimeric c-K-*ras*/c-H-*ras* genes into NIH3T3 cells. The chimeras contained the first coding exons of the c-K-*ras* gene from tumors 3 or 28 (data not shown). These results demonstrate the validity of the RNAse A mismatch cleavage method for the diagnostic detection of *ras* genes containing single base point mutations in primary tumors.

c-K-*ras* Mutational Frequency in Primary Human Colon Tumors

Our results indicate that about 40% (27 of 68) of primary colon tumors contain c-K-*ras* genes mutant at position 12 (9). Mutant c-K-*ras* genes were not detected in RNA prepared from normal colonic tissue from the same patients and no mutations were found at other positions within the first coding exon. Thus, the location of somatic point mutations in the c-K-*ras* gene is specific for spots that activate its transforming potential (i.e. codon 12). Because the RNAse A mismatch cleavage method is not dependent on the position of the mutation in the gene transcript covered by the RNA probe, this result argues against the hypothesis that mutations in *ras* genes are a secondary consequence of the higher mutation rate of tumor cells (19). If this were the case, random mutations in other regions of the gene should have been detected. Instead, our results strongly support the concept that *ras* somatic mutational activation is directly associated with the tumorigenesis process.

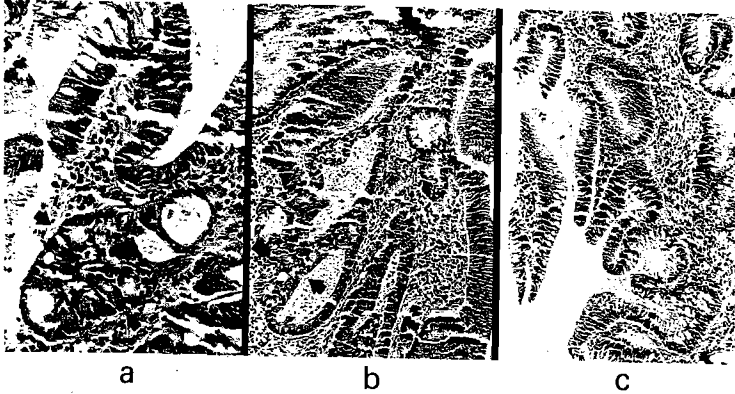
The RNAse A mismatch cleavage method is more sensitive in detecting c-K-*ras* oncogenes in primary tumors than the NIH3T3 focus formation assay. Parallel experiments showed that DNA from only 20% of the tumors was able to induce the morphological transformation of NIH3T3 cells. Unspecific degradation of tumor DNA (due to necrosis or insufficient preservation of frozen tumor tissue) might lead to a decrease in its transfection efficiency to levels below detection in the focus assay. It is noteworthy that we were able to detect mutant c-K-*ras* oncogenes with our method in frozen tumors which included contaminating normal stromal tissue, without prior selection for carcinoma tissue through histopathological analysis. We estimate that our method can detect the presence of c-K-*ras* oncogenes in solid tumors containing a minimum of 5-10% mutant transcripts in the total tumor RNA population.

No significant differences were observed between the mutation frequency at codon 12 of the c-K-*ras* gene and the degree of differentiation or stage of progression of the tumors. Mutant genes were found in tumors ranging from early stages of progression to very advanced stages of disease (Table 1). However, a striking difference was apparent between the position of the mutation at codon 12 of the c-K-*ras* gene and the degree of invasiveness of the tumors. While 4 of 9 tumors mutant at the first position of codon 12 (NGT) were classified as being at an early stage of progression (Duke's O and A), 16 of the 18 tumors mutant at the second position of the triplet (GNT) were classified as being more invasive (Duke's B, C, and D). Whether these differences are relevant in a clinical, prognostic context remains to be determined.

Mutational Activation of c-K-*ras* Genes in Colon Tumors at Early Stages of Progression

Another interesting finding derived from our studies was the detection of mutant c-K-*ras* oncogenes in premalignant villous adenomas. Villous adenomas or villoglandular polyps are relatively common types of benign solitary tumors which emerge directly from the colon mucosa as large superficial neoplasms most frequently in the rectum and rectosigmoid area. The incidence of malignant change of these types of benign tumors is higher than in adenomatous polyps. Villous adenomas frequently contain focal areas of malignant conversion to carcinoma cells, but are considered malignant only when infiltrating activity can be demonstrated by microscopic analysis (20).

Of the eight colon tumors associated with villous adenomas that we have analyzed, seven contained mutations at codon 12 of the c-K-*ras* gene, five in the first position, and two in the second (Table 1). The eight tumors analyzed were collected at different stages of progression. Tumors 3 and 28 were obtained at a very early stage of development. Tumor 3 was classified as a moderately differentiated adenocarcinoma arising within a villous adenoma, but the carcinoma



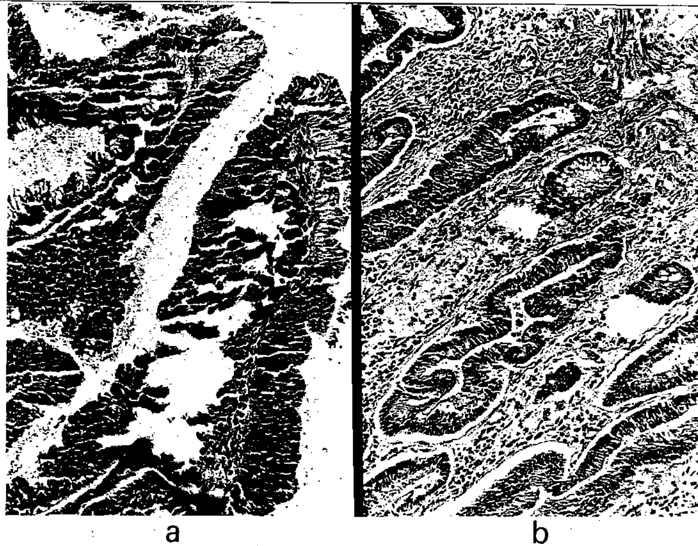


FIGURE 4. Histopathological Analysis of Premalignant Human Primary Colon Tumors

A) Histological photograph of villous adenoma 28. The features of a villoglandular polyp are demonstrated in an frozen section from the area adjacent to the tissue analyzed by the RNAse A mismatch cleavage method. Villi are apparent in panel c (x200) and complex glandular structures consistent with carcinoma *in situ* (CIS) can be seen between the black arrows in two areas of the polyp in frames a and b (x400 and x200, respectively).

B) Histological photograph of tumor 3. The overall diagnosis was of an invasive adenocarcinoma arising from a villoglandular polyp. The material remaining from the sample used for RNA and DNA preparation was prepared for frozen section. The section demonstrates villi in Frame a (x200). No invasive adenocarcinoma or carcinoma *in situ* is apparent (Frame b).

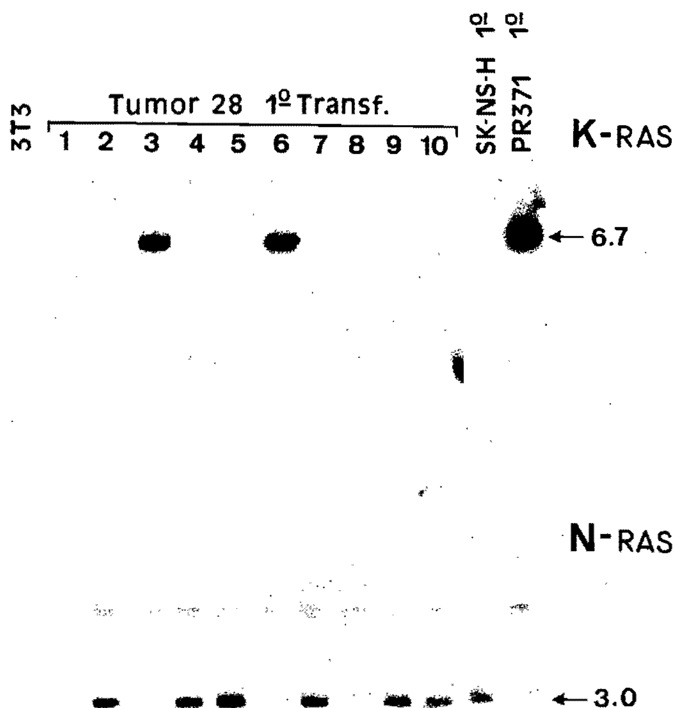


FIGURE 5. Southern Blot Hybridization Analysis of NIH3T3 Transformants Induced by DNA from Villous Adenoma 28.

Ten micrograms of high molecular weight DNA from NIH3T3 primary transformants induced by DNA from villous adenoma 28 (lanes 1 through 10), from NIH3T3 cells (3T3), from a NIH3T3 primary transformant induced by DNA from the human neuroblastoma cell line SK-NS-H, and from a NIH3T3 primary transformant induced by DNA from the human lung carcinoma cell line PR371, were digested with either *Eco*Rl (K-*ras*) or *Pst*I (N-*ras*) and electrophoresed through a 1% agarose gel. The DNA fragments were transferred to a nitrocellulose filter and hybridized to either ^{32}P-labeled pKRSm2.6 K-*ras* specific probe or to pANlNgly N-*ras* specific probe.

did not invade beyond the submucosal layer. Histopathological analysis of tumor 28 revealed a villous adenoma with focal areas of non-invasive carcinoma *in situ* and was classified accordingly as a pre-malignant villous adenoma.

Fragments of tumors 3 and 28 adjacent to the tissue used for RNA and DNA preparation were resubmitted for microscopic analysis. One of two fragments of tumor 28 contained carcinoma *in situ* (Figure 4A), while the other fragment from tumor28 and the fragment from tumor 3 (Figure 4B) contained only villous adenoma tissue. These results confirm the original classification of these tumors and indicate that mutant c-K-*ras* genes were present in the premalignant adnomatous tissue.

We also have analyzed six adenomatous polyps, only one of which scored positive in the RNAse A mismatch cleavage assay (Table 1 and K.F., C.A., M.P. in preparation). Interestingly, the polyp which was positive for a mutant c-K-*ras*

gene by our assay was the only one that already contained carcinoma tissue. Although interpretation of these results will have to await further studies, a possibility is that while somatic *ras* mutational activation may be involved in the generation of premalignant villous adenomas, it may occur after of during the transition from adenoma to carcinoma in adenomatous polyps or tubular adenomas.

Coexistence of c-K-*ras* and N-*ras* Oncogenes in Villous Adenoma 28

Our studies also revealed the simultaneous presence of both c-K-*ras* and N-*ras* activated oncogenes in human colon tumors (9). RNAse A mismatch cleavage analysis of tumor 28 revealed the presence of a mutant c-K-*ras* oncogene in this villous adenoma which was confirmed by cloning and sequencing (Figure 3). However, parallel analysis of the transforming activity of tumor DNA by the NIH3T3 focus formation assay revealed the presence of a transforming gene in this tumor which was identified by Southern blot hybridization analysis of primary transformants as the N-*ras* oncogene.

Repeated analysis by the NIH3T3 focus assay (Figure 5) with DNA prepared from different tumor fragments confirmed the simultaneous presence of N-*ras* and c-K-*ras* oncogenes in villous adenoma 28. Thus, twelve out of sixteen total NIH3T3 transformants contained N-*ras* sequences, while four contained c-K-*ras* sequences (an example of these experiments is given in Figure 5). Although we cannot rule out the possibility that tumor 28 may have originated from two distinct cell populations, each one containing a different mutated *ras* oncogene, we interpret this finding as indicative of the accumulation of mutations in *ras* genes during the process of tumor progression. Therefore, mutational activation of *ras* proto-oncogenes may also occur at late stages of tumor development.

In summary, our studies have revealed a relatively high incidence of mutant c-K-*ras* oncogenes in human colon cancer supporting the concept that somatic *ras* mutational activation is playing an important role during the process of human tumor development and progression. In addition, the RNAse A mismatch cleavage method provides an alternative molecular diagnostic tool which could have prognostic application in human cancer.

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