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A human gastric carcinoma contains a single mutated and an amplified normal allele of the Ki-*ras* oncogene

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ABSTRACT

The DNA from various human tumors and tumor cell lines was screened for the presence of mutated ras oncogenes with synthetic oligonucleotide probes, as well as with the NIH/3T3 cell transfection assay. Among the various mutations found we discovered two novel Ki-ras mutations in codon 12: gly to ala and gly to ser. A gastric carcinoma was found to possess a single mutated Ki-ras allele (gly-12 to ser), as well as a 30-50 fold amplified normal allele. This implies that two activating steps must have occurred in this malignancy.

INTRODUCTION

The human ras genes have attracted a great deal of attention because of their possible role in the development of cancer. The gene family consists of three functional ras genes, Ha-ras, Ki-ras and N-ras which all three encode 21kD proteins closely related to each other (1,2). The proteins, which probably are present in all cells, are bound to the inner surface of the plasma membrane, bind GDP/GTP (3) and possess GTP-ase activity (4,5). Although their normal functions are unknown it has been suggested that, like G-like proteins, they transduce receptor-mediated growth control signals into the cell (4,5). In approximately fifteen percent of the human tumors one of the ras genes was found to be altered into a transforming gene detectable in the NIH/3T3 cell transfection assay (7). The first altered ras gene to be identified was the Ha-ras gene in the EJ bladder carcinoma cell line. It was found that this gene was activated by a single point mutations resulting in the substitution of glycine by valine at position 12 of the protein sequence (6). Other activated ras genes analysed subsequently were found to contain point mutations which alter either glycine at position 12 or glutamine at position 61 (7). Recently, alterations of the glycine residue at position 13 of N-ras were found in acute myeloid leukemia (8). In naturally occurring tumors no other mutations have been detected as yet,

although it has been shown in vitro that substitutions at codons 59 and 63 (9), as well as small deletions or insertions around codon 12 also generate transforming genes (10). Furthermore, when expressed at high levels a normal ras gene can also transform NIH/3T3 cells (11,24). Our knowledge about the occurrence of activated ras oncogenes and more specifically about the type of activating mutations is still very limited, mainly due to the laboriousness of the existing biological assays. Therefore, we have used in addition to the NIH/3T3 cell assay, synthetic oligonucleotide probes to detect and analyse activated ras oncogenes in several human tumors and tumor-derived cells lines. In one case of a gastric carcinoma we found both a mutated Ki-ras allele at position 12 as well as an amplified normal allele.

MATERIALS AND METHODS

Tumor cells and DNA transfections

DNA was transfected into NIH/3T3 cells as described (12) and foci were scored after 14 days. The colon-carcinoma cell lines described in this paper were obtained from Dr J. ten Kate and Dr. P. Meera Khan.

Synthetic oligonucleotide probes

The oligomers were synthesized by the solid phase triester method (13) ^{32}P labeling of the N-ras oligomer occurred by a primer extension reaction using an 8-mer primer as described (14). The Ki-ras oligomers were end-labeled using γ-^{32}P-ATP (5000 Ci/mMol Amersham U.K.) and T4 polynucleotide kinase. To that end 150 µg oligomer was incubated in a 10 µl reaction with 50 µCi of γ-^{32}P-ATP in 50 mM Tris, 10 mM MgCl, 5 mM dithiotheitrol and 0.1 mM EDTA at 37°C for 30 min. The incubation mixture was separated on a 10% sequence gel which can separate the labeled 20-mer for its unlabeled precursor.

Agarose gel electrophoresis and direct gel hybridization

Agarose gel electrophoresis and direct gel hybridization were as described (14). Hybridization with the N-ras oligomer probes was performed in 5x SSPE (1x SSPE=10 mM sodium phosphate pH 7.0, 0.18 M NaCl and 1 mM EDTA), 0.3% sodium dodecylsulfate (SDS) and 100 µg/ml sonicated, denatured E.coli carrier DNA (16 hr at 50°C). Hybridized gels were washed as follows: twice in 2xSSPE, 0.1% SDS at room temperature, once in 5xSSPE 0.1% SDS at 52°C (15 min) and once the same solution at 59°C for 5 min. The Ki-ras oligomers were hybridized in a solution containing 3.0M tetramethylammonium chloride, 50 mM Tris HCl, pH 7.5, 2 mM EDTA, 0.3% SDS and 30 µg/ml carrier DNA for 16 hr at 53°C. The filters were washed in the same solution at room temperature and subsequently for 15 min at 58°C.

RESULTS

DNA transfection analysis

Using transformation of NIH/3T3 cells as an assay for the presence of transforming genes we have analysed the DNA from various human tumors. Two of these were found to be positive in this assay, a gastric carcinoma DNA inducing 2 foci per 100 µg of DNA (100-fold lower than activated Ha-ras-containing DNA from the bladder carcinoma cell line EJ) and a bladder carcinoma which induced 15 foci per 100 µg of DNA. Secondary transfectants were isolated and subsequent Southern blotting analysis showed that the gastric carcinoma DNA contained an activated Ki-ras gene and the bladder carcinoma DNA an activated N-ras gene.

Oligonucleotide hybridization of the N-ras gene

To analyse the transfected N-ras genes derived from the bladder carcinoma we used synthetic 20-mer oligonucleotide probes. With these probes we are able to detect single-base-pair substitutions in the codons 12, 13 and 61 of the N-ras genes based on the fact the a fully matched hybrid between the oligomers and genomic DNA is more stable than a single-base-pair-mismatched hybrid. For detection of the mutations, mixtures of oligomers differing at only one position in a codon were used (Fig. 1). One oligomer of a mixture will then be able to hybridize and form a fully matched hybrid with each of the possible mutations in the varied base pair. For instance, the oligomer mixture N61-p2 consists of three oligomers, one of which will

N12-wt	gly	GGAGCAggtGGTGTTGGGAA
N61-wt	gln	TACTCTTCttgTCCAGCTGT
p1	glu	--------ttc---------
	lys	--------ttt---------
p2	arg	--------tcg---------
	leu	--------tag---------
	pro	--------tgg---------
p3	his	--------gtg---------
	his	--------atg---------
K12-wt	gly	CCTACGCCaccAGCTCCAAC
p1	cys	--------aca---------
	ser	--------act---------
	arg	--------acg---------
p2	val	--------aac---------
	asp	--------atc---------
	ala	--------agc---------
K61-wt	gln	TACTCCTCttgACCTGCTGT

Fig.1. Sequences of the 20-mer probes of the N-ras and Ki-ras gene. Sequences are from Taparowsky et al. (23) Capon et al., (16).

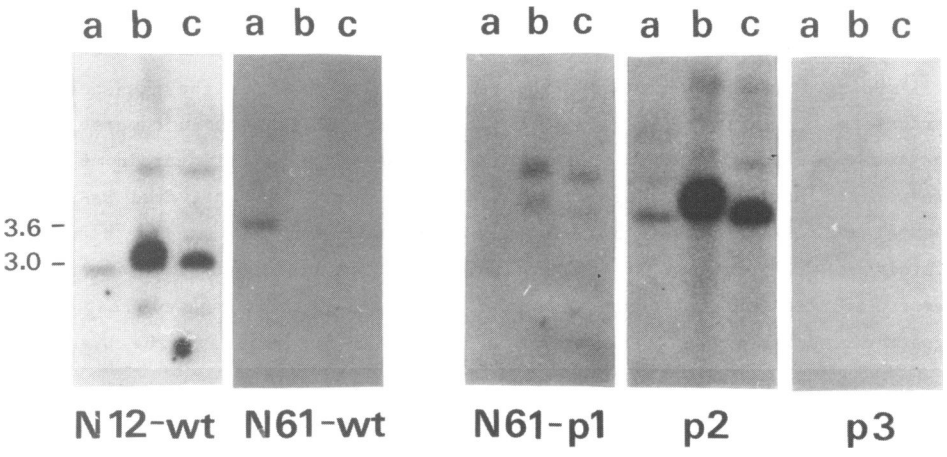

Fig.2. Hybridization of synthetic oligomers to genomic DNA of an human bladder carcinoma. 10 µg of DNA was digested with PstI and electrophoresed through 0.5% agarose. The different lanes contain DNA from the original bladder carcinoma (a) and DNAs from NIH/3T3 cell transfectants from this tumor (B and C); lane B was slightly overloaded). The various panels represent autoradiograms of identical gels after hybridization with various probes as indicated. Hybridization occurs to either a 3.0 kb (N12 probe) or a 3.6 kb (N61-probes) PstI fragment.

form a fully matched hybrid with an N-ras gene mutated at the second base of codon 61.

PstI-digested DNA from two independently isolated bladder carcinoma transfectants and from the original tumor was hybridized to a probe corresponding to the normal N-ras gene around codon 61 (N61-wt) and was washed under conditions in which only a fully matched hybrid is stable. In the resulting autoradiograph, a hybridizing signal was detected with the DNA from the original tumor but not with the DNAs of the transfectants (Fig. 2). This shows that the tumor DNA contains at least one N-ras allele with a normal sequence around codon 61 but that the transforming N-ras gene is mutated in this region. Hybridization analysis with the mutant probes for codon 61 showed strong hybridization of the N61-p2 group to the DNAs of the transfectants and a weak hybridization to the original tumor DNA (Fig.2). The very intense signal with DNA of the transformants showed that the transfected N-ras genes were amplified. This result implies that the transfected N-ras gene as well as one of the N-ras alleles in the original tumor contains a mutation at the second position of codon 61. Further analysis with individual members of the N61-p2 mixture showed that the codon 61

sequence of the activated N-ras gene reads CGA instead of CAA (not shown), and that consequently gln-61 had been replaced by arg.

Oligonucleotide hybridization of the K-ras gene

To analyse the transfected Ki-ras gene derived from the gastric carcinoma we have used a similar approach as for the N-ras gene described above. Mutation-specific Ki-ras probes (see Fig.1) were hybridized to genomic DNA of both the 3T3 transfectant and the original tumor. In addition, we have hybridized the probes to DNA of various colon carcinoma cell lines of which only SW480 had been reported to contain an activated Ki-ras allele with a mutation in codon 12 (GGT GTT, 15,16). A variant hybridization procedure was used. Instead of 0.9 M NaCl in the hybridization and washing buffers we used 3.0 M tetramethylammonium chloride. This reagent can, in the correct molar concentration, render the stability of a hybrid independent of its CG content (17). Thus a 20 base pair hybrid with 60% CG has the same stability as one with 40% CG (Tm=60°C). Furthermore the difference in the stabilities of a perfect hybrid and of a single-base-pair-mismatch hybrid is larger (Δ Tm= ~ 10°C) compared to those in 0.9 M NaCl (Δ Tm= ~ 5°C). This procedure, first developed by Wood et al. (18) and R.L. Cate (pers.comm.), is a very useful alternative for the hybridization in high salt. When PstI-digested DNA from the gastric carcinoma transfectant was hybridized to probes for the normal codons 12 and 61 we obtained a positive signal only with the K61-wt probe (not shown). This suggests a mutation in or around codon 12 of the Ki-ras gene. Direct hybridization of a PstI digest of DNA from the original tumor with both the normal or the mutant probes for codon 12 resulted in a very strong hybridization signal with the K12-wt probe, a weak signal with the K12-p1 probes and no hybridization signal with the K12-p2 probes (Fig. 3). This implies that the gastric tumor DNA contains one (or possibly a few) Ki-ras 12 allele with a mutation in the first base of codon 12, as well as a highly amplified normal K-ras allele. From hybridization with serial dilution we estimate the amplification to be 30 to 50-fold (not shown). To determine the exact mutation in codon 12 we have rehybridized the tumor DNA with the individual members of the K12-p1 mixture. It was found that the sequence of the mutated allele corresponds to the sequence AGT in stead of GGT in codon 12. This implies that serine has replaced glycine. To exclude the possibility that the hybridization with the AGT-specific oligomer probe is due to aspecific hybridization to the highly amplified normal allele we have hybridized DNA from gastric carcinoma transfected 3T3 cells with the K12-AGT (ser) probe as well as with the K61-wt probe. Both resulted in

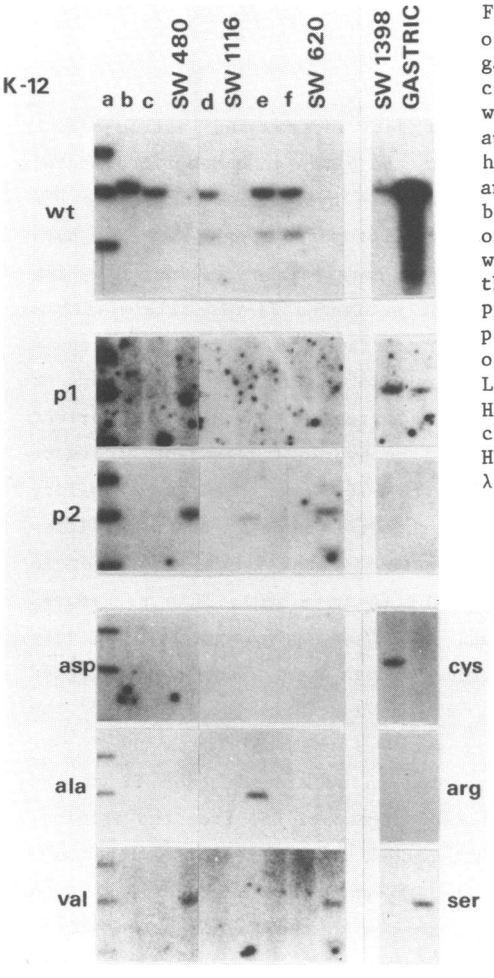

Fig.3. Hybridization of synthetic oligomers to genomic DNA of a human gastric carcinoma and of various colon carcinoma cell lines. DNA was digested with EcoRI. The top three panels show autoradiograms of identical gels hybridized with the K12-wt, K12-p1 and K12-p2 probes, respectively. The bottom three panels show autoradiograms of the same gels after hybridization with individual members of either the K12-p2 mixture (left part of the panel) or the K12-p1 mixture (right part of the panel). Hybridization occurs to a 6.7 kb EcoRI fragment. Lane a, contains DNA of the cell line HL60; lanes b-f contain DNA of the colon carcinoma cell lines SW48, SW948, HT29, COLO-205, LOB 205. Marker: phage λ digested with HindIII.

hybridization signals of equal intensity (not shown) indicating that the transfectant DNA contains the same G to A mutation as found in the original tumor DNA. In addition to the gastric carcinoma DNA we have analysed the DNA from various colon carcinoma cell lines for the presence of Ki-ras 12 mutations (Fig.3). Out of 7 cell lines analysed, three (SW480, SW620 and SW1116) contained DNA that did not hybridize with the K12-wt oligomers and hence, did not possess a normal Ki-ras allele. The K12-p1 probe mixture gave a positive hybridization signal with SW1398 DNA whereas the K12-p2 probe mixture showed positive signals with the DNAs of SW480, SW620 and SW1116. In all cases the exact basepair change was determined with individual oligomers

TABLE I: RAS GENE MUTATIONS

DNA	ras gene	mutated codon	amino acid
bladder ca.	N	$CAA_{61} \rightarrow$ CGA	arg
gastric ca.	Ki	$GGT_{12} \rightarrow$ AGT	ser
SW480	Ki	$GGT_{12} \rightarrow$ GTT	val
SW620	Ki	$GGT_{12} \rightarrow$ GTT	val
SW1116	Ki	$GGT_{12} \rightarrow$ GCT	ala
SW1398	Ki	$GGT_{12} \rightarrow$ TGT	cys

(Fig.3 and Table 1). From these results we conclude that SW480, SW620 and SW1116 possess homozygous mutations changing gly-12 to val, val and ala, respectively, and SW1398 a heterozygous mutation changing gly-12 to cys.

DISCUSSION

Specific oligonucleotides have shown to be a useful tool in the analysis of activated ras oncogenes (8,14). In combination with the NIH/3T3 cell transfection assay, it is not only possible to identify mutations at already known sites in codons 12 or 61 of the three ras oncogenes, but also to discover novel mutations. In this way we recently detected mutations in codon 13 in the N-ras gene of acute myeloid leukemia cells (8). We now report the detection of two Ki-ras 12 mutations not yet reported, one resulting in a gly to ser change in a gastric carcinoma, and another resulting in a gly to ala substitution in a colon carcinoma cell line.

Our most striking observation was that the gastric carcinoma possessed, apart from the ser-12-mutated Ki-ras allele, also a highly amplified normal Ki-ras gene. Since high levels of normal ras can also transform cells (11,24), it is reasonable to assume that amplification of the normal Ki-ras allele has contributed to the development of this gastric carcinoma. Ras amplification has also been detected in a bladder carcinoma (19) and in the breast carcinoma cell line MCF-7 (20). However, in these cases no additional mutated ras allele was found. Possibly, neither the serine mutation nor the amplification itself were sufficient for tumor development and the second activating step occurred during progression of the tumor. The second step could be either the amplification of the remaining normal allele or the mutation of one of the already amplified normal alleles. Another possibility, however, that cannot be excluded is that the tumor consists of two

different cell types, one with a K-ras mutation and one with a K-ras amplification. It is noteworthy that the same gly to ser mutation is also present in the viral Ki-ras gene but in this case in combination with an alteration at position 59. Whether the two ras gene activating steps in the gastric carcinoma are sufficient for tumor development is unknown. A similar two step activation could have occurred in a human lung carcinoma passaged through nude mice, where both a mutated and an amplified Ki-ras allele were present (21). In this case the mutated allele is amplified. (Y. Taya, personal communication).

A further observation is that lines SW480 and SW620 contain the same activating mutation. These two cell lines were independently derived from the same patient, SW480 from a primary colon carcinoma and SW620 from a metastatic tumor (22). This may indicate that the mutation was already present in the primary tumor.

A final observation is that the alanine and valine mutations in Ki-ras are homozygous, like two arginine mutations in Ki-ras 12 reported thus far (25). From a third Ki-ras 12 arg mutation in a squamous cell lung carcinoma it is not clear whether the mutation is present in only a part of the tumor or whether the mutation is heterozygous. In contrast, the cysteine mutations are heterozygous both in the SW1398 cell line and in the cell line Calu-1 (16). Possibly, some Ki-ras mutations are not sufficiently dominant and cells containing such mutations are more likely to lose their normal allele and become homozygous for the mutated Ki-ras as a result of selection. Further analysis is necessary to substantiate this hypothesis.

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