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A POINT MUTATION AT CODON 13 OF THE N-ras ONCOGENE IN A HUMAN STOMACH CANCER

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Summary: A surgically removed human stomach cancer with the histological diagnosis of poorly differentiated adenocarcinoma contained an activated N-ras oncogene detected by an in vivo selection assay in nude mice using transfected NIH3T3 cells. Analysis using synthetic 20-mer oligonucleotide probes revealed a point mutation from G to C at the first letter of codon 13 of the N-ras gene resulting in the substitution of arginine for glycine. This is the first observation of an activated N-ras oncogene in human stomach cancers.

One important approach to detect human transforming genes has involved DNA transfection. Human cellular transforming genes can be detected in tumors by their ability to induce the transformation of NIH3T3 cells (for review 1-3), a continuous murine cell line that is contact-inhibited and highly susceptible to DNA transfection (4). The use of in vitro focus forming assays to detect morphological transformation of NIH3T3 cells has shown that between 10 and 20% of human tumors contain activated forms of either c-H-ras, c-K-ras or N-ras (5-7). However, only an activated ras gene, which is a c-K-ras gene, has been detected so far in human stomach cancers (8). Activation of ras genes in most of the human tumors has been shown to result in a point mutation in either codon 12 or 61 of the gene (for review 5,6). Recently, the use of a direct in vivo selection assay has detected an unique form of a mutation at codon 13 of the N-ras gene that has not been reported before (9).In the present communication, we report activation at codon 13 of the N-ras gene in a human stomach cancer.

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MATERIALS AND METHODS

Transfection assay: High-molecular weight DNA was extracted from a surgically removed human stomach cancer (GC-1) with the histological diagnosis of poorly differentiated adenocarcinoma. The transforming activity of the DNA was assayed by transfection of NIH3T3 cells using an *in vivo* selection method as described (9). Briefly, 20 μg cellular DNA and 300 ng pSV2Neo were precipitated with calcium phosphate into each of five 60-mm plates seeded 1 day previously with 2×10^5 NIH3T3 cells. After 20-24 hr the co-precipitate was washed off and the cells were incubated in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% calf serum) for 12-24 hr. Each dish was trypsinized and cells were divided into three dishes containing selective medium with 400 μg/ml G418. After 7-10 days of growth in selective medium, 1.5×10^6 cells were then injected into each of two inguinal subcutaneous sites of nude mice (BALB/c-*nu*/*nu* mice, 5 weeks-old female, background congenitally athymic).

Southern blot analysis: DNAs isolated from tumors in nude mice were subjected to Southern blot analysis (10) for detection of human repetitive sequences and human proto-oncogene sequences. Ten micrograms of high-molecular weight DNAs were digested with restriction endonucleases, electrophoresed through 0.8% agarose gels, and blotted to nylon filters. The resulting blot was hybridized with a nick-translated ^{32}P-labeled DNA probe (7).

Synthetic oligonucleotide probes: Synthesis of oligonucleotide probes was carried out as described previously (9,11). The probes N13-wt (5'-GGAGCAGGTGGTGTTGGGAA-3'), N13-1C (5'-GGAGCAGGTCGTGTTGGGAA-3'), and N61-wt (5'-TACTCTTCTTGTCCAGCTGT-3') were synthesized using a 20-mer synthesized template and a 8-mer primer complementary to the 3' end of the 20-mer. This primer-template mixture was incubated with [α-^{32}P] dGTP, [α-32P]dTTP, cold dCTP and dATP, and DNA polymerase I. Because of the 5' phosphate groups on the 8-mer primer, the labeled oligomer was separated from the unphosphorylated template on a 10% sequence gel.

Direct gel hybridization: Ten micrograms of DNA was digested with PstI and electrophoresed on a 0.5% agarose gel. The gels were denatured in 0.4 M NaOH, 0.8 M NaCl, neutralized in 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, and dried. The dried gels were hybridized at 50°C with N61-wt or at 53°C with the other probes in 5 x SSPE (1 x SSPE is 10 mM sodium phosphate pH 7.0, 0.18 M NaCl, 1 mM EDTA), 0.3% SDS and 10 μg/ml sonicated salmon sperm DNA. Hybridized gels were washed in 2 x SSPE, 0.1% SDS at room temperature, in 5 x SSPE, 0.1% SDS at 53°C for 15 min and finally in the same solution at 59°C for N61-wt or at 63°C for the other probes for 5 min (9). Gel membranes were autoradiographed for 3-5 days using intensifying screens.

RESULTS

Identification of the N-ras oncogene: The resulting tumors arose 30-40 days after inoculation of transfected NIH3T3 cells in two of four mice. Eight normal human DNAs tested in the same way failed to give any tumors within 80 days. The two transformants (GC1-T1 and GC1-T2) were shown to contain a large number of human repetitive sequences hybridized to the probe Blur-8 (Fig. 1A). In view of evidence relating transforming genes of a number of human tumors to *ras* genes, we analysed the trans-

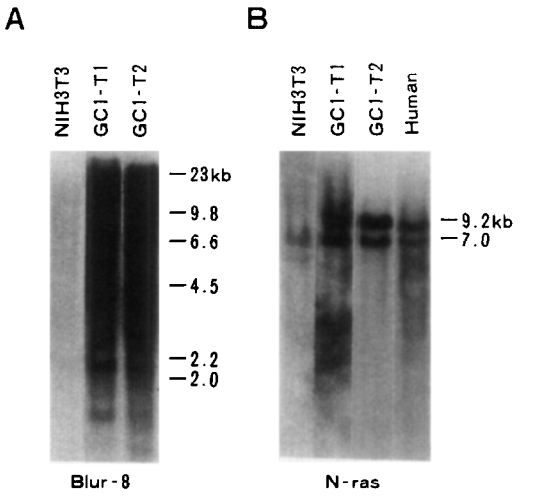

Figure 1. Identification of the N-ras oncogene in transformant DNAs derived from the CC-1 DNA. Ten micrograms of high-molecular weight DNA was digested with EcoRI, electrophoresed, blotted, and hybridized (A) to ^{32}P-labeled human repetitive DNA fragments purified from Blur-8 and (B) to ^{32}P-labeled N-ras-specific DNA fragments. Normal NIH3T3 DNA and normal human DNA were used as controls.

formant DNAs for these oncogene sequences. Human N-ras DNA fragments were detected in both transformant DNAs (Fig. 1B), suggesting that the transforming activity observed in the CC-1 DNA is derived from an activated N-ras gene.

Analysis of the N-ras oncogene by oligomer probes: To analyse the N-ras oncogene detected, oligomer probes were used. Using these probes we are able to detect single base pair (bp) substitutions in the N-ras gene based on the fact that a fully matched hybrid between the oligomers and the genomic DNA is more stable than a 1-bp mismatched hybrid (11). In the following hybridization experiments, we used two transformant DNAs as controls. The two transformant DNAs from tumor samples AML2-T1 and RAEB1-T1, which were obtained other transfection experiments, forms a fully match hybrid with the N13-wt and the N13-1C, respectively. The point mutation at codon 61 of the N-ras gene in the AML2-T1 DNA and that at codon 13 of the N-ras gene in the RAEB1-T1 DNA were previously detected by nucleotide sequence analysis (unpublished results). The probe N13-1C, corresponing to a point mutation from G to C at the first

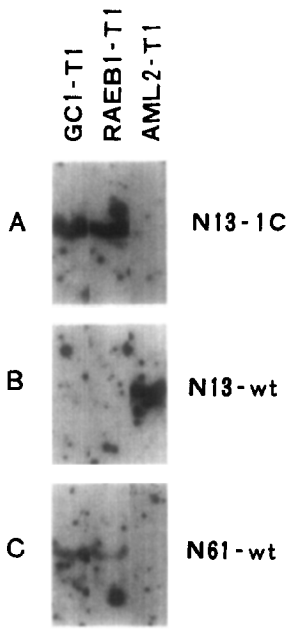

Figure 2. Analysis of the N-ras oncogene by oligomer probes. Ten micrograms of DNA was digested with PstI and electrophoresed. The gels were denatured, neutralized, dried, and hybridized (A) to N13-1C, (B) to N13-wt, and (C) to N61-wt. Hybridization occurred to either a 3.0 kb (N13-1C and N13-wt probes) or a 3.6 kb (N61-wt probe) PstI fragment.

letter of codon 13, formed a fully matched hybrid with the DNAs from GC1-T1 and RAEB1-T1 (Fig. 2A). In contrast, the oligomer N13-wt, corresponding to the normal sequence of the N-ras gene at codon 13, formed a fully matched hybrid with the AML2-T1 DNA but with none of the other DNAs (Fig. 2B). Further, the N61-wt probe, corresponding to the normal sequence at codon 61 formed a fully matched hybrid with the GC1-T1 and RAEB1-T1 but not with AML2-T1 (Fig. 2C). These results suggest that the GC1-T1 DNA contains a point mutation from G to C at the first letter of codon 13 of the activated N-ras gene.

DISCUSSION

In stomach cancers, the percentage of samples capable of transforming NIH3T3 cells upon transfection is much lower than those reported previously in other solid cancers (12). Only a few transforming genes so far reported in stomach cancers were a c-raf gene (13), a c-K-ras

gene (8), and two hst genes (12). Although most of the transforming genes detected by NIH3T3 transfection assays are members of ras gene family in various cancers (for review 5,6), only an activated c-K-ras gene has been detected in human stomach cancers (8). The present study is the first observation of an activated N-ras gene in a stomach cancer.

In a number of activated ras genes in human tumors that have been analysed by NIH3T3 transfection assays using an in vitro focus formation method, activation has been shown to result in a single nucleotide transition in either codon 12 or 61 of the gene (for review 5,6). However, in vitro mutagenesis experiments have shown that mutations at codon 13, 59 and 63 can also lead to transforming activity (14). Bos et al. have detected a mutation at codon 13 of the N-ras gene in four leukemia samples by in vivo selection assays (9). The N-ras genes in the four cases had a GAT or GTT triplet at codon 13, whereas the N-ras gene presented here contained a CGT codon 13. A standard in vitro focus formation assay revealed that NIH3T3 cells transfected with the cloned N-ras gene containing a CGT codon 13 did not show a striking change in morphology, and that they showed relatively slow proliferation (data not shown). Our results indicate the possibility of detection of activated ras genes with mutations other than those at codon 12 and 61 by transfection assays using an in vivo selection method in stomach cancers.

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