Amino-acid Substitution at Codon 13 of the N-ras Oncogene in Rectal Cancer in a Japanese Patient

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The activation of proto-oncogenes in colorectal cancers in Japanese patients was studied using a mouse NIH3T3 cell transfection assay system. Of thirty-five colorectal cancers examined, one rectal cancer showed an unusually high transformation efficiency and, in this rectal cancer, the N-ras oncogene was found to be activated. Nucleotide sequence analysis of the activated N-ras showed a single G → C point mutation at the first letter of codon 13, resulting in the coding of arginine instead of glycine. This amino-acid substitution at codon 13 may be responsible for the efficient induction of transformants of NIH3T3 cells in vitro.

Key words: Rectal cancer — N-ras gene — Codon 13 mutation

Studies by DNA-mediated gene transfer to mouse NIH3T3 cells have made possible the detection of transforming genes in human tumors. Approximately 20% of the DNAs of the human tumors examined were found to contain a mutationaly activated form of a ras family gene, either H-ras, K-ras or N-ras.1-4 In almost all cases, the activated ras gene was found to involve an amino acid substitution, of either glycine at position 12 or of glutamine at position 61, in the p21 protein. In addition, amino acid substitutions at positions 13, 59 and 63, produced by in vitro mutagenesis, were found to result in activation.5 Recently, alterations at codon 13 of N-ras have been found in human acute myeloid leukemia.6 However, there have been no reports about a mutation of codon 13 in other malignancies.

We examined the oncogenes of fresh samples of human colorectal cancers in an NIH3T3 transfection assay system and found an activated N-ras oncogene with a mutation of codon 13 in a rectal cancer.

MATERIALS AND METHODS

Tumor Materials Fresh colorectal cancer tissues were obtained at the time of their operation from patients at the National Cancer Center Hospital (Tokyo).

Cells Mouse NIH3T3 and al-1 cells were provided by Dr. M. Wigler, al-1 being a secondary transformant of NIH3T3 cells containing a high copy number of human activated H-ras from T24 bladder carcinoma cells.7 The NIH3T3 and the transformed cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% and 5% calf serum, respectively, in a humidified CO₂ incubator at 37°. The medium was changed twice a week.

DNA Transfection High-molecular-weight DNAs were extracted from pressed, frozen cancer tissues.

The DNA was transfected into NIH3T3 cells using the calcium phosphate precipitation method.8 For this, 2.5 X 10⁵ NIH3T3 cells were seeded onto a 10 cm Petri dish and, 48 hr later, were transfected with 30 μg of DNA. In each assay, a 60 μg sample of DNA was tested on two recipient cultures. Transformed foci were obtained after 14 to 21 days. Control assays using DNA from al-1 (positive control) and salmon testis DNA (negative control) were included in each experiment.

Southern Blot Analysis Cellular DNAs were digested with restriction endonucleases (Toyobo Co.) using a threefold excess of enzyme, as recommended by the supplier. The digested DNA (10 μg/lane) was subjected to electrophoresis in 0.7% agarose gel and blotted onto nitrocellulose filters by the method of Southern.9 For the detection of human Alu repeated DNA sequences, hybridization was performed in 50% formamide-0.8M NaCl-0.1M sodium PIPES (pH 6.8)-10% dextran sulfate-100 μg/ml of denatured salmon testis DNA-5 × Denhardt’s solution-5mM EDTA (pH 8.0)-0.1% SDS, at 42° for 16 hr with a nick-translated Alu-sequence. Similar conditions were used with probes other than the Alu-sequence, except that the concentration of NaCl was 0.65M, not 0.8M. The filters were washed 4 times with 2×...
SSC (1×SSC: 0.15M sodium chloride-0.015M sodium citrate)-0.1% SDS-0.2% sodium pyrophosphate, at 50° for 20 min periods.

Probes The following plasmid clones were used: HiHi380 (v-K-ras), BS9 (v-H-ras), BLUR8 (Alu-repetitive sequence), p6al (partial cDNA of human N-ras) and pP485-4 (human N-ras first exon including flanking sequences). Each plasmid was digested with appropriate enzymes, and probe fragments were isolated from agarose gels by electro-elution. The probes were labeled with \([\alpha^{32P}]dCTP\) (Amersham) by nick translation. The specific activities were approximately 10^6 cpm/ug.

DNA Sequence Analysis The DNA of a primary transformant (lane c in Figs. 1 and 2) was digested completely with EcoRI and then enriched by size-fractionation using sucrose gradient centrifugation. A genomic library was constructed using the phage vector EMBL4. This library was screened with the probe specific for the N-ras first exon, a 0.9 kbp PvuII fragment from pP485-4, by plaque hybridization. The DNA fragments to be sequenced were cloned into phage M13mp18 and propagated in E. coli JM 101 cells. Single-stranded DNA containing the inserts was prepared from recombinant phages and sequenced by the dideoxynucleotide chain-termination method with \([\alpha^{32P}]dATP\) (Amersham).

RESULTS

Transforming Activities of DNAs from Japanese Colorectal Cancers High-molecular-weight DNAs were isolated from the colorectal cancer tissues of thirty-five patients and transfected into NIH3T3 cells. About 25% of the colorectal cancer DNAs tested gave morphological transformants. The average

Fig. 1. Detection of human DNA sequences in NIH3T3 primary (A) and secondary (B) transformants derived from a rectal cancer. DNA (10 μg) from each transformant was digested with EcoRI, electrophoresed in 0.7% agarose gel and blotted onto nitrocellulose. The filter was probed with BLUR8. Lane a, DNA of NIH3T3 cells; lane b, DNA from al-1; lanes c–f, DNAs of primary transformants; lanes g and h, secondary transformants derived from the DNA shown in lane c. Lanes i and j, DNAs of secondary transformants derived from the DNA shown in lane d. DNA fragments of HindIII-digested DNA served as molecular weight standards (labeled in kbp).
transformation efficiency was about 0.01 focus/µg of donor DNA, which was much lower than that observed with the DNA from al-l cells (about 0.4 focus/µg). However, a DNA sample obtained from the rectal cancer tissue of a 79-year-old female patient showed an exceptionally high transformation efficiency of 0.48 focus/µg: 120 µg of DNA induced fifty-eight foci. This transformation efficiency was comparable with that of the DNA from al-l cells.

The transformation efficiencies of human solid tumors and human tumor cell lines have been found to be about one-fifth of that of al-l cells. Thus, the unusually high transformation efficiency of this rectal cancer prompted us to investigate its activated oncogene. When 120 µg samples of DNA from two of the fifty-eight primary transformants, which were Alu sequence-positive (see next subsection), were subjected to a second transfection assay, they induced fifty and fifty-six foci, respectively.

Detection of Repeated Human DNA Sequence To examine whether the transforming activity was due to the presence of human sequences, we tested fourteen primary transformants, and five and four secondary transformants, respectively, obtained from two different primary transformants for Alu-sequences, by Southern blotting using BLUR8 as the probe. All the DNAs tested contained human repetitive sequences (some are shown in Fig. 1). These DNAs each gave different hybridization bands, indicating that the foci were transformed independently.

Detection of the Dominant Transforming Gene First we tested whether the transforming activity of this rectal cancer was due to any member of ras family oncogenes using v-K-ras, v-H-ras and N-ras as probes. No extra sequences of the H-ras or K-ras oncogene, other than those which were mouse-specific, were detected in these transformants (data not shown). However, all the primary and secondary transformants tested had human N-ras sequences in addition to mouse sequences (Fig. 2). The N-ras probe, a SalI-NcoI fragment of p6al (human cDNA) which covers exons 2, 3 and 4, hybridizes to 9.2

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Fig. 2. Detection of sequences related to N-ras in NIH3T3 primary and secondary transformants derived from a rectal cancer. A Southern blot of EcoRI-digested DNAs was probed with a SalI-NcoI fragment of p6al, cDNA of human N-ras. Lane a, DNA of NIH3T3 cells; lane b, DNA of human placenta; lanes c–j and the molecular weight standards are the same as for Fig. 1.
kilobase pair (kbp) and 7 kbp DNA fragments in EcoRI-digested human DNA. The former includes exons 1 and 2, and the latter exons 3 and 4. However, the size of the N-ras hybridized DNA fragments, present in the EcoRI-digested DNA of the transformants, varied from 5 to 23 kbp. This size heterogeneity was probably due to the loss and gain of restriction enzyme sites in the flanking sequences of the human N-ras gene upon its integration into mouse DNA. Similar observations on N-ras have been reported.

DNA Sequence Analysis of Activated N-ras

Since activations of human N-ras by amino acid substitution at positions 12, 23, and also 13 have been reported, we analyzed the nucleotide sequences of exons 1 and 2, which could have included codons 1–96 of this activated N-ras gene, and compared the sequences with human N-ras protooncogene. The first exon was contained within a 300-bp HindIII fragment, and the second within a 450-bp PstI-BstEII fragment. The nucleotide sequence was identical to the normal human N-ras sequence with one exception. As shown in Fig. 3, the first exon had a single base change within the 13th codon of the predicted p21 protein. Instead of the normal triplet of GGT at codon 13, G → C transversion was detected at the first nucleotide. This should result in an alteration of glycine to arginine at this position in the p21 protein.

DISCUSSION

The DNAs from many human tumor cell lines and tumors have been shown to contain oncogenes responsible for inducing the malignant transformation of NIH3T3 cells. Activated ras family genes with point mutations mainly at codons for amino acids 12 and 61 of p21 protein have been detected by various researchers.

Activation of human N-ras, by amino acid substitution at position 12, was found in teratocarcinoma cell line PAI and in acute myeloid leukemia cells. Amino acid substitutions at position 61 were found in neuroblastoma SK-N-SH cells, lung carcinoma SW-1271 cells, fibrosarcoma HT1080 cells, rhabdomyosarcoma RD301 cells, pro-myelocytic HL 60 cells, rectal carcinoma 7060 cells and a bladder carcinoma. Amino acid substitution at position 13 was found in acute myeloid leukemia. Although activations of N-ras were found in fresh tissue of human colon cancers, the mechanism of activation has not been reported. In the present study, we found N-ras to be activated in a rectal cancer, and this activation to be due to a mutation within codon 13. Codon 13 of normal N-ras is GGT and encodes glycine, but that of activated N-ras is CGT and encodes arginine.

Recently, Bos et al. detected changes in codon 13 of N-ras in five cases of human acute myeloid leukemia by using an in vivo selection assay in nude mice and by the oligomer hybridization method. They observed two types of mutation in the second letter of codon 13: mutations from GGT to GAT and GTT, resulting in replacements of glycine by aspartic acid and valine, respectively.
claimed that these N-ras codon 13 mutants have limited ability for inducing foci in an NIH3T3 cell transfection system. In contrast, we found the N-ras, activated by replacement by arginine at codon 13, to be as efficient in producing transformants as al-1 cells, which have several copies of activated H-ras.\textsuperscript{39} The N-ras in the original rectal cancer DNA was not amplified and, in its flanking sequences, showed the same sites to be susceptible to the restriction enzymes, EcoRI, PstI, HindIII or PvuII, as those in human placenta DNA, indicating that there was no rearrangement within the region at least 5 kbp upstream of exon 1 (data not shown). It follows that differences in transforming activities in mutants of codon 13 could be due to the type of amino acid substitution. Furthermore, neither amplification nor rearrangement of c-K-ras-2, c-H-ras-1, c-myc or c-raf was detected (data not shown). It is very likely that the point mutation at codon 13 of N-ras was present in the original tumor, since the transforming efficiency of the DNA sample from the original tumor was quite high.

Barbacid and his collaborators found a new oncogene, trk, showing homology with tyrosine kinase, in fresh tissue taken from a human colon carcinoma.\textsuperscript{81} Since colorectal cancer is very common in Western countries and its incidence in Japan is also increasing, it is important to carry out the molecular analysis of its oncogene activation.

The present study suggested that mutants of codon 13 in N-ras may also be found in other neoplasmas, and may not be limited to acute myeloid leukemia or hematopoietic malignancies.

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