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A novel combination of *K-ras* and *myc* amplification accompanied by point mutational activation of *K-ras* in a human lung cancer

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Amplifications of two oncogenes, *c-K-ras-2* and *c-myc*, were found in a human lung giant cell carcinoma (LGCC) Lu-65, which is maintained in nude mice. The extent of *c-K-ras-2* and *myc* amplifications were estimated to be 10- and 8-fold, respectively, by means of the Southern hybridization procedure. In addition, NIH3T3 cells were transformed by transfection of Lu-65 DNA and the transforming gene was identified as *c-K-ras-2*. *c-K-ras-2* genes were cloned from a gene library of Lu-65 and a single point mutation causing a substitution of cysteine for glycine in codon 12 was found by DNA sequencing. It was concluded that the amplification of the *c-myc* and *c-K-ras-2* genes are accompanied by point mutational activation of *c-K-ras-2* in the human LGCC Lu-65. This is the first report of multiple gene amplification accompanied by a point mutation of oncogenes in human cancer cells, providing further support for the idea that co-operation of at least two activated cellular oncogenes is required for carcinogenesis.

Key words: oncogene/gene amplification/*K-ras* gene/*myc* gene/lung cancer

Introduction

Recent studies on oncogenes have shown that the activation of at least two cellular oncogenes is necessary for carcinogenesis. Transfection of normal rodent cells with oncogenes strongly suggested that two oncogenes, the *myc* and *ras* genes, must work co-operatively for the induction of cancer (Land *et al.*, 1983a; Ruley, 1983; Newbold and Overell, 1983). In addition, the activation of two oncogenes, *myc* and *Blym-1* (Goubin *et al.*, 1983; Diamond *et al.*, 1983) or *myc* and *N-ras* (Murray *et al.*, 1983), was found in some hematopoietic tumors. Therefore, it was of interest to investigate whether two oncogenes are activated in other kinds of human cancer. Here we report the activation of a novel combination of oncogenes, which was discovered in a human lung giant cell carcinoma (LGCC) during studies of the structural alterations of oncogenes in human cancer cells. We found that in this LGCC amplification of the *c-myc* and *c-K-ras* genes is accompanied by a point mutation in the 12th amino acid of the *c-K-ras* gene product.

Results

Amplification of two oncogenes, c-K-ras-2 and c-myc

Figure 1A shows that *c-K-ras* is amplified ~10-fold in LGCC Lu-65, which was maintained in nude mice. Similar degrees of amplification were observed in samples of DNA of Lu-65 at various passage levels (3, 7 and 8) in nude mice (data not shown). Amplification of another oncogene, *c-myc*, was also found in the same LGCC Lu-65 (Figure 1D), its amplification being ~8-fold, as estimated in similar experiments to those in Figure 1A, lane 7a – d (data not shown). No amplification of *H-ras* (Figure 1B), *N-ras* (Figure 1C), *src* or *mos* was observed (data not shown). From the calculated size of the hybridized fragments, the amplified *K-ras* was concluded to be *c-K-ras-2*, the only active *c-K-ras* gene (McGrath *et al.*, 1983; Shimizu *et al.*, 1983). No amplification of *K-ras*, *H-ras*, *N-ras* or *myc* was detected in other lung cancers or stomach cancers (Figure 1).

NIH3T3 cells are transformed by Lu-65 DNA

The transforming activity of Lu-65 DNA was examined by transfection assay as described previously (Sekiya *et al.*, 1983). When NIH3T3 cells were transfected with 200 μg of Lu-65 DNA, a clear focus was observed, whereas no focus was seen in the control. The same result was obtained in a repeat experiment. The presence of the human DNA sequence in this transformant was shown by hybridization with BLUR8 DNA (Figure 2).

An attempt was made to identify the transforming gene of Lu-65, as shown in Figure 3. Hybridization of the DNA of NIH3T3 cells transformed by Lu-65 DNA clearly demonstrates the presence of both the human (6.7 and 3.0 kb) and mouse (12 kb) *c-K-ras* gene in NIH3T3 cells transformed by Lu-65 DNA. In contrast, no human *N-ras* gene was found in the same cells (Figure 3, right). These results together with the finding that the *c-K-ras-2* gene is amplified in Lu-65 strongly suggest that the transforming gene of Lu-65 is *c-K-ras-2*. The copy number of the integrated human *c-K-ras* gene in this NIH3T3 transformant was estimated as one, or at most two per diploid cell. Therefore, point mutational activation in the Lu-65 *c-K-ras-2* was expected.

c-K-ras-2 gene of Lu-65 has a point mutation

This possibility was tested by cloning the *c-K-ras-2* gene from Lu-65. First, a gene library was constructed as described (Maniatis *et al.*, 1978). Fifteen to twenty kb fragments of Lu-65 DNA obtained by partial digestion with *Eco*RI were ligated to the arms of Charon 4A phage DNA and then packaged *in vitro*. The 120 000 phages of this library were screened by plaque hybridization as described before (Taya *et al.*, 1982) using a ^{32}P-labelled 0.8-kb *Eco*RI-*Xba*I fragment of *v-K-ras* (Ellis *et al.*, 1981). Four λ-phage clones with *c-K-ras-2* exons were obtained and two of them were confirmed to have exon 1 by comparison of their restriction map with that reported for *c-K-ras-2* (Figure 4) (McGrath *et al.*, 1983; Shimizu *et al.*, 1983). After sub-cloning of the 6.7-kb *Eco*RI fragment of λ GK-2 into pBR322, the DNA sequence of exon 1 was determined to be as shown in Figures 5 and 6 by the M13-dideoxy method. As expected, a single point mutation with a substituion of cysteine for glycine was found in codon

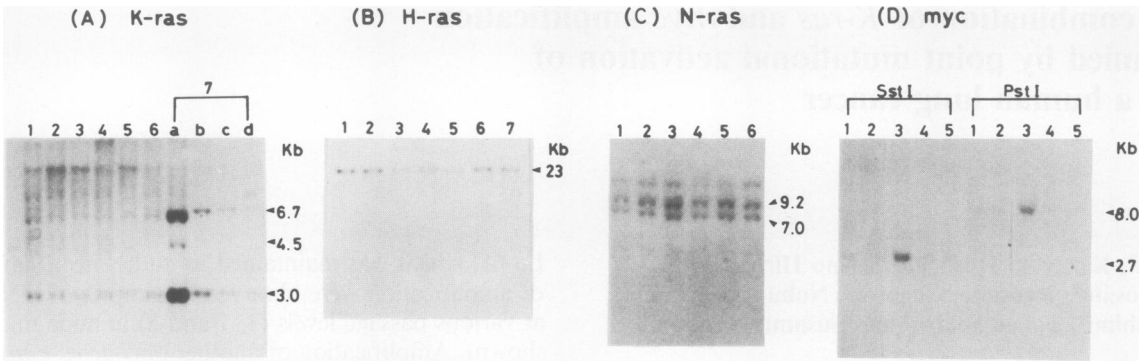

Fig. 1. Southern blot analysis of oncogenes in human normal and cancer DNA. **(A)** and **(B) lane 1**, placenta; **lane 2**, normal stomach; **lanes 3** and **4**, stomach adenocarcinomas Y15 and Y5; **lane 5**, lung adenocarcinoma L-27; **lane 6**, small cell lung carcinoma of a patient; **lane 7**, LGCC Lu-65. **(C) lanes 1, 2** and **3**, stomach adenocarcinomas Y8, Y15 and Y16, respectively; **lanes 4, 5** and **6**, lung adenocarcinoma L-27, lung small cell carcinoma Lu-134 and LGCC Lu-65, respectively. **(D) lane 1**, placenta; **lane 2**, stomach adenocarcinoma Y16; **lanes 3, 4** and **5**, LGCC Lu-65, Lu-116 and lung adenocarcinoma L-27, respectively. Samples of 2.5 μg of DNA, **(A) lane b**, 0.5 μg; **c**, 0.25 μg; **d**, 0.125 μg, were digested with *Eco*RI **(A, B** and **C)** or *Sst*I or *Pst*I **(D)**, separated by electrophoresis in 0.8% agarose gels and analyzed by Southern blotting. The probes used were a 1-kb *Eco*RI fragment of HiHi-3 (*K-ras*) (Ellis *et al.*, 1982), a 0.46-kb *Eco*RI fragment of BS-9 (*H-ras*) (Goubin *et al.*, 1983), a mixture of 1.0-kb *Hind*III fragment of pNPl and 1.8-kb *Xba*I-*Eco*RI fragment of pNP5 (*N-ras*), and a *Sal*II-*Pst*I fragment containing the 3′ end of *v-myc* (Alitalo *et al.*, 1983).

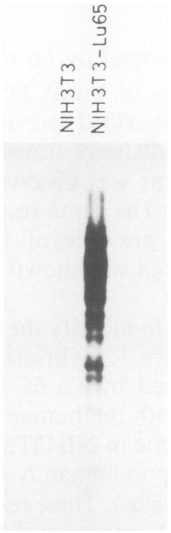

Fig. 2. Detection of human DNA sequences in transformed NIH3T3 cells after transfection with Lu-65 DNA. Samples of 2.5 μg of DNAs prepared from NIH3T3 cells or primary transformants by Lu-65 DNA transfection were digested with *Eco*RI, separated by electrophoresis on 0.8% agarose gel and analyzed by Southern blotting. The probe used was a 300-bp *Bam*HI fragment of BLUR8.

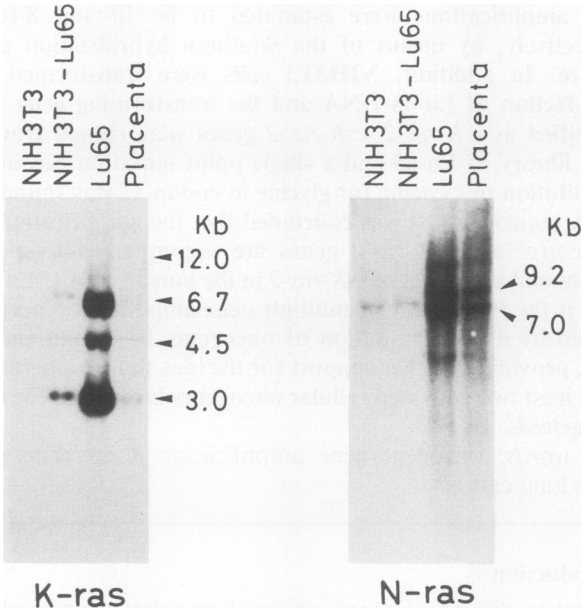

Fig. 3. Detection and identification of the Lu-65 transforming gene. Samples of 2.5 μg of DNA from four kinds of indicated cells were digested with *Eco*RI and analyzed by Southern blotting using ^{32}P-labelled *K-ras* **(A)** or *N-ras* **(B)** probes as described in the legend to Figure 1. NIH3T3-Lu-65 means NIH3T3 cells transformed by Lu-65 DNA.

12. The type of mutation was the same as that previously reported for a human lung carcinoma cell line Calu-1 (McGrath *et al.*, 1983; Shimizu *et al.*, 1983) and a lung adenocarcinoma PR-371 that has been maintained in nude mice (Nakano *et al.*, 1984). The transforming capacity of such a mutated *c-K-ras-2* gene has already been demonstrated (McGrath *et al.*, 1983; Shimizu *et al.*, 1983). Therefore, the transformation of NIH3T3 cells by Lu-65 DNA was thought to be caused by the *c-K-ras-2* gene which has a single point mutation in codon 12.

Thus it was concluded that the amplification of the *c-myc* and *c-K-ras-2* genes is accompanied by point mutational activation of *c-K-ras-2* in the human LGCC Lu-65. As far as we know, this is the first report of multiple gene amplification with a point mutation of an oncogene in human cancer cells.

Discussion

Activation of two oncogenes has been described previously by two groups. An active *N-ras* gene has been found to co-exist with an amplified *c-myc* gene in the HL-60 human promyelocytic leukemia cell, and with a translocated *myc* gene in an American Burkitt's lymphoma, although *N-ras* is not amplified in these cells (Murray *et al.*, 1983). Other examples of co-operation of two oncogenes were found in chicken lymphoma and Burkitt's lymphoma where *Blym-1* co-exists with a leukosis virus-activated and translocated *c-myc* gene, respectively (Goubin *et al.*, 1983; Diamond *et al.*, 1983). Our finding of activation of two oncogenes in another human

cancer provides strong support for the idea that the co-operation of at least two cellular oncogenes is required for carcinogenesis (Land *et al.*, 1983b), since we examined lung cancer cells, whereas hematopoietic tumors have been examined previously.

We have no direct evidence that *K-ras* and *myc* were amplified in the original tumor tissue of the patient. However, no significant difference was observed in the extent of amplification of *c-K-ras-2* and *c-myc* in Lu-65 DNAs from cells at several passages (3, 7 and 8 passages) in nude mice, and tumors propagated in nude mice maintain the morphological characteristics and functions of the original tumor well (Shimosato *et al.*, 1979). Therefore, it is very likely that *c-K-ras-2* and *c-myc* in Lu-65 were already amplified in the tumor of the patient. It should be noted that amplification of *myc* or *N-myc* has been demonstrated in some original tumors from patients (Favera *et al.*, 1982; Schwab *et al.*, 1983; Brodeur *et al.*, 1984; Lee *et al.*, 1984).

The fact that the only λ-phage clone sequenced here had a point mutation in exon 1 suggests the presence of the same mutation in most of the amplified *c-K-ras-2* genes of Lu-65, although further experiments are necessary to confirm this assumption. It is possible that the amplified *c-myc* genes of Lu-65 are also accompanied by point mutations, deletions and duplications as have been shown in translocated and in insertionally mutagenized *c-myc* genes (Rabbitts *et al.*, 1983,1984; Taub *et al.*, 1984a, 1984b; Westaway *et al.*, 1984). Studies are in progress to test this possibility.

LGCC is a variant of large cell carcinoma. Its growth in patients is usually rapid and some cells have a very large amount of cytoplasm as well as multiple nuclei. It is especially intriguing that LGCC frequently produces granulopoietic colony-stimulating factor (Kameya *et al.*, 1982; Asano *et al.*, 1977).

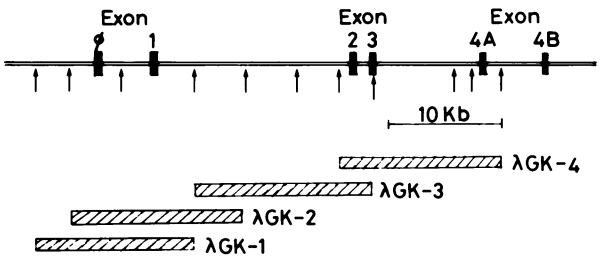

Fig. 4. Four λ-phage clones with *c-K-ras-2* exons obtained from a Lu-65 gene library. Hatched bars indicate the insert DNA regions in isolated λ-phage clones. Arrows are *Eco*RI sites. This map was constructed by comparison with the reported maps of *c-K-ras-2* (Shimizu *et al.*, 1983; McGrath *et al.*, 1983).

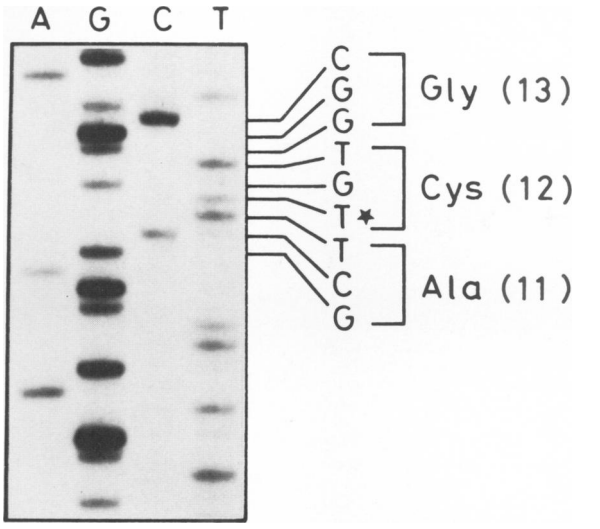

Fig. 5. Autoradiogram of the sequencing gel area where single base substitution was found in *c-K-ras-2* of Lu-65. The *Stu*I-*Sau*3A fragment, which should contain most of exon 1 of *c-K-ras-2* (McGrath *et al.*, 1983) and its 5'-flanking intron region, was cloned into M13mp8 (Messing *et al.*, 1981; Messing and Vieira, 1982) from the plasmid clone derived from GK-2 and its sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977). The asterisk indicates the thymine which was substituted for guanine of normal exon 1. The bracketed numbers are the predicted order of amino acid residues from the N-terminal of *c-K-ras-2* protein.

Materials and methods

Human cells

Lu-65, Lu-116, Lu-134 and L-27 have been maintained in nude mice. Stomach adenocarcinoma Y5, Y8, Y15, Y16, small cell lung carcinoma of the patient and normal stomach were obtained at the time of surgery. The use of materials of human origin which is described was carried out under the authorization of the Institute.

Isolation of total cellular DNA

High-mol. wt. DNAs were isolated as described by Gross-Bellard *et al.* (1973). Tissues were homogenized with a Potter homogenizer using a buffer containing proteinase K.

Southern blotting

The digested DNAs were separated by agarose gel (0.8%) electrophoresis and transferred to nitrocellulose paper as described (Southern, 1975). ^{32}P-Labelled probes were prepared by nick-translation (Rigby *et al.*, 1977). Hybridization was performed at 42°C for 16 h in 50% formamide-10% dextran sulfate (0.8 M) NaCl (100 mM) Pipes (pH 6.8) (100 μg/ml) denatured herring testis DNA-5 x Denhardt's solution as described previously (Sekiya *et al.*, 1983), and then the filters were washed with 0.2 x SSC, 0.1% SDS at 50°C.

DNA probes

The following plasmid clones were used in this study: HiHi3 (*K-ras*) (Ellis *et al.*, 1981); BS9 (*H-ras*) (Ellis *et al.*, 1980); pNPl and pNp5 (*N-ras*, gifts from

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                1                                  10                                  20
                Met Thr Glu Tyr Lys Leu Val Val Val Gly Ala Cys Gly Val Gly Lys Ser Ala Leu Thr
AGGCCTGCTGAAA ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA GCT TGT GGC GTA GGC AAG AGT GCC TTG ACG
                                                       GGT
                                                       Gly
   StuI
                21                                 30
                Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr Asp
                ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC GAA TAT GAT C
                                                                 Sau3A
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Fig. 6. Nucleotide sequence of exon 1 of *c-K-ras-2* in Lu-65. The sequence of *Stu*I-*Sau*3A fragment mentioned in the legend to Figure 5 is shown. The predicted amino acid sequence is also shown. The position where the sequence of Lu-65 differs from that of normal human cells is shown in a box. In this position glycine is coded by normal *c-K-ras-2*.

Dr K.Shimizu); pV*myc* (*myc*, gifts from Dr T.Yamamoto), BLUR8 (Jelinek *et al.*, 1980).

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