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ACTIVATION OF THE c-K-ras ONCOGENE IN A HUMAN PANCREAS CARCINOMA

Hisamaru Hirai[1], Tetsuro Okabe, Yumi Anraku, Michio Fujisawa, Akio Urabe and Fumimaro Takaku

The Third Department of Internal Medicine,
Faculty of Medicine, University of Tokyo,
7-3-1 Hongo, Tokyo 113, Japan

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Summary: The human pancreas carcinoma cell line T3M-4 contains activated c-Kirsten(K)-ras oncogene detectable by the DNA-mediated gene transfer technique using NIH/3T3 cells. DNA fragments containing coding lesions have been cloned, and nucleotide sequence analysis suggests that the T3M-4 oncogene has been activated by a single nucleotide transition from A to C in the second exon, which results in the substitution of histidine for glutamine in coden 61 of the predicted amino acid sequence. The quantity analysis of c-K-ras oncogene in the DNA and RNA of T3M-4 cells revealed that the c-K-ras gene was amplified and overexpressed in T3M-4 cells. These findings indicate that the T3M-4 c-K-ras oncogene is activated by different mutational events. © 1985 Academic Press, Inc.

Several oncogenes of human malignant tumors have been detected by their ability to induce morphological transformation of NIH/3T3 cells (for review 1-3). Most oncogenes so far detected belong to the ras gene family (for review 2,3), which consists of three proto-oncogenes, c-Harvey(H)-ras, c-Kirsten(K)-ras, and N-ras. Molecular cloning and nucleotide sequence analysis of the ras genes from human tumors revealed that a single point mutation in coden 12 or 61 of the predicted p21 protein is responsible for the transforming activity. We report here the cloning and identification of the lesion responsible for activation of c-K-ras oncogene from human pancreas carcinoma cell line T3M-4. The c-K-ras gene is amplified and overexpressed in T3M-4 cells. Thus, the T3M-4 c-K-ras gene is activated by different mutational events.

MATERIALS AND METHODS

Cells and transfection assay: The T3M-4 cell line was established from a Japanese male patient with primary pancreatic exocrine adenocarcinoma (4).

[1]To whom correspondence should be addressed.

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T3M-4 cells produce carcinoembryonic antigen *in vitro* in culture and *in vivo* in nude mice (4). DNA transfection assays using NIH/3T3 cells were performed by the calcium phosphate precipitation methods (5), as described previously (6).

Analysis of DNAs from transformed cells: Twenty µg of high molecular weight DNA were digested with appropriate restriction endonucleases, electrophoresed through 0.8% agarose gels and blotted to nitrocellulose filters as described by Southern (7). The resulting blots were hybridized with radioactive probes.

RNA dot blot hybridization: Poly(A)$^+$RNA doubling dilutions were prepared and denatured, and 5 µl samples (1 µg poly(A)$^+$RNA per original spot) were spotted onto a nitrocellulose filter (8). Hybridization was carried out as described by Thomas (8).

Molecular cloning: DNA from the NIH/3T3 secondary transformant was digested to completion with the restriction endonuclease EcoRI, and fragments of different sizes were fractioned by agarose gel electrophoresis and purified by the electroelution method. DNA of the λgtWES·λB phage vector was digested with EcoRI and purified by preparative sucrose gradient centrifugation. Purified phage arms and cellular DNA fragments were ligated at 1:1 molar ratio by T4 DNA ligase and packaged *in vitro* into phage particles (9). Positive plaques were identified by *in situ* hybridization of phage plaques (10).

Nucleotide sequence analysis: Sequences were determined by the chain terminator method (11) after subcloning suitable restriction enzyme-generated DNA fragments into phage M13 mp10 and mp11 replicative form DNAs.

RESULTS

Identification of the T3M-4 oncogene: When high molecular weight DNA of T3M-4 cells was subjected to transfection analysis using NIH/3T3 cells, a relatively high level of focus formation was observed (efficiency, 0.06 focus per µg DNA). The transforming activity was increased 1.5 fold by a second cycle of transfection using high molecular weight DNA of primary NIH/3T3 transformants. All the transformants obtained were transplantable to *nude* mice. To confirm that the foci observed were induced by human DNA, the DNAs of transformants were subjected to Southern blotting analysis using a probe specific for human *Alu*-family repetitive sequences (12). Primary transformants demonstrated numerous bands containing human repetitive sequences (Fig. 1A), and secondary transformants retained a relatively few *Alu*-related fragments (Fig. 1B). In view of evidence relating transforming genes of a number of human tumors to *ras* genes (2,3), we analyzed T3M-4 transformant DNAs for sequences homologous to these *onc* genes. Neither N-*ras* (13) nor H-ras probes (14) detected any fragments other than their endogenous mouse related fragments (data not shown). The K-*ras* probe (14) specifically

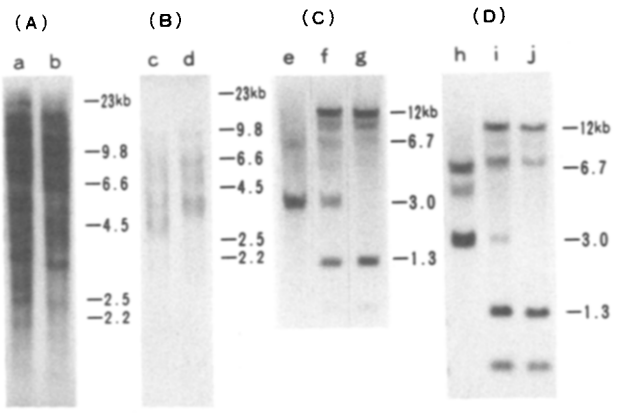

Figure 1. Identification of the T3M-4 oncogene by Southern blot analysis. Twenty μg of high molecular weight DNA was digested with EcoRI, electrophoresed, blotted, and hybridized to ^{32}P-labeled human repetitive sequence DNA fragment purified from Blur-8 (12) (A and B) or to ^{32}P-labeled v-K-ras-specific DNA fragment purified from pHiHi-3 (14) (C and D). Lane a, NIH/3T3 primary transformant (29L4-8) DNA; lanes b and f, NIH/3T3 primary transformant (29L4-12) DNA; lane c, NIH/3T3 secondary transformant (32L4-6) DNA; lanes d and i, NIH/3T3 secondary transformant (32L4-2) DNA; lanes e and h, T3M-4 DNA; lanes g and j, NIH/3T3 DNA.

detected the presence of human c-K-ras EcoRI-fragments of 6.7, 4.7 and 3.0 kb in DNAs of T3M-4 transformants (Fig. 1C and D).

c-K-ras amplification and overexpression in T3M-4 cells: We analyzed the quantity of c-K-ras in the DNA of T3M-4 cells by digestion with restriction endonucleases. The signals obtained in the experiment illustrated by Fig. 2A indicated that c-K-ras is amplified approximately 4-fold in T3M-4 cells. However, we observed that neither N-ras nor c-H-ras gene is amplified in T3M-4 DNA (data not shown). For the expression of c-K-ras in the T3M-4 cell line, the analysis by RNA dot blot hybridization was performed. The expression of c-K-ras in T3M-4 cells was enhanced 4- to 8-fold greater than in normal human pancreatic cells or placental cells (Fig. 2B). The comparative RNA dot hybridization did not detect any difference in the expression level of c-H-ras or N-ras gene among those cells (data not shown).

Molecular cloning and nucleotide sequence of the coding exons of the T3M-4 oncogene: The structure of the human c-K-ras gene has been reported (15,16), and the nucleotide sequence of the coding lesions of c-K-ras gene has been determined (15-17). We cloned four EcoRI-fragments containing coding exons

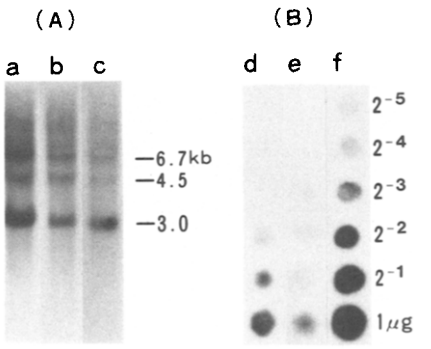

Figure 2. c-K-ras amplification and overexpression in T3M-4 cells. (A) Southern blot analysis with K-ras-specific probe (pHiHi-3). EcoRI-digested DNA was electrophoresed, blotted, and hybridized. Lane a, 20 μg of T3M-4 DNA; lane b, 5 μg of T3M-4 DNA; lane c, 20 μg of human placental DNA. (B) Dot blot analysis with K-ras-specific probe (pHiHi-3). Lane d, poly(A)$^+$RNA from human placental cells; lane e, poly(A)$^+$RNA from normal pancreatic cells; lane f, poly(A)$^+$RNA from T3M-4 cells.

(Fig. 3) because human c-K-ras gene spans a lesion of at least 30 kb (15,16). The small fragments of the cloned inserts were subcloned into M13 vectors and were sequenced. Sequence comparisons of the coding exons from the T3M-4 and the normal c-K-ras gene revealed a single nucleotide difference in coden 61. The T3M-4 c-K-ras encodes histidine (CAC) in coden 61 while normal c-K-ras encodes glutamine (CAA) (Fig. 4).

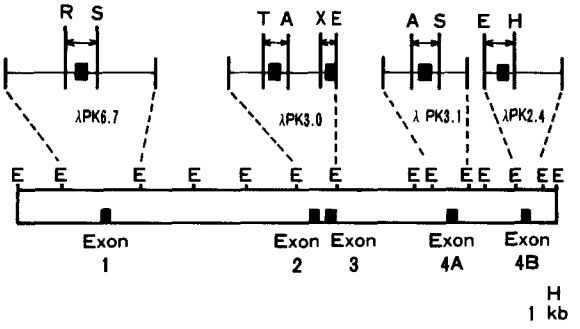

Figure 3. Four DNA fragments cloned from the NIH/3T3 secondary transformant (32L4-2) DNA. The restriction map of c-K-ras gene and the localization of coding exons are based on the data of Shimizu et al. (15). λPK6.7, λPK3.0 and λPK3.1 were cloned by v-K-ras-specific fragment from pHiHi-3, and λPK2.4 was cloned by v-K-ras-specific fragment from pKBE-2 (14). Exons 1-4B are denoted by solid blocks. Arrows indicate lesions sequenced. Restriction endonuclease sites are: E, EcoRI; R, RsaI; S, Sau3A; T, TaqI; A, AluI; X, XbaI; H, HindIII.

Exon 1

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1   2   3   4   5   6   7   8   9   10  11  12  13  14  15
Met Thr Glu Tyr Lys Leu Val Val Val Gly Ala Gly Gly Val Gly
ATG ACT GAA TAT AAA CTT GTG GTA GTT GGA GCT GGT GGC GTA GGC

16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
Lys Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe Val Asp
AAG AGT GCC TTG ACG ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC

31  32  33  34  35  36  37
Glu Tyr Asp Pro Thr Ile Glu
GAA TAT GAT CCA ACA ATA GAG
                           ↑
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Exon 2

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38  39  40  41  42  43  44  45  46  47  48  49  50  51  52
Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly Glu Thr Cys Leu
GAT TCC TAC AGG AAG CAA GTA GTA ATT GAT GGA GAA ACC TGT CTC

53  54  55  56  57  58  59  60 |61| 62  63  64  65  66  67
Leu Asp Ile Leu Asp Thr Ala Gly|His|Glu Glu Tyr Ser Ala Met
TTG GAT ATT CTC GAC ACA GCA GGT|CAC|GAG GAG TAC AGT GCA ATG
                               |↑A|
                               |Gln|

68  69  70  71  72  73  74  75  76  77  78  79  80  81  82
Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys Val Phe
AGG GAC CAG TAC ATG AGG ACT GGG GAG GGC TTT CTT TGT GTA TTT

83  84  85  86  87  88  89  90  91  92  93  94  95  96  97
Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His His Tyr
GCC ATA AAT AAT ACT AAA TCA TTT GAA GAT ATT CAC CAT TAT AG
                                                       ↑
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Figure 4. The DNA sequence and predicted amino acid sequence of the entire first and second exons of the T3M-4 c-K-ras oncogene. A single base change (A to C) and the consequent amino acid change of glutamine to histidine are boxed. The known RNA splice sites are marked by arrows (15-17).

DISCUSSION

In a number of human tumors, ras genes have undergone point mutations that confer to them oncogenic potential in the NIH/3T3 assay (15,17-24). In each case so far analyzed, the lesion responsible for transforming capability in the NIH/3T3 assay has been localized to a single point mutation in coden 12 or 61 of a member of ras genes (15,17-24). It remains to be determined how point mutations in coden 12 or 61 can so markedly affect normal ras functions as well as whether the mechanism of cell transformation by activation in coden 12 is the same as that by activation in coden 61. However, almost all of the c-K-ras genes activated have been found changed in coden 12 (15, 17,23,24). The T3M-4 c-K-ras oncogene was activated by the single base substitution in coden 61 and by the amplification and overexpression. The am-

plification and overexpression may play an additional role in the transforming activity of the T3M-4 c-K-ras gene.

Another suggestion is that the amplification and enhanced expression of cellular oncogenes may contribute to the maintenance of the neoplastic phenotype (25). The amplification and enhanced expression of cellular oncogenes have been found in several tumor cells (25-28). However, any mutational configuration of these amplified oncogenes, such as a rearrangement or a single point mutation, has not been detected (25). In the present case, it has not been understood whether both alleles suffer the point mutation or whether the mutated allele is amplified. Nonetheless, our observation of the oncogene activation by different mutational events may provide circumstantial evidence for multi-step process of carcinogenesis.

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