Four human carcinoma cell lines with novel mutations in position 12 of c-K-ras oncogene

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Received 9 September 1985; Revised and Accepted 9 December 1985

ABSTRACT

We have used synthetic oligonucleotides to probe for mutations affecting amino acid 12 of the c-K-ras gene in human cell line DNA. Of seven carcinoma cell lines tested, four were found to contain a mutation at this position. In each the nucleotide G was replaced with an A resulting in a Gly to Asp substitution in three cases (cell lines A427, A1165 and A1663) and Gly to Ser in the fourth (A549). Neither of these substitutions have been previously reported in either human tumor or human tumor-derived cell line DNA's. These results indicate that association between mutations involving position 12 of the human c-K-ras oncogene and carcinomas may be stronger than previously recognized.

INTRODUCTION

Activation of ras oncogenes, measured by their ability to transform NIH 3T3 cells or induce tumorigenesis, frequently involves a mutation in amino acid position 12 or 61 (1-12). Recent data demonstrated that mutations in at least position 12 of the mouse ras oncogene is a very early event in tumorigenesis and that G to A mutations in the 12th codon of H-ras is concomitant with and presumably contributing to initiation of carcinogenesis (13). Since the discovery of the NIH 3T3 transformation assay, a large number of cell lines and primary tumors, in particular carcinomas, have been reported to contain activated c-K-ras genes (4, 9-12, 14-17). Cloning and sequencing of the transforming c-K-ras and/or detection of restriction fragment length polymorphism (RFLP) demonstrated that lung carcinoma cell lines Calu-1 and PR371 contain a Gly12 to Cys (4,9), colon adenocarcinoma cell line SW480 a Gly12 to Val (10) and lung carcinoma cell line PR310 a Gln61 to His substitution (11). Santos et al. (12) using RFLP have found two cell lines (A2182 and A1698) and one tumor DNA (LC10), all from carcinoma origin, containing a Gly12 to Arg substitution.

The use of the NIH 3T3 transformation assay and RFLP techniques for detection of mutations in tumor and cell line DNAs has its limits. In the
transformation assay, the mutations leading to an obvious transformed phenotype might not represent the ones commonly causing activation in tumors; amino acid substitutions resulting in a relatively weak transforming ras oncogene could escape detection. The RFLP techniques have strong limitations since not all possible mutations result in the gain or loss of restriction sites.

Recently oligonucleotide technology has been developed as an alternative approach for the detection of point mutations in eukaryotic genes (13, 18-22). The strength of this technology is in its capability to unambiguously demonstrate the presence of any point mutation in a given gene. This would allow detection of ras mutations that lead to a protein with a weakly transforming activity and would go undetected in the NIH 3T3 assay. Hybridization of ras oligonucleotide probes to tumor DNA could establish the frequency of ras activation in human tumorigenesis. This information is important for studies on the mechanisms of oncogenesis and therefore of potential clinical relevance.

In the present studies, we have used synthetic oligonucleotides to detect point mutations in codon 12 of the c-K-ras gene in human cell lines. Analysis of cell lines A549 and A427, previously shown to contain an activated c-K-ras oncogene by the NIH 3T3 transfection assay (16,17), demonstrated the presence of mutations at this position. The analysis of five other carcinoma cell lines, in which there is, to our knowledge, no published evidence of c-K-ras activation, resulted in the identification of two cell lines with a mutation within codon 12 of the c-K-ras gene. In total, of seven carcinoma lines studied, four contained mutations at this site. All of the mutations consisted of G to A substitutions; in contrast, 5 non-carcinoma cell lines analyzed by the same procedure were found to be wild type at c-K-ras codon 12.

MATERIALS AND METHODS

Cell lines

Cell lines were obtained from Frederick Cancer Research Facility (NIH). All, except AG3344, A101D, A1165 and A1663 are deposited in the American Type Culture Collection (ATCC). AG3344 is available from the Human Genetic Mutant Cell Culture Repository. Cell lines A1165 and A1663 originated from S. Aaronson's laboratory, while cell line A101D was derived by Dr. L.E. Hooser.
Cell line DNA isolation

The DNAs were isolated as described (15). After isolation, DNAs were digested with Eco RI (BRL) as recommended by the supplier. Digested DNAs were phenolized, ethanol precipitated and dissolved in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

Oligonucleotide synthesis

Oligonucleotides K-ras 12 I and II (Fig.1) were synthesized as a mixture by the phosphotriester method using a Biosearch SAM I synthesizer and purified through Sephadex G75 chromatography. The other oligonucleotides were prepared using an Applied Biosystems model 380 synthesizer as specified by the supplier and purified by denaturing 12% polyacrylamide gel electrophoresis and chromatography on Sephadex G25. Oligonucleotides were sequenced by a modified Maxam and Gilbert protocol as described (23).

Labelling of oligonucleotides

Oligonucleotides were kinased using \( ^{32} \text{P}-\text{ATP} \) (Amersham, 5000 Ci/mmol) as described (23). The kinased probes were separated from the unincorporated ATP by chromatography on Sephadex G25 in TNE (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Specific activities were greater than 2x10^6 cpm/pmol.

The complementary strand of oligonucleotides K-ras 12 A, T and G were synthesized and labelled for use as probes as described previously (20, 21) using Klenow polymerase and \( ^{32} \text{P}-\text{dTTP} \) (Amersham, 3000 Ci/mmol) and subsequently purified by gel electrophoresis in a 12% denaturing polyacrylamide gel. The oligonucleotides were eluted from the gel with water and used directly for hybridizations.

Agarose gel electrophoresis and hybridizations

Ten ug of Eco RI digested DNA was loaded on a 0.5% (w/v) agarose (IBI) gel and run in 1xTBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) at 20 V. After electrophoresis the gels were processed as described (19). Briefly, the gels were treated first in denaturing solution (0.4 M NaOH, 0.8 M NaCl) for 45 min at room temperature, and then neutralized in 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl for 30 min at room temperature, placed on a piece of 3MM Whatman paper and dried on a gel drier (Biorad). The dried gels were re-hydrated in distilled water before use in the hybridization.

Gels were pre-hybridized in Seal-A-Meal bags in 5 x SSPE (1 x SSPE=10 mM sodium phosphate pH 7.0, 180 mM NaCl and 1 mM EDTA) containing 0.1% SDS and 200 ug/ml yeast RNA in a waterbath at 65°C for 2 h. Hybridizations were
done at 48°C for 10 to 14 h in 5 x SSPE containing 0.1% SDS, 50 μg/ml denatured salmon sperm carrier DNA and approximately 1 ng/ml of probe. The gels were subsequently washed in 2 x SSPE, 0.1% SDS at room temperature with slow shaking for 90 min, followed by washing in 5 x SSPE at 48°C for 15 min and at 66°C for two minutes. After exposure to X-ray film (Kodak XAR-5) gels were washed at 65°C for 30 min in 1 x SSPE, 0.1% SDS to remove the hybridized oligonucleotides. Gels were subsequently re-hybridized as described above.

RESULTS AND DISCUSSION

High molecular weight DNAs were extracted from cell lines and digested to completion with Eco RI. Digests were loaded on agarose gels and electrophoresed, followed by processing and sequential hybridizations of the gel to radioactive oligonucleotide probes (20 mer) containing either the wild type or mutant sequence around codon 12 of c-K-ras oncogene (see Fig.1). The cell line HT1080, containing a mutation in c-N-ras 61 (21), was assumed to have a wild type c-K-ras oncogene and was included as a control.

In initial experiments, Eco RI digested DNAs from cell lines A1165, A549, A427, A431, A498, A1663, HTB-52 and HT1080 were hybridized to the K-ras 12 WT oligonucleotide probe (see Fig.2A). All of the DNAs tested with the exception of A549 showed a 6.7 kb band containing the wild type exon I of c-K-ras (24). When replica panels of the same DNAs were hybridized to K-ras 12 I, a 6.7 kb Eco RI fragment was visible only in A549 (Gel I, I) indicating the presence of a mutation in position I of codon 12. Upon hybridization to K-ras 12 II (Gel I, II) stronger bands were visible in the A1165, A427 and A1663 than in the wild type DNA (HT1080), suggesting the presence of a mutation in position II of codon 12 in the DNA of these cell lines. Gels were washed at low salt and high temperature to remove the hybridized oligonucleotides and subsequently re-hybridized to the individual oligonucleotides of the K-ras 12 II series (Fig.2B). The autoradiogram shows that DNAs of cell lines A1165, A427 and A1663 contain a hybridizing 6.7 kb Eco RI fragment indicating that these DNAs contain a G to A mutation in position II of codon 12 of one of the c-K-ras alleles. The segments of gel I were again washed at low salt and high temperature and re-hybridized with the individual oligonucleotides from the series K-ras 12 I (Fig.2C); a strong hybridizing band was visible in A549 upon hybridization with the oligonucleotide probe K-ras 12 II but not with K-ras 12 IA or IG. This demonstrates that the mutation is a G to A transition in position I of codon 12.
Fig. 1. K-ras 12 oligonucleotide series. Listed is the wild type sequence of the c-K-ras gene region covering nucleotides 25 to 44 (4) and the oligonucleotide series K-ras 12 used in the hybridization experiments. Amino acids encoded by codon 12 in the wild type and mutant oligonucleotides are also shown. The primer was used for synthesis of the complementary strand of some of the oligonucleotides.

Since it appears that oligonucleotide K-ras 12 IIG gives some slight background bands, we used the labelled complementary strand of K-ras 12 IIA, T and G to confirm our results. The probes were synthesized and labelled using the primer shown in Fig. 1, hybridized to the individual oligonucleotides and extended using Klenow polymerase and $^{32}$P-dTTP. The results of the hybridization (Fig. 2C, gel II) shows that the complementary strand for K-ras 12 IIT (IIA*) gives a band of 6.7 Kb in the A1663 lane, with no detectable background bands, confirming the results obtained with the kinased K-ras 12 IIT probe. The same experiment was done for gel I (data not shown), using the labelled complementary strand of oligonucleotides K-ras 12 IIA, IIT and IIG. On the autoradiogram, bands were only visible in DNAs A1165 and A427 of the panel hybridized to the probe complementary to K-ras 12 IIT, but not in the panels hybridized with the other two oligonucleotides probes. These results establish that mutations affecting
Fig. 2. Autoradiograms of the oligonucleotide probes hybridized to genomic DNA digests on agarose gels.

High molecular weight DNAs from cell lines a, A1165; b, A549; c, A427; d, HT1080; e, A431; f, A498; g, A1663 and h, HTB-52 were extracted, digested with Eco RI as described in Materials and Methods and run on two separate gels (gel I and gel II). \(^{32}P\) labelled Hind III marker (m) was included as a molecular weight standard. The size of the marker fragments is indicated by the numbers at the left margin. DNA samples m, a, b, c and d were loaded 3 times in the same order on gel I; samples m, e, f, g, h and d were loaded 3 times in the same order on gel II.

After electrophoresis each gel was treated as described in Materials and Methods, cut into three identical sections (arrows) and each
individual section was hybridized (A) to either K-ras 12 WT, K-ras 12 II (WT, I and II respectively) kinased oligonucleotide probe. After hybridization the membranes were washed and the sections of each gel realigned to form the original gel followed by exposure to X-ray film. After exposure the gels were washed in low salt buffer to remove the hybridized oligonucleotide and re-hybridized (B) to either oligonucleotide probes K-ras 12 IIA, K-ras 12 IIT or K-ras 12 IIG (IIA, IIT or IIG respectively); after washing and X-ray film exposure, the gels were again washed in low salt buffer as described above and re-hybridized (C, gel I) to either oligonucleotide K-ras 12 IA, K-ras 12 IT or K-ras 12 IG (IA, IT and IG respectively) and (C, gel II) to the complementary strand of the oligonucleotide K-ras 12 IIA, K-ras 12 IIT or K-ras 12 IIG (IIA*, IIT* and IIG* respectively) followed by washing and film exposure as above.

c-K-ras codon 12 in cell lines A1663, A1165 and A427 can be unambiguously demonstrated using both strands of the oligonucleotides as a probe.

Gel II was further used for detection of point mutations in position 61 of c-K-ras gene. Upon hybridization to an oligonucleotide (20 mer) containing the wild type sequence around position 61 of c-K-ras, a 3.0 kb Eco RI fragment containing the second exon of c-K-ras (24) was observed, but no band could be detected at this position when oligonucleotides containing base changes in position I, II or III of codon 61 were used as hybridization probes (data not shown). We thus conclude that cell lines A431, A498 and HTB-52 are wild type at both position 12 and 61 of c-K-ras.

Table 1. Summary of the results from the hybridization experiments.

<table>
<thead>
<tr>
<th>Carcinoma:</th>
<th>Cell line</th>
<th>Codon 12, position*</th>
<th>Mutation</th>
<th>Amino acid at codon 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung Adenocarcinoma</td>
<td>A549</td>
<td>I</td>
<td>G to A</td>
<td>Ser</td>
</tr>
<tr>
<td>Lung</td>
<td>A427</td>
<td>II</td>
<td></td>
<td>Asp</td>
</tr>
<tr>
<td>Liver Adenocarcinoma</td>
<td>HTB 52</td>
<td>I</td>
<td>G to A</td>
<td>Asp</td>
</tr>
<tr>
<td>Pancreas</td>
<td>A1165</td>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermoid carcinoma of vulva</td>
<td>A431</td>
<td>I</td>
<td>G to A</td>
<td>Asp</td>
</tr>
<tr>
<td>Bladder</td>
<td>A1663</td>
<td>II</td>
<td>G to A</td>
<td>Asp</td>
</tr>
<tr>
<td>Kidney</td>
<td>A498</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide substitutions at the first or second position of c-K-ras codon 12 are indicated by a +.
Using the oligonucleotide hybridization technique, we demonstrated point mutations in codon 12 of c-K-ras in 4 of 7 carcinoma cell lines tested. In contrast, 5 human non-carcinoma lines, A673 (rhabdomyosarcoma), A172 (glioblastoma), AG 3344 (lymphoblast), CCL 137 (embryonic lung) and A101D (melanoma) hybridized to the wild type but not the mutant oligonucleotide probes (data not shown). These results are summarized in Table 1.

Three of the mutations, those in the cell lines A427, A1165 and A1663, resulted in a G to A transition in the second position of the 12th codon causing a Gly to Asp substitution. A number of observations confirm that these cell lines are distinct from each other (data not shown): 1. The same DNA preparations used for this study showed polymorphism between cell line A472 and the other cell lines, A1165 and A1663, for another oligonucleotide probe. 2. The three cell lines show a great number of qualitative and quantitative differences in protein expression, as judged by two dimensional protein gel electrophoresis. 3. Considerable differences in morphology and doubling time were evident. The fourth mutation, in cell line A549, consists of a G to A transition in the first position of the same codon, resulting in a Gly to Ser replacement. Neither of these mutations have been previously identified in DNA's from either human tumors or human tumor-derived cell lines. The Gly\textsuperscript{12} to Ser replacement is present among other changes in Kirsten murine sarcoma virus (25) and the Gly\textsuperscript{12} to Asp substitution have been found in the c-K-ras gene of radiation induced mouse tumors (14). These findings argue that the association of K-ras activation with human carcinomas may be much greater than previously recognized.

Until recently the identification of mutations in ras genes has been accomplished using either RFLP or sequencing of the cloned gene after selection of the transforming gene using the NIH 3T3 transformation assay. The first procedure, RFLP, does not allow random detection of mutations in a given gene since not all mutations result in altered restriction sites. Therefore, only a limited set of mutations, if any, can be detected. The second procedure, the cloning and subsequent sequencing of the transforming gene, is time consuming and laborious, and in addition is potentially subject to bias since the more powerful transforming mutation might not be the most prevalent in tumors. In this study we found a high frequency of aspartic acid rather than glycine in position 12 of the human c-K-ras gene. The fact that these and the Gly\textsuperscript{12} to Ser mutation have not been previously reported could be explained if these mutant genes were relatively weak.
transformants of NIH 3T3 cells. Conflicting reports on the presence of activated c-K-ras oncogene in cell line A549 by the use of the NIH-3T3 assay (17, 26) could also be explained by the difficulty of obtaining transformant from mutants with relatively low transforming potency. This hypothesis is strengthened by the findings of Fasano et al. (27), who in vitro mutagenesis experiments in the H-ras gene demonstrated that replacement of Gly\(^{12}\) to Ser or Asp produced a gene with less than 50% of the potency in focus induction when compared with a glycine to valine amino acid change. Alternatively, the failure to produce transformants by a mutated c-K-ras gene might be related to secondary mutations affecting transcription levels of this gene. Further studies are required to determine the frequency of these type of ras activating mutations in carcinomas. Oligonucleotide probes, as the ones used in these studies, will become an important tool to elucidate the role of the ras oncogene family in human tumorigenesis.

ACKNOWLEDGEMENTS

We thank Dr. Hans Bos for advice in the early stages of this work and for providing some of the oligonucleotides used in this study, and Drs. Nora Heisterkamp and John R. Stephenson for helpful discussions and critical reading of the manuscript. This work was supported by Oncogene Science, Inc.

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REFERENCES