No Evidence for Point Mutations in Codons 12, 13, and 61 of the ras Gene in a High-Incidence Area for Esophageal and Gastric Cancers


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ABSTRACT

The molecular mechanisms underlying the induction of esophageal and gastric cancer are not yet understood. It is possible that different etiological factors from geographically distinct areas play a role in the onset of these cancers. Twenty-seven primary esophageal and 11 gastric cancers originating from the high-incidence areas of South Africa were analysed for the presence of ras protooncogene mutations. We found no evidence for mutations in codons 12, 13, or 61 or the H-ras, K-ras, and N-ras genes in these primary cancers.

Our results indicate that etiological factors such as fungal contamination of basic foodstuffs in a high-incidence area for these cancers do not play a role in the activation of ras genes and that mutations in these genes are not directly involved in the development of primary esophageal and gastric cancers in the South African population.

INTRODUCTION

Certain areas of South Africa are characterized by having a very high incidence of esophageal cancer (1-3). However, in spite of intensive efforts, the molecular mechanisms underlying the induction of esophageal cancer are not yet understood. Comparisons of the dietary and cultural habits of people from geographically distinct high-incidence areas have revealed very little in common with all groups to suggest a common induction mechanism. It is, therefore, possible that different etiological factors play a role in the distinct areas, for example, alcohol and tobacco smoking in France (4) and fungal contamination of foodstuffs in China and South Africa (5-9). Studies of fungal contamination of food suggest that mycotoxins (or other factors derived from these contaminating organisms) may be involved in tumorigenesis (6-12). One possible mechanism for tumorigenesis by aflatoxins is by DNA adduct formation, a process which introduces point mutations into DNA (13-16). There is evidence that the ras family of protooncogenes are targets for a number of carcinogens (13, 17-19), among which may be aflatoxins (13, 14).

Point mutations which lead to the production of mutant ras p21 protein, and therefore, transformed phenotype, have been found to occur spontaneously in codons 12, 13, and 61 of transformed ras genes (20). Site-directed mutagenesis has shown that, in addition to these positions, single amino acid substitutions at positions 59, 63, 116, and 119 (for example) can also activate the ras gene (20). In naturally occurring tumors, precise mutation sites vary among tumor types but may be as high as 40%, as found in gastrointestinal tumors (20). Since the areas which show a high incidence of esophageal cancer in South Africa also show a high incidence of fungal contamination of basic foodstuffs (8), we sought to determine whether esophageal cancer in these areas was a function of ras gene mutations mediated by mycotoxins (or other environmental carcinogens).

Some regions and ethnic groups of South Africa also have a high incidence of gastric cancer. It has recently been estimated that the incidence in males in the Western Cape (African Negro/Xhoi San/Malaysian/Western European) is approximately 89/100,000/4 years, which is nearly 7 times higher than the incidence in Caucasian males. Since ras gene mutations are often found in gastrointestinal (mainly colon) tumors and some have been found in gastric cancers (21-24), we also investigated this cancer in local patients.

This report concerns our search for point mutations in H-, K-, and N-ras genes at codons 12, 13, and 61, in a series of 27 esophageal and 11 gastric primary tumor samples from patients with the disease originating from the high-incidence areas of South Africa.

MATERIALS AND METHODS

Extraction of DNA from Tissue. Human esophageal (squamous carcinomas) and gastric (from body of stomach) tumor samples were obtained from Tygerberg and Frere Hospitals (fresh or paraffin embedded), South Africa. Fresh tumor tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until DNA was extracted (25). Extraction of DNA from paraffin-embedded tumor tissue was done as described elsewhere (26). Patients were all Xhosas, resident originally in the Transkei (esophageal cancer) or people of mixed ancestry (African Negro/Xhoi San/Malaysian/Western European) from the Western Cape (gastric cancer).

Synthetic Oligonucleotides. Oligonucleotides to screen for the presence of specific point mutations of ras genes were purchased from DuPont (West Germany) (codons 12 and 61) and Clontech (USA) (codon 13). The oligonucleotide probes were 5'-end labeled with [32P]ATP (Amersham; specific activity, 5000 Ci/mmol; 1 Ci = 37 GBq) and T4 polynucleotide kinase (Amersham). Labeled probes were routinely purified on a 10% polyacrylamide/7 M urea-sequencing gel. Other oligonucleotide primers were synthesized by Beckman Model System I plus DNA synthesizer. The sequences for the primers are given in "Results" (Table 1).

PCR. Genomic DNA amplification with Taq polymerase (New England Biolabs) was done as described by Saiki et al. (27). The reaction mixture (100 µl) was incubated at 95°C for 5 min, followed by a 10-s centrifugation in an Eppendorff microfuge, after which 2 units of Taq polymerase (Biolabs) was added. The mixture was overlaid with 40 µl of mineral oil and the cap of the tube was sealed with a hot spatula. The heating cycle was 55°C for 1.5 min, 95°C for 1 min, and 40°C for 2 min. This cycle was repeated 30 times. After the last cycle, all samples were incubated for an additional 10 min at 55°C to ensure that the final extension step was complete.

Dot-Blot Hybridization. An aliquot of each PCR mixture, containing 100 ng of the original DNA, was adjusted to 0.4 M NaOH-25 mM EDTA in a 200-µl volume and applied under vacuum to a Hybond-N nylon filter (Amersham) in a dot-blot apparatus (26). Each filter was prehybridized in 8 ml solution consisting of 5x SSPE (1x SSPE = 10 mM sodium phosphate, pH 7.0-0.18 M NaCl-1 mM EDTA), 0.5% Hybridization. An aliquot of each PCR mixture, containing 100 ng of the original DNA, was adjusted to 0.4 M NaOH-25 mM EDTA in a 200-µl volume and applied under vacuum to a Hybond-N nylon filter (Amersham) in a dot-blot apparatus (26). Each filter was prehybridized in 8 ml solution consisting of 5x SSPE (1x SSPE = 10 mM sodium phosphate, pH 7.0-0.18 M NaCl-1 mM EDTA), 0.5% T4 polynucleotide kinase (Amersham). Labeled probes were routinely purified on a 10% polyacrylamide/7 M urea-sequencing gel. Other oligonucleotide primers were synthesized by Beckman Model System I plus DNA synthesizer. The sequences for the primers are given in "Results" (Table 1).

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Electrophoresis was carried out on 1.7% agarose gel and stained with ethidium bromide. Arrow, DNA fragments with a length of 108 base pairs.

**Results**

Construction of Oligonucleotide Primers Used for the Amplification of ras Oncogenes. Primers used to selectively amplify one of the ras genes in the area of codons 12, 13, or 61 are given in Table 1. A 10-µg aliquot of the PCR product was routinely analyzed on a 1.7% agarose gel (Fig. 1) to confirm that the amplification step was successful, since polymerase inhibitors have been found which may cause problems with the reaction (28, 29). The PCR product of some samples was digested with an appropriate restriction enzyme and analyzed on a 1.7% agarose gel, as another means to confirm that the correct sequence was amplified (results not shown).

### Table 1 List of Oligonucleotide Primers Used to Analyze ras Gene Mutations (codons 12 and 61)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Target</th>
<th>Fragment (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-512</td>
<td>GAC CCC GGG CCG CAG</td>
<td>H-ras 12</td>
<td>104</td>
</tr>
<tr>
<td>H-312</td>
<td>CTG GAT CAG CTT GAT</td>
<td>H-ras 12</td>
<td>108</td>
</tr>
<tr>
<td>K-512</td>
<td>GAC TGA ATA TAA ACT TGT GG</td>
<td>K-ras 12</td>
<td>109</td>
</tr>
<tr>
<td>K-312</td>
<td>CTA TTG TTG CAT ATT CG</td>
<td>K-ras 12</td>
<td>108</td>
</tr>
<tr>
<td>N-512</td>
<td>GAC TGA GTA CAA ACT GTG GG</td>
<td>N-ras 12</td>
<td>109</td>
</tr>
<tr>
<td>N-312</td>
<td>CTC TAT GGT GGG ATC ATA TT</td>
<td>N-ras 12</td>
<td>103</td>
</tr>
<tr>
<td>H-561</td>
<td>AGA GGT GCC TGT TGG ACA TC</td>
<td>H-ras 61</td>
<td>73</td>
</tr>
<tr>
<td>H-361</td>
<td>CGA ATG TAC TGG TCC CGC AT</td>
<td>H-ras 61</td>
<td>128</td>
</tr>
<tr>
<td>K-561</td>
<td>TTC CTA CAG GAA GCA AGT AG</td>
<td>K-ras 61</td>
<td>128</td>
</tr>
<tr>
<td>K-361</td>
<td>CAC AAA GAA AGC CCT CCC CA</td>
<td>K-ras 61</td>
<td>128</td>
</tr>
<tr>
<td>N-561</td>
<td>GGA GAA ACC TCT TGG GTA</td>
<td>N-ras 61</td>
<td>103</td>
</tr>
<tr>
<td>N-361</td>
<td>ATA CAC AGA GGA AGC CTT CG</td>
<td>N-ras 61</td>
<td>73</td>
</tr>
</tbody>
</table>

* Primes, synthetic oligonucleotides used to amplify DNA segments in *vitro*. Numbers prefixed with 5 or 3 refer to primers 5' or 3' of target sequence and suffixes of 12 or 61 refer to codons 12 or 61.

**Analysis of Tumor DNA for the Presence of Activating ras Mutations.** The sensitivity of the analysis in our approach is enhanced considerably by increasing the ratio of specific target sequences to all other genomic sequences. DNA from 27 esophageal and 11 gastric tumors was screened for mutations in codons 12 and 61 of the H-ras, K-ras, and N-ras genes, following polymerase chain reaction amplification. An aliquot of the amplified DNA was spotted onto a nylon membrane and hybridized to the various mutation-specific oligomers. The plasmid pHiHi-3 (mutant K-ras 12) (30), which was predetermined by using the control plasmids (as obtained from the suppliers) in separate hybridization experiments. No hybridization was obtained with these probes (results not shown) except to the positive control plasmid pN-ras (HT) which hybridized strongly to the mutant probe at codon 61 (Fig. 2). In order to exclude the possibility that only one of the alleles in the tumor samples is mutated and, hence, that the sample will test positive for the normal probe, the dot-blot hybridization procedure on the same amplified DNA was repeated, using the mutant probes N-709, N-710, N-711, N-712, N-713, N-714, and N-715 (as obtained from the positive control plasmid pN-ras (HT) which hybridized strongly to the mutant probe at codon 61 (Fig. 2). These results indicate that the mutation-specific probes hybridized exclusively to those DNA sequences that fully matched the corresponding ras gene and that the tumor samples analyzed contained no point mutations in codon 61 of the N-ras gene. Note that normal (contaminating) cells may have an influence on the results, but it is possible to clearly identify a mutation present where mutant cells represent only 10% of the total cell number by using this standard protocol for detecting point mutations (33).

The procedure of amplification followed by hybridization with sequence-specific oligonucleotide probes was repeated for the same tumor samples and no point mutations were found in codons 12, 13, or 61 of the H-ras, K-ras, and N-ras genes.

![Fig. 1. DNA yield after PCR amplification. Amplification of K-ras codon 12 was done as described in "Materials and Methods" using 1 μg of esophageal DNA (samples 1–8) and primers K512 and K312. After 30 cycles of PCR, electrophoresis was carried out on 1.7% agarose gel and stained with ethidium bromide. Arrow, DNA fragments with a length of 108 base pairs.](image)

![Fig. 2. Dot-blot hybridization of a normal oligomer probe to PCR-amplified DNA. DNA from 11 gastric (filter A) and 27 esophageal (filter B) tumor samples was amplified with primers N-561 and N-361 in *vitro* by PCR and hybridized with oligonucleotide probe N-708, which identifies normal sequences for codon 61 of the N-ras gene. Grid areas B2 (filter A) and C8 (filter B) contain 100 ng of the plasmid pN-ras (HT) (mutant N-ras 61).](image)

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were amplified with primers N-561 and N-361 by PCR and hybridized with
cell line DNA to transform cultured cells. In this study, we
differ vastly. That the two studies done on geographically
gastric cancer from geographically distinct regions suggest a
descendants model mycotoxins by inhabitants of high- and low-risk areas for esophageal
acids and nitrate by inhabitants of high- and low-risk areas for esophageal
of nitroproline and its and lipotrope deficiencies in populations at risk for esophageal
title={ras GENE POINT MUTATIONS AND ESOPHAGEAL AND GASTRIC CANCER}

discussion

esophageal tissue samples (35, 36), it seems likely that the
detected no ras gene mutations in codons 12, 13, or 61 in primary esophageal
cancers.

It is important to note that a similar study was recently done in
France, where a high-incidence area for esophageal cancer is
found (34). No mutant ras genes were detected. The authors state that their results do not imply that other high-incidence areas should have the same etiology, since it is clear that diet and lifestyles among French Caucassians, Chinese (in Linxian County, People’s Republic of China), and South African Xhosas differ vastly. That the two studies done on geographically distinct high-incidence areas should come to the same conclusion is important.

In spite of the fact that DNA adducts have been detected in esophageal tissue samples (35, 36), it seems likely that the activation of ras genes by point mutations is not directly involved in the development of this cancer but does not exclude the involvement of ras by other mechanisms.

gastric cancer. Gastric cancers containing activated K-ras (21), N-ras (22, 24), and H-ras (23) have thus far been detected. These have been detected primarily by the ability of tumor or cell line DNA to transform cultured cells. In this study, we found that oligo screening of ras genes after PCR amplification detected no ras gene mutations in codons 12, 13, or 61 in genomic DNA from 11 primary gastric cancers from our local high-incidence population. As far as we can ascertain, there have been no other reports on extensive oligonucleotide screening for ras mutations in gastric cancers. This would tend to confirm the results of Sakamoto et al. (36) who suggested that DNA from gastric cancers generally has a lower transformation efficiency than other human tumors. Some of these transformants are also not due to ras, but other transforming oncoproteins, such as kst (36). Our results suggest that ras gene mutations are not involved in the high incidence of gastric cancer found in the Western Cape region of South Africa among people of mixed ancestry. The results of this study and others which show a low transformation efficiency and low ras mutation rate in gastric cancer from geographically distinct regions suggest a common, as yet unidentified etiology for this cancer.

references


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