TP53 Gene Mutation Profile in Esophageal Squamous Cell Carcinomas

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ABSTRACT

Esophageal squamous cell carcinoma is a form of cancer occurring most commonly in males, particularly those living in some areas of Asia, Africa, and western Europe. In some of these tumors, a sequence alteration has been identified in the coding region of the TP53 gene which is known to inactivate the tumor suppressor function of its product. Using a GC clamp (i.e., a GC rich domain) denaturing gradient gel electrophoresis assay we have been able to identify sequence modifications in 27 of the 32 tumor samples analyzed (84%). Most of the mutations occur in exon 6, a region of the gene which has not previously been reported as being a hot spot for the mutations of other cancers. Twelve of the mutations reported here have not been described in other types of tumors and these consist mostly of frameshift or splice mutations.

The distribution of mutations [transitions (45%), transversions (34%), and frameshift (21%)] suggests that the etiological contribution of genotoxic factors might be complex and might associate different exogenous and endogenous mutagen exposures.

INTRODUCTION

The TP53 gene is probably the most commonly mutated gene in human cancers (1, 2). One of the several lines of evidence of TP53 involvement in human malignancies is the loss of heterozygosity in 17p associated with mutations in the other TP53 allele (3). The normal allele of this gene encodes a 375 amino acid nuclear phosphoprotein involved in the regulation of cell proliferation (4). It has been proposed also that the p53 function can be inactivated in a single allele, i.e., with a dominant negative effect (5). TP53 genes contain five highly conserved domains which are the most frequent sites of mutations in many human tumors (6, 7). Extensive analysis has shown about 1600 molecular defects in more than 35 cancer types (1, 2). Most of these mutations are somatic, but germline mutations have been described in the familial Li Fraumeni syndrome (8, 9). Mutated alleles of TP53 have been found in solid tumors of the colon (10), lung (11), breast (12), ovary (13), brain (14), liver (15, 16), and in hematopoietic tissues (17). These various mutant alleles code for proteins that have altered growth-regulatory properties (3, 5). Interestingly, the pattern of point mutations in TP53 gene varies in different human cancers and each pattern appears to associate with the cancer of a particular organ (1, 2). It has also been observed that specific etiological agents can produce specific mutations. For example, the G→T transversion in codon 249 of hepatocellular carcinomas can be the result of the interaction of aflatoxin B with DNA in certain areas (18). Other chemical carcinogens such as nitrosamine are also able to produce a high incidence of organ-specific tumors each with a particular pattern of mutations in the TP53 gene (19). Cancer of the esophagus occurs with a high frequency in certain regions of the world such as China, Iran, South Africa, and France (20). In western Europe and North America, the classical etiological factors of esophageal cancer are tobacco and alcohol consumption. Because in the western part of France, Brittany, the incidence rate of esophageal cancers is high (27/100,000) (21), we have undertaken a systematic screening of point mutations in the TP53 gene in a series of 32 esophageal squamous cell carcinomas in patients of a same ethnic origin. For this study, we have used a GC clamp denaturing gradient gel electrophoresis assay to analyze the conserved domains of the gene and we have determined the spectrum of mutations in the TP53 gene. The particular profile described in this work might reflect the genotoxic effects of different environmental factors in this type of cancer.

MATERIALS AND METHODS

Patients. Thirty-two patients with an esophageal squamous cell carcinoma were included in this study. Most of them had history of smoking and excessive alcohol consumption (Table 1). For each patient, biopsy specimens were taken from the tumor, and whole blood samples were collected. DNA were prepared from peripheral leukocytes and from biopsy specimens after proteinase K digestion and phenol-chloroform extraction as described elsewhere.

All the patients were seen at the time of diagnosis and none had chemotherapy or radiation therapy prior to the time the biopsies were taken from the tumor.

The tumors were staged according to the new TNM classification by the International Union Against Cancer (22, 23) (Table 1).

Primers. Eight couples of primers have been used to study exons 2–8 containing the 5 domains highly conserved through evolution, as follows:

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5i5 GC (GC) - CT TGC AGC AGC ACT GCC T</td>
</tr>
<tr>
<td></td>
<td>2i3 CGG GGT GGT GCC CTG CCC TT</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 1 to 25);</td>
</tr>
<tr>
<td>Exons 2–4 (partial),</td>
<td>2i5 CAG GTG ACC CAG GTG TGG AAG</td>
</tr>
<tr>
<td></td>
<td>4i3 GAA TCC CAA AGT TCC AAA CA</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 26–84);</td>
</tr>
<tr>
<td>Exon 4 (partial),</td>
<td>4i5 GC (GC) - CC AGC AGC TCC TAC ACC GGC</td>
</tr>
<tr>
<td></td>
<td>4i3 GAA TCC CAA AGT TCC AAA CA</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 84–125);</td>
</tr>
<tr>
<td>Exon 5 (partial),</td>
<td>5i5 GC (GC) - CT TTC AAC TCT GTC TCC TTC</td>
</tr>
<tr>
<td></td>
<td>5i3 CCT GGG CAA CCA GCC CTG TC</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 126–148);</td>
</tr>
<tr>
<td>Exon 6,</td>
<td>5i5 TGG CCA AGA CCT GCC CTG TG</td>
</tr>
<tr>
<td></td>
<td>5i3 GCC AGC AGC TCC TAC ACC GGC</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 148–186);</td>
</tr>
<tr>
<td>Exon 7,</td>
<td>6i5 CCC CAC GCC TCT AGT TCC TC</td>
</tr>
<tr>
<td></td>
<td>6i3 GC (GC) - TG CTC ACC CGG AGG GCC ACT GA</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 187–224);</td>
</tr>
<tr>
<td>Exon 8,</td>
<td>7i5 GC (GC) - T TGC CAG ACC AGC ACT TCC CCC GAA G</td>
</tr>
<tr>
<td></td>
<td>7i3 GGG CAC AGC AGG CCA GTG TG</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 225 to 261);</td>
</tr>
<tr>
<td>Exon 9,</td>
<td>8i5 GC (GC) - GGA CAG GTA GGA CCT GAT TTC</td>
</tr>
<tr>
<td></td>
<td>8i3 AAT CTG AGG CAT AAC TGC AC</td>
</tr>
<tr>
<td></td>
<td>(this PCR product explores codon 262–306).</td>
</tr>
</tbody>
</table>

The presence of (GC) in a primer indicates the position of a GC clamp.
mM MgCl₂, 200 μM of each deoxynucleotide triphosphate, 50 pmol of each
Perkin Elmer Cetus DNA thermocycler 9600 (2 min at 94°C followed by 5
cycles of 30 s at 94°C, 30 s at 54°C, 30 s at 72°C and 30 cycles of 30 s at 94°C
for 2 min at 94°C and hybridized for 15 min at 70°C to create heteroduplexes.
After these cycles, the samples were denaturated for sequencing. Single-strand DNA was obtained in the asymmetric PCR.

A 20-μl aliquot of the PCR product was electrophoresed in a 6.5% polyacrylamide gel (37, 5:1) with a linearly increasing denaturing gradient from 50-100% (100% denaturating: 7 M urea and 40% formamide V/V) at 75 V for 7-9 h at constant 60°C temperature, depending on the melting properties. The melting temperature of a DNA fragment is dependent on its sequence and fragments differing by only a single base pair, these fragments will melt at different temperatures, depending on the melting map of the amplified DNA.

Electrophoresis. A 20-μl aliquot of the PCR product was electrophoresed on a simple nondenaturing 8% polyacrylamide gel electrophoresis; this is due to the creation of an heteroduplex between the wild type and the mutated allele.

Amplification. Approximately 300 ng of genomic DNA were amplified in a 100-μl reaction mixture containing 10 μM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each deoxynucleotide triphosphate, 50 pmol of each primer, and 2 units of Taq polymerase. Thirty-five cycles were performed in a Perkin Elmer Cetus DNA thermocycler 9600 (2 min at 94°C followed by 5 cycles of 30 s at 94°C, 30 s at 54°C, 30 s at 72°C, and 30 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C). After these cycles, the samples were denaturated for 2 min at 94°C and hybridized for 15 min at 70°C to create heteroduplexes.

Electrophoresis. A 20-μl aliquot of the PCR product was electrophoresed in a 6.5% polyacrylamide gel (37, 5:1) with a linearly increasing denaturing gradient from 50-100% (100% denaturating: 7 M urea and 40% formamide V/V) at 75 V for 7-9 h at constant 60°C temperature, depending on the melting map of the amplified DNA.

Computer analyses were performed using Melt 87, Melt Map Programs, generously provided by Lerman. The optimal times of migration for electrophoresis were determined for each PCR product.

Following electrophoresis the gels were stained with ethidium bromide and photographed under UV transillumination.

DNA Sequencing. PCR products displaying an altered mobility in the gel were sequenced. Single-strand DNA was obtained at the asymmetric PCR. Each single-strand product was concentrated using Centricron 100 (Amicon, Danvers, MA) and the reaction products were used for sequencing by the dideoxy nucleotide chain termination method.

The nomenclature used to design the mutations is detailed by Beaudet (24).

Controls. Different types of controls have been carried out in order to verify that the mutated alleles that we have found are not artefacts of the sequencing assay or due to the theoretical possibility of error rates of the Taq polymerase. (a) The DGGE² analyses have been performed twice each time a modified pattern of migration of the PCR product was detected. (b) All the sequencing data were obtained by sequencing from another aliquot of the tumor DNA, and this means that all these mutations were characterized on a different sample that the first one in which the mutation was initially identified.

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² The abbreviations used are: DGGE, denaturing gradient gel electrophoresis.

All sequencing data were controlled and confirmed twice. (c) Each time the mutated allele creates or destroys a restriction site a confirmation of its presence was performed by restriction analysis of the PCR product and characterization of the novel restriction pattern. For each mutated allele we have looked for the creation or the destruction of a restriction site on the mutated sequence. For P278T, the nucleotide change creates a restriction site; for the H193L, the mutation creates a AluI site. The confirmation of the presence of the mutated sequence was obtained after amplification and enzyme digestion. Some frame-shift mutations corresponding to 2 or 3 base pair deletion/insertion are detected on a simple nondenaturing 8% polyacrylamide gel electrophoresis; this is due to the creation of an heteroduplex between the wild type and the mutated allele. All the deletions/insertions described here have been confirmed in such a way.

RESULTS

We have developed the denaturing gradient gel electrophoresis assay to detect any single base change that occurs within a DNA fragment, whereby such a change alters that fragments melting properties. The melting temperature of a DNA fragment is dependent on its sequence and fragments differing by only one nucleotide will usually exhibit differences in their melting behavior. In practice, melting is achieved by electrophoresis through a polyacrylamide gel containing an increasing concentration of denaturing agents. If two fragments differ by a single base pair, these fragments will melt at different positions in the gel (25–27). This is illustrated by the DGGE analysis of exon 5 (Fig. 1). Due to extensive reports on the functional importance of highly conserved domains in gene products and the high frequency of mutations in the corresponding region of the gene, we have developed an assay which allows complete analysis of these regions, i.e., the five exons (2, 4, 5, 7, and 8) of the gene that encode for these five conserved domains and the exons 3 and 6.

Our DGGE conditions have permitted the identification of an altered pattern of migration in 27 samples among 32 (84%). Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Smoking</th>
<th>Tumor Extension</th>
<th>Stage</th>
<th>Exon</th>
<th>Nucleic acid</th>
<th>Event</th>
<th>Mutation</th>
<th>(amino acid no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>57</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>III</td>
<td>8</td>
<td>GAG → AAG</td>
<td>Transition</td>
<td>E255K (glutamic acid → lysine)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>53</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>III</td>
<td>8</td>
<td>CCT → TCT</td>
<td>Transition</td>
<td>P278S (proline → serine)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>73</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>IIA</td>
<td>8</td>
<td>CAT → CGT</td>
<td>Transition</td>
<td>H214R (histidine → arginine)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>60</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>IIA</td>
<td>8</td>
<td>del GTA</td>
<td>Frameshift</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>67</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>IIA</td>
<td>8</td>
<td>dupli ACGAG</td>
<td>Frameshift</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>57</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>IIA</td>
<td>8</td>
<td>CCT → TCT</td>
<td>Transition</td>
<td>P278S (proline → serine)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>55</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>IIA</td>
<td>8</td>
<td>CCT → ACT</td>
<td>Transition</td>
<td>P278T (proline → threonine)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>77</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>III</td>
<td>8</td>
<td>-2A → T</td>
<td>Transition</td>
<td>Acceptor splice site</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>56</td>
<td>+</td>
<td>T₂N₂M₀</td>
<td>III</td>
<td>8</td>
<td>-3A → T</td>
<td>Transition</td>
<td>Acceptor splice site</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 p53 mutations in our series of human esophageal carcinomas
illustrates the molecular defects we have found. We have identified 10
transversions (34%), 13 transitions (45%), and 6 frameshift mutations
(21%). Two missense mutations (P278T and P278S) were found in
one sample as well as a polymorphism (E271E) and a mutation
(Y220C) in another tumor sample. Of these mutations, 12 have not
been reported to date: two of these were transversions which affect the
two lower bands are homoduplexes of normal and mutant DNA.

The mix of the PCR product of the samples has not shown any
formation in denaturing gradient gels, the presence of a possible
defects were located in exon 5 (25%), 13 were located in exon 6
(46%), and 8 were located in exon 8 (29%) (Fig. 3). These data clearly
underline a cluster of mutations in exon 6 of the

tumor samples. We note also that 5 frameshift mutations were situated
in exon 5. Due to the frequent loss of heterozygosity in esophageal
cancer cells (30, 31), all our samples have been mixed with the PCR
product of wild-type p53 DNA in order to detect, by heteroduplex
formation in denaturing gradient gels, the presence of a possible
mutation on one allele of the tumor, the other allele having been lost.

In this work, by screening the 7 exons (2–8) of the gene that code
for the 5 conserved domains of the p53 protein, we succeeded in
identifying mutations in 27 of 32 esophageal squamous cell carcino-
mas. This corresponds to 84% of the cancers, and is, to our knowl-
edge, the highest incidence of TP53 mutations reported in human
malignancies.

Our results also suggest that somatic mutation in this type of tumor
might be much more frequent than previously reported. This may be
due to the fact we have used one of the most powerful assays for
detection of mutations in a human gene. The denaturing gradient gel
electrophoresis initially described by Lerman (25) and improved by
Sheffield (26) by his addition of a GC clamp at one end of DNA
fragment has already allowed the successful screening of other genes
responsible for human diseases such as globin (37), rhodopsin (38), or
cystic fibrosis transmembrane conductance regulator (39). However,
in five tumors we have not been able to detect any sequence abnor-
mality. To explain this lack of mutation, several potential explanations
could be proposed: (a), we have screened only those exons which
encode the conserved domains of the tumor suppressor gene, while
exons 9, 10, and 11 have not been studied; (b) some mutations could
be located in the intronic regions of the gene (1); (c) because tumor
samples contain inflammatory and stromal cells with normal DNA, a
mutation might be hidden by the wild type sequence of the
TP53 gene. Despite these five tumors with undetected mutations, at this time, our
data are highly suggestive that in esophageal cancer, mutation of the
TP53 gene might occur with considerable frequency. This work also
highlighted the location of a highly mutated region, exon 6 (codon
187–224) in which 45% of the mutations were found. These data
would indicate that, although this region does not encode part of a
conserved domain, it should be considered a hot spot in esophageal
tumors.

Esophageal cancer is one of the six most common cancers world-
wide with very significant variation in its geographical distribution. In
western Europe and in the United States, alcohol and tobacco use are
closely linked with esophageal carcinoma. Each of these is associated

Fig. 1. DGGE analysis of exon 5. Lane 1, del GTCA, 165; Lane 2, dupli ACATGACCG, 168; Lane 3, CAT → CT, H179L; Lane 4, del C, 159; Lane 5, ins CCCCT, 143; Lane 6, del TGA, 169; Lane 7, normal. DGGE analysis of amplified PCR product of exon 5: Lanes 1–6 refer to different squamous cell carcinoma samples carrying different mutations. The control DNA (Lane 7) is from a genomic DNA. The PCR product was electrophoresed in a 50–100% gradient denaturing gel for 8 h. The two upper bands are heteroduplexes and the two lower bands are homoduplexes of normal and mutant DNA.

Fig. 2. Sequencing data of four novel mutations.
with an increased risk of cancer of the esophagus. Both factors have a synergistic effect on carcinogenesis in the upper alimentary tract, and their combined use is associated with even higher risk. Alcohol can increase the susceptibility of the esophageal epithelium towards carcinogenesis in the upper alimentary tract, affecting DNA and cancer development. In our study, all the patients originated from Brittany, an area of western Europe where epidemiological studies have shown an increase in the risk of developing esophageal cancer from alcohol consumption (41).

Of particular importance also is the profile of mutations we have found in the TP53 gene of these patients. The spectrum of mutations observed in this series of 27 esophageal cancers of identical ethnic origin is quite particular with 45% of the DNA lesions being transitions, 34% transversions, and 21% being composed of deletions, duplications or small insertions. It is now well established that the pattern of mutations vary greatly in human neoplasms and is in part related to organ site. For example, G:C to A:T transitions predominated in colon lymphoid or brain tumors, and most of these occur at CpG dinucleotides (42). On the other hand, G:C to T:A transitions are the most frequent substitutions observed in cancers of the lung and the liver. These transversions are known to be induced by benzo[a]pyrene and aflatoxin B associated with the etiology of lung and liver tumors, respectively. These results have been confirmed in vitro showing that the TP53 nucleotides identified as mutational hotspots in lung and liver cancers are targets for benzo[a]pyrene and aflatoxine B.

It has been shown in rats (19) that esophageal tumors induced by alkylating nitroso compounds contain 78% transitions clustered in exon 6 of the gene. An article by K. H. Vihikancas (43) has reported that such a mutational lesion in the encoded tumor suppressor protein is a very frequent, and perhaps obligate, step in the development of the carcinogenesis of the esophagus. The particular profile of mutations observed by us in the TP53 gene in part contradicts the pattern previously reported by Hollstein (32) in which the occurrence of transversions was reported, at least in the first tumors analyzed, as being exceptionally frequent; however, in this series of 15 squamous cell carcinoma samples from the western part of France, 3 were carrying a G:C > T:A transition.

This molecular analysis of TP53 in esophageal neoplasms could suggest that these different profiles of mutations could be the result of exposure to several genotoxic environmental factors. For example, nitrosamine could cause transitions and benzopyrene transversions, and other genotoxic agents of esophageal cancer could be responsible for deletional-insertional events.

Since the definitive role and exact list of genotoxic agents capable of inducing mutations have yet to be deduced, it is noteworthy that our data are highly suggestive that esophageal tumors in this part of the world (western Europe) could be due to the synergistic effect of different carcinogens. These data may be helpful in defining the chemicals agents capable of producing the spectrum of mutations we have described as well as in understanding the etiology of human esophageal cancer.

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