TP53 Gene Mutation Profile in Esophageal Squamous Cell Carcinomas

M. P. Andrêzet, M. Robaszkiewicz, B. Mercier, J. B. Nousbaum, J. P. Bail, E. Hardy, A. Volant, P. Lozac'h, J. F. Charles, H. Gouérou, and C. Féric


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 carcinoma 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 was 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:

exon 2,
2i5 GC (GC) - CT TGC AGC AGC CAG ACT GCC TC
2i5 TGC CAG GTT GGT GCC CTG CTC TT
(this PCR product explores codon 1 to 25);

Exons 2–4 (partial),
2i5 CAG GTG ACC CAG GTT TGG AAG
4i3 GC (GC) - GGA AGG GAC AGA AGA TGA CAG
(this PCR product explores codon 26–84);

Exon 4 (partial),
4i5 GC (GC) - CC AGC AGC TCC TAC ACC GGC
4i5 TGG CCA AGA CCT GCC CTG TG
(this PCR product explores codon 84–125);

Exon 5 (partial),
5i5 GC (GC) - CT TTC AAC TCT GTC TCC TTC
5i5 TGG CCA AGA CCT GCC CTG TG
(this PCR product explores codon 126–148);

Exon 6,
6i3 GC (GC) - TG CTC ACC CGG AGG GCC ACT GA
6i3 GAA TCC CAA AGT TCC AAA CA
(this PCR product explores codon 187–224);

Exon 7,
7i3 GC (GC) - T TGC CAC AGG TCT CCC CAA G
7i3 GGG CAC AGC AGG CCA GTG TG
(this PCR product explores codon 225 to 261);

Exon 8,
8i5 GC (GC) - GGA CAG GTA GGA CCT GAT TTC
8i5 TGG CCA AGA CCT GCC CTG GC
(this PCR product explores codon 262–306).

Received 6/9/93; accepted 9/30/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by the Ligue Nationale contre le Cancer, Comité du Finistère.
**Table 1: p53 mutations in our series of human esophageal carcinomas**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Drinking</th>
<th>Smoking</th>
<th>TNM Extension</th>
<th>Stage</th>
<th>Exon</th>
<th>Nucleic acid</th>
<th>Event</th>
<th>Mutation (amino acid no.)</th>
</tr>
</thead>
</table>
| 1       | F    | 57   | +        | +       | T,N,M,I      | III   | 8    | GAG → AAG   | Transition | E255K (glutamic acid → lysine)
| 2       | M    | 53   | +        | +       | T,N,M,I      | III   | 1    | CTC → TCT  | Transition | P278S (proline → serine)
| 3       | M    | 73   | +        | +       | T,N,M,I      | III   | 5    | CAT → CTT  | Transition | R273C (histidine → arginine)
| 4       | M    | 60   | +        | +       | T,N,M,I      | III   | 6    | del GTA    | Frameshift | 165
| 5       | M    | 67   | -        | -       | T,N,M,I      | III   | 8    | CCAACG     | Duplication | 5 dupli AATAGACG
| 6       | M    | 63   | -        | -       | T,N,M,I      | III   | 5    | dEDGE      | Frameshift | 158
| 7       | M    | 55   | +        | +       | T,N,M,I      | III   | 8    | CCT → TCT  | Transition | P278S (proline → serine)
| 8       | M    | 77   | -        | +       | T,N,M,I      | III   | 8    | CCT → ACT  | Transition | P278T (proline → threonine)
| 9       | M    | 56   | -        | +       | T,N,M,I      | III   | 8    | CCAACG     | Duplication | 5 dupli AATAGACG
| 10      | M    | 71   | +        | +       | T,N,M,I      | I     | intron 7 | -2A → T    | Transition | R273C (arginine → cysteine)
| 11      | M    | 62   | +        | +       | T,N,M,I      | I     | 8    | CGT → TGT | Transition | R273C (arginine → cysteine)
| 12      | M    | 74   | -        | +       | T,N,M,I      | IIA   | intron 5 | -2A → T    | Transition | R273C (arginine → cysteine)
| 13      | M    | 62   | -        | +       | T,N,M,I      | IIA   | 6    | ATC → TTC  | Conversion | H195X (isoleucine → phenylalanine)
| 14      | M    | 68   | +        | +       | T,M,N,I      | III   | 3    | CAT → CTT  | Transition | H195L (histidine → leucine)
| 15      | M    | 76   | +        | +       | T,N,M,I      | III   | 5    | CAT → CTT  | Transition | H195L (histidine → leucine)
| 16      | M    | 62   | -        | +       | T,N,M,I      | IIA   | 5    | CAT → CTT  | Transition | H195L (histidine → leucine)
| 17      | M    | 58   | -        | +       | T,N,M,I      | IIA   | 6    | del NT     | Frameshift | 215
| 18      | M    | 59   | -        | +       | T,N,M,I      | IIA   | 6    | CAT → CTT  | Transition | H195L (histidine → leucine)
| 19      | M    | 64   | -        | +       | T,N,M,I      | IIA   | 6    | TAF → TGT | Transition | Y205C (tyrosine → cysteine)
| 20      | M    | 61   | unknown  | unknown | T,N,M,I      | IIA   | 8    | CGT → TGT | Transition | R273C (arginine → cysteine)
| 21      | M    | 61   | unknown  | unknown | T,N,M,I      | IIB   | 5    | del C      | Frameshift | 159
| 22      | M    | 70   | -        | +       | T,N,M,I      | IIA   | 6    | ATC → AGC | Transition | H195S (isoleucine → serine)
| 23      | M    | 80   | -        | +       | T,N,M,I      | I     | 8    | CCT → CTT  | Transition | P278R (proline → arginine)
| 24      | M    | 79   | -        | +       | T,N,M,I      | IIA   | 5    | ins CCCCTT | Frameshift | 142
| 25      | M    | 62   | -        | +       | T,N,M,I      | IIA   | 6    | AGA → TGA  | R290X (arginine → stop codon)
| 26      | M    | 52   | -        | +       | T,N,M,I      | III   | 6    | TAF → TGT | Transition | Y205C (tyrosine → cysteine)
| 27      | M    | 66   | -        | +       | T,N,M,I      | III   | 6    | TAF → TGT | Transition | Y220C (tyrosine → cysteine)
| 28      | M    | 63   | -        | +       | T,N,M,I      | I     | 6    | TAF → TGT | Transition | Y220C (tyrosine → cysteine)
| 29      | M    | 29   | -        | +       | T,N,M,I      | I     | 6    | TAF → TGT | Transition | Y220C (tyrosine → cysteine)
| 30      | M    | 64   | -        | +       | T,N,M,I      | III   | 5    | del TGA    | Frameshift | 169
| 31      | M    | 47   | +        | +       | T,N,M,I      | III   | 8    | CAT → CTT  | Transition | H193L (histidine → leucine)
| 32      | M    | 58   | -        | -       | T,N,M,I      | III   | 6    | GAG → TGA  | Frameshift | 169

*Previously described mutations (Refs. 13, 32, 33, 44-47).

**Amplification.** Approximately 300 ng of genomic DNA were amplified in a 100-μl reaction mixture containing 10 μM Tris-HCl (pH 8.3), 50 μM 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 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 denatured 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% denaturing: 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 Centricon 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 et al. (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.²

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 RsaI site; for the H193L, the mutation creates an Alul 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 nonthermalizing 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
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
THE TWO LOWER BANDS ARE HOMODUPLEXES OF NORMAL AND MUTANT DNA.

DISCUSSION

ACQUIRED MUTATIONS IN THE TP53 GENE HAVE BEEN DETECTED IN EPITHELIAL,

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 CARCINOMAS. THIS CORRESPONDS TO 84% OF THE CANCERS, AND IS, TO OUR KNOWLEDGE, 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 DENUATING 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 GLOBLIN (37), RHODOPIN (38), OR CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (39). HOWEVER, IN FIVE TUMORS WE HAVE NOT BEEN ABLE TO DETECT ANY SEQUENCE ABNORMALITY.

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
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 carcinogens by several mechanisms. Ethanol can enhance the activation of procarcinogens to carcinogens through microsomal enzyme induction. Ethanol can interfere with the cellular DNA repair mechanisms and the immune system. Alcohol by itself may increase the risk of carcinogenesis in the upper alimentary tract, nitrosamines, polycyclic hydrocarbons, fermentation oils, and other mutagenic compounds. Increased exposure to carcinogens by smoking and excessive alcohol consumption contributes to mutagenic events 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 mutational spectrum of TP53 is the most frequent and mutation the most frequent in human and murine p53. Oncogene, 1: 71–78, 1987.


It has been shown in rats (19) that esophageal tumors induced by alkylating nitroso compounds contain 78% transitions clustered in exons 6 of the gene. An article by K. H. Vähäkangas (43) has reported the mutational spectrum of TP53 mutations in lung cancer of underground miners exposed to radon: 2 deletions were found in 7 patients, although deletions have rarely been reported in lung cancers or other forms of cancer. In fact, less than 40 deletions-insertions have been reported among the 400 TP53 DNA lesions so far published (1).

The results we have obtained in this work in which we have been able to identify 84% of samples carrying a mutated allele in the TP53 gene of esophageal tumors is of importance since this could indicate that such a mutational lesion in the encoded tumor suppressor protein is a very frequent, and perhaps in step with 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.

ACKNOWLEDGMENTS

We are indebted to Professor T. Soussi for providing us with the genomic sequences of the TP53 gene and for reading the manuscript. We thank Chantal Baelcl for assistance in manuscript preparation and Annie Le Menn for the mounting of photographs.

REFERENCES


TP53 Gene Mutation Profile in Esophageal Squamous Cell Carcinomas

M. P. Audrézet, M. Robaszkiewicz, B. Mercier, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/23/5745

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.