

## Analysis of *p53* gene polymorphisms in Romanian patients with squamous cell oesophageal carcinoma

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### Abstract

The mutations of the *p53* tumor suppressor gene are a major step in carcinogenesis and the most common genetic defects known to occur in diverse human carcinomas. The frequency of *p53* gene mutations in human esophageal carcinoma is variable (ranging from 38% to 69%). In this study we evaluated the alterations of the *p53* gene by DNA sequencing analysis and immunofluorescence. Exons 5 to 8 of the *p53* gene were amplified by PCR using specific primers and were analyzed by direct sequencing. We have revealed multiple nucleotide polymorphisms both in the tumoral tissue and in the blood samples. By matching genetic data with the clinical data, the development of changes in *p53* gene could be correlated with the early stages (I-IIa) of squamous cell oesophageal carcinoma. The more advanced stages of cancer could be correlated with shifts in the polarity of the corresponding protein which could lead to conformational changes and alterations of its response capacity.

**Keywords:** *p53* gene, genetic mutations, squamous cell oesophageal carcinoma

### Introduction

Esophageal carcinoma is a malignant neoplasm relatively common on a worldwide scale with an average of 300,000 new cases arising each year [1]. Esophageal cancer has a worse prognosis compared with other digestive cancers because of a high frequency of lymph node metastasis and recurrence rate. Multimodality treatments including surgery, chemotherapy and radiotherapy are often required to counter the disease [2].

In Romania, the incidence of this type of cancer is 5.3/100,000 population in males and 0.7/100,000 in females, with mortality rates of 4.8/100,000 and 0.5/100,000 in males and females, respectively. Esophageal cancer is a treatable but rarely curable cancer, as many patients have advanced-stage disease at the time of diagnosis [3].

Carcinogenesis is a multistep process and carcinoma progresses are a result of accumulated genetic alterations that cause the cells to escape normal controls over cell growth and differentiation [4]. Mutation of the *p53* tumor suppressor gene is a major step in carcinogenesis and the most common genetic defect known to occur in diverse human carcinomas [5-8]. The frequency of the *p53* gene mutations in human esophageal carcinoma is variable (ranging from 38% to 69%), and no general conclusions have been reached with respect to the relationship between the *p53* gene status of esophageal carcinoma and its clinicopathological findings [9]. Most *p53* mutations are localized in the protein DNA binding

domain (residues 100–300) leading to a bias of *p53* mutation analysis, as more than 80% of *p53* mutation studies focus on exons 5–8 (residues 126–306) [10,11].

In this study we evaluated the alterations of the *p53* gene and modifications in the corresponding protein expression by DNA sequencing analysis and immunofluorescence.

## Materials and Methods

### *Patients*

Samples of tumour tissue and blood were obtained from ten patients with oesophageal squamous cell cancer (ESCC) from “Fundeni Clinical Institute”, Bucharest. All cases were diagnosed as ESCC by histopathology in different tumoral stages (I-IV). The average age of the patients at the time of surgery was 55 years (range: 40-73), 80% of the studied patients were men and 20% - women.

### *DNA extraction*

DNA from tumoral tissue samples was isolated with Wizard Genomic DNA Purification Kit (Promega). DNA from blood samples was extracted with QIAmp®DNA Blood Minikit (Qiagen). Next, the DNA precipitate was rehydrated with 50µL DNA Rehydration Solution from each kit. DNA quantity and purity was determined spectrophotometrically (A260/280 ratio).

### *Primer sequences and PCR amplification*

Polymerase chain reaction (PCR) was used to amplify two fragments belonging to the *p53* gene (408pb and 610pb). Table 1 shows the primer sequences used for *p53* exons 5-8 [12]. The first primer pair was designed to amplify a fragment of DNA including exon5, exon6 and intron 5 of the *p53* gene and the second primer pair was designed for the amplification of a DNA fragment including exon7, exon 8 and intron 7.

The reaction mixture (25 µl) included 1X buffer Taq polymerase, 1.5mM MgCl<sub>2</sub>, 0.4 mM dNTP mix, 0.4 µM primer, template DNA (10-100ng) and Taq DNA polymerase (1unit, Promega). The amplicons were purified with the Wizard PCR Preps DNA Purification System (Promega).

Table1. Primer Sequence

| <i>SDHB gene exon</i> | <i>Primer Sequence</i>                        | <i>Amplicon (bp)</i> | <i>Tm</i> |
|-----------------------|---|----------------------|-----------|
| 5_6                   | TTCCTCTTCCTGCAGTACTC<br>AGTTGCAAACCAGACCTCAG  | 408                  | 65        |
| 7_8                   | AGGTTGGCTCTGACTGTACC<br>ATTGTCCTGCTTGCTTACCTC | 610                  | 61        |

### *DNA sequencing*

PCR products were sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in an automated Genetic Analyzer (Applied Biosystems 3130) following the manufacturer's instructions. The resulted sequences were analyzed with BLAST program (on-line application, <http://blast.ncbi.nlm.nih.gov/>) and then compared with sequences of the *p53* human gene from GenBank database.

### *Immunofluorescence*

A series of five-micron sections from tissues, previously conserved at 80°C, were cut using a cryotome (Shandon Cryotome, Thermo Scientific), and were fixed in acetone for 10 min. Nonspecific binding of IgG was blocked using 10% normal goat serum in a humidity

chamber, for 60 min at room temperature. Next, the slides were rinsed with PBS solution and incubated with the mouse monoclonal primary antibody against human P53 protein (Santa Cruz) at a 1:100 dilution, for 60 min at 4°C. After a new washing step with PBS solution slides were incubated with goat anti-mouse IgG-FITC secondary antibody (Santa Cruz), diluted 1:150, for 60 min at room temperature. Slides analysis was performed using an Olympus CX 31 immunofluorescence microscope.

## Results and Discussions

The amplified fragments of the *p53* gene, obtained from the genomic DNA isolated from tumour and blood samples, were verified through agarose gel electrophoresis (2%). The obtained profiles corresponding to the tumour samples are presented in figure 1 and 2.

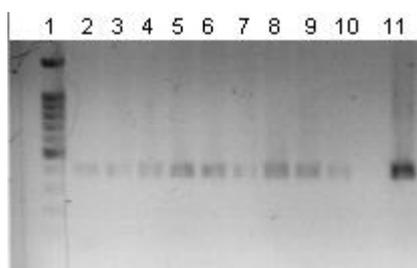


Figure 1. *p53* gene target fragments; 408pb in case of the DNA fragments amplified with the exons 5-6 primer set. Line 1- 100bp molecular mass marker; Lines 2-11 - *p53* gene amplified fragments from oesophageal cancer samples.

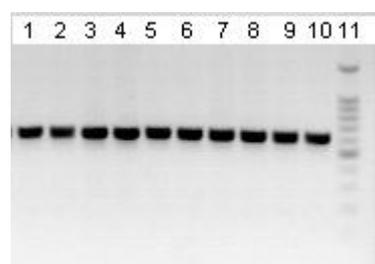


Figure 2. *p53* gene target fragments; 610pb in case of the DNA fragments amplified with the exons 7-8 primer set; Lines 1-10- *p53* gene amplified fragments from oesophageal cancer samples. Line 11- 100bp molecular mass marker;

The purified PCR products were directly sequenced on an ABI 3130 sequencer. The sequences obtained from blood and tumour samples were compared with the *p53* gene sequences from the GenBank database. Multiple nucleotide polymorphisms, both in the tumour tissue and in the blood samples, were discovered. From ten patients analyzed, four (40%) presented *p53* polymorphisms, which are showed in the following graphs (Fig. 3-6).

In the exon 5 nucleotide sequence was observed one polymorphism in patient 7, (Fig. 4). This polymorphism corresponds to a missense heterozygous point mutation localized in the 175 codon (CGC→CAC). Normally, the amino acid in position 175, in the protein chain, is arginine, but due to this genetic mutation arginine is replaced with histidine, both amino acids being basic and polar.

During the analysis of the exon 6 sequence, two heterozygous point mutations were noticed: i) a silent point mutation in 213 codon CGA→CGG; this mutation was found both in tumour and blood samples of patient 6 and the codified amino acid is arginine, (Fig. 3); ii) a missense point mutation in 220 codon TAT→TGT, in the tumour samples of patient 11; TAT codon is coding for tyrosine and TGT is coding for cysteine, (Fig. 6). Tyrosine and cysteine are both neutral amino acids, but cysteine is a less polarized amino acid then tyrosine.

Another type of *p53* modification, showed in figure 5, developed inside the intron 7 was evidenced in patient 8 (Fig. 5). Two polymorphisms have been found corresponding to the position + 18437 - T/C and to the position +18457 - T/G.

The correlations between genetic data and clinical data revealed that the modifications in *p53* gene already appear in the early stages (I-IIa) of squamous cell oesophageal carcinoma. The more advanced stages of cancer could be associated with shifts in the polarity of the

corresponding protein which could lead to conformational changes and alterations of its response capacity (Table 2).

In our previous studies on esophagus cancer [13] we found a major change in the *p53* gene of one patient: the transformation of the 196 CGA codon in a STOP (TGA) one. It should be noticed that this change could be produced in an early stage of cancer development.

Mutations and deletions of the *p53* gene are the most common genetic alterations in human cancers. More than 85% of known, cancer-related *p53* mutations are missense mutations. For several cancers, mutations of the *p53* and over-expression of the mutant P53 protein (nuclear accumulation) are associated with advanced tumor stage and poor survival [14]. Missense mutations occurring *in vivo* could lead to single amino acid substitutions, which results in changes of P53 conformation. The consequences of these changes might contribute to tumor progression and to a poor prognosis of the disease [15].

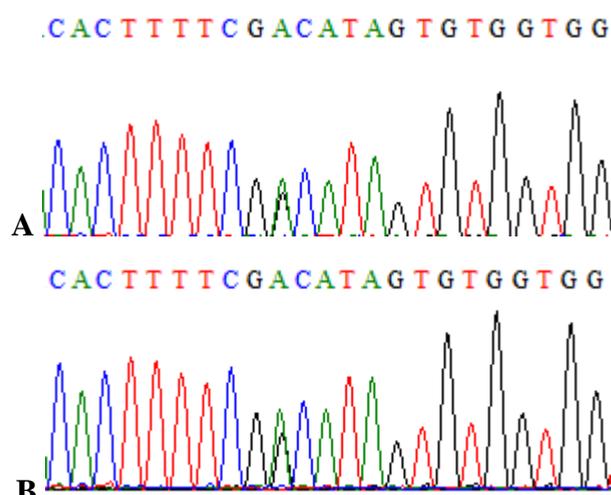


Figure 3. Partial nucleotide sequence of *p53* exon 6 gene in patient 6. A. Blood sample. B. tumour tissue.

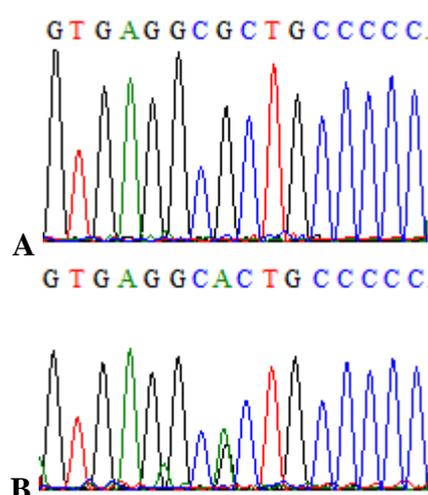


Figure 4. Partial nucleotide sequence of the *p53* exon 5 gene in patient 7. A. Blood sample. B. tumour tissue.

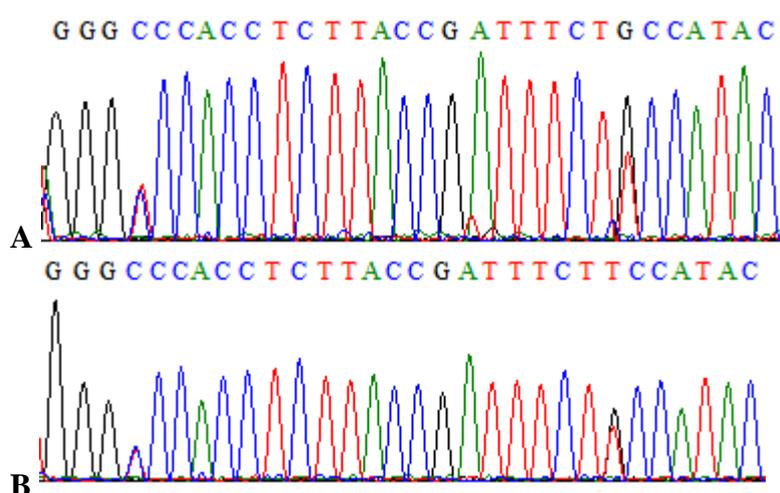


Figure 5. The heterozygous point mutations detected in intron 7 of the *p53* gene in patient 8. A. Blood sample. B. tumour tissue.

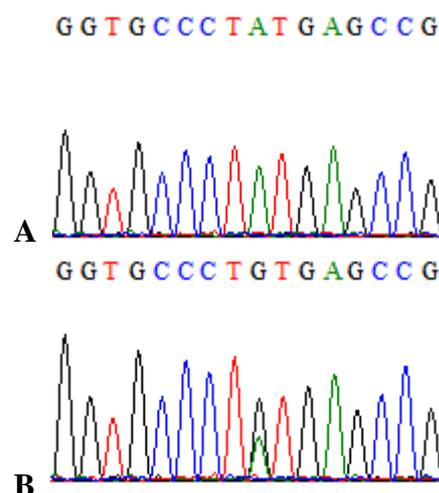


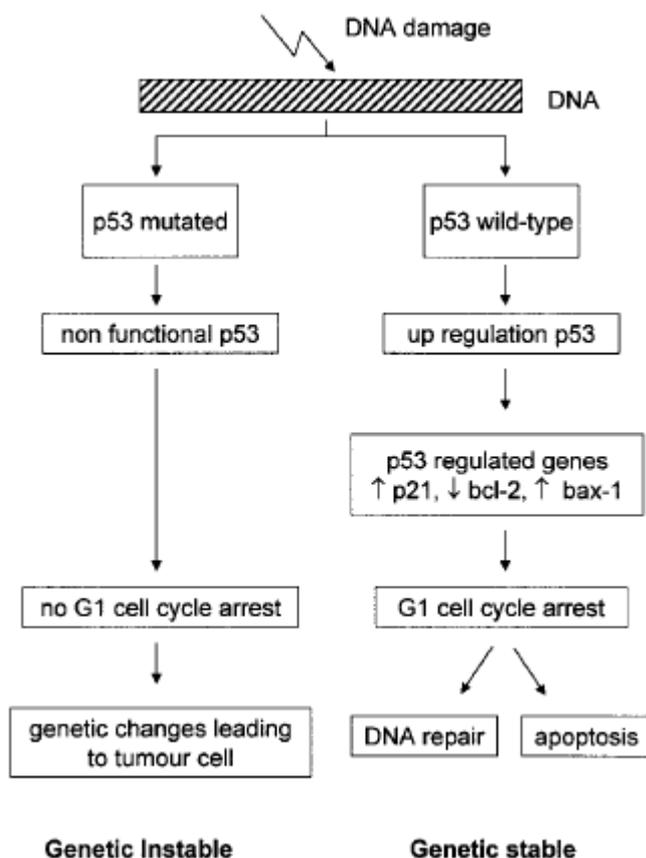
Figure 6. Partial nucleotide sequence of the *p53* exon 6 gene in patient 11. A. Blood sample. B. tumour tissue.

**Table 2.** *p53* gene mutations found in patients with different tumoral stages of ESCC .

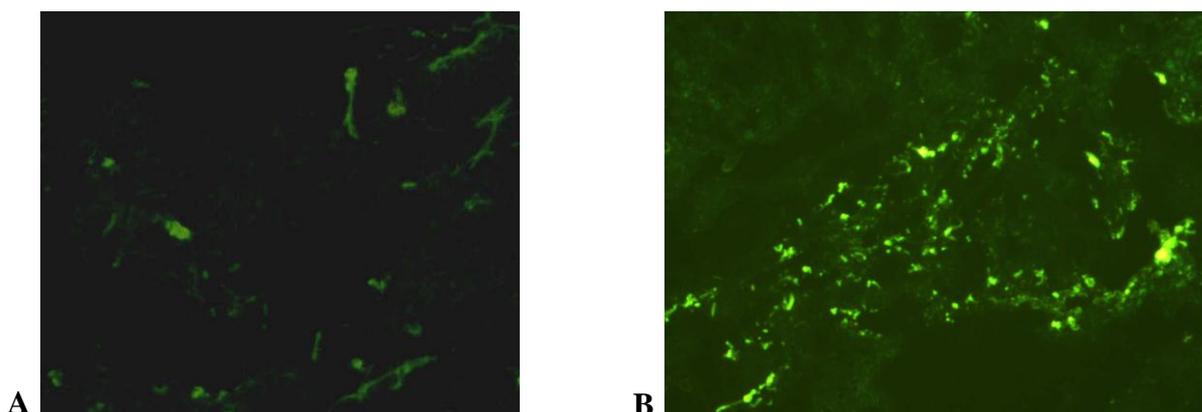
Analysis of *p53* gene polymorphisms in Romanian patients  
with squamous cell oesophageal carcinoma

| Patient | Tumoral stage | <i>p53</i> polymorphisms                | Amino acid change |
|---------|---------------|---|-------------------|
| 6       | IIa           | codon 213 CGA→CGG                       | Arg→Arg           |
| 7       | Ib            | codon 175 CGC→CAC                       | Arg→His           |
| 8       | I             | intron7,<br>+ 18437, T/C<br>+18457, T/G | -                 |
| 11      | IV            | codon 220 TAT→TGT                       | Tyr→Cys           |

The P53 protein functions in a homotetrameric complex as a transcription factor that induces expression of genes that facilitate cell cycle arrest, DNA repair, and apoptosis (Fig. 8). One mutant P53 protein, in the tetrameric P53 complex, could abolish the function of the entire complex. Further, most of the mutant proteins have a much longer half-life than the wild-type protein. This means that when a cell harbors one inactivating *p53* mutation, the expression of this protein could increase relative to the product of the wild-type allele, and the activity of the wild-type protein will be inhibited by complexing with the mutant protein (dominant-negative). The increased cellular P53 concentration can be visualized by immunohistochemistry. Comparing the results of other immunohistochemical studies, it is clear that in more than 50% of esophageal adenocarcinomas, pronounced P53 overexpression is present [16,17]. Our study confirms that previous data, the analysis of ESCC Romanian patients revealed an overexpression in P53 protein in tumor tissue (Fig.9).



**Figure 8.** *p53* and cell cycle regulation. With DNA damage due to the DNA changes an up-regulation of wild-type P53 protein could be evidenced. This leads to an increased transcription of *p53*-regulated genes (e.g., *p21*, *bax-1*, *bcl-2*), which inhibit the cell cycle. This facilitates DNA repair, or the cell entrance in the apoptotic pathway. Thus *p53* provides genomic stability. Mutations in the *p53* gene render the *p53* protein inactive, and the damaged DNA is transmitted [18].



**Figure 9.** Immunofluorescence staining for p53 protein in ESCC.  
A. Negative expression in normal tissue sample. B. Overexpression of p53 in tumour tissue sample.

## Conclusions

The *p53* mutations are localized in the protein DNA binding domain, leading to a bias of *p53* mutation analysis, as more than 80% of *p53* mutation studies focus on exons 5–8 (residues 126–306) [10,11].

We have found *p53* polymorphisms in 40% of the analyzed patients. The polymorphisms correspond to heterozygous point mutation found in exon 6 (silent mutation - codon 213 and missense mutation - codon 220), exon 5 (missense mutation - codon 175) and intron 7 (positions + 18437, T/C and +18457, T/G).

The mutations of *p53* gene can determine disruption of P53 protein conformation and in loss of its function. The consequences of these changes might contribute to tumor progression and to a poor prognosis.

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Analysis of *p53* gene polymorphisms in Romanian patients  
with squamous cell oesophageal carcinoma

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