P53 GENE SNP INVESTIGATION IN SQUAMOUS CELL OESOPHAGEAL CARCINOMA

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Abstract*:* Squamous cell cancer (ESCC) and adenocarcinoma (EADC) are the two main subtypes of oesophageal cancer. Genetic alterations associated with the development of oesophageal cancer are often observed. The aim of this study was to identify p53 gene polymorphisms in several patients with oesophageal squamous cell carcinoma. Genomic DNA was isolated from normal-, tumor- and blood-samples from patients with ESCC. PCR amplification of two p53 gene fragments was performed using two sets of primers DNA samples were sequenced and then analyzed with BLAST program Exons 5, 7 and 8 did not present mutations for the studied patients, but exon 6 featured one modification in two different patients. The mutations of p53 gene can determine the modification of its proteine function and the loss of its antioncogene role, which reflects the abnormal proliferation of the cells implicated in this type of cancer.

INTRODUCTION

Esophageal cancer is the 6th most common cancer globally and it also is one of the most interesting tumor types because of the great diversity in its incidence worldwide and in its pathological type (Shao *et al*., 2008; Alidina *et al*., 2004, Li, 1982). Oesophageal squamous cell cancer (ESCC) and adenocarcinoma (EADC) are the two main subtypes of oesophageal cancer. Other oesophageal malignant neoplasms are rare. It has been demonstrated that the squamous cell carcinoma is encountered in its highest percentage, 70% of oesophageal cancers, in some regions of China (Gamliel, 2000). In the last few decades, there has been an increased incidence of newly diagnosed adenocarcinoma (Blot *et al*., 1991; Devesa *et al*., 1998; Heitmiller and Sharma, 1996) of the distal esophagus, with a prevalence of 10 % /year due to GERD. In Europe and the USA the overall incidence does not exceed 10/100 000/year in men and 2/100 000/year in women and the pathological type is mainly the adenocarcinoma (Shao *et al*., 2008).

The incidence of this type of cancer (ESCC) is greatly reduced in Romania, 1.78/100 000/year. There has been some progress in the last two decades, leading to a better understanding of the molecular biology of esophageal cancer, a more correct staging with a better selection of the patients, the improvement of the surgical techniques and multimodal treatments. Esophageal cancer remains a malignancy with high morbidity and mortality despite improvements in diagnosis, staging, and surgical therapy (Popescu *et al*., 2008). In our country the survival rate is less than 10% at 2 years because of the patients are diagnosed more often in advanced tumoral stages, with early lymph node metastases.

The study of predictive factors (such as molecular and biological markers) is useful for the selection of patients who benefit of non-surgical treatment, such as chemoradiotherapy (estimating a complete pathologic response, allowing the election of the chemotherapy agent in patients with systemic disease or allowing the introduction into practice of targeted agents acting against specific molecular mechanisms associated with an unfavourable prognosis), (Popescu *et al*., 2008).

Similarly to other types of gastrointestinal cancers, in the case of oesophageal cancer, genetic errors appear in normal cells during the transformation into tumoral cells (Wafik, 2003). The damage of cell cycle control in the G1 phase through the inactivation of *p16*, amplification of D1 cyclin, and activation of oncogene-like receptor for epidermal growth factor (EGFR) and *c-myc* (Mandard *et al*., 2000, Montesano *et al*., 1996) are included (McCabe and Dlamini, 2005) among the genetic alterations associated with the development of oesophageal cancer mutations of p53 gene.

The studies on the structure and functions of the p53 gene and protein reveal that p53 has an important role in tumor pathogenesis as a component of the regulatory network of cell cycle control, DNA integrity and programmed cell death (Kwok Pui-Yan, 2003; Guimaraes and Hainaut, 2002). The most frequent genetic modifications that affect p53 gene are located between exons 5-9, corresponding to a highly conserved central domain of the protein (Krauss, 2003; Ireland et al., 1997; Li et al., 2005).

Our study was focused on p53 gene polymorphisms in Romanian patients diagnosed with an advanced stage of oesophageal squamous cell carcinoma.

MATERIALS AND METHODS

Tumor samples: Samples from normal tissue, tumor tissue and blood were obtained from patients with oesophageal squamous cell cancer who had undergone clinical surgery at the Department of Surgery, "Fundeni Clinical Institute of Digestive Diseases and Hepatic Transplantation" in Bucharest. All cases were diagnosed as ESCC by histopathology in advanced tumoral stages (stage II and III).

DNA extraction: DNA from normal and tumoral tissue samples was isolated with Wizard Genomic DNA Purification Kit from *Promega* and the DNA from blood samples was extracted with QIAmp®DNA Blood Minikit *(Qiagen).* Then, the DNA precipitate was rehydrated with 50µL DNA Rehydratation Solution from each kit. The DNA sample was stored at 4°C overnight. The purity and concentration of DNA was determined by spectrophotometry.

DNA amplification: Polymerase chain reaction (PCR) was used to amplify two fragments from the p53 gene (408pb and 610pb). One fragment (408pb) included exon5, exon6 and intron 6 and was amplified with the primer set: exon 5-6 FW (5'-TTC CTC TTC CTG CAG TAC TC-3')/ RW (5'-AGT TGC AAA CCA GAC CTC AG-3'). The second fragment (610pb) included exon7, exon 8 and intron 8 and was amplified with another set of primers: exon 7-8 FW (5`-AGG TTG GCT CTG ACT GTA CC-3`) and RW (5`-ATT GTC CTG CTT GCT TAC CTC-3`). The 25 µl of reaction mixture contained 1X buffer Taq ploymerase, 1.5mM $MgCl_2$, 0.4 mM of dNTP mix , 0.4 µM of each primer, template DNA (10-100ng) and Taq DNA polymerase (1unit, *Promega*). PCR conditions were 95°C for 1 min, 30 cycles of 95°C for 30 sec, 65°C for 45 sec and 72°C for 90 sec and a final cycle of 72°C for 7 min. The amplicons were purified with the Wizard PCR Preps DNA Purification System from Promega.

DNA sequencing: PCR products were sequenced using the ABI PRISM ® BigDye ™ Terminator Cycle Sequencing Ready Reaction kit (*Applied Biosystems*) on an automated DNA sequencer (*Applied Biosystems* 310) following the manufacturer's instructions. DNA sequences of both strands were obtained using the same primer set as the PCR amplification (forward and reverse). DNA sequences obtained for p53 gene were processed with BioEdit v.7.0 software. The resulting sequences were analyzed with the BLAST program (on-line application, http://blast.ncbi.nlm.nih.gov/) and then compared with sequences of the p53 human gene from the GenBank database.

RESULTS AND DISCUSSION

In order to determine p53 polymorphisms in exons 5-8, from squamous cell oesophageal cancer cases that were included into the study, a series of PCR assays were performed with designated primers. We amplified the fragments of p53 gene from the isolated genomic DNA from normal and tumoral samples. The amplicons obtained were verified through agarose gel electrophoresis (2%). The profiles obtained are presented in figure 1.

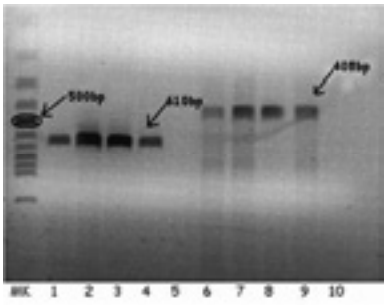

Figure 1. The p53 gene DNA fragments; 408pb for DNA fragments amplified with primer set exons 5-6 and 610pb for DNA fragments amplified with primer set exons 7-8 MK-molecular mass marker of 100 base pairs; 1-5 Amplified fragments for exon 5-6 from oesophageal cancer samples; 5-Negative control; 6-10 Amplified fragments for exon 7-8 from oesophageal cancer samples; 10- Negative control.

After processing the electrophoresis profiles, we performed a successful sequencing method for the amplified fragments. The sequences obtained were analyzed with the BioEdit software. By comparing the fragments obtained from normal and tumoral samples with the p53 gene sequences from the GenBank database, we have discovered single nucleotide polymorphisms in the tumoral tissue.

Nucleotide sequence from p53 gene analyses performed on the study cases revealed two heterozygous point mutations in the tumoral tissue samples in the exon 6 (Figure 2A). In the other analyzed exons (exon 5, exon 7 and exon 8) we did not determine any polymorphisms.

Both mutations identified in exon 6, can modify codon 196 (CGA) of the p53 gene, that normally codes for arginine. The CGA codon transformed into the TGA codon and the switch between nucleotide C and T determined a change of codon 196 into a STOP codon (Figure 2B). This mutation has been described in the literature (Krauss, 2003) as rare, but it can be encountered in different types of cancers. Because of this mutation, the p53 protein may have a translation error and therefore could result in a truncated protein.

Most known mutations in the p53 gene have been limited to codons 97-292 (Ireland et al., 1997; Greenblatt et al., 1994), that translates into a core domain of the p53 protein that binds with DNA and is highly conserved. The mutation we identified in this study is relevant, due to the fact that the p53 protein has a significant role in cancer (Hollstein et al. 1991; Cho et al., 1994). Its genetic alteration that conducts to a truncated protein can be related to an uncontrolled specific proliferation in cancer.

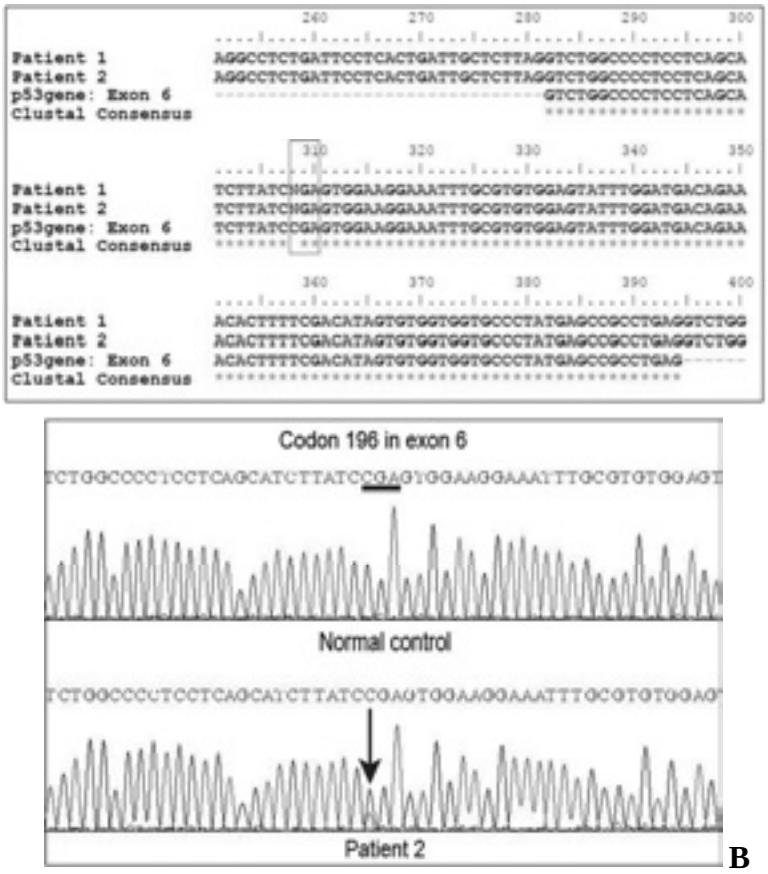

Figure 2. Direct sequencing of the p53 suppressor gene in squamous cell oesophageal carcinoma samples. A: Alignment of tumoral sequences of exon 6 of the p53 gene from two of the analyzed patients, nucleotide N can be C or T. B: A heterozygous point mutation in patient 2.

CONCLUSIONS

Taking into account that the p53 protein functions are of great significance in the normal cell functions modulation, the coding sequence genetic alteration can have an important role in tumoral malignancy.

The most frequent mutations of p53 are located between exons 5-9, corresponding to a highly preserved domain of the protein, DNA binding domain. In this study, we have found mutations located in exon 6 identified in the tumoral samples belonging to two of the analyzed patients. These mutations can lead to a truncated protein synthesis, codon 196 can become a STOP codon (CGA→TGA).

Further studies are needed to examine whether the observed mutation of the p53 suppressor gene is correlated with other mutations from different genes with a relevant role in cancer development.

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