EXPRESSION LEVELS OF p53 MESSENGER RNA DETECTED BY REAL TIME PCR IN TUMOR TISSUE, LYMPH NODES AND PERIPHERAL BLOOD OF PATIENTS WITH NON-SMALL CELL LUNG CANCER - NEW PERSPECTIVES FOR CLINICOPATHOLOGICAL APPLICATION

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ABSTRACT
The importance of p53 mutations in the pathogenesis of lung cancer is well established, but it is still controversial whether the presence of p53 mutations or overexpression of P53 protein are of clinical significance. The aim of this study was to define the levels of expression of p53 mRNA in tumor, lymph nodes and peripheral blood of patients with non-small cell lung cancer, detected by relative quantification real-time PCR and to analyze the association with clinicopathological data. We applied real-time PCR to define the levels of expression of p53 mRNA in tumor, lymph nodes and blood of 25 patients with non-small cell lung cancer, which underwent an operation at the Department of Thoracic Surgery of the University Hospital for Pulmonary Diseases in Sofia. As a control group 25 healthy volunteers were used. The method of relative quantification was applied for the analysis. We detected a high expression of p53 mRNA in 90.9% of the tumors and in 73.7% of the lymph nodes. A trend for a correlation between the high levels of p53 expression in the tumor and lymph nodes was also established (p<0.19). We did not find any correlation between p53 expression in tumor tissue and peripheral blood.

The expression analysis of p53 messenger RNA, detected by real-time PCR, can supplement the knowledge of p53 as a biomarker for lung cancer diagnosis and pathogenesis. The method is more sensitive than the currently used methods for p53 expression analysis and thus provides opportunities for a more accurate clinical application of molecular markers.

Keywords: p53, expression analysis, clinical implication, non-small cell lung cancer

Introduction
Lung cancer remains the leading cause of death due to cancer in industrialized countries, accounting for more than 30% of cancer associated deaths (2). Despite improvements in the diagnosis and therapy of lung cancer in the past two decades, the overall 5-year survival rate remains less than 15% (12).

Lung cancer, like many other cancers, arises through a multi-step process of genetic alterations. These include mutational, chromosomal and epigenetic changes leading to the activation of proto-oncogenes or inactivation of tumor suppressor genes and mutator genes (6). One major genetic alteration, detected in lung cancer, includes point mutations in the p53 tumor suppressor gene (9, 14). The rate of p53 mutations in non-small cell lung cancer varies from 30% to 50% of the cases and are clustered in ‘hot spots’ at codons 157, 158, 248, 249, 273 and 282 within exons 5 and 8, where the four evolutionarily conserved domains of the p53 gene are located (5, 7, 10). These types of mutations are typical for both smokers and nonsmokers.

Missense mutations of the p53 gene are most common and usually but not always prolong the half-life of the protein from minutes to hours. This results in nuclear accumulation of the P53 protein, which can be detected by immunohistochemistry (13). It has been shown that p53 mutations in lung cancer are different from those in other cancers and that an excess of G to T transversions is characteristic for these tumors (8, 11). The genetic profile of p53 mutations in lung cancer and adenocarcinoma differ in their characteristics and location. This is not attributed to the different exposure to cigarette smoke as the number of the pack/years in the investigated patients is approximately the same (4).

There are a lot of studies that deal with the analysis of p53 expression detected by immunohistochemistry or highlight on the mutational spectra of the gene, but it is still controversial whether the presence of p53 mutations or overexpression of P53 protein are of clinical significance for lung cancer pathogenesis and prognosis.

The aim of this study was to define the levels of expression of p53 mRNA in tumor tissue, lymph nodes and peripheral blood of patients with non-small cell lung cancer, detected by relative quantification real-time PCR. As part of the study we also looked for correlations with the clinicopathological characteristics of the patients. This could enlarge the knowledge concerning the role of p53 in lung carcinogenesis.
Materials and Methods

Patients and tissue specimens

The study was approved by the Ethics Committee of the Medical University of Sofia. This study includes twenty-five patients with non-small cell lung cancer diagnosed and operated between November 2006 and February 2007 at the Department of Thoracic Surgery, University Hospital for Pulmonary Diseases, Sofia and 25 healthy volunteers. All participants have signed an informed consent before the enrollment. Their clinical records and histopathological diagnoses were fully reviewed and the patients were followed up within a year after operation (until 31.03.2008). Twenty-one patients were men and four were women. The mean age of the patients was 60.00 years (range 50-72 years).

Histopathological classification and differentiation of the tissue samples was done by a pathologist according to the criteria of World Health Organization, 1981: thirteen patients were with squamous cell lung cancer, five with adenocarcinoma, three with bronchoalveolar carcinoma and four with adenosquamous carcinoma. Four of the patients had well differentiated tumors, ten had cancers with moderate differentiation and eleven were with poor differentiation.

TNM staging was assessed by surgical reports according to the post-surgical pathological international system adopted by the International Union Against Cancer - 20% of the patients were in stage I, 16% in stage II, 56% in stage III and 8% in stage IV.

The participants’ smoking history (number of cigarettes per day and duration of smoking) was obtained from preoperative personal interviews. As non-smokers were defined subjects with no previous history of smoking and smokers were defined as both former and current smokers. All patients with lung cancer were current smokers. At the time of operation samples of the tumor tissue, adjacent non-cancerous tissue and hilar lymph nodes were taken in sterile tubes and transported on dry ice within 15-20 minutes after the resection. Consequently they were stored at -80ºC until the time of analysis. In total seventy-five samples of solid tissues were analysed – twenty-five from tumor tissue, twenty-five from adjacent non-cancerous lung tissue and twenty-five from hilar lymph nodes. For the purpose of the investigation fifty samples of blood were taken preoperatively from twenty-five patients and twenty-five healthy volunteers. Three milliliters of peripheral blood were drawn in sterile conditions in vacuum containers, with potassium EDTA. The blood was submitted to total RNA extraction within 15 min after the drawing procedure.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the surgically removed tumor, adjacent non-cancerous tissue and lymph nodes and from the peripheral blood using TRIZOL Protocol for RNA extraction from blood and solid tissues.

RNA isolates for analysis were electrophoretically intact. Those with 260/280nm absorption ratio > 1.8 were immediately subjected to cDNA synthesis. First strand cDNA was synthesized from total RNA with the TaqMan Reverse Transcription Reagents using random primers (Cat. No. 8080234, Applied Biosystems) employing Technne apparatus TC 412 following the manufacturer’s manual.

Use of Q-PCR

Q-PCR was performed in Real-time PCR System 7500. Each reaction mixture contained: Universal Mastermix 10µl, forward primer and reverse primers, TaqMan probe 1µl, (Hs00153349_m1 TP53, Hs99999903_m1 ACTB, Hs01591644_m1), cDNA 4µl and 5 µl PCR water. PCR amplification was carried out at the following conditions: 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s and extension at 60°C for 60 s. PCR products were quantified by measuring the fluorescence intensity at the end of each amplification cycle.

The level of each mRNA was normalized to β-actin for the blood samples and Pol2α for the solid tissues and presented as expression ratio. The expression ratio of a particular mRNA marker in each subject was represented as the mean of three independent experiments. The relative comparative quantitation method was applied to determine the gene expression levels.

Results and Discussion

The most important point in this method was the choice of a normal referent control. For the tumor tissue and lymph nodes we accepted as a control the adjacent non-cancerous lung tissue. As a control group of the blood samples of the patients served the blood samples of the healthy volunteers. The values of RQ within the range (RQ ± 2SD) in the corresponding referent group were accepted as normal. RQ for p53 expression which was more than 2 standard deviations from the median RQ in the control group was considered as overexpression (high level of expression). RQ, smaller than 2 standard deviations from median RQ in the control group was considered as underexpression (low level of expression).

High expression levels of p53 were detected in 90.9% of the tumors (Fig. 1), 73.7% of the lymph nodes and in 5.9% of the blood samples. With normal expression were 9.1% of the tumors, 15.8% of the lymph nodes and 76.5% of the blood samples. Low levels of expression were detected in 17.6% of the blood samples and 10.5% of the lymph nodes. In three tissue samples, 6 lymph node samples and 7 blood samples real time PCR analysis detected Ct after the 37th cycle of the reaction, thus were excluded from the analysis.

The inability of the routine staging system to characterize the biological behavior of the tumor is a major obstacle for accurate cancer staging. Thus there is a demanding call for biomarkers that can help for the thorough interpretation of the prognostic factors in lung cancer-TNM classification.

Although there are a lot of studies, dealing with the mutational spectrum of p53 gene, as well as IHC studies for p53 protein expression in lung cancer specimens, there are no studies for p53 mRNA expression detected by real-time PCR until now.
Our real-time PCR analysis showed higher rates of overexpression in the tumor tissue – 90.9%, which may implicit that there is indeed a higher rate of p53 expression than it was actually detected by immunohistochemistry until now. Keeping in mind that p53 is a marker for a bad prognosis (3) in lung cancer patients independently of tumor stage, we can say that real-time PCR is a method for detection with an important clinical application. It can help us improve the clinical approach and therapy in patients subdividing them into different risk groups, determining the necessity of a more aggressive therapy and disease control.

Because of the limited number of patients in our study this correlation should be confirmed in larger studies. Having in mind that our group represents predominantly patients with squamous cell lung cancer our results are confined to this group of patients. In comparison the immunohistochemical analysis of p53 expression was unable to define a correlation between p53 expression and the tumor stage (1).

Comparing the levels of expression of p53 mRNA in the tumor tissue and in the lymph nodes we found a trend for significant relation between the overexpression of p53 in the tumor and in the lymph nodes \( (p<0.19) \). This confirms the fact that p53 mRNA could be used as a marker for micrometastasis in lymph nodes.

In the present study we defined for the first time the ranges of expression of p53 mRNA in peripheral blood of lung cancer patients and in healthy volunteers in order to assess the clinical importance of p53 as a non-invasive biomarker for lung cancer diagnosis. Most of our lung cancer patients had expression values of p53 mRNA in peripheral blood that was comparable to the levels of expression of p53 mRNA in healthy volunteers except one who had a higher expression.

**Conclusions**

Our results showed high incidence of p53 overexpression in tumor tissues of patients with non-small cell lung cancer. This was associated with the overexpression of p53 mRNA in corresponding lymph nodes and a trend to be associated with the advanced stages.

**REFERENCES**