The detection of quantitative serum p53 protein in lung cancer

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ÖZET

Akciğer kanserinde kantitatif serum p53 proteini saptanması

Tüm insan tümörlerinin yarısından fazlasında hücre içinde biriktiği gösterilen p53 proteininin, çeşitli maligniteleri olan hastaların serumlarında değişken olarak bulunduğu bildirilmiştir. Bu çalışmada, akciğer kanserli hastaların serumlarında p53 proteininin saptanması ve akciğer kanserinde p53 değişimlerinin belirleyicisi olarak değerinin doğrulanması amaçlanmıştır. Yeni tanı almış 94 akciğer kanserli hastada serum p53 proteinin saptanmasında pantropik kantitatif ELISA tekniği kullanıldı. Serum örnekleri herhangi bir tedavi almadan önce, başvuru sırasında alındı. Otuzdört sağlıklı gönüllüden oluşan kontrol grubunda; serum p53 proteini saptanmadı. Serum p53 proteini; 94 hastanın sadece 3 (%3.2)'ünün serumlarında vardı. Küçük hücreli dışı akciğer kanserli grupta 72 hastanın 2 (%2.8)'sinde ve küçük hücreli akciğer kanseri olan 22 hastanın 1 (%4.5)'inde serum p53 proteini saptandı. Serum p53 proteini düzeyleri pozitif olan örneklerde 1-31.25 U/mL arasında değişiyordu. Serumlarında p53 proteini saptananlar ileri evre hastalığı olan ve kötü prognozlu hastalardı. Sonuç olarak; serum p53 protein düzeyinin prognostik önemi p53 proteini pozitif olan hastaların sayısının az olması nedeniyle belirlenemedi. Kantitatif olarak ELISA ile serum p53 proteini analizinin kullanımı akciğer kanserli hastalarda kolay uygulanabilir bir teknik olmasına karşın, p53 proteini değişikliklerini değerlendirmede bir belirleyici olarak sınırlı bir kullanıma sahiptir.

Anahtar Kelimeler: *Serum p53 proteini, akciğer kanseri, kantitatif ELISA.*

SUMMARY

The detection of quantitative serum p53 protein in lung cancer

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p53 protein, which accumulates intracellularly in over half of all human tumors, has been reported to be variably present in the sera of patients with various malignancies. In this study, it was aimed to detect p53 protein in the sera of lung cancer patients, and to verify its value as a marker of p53 alterations in lung cancer. A pantropic quantitative ELISA technique was used to detect serum p53 protein of 94 newly diagnosed patients with lung cancer. Serum samples were collected on admission before any treatment. There was no detectable serum p53 protein in the control group including 34 healthy volunteers. Serum p53 protein was present in only 3 (3.2%) of 94 patients. In nonsmall cell lung cancer (NSCLC) group, se-

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rum p53 protein had been detected in 2 (2.8%) of 72 patients, and it was detectable in 1 (4.5%) of 22 patients in SCLC group. Serum levels of p53 protein ranged from 1 U/mL to 31.25 U/mL in positive samples. Patients who had p53 protein in their serum samples, were at late stage and had poor prognosis. In conclusion; prognostic value of detectable serum p53 protein levels could not be define, because of the small number of p53 positive patients. The use of quantitative serum p53 protein analysis with ELISA is of very limited value as a marker in evaluating p53 changes in lung cancer patients, despite the fact that is an easy technique to perform.

Key Words: *Serum p53 protein, lung cancer, quantitative ELISA.*

Lung cancer is the leading cause of cancer death throughout the world with more than one million annual deaths (1). In the United States, it is the leading cause of cancer death in both men and women and accounts for 28% of all cancer deaths (2). Lung cancer is the most common cancer in the world today, accounting for 18% of cancers of men worldwide, and 21% of cancers in men in developed countries (3).

Lung cancer is the result of multistep accumulation of genetic and molecular alterations highly related to tobacco carcinogens, involving key mechanisms of proliferation and apoptosis (4). The p53 tumor suppressor gene is clearly a component in biochemical pathways central to human carcinogenesis; p53 protein alterations due to missense mutations and loss of p53 protein by nonsense or frameshift mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells (5). p53 mutations are found in 50-55% of all human cancers (6). Mutations in the p53 gene are very common in lung cancer, occuring in 90% of small-cell lung cancers (SCLC), and in 50% of non small-cell lung cancers (NSCLC) (7,8).

The p53 gene consists of 11 exons, and most p53 mutations occured in the regions of the gene which are highly conserved through evolution, primarily in exons 5-8. However, evaluation of only exons 5-8 is likely to underestimate the prevalence of p53 mutations, especially non-missense mutations (5). In the p53 gene, mutations may occur in 90 of 393 codons required for the synthesis of the protein, and this makes diagnosis more difficult because the region to be analyzed extends over almost the entire gene. The molecular analysis is unsuited for routine diagnostic analysis (9).

p53 protein is normally undetectable because of its short half-life, but the mutant protein is more stable with a half-life of 4-12 hours, and it accumulates in the nucleus. It is possible to detect this nuclear accumulation by immunohistochemistry. Positive immunostaining is usually indicative of abnormalities of the p53 gene and its product, but it is highly dependent of the type of p53 mutation. It is a sensitive method for detection of missense mutation. The mutations that abolish p53 expression (splicing signal mutations, nonsense mutations, insertions, or deletions) do not produce the protein and therefore give a negative result (10,11). Enzyme-linked immunosorbent assay (ELISA) has been also used to detect p53 protein overexpression in tumor tissues (12-14).

p53 antibodies is an important area of interest in p53 studies. This humoral response is predominately associated with p53 gene missense mutations and p53 accumulation in the tumor, but the sensitivity of this detection is only 30% (15). The exact mechanism that leads to the formation of these antibodies is not clear, and accumulation of p53 in the tumor shows a good correlation with the presence of p53 antibodies. However, p53 protein release in to the bloodstream, followed by an immune response, can be a one of the possible explanations, p53 protein detection in the serum samples of cancer patients has been the subject of only a few investigations, concerning patients with colon, lung, bladder, pancreatic, breast, gynaecological cancer, and malignant lymphomas. The results of these studies are highly vari-

able, ranging from 1 to 64% (16-26). Serum p53 protein has been detected previously in 1.8-13% of patients with NSCLC and SCLC (17-20).

In the present study, the objective was to detect quantitatively p53 protein in the sera of lung cancer patients with ELISA technique, to verify its value as a marker in p53 alterations in lung cancer.

MATERIALS and METHODS

Patients

Venous blood samples were drawn on admission before any treatment from 94 newly diagnosed patients with lung cancer (age ranged: 38-84 years), including 72 cases of non small-cell and 22 cases of small-cell lung cancer. We also obtained serum samples from 34 healthy volunteers as controls. The serum was separated by centrifugation, divided small aliquots and stored at -70°C until analysis. Tumor stage was defined according to the TNM classification system in NSCLC patients and to Veterans Administration Lung Study Group criteria as limited and extensive disease in SCLC patients (27,28).

Enzyme-Linked Immunosorbent Assay for Quantitative Detection of Human p53

Serum p53 protein level was measured with a pantropic human p53 quantitative ELISA (BMS256-BenderMedSystems, Austria) according to the manufacturer's protocol. Principles of the test are as follows; an anti-p53 monoclonal coating antibody (murine), selective for either mutant and normal p53 protein, is adsorbed onto microwells. p53 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin conjugated monoclonal anti-p53 antibody is added and binds to p53 captured by the first antibody. Following incubation unbound biotin-conjugated anti-p53 is removed during wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-p53. After the incubation unbound streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of p53 present in the sample. The reaction is terminated by the addition of acid and absorbance is measured at 450 nm (Awareness Technology Inc. spectro-photometer is used in our assay).

A standard curve is prepared from seven p53 standard dilutions and p53 sample concentration determined. According to the manual for this ELISA kit, a cut-off level of p53 protein was 0.5 U/mL.

RESULTS

The mean age of patients was 60 (range 38-84). Detailed characteristics of the patients are shown in Table 1. There was no detectable serum p53 protein in the control group. Serum p53 protein was present in only 3 (3.2%) of 94 patients. In NSCLC group, serum p53 protein

Table 1. Clinical characteristics of patients.

Clinical feature	Number of patients (%)
Age	
< 60 years	47 (50)
≥ 60 years	47 (50)
Gender	
Female	4 (4.3)
Male	90 (95.7)
Histopathological type	
Non small-cell	72 (76.6)
Squamous cell	36 (38.3)
Adenocarcinoma	14 (14.9)
Bronchioloalveolar	1 (1.1)
Large cell	2 (2.1)
Non small-cell	19 (20.2)
Small-cell	22 (23.4)
Disease stage	
IB	1 (1.1)
IIB	5 (5.3)
IIIA	19 (20.2)
IIIB	21 (22.3)
IV	26 (27.7)
Limited disease	11 (11.7)
Extensive disease	11 (11.7)
Performance status (ECOG)	
≤ 2	80 (85.1)
> 2	14 (14.9)
Smoking history	
Smoker	89 (94.7)
Nonsmoker	5 (5.3)

Table 2. Clinical characteristics of patients which were positive for serum p53 protein and their serum level.

Patient	1	2	3
Gender	Male	Male	Male
Age	69	66	59
Smoking history (pocket year)	30	80	15
Performance status	0	2	3
Histopathological type (differantiation)	SCC (low)	SCC (intermediate)	Small-cell
Disease stage	III A	IV	Extensive disease
Survival (month)	9	4	2
Serum p53 protein level	1 U/mL	31.25 U/mL	1 U/mL

SCC: Squamous cell cancer.

had been detected in 2 (2.8%) of 72 patients. It was detectable in 1 (4.5%) of 22 SCLC patients. Serum levels of p53 protein ranged from 1 U/mL to 31.25 U/mL in positive samples. Two of these patients had squamous cell carcinoma (SCC) of the lung. One of them was at stage IIIA, nevertheless he had a worse prognosis with an early detected bone metastasis, presented by a pathological fracture of right humerus, and the other patient had a stage IV disease, he was dead 4 months after the diagnosis. The third patient had a small-cell lung cancer with extensive disease. Their clinical features and serum p53 protein levels are shown in Table 2.

DISCUSSION

p53 gene as a frequent target in human cancers, and its protein product have been the center of intensive study from its discovery. The mutational spectra at the p53 locus in different tissue type indicates a strong role for diverse environmental mutagens. Cigarette smoking is thought to be responsible for 90% of lung carcinomas in men and 78% in women. The prevalent mutation in lung cancer is G:C→T:A transversion, positively correlated with lifetime cigarette consumption, and this observation is compatible with the role of exogenous carcinogens in lung cancer (29,30). Mutations in the p53 gene are very common in lung cancer, occuring in 90% of SCLC, and in 50% of NSCLC (7,8). Methods used for detecting p53 alterations have advantages and disadvantages specific for each of them. The molecular analysis, which seems to be the most accurate technique for identification of p53 mutations, is laborious and methods of analysis of mutations can bias conclusions regarding the prevalence and nature of p53 mutations (5,9). Immunohistochemistry is a sensitive method for the detection of missense stabilizing mutation in lung cancer, but 15-20% of the mutations belong to the class of splicing abnormalities with frameshift or stop codon, and generates false-negative results.

On the other hand, differences in fixation methods, in antibodies and interobserver variability caused by scorring system are disadvantages of this method (10,11,31). These two methods also require tumor tissue.

Serological analysis with ELISA is simple, there is no requirement of tumor tissue and rapid evaluation of many samples is possible. This method (ELISA) was used to detect circulating antibodies in cancer patients extensively from the first described antibodies against human p53 protein in breast cancer patients sera by Crawford until today (32). The lack of sensitivity is an important disadvantage of this assay. In only 20-40% of patients with p53 mutations will develop anti-p53 antibodies. The average frequency of anti-p53 antibodies in lung cancer is 17% (15).

Another area of serological analysis with ELISA is serum p53 protein evaluation. p53 protein detection in the serum samples of cancer patients has been the subject of only a few investigations in the literature, concerning patients with colon, lung, bladder, pancreatic, breast, gynaecologi-

cal cancer, and malignant lymphomas (16-26). In Table 3, reports of p53 protein detection by ELISA in serum and plasma samples of different groups of cancer patients are shown. The results of these studies reflect that serum p53 protein detection rate is variable in different groups of cancer patients, and also each of these studies has used different ELISA kits with different cut-off levels. In the present study, we examined the serum p53 protein status of lung cancer patients by pantropic quantitative p53 protein ELISA kit. We found detectable levels of serum p53 protein in 3.2% of patients with lung cancer. Serum p53 protein had been detected in 2 (2.8%) of 72 NSCLC patients, and in 1 (4.5%) of 22 SCLC patients. This low frequency of positive serum p53 protein levels is compatible with previous reports including lung cancer patients. Luo et al. showed that 3 (13.1%) of 23 patients with lung cancer (including 2 SCLC, 21 NSCLC) had positive serum p53 protein levels by using a mutant specific ELISA. Two of them had a diagnosis of squamous cell carcinoma and one patient had an adenocarcinoma of the lung. They also found that these patients had increased levels of p53 protein in tumor tissue on immunohistochemical staining, and/or mutations in the p53 gene (18).

In another study reported by Fontanini et al, they found detectable levels of serum mutant p53 protein in 13% of patients, and the concentrations of mutant p53 were significantly higher in those patients with lymph node involvement and late stage disease (17). Segawa et al, also showed that only in 2 (5.5%) of 36 SCLC patients had an elevated serum p53 protein level with a pantropic quantitative ELISA, and serum p53 protein levels were quite low both in patients with benign lung diseases, and the difference in levels between these groups was not significant (20). In the study of Levesque et al, they used a quantitative immunoassay, and detected only two positive sera from 114 lung cancer patients (19). The highest rate of detectable p53 protein levels in the literature is in the malignant lymphomas with 37-64% rates of positivity. In patients with high grade and Hodgkin lymphomas, serum and plasma p53 protein levels were frequently high. Lymphomas comprise a heterogenous group of malignant tumors, ranging from indolent to aggressive cases, and results of p53 protein studies confirmed that serum/or plasma p53 protein levels were higher in patients with more aggressive course of disease (24,25). Suwa et al found that pancreatic adenocarcinomas with distant metastases showed significantly higher serum p53 concentrations than tumors without metastases (22). In the present study, our three patients having detectable level of p53 protein in their sera, were at late stage and had poor prognosis. These results suggest that serum

Tablo 3. Details of reports on p53 protein detection by ELISA in cancer patients.

	Specificity of detected p53 protein	Material	Malignancy	No. of p53 (+)/total no. of patients	Cut-off level
Lehtinen et al. (24)	Mutant	Plasma	Malignant lymphoma	30/81	30 pg/mL
Trumper et al. (25)	Mutant	Serum	Hodgkin lymphoma	21/33	50 pg/mL
Suwa et al. (22)	Mutant	Serum	Pancreatic carcinoma	23/104	0.05 ng/mL
Morita et al. (21)	Mutant	Serum	Bladder cancer	1/100	50 pg/mL
Barbati et al. (23)	Pantropic*	Serum	Ovarian cancer	9/39	200 pg/mL
			Endometrial cancer	7/43	
			Cervical cancer	8/56	
Luo et al. (16)	Mutant	Plasma	Colon carcinoma	7/22	0.05 ng/mL
Luo et al. (18)	Mutant	Serum	Lung cancer	3/23	0.05 ng/mL
Segawa et al. (20)	Pantropic	Serum	Small-cell lung cancer	2/36	10 pg/mL

* Pantropic- selective either for mutant and wild-type p53 protein.

p53 protein may be raised and became detectable during the progression of the disease. The exact mechanisms for this remain unclear, but this increase can be the result from the destruction of tumor cells. The mechanism that leads to the formation of circulating anti-p53 antibodies is poorly understood, and by which p53 (wild type/mutant) is presented to the immune system is unknown. It is not clear whether p53 mutation is really required for the production of these antibodies or whether the sole accumulation of p53 protein can lead to this humoral response (15).

This immune response depends on several factors such as the mutation type and complex formation with heat shock protein hsp 70 (33). Anti-p53 antibodies is not specific for only mutant p53, they can recognize the wild-type also (34,35). Thus, pantropic p53 quantitative ELISA can give more accurate knowledge of serum p53 protein status which is either the cause, and perhaps the result of complex interaction of humoral immunity in cancer patients. The presence of anti-p53 antibodies could accelerate the clearance of p53 from the serum or influence the ability of ELISA to identify p53 by masking relevant epitopes, causing a false-negative results. Another contributing cause of false-negative results can be uncharacterized interactions between p53 and other serum proteins (16,17).

The value of quantitative serum p53 protein analysis with ELISA in lung cancer patients is very limited in evaluating p53 changes, despite it is an easy technique to perform. This study confirms that serum p53 protein detection rate is low in lung cancer patients. It can be proposed to combine this method either with immunochemical, mutational analysis or detection of serum anti-p53 antibodies for an increased yield of detecting p53 alterations, but this effort can be time consuming, and has no additional benefit on p53 studies. It can also be proposed to make measurements of serum p53 protein before and after chemotherapy to verify if this protein becomes detectable after destruction of tumor cells.

CONCLUSION

It seems that the detection of serum p53 protein is not a prognostic marker in lung cancer patients. However, further investigations concerning the complex molecular mechanisms and causes of these low detectable levels of serum p53 protein, which are possibly related to the tumor immunology and host defense mechanisms, will be helpful to understand different steps of cancer development and progression.

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