p53 Protein in Non-Small Cell Lung Cancer as Quantitated by Enzyme-linked Immunosorobt Assay: Relation to Prognosis

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ABSTRACT

The prognostic value of p53 protein in tumor extracts as measured by ELISA was studied retrospectively in 228 non-small cell lung cancer (NSCLC) patients. The assay measures both wild-type and mutated p53. The specimens on which this study was performed have been used earlier to analyze the prognostic impact of components of the plasminogen activation system, which enabled an analysis of relationships between these components and p53 protein. The median of the p53 protein values in the 228 patients was 0.10 (range, 0–0.70) ng/mg protein. Survival analysis comparing patients with p53 levels below versus above the median showed no significant difference (P = 0.67). When analyzing the histological types, adenocarcinoma (n = 106), squamous cell carcinoma (n = 84), and large cell carcinoma of the lung (n = 38) separately, similarly, no significant differences in survival between patients having low versus high tumor p53 levels were found. When comparing levels of p53 protein in the three histological types, a significant difference (P < 0.0001) was found, with adenocarcinomas having the lowest levels. There was a weak positive correlation (r = 0.22) between p53 protein and plasminogen activator inhibitor type 1 (PAI-1). Multivariate analysis proved no impact of p53 on survival; tumor size, PAI-1, and lymph node involvement were the only variables with significant influence on survival. These data indicate that p53 protein quantitated with a sandwich ELISA in tumor extracts from NSCLC has no prognostic value, but the observed statistically significant difference of p53 protein content between histological subgroups may be related to differences in etiology and biology in different NSCLC subtypes. In addition, the weak association found between p53 protein and the independent prognostic marker PAI-1 could suggest yet undefined interactions in lung cancer.

INTRODUCTION

The p53 tumor-suppressor gene has been called “the guardian of the genome” (1). It has been demonstrated that the p53 protein is able to block the division of cells with DNA damage (2). The p53 suppressor gene is mutated commonly in human cancer (3), and inactivation of p53 protein tumor-suppressor activity seems to be one of the most common molecular steps in the development of cancer (4). p53 mutations have been identified frequently in human lung cancers (5–8); the prevalence was reported to be highest in small cell carcinomas (>70%) and lowest in adenocarcinomas (33%; Ref. 3). A number of studies have shown a very strong association between high-level expression of the p53 protein in tumors and point missense mutations in the p53 gene (9), the overexpression of mutant p53 protein being linked to a prolonged half-life of the protein compared with wild-type p53 (3). Recently, Lane (9) suggested a new hypothesis for the stability of mutant p53 in tumor cells: cells lacking wild-type p53, but containing mutant p53, respond with persistent increased p53 levels when exposed to DNA damage. In contrast, cells containing wild-type p53 also respond to DNA damage by increasing p53 protein levels, but the levels return to normal after growth arrest or apoptosis, as a result of effective elimination of the inducing signal from the cellular environment (9).

Lung cancer is one of the most frequent cancers and is known to be associated with exposure to genotoxic agents (10), accounting for more than 160,000 new cases annually in the United States. The disappointing results, despite intensive attempts to improve therapy, reflect how urgent it is to obtain a better understanding of the cellular and molecular biology of lung cancer. Several components and genetic changes have been studied, such as proto-oncogenes, different molecular markers, growth factors, and surface antigens, but none of these studies have changed clinical practice yet (11). The presence of p53 mutations identified by PCR (12, 13) or immunohistochemistry (14–18) has been studied in lung cancer, mainly in NSCLC,2 and different results on the prognostic value of p53 have been reported (12–19).

To investigate the prognostic impact of p53 protein overexpression in NSCLC, we studied tumor tissue extracts from 228 NSCLC patients measuring p53 protein content by a pan-p53 ELISA. Previously, we studied the prognostic impact of components of the plasminogen activation system in the same patients. The plasminogen activation system is a proteolytic enzyme system known to be involved in cancer invasion and metastasis (20–23). In NSCLC, PAI-1 is an independent prognostic marker in AC (24), and the uPAR is an independent prognostic marker in SC (25). We have hypothesized that this variation in prognostic impact of uPAR and PAI-1 between AC

2 The abbreviations used are: NSCLC, non-small cell lung cancer; PAI-1, plasminogen activator inhibitor type 1; uPAR, urokinase-type plasminogen activator receptor; SC, squamous cell lung carcinoma; LC, large cell lung carcinoma; AC, adenocarcinoma of the lung.
and SC could reflect differences in tumor and stroma cell interactions with regard to the plasminogen activation system (26).

The aim of the present study was to quantitate p53 protein in SC, AC, and LC, correlating it to clinical features, especially the stage of disease and survival, and to components of the plasminogen activation system.

MATERIALS AND METHODS

Tumor Tissue and Tissue Extracts. The present study includes tumor tissue from 228 patients having surgical resection for NSCLC at Bispebjerg Hospital within the period 1986–1992. p53 could not be measured in 14 of the 228 samples due to an insufficient amount of material. Tumors and tumor extracts were all stored at −80°C, and extraction was performed using a Triton X-100-containing buffer, as described elsewhere (24). Tissue sections were made from all specimens and evaluated by our pathologist (D. F.) before the extraction procedure to ensure that specimens contained tumor tissue. Only specimens containing tumor cells were included. No effort was made to quantitate the amount of tumor cells present.

Clinical Data. For all patients, data on age, sex, histopathology, tumor size, number of tumor-positive mediastinal lymph nodes, stage, type of surgery, and overall survival (by January 1, 1994) were registered (Table 1). The histological classification of the tumors was based on the WHO classification (28). All diagnoses were performed by the same lung pathologist (D. F.). Patients were followed up at the outpatient department after surgery, as described earlier (24). Data on survival were obtained from the Danish Death Registry and calculated from the day of surgical resection (24, 25). There was no difference in survival between the three histological subtypes.

ELISAs. To measure levels of p53 protein in tumor extracts, a pantropic, p53-quantitative ELISA was used (Oncogene Science, Cambridge, MA), measuring both wild-type and mutant p53. The assay is a sandwich ELISA with the monoclonal antibody PAb 1801 as a catching antibody and a rabbit p53 antibody for detection. The ELISA has been characterized by the manufacturer; the interassay and intraassay variations are reported to be 15% and 6%, respectively; and the sensitivity is 10 pg/ml (Oncogene Science). The ELISA was used according to the vendor’s instructions. Due to the possible influence of the extraction buffer on the signal in the ELISA, we compared standard curves with and without the extraction buffer. Both curves showed linearity, but the extraction buffer caused a reduction in the signal of 25%. Protein content of the tumor extracts was determined by a Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using BSA as standard. All p53 protein values are given as ng/mg protein.

Statistical Methods. For data base management, descriptive statistics, and survival analysis, the SPSS PC software (version 4.01; SPSS Inc.) and the BMDP statistical software (BMDP Statistical Software, Los Angeles, CA) were used. Life table analysis of overall survival was calculated by the product limit method (Kaplan-Meier), and the log rank test was used to test the statistical significance of differences between subgroups of patients. The Cox proportional hazards model was used for univariate and multivariate survival analysis (29). The Mann-Whitney and the Kruskal-Wallis tests were used to test differences in content of p53 protein between two or more groups.

RESULTS

Distribution of p53 Levels. p53 protein levels were measured in 214 extracts, the median value being 0.10 (range, 0–0.70) ng/mg protein. Eighteen extracts had no detectable amounts of p53 protein (<10 pg/ml). Fig. 1A shows the distribution of p53 protein in all 214 patients. In 78 SC extracts, the median value of p53 protein was 0.23 (range, 0–0.70) ng/mg protein, for LC and AC, the median values were 0.17 (range, 0–0.46) ng/mg protein and 0.036 (range, 0–0.53) ng/mg protein, respectively. p53 protein content in the three subgroups of NSCLC was compared, and differences were tested by nonparametric, one-way ANOVA (Kruskal-Wallis), demonstrating a significant difference (P < 0.0001) between the histological subtypes, with the lowest values in AC. Fig. 1B shows the distribution of p53 protein in each of the three subgroups.

Prognostic Significance of p53. The median value of the p53 protein was used as the cutoff value to divide the patients into groups with low and high levels. Life table analysis was performed using Kaplan-Meier plots and log rank tests for evaluation of the significance of the differences. There was no
significant difference between patients with low versus high p53 protein ($P = 0.67$). Because p53 mutations have been reported to be early events in lung cancers (3), stage I patients were analyzed separately. The group consisted of 131 stage I patients, and the median value in this subset was the same as in the total population: 0.10 ng/mg protein. No difference in survival was observed comparing patients with p53 values below and above the median ($P = 0.64$; Fig. 2). Similar nonsignificant differences were observed when comparing survival in patients with low versus high p53 values within each of the three cell types, using the median as the cutoff value in each of the subgroups, respectively: SC, $P = 0.87$; LC, $P = 0.77$; and AC, $P = 0.50$.

Comparing survival of AC patients without detectable p53 protein (17 patients) with that of patients having p53 protein showed no significant difference ($P = 0.61$).

Other Prognostic Factors. In addition to common clinical features, such as histology, age, sex, tumor size, lymph nodes, stage of disease, and surgical resection, p53 was related to three components of the plasminogen activation system: uPA, PAI-1, and uPAR measured in tumor extracts (24, 25). Correlations between p53 protein level and other variables (listed in Table 1) were studied using the $\chi^2$ test (Table 2). As already shown with the Kruskal-Wallis test, adenocarcinomas contained significantly less p53 than SC and LC. Furthermore, uneven distributions were observed in relation to age, tumor size, stage, uPA, PAI-1, and uPAR. A Mann-Whitney test, however, comparing levels of p53 protein in stage I patients with those in stages II and III was not significant ($P = 0.49$).

The correlations between p53 protein and uPA, PAI-1, and uPAR were studied further in linear regression analysis. A weak positive correlation was found between p53 protein and PAI-1 ($r = 0.22; P = 0.001$), whereas there was no significant correlation with uPA or uPAR.

Multivariate Analysis. For all patients, the relationship between p53 protein, histology, uPA, PAI-1, uPAR, and other possibly prognostic variables, such as age, sex, tumor size, lymph node involvement, and survival, was studied by the Cox multivariate analysis (Table 3). Variables were eliminated from the model stepwise in a backward fashion and reincluded only if $P < 0.05$. This analysis left tumor size, lymph node involvement, and PAI-1 as the only significant variables, with relative risks of 2.51 (95% confidence interval, 1.72–3.65), 1.31 (95% confidence interval, 1.02–1.67), and 1.6 (95% confidence interval, 1.1–2.33), respectively.

DISCUSSION

In this retrospective study of p53 protein content in tumor tissue from NSCLCs, we were unable to demonstrate any prognostic value of p53 protein as measured by ELISA. When analyzing the data for the histological subgroups separately, including data on 84 SC, 106 AC, and 38 LC, no prognostic...
impact of p53 protein in any of the three subgroups was observed. However, a highly significant \( (P < 0.00001) \) difference between levels of p53 protein in AC compared with SC and LC was found. In a multivariate Cox regression analysis, in which standard clinical parameters together with components of the plasminogen activation system (uPA, uPAR, and PAI-1) were included, PAI-1 was found to be an independent prognostic factor, with tumor size and lymph node involvement as the only other factors showing prognostic significance. A linear regression analysis showed a weak but significant association between p53 protein and PAI-1. Although PAI-1 could turn out to be a useful biochemical prognostic factor, p53 does not seem to be useful in such a clinical context.

The ELISA used is a pantrropic p53 ELISA, which measures both wild-type and mutated p53 protein. The catching monoclonal antibody PAb 1801, used in the present ELISA, has been shown in prior immunohistochemistry studies to stain lung tumor tissue but not normal lung epithelial tissue (15). PAb 1801 also has been used in immunoblots, resulting in positive reactions only, when used as a probe against a lysate from a p53-positive and not a p53-negative cell line (30).

PAb 1801 has been used to quantitate p53 immunoreactivity on histological sections of NSCLC, and the staining intensity was correlated with patient survival (14–18). Some of these studies indicate an inverse prognostic impact of p53 (15, 16, 18), whereas other studies (14, 17) showed no relationship between p53 protein staining and survival. These conflicting results suggest that if an association exists, it is probably weak (11).

The p53 ELISA used in the present study, however, does not distinguish between wild-type and mutant protein and, therefore, does not provide insight as to whether p53 mutations or their functional consequences have a prognostic role. p53 levels may be high due to increased stability of the mutant protein, normal protein accumulation as a result of viral binding, or DNA damage from genotoxic agents.

\( p53 \) is a tumor suppressor gene that is present in mutated forms in most cancers (3). \( p53 \) mutations have been reported to...
have different prevalence in different subtypes of NSCLC (8, 31, 32), being lowest in AC, approximately 33%, close to the prevalence in other adenocarcinomas but different from other lung cancer histologies, and being 65% in SC and 60% in LC (3). This difference is also confirmed in the present investigation, showing a significant difference between levels of p53 protein in AC compared with SC and LC. These findings support further the hypothesis (25) that NSCLC subtypes are biologically different.

The weak association between p53 protein and PAI-1 protein made us speculate whether these two molecules are related biologically. Recently, Dameron et al. (33) reported a link between the loss of p53 and the loss of angiogenic inhibition in \textit{in vitro} experiments. At the same time, it is known that the angiogenic inhibitor angiotain shares more than 98% of its amino acid sequence with an internal fragment common to both human and mouse plasminogen (34). Plasminogen can be activated to plasmin by tissue-type PA or uPA; PAI-1 is an inhibitor of both activators. A link could exist between the plasminogen activation system and p53, \textit{e.g.}, both molecules have influence on angiogenic inhibitors, but further research is needed to clarify this hypothesis.

The finding that p53 protein has no prognostic role when measured by ELISA in tumors in which components of the plasminogen activation system are found to be prognostic suggests that determination of p53 in lung cancer tissue is not useful as a prognostic assay. The fact that levels of p53 protein are significantly higher in SC than in AC may suggest that p53 plays an important role in SC carcinogenesis.

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