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p53 immunostaining is correlated with reduced survival and is not correlated with gene mutations in resected pulmonary large cell carcinomas

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Abstract

Malignancy of pulmonary large cell carcinomas (LCC) increases from classic LCC through LCC with neuroendocrine morphology (LCCNM) to large cell neuroendocrine carcinomas (LCNEC). However, the histological classification has sometimes proved to be difficult. Because the malignancy of LCC is highly dependent on proteins with functions in the cell cycle, DNA repair, and apoptosis, *p53* has been targeted as a potentially useful biological marker. *p53* mutations in lung cancers have been shown to result in expression and protein expression also occurs in the absence of mutations. To validate the importance of both *p53* protein expression (by immunostaining) and *p53* gene mutations in lung LCC (by PCR-single strand conformational polymorphism analysis of exons 5, 6, 7, and 8) and to study their relationships with clinical factors and sub-classification we investigated the correlation of *p53* abnormalities in 15 patients with LCC (5 classic LCC, 5 LCNEC, and 5 LCCNM) who had undergone resection with curative intent. Of these patients, 5/15 expressed *p53* and none had mutant p53 sequences. There was a negative survival correlation with positive *p53* immunostaining (P = 0.05). After adjustment for stage, age, gender, chemotherapy, radiotherapy, and histological subtypes by multivariate analysis, *p53* expression had an independent impact on survival. The present study indicates that *p53* assessment may provide an objective marker for the prognosis of LCC irrespective of morphological variants and suggests that *p53* expression is important for outcome prediction in patients with the early stages of LCC. The results reported here should be considered to be initial results because tumors from only 15 patients were studied: 5 each from LCC, LCNEC and LCCNM. This was due to the rarity of these specific diseases.

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Introduction

Because there is a significant difference in survival time for patients with large cell carcinoma (LCC), Travis et al. (1) recently proposed to subdivide LCC into three subtypes: classic LCC, LCC with neuroendocrine morphology (LCCNM) and large cell neuroendocrine carcinoma (LCNEC), although the LCCNM subtype has not yet been recognized officially in the current WHO classification (2004). Previous reports and data from our group have shown that the malignancy of pulmonary LCC increases in the following order: from classic LCC through LCCNM to LCNEC (2-7). These highly malignant diseases are characterized by rapid and disseminated tumor growth in the majority of patients, and share morphological and functional features (8-11). Since the degree of malignancy of LCC correlates well with histological type, it is essential to be able to distinguish between types. At present, LCC sub-classification depends on the evaluation of histological criteria such as neuroendocrine (NE) morphology, the number of mitoses, the presence or absence of necrosis, and immunohistochemical (IHC) staining (1,12,13). However, these parameters are influenced by different IHC and tissue sampling methods. Therefore, it is often difficult to achieve a specific diagnosis based on available criteria. Among experienced lung pathologists, disagreement commonly occurs when differentiating between LCC lung tumors (14). Thus, it is clear that alternative objective markers are needed to assist in their classification.

Recently, advances in molecular biology and genetics have raised the possibility of new diagnostic techniques and treatment to be applied in clinical oncology for LCC (15-20). The results indicate that LCC belongs to a group of non-small cell lung carcinomas, which suggests the possibility that gene abnormalities may be objective markers for the classification of LCC. While genetic analysis of LCC has been conducted only very recently, the inactivation of recessive tumor suppressor genes associated with chromosomal deletion, such as *p53* (17p), has been implicated in the genesis of human cancer in a number of studies (21-26). The normal *p53* protein has important functions in cell cycle checkpoints and modulates important events such as G1 arrest, DNA repair, and apoptosis (24-26). Alterations in the *p53* gene could play crucial roles in the genesis of carcinomas and have been shown to represent one of the most common molecular biological changes in lung carcinomas, including LCC (25,26). More than 85% of the mutations identified have had their structures determined (25,26). Most mutant *p53* proteins produced by missense mutations are resistant to degradation and thus have prolonged half-lives, allowing their detection by IHC staining. Accordingly, many investigators have used this approach as a screening method to identify *p53* alterations in tumor samples (25-28). On the other hand, null mutations (complete absence of gene product expression) and nonsense mutations (premature stop codons and shortened protein products) are less common in exons 5-8 but predominate outside these exons, and often may not be detectable by IHC (26,29-32). In addition, protein expression has been described in the absence of mutations (33,34).

Overall, however, published data provide limited comparative information about molecular alterations among the three lung LCC types. A more extensive analysis of LCC at the DNA level is thus warranted to correlate genetic alterations with histological types and patient prognosis. Therefore, to clarify the relationship between *p53* mutations, especially the type of mutation, and IHC staining as well as prognosis, we have investigated both *p53* protein expression (by IHC) and *p53* gene mutations by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis of exons 5, 6, 7, and 8 (35).

Patients and Methods

Tumor samples and clinicopathological data

p53 alterations were examined in a series of 15 LCC (5 classic LCC, 5 LCNEC and 5 LCCNM) that were not accompanied by other primary malignancies and were resected consecutively from 1982 to 2002 at the University Hospital, University of São Paulo. The 15 patients included 11 men and 4 women, and the median patient age at surgery was 58 years (range: 38-75 years; Table 1). All 15 patients had full-preoperative tumor staging (T stage), and were considered to have potentially curable tumors by surgical resection. However, after pathological evaluation when more lymph nodes were sampled (N stage), 4 patients were in N1 final stage, with the remaining 11 patients in N0. Postoperative pathological final staging (P stage) resulted in 9 stage I cases and 6 stage II cases. For survival analysis, the median complete follow-up time was 18 months (range: 3 to 43 months). All patients underwent postoperative chemotherapy (scheme 1 (until 1985) - cyclophosphamide; scheme 2 (after 1985) - cisplatin + vincristine + mitomycin), 5 of them with associated radiotherapy. The major criteria for adjuvant therapy were the anaplastic or NE phenotype and the aggressive nature of the disease. No standardized adjuvant treatment protocol was followed because there was a long interval between the first and the last cases, with a correspondingly broad variation of protocols used within this period.

Tumor tissue samples were obtained at surgical treatment of LCC and fixed in 10% formalin. For each case, one or two slides of the main tumor were selected by light microscopy. Acceptable sections were those that represented the predominant histological pattern of LCC identified in the majority of slides, with at least 10 microscopic fields at a magnification of 250X. Their respective paraffin-embedded blocks were sectioned at 3 µm and stained with hematoxylin and eosin. Two pathologists (AMA and VLC) reviewed these slides separately in an independent and blind fashion and agreed with the previous histological criteria for the diagnosis of LCC. To minimize discrepancies, the criteria evaluated for tumor classification were agreed upon during a series of preliminary discussions between the two pathologists and after informal review of the cases.

The 15 tumors were classified either as LCNEC or LCCNM if there was evidence of all of the following: 1) NE morphology, such as organoid nesting, palisading rosettes, and trabeculae, 2) a high mitotic rate of ≥11 per 2 mm^2 (10 high-power fields), 3) necrosis (often a large zone), 4) cytological features of a non-small cell carcinoma, i.e., large cell size, low nuclear to cytoplasmic ratio, vesicular or fine chromatin, and/or frequent nucleoli (Figure 1A-C). LCC without NE morphology were classified as classic LCC. Thus, we examined and compared the clinicopathological and biological differences of classic LCC, LCNEC, and LCCNM.

The distinction between LCNEC and LCCNM was obtained by NE immunoexpression, which is positive for LCNEC, but negative for LCCNM. To detect NE immunoexpression (1-3), we used a polyclonal anti-chromogranin antibody (Dako A/S, Glostrup, Denmark) at 1:1,600 dilution and a monoclonal anti-synaptophysin antibody (Dako) at 1:100 dilution. Examples of tumor staining for chromogranin and synaptophysin are illustrated in Figure 1D.

p53 mutation analysis

DNA extraction. DNA was extracted from three 10-µm sections of the paraffin block that best represented each tumor (previously selected from hematoxylin and eosin-stained slides). Disposable microtome blades were used, and instruments were cleaned with xylene after each tissue section to avoid

cross-contamination. Three baths in 500 µL xylene at 95ºC followed by three baths in 500 µL 99% ethanol were used to dewax the tissue. Next, the tissue samples were centrifuged for 5 min at 13,000 rpm and 4ºC. The resulting pellets were incubated overnight at 37ºC in 500-µL digestion buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 25 mM disodium EDTA, 0.5% sodium dodecyl sulfate) containing 10 µL proteinase K (200 µg/mL final concentration). The enzyme was inactivated by heating the specimens for 10 min at 95ºC, protein was removed by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and nucleic acid was precipitated from the aqueous phase by adding 1/4 volume of 8 nM ammonium acetate and an equal volume of isopropanol. The nucleic acid samples were then incubated at -20ºC for 1 h and centrifuged for 10 min at 13,000 rpm and 4ºC. The resulting pellets were washed in 70% ethanol for salt removal, air dried, and resuspended in 50 µL TE/RNAse (36).

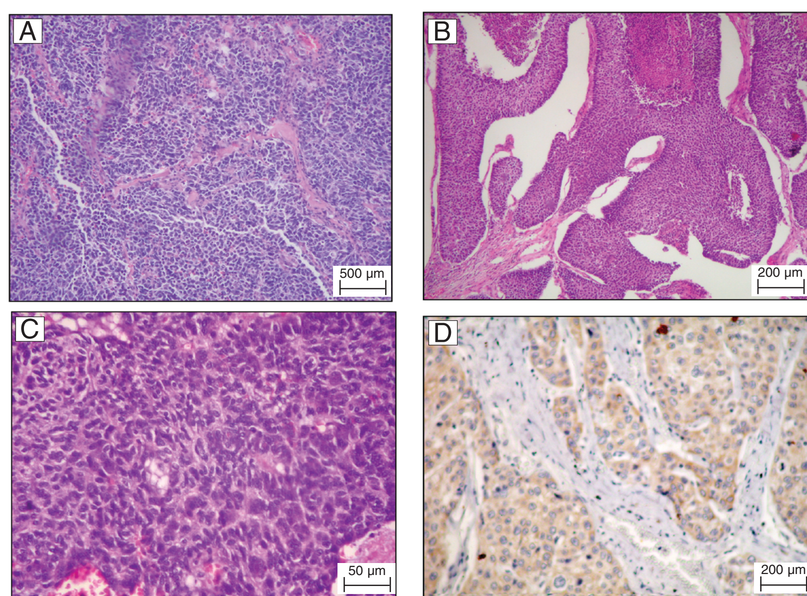

Figure 1. Histological patterns of large cell carcinomas. *Panel A* shows the classical large cell carcinoma (LCC), a large cell tumor with clearly visible nucleoli, lower nuclear-cytoplasmic ratio and absence of neuroendocrine features (hematoxylin and eosin, 100X). *Panels B* and *C* show neuroendocrine morphology in LCC with organoid nesting, palisading, and rosettes. Numerous mitoses are seen in LCC with neuroendocrine morphology (B). Cytologic features in large cell neuroendocrine carcinoma include large cell size, fine chromatin and clearly visible nucleoli (C; hematoxylin and eosin, Panel B 40X and Panel C 400X). *Panel D* shows an immunohistochemistry reaction (magnification 200X) with tumor cells stained for antichromogranin A antibody and only large cell neuroendocrine carcinoma tumors stained for chromogranin.

Polymerase chain reaction amplification

The PCR amplification and non-isotopic detection of exons 5 to 8 of the *p53* gene were described by Soong and Iacopetta (37) and will be summarized here. The PCR assays were carried out in 25-µL reaction buffer containing 0.2 mM deoxynucleotide triphosphates, 2.5 mM magnesium chloride, each primer at a concentration of 0.4 µM, 0.25 units *Taq* polymerase, and 1 µL extracted DNA. Thirty-five thermal cycles of 30 s at 94ºC, 1 min at 60ºC, and 1 min at 72ºC were completed, and the cycling was concluded by a 10-min extension at 72ºC, all in a thermocycler Gene Amp PCR System 2400 (Perkin Elmer, Foster City, CA, USA). Procedures to prevent contamination were followed.

Single-strand conformational polymorphism screening for *p53* mutations

Single-stranded DNA for SSCP analysis was produced by combining equal 5-µL volumes of PCR product and formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heating at 95ºC for 10 min. The DNA was subjected to electrophoresis at 200 V for 2 h at 4ºC within a Mighty-small apparatus (Pharmacia, Uppsala, Sweden) containing 15% polyacrylamide gel with 5% glycerol. Silver staining was done by the method of Bassam et al. (38). The gels were soaked for 5 min in 10% ethanol, 5 min in 1% nitric acid, and 10 min in an impregnating solution (0.1 g silver nitrate and 150 µL formaldehyde in 100 mL water), then soaked in a developing solution (3 g sodium carbonate, 150 µL formaldehyde, and 100 µL sodium sulfate in 100 mL water) until bands

were visible, and then fixed in 10% acetic acid for 5 min. The gels were analyzed under white light with a computerized imaging system (gel Doc 1000, BioRad, North Ryde, Australia) and compared with wild-type *p53* samples from normal tissue extracted and amplified by the same methods to detect *p53* mutations, which would appear as extra bands or mobility shifts.

p53 protein analysis

The presence of *p53* protein was detected by IHC staining using the avidin-biotin immunoperoxidase complex technique, pressure-cooking antigen retrieval, biotinylated rabbit antimouse IgG (Dako; dilution 1:400) streptavidin combined *in vitro* with biotinylated horseradish peroxidase (Dako; dilution 1:1000), diaminobenzidine tetrahydrochloride, and hematoxylin counterstaining. The antibody used was mouse monoclonal anti-human *p53* protein (DO7; Dako; dilution 1:40), whose specificity and sensitivity in non-small cell lung cancer vary from 35 to 75% and from 75 to 100%, respectively (25-28). Brownish nuclear staining was considered to be evidence of *p53* antigen expression by cells. In addition, we quantified the staining as follows. First, we selected the region with the highest expression at low magnification. Then, at 400X, we used an eyepiece coupled to a systematic point-sampling grid with 100 points and 50 lines to count the fraction of points overlaying positively stained structures and we averaged this over 10 microscopic fields to obtain a percentage of the resulting stained structures (39,40).

Statistical analysis

To assess any correlations between frequencies or types of mutation, immunohistochemistry and clinicopathological data, Pearson chi-square and independent-sample *t*-test procedures were used, with $P < 0.05$ indicating a significant difference. Survival curves were created by the Kaplan-Meier method, and the statistical significance of differences was calculated by the log-rank test. Multivariate analyses were performed to identify independent prognostic factors and to assess the relative risk using the Cox proportional hazards model with the Statistical Package for Social Science (Norusis MJ, SPSS for Windows (10.0), Chicago, IL, USA, SPSS Inc.; 2002). In this model, seven factors potentially related to survival (age at surgery, sex, histology, P stage, chemotherapy, radiotherapy, and *p53* status) were included, and the model selection to identify the subset of significant variables was based on the stepwise method for backward selection.

Results

p53 mutation

No mutations were detected; electrophoresis of the amplified products of exons 5, 6, 7, and 8 of the *p53* gene revealed no abnormalities of band migration in any of the 15 cases studied (Figure 2A-D).

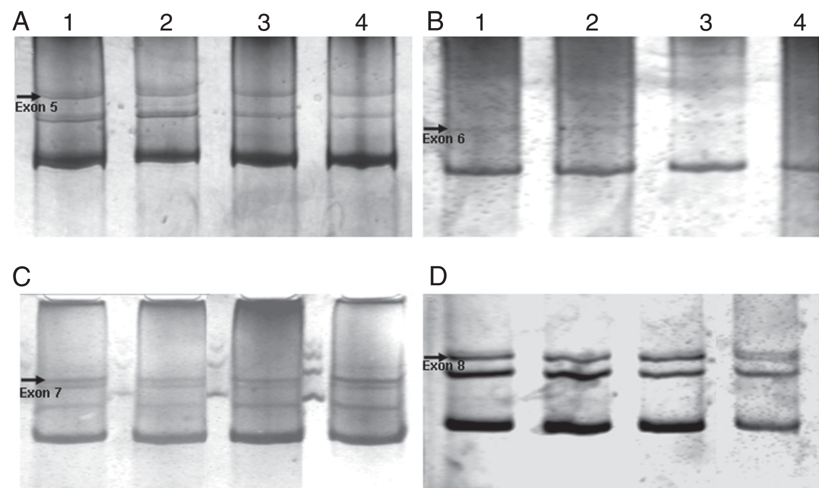

Figure 2. *Panels A* to *D* show electrophoresis of the amplified products of exons 5, 6, 7, and 8 of the *p53* gene, respectively, which revealed no abnormalities of band migration in any of the 15 cases studied in *lanes 1*, *2*, and *3* of tumors and *lane 4* of normal tissue.

Table 1. Characteristics of patients with resected large cell pulmonary carcinomas.

	p53 negative	*p53* positive	Total
Number of cases	10	5	15
Age (years)	58 (38-75)	55 (50-63)	56 (38-75)
Gender			
Male	8 (53.3%)	3 (20%)	11 (73.3%)
Female	2 (13.3%)	2 (13.3%)	4 (26.7%)
P stage			
I	6 (40%)	3 (20%)	9 (60%)
II	4 (26.7%)	2 (13.3%)	6 (40%)
III	0	0	0
N stage			
N0	7 (46.7%)	4 (26.6%)	11 (73.3%)
N1	3 (20%)	1 (6.7%)	4 (26.7%)
N2	0	0	0
N3	0	0	0
T stage			
T1	3 (20%)	4 (26.7%)	7 (46.7%)
T2	7 (46.7%)	1 (6.6%)	8 (53.3%)
T3	0	0	0
T4	0	0	0
Histological subtypes			
LCC	4 (26.7%)	1 (6.7%)	5 (33.3%)
LCNEC	3 (20%)	1 (13.3%)	5 (33.3%)
LCCNM	3 (20%)	1 (13.3%)	5 (33.3%)
Radiotherapy	7 (46.7%)	3 (20%)	10 (66.7%)
Chemotherapy	10 (66.7%)	5 (33.3%)	15 (100%)
Follow-up (months)	18 (3-42)	18 (4-43)	18 (3-43)
Overall survival (months)	12.6 (3-42)	12.8 (4-43)	12 (3-43)

Data reported as median (range) or number with percent of total in parentheses. P stage = postoperative pathological final staging; N stage = lymph node sampled after pathological evaluation: T stage = preoperative tumor staging; LCC = large cell carcinoma; LCNEC = large cell neuroendocrine carcinoma; LCCNM = large cell carcinoma with neuroendocrine morphology.

Table 2. Histological subtypes and results of *p53* expression and mutation.

Histological subtypes	*p53* expression		*p53* mutation
	Mean	Range	
LCC	9.3	0-46.50	No mutation
LCNEC	14.96	0-61.70	No mutation
LCCNM	21.28	0-80	No mutation

The unit of *p53* expression "% of points" indicates the number of points overlying the phenomena of interest divided by the total number of points overlying the tumor. For abbreviations, see legend to Table 1.

p53 expression and relationship with clinicopathological parameters

Tables 1 and 2 show clinicopathological parameters. Five cases (33.3%) were positive for *p53*. Among the positive cases, immunostaining was present in more than 60% of the cells in 3 cases (2 LCNEC and 1 LCCNM). Tumors of male patients younger than 65 years tended to be more *p53*-positive (P = 0.01). No statistically significant relationship was demonstrated between *p53* and N stage or T stage.

Survival and *p53* status

Examination of the relationships between *p53* expression by the cut-off point of 60% positive selected taking into account the value above the median and overall survival, using the Kaplan-Meier method and the log-rank test, revealed a shorter survival period for the 2 LCC patients with *p53* expression higher than 60% compared to those with lower expression. This number was obtained by the median value of *p53* expression in the subtypes and adopted as cut-off. The median survival for patients with *p53* expression higher than 60% was 4 and 24 months for patients with *p53* expression lower than 60% and this difference was statistically significant (P = 0.02; Figure 3). We also found that the prognostic information provided by *p53* was maximized when this variable was used as the continuous one (likelihood ratio = 48.31), that is, one without the usual cut-off point at 60% of the tumor tissue (likelihood ratio = 36.87). There was no difference in survival at the N stage, P stage, T stage, and histological types (classic LCC, LCNEC, and LCCNM).

Multivariate analysis of age, sex, histology, P stage, chemotherapy scheme, radiotherapy, and the immunoexpression of *p53* was performed to examine the interrelationship of possible prognostic factors and survival (Table 3). In all cases, *p53* immunoex-

pression was an independent prognostic factor with statistical significance, predicting poor survival for P stage II patients with a relative risk of 16.103 (P = 0.05; Table 3).

Discussion

In the present study we determined *p53* protein expression by IHC and looked for mutations in *p53* exons 5, 6, 7, and 8 by PCR-SSCP analysis in 15 specimens of resected LCC (classic LCC, LCNEC, and LCCNM). The series is small because LCC of the lung are rare, representing approximately 9% of all lung cancers in most studies.

Although we did not detect *p53* mutations in our 15 cases of localized LCC, IHC staining indicated that *p53* protein was expressed in 5 of the 15 tumors (33.3%). Among these tumors, the histological types LCNEC and LCCNM presented the same expression. A possible explanation for this low frequency of mutations is due to the small number of tumors included in each category and the rarity of these tumors. In our study and in most of the other studies in the literature with low-*p53* mutation rates (26,29-32), exons 5 through 8 were examined. However, when primers to amplify the region extending from exons 4 to 8 and exon 11 (9 and 10 not analyzed) were used to evaluate a series of 144 localized non-small cell lung carcinomas (107 adenocarcinomas and 37 squamous cell carcinomas) (33) *p53* mutations were found in 45% of the cases, suggesting that exons 4 and 11 ought to be evaluated in addition to exons 5 through 8.

To interpret our results, it is important to understand the situations that can cause a tumor to be *p53* positive or *p53* negative by IHC. As mentioned before, *p53* normally has too short a half-life to be detected by IHC (35). Mutations are the most common *p53* mutations, and because they stabilize *p53* protein, they are detected by IHC (35). Nonsense or frame shift mutations, gene

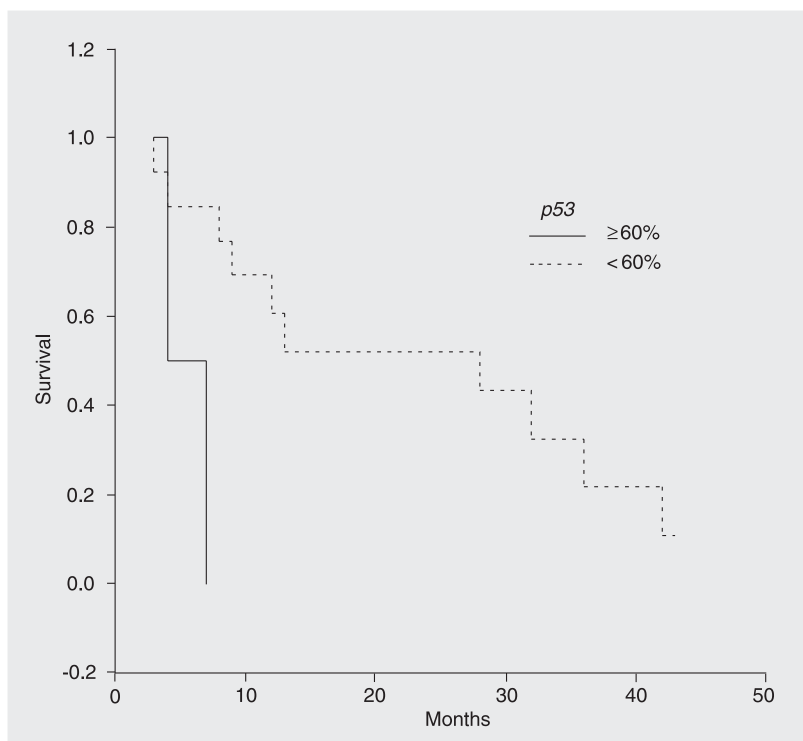

Figure 3. Kaplan-Meier overall survival curves of patients with resected pulmonary large cell carcinomas show that the amount of *p53* immunoexpression is inversely related to survival. >60% indicates that more than 60% of the tumor cell structure reacted with monoclonal antibodies to *p53*. The >60% group contained tumors from 2 patients and the <60% group consisted of tumors from 13 patients. Fifteen patients were *p53* negative and are included in the <60% group.

Table 3. Cox proportional hazard model analysis of survival time.

Variables	Coefficient B	SEM	P	Exp (B)	95%CI for Exp (B)	
					Lower	Upper
Female gender	-0.704	1.044	0.500	0.495	0.64	3.824
Age	-0.517	1.148	0.652	0.596	0.063	5.652
P stage II	-1.602	1.528	0.294	0.201	0.010	4.023
Histology						
LCNEC	-1.043	0.990	0.291	0.352	0.051	2.452
LCCNM	0.181	1.104	0.870	1.198	0.138	10.431
CHT	–	–	–	–	–	–
RT	-0.393	1.098	0.836	0.675	0.16	27.887
p53 >60%	2.779	1.416	0.05	16.103	1.003	256.563

SEM = standard error of the mean; Exp = risk; LCNEC = large cell neuroendocrine carcinoma; LCCNM = large cell carcinoma with neuroendocrine morphology; CHT = chemotherapy; RT = radiotherapy.

deletions, and mutations that cause truncation of the protein may not be detected by IHC. The IHC method can also detect *p53* that has been temporarily stabilized by some mechanism other than mutation (25). Stabilized *p53* that is detected by the IHC method is inactive and does not normally function in the control of cell cycle and apoptosis induction (35).

Our results confirmed other reports that LCC and its variants LCNEC and LCCNM have a high frequency of *p53* expression but are not correlated with gene mutations (19-21,23). Moreover, *p53* mutations occurred at the same frequency in LCC, LCNEC and LCCNM. Inactivating *p53* mutations (mostly missense mutations) are detected in up to 50% of LCC (31). These findings suggest that since LCC is a poorly differentiated tumor originating from the same stem cells, it should share the molecular and genetic alterations of their variants LCNEC and LCCNM (22) when exposed to the same carcinogens.

Reported clinical prognostic criteria for LCC are not different from those for other non-small cell lung cancers. The major criteria are performance status at diagnosis and the disease extension reflected by tumor, node, metastasis, and stage. However, in the present study, we found a negative survival correlation with positive *p53* immunostaining. These findings suggest that expression of *p53* protein determined by immunostaining may contribute to an adverse outcome due to the ability of *p53* to act as a dominant oncogene, or alternatively, that *p53* expression may reflect ongoing DNA damage in the tumor as a marker of a more aggressive behavior. When adjusted for stage, age, gender, and histological subtypes by multivariate analysis, there was an independent impact of *p53* expression on survival.

The present study indicated that assessment of *p53* could provide an objective marker that can distinguish LCC prognosis irrespective of morphological variants. These data also suggest that *p53* expression and absence of gene mutations are important for outcome prediction in patients with early stages of LCC. The results reported here should be considered to be initial results because tumors from only 15 patients were studied: 5 each from LCC, LCNEC and LCCNM. This was due to the rarity of these specific diseases.

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