p53 Mutations in Defined Structural and Functional Domains Are Related to Poor Clinical Outcome in Non-Small Cell Lung Cancer Patients[1]

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ABSTRACT

The prognostic value of *p53* status in non-small cell lung cancer has been investigated in 148 patients with clinical stage I-IIIB disease. Tumor tissues were examined for mutations in exons 4–9, with emphasis on defined structural and functional domains. Eighty-four mutations were detected in 83 (54%) of the patients. Eighty-eight percent of the mutations were within exons 5–8, and 12% of the mutations were within exons 4 and 9. Missense mutations occurred in 67% of the tumors, and 30% were null mutations (10% stop mutations, 15% frameshift mutations, and 5% splice site mutations). Patients with mutations in *p53* had a significantly higher risk for lung cancer-related death and for death from all causes than those with wild-type *p53* [hazard ratio (HR) = 2.09 and 95% confidence interval (CI) = 1.20–3.64 and HR = 1.69 and 95% CI = 1.06–2.70, respectively]. Mutations in *p53* related to even still poorer lung cancer-related prognosis were found at the following locations: (*a*) exon 8 (HR = 3.5; 95% CI, 1.59–7.71)]; (*b*) the structural domains L2 + L3 (HR = 2.36; 95% CI, 1.18–4.74), and (*c*) codons involved in zinc binding (HR = 11.7; 95% CI, 3.56–38.69). Together, the biologically functional group of severe flexible mutants (codons 172, 173, 175, 176, 179, 181, 238, 245, and 267) and severe contact mutants (248, 282) were significantly related to shorter lung cancer-related survival (HR = 4.16; 95% CI, 1.93–8.97). Squamous cell carcinoma was the dominant histological type in tumors involved in poor prognosis in exon 8 (HR = 3.19; 95% CI, 1.07–9.45). These results indicate that mutations in defined structural and functional domains of *p53* may be useful molecular biological markers for prognosis and treatment strategy in non-small cell lung cancer patients.

INTRODUCTION

Lung cancer is the most common cancer worldwide and is predicted to remain a major cause of cancer deaths in the next century because of contributions from the East European and developing countries (1–4). The survival rate for NSCLC[4] is related to clinical stage but remains poor across all stages (5). Although prognosis is generally poor, there are now somewhat more promising outlooks for treatment of these patients (6–8). Genetic markers may be used in addition to staging for assigning NSCLC patients to subgroups for clinical trials and more intense adjuvant therapy.

Alterations in proto-oncogenes (*K-ras*, *c-myc*, and *HER2/neu*) and tumor suppressor genes (*Rb*, *p16*, and *p53*) have been reported to play crucial roles in lung tumor development. Alterations in the *p53* gene are the most common genetic change in lung cancer: occurring in about 80% in small-cell lung cancer and in about 50% in NSCLC (9, 10). The *p53* gene is of critical importance in cell cycle control, DNA repair, and programmed cell death (reviewed by Levine in Ref. 11). Alterations in the *p53* gene play an important role in the development of human lung cancer, occurring at an early stage in tumor development (12–15).

X-ray crystallographic structure of p53 shows that the central portion consists of three loops involved in DNA binding: a β sandwich that serves as a scaffold for two large loops (L2 and L3) that interact with the minor grove; and a loop-sheet-helix motif that interacts with the major grove (16). The loops are held together partly by a tetrahedraly coordinated zinc atom. Four amino acid residues are bound to the zinc atom: Cys-176 and His-179 (L2 loop); and Cys-238 and Cys-242 (L3 loop). Most of the mutations are located within this central DNA-binding domain (residues 102–292) of *p53* and are particularly common in the four highly conserved domains in this region. Lung cancer studies indicate that 10–20% of the mutations occur outside exons 5–8, mainly in exon 4 (17, 18). Missense mutations are the most common (about 90%; Ref. 9).

Several reports have studied the association between *p53* mutations and prognosis in NSCLC, but the results are still controversial (19, 20). However, only a few lung cancer studies have analyzed mutations and prognosis in relation to affected exons and domains. One study reports that *p53* mutations in exon 8 correlated most strongly with survival (21). There is also

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[4] The abbreviations used are: NSCLC, non-small cell lung cancer; HR, hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma; AC, adenocarcinoma; wt, wild-type.

Table 1 Patient and tumor characteristics, related to the presence or absence of *p53* mutations

	Portion of mutations in each group		
	No./all cases	Percentage	*P*
Gender			0.01
Male	68/114	60	
Female	12/34	35	
Age (yrs)			
≤59 (males)	12/24	50	NS[a]
≤59 (females)	4/7	57	
>60 (males)	56/90	62	0.03
>60 (females)	8/27	30	
Histological type			0.01
SCC	44/66	67	
AC	28/68	44	
Large cell carcinoma	8/14	57	
Differentiation grade			NS
Well	15/33	50	
Moderate	30/50	60	
Poor	28/52	58	
Tumor status			NS
T_1	18/32	56	
T_2	50/97	52	
T_3	8/12	67	
T_4	0/2	0	
Nodal status			NS
N_0	55/105	52	
N_1	9/20	45	
N_2	12/18	67	
Smokers			0.05
Yes	71/125	57	
No[b]	1/6	17	

[a] NS, not significant.
[b] <1 pack-year.

an observation indicating that mutations in exon 5 are associated with poor prognosis (22). To clarify this further, we have, through an extensive analysis of exons 4–9 and relevant clinical-pathological information, focused on the prognostic role of *p53* mutations at different locations in defined structural and functional domains.

PATIENTS AND METHODS

Patients. This prospective study includes lung cancer patients admitted to the university hospital surgery departments of The Norwegian National Hospital and Haukeland Hospital from 1988–1996. Patients were selected consecutively as often as practicably possible, whenever sufficient tumor tissue was available for DNA analysis in addition to the amount required for histopathological examination. Only patients (148 subjects) with histologically confirmed NSCLC participated in the study. Informed consent was obtained from the patients. They had no earlier malignancies. No adjuvant therapy was given, except for one patient who received postsurgical radiation therapy.

The patients were in clinical stage I (68%), stage II (25%), stage IIIA (13%), and stage IIIB (3%) at the time of surgery. Patient and tumor characteristics are listed in Table 1. Information on tobacco consumption and other clinical data were obtained from a questionnaire from the patient and the treating physician before surgery. The hospital records were reviewed with regard to clinical information, without knowledge of the *p53* status of the tumors. The following information was recorded: (*a*) revealing symptoms; (*b*) history of cardiovascular disease; (*c*) pulmonary function status; (*d*) surgery procedure; (*e*) primary adjuvant therapy, including radiotherapy; and (*f*) date of death. All patients were traced in the National Register and The National Bureau of Statistics until the end of 1997 for date of death, whenever this information was unavailable from hospital records. Median follow-up time was 35 months. For survival analysis, the outcomes were divided into three categories: (*a*) death caused by lung cancer; (*b*) death from other causes without signs of relapse; and (*c*) still alive on December 31, 1997. These clinical data were analyzed together with the prospectively recorded data shown in Table 1.

Tumor Classification. The tumor-node-metastasis (TNM) staging and clinical staging were assessed from surgery and pathology reports according to the postsurgical pathological international grading system adopted by the International Union against Cancer and the American Joint Committee on Cancer (23, 24). The histopathological features of the surgical specimens listed in Table 1 were classified by two of us (V. S. and A. O. M.) according to the WHO criteria (25). Adenosquamous carcinomas were assigned to either the SCC or AC group, according to the prevailing histological component.

Tumor Specimens and DNA Extraction. Tumor tissue was collected at the time of surgery and cut into pieces. One piece was fixed in 4% buffered formalin for histopathological examination; an adjacent piece was snap-frozen in liquid nitrogen and stored at −80°C until cut on a microtome and evaluated by light microscopy, ensuring that only tissue with more than 80% tumor cells was used for further DNA extraction.

***p53* Mutational Analysis.** Tumor DNA was screened for mutations in exons 4–9 by a modified single-strand conformational polymorphism method (17) and/or by chemical cleavage using fluorescence-labeled primers and capillary electrophoresis (ABI Prism 310) essentially as described by Verpy *et al.* (26). Samples with aberrantly migrating bands in the screening step were sequenced as described previously (17).

Statistical Analysis. Comparison between different groups was made using the χ^2 test. For analysis of lung cancer-related survival, Kaplan-Meier curves were constructed. The equality of survival curves for different subgroups was evaluated by use of the log-rank method. All *P*s were estimated from two-sided statistical tests. Relative hazards of dying from lung cancer were estimated by use of Cox's proportional hazards model. In the multivariate models, the following features were taken into consideration: (*a*) age at surgery; (*b*) tumor and node status; (*c*) tobacco consumption; (*e*) histopathological features; (*f*) distribution of mutations along exons; and (*g*) functional or structural domains of DNA binding sites of the p53 protein. For lung cancer-related survival analysis, death from lung cancer was considered to be the event of interest, and all other deaths were treated as censoring points. For overall survival, all causes of death were included in the analysis. This study refers to lung cancer-related survival unless otherwise stated. Statistical analysis was carried out using the statistical software program package SPSS for Microsoft Windows version 6.0.

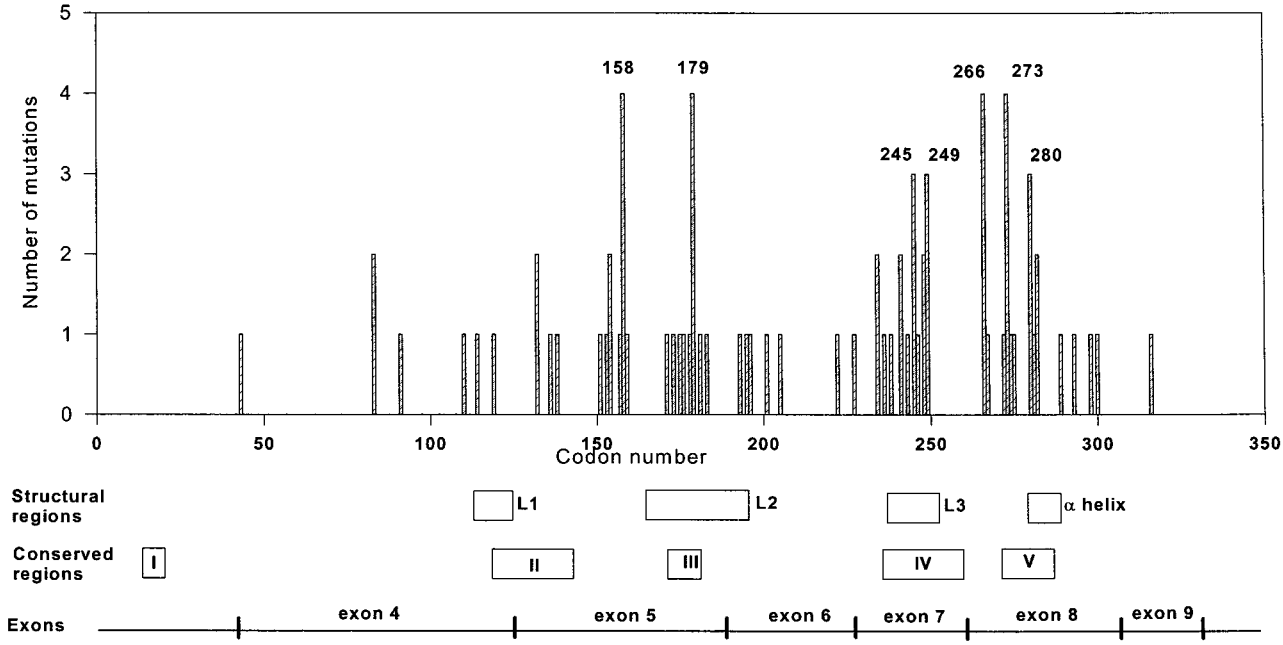

Fig. 1 Distribution of *p53* mutations in tumors of NSCLC patients. The relationship to structural and conserved regions in the core domains and the relationship to exons 4–9 were analyzed in this study.

RESULTS

Of 148 NSCLC patients, 83 (54%) had *p53* mutations within exons 4–9. In one tumor, there were two missense mutations. The p53 status in relation to clinicopathological information for these cases is shown in Table 1. A higher frequency of *p53* mutations was found among men (60%) than among women (35%), and *p53* mutations were seen more frequently in SCC (67%) than in AC (44%) and large cell carcinoma (57%).

Seventy-three (88%) of the mutations were within exons 5–8, and 10 (12%) of the mutations were within exons 4 and 9. The highest mutational frequencies were found in exons 5, 7, and 8 (33%, 22%, and 27%, respectively). The *p53* mutation spectrum is illustrated in Fig. 1. Both the frequency and distribution of *p53* mutations in NSCLC are in line with most previous lung cancer studies. Among the 84 mutations identified, 56 (67%) were missense mutations, 25 (30%) were null mutations including 8 (10%) nonsense (stop) mutations, 13 (15%) frameshift mutations (of which 9 were deletions, and 4 were insertions) and 5% splice site mutations. We identified 42 of 64 (66%) transversions and 22 of 64 (34%) transitions. G to T transversions were observed in 24 (38%) of the cases in this cohort, and G to A transitions were observed in 10 (16%) of the cases in this cohort. Of 80 patients with mutations affecting the coding region, 42 (53%) had mutations in conserved regions I-V of the gene. In the zinc-binding loops L2 and L3 of the p53 protein, the number of mutations was 13 (16%) and 14 (18%), respectively. Five patients had mutations in the H2 α-helix, and 11 patients had mutations in the S10 domain (of which six patients had mutations at residues 271–274). Furthermore, our analysis identified 10 mutations in four codons (codons 241, 248, 273, and 280) of the seven codons related to amino acids important in direct DNA binding (codons 120, 241, 248, 273, 276, 280, and 283).

The lung cancer-related survival analysis included 80 NSCLC patients with *p53* mutations affecting the coding region and 68 patients with wt *p53*. The major significant predictors for poor survival in the proportional hazards regression analysis were tumor size (T_2 and T_3 compared to T_1), nodal status (N_2 compared to N_0), and age above 60 years at the time of surgery, notably among those patients with SCC (Table 2). Overall survival was also reduced across all stages (HR = 1.69; 95% CI, 1.06–2.70), and the main predictors for overall survival were also tumor size and nodal status (data not shown). As shown by the Cox regression model, survival was not significantly associated with smoking habits, pulmonary ventilation capacity, airway obstruction, coexisting cardiovascular disease, tumor location (left/right), or whether or not there were revealing symptoms before the surgical procedure.

Univariate analysis showed poorer lung cancer-related survival for patients with *p53* mutations across all clinical stages. This was particularly evident for patients with mutations in exon 8, whereas for exon 9, the numbers were too low for evaluation. Kaplan-Meier survival estimate curves for lung cancer-related survival in patients with and without *p53* mutations and for locations of these mutations within exons are depicted in Fig. 2. In the multivariate model, patients with mutations in the *p53* gene had poor survival compared with patients without wt *p53*: the HR for dying from lung cancer was 2.09 (95% CI, 1.20–3.64). As shown in Table 2, the HR was even more pronounced for patients with mutations in exon 8 (HR = 3.50; 95% CI, 1.59–7.71). No significant differences were observed in survival for patients with mutations in exons 5 and 7 compared to

Table 2 Multivariate Cox regression analysis predicting lung cancer-related survival of 148 patients treated by surgery for NSCLC

	NSCLC (n = 148)			AC (n = 68)			SCC (n = 66)		
Variables	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age at surgery			0.02			0.81			0.07
0–59 yrs	Ref.[a]			Ref.			Ref.		
60+ yrs	2.42	1.14–5.17		1.15	0.38–3.48		6.60	1.09–39.95	
Gender			0.26			0.36			0.72
Female	Ref.			Ref.			Ref.		
Male	1.79	0.77–4.19		1.88	0.49–7.20		0.86	0.25–2.97	
Smoking (pack-years)			0.86			0.78			0.57
0	Ref.			Ref.					
1–19	1.81	0.22–15.00		2.22	0.19–28.89		Ref.		
20–39	1.87	0.23–16.17		1.77	0.20–23.73		1.51	0.28–2.68	
40+	2.07	0.23–18.45		3.42	0.21–57.04		2.12	0.29–3.56	
Tumor status			<0.01			0.01			0.05
T_1	Ref.			Ref.			Ref.		
T_2	2.90	1.30–6.47		1.17	0.28–4.83		2.97	0.80–11.00	
T_3	10.25	3.53–29.75		10.23	1.91–54.60		21.53	3.60–128	
Nodal status			0.01			0.03			0.95
N_0	Ref.			Ref.			Ref.		
N_1	1.70	0.75–3.90		2.27	0.48–10.74		0.82	0.15–4.54	
N_2	2.66	1.31–5.44		4.83	1.44–16.17		1.25	0.41–3.76	
Mutant exon of p53 status[b]			0.02			0.82			0.11
wt	Ref.			Ref.			Ref.		
Exon 4	2.25	0.63–8.00		1.56	0.17–14.6		2.02	0.23–17.71	
Exon 5	1.43	0.68–3.04		0.99	0.27–3.60		1.75	0.60–5.14	
Exon 6	3.17	0.59–16.97		[c]			3.62	0.35–37.23	
Exon 7	1.77	0.83–3.78		1.81	0.53–6.21		1.28	0.37–4.40	
Exon 8	3.50	1.59–7.71		2.03	0.51–8.20		3.19	1.07–9.45	
Exon 9	5.76	1.57–21.08		[c]			[c]		

[a] Ref., reference group.
[b] Number of patients: 6 patients, exon 4; 26 patients, exon 5; 5 patients, exon 6; 18 patients, exon 7; 22 patients, exon 8; and 3 patients, exon 9.
[c] Value not estimated because there were fewer than three patients in the category.

those with wt *p53*. Among patients with missense mutations, the HR was 2.00 (95% CI, 1.10–3.63), whereas for patients with null mutations (*i.e.*, stop, frameshift, and splice site mutations), the corresponding figure was 2.34 (95% CI, 1.14–4.79). Among the histopathological subtypes, SCC had a significantly increased HR only for mutations in exon 8 (HR = 3.19; 95% CI, 1.07–9.45).

Mutations in defined domains of the *p53* gene are clearly related to an increased risk of dying from lung cancer-related disease. The HRs for mutations involving the L2 loop and the L2 + L3 loops together were 3.97 (95% CI, 1.51–10.45) and 2.36 (95% CI, 1.18–4.74), respectively. There was no increased risk associated with mutations in the L3 loop. The most severe effect on survival was shown for mutations located in the codons involved in the binding of the zinc atom (HR = 11.7; 95% CI, 3.6–38.7).

For prognostic evaluation, *p53* mutations have been classified into groups according to possible biological functions (27): (*a*) severe flexible mutants and severe contact mutants; (*b*) scrambled mutants; and (*c*) mild contact mutants and mild flexible mutants (see Table 3). The group of severe flexible mutants and severe contact mutants was significantly related to reduced survival (HR = 4.16; 95% CI, 1.93–8.97).

The analysis of overall survival for the variables above gave results that were similar to those for lung cancer disease-free survival, although slightly less significant.

DISCUSSION

Clinical parameters such as tumor size and lymph node status have been used to characterize lung cancer phenotypes in relation to prognosis. We confirm in this study that tumor size and nodal status are important predictors for both overall and lung cancer-related survival, which again correlates with clinical stage. An independent factor for poor prognosis was also age > 60 years at the time of surgery. However, it is important to establish prognostic factors that will define subgroups of patients.

In this study, the mutations in *p53* were identified by sequencing all samples that had aberrant migration bands detected by the modified single-strand conformational polymorphism and/or chemical cleavage method. They both detect nearly all *p53* mutations (17). *p53* mutations were detected in 54% of the specimens examined, and a predominance of G to T transversions were observed (31% of all mutations).

We present data consistent with previous studies in which mutations in *p53* were found to be a strong and independent prognostic factor in lung cancer (18, 19, 28, 29), although these findings were not confirmed by others (21). Regions outside exons 5–8 were sequenced in only one of these studies (19). In the present study, significantly poorer prognosis was observed for *p53* mutations within exon 8 in SCC. This observation is in line with a previous study (21). Furthermore, we found that patients with mutations in exon 5, 6, and 7 had about the same

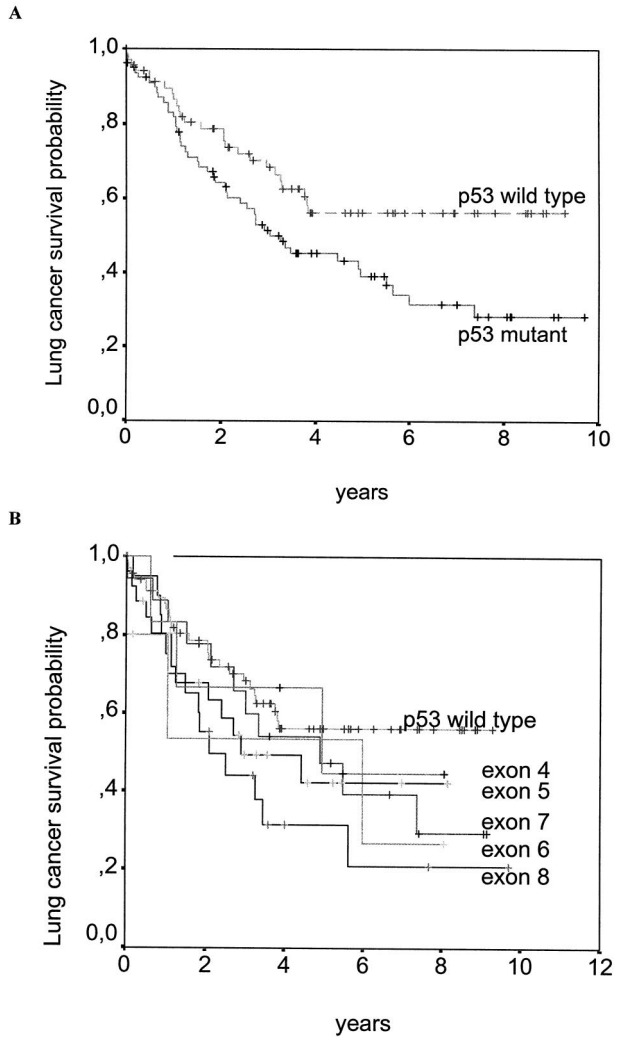

Fig. 2 Kaplan-Meier lung cancer-related survival curves for NSCLC patients in clinical stage I–IIIB according to *p53* status. *A*, patients (n = 84) with *p53* mutations in exons 4–9 (*p53 mutant*) and patients (n = 64) with wt *p53*. Patients with mutations had significantly worse survival (P = 0.022). *B*, patients with mutations classified according to location within exons 4–8. Patients with mutations in exon 8 had significantly worse survival than those with wt *p53* (P = 0.016). Exon 9 is omitted due to the small number of patients in the group.

survival rate. We could not confirm the results from a recent study demonstrating exon 5 mutations as an independent significant poor prognostic factor in NSCLC (22). In that study, the *p53* mutation frequency was only 21%.

Missense mutations have been reported to be associated with poor lung cancer-related survival in clinical stage I patients, as shown by analysis of Kaplan-Meier survival plots (18). Our Cox regression model, with 67% of the patients in clinical stage I, did not show such an association. In contrast, it has been shown previously that null mutations are associated with poor lung cancer-related survival among patients mainly in clinical stage III (30). In that study (30), there was a low frequency of *p53* mutations (25%) including null mutations (4%). Kaplan-Meier survival plots of our data also indicate that null mutations are significantly associated with poor prognosis for all stages combined (P = 0.03) and clinical stage I patients (P = 0.008) compared to patients without mutations. However, null mutations did not remain an independent prognostic factor in our multivariate analysis, whatever survival end point was considered.

Thirty-four percent of the *p53* mutations occurred in the L2 and L3 domains. They interact to provide for DNA contacts and are partly held together by a zinc atom, thus playing an important role in DNA binding (31). We show here for the first time that mutations occurring in these domains in NSCLC patients are clearly related to poor prognosis; the HR for mutations involving the L2 loop and the L2 + L3 loops together was 3.97 (95% CI, 1.51–10.45) and 2.36 (95% CI, 1.18–4.74), respectively. A novel finding was also that patients with mutations in the biologically functional domains of severe flexible and severe contact mutants had significantly reduced survival (HR = 4.16). Patients with mutations in the H2 α-helix did not have shorter survival, as was suggested in an earlier study (21). Mutations affecting L2 and L3 have been observed in breast cancer patients with shortened survival (27), and colorectal cancer mutations affecting L3 were also associated with increased risk of cancer-related death (32). The most striking observation in the present study was a HR of 11.7 in patients with mutations affecting the four residues binding the zinc atom (codons 176, 179, 238, and 242) that bridges loops L2 and L3. These mutations may destabilize this portion of the DNA-protein binding domain and p53 functions, resulting in more aggressive tumors and shorter survival.

Several studies have examined p53 structure/function relationships. Both loss of normal function of wt p53 and gain of oncogenic properties of p53 may be possible reasons for decreased survival (33–35). Hinds *et al.* (35) have shown that the mutant of codon 175 has biological properties (transformation frequency and transactivation) that differ from those of the mutant for codon 273. Replacement of cysteine with serine in the residue that binds zinc is shown to markedly reduce *in vitro* DNA binding, block transcription activation, and enhance transformation by *p53* (36). A recent study of *p53* hot spot data supports the concept that within the conserved DNA binding region, there are amino acid residues and sequence motifs that are essential to p53 structure and function, and other sequences that are less critical (37). It also appears that specific amino acid substitution may be important (38). Because studies have shown that there are many different somatic *p53* mutations that could have different functions, additional studies are required to clarify the mechanisms of mutant *p53*.

In conclusion, these results indicate that the *p53* mutational status constitutes a prognostic factor that may provide a basis for prognostic evaluation and for stratifying patients biologically in future lung cancer studies.

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Table 3 Cox regression analysis of lung cancer-related survival by distribution of mutations within various domains of the *p53* gene

Location	No. of patients	Codons	HR	95% CI
L2 loop	13	L2 = 163–195	3.97	1.51–10.45
The other mutants			1.86	1.07–3.22
Loops L2 + L3	27	L2 = 163–195	2.36	1.18–4.74
The other mutants		L3 = 236–251	1.87	1.05–3.35
Severe flexible mutants and severe contact mutants	17	172, 173, 175, 176, 179, 181, 238, 245, 238, and 267 and 248 and 282	4.16	1.93–8.97
The other mutants			1.69	0.96–2.97
Zinc atom binding residues	6	176, 179, 238, and 242	11.7	3.56–38.69
The other mutants			1.86	1.08–3.21

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