

# Lung Cancer Pathogenesis Associated With Wood Smoke Exposure\*

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**Background:** Tobacco is considered the most important cause of lung cancer, but other factors could also be involved in its pathogenesis. The aim of the present work was to establish an association between wood smoke exposure and lung cancer pathogenesis, and to analyze the effects of wood smoke on p53 and murine double minute 2 (MDM2) protein expression.

**Design:** Blood samples were obtained from 62 lung cancer patients, 9 COPD patients, and 9 control subjects. Of the 62 lung cancer patients, 23 were tobacco smokers (lung cancer associated with tobacco [LCT] group), 24 were exposed to wood smoke (lung cancer associated with wood smoke [LCW] group), and 15 could not be included in these groups. Western blot assays were performed to identify the presence of p53, phospho-p53, and murine double minute 2 (MDM2) isoforms in plasma samples. Densitometric analysis was used to determine the intensity of p53, phospho-p53, and MDM2 bands.

**Results:** Approximately 38.7% of the lung cancer patients examined had an association with wood smoke exposure, most of them women living in rural areas. Adenocarcinoma was present in 46.7% of these patients. The p53 and phospho-p53 proteins were significantly increased in LCW samples ( $56,536.8 \pm 4,629$  densitometry units [DU] and  $58,244.8 \pm 7,492$  DU, respectively [ $\pm$  SD]), in comparison with the other groups. The 57-kD MDM2 isoform plasma concentration was very high in LCW and LCT samples ( $75,696.4 \pm 11,979$  DU and  $78,551.7 \pm 11,548$  DU, respectively). MDM2-p53 complexes were present in a high concentration in control and COPD subjects. This allows p53 degradation and explains the low concentrations of p53 found in these groups. MDM2-phospho-p53 complexes were observed in COPD but not in the other samples. This correlates with the low concentration of p53 observed in the COPD group ( $13,657 \pm 2,012$  DU), and could explain the different clinic evolution of this smoker population in comparison with the LCT subjects.

**Conclusion:** This study suggests that there is a possible association of lung cancer with wood smoke exposure. Likewise, our findings demonstrate that wood smoke could produce similar effects on p53, phospho-p53, and MDM2 protein expression as tobacco. (CHEST 2005; 128:124-131)

**Key words:** COPD; lung cancer; murine double minute 2; p53; wood smoke.

**Abbreviations:** BaP = benzo[a]pyrene; DU = densitometry unit; LCT = lung cancer associated with tobacco; LCW = lung cancer associated with wood smoke; MDM2 = murine double minute 2; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Lung cancer is one of the major causes of cancer death in the world.<sup>1</sup> In Mexico, this neoplasm has a mortality rate of 125.2 per 100,000 in men and 48.8 per 100,000 in women.<sup>2,3</sup> The high lung cancer mortality rate is related to difficulties in early diagnosis due to a lack of symptoms associated with early disease, as well as a poor sensitivity of the classical methods for early detection.

The p53 gene is the most frequently mutated gene found in human cancers.<sup>4</sup> p53 mutations are present in approximately 50% of the non-small cell lung cancers and approximately 90% of the small cell lung cancers.<sup>5</sup> The p53 gene encodes a protein that functions as a transcription factor.<sup>6</sup> The downstream genes regulated by p53 are involved in DNA repair, apoptosis, inhibition of angiogenesis, reentry into cell cycle,

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oxidative stress, and determination of cell fate.<sup>7</sup> The activation of the p53 protein occurs through phosphorylation of the serine residues near the C-terminal domain in response to cellular stress produced by DNA damage.<sup>8</sup> p53 degradation proceeds through an auto-regulatory feedback loop.<sup>6,9</sup> p53 stimulates murine double minute 2 (MDM2) transcription; MDM2 then binds to the p53 unphosphorylated N-terminal domain promoting its degradation by the ubiquitin-proteasome pathway, lowering p53 cell concentration and MDM2 expression.

Lung cancer has been associated with tobacco smoking.<sup>10,11</sup> Tobacco smoke contains approximately 60 carcinogenic compounds. Among them, benzo[*a*]pyrene (BaP) and N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are the most important carcinogens present in tobacco smoke, since they are the main initiators of human lung cancer by inducing multiple genetic alterations including p53 gene mutations.<sup>12</sup>

Although tobacco smoke is considered the major cause of lung cancer, other factors may be involved in its pathogenesis. In developing countries, wood and other solid fuels are used for cooking and heating. Exposure to biomass smoke has been associated with respiratory diseases such as chronic bronchitis, emphysema, and asthma.<sup>13,14</sup> However, there is not enough evidence that wood smoke exposure participates in lung cancer onset in nonsmoker patients. The aim of the present work was to analyze the correlation between lung cancer and wood smoke exposure, and the possible effects of smoke on p53 and MDM2 protein expression.

### Patients

Blood samples were collected in 5-mL lithium heparin-coated tubes from 62 patients with primary lung cancer between March 2003 and July 2004. The diagnosis was established histologically by the analysis of tumor samples obtained by bronchoscopy or percutaneous needle biopsy, and/or sputum cytology. Patients were staged prior to treatment according to Mountain.<sup>15</sup> Lung cancer patients were classified into the following two groups: (1) lung cancer associated with tobacco (LCT), consisting of 6 women and 17 men who were current smokers for > 10 years (mean smoking history,  $23.65 \pm 15.5$  pack-years; range, 11 to 56 pack-years); and (2) lung cancer associated with wood smoke (LCW), consisting of 22 women and 2 men who were not tobacco smokers but had been exposed to domestic wood smoke for a mean duration of  $44 \pm 17.5$  years ( $195.4 \pm 92.8$  h/yr). These patients used traditional "three-stone" stoves in their kitchens without a chimney. The LCW patients were screened for additional carcinogens associated with occupational exposure or passive tobacco smoke. Fifteen lung cancer patients were not included in these groups: 3 were passive smokers, 3 were smokers exposed to wood smoke, and in 9 cases, no association could be established. Clinical data are given in Table 1.

Nine smoker patients with COPD were also examined. COPD diagnosis was confirmed by medical history and the results of spirometry. American Thoracic Society criteria were used: history of productive cough for 3 consecutive months each year for the past 2 years, with an FEV<sub>1</sub> < 80% of the predictive value, an FEV<sub>1</sub>/FVC ratio < 70%, and a reversibility in FEV<sub>1</sub> < 10% after inhalation of 400 µg salbutamol (Table 2).<sup>16</sup> This group consisted of five women and four men who were current smokers for > 10 years (mean smoking history,  $27.95 \pm 18.2$  pack-years; range, 10 to 70 pack-years) without exposure to wood smoke. Subjects with a history of asthma, atopy, or allergy were excluded from the study. None of the COPD patients had emphysema detected on a CT scan. Nine healthy nonsmoker volunteers, five women and four men with normal spirometry values, with no signs of infective respiratory disease during the past 3 weeks, no exposure to wood smoke, and no history of asthma, atopy, or allergy were used as control subjects.

**Table 1—Clinical Characteristics of Lung Cancer Patients (n = 62)\***

Characteristics	All Patients	LCT Group	LCW Group	Other
Subjects	100 (62)	37.1 (23/62)	38.7 (24/62)	24.2 (15/62)
Gender				
Women	56.5 (35/62)	17.1 (6/35)	62.9 (22/35)	20 (7/35)
Men	43.5 (27/65)	63 (17/27)	7.4 (2/27)	29.6 (8/27)
Histologic types				
AC	72.6 (45/62)	28.9 (13/45)	46.7 (21/45)	24.4 (11/45)
SCLC	17.7 (11/62)	45.4 (5/11)	27.3 (3/11)	27.3 (3/11)
SCC	9.7 (6/62)	83.3 (5/6)		16.7 (1/6)
Stage (TNM)				
IB	4.8 (3/62)	33.3 (1/3)	66.6 (2/3)	
IIA	3.2 (2/62)	100 (2/2)		
IIB	4.8 (3/62)	33.3 (1/3)	66.6 (2/3)	
IIIA	9.7 (6/62)	50 (3/6)	33.3 (2/6)	16.7 (1/6)
IIIB	21 (13/62)	15.4 (2/13)	61.5 (8/13)	23.1 (3/13)
IV	56.5 (35/62)	40 (14/35)	31.4 (11/35)	28.6 (10/35)

\*Data are presented as % (No./total patients). AC = adenocarcinoma; SCLC = small cell lung cancer; SCC = squamous cell carcinoma.

**Table 2—Spirometry Values of Control, COPD, and Lung Cancer Subjects\***

Variables	Control Group	COPD Group	LCT Group	LCW Group
Age, yr	59.2 ± 8.3 (51–75)	52.9 ± 7.7 (44–57)	58 ± 9.6 (36–72)	58.7 ± 12.4 (34–76)
FEV <sub>1</sub> , % predicted	97.6 ± 4.9 (87–100)	61.3 ± 10 (46–74)	67.3 ± 29.7 (28–122)	70.8 ± 29.2 (28–129)
FEV <sub>1</sub> /FVC, %	79.8 ± 5.8 (71–90)	49.3 ± 11 (34–64)	75.6 ± 8.9 (64–89)	76.8 ± 11.4 (51–91)
FEV <sub>1</sub> reversibility, %	ND	5.6 ± 2.4 (2–9.5)	ND	ND

\*Values given as mean ± SD (range). ND = not done.

Venous blood samples from cancer, COPD, and control subjects were centrifuged, and plasma protein content was measured by the bicinchoninic acid protein assay (Pierce Chemical Company; Rockford, IL).<sup>17</sup> Samples were then stored at –70°C until used. Informed consent was obtained from each patient, and the protocol was approved by the local Ethical and Research Committees.

#### Western Blot Assay

Presence of p53, phospho-p53 and MDM2 isoforms was examined in cancer and control samples. Cancer samples from patients with an advanced stage of the disease (stages IIIB and IV), were used for this assay. Electrophoresis was carried out using 15 µg of protein per lane in 8% sodium dodecyl sulfate polyacrylamide gels under reducing conditions with 5% 2-mercaptoethanol boiled during 5 min. Proteins were then transferred to polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk in 100 mmol/L Tris-HCl buffer, pH 7.5, with 150 mmol/L NaCl and 0.1% Tween 20 (Tris buffered saline solution plus Tween 20) for 1 h. At this time, membranes were incubated with the following monoclonal antibodies: 5 µg/mL anti-p53 (clone Pab 240), 5 µg/mL antiphospho-p53 (serine 392) [both from Calbiochem-Novabiochem International; San Diego, CA], and 1 µg/mL MDM2 (SMP 14; Santa Cruz Biotechnology; Santa Cruz, CA). The MDM2 antibody recognizes all MDM2 isoforms and MDM2 complexes. Unbound antibodies were removed by washing with TTBS buffer, and primary antibodies were detected (VectaStain ABC kit; Vector Laboratories; Burlingame, CA). Bands identified in the Western blot assay were analyzed by densitometry (Kodak Digital Science ID Image Analysis Software; Eastman Kodak; Rochester, NY), which measures the surface and intensity of bands. Results were expressed as densitometry units (DU).

#### Statistical Analysis

Densitometry results were analyzed using the Mann-Whitney *U* test, and expressed as mean ± SD; *p* ≤ 0.05 was considered significant.

## RESULTS

Sixty-two patients with primary lung cancer were examined prior to chemotherapy (Table 2). Approximately 38.7% of the patients had an association with wood smoke (24 of 62 subjects), and 37.1% had an association with tobacco smoke (23 of 62 subjects).

Lung cancer incidence was higher in women (56.5%, 35 of 62 subjects) than in men (43.5%, 27 of 62 subjects) in the population examined. A high percentage of lung cancer was associated with wood

smoke exposure (62.9%, 22 of 62 subjects) in comparison to the percentage associated with tobacco smoke (17.1%, 6 of 35 subjects) in women population. Sixty-three percent of men (17 of 27 subjects) were tobacco smokers, while 7.4% of men (2 of 27 subjects) were exposed to wood smoke.

Adenocarcinoma was the main histologic type observed in lung cancer patients (72.6%, 45 of 62 subjects). This histologic type was present in 28.9% (13 of 62 subjects) of the LCT group and in 46.7% (21 of 45 subjects) of the LCW group. Adenocarcinoma was present in 28 women, 19 associated with wood smoke exposure and 5 associated with tobacco. Adenocarcinoma was also present in 17 men; 8 were tobacco smokers and 2 were exposed to wood smoke (Table 3). Clinical staging showed that most of the patients examined had advanced disease: stage IIIB (21%, 13 of 62 subjects) and stage IV (56.5%, 35 of 62 subjects).

The Western blot assay showed the presence of p53 as a 50-kD band in all samples examined (Fig 1, *top*). The densitometry analysis demonstrated a significant increase in p53 protein expression in lung cancer patients in comparison with the other groups (*p* = 0.0004). The highest net intensity was found in the LCW group followed by the LCT group (56,536.8 ± 4,629 DU and 40,521.2 ± 8,804 DU,

**Table 3—Lung Cancer Histologic Type Distribution (n = 62)\***

Variables	Women	Men
Adenocarcinoma		
Tobacco smoker	5	8
Wood smoke	19	2
Tobacco and wood	0	2
Passive smoker	2	0
NA	2	5
Squamous cell carcinoma		
Tobacco	1	4
Passive smoker	1	0
Small cell lung cancer		
Tobacco	0	5
Wood smoke	3	0
Tobacco and wood	1	0
NA	1	1

\*Data are presented as No. NA = no association.

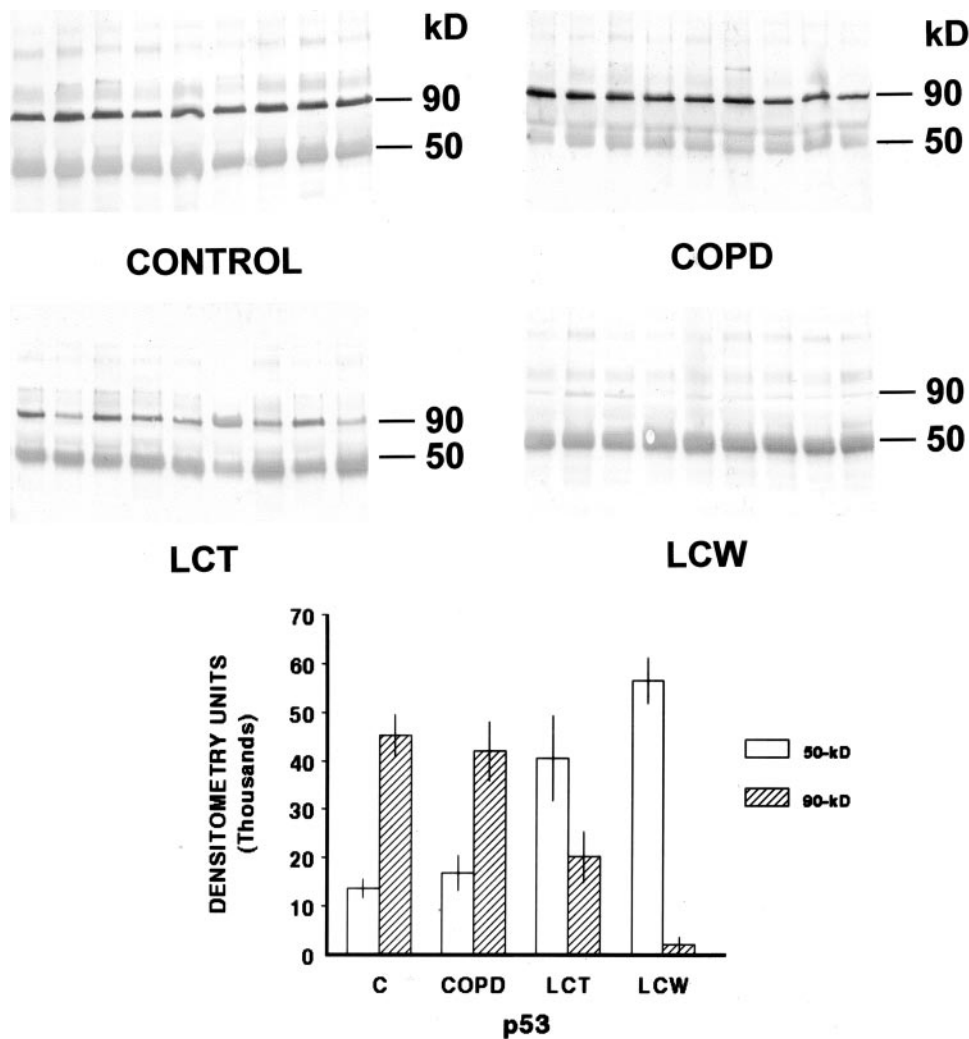


FIGURE 1. *Top*: Western blot plasma detection of p53. The p53 protein was detected as a band of 50 kD in all samples. A band of 90 kD (p53-MDM2 complexes) was observed with high intensity in control and COPD subjects. Molecular weight markers are listed on the right. *Bottom*: The p53 densitometry analysis showed a significant increase in p53 (50-kD band) protein expression in LCW and LCT samples. The 90-kD band (p53-MDM2 complexes) net intensity was significantly higher in control and COPD than in lung cancer samples. Bars represent the SD.

respectively;  $p = 0.002$  between lung cancer groups) [Fig 1, *bottom*]. There were no significant differences among COPD and control groups ( $13,657 \pm 2,012$  DU and  $16,925.8 \pm 3,611$  DU, respectively). A 90-kD band was also observed in all plasma samples. This band may correspond to p53-MDM2 complexes (see below). The net intensity was significantly higher in control and COPD samples ( $45,306.8 \pm 4,227$  DU and  $42,004.7 \pm 6,056$  DU, respectively), than in LCT and LCW groups ( $20,233.1 \pm 5,226$  DU and  $2,092 \pm 1,526$  DU;  $p < 0.00045$ ).

Phospho-p53 was observed in control, COPD, and lung cancer samples as a 50-kD band (Fig 2, *top*). The densitometric analysis showed a significant in-

crease in phospho-p53 protein (50-kD band) in LCW ( $58,244.8 \pm 7,492$  DU) in comparison with LCT ( $39,322.1 \pm 7,932$  DU;  $p = 0.0004$ ) [Fig 2, *bottom*]. Phospho-p53 protein expression was significantly lower in control and COPD subjects ( $27,533.4 \pm 3,889$  DU and  $25,503.6 \pm 4,601$  DU, respectively) than in LCT and LCW cancer patients ( $p < 0.002$ ). A 90-kD band was also observed in the COPD group with a net intensity of  $42,004.7 \pm 6,056$  DU. This band was detected with a very low intensity in LCT and LCW groups ( $1,249 \pm 585$  DU and  $1,205 \pm 762$  DU, respectively), but not in control samples. It is possible that this band corresponds to complexes between MDM2 and p53 phosphorylated in its C-terminal domain.

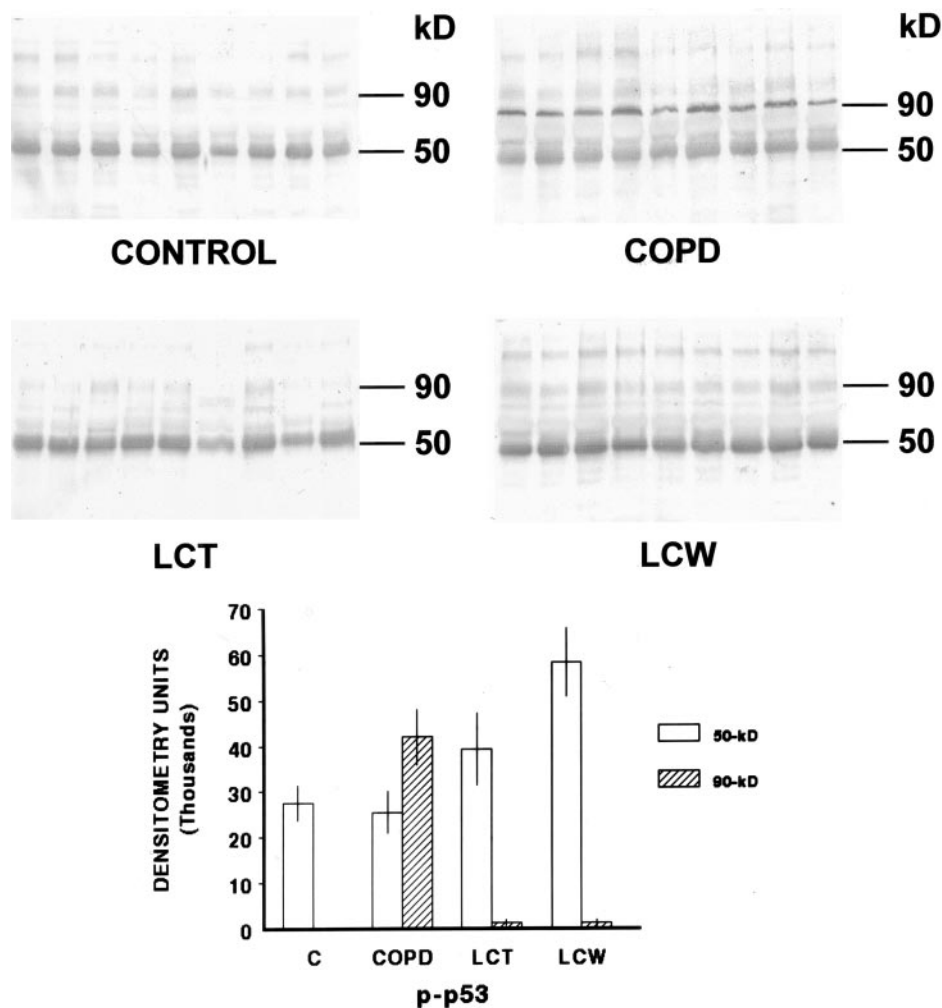


FIGURE 2. *Top*: Phospho-p53 Western blot analysis. Phospho-p53 was identified as a band of 50 kD in all samples examined. A band of approximately 90 kD was observed with a high intensity in COPD subjects. Molecular weight markers are listed on the right. *Bottom*: Phospho-p53 densitometry analysis demonstrated that LCW samples had a significant increase in phospho-p53 protein expression in comparison with the other groups. The 90-kD band (phospho-p53 complexes) was observed with a high net intensity in COPD patients. Bars represent the SD.

To determine the presence of MDM2 isoforms in plasma from lung cancer, control, and COPD subjects, a Western blot assay using an antibody that recognizes all MDM2 isoforms and MDM2 complexes was performed. This assay revealed the presence of the 57-kD MDM2 isoform in all samples examined (Fig 3, *top*). The densitometry study showed that LCT and LCW ( $78,551.7 \pm 11,548$  DU and  $75,696.4 \pm 11,979$  DU, respectively) showed the highest expression of this protein in comparison with the control and COPD groups ( $21,452.6 \pm 5,345$  DU and  $23,078 \pm 4,733$  DU, respectively;  $p < 0.0005$ ) [Fig 3, *bottom*]. The 90-kD MDM2 isoform was also detected in all samples but with a lower intensity than the 57-kD isoform (Fig 3, *top*). This MDM2 isoform was significantly increased in

LCT and LCW groups ( $11,505.8 \pm 6,009$  DU and  $12,065.3 \pm 4,405$  DU, respectively) in comparison with control and COPD samples ( $2,335.6 \pm 1,861$  DU and  $3,611.5 \pm 1,077$  DU,  $p < 0.007$ ) [Fig 3, *bottom*]. The 76-kD MDM2 isoform had a low intensity in control and COPD patients but not in lung cancer patients (Fig 3, *top*). The protein expression of this isoform was significantly higher in COPD than in control samples ( $10,146.7 \pm 3,275$  DU and  $1,581.6 \pm 836$  DU, respectively;  $p = 0.0004$ ) [Fig 3, *bottom*].

## DISCUSSION

Tobacco smoke is still considered the main cause of lung cancer. After decades of antismoking cam-

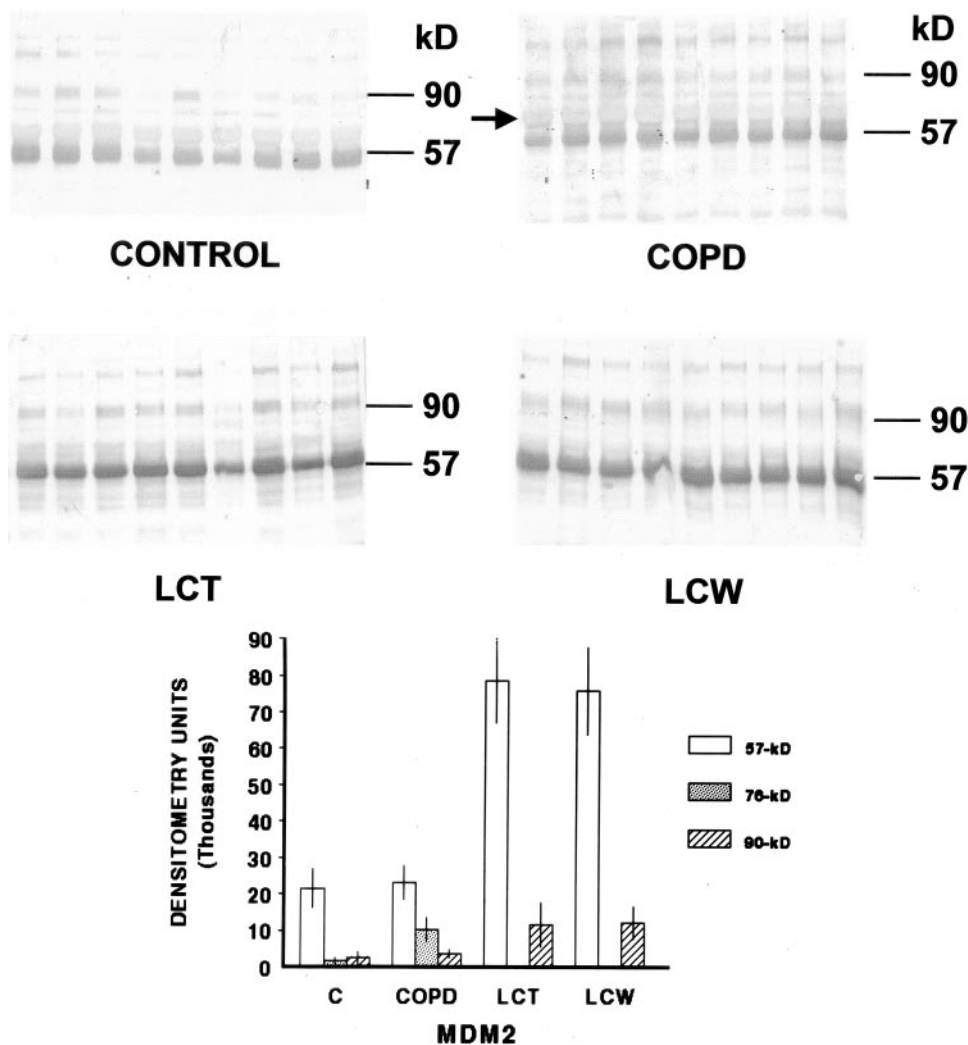


FIGURE 3. *Top*: MDM2 Western blot assay. The 57-kD isoform was detected in all samples. The highest intensity was observed in LCT and LCW samples. The 90-kD isoform was observed mainly in lung cancer patients. The 76-kD isoform was present in COPD subjects but not in the other groups (arrow). Molecular weight markers are listed on the right. *Bottom*: Densitometry analysis demonstrated a significant increase in the 57-kD MDM2 isoform protein expression in LCT and LCW samples. The 90-kD isoform was detected with a very low intensity in all samples with a significant increase in lung cancer groups. Protein expression of the 76-kD isoform was significantly higher in COPD than in control subjects. Bars represent the SD.

paigms, a constant increase in lung cancer cases around the world could be an indicator that factors other than tobacco smoking are involved in this disease. In this work, we examined 62 primary lung cancer patients who were about to receive chemotherapy treatment and follow-up during the evolution of the disease. We found that 38.7% of the lung cancer patients examined were nonsmokers with a history of continuous wood smoke exposure for > 10 years. They were mostly women living in rural areas in poverty conditions. In our country, women are exposed to wood smoke for many hours per day. That could explain the higher incidence of lung cancer associated with wood smoke in women.

Adenocarcinoma was the most frequent lung cancer histologic type observed in our smoker and nonsmoker patients in both genders. These results are comparable with those obtained by other authors.<sup>18,19</sup> These works point out that there is an increase in adenocarcinoma in comparison with other histologic types, particularly squamous cell carcinoma, in smoker lung cancer patients in recent years. This increase was similar in both sexes.<sup>20</sup>

The change in the distribution of the histologic types could be related to modifications in cigarette smoke composition with low tar and nicotine. Smokers who consumed these type of cigarettes need to smoke more cigarettes per day with a deeper inha-

lation in order to maintain blood nicotine levels.<sup>21</sup> Moreover, it has been reported that tobacco smoke has higher levels of NNK and lower concentrations of BaP than used before.<sup>12</sup> In this context, Hoffman et al<sup>22</sup> demonstrated that the NNK induces adenocarcinomas while BaP produces squamous cell carcinomas in laboratory animals. Cigarette design might be also involved in the increasing predominance of adenocarcinoma over squamous cell carcinoma. The particle size in the smoke inhaled from filter cigarettes is smaller in comparison with the particles size from the smoke of nonfilter cigarettes. The small size of the inhaled particles and a deeper inhalation cause particle deposition in the alveolar regions, which corresponds to the particle distribution pattern observed in adenocarcinoma.<sup>18</sup> These observations could be an explanation for the high adenocarcinoma frequency that developed in our LCT group. Likewise, adenocarcinoma was the main histologic type in wood smoke-exposed patients. Although wood smoke, like tobacco smoke, contains BaP, it is possible that the way in which wood smoke is inhaled causes different effects on the respiratory epithelium.<sup>23</sup> Additionally, the NNK or another carcinogen that induces adenocarcinoma could be present in wood smoke. This proposal needs future research.

It is well to know that BaP and NNK produce p53 mutations characterized by G to T transversions.<sup>12,24</sup> In fact, an increase in the frequency of this type of mutation in lung cancer on smoker (30%) in comparison with nonsmoker subjects (12%) has been observed. In our study, we detected p53 in all plasma samples examined by immunoblotting. It was not surprising to detect p53 in samples from COPD and control subjects, since this protein is present in normal cells. An increase in p53 concentration was expected in plasma from lung cancer patients because these subjects had an advanced stage of the disease with an active metastatic process. Our plasma results are similar to those reported by other authors<sup>25,26</sup> in lung cancer tissue. Interestingly, the highest p53 protein expression was observed in lung cancer associated with wood smoke.

Further, phospho-p53 of the C-terminal domain was also analyzed in order to determine p53 functional state. We found an increase in phospho-p53 in lung cancer samples, particularly in wood smoke-exposed patients, in comparison with control and COPD subjects. This results demonstrated that p53 was functional active. However, the other hand, the 57-kD MDM2 isoform but not the 76-kD and 90-kD MDM2 isoforms was increased in the plasma from lung cancer patients. The increase in MDM2 could be due to a high phosphorylation of the p53 N-terminal domain that prevents the formation of

MDM2-p53 complexes with an accumulation of both molecules. Moreover, a band of approximately 90-kD that corresponds to these complexes was observed with a low intensity in the p53 Western blot from lung cancer samples in comparison with the other groups.

Likewise, a 90-kD band with high intensity was also detected in COPD but not in the other samples of the phospho-p53 Western blot assay. This band may correspond to phospho-p53-MDM2 complexes. Interestingly, the COPD group as well as the LCT subjects were exposed to the same tobacco carcinogens, but the COPD patients evolved in a different direction. It is possible that p53 phosphorylation in COPD subjects occurs mainly in the C-terminal domain without any interference in p53 degradation.

In summary, wood smoke produces similar changes in p53, phospho-p53, and MDM2 protein expression as tobacco smoke. Therefore, it is possible that besides the BaP, wood smoke may contain other carcinogens such as NNK that could participate particularly in lung adenocarcinoma induction.

Our findings suggest that wood smoke, like tobacco smoke, could be involved in lung cancer pathogenesis. Therefore, it is important to consider wood smoke exposure as a possible risk factor for the development of lung cancer in nonsmoker subjects.

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