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Redox Imbalance in Lung Cancer of Patients with Underlying Chronic Respiratory Conditions

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Chronic respiratory diseases such as obstructive pulmonary disease (COPD) and oxidative stress may underlie lung cancer (LC). We hypothesized that the profile of oxidative and antioxidant events may differ in lung tumors and blood compartments of patients with non-small cell LC (NSCLC) with and without COPD. Redox markers (immunoblotting, ELISA, chemiluminescence, 2D electrophoresis and proteomics) were analyzed in blood samples of 17 control subjects and 80 LC patients (59 LC-COPD and 21 LC) and lung specimens (tumor and nontumor) from those undergoing thoracotomy (35 patients: 23 LC-COPD and 12 LC). As smoking history was more prevalent in LC-COPD patients, these were further analyzed *post hoc* as heavy and moderate smokers (cutoff, 60 pack-years). Malondialdehyde (MDA)–protein adducts and SOD1 levels were higher in tumor and nontumor samples of LC-COPD than in LC. In tumors compared with nontumors, SOD2 protein content was greater, whereas catalase levels were decreased in both LC and LC-COPD patients. Blood superoxide anion levels, protein carbonylation and nitration were greater in LC and LC-COPD patients than in the controls, and in the latter patients compared with the former. Systemic superoxide anion, protein carbonyls and nitrotyrosine above specific cutoff values best identified underlying COPD among all patients. Smoking did not influence the study results. A differential expression profile of oxidative stress markers exists in blood and, to a lesser extent, in the tumors of LC-COPD patients. These findings suggest that systemic oxidative stress and lung antioxidants (potential biomarkers) may predispose patients with chronic respiratory diseases to a higher risk for LC.

INTRODUCTION

Cigarette smoke continues to be the main etiologic factor of lung cancer (LC), a disease that remains the leading cause of cancer deaths worldwide. Importantly, the risk for LC increases in patients with underlying respiratory conditions such as chronic obstructive pulmonary disease (COPD), which also constitutes a major cause of morbidity and mortality in developed countries (1–7). Interestingly,

in patients with moderate-to-severe COPD, especially in those with emphysema, the prevalence of LC can go up as high as five-fold as compared with smokers without the disease (8–12). Elucidation of the biological mechanisms that may predispose patients with chronic respiratory diseases to a higher incidence of LC is required.

Oxidative and nitrosative stress have been shown to favor carcinogenesis

through the activation of cellular processes that result in neoplastic transformation or the induction of DNA mutations (13). In keeping with this, cell viability and growth seemed to be favored by a continuous process of DNA adducts and posttranslational modifications of proteins and lipids driven by oxidants (14). Proteins, DNA and lipids are the main cellular targets for the action of those oxidants that escape the tissue antioxidant capacity (8,11,12,15)

Reaction of oxidants with lysine, arginine, proline and threonine residues of the protein side-chains leads to the formation of reactive carbonylation derivatives (aldehydes and ketones) that constitute a widely studied form of protein oxidation. Reactive carbonyl groups may also be the result of Michael Addition reactions of

lysine, cysteine or histidine residues with α-, β-unsaturated aldehydes (for example, MDA) synthesized during the peroxidation of polyunsaturated fatty acids of the membranes (16,17,18). Furthermore, the highly reactive species peroxynitrite, formed by the near-diffusion limited reaction between nitric oxide and superoxide anion, may also directly oxidize proteins or nitrate tyrosine residues leading to nitrosative stress. Additionally, the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), 2' a marker of DNA oxidation, was demonstrated to induce mutagenic changes aside from interfering with gene expression (19–22).

Previous investigations have attempted to explore whether oxidative stress develops in patients with LC. For instance, structural and functional proteins were more severely nitrated in the lung tumor lesions than in the nontumor lungs of patients (9). Protein nitration and oxidation levels were also increased in plasma proteins of patients with LC (10). Increased systemic oxidative stress was also demonstrated in patients with advanced LC before and after chemotherapy (14,15). More recently, levels of several oxidative stress markers were also increased in the normal airway epithelium distant to the neoplasm and blood of patients with LC (23). Interestingly, in mice, a reduction in tumor burden was also demonstrated in response to the inhibition of superoxide dismutase (SOD)1 by the small molecule ATN-2, while it also favored cell death in different NSCLC cell lines (24). Very recently, SOD2 upregulation and catalase downregulation were also shown to act as potential biomarkers of tumor progression in several cancer types in actual patients (25). Taken together, these findings suggest that oxidative damage and antioxidant dysregulation induce and promote carcinogenesis in different experimental models. However, in those investigations (9,10,25,26), the presence of underlying respiratory conditions was not considered. Moreover, studies in

which lung tumor lesions and nontumor parenchyma and blood compartments were analyzed from the same patients with underlying respiratory diseases are also lacking.

On this basis, we hypothesized that oxidative stress may be a predisposing biological mechanism for LC in patients with underlying chronic respiratory diseases such as COPD. Moreover, the study also sought to explore whether the profile of oxidative stress and antioxidant events may differ in the lung tumor lesions and blood compartments of patients with NSCLC with and without COPD. Moreover, it was also hypothesized that levels of redox markers may be differentially expressed in tumor lesions versus the surrounding nontumor parenchyma in both groups of patients. Accordingly, the study objectives were: 1) to investigate levels of markers of protein oxidation and nitration and antioxidant systems in the tumor and nontumor lung parenchyma and blood of patients with NSCLC with and without COPD; 2) to identify the markers that may predict underlying COPD from all LC patients; and 3) to determine whether smoking history may influence the expression profile of the target redox markers in LC patients with COPD.

MATERIALS AND METHODS

Study Design and Patients

This is a prospective cross-sectional study in which patients were recruited consecutively from the Lung Cancer Clinic of the Respiratory Medicine Department at Hospital del Mar in Barcelona, Spain. For the purpose of the investigation, 80 Caucasian patients with LC were recruited consecutively from the weekly LC board meeting before having received any treatment for their lung neoplasm. These patients were further subdivided *post hoc* into two groups according to the presence of underlying COPD, which was diagnosed on the basis of current guidelines (27,28). The LC-COPD group had

59 patients with LC who also had COPD (57 males; 2 females). The LC group had 21 patients with LC without COPD (14 males; 7 females). A group of control subjects (n = 17, 8 males; 9 females) who underwent bronchoscopy for nontumor lesions was also recruited in the study, and blood samples were drawn on the same day. In all 80 patients, blood samples were obtained at the time of diagnostic confirmation for LC. Moreover, from the same study cohort, in the group of patients who underwent thoracotomy for the surgical resection of their lung neoplasms using a clinical indication according to guidelines for diagnosis and management of lung cancer (29), specimens from the tumor and nontumor lung parenchyma were also obtained in all cases (n = 35, 44%) and were further subdivided *post hoc* as follows: the LC-COPD group had 23 patients with LC who also had COPD (all males); and the LC group had 12 patients with LC without COPD (8 males; 4 females). In these two groups of patients, blood and lung specimens were available for the study (n = 35).

Histological diagnosis and staging (tumor, node, metastasis, [TNM]) (30,31) of LC were confirmed in all patients. Exclusion criteria were as follows: chronic cardiovascular disorders, chronic metabolic diseases, clot system disorders, signs of severe bronchial inflammation and/or infection (bronchoscopy), current or recent invasive mechanical ventilation and chronic oxygen therapy. In the present investigation, approval was obtained from the Institutional Ethics Committee on Human Investigation (Hospital del Mar–IMIM) in accordance with the World Medical Association Guidelines (Helsinki Declaration of 2008) for research on human beings. Informed written consent was obtained from all patients.

Clinical Assessment

Lung function parameters were assessed in all patients following standard procedures. Body composition

evaluation included the assessment of body mass index (BMI). Nutritional parameters were also evaluated through conventional blood tests.

Sample Collection

Blood sample specimens were obtained in all the recruited patients (n = 97) from the arm vein after an overnight fasting period, and conventional analytical parameters were analyzed together with the target study redox markers. Moreover, in all patients undergoing thoracotomy (n = 35), lung specimens were obtained from both tumor and nontumor surrounding parenchyma during the surgery, in which standard technical procedures were followed by the specialized thoracic surgeons. In all cases, the expert pathologist selected a fragment of lung tumor and nontumor specimens approximately 10×10 mm^2 from the fresh samples after a careful collection of the specimens required for diagnosis purposes. Importantly, a minimum amount of 50% of cancer cells was similarly identified in all tumor types from all the study patients. The remaining cell components were inflammatory and stromal cells in all the analyzed tumors.

Sample Preservation

Lung specimens (tumor and nontumor) were snap-frozen in liquid nitrogen and stored at –80°C until further use. Blood samples were centrifuged (588g at 4°C for 15 min) and were immediately frozen at –80°C until further analyses.

Molecular Biology Analyses

Immunoblotting of 1D electrophoresis. Protein levels of the different molecular markers in lung specimens were explored using methodologies previously published (23,32,33). The following antibodies were used to detect the different molecular markers: total protein carbonylation (OxyBlot Protein Oxidation Detection Kit, Chemicon International Inc.), malondialdehyde protein adducts (anti-MDA–protein adducts antibody, Academy Bio-Medical Company Inc.), total protein nitration

(anti-3-nitrotyrosine antibody, Invitrogen), SOD1 and SOD2 (anti-SOD1 and SOD2 antibodies, Santa Cruz Biotechnology), catalase (anti-catalase antibody, Calbiochem Merck Group) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, anti-GAPDH antibody, Santa Cruz).

Identification of carbonylated proteins in lung specimens. Two-D electrophoresis and silver staining. Carbonylated lung proteins were separated and identified in the samples as published elsewhere (23,34).

Identification of carbonylated proteins in lung specimens—mass spectrometry. Identification of carbonylated proteins was conducted in the Proteomics Laboratory at Universitat de Barcelona following the quality criteria established by ProteoRed standards (Instituto Nacional de Proteómica) and procedures previously published (23,34).

Protein carbonyl enzyme-linked immunosorbent assay (ELISA) in blood. Plasma levels of protein carbonylation were determined using the OxiSelect protein carbonyl ELISA kit (Cell Biolabs Inc.) following the manufacturer's instructions and previous studies (23,35).

Protein nitrotyrosine ELISA in blood. Plasma from all individuals was used in this assay (Nitrotyrosine ELISA, Cell Biolabs Inc.) following the manufacturer's instructions and previous studies (23,35).

Glutathione ELISA in blood. Plasma levels of the ratio of oxidized (GSSG) to reduced glutathione (GSH) were determined using the OxiSelect total glutathione GSSG/GSH ELISA kit (Cell Biolabs Inc.) following the precise manufacturer's instructions and previous methodologies (34).

Measurement of superoxide anion radicals using lucigenin-derived chemiluminescence in blood. Superoxide anion concentrations were measured following methodologies formerly described (23,36).

Statistical Analyses

All statistical analyses were performed using the software statistical package for the social sciences (SPSS) 15.0 (IBM). Data are expressed as mean (standard

deviation). The normality of the study variables was explored using the Shapiro-Wilk test. Variables detected in either blood or lung sample specimens from both groups of patients were analyzed independently using appropriate statistical approaches.

The LC-COPD group of patients was further subdivided into those with moderate (n = 24) and heavy (n = 35) exposures to cigarette smoke to determine the potential influence of smoking history on the study results. Median value of 60 pack-years in both subgroups of LC-COPD patients was used as the cutoff value to subdivide these patients according to their smoking history.

Furthermore, comparisons of oxidative stress markers were also explored in both lung tumor and nontumor specimens of LC (n = 12) and LC-COPD (n = 23) patients who underwent thoracotomy. Again, a *post hoc* analysis according to their smoking history was also conducted in LC-COPD patients, in both tumor and nontumor lung specimens: moderate smokers (n = 8) and heavy smokers (n = 15).

In the different sets of comparisons among the study groups, for the quantitative variables, differences between groups were assessed using one-way analysis of variance (ANOVA) and Tukey *post hoc* analysis was used to adjust for multiple comparisons. Differences in qualitative variables between the study groups were explored using the χ^2 test. Statistical significance was established at $P \le 0.05$.

The potential predictive value of all the study variables was evaluated using receiver operating characteristic (ROC) curves and their associated areas under the curve with 95% confidence intervals (CI). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with 95% CI were also calculated for the best cutoff points of the study variables that best identified underlying COPD among all LC patients (superoxide anion, protein carbonyls and nitrotyrosine in blood). Furthermore, multivariate logistic

regression analyses were performed with categorized variables of the biological parameters protein carbonylation and nitrotyrosine to estimate the variables potentially related to COPD among all LC patients.

RESULTS

Clinical Characteristics

Tables 1 and 2 illustrate all clinical and functional variables of all control subjects, LC-COPD and LC patients recruited in the study (N = 97) and of those undergoing thoracotomy from which lung specimens were obtained (N = 35), respectively. As expected, the number of LC-COPD patients was greater than that of LC or controls (three-fold), and male patients were predominant over females in LC and LC-COPD patients (Tables 1 and 2). No significant differences were observed in age or body composition between LC or LC-COPD patients and control subjects, as well as between LC-COPD and LC patients (Tables 1 and 2). The proportions of current smokers and ex-smokers were significantly higher in both LC and LC-COPD patients compared with controls, and also between LC-COPD as a group and LC patients (Table 1). Furthermore, the number of pack-years was significantly greater in LC-COPD as a group as compared to control subjects and LC patients, and also in heavy smokers as compared to the moderate subgroup of patients (Tables 1 and 2). As expected, according to the patients' classification, lung function parameters were significantly reduced in LC-COPD patients compared with control individuals and LC patients (Tables 1 and 2). No significant differences were observed in lung function parameters between heavy and moderate smokers in LC-COPD group (Tables 1 and 2). Furthermore, no significant differences were found in TNM staging or histological diagnosis between the study groups of patients (LC and LC-COPD) (Tables 1 and 2). Blood total leucocytes and neutrophils showed greater levels

in LC and LC-COPD patients than in the controls as well as in LC-COPD compared with LC patients (Tables 1 and 2). Moreover, blood total lymphocyte counts were higher in LC-COPD heavy smokers than in moderate smokers (Table 1). Levels of C-reactive protein (CRP) were increased in LC and LC-COPD patients compared with those in the controls, and in LC-COPD compared with LC patients (Tables 1 and 2). No differences were observed in albumin, total proteins, fibrinogen, globular sedimentation velocity (GSV) or ceruloplasmin among the study groups (Tables 1 and 2). Previous body weight loss was more prominent in LC and LC-COPD patients compared with control subjects, and it was also greater in patients with LC-COPD, especially in the heavy smokers, than in LC patients (Table 1). However, no significant differences in body weight were observed in patients undergoing thoracotomy (Table 2).

Molecular Markers of Redox Balance and Oxidized Proteins

Lung specimens. No differences were found in levels of reactive carbonyls between tumor and nontumor lung specimens in any of the study groups of patients, even when LC-COPD patients were subdivided into heavy and moderate smokers (Figures 1A, B). MDA–protein adduct levels were significantly greater in both tumor and nontumor lung specimens in LC-COPD patients than in LC, and smoking exposure did not have any significant influence on these results (Figures 1C, D). Structural and functional proteins were carbonylated in the lung of LC and LC-COPD in both tumor and nontumor lesions (Table 3). Other nonparenchymal proteins were also oxidized in the same specimens (Table 3). Protein tyrosine nitration levels were significantly increased in the tumor lesions compared with nontumor lungs in LC patients (Figures 2A, B). Furthermore, protein tyrosine nitration was also significantly higher in nontumor lungs of LC-COPD

patients compared with nontumor specimens of LC patients (Figures 2A, B). Additionally, no significant differences were found in LC-COPD heavy smokers compared with moderate smokers (Figures 2A, B).

Protein levels of the antioxidant enzyme SOD2 were significantly increased in the tumor lesions compared with the nontumor lungs in both LC and LC-COPD patients (Figures 3A, B). In the *post hoc* analysis of the latter group, heavy smokers were those exhibiting greater levels in lung tumors versus nontumor specimens (Figures 3A, B). SOD1 protein levels did not differ between tumor and nontumor lungs in either LC or LC-COPD patients (Figures 3C, D). However, SOD1 protein levels were significantly higher in both nontumor and tumor samples in LC-COPD patients than in LC group (Figures 3C, D). No differences were observed between heavy and moderate smokers in the LC-COPD group of patients (Figures 3C, D). Catalase protein content was significantly decreased in the tumors compared with nontumor specimens in both LC and LC-COPD groups (Figures 3E, F). Moreover, catalase protein levels were also reduced in the tumors compared with nontumor specimens in both moderate and heavy smokers LC-COPD patients in the *post hoc* analysis (Figures 3E, F).

Blood. Superoxide anion, protein carbonylation and nitration levels were significantly increased in the blood of both LC and LC-COPD patients compared with control subjects (Figures 4A–C, respectively). Moreover, levels of these three oxidative stress markers were also significantly greater in the blood of LC-COPD patients than in LC (Figures 4A–C, respectively). No significant differences were observed in GSSG/GSH levels among the study groups (Figure 4D). Furthermore, no differences between heavy and moderate smokers LC-COPD patients were observed in the *post hoc* analysis of the systemic markers of oxidative stress analyzed in the study (Figures 4A–D, respectively).

Table 1. Clinical and functional characteristics of all the study patients.[a,b]

Anthropometric variables	Controls, N = 17	LC, N = 21	LC-COPD, N = 59	LC-COPD, moderate smokers, N = 24	LC-COPD, heavy smokers, N = 35
Age, years	61 (13)	63 (12)	67 (10)	69 (10)	66 (10)
Male/female, N	8/9	14/7	57/ 2	23/1	34/1
BMI, kg/m^2	26 (3)	27 (4)	25 (4)	25 (4)	26 (5)
Smoking history					
Current: N, %	3, 18	11, 52[¶¶¶]	36, 61[¶¶¶,**]	11, 46	25, 71[§§]
Ex-smoker: N, %	2, 12	6, 29[¶¶¶]	23, 39[¶¶¶,**]	13, 54	10, 29[§§]
Never smoker: N, %	12, 70	4, 19[¶¶¶]	0, 0[¶¶¶,**]	0, 0	0, 0
Pack-years	35 (19)	51 (23)	64 (22)[¶,*]	45 (8)	77 (18)[§§§]
Lung function testing					
FEV$_1$, % predicted	102 (14)	91 (9)[¶]	58 (13)[¶¶¶,***]	59 (13)	56 (13)
FEV$_1$/FVC, % predicted	80 (6)	76 (6)	61 (8)[¶¶¶,***]	61 (6)	61 (9)
DLco, % predicted	97 (11)	89 (13)	68 (18)[¶¶¶,***]	67 (18)	69 (18)
Kco, % predicted	91 (12)	88 (10)	79 (20) [¶¶,*]	79 (20)	79 (21)
TNM staging					
Stage IA: N, %	NA	5, 24	8, 13	4, 16	4, 11
Stage IB: N, %	NA	1, 5	9, 16	5, 20	4, 11
Stage IIA: N, %	NA	0	5, 8	1, 5	4, 11
Stage IIB: N, %	NA	3, 14	2, 3	1, 5	1, 4
Stage IIIA: N, %	NA	4, 19	10, 18	3, 12	7, 20
Stage IIIB: N, %	NA	4, 19	6, 10	1, 4	5, 14
Stage IV: N, %	NA	4, 19	19, 32	9, 38	10, 29
Histological diagnosis					
Squamous cell carcinoma: N, %	NA	7, 33	23, 39	10, 42	13, 37
Adenocarcinoma: N, %	NA	10, 48	23, 39	9, 37	14, 40
Others: N, %	NA	4, 19	13, 22	5, 21	8, 23
Blood parameters					
Total leucocytes/μL	6 × 10^3 (2.1 × 10^3)	9 × 10^3 (2.1 × 10^3)[¶¶]	8 × 10^3 (2.1 × 10^3)[¶¶]	8 × 10^3 (2.2 × 10^3)	9 × 10^3 (1.9 × 10^3)
Total neutrophils/μL	4 × 10^3 (1.9 × 10^3)	6 × 10^3 (2.2 × 10^3)[¶¶]	6 × 10^3 (1.7 × 10^3)[¶¶]	6 × 10^3 (1.7 × 10^3)	6 × 10^3 (1.7 × 10^3)
Total lymphocytes/μL	2 × 10^3 (559)	2 × 10^3 (622)	2 × 10^3 (664)	1 × 10^3 (614)	2 × 10^3 (660)[§]
Albumin, g/dL	4.0 (0.4)	4.3 (0.3)	4.1 (0.4)	4.1 (0.4)	4 (0.4)
Total proteins, g/dL	7.2 (0.5)	7.5 (0.5)	7.4 (0.5)	7.3 (0.5)	7.4 (0.5)
Fibrinogen, mg/dL	405 (57)	474 (97.6)	445 (148.6)	419 (148.2)	469 (148)
CRP, mg/dL	0.4 (0.3)	1.3 (1.3)[¶]	4.8 (6.5)[¶¶¶,**]	5.75 (6.6)	4.1 (6.3)
GSV, mm/h	16.1 (11.1)	20.1 (9.7)	29.6 (29)	30 (32)	29 (27)
Ceruloplasmin, g/dL	22.8 (4.6)	25.7 (4.8)	30.3 (7.2)	34 (9)	29 (5.8)
Body weight loss, kg					
0: N, %	0, 0	19, 90.4[¶¶¶]	38, 65[¶¶¶,***]	18, 75	20, 57[§§§]
1–4: N, %	0, 0	1, 4.8[¶¶¶]	6, 10[¶¶¶,***]	2, 9	4,11[§§§]
5–8: N, %	1, 100	1, 4.8[¶¶¶]	13, 22[¶¶¶,***]	4, 16	9, 26[§§§]
9–12: N, %	0, 0	0, 0[¶¶¶]	2, 3[¶¶¶,***]	0, 0	2, 6[§§§]

FEV$_1$, forced expiratory volume in the first second; FVC, forced vital capacity; DLco, carbon monoxide transfer; Kco, Krogh transfer factor; NA, not applicable.

[a]Continuous variables are presented as mean (standard deviation), while categorical variables are presented as the number of patients in each group and percentage of the total population.

[b]Statistical analyses and significance: [¶]$p < 0.05$, [¶¶]$p < 0.01$, [¶¶¶]$p < 0.001$ between any of the study patients (LC and LC-COPD as a group) and the controls; [*]$p < 0.05$, [**] $p < 0.01$, [***]$p < 0.001$ between LC-COPD patients as a group and LC patients; [§]$p < 0.05$, [§§]$p < 0.01$, [§§§]$p < 0.001$ between LC-COPD heavy smokers and LC-COPD moderate smokers in the *post hoc* analyses.

Table 2. Clinical and functional characteristics of the study patients undergoing thoracotomy.[a,b]

Anthropometric variables	LC, N = 12	LC-COPD, N = 23	LC-COPD, moderate smokers, N = 8	LC-COPD, heavy smokers, N = 15
Age, years	64 (12)	66 (9)	68 (9)	65 (9)
Male/female, N	8/4	23/0	8/0	15/0
BMI, kg/m^2	27 (3)	26 (5)	25 (5)	25 (5)
Smoking history				
Current smoker: N, %	6, 50	14, 61	3, 37	11, 73
Ex-smoker: N, %	4, 33	9, 39	5, 63	4, 27
Never smoker: N, %	2, 17	0, 0	0, 0	0, 0
Pack-years	41 (14)	60 (18)**	41 (6)	70 (14)[§§§]
Lung function testing				
FEV$_1$, % predicted	86 (7)	60 (12)***	61 (12)	60 (14)
FEV$_1$/FVC, % predicted	74 (3)	61 (7)***	60 (7)	62 (8)
DLco, % predicted	96 (15)	74 (20)**	69 (19)	76 (20)
Kco, % predicted	91 (12)	78 (17)*	73 (11)	80 (20)
TNM staging				
Stage IA: N, %	3, 25	7, 30	3, 38	4, 27
Stage IB: N, %	3, 25	2, 9	1, 13	1, 7
Stage IIA: N, %	0, 0	6, 26	2, 25	4, 27
Stage IIB: N, %	3, 25	2, 9	2, 25	0, 0
Stage IIIA: N, %	3, 25	6, 26	0, 0	6, 39
Stage IIIB: N, %	0, 0	0, 0	0, 0	0, 0
Stage IV: N, %	0, 0	0, 0	0, 0	0, 0
Histological diagnosis				
Squamous cell carcinoma: N, %	3, 25	9, 39	5, 62	4, 27
Adenocarcinoma: N, %	6, 50	13, 57	3, 38	10, 67
Others: N, %	3, 25	1, 4	0, 0	1, 6
Blood parameters				
Total leucocytes/µL	8×10^3 (2.3×10^3)	10×10^3 (2.4×10^3)*	9×10^3 (2.4×10^3)	1×10^4 (2.5×10^3)
Total neutrophils/µL	5×10^3 (2.2×10^3)	7×10^3 (2.6×10^3)*	7×10^3 (2.1×10^3)	7×10^3 (2.8×10^3)
Total lymphocytes/µL	2×10^3 (497)	2×10^3 (527)	2×10^3 (386)	2×10^3 (598)
Albumin, g/dL	4.3 (0.2)	6.1 (9.3)	10.4 (16)	4 (0.5)
Total proteins, g/dL	7.2 (0.6)	7.2 (0.4)	7.1 (0.3)	7.2 (0.5)
Fibrinogen, mg/dL	412 (191)	430 (93)	391 (67)	459 (102)
CRP, mg/dL	1.3 (1)	4.9 (7.4)*	3 (4.4)	5.5 (8.4)
GSV, mm/h	22.4 (12.3)	32.2 (25)	23.4 (22.9)	35.3 (25.8)
Ceruloplasmin, g/dL	25.8 (4)	29.9 (8.5)	24.5 (2.1)	31.2 (9)
Body weight loss, kg				
0: N, %	11, 92	18, 78	6, 74	12, 80
1–4: N, %	1, 8	2, 9	1, 13	1, 7
5–8: N, %	0, 0	3, 13	1, 13	2, 13

FEV$_1$, forced expiratory volume in the first second; FVC, forced vital capacity; DLco, carbon monoxide transfer; Kco, Krogh transfer factor.
[a]Continuous variables are presented as mean (standard deviation), while categorical variables are presented as the number of patients in each group and percentage of the total population.
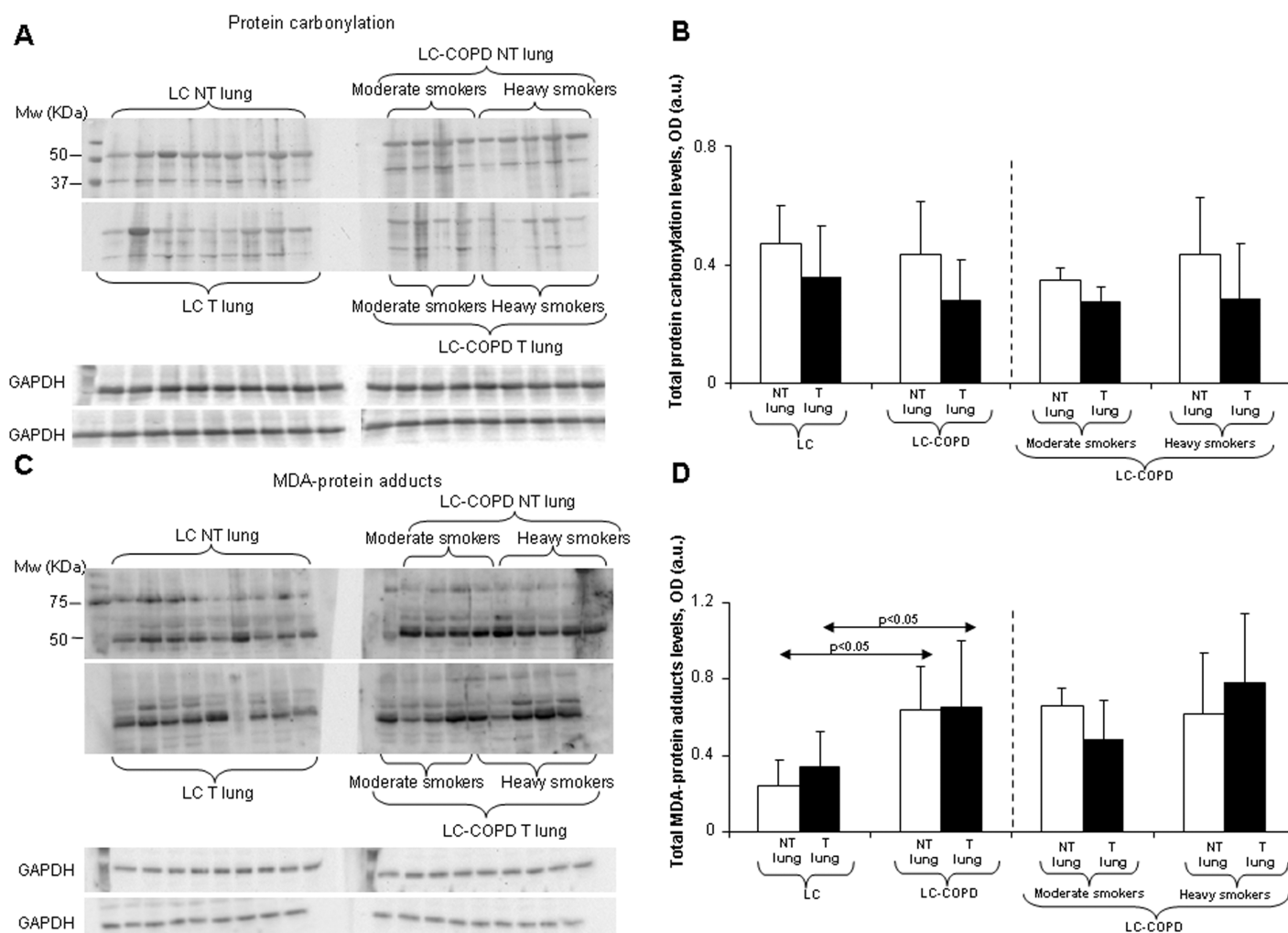[b]Statistical analyses and significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, between LC-COPD patients as a group and LC patients; [§]$p < 0.05$, [§§]$p < 0.01$, [§§§]$p < 0.001$, between LC-COPD heavy smokers and LC-COPD moderate smokers in the *post hoc* analyses. Comparisons of the clinical and physiological variables between LC-COPD as a group and LC patients, as well as between the two groups of LC-COPD patients (moderate and heavy smokers) were assessed using the Student *t* test. Differences between the study groups in the qualitative variables were assessed using the χ^2 test.

Sensitivity and Specificity of Redox Markers

The study variables that best identified underlying COPD among all LC patients were systemic superoxide anion, protein carbonylation and nitration levels. The sensitivity and specificity of these variables were relatively high above the cutoff values at 3.56 nmol/L, 0.56 nmol/mg and 42.88 nmol/L, respectively, among all LC patients in the study (Table 4A).

Figure 1. (A) Immunoblots of total protein carbonylation in the lung tumor (T) and nontumor (NT) of LC and LC-COPD patients. (B) Mean values and standard deviation (optical densities (OD) expressed in arbitrary units (a.u.)) of total protein carbonylation levels in lung did not significantly differ between tumor (T) and nontumor (NT) lung samples and between any of the study groups of patients. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. (C) Immunoblots of MDA–protein adducts in the lung tumor (T) and nontumor (NT) of LC and LC-COPD patients. (D) Mean values and standard deviation (OD expressed in a.u.) of total MDA–protein adduct levels in the lung were greater in LC-COPD as a group in both tumor (T) and nontumor (NT) lung samples compared with LC patients. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. MW, molecular weights; KDa, kilodaltons.

Furthermore, a multivariate model, with a high predictive value of the study variables that best identified underlying COPD among all LC patients was obtained when protein carbonylation and nitrotyrosine were analyzed together. The equation representing the multivariate model is shown in Table 4B.

DISCUSSION

The reported findings confirm the study hypothesis to a great extent. Protein oxidation levels, as measured by MDA–protein adducts, and SOD1 protein content were significantly increased in the lung tumor specimens of LC-COPD patients as compared with LC patients. Protein content of SOD2

was increased while catalase levels were decreased in lung tumors of both LC-COPD and LC patients. Additionally, in the subgroup of LC-COPD heavy smokers compared with moderate smokers, a rise in SOD2 levels together with a decrease in catalase content were observed in the lung tumors. Moreover, in the LC-COPD patients

Table 3. Identified carbonylated proteins in the lung specimens from the study patients.[a]

Identified carbonylated protein	Accession no.	Mass	MASCOT score
Lung tumor lesions			
α-Enolase, LC-COPD and LC	ENOA_HUMAN	47.48	163
Protein DJ-1, LC-COPD and LC	PARK7_HUMAN	19.9	384
α₂-Macroglobulin, LC-COPD and LC	A2MG_HUMAN	163.2	99
Electron transfer flavoprotein subunit alpha, mitochondrial, LC-COPD	ETFA_HUMAN	35.1	490
Triosephosphate isomerase, LC-COPD	TPIS_HUMAN	26.7	418
Proteasome activator complex subunit 1, LC-COPD	PSME1_HUMAN	28.7	165
Thymidine phosphorylase, LC-COPD	TYPH_HUMAN	50.3	80
26S protease regulatory subunit 6A, LC-COPD	PRS6A_HUMAN	49.20	642
60 kDa heat shock protein, mitochondrial, LC-COPD	CH60_HUMAN	61.2	80
Actin, cytoplasmic 1, LC-COPD	ACTB_HUMAN	41.7	90
5′-AMP-activated protein kinase subunit γ-2, LC-COPD	AAKG2_HUMAN	63.2	63
Cofilin-1, LC	COF1_HUMAN	18.5	57
Transthyretin, LC	TTHY_HUMAN	15.9	405
Ferritin light chain, LC	FRIL_HUMAN	20.06	92
Nontumor lung specimens			
Protein DJ-1, LC-COPD and LC	PARK7_HUMAN	19.9	309
Transthyretin, LC-COPD and LC	TTHY_HUMAN	15.9	68
Vimentin, LC-COPD and LC	VIME_HUMAN	53.6	172
Actin, cytoplasmic, LC-COPD and LC	ACTB_HUMAN	41.7	160
Serotransferrin, LC-COPD and LC	TRFE_HUMAN	79.29	169
Triosephosphate isomerase, LC-COPD and LC	TPIS_HUMAN	26.7	82
Serum amyloid P-component, LC-COPD and LC	SAMP_HUMAN	25.5	123
Glutathione S-transferase, LC-COPD and LC	GSTP1_HUMAN	23.6	80
ATP synthase subunit beta, LC-COPD and LC	ATPB_HUMAN	56.5	118
Carbonic anhydrase 1, LC-COPD and LC	CAH1_HUMAN	28.9	57
Apolipoprotein A-I, LC-COPD and LC	APOA1_HUMAN	30.8	83
Actin, cytoplasmic 1, LC-COPD and LC	ACTB_HUMAN	41.7	59
Hemoglobin subunit beta, LC-COPD and LC	HBB_HUMAN	16.0	62

[a]Database: SwissProt 56.0. Protein scores greater than 56 are significant ($p < 0.05$).

compared with LC patients, systemic levels of species superoxide anion, protein carbonylation and nitration levels were significantly greater while levels of the antioxidant glutathione were decreased. Importantly, no significant differences between moderate and heavy smokers were seen in any of the markers analyzed in the blood samples when LC-COPD patients were further subdivided according to their smoking history. These findings suggest that underlying COPD itself rather than chronic cigarette smoke exposure accounts for the differential pattern of redox balance expression detected in the lung tumors and especially in the blood compartment of LC-COPD patients with respect to LC group. Importantly, as expected from previous epidemiologic studies (1,2,5,37–39), it is also confirmed from this series of patients that the number of LC with underlying COPD is greater than that of patients with LC without any underlying respiratory condition.

To our knowledge, this is the first investigation trying to explore the expression profile of oxidative stress markers in lung and blood specimens in LC patients with and without a chronic respiratory disease of very high prevalence such as COPD. Moreover, the expression of the redox markers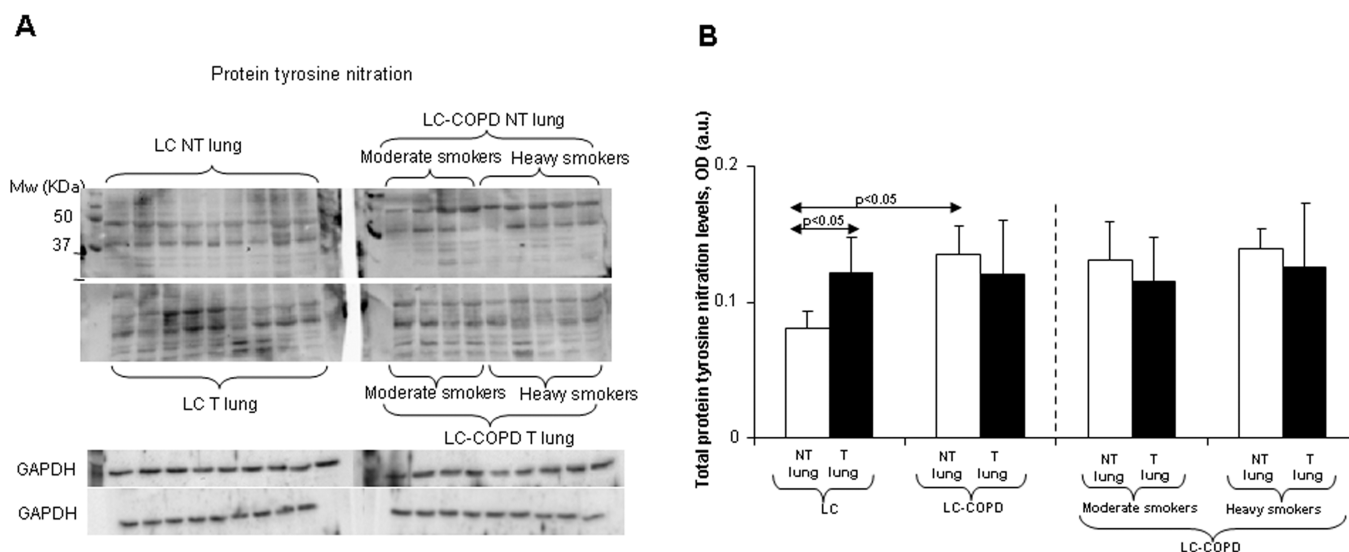 was also analyzed in the surrounding nontumor parenchyma of the same patients. This approach was taken to better characterize the expression of the target redox events in the actual tumor lesions of each group of patients. Several well-validated markers of oxidative stress and antioxidant systems have been analyzed in the biological specimens obtained from the patients.

Interestingly, in a previous study (23), protein carbonylation levels, as measured by MDA–protein adducts, were also increased in the normal epithelium of patients with LC with and without COPD. Other investigations have also demonstrated a rise in different redox markers in lung tissues or blood of patients with LC (9,10,23,25,26), thus the results reported herein are coherent with existing knowledge in the field. Importantly, in the current study, MDA–protein adduct levels were significantly greater in the lung tumor and nontumor parenchyma of patients with LC-COPD than in the corresponding lung specimens of LC patients with no COPD. Hence, COPD per se, independent of smoking history, induced an increase in protein oxidation levels that may have contributed to the development of LC in the patients.

As shown in Table 3, a variety of structural and functional proteins were carbonylated in the lung tumors and nontumor parenchyma in both groups of patients. Previous investigations have reported that proteins such as cofilin (24), vimentin (40) and α₁-antitrypsin (41,42) were also carbonylated in the lungs in other experimental models. In addition, the same proteins were also more oxidized in the normal epithelium of the airways distant to the neoplasm in patients with LC (23). Importantly, the function of these proteins was altered as a result of the oxidative posttranslational modifications, which may have led to lung destruction and emphysema (41,42).

Several proteins including vimentin, actin and carbonic anhydrase-1 were identified to be tyrosine nitrated in the lung tumors of patients in a previous

Figure 2. (A) Immunoblots of total protein tyrosine nitration in the lung tumor (T) and nontumor (NT) of LC and LC-COPD patients. (B) Mean values and standard deviation (optical densities (OD) expressed in arbitrary units (a.u.)) of total protein tyrosine nitration levels were significantly increased in the tumor (T) lesions compared with nontumor (NT) lungs in LC patients. Total protein tyrosine nitration was also significantly higher in nontumor (NT) lung specimens of LC-COPD as a group compared with nontumor (NT) parenchyma of LC patients. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. MW, molecular weights; KDa, kilodaltons.

study (9). Whether patients might also have had underlying COPD was not analyzed in that investigation (9). In the present study, protein tyrosine nitration levels were significantly greater in the cancer lesions compared with the non-tumor parenchyma of the LC group but not LC-COPD patients. Nonetheless, protein nitration levels were higher in the nontumor lung of LC-COPD than LC patients. These findings may account for the lack of a rise in protein tyrosine nitration levels in tumors compared with nontumor specimens in LC-COPD patients. Indeed, protein nitration levels were similar in the tumors of both groups of patients. These findings suggest that COPD per se induces a rise in protein tyrosine nitration levels in the lungs of the patients, and these levels may be involved in the structural alterations observed in emphysema but are not necessarily involved in lung carcinogenesis. Future studies should elucidate whether nitrosative stress contributes to lung carcinogenesis in patients.
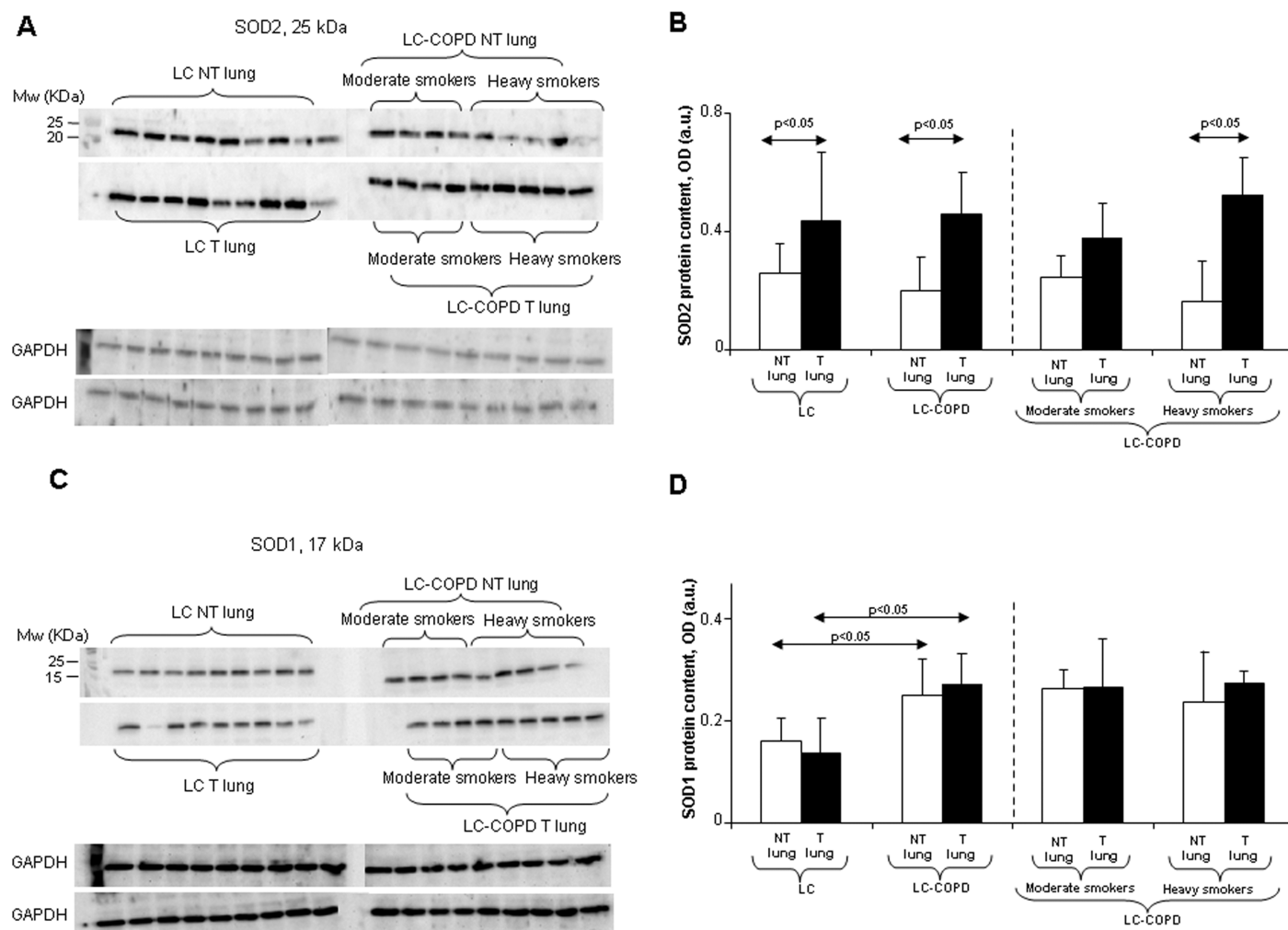
Importantly, a significant rise in mitochondrial SOD2 protein content was observed in the tumors compared with nontumor parenchyma in both groups of patients. Additionally, when patients with LC-COPD were analyzed separately on the basis of their smoking history, the heaviest smokers were those exhibiting the actual increase in SOD2 levels in the lung tumors. These findings suggest that chronic exposure to cigarette smoke may further enhance the rise in SOD2 content observed in the tumors of those patients. Taken together, it would be plausible to conclude that SOD2 may be a key survival mechanism for the cancer cells to proliferate in the tumors. In fact, a recent study (24) demonstrated that inhibition of SOD activity reduced tumor burden in mice and promoted cell death in several lines of NSCLC lines of cells. Furthermore, SOD2 was shown to favor cell migration and invasiveness of tumors in other investigations (43,44). More recently, mRNA and protein levels of SOD2 were also significantly increased in lung tumors and other cancer types in patients (25). The authors concluded that SOD2 may even be considered as a biomarker for cancer progression from tumor growth to metastasis.

In summary, SOD2 overexpression seems to be involved in tumorigenesis in patients with LC, particularly in those with COPD who have a history of heavy smoking, as shown in the current study. Hence, drugs targeted to block SOD2 activity could be of interest in clinical settings.

Protein content of SOD1 did not differ between tumor and nontumor parenchyma in any of the study groups. Nonetheless, levels of SOD1 were, in general, much greater in the patients with underlying COPD in both tumor and nontumor lung specimens than in LC patients with no COPD. Interestingly, cigarette smoke did not influence SOD1 levels in any of the LC-COPD patients. In view of these findings, SOD1 seems to participate in antioxidant defense of the lungs in COPD patients regardless of the presence of LC rather than in carcinogenesis.

The antioxidant enzyme catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen, thus protecting the cells from oxidative damage. In the present study, catalase protein levels were reduced significantly
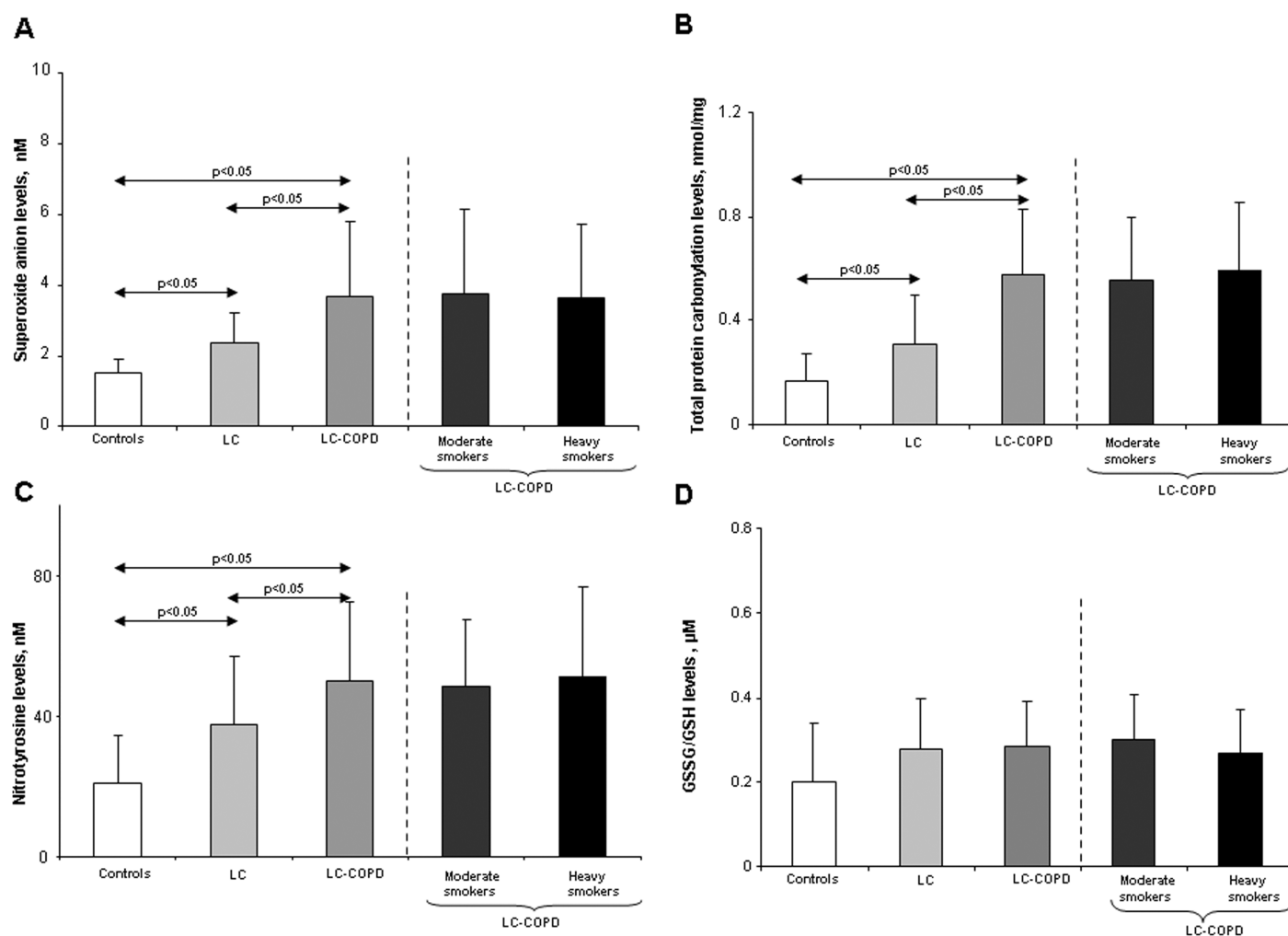
Figure 3. (A) Immunoblots of SOD2 in the lung tumor (T) and nontumor (NT) of LC and LC-COPD patients. SOD2: superoxide dismutase 2; LC: lung cancer; COPD: chronic obstructive pulmonary disease; MW: molecular weights; KDa: kilodaltons. (B) Mean values and standard deviation (optical densities (OD) expressed in arbitrary units (a.u.)) of SOD2 protein content were significantly greater in the tumor lesions (T) compared with nontumor (NT) lungs in LC patients and LC-COPD as a group as well as in LC-COPD heavy smokers. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. (C) Immunoblots of SOD1 in the lung tumor (T) and nontumor (NT) of LC and LC-COPD patients. (D) Mean values and standard deviation (OD expressed in a.u.) of SOD1 protein content were significantly increased in both tumor (T) and nontumor (NT) lung specimens of LC-COPD patients compared with LC patients. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. (E) Immunoblots of catalase in the lung tumor (T) and nontumor (NT) of LC and LC-COPD patients. (F) Mean values and standard deviation (OD expressed in a.u.) of catalase protein content was significantly reduced in the tumor lesions (T) compared with nontumor (NT) lungs in both LC-COPD and LC patients, as well as in LC-COPD heavy smokers. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. SOD2, superoxide dismutase 2; MW, molecular weights; KDa, kilodaltons.

in tumors compared with nontumor parenchyma in both groups of patients. Furthermore, in LC-COPD patients, the heaviest smokers were, indeed, those showing the decrease in catalase levels in the tumor lesions compared with the nontumor lungs. These findings are consistent with that previously reported in the literature in other models such as the contribution of catalase deficiency to mammary tumorigenesis in rodents (45) and cancer in patients (25). Collectively, it would be possible to conclude that catalase depletion seems to be involved in cancer development, especially in LC-COPD patients who are also heavy smokers.

Superoxide anion and protein carbonylation and nitration levels were significantly increased in the blood of both groups of patients as compared

Figure 4. (A) Mean values and standard deviation of total blood superoxide anion measured by lucigenin-derived chemiluminescence (20 micromol/L) were significantly higher in both LC and LC-COPD compared with controls. Blood superoxide anion levels were also greater in LC-COPD compared with LC patients. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. (B) Mean values and standard deviation of total blood protein carbonylation as measured by ELISA (nmol/mg) were significantly higher in both LC and LC-COPD patients compared with controls. Total blood protein carbonylation levels were also increased in LC-COPD compared with LC patients. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. (C) Mean values and standard deviation of blood protein tyrosine nitration as measured by ELISA (nmol/L) were significantly greater in LC and LC-COPD patients compared with controls. Blood nitrotyrosine levels were also higher in LC-COPD compared with LC patients. Significant p values ($p < 0.05$) that correspond to comparisons between the different groups of patient and types of samples (blood and lung specimens) are indicated using an arrow in each case. The absence of any statistical sign means that no significant differences were found between groups for the different study comparisons. (D) Mean values and standard deviation of GSSG/GSH as measured by ELISA (μmol/L) in the blood did not significantly differ among the study groups of patients. The absence of any statistical sign means that no significantly differences were found between groups for the different study comparisons.

to the control subjects, as well as in patients with LC-COPD as compared to LC patients. Moreover, when LC-COPD patients were further subdivided *post hoc* according to their smoking history, no differences in the levels of these three

oxidative stress markers were observed between them, suggesting that the rise in protein oxidation and nitration along with superoxide anion was rather associated with underlying COPD. These findings are in agreement with previous

studies in which several markers of oxidative stress were also shown to be increased in patients with only COPD (23,35,46). Moreover, in other studies (10,15,23), patients with LC also exhibited a rise in systemic protein oxidation

Table 4A. Markers with predictive value in blood for LC-COPD with respect to LC patients.

	ROC[a] area (95% CI)	Best cutoff	Sensitivity[b] (%) (95% CI)	Specificity[b] (%) (95%CI)	NPV[b] (%) (95% CI)	PPV[b] (%) (95% CI)
Superoxide anion	0.655 (0.517–0.793)	3.56 nmol/L	69.2 (53.6–81.4)	100 (84.5–100)	43.8 (30.7–57.7)	100 (75.8–100)
Protein carbonylation	0.797 (0.683–0.991)	0.56 nmol/mg	54.2 (41.7–66.3)	94.4 (74.2–99.0)	38.6 (25.7–53.4)	97.0 (84.7–99.5)
Nitrotyrosine	0.682 (0.542–0.821)	42.88 nmol/L	65.2 (50.8–77.3)	70.0 (48.1–85.5)	46.7 (30.2–63.9)	83.3 (68.1–92.1)

[a]ROC curve area in blood for LC-COPD with respect to LC patients. [b]Sensitivity, specificity, PPV and NPV, all expressed as percentage, with 95% CI, were calculated in all patients with LC-COPD (N = 59) for the best cutoff points of the study variables with respect to LC patients without COPD (N = 21).

Table 4B. Equation of the multivariate model with a high predictive value for the factors related to COPD in LC patients.[a]

$$P_i = 1/1 + e^{-(-1.162 + (2.801 \cdot \text{protein carbonylation}) + (1.796 \cdot \text{nitrotyrosine}))}$$

[a]Excluding patients with superoxide anion >3.56 nmol/L (because all of them are always LC-COPD, specificity 100%), a multivariate model with a high predictive value for the factors related to COPD in LC patients was obtained when protein carbonylation and nitrotyrosine were analyzed together. P_i is the probability of underlying COPD in patients with LC, e is the Euler's number, protein carbonylation = 0, if levels of protein carbonylation are below 0.56 nmol/mg, or protein carbonylation = 1 when levels are equal or above 0.56 nmol/mg and nitrotyrosine = 0, if levels of nitrotyrosine are equal or below 42.88 nmol/L or nitrotyrosine = 1 if levels are above 42.88 nmol/L.

and nitration levels together with the identification of the corresponding oxidized and nitrated plasma proteins (10,23). However, whether LC patients also had concomitant COPD was not explored in those studies (10,23). Consistent with the findings in the present study, LC patients with underlying COPD in a previous report (23) also showed a greater increase in systemic protein oxidation and superoxide anion than patients with only LC.

Importantly, glutathione transferases, which catalyze the conjugation of electrophilic compounds to the powerful antioxidant glutathione, are key in the detoxification of carcinogens and polycyclic aromatic hydrocarbons (47). Several mutations or deletions of those enzymes have been shown to increase susceptibility to develop cancers in patients (48,49). Recently, a meta-analysis has put forward that a specific genotype of glutathione transferases increase the risk to develop LC in Asian populations (49). In the current investigation, however, levels of oxidized glutathione as expressed by the ratio of GSSG to GSH did not significantly differ between the study groups. In addition, smoking history did not influence the results encountered in LC-COPD patients as no differences were detected between moderate and heavy smokers in this group. Taken together, these findings suggest that oxidative damage through protein oxidation and nitration and superoxide anion formation may contribute to a greater risk of lung carcinogenesis, especially in patients with underlying COPD.

In line with this, a predictive model of the study variables that best predicted the presence of underlying COPD among all LC patients was obtained in the current investigation. In this regard, systemic levels of superoxide anion, protein carbonyls and nitrotyrosine above a specific threshold value in each case (Table 4A) were predictive of underlying COPD among all patients with LC in the study. Furthermore, as a result of a multivariate model analysis, an equation was obtained in which blood superoxide anion and protein carbonyl levels showed a high predictive value for underlying COPD among all LC patients (Table 4B). Despite that, these results should be taken cautiously, as it would also be possible to conclude that underlying COPD may predispose patients to a higher risk of LC development via increased levels of oxidative events, especially of the blood compartment. In fact, increased oxidative stress was shown to be a powerful carcinogenic mechanism in several models (50,51). Future investigations should focus on the elucidation of the specific cellular pathways that are damaged oxidatively in patients with LC and underlying COPD as this will contribute to the identification of specific markers that may help predict LC development in COPD. Prospective screening studies, in which COPD patients should be followed up for several years will help identify the biomarkers with the greatest predictive value for LC in this population.

Study Limitations

A first limitation in the study refers to the relatively lower number of lung specimens analyzed in the study compared with the number of blood samples. Nonetheless, for ethical reasons, tumor and nontumor lung specimens could only be obtained from patients undergoing thoracotomy for the treatment of their lung neoplasm from the established cohort of patients that participated in this investigation, from whom blood samples had been obtained in all cases.

A second limitation is the relatively low numbers of females in both groups of patients. This is because the prevalence of LC in female patients, especially in patients with underlying COPD is still very low in our geographical region (6,52).

CONCLUSION

A differential expression profile of oxidative stress markers has been identified in the lung tumors and blood compartments in patients bearing a chronic respiratory condition as compared to those without it. Modifications in protein content of antioxidants such as SOD2 and catalase may be regarded as important biomarkers for LC progression in the patients, especially in heavy smokers with concomitant COPD. Systemic levels of superoxide anion, protein carbonylation and nitration exhibited a predictive value for underlying COPD among all patients with LC. These findings suggest that redox imbalance, especially that encountered in blood, may predispose patients with chronic respiratory conditions to a higher risk for LC.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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