Widespread Expression of Thioredoxin and Thioredoxin Reductase in Non-Small Cell Lung Carcinoma

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ABSTRACT

We investigated the expression of thioredoxin (Trx) and thioredoxin reductase (TrxR) in 89 non-small cell lung carcinomas. Additionally, immortalized human bronchial epithelial cells (BEAS 2B) and four human lung carcinoma cell lines (A549, SK-MES-1, CALU-6, and A427) were studied by Western blot and reverse transcription-PCR for the synthesis of Trx and TrxR protein and mRNA expression in vitro. The histological samples were also studied for immunohistochemical p53 and proliferating cell nuclear antigen expression and apoptosis. In non-neoplastic lung, Trx and TrxR expression was seen in bronchial epithelial cells and alveolar macrophages, metaplastic alveolar epithelial cells, and chondrocytes of the bronchus. In non-small cell lung carcinomas, there was a widespread expression of Trx and TrxR with only three and eight cases negative, respectively. Trx and TrxR expression was located in both cytoplasmic and nuclear compartments of the cells. There was a statistical association between cytoplasmic and nuclear Trx or TrxR expression. Grade I-II tumors showed stronger cytoplasmic and nuclear Trx and TrxR immunoreactivity than grade III tumors. No association was found between p53 and proliferating cell nuclear antigen expression and Trx or TrxR immunoreactivity. However, apoptosis was inversely associated with nuclear Trx and TrxR positivity. In the cell lines studied, both non-neoplastic BEAS 2B cells and all of the carcinoma cell lines expressed Trx and TrxR proteins and mRNA. The results show that these redox-regulating proteins are highly expressed in lung carcinomas taking part in activation of transcriptional factors and regulation of apoptosis in non-small cell lung carcinoma. In high-grade tumors, Trx and TrxR expression is diminished, suggesting loss of redox regulation in tumors with low differentiation.

INTRODUCTION

Lung cancer is still one of the leading causes of cancer death in Western countries. Although its incidence has been declining, it still ranks second and fifth in the cancer death of men and women in Finland (Finnish Cancer Registry 1995). The main reason for the development of lung cancer is smoking, but other factors, such as pollution, radiation, and asbestos exposure, also play a role (1–3). The prognosis of lung cancer is dependent on the stage of the disease and the histology of the tumor (4). There are also several biological factors that may influence the prognosis, such as p53 expression, cell proliferation, and apoptosis (5, 6). All of these factors have been shown to be influenced also by the redox state of the cells. The redox state may also influence the resistance of the cancer cells to chemotherapy, thus affecting the prognosis of the patients (7, 8).

One important family of proteins affecting the redox state of the cells is the Trx1-TrxR system (9). Trx is a small Mr 11,500 disulfide-reducing enzyme with a redox-active Cys-Gly-Pro-Cys active site (10). It has been shown to regulate p53 expression and facilitate p53-dependent induction of p21 (11). It regulates the expression of transcriptional factors such as TF111C, nuclear factor-κB, Jun/Fos, and AP-1 through redox control of these proteins, thus influencing their binding to DNA (11–14). Human Trx is identical to adult T-cell leukemia-derived factor, which is secreted by human T-cell lymphotropic virus-1-transformed lymphocytes and which has growth-promoting effects on transformed cells (15, 16). Consequently, Trx also promotes cell proliferation, and in transfection experiments, it has been shown to increase the growth rate and colony formation of nonmalignant and malignant cells (17). In addition, Trx increases oxidant and drug resistance of various cells, and it may be up-regulated during drug exposure and be associated at least with cisplatin resistance (8).

The function of Trx as a disulfide reductase in mammalian cells is generally dependent upon the activity of the selenoenzyme TrxR (9, 18). These enzymes catalyze the NADPH-dependent reduction of the active disulfide site of Trx to a dithiol. In humans, there are three known isoforms of human TrxRs, namely TR1, TR2, and TR3 (19). All of the human TrxRs have the conserved COOH-terminal sequence Gly-Cys-

³ The abbreviations used are: Trx, thioredoxin; TrxR, Trx reductase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HPF, high power field; RT-PCR, reverse transcription-PCR.

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tumors, their expression was correlated also with clinical data, influence of these proteins on the biological behavior of the optosis, as determined by the TUNEL method. To study the cells, as determined by PCNA immunohistochemistry and ap-

lung carcinoma samples. The expression of the proteins was compared in any tumor. There is one recent study (24) showing that Trx is up-regulated in gastric carcinomas. In that study (24), Trx was associated with increased proliferation and decreased apoptosis. Contradictory findings have also been observed because, at least in hepatoma cells, high Trx expression was associated with decreased cell proliferation (25). A functional Trx system requires active TrxR in the cells, but as to our knowledge no immunohistochemical studies have been conducted on TrxR in malignant tumors; neither has Trx and TrxR expression been compared in any tumor.

This study was undertaken to investigate the immunohis-
tochemical expression of Trx and TrxR in 89 non-small cell lung carcinoma samples. The expression of the proteins was correlated with p53 expression and proliferation of the tumor cells, as determined by PCNA immunohistochemistry and apoptosis, as determined by the TUNEL method. To study the influence of these proteins on the biological behavior of the tumors, their expression was correlated also with clinical data, such as survival of the patients.

**MATERIALS AND METHODS**

**Tissue Specimens.** Samples (n = 89) of non-small cell lung carcinoma were retrieved from the files of the Department of Pathology, Oulu University Hospital between 1978 and 1991. All of the material had been fixed in 10% buffered formalin and embedded in paraffin. The diagnosis of all of the cases was based on standard H&E staining complemented with special immunohistochemical stains when necessary. The diagnosis was made according to the WHO classification of lung tumors (4). There were 57 squamous cell carcinomas, 27 adenocarcinomas, 4 bronchioloulveolar carcinomas, and 1 large cell carcinoma in the material. Of a total of 81 graded tumors, 13 were of grade I, 39 of grade II, and 29 of grade III. There were seven females and 82 males in the study.

**Cultured Cells.** Immortalized human bronchial epithelial cells, BEAS 2B, were obtained from the National Cancer Institute, Laboratory of Human Carcinogenesis (Dr. C. Harris, Bethesda, MD). Human lung carcinoma A549, SK-MES-1, CALU-6, and A427 cells were obtained from American Type Culture Collection (Rockville, MD). The BEAS 2B cells were grown on uncoated plastic flasks (Nalge Nunc International, Roskilde, Denmark) in bronchial epithelial cell growth medium (Clonetics) at 37°C in a 5% CO₂ atmosphere. Lung carcinoma cells were grown in α-MEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in similar conditions as described for BEAS 2B cells. Mesothelioma cells were used for the investigation of Trx and TrxR expression in isolated mitochondria. For this purpose, M14K mesothelioma cells (26) were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum and 0.03% glutamine.

**Immunohistochemistry for Trx and TrxR.** Four-µm thick sections were cut from a representative paraffin block. The sections were first deparaffinized in xylene and rehydrated in descending ethanol series. To enhance immunoreactivity, the sections were incubated in 10% citrate buffer (pH 6.0) and boiled in a microwave oven for 2 min at 850 W and after that for 8 min at 350 W. Endogenous peroxidase activity was eliminated by incubation in 0.1% hydrogen peroxide in absolute methanol for 10 min. After incubation with the affinity-purified goat polyclonal human Trx antibody with a dilution of 1:200 overnight at 4°C (American Diagnostica, Greenwich, CT), a biotinylated secondary goat antibody was applied (dilution, 1:400), followed by the avidin-biotin-peroxidase complex (all from Dakopatts, Glostrup, Denmark). The color was developed using 3,3’-diaminobenzidine, and the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Replacement of the primary antibody by PBS at pH 7.2 and goat IgG immunoglobulin isotype (Zymed Laboratories, Inc., San Francisco, CA) were used as a negative controls.

The antibody to TrxR was the γ-globulin fraction of a polyclonal rabbit antitrat antibody directed against cytosolic TrxR in rat liver (27) and has been characterized previously (28). It was used with a dilution of 1:1000. The immunostaining procedure was mainly similar to the procedure described above, except that the secondary antibody was a biotinylated antirabbit antibody and that the color was developed with aminoethylcarbazol. The negative controls consisted of PBS at pH 7.2 and rabbit immunoglobulin isotype (Zymed Laboratories, Inc.).

Trx and TrxR immunoreactivities in lung carcinoma biop-
sies were assessed semiquantitatively by grading the staining intensity of the tumor cells and the extent of positive staining of the tumor cells. A combined score was then divided into four groups: negative (−), weak (+), moderate (++) and strong (+++). Two investigators performed the analysis independently, and the interobserver repeatability was good (κ = 0.77 for Trx and κ = 0.57 for TrxR) as measured using the Cohen’s κ statistics, as described earlier (29).

**Immunohistochemistry for p53 and PCNA.** For p53 immunohistochemistry, polyclonal antibody CM1 (dilution, 1:1000; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) and biotinylated rabbit antibody (1:400; Dakopatts) were used. This was followed by the avidin-biotin-peroxidase complex (Dakopatts). Diaminobenzidine was used as chromogen. P53 positive cases were divided into five groups: 0 = no cells positive; 1 = <1% of cells positive; 2 = 1–5% of cells positive; 3 = 6–10% of cells positive; 4 = 11–40% of cells positive; and 5 = >40% of cells positive.

For PCNA immunostaining, the monoclonal antibody PC10 (Dako, Glostrup, Denmark) was used with a dilution of 1:50. The immunostaining procedure was similar to that with
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1% of cells positive; 2.5–5% of cells positive; and 51–75% of cells positive; and 5 1–10% of cells positive; 3 11–50% of cells positive; 4 51–75% of cells positive; and 5 >75% of cells positive.

3'-end Labeling of DNA in Apoptotic Cells. To detect apoptotic cells, in situ labeling of the 3'-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD), as described previously (6). After being deparaffinized and rehydrated in ethanol, the sections were incubated with 20 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. The endogenous peroxidase activity was blocked by incubating the slides in 2% hydrogen peroxide in PBS (pH 7.2). The slides were then treated with terminal transferase enzyme and digoxigenin-labeled nucleotides, after which amidogixigenin-peroxidase solution was applied on the slides. The color was developed with diaminobenzidine, after which the slides were lightly counterstained with hematoxylin. For control purposes, we used tissue sections from hyperplastic lymph nodes showing an increased number of apoptotic B cells within germinal centers and a low number of apoptotic T cells in the interfollicular areas.

Cells were defined as apoptotic if the whole nuclear area of the cell labeled positively. Apoptotic bodies were defined as small positively labeled globular bodies in the cytoplasm of the tumor cells that could be found either singly or in groups. To estimate the apoptotic index (the percentage of apoptotic events in a given area), apoptotic cells and bodies were counted in 10 HPFs, and this figure was divided by the number of tumor cells in the interfollicular areas.

Western Blot for Trx and TrxR. The cell pellets and isolated cell fractions were resuspended in sterile water, mixed with the electrophoresis sample buffer, and boiled for 5 min at 95°C. The protein concentration was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), as described previously (6). After being deparaffinized and rehydrated in ethanol, the sections were incubated with 20 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. The endogenous peroxidase activity was blocked by incubating the slides in 2% hydrogen peroxide in PBS (pH 7.2). The slides were then treated with terminal transferase enzyme and digoxigenin-labeled nucleotides, after which amidogixigenin-peroxidase solution was applied on the slides. The color was developed with diaminobenzidine, after which the slides were lightly counterstained with hematoxylin. For control purposes, we used tissue sections from hyperplastic lymph nodes showing an increased number of apoptotic B cells within germinal centers and a low number of apoptotic T cells in the interfollicular areas.

The proportion of PCNA-positive cells was counted in at least six HPFs. PCNA positivity was classified as follows: 1 = <1% of cells positive; 2 = 1–10% of cells positive; 3 = 11–50% of cells positive; 4 = 51–75% of cells positive; and 5 = >75% of cells positive.

RT-PCR for Trx and cTrxR. Expression of Trx and the cytosolic TrxR mRNAs in the cultured cells was investigated by using RT-PCR. The oligonucleotide primers were designed according to cDNA sequence data published earlier (6). Total cellular RNA was extracted from the cells using a kit for RNA isolation (RNEasy; Qiagen, Hilden, Germany) and the respective antisense primer (20 pmol of the Trx primer and 5 pmol of the cTrxR primer) at 42°C for 45 min in a 20-μl reaction mixture containing 1 unit of RNase inhibitor (5 Prime-3 Prime, Boulder, CO). The cDNA was PCR-amplified in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT) using 1 unit of DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland) and the respective sense primer (20 pmol of the Trx primer and 5 pmol of the cTrxR primer) in a 100-μl reaction volume containing 1.5 mM MgCl₂. The thermal profile involved 35 or 27 cycles (Trx and cTrxR, respectively) of denaturation at 94°C for 50 s, primer annealing at 60°C or 64°C (Trx and cTrxR, respectively) for 50 s, and extension at 72°C for 1 min 30 s. PCR products (Table 1) were electrophoresed in an ethidium bromide-stained 2% agarose gel (SeaKem, Rockland, ME). The sequence of the Trx amplification product was confirmed using dRhodamine Terminator Cycle Sequencing Kit and ABI Prism 377 automatic DNA Sequencer (Perkin-Elmer Corp., Foster City, CA). Negative controls were established by substituting the RNA sample with water in each experiment and by leaving the reverse transcriptase enzyme out of the RT-reaction for each of the samples.

Table 1 Oligonucleotide primers

<table>
<thead>
<tr>
<th>Product</th>
<th>Sense Sequence</th>
<th>cDNA nt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trx bp</td>
<td>5'-TGAAGCAGATCGAGACAGAC-3'</td>
<td>305</td>
</tr>
<tr>
<td>cTrxR bp</td>
<td>5'-TGCTTCCCGACGTTAGCAC-3'</td>
<td>420</td>
</tr>
</tbody>
</table>

* nt, nucleotides; numbering is started from the ATG initiation codon.
supernatant was collected and stored at −80°C for further Western blot analysis. Mitochondrial and cytosolic protein fractions were isolated as described by Yang et al. (33). The detached cells were washed twice with ice-cold PBS and resuspended with five volumes of buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium-EDTA, 1 mM sodium-EGTA, and 1 mM DTT] containing 250 mM sucrose and a mixture of protease inhibitors (Complete; EDTA-free; Boehringer Mannheim). The cells were homogenized with 10 strokes of a teflon homogenizer, and the homogenates were centrifuged once at 750 × g for 10 min at 4°C. The pellets (membrane fraction-including nuclei) were stored at −80°C, and supernatants were centrifuged first at 750 × g for 10 min and then at 10,000 × g at 4°C. The resulting mitochondrial pellets were suspended in buffer A containing 250 mM sucrose and frozen at −80°C. The protein contents of the fractions were determined by Bio-Rad protein assay, and the fractions were processed further as described for Western blotting to detect Trx and TrxR.

**Statistical Analysis.** SPSS (7.5) for Windows (Chicago, IL) was used for statistical analysis. The significance of associations was determined using Fisher’s exact probability test. The survival of the patients in relation to Trx immunoreactivity was assessed by the log rank test. Probability values (P < 0.05) were considered statistically significant.

**RESULTS**

**Trx Immunoreactivity.** The immunoreactivity for Trx was diffuse and cytoplasmic, but also nuclear immunoreactivity could be observed. Generally, the tumor tissue stained homogeneously, but in some cases differences in the staining intensity of various areas could be detected. All of the lung carcinomas (except for three cases) showed positive Trx reactivity (Fig. 1, A and B). All together, 20 cases showed strong positivity, 41 cases showed moderate positivity, and 32 cases showed weak positivity (Table 2). Nuclear staining in lung carcinomas was frequent; 46 cases showed strong positive nuclear staining. There was no significant difference in cytoplasmic or nuclear staining between squamous cell carcinoma and adenocarcinoma.

Of the non-neoplastic tissues obtained from five samples of non-neoplastic lung, bronchial epithelial cells and cells of the mucous and serous glands in the walls of the bronchial ducts also expressed positivity. Interestingly, in bronchial epithelial cells, the staining was often apical, and ciliary structures expressed exceptionally strong immunoreactivity. In bronchial epithelial and mucous glands, nuclear positivity was occasionally detected. Cartilaginous cells in the walls of the bronchi expressed Trx. Positivity was especially seen in the nuclei of the chondrocytes (Fig. 1C). Cytoplasmic positivity was occasionally observed in fibroblasts and endothelial cells. In lung tissue, strong Trx immunoreactivity was seen in alveolar macrophages (Fig. 1D). Pneumocytes were usually negative except in proliferating type II pneumocytes or metaplastic pneumocytes. Lymphocytes and neutrophils were negative.

**TrxR and Its Association with Trx Immunoreactivity.** As with Trx positive, immunoreactivity for TrxR was seen in the

**Table 2**  
Trx expression in non-small cell lung carcinoma

<table>
<thead>
<tr>
<th>Trx</th>
<th>Squamous cell carcinoma</th>
<th>Adenocarcinoma</th>
<th>Others</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or weak Cytoplasmic</td>
<td>22</td>
<td>10</td>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>Nuclear</td>
<td>25</td>
<td>14</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>Strong Cytoplasmic</td>
<td>35</td>
<td>17</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td>Nuclear</td>
<td>32</td>
<td>13</td>
<td>3</td>
<td>48</td>
</tr>
</tbody>
</table>
cytoplasmic and nuclear compartments. Of the carcinomas, 47 cases showed no or weak positivity and 37 cases showed moderate or strong positivity. Only eight cases were negative (Fig. 2, A and B; Table 3). Nuclear positivity was negative or weak in 41 cases and moderate or strong in 43 cases. There was a statistically significant association between cytoplasmic and nuclear TrxR immunoreactivity \( (P = 0.0001; \text{Fisher’s exact test}) \). There was no statistical difference in either cytoplasmic or nuclear positivity of TrxR and squamous cell carcinoma or adenocarcinomas \( (P = 0.20 \text{ and } P = 0.26, \text{respectively}) \). A significant association was found between cytoplasmic Trx and TrxR positivity \( (P = 0.04) \) and between nuclear Trx and TrxR immunoreactivity \( (P = 0.02) \).

In nonmalignant lung tissues from five healthy non-neoplastic lung and from nonmalignant tissue adjacent to tumor areas, positive TrxR expression was seen in bronchial epithelial cells and the epithelium of serous and mucous glands, in alveolar macrophages, and in metaplastic alveolar epithelial cells (Fig. 2, C and D). Normal alveolar epithelium appeared negative. Endothelial cells and a subpopulation of inflammatory lymphocytes were positive. As with Trx, chondrocytes of the bronchial epithelium were also positive for TrxR.

**Table 3**  
<table>
<thead>
<tr>
<th>TrxR</th>
<th>Squamous cell carcinoma</th>
<th>Adenocarcinoma</th>
<th>Others</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or weak</td>
<td>Cytoplasmic</td>
<td>32</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nuclear</td>
<td>25</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>Strong</td>
<td>Cytoplasmic</td>
<td>21</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Nuclear</td>
<td>28</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 4**  
<table>
<thead>
<tr>
<th>Trx immunoreactivity cytoplasmic/nuclear</th>
<th>Grade I and II</th>
<th>Grade III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or weak</td>
<td>16/18</td>
<td>16/20</td>
<td>32/38</td>
</tr>
<tr>
<td>Strong</td>
<td>36/34</td>
<td>13/9</td>
<td>49/43</td>
</tr>
<tr>
<td>Total</td>
<td>52/52</td>
<td>29/29</td>
<td>81/81</td>
</tr>
</tbody>
</table>

\( ^a \text{Cytoplasmic, no or weak vs. strong, } P = 0.028; \text{nuclear, no or weak vs. strong, } P = 0.0029. \)

**Trx and TrxR Immunoreactivity in Association with p53 Expression, PCNA Positivity, Histological Parameters, and Survival of the Patients.** Positive p53 expression was found in 43 cases. Strong PCNA expression was found in 36 cases. The mean apoptotic index in the tumors was \( 1.29 \pm 1.19\% \). The apoptotic index in grade I tumors was \( 0.94 \pm 0.54\% \), in grade II tumors it was \( 1.13 \pm 1.22\% \), and in grade III tumors it was \( 1.59 \pm 1.29\% \). Although grade III tumors had a higher apoptotic index, the difference in the apoptotic index between grade I-II and grade III tumors did not reach statistical significance \( (P = 0.08) \). Trx positivity (overall/nuclear) was not associated with p53 immunoreactivity \( (P = 0.24/0.19) \), PCNA positivity \( (P = 0.64/0.20) \), or survival of the patients \( (P = 0.14/0.96) \). Patients with grade I or II tumors had a higher Trx positivity than patients with grade III tumors did \( (P = 0.02 \text{ for nuclear and } P = 0.036 \text{ for overall positivity by Fisher’s exact test; Table 4}) \). There was a significant inverse association between the extent of nuclear Trx positivity and apoptosis \( (P = 0.028) \) but not with apoptosis and cytoplasmic Trx positivity \( (P = 0.114; \text{Table 5}) \).

As with Trx, cytoplasmic or nuclear TrxR positivity was not associated with p53 immunoreactivity \( (P = 0.22/0.37) \), tumor cell proliferation \( (P = 0.16/0.67) \), or survival of the patients.
patients ($P = 0.82/0.16$). Patients with grade I and II tumors had a stronger TrxR expression than patients with grade III disease did ($P = 0.02$ for cytoplasmic and $P = 0.03$ for nuclear positivity; Table 6). There was a significant inverse association between nuclear TrxR positivity and apoptosis ($P = 0.033$) but not with cytoplasmic TrxR positivity and apoptosis (Table 7).

**Table 5**  TrxR expression and tumor grade

<table>
<thead>
<tr>
<th>TrxR immunoreactivity</th>
<th>Grade I and II</th>
<th>Grade III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or weak</td>
<td>22/25</td>
<td>20/14</td>
<td>42/39</td>
</tr>
<tr>
<td>Strong</td>
<td>27/24</td>
<td>8/14</td>
<td>35/38</td>
</tr>
<tr>
<td>Total</td>
<td>49/49</td>
<td>28/28</td>
<td>77/77</td>
</tr>
</tbody>
</table>

*a Cytoplasmic, no or weak vs. strong, $P = 0.021$; nuclear, no or weak vs. strong, $P = 0.62$.

**Table 6**  Trx expression and apoptosis

<table>
<thead>
<tr>
<th>Trx immunoreactivity</th>
<th>No. of cases</th>
<th>Apoptotic index</th>
<th>$P$</th>
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</thead>
<tbody>
<tr>
<td>No or weak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>34</td>
<td>1.49 ± 1.23%</td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td>40</td>
<td>1.55 ± 0.76%</td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>54</td>
<td>1.07 ± 1.11%</td>
<td>$0.11$</td>
</tr>
<tr>
<td>Nuclear</td>
<td>48</td>
<td>0.97 ± 0.82%</td>
<td>$0.021$</td>
</tr>
</tbody>
</table>

**Table 7**  TrxR expression and apoptosis

<table>
<thead>
<tr>
<th>TrxR immunoreactivity</th>
<th>No. of cases</th>
<th>Apoptotic index</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or weak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>46</td>
<td>1.20 ± 1.14%</td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td>40</td>
<td>1.58 ± 1.46%</td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>37</td>
<td>1.39 ± 1.24%</td>
<td>$0.46$</td>
</tr>
<tr>
<td>Nuclear</td>
<td>43</td>
<td>1.01 ± 0.76%</td>
<td>$0.028$</td>
</tr>
</tbody>
</table>

**Conclusions.** Trx and TrxR are major proteins taking part in the regulation of the redox state of cells (9, 34, 35). In this study, we investigated their immunohistochemical expression in a large set of non-small cell lung carcinomas. Because Trx has been shown to influence cell growth, apoptosis, and p53 expression, we also investigated these parameters and their association with Trx and TrxR expression in these tumors. Additionally, one non-neoplastic cell line (BEAS 2B) and four malignant lung carcinoma cell lines were studied for the expression of Trx and TrxR protein expression by Western blot and mRNA expression by RT-PCR.

Both our immunohistochemical experiments in tissue samples and cell line studies showed that Trx and TrxR are expressed in non-small cell lung carcinomas with only a few cases being negative. Thus, the results indicate that the Trx-TrxR system is actively operating in neoplastic tissues of the lung. Similar observations have been done in cultured cells (17, 34, 36, 37) and tissue homogenates (23), but no corresponding studies have been conducted on lung cancer. In gastric carcinomas, Trx expression was associated with increased proliferation and decreased apoptosis (24). In our study, however, there was no association between PCNA positivity and Trx or TrxR expression in non-small cell lung carcinoma. These results suggest that the association and regulation of Trx and TrxR expression with proliferation might be different in various tumors. On the other hand, PCNA positivity is not necessarily associated with proliferation but indicates genomic damage of the cells (38).

TrxR expression was associated with a decreased extent of apoptosis in the tumors, whereas cytoplasmic positivity did not. The results indicate that Trx and TrxR take part in apoptosis regulation in non-small cell lung carcinoma also and that this effect possibly is mediated through regulation of transcriptional factors. One candidate for this effect might be p53, which has been shown to be regulated by
Thioredoxin and Thioredoxin Reductase in Lung Cancer

Thioredoxin (Trx) and thioredoxin reductase (TrxR) are important redox enzymes. They function to regulate the redox balance in the cell, and their expression can be linked to tumor grade and apoptosis. In this study, the expression of Trx and TrxR was analyzed in non-small cell lung carcinomas (NSCLCs) and found to be associated with tumor grade and apoptosis.

Wild-type p53 protein is known to increase apoptosis through regulation of Bax (39). In our material, we did not find any association between p53 positivity and Trx or TrxR expression. However, positive p53 immunohistochemistry mostly indicates accumulation of mutated p53 protein that many times has lost some of the functions of the wild-type protein. Consequently, our results do not necessarily tell about the relationships between Trx or TrxR and wild-type p53 protein. However, it indicates that putative p53 mutations, as reflected by accumulation of p53 protein, do not influence Trx or TrxR expression to a significant degree and vice versa.

There was no difference in the expression of Trx and TrxR between squamous cell carcinoma and adenocarcinomas, suggesting that their expression is not linked to histological differentiation in non-small cell lung carcinomas. However, tumors with a lower grade showed a stronger Trx and TrxR expression than high-grade tumors. Such an association might indicate that the expression of Trx and TrxR could be connected to a higher cellular differentiation of these tumors and that in tumors with a lower differentiation there would be a gradual loss in redox regulation. Such a loss might lead to an increased cellular and genomic damage in these tumors, thus contributing to a greater genomic instability of the tumor cell population. In fact, free radicals have been shown to cause genomic damage (40). Loss of Trx and TrxR expression might also lead to derangements in the regulation of transcriptional factors and might in this way influence tumor cell proliferation and apoptosis in high-grade tumors. In line with this, tumors of a high grade displayed a higher extent of apoptosis than did low-grade tumors. Similar associations with tumor grade and apoptosis have been shown in other carcinomas (41). It has also been suggested that the TUNEL method may be partly unspecific for detecting apoptosis and may also indicate genomic damage (41). However, there has usually been a significant correlation between light microscopic detection of apoptosis and the TUNEL method (41).

There was a strong association between the expression of Trx and TrxR in non-small cell lung carcinoma. This is not a surprising finding because TrxR is known to function together with Trx (19). Analogous to this, the expression of Trx and TrxR was also similar in non-neoplastic tissues. Strong Trx and TrxR expression was found in bronchial epithelial cells and alveolar macrophages, but alveolar epithelial cells were usually negative except for regenerating type II pneumocytes. These results are similar to the distribution of TrxR and Trx observed in adult rats (28). The distribution of Trx and TrxR expression in non-neoplastic lung suggests that the Trx-TrxR system possibly takes part in the defense of the lung to outside noxious stimuli. Such noxious stimuli might induce formation of reactive oxygen metabolites, the neutralization of which the Trx-TrxR system also takes part, along with superoxide dismutases and enzymes associated with glutathione metabolism.

In conclusion, our results show strong Trx and TrxR expression in non-small cell lung carcinoma both in vitro and in vivo, suggesting that these proteins take part in regulating the redox balance in these tumors and, through this influence, the activity of transcriptional factors and tumor growth in non-small cell lung carcinoma. They are also expressed in many cells of the non-neoplastic lung such as bronchial epithelial cells, alveolar macrophages, and regenerating type II pneumocytes, suggesting a part in cellular defense against outside noxious stimuli. The histochemical analysis of Trx and TrxR may prove a useful marker of tumor treatment because many clinically useful drugs target the TrxR with its essential selenocysteine residue (42).

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Widespread Expression of Thioredoxin and Thioredoxin Reductase in Non-Small Cell Lung Carcinoma

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