Thioredoxin Expression Is Associated with Lymph Node Status and Prognosis in Early Operable Non-Small Cell Lung Cancer[1]

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ABSTRACT

Purpose: **Thioredoxin (TRX), a low molecular weight protein, exerts reduction-oxidation control over a number of transcription factors involved in cell activation and proliferation. High TRX mRNA levels have been found in lung carcinomas, a trait associated with a growth and survival advantage.**

Experimental Design: **In this study, we examined the immunohistochemical expression of human TRX in normal lung and in 102 primary non-small cell lung carcinomas.**

Results: **In normal lung, the staining for TRX was cytoplasmic in the respiratory bronchial epithelium, alveolar epithelium, and alveolar macrophages. Bronchial glandular cells demonstrated a mixed nuclear and cytoplasmic staining. In lung carcinomas, the pattern of expression for TRX was predominantly cytoplasmic and only occasionally nuclear. A strong association between absence of TRX expression and regional lymph node negativity was observed ($P = 0.004$). High proliferation index, as detected with Ki-67 antibody, was associated with high TRX expression ($P = 0.02$). A significant correlation between high cytoplasmic p53 reactivity and low TRX expression was observed ($P = 0.04$). No association with grade, tumor stage, histology, or bcl-2 was noted. A significant coexpression of TRX with human activator protein endonuclease 1 was recorded ($P = 0.04$). Absence of TRX expression was associated with a better outcome ($P < 0.05$).**

Conclusions: **We conclude that overexpression of TRX in non-small cell lung carcinomas is indicative of a more aggressive tumor phenotype and is associated with bad prognostic features and possibly with a poorer outcome.**

INTRODUCTION

TRXs[4] are low molecular weight redox proteins found in both prokaryotic and eukaryotic cells (1). Human TRX is a M_r 11,500 protein with 27% amino acid identity to *Escherichia coli* TRX. It contains three additional Cys residues not found in bacterial TRX that give it unique biological properties (2). TRX was originally studied for its ability to act as a reducing cofactor for ribonucleotide reductase, the first unique step in DNA synthesis (3). Human TRX was subsequently shown to exert redox control over a number of transcription factors including NF-κB, human transcription factor-1C, BZLF1, the glucocorticoid receptor, and indirectly through the nuclear redox protein HAP1/Ref-1, AP-1 (4–8). TRX modulates the binding of these transcription factors to DNA and thus regulates gene transcription. TRX expression is induced by a variety of forms of stress, including viral infection, mitogens, X-ray and UV irradiation, hydrogen peroxide, and postischemic reperfusion (9). Regulation of the intracellular redox environment is critical for cell viability, activation, and proliferation.

Recent studies (10, 11) have shown that TRX offers a survival as well as a growth advantage to tumors *in vivo*, unlike bcl-2, which offers only a survival advantage. It has been reported (12) that almost half of human primary lung cancers overexpress TRX mRNA as compared with normal lung tissue from the same subjects. In a recent study (13), we reported that nuclear localization of HAP1/Ref-1 is associated with good prognosis in early operable NSCLC. HAP1, apart from being identified as a DNA repair protein, has also been shown to regulate the DNA-binding activity of several oxidized transcription factors, including AP-1 (14). There is evidence (15) of increased expression of the transcription factor complex AP-1 in adenocarcinomas and squamous cell carcinomas of the lung, although the significance of this finding is still unclear. Because AP-1 transcriptional activity is regulated by a direct association between TRX and HAP1, assessment of their expression in

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[4] The abbreviations used are: TRX, thioredoxin; redox, reduction-oxidation; AP-1, activator protein-1; HAP1, human activator protein endonuclease 1; NSCLC, non-small cell lung cancer; wtp53, wild-type p53; PI, proliferation index; NF-κB, nuclear factor κb.

NSCLC may provide further information in elucidating the pathways involved during human lung carcinogenesis.

In this study, we examine the immunohistochemical expression of TRX in normal lung and in lung carcinomas. Our aim is to describe the pattern of expression and relate this to other biological factors including tumor-node stage, histology, Ki-67, p53, bcl-2, and HAP1/Ref-1.

MATERIALS AND METHODS

Patients and Tumors. Samples from normal lung (n = 12) and a consecutive series of tumor specimens from 102 patients with operable ($T_{1,2}N_{0,1}M_0$) NSCLC were obtained from the archives of the Department of Cellular Pathology at John Radcliffe Hospital (Oxford, United Kingdom). Sixty-eight patients had squamous carcinomas, and 34 patients had adenocarcinomas. All of the patients were treated with surgery alone and survived for at least 60 days after being operated on, which excludes perioperative mortality-related bias. No chemotherapy or radiotherapy was given before or after surgery. The ages of the patients ranged from 45–75 years (median age, 62 years). Eighty-one patients were male, and 21 were female. Follow-up ranged from 2–96 months.

Immunohistochemistry for TRX. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections cut onto silane-coated slides. Cellular expression of TRX was determined using a standard avidin-biotin-peroxidase complex technique and an anti-TRX monoclonal antibody. TRX antiserum was kindly provided by Garth Powis (Arizona Cancer Center, Tucson, AZ). Before incubation with TRX (used in a 1:500 concentration), sections were subjected to two microwave irradiations for 5 min each in a heat-stable glass dish filled with 10 mM citrate buffer (pH 6.0) for antigen retrieval. All of the incubations were performed at room temperature, and all washings between incubations were in Tris-buffered saline. Negative controls consisted of substitution of the primary antibody with a nonspecific IgG immunoglobulin.

Assessment of TRX Expression. Assessment of TRX expression was performed independently by two experienced observers (magnification, ×240). Cases of discordant interpretation between the two observers were resolved after consensus over a conference microscope. TRX expression was calculated at the whole tumor area and reported as a percentage of positive cells. As cutoff points, we used the median percentage of cells with strong reactivity to distinguish between two groups of cases with high (>median) or low (<median) reactivity. The nuclear and the mixed (nuclear and cytoplasmic) reactivity were assessed separately.

Other Immunohistochemical Techniques. Nuclear p53 expression was assessed on 8-μm cryostat sections with the alkaline-phosphatase/anti-alkaline phosphatase technique, using the DO-7 antibody, as described previously (16). This antibody is considered to be a marker of mutant p53 protein, although wtp53 protein may also be detected. Cytoplasmic-perinuclear p53 was also assessed in cryostat sections, using the polyclonal antibody 248 (17). This antibody recognizes the cytoplasmic wtp53 protein (17). Samples with positive staining in <10% of cancer cells or with weak diffuse staining were considered negative.

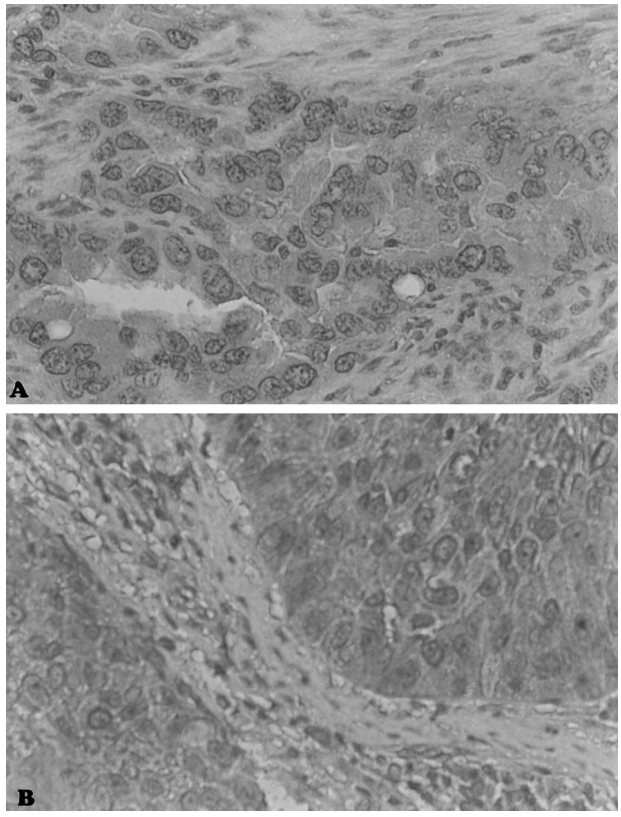

Fig. 1 Streptavidin-biotin immunoperoxidase staining (×240). *A*, lung carcinoma showing mixed nuclear and cytoplasmic staining; *B*, lung carcinoma showing cytoplasmic staining.

Cellular expression of HAP1 was determined using a standard avidin-biotin-peroxidase complex technique and an anti-HAP1 rabbit polyclonal antibody, as described previously (18). Cases bearing nuclear HAP1 positivity at >25% of cancer cells were considered positive.

PI was assessed with the Ki-67 monoclonal antibody, as described previously (19). Ki-67 assessment was based on the percentage of stained nuclei; 0–40% was considered low PI (PI1), and >40% was considered high (PI2).

bcl-2 staining was performed on frozen sections with the alkaline-phosphatase/anti-alkaline phosphatase method, using a monoclonal antibody that is specific for bcl-2 (clone 100, raised to a synthetic peptide; Ref. 20). A strong diffuse expression was used to define positive cases, as described previously (20). The sections were analyzed in a blinded fashion, and the results of the immunohistochemistry, tumor status, and patient outcome were subsequently correlated.

Statistics. The relationships between different tumor variables were examined using Fisher's exact test, χ^2 test, or the unpaired two-tailed t test, as appropriate (TRX was taken as a continuous or categorical variable). Survival curves were plotted using the Kaplan-Meier method, and statistical differences between life tables were determined by a log-rank test. A Cox proportional hazards model was used to assess the effect of different tumor variables on overall survival. The statistical

analysis was performed using the Stata package (release 3.1; Stata Corp., College Station, TX).

RESULTS

TRX Expression in Normal Lung. Respiratory bronchial epithelium showed cytoplasmic staining with strong TRX expression adjacent to the basement membrane of the epithelium. Alveolar epithelium and alveolar macrophages also showed a cytoplasmic staining. Mucus-secretory glands showed a mixed nuclear and cytoplasmic staining.

TRX Expression in Lung Carcinomas. The pattern of expression of the cancer cells was predominantly cytoplasmic, with a varying percentage of cells bearing nuclear reactivity (Fig. 1). Membranous and/or perinuclear expression was also present occasionally (Fig. 1). Stromal fibroblasts and infiltrating macrophages showed occasional cytoplasmic reactivity. The percentage of cancer cells expressing TRX was counted in all of the optical fields, and the median and mean values were recorded. The median percentage of cells with mixed cytoplasmic and nuclear reactivity was 50% (range, 0–100; mean, 41%). The median percentage of cells with nuclear reactivity was 0% (range, 0–90; mean, 11%). Of 102 examined cases, 40 (39%) showed positive mixed staining in >50% of cancer cells, and 62 (61%) showed positivity in <50% of the cells. Thirty-nine of these 62 cases (38%) showed TRX expression in a substantial percentage of cancer cells (10–50%), whereas 23 (23%) were practically negative. We therefore distinguished three categories of high, low, and negative reactivity. A similar analysis was performed, taking into account the nuclear expression (data not shown).

Relationship of TRX Expression to Different Tumor Characteristics. Table 1 shows the relationships between TRX expression and different histological and molecular variables. A strong association between negative TRX expression and absence of regional lymph node involvement was observed ($P = 0.004$). PI, as detected by Ki-67 antibody expression, was significantly higher in cases with high TRX expression ($P = 0.02$). No association with tumor stage, histology, grade, or the extent of necrosis was recorded. wtp53 expression was more frequent in cases with negative/low TRX reactivity ($P = 0.04$), but no association of p53 nuclear accumulation with TRX was observed. A significant coexpression of TRX with HAP1 was also noted ($P = 0.04$). No association of the percentage of cells with nuclear TRX expression with any of the examined variables was observed.

Survival Analysis. Univariate analysis showed that TRX-expressing cases (high or low TRX expression) had significantly poorer survival compared with TRX-negative tumors ($P < 0.05$; Fig. 2). However, in a multivariate model, tumor stage and node stage were the only variables that maintained an independent prognostic significance ($P = 0.001$ and t ratio = 3.4 and $P = 0.0004$ and t ratio = 3.71 for tumor stage and node stage, respectively). Excluding node stage (which was strongly associated with TRX expression), TRX expression approached independent significance ($P = 0.07$, t ratio = 1.81).

Table 1 Relationships between different patterns of TRX expression and various tumor parameters (χ^2 test)

Parameters (Overall, $n = 102$)	TRX negative ($n = 23$)	TRX low ($n = 39$)	TRX high ($n = 40$)	P (χ^2 test)
T stage				
T_1	10	16	20	0.71
T_2	13	23	20	
N stage				
N_0	22	22	25	0.004
N_1	1	17	14	
Grade				
1/2	9	21	17	0.47
3	14	18	22	
Histology				
Squamous	19	23	26	0.15
Adenosquamous	4	16	14	
Ki-67				
Low	6	19	8	0.02
High	17	20	31	
Necrosis				
Limited/no	9	18	16	0.83
Extensive	14	21	23	
HAP1				
Negative	11	9	6	0.04
Positive	7	13	19	
bcl-2				
Negative	17	34	29	0.23
Positive	6	5	11	
P53 (DO-7)				
Negative	15	28	27	0.84
Positive	8	11	13	
p53 (pAb258)				
Negative	6	8	18	0.04
Positive	17	31	21	

DISCUSSION

TRX is a multifunctional protein with a redox-active disulfide/dithiol in the active site; it operates together with NADPH and TRX reductase as a general protein disulfide-reducing system (21). The TRX system (TRX, TRX reductase, and NADPH) is widely conserved in almost all species from bacteria to higher eukaryotes, playing a major role in cell activation and cell growth promotion.

In this study, we observed that in both normal lung and lung carcinomas the pattern of TRX immunohistochemical expression was predominantly cytoplasmic; however, occasional nuclear reactivity was also present in some cases. These differences in the intracellular localization may be relevant to the multiple functions of TRX. An important finding of this study was the observed significant association of TRX overexpression with regional lymph node involvement. This observation is in keeping with the proposed role of TRX as a growth promoter in some human cancers, including lung cancer (10–12). Experimental data (10) have shown that transfection of human MCF-7 breast cancer cells with TRX increases their colony formation in soft agarose. Transfection of the MCF-7 cells with a dominant-negative inactive mutant TRX causes inhibition of colony formation and complete inhibition of tumor formation when the cells are inoculated into *scid* mice (10). Therefore, although the role that TRX may play in malignant transformation of cells is unknown, overexpression of TRX in lung carcinomas and subsequent redox regulation and activation of a number of intracellular proteins that control cell growth and proliferation may be indicative of a more aggressive tumor phenotype and thus the

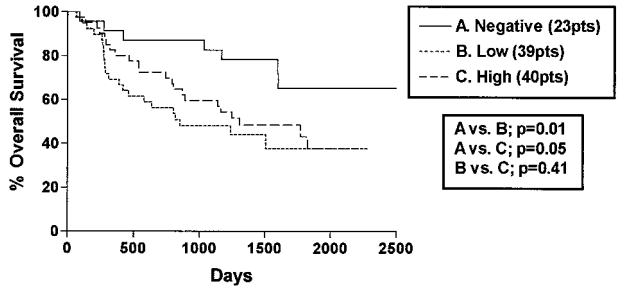

Fig. 2 Survival curves of patients with NSCLC, according to TRX expression.

association of TRX overexpression with nodal positivity and a poorer outcome in our cases. This is supported further by the observed significant association between TRX overexpression and a high PI, as measured by Ki-67 expression.

We observed a significant inverse association between TRX negativity and cytoplasmic wtp53 positivity. However, this finding should be interpreted with caution because the precise role played by wtp53 protein in the cytoplasm is unclear, and immunohistochemistry for p53 with the available antibodies is frequently unreliable (16, 17, 22). It is possible that TRX may have a completely different role in the cytoplasm and the nucleus with respect to p53 function. This is something that has been reported (23) in the case of the redox regulation of NF-κB function by TRX; the action of TRX in the cytoplasm and in the nucleus has been suggested to be distinct, with TRX inhibiting prooxidant-mediated activation of NF-κB in the cytoplasm and potentiating DNA binding in the nucleus. Ueno *et al.* (24) reported recently that TRX enhances the sequence-specific DNA binding activity of p53 directly or indirectly (through HAP1), which may indicate a functional coupling between TRX and HAP1 in the p53 activation system. Whether the observed coexpression between HAP1 and TRX in our study is relevant to a role in p53 activation is unclear. Consideration of redox regulation of the transcription factors with respect to cellular compartments might therefore be an important issue for understanding the mechanisms of redox regulation of gene expression.

Assessment of TRX expression may also be of importance in planning anticancer therapies. Several recent reports (25) have demonstrated that TRX is involved in drug resistance against anticancer drugs such as cisplatin, etoposide, and doxorubicin. TRX-transfected cells are more resistant to such anticancer drugs, and TRX antisense-transfected cells are more sensitive to the same anticancer agents. Cisplatin and etoposide represent the most active agents for the treatment of NSCLC. Hence, absence of TRX expression may be indicative of a drug-sensitive and less aggressive tumor phenotype, and specifying anticancer treatment according to TRX levels could be of value. Additionally, the use of drugs that inhibit the redox activity of TRX or inhibit the enzyme responsible for the reduction of TRX, the flavoprotein TRX reductase, might also offer a novel approach to treating some forms of human cancer.

In conclusion, we have shown that in lung carcinomas the immunohistochemical pattern of TRX expression is predominantly cytoplasmic and that TRX positivity is associated with bad prognostic features such as regional lymph node involvement and high PI. It seems that in NSCLC, TRX overexpression is indicative of a more aggressive tumor phenotype, which is possibly associated with a poorer outcome.

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