Histopathology 2009, **54**, 313–320. DOI: 10.1111/j.1365-2559.2009.03381.x

Expression profiles of thioredoxin family proteins in human lung cancer tissue: correlation with proliferation and differentiation

Aristi P Fernandes, Arrigo Capitanio, Markus Selenius, Ola Brodin,[1] Anna-Klara Rundlöf & Mikael Björnstedt

Division of Pathology, Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, and [1]Division of Oncology, Karolinska University Hospital, Stockholm, Sweden

Date of submission 7 January 2009
Accepted for publication 15 February 2009

Fernandes A P, Capitanio A, Selenius M, Brodin O, Rundlöf A-K & Björnstedt M
(2009) *Histopathology* **55**, 313–320

Expression profiles of thioredoxin family proteins in human lung cancer tissue: correlation with proliferation and differentiation

Aims: Lung cancer is one of the most common causes of cancer lethality worldwide. Despite recent progress, long-term survival remains poor. The aim of this study was to explore the expression pattern of the thioredoxin superfamily of proteins as potential new diagnostic and/or predictive markers.

Methods and results: The expression of thioredoxin 1 (Trx1), thioredoxin reductase 1 (TrxR1), the isoforms TrxR1-v.2,3,5, glutaredoxin 1 (Grx1) and glutaredoxin 2 (Grx2) was examined by immunohistochemistry on paraffin-embedded sections from 42 cases of non-small cell lung cancer patients. Additional cases of lung cancer from tissue microarray were examined and the immunoreactivity was compared. All proteins except TrxR1 showed a significant correlation with the degree of differentiation in adenocarcinoma. Trx1 and TrxR1-v.2,3,5 also showed a significant correlation with differentiation in squamous carcinoma. Furthermore, Grx1 and Grx2 showed a clear inverse correlation with proliferation. The proliferation rate was further analysed *in vitro* in stably transfected Grx2 overproducing cells, showing that the proliferative effect of Grx2 is strictly dependent on subcellular localization.

Conclusions: The thioredoxin family of proteins is important for growth and differentiation of lung cancer cells. The correlation with differentiation and proliferation of these enzymes makes them promising predictive/diagnostic markers.

Keywords: diagnostic markers, differentiation, lung cancer, proliferation, thioredoxin system

Abbreviations: EGFR, epidermal growth factor receptor; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; mGrx2a, mitochondrial Grx2a; NADPH, nicotinamide adenine dinucleotide phosphate; PCR, polymerase chain reaction; TMA, tissue microarray; Trx, thioredoxin; TrxR, thioredoxin reductase; TTF-1, thyroid transcription factor-1

Introduction

Lung cancer is the most common cause of cancer lethality.[1] The resistance to chemotherapy can be both primary and secondary. However, most tumour types acquire secondary drug resistance. Today the 5-year survival rate is no better than 15%. The molecular mechanisms behind the resistance to chemotherapy of these tumours are known to a limited extent. However, there is clear evidence that it is linked to increased defence against free radicals, and several studies have further shown strong indications that cellular redox systems are involved.

The thioredoxin and glutaredoxin systems are two multifunctional redox active protein disulphide reductase systems, widely distributed in nature.[2,3] The thioredoxin system comprises thioredoxin (Trx),

Address for correspondence: A Fernandes, Division of Pathology, Department of Laboratory Medicine, Karolinska Institutet, F46, Karolinska University Hospital Huddinge, 141 86 Stockholm, Sweden. e-mail: aristi.fernandes@ki.se
A.P.F. and A.C. contributed equally to this work.

thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH), whereas the glutaredoxin system consists of NADPH, glutathione reductase (GR), glutathione (GSH) and glutaredoxin (Grx). Both redox systems are involved in several important biological functions such as apoptosis, defence against oxidative stress, synthesis of DNA, thiol redox control of enzymes, as well as regulation of receptors and transcription factors.[4] All redox proteins belonging to the thioredoxin and glutaredoxin systems exist as isoenzymes, where Trx1, TrxR1 and Grx1 are mainly cytosolic, and Trx2, TrxR2 and Grx2 predominantly localize to the mitochondria.[5,6] Several of these proteins are also further subjected to alternative splicing, generating different protein variants from the same gene. TrxR1 can result in five protein variants,[7] TrxR1-v1 to TrxR1-v5, and Grx2 is believed to give rise to three forms, Grx2a–c.[8]

Numerous reports have implicated the thioredoxin system in tumour growth and resistance.[9] It has, for example, been shown that TrxR1 deficiency can reverse the morphology and tumorigenicity of lung carcinoma cells.[10] Using TrxR1 knock down cells, it has also been suggested that TrxR1 is essential for tumour growth in mice. In squamous lung cancer the expression of Trx1 and TrxR1 are elevated, especially in highly differentiated cells.[11,12] High levels of Trx1 have further been linked to chemotherapeutic resistance.[13] It has therefore been suggested that TrxR and Trx are prime targets for cancer therapy.[14,15] TrxR is known to be a drug target for several cytostatic agents through nucleophilic attacks on the active site.[9,16] Recently, TrxR has also been shown to be a target for the chemopreventive agents Resveratrol[17] and Motexafin gadolinium.[18] Flavinoids have also been shown to target TrxR.[19]

The differentiation of tumour cells is generally directly correlated to prognosis. The involvement of antioxidant proteins in the control of differentiation has previously been reported.[20–22]

Taken together, there is increasing evidence of, and understanding of the implications of thioredoxin and glutaredoxin systems' involvement in tumour development and resistance. We aimed to map Grx1, Grx2, Trx1, TrxR1 and its isoforms in human lung cancer, and thus explore the prognostic and diagnostic potential of these proteins.

Materials and methods

CLINICAL MATERIAL

Paraffin blocks of 42 tumours from patients with lung cancer (adenocarcinoma $n = 24$, large cell carcinoma $n = 6$, and squamous cell carcinoma $n = 12$) were randomly selected from the archive at the Laboratory for Clinical Pathology, Karolinska University Hospital in Huddinge, Sweden, following approval from the regional ethics committee. Tissue microarray (TMA) sections (1 mm in diameter) including tissue material from 127 patients with lung cancer (adenocarcinoma $n = 90$, large cell carcinoma $n = 6$, squamous cell carcinoma $n = 20$, and small cell carcinoma $n = 11$) were kindly provided by M. Barbareschi (Department of Pathology, S. Chiara General Hospital, Trento, Italy). The TMA samples were completely anonymous; individual samples could not be tracked and no information was provided concerning the identities of the patients.

The tumours were graded according to the American Joint Committee on Cancer,[23] where grade I represents highly, grade II moderately and grade III poorly differentiated as assessed by nuclear polymorphism, degree of anisokariosis, architectural disorder, gland formation and mucus production. Large cell carcinomas were all assessed as poorly differentiated (grade III).

PREPARATION OF PARAFFIN-EMBEDDED CELLS

Grx2-overexpressing HeLa cell lines were cultured in RPMI 1640 medium at 37°C and 5% CO_2. Cells were collected with a cell scraper and centrifuged at 300 g for 7 min. The supernatant was discarded and the pellet resuspended in 200 μl of human blood plasma. Fibri-prest2 (0.5 ml; Diagnostica, Stago, Asnieres, France) was added and incubated at room temperature for 5 min. The resulting pellet was then fixed in 4% formalin overnight, thereafter embedded in paraffin and cut in 3 μm thick sections and stained for Mib-1 (see below).

IMMUNOHISTOCHEMISTRY

For immunohistochemistry, 3-μm tissue sections were deparaffinized in xylol and rehydrated in decreasing concentrations of ethanol. The tissue sections were heated in a microwave oven for 10 min in 0.01 M citrate buffer, pH 6.0, for antigen retrieval. The automatic Dako TechMate 500 was used for staining (Dako, Glostrup, Denmark). Tissue slides were stained with primary antibodies against TrxR1 (Upstate, Billerica, MA, USA), 1:1000; TrxR1-v.2,3,5,[22] 1:250; Grx1 (IMCO, Stockholm, Sweden), 1:50; Grx2 (Histo-Line, Seoul, Korea), 1:50; and Trx1 (IMCO), 1:100, diluted in ChemMate antibody diluent (Dako), for 25 min at room temperature. For visualization Dako ChemMate Detection kit Peroxidase/Diaminobenzidine (Dako) was

used. The sections were counterstained with haematoxylin. The specificity of the primary antibody reaction was confirmed by pre-incubation for 1 h at 37°C of the antibody with the specific antigen before staining. In addition to the redox enzymes, the sections were stained for the proliferation marker Mib-1 (equivalent to Ki67) (stained by the accredited laboratory facility at Karolinska University Hospital).

MATHEMATICAL DETERMINATION OF IMMUNOREACTIVITY

Using the Hue Saturation and Intensity mathematical model,[24] the saturation of a given colour was used to compare different degrees of positivity of immunohistochemically stained sections. The method consisted of the following steps: selection of the range of the colour (here, brown, in the range 30–45°) and measurement of the saturation of the hue in the tumour cells relative to surrounding non-tumorous tissue in the same section [scale 0–1, 0, completely negative, 1, weak positivity (saturation between 0.01 and 0.33), 2, moderate positivity (saturation between 0.34 and 0.66), 3, strong positivity (saturation between 0.67 and 1)]. The image analysis software used is described by Cunnea *et al.*[25] Results were analysed using the STATISTICA software (StatSoft Inc., Tulsa, OK, USA).

DNA EXTRACTION FROM PARAFFIN-EMBEDDED MATERIAL

Genomic DNA was purified from paraffin-embedded human lung tumour material (the TMA cases were not examined). Sections were deparaffinized according to standard protocol, scraped into an Eppendorf tube and purified using the QIAamp DNA Micro kit (Qiagen, Solna, Sweden) according to the manufacturer's protocol.

NESTED POLYMERASE CHAIN REACTION AND SEQUENCING

Nested polymerase chain reaction (PCR) amplifications were performed with primers amplifying parts of exon 18, 19 and 21 of epidermal growth factor receptor (EGFR) as described.[26] The PCR mixture contained sample DNA, 0.2 μM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate, 2.5 units of Taq DNA polymerase (Qiagen), 1× Q solution (Qiagen) and 1× PCR buffer containing 1.5 mM $MgCl_2$. The following PCR programme was used – initial denaturation at 95°C for 4 min, followed by 35 cycles as follows: 94°C for 30 s, 51°C for 30 s, and 72°C for 60 s. The final extension was at 72°C for 10 min. For purification of the PCR products the MiniElute PCR purification kit was used (Qiagen). The size of the products was confirmed by electrophoresis on a 1% agarose gel before DNA sequence analysis (Cybergene AB, Stockholm, Sweden).

STATISTICAL EVALUATION

For statistical analysis Wilcoxon matched pairs test was used and correlation analysis was performed by using the Spearman rank correlation test with a significance level at $P < 0.05$.

Results

IMMUNOHISTOCHEMICAL CHARACTERIZATION

To investigate the expression pattern of members of the thioredoxin superfamily, 42 cases of variously differentiated lung cancer were selected from archived material. A summary of patient characteristic is presented in Table 1. Using antibodies for Trx1, TrxR1, TrxR1-v.2,3,5, Grx1 and Grx2, we determined the expression patterns for each protein in all sections. All of the above examined proteins were localized in the cytosol except for Grx2, which was predominantly localized in the mitochondria. To measure and compare the different degrees of positivity of the expression patterns of the thioredoxin family proteins, we based

Table 1. Characteristics of 42 lung cancer patients

Patient characteristics	Total (n = 42)	Women (n = 26)	Men (n = 16)
Age			
Mean	66	65	68
Min.	31	31	57
Max.	78	78	78
Histology			
Adenocarcinoma	24	19	5
Squamous carcinoma	12	5	7
Large cell carcinoma	6	2	4
Smoking			
Smoker	26	18	8
Ex-smoker	14	6	8
Never smoked	2	2	0

the evaluation on the hue saturation of the immunoreactivity of the tumour tissue compared with that of the non-tumour tissue of the same section. The expression levels of all proteins except for TrxR1 were significantly elevated in the tumour area of the section. A representative figure is shown for Trx1, illustrating the immunoreactivity in adenocarcinoma with different degrees of differentiation (Figure 1).

CORRELATION OF INTENSITY OF IMMUNOREACTIVITY OF REDOX ENZYMES AND GRADING

As seen in Figure 2, all redox proteins showed a higher intensity of reactivity in the adenocarcinoma area compared with the surrounding non-neoplastic tissue from the same section. Apart from the increased expression of the enzymes examined in the tumour sections, Trx1, TrxR1-v.2,3,5, Grx1 and Grx2, all showed a significant correlation with the degree of differentiation in the adenocarcinoma cases (Figure 2A). The highly differentiated tumours resulted in the maximum intensity of immunoreactivity of the redox proteins. To confirm further the expression patterns and correlation with differentiation, an additional 127 cases were analysed from TMA. The TMA supported these findings with an almost identical pattern (Figure 2B). A combined summary of all data for the adenocarcinomas is presented in Figure 2C. Of note is the staining against TrxR1-v.2,3,5, which had a much stronger correlation with differentiation and was not seen for the total TrxR1.

The results from the squamous, large cell and small cell carcinoma cases are listed in Table 2. The general expression pattern showed a lower immunoreactivity in these sections compared with adenocarcinoma, probably due to higher grading. Significant differences compared with non-neoplastic tissue are shown in Table 2. A significant correlation with differentiation in the squamous cell carcinomas was observed only for TrxR1-v.2,3,5 ($P < 0.05$) and Trx1, which had a high degree of correlation ($P < 0.001$). No correlation test could be performed on large cell and small cell carcinomas, since all of them were graded as poorly differentiated (grade 3).

PROLIFERATION AND INTENSITY OF IMMUNOREACTIVITY OF REDOX PROTEINS

The 42 sections were stained with the proliferation marker Mib-1. Correlation of Mib-1 with the redox enzymes displayed a significant correlation with both Grx1 and Grx2 ($P < 0.01$ and 0.001, respectively) (Figure 3), where higher proliferation resulted in decreased levels of these enzymes.

PROLIFERATION INDEX IN GRX2-OVEREXPRESSING CELLS

Cells stably overexpressing Grx2 were used to verify the results from immunohistochemistry, showing a high degree of correlation between Grx2 and the proliferation rate. Control mock transfected cells, cells over-

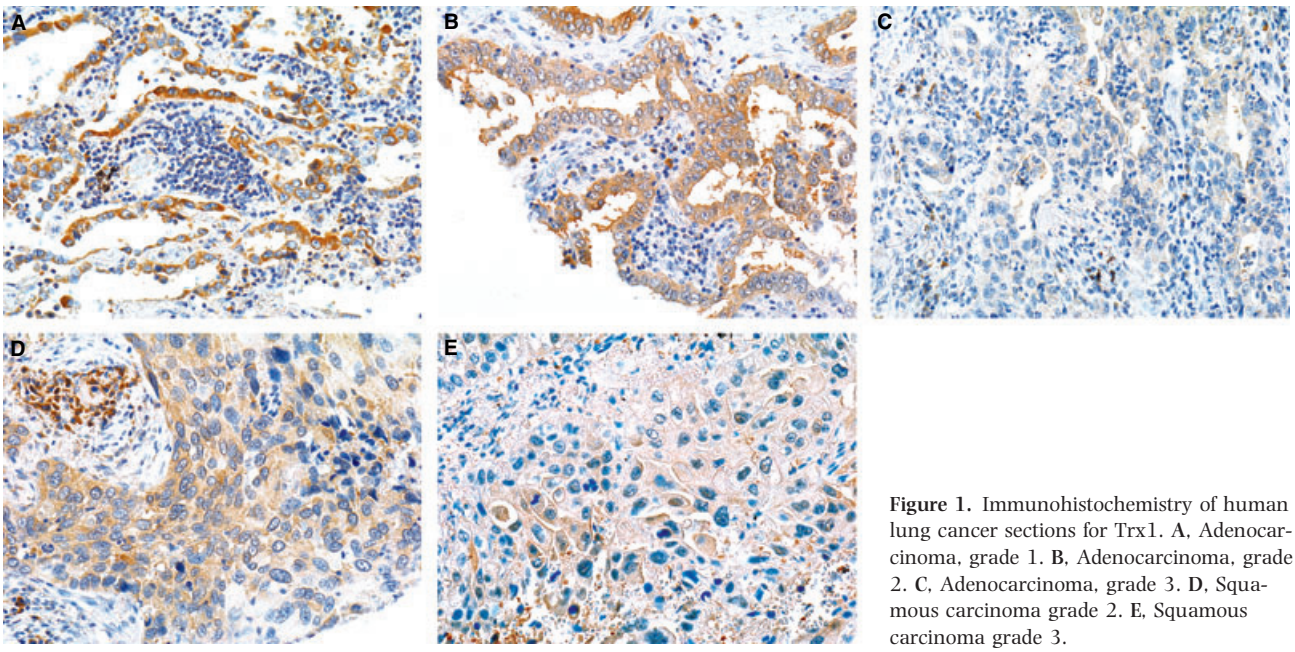

Figure 1. Immunohistochemistry of human lung cancer sections for Trx1. **A**, Adenocarcinoma, grade 1. **B**, Adenocarcinoma, grade 2. **C**, Adenocarcinoma, grade 3. **D**, Squamous carcinoma grade 2. **E**, Squamous carcinoma grade 3.

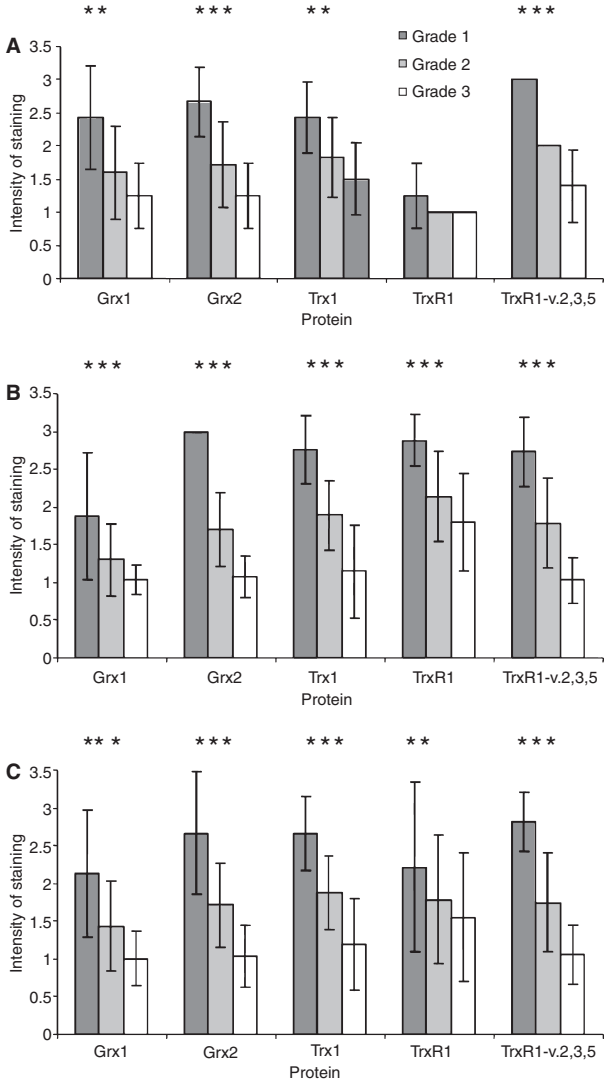

Figure 2. Correlation of the different grades in adenocarcinoma tissue with the intensity of immunoreactivity of the redox proteins (Grx1, Grx2, Trx1, TrxR1, TrxR1-v.2,3,5). The staining intensity was calculated by comparison with the non-neoplastic tissue within the section (set to 0). **A**, Results from immunohistochemistry of 24 tissue sections. **B**, Results from immunohistochemistry staining of tissue microarray (TMA) material of 90 cases. **C**, Combined results from tissue sections and TMA material. Protein expression was correlated with tumour grade using the Spearman rank correlation test. Stars denote statistical significance of expression in the tumour material correlated with grade: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

expressing mitochondrial Grx2a (mGrx2a), and cells overexpressing Grx2a lacking the mitochondrial translocation signal, resulting in elevated cytosolic levels of Grx2a, were compared (Figure 4). The results show that there were no significant differences between the control cells (42% positivity) and cells overexpressing the cytosolic form of Grx2a (50% positivity). However, the mGrx2a showed a marked increase in proliferation of >90% positivity, indicating that cytosolic Grx2a is overexpressed in lung cancer.

EGFR

Mutational analysis was performed by PCR followed by sequencing in order to examine potential mutations in the EGFR exons 18, 19 and 21, all known to have specific alterations connected to drug resistance in lung cancer. The PCR and sequence determination confirmed that no mutations were present in the sections examined (data not shown).

Discussion

In recent decades substantial progress has been achieved in the diagnosis and treatment of several human cancers. However, the result for lung cancer remains poor and, as a consequence, lung cancer is now the leading cause of cancer death worldwide, although lung cancer is not the predominant malignancy.[1] There is clearly a need for improved knowledge about lung cancer at all levels, from basic science to clinical investigations.

This study has described the expression patterns of thioredoxin family proteins in a large clinical sample of human lung tumours. There was a clear inverse correlation between the intensity of immunoreactivity of all proteins except for TrxR1 and tumour grade in adenocarcinoma cases. The strongest intensity was observed in highly differentiated tumours, whereas the weakest was observed in poorly differentiated tumours. This negative correlation between grade and immunoreactivity is supported by previous data suggesting a correlation between epithelial phenotype and expression of Trx and TrxR.[21,27] Neither Trx1 nor TrxR1/TrxR1-v2,3,5 correlated with proliferation index in our material, although several other studies have suggested that high levels of Trx1 in other tumour cells are associated with a more aggressive phenotype with increased proliferation and decreased apoptosis.[28–30] This demonstrates the complexity and diversity in the regulation of proliferation in various tumour cells.

Immunohistochemistry is widely used in the diagnosis of primary and metastatic tumours of the lung.[31] However, there is no single prognostic or diagnostic marker available. Thyroid transcription factor-1 (TTF-1) is highly specific for lung histogenesis and is used to detect lung metastasis throughout the body and also to distinguish between primary and secondary tumours in the lung. However, TTF-1 expression is not primarily used to correlate differentiation and grade. During lung carcinogenesis several immunohistochemical markers

Protein	Squamous G2	Squamous G3	LC G3	SCLC G3
Grx1	2.43 ± 0.53*	0.8 ± 0.45	1.83 ± 0.98*	0
Grx2	0.50 ± 0.52*	0	0.67 ± 0.82	1.00 ± 0
Trx1	1.71 ± 0.78***	0.83 ± 0.98	2.08 ± 0.51**	1.30 ± 0.48**
TrxR1	1.58 ± 0.77***	1.2 ± 0.92*	1.64 ± 1.21*	1.80 ± 0.42**
TrxR1-v.2,3,5	1.25 ± 1.02***	0.8 ± 0.79*	1.67 ± 0.71**	1.29 ± 0.49*

Table 2. Intensity of immunoreactivity of redox proteins in lung cancer compared with surrounding non-neoplastic tissue

For statistical analysis Wilcoxon matched pairs test was used (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

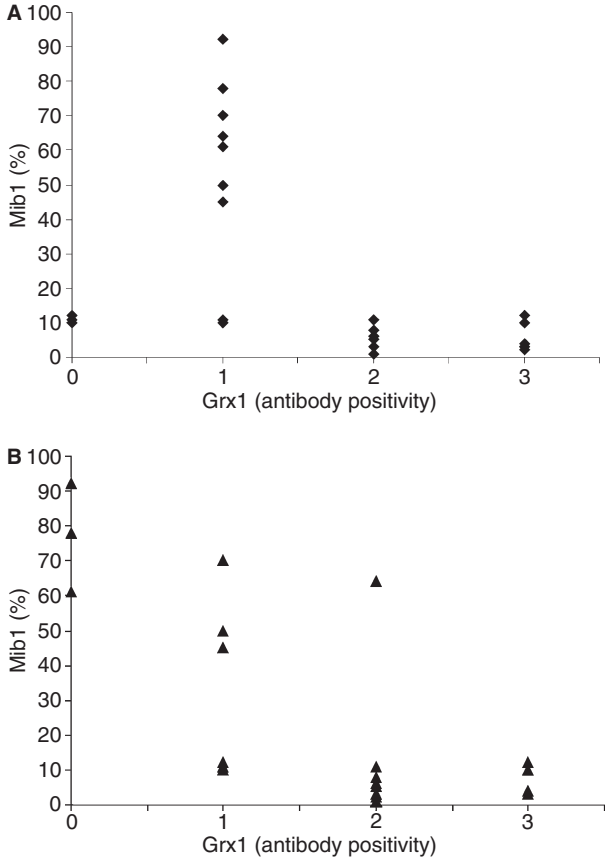

Figure 3. Grx1 and Grx2 expression in adenocarcinoma correlated with proliferation rate. Percentage of Mib-1 immunoreactivity in 24 tissue sections was compared with expression of Grx1 (**A**) and Grx2 (**B**). Scale of antibody positivity: 0, completely negative; 1, weak positivity; 2, moderate positivity; 3, strong positivity. Grx1 and Grx2 showed a significant ($P < 0.01$ and 0.001, respectively) negative correlation with proliferation, as measured by Mib-1 reactivity.

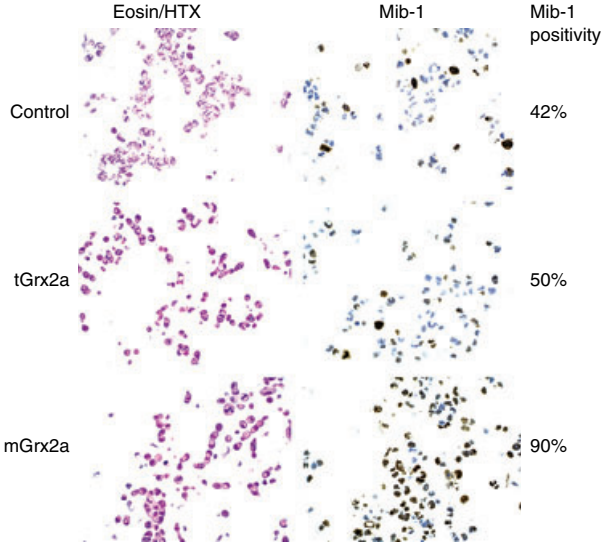

Figure 4. Cells stably overproducing Grx2a were characterized in terms of proliferation. Cells were stained for Mib-1. **A**, Mock-transfected HeLa cells (served as control). **B**, tGrx2a, cells overexpressing Grx2a but without the sequence essential for mitochondrial translocation. **C**, mGrx2a, cells overexpressing the intact mitochondrial Grx2a.

are often observed, including Ki67, cyclin D1, hTERT and survivin.[32] The thioredoxin family proteins are also important in the carcinogenic process and are suggested to be part of the resistant phenotype expressed by tumour precursors and overt cancers.[33,34]

In a previous study Trx and TrxR were shown to correlate inversely with grade, but the predominant tumour investigated by these authors was squamous carcinoma.[11] In the present study, the predominant tumour type was adenocarcinoma and the inverse correlation was most pronounced in this phenotype. Furthermore, we found the strongest correlation between grade and intensity of immunoreactivity with the antibody against the isoforms TrxR1-v2,3,5, indicating that one or more of these isoforms has different effects than classical TrxR1. This in turn suggests that one of the isoforms has a tumorigenic function and thus might be used as a prognostic tool. Cancer-specific alternative splicing has been shown for >300 genes, although the divergent patterns of protein variants are largely unknown.[35]

Proliferation, as measured by Mib-1, was inversely correlated with the expression of Grx1 and Grx2 in the lung cancer sections examined. HeLa cells stably transfected to overexpress the mitochondrial form of Grx2 (Grx2a), however, showed a marked increase in proliferation. These cells have previously also been shown to protect against apoptosis by inhibiting the release of cytochrome *c*. The discrepancy in proliferation between the tumour sections and the overproducing cells may be explained by the results with cells overexpressing a form of Grx2a lacking the mitochondrial localization signal, rendering the Grx2a in these cells cytosolic. The proliferation rate of these cells was not affected compared with control cells. This, in turn, was a surprising and interesting finding, showing inconsistency between the two variants in terms of proliferation even though they only differ in localization. In a previous report it was suggested that there is a transcript variant of Grx2 that is tumour specific (Grx2c).[8] This variant is also believed to be located in the cytosol and to have exactly the same sequence as the one overproduced in these cells. We might therefore have a situation in these tissues where the mitochondrial form is suppressed to varying degrees, such that the actual protein detected is the tumour-specific form (Grx2c), which is not located in the mitochondria and does not contribute to enhanced proliferation. This is an intriguing thought, which merits further study.

Our data clearly demonstrate a strong correlation between differentiation and the expression of thioredoxin family proteins. The observations are in keeping with previously published data on cell lines and experimental models, and provide further evidence for the importance of the thioredoxin family of proteins in epithelial differentiation. Our results thus suggest a great potential for these enzymes as tools in the diagnosis and classification of lung tumours.

Acknowledgements

This study was supported by Radiumhemmets forskningsfonder (Radiumhemmets Research Society), Läkaresällskapet (Swedish Medical Association), Cancerfonden (Swedish Cancer Society), Cancer och allergifonden (Swedish Cancer and Allergy Society), ALF (Stockholm County Council) and Hjärt-Lung fonden (the Swedish Heart Lung Foundation).

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J. Clin.* 2005; **55;** 74–108.
2. Arnér ES, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* 2000; **267;** 6102–6109.
3. Fernandes AP, Holmgren A. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid. Redox Signal.* 2004; **6;** 63–74.
4. Lillig CH, Holmgren A. Thioredoxin and related molecules—from biology to health and disease. *Antioxid. Redox Signal.* 2007; **9;** 25–47.
5. Gromer S, Urig S, Becker K. The thioredoxin system – from science to clinic. *Med. Res. Rev.* 2004; **24;** 40–89.
6. Lillig CH, Berndt C, Holmgren A. Glutaredoxin systems. *Biochim. Biophys. Acta* 2008; **1780;** 1304–1317.
7. Rundlöf AK, Janard M, Miranda-Vizuete A, Arnér ES. Evidence for intriguingly complex transcription of human thioredoxin reductase 1. *Free Radic. Biol. Med.* 2004; **36;** 641–656.
8. Lonn ME, Hudemann C, Berndt C *et al.* Expression pattern of human glutaredoxin 2 isoforms: identification and characterization of two testis/cancer cell-specific isoforms. *Antioxid. Redox Signal.* 2008; **10;** 547–557.
9. Urig S, Becker K. On the potential of thioredoxin reductase inhibitors for cancer therapy. *Semin. Cancer Biol.* 2006; **16;** 452–465.
10. Yoo MH, Xu XM, Carlson BA, Gladyshev VN, Hatfield DL. Thioredoxin reductase 1 deficiency reverses tumor phenotype and tumorigenicity of lung carcinoma cells. *J. Biol. Chem.* 2006; **281;** 13005–13008.
11. Soini Y, Kahlos K, Napankangas U *et al.* Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin. Cancer Res.* 2001; **7;** 1750–1757.
12. Park JH, Kim YS, Lee HL *et al.* Expression of peroxiredoxin and thioredoxin in human lung cancer and paired normal lung. *Respirology* 2006; **11;** 269–275.
13. Kim SJ, Miyoshi Y, Taguchi T *et al.* High thioredoxin expression is associated with resistance to docetaxel in primary breast cancer. *Clin. Cancer Res.* 2005; **11;** 8425–8430.
14. Arnér ES, Holmgren A. The thioredoxin system in cancer. *Semin. Cancer Biol.* 2006; **16;** 420–426.
15. Nguyen P, Awwad RT, Smart DD, Spitz DR, Gius D. Thioredoxin reductase as a novel molecular target for cancer therapy. *Cancer Lett.* 2006; **236;** 164–174.
16. Witte AB, Anestål K, Jerremalm E, Ehrsson H, Arnér ES. Inhibition of thioredoxin reductase but not of glutathione reductase by the major classes of alkylating and platinum-containing anticancer compounds. *Free Radic. Biol. Med.* 2005; **39;** 696–703.
17. Hu Y, Rahlfs S, Mersch-Sundermann V, Becker K. Resveratrol modulates mRNA transcripts of genes related to redox metabolism and cell proliferation in non-small-cell lung carcinoma cells. *Biol. Chem.* 2007; **388;** 207–219.
18. Hashemy SI, Ungerstedt JS, Zahedi Avval F, Holmgren A. Motexafin gadolinium, a tumor-selective drug targeting thioredoxin reductase and ribonucleotide reductase. *J. Biol. Chem.* 2006; **281;** 10691–10697.
19. Lu J, Papp LV, Fang J, Rodriguez-Nieto S, Zhivotovsky B, Holmgren A. Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. *Cancer Res.* 2006; **66;** 4410–4418.
20. Oh JE, Karlmark Raja K, Shin JH, Hengstschlager M, Pollak A, Lubec G. The neuronal differentiation process involves a series of antioxidant proteins. *Amino Acids* 2005; **29;** 273–282.
21. Sun X, Dobra K, Björnstedt M, Hjerpe A. Upregulation of 9 genes, including that for thioredoxin, during epithelial differentiation of mesothelioma cells. *Differentiation* 2000; **66;** 181–188.

22. Rundlöf AK, Fernandes AP, Selenius M *et al.* Quantification of alternative mRNA species and identification of thioredoxin reductase 1 isoforms in human tumor cells. *Differentiation* 2007; **75;** 123–132.
23. Greene FL, Page DL, Fleming ID *et al. AJCC cancer staging manual*, 6th edn. New York, NY: Springer-Verlag, 2002.
24. Gonzales R, Woods R. *Digital image processing*. Englewood Cliffs, NJ: Prentice Hall, 1992.
25. Cunnea P, Fernandes AP, Capitanio A, Eken S, Spyrou G, Björnstedt M. Increased expression of specific thioredoxin family proteins; a pilot immunohistochemical study on human hepatocellular carcinoma. *Int. J. Immunopathol. Pharmacol.* 2007; **20;** 17–24.
26. Rosell R, Ichinose Y, Taron M *et al.* Mutations in the tyrosine kinase domain of the EGFR gene associated with gefitinib response in non-small-cell lung cancer. *Lung Cancer* 2005; **50;** 25–33.
27. Nalvarte I, Damdimopoulos AE, Nystom C *et al.* Overexpression of enzymatically active human cytosolic and mitochondrial thioredoxin reductase in HEK-293 cells. Effect on cell growth and differentiation. *J. Biol. Chem.* 2004; **279;** 54510–54517.
28. Kakolyris S, Giatromanolaki A, Koukourakis M *et al.* Thioredoxin expression is associated with lymph node status and prognosis in early operable non-small cell lung cancer. *Clin. Cancer Res.* 2001; **7;** 3087–3091.
29. Grogan TM, Fenoglio-Prieser C, Zeheb R *et al.* Thioredoxin, a putative oncogene product, is overexpressed in gastric carcinoma and associated with increased proliferation and increased cell survival. *Hum. Pathol.* 2000; **31;** 475–481.
30. Matsutani Y, Yamauchi A, Takahashi R *et al.* Inverse correlation of thioredoxin expression with estrogen receptor- and p53-dependent tumor growth in breast cancer tissues. *Clin. Cancer Res.* 2001; **7;** 3430–3436.
31. Jagirdar J. Application of immunohistochemistry to the diagnosis of primary and metastatic carcinoma to the lung. *Arch. Pathol. Lab. Med.* 2008; **132;** 384–396.
32. Pankiewicz W, Minarowski L, Niklinska W, Naumnik W, Niklinski J, Chyczewski L. Immunohistochemical markers of cancerogenesis in the lung. *Folia Histochem. Cytobiol.* 2007; **45;** 65–74.
33. Björkhem-Bergman L, Torndal UB, Eken S *et al.* Selenium prevents tumor development in a rat model for chemical carcinogenesis. *Carcinogenesis* 2005; **26;** 125–131.
34. Björkhem L, Teclebrhan H, Kesen E, Olsson JM, Eriksson LC, Björnstedt M. Increased levels of cytosolic thioredoxin reductase activity and mRNA in rat liver nodules. *J. Hepatol.* 2001; **35;** 259–264.
35. Xu Q, Lee C. Discovery of novel splice forms and functional analysis of cancer-specific alternative splicing in human expressed sequences. *Nucleic Acids Res.* 2003; **31;** 5635–5643.

