Overexpression of the TXNDC5 Protein in Non-small Cell Lung Carcinoma

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Abstract. *Thioredoxin domain containing protein 5 (TXNDC5) is a member of the thioredoxin (Trx) domain-containing family of proteins that have been implicated in cancer progression. The expression of TXNDC5 in non-small cell lung carcinoma (NSCLC) tumours compared to patient-matched normal lung tissue was determined and cell line models were used to determine if expression was regulated by hypoxia. Patients and Methods: Samples of tumour and normal lung tissue were taken during surgery and immediately frozen. The expression of TXNDC5 was determined by Western blotting and immunohistochemistry. To analyse the effect of hypoxia on TXNDC5 expression NSCLC cell lines were used. Results: Tumours from 18/29 (62%) individuals exhibited an increase in TXNDC5 expression compared to normal lung tissue ($p<0.05$). TXNDC5 expression was not elevated by hypoxia. Conclusion: TXNDC5 is up-regulated in the majority of resected human NSCLC. Cell line data indicates that the expression of TXNDC5 in tumour cells is not regulated by hypoxia.*

Lung cancer is the leading cause of cancer death worldwide. Non-small cell lung carcinoma (NSCLC) accounts for 80% of all cases. The five year survival rate in Europe is 8% (1) and the median survival after diagnosis is 4-5 months if left untreated (2). Although biomarker-led targeted therapy with, for example, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor may improve upon chemotherapy, this is in a minority of patients with advanced disease. There is a continuing need to determine the molecular basis of NSCLC and so identify further drug targets for prevention and therapy.

A family of proteins emerging as potential targets in the treatment of cancer are the thioredoxin (Trx)-family of proteins. These proteins catalyse the formation of native disulfide bonds and play a critical role in regulating redox homeostasis in the cell (3). The best-characterised member of this family is protein disulphide isomerase (PDI), a ubiquitously expressed endoplasmic reticulum (ER)-resident protein involved in protein folding. PDI possesses two domains with sequence and structural homology to Trx and thiol-disulfide bond exchange activity (3).

A less studied member of the Trx family is thioredoxin domain containing protein 5 (TXNDC5) (synonyms include EndoPDI, endoplasmic reticulum protein 46 (ERP46), mammalian gene collection 3178 (MGC3178)). Originally identified as an ER-directed protein, it possesses three Trx domains and a KDEL (Lys-Asp-Glu-Leu) C-terminal ER localisation motif (4-6). Consistent with the role of thioredoxin proteins in redox homeostasis TXNDC5 expression is up-regulated by hypoxia and protects endothelial cells from hypoxia-induced cell death (6).

The role of Trx proteins in cancer is an area of growing interest. PDI is highly expressed in invasive glioma cells and plays a role in tumour cell invasion (7). Other members of the family, Trx, peroxiredoxin (Prx) and Trx activating enzyme, thioredoxin reductase (TrxR) have been reported to be expressed in a variety of different tumours including NSCLC and expression of these proteins is elevated by hypoxia (8, 9). Trx can stimulate growth by enhancing the activity of growth factors and has been found to stimulate growth in a variety of tumour cell lines (10, 11). It is associated with aggressive tumour growth, resistance to

Abbreviations: TXNDC5: thioredoxin domain containing protein 5, NSCLC: non-small cell lung carcinoma, Trx: thioredoxin, Prx: peroxiredoxin, TrxR: thioredoxin reductase.

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Key Words: Non-small cell lung cancer, TXNDC5, thioredoxin, protein disulphide isomerase.

standard therapy and decreased patient survival and is a valid cancer drug target (3).

In contrast to Trx relatively little is known about the role of TXNDC5 in cancer. TXNDC5 mRNA expression is increased in cancer of the cervix, uterus, colon, stomach, prostate, liver and lung (4, 6, 12-13). That this increased expression may support the tumour cell phenotype has been shown in gastric cancer cell lines where TXNDC5 can enhance cell growth, survival and invasion while silencing TXNDC5 has been reported to restrain the growth and proliferation in these cells (14).

In order to identify novel biomarkers and therapeutic targets for cancer treatment isolating proteins differentially expressed between tumour and patient matched normal tissue is a key strategy. Thus the aim of the present study was to determine the expression of TXNDC5 protein in NSCLC, using fresh-frozen tumour tissue and patient-matched normal tissue collected during surgery for NSCLC. In addition, cell line models were used to investigate whether TXNDC protein expression is regulated by hypoxia in NSCLC cells.

Patients and Methods

Patients and tissue samples. The study was approved by the South West 4 Research Ethics Committee. Patients with suspected lung cancer were identified from an elective thoracic surgery list at the Bristol Royal Infirmary, Bristol, UK and provided informed consent. The study cohort comprised 29 patients; 72% were male and the average age of the cohort was 64.6 years. Ten percent of patients were non-smokers.

The study research nurse was present at surgery to collect the tissue, which was surplus to diagnostic requirements. Three separate samples of the tumour and adjacent normal tissue from the resection margin were taken and flash frozen in liquid nitrogen as quickly as possible (average time taken to freeze samples after surgical removal was 15.9 minutes; range 8-25 minutes). Only NSCLC tumour tissue was used in the study. Using a frozen section a pathologist made an assessment of the proportion of the sample that was tumour and recorded the histology and stage determined according to the TNM classification (15). All the samples (normal, designated N1-3 and tumour, designated T1-3) were stored at –80˚C until required for analysis. Separate samples were prepared for immunohistological analysis by formalin-fixation and paraffin-embedding.

Preparation of human tissue lysates. The frozen tissue samples were homogenised in ice cold 'extraction buffer' (50 mM Tris pH 7.5, 120 mM NaCl, 1% (v/v) Nonidet-P40, 40 mM β-glycerophosphate, 1 mM benzamidine, 1 mM EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, 5 mM Na_3VO_4, 2 mM phenylmethylsulphonyl fluoride). Using a polytron homogeniser the samples were pulsed for 10 sec followed by 30 sec incubation on ice; repeated three times. Tissue homogenates were agitated on a rotator at 4˚C for 30 min and rested on ice for 15 min before centrifugation twice at 16,000 × g for 15 min to remove insoluble material. The protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA). The samples were stored at –80˚C.

Cell culture and hypoxic incubation. NSCLC cell line, A549 was obtained from the American Tissue Culture Collection (Rockville, MD, USA). NSCLC cell line, Hcc95 was kindly supplied by Professor M. Seckl (Imperial College London). The A549 and Hcc95 cells were cultured in DMEM (#D5796 Sigma, Poole, UK) supplemented with 10% foetal bovine serum (FBS, Invitrogen, Paisley, UK), 20000 U/ml penicillin, 7 mM streptomycin and 200 mM glutamine. The cell lines were grown at 37˚C in a humidified atmosphere supplemented with 5% (v/v) CO_2.

Cells in experimental plates, at 90% confluence, were exposed to hypoxic conditions, for the times indicated in the text in a humidified chamber at 37˚C by incubation in a gas mixture containing 1% O_2, 94% N_2 and 5% CO_2. Following incubation the cells were transferred to ice where they were washed twice with ice cold PBS and scraped into NP40 extraction buffer. The cell extracts were incubated on ice for 20 min and then centrifuged at 16,000 × g for 5 min to remove insoluble material. The protein concentration was determined by BCA assay. The samples were stored at –80˚C.

Western blotting analysis. The human lung tissue samples (15 μg protein) and NSCLC cell line lysates (20 μg protein) were solubilised by boiling in Laemmli sample buffer and separated by SDS-PAGE using 4-12% Bis-Tris gradient gels (Invitrogen). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Watford, Hertfordshire, UK). The membranes were blocked using 5% (w/v) bovine serum albumin in TBS-T (20 mM Tris pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween-20) for 1 hour. The membranes were washed, then incubated with primary antibody (1 μg/ml) overnight in TBS-T containing 5% w/v bovine serum albumin, before washing and incubating with the appropriate secondary antibodies, diluted in TBS-T, for 1 hour. Goat polyclonal anti-TXNDC5 (#AF4840) was purchased from R&D Systems (Abingdon, Oxfordshire, UK), mouse monoclonal anti-F_1-ATPase (an antibody raised to the F_1 subunit of adenosine triphosphosphate synthase) from Santa Cruz (Santa Cruz, CA, USA), rabbit polyclonal anti-Trx-1 from Cell Signalling (Boston, MA, USA), mouse monoclonal anti-hypoxia-inducible factor α (Hif-1α) from BD Biosciences (San Jose, CA, USA) and mouse monoclonal α-tubulin from Sigma. Donkey horse radish peroxidase (HRP) conjugated anti-mouse IgG, anti-rabbit IgG and anti-goat IgG antibodies were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, ME, USA). The immunoblots were visualized using an enhanced chemiluminescence detection system (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantified by densitometry (Quantity One Software, BioRad, Hemel Hempstead, Hertforshire, UK). Parallel blotting with an antibody towards the mitochondrial F1-ATPase protein controlled for protein loading. Protein from the tissue of individual patients was run on different gels such that the absolute levels of TXNDC5 expression between patients was not compared.

Immunohistochemistry. Two μm sections were cut from the formalin-fixed and paraffin-embedded normal and tumour lung tissue and were mounted on glass slides. TXNDC5 was detected using a mouse polyclonal antibody (cat #SAB1400718, Sigma). Automated immunohistochemistry was performed using a BOND-III system (Leica Microsystems, Bannockburn, IL, USA). The slides were covered with BOND Universal Covertiles and placed into the BOND-III instrument. The following steps were performed by the instrument according to the manufacturer's instructions (all reagents were purchased from Leica Microsystems). The slides were deparaffinised using BOND Dewax Solution at 50˚C for 30 min.

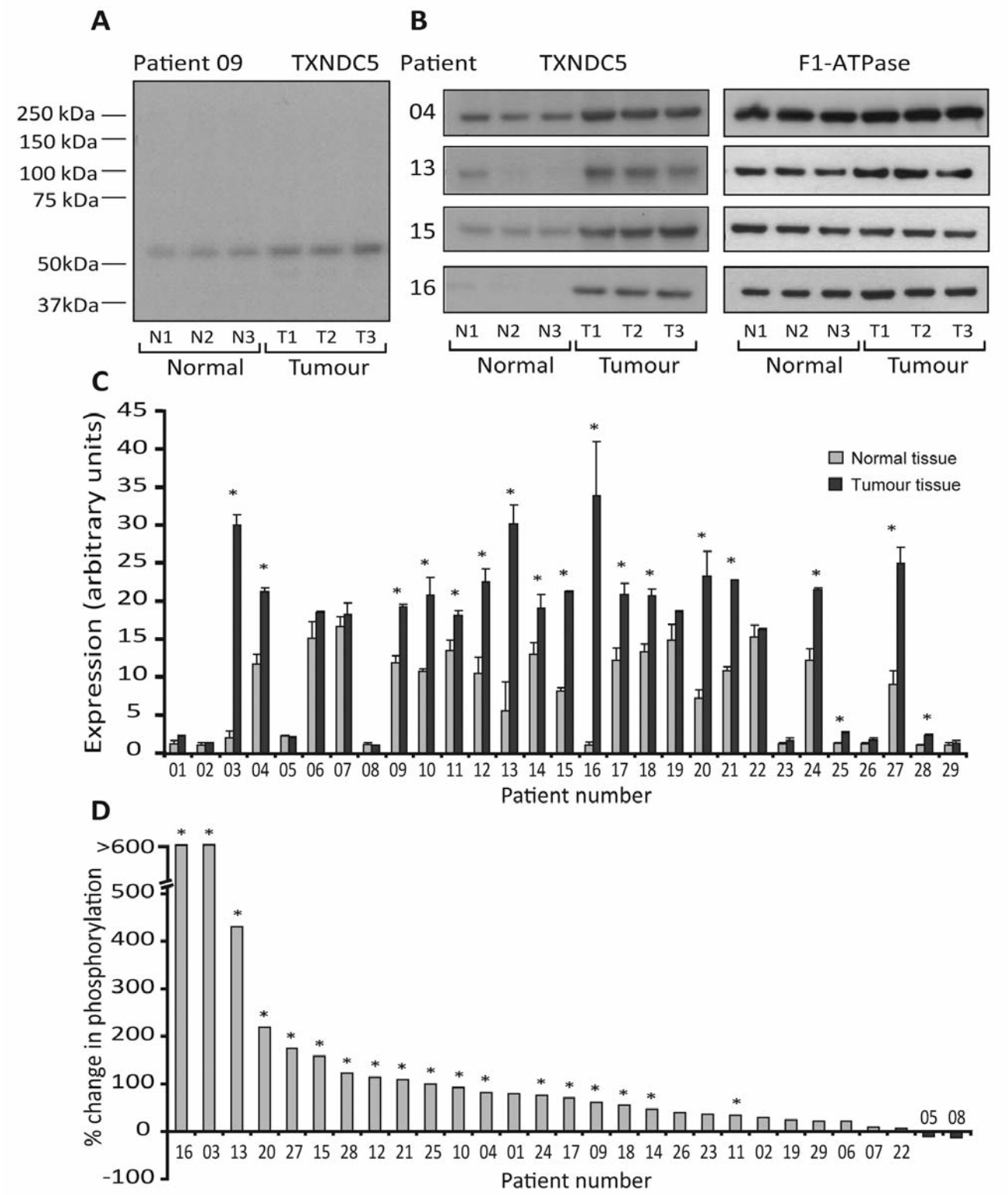

Figure 1. *TXNDC5 expression in NSCLC tissue in comparison to normal lung tissue. A and B: Representative Western blots. N1, 2, 3 and T1, 2, 3 triplicate samples from the same patient's normal and tumour lung tissue respectively. F1-ATPase, loading control. C: Western blotting data quantified by densitometry. Each bar average expression over samples N1-3 or T1-3 ±SEM. *indicates p<0.05 difference in expression between the normal and tumour samples determined by Kruskal-Wallis test. D: Percentage change in TXNDC5 expression in tumour samples in comparison to patient-matched normal, shown in descending order.*

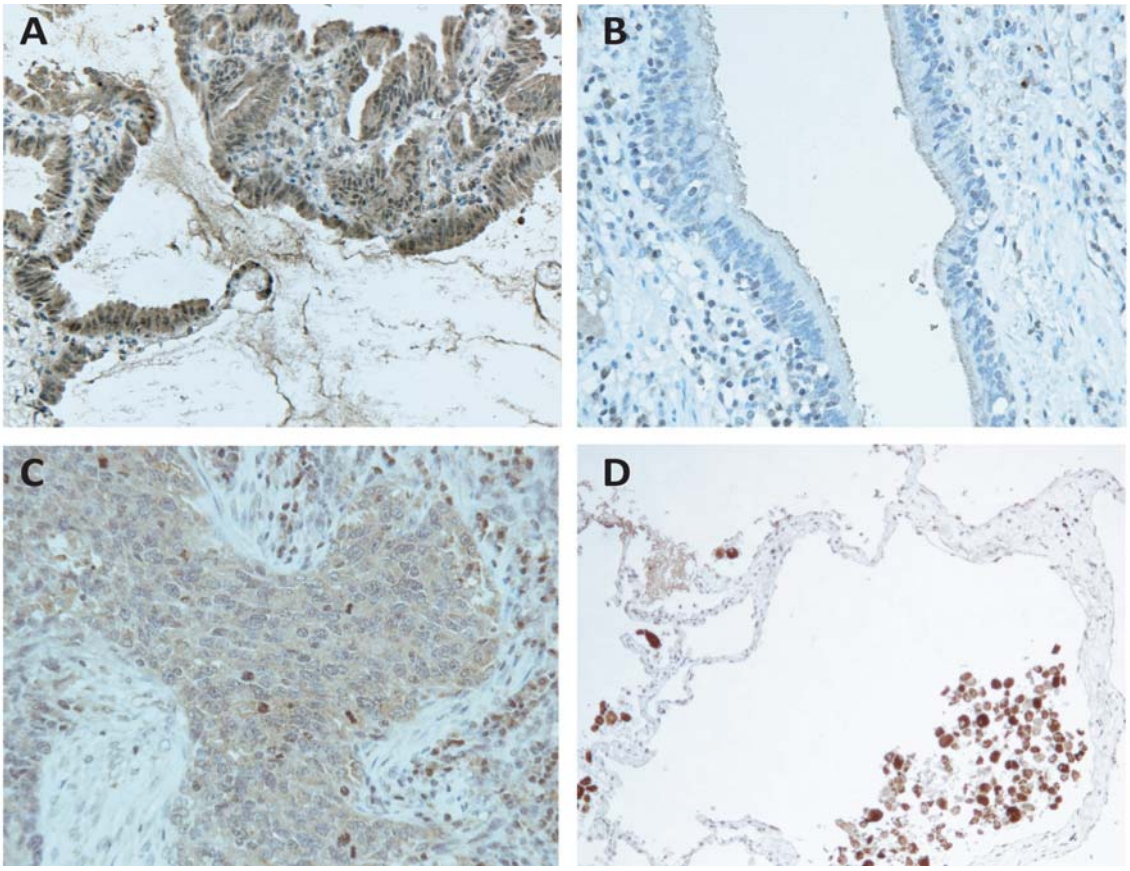

Figure 2. *TXNDC5 immunostaining in normal and tumour human lung tissue. TXNDC5 protein expression in NSCLC tissue and patient-matched normal lung tissue. A: Diffuse cytoplasmic TXNDC5 immunoreactivity in the tumour epithelium of patient 16, (×200 obj). B: Histologically normal lung tissue from patient 16, negative for TXNDC5 immunoreactivity (×300 odj). C: TXNDC5 immunoreactivity in tumour epithelium from patient 03 (×400 obj). D: Histologically normal lung epithelium from patient 03 showing no staining for TXNDC5 (×100 obj). The cluster of cells positive for TXNDC5 are macrophages.*

Antigen retrieval was achieved by heating at 90˚C for 40 min in a high pH buffer (BOND Epitope Retrieval Solution 2). This was followed by a peroxide block for 5 min at room temperature before incubation with a 1:50 dilution of anti-TXNDC5 antibody in BOND antibody diluent. The slides were then incubated with Post Primary reagent for 8 min followed by washing with BOND Wash Solution for 6 min. The slides were incubated with BOND Polymer HRP for 8 min at room temperature followed by a further wash step. The slides were incubated with DAB (3,3'-diaminobenzidine tetrahydrochloride) for 10 min followed by haematoxylin counterstaining for 5 min and finally mounting. Positive staining was identified as those tissue cells containing clear brown staining. For negative controls the primary antibody was omitted.

Results

The tissue samples were confirmed to consist of at least 90% tumour tissue. Fifty three percent of the tumours were adenocarcinomas, 40% squamous cell, 3% bronchiolalveloar carcinomas and 3% other. 8% were Stage Ia, 62% Stage Ib, 4% Stage IIa, 19% Stage IIb and 8% Stage IIIa. (T1, T2 and T3). The expression of TXNDC5 analysed by Western blotting is shown in Figure 1A and 1B.

TXNDC5 migrated at the expected molecular weight of 55kDa with no other proteins being visibly detected by the antibody (Figure 1A). In patients 13 and 16 the level of TXNDC5 expression in the normal tissue was low whereas in patients 04 and 15 TXNDC5 expression in the normal tissue was relatively high. However in all the patients shown, the expression of TXNDC5 was higher in the tumour samples when compared to the patient-matched normal tissue, whereas that of the control protein (F1-ATPase) was relatively unchanged (Figure 1A and B).

The immunoblots of the samples from all 29 patients quantified using densitometry are presented in Figure 1C. TXNDC5 protein expression was up-regulated in the tumours of 62% (18/29) of the patients when compared to the matched normal lung tissue (Figure 1C; Kruskal-Wallis test, $p<0.05$). The data presented in Figure 1C were normalised by calculating the percentage change in

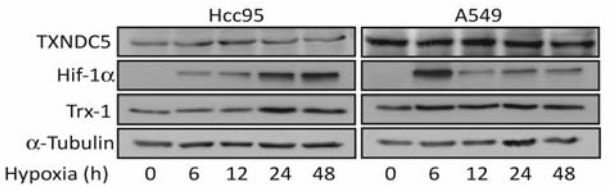

Figure 3. *Effect of hypoxia on TXNDC5 expression. Western blot of A549 and Hcc95 cells incubated under hypoxic conditions. An anti-α-tubulin antibody was used as a control for protein loading.*

expression of TXNDC5 for each patient and the results are shown in Figure 1D where the patients are placed in order of the highest percentage increase to the lowest. In patients in which there was an increase in expression ($p<0.05$), the mean increase was 343% (SEM 164).

To consolidate the data obtained by Western blotting and to analyse cellular expression, the immunohistochemical localization of TXNDC5 was determined using tumour and patient-matched normal tissue (Figure 2). In the tumour tissue TXNDC5 was primarily localised to the tumour cells which exhibited moderate diffuse cytoplasmic immunoreactivity (Figure 2A and 2C). Consistent with the expression levels observed by Western blotting (Figure 1), in the histologically normal lung tissue relatively little or no TXNDC5 immunoreactivity was observed in the epithelium (Figure 2B and 2D). In both tumour and normal tissue TXNDC5 immunoreactivity was observed in macrophages (Figure 2D). No staining was observed in the absence of the TXNDC5 antibody (data not shown).

To investigate whether the expression of TXNDC5 in NSCLC cells is regulated by hypoxia the expression of TXNDC5, Trx1 and the Hif-1α was determined by western blotting in the A549 and Hcc95 cells. Hypoxia had no detectable effect on TXNDC5 expression in either the A549 or Hcc95 cells whereas the expression of Hif-1α and Trx 1 proteins was up-regulated (Figure 3). Continued growth of the control culture under normoxia over the 48 h did not affect the expression of the proteins analysed (not shown).

Discussion

To our knowledge only one previous study has considered the expression of TXNDC5 in lung tumours. In this particular study tissue-array analysis showed mRNA abundance to be greater in lung tumour tissue than in normal tissue in each of six individuals (6). In the present study the protein expression of TXNDC5 in the NSCLC tissue in comparison to the patient-matched normal tissue was up-regulated in 62% of patients. Immunohistochemistry demonstrated that the increase in expression in the tumour tissue was due to an increase in expression in the tumour cells themselves, relative to the expression in the normal epithelium. TXNDC5 mRNA expression has also been found to be up-regulated in other cancer types, suggesting that the increased TXNDC5 protein expression observed here was at least in part a result of increased TXNDC5 gene transcription and not wholly accounted for by an increase in translation or protein stabilization. In colorectal adenoma, the increase was seen in 22/26 (85%) of early stage tumours (4). Taken together with the present study on relatively early stage NSCLC, this suggests that the dysregulation of TXNDC5 protein expression may be an early event in the pathogenesis of tumours of both the colon and lung. TXNDC5 expression has also been reported to be increased in poorly differentiated hepatocellular carcinoma but not in well differentiated tumours (12) consistent with a causative role in the development of more aggressive tumours.

Although, TXNDC5 expression has been reported to be induced by hypoxia in cultured endothelial cells (6), hypoxia had no detectable effect on TXNDC5 expression in either the A549 or the Hcc95 NSCLC cells under conditions where Hif-1α and Trx-1 were both up-regulated (Figure 3).

TrxR is also up-regulated in response to hypoxic conditions and an inhibitor of TrxR (ethaselen) has been found to reduce tumour size in mouse A549 tumour xenografts in combination with cisplatin (16). TrxR was therefore suggested to be a good target for cancer chemotherapy, however this may only be the case for a subset of cells in the tumour that have become exposed to hypoxic conditions. TXNDC5 may represent a preferable target on the basis that its up-regulation may occur *via* a hypoxic-independent mechanism.

There is a clear need for new and effective approaches to treat NSCLC. Combined with the emerging role of the thioredoxin family of proteins in cancer and the fact that we show TXNDC5 was found to be so frequently up-regulated in human NSCLC suggests that it should be further investigated as a novel beneficial therapeutic target in a large proportion of patients.

Acknowledgements

We are grateful to the Wellcome Trust for partial funding of this work. We would also like to thank Mr J.A. Morgan FRCS (University Hospitals Trust, Bristol) for his assistance in providing tissue, Prof. C. Paraskeva (University of Bristol) for help and useful discussions and Prof. Michael Seckl (Imperial College, UK) for the gift of NSCLC cell lines. This manuscript is dedicated to the memory of Mr Morgan.

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Received January 12, 2011
Revised April 5, 2011
Accepted April 7, 2011