Thioredoxin system protein expression in carcinomas of the pancreas, bile duct and ampulla.

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Abstract

Background: Pancreatic cancer (PC), including the ampulla and bile duct, is very aggressive, and thus difficult to treat with effective therapies. The current treatment options have failed to improve PC five-year survival rates over the last 30 to 40 years, which remain very low, at ~3%; there is, therefore, an urgent need to identify new targets and treatment modalities (1). Methods: The protein expression of thioredoxin (Trx), thioredoxin reductase (TrxR) and thioredoxin interacting protein (TxNIP) was assessed in two cancer patient cohorts by standard immunohistochemistry using tissue microarrays. The first cohort was composed of 85 pancreatic adenocarcinomas (PAD) and the second of 145 cancers of the bile duct and ampulla. Results: In the PAD cohort, high cytoplasmic TrxR expression significantly associated with lymph node metastasis (P = 0.033). High expression of cytoplasmic (P = 0.018) and nuclear (P = 0.006) Trx were significantly associated with better overall survival, with nuclear Trx expression remaining significantly associated with survival in multivariate Cox-regression (Hazard Ratio (HR) 0.316; 95% Confidence Interval (95% CI) 0.174-0.573; P < 0.0001) when potentially confounding factors were included (gender, age, tumour size, tumour grade, tumour stage, lymph node status, perineural and venous invasion). In cancers of the bile duct and ampulla, high expression of nuclear TrxR and high cytoplasmic TxNIP were associated with patients aged above 60 years (P = 0.024 and P = 0.049 respectively). Associations were also observed between high nuclear TrxR expression and the presence of venous (P = 0.001) and perineural (P = 0.021) invasion. Low cytoplasmic TxNIP expression was also associated with the presence of perineural invasion (P = 0.025). High expression of cytoplasmic TxNIP was significantly associated with better overall survival (P = 0.0002), which remained significant in multivariate Cox-regression analysis (HR 0.548; 95% CI 0.340-0.882; P = 0.013) when potentially confounding factors were included (tumour grade, stage, lymph node status, perineural and venous invasion). Conclusion: Current findings demonstrate the prognostic importance of Trx system protein expression in pancreatic, bile duct and ampullary cancers, with expression of certain members potentially being involved in disease progression. Current findings warrant a larger follow-up study.

Background

Worldwide, pancreatic cancer (PC) is the seventh leading cause of cancer deaths, with 331,000 deaths per year (2). In the United Kingdom, there were 9,921 new PC cases registered in 2015, and 9,263 PC-related deaths in 2016, making it the sixth leading cause of cancer deaths, and responsible for 6% of all cancer deaths (1). Cancers of the ampulla are less common in the United Kingdom than those of the bile duct and of PCs, with 3,258, 12,638, and 62,310 cases registered between 1998 and 2007 respectively (3).

Patients with bile duct and ampullary cancers have much higher survival rates than PAD patients (five-year survival of 27%, 37% and ~3% respectively), with variation in survival partly explained by differences between both cohorts in terms of tumour resectability (49%, 78% and 10-20% respectively) (1, 4-6). Early diagnosis, due to early symptoms, may explain the higher resectability rate and better survival outcomes of bile duct and ampullary patients over PAD patients. However, after tumour resection, several factors, such as genetic, embryologic, anatomic and histologic factors, have been related to survival for pancreatic, bile duct and ampullary cancer patients, with high tumour stage, large tumour size and lymph node metastases seeming to adversely affect survival regardless of the origin of these cancers (7).

The main treatments for patients with carcinomas of the pancreas, bile duct and ampulla are surgery, chemotherapy and radiotherapy. However, the treatment modalities for PC differ between Europe, including the United Kingdom, and the United States (8). Adjuvant radiotherapy is more commonly employed in the United States than in Europe and the United Kingdom, while combination chemotherapy is used more commonly in Europe and the United Kingdom than in the United States (8). Nevertheless, the current treatment modalities for PC have failed to improve the five-year survival rate over the last 30 to 40 years and in the United Kingdom remains very low, at ~3% (1).

For radiation therapy, a number of resistance mechanisms may explain the lack of treatment success, one of these being inherent or acquired expression of redox proteins i.e. proteins that can scavenge certain chemotherapy- and/or radiation-induced radicals, mainly intracellular reactive oxygen species (ROS), making the treatment less effective. Redox proteins have, therefore, been studied widely for the role they play in regulating therapeutic response of tumour cells, in addition to assessing the

association of their expression with clinicopathological criteria and/or patient survival parameters (9).

The Trx system is an important family of redox related proteins that can regulate redox homeostasis and affect the redox state of different signaling molecules, thereby regulating numerous downstream pathways involved in regulation of cell growth, apoptosis, gene transcription, cell cycle progression and oxidative stress (10-12).

The Trx system consists of thioredoxin (Trx), the activating enzyme thioredoxin reductase (TrxR) and the endogenous inhibitor of the system, thioredoxin-interacting protein (TxNIP). Thioredoxins are a class of low molecular weight redox proteins characterised by a conserved active site (-Cys-Gly-Pro-Cys) found in all Trx family proteins (13, 14).

Trx proteins are 12 kDa proteins that include cytosolic thioredoxin-1 (Trx1), mitochondrial thioredoxin-2 (Trx2) and a larger thioredoxin-like protein (p32TrxL). Cytosolic Trx1 is the most widely studied isoform and acts as the major disulphide reductase of proteins in living cells (14). Reduced Trx1, the bioactive form, binds to apoptosis signal-regulating kinase 1 (ASK-1), a key apoptotic regulator whose activation is essential for tumour necrosis factor α (TNF α)-induced apoptosis, and transforms it to inactive form which protects cells against apoptosis (15). The growth and apoptotic regulatory effects of Trx may also be explained by the selective activation of a number of transcription factors such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), glucocorticoid receptor, TP53, AP1 (activator protein 1) and AP-2 (activator protein 2) (14).

TrxR is a member of the homodimeric pyridine nucleotide-disulphide oxidoreductase family and exists in two forms: cytosol (TrxR1) and mitochondria (TrxR2) (16). TrxR is the only known enzyme that can reduce oxidised Trx by obtaining reducing equivalence from NADPH (17). As an activator of Trx, TrxR therefore plays an important role in the pathways reliant on Trx activity, with its inhibition causing oxidation of Trx leading to activation of p38 and JNK, and downstream apoptosis (18).

TxNIP, also known as vitamin D3 up-regulated protein 1 (VDUP1) or Trx binding protein 2 (TBP2), is a stress-responsive protein that inhibits Trx activity by preventing the recycling of oxidised Trx to the reduced, bioactive, form (16). TXNIP can inhibit the activity of Trx by two pathways. First, oxidised TxNIP ‘Cys 247’ binds reduced Trx ‘Cys 32’ and acts as a competitive inhibitor to remove Trx from

those proteins whose function are inhibited by separation from Trx, such as ASK-1. Second, overexpression of TxNIP, by factors such as disturbed flow and high glucose, resulting in decreased activity of TrxR, leading to an increase in oxidative stress and apoptosis (19).

Numerous studies have investigated the relationship of redox proteins, including the Trx system, with clinicopathological criteria and patient survival outcomes in different types of cancer, including ovarian, breast, gastro-oesophageal, colorectal and brain cancers (20-26). However, expression of Trx system proteins has never been previously studied in any robust way, or associated with clinical characteristics, in PC patient tumours. One previous study demonstrated that pancreatic ductal adenocarcinoma (PDAC) tissues were immunohistochemically more positive for Trx expression (24/32 cases) than pancreatic cystadenocarcinoma or normal pancreas tissues but there was no link to/with clinicopathological criteria or patient survival outcomes (27).

The current study set out to determine if Trx system proteins (Trx, TrxR and TxNIP) were expressed in carcinomas of the pancreas, bile duct and ampulla, and if expression correlated with clinicopathological criteria and/or patient survival.

Methods

Clinical samples

Assessment of expression of the Trx system was conducted using tissue microarrays (TMA's) comprised of tumour tissues collected from patients treated at Nottingham University Hospitals between 1993 and according to REMARK criteria (28). This study has ethical approval from the Nottingham Research Ethics Committee. In total 230 patients were included in the current study: 85 patients with pancreatic adenocarcinoma (PAD) and 145 patients with bile duct and ampullary tumours. 61% of patients with pancreatic adenocarcinoma were male (52/85) with the age of the patients ranging from 35 to 81 years and a median age of 66. 56% of patients with bile duct and ampullary tumours were male (81/145) with the age of the patients ranging from 39 years to 85 years with a median age of 65. Table 1 shows the clinicopathological characteristics for both cohorts.

For both patient cohorts survival was calculated from the date of surgery to the date of death, or from the date of surgery to the last date known to be alive for those patients censored. The median

survival time was 18.2 months for PAD cohort and 18.0 months for bile duct and ampullary tumours cohort.

In the pancreatic cohort 53.7% (36/67) of patients received adjuvant chemotherapy (data was not available for 18 patients), of the 36 patients that did receive adjuvant chemotherapy 52.8% (19/36) received fluorouracil/folinic acid and 13.8% (5/36) received gemcitabine chemotherapy. In the bile duct and ampullary cohort 28.7% (27/94) of patients received adjuvant chemotherapy (data was not available for 51 patients), of the 27 patients that did receive adjuvant chemotherapy 48.1% (13/27) received fluorouracil/folinic acid and 18.5% (5/27) received gemcitabine chemotherapy in the context of clinical trials.

Western blotting

The specificities of Trx, TrxR and TxNIP antibodies were initially assessed by Western blotting using pancreatic cell lysates from PANC-1, MIA PaC-2 and BxPc-3 pancreatic cell lines and breast cancer cell lysates of from breast cancer cell lines, MDA-MB-231 and MCF-7, that were used as positive controls. All cell lines were originally obtained from ATCC with authentication conducted every 4-6 months using Short Tandem Repeat (STR) profiling test. Sub-confluent cells were harvested and resuspended in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail (Thermo) and EDTA solution (Thermo). Lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% (w/v) milk powder in 0.1% PBS/Tween, the nitrocellulose membrane was incubated with primary antibody at 4°C overnight. The primary antibodies used in this study were: rabbit anti- human Trx antibody (1:5000 dilution; Ab133524, Abcam), mouse anti- human TrxR antibody (1:1000 dilution; Ab16847, Abcam) and rabbit anti- human TxNIP antibody (1:1000 dilution; Ab188865, Abcam). Mouse anti human β-actin antibody (1:2000 dilution; Ab8226, Abcam) was used as internal control. Secondary antibody was anti-mouse HRP or anti-rabbit HRP-conjugated antibody (Dako), at room temperature for 1 hour. Membranes were developed with Amersham ECL reagent on hyperfilm (GE Healthcare).

Tissue microarray and immunohistochemistry

Antibody concentrations to be used in IHC were optimised, by IHC as decribed later, using three full-

face PC tissue sections from each of the three PC types i.e. 3 pancreatic adenocarcinomas, 3 ductal adenocarcinomas and 3 ampullary adenocarcinomas. The optimization of antibody concentration was carried out using a Novolink Novocastra polymer detection kit (Leica, Denmark), each using three different concentrations, including one as recommended by the manufacturer in their datasheet. Additional concentrations were tried if initial concentrations did not give appropriate immunohistochemical staining. As antibodies of the Trx system were previously optimised in BC tissues (Zhang, 2014), breast tumour composite section were used as as positive and negative controls. Negative controls omitted the primary antibody from the procedure. By comparing staining patterns from each marker with the negative control, asessed by a specialist pathologist, the appropriate concetration of each antibody (anti-Trx, anti-TrxR and anti-TxNIP antibodies) was determined.

TMA construction and immunuhistochemical process has been previously described (29). Briefly, TMA slides were initially deparaffinised in xylene, followed by rehydration in ethanol and water. Antigen retrieval was performed in 0.01molL^{-1} sodium citrate buffer (pH=6.0) in a microwave for 10 minutes at 750W and 10 minutes at 450W. Tissue was treated with peroxidase block, washed with Tris-buffered saline (TBS), and then treated with protein block solution. Anti-Trx, anti-TrxR and anti-TxNIP antibodies were diluted 1:2000, 1:100 and 1:250 respectively, and applied to the tissue for one hour at room temperature (for anti-Trx and anti-TxNIP) or overnight at 4°C (for anti-TrxR). Following antibody incubation, tissue was washed with TBS prior to application of post primary solution, washed with TBS followed by application of Novolink polymer solution. Immunohistochemical reactions were developed using 3,3’ diaminobenzidine as the chromogenic substrate and tissue was counterstained with heamotoxylin prior to dehydration in ethanol and fixation in xylene. Breast tumour composite sections, comprised of 6 stage 1 breast tumours of grade 1 to 3, were included as positive controls with each run, with the negative control having primary antibody substituted for PBS.

All cores were assessed semi-quantitatively using an immunohistochemical H-score using a HPF Nikon Eclipse E600 microscope at 200x magnification. Staining intensity was assessed as; none (0), weak (1), medium (2) and strong (3) over the percentage area of each staining intensity. H scores were

calculated by multiplying the percentage area by the intensity grade (H score range 0–300). Each core was assessed individually by two individuals, including one specialist histopathologist, blinded to clinical data, and a consensus agreed. An average H-score was generated by taking the mean H-score of the three cores (n=187; n=72 PAD cohort, n=115 bile duct and ampullary cohort) or two cores (n=43; n=13 PAD cohort, n=30 bile duct and ampullary cohort) available for each patient included in this study (n=230).

Statistical analysis

The relationship between categorised protein expression and clinicopathological variables was assessed using Pearson Chi Square (χ^2) test of association. Survival curves were plotted according to the Kaplan-Meier method and significance determined using the log-rank test. Multivariate survival analysis was performed by using the Cox Proportional Hazards regression model. All differences were deemed statistically significant at the level of $P < 0.05$. Statistical analysis was performed using SPSS 23.0 software (IBM Corporation). Stratification cut points were determined using X-Tile software (Yale School of Medicine) and were determined prior to statistical analyses (30).

Results

Antibody specificity was determined prior to immunohistochemical staining (supplementary figure 1). Figure 1 shows representative photomicrographs of different staining patterns, i.e. weak, moderate or strong staining of cytoplasmic or nuclear expressision, of Trx system protein expression from PAD TMAs. Supplementary figure 2 shows representative photomicrographs of staining of Trx system protein expression from the carcinomas of the pancreas, bile duct and ampulla TMAs.

In the PAD cohort, cytoplasmic Trx had a median H-score of 210 with values ranging from 100 to 300; nuclear Trx had a median H-score of 225 and ranged from 50 to 300; cytoplasmic TxNIP had a median H-score of 150 and ranged from 0 to 267; cytoplasmic TrxR had a median H-score of 75 and ranged from 0 to 225; nuclear TrxR had a median H-score of 75 and ranged from 0 to 225. The X-tile cut point for cytoplasmic Trx was 160, nuclear Trx was 234, cytoplasmic TxNIP was 217, cytoplasmic TrxR was 58 and nuclear TrxR was 58; with 82.0% (61/74), 45.2% (33/73), 17.5% (14/80), 61.8% (47/76) and 61.8% (47/76) having high protein expression respectively.

In the bile duct and ampullary carcinoma cohort, cytoplasmic Trx had a median H-score of 150 and ranged from 0 to 300; nuclear Trx had a median H-score of 166.7 and ranged from 0 to 300; cytoplasmic TxNIP had a median H-score of 166.7 and ranged from 0 to 300; cytoplasmic TrxR had a median H-score of 66.7 and ranged from 0 to 250; nuclear TrxR had a median H-score of 66.7 and ranged from 0 to 250. The X-tile cut point for cytoplasmic Trx was 142, nuclear Trx was 133, cytoplasmic TxNIP was 85, cytoplasmic TrxR was 167 and nuclear TrxR was 75; with 60.2 % (77/128), 63.3% (81/128), 75.6% (99/131), 10.7% (14/131) and 47.4% (63/133) having high protein expression respectively.

The correlation between expression levels of the proteins with one another was assessed using the Spearman rank correlation coefficient. In the PAD cohort, cytoplasmic TxNIP expression had a statistically significant, albeit weak, correlation with cytoplasmic TrxR ($r = 0.234$, $P = 0.038$) and nuclear TrxR expression ($r = 0.241$, $P = 0.032$). In addition, cytoplasmic TrxR expression was strongly correlated nuclear TrxR expression ($r = 0.711$, $P < 0.001$). Nuclear expression of Trx was also correlated with cytoplasmic Trx expression ($r = 0.549$, $P < 0.001$).

In the bile duct and ampullary tumours, cytoplasmic Trx expression had a statistically significant and strong correlation with nuclear Trx expression ($r = 0.653$, $P < 0.001$), cytoplasmic TrxR expression ($r = 0.436$, $P < 0.001$) and nuclear TrxR expression ($r = 0.328$, $P < 0.001$). In addition, nuclear Trx expression correlated with cytoplasmic TrxR expression ($r = 0.2$, $P = 0.25$) and nuclear TrxR expression ($r = 0.376$, $P < 0.001$). Cytoplasmic TrxR expression correlated strongly with nuclear TrxR expression ($r = 0.653$, $P < 0.001$).

Associations with clinicopathological criteria

Levels of protein expression were assessed in light of clinicopathological criteria in the PAD and the bile duct and ampullary cancer cohorts to determine associations. In the PAD cohort, the only association observed was between high cytoplasmic TrxR expression and lymph node metastasis ($\chi^2 = 4.533$, d.f. = 1, $P = 0.033$) (Supplementary Tables 1 and 2).

In bile duct and ampullary cancers, high expression of cytoplasmic TxNIP and of nuclear TrxR and

were associated with patients aged above 60 years (χ^2 = 3.892, d.f. = 1, P = 0.049 and χ2 = 5.091, d.f. = 1, P = 0.024 respectively) (Table 2). Associations were also observed between high nuclear TrxR expression and the presence of venous invasion (χ^2 = 10.548, d.f. = 1, *P* = 0.001) and the presence of perineural invasion (χ^2 = 5.314, d.f. = 1, *P* = 0.021). Low TxNIP expression associated with the presence of perineural invasion (χ^2 = 5.044, d.f. = 1, *P* = 0.025) (Table 3).

Relationship with clinical outcome

In the PAD cohort, high expression of both cytoplasmic and nuclear Trx were significantly associated with better overall survival (*P* = 0.018 and *P* = 0.006 respectively) (Figure 2, panel A and B). Cytoplasmic TrxR, nuclear TrxR and cytoplasmic TxNIP expression showed no association with overall survival (Figure 2 panel C, D and E).

In multivariate Cox-regression, potentially confounding factors of gender, age, tumour size, grade, stage, lymph node status, perineural and venous invasion were included even though not independently associated with survival; with individual Kaplan-Meier statistics of *P* = 0.380, *P* = 0.694 *P* = 0.419, *P* = 0.820, *P* = 0.349, *P* = 0.063, *P* = 0.163 and *P* = 0.491 respectively. Nuclear Trx expression remained significant for survival in multivariate analysis (Hazard Ratio (HR) = 0.316; 95% Confidence Interval (95% CI) = 0.174-0.573; *P* < 0.001) whilst cytoplasmic Trx was not significant (HR = 0.5; 95% CI = 0.218 -1.146; *P* = 0.102) (Table 4 panel A and B).

In cancers of the bile duct and ampulla, cytoplasmic Trx, nuclear Trx, cytoplasmic TrxR and nuclear TrxR expression showed no association with overall survival (Figure 3, panel A, B, C and D). However, high expression of cytoplasmic TxNIP significantly associated with better overall survival (*P* = 0.0002) (Figure 3, panel E), which remained significant in multivariate Cox-regression analysis (HR = 0.548; 95%CI = 0.340-0.882; *P* = 0.013) (Table 5). In the multivariate Cox-regression for this cohort the potential confounding factors of patient grade, stage, lymph node status, perineural and venous invasion were included and were significantly associated with survival, with individual Kaplan-Meier statistics of *P* = 0.011, *P* = 0.004, *P* = 0.003 *P* = 0.001 and *P* = 0.012 respectively.

As Trx and TrxR were expressed in both nucleus and cytoplasm, data were also analysed by grouping

patients into combinations based upon expression profiles, i.e. low nuclear staining with low cytoplasmic, low nuclear with high cytoplasmic, high nuclear with low cytoplasmic and high nuclear with high cytoplasmic. In the PAD cohort, no significant correlation was observed in the combination analysis between nuclear and cytoplasmic expression of Trx or TrxR.

Equally, in the bile duct and ampullary carcinoma cohort, no significant correlation was observed from the analysis of combined nuclear and cytoplasmic TrxR expression. However, low nuclear with high cytoplasmic expression of Trx (n=14) showed longer overall survival than other three subgroups (n=114); either against each separate subgroup (P = 0.017) (Figure 4A) or when the three subgroups were combined together (P = 0.002) (Figure 4B).

Discussion

The Trx system regulates the redox state of different signalling molecules and, as a result, can regulate cell growth, apoptosis, gene transcription, cell cycle progression and ability to deal with oxidative stress (10-12). Several studies have reported associations between Trx system protein expression and clinicopathological criteria and patient survival outcome in different cancer types (20-26). Nevertheless, the prognostic significance of expression of the Trx system has never been previously assessed in PC patient tumours. One previous study demonstrated that PDAC tissues were immunohistochemically more positive for Trx expression (24/32 cases) than pancreatic cystadenocarcinoma or normal pancreas tissues (27), suggesting a possible association of Trx expression with malignant potential of PDAC.

Current findings show that high cytoplasmic TrxR expression was associated with lymph node metastasis (P = 0.049). A previous study, albeit in a different cancer type (50 patients with oral squamous cell carcinoma), described an association between low TrxR expression and lymph node metastasis (P = 0.027) (31). It may well be, therefore, that TrxR plays a role in regulating lymph node metastasis. TrxR expression was also of interest in the bile duct and ampulla cancer cohort, with current data showing that high nuclear TrxR expression was associated with the presence of venous (P = 0.001) and perineural invasion (P = 0.021), again suggesting that in cancers of the pancreas, bile duct and ampulla, TrxR may be involved in tumour invasion.

Low cytoplasmic TxNIP expression was also significantly associated with the presence of perineural invasion ($P = 0.025$) in the bile duct and ampulla cancer cohort. In agreement with such findings, a previous study also reported an association between high TxNIP and absence of perineural invasion ($P = 0.030$) in 140 gastro-oesophageal adenocarcinoma patients (23).

In the PAD patients, current findings demonstrate that high expression of cytoplasmic Trx ($P = 0.018$) and nuclear Trx ($P = 0.06$) is significantly associated with better overall survival, with nuclear Trx expression remaining significantly associated with survival in multivariate Cox-regression analysis ($P < 0.0001$). Such a finding, in light of expression in other tumour types, is somewhat unexpected. Raffle and colleagues observed that high expression of Trx was significantly associated with poor overall survival ($P = 0.004$) in 12 colorectal cancer patients (32). In 154 ovarian cancer patients, low cytoplasmic expression of Trx was significantly associated better progression-free survival ($P = 0.032$), whereas Trx nuclear expression did not ($P = 0.455$) (20). And in 65 gastric cancer patients high expression of Trx was significantly associated with poor recurrence-free ($P = 0.008$) and overall survival ($P = 0.015$) (33). However, a study in 174 Hodgkin lymphoma patients produced data similar to current findings, showing that high cytoplasmic Trx expression associated with better failure-free survival ($P = 0.049$) and which remained significant in multivariate Cox-regression analysis ($P = 0.023$) (34). The relative importance of expression may, therefore, vary from tumour type to tumour type with no overall generalisations possible. Current data also show no relationship between cytoplasmic or nuclear TrxR expression with overall survival, unlike a study in 98 locally advanced breast cancer that showed that high expression of TrxR ($P = 0.021$) and TxNIP ($P = 0.021$) were significantly associated with better distant metastasis-free survival (22).

In the bile duct and ampulla carcinoma cohort, current data reveal a strong association between high cytoplasmic TxNIP expression and better overall survival, which remained significant in multivariate Cox-regression analysis. Such data are comparable with the previous breast cancer study mentioned above, that showed high expression of TxNIP was significantly associated with distant metastasis-free survival ($P = 0.021$) and overall survival ($P = 0.037$) (22). Lim and colleagues also observed a significant correlation between high expression of TxNIP and longer relapse-free survival ($P = 0.036$)

in 65 gastric cancer patients (33). Furthermore, high expression of TxNIP was significantly associated with a better disease specific survival (P = 0.016) in 66 gastro-oesophageal adenocarcinomas patients (23), with a recent study also showing that high-grade meningioma patients with strong TXNIP expression (n=37) had longer recurrence-free time (P = 0.02) than those with weak expression of TxNIP (n=28) (25).

Althought carcinomas of the pancreas, bile duct and ampulla have overlapping symptoms and a common treatment, such differences between the results of two cohorts may be due to the significant differences in their survival, and accumulating evidence displaying differences in their biology, including their molecular profiles (35).

A further important aspect to note is that this study describes the protein expression of Trx system family members and not their relative activity levels. Determining the activity of Trx system family members may be possible as part of future in vitro studies, using Trx/TrxR activity assays such as the insulin reduction assay (Kunkel et al., 1997).

Further analysis, in the bile duct and ampullary carcinoma cohort, showed that low nuclear with high cytoplasmic expression of Trx showed longer overall survival than other three subgroups; either against each subgroup (P = 0.017) or when the three subgroups were combined (P = 0.002). In contrast to the current findings, Woolston and colleagues investigated the associations between Trx expression and ovarian cancer patients' survival, and observed that high nuclear with low cytoplasmic expression of Trx demonstrated a significantly better overall survival either against each separate subgroup (P = 0.04) or when the three subgroups were combined together (P = 0.004). Furthermore, high nuclear with low cytoplasmic expression of Trx were associated with better progression-free survival either against each separate subgroup (P = 0.025) or when the three subgroups were combined together (P = 0.003) (20).

A number of studies have examined the differences in the subcellular localization of Trx protein in the mammalian cell, suggesting that Trx protein was in reduced state within the nucleus more than in the cytoplasm, and was imported from the cytoplasm during oxidative stress, both in normal and malignant cancer cells (36-40). Furthermore, a previous study has demonstrated that nuclear Trx

protein offers better protection against oxidative stress than cytoplasmic or mitochondrial Trx protein in human colonic epithelial cells (41). Taken together with previous studies, current data suggest that the Trx system plays a role in the activation of nuclear transcription factors that potentially govern cellular responses to oxidative stress.

In summary, this study demonstrates that high nuclear and high cytoplasmic expression of Trx is associated with better overall survival in PAD patients, and that high cytoplasmic TxNIP expression is associated with better survival in bile duct and ampullary cancer patients, with each being potentially important independent prognostic factors.

Conclusion

Trx system protein expression is important in pancreatic, bile duct and ampullary cancers, with expression of certain members potentially being involved in disease progression. The current findings warrant a larger follow-up study with increased numbers of patients.

Abbreviations

PC: Pancreatic cancer; **Trx:** thioredoxin; **TrxR:** Thioredoxin reductase; **TxNIP:** Thioredoxin interacting protein; **PAD:** Pancreatic adenocarcinomas; **HR:** Hazard Ratio; **ROS:** Reactive oxygen species; **Trx1:** Cytosolic thioredoxin-1; **Trx-2:** Mitochondrial thioredoxin-2; **p32TrxL:** Larger thioredoxin-like protein; **ASK-1:** Apoptosis signal-regulating kinase 1; **TNF α:** Tumour necrosis factor α; **NF-κB:** Nuclear factor kappa-light-chain-enhancer of activated B cells**; AP-1:** Activator protein 1**; AP-2:** Activator protein 2; **TrxR1:** Cytosolic TrxR; **TrxR2:** Mitochondrial TrxR2; **VDUP1:** Vitamin D3 up-regulated protein 1; **TBP2:** Thioredoxin binding protein 2; **PDAC:** Pancreatic ductal adenocarcinoma; **TMA:** Tissue microarrays; **STR:** Short Tandem Repeat; **TBS:** Tris-buffered saline; **χ^2:** Chi Square.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the National Regional Ethics Service Committee East Midlands - Nottingham 1 for the use of anonymised archival specimens and the requirement for patient/relative consent was waived by the Ethics Committee.

Consent for Publication

NA

Availability of data and material

Additional data has been submitted as supplementary information with original H-scores and images being held in a secure database. Such information can be made available via contacting the corresponding author.

Competing interests:

The authors declare that they have no conflicts of interest.

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Authors' contributions

KSA carried out the staining; AMZ and KSA carried out the scoring; AMZ, DNL, SJS, SGM carried out data collection; SJS, KSA, SGM participated in data analysis; KSA, SJS, SGM participated in design and coordination of the study. SGM conceived of the study.

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NA

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Tables

Table 1. Clinicopathological variables of the PAD cohort of 85 patients and the bile duct and ampullary cohort of 145 patients.

Characteristics	PAD cohort=(85)	Bile duct and Ampullary cancer cohort (n=145)
	Frequencies (%)	Frequencies (%)
Age		
≤ 60 years	27 (31.8)	46 (31.7)
> 60 years	56 (65.9)	98 (67.6)
Missed	2 (2.4)	1 (0.7)
Gender		
Male	52 (61.2)	81 (55.9)
Female	33 (38.8)	64 (44.1)
Missed	0 (0)	0 (0)
Tumour size		
≤ $2cm^2$	6 (7.1)	56 (38.6)
> $2cm^2$	77 (90.6)	87 (60)
Missed	2 (2.4)	2 (1.4)
Tumour stage		
I	1 (1.2)	3 (2.1)
II	18 (21.2)	30 (20.7)

III	62 (72.9)	106 (73.1)
IV	3 (3.5)	5 (3.4)
Missed	1 (1.2)	1 (0.7)
Lymph node stage		
Negative	28 (32.9)	52 (35.9)
Positive	54 (63.5)	85 (58.6)
Missed	3 (3.5)	8 (5.5)
Venous invasion		
Absent	30 (35.3)	55 (37.9)
Present	54 (63.5)	88 (60.7)
Missed	1 (1.2)	2 (1.4)
Prenural invasion		
Absent	15 (17.6)	60 (41.4)
Present	69 (81.2)	84 (57.9)
Missed	1 (1.2)	1 (0.7)

Table 2. Associations between Trx and TxNIP protein expression and clinicopathological variables in the bile duct and ampullary carcinoma cohort. The number of observations for the cohort is shown for each clinicopathological variable; the table does not include the number of observations where clinicopathological data were not available. The frequency of observed clinicopathological variables is noted next to the variable subgroup. The P-values are resultant from Pearson Chi Square test of association ($\chi 2$) or Fisher's Exact test in a 2×2 table if a cell count was less than 5 (indicated by *). Significant P-values are indicated by bold font.

Variable	Trx (cytoplasmic)			Trx (nuclear)			T	
	Low	High	P-value	Low	High	P-value	Low	H
Age								
≤ 60 years	17 (13.1)	25 (19.2)	0.865	18 (13.8)	24 (18.5)	0.401	16 (11.9)	30
> 60 years	37 (28.5)	51 (39.2)		31 (23.8)	57 (43.8)		17 (12.7)	71
Gender								
Male	26 (19.8)	47 (35.9)	0.144	25 (19.1)	48 (36.6)	0.402	17 (12.6)	57
Female	28 (21.4)	30 (22.9)		24 (18.3)	34 (26.0)		17 (12.6)	44
Tumour size								
≤ $2cm^2$	16 (12.3)	32 (24.6)	0.187	18 (13.8)	30 (23.1)	0.917	11 (8.20)	39
> $2cm^2$	37 (28.5)	45 (34.6)		30 (23.1)	52 (40.0)		23 (17.2)	61
Tumour stage								
1	1 (0.8)	2 (1.5)	0.778	2 (1.5)	1 (0.8)	0.329	0 (0.0)	3
2	11 (8.4)	16 (12.2)		12 (9.2)	15 (11.5)		5 (3.7)	23
3	41 (31.3)	55 (42.0)		32 (24.4)	64 (48.9)		28 (20.7)	71
4	1 (0.8)	4 (3.1)		3 (2.3)	2 (1.5)		1 (0.7)	4
Node status								
Negative	17 (13.5)	29 (23.0)	0.456	17 (13.5)	29 (23.0)	0.937	27 (20.9)	22
Positive	35 (27.8)	45 (35.7)		29 (23.0)	51 (40.5)		40 (31.0)	40
Venous invasion								
Absent	21 (16.2)	29 (22.3)	0.821	22 (16.9)	28 (21.5)	0.186	11 (8.2)	41
Present	32 (24.6)	48 (36.9)		26 (20.0)	54 (41.5)		23 (17.2)	59
Perineural invasion								
Absent	21 (16.0)	36 (27.5)	0.371	26 (19.8)	31 (23.7)	0.088	9 (6.7)	49
Present	33 (25.2)	41 (31.3)		23 (17.6)	51 (38.9)		25 (18.5)	52

Table 3. Associations between TrxR protein expression and clinicopathological variables in the bile duct and ampullary carcinoma cohort. The number of observations for the cohort is shown for each clinicopathological variable; the table does not include the number of observations where

clinicopathological data were not available. The frequency of observed clinicopathological variables is noted next to the variable subgroup. The P-values are resultant from Pearson Chi Square test of association ($\chi 2$) or Fisher's Exact test in a 2×2 table if a cell count was less than 5 (indicated by *). Significant P-values are indicated by bold font.

Variable	TrxR (cytoplasmic)			TrxR (nuclear)		
	Low	High	P-value	Low	High	P-value
Age						
≤ 60 years	41 (30.6)	4 (3.00)	0.773*	30 (22.4)	15 (11.2)	0.024
> 60 years	79 (59.0)	10 (7.50)		41 (30.6)	48 (35.8)	
Gender						
Male	68 (50.4)	8 (5.90)	0.946	37 (27.4)	39 (28.9)	0.219
Female	53 (39.3)	6 (4.40)		35 (25.9)	24 (17.8)	
Tumour size						
≤ $2cm^2$	47 (35.1)	5 (3.70)	0.802	30 (22.4)	22 (16.4)	0.385
> $2cm^2$	73.1 (54.5)	9 (6.70)		41 (30.6)	41 (30.6)	
Tumour stage						
1	3 (2.2)	0 (0.0)	0.100	2 (1.5)	1 (0.7)	0.461
2	23 (17.0)	4 (3.0)		14 (10.4)	13 (9.6)	
3	92 (68.1)	8 (5.9)		55 (40.7)	45 (33.3)	
4	3 (2.2)	2 (1.5)		1 (0.7)	4 (3.0)	
Node status						
Negative	9 (7.0)	38 (29.5)	0.326	43 (33.3)	6 (4.7)	0.522
Positive	22 (17.1)	60 (46.5)		73 (56.6)	7 (5.4)	
Venous invasion						
Absent	44 (32.8)	5 (3.7)	0.944	35 (26.1)	14 (10.4)	0.001
Present	76 (56.7)	9 (6.7)		36 (26.9)	49 (36.3)	
Perineural invasion						
Absent	48 (36.5)	9 (6.7)	0.077	37 (27.4)	20 (14.8)	0.021
Present	73 (54.1)	5 (3.7)		35 (25.9)	43 (31.9)	

Table 4. Multivariate Cox Regression analysis of factors associated with overall survival in carcinomas of the pancreas. Exp (B) is used to denote hazard ratio, and 95% CI is used to denote 95% confidence interval. Significant P-values are indicated by bold font.

A

PAD cohort	P value	EXP(B)	95.0% CI for EXP(B)	
			Lower	Upper
Cytoplasmic Trx expression	0.102	0.5	0.218	1.146
Gender	0.579	1.178	0.662	2.096
T stage	0.493	0.772	0.368	1.619
Node status	0.113	1.727	0.878	3.398
Venous invasion	0.833	1.068	0.581	1.962
Prenural invasion	0.305	1.521	0.682	3.392
Tumour size	0.221	1.825	0.697	4.784
Patient age	0.804	0.929	0.518	1.666
Grade	0.874	1.045	0.608	1.796

B

PAD cohort	P value	EXP(B)	95.0% CI for EXP(B)	
			Lower	Upper
Nuclear Trx expression	<0.0001	0.324	0.177	0.592
Gender	0.182	1.482	0.831	2.64
T stage	0.99	1.004	0.497	2.031
Node status	0.015	2.353	1.178	4.698
Venous invasion	0.292	1.412	0.744	2.679
Prenural invasion	0.985	1.008	0.458	2.217
Tumour size	0.132	2.202	0.788	6.155
Patient age	0.601	1.176	0.642	2.154
Grade	0.644	1.135	0.664	1.94

Table 5. Multivariate Cox Regression analysis of factors associated with overall survival in carcinomas of the bile duct and ampulla. Exp (B) is used to denote hazard ratio, and 95% CI is used to denote 95% confidence interval. Significant P-values are indicated by bold font.

Bile duct and ampullary cancer cohort	P value	EXP(B)	95.0% CI for EXP(B)	
			Lower	Upper
TxNIP expression	0.013	0.548	0.34	0.882
T stage	0.063	1.634	0.974	2.741
Node status	0.054	1.586	0.992	2.537
Venous invasion	0.916	0.975	0.606	1.568
Prenural invasion	0.251	1.309	0.827	2.073
Grade	0.05	1.5	0.999	2.251

Additional Files

Legend of supplementary tables:

Supplementary Table 1: Associations between Trx and TxNIP protein expression and various clinicopathological variables in the PAD cohort. The number of observations for the cohort is shown for each clinicopathological variable; the table does not include the number of observations where clinicopathological data were not available. The frequency of observed clinicopathological variables is noted next to the variable subgroup. The P-values are resultant from Pearson Chi Square test of association ($\chi 2$) or Fisher's Exact test in a 2×2 table if a cell count was less than 5 (indicated by *).

Supplementary Table 2: Associations between TrxR protein expression and various clinicopathological variables in the PAD cohort. The number of observations for the cohort is shown for each clinicopathological variable; the table does not include the number of observations where clinicopathological data were not available. The frequency of observed clinicopathological variables is noted next to the variable subgroup. The P-values are resultant from Pearson Chi Square test of association ($\chi 2$) or Fisher's Exact test in a 2×2 table if a cell count was less than 5 (indicated by *).

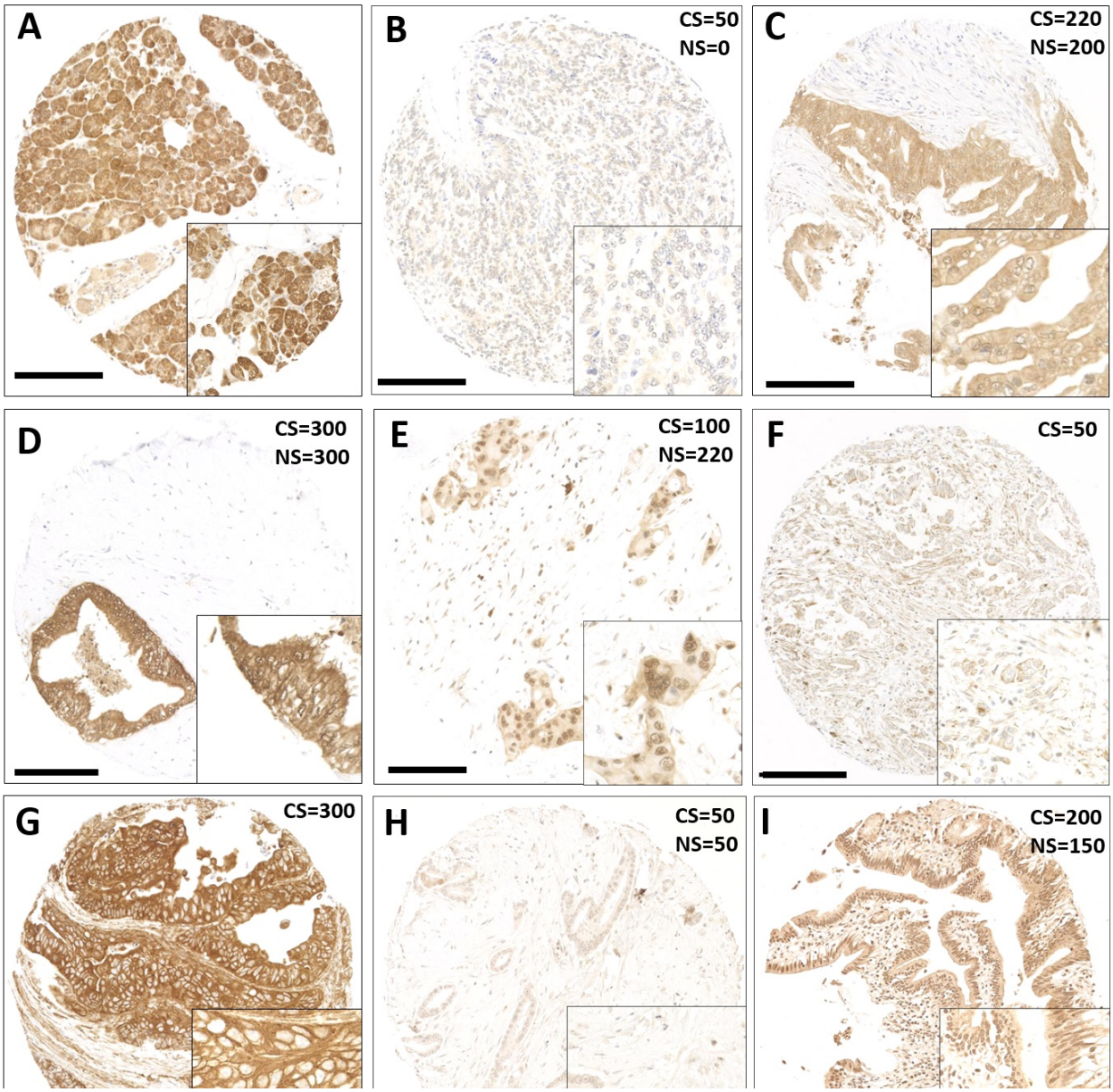
Legend of supplementary figures:

Supplementary Figure 1: Specificity of Trx, TrxR and TxNIP antibodies. Lysates from (1)

PANC-1, (2) MIA Paca-2 and (3) BxPc-3, (4) MDA-MB-231 and (5) MCF-7 cancer cell lines were subjected to ECL-Western blot to assess the specificity of Trx, TrxR and TxNIP antibodies.

Supplementary Figure 2: Representative photomicrographs of protein expression. A, B and C are negative controls of PC tissue sections representing PAD, bile ductal and ampullary carcinomas respectively. D, E and F are expression of Trx, TrxR and TxNIP in PADs respectively. G, H and I are expression of Trx, TrxR and TxNIP in bile ductal carcinomas respectively. J, K and L are expression of Trx, TrxR and TxNIP in ampullary carcinomas respectively. Photomicrographs are at 10x magnification with 20x magnification inset box where scale bar shows 200µm.

Figures



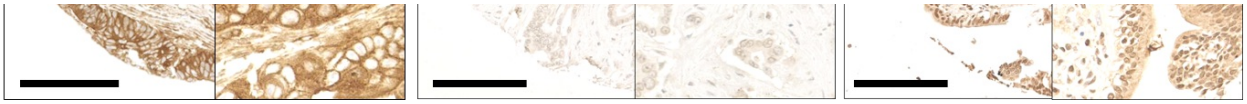
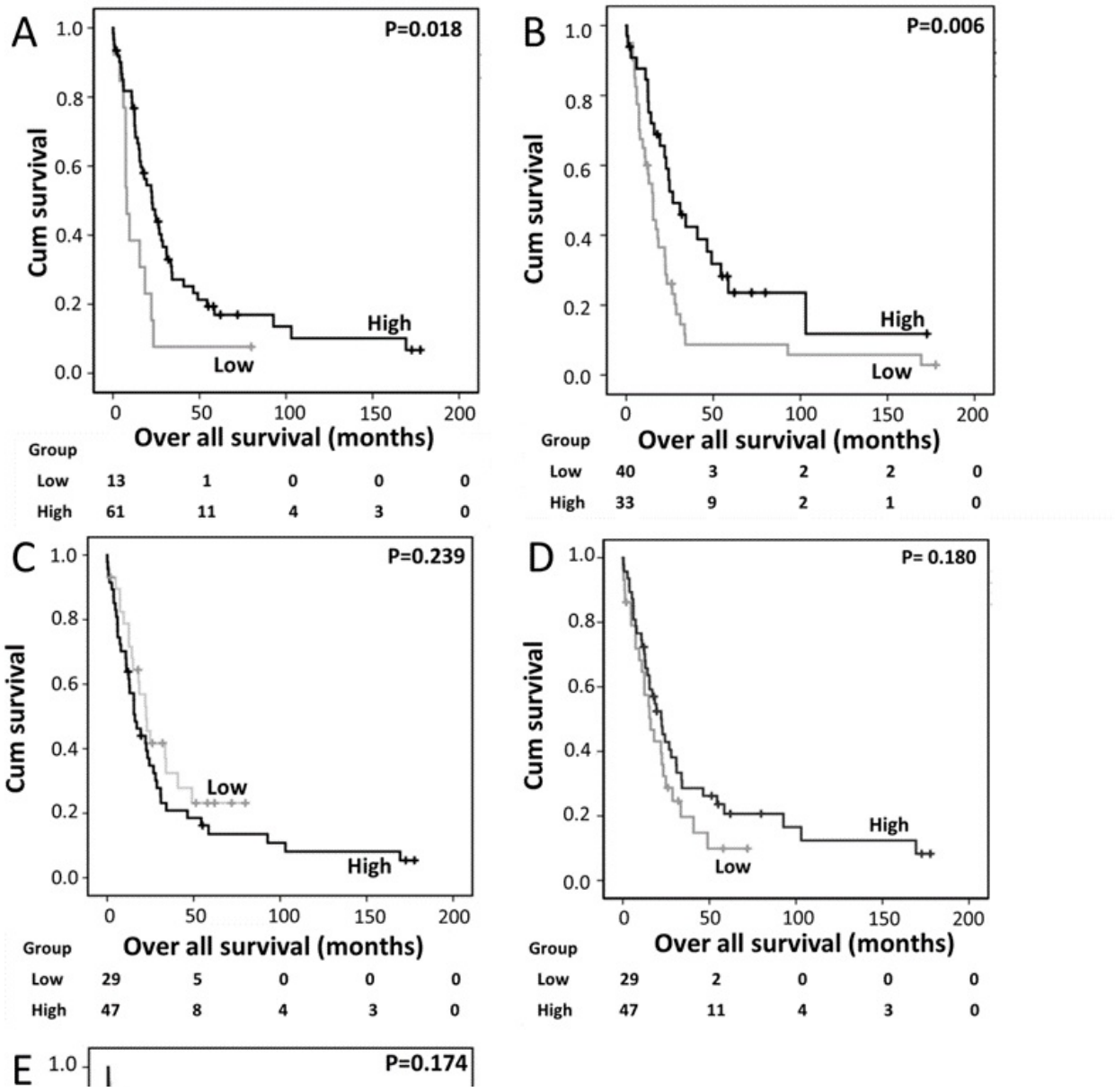


Figure 1

Representative photomicrographs of different staining patterns of Trx system protein expression from PAD TMAs. (A) benign pancreatic tissue, (B) weak staining of Trx, (C) moderate staining of Trx, (D) strong staining of Trx, (E) weak cytoplasmic staining with moderate nuclear staining of Trx, (F) weak cytoplasmic staining of TxNIP, (G) strong cytoplasmic staining of TxNIP, (H) weak staining of TrxR, (I) moderate staining of TrxR. Photomicrographs are at 10x magnification with 20x magnification inset box where scale bar shows 200µm. Abbreviation: CS=Cytoplasmic H-score, NS= Nuclear H-score.



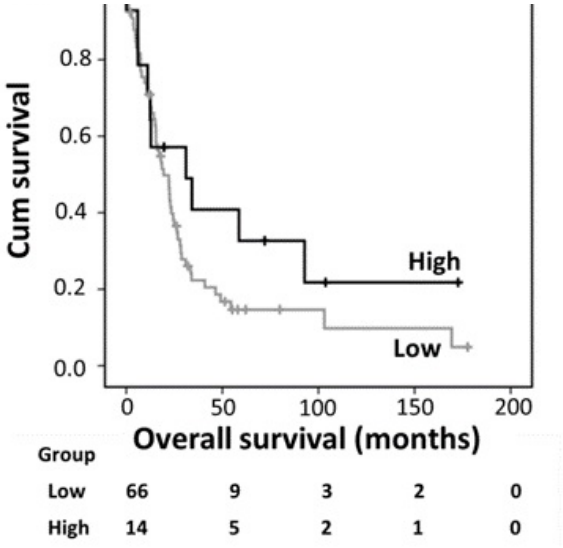
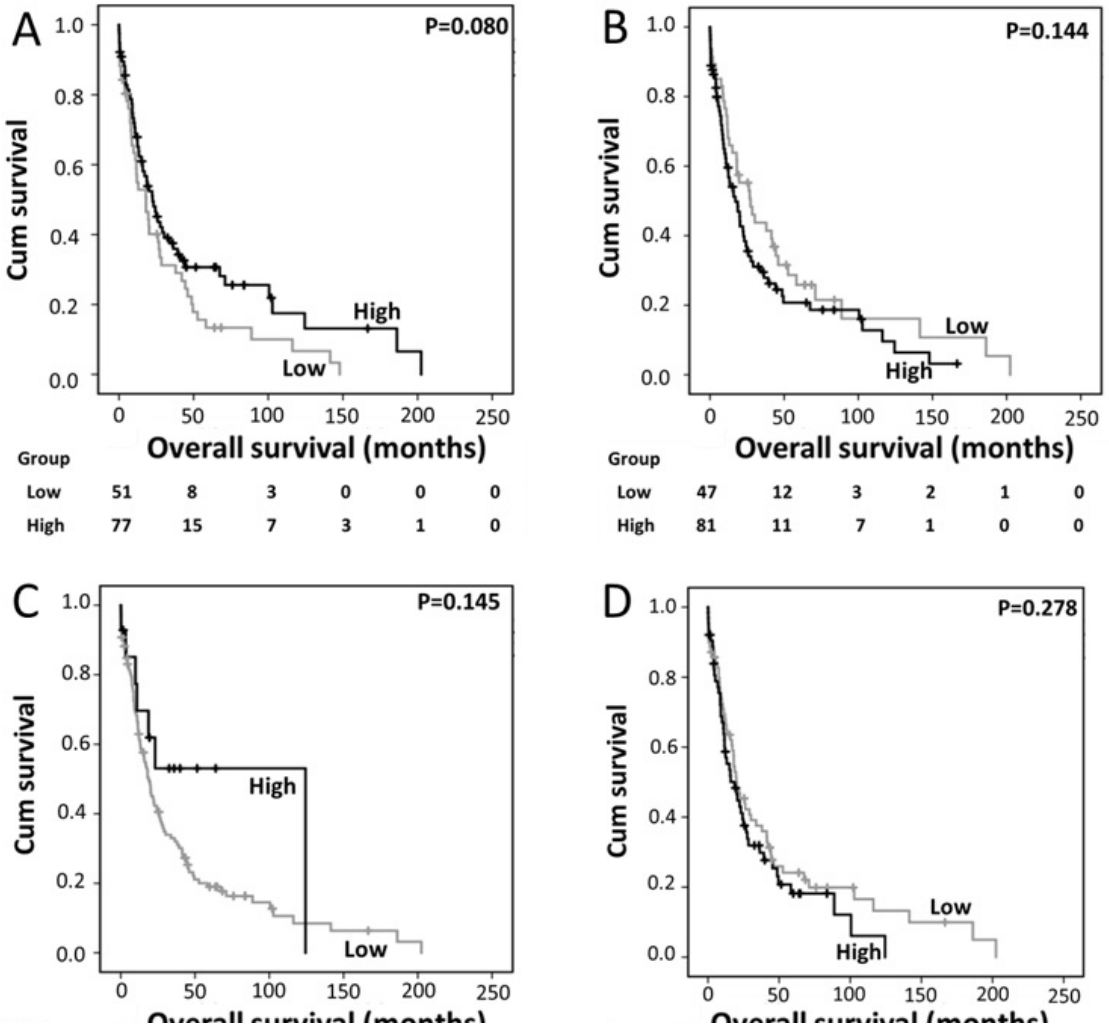


Figure 2

Kaplan-Meier analysis of overall survival showing the impact of Cytoplasmic Trx (A), nuclear Trx (B), cytoplasmic TrxR (C), nuclear TrxR (D) and cytoplasmic TxNIP (E) expression in the PAD cohort with significance determined using the log-rank test.



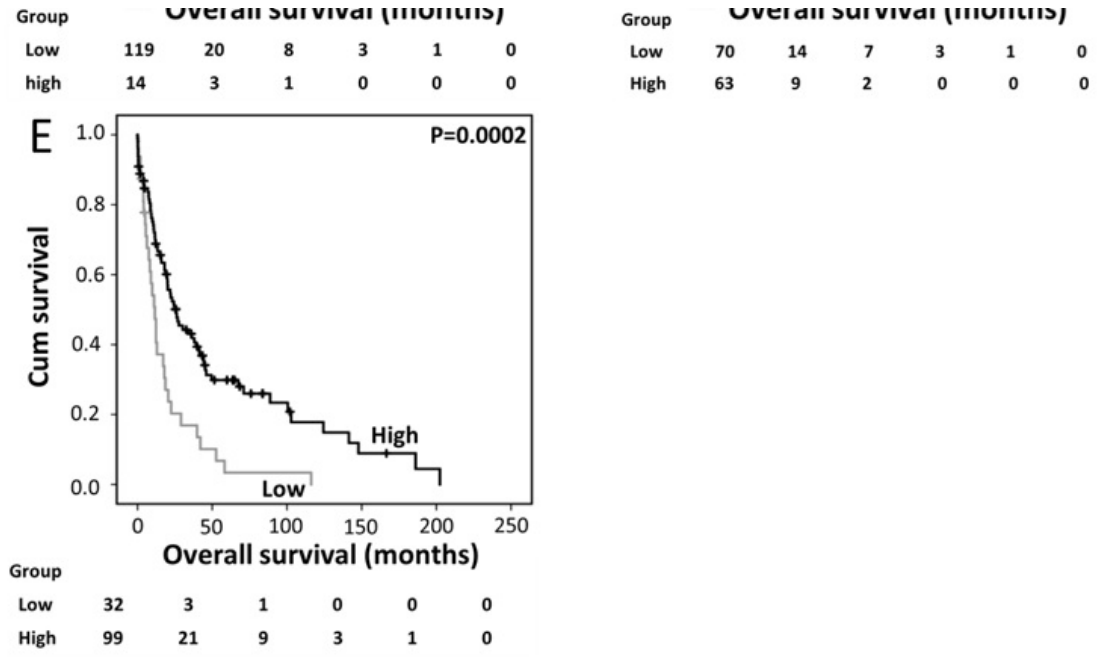


Figure 3

Kaplan-Meier analysis of overall survival showing the impact of Cytoplasmic Trx (A), nuclear Trx (B), cytoplasmic TrxR (C) and nuclear TrxR (D) and cytoplasmic TxNIP (E) expression in the bile duct and ampullary carcinoma cohort with significance determined using the log-rank test.

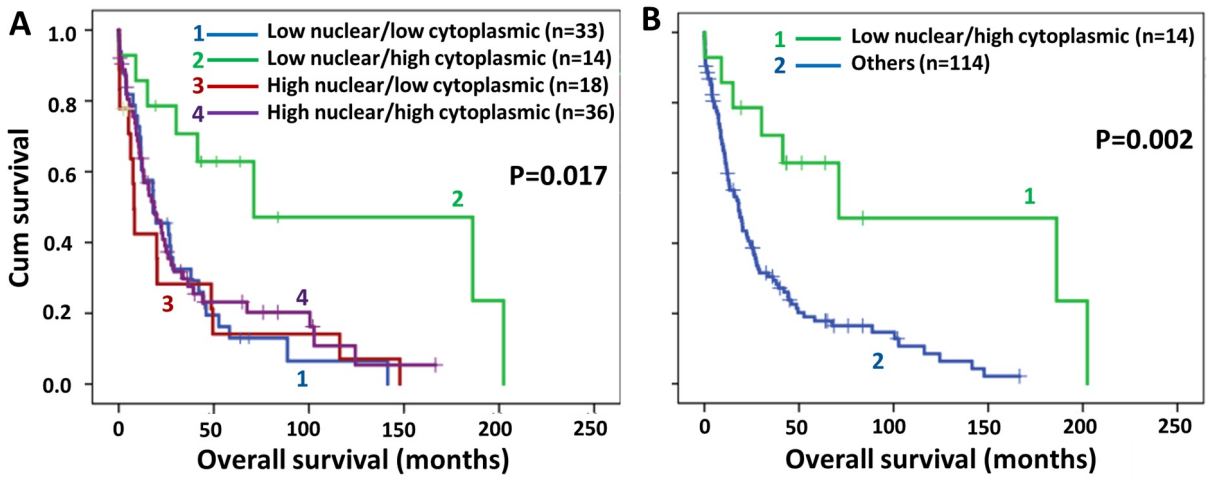


Figure 4

Kaplan–Meier overall survival analysis between different subgroups of Trx expression in the bile duct and ampullary cohort. Kaplan-Meier analysis of overall survival based upon expression profiles. Low nuclear/high cytoplasmic expression showed longer overall survival than other three subgroups either against each separate subgroup (A) or when the three subgroups were combined (B).

Supplementary Files

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