Research Paper

Thioredoxin interacting protein and its association with clinical outcome in gastro-oesophageal adenocarcinoma

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A B S T R A C T

The overall prognosis for operable gastro-oesophageal adenocarcinoma remains poor and therefore neoadjuvant chemotherapy has become the standard of care, in addition to radical surgery. Certain anticancer agents (e.g. anthracyclines and cisplatin) generate damaging reactive oxygen species as by-products of their mechanism of action. Drug effectiveness can therefore depend upon the presence of cellular redox buffering systems that are often deregulated in cancer. The expression of the redox protein thioredoxin interacting protein, was assessed in gastro-oesophageal adenocarcinomas. Thioredoxin interacting protein expression was assessed using conventional immunohistochemistry on a tissue microarray of 140 adenocarcinoma patients treated by primary surgery alone and 88 operable cases treated with neoadjuvant chemotherapy. In the primary surgery cases, high thioredoxin interacting protein expression associated with a lack of lymph node involvement (p = 0.005), no perineural invasion (p = 0.030) and well/moderate tumour differentiation (p = 0.033). In the neoadjuvant tumours, high thioredoxin interacting protein expression was an independent marker for improved disease specific survival (p = 0.002) especially in cases with anthracycline-based regimes (p = 0.008). This study highlights the potential of thioredoxin interacting protein as a biomarker for response in neoadjuvant treated gastro-oesophageal adenocarcinoma and may represent a useful therapeutic target due to its association with tumour progression.

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Introduction

The overall prognosis for patients with gastro-oesophageal adenocarcinomas remains poor and although radical surgery plays a critical role in the management of operable disease, additional therapy is required to improve patient outcomes. The United Kingdom Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial demonstrated a 25% reduction in the risk of death and a significant improvement in 5 year survival in patients given neoadjuvant chemotherapy compared to those treated with surgery alone [1]. Similar survival benefits have also been demonstrated in the MRC OEO2 trial of surgical resection with or without neoadjuvant che-

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reduction to a semiquinone free radical, in the presence of oxygen. After dismutation, resultant hydrogen peroxide can be converted into the highly damaging hydroxyl radical. The semiquinone radical can also intercalate and damage DNA [8,9].

Multiple mechanisms maintain the redox state of cells and can protect them from oxidative stress. In cancer, these systems are often deregulated/overexpressed to compensate for the increased oxidative stress caused by the accelerated proliferation, altered metabolic activity and persistent growth promoting signalling of tumours [10,11]. One of the key systems involved is the thioredoxin system, exerting its main function as an antioxidant via thioredoxins interaction with its downstream peroxiredoxins [12,13]. Thioredoxin interacting protein (TxNIP), also known as vitamin D3 upregulated protein-1 (VDUP-1), is best known as a competitive inhibitor for thioredoxin and is down regulated in a number of cancers [14,15]. It has been previously demonstrated, via RT-PCR, that Thioredoxin interacting protein gene expression is significantly lower in colorectal and gastric cancers than in adjacent normal tissues [16].

Thioredoxin interacting proteins importance in anthracycline based chemotherapy response has recently been demonstrated, via immunohistochemistry, in locally advanced primary breast cancer [17]. It was found to be an independent prognostic factor for distant metastasis free survival and overall survival. Therefore, the current study was conducted to examine the prognostic and predictive value of Thioredoxin interacting protein expression in gastro-oesophageal adenocarcinoma patients treated with reactive oxygen species generating chemotherapy agents, including a significant proportion with anthracycline based regimes, and as a comparison, in cases with primary surgery alone.

Materials and methods

Clinical samples

The study is reported according to REMARK criteria [18]. Tissue was obtained from patients treated at Nottingham University Hospitals Trust between 2001 and 2008. The cohort consisted of two sets of patients, 140 adenocarcinoma primary surgery cases that had not been exposed to neoadjuvant treatment and 88 operable adenocarcinoma cases that had at least one cycle of neoadjuvant chemotherapy [19]. During the study period, patients in the neoadjuvant arm were treated with either neoadjuvant ECF [Epirubicin (50 mg/m²), Cisplatin (60 mg/m²) and continuous infusional 5-FU (200 mg/m² per day)] or ECX [Epirubicin (50 mg/m²), Cisplatin (60 mg/m²) and capetebine (625 mg/m² p.o. b.d continuously)] chemotherapy, up to three cycles prior to surgery, or CF [cisplatin (80 mg/m²) and infusional 5-FU (1000 mg/m² daily for 4 days)] chemotherapy up to two cycles prior to surgery. The remaining three patients had either Carbo/F or CX. The tumours analysed in the neoadjuvant arm were from surgical samples post neoadjuvant treatment.

Median follow up was 28.4 and 27.3 months and median time to recurrence was 9.1 and 10.2 months for the neoadjuvant and primary surgery cohorts respectively. Disease specific survival was calculated from the date of diagnosis until 26th November 2010 when any remaining survivors were censored. Tumour regression grade (TRG) was defined as per Mandard’s criteria [20]. Tables 1 and 2 show the full clinicopathological characteristics of the patient cohort. The conduct of this study was approved by the Ethics Committee of Nottingham University Hospitals.

Tissue microarray

TMAs were constructed as described previously [21]. In short, area-specialised histopathologists identified and marked representative areas on haematoxylin and eosin stained slides from formalin-fixed paraffin-embedded tumour blocks, for tissue microarray construction. Triplicate tissue cores (0.6 mm) were taken from the marked areas and arrayed into separate recipient paraffin block using a manual tissue-arrayer (Beecher Instruments, Silver Spring, MD, USA).

| Table 1 | Clinical characteristics of patients. |
|---------|--------------------------------------|
|                     | Neoadjuvant chemotherapy (N=88) | Surgery (N=140) |
| Total number of patients | 88 (%) | 140 (%) |
| Median Age (years) | 63 (74) | 74 (74) |
| Sex | Male (63) | 73 (74) | Female (15) | 15 (26) |
| Site of tumour | Gastric (20) | 23 (91) | GOJ (41) | 46 (37) |
| Surgery | Total gastrectomy (22) | 25 (69) | Oesophagectomy/ oesophagogastrectomy (61) | 69 (14) |
| Disease Specific Survival | Alive (30) | 34 (42) | Died due to cancer (48) | 55 (37) |
| Recurrence | None (39) | 44 (86) | Recurrence (49) | 56 (38) |
| Unknown | – | 1 (1) |

| Table 2 | Pathological criteria of patients showing association with Thioredoxin interacting protein expression * = p < 0.05 ** = p < 0.01. |
|---------|-------------------------------------------------|
|                     | Neoadjuvant Chemotherapy (N=88) | Surgery (N=140) |
| T stage | T0,1,2 (26) | 0.365 | 62 (44) | 0.113 |
|          | T3,4 (61) | 0.738 | 78 (56) |
|          | Tx (1) | – | – |
| N stage | N0 (22) | 0.046* | 33 (24) | 0.005** |
|          | N1 (66) | 0.365 | 78 (56) |
| M stage | M0/MNx (83) | N/A | 138 (99) | N/A |
|          | M1 (5) | 0.162 | 2 (1) |
| Overall stage | 1,2 (26) | 0.484 | 61 (44) | 0.162 |
|          | 3,4 (62) | 0.484 | 79 (56) |
| Tumour regression grade response after chemotherapy | 1,2,3 (39) | 0.300 | 34 (24) | 0.970 |
|          | 4,5 (49) | 0.300 | 105 (75) |
|          | Unknown | – | 1 (1) |
| Vascular invasion | No (40) | 0.404 | 44 (31) | 0.338 |
|          | Yes (48) | 0.005** | 96 (69) |
| Perineural invasion | No (75) | 0.469 | 75 (54) | 0.030* |
|          | Yes (13) | 0.033* | 65 (46) |
| Differentiation of tumour | well/moderate (42) | 0.738 | 62 (44) |
|          | poor (46) | 0.738 | 78 (56) |
**Immunohistochemistry**

Immunohistochemistry was performed as previously described [22] with the following modifications. Microwave antigen retrieval in pre-warmed 0.01 mol/L sodium citrate buffer (pH6) was conducted for 10 min at 450 W. The primary antibody was diluted in blocking solution, and incubated for 60 min at room temperature (Thioredoxin interacting protein 1:3000 (MBL International Corporation, Woburn, USA)). Blocking, secondary and ABC reagents were supplied in kit form from Vector Labs (Universal PK-6200 or Goat PK-6105, DAB SK-4100 (Vector Laboratories, Burlingame, USA)) followed by 3,3’-diaminobenzidine (DAB) substrate (K3468 (Dako, Glostrup, Denmark)). Primary antibody was omitted for negative controls. Gastric tissue and historically used breast composite blocks composed of six stage I breast carcinomas, including grade I, II and III tumours were used for initial antibody optimisations and positive controls. Antibody specificity was assessed by Western blotting on gastric and breast cancer cell lysates prior to commencing the study.

**Assessment of expression**

Assessment of expression of protein staining was conducted independently by the specialist gastro-intestinal histopathologist.

![Photomicrographs of immunohistochemical Thioredoxin interacting protein expression patterns at 10× magnification in tumours after receiving neoadjuvant chemotherapy (left panels) or from primary surgery alone (right panels). Scale bars denote 500 μm.](image-url)
(IS) and the lead scientist (CW) blinded to the study end points. Staining intensity was divided into four grades, none (0), weak (1) moderate (2) and strong (3). Semiquantitative H scores were calculated by multiplying the percentage of positive tumour cells by the staining intensity, giving a range between 0 and 300. The median H score for the three replicate cores for each case was then calculated and used in the final assessments. To stratify the cohort of patients into low- vs. high-expressing tumours, the median of the two study populations was calculated and applied (Neoadjuvant 77, Primary surgery 55).

Statistical analysis

SPSS version 15.0 statistical software package was used with patients categorised into two groups, low and high (or negative and positive), depending on whether their staining intensities fell above or below the cut-off values. The Mann Whitney U test was used to examine the Thioredoxin interacting protein expression levels between the primary and neoadjuvant groups. The association between protein expression and clinicopathological criteria was assessed by univariate analysis using a Pearson χ² test. Survival analysis comparing protein expression levels with recurrence and overall survival were conducted using Kaplan-Meier curves and the statistical significance evaluated using the log rank test. Cox proportional hazards model was used to test the statistical independence and significance of Thioredoxin interacting protein versus available clinical parameters. Agreement between the two independent observers was assessed using intraclass correlations (0.850).

Results

Thioredoxin interacting protein expression and pathological associations

Fig. 1 demonstrates the diffuse, granular and sometimes heterogeneous cytoplasmic immunostaining for Thioredoxin interacting protein in the tumour samples. In certain tumour cases, there was also heterogeneous staining between the triplicate cores. This had previously been observed in a selection of whole mount sections for this marker but triplicate cores allow for this potential heterogeneity to be represented. From Fig. 2 it can be seen that the levels of Thioredoxin interacting protein expression significantly differed between the treatment approaches when comparing the continuous data from each cohort using the Mann Whitney U test (p=0.007). There was a higher median of expression in the tumours that had received neoadjuvant chemotherapy (77) than the untreated primary surgery cases (55).

Thioredoxin interacting protein associations in tumours receiving primary surgery only

Table 2 shows the associations between Thioredoxin interacting protein expression and pathological features in the cases that had primary surgery. A significant association was seen with high Thioredoxin interacting protein expression and a lack of lymph node involvement (N stage: χ² 8.027, df=1, p=0.005), no perineural invasion (χ² 4.705, df=1, p=0.030) and well/moderate tumour differentiation (χ² 4.521, df=1, p=0.033).

In the primary surgery cases, no significance was observed between Thioredoxin interacting protein expression and disease specific survival (p=0.507) or risk of recurrence (p=0.722).

Discussion

The results presented in the current study have demonstrated that a high expression level of Thioredoxin interacting protein is an independent determinant of good disease specific survival in gastro-oesophageal patients that have received neoadjuvant chemotherapy (p=0.002). The predictive power of Thioredoxin interacting protein appears more relevant to those neoadjuvant cases that received treatment containing anthracyclines compared to those in the CF treatment group (5-FU/cisplatin) but further validation studies would be required to confirm this. Woolston et al., have previously shown the potentially predictive/prognostic role of redox protein expression in tumours taken pre and post neoadjuvant anthracycline based chemotherapy in locally advanced primary breast cancer patients [17]. High Thioredoxin interacting protein was an independent prognostic factor for an improved disease free- and overall survival as well as a significant increase in expression between paired pre- and post-chemotherapy tumour samples.
Lim et al., demonstrated that a low Thioredoxin and high Thioredoxin interacting protein gene expression in 68 stage III gastric cancer cases had a better recurrence free survival. In 85% of gastric cancers that they went on to examine by immunohistochemistry, 85% under expressed Thioredoxin interacting protein compared to normal tissues with a tendency for this under expression to occur in two poor prognostic groups: diffuse histology and high stage [23].

It may well be that it is the differences in tumour site between groups (i.e. 23 vs. 91% gastric) that is influencing the expression differences seen between the neoadjuvant and primary surgery groups; however, 5-FU could also be contributing to the expression differences. 5-FU upregulated Thioredoxin interacting protein levels in a gene expression study [24] and is known to trigger reactive oxygen species generation through as yet undetermined mechanisms [25]. 5-FU also decreased Focal adhesion kinase protein expression and up-regulated Thioredoxin interacting protein expression in 293 kidney cells [26]. The higher overall expression of Thioredoxin interacting protein seen in tumours examined after neoadjuvant therapy compared to primary surgery (p=0.016) may be indicative of redox pathways being activated in response to therapy but further work is required in this area, potentially by examining the expression of TxNIP in normal tissue at different sites from which the tumours arise.

The effects of Thioredoxin interacting protein could be via thioredoxin inhibition, allowing the reactive oxygen species generated by the cytotoxic agents to exert their damaging effects. An increased level of reactive oxygen species has been observed in fibroblasts with increased levels of Thioredoxin interacting protein [27] and preventing thioredoxin interacting with PAG or ASK-1 can sensitise cells to oxidative stress [28]. However, in contrast, gene silencing of Thioredoxin interacting protein in melanoma cells reduced reactive oxygen species production [29] suggesting a potential tumour specific effect. In gastric cancer, primary tumours expressing high levels of thioredoxin have been shown to have a

| Neoadjuvant treated patients |  |
|-----------------------------|---|
| Thioredoxin interacting protein |  |
| Hazard ratio | 95% CI | Significance |
| Protein | 0.4 | 0.2–0.7 | 0.002* |
| T stage | 3.0 | 1.1–7.8 | 0.028* |
| N stage | 3.0 | 1.3–6.7 | 0.009* |
| Vascular invasion | 2.5 | 1.3–5.0 | 0.009* |
| Anthracycline based patients |  |
| Thioredoxin interacting protein |  |
| Hazard Ratio | 95% CI | Significance |
| Protein | 0.3 | 0.1–0.7 | 0.008* |
| T stage | 8.4 | 1.0–66.6 | 0.045* |
| N stage | 2.2 | 0.7–6.9 | 0.159 |
| Vascular invasion | 2.5 | 0.7–8.1 | 0.136 |

Table 3 Cox proportional hazards analysis for disease specific survival in the neoadjuvant treated patients and anthracycline based neoadjuvant treated patients. HR = Hazard ratio, CI = Confidence Interval, * = p < 0.05.

Fig. 3. Kaplan Meier survival curves. (A) High expression of Thioredoxin interacting protein (TxNIP) significantly associated with a better disease specific survival (p=0.016). This was more apparent in the cases that had (B) anthracyclines included in their neoadjuvant treatment regime (p=0.022) compared to those (C) without (p=0.561).
higher proliferative rate and lower rate of spontaneous cell death [30] and knock-out of Thioredoxin interacting protein in a mouse model induced Helicobacter pylori related gastric cancer [31]. Forced expression of Thioredoxin interacting protein in stomach cancer and promyelocytic leukaemia cell lines inhibited their proliferation [14]. Thioredoxin interacting protein is also regulated by class II HDACs such as HDAC10. HDAC10 transcriptional down-regulation of Thioredoxin interacting protein leads to altered reactive oxygen species signalling in human gastric cancer cells [32]. It appears, from such reports and from current results, that indirect modulation of Thioredoxin interacting protein by agents such as HDAC inhibitors may represent a promising therapeutic approach.

In the primary surgery cohort of patients, although not significant for survival or recurrence, Thioredoxin interacting protein did show significant association with prognostic features such as lymph node involvement (N stage p = 0.005), perineural invasion (p = 0.030) and differentiation status of the tumour (p = 0.033). M stage cases were not included in the analyses as only 6% of primary surgery cases and 1% of neoadjuvant cases had distant metastases reported. Thioredoxin interacting protein has been demonstrated to suppress tumour growth and metastasis in transplantation models [33] and suppress cell invasiveness and tumour metastases of melanoma B16-F10 in in vivo models possibly via its association with the β domain of von Hippel-Lindau protein [34]. Loss of Thioredoxin interacting protein has been demonstrated to be enough to induce malignancy, forming hepatocellular carcinomas in mice [35]. In the case of differentiation, Thioredoxin interacting protein is thought to play a role in epithelial differentiation in the GI tract [36] with Thioredoxin expression predominantly seen in undifferentiated gastric cancers [37]. Such reports support current findings where high Thioredoxin interacting protein expression associates with better tumour differentiation, lack of lymph node involvement and no perineural invasion.

In summary, this study highlights the potential of Thioredoxin interacting protein as a biomarker for response to reactive oxygen species generating chemotherapy agents used in the neoadjuvant setting in gastro-esophageal adenocarcinoma. It also indicates its role in tumour progression through its associations with pathological criteria.

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