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# Tissue Distribution and Activity of Thioredoxin Reductase in Pancreatic Carcinoma

**Faezeh Sadrabadi Haghighi*#, Mohsen Aliakbarian**#, Alireza Khooei***, Seyed Isaac Hashemy**♦**

*Department of Clinical Biochemistry, Faculty of Medicine, Mashhad University of Medical Science, Mashhad, Iran
**Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
***Department of Pathology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
#These authors equally contributed to this work.

## Abstract

**Background:** Thioredoxin, NADPH, and thioredoxin reductase form the thioredoxin system which exists in all living cells. Oxidants have a major role in cancer pathogenesis; therefore, it is necessary to study the role of redox-active compounds such as thioredoxin reductase to increase our knowledge about the molecular mechanisms involved in cancer pathogenesis and ultimately design more effective treatments. The research on the role of the thioredoxin system in pancreatic cancer is limited; hence, we intend to compare the tissue distribution and activity of thioredoxin reductase in pancreatic cancer with healthy tissues located at the tumor margins.

**Methods:** A total of 29 patients with pancreatic cancer participated in this study. The tissue distribution was determined by immunohistochemistry analysis. We used a commercial ELISA kit to determine enzyme activity.

**Results:** There was no significant difference between the tumor tissue and its normal surrounding tissue in terms of thioredoxin reductase activity ($P$=0.56). However, there was a significant difference when we considered the different disease stages. A significant relationship also existed between the staining intensity of thioredoxin reductase and disease stage ($P$=0.022).

**Conclusion:** There was no observed difference between the pancreatic cancer tissue and its healthy margin in terms of thioredoxin reductase activity and tissue distribution. This finding did not support its possible role in pancreatic cancer pathogenesis.

***Keywords:*** Oxidative stress, Thioredoxin reductase, Pancreatic cancer, Immunohistochemistry, Antioxidant

♦**Corresponding Author:**
Seyed Isaac Hashemy; MD, PhD
Surgical Oncology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
Tel: +98-9158112664
Email: hashemyi@mums.ac.ir

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## Introduction

An estimated 53070 patients were diagnosed with pancreatic cancer in the US in 2016 with 41780 deaths due to this disease in the same year.[1, 2] Overall, pancreatic cancer has the worst prognosis among malignancies with a 5-year survival rate of only 7.7%.[2] Although pandemic, pancreatic cancer is difficult to treat and its exact cause is not known.

Thioredoxin (Trx), together with Trx reductase (TrxR) and NADPH, form the thioredoxin system. A study of the activity and role of Trx shows that Trx is the main protein disulfide reducing agent in cells.[3] The most common description of the Trx system is its role as a protein-disulfide reductase. TrxR acts by removing electrons from NADPH and transferring them to the Trx active site as a general disulfide reductase. Various functions of Trx and TrxR have been recognized in numerous biological systems.[4] However, the role of cytosolic Trx (Trx1) in cancer is a debatable issue, with two different points of view. According to the first view, the increased level of Trx1 acts as a stimulus for cancer cell growth.[5] Based on the second view, the increased expression of Trx1 is a response to a significant increase in oxidative stress.[6, 7] Oxidative stress, which is due to the disruption of the oxidant-antioxidant balance in favor of oxidants, is a very well-known mechanism in cancer pathogenesis[8-11] and many other diseases.[12-14]

Multiple studies have shown overexpression of the Trx-interacting protein (TXNIP) in non-small cell lung cancer,[15] melanoma,[16] thyroid cancer,[17] breast cancer,[18] cervical cancer,[19] and colon cancer.[20] Additionally, Trx1 leads to the production of hypoxia-induced factor (HIF-1α), which increases the production of vascular endothelial growth factor (VEGF), and leads to tumor angiogenesis and drug resistance.[21] High levels of Trx and TrxR have been reported in many cancer cells,[22, 23] including neoplastic liver cells, which indicates that increased cytosolic TrxR (TrxR1) is of great importance for the survival and promotion of cancer progression.[24]

The Trx system is assumed to have a crucial role in numerous cancers via a variety of routes, which could be studied in order to obtain better results from preventive and therapeutic measures against cancer.[25] Given the importance of TrxR and its association with cancer, we aim to compare the tissue distribution and activity of TrxR in pancreatic cancer tissues and its healthy margins.

## Materials and Methods

### *Tissue samples*

We obtained samples from 29 pancreatic cancer patients who referred to the Department of Surgery, Imam Reza Hospital, Mashhad, Iran from 2016 to 2017. We recorded the tumor size, lymph node involvement, and metastasis, as well as tumor staging (Table 1) for each patient.

The control group comprised healthy tissue located at the tumor margins. All patients with pancreatic cancer who were surgical candidates and consented to have surgery, and agreed to participate in the study were included in the research. During the surgery, two samples (one for pathology and immunohistochemistry and the other for enzyme activity) were separated from the tumor tissue of each patient. In addition, two samples were prepared from the normal tissue located adjacent to the tumor, and with a margin of at least 2 cm from the tumor tissue.

The Research Council at Mashhad University of Medical Sciences approved this study.

### *Tissue distribution of TrxR according to immunohistochemistry analysis*

Paraffin-embedded blocks were sectioned and mounted on frost-free slides. The 3 µm sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Slides were washed with 1× PBS, and endogenous peroxides were blocked with 1.5% hydrogen peroxide in 1× PBS for 20 min at 25°C. After three, 5 min washes in 1× PBS, the slides were incubated in blocking solution (1× PBS with 0.1% Triton X-100, 3% bovine serum albumin) with 5% normal donkey serum for 10 min at 25°C. The control (no primary antibody) and experimental slides were incubated

overnight at 4°C in either blocking solution alone or blocking solution with anti-TXNRD1 antibody (ab16840; Abcam) at a 1:400 dilution. Biotin-conjugated secondary antibody (Dako Corp.) at a dilution of 1:200 was added and slides were incubated at 25°C for 30 min, then washed 3 times with 1× PBS. The ABC Peroxidase Staining kit from Leica Biosystems Company (1:100 dilution each of Reagents A and B in 1× PBS) was applied at 25°C for 30 min. After 3 washes with 1× PBS, staining was visualized with peroxidase-sensitive 3,3'-diaminobenzidine tablets. Exposure times were synchronized so that all tissue samples within an antibody group were exposed to DAB for the exact same time. All slides were counterstained with 0.1% methyl green for 3 min at 60°C, dehydrated in ethanol, cleared in xylene, and mounted with permount. Images were obtained at 200× using an Olympus BX40 microscope, and the score of tissue distribution was obtained by multiplication of the intensity score and the score of positively stained cells (Table 2 and Figure 1).

### *TrxR activity*

Initially, the samples were washed with PBS

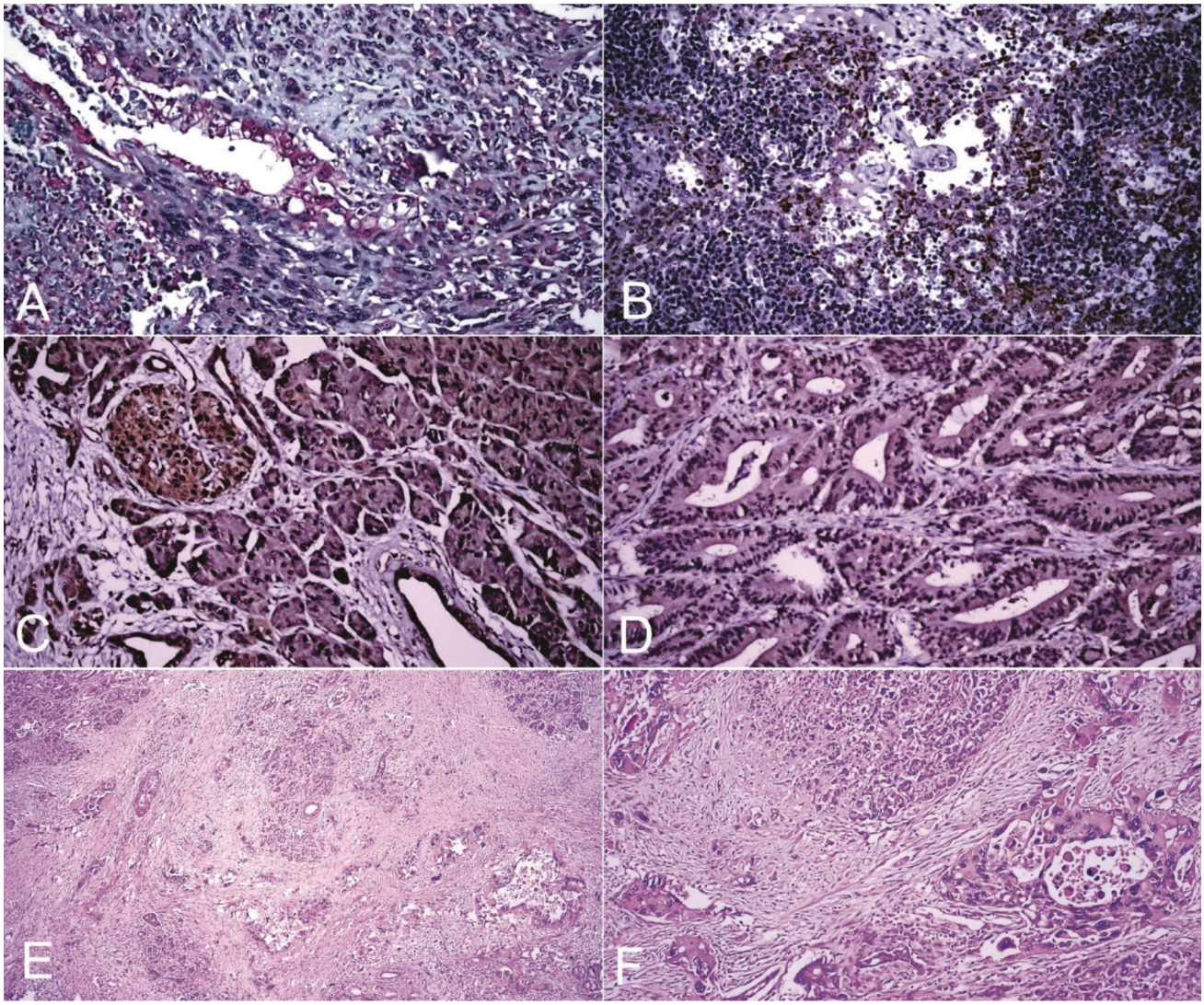


**Figure 1.** Immunohistochemical localization of thioredoxin reductase (TrxR) in pancreatic cancer tissue and healthy surrounding tissue. (A) Moderate staining of tumor cells at 200× magnification. (B) Moderate staining of normal cells at 200× magnification. (C) Strong staining of normal cells at 40× magnification. (D) Moderate staining of tumor cells at 40× magnification. (E) Invasive ductal carcinoma with a high grade atypical nature that induced stromal desmoplasia, H&E staining (100×). (F) Invasive ductal carcinoma with infiltrative nature that destroyed the pancreatic acini, H&E staining (200×).

solution (pH 7.4) to remove any clots and red blood cells. Next, the samples were cut into very small pieces and homogenized in 5 mL of buffer per gram of tissue (50 mM potassium phosphate that included 1 mM EDTA). Afterwards, this product was centrifuged at 10000 rpm for 15 min at 4°C. The liquid above the product was removed for testing and maintained at -80°C until laboratory analysis. Measurement of the enzyme activity level was carried out using the Thioredoxin Reductase Colorimetric Assay Kit (item no.10007892; Caymans Chemical Company, USA). This experiment is based on the reduction of DTNB to TNB by NADPH. TNB, a yellow product, is measured at a wavelength of 412 nm. The measurement of TrxR activity by DTNB reduction in the presence or absence of aurothiomalate, an inhibitor specific to TrxR, allows us to calculate the rate of DTNB reduction conducted independent of TrxR. For example, in the presence of glutathione. We considered the difference between these two results (presence or absence of aurothiomalate) to be the rate of DTNB reduction associated with TrxR activity.

### *Statistical analysis*

Data was analyzed using SPSS 21.0. The results are shown as mean±SD, and a *P*-value of ≤0.05 was considered to be statistically significant.

## Results

There were 29 participants (20 male and 9 female) who met the inclusion criteria.

In terms of tumor size, 10 patients had stage T1 tumor, 8 were stage T2, 7 were stage T3, and 1 patient had a T4 tumor. In terms of regional lymph node involvement, 14 patients were N0 and 12 patients were N1. Only one subject showed evidence of metastasis that had spread to distant organs, and 25 patients were M0.

We assessed the percentage of stained cells in patients' healthy tissues and observed that 66.7% had a score of 3 and 33.3% had a score of 4. The percentage of stained cells in the tumor tissues according to score was as follows: score 1 (4%), score 2 (8%), score 3 (28%), and score 4 (60%).

**Table 1.** TNM staging of study patients. With regards to T, N, and M staging in the present study, the status of overall disease staging is shown.

| Stage | Number | Percent |
|---|---|---|
| IA | 7 | 24.1 |
| IB | 6 | 20.7 |
| IIA | 2 | 6.9 |
| IIB | 10 | 34.5 |
| IV | 1 | 3.4 |
| Missing data | 3 | 10.3 |
| Total | 29 | 100 |

The average scores for positively stained cells in the healthy tissues was 3.33±0.516, whereas in tumor samples it was 3.44±0.821.

The scores for staining intensity in the samples of healthy tissues were 1 (16.7%), 2 (33.3%), and 3 (50%). The scores of staining intensity in the tumor tissue samples were 1 (48%), 2 (28%), and 3 (24%). The mean staining intensity scores in healthy tissues was 2.33±0.816 and for tumor samples, it was 1.76±0.831.

The tissue distribution of TrxR was determined by multiplying the percentage of stained cells and staining intensity for each sample. The TrxR distribution ranged from 1 to 12 in tumor tissues, as follows: 1 (3.4%), 2 (6.9%), 3 (17.2%), 4 (13.8%), 6 (10.3%), 8 (13.8%), and 12 (20.7%). The tissue distribution of TrxR in the healthy tissues was within the range of 3 to 12, as follows: 3 (16.7%), 6 (16.7%), 8 (16.7%), 9 (33.3%), and 12 (16.7%).

The comparison of the numbers of positively stained cells in the tumor tissue of pancreatic cancer and their healthy margins showed no statistically significant difference (*P*=0.293). The staining intensities of TrxR in the tumor tissue of pancreatic cancer and the healthy tissue adjacent to the tumor were also not statistically different (*P*=0.316). The tissue distributions of TrxR in the tumor tissue of pancreatic cancer and their healthy margins did not statistically differ (*P*=0.180).

We did not observe any correlation between the percentage of positively stained cells and the disease stage (*P*=0.75). However, there was a statistically significant relationship between the staining intensity of tumor tissues and the disease

**Table 2.** The scoring system for staining intensity and abundance of positive cells according to immunohistochemistry analysis of the pancreatic cancer tumor tissues and their healthy margins.

| Score | Staining intensity | Abundance of positive cells |
|---|---|---|
| 1 | Weak | Staining in ≤25% of cells |
| 2 | Moderate | Staining in 26%–50% of cells |
| 3 | Strong | Staining in 51%–75% of cells |
| 4 | Not applicable | Staining in ≥76% of cells |

stage (*P*=0.022).

The enzyme activity level in healthy tissues ranged from 0.017 to 0.128 µmol/min/mL (0.024±0.020 µmol/min/mL). The enzyme activity in the tumor tissues was within the range of 0.017 to 0.190 µmol/min/mL (0.041±0.039 µmol/min/mL). We did not observe any statistically significant difference in TrxR activity in the tumor tissue and its normal surrounding environment (*P*=0.56). However, this difference was significant when considered different disease stages (data not shown).

## Discussion

This study enrolled 29 pancreatic cancer patients who referred to Imam Reza Hospital, Mashhad, Iran and were candidates for surgery. This study aimed to measure and compare the activity and tissue distribution of TrxR in tumor tissue and its healthy surrounding tissue.

In this study, the patients' ages ranged between 21 and 82 years (mean=54.39 years, SD=15.25 years). The risk of pancreatic cancer increases with age, and epidemiological studies have shown that 90% of these cancers occur in people over 40 years of age.[2] In our study, 90% of patients were over 40 years of age. There were 20 male subjects (69%) and 9 female subjects (31%) enrolled in this study, which shows the greater likelihood of males to be diagnosed with pancreatic cancer compared to females. Of note, the higher incidence of pancreatic cancer in men is not an inherent problem associated with gender but is largely related to the fact that men are further dealing with risk factors. Currently, according to epidemiological studies, it has been found that the prevalence of this cancer in men is greater than women.[2]

There was no significant difference when we compared the cancer stage to gender. Considering the recent epidemiologic studies, it could be concluded that there is no relationship between stage and gender, and its distribution is homogenous.[2]

We assessed the activity level of TrxR in healthy and tumor tissues. Although the average level of activity in the tumor tissue was greater than healthy tissue, this difference was not statistically significant (*P*=0.56). Other studies that measured the activity level of TrxR in colorectal cancer reported that the activity level of TrxR in tumor tissues increased relative to normal tissues.[26] In our study this difference was not significant except when we separately considered the different stages, which led to a significant difference. A similar study with a larger sample size might clarify the results.

We assessed the tissue distribution of TrxR in the pancreatic tumor tissue and its healthy margins. There was a statistically significant positive correlation between staining intensities of tumor and stage (*P*=0.022). It could be stated that the greater development of the tumor might lead to a higher expression of the enzyme by the tumor, or tumor cells with more enzymes have a greater proliferative nature. It could be hypothesized that with tumor development, more enzyme would be expressed to deal with the environment's oxidative stress.

The above-mentioned results indicated that the TrxR expression in pancreatic tumor tissue showed no significant difference with healthy tissue. Numerous studies have suggested high rates of Trx expression in tumor tissues from numerous human malignancies such as the lung, liver, colorectal, breast, and malignant melanoma.[16, 26, 27] Investigations of TrxR expression in pancreatic tumor tissue and its

comparison with the healthy tissue had not been conducted until now. Despite the fact that an increase in TrxR expression has been shown in other tumors, there was no significant difference found in the current study. Therefore, we might conclude that TrxR overexpression did not occur in pancreatic cancer and it might not be a good biomarker for pancreatic cancer. In this study, we used healthy tissue at the tumor margin as the healthy control tissue, which might explain our results since a higher level of TrxR expression compared to the completely normal pancreas would be a possibility. Lack of access to healthy pancreatic tissue due to ethical reasons and uncertainty over the similarity of the healthy tissue with our control samples in terms of TrxR expression were among the limitations of this study.

Taken together, TrxR does not seems to have overexpression or increased activity in pancreatic cancer. These findings do not support a role for this protein in pancreatic cancer pathogenesis.

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## Conflict of Interest

None declared.

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