Expression of thioredoxin-1 (TXN) and its relation with oxidative DNA damage and treatment outcome in adult AML and ALL: A comparative study

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Objective: Thioredoxin-1 (TXN) is a key element in the elimination of reactive oxygen species as well as activation of tumor suppressor genes and DNA repair enzymes. Several studies showed that TXN was over expressed in solid tumors and this was correlated to poorer prognosis. However, TXN expression has been insufficiently studied, particularly in newly diagnosed adult acute leukemia.

Methods: This study was designed to evaluate the gene expression of TXN in acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) adult patients and to investigate its association with oxidative DNA damage. The expression of TXN was analyzed using quantitative reverse transcriptase-polymerase chain reaction while oxidative DNA damage was evaluated by measuring serum 8-hydroxy-2-deoxyguanosine (8-OHdG) by enzyme-linked immunosorbent assay and strand breaks by the comet assay.

Results: We found that TXN was under expressed in both AML and ALL groups (P < 0.001 for both) as compared to the control group. Also TXN expression level was negatively correlated with serum 8-OHdG and tail moment in both AML (P = 0.042 and 0.047, respectively) and ALL (P < 0.001 and P = 0.02, respectively) while it showed no correlation with treatment outcome in either groups.

Discussion: This study suggests that TXN expression is hindered in adult acute leukemia which augments oxidative DNA damage and hence mutagenesis.

Conclusion: This study provides a new insight into the pathogenesis of acute leukemia and suggests TXN as a new screening test for the risk for acute leukemia.

Keywords: Thioredoxin, TXN, AML, ALL, Oxidative DNA damage, 8-OHdG, Strand breaks

Introduction
Leukemia, the serious hematologic malignancy, accounts for around 300,000 new cases each year representing 2.8% of all new cancer cases and 222,000 deaths. The high ratio of deaths/cases (74%) reflects the aggressiveness and poor prognosis of leukemia in many countries due to the lack of complex treatment regimes. There is a significant geographic variation in mortality rates due to better survival (and hence lower mortality) in developed countries where survival rate reaches up to twice the rate in developing countries; however, the geographic variation in the range of incidence rates is little.¹,² The accumulation of oxidative DNA damage is one of the major contributors to malignant transformation in acute leukemia either through disrepair or incomplete repair of DNA specially if combined with a deficient apoptotic pathway.³ Oxidative stress which is the bio-molecular damage caused by attack of reactive oxygen species (ROS) upon the components of the cell as a result of the imbalance between production of ROS and their elimination mechanisms known as antioxidants.⁴ A variety of specific and efficient antioxidants exist to control the balance between production of ROS and their elimination mechanisms known as antioxidants.⁴ A variety of specific and efficient antioxidants exist to control the 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functional damage that may induce somatic mutations and malignant transformation. The role of oxidative stress in carcinogenesis is controversial. ROS can act either anti-carcinogenically by arresting cell-cycle or promoting apoptosis or necrosis, and inhibiting angiogenesis or, conversely, pro-carcinogenically by to promoting proliferation, invasiveness, angiogenesis, and metastasis as well as suppressing apoptosis.

Human cellular components and DNA are constantly exposed to oxidative attacks leading to many lesions. One of these lesions is 8-hydroxyguanosine which is mediated by OH attack and its hydrolysis product 8-hydroxy-2-deoxyguanosine (8-OHdG) that is considered as the most common endogenous DNA base modification. The 8-OHdG is used as a fingerprint of radical DNA attack and has been implicated in cancer progression. For example, in breast cancer, 8-OHdG has been reported to be increased 8- to 17-fold in breast primary tumors compared with non-malignant breast tissue. In addition, ROS can activate specific signaling pathways that contribute to tumor development through the regulation of cellular proliferation, angiogenesis, and metastasis. For example, ROS enhance melanoma cells trans-endothelial migration during intravasation through the increasing expression of thioredoxin-interacting protein (TXNIP) and inhibition of TXN expression. Also, a genetic influence on 8-OHdG production was seen for polymorphisms in thioredoxin reductase 1 (TXNRD1) and 2 (TXNRD2).

The thioredoxin (TRX) system is one of the major antioxidant systems. It is integral to maintaining the intracellular redox state. TXN is a major member of the thioredoxin system. It is a low-molecular-weight (10–12 kDa) cellular redox protein located in the nucleus and cytoplasm. It was previously identified as an autocrine growth factor in transformed lymphoid cells. It has several functions including the regulation of the activity of various antioxidant enzymes. Its structure includes a conserved Cys-Gly-Pro-Cys redox catalytic site. Thioredoxin reductase (TXNR) transfers reducing equivalents from NADPH to TXN and reduces the active site disulfide of oxidized TXN (TXN-S2) to a dithiol (SH2). In mammals, both TXN and TXNR are expressed as different isoforms. They are predominantly expressed in the cytosol. TXNIP binds TXN and inhibits its dithiol reductase activity. The TXN coding gene is a protooncogene that stimulates tumor growth and inhibits apoptosis whether spontaneous or drug induced. Overexpression of the TXN gene is also associated with both hypoxia-induced factor-1α (HIF-1α) levels and HIF-1 transactivation gene overexpression in cancer cells, resulting in increased vascular endothelial growth factor production and excessive tumor angiogenesis. In addition, overexpression of TXN has been correlated with aggressive tumor growth, and decreased survival in patients with solid tumors. It is thought that TXN has an important role in maintaining the transformed phenotype of some human cancers as well as their resistance to chemotherapy and is thus a promising target for cancer drug development. Also, TXNR was up-regulated in many cancer cells and may contribute to chemoresistance.

TXN overexpression has been implicated in increased tumor growth in various solid tumor types and model systems. However, there is a lack of supporting experimental evidence about the pathophysiological and therapeutic significance of the TXN family in different types of acute leukemia. Recent preclinical in vitro data and clinical data in solid tumors strongly support that the development of drugs acting via the TXN system is a promising route for cancer therapy.

Subjects and methods
Subjects
From May 2013 to June 2014, a total of 55 patients with acute leukemia were recruited from Clinical Hematology and Stem Cell Transplantation Unit, Ain Shams University Hospitals (ASUH), Cairo, Egypt (30 acute myeloid leukemia (AML) patients, 15 males and 15 females with the mean age of 45 years and 25 acute lymphoid leukemia (ALL) patients, 15 males and 10 females with the mean age of 34 years). The diagnosis was made based on the morphologic findings from Wright-Giemsa-stained smears of bone marrow aspirates and immune-phenotyping analyses of leukemia cells. Written informed consent was obtained from every patient with their personal signature. The consent informed the patients that their blood samples will be used for laboratory analysis for medical research purpose with a brief description for the aim of the study. In April 2013, the protocol for this study was approved by the Committee of Medical and Pharmaceutical Ethics of ASUH in accordance with the Helsinki Declaration of 1975, as revised in 2008. The study also included 20 healthy age- and sex-matched volunteers who served as healthy control.

Blood sampling
Peripheral blood (PB) samples were withdrawn from the patients. The PB samples were divided into two aliquots. The first aliquot of blood was collected on vacutainer tube containing ethylenediamine tetra-acetic acid, disodium salt (final concentration of 1.5 mg/ml) (Na2 EDTA), which was further divided into other two aliquots for RNA isolation and comet assay. The second aliquot was collected on a plain vacutainer tube for the assay of serum 8-OHdG.
Methods

RNA isolation and real-time quantitative RT-PCR
Total RNA was extracted from whole blood using the QIAamp RNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and stored at −80°C until use. After treatment with DNA-free (Ambion, Austin, TX, USA) to remove chromosomal DNA, the mRNA expression levels of TXN and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using the Stratagene MX 3000P thermal cycler (Stratagene, CA, USA). The quantitative RT-PCR amplification was performed using the one-step QuantiFast Probe Assay for TXN (Cat. no.:QF00478121) and for GAPDH (Cat. no.:QF00531132), (Qiagen, Hilden, Germany). All reactions were performed using 25 μl samples containing 50 ng RNA. The reaction protocol used involved heating for 10 minutes at 50°C, followed by 5 minutes at 95°C followed by 35 cycles of amplification (10 seconds at 95°C and 30 seconds at 60°C). Analysis was performed using the MxPro-Mx3000P software (Stratagene). The expression levels of the TXN gene in unknown samples were normalized and analyzed by the 2^−ΔΔCt method, where ΔΔCt = (CtTXN−CtGAPDH)sample − (CtTXN−CtGAPDH)calibrator. The average ΔΔCt (CtTXN−CtGAPDH) from control samples combined was defined as 1.0, as calibrator.

Comet assay

The alkaline comet assay (comet assay kit, Trevigen, MD, USA) was applied under yellow light to avoid DNA damage that may arise from fluorescent white light as outlined. Equal volumes of blood diluted with PBS and 1% low melting point agarose (LMPA) were layered on slides pre-coated with normal melting point agarose, covered with a coverslip and placed on a slide tray on ice packs for 5 minutes till agarose hardened. Cover slip was then removed and a third layer of LMPA was added to the slide. Cover slip was then replaced and the slide was returned to the slide tray for another 5 minutes until the agarose layer hardened. Viability test was performed then slides were incubated for 2 hours at 4°C in a freshly prepared lysis solution (comet assay kit) to remove membranes and histones from the DNA. Nucleoids were then treated with alkaline buffer (pH 13, T 4°C, comet assay kit) for 20 minutes to allow denaturation and DNA unwinding. Electrophoresis in an alkaline buffer (pH 13, T 4°C, comet assay kit) for 30 minutes allowed migration of loops of DNA whose supercoiling was relaxed by a strand break to migrate towards the anode. Slides were then soaked in a neutralization buffer (Tris base 400 mM pH 7.5) for 10 minutes, washed twice in distilled H2O for 10 minutes and finally air-dried to bring cells into a single plane to facilitate observation. After staining with 1× ethidium bromide, single nucleoid migration profiles were analyzed with fluorescence microscopy (Leica DMRXA) connected to a camera (Leica DFC340 FX) and a computerized image analysis system (COMET IV software, Perceptive). The software computed all major measurement parameters, including (1) tail length: distance from center of nucleoid mass to distal tail end, (2) tail intensity (TI): relative fluorescence intensity of comet tail, a measure of percentage of DNA in the tail, and (3) tail moment (TM): essentially the product of tail length and TI. For our purposes, TM was selected to measure DNA fragmentation.

Determination of serum 8-OHdG by ELISA

Serum 8-OHdG levels were determined by the enzyme-linked immunosorbent assay (ELISA) using commercially available kit: human 8-OHdG immunoassay (Glory Science, TX, USA). All ELISA procedures were done by the Hyprep automated ELISA system (Hyperion, Inc., Miami, FL, USA) according to the manufacturer’s instructions.

Statistical analysis

Differences in expression levels of TXN, sr-8-OHdG, and TM between different groups were analyzed using the Mann–Whitney U test and the Kruskall–Wallis test. Correlation of TXN expression level with sr-8-OHdG and TM in different groups was analyzed using the Spearman test. Statistical significance was accepted when the P values were less than 0.05. All of the analyses were performed by the SPSS 17.0 software.

Results

Clinical characteristics of the studied groups are summarized in Table 1.

TXN was under expressed in AML and ALL groups as compared to the control group

For AML patients, the median fold expression level of TXN was 0.514 (range, 0.11–0.96), whereas for ALL patients, it was 0.563 (range, 0.28–0.99) which is significantly lower than normal expression level indicating under expression (P < 0.001 for both AML and ALL). Of note, there was no significant difference between the fold expression levels of TXN in AML and ALL groups (P = 0.146; Fig. 1).
Serum 8-OHdG was significantly higher in AML and ALL groups as compared to the control group

For AML patients, the median serum 8-OHdG level was 679.5 (range, 452.73–1055) and for ALL patients, it was 634.2 (range, 486.15–895) which was significantly higher than the control group (median = 155.9, range = 134.5–227.5) (P < 0.001 for both), while there was no significant difference between AML and ALL groups (Fig. 2).

The TM was significantly higher in AML and ALL groups as compared to the control group and in AML group compared to ALL group

For AML patients, the median TM was 25.9 (range, 21.14–32.4) and for ALL patients, it was 22 (range, 15.6–30) which was significantly higher than the control group (median = 5.75, range = 4.6–7.7) (P < 0.001 for both). Also, TM in the AML group was significantly higher than the ALL group (P < 0.001; Fig. 3).

Serum 8-OHdG and TM were positively correlated in both AML and ALL groups

Our results showed strong positive correlation between serum 8-OHdG and TM in AML and ALL groups (P = 0 and P = 0.002, respectively; Fig. 4A and B)

Expression levels of TXN were negatively correlated with serum 8-OHdG and TM in AML and ALL groups

The TXN fold expression showed strong negative correlation with serum 8-OHdG in both AML and ALL groups (P = 0.042 and P = 0, respectively; Fig. 5A and B). Similar results were shown with TM (P = 0.047 and 0.02, respectively; Fig. 6A and B)

Expression levels of TXN were not correlated with EFS or OS in AML and ALL groups

The TXN fold expression showed no correlation with event-free survival (EFS) or overall survival (OS) in both AML (P = 0.74 and 0.28, respectively) and ALL (P = 0.68 and 0.89, respectively).

Table 1 Clinical characteristics of the studied groups

| Characteristic | Control | Median | Range       | AML | Median | Range       | ALL | Median | Range       |
|---------------|---------|--------|-------------|-----|--------|-------------|-----|--------|-------------|
| Age (years)   |         | 41.5   | 20–65       |     | 45     | 18–70       |     | 31     | 18–79       |
| TXN fold expression |     | 0.514a | 0.11–0.96   |     | 0.563a | 0.28–0.99   |     |        |             |
| Sr 8-OHdG     |         | 155.9  | 134.5–227.5 |     | 679.5a | 452.73–1055 |     | 634.2a | 486.15–895  |
| TM            |         | 5.75   | 4.6–7.7     |     | 25.9a  | 21.14–32.4  |     | 22b,15b | 15.6–30     |
| EFS (months)  |         | –      | –           |     | 3.6    | 0–12        |     | 4.2    | 0–12        |
| OS (months)   |         | –      | –           |     | 7.9    | 1–18        |     | 10.7   | 1–18        |

aSignificantly different from the control group at P < 0.001.
bSignificantly different from the AML group at P < 0.001.
Discussion

For several decades, now, many researchers worldwide dedicated their research to achieve early diagnosis and treatment of malignancies. The discovery of novel diagnostic and therapeutic strategies for cancer is the only way to shift malignancy from a non-curable to a curable disease. Although the understanding of the mechanisms of carcinogenesis by oxidative stress has advanced greatly in the last two decades, future studies will be necessary to identify conditions that better predict the risk of transformation into an active disease that requires treatment.\(^{24}\)

Since its definition by Sies et al.,\(^ {25}\) oxidative stress was implicated in the pathogenesis of many diseases including cancer.\(^ {26}\) High rates of both lipid peroxidation and DNA base lesions that result in genomic instability, such as strand breaks, base modifications, and DNA–protein cross linkages, have been found in the majority of cancerous tissues. Oxidative DNA damage plays an essential role in the initiation of carcinogenesis and malignant transformation.\(^ {27}\) Generally, in response to increased ROS levels, cellular antioxidant molecules such as thioredoxin are upregulated to counteract their detrimental effect. However, cancer cells take advantage of upregulated antioxidant molecules to protect themselves against ROS-induced cell damage.\(^ {28}\) Oxidative stress was found to closely precede cancer and is responsible for the molecular alterations underlying the pathogenesis of cancer and as a result the byproducts of oxidative stress have been proposed to be promising clinical markers of cancer.\(^ {29}\) Based on the previous work, chronic lymphoid leukemia cells were found to accumulate ROS and reactive nitrogen species (RNS) due to deficiency in cellular antioxidants, including TXN.\(^ {29}\) Previously, TXN was found to be over expressed in solid tumors, and this was correlated with tumor aggressiveness and poorer prognosis.\(^ {21}\) However, TXN expression was not sufficiently studied in acute leukemia and we claim that this is the first study studying the fold expression of TXN in Egyptian patients newly diagnosed with acute leukemia.

In our study, we found that TXN was surprisingly under expressed in both AML and ALL; however, there was no significant difference between the two groups. This indicates that the pathogenesis of acute leukemia is quite different from other solid tumors where antioxidant molecules including TXN have been shown to be up-regulated, correlating with increased tumor proliferation, survival as well as chemoresistance.\(^ {30}\) This finding gives a new insight into the pathogenesis of acute leukemia. Being critical redox regulator, TXN is involved in a number of redox signaling pathways by activating numerous...
redox sensitive transcription factors that are essential for various cellular processes. TXN is usually present in the cytoplasm, but under certain circumstances it is translocated to the nucleus where it activates redox sensitive transcription factors. Several studies have reported the involvement of TXN in regulating the activity of the tumor suppressor gene p53 by enhancing the sequence-specific DNA-binding activity of p53.\textsuperscript{31} Also, TXN has also been shown to be positively correlated with p53-induced DNA repair in breast cancer.\textsuperscript{32} In addition, previous studies have shown that TXN is implicated in the regulation of the activity of phosphatase and tension homolog gene (PTEN) which is a redox sensitive tumor suppressor protein that is inactivated by H\textsubscript{2}O\textsubscript{2}-induced ROS generation.

TXN binds to an inactive oxidized form of PTEN and reduce it, leading to the activation of PTEN.\textsuperscript{33} From the previous, we can conclude that under expression of TXN in the leucocytes may result in inactivation of both p53 and PTEN allowing uncontrolled cell growth and proliferation giving rise finally to the development of acute leukemia which can be regarded as a novel mechanism of the pathogenesis of acute leukemia which requires further investigation and research to be confirmed. However, these results are quite different from the results of Zhou \textit{et al}.\textsuperscript{34} who found that TXN was overexpressed in relapsed AML. This difference might be attributed to the difference in the study population in both studies concerning the status of AML (\textit{de novo} or relapsed) as well as the ethnic differences.
Members of the TRX system regulate apoptosis through a wide variety of mechanisms. TRX proteins have antiapoptotic effect by scavenging intracellular ROS through the dithiol at the active site. Also, they interact with apoptosis signal-regulating kinase 1. TXNIP is a growth suppressor and its expression is suppressed in human T-cell leukemia virus type-1 transformed cells. Up-regulation of TXNIP caused an increase in ROS production and induced apoptosis in AML in vitro and in vivo. In light of these findings, the under expression of TXN is supposed to induce apoptosis. However, such a claim requires performing apoptosis assays in order to confirm or deny it and clarify the possible effects of TXN under expression on the apoptotic machinery and the resulting consequences regarding prognosis, response to chemotherapy and treatment outcome.

Another interesting finding in our study is that serum 8-OHdG was significantly higher in both AML and ALL groups than the control group and that TXN fold expression was negatively correlated with serum 8-OHdG in both AML and ALL. 8-OHdG is produced by oxidative action of ROS and RNS, including hydroxyl radical and peroxynitrite on DNA. TXN provides the electrons to thiol-dependent peroxidases (peroxiredoxins) to remove ROS and RNS rapidly in order to protect DNA from ROS and RNS attacks which causes the generation of 8-OHdG. Also, TXN is involved in DNA and protein repair by reducing ribo-nucleotide reductase, methionine sulfoxide reductases, and regulating the activity of many redox sensitive transcription factors. Thus, under expression of the TXN gene and the subsequent TXN deficiency results in the accumulation of ROS and RNS, including hydroxyl radical and peroxynitrite and accumulation of oxidative DNA damage as well as lack of DNA repair enzymes which finally lead to significant increase in oxidative DNA damage represented by increased serum 8-OHdG in the studied groups.

Also, TM was significantly higher in both AML and ALL groups compared to the control group and TXN fold expression was negatively correlated with TM in both AML and ALL. This finding highlights the excessive DNA strand breaks in the studied groups. Both nuclear and mitochondrial DNA are subjected to deleterious ROS-mediated modifications which if not repaired by base excision repair (BER) or nucleotide excision repair can lead to disease-causing mutations which can finally lead to cancer. As a result, failure of DNA repair due to inactivation of DNA repair enzymes by ROS results in DNA strand breaks. ROS predominantly causes DNA base or sugar damage forming single strand breaks (SSBs). However, double strand breaks (DSBs) can also arise after replication past ROS-induced lesions, or when they arise in close proximity. Although DSBs arise at a lower frequency than SSBs, they are biologically significant if unrepaired or misrepaired. There are multiple, overlapping pathways for BER and SSB repair, such that loss of proteins involved in the repair pathways tends to slow but not completely abolish repair. DSBs repair is much slower and loss of the major DSB rejoining pathway is extremely significant. Kim et al. proved that TXN affects BER activity. In order to understand the role of TXN, they constructed a TXN knockdown system, where DNA damage in terms of relative intensity of the DNA tail was markedly higher in the TXN knocked down cells compared with that in the wild-type cells. In addition, they observed a modulatory role of TXN in the BER pathway through the p53 downstream gene and DNA-damage-inducible protein 45 α (Gadd45α). P53 level and function decreased in the TXN knocked down cells which also showed decreased Gadd45α expression and interaction of Gadd45α with apurinic/apyrimidinic endonuclease 1 (APE1). In conclusion, the redox factor TXN might take part in the control of APE1 function by modulating the p53-mediated BER through the protein–protein interaction between Gadd45α and APE1, providing insight into the novel role of TXN in modulation of BER. And so our results confirm these findings where the under expression of TXN was accompanied by defective DNA repair mechanisms such as BER resulting in a significant increase in DNA strand breaks.

In addition to this, the TM in AML patients was significantly higher than in ALL patients. This can be partly explained by the findings of Sallmyr et al. where they stated that the genetic changes in myeloid malignancies lead to increased production of endogenous sources of DNA damage, such as ROS, that can drive a cycle of genomic instability leading to DSBs and changed repair that can lead to induction of genomic changes. Evidence is coming to light that defects in a main repair pathway for DSBs, non-homologous end-joining, lead to up-regulation of alternative repair mechanism that can create chromosomal deletions and translocations.

Moreover, we found that serum 8-OHdG and TM were positively correlated in both AML and ALL. In the studied groups, 8-OHdG increased as a result of TXN under expression and the resulting accumulation of ROS and increased oxidized DNA lesions. The consequent deficiency in DNA repair mechanisms results in increased SSB and DSB resulting in increased TM. These findings are in accordance with the findings of Bridge et al. previously mentioned.

Finally, we found that TXN expression level showed no correlation with EFS or OS in both AML and ALL groups and therefore, we cannot claim that it could be used to predict treatment outcome in acute leukemia.
Admitting that the number of cases and the follow-up period in this study were limited, we hope this study would prompt other scientists to investigate the implications of TXN expression in acute leukemia to confirm our observations in a larger series of patients.

Conclusion

In summary, this study investigated the relationship between TXN expression and the oxidative DNA damage in adult acute leukemia in Egyptians. We have shown that TXN was under expressed in adult acute leukemia patients which is associated increased oxidative DNA damage in these patients. Therefore, recombinant TXN can be incorporated in leukemia treatment regimens as a new approach to cut off oxidative DNA damage and further mutagenesis. New investigational approaches, as part of well-controlled trials, would be needed to develop a new treatment strategy in adult acute leukemia.

Acknowledgements

We would like to thank all the patients who participated in this study. We also acknowledge the Immunology Laboratory, Clinical Pathology Department, Faculty of Medicine, Ain Shams University, for their great help with the RT-PCR work.

Disclaimer statements

Contributors

Immunology laboratory staff, Clinical Pathology Department, Faculty of Medicine, Ain Shams University, helped with the RT-PCR work.

Funding

None.

Conflicts of interest

All authors have no conflict of interest to report.

Ethics approval

In April 2013, the protocol for this study was approved by the Committee of Medical and Pharmaceutical Ethics of Ain Shams University Hospitals in accordance with the Helsinki Declaration of 1975, as revised in 2008.

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