Prognostic Relevance of Ww-Oxidoreductase Gene Expression in Patients with Acute Lymphoblastic Leukemia

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Abstract

Background: The WWOX gene (WW-Oxidoreductase) gene is frequently lower expressed in variety of tumor.

Methods: Screening for WWOX gene expression was assessed using real-time reverse transcriptase polymerase chain in 50 ALL cases and 50 healthy control.

Results: WWOX gene was significantly lower in ALL cases (0.4323 ± 1.38925) when compared with healthy control (10.501 ± 9.0338) (p<0.001). No significant differences were found between high and low WWOX gene expression regarding clinical data, age, sex, hematological data. Patients who highly expressed WWOX gene achieved CR at significantly higher rates in ALL (p=0.002), and had significantly lower frequency refractory disease in ALL patients (p=0.017). Higher expression WWOX gene patients have statistically longer OS (p=0.049) when compared with low expression WWOX gene patients.

Conclusion: Our result suggested that WWOX gene was a predictor for better outcome, could be a useful target for immunotherapy and might represent a candidate marker for monitoring of minimal residual disease.

Keywords: WWOX, ALL; Hematological data; Tumor suppressor gene

Introduction

Acute lymphoblastic leukemia (ALL) is a form of leukemia, or cancer of the white blood cells characterized by the overproduction of cancerous, immature white blood cells—known as lymphoblasts [1]. Malignant, immature white blood cells continuously multiply, causing damage and death by inhibiting the production of normal cells—such as red and white blood cells and platelets—in the bone marrow and by spreading (infiltrating) to other organs. Acute Lymphoblastic leukemia (ALL) encompasses a group of lymphoid neoplasms that morphologically and immunophenotypically resemble B-lineage and T-lineage precursor cells. These neoplasms may present predominantly as a leukemic process, with extensive involvement of the bone marrow and peripheral blood or may be limited to tissue infiltration, with absent or only limited (less than 25%) bone marrow involvement [2]. The latter cases are typically designated as Lymphoblastic lymphomas (LBLs). ALL and LBLs appear to constitute a biologic continuum, although they may show distinct clinical features. The current World Health Organization Classification of hematopoietic neoplasms designates these disorders as B- or T-lymphoblastic leukemia/lymphoma [3]. The WW domain-containing Oxidoreductase (WWOX) gene is located at 16q23.3-24.1, a region that spans the second most common human fragile site, FRA16D [4]. The name for this newly identified gene comes from the fact that it has two WW domains coupled to a region with high homology to the short chain dehydrogenase/ reductases family of enzymes [5]. Genomic analysis has revealed that WWOX contains 9 exons encoding an mRNA that is 2.2 kb long, which encodes a 46 kDa WWOX protein containing 414 amino acids [4]. The full-length WWOX, which encodes a 414 amino acid protein, possesses two typical N-terminal WW domains (first domain, amino acids 17-49; second domain, amino acids 58-90), a C-terminal short-chain dehydrogenase reductase (SDR) domain, and a nuclear localization sequence (NLS) [6]. Additionally, an NSYK (Asn-Ser-Tyr-Lys) motif for binding with sex steroid hormones, a nuclear localization signal (NLS) (GKRKRV), and a mitochondria-targeting sequence in the ADH/SDR domain have been defined in WWOX [7]. The first N-terminal WW domain is needed for the classical WW-PXXY interaction [6]. The first WW domain binds target proteins containing the proline-rich PXXYmotif (s) during signal transduction. For example, WWOX interacts with p73, activator protein 2y (AP-2y), ErbB4, ezrin, small integral membrane protein of the lysosome/late endosome (SIMPLE), and c-JUN transiently over expressed WWOX blocks the nuclear accumulation of p73, AP-2y, and c-JUN in vitro [8]. While presumed that mutation of p73 might lead to production of defective p73 protein and this might have a role in the process of leukemogenesis of ALL [1]. In the Wnt/β-catenin pathway, transiently over expressed WWOX prevents nuclear import of dishevelled [6]. Similarly, in the HGF/MET pathway, ectopic WWOX inhibits the MET C-terminal fragment for nuclear translocation and suppression of the downstream gene expression [6]. The proteins with the SDR domain are involved in oxidation and reduction of various substrates such as lipid hormones, sugars, alcohols and retinoids [6]. The gene is also called FOR, which stands for FRA16D Oxidoreductase. Alternative spliced WWOX transcripts (variants 1-8) encode proteins that share N-terminal WW domains in common but differ at their C-terminus, with variant 3 having a truncated Oxidoreductase domain. It has been suggested that proteins encoded by these variants interfere with normal WWOX function in a dominant negative fashion [7-9]. Recently, tumor suppressor’s p53

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Received September 18, 2015; Accepted October 10, 2015; Published October 14, 2015

Citation: Elbossaty WF, Malaf C, Elghanam DM (2015) Prognostic Relevance of Ww-Oxidoreductase Gene Expression in Patients with Acute Lymphoblastic Leukemia. J Cancer Sci Ther 7: 302-307. doi:10.4172/1948-5956.1000367

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and WWOX were shown to regulate the apoptosis of glioblastoma cells [10]. WWOX may act as an alternative receptor for sex steroid hormones, since its SDR domain possesses an NSYK motif capable of interacting with androgen and estrogen [10]. Under stress conditions, WWOX is activated via phosphorylation of p53, which stabilizes p53 and its apoptotic function [10] also, WWOX binds Disheveled proteins (Dvl), which are key components in Wnt/b-catenin signaling pathway. No PpY motifs in Dvl. Transiently over expressed WWOX sequesters Dvl-2 in the cytoplasm and there by blocks Dvl-2 mediated TCF transcriptional activity [10]. There are now approximately 100 reports concerning the correlation of the loss of WWOX expression with cancer development, including some reporting association of WWOX absence with poor prognosis and outcome in various cancer types. Ectopically over expressed Wwox has been reported to promote apoptosis [10], tumor suppression, suppression of anchorage-independent growth. Low, undetectable expression or aberrant transcripts of WWOX were found in tumor suppressor, suppression of anchorage-independent growth. Low, undetectable expression or aberrant transcripts of WWOX were found in tumors with poor prognosis and outcome in various cancer types Ectopically over expressed Wwox has been reported to promote apoptosis [10], tumor suppression, suppression of anchorage-independent growth.

Materials and Methods

Fifty patients (33 males and 17 females), newly diagnosed with ALL, were selected from oncology center, Mansoura university with written informed consent. The mean age was 38.40 ± 13.44 years, in addition, a control group of fifty healthy age and gender matched, were selected from oncology center, Mansoura university with written informed consent. The mean age was 38.40 ± 13.44 years, in addition, a control group of fifty healthy age and gender matched patients, newly diagnosed with ALL, were selected from oncology center, Mansoura university with written informed consent. The mean age was 38.40 ± 13.44 years, in addition, a control group of fifty healthy age and gender matched, were selected from oncology center, Mansoura university with written informed consent. The mean age was 38.40 ± 13.44 years, in addition, a control group of fifty healthy age and gender matched, were selected from oncology center, Mansoura university with written informed consent. The mean age was 38.40 ± 13.44 years, in addition, a control group of fifty healthy age and gender matched, were selected from oncology center, Mansoura university with written informed consent. The mean age was 38.40 ± 13.44 years, in addition, a control group of fifty healthy age and gender matched, were selected from oncology center, Mansoura university with written informed consent.

End points

Complete remission (CR) is defined as acellularity of more than 20% with fewer than 5% blasts in bone marrow (BM) after induction chemotherapy, and relapse is defined by the appearance of one of the following more than 50% lymphoblasts in asingle BM aspirate [13]. progressive repopulation of lymphoblasts in excess of 5% culminating in more than 25% on two or more BM samples separated by 1 week or more [14]. More than 25% lymphoblasts in the BM and 2% or more circulating lymphoblasts [15]. Leukemic cell infiltration in extramedullary organs for example, central nervous system or in CSF with cell count greater than 5 WBCs/mm. Disease -free survival (DFS) was defined only for those patients achieving a CR. It was measured from the CR date until date of relapse or death, regardless of cause, censoring for patients alive at least follow up.

RNA extraction and cDNAsynthesis

RNA was isolated from 1 ml ALL peripheral blood by RNA blood mini kit (Qiagen, Hilden, Germany) following the manufacturer instructions. Subsequently 0.5 μg RNA was reverse transcribed in to cDNA in 20 μl reverse transcriptase buffer containing 10 mMol/L DTT, 0.5 mMol/L each of dATP, dGTP, dCTP and dTTP, 200 units of Moloney murine leukemia virus reverse transcriptase,5 units of RNase inhibitor,and 5 mMol of random hexamers (MBI Fermentas, st.Leon-Rot, Germany) and applied to 7000 sequence detection system at 25°C for 10 min, 370°C for 120 min and 850°C for 5 min.

Quantitative real - time (QRT-PCR)

The mRNA expression level of WWOX and the endogenous housekeeping gene GA PDH as a reference were quantified by the RT - QPCR method using ABI prism 7000 real-time PCR sequence detection system (Applied Biosystems, Foster city,CA).the sequence of primers and probes of WWOX and PCR products were amplified and detected using dual fluorescent non-extendable probes labeled with 6-carboxy-fluorescien (FAM), reporter and 6-carboxyfluorescien methylrhodamine (TAMRA), quencher at 5'-end and 3'-end, respectively. 4μl of cDNA in each PCR reaction in a final volume of 20 μl containing 900nmol/L of sence and antisense primers (Table 1), 200 nmol/L of the TaqMan probe, 5 mMol/L MgCl2, KCl, and Tris- HCl, 0.2 mMol/d dATP, dCTP, dGTP, dTTP and 0.5 units of AmpliTaq DNA polymerase PCR program was 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds for each patient and control. The relative mRNA expression level of WWOX was calculated using the comparative cycle method. Briefly the target PCR Ct values, that is, the cycle number at which emitted fluorescence exceeds 10x the standard deviation of baseline emissions are normalized to the GAPDH Ct value. Relative mRNA expression levels were calculated using the 2-△△Ct method.

Statistical analysis

Data were analyzed using chi-square or Fisher exact tests to analyze the expression of mRNA levels of WWOX gene between different groups. Survival curves were plotted using the Kaplan-Meier method, and differences were analyzed using log-rank tests. A p-value <0.05 was considered significant. Logistic regression was carried out using odd ratio. Proportional hazards models were constructed to determine whether WWOX was associated with outcome when adjusting for other prognostic variables. All data analyses were done using SPSS software version 22.

Results

Expression of WWOX mRNA in ALL patients

In this study, the expression of WWOX mRNA was investigated in the 50 ALL patients and 50 cases of healthy controls by using real-time PCR. WWOX low expression was found in 25 (50%) of 50 patients, the difference between WWOX expression in ALL patients and healthy controls was found to be statistically significant (p<0.001) (Table 2).

Table 1: Sequence of primers and probe for WWOX gene expression.

| Primers | Primer Sequence |
|----------|-----------------|
| Forward primer | 5'-CCCCAGGAGAATTTCCAGATT-3' |
| Reverse primer | 5'-CAGTGGAGACACCTGGATGATT-3' |
| TaqMan® Probe | 5'-FAM TACAATGTTGCGACCTGACTGT TAMRA-3' |

Prognostic relevance of WWOX expression

High expression WWOX had a statistically higher CR (23 of 25; 92% vs 15 of 25; 60%; p=0.002), lower refractory (0 of 25; 0% vs. 10 of 25; 40%; p= 0.017) (Table 4). Kaplan –Meier analysis demonstrated significantly longer OS and DFS in highly expression WWOX (86.7% vs. 56.6%; 37.034 months vs. 22.998 months; p= 0.041, 83.6% vs. 76.9%; 37.464 months vs. 34.333 months; p= 0.883) (respectively in OS and DFS).

No PPxY motif is in Dvl. Transiently over expressed WWOX sequesters Dvl-2 in the cytoplasm and there by blocks Dvl-2-mediated TCF transcriptional activity [10]. There are now approximately 100 reports concerning the correlated of the loss of WWOX expression with cancer development including some reporting association of WWOX absence with poor prognosis and outcome in various cancer types. Ectopically over expressed Wwox has been reported to promote apoptosis [10], tumor suppression, suppression of anchorage-independent growth. Low, undetectable expression or aberrant transcripts of WWOX were reported in several tumor cell lines of different origins [11,12].
comparison to low expression WWOX patients (Table 5, Figures 1-3). Applying to odd ratio of high to low WWOX expression showed that highly WWOX expressers were 2.1 times more likely to have complete remission (95% CI:0.03-4.61; p=0.01), 2.46 times more likely to relapse (95% CI:0.187-2.41;p=0.0494) and 0.05 times more likely to refractory (95% CI:0.01-0.32; p=0.2) than low WWOX expressers (Table 6). Multivariate analysis confirmed the prognostic value of WWOX expression as independent predictor for longer OS (HR=0.347, 95% CI: 0.095-0.872; p=0.01) (Table 7). Taken together, these results confirm the prognostic value of WWOX in ALL.

Discussion

The WWOX gene was identified recently as a tumor suppressor gene at 16q23.3–24.1, a chromosome region that spans the common fragile site FRA16D. Several studies have revealed alterations of WWOX in several types of human cancers. The present study demonstrated that a relatively low expression of WWOX correlates with complete remission and relapse diagnosis statuses in ALL patients, supporting the hypothesis that the occurrence of ALL is a progressive and multi-staged process, similar to that of other tumors. In the present study, significant amount of low expression of WWOX mRNA were reported in 50% (25 of 50) of ALL. Chen X et al. found that low level of mRNA WWOX gene was detected in about 48.2% from ALL cases. Epigenetic changes contribute greatly to leukemia development. DNA methylation is a well-studied mechanism in epigenetic. The hypermethylation of numerous genes has been detected in various types of tumors and hematological neoplasms [16]. Previous studies have shown that DNA methylation is the most commonly detected alteration in ALL [17]. DNA methylation often results in the silencing of tumor suppressor genes, although the gene sequence may not have been changed. This mechanism has been identified as leading to the loss of function of numerous tumors suppressor genes in various types of tumor cells [18]. Based on these observations, it has been postulated that the inactivation of WWOX is driven by homozygous deletions or methylation status.

Table 2: WWOX gene expression in studied groups.

| Variables | Control (n=50) | ALL (n=50) | P |
|-----------|---------------|------------|---|
| WWOX ΔΔCT Median (Range) | 31.07 (20.2-32) | 33.060 (21.9-41) | 0.011* |
| Mean ± SD | 28.17 ± 5.4015 | 33.028 ± 4.22918 | |
| Expression Median (Range) | 14.846 (0.0139-20.1) | 0.005.8203 (5.04X10-6-6.88) | <0.001** |
| Mean ± SD | 10.501 ± 9.0338 | 0.43239 ± 1.38925 | |

Table 3: Patient’s characteristics according to WWOX expression status.

Table 4: Comparison between high and low WWOX expressers regarding outcome measures.

Table 5: Survival analysis in studied cases.

J Cancer Sci Ther ISSN: 1948-5956 JCST, an open access journal Volume 7(10) 302-307 (2015) - 304

Citation: Elbossaty WF, Malak C, Elghanam DM (2015) Prognostic Relevance of Ww-Oxidoreductase Gene Expression in Patients with Acute Lymphoblastic Leukemia. J Cancer Sci Ther 7: 302-307. doi:10.4172/1948-5956.1000367
status of WWOX may aid in the development of future treatment approaches for ALL.

Conclusions and Future Directions

As far as we can tell, this is the first investigation regarding the role of the WWOX gene in the pathogenesis of human leukemia. Because the loss of WWOX expression is common but the gene deletion is not so frequent, epigenetic changes of the WWOX gene such as promoter methylation might also be involved in the pathogenesis of leukemia. Future investigation into the epigenetic regulation of the WWOX gene will shed more light into the early event leading to the loss of the WWOX tumor suppressor gene and provide new therapeutic
opportunities for acute leukemia with the emerging drugs that reverse the cancer associated epigenetic alteration.

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