Original Article

Expression of UBE2Q2, a Putative Member of the Ubiquitin-conjugating Enzyme Family in Pediatric Acute Lymphoblastic Leukemia

Atefeh Seghatoleslam PhD•[1], Ahmad Monabati MD[2], Farzaneh Bozorg-Ghalati MSc[1], Mohsen Nikseresht PhD[3], Mohammad-Reza Bordbar MD[4], Mostafa Rahvar MSc[1], Ali Akbar Owji PhD[1]

Abstract

Background: Acute lymphoblastic leukemia (ALL) is a cancer of the white blood cells most commonly found in childhood with a peak incidence at 2–5 years of age. The ubiquitin degradation pathway facilitates degradation of damaged proteins and regulates the growth and stress response. This pathway is activated in various cancers, including ALL. It has been previously reported that the newly characterized human gene UBE2Q2, a putative member of the ubiquitin-conjugating enzyme family, is over-expressed in the tumor mass and invasive epithelium in head and neck squamous cell carcinoma and breast cancer.

Methods: Here, we have used quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to assess expression of the UBE2Q2 gene in bone marrow samples of 20 children with ALL. Whole blood samples of 20 normal children were used as control specimens.

Results: RT-PCR revealed the expression of UBE2Q2 mRNA in 80% of the bone marrow samples from ALL patients as well as in 85% of leukemic normal peripheral blood cells. According to the results of quantitative RT-PCR, the levels of UBE2Q2 mRNA expression in the bone marrow cells of 11 out of the 20 children with ALL (55%) were significantly higher (> 2–47 fold) than those in blood cells of normal children.

Conclusion: Our data suggest that the newly characterized human gene, UBE2Q2, may have implications for the pathogenesis of ALL and could be used for molecular diagnosis purposes in the future.

Keywords: Acute lymphoblastic leukemia, ubiquitin conjugating enzyme, UBE2Q2, real-time PCR

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder that originates in a single B or T lymphocyte progenitor. The disease is most common in children but can be seen in individuals of any age. ALL has many subtypes and can be classified by immunologic, cytogenetic, and molecular genetic methods.[1] One of the pathways which has been mostly studied in different cancers is the ubiquitin degradation pathway. Ubiquitin-mediated degradation is a complex process comprised of well-defined steps that involve ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). Proteins tagged with ubiquitin are subsequently recognized by the proteasome for digestion and fragmentation. The enzymatic nature, multitude of E2s and E3s, and their specific substrate recognition predestines them as therapeutic targets.[2] Timed degradation of cellular regulatory proteins by this pathway also plays a critical role in controlling cellular growth and proliferation. Substrates of this pathway include tumor suppressors, cell cycle proteins, transcription factors, and tyrosine kinase receptors.[3,4] There is evidence that de-regulation of the ubiquitin-proteasome pathway can lead to cancer development through the mechanisms that control the stability of important proteins, such as those involved in regulating transcription and growth factors.[5–7] Accordingly, some members of the E2 family of human ubiquitin-conjugating enzymes have been shown to be overexpressed in hepatocellular carcinoma[8] and leukemia.[9] UBE2Q2 (also named LOC92912) was initially identified as a sequence that upregulated in hypopharyngeal tumors subjected to differential display and microarray techniques.[10,11] Further characterization of UBE2Q2 conducted by our group has shown that the gene is upregulated in about 85% of hypopharyngeal tumors, in the invasive epithelium and cancer islets of the tumor samples.[12] The gene is located on chromosome 15, encodes a protein of 375 amino acids, and is a novel putative member of the E2 ubiquitin-conjugating enzyme family.[12] With subsequent studies on breast cancer, we have shown that UBE2Q2 mRNA is over-expressed in 71.4% of tumor samples compared to corresponding normal tissue.[13] In a parallel study we have observed that this gene is up-regulated in invasive ductal carcinoma when compared to the in situ part of the breast (unpublished data). The present study quantitatively evaluates the expression of UBE2Q2 gene, as a newly identified gene, in ALL.

Materials and Methods

The study included 20 bone marrow aspirations from 3–14 year-old patients diagnosed with childhood ALL and 20 EDTA

Authors' affiliations: [1]Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. [2]Department of Pathology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. [3]Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran. [4]Department of Hematology and Oncology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.
•**Corresponding author and reprints:** Atefeh Seghatoleslam PhD, Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz 71345-1167, Iran. Tel: +98-711-230-3029, Fax: +98-711-230-3029,
E-mail: seghatolea@sums.ac.ir
Accepted for publication: 16 November 2011

Clinicopathologic parameters	Normal	ALL
Subjects (*n*)	20	20
Age (yr)	3 – 14	3 – 14
Immunophenotype	—	—
Early pre B cell	—	1
Pre B cell	—	7
Mature B cell (Burkitt›s)	—	1
Common type	—	6
T-cell	—	5
WBC× 10^3/μL	11.43 ± 0.66	367.59 ±182.63
RBC× 10^6 /μL	4.22 ± 0.13	2.76 ± 0.12
HB (g/dL)	12.24 ± 0.30	7.6 ± 0.38
PLT × 10^3/μL	321 ± 26.34	55 ± 5.62
Blast (%)	—	91.5 ± 1.23
Lym (%)	—	6.05 ± 1.17
WBC= white blood cell; RBC= red blood cell; HB= hemoglobin; PLT= platelet count; Lym= lymphocyte		

peripheral blood samples collected from normal healthy children as controls. This study enrolled only patients who were new cases of ALL and had not taken any therapeutic medications at the time of sampling, as the main inclusion criteria. Patients were selected from those who attended the Hematology and Oncology Clinics of Shahid Faghihi Hospital, affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. In all cases, sample collection was performed according to the rules governed by the Ethics Committee of Shiraz University of Medical Sciences. Immunocytochemistry of the leukemia samples was performed; those confirmed as ALL were examined for detection of ALL typing. All samples were cell counted; clinicopathological significance was determined and prepared for RNA extraction.

RNA extraction

Peripheral blood or bone marrow samples were collected in EDTA. White blood cells were immediately isolated using Ficoll density centrifugation and the cells were lysed in Tripure isolation reagent (Roche Applied Science, Germany). Total RNA was extracted following the manufacturer's instructions. The quantity and purity of total RNA were determined by ultraviolet spectrophotometer. The integrity of RNA was confirmed by agarose gel electrophoresis.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was prepared from 5 μg of RNase-free DNase-treated RNA (random primer or random hexamers) with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, EU). The primer sequences were 5′-CCGTGGGTAGT-GGTTGATCT-3′ and 5′-AGCGATTCCGCATCGTCAGT-3′ for UBE2Q2, in addition to 5′-GAAGGCTGTGGTGCTGATGG-3′ and 5′-CCGGATATGAGGCAGCAGTT-3′ for the RPLP0 gene as an internal control. PCR conditions were optimized to achieve the best annealing temperature for all primers as follows: initial denaturation (94°C, 5 min); 30 cycles for amplification (denaturation: 94°C, 30 sec, annealing: 62.5°C, 30 sec, extension: 72°C, 1 min) and final extension (72°C, 7 min). After each run, PCR products were loaded on a 1.5% agarose gel that contained gel red (Biotium, USA) along with a DNA marker of 50-1000 bp (Fermentas) and run at 100 volts. For this purpose, 12 μL of each PCR product was mixed with 5 μL of loading dye and loaded on the gel. A single specific band was observed for each of the UBE2Q2 and RPLP0 genes.

Real Time PCR

Quantitative RT-PCR was performed by the ABi thermal cycler (Applied Bio System, USA). The PCR reaction mixture contained 2 μL of cDNA (approximately 150 ng), 1 μL of 10 μmol/L of each of the forward and reverse primers, and 12.5 μL of ABi SYBR Green Super Mix in a total volume of 25 μL. The samples were loaded on optical 8-tube stripes (0.2 mL) in duplicate or triplicate. All incubations included an initial denaturation at 95°C for 10 min and 40 cycles (15 sec at 95°C, and 1 min at 60°C), followed by a melting curve (60–95°C). For all runs, a set of five-fold serial dilutions of the internal standard was used to generate a standard curve. Product specificity was confirmed by agarose gel (2.0% w/v) electrophoresis of the amplified products and by melting curve analysis (Tm). All quantitative real-time PCR assays were linear within this concentration range, with correlation coefficients of $r^2 > 0.999$. Data were analyzed by the ratio = $2^{-\Delta\Delta ct}$ method.[14]

Statistical analysis

Statistical analysis of the differences in the expression levels of UBE2Q2 mRNA was done by Mann-Whitney rank-sum test using SPSS 10 analytic software (SPSS, Inc., Chicago). The differences are considered significant at $P < 0.05$. The data are expressed as mean ± SEM.

Results

### Clinicopathological data of normal and ALL samples

The major laboratory findings and clinicopathological information of peripheral blood samples related to 20 normal and 20 patients with ALL are presented in Table 1.

### UBE2Q2 gene expression in ALL and normal peripheral blood by RT-PCR

Electrophoretogram related to the RT-PCR products of the UBE2Q2 gene in the bone marrow cells of ALL patients and blood cells of normal subjects are shown in Figure 1. The specific single bands of 317 bp for UBE2Q2 and 102 bp for RPLP0 were observed. Our RT-PCR data revealed that 16 out of 20 leukemic marrow samples (80%) and 17 out of 20 normal blood samples (85%) expressed UBE2Q2 mRNA.

UBE2Q2 gene expression in ALL and normal peripheral blood by quantitative RT-PCR

Relative expression of the levels of UBE2Q2 mRNA in cancer-
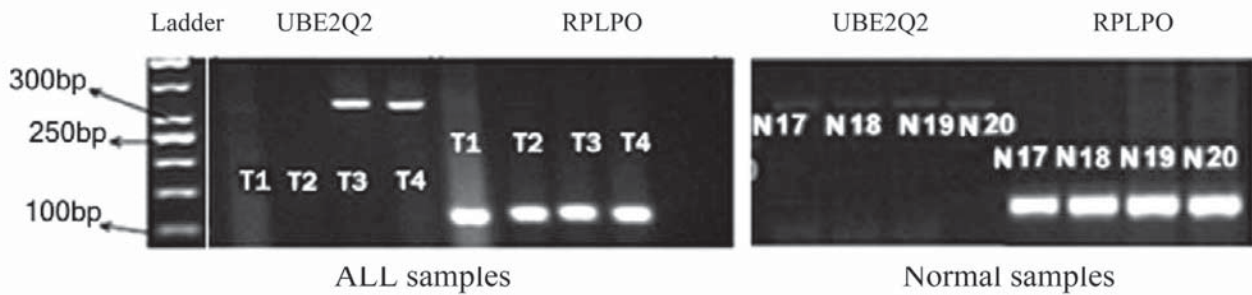
Figure 1. Representative RT-PCR results of UBE2Q2 gene expression in ALL patients (left panel) and normal subjects (right panel). Internal control, RPLPO (lanes 5–8 in both left and right panels). Lane 2, left panel is an ALL sample that has no detectable UBE2Q2 expression.
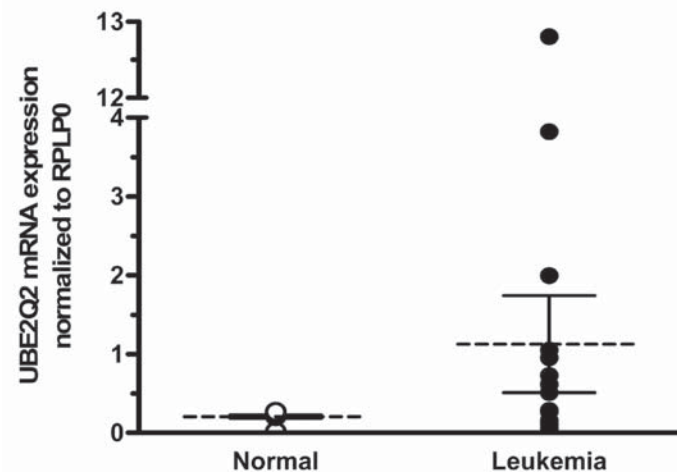
Figure 2. Quantitative RT-PCR results using specific primers for UBE2Q2. RPLP0 is the internal control. Relative expression (mean ± SEM) is 0.25 ± 0.002 for normal and 1.387 ± 0.67 for ALL samples. The difference between both groups is significant at P= 0.001 (Mann-Whitney test).

ous bone marrow cells and normal blood cells are presented in Figure 2. Accordingly, the transcript was found to be over-expressed in 11 out of the 20 ALL bone marrow samples (55%), in a range from 2–47 fold as compared to the mean of the control samples. In four patients (20%), the expression of UBE2Q2 mRNA was equivocal (1/2 to 2) and downregulation of mRNA for UBE2Q2 (<1/2 fold) was observed in five of the leukemic patients (25%) when compared to normal subjects. The difference between the mean level of UBE2Q2 expression in the ALL (1.387 ± 0.67) and normal (0.25 ± 0.002) subjects was statistically significant at P = 0.001 (Mann-Whitney test).

Discussion

This study has aimed to investigate the expression pattern of the newly characterized human gene, UBE2Q2, in ALL. The protein encoded by this gene contains a C-terminal ubiquitin-conjugating enzyme domain. This domain is highly conserved among species, suggesting that it has an important biological function. UBE2Q2 gene has been shown to be upregulated at both protein and mRNA levels in hypopharyngeal head and neck squamous cell carcinoma[12] and breast cancer.[13] The gene has also been reported to alter cell cycle progression in cultured cells.[15]

UBE2Q2 belongs to the E2 family of enzymes and potentially has ubiquitin-conjugating enzyme activity. The involvement of the components of the ubiquitin-proteasome pathway in leukemia has been assessed in several studies as follows: downregulation of ubiquitin gene expression during differentiation of human leukemia cells,[16] over-expression of CDC34 ubiquitin-conjugating enzyme in pediatric acute lymphoblastic leukemia,[9] the involvement of ubiquitin-conjugating enzyme UbcH5A and UbcH6 in degradation of some proteins in leukemia,[17] over-expression of ubiquitin-conjugating enzyme E2N (CCRF-CEM) in T-cell ALL,[18] and induction of ubiquitin-conjugating enzyme UBCH8 in AML patients treated through chemotherapy.[19]

The proteasome and its upstream system of ubiquitin-conjugating enzymes is responsible for 80% of the cell's protein degradation, and therefore has a major role in cellular homeostasis.[5] There is a delicate network of thousands of proteins whose interactions in the human cell are tightly regulated; imbalances in this network lead to diseases such as cancer and various genetic disorders. On the other hand, gene expression analysis has the potential to define prognostically distinctive ALL subsets, to offer insights into leukemogenesis and to identify potential therapeutic targets. In addition gene expression profiling can identify patients with favorable and unfavorable early responses to therapy. Flotho et al.[20] analyzed gene expression in the leukemia cells of 187 children with newly diagnosed ALL and reported a group of genes associated with ALL. Similarly, genetic profiling was utilized by Winter et al.[21] to predict induction failure in T-ALL patients, and by Bhojwani et al.,[22] and Cortez et al.[23] to predict early response and outcome in high-risk childhood ALL. Thus, genetic profiling may identify

patients with poor initial responses. By identifying such patients at diagnosis, alternative treatments could be incorporated earlier in treatment, being probably more effective in preventing or overcoming relative chemoresistance.

In this study, expression of UBE2Q2 relative to that of housekeeping gene, RPLP0, was investigated. For this purpose, RT-PCR and real-time PCR methods were applied. According to our RT-PCR results, the gene was expressed in a large proportion, but not all cells from both normal and leukemia samples. Real-time PCR data revealed that most of the leukemic cells expressing UBE2Q2 showed higher levels of UBE2Q2 mRNA when compared to the mean expression levels of UBE2Q2 mRNA in the normal blood cells that tested positive for the transcript. Correlation analysis by RT-PCR showed a significant correlation between expression levels of mRNAs that encoded UBE2Q2 in children diagnosed with ALL and normal children. As shown by the results, significant upregulation (2–47 fold) was observed for UBE2Q2 gene in ALL samples when compared to the normal ones. This result agreed with other reports concerning the upregulation of this gene in different cancers, including Head and Neck Squamous Cell Carcinoma[12] and breast cancer.[13]

In the present study, the control group was comprised of peripheral blood samples from age-matched healthy subjects that mostly contained mature and differentiated cells. Our test group consisted of acute leukemia bone marrow aspirates that contained mainly blast cells. A more appropriate control group could be constructed from bone marrow aspirates of age-matched healthy subjects. However, we were not able to collect such samples due to human and ethical considerations. Thus, because of the novelty of the subject and lack of adequate information about UBE2Q2 we found it worthy to carry out this project.

The proteasome-ubiquitination pathway is a central part of the cell's homeostasis so the rewards of developing agents that can specifically interfere with the E2 ubiquitin-conjugating enzyme system will remarkably be high in the area of cancer treatment. However, while the necessity of continual investigation on this gene and on a larger number of samples still exists, our data suggest that it might be a potentially useful tool in molecular diagnosis purposes and a drug target for treating ALL in the future. The results of our previous studies have suggested that actin[12] and other cytoskeleton proteins[24] may be potential substrates for the protein product of the UBE2Q2 gene. However, finding the link between the product(s) of this gene and cancer development is a potential outlook for future studies.

Acknowledgments

References

1. Lichtman M, Beutler E, Kipps T, Seligsohn U, Kaushansky K. Acute Lymphoblastic Leukemia. Malignant diseases. In: Lichtman MA, Williams WJ, eds. Williams Hematology, 7th ed. Part IX. Chapter 91. New York: McGraw-Hill; 2007: 1321 – 1325.
2. Nandi D, Tahiliani P, Kumar A, Chandu D. The ubiquitin-proteasome system. *J Biosci*. 2006; **31:** 137 – 155.
3. Hershko A. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. *Cell Death Differ*. 2005; **12:** 1191 – 1197.
4. Vodermaier HC. APC/C and SCF: controlling each other and the cell cycle. *Curr Biol*. 2004; **14:** R787 – R796.
5. Schwartz AL, Ciechanover A. Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. *Annu Rev Pharmacol Toxicol*. 2009; **49:** 73 – 96.
6. Schwartz AL, Ciechanover A. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med*. 1999; **50:** 57 – 74.
7. Lipkowitz S. The role of the ubiquitination-proteasome pathway in breast cancer: ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer. *Breast Cancer Res*. 2003; **5:** 8 – 15.
8. Ieta K, Ojima E, Tanaka F, Nakamura Y, Haraguchi N, Mimori K, et al. Identification of overexpressed genes in hepatocellular carcinoma, with special reference to ubiquitin-conjugating enzyme E2C gene expression. *Int J Cancer*. 2007; **121:** 33 – 38.
9. Eliseeva E, Pati D, Diccinanni MB, Yu AL, Mohsin SK, Margolin JF, et al. Expression and localization of the CDC34 ubiquitin-conjugating enzyme in pediatric acute lymphoblastic leukemia. *Cell Growth Differ*. 2001; **12:** 427 – 433.
10. Cromer A, Carles A, Millon R, Ganguli G, Chalmel F, Lemaire F, et al. Identification of genes associated with tumorigenesis and metastatic potential of hypopharyngeal cancer by microarray analysis. *Oncogene*. 2004; **23:** 2484 – 2498.
11. Lemaire F, Millon R, Young J, Cromer A, Wasylyk C, Schultz I, et al. Differential expression profiling of head and neck squamous cell carcinoma (HNSCC). *Br J Cancer*. 2003; **89:** 1940 – 1949.
12. Seghatoleslam A, Zambrano A, Millon R, Ganguli G, Argentini M, Cromer A, et al. Analysis of a novel human gene, LOC92912, overexpressed in hypopharyngeal tumors. *Biochem Biophys Res Commun*. 2006; **339:** 422 – 429.
13. Nikseresht M, Seghatoleslam A, Monabati A, Talei A, Bozorg Ghalati F, Owji AA. Over expression of the novel human gene, UBE2Q2, in breast cancer. *Cancer Genetic cytogenet*. 2010; **197:** 101 – 106.
14. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; **29:** e45.
15. Seghatoleslam A, Zambrano A. Novel human gene, LOC92912, alters cell cycle progression in cultured cells. *Iran J Med Sci*. 2009; **34:** 277 – 284.
16. Shimbara N, Sato C, Takashima M, Tanaka T, Tanaka K, Ichihara A. Down-regulation of ubiquitin gene expression during differentiation of himan leukemia cells. *FEBS Lett*. 1993; **322:** 235 – 239.
17. Gu H, Roizman B. The degradation of promyelocytic leukemia and Sp 100 proteins by herpes simplex virus 1 is mediated by the ubiquitin-conjugating enzyme UbcH5a. *Proc Natl Acad Sci U S A*. 2003; **100:** 8963 – 8968.
18. Gez S, Crossett B, Christopherson RI. Differentially expressed cytosolic proteins in human leukemia and lymphoma cell lines correlate with lineages and functions. *Biochim Biophys Acta*. 2007; **1774:** 1173 – 118319.
19. Buchwald M, Pietschmann K, Müller JP, Böhmer FD, Heinzel T, Krämer OH. Ubiquitin conjugase UBCH8 targets active FMS-like tyrosine kinase 3 for proteasomal degradation. *Leukemia*. 2010; **24:** 1412 – 1421.
20. Flotho C, Coustan-Smith E, Pei D, Cheng C, Song G, Pui CH, et al. A set of genes that regulate cell proliferation predicts treatment outcome in childhood acute lymphoblastic leukemia. *Blood*. 2007; **110:** 1271 – 1277.
21. Winter SS, Jiang Z, Khawaja HM, Griffin T, Devidas M, Asselin BL, et al. Identification of genomic classifiers that distinguish induction failure in T-lineage acute lymphoblastic leukemia. *Blood*. 2007; **110:** 1429 – 1438.
22. Bhojwani D, Kang H, Menezes RX, Yang W, Sather H, Moskowitz NP, et al. Gene expression signatures predictive of early response and outcome in high-risk childhood acute lymphoblastic leukemia: a Children's Oncology Group Study. *J Clin Oncol*. 2008; **26:** 4376 – 4384.
23. Cortez MA, Scrideli CA, Yunes JA, Valera ET, Toledo SR, Pavoni-Ferreira PC, et al. mRNA expression profile of multidrug resistance genes in childhood acute lymphoblastic leukemia. Low expression levels associated with a higher risk of toxic death. *Pediatr Blood Cancer*. 2009; **53:** 996 – 1004.
24. Melner MH, Ducharme NA, Brash AR, Winfrey VP, Olson GE. Differential expression of genes in the endometrium at implanttation: upregulation of a novel member of the E2 class of ubiquitin-conjugating enzymes. *Biol Reprod*. 2004; **70:** 406 – 414.