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Evaluating the role of Ubiquitin D gene expression in types of leukemia

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ABSTRACT

Using animal models to develop new treatments is essential, especially in diseases like cancer. In this study, we induced leukemia by intravenous injection of cancer cells (BCL1 cell line) and the examination of cell markers in the animal's blood to study the changes in the expression of the UBD gene as a biomarker for diagnosing and examining the progress of the disease. For this purpose, five million BCL-1 cells were injected into the tail vein of BALBIe mice of the same breed. Fifty mice were killed after four weeks, and we examined peripheral blood cells and histological changes. Then RNA of the samples was extracted, and cDNA synthesis was done with the help of MMuLV enzyme, Oligo dT, and Random hexamer primers. Specific primers for UBD were designed using Primer Express software, and the expression level of the UBD gene was measured by the method. The results showed that in the CML group, the lowest expression level was 1.70 times, and in the ALL group, the highest expression level was 7.97 times compared to the control group. The average increase in UBD gene expression was 3.21 times in the CLL group and 4.94 times in the AML group. The UBD gene can be further investigated so that it may be used as a proposed biomarker for the diagnosis of leukemia. Therefore, the evaluation of the expression level of this gene can be used to diagnose leukemia. However, more studies than the currently applied methods are needed in cancer diagnosis, which has many errors compared to the technique used in this study, and to prove its accuracy and sensitivity.

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Introduction

Leukemia is one of the most common types of malignancy in children, with an incidence of approximately 40 per million people, which accounts for 30% of all cancer cases in children less than 15 years of age (1). This disease creates the effect of reproduction and incomplete development of white blood cells and bone marrow. In this disease, the bone marrow abnormally produces many blood cells (2). These blood cells do not function adequately compared to normal cells and cause the production of normal white blood cells to stop and minimize the ability of a person to fight diseases. Bone marrow leukemia is divided into two types, acute and chronic, so there are four types of leukemia (ALL, CLL, AML, and CML)(3). One of the common symptoms of this disease is weight loss and anorexia, but 40% of patients are asymptomatic. In this category of patients, only the abnormal count of blood cells is effective in diagnosis (4).

Leukemia is the second leading cause of death in some countries. The disease is seen in all age groups, even children, so about 32% of children's cancers are related to leukemia. The history of abortion and the risk of leukemia have also been examined in several studies, and contradictory results have been obtained (5). Acute leukemia may affect pregnancy and the fetus. Impairment and stagnation of fetal growth have been observed in mothers who did not undergo chemotherapy, as well as premature birth, spontaneous abortion, and stillbirths are common in acute leukemia (6). Fetal malformation occurs in 10% of deliveries that received chemotherapy in the first trimester of pregnancy. Several indirect factors, including exposure to infectious agents, and exposure to pets, are associated with leukemia. It seems that leukemia, like other cancers, is a multifactorial disease where environmental and genetic factors interact with each other in its occurrence (7).

Studies have shown that the amount of ubiquitin-D (UBD), also known as FAT10, is increased in cancer cells (5, 8, 9). The relationship between UBD expression with cell differentiation and advanced stages of cancer indicates that UBD is probably involved in the progression of carcinogenesis (10). In general, it seems that UBD can be an essential indicator in predicting, diagnosing, and checking cancer progress and as an informative factor in the early stages of the disease (11). So far, the relationship between the expression of this gene and the possibility of leukemia has not been evaluated in any study; therefore, the purpose of this study is to investigate UBD as a biomarker for early diagnosis and to investigate its role in an animal model of leukemia with the real time PCR technique.

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Table1. The sequence of UBD and GAPDH gene primers for real time PCR technique.

Gene		Primer Sequence	Molecular Weight
UBD	Forward	GCTGCATGCAAAGTCCTTTTTC	72bp
	Reverse	GAAGCATTGGGAGCCATCTC	
GAPDH	Forward	ATGGAGAAGGCTGGGGCT	124bp
	Reverse	ATCTTGAGGCTGTCATACTTCTC	

Materials and Methods

Leukemia induction in BALB/c mice

To induce leukemia, BCL-1 cells (Thermo Fisher Scientific, USA) were prepared and propagated in RPMI (Gibco medium) (Thermo Fisher Scientific, USA) and 10% fetal bovine serum or (Gibco FBS) (Thermo Fisher Scientific, USA) in an incubator with a temperature of 37°C and 5% carbon dioxide. When used for injection, the cells were washed and dissolved in an FBS-free medium and counted with trypan blue until the viable cells were more than 99%.

In this study, 50 eight-week-old female BALB/c mice were used. Mice were divided into two test groups (received five million BCL1 cells) and control (received the same medium volume).

Preparation of mouse peripheral blood

The blood of mice was collected using a syringe containing the anticoagulant heparin. Then, two milliliters of lysing solution containing ammonium chloride, potassium carbonate, and ethylene diamine tetra-acetic acid (EDTA) were added to remove red blood cells. After two minutes, FBS was added and centrifuged. To count the cells in each milliliter, a sample of the suspension was taken with a sampler and counted with a Neobar slide.

Also, before removing the red blood cells, a drop of blood was poured on the slide, and a spread was prepared. After drying and fixing with methanol, it was stained with Giemsa dye. Then in the area where the red blood cells were not compressed, three hundred leukocytes and the percentage of lymphocytes, neutrophils, monocytes, eosinophils, and basophils were counted using a cell counter.

Primer design for real-time PCR

To amplify the sequence of the UBD gene and GAPDH gene, a specific primer pair was designed for each gene. Primers were designed using Gene runner 5 and 3.0.1 Primer Express software. To prevent genomic DNA replication, primers were designed to amplify exonic junctions. The sequence of designed primers is shown in Table 1.

Real-time PCR reaction

To extract RNA from blood, 100 microliters of whole blood were first placed in a 1.5ml microtube. RNA extraction and cDNA synthesis were performed by RNX-Plus method and Vivantis kit.

UBD and GAPDH genes were amplified to measure gene expression with qPCR reactions. The final volume for each reaction was 20µl, including 100ng of Power SYBR® Green PCR Master, 1µl of cDNA, 10µl of Mix (Applied Biosystems, USA), 10mmol/µl primers, and 6µl of nuclease-free water. The temperature protocol was performed as denaturation at 95˚C for 3 minutes followed by 45 cycles, denaturation at 95˚C for 5 seconds, and annealing at 60˚C for 30 seconds. The expression of relative genes was determined by using the ΔΔct method. The samples of healthy mice were considered a normal group. Proliferation and Melting Curves were analyzed using the Applied Biosystems 7500 device. Then, a gene expression graph was drawn using Prism 5 GraphPad software.

Results

Differential count of peripheral blood leukocytes

From the peripheral blood of mice, the spread was prepared and stained by the Giemsa method, and then a differential count of leukocytes was done, the results of which are reported in percentage. As seen in Table 2, a significant difference was observed between the percentage of lymphocytes in the control and case groups (p=0.02). Also, the reduction of neutrophils (about 10% in the four-week group compared to the control) was significant, and the ratio of lymphocytes to neutrophils increased from 4.93 in the control group to 8.82 in the case group. No statistically significant difference was observed in the percentage of monocytes.

UBD gene expression level

In this research, a melting curve diagram was drawn for UBD and GAPDH genes (Figure 1) to check the specificity of primers by Cyber Green fluorescence dye, ensure the amplification of specific fragments, and prevent the absence of non-specific fragments in the PCR product. It confirms the correct connection primers to the UBD gene,

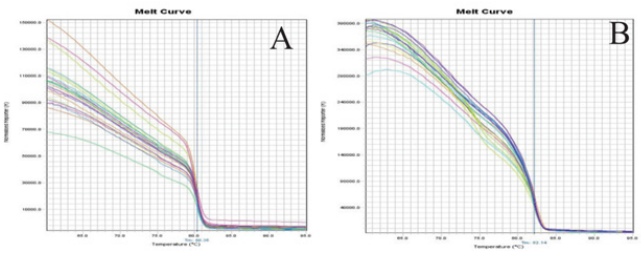

Figure 1. Melting curve diagram of GAPDH and UBD genes; A: GAPDH gene melting curve, B: Melting curve of the UBD gene.

Table 2. Differential count of lymphocytes, neutrophils, and monocytes in the blood of control and BCL-1 treated mice after two and four weeks (mean ± SD).

Cell Group	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)	Lymphocyte/Neutrophil
Control (n=50)	81.97 ± 0.53	16.62 ± 1.01	1.45 ± 0.17	4.93
Case (n=50)	87.78 ± 1.60*	9.96 ± 1.72	1.94 ± 0.48	8.82

* means statistical difference with the control group in P<0.05.

and the PCR product is precisely for the desired gene. After the amplification reaction, the ct of the samples was calculated by the device and converted to relative quantification or RQ. The gene expression was measured by the ΔΔCt method (Figure 1).

The expression levels of diseased mouse samples were compared with standard models, in which the results obtained are compared to the level of expression of the same gene in normal tissue. The RQ of the samples was removed from the device, and the results were drawn with Prism 5 GraphPad software (Figure 2).

Examining the results of the ubiquitin-D gene in normal samples and samples of mice with leukemia by Real-Time PCR

As the results show, the expression level of the UBD gene in patient samples has increased expression compared to healthy samples. This increase in expression indicates the highest growth in expression in sample number 45. The increase in expression is approximately 11 times that of the normal sample. In sample 43, there is the lowest increase in expression, this sample was in the CML group, and its expression increased very little compared to the normal sample.

In examining the type of leukemia in the samples, it was found that the mice in the CML group had the lowest expression (1.70 times). The ALL group had the highest expression (7.97 times) on average compared to normal and the average increase in UBD gene expression in individuals. It was found to be 3.21 times in the CLL group and 4.94 times in the AML group ($p<0.0001$). Therefore, it can be stated that the disease group is very effective in the level of UBD gene expression and plays an essential role in identifying leukemia. There is a significant relationship between the type of leukemia and the level of UBD gene expression (Figure 3).

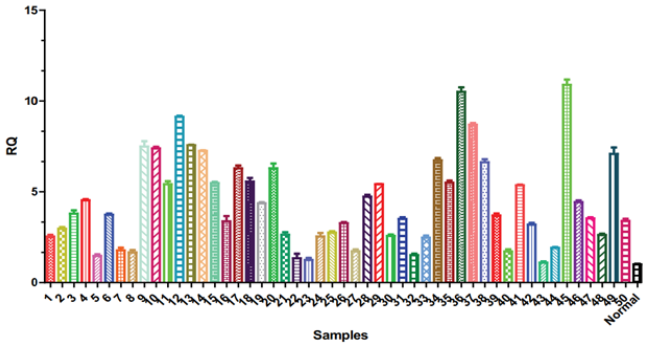

Figure 2. Analysis of UBD gene expression in the patient group compared to the control group. RQ shows the level of UBD gene expression compared to the normal sample. The numbers indicate the sample number.

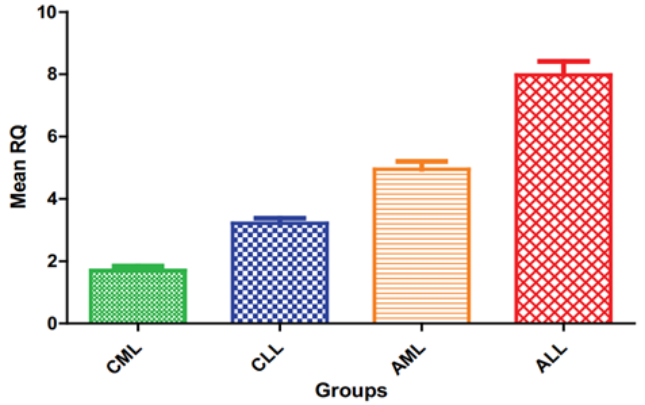

Figure 3. The level of ubiquitin-D gene expression in mice with leukemia according to disease group.

Discussion

Leukemia is one of the five most common cancers worldwide, which is also more common in children. Various methods are used to treat blood cancer (12). The most common treatments at present are radiation therapy and chemotherapy. These methods and their advantages will also have many side effects on the patient (13). Therefore, the need for new treatments with better effectiveness and fewer side effects is quite noticeable. Animal models play a vital role in investigating the efficacy and side effects of drugs, the course of the disease, and the immune system's responses (14). Creating models of cancer diseases is one of the most essential and standard methods in studying these diseases worldwide (15).

In this study, UBD mRNA was identified in the blood samples of mice with leukemia for the first time. Studies have shown that UBD is essential as an index in diagnosing leukemia, especially for investigating the type of leukemia (ALL, CLL, AML, and CML). Our association between UBD expression with cell differentiation and stages of cancer progression suggests that UBD is likely to be involved in the advancement of carcinogenesis. Yan *et al*. (16) investigated the amount of UBD in the tissue of people with colon cancer with the help of qPCR and tissue microarray (TMA), western blotting, and immunohistochemical staining. Their studies showed that the increase in the cytoplasmic amount of UBD directly correlates with the stages of the disease, and the development of metastasis depends. They also stated that the life expectancy of people with UBD-negative colon cancer is much higher than that of UBD-positive people. Ren's *et al*. study (17) showed that the level of FAT10 expression through the TNF receptor (TNFR1) and the pathway of NF-kβ increases. Therefore, treatment with TNF-α can also inhibit the expression of FAT10. Qing *et al*. (18) investigated the dependence of increased FAT10 expression and colon cancer stage. They used the comparison of different cells with cells in various phases of cancer, and the techniques they used were immunohistochemistry and fluorescent microscopy. Their studies showed that the increase in FAT10 expression starts from the serrated adenoma stage and continues until the villous, villotubular, and invasive adenocarcinoma stages. Oliva *et al*. (19) investigated the inhibition of increased expression of UBD by TNF α and INFγ. They stated that combined treatment with TNFα and INFγ leads to more control of disease progression. The results showed that the amount of mRNA related to UBD increased in cancer tissues and surrounding tissues. Increased expression of UBD was seen, especially in the samples of people in stages II and III of the disease (20,21).

For the first time, this study has investigated the level of UBD gene expression in the blood of mice with leukemia compared to healthy mice. This study's results can effectively improve the prognosis of cancer. The present study and previous reports show that correct prediction of outcomes in cancer patients is a complex and clinical

problem. Despite the extensive studies conducted on cancer worldwide, the accuracy of the determining factors is still questionable. In addition, the progress of the disease is accompanied by an increased risk of death due to this disease. It seems necessary to conduct future studies to determine the role of various clinical and pathological factors to know the prognosis of leukemia.

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