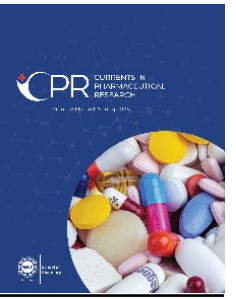
Currents in Pharmaceutical Research (CPR)
Volume 1 Issue 1, Spring 2023
ISSN$_{(P)}$: 3007-3235 ISSN$_{(E)}$: 3007-3243
Homepage: https://journals.umt.edu.pk/index.php/cpr

Article QR



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DOI:	https://doi.org/10.32350/cpr.11.02
History:	Received: April 14, 2023, Revised: May 10, 2023, Accepted: May 27, 2023, Published: June 28, 2023
Citation:	Saeed B, Busharat M, Farooq F, Ikram M, Uddin Z, Shah N, Amirzada MI. Assessment of human interleukin-24 expression in thalassemic patients and investigation of current prescription pattern in Thalassemia. *Curr Pharma Res*. 2023;1(1):05–15. https://doi.org/10.32350/cpr.11.02
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Conflict of Interest:	Author(s) declared no conflict of interest

A publication of
The School of Pharmacy
University of Management and Technology, Lahore, Pakistan

Assessment of Human Interleukin-24 Expression in Thalassemic Patients and Investigation of Current Prescription Pattern in Thalassemia

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ABSTRACT

Interleukin (IL)-24 or melanoma differentiation-associated gene-7 (MDA7) belongs to the family of cytokines initially obtained from malignant tissues and manifests itself in various types of cells (of a large size). Thus, they play a crucial part in anemias. The current study aims to analyze the activation and involvement of IL-4 in thalassemia patients. For this purpose, a study population of 50 patients was randomly selected to study the prescription pattern and blood samples for the activation of IL-24 by utilizing the RT-PCR procedure. Extraction of mRNA was followed by cDNA synthesis, further followed by real-time polymerase chain reaction (RT-PCR). Based on RNA extraction, 31 samples were selected to study the expression of MDA-7 gene in blood samples. Patients were randomly divided into two groups based on the number of transfusions at the time of blood sampling. Group 1 comprised patients with more than 100 transfusions, while Group 2 comprised patients with less than 100 transfusions. Prescription patterns showed the recommendation of approximately the same classes of drugs which are particularly used for thalassemic patients. The findings from gel electrophoresis were analyzed visually. These showed the IL-24 expression in the majority of patients included in Group 1, while no expression was found in individuals included in Group 2. The results revealed the relationship of IL-24 with thalassemia owing to its expression; however, the exact mechanism and contributing factors still remain unknown. Hence, further investigation is needed to rule out the involvement of IL-24 in thalassemia.

Keywords: electrophoresis, Interleukin-24, prescription pattern, RT-PCR, thalassemia

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1. INTRODUCTION

Thalasemia is a hereditary disorder associated with defective synthesis of α and β globin chains of hemoglobin [1]. The two types of thalassemia, namely α and β thalassemia, are further classified into major, minor, intermediate, and sickle cell anemias [2]. β thalassemia is a common, inherited, congenital disorder of hemoglobin production, resulting in hemolytic anemia and multiorgan involvement [3]. Each year, nearly 60,000 beta thalassemia children are born worldwide, while carriers are estimated to be around 90 million people (1.5% of the global population) [4]. Decreased oxygen carrying capacity, long-lasting inflammation, dysfunction of endothelium, and overloading of iron have been verified as interconnected progressions in thalassemia [5].

The current treatment of thalassemia is challenging as it is a hereditary disease and only symptomatic treatment can be followed to provide relief to the patient. The strategy often followed is blood transfusion along with multivitamins and folic acid supplements [6, 7]. Occasionally, the removal of the spleen becomes necessary due to its enlargement. Iron antidotes are also given against iron overload. The best but the most expensive treatment plan is to transplant the bone marrow, so that the patient can be able to synthesize their blood [8]. Unfortunately, bone marrow transplant rejection rate rises due to significant risk aspects including older stages of life, progressive types of illnesses, immune response to transfusion, and decreased severity conditioning [9].

Cytokines are minor, solvable combinations of proteins and carbohydrates having a molecular mass of less than 30 kilodaltons. Cytokines show their effect by modifying the properties of similar, neighboring, or other tissues and may have pleiotropic, opposing, unnecessary, or synergistic influence on their functions [10].

Interleukin-24, also known as Melanoma Differentiation Associated Gene 7 (MDA-7), is related to the IL-10 family of cytokines. IL-24 can cause the suppression of growth and produces apoptosis in many types of tissues. It also plays an important role in increasing cell death. The mechanism behind the use of IL-24 as an anticancer drug is still unknown [11]. Recently, the role of cytokines in thalassemia has been focused. Recurrent blood transfusions resulting in iron overload induce an increased production of cytokines in thalasemia patients [12]. The expression of

interleukin-6 and interleukin-8 has been assessed in thalassemia [13]. A new solution to the disease is now provided by checking the quantification and expression of these soluble antigens and cytokines in the serum that shows related effects in modifying the different stages of human response.

Unlike other cytokines, the role of IL-24 has not been evaluated in thalassemia; rather, its expression was checked in anemia. Hence, to determine whether it plays a significant role in the destruction of erythrocytes in thalassemia or not, the current study focuses on investigating the expression of IL-24 in thalassemia.

2. MATERIALS AND METHODS

2.1. Study Design

The cross-sectional and descriptive research design was adopted for this study. It has been conducted by Kids Blood Diseases Organization (KBDO), Mansehra, KPK, Pakistan. Patient who were diagnosed with thalassemia were selected for research purposes. Due to the involvement of human blood, ethical approval was necessary. Thus, approval was obtained from the ethical team of the Pharmacy Department, CUI Abbottabad campus and from the Medical Director KBDO, Mansehra.

2.1.1. Inclusion Criteria. According to the inclusion criteria, patients with known thalassemia and patients with available treatment regimens were selected for this study.

2.1.2. Exclusion Criteria. Patients with any comorbidity that may affect the results of the research were excluded from the study.

2.2. Materials

The materials used in this research included a DNA ladder, agarose gel (1%), cDNA first strand synthesis kit, TAE buffer, master mix (Syber green), RNA mini-preps kit for RNA extraction, 96-100% ethanol, microcentrifuge tubes, pipette tips and EDTA tubes, oven, PCR machine, vortex mixer, Lvis plates, microcentrifuge, ultraviolet range spectrophotometer, incubator and gel electrophoresis.

2.3. Collection of Blood Samples

The patients who come to receive blood at KBDO center, Mansehra were selected based on the inclusion criteria of this study. After getting

informed permission from them or their guardians, 1cc blood samples were obtained in EDTA tubes.

2.4. Procedure

RNA was extracted from the newly anticoagulated blood of thalassemic patients with RNA extraction kit. After extraction, the quantity of nucleic acid in the sample was assessed by checking absorption at two different readings of 260 and 280 nanometers using Lvis plates in a UV visible spectrophotometer. After quantification, the pulled-out RNA was stored at a very low temperature of -80◦C for the further processing and production of cDNA.

2.5. Synthesis of cDNA from Messenger RNA

G-Script first strand synthesis kit was used to synthesize DNA from RNA. Then, the end product was stored at a very low temperature of -20 degree Celsius for PCR.

2.6. Real Time-PCR 1

PCR conspire 2400 thermocycler was used to run the PCR procedure on synthesized cDNA in three steps namely denaturation, annealing, and extension. After PCR, IL-24 primers (forward and reverse) were added.

2.7. Gel Electrophoresis

A 5-microliter final product of PCR was added in agarose gel of 1%. After loading and movement of synthesized samples in agarose, the gel was detached and then examined under ultraviolet light for to identify bands.

2.8. Statistical Analysis

Statistical Package for Social Sciences or SPSS (version 24) was used to calculate the statistical results.

3. RESULTS

3.1. Study Population

Thalassemic blood samples were obtained from 50 patients from Kids Blood Diseases Organization (KBDO), Mansehra, KPK, Pakistan with an interval of 3 months. However, after RNA quantification, blood samples of 19 patients were not processed any further due to decreased RNA purity. The remaining 31 extracted RNAs were included in the next steps to assess

the comparative mRNA expression of IL-24. Both male and female patients were involved in this study.

3.2. Patient Characteristics

3.2.1. Gender Distribution. Blood samples were obtained from a total of 50 thalassemic individuals which included 21 female and 29 male patients.

3.2.2. Distribution of Age Group. As far as the age distribution of thalassemic patients is concerned, 17 thalassemic blood samples were obtained from individuals aged 3 to 8 years, 21 such samples were obtained from individuals aged 9 to 14 years, 9 samples were obtained from individuals aged 15 to 20 years, and 3 samples were obtained from individuals aged 21 to 26 years (Table 1).

Table 1. Age Group Distribution

Groups	Group 1 (3-8 years)	Group 2 (9-14 years)	Group 3 (15-20 years)	Group 4 (21-26 years)
No. of patients	17	21	09	03

3.3. Patients Current Treatment

Prescription patterns of all the 50 patients included in this research were studied. Since the treatment of thalassemia is very difficult, so most of the drugs are prescribed for symptomatic relief and preventive measures. Hence, almost all patients were administered the same medicines to improve their quality of life. Accordingly, treatments given to thalassemic patients included blood transfusion, iron antidotes, vitamins (B complex), folic acid supplementation, and cephalosporins (cefixime and ceftriaxone).

3.4. Expression of IL-24

Out of the 50 patients, 31 were analyzed for IL-24 gene expression as their nucleic acid concentration was high enough to process further for PCR and gel electrophoresis. Of the 31 selected patients, 25 were from Group 1 (comprising patients who had more than 100 transfusions in life), while only 6 patients were from Group 2 (comprising patients with the number of transfusions less than 100). This group contained very few (6 number) patients because nucleic acid concentration in group samples was very low. Figure 1 shows the results of the expression of IL-24 in both groups.

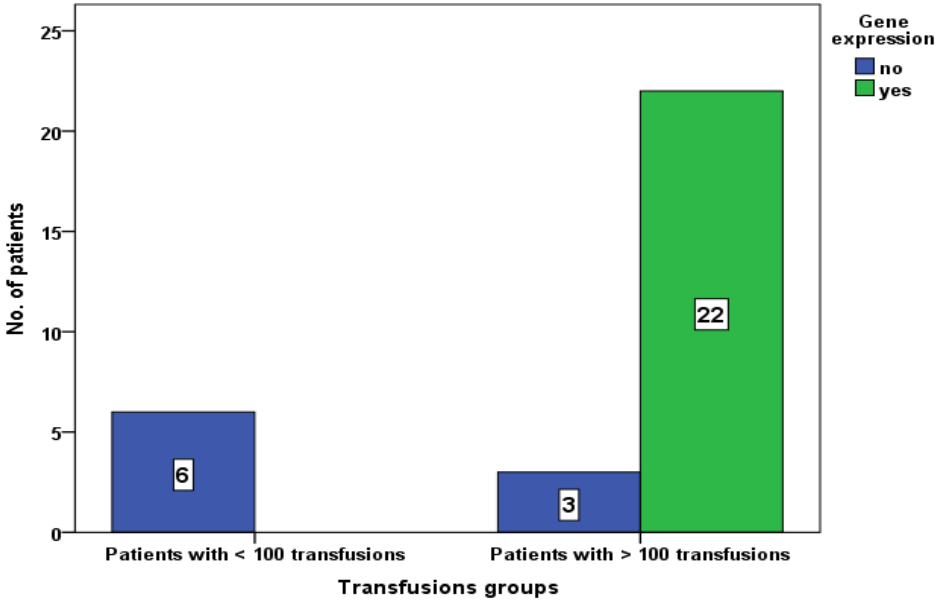

Figure 1. Gene Expression of IL-24 vs. Total Number of Transfusions

3.5. Gel Electrophoresis

After running the samples in electrophoresis, the gel was investigated visually for checking the expression in the bands. The results demonstrated that patients with more than 100 transfusions had a much greater expression of IL-24 levels as compared to the patients with less than 100 transfusions.

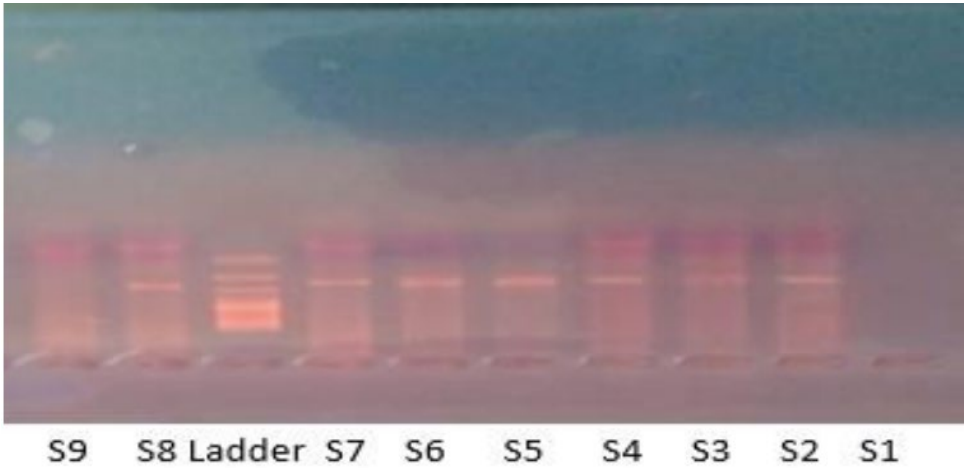

Figure 2. Gel Electrophoresis Imaging of IL-24 in Thalassemia from Samples 1-9.

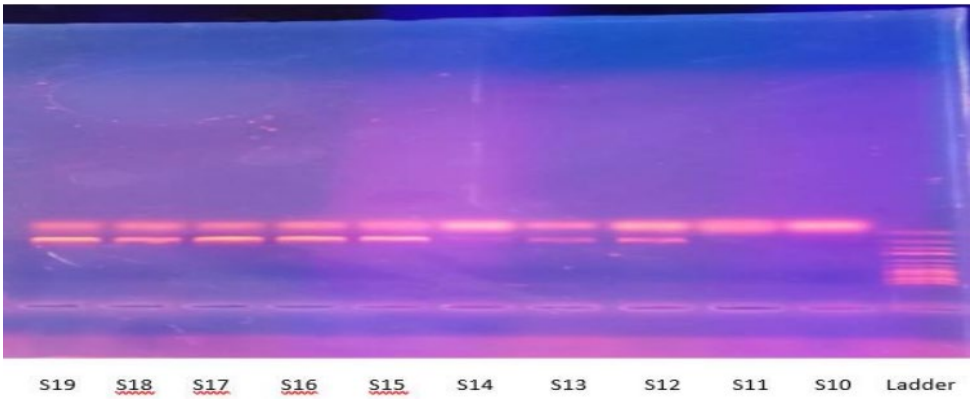

Figure 3. Gel Electrophoresis Imaging of IL-24 in Thalassemia from Samples 10-19.

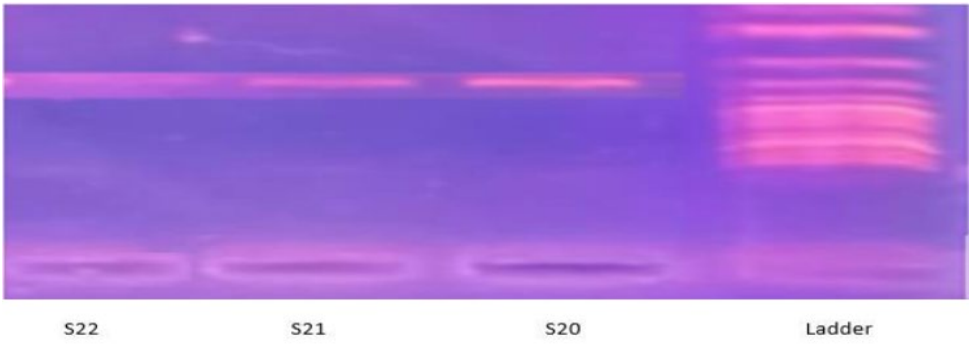

Figure 4. Gel Electrophoresis Imaging of IL-24 in Thalassemia from Samples 20-22.

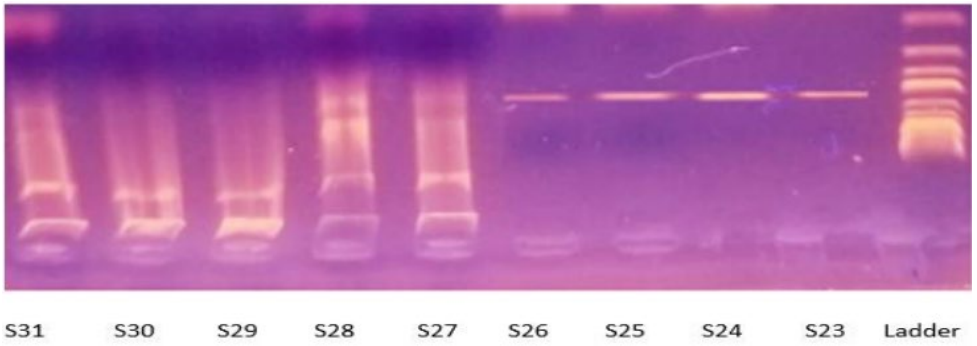

Figure 5. Gel Electrophoresis Imaging of IL-24 in Thalassemia from Samples 23-31.

3.6. Prescription Patterns

The evaluation of prescription patterns revealed that the range of drugs prescribed to patients was very short, since no drug of choice for thalassemia treatment is available. This is why the patients need symptomatic treatment and drugs are used only for symptomatic relief. Almost all the patients were prescribed similar types of medicines including folic acid, deferoxamine, and vitamin B complex. For infections, cephalosporins (cefixime and ceftriaxone) were prescribed.

4. DISCUSSION

β-thalassemia is caused due to a reduction in or the lack of hemoglobin beta chain [1]. Different biological and chemical changes are recognized in abnormal thalassemic red blood cells, such as the presence of a high amount of calcium, as well as modifications in cholesterol and phospholipids of the cell membrane. Most of these alterations express high oxidation induced damage to the cells [3].

Agarwal *et al*. found that quick stimulation of mRNA for interleukin-1, tumor necrosis factor, interleukin-6, and interleukin-8 was achieved by activated monocytes when these defensive cells were activated by antigens [14]. Dore et al. suggested that the hyperactivity of thalassaemic macrophages related to chronic haemolysis was the main cause of the increment in cytokines, such as IL-8 [15]. Rougier et al. showed that bone marrow stromal cells might represent a major source of cytokines and regulate the local production of IL-6 and IL-8 in human bone marrow [16]. The high uptake of iron from intestine because of decreased production of RBCs and iron obtained from donated blood stores in many tissues result in iron induced injuries, particularly in hepatic, cardiac, and endocrine cells [15].

The current study focused to check if there is any relationship between thalassemia and IL-24. The evaluation of prescription patterns revealed that most of the patients received the same treatment regimen since thalassemia can only be treated temporarily by either continuous blood transfusions or it can be treated permanently by bone marrow transplant. Most of patients received iron antidotes, such as deferoxamine for iron toxicity, folic acid, and vitamin B.

Since erythropoiesis is inadequate in thalassaemia, the bone marrow experiences compensatory stress. The elevated plasma levels of interleukins

may be attributed to the increased activity of bone marrow and stromal cells. The increased cytokine levels may also enhance neutrophil and macrophage chemotactic and phagocytic activity [13].

In order to define the exact mechanism of action of IL-24, it needs to be studied on the molecular level.

4.1. Conclusion

In the current study, IL-24 expression was checked between two groups of thalassemic patients based on the number of transfusions they had received. The group with patients who had received more than 100 transfusions revealed the expression of IL-24 with few exceptions. While, the group with patients who had received less than 100 transfusions showed no expression of IL-24. The expression of IL-24 revealed its probable role and importance in thalassemia. However, further study is needed to disclose its exact role and mechanism of action at the molecular level based on which it takes effect in thalassemia.

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