Analysis of GATA-1, FoxO3 and pRb Gene expression and assessment of longevity associated FoxO3 rs2802292 polymorphism in transfusion dependent β-thalassemia major patients

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Abstract- The hallmark of β-thalassemia is thought to be ineffective erythropoiesis, which can be caused by a variety of internal and external factors. Several proteins including GATA-1, FoxO3 and pRb are involved in the regulation of normal erythropoiesis to produce functional RBCs and dysregulation of these proteins are reported to be one of the several causes of inefficient erythropoiesis, hemoglobinopathies, or leukemic transformation in hematological illnesses. The aim of this study is to evaluate the expression profiles of GATA-1, FoxO3 and pRb in β-thalassemia major patients in comparison to healthy controls. The present study also aimed to evaluate the longevity associated FoxO3 (rs2802292) polymorphisms in β-thalassemia major patients in Karachi, Pakistan. The reported study was a case control study for which 50 Healthy and 50 β-thalassemia major patients were enrolled after informed consent. The expression analysis of GATA-1, FoxO3 and pRb was done using RT-qPCR and 2 –ΔΔCt method was used to calculate relative quantification of the target genes. FoxO3 polymorphism (rs2802292) was genotyped using ARMS-PCR. The distribution of blood groups in β-thalassemia major patients was observed to be O>B>A. A decrease in BMI with an increase of the ages in the β-thalassemia major patents was also observed as part of the study. When assessing FoxO3 polymorphism rs2802292 (G/T), only 2% of the healthy individuals and no β-thalassemia major patients carrying the allele G. 98% of the study cohort showed homozygosity towards allele T. Expression analysis showed a downregulation of all three studied markers GATA-1, FoxO3 and pRb in β-thalassemia patients. This study indicates the dysregulation of crucial erythropoiesis markers GATA-1, FoxO3 and pRb as an important cause of impaired erythropoiesis in β-thalassemia major patents. Furthermore, a homozygous mutation toward TT allele in FoxO3 rs2802290 anticipates an equally high risk of developing cardiovascular diseases, cancers, and diabetes in our population regardless of experiencing hematological disorders.

Keywords: β-thalassemia, ineffective erythropoiesis, GATA-1, FoxO3, pRb, expression analysis.

I. Introduction

Hematopoiesis is the fundamental and intriguing process progressively performed throughout the life by hematopoietic stem cells (HSC) to keep the hematopoietic function stable [1]. Erythropoiesis is the process of generation, development and maturation of red blood cells that is evolved to fulfill the organism's needs for increased oxygen-carrying capability. The process of erythropoiesis involves a series of well-defined stages including the formation of progenitor colony forming unit erythroid (CFU-E) which give rise to red blood cells after undergoing multiple stages of differentiation. This leads to the formation of erythroblasts, which are immature red blood cell precursors including basophilic, polychromatophilic erythroblasts, and normoblasts [2]. In the final stage of erythropoiesis, erythroblasts undergo terminal maturation, where they undergo changes in cell morphology. This includes a reduction in cell size, the disappearance of nucleoli and the nucleus, and the accumulation of hemoglobin to form mature red blood cells (erythrocytes) [3]. The complex and multistage process of erythropoiesis involves diverse signaling mechanisms, growth factors, transcription factors etc. to produce functional RBCs. Any changes in the levels of these markers can cause a range of serious problems including inefficient differentiation, oxidative stress and altered cell cycle leading towards excess apoptosis of immature RBCs. Therefore, optimal regulation of all factors involved in erythropoiesis is extremely crucial [4]. Anemia and inefficient erythropoiesis (IE) are the symptoms of a number of disorders caused by multiple pathogenic conditions which disturb RBC production or their functions [5]. Under physiological condition, the bone marrow ensures a uniform level of nucleated erythroid precursors, which is necessary to generate an adequate number of enucleated RBCs to fulfil the tissue oxygen requirement. Erythroid precursors multiply quicker but fail to mature more regularly in stages of ineffective erythropoiesis [6].

Ineffective erythropoiesis can give rise to multiple complications in humans; however, it is considered as a hall mark for β-Thalassemia which is categorized as an inherited condition and is one of the most prevalent hemoglobinopathy throughout the globe [5]. The disease is caused by autosomal mutation in the β-globin gene and thus results in total absence or very low production of β-globin in β-Thalassemia patients [7]. This in turn leads towards an imbalance between α/β-globin chain synthesis and results in buildup of free α-globin chains which forms highly toxic aggregates [8].

Several molecular factors including transcription regulators and hormones are involved in the complex process of erythropoiesis. This study has focused on three essential proteins GATA-1, FoxO3 and pRB which are reported to be substantially involved in this regulation.

GATA1 is a zinc finger DNA binding master regulator of erythropoiesis, controlling the development, maturation, and survival of red blood cells. GATA-1 interacts with diverse co-regulators to function effectively; however, some mutations in GATA-1 prevent its binding with interacting regulators and cause severe hemoglobinopathies including X-linked thrombocytopenia with thalassemia [9, 10]. GATA-1 is a caspase-3 target, yet it is impressively unaffected by cleavage events that occur during erythroid development due to the safeguard role of HSP70 which stops GATA-1 from being cleaved by caspase-3 [11]. In β-Thalassemia, during the course of erythroblast formation, HSP70's localization is altered and restricted to the cytoplasmic compartment by an interaction with free α-globin chains. This crucial event causes GATA-1 to become dysfunctional, which in turn triggers apoptotic processes and terminates end-stage development and leading towards ineffective erythropoiesis [12]. The impact of various mutations in GATA-1 on clinical conditions, protein homeostasis, and dysregulation have been extensively investigated; however, GATA-1's mRNA expression profiling in β-Thalassemia patients has not been examined. [13]. It has been suggested that insufficient expression of the GATA-1 gene puts off the balance between erythroid proliferation, survival, and differentiation, causing an abnormal buildup of immature erythroid progenitor cells [14].

In mammals, normal and stress related erythropoiesis is controlled by a diverse set of regulatory proteins, among which FoxO3 is the most essential as demonstrated by the quick fatality of FoxO3 mutant mice in response to an early erythroid oxidative stress [15]. GATA-1 promotes the expression of FoxO3 throughout the erythroid maturation period. This modulation is essential in the regulation of autophagy during terminal erythroid maturation [16, 17]. Previous data suggest that FoxO3 regulates erythroid gene expression by increasing the expression of other transcription factors of erythropoiesis. The expression of almost one third of genes which are required at various stages of terminal erythroblast maturation are reported to be down regulated if the activity of FoxO3 is lost [17]. Broader in magnitude, FoxO3 controls biological activities vital to sustained well-being, including but not limited to substrate oxidation, protein synthesis, cell proliferation, and apoptosis induction [18]. Various single nucleotide polymorphisms (SNPs) of FoxO3 have been found associated with multiple diseases and longevity. One of those SNPs, the allele "G" of rs28022292 is particularly found to be associated with longevity and healthy aging in individuals [19].

During erythropoiesis a cell-cycle regulated transition from early erythropoiesis to late phase erythropoiesis is observed [20]. The G1-to-S phase shift, which is particularly crucial for erythropoiesis, is controlled by retinoblastoma protein (pRb). This protein regulates many biological development processes in multiple cell types, and control gene expression at different levels of cell cycle [21]. Studies have revealed that dysregulation of pRb protein from hematopoietic stem cells results in myeloprolifirative disease [22]. Cell-specific deletion has shown that pRb and several of its downstream E2F mediators are intrinsically necessary for normal erythroid development and that Rb-deficient embryos exhibit significant anemia [23].

Dysregulation of either of these proteins can have serious effects on β-Thalassemia patients. The study therefore aims to investigate the comparative gene analysis GATA-1, FoxO3 and pRb in β-Thalassemia major patients. This Study also focuses on to analyze the impact of Fox03 polymorphism rs2802292 on β-thalassemia major patients.

II. Materials and Methods

Subject Recruitment and sample collection:

This study was designed as a case-control study. A total of 100 study subjects were enrolled voluntarily, which were divided into two groups i.e 50 Healthy (control) group and 50 β-Thalassemia major patients' group. Participants from both genders were included with an age limit of 10-35 years. Participants having any other co-morbidity were excluded from the study. This study was approved by the Institutional Review Board (IRB) of Dow University of Health Sciences [IRB-1709/DUHS/Approval/2020]. 1ml of peripheral blood was collected from each participant and was subjected to RNA extraction.

RNA Extraction and Quantification:

RNA was extracted using the Trizol Reagent [Sigma-Aldrich] according to the manufacturer instructions. To measure the purity and the total concentration of extracted RNA, a Nanodrop microvolume Spectrophotometer was used. All the RNA samples were suspended in TE buffer and stored at -40°C until further use.

DNAse Treatment and Reverse Transcription:

Extracted RNA was treated with DNAse-I, RNAse-free to eliminate trace quantities of DNA prior cDNA synthesis. 500ng of purified RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit using random hexamer according to the manufacturer guidelines.

FoxO3 Polymorphism Analysis:

ARMS-PCR was performed to screen patients for the SNP (rs2802292; G /T) in the FoxO3 gene on chromosome 6. ARMS-PCR was set for 35 cycles at 95°C, 62°C and 72°C for denaturation, annealing and extension, respectively. All the reactions amplified using 2X dream Taq PCR master mix in a total volume of 10µL.The PCR products were visualized using 2% (w/v) agarose gel electrophoresis. Primers for ARMS-PCR re mentioned in Table-1

Table-1: List of all the primers used in this study.

Expression Analysis Primers (RT-qPCR)		
Gene	**Forward Primer**	**Reverse Primer**
GATA-1	CCAGTTTGTGGATCCTG	ACCCCTGATTCTGGTGTGG
pRb	TCTACCTCCCTTTCCCTGTTT	AGTCATTTTTGTGGGTGTTGG
FoxO3	GCAAGCACAGAGTTGGATGA	CAGGTCGTCCATGAGGTTT
Polymorphisms Analysis Primers (ARMS-PCR)		
FoxO3 (F1-G)	CACAAGAGCTCAGGGCTGGGA **G**	TCTGTTTGCAGGAACACAGAC T
FoxO3 (F1-T)	CACAAGAGCTCAGGGCTGGGA **T**	

Expression Analysis:

All the primers used for expression analysis are listed in Table-1. The differential expression levels of FoxO3, GATA-1, pRb, and GAPDH (endogenous control) were determined by real-time quantitative RT-PCR analysis. All qPCR reactions were completed in duplicates with a total volume of 10µL in using Invitrogen SYBR Green master mix. QuantStudio7 Flex Real

Time PCR Detection System was used to measure the Ct values. $2^{-\Delta\Delta Ct}$ method was used to calculate relative quantification of the target genes.

Statistical Analysis:

SPSS version 24 was used for statistical analysis of all the data collected from above mentioned experiment. Pearson's Chi square was used for ARMS-PCR data while gene counting method was used to calculate genotype frequency and allele frequency of healthy and β-thalassemia major patients. The Wilcoxon paired sample test was used to statistically evaluate the data obtained from expression analysis.

III. RESULTS

Demographic characteristics of the study participants:

In this study a total of 100 participants (50 β-thalassemia patients; 50 healthy controls) were enrolled. β-thalassemia patients were further divided into male and female groups where 22 were males and 28 were females. The average age of male group was found to be 25 years whereas female group was 26 years. A structured questionnaire was introduced to the study participants for collection of socio-demographic and clinical characteristics of the subjects. All the demographic and clinical characteristics for β-thalassemia patients are listed in Table-2.

Table-2: Demographic and clinical details of all the study participants.

Variables	β-Thalassemia major patients	
Demographic Details		
Gender	Male (n)	Female (n)
	22	28
Age (years)	25 years	26 years
Clinical History		
BMI (Kg/m^2)	21.8	19.9
Hemoglobin(g/dL)	9.147	8.588
WBC (x10^9/l)	7.322	8.035
Platelets(x10^9/l)	212.55	230.77
Blood Groups		
A	5	6
B	9	7
AB	-	-
O	8	14
Frequency of transfusions/month		
Twice	6	14
Multiple	5	6

Body Mass Index of β-thalassemia patients:

Table-3: BMI data of β-thalassemia major patients

BMI	Age (Years)	≥18 Kg/m^2 Normal range (%)	<18 Kg/m^2 Underweight (%)
	10-20	80	20
	21-30	34.46	65.5
	>30	22.22	77.77

β-thalassemia patients were categorized in three groups based on their ages (10-20; 21-30 and > 30 years of age). Since no obese patients were found, two groups of β-thalassemia were formed based on their BMI (Underweight < 18 kg/m^2 and normal range ≥18 kg/m^2). Results of this analysis are presented in Table-3. According to the findings a clear trend of decreasing BMI with increasing age is observed. In age group 10-20 years only 20 % of the patients were noted to be underweight that raised to 65 % and 78% respectively in age groups of 21-30 and > 30 years.

Screening for SNP:

The SNP (rs2802292; G/T) in the FoxO3 gene on chromosome 6 was investigated using ARMS-PCR. Amplified products of ARMS-PCR for healthy and diseased individuals were resolved in 2% agarose gel. 100bp DNA ladder was used as reference. The lack or presence of bands reveals whether the sample is homozygous or heterozygous with respect to allele.

Results of the ARMS-PCR for healthy subject are presented in Figure-1A. Each sample shows homozygosity towards mutant allele T. Only 1 sample H18 in lane 2 shows homozygosity toward wild type allele G. No heterozygous trait was found in any healthy sample.

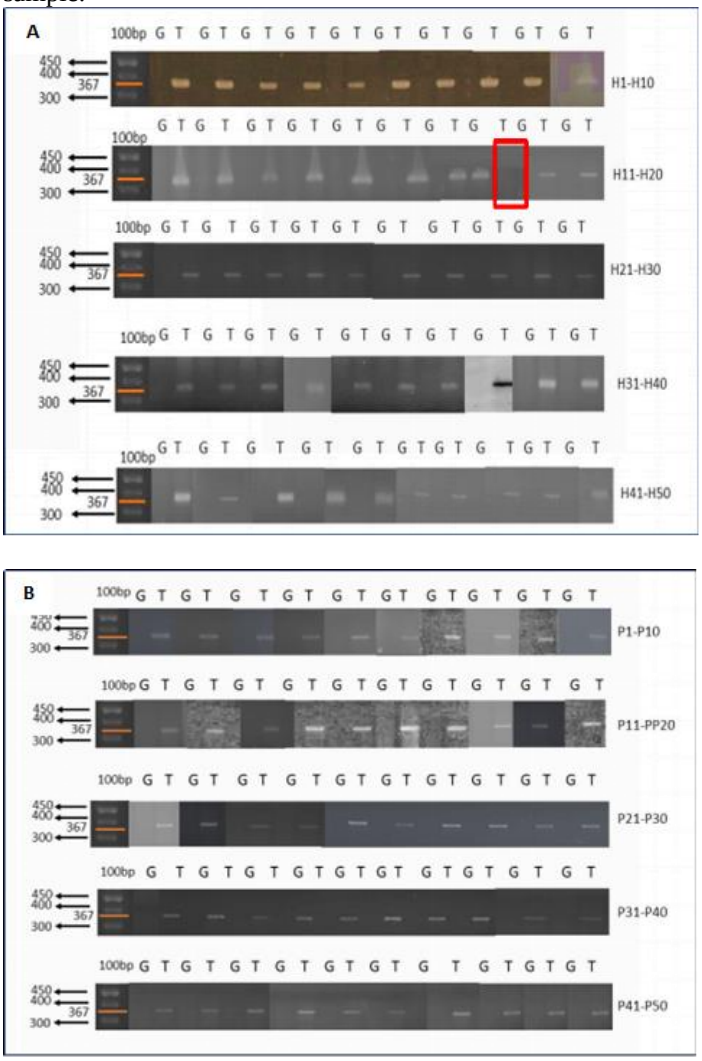

Figure-1: ARMS-PCR for Healthy (A) and β-thalassemia major patients (B); SNP (rs2802292; G/T).

Results of ARMS-PCR for β-thalassemia patients are presented in Figure-1B. All analyzed samples of β-thalassemia patients showed

homozygosity towards mutant allele T. None of the samples show homozygosity towards wild type allele G or heterozygosity towards G and T alleles.

Allele Frequency:

Table-4: Allele frequency data of healthy participants and β-thalassemia patients

Allele	Healthy	Patients	OR	95% Cl	p-value
G	2/100	0/100	0.1960	0.2418 to 107.625	0.2949
T	98/100	100/100	5.1015	0.0093 to 4.1353	0.2949

Table-4/Figure-2 show the allele frequencies of the SNP (rs2802292; G/T) in healthy individuals and patients with β-thalassemia. The results showed that in our study cohort none of the β–thalassemia major patients were found to have wild type allele G while mutant allele T was found in all β–thalassemia major patients. Similarly, in healthy subjects only 2% individuals were found to have wild type allele G while 98% individuals were found to have mutant allele T. Allele T had an odds ratio of 5.1015, whereas allele G had an odds ratio of 0.1960.

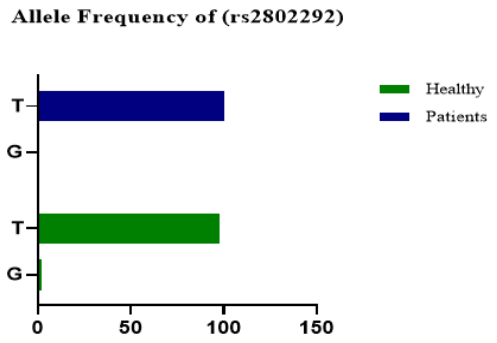

Figure-2: Graphical representation of distribution of alleles in healthy population and β-thalassemia major patients.

Gene Frequency:

Table-5: Genotype frequency of healthy subjects and β-thalassemia major patients.

FoxO3	**Percentage n%**				
Genotype	**Healthy (n)**	**Patients(n)**	**OR**	**95% cl**	**p-value**
GG	1(2%)	0 (0%)	0.3267	0.0130 to 8.2151	0.4966
TT	49 (98%)	50 (100%)	3.606	0.1217 to 76.9532	0.4966
GT	0 (0%)	0 (0%)	1.000	0.0195 to 51.3856	1.0000
Total	50	50			

The genotype frequency of the SNP (G/T) of the FOXO3 gene in healthy individuals and patients with β-thalassemia is shown in Table-5/ Figure-3. It was found that in our study cohort only 2% of the healthy population was homozygous wild type (GG) while other 98% was homozygous mutant (TT). On the other hand, 100% of the β-thalassemia patients were homozygous mutant (TT). The odds ratios were calculated to be 0.3267 for the wild type (GG), 3.0606 for the mutant type (TT), and 1.000 for the heterozygous (GT) condition.

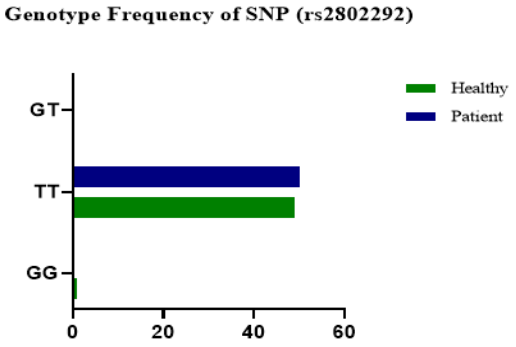

Figure-3: Graphical representation of distribution of genotypes in healthy and β-thalassemia major patients.

Expression profiling of FoxO3, GATA-1 and pRb:

Expression analysis of GATA-1, FoxO3 and pRb showed a downregulation of all three biomarkers in β-thalassemia major patients when compared with the healthy individuals (Figure-4). However, when statistically evaluated this data was found to be insignificant as the p-values for all three biomarkers was p=>0.05. P-values were calculated using Relative Quantity (RQ) of these selected biomarkers in β-thalassemia patients as compared to healthy individuals. RQ values were calculated using $2^{-\Delta\Delta Ct}$ method.

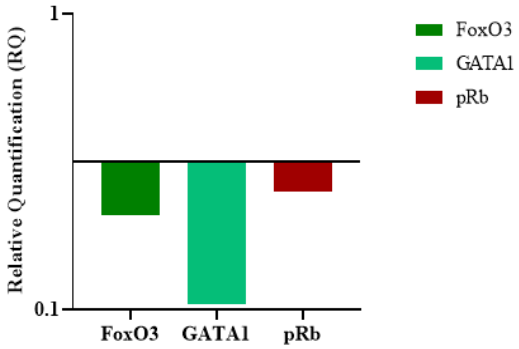

Figure-4: FoxO3, GATA-1 and pRb mRNA expression analysis in β-thalassemia major patients.
*p>0.05

IV. Discussion

Defective erythropoiesis leads to various pathological conditions, resulting in impairment of whole regulatory cascade and altered expression of associated genes. Amongst the reported haemoglobinopathies, β-thalassemia major is the second most prevalent blood disorder in the world [24]. Every year, 330,000 infants are born with β-thalassemia [25]. The pathology of this disease is characterized as decreased red blood cell survival and hemoglobin production due to defected beta globin chains. These

defected globin chains form unstable homo-tetramers which accumulates as inclusion bodies, precipitates earlier in the erythrocyte life span and cause a damage to the red blood cells maturation [26]. The ineffective erythropoiesis in β-thalassemia is due to aberrant differentiation of erythroid progenitors, accelerated erythroblast death rate and a relatively low production of reticulocytes in comparison to the volume of the erythroid progenitor present [27, 28].

This study aimed to analyze the comparative mRNA expression of essential regulatory proteins of erythropoiesis GATA-1, FoxO3, and pRb. This Study also focuses on analyzing the impact of FoxO3 polymorphism rs2802292; G/T on β-thalassemia major patients.

A total of 50 β-thalassemia major patients were recruited from Husaini blood bank, Karachi and their blood samples were collected. Out of 50 patients, 28 were female and 22 were male with an average age of 26 and 25 years respectively. When compared with the standard range, low levels of hemoglobin and red cell count was observed both in male and female group of β-thalassemia patients. This is in line with some other studies and gives a rough estimate of anemia and ineffective erythropoiesis in β-thalassemia major patients [17, 18]. This ineffective erythropoiesis might lead to low levels of BMI and is indicative of underline growth problems in β-thalassemia patients. Our results show a clear trend of decreasing BMI with increasing age. Some other studies also reported similar consequences which indicates that β-thalassemia patients might have increased metabolic disorders [29, 30].

The distribution of blood groups in β-thalassemia patients observed in this study was O >B > A. A similar pattern of blood group distribution among β-thalassemia patients has been reported in previous studies [31, 32]. The knowledge of blood group prevalence and distribution is of critical importance in the management of hospital blood banks as well as in the formulation of transfusion policies for β-thalassemia patients. Numerous studies associate prevalence of blood groups with various diseases [33]. ABO blood groups have also been analyzed as an indicative of the possible link between ABO blood groups and the type of population at risk of suffering from thalassemia [31]. Since the patients of β-thalassemia major need continuous blood transfusion for their survival. Hemoglobin levels in patients of β-thalassemia major are used to keep normal through blood transfusion [34]. Out of 50 diseased cohort enrolled in this study, six male patients underwent for blood transfusion twice a month while five male patients underwent for blood transfusion multiple times in a month. Similarly, in female patient cohort, fourteen underwent for blood transfusion twice a month and six underwent multiple times for blood transfusion in a month. This blood transfusion therapy in β-thalassemia major patients is scheduled according to the blood profile of the individual and is needed to keep the hemoglobin levels stable [34].

mRNA expression of important erythropoietic markers GATA-1, FoxO3, and pRb were analyzed through qRTPCR. The expression of respective gene markers was compared between healthy individuals and β-thalassemia major patients and the data was presented as relative quantification of the mRNA transcript. In this study 10% downregulation of GATA-1 mRNA transcript was observed in β-thalassemia major patients as compared to the healthy subjects. This data is in accordance with the results of Gutiérrez et al. who reported a 40% decrease in the GATA-1 mRNA transcript levels in Diamond Blackfan anemia [35]. These lower levels of GATA-1 expression have been found associated with impaired hematopoiesis due to a single nucleotide mutation in GATA-1 gene present 24 nucleotides upstream of the canonical splice site in intron 5 and affects canonical splicing of its mRNA [13]. In 2014, Moriguchi and colleagues created a mouse line with a GATA-1 knockdown allele, resulting in a significant reduction in GATA-1 mRNA expression to approximately 5% of the wildtype level. Hemizygous (GATA-1) male embryos carrying this knockdown allele did not survive past embryonic day 10.5 (E10.5) due to severe anemia, underscoring that a mere 5% of GATA1 production is inadequate to sustain embryonic erythropoiesis [36]. Another independent study reported the development of oncogenic conditions in partial knock down mice model of GATA-1 due to the accumulation of erythroid progenitors [37]. Results of current study and cited literature direct that low levels of GATA-1 gene expression in β-thalassemia patients are associated with impaired differentiation of erythroid progenitors and hence give rise to ineffective erythropoiesis.

The FoxO3 is one of the major active fork head box O (FoxO) class transcriptional factors of cellular homeostasis that acts as a crucial mediator of terminal erythroid differentiation, enucleation of RBCs and management of antioxidant gene transcription [16]. As erythroid cells get matured and accumulate hemoglobin, they become increasingly susceptible to oxidative damage. Under physiological erythropoiesis, activation of FoxO3 is projected to provide a protective mechanism against excess reactive oxygen species (ROS) [38]. Impact of FoxO3 is facilitated through the increased transcription of the cascading enzymes responsible for scavenging ROS through superoxide dismutase 2 and catalase enzymes. However, in pathological erythropoiesis, which is further characterized by abnormally elevated ROS, heme, and free iron levels, impaired FoxO3 mRNA levels have been reported [39-42]. Results of our study also revealed a 43.3% down regulation of FoxO3 transcript in β-thalassemia major patients as compared to the healthy subjects. These observations strengthen the results of an increased ROS levels and decreased antioxidant activity among our β-thalassemia major patients' cohort in comparison to the healthy individuals [43]. Furthermore, to its protective role in erythropoiesis, FoxO3 and its various single nucleotide polymorphisms (SNPs) are known to be involved in healthy aging [44]. Particularly the SNP rs2802292 shows capability to increase the expression of FoxO3 and enhance the functioning of the core component responsible for responding to various stress cues during the aging process [45]. Homozygosity for the G-allele of FoxO3 rs2802292 is associated with lower prevalence of cancer and cardiovascular diseases along with greater insulin sensitivity in various populations [45-50]. Willcox shown that G allele of FoxO3 SNPrs2802292 protects against risk of death from CHD [51]. Chen R., et al., reported an increased risk of mortality in men with cardiometabolic disease having homozygous TT allele [52]. In the present study, FoxO3 polymorphism (rs2802292; G/T) was evaluated in healthy controls and β-thalassemia major patients. All β-thalassemia major patients and 98% of healthy subjects were found to be homozygous mutant (TT). Results of our study predicted an equally high risk of developing cardiovascular diseases, cancers, and diabetes in our population regardless of enduring hematological disorders.

Regulated cell cycle progression plays a pivotal and indispensable role during erythropoiesis. The Retinoblastoma protein (pRb) is a crucial cell cycle regulator that controls the G1-to-S phase shift [23]. Deregulated synchronization of cell cycle exit and mitochondrial biogenesis has been linked to ineffective terminal differentiation in erythroid with inactivated pRb. [53]. Retinoblastoma protein is an intrinsic regulator, and its deficiency causes DNA damage-induced G1 and disrupted intra-S-phase checkpoints, leading in G2/M arrest. Downregulation of pRb has been reported in numerous cancers including liver cancer, Osteosarcoma, breast cancer and colorectal cancer [54-56]. Several studies have associated the occurrence of ineffective erythropoiesis due to inactivation of pRb protein, however no study reports the dysregulation of pRb in β-thalassemia major patients. Our study displayed ~ 23% downregulation of pRb gene in β -thalassemia major patients. Steven et al associated lower levels of pRb with shorter survivor of patients with Acute Myelogenous Leukemia, 12 weeks versus 40 weeks in patients with higher levels of pRB. [57]. These results further accenting a higher risk for shorter life span in β -thalassemia major patients.

V. CONCLUSION

β –thalassemia is the second most prevalent haemoglobinopathy in the world. This study focused on studying and understanding some of the underlying mechanisms associated with β –thalassemia at the molecular level. Three biomarkers GATA-1, FoxO3 and pRb were selected for the study. Our research provided significant understanding of dysregulation of these genes and their possible impacts on the pathophysiology of β-Thalassemia. Furthermore, results of the present study directed that the study population have an equally significant likelihood of getting diabetes, malignancies, and cardiovascular diseases and a short life span regardless of experiencing hematological abnormalities.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Review Board (IRB) of Dow University of Health Sciences [IRB-1709/DUHS/Approval/2020].

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