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Homeobox *A5* and *A9* expression and beta-thalassemia

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ABSTRACT

Background and aim: The pathogenesis of β-thalassemia has been attributed to ineffective erythropoiesis. The function of Hox genes in normal haematopoiesis has been widely studied using gene expression analysis. The aim of this study is to evaluate the expression of *HoxA9*, and *HoxA5* genes in beta-thalassemia.

Materials and methods: Children with thalassemia major, thalassemia intermediate, and age and sex-matched healthy controls (n = 50/group) were enrolled. Detection of *HoxA5* and *HoxA9* mRNA expression was performed by real-time polymerase chain reaction (RT-PCR).

Results: Expression of *HoxA9* increased in a direct linear trend (median 0.5 in controls, 2.4 in intermediate disease, 4.1 in major disease, $p = 0.001$) and generally correlated with the red cell count, haematocrit, ferritin and levels of beta-globin. In those with thalassemia major, the relative change of *HoxA9* was linked to transfusion history, the white blood cell count, ferritin, and beta-globin (all $r > 0.5$, $p < 0.001$). Levels of *HoxA9* were superior to *HoxA5* in differentiating controls from thalassemia intermedia, whilst both differentiated major from the intermediate disease.

Conclusion: This study highlights the importance of HoxA genes in early identification of patients, at high risk of developing complications, as it allows specific measures to delay the progression of the disease. HoxA gene expression is a promising diagnostic and prognostic marker in patients with β-thalassemia.

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Introduction

β-thalassemia is a spectrum of hereditary blood disorders characterized by defects in the synthesis of the β chains of haemoglobin resulting in a range of phenotypes from severe anaemia to clinically asymptomatic individuals [1,2]. The severity of symptoms is related to the extent of absent production of β-globin chain. The genotypic variability of β-globin synthesis is designated as β(+) for decreased production and β(0) for absent production. The phenotypic variability is designated as either minor, intermediate, or major. β-thalassemia minor is heterozygosity with one unaffected beta-globin gene and one affected, either β(+) or β(0). Homozygosity or compound heterozygosity with β(+) or β(0) causes intermediate and major. These are distinguished clinically by the severity of anaemia and not by genotype [3]. The molecular defects in β-thalassemia result in absent or reduced β-chain production. Alpha chain synthesis is unaffected, and hence there is an imbalanced globin chain production leading to an excess of α chains. In the absence of their partners, they are unstable, and they precipitate in the red cell precursors, giving a rise to large intracellular inclusions, which interferes with red cell maturation [4]. Hence, the pathogenesis of β-thalassemia has been attributed to ineffective erythropoiesis due to intramedullary apoptosis and delayed maturation of erythroid progenitor cells [5].

Data from zebrafish point to Homeobox (Hox) genes having an important role in normal haematopoiesis related to haematopoietic stem cells (HSCs) and early haematopoietic progenitors [6]. The Hox genes contain several clusters (A-D). Each cluster consists of paralog groups with nine to eleven members assigned on the basis of sequence similarity and relative position within the cluster [7]. The *HOXA* family encodes proteins that contain the DNA-binding homeobox motif and controls the early patterns of embryo segmentation. Although *HOX* expression is typically inhibited in adults, reactivation may occur with various homoeostatic cellular processes including haematopoiesis. Hox genes are required for the maintenance of progenitor or stem cell status, promoting their proliferation. *HoxA9* is the most preferentially expressed Hox gene in human CD34+ HSCs and early haematopoietic progenitors [8]. *HoxA5* has two effects on erythropoiesis: it causes a predominance of mature erythroid lineage cells and the partial apoptosis of erythroid progenitors. RNA-seq indicates that multiple biological processes including erythrocyte homoeostasis, cell metabolism, and apoptosis are modified by *HoxA5* [9]. We hypothesized roles for *HoxA9* and *HoxA5* in β-thalassemia.

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Materials and methods

We tested our hypothesis on 100 children suffering from β-thalassemia (50 thalassemia major, 50 thalassemia intermediate) and 50 healthy children as controls not suffering from any haematological disorders. Patients were selected from the Hematology Unit, Pediatric Department, Menoufia University Hospital. An informed written consent was obtained from all legal guardians of the children participating in this study. The protocol was approved by the Ethical Committee of Medical Research, Faculty of Medicine, Menoufia University. All studied subjects were subjected to complete history recording (including personal history, history of blood transfusion and splenectomy, thorough clinical examination stressing on the presence of pallor, jaundice, abnormal growth, and abnormal facies).

Five millilitres (ml) of venous blood were withdrawn from the cubital vein and divided as follows: 2 ml of blood were put in a tube containing EDTA for Complete Blood Count (CBC) measured with Pentra – 80 automated blood counter (ABX – France, Rue du Caducee-Paris, Montpellier, France), estimation of Hb F% by Hb electrophoresis (automated analyser (minilite) MNL320350-Italy) and for detection of beta-globin chain, *HoxA9*, *HoxA5* mRNA expression by RT-PCR. The remaining 3 ml blood was transferred into the plain tube, separated by centrifugation, and stored at −20 °C for determination of serum ferritin by ELISA (Ramco Laboratories Inc., Stafford, Texas, USA).

RNA was extracted from fresh EDTA treated blood sample using Direct-zol RNA (Zymo Research Corp, California, USA) for purification of total cellular RNA according to the manufacturer's instructions. Single-stranded cDNAs were created utilizing QuantiTect Reverse Transcription Kit (Qiagen, Applied Biosystems, USA): 10 ng of extracted RNA was added to each tube containing reverse-transcription master mix, to achieve reverse-transcription reaction of 20 µl total volume and stored on ice. The programming of cycler condition was to hold for 1 h at 42 °C, hold for 5 min at 95 °C to inactivate Quantiscript Reverse Transcriptase then for 5 min at 4 °C. The reverse-transcription reactions were stored at – 20 °C for real-time PCR.

Second Step-PCR was done with cDNA Amplification with SYBR Green II with low ROX for detection of *HoxA9* and *HoxA5* genes expression (QuantiTech). The reaction mix for each gene was prepared as follow: 10 µl of 2x QuantiTect SYBR Green PCR Master Mix with low ROX, 1 µl for each forward and reverse primers of the gene, 3 µl of template cDNA, 5 µl of RNase-free water to give a final total reaction volume of 20 µl.

The forward (F) and reverse (R) primers for each gene were: beta-globin primer sequence: Forward primer 5`ATCCTGAGAACTTCAGGCTCCTGGG-3`, reverse primer 5` GAGCTTAGTGATACTTGTGGGCCAG-3`, *HoxA5* (F:CG CAAGCTGCACATTAGTCACG, R:GAGAGGCAAAGGGCAT GAGCTA), and *HoxA9* (F:GCCGGCCTTATGGCATTAA, R: TGGAGGAGAACCACAAGCATAGT). Beta-actin was used as the endogenous control gene.

The programming of real-time cycler was to hold for 15 min at 95°C for initial denaturation, 45 cycles at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 34 s with a final extension at 72°C for 10 min. Melting curve analysis of the PCR products was performed using 7500 software version 2.0.1, the melting curve cycling program is 95°C for 15 s, 55°C for 1 min fluorescence data collection, 95°C for 30 s and 55°C for 15 s. Data analysis was conducted using Applied Biosystems 7500, software version 2.0.1. The Comparative Ct method has been calculated from the following equation: Relative gene expression = *Relative quantitation* (see tables) *(RQ)* = [2] -[Δ] [Δ] Ct, where; Δ ΔCt = ΔCt sample – ΔCt reference. Here, Δ Ct sample is the Ct value for any sample normalized to the endogenous housekeeping gene and Δ Ct reference is the Ct value for the calibrator also normalized to the endogenous housekeeping gene. The relative quantitation (RQ) value determines the change in expression in nucleic acid sequence in test sample (target) relative to the same sequence in the control sample.

Data were analysed by SPSS v22.0. The Kolmogorov–Smirnov test was used to verify the normality of distribution. Two types of statistics were done: Descriptive statistics in which percentage (%), mean and standard deviation (SD) were used while for analytical statistics: Chi-square test ($\chi 2$) was used to study the association between two qualitative variables, Fisher exact test for 2×2 tables, Student t-test: used for comparison between two groups having quantitative parametric variables, Mann–Whitney test (nonparametric test): used for comparison between two groups, ANOVA (F) test (parametric test): used for comparison between more than two groups, with linear trend estimation to describe the behaviour of the observed data, Kruskal–Wallis (H) test (nonparametric test): used for comparison between three or more. In view of our small sample size, and multiple analyses with potential conflict, a p-value ≤0.01 was considered significant.

Results

Demographic features showed a non-significant statistical difference as regards gender, 58% boys and 42% girls in each group, age 7.3 (3.4), 8.5 (4.10) and 8.1 (4.1) in the control, intermediate and major groups, respectively, (P value = 0.306). The children with major disease had more units of blood transfused/year (9.4 [3.3] v 2.5 [0.7]), more jaundice (82% v 16%), and more organomegaly (80% v 10%)(all $p < 0.001$). Almost all children (96%) with the major disease had

undergrowth, 60% had facies, and none of the children with intermediate disease had these features.

As expected, there were numerous abnormalities in full blood count indices, ferritin and the RQ of beta-globin in the health/intermediate/major disease trend, and many differences between the groups. The RQ of *HoxA5* and Hb F were higher in thalassemia intermedia than in the two other groups (Table 1). In the thalassemia intermedia group, the RQ of *HoxA9* correlated with levels of haemoglobin, and the relative expression of *HoxA5* and *HoxA9* failed to correlate significantly (Table 2). In the thalassemia major group, the RQ of *HoxA5* correlated with the RQ of beta-globin, whilst the RQ of *HoxA9* correlated with the number of units transfused, the white blood cell count, ferritin and the RQ of beta-globin. There was a weak but not-significant inverse relationship between *HoxA5* and *HoxA9* (Table 3).

Receiver Operator Curve (ROC) analysis, sensitivity, specificity, positive and negative predictive values are shown in Table 4. The area under the curve in separating intermediate thalassemia from the controls was greater using *HoxA9* than using *HoxA5* (95% CIs fail to overlap). However, the difference in the area under the curve in separating thalassemia intermediate from thalassemia major using *HoxA9* or *HoxA5* was not significant (95% CIs overlap).

Table 2. Correlations between RQ of *HoxA5*, RQ of *HoxA9* with different parameters in patients with thalassemia intermediate (n = 50).

	RQ of *HoxA5*		RQ of *HoxA9*	
	r_s	p	r_s	p
No. of transfusions (year)	−0.21	0.148	0.06	0.699
WBCs	0.04	0.773	−0.02	0.913
RBCs	−0.08	0.569	−0.30	0.032
Hb	−0.18	0.215	−0.38	0.006
HCT	−0.13	0.363	−0.26	0.069
MCV	−0.10	0.474	−0.24	0.090
MCH	−0.27	0.053	−0.17	0.227
MCHC	−0.22	0.122	−0.04	0.778
RDW	−0.02	0.911	0.02	0.999
Hb F%	−0.16	0.272	0.07	0.650
Ferritin	−0.36	0.011	0.1	0.493
RQ of β-globin	0.05	0.737	−0.20	0.081
RQ of *HoxA5*	– – –	– – – -	0.13	0.360

r_s: Spearman coefficient. RQ=relative quantitation.

Table 3. Correlation between RQ of *HoxA5*, RQ of *HoxA9* with different parameters in thalassemia major (n = 50).

	RQ of *HoxA5*		RQ of *HoxA9*	
	r_s	p	r_s	p
No. of transfusions (year)	−0.29	0.038	0.60	<0.001
WBCs	−0.18	0.265	0.52	<0.001
RBCs	−0.13	0.374	0.20	0.165
Hb	−0.26	0.072	0.28	0.051
HCT	−0.16	0.256	0.20	0.160
MCV	−0.26	0.071	−0.04	0.806
MCH	0.31	0.080	0.08	0.597
MCHC	−0.18	0.212	0.19	0.194
RDW	0.24	0.095	−0.04	0.806
Hb F	0.16	0.279	−0.23	0.112
Ferritin	−0.23	0.101	0.73	<0.001
RQ of β-globin	0.39	0.005	− 0.57	<0.001
RQ of *HoxA5*	– – – -	– – –	−0.25	0.035

r_s: Spearman coefficient. RQ=relative quantitation.

Discussion

Homeobox genes encode a group of transcription factors with a highly conserved 60 amino acid DNA-binding motif, the role of *Hox* in normal haematopoiesis is primarily at the level of haematopoietic stem cell function [6–9]. Nevertheless, there is a scarcity in the published literature regarding the role of *HoxA* in thalassemia. Thus, we conducted the present case–control study to determine *HoxA* expression among β-thalassemia patients and to investigate the relation of their expression and different laboratory investigations.

Our principal result is the trend increase in the RQ of *HoxA9* from health to intermediate disease and major disease that broadly correlates with the increase in HbF and ferritin, and decrease in red blood cell count, haemoglobin, haematocrit and the RQ of β-globin. Many of these are to be expected and are reflective of the general pathology of the disease. For example, the increase in ferritin almost certainly reflects the need for increased blood transfusion over the disease spectrum [10,11]. The marked increase in the RQ of *HoxA5* in children with thalassemia intermediate is difficult to explain but is likely to reflect a disease process specific to this subgroup. This group also has the highest levels of HbF, leading to the speculation of a pathophysiological link.

Table 1. Comparison between the three studied groups according to laboratory investigation and gene expression.

Metric	Controls (n = 50)	Intermediate cases (n = 50)	Major cases (n = 50)	P value
WBCs (x10^3/ul)	4.8 ± 0.4	4.1 ± 1.3[ab]	9.2 ± 1.4 [c]	<0.001
RBCs (x10^6/ul)	4.8 ± 0.4	3.2 ± 0.7 [ab]	2.8 ± 0.5 [c]	<0.001
Hb (g/L)	117 ± 9	85 ± 15 [ab]	71 ± 11 [c]	<0.001
HCT (%)	35.4 ± 2.4	25.2 ± 5.0[ab]	20.3 ± 3.4 [c]	0.001
MCV (FL)	73 ± 4	73. ± 4	72 ± 5	0.126
MCH (Pg)	26.4 ± 4.2	25.8 ± 1.9	25.5 ± 2.1	0.158
Hb F%	0.1 (0.05–0.4)	39.8 (28–48)[ab]	10.05 (3–15.1) [c]	0.081
Ferritin (ng/ml)	43 (35–57)	195 (170–280) [ab]	1824 (1500–3227) [c]	<0.001
RQ of β-globin	1.4 (1.2–1.9)	0.7 (0.5–0.9) [ab]	0.2 (0.1–0.3) [c]	0.008
RQ of *HoxA5*	1.0 (0.2–1.6)	6.8 (3.5–8.9) [ab]	1.1 (0.2–2.9)	0.501
RQ of *HoxA9*	0.5 (0.4–0.8)	2.4 (2.0–3.0) [ab]	4.1 (2.4–7.1) [c]	0.001

Data mean (SD) or median (IQR). p value for linear trend estimation for comparing between different groups. In subgroup analysis – a: significant difference intermediate v major groups b: significant difference intermediate v controls, c: significant difference Major v controls at $p < 0.05$. RQ=relative quantitation.

Table 4. Agreement (sensitivity, specificity) for different parameters to predict thalassemia intermediate cases vs control and thalassemia major cases vs thalassemia Intermediate cases.

	AUC (95% CI)	Cut off	Sensitivity	Specificity	PPV	NPV
Intermediate cases vs control.						
RQ of *HoxA5*	0.91 (0.86–0.97)	>3.0	82.0	86.0	85.4	82.7
RQ of *HoxA9*	0.99 (0.98–1.0)	>1.75	92.0	90.0	90.2	91.8
Major cases vs intermediate cases						
RQ of *HoxA5*	0.87 (0.81–0.95)	≤4.47	94.0	74.0	78.3	92.5
RQ of *HoxA9*	0.79 (0.71–0.89)	>3.28	62.0	96.0	93.9	71.6

AUC: Area under the curve CI: Confidence Intervals. NPV: Negative predictive value PPV: Positive predictive value.

However, there were strong links between the RQ of *HoxA9* and (inversely) haemoglobin in intermediate disease, and with the transfusion history, ferritin, the white cell count and (inversely) the RQ of β-globin in major disease. These too may well be spurious (e.g., ferritin levels merely reflect transfusion history), and so we must be careful in attributing a causative mechanism. In this disease group, the two HOXA indices correlated weakly. *HoxA9* was better at defining intermediate cases from the controls, but both the HOXA types were equally effective at separating major from intermediate thalassemia.

The genes of the *HOXA* cluster are especially highly transcribed in haematopoietic system helping in the differentiation and regeneration of haematopoietic stem cells, their expression gradually declining during maturation [12]. A case report of a patient with a deletion involving *HOXA* showed unusual features of multiple episodes of oxyhaemoglobin desaturation [13]. The fusion between protein nucleoporin 98 (NUP98) and *HoxA9* has been found to stimulate the proliferation of HSCs by activating the expression of other HOX genes including HOXA7, MEIS1 and PBX3 [14,15]. One plausible explanation for the *HoxA9* overexpression in β-thalassemia is that it is one of the most highly expressed Hox genes in the haemopoietic stem cell (HSC) compartment, and thus, it could potentially be the major determinant of physiologic HSC self-renewal. This is reinforced by evidence that overexpression of *HoxA9* can enhance HSC regeneration *in vivo*, thus implicating rapidly HSC self-renewal in thalassemia cases [16].

In contrast to our data on *HoxA9*, we found little evidence of a value in *HoxA5*. In colorectal cancer, it negatively correlates with the proliferation and angiogenesis-related genes and may act as a tumour suppressor, inhibiting the activity of the Wingless pathway through the regulation of β-catenin inhibitory proteins to decrease the potential for self-renewal and differentiation characteristics of cancer cells [17]. Although *in vitro* data points to the activation of *HoxA5* signalling playing an important role in CD34 +ve haematopoiesis [18], our clinical data does not support this hypothesis. In a mouse model, *HoxA5* drives the cell cycle and arrests erythroid progenitor cells in G0 phase, although the HSC pool shrinks after overexpression of *HoxA5*, HSCs sustain the abilities of self-renewal and multipotency [9]. Our data does not support this conclusion in our own species. Increased activation of the Jak2/STAT5 pathway promotes disproportionate proliferation of erythroid progenitors [5]. It is not clear whether *HoxA5* can expand HSCs, but knock-in of human *HoxA5* caused an increase in the number of myeloid progenitors and blocked erythroid differentiation [18].

We recognize certain limitations. A case–control study has inherent limitations of possible misclassification and ascertainment bias. In addition, the study was a small single-centre experience and therefore the results cannot be generalized to the general population. Nevertheless, our data represents an advance in biomedical science because as it draws attention to the possible use of *HoxA* gene analysis in the identification of patients with thalassemia and in differentiating between its two subtypes of major and intermediate thalassemia.

Summary table

What is known about this subject

- The molecular defects in β-thalassemia result in absent or reduced β-chain production resulting in clinical sequelae such as iron overload and splenectomy.
- The pathogenesis of β-thalassemia has been attributed to intramedullary apoptosis and delayed maturation of erythroid progenitor cells.
- Homeobox genes encode a group of transcription factors which have a role in normal haematopoiesis.

What this paper adds:

- *HoxA9* levels, but not *HoxA5* levels, increase with disease severity of thalassemia, and inversely with expression of β-globin
- The *HoxA9* and *HoxA5* expression may be used for differentiation between β-thalassemia major and intermediate, but *HoxA9* is preferred in differentiating intermediate thalassemia from healthy children.

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Disclosure statement

The authors declare that there is no conflict of interest.

References

[1] Kattamis A, Forni GL, Aydinok Y, et al. Changing patterns in the epidemiology of beta-thalassemia. Eur J Haematol. 2020;105:692–703.

[2] Thein SL. Molecular basis of beta thalassemia and potential therapeutic targets. Blood Cells Mol Dis. 2018;70:54–65.

[3] Origa R, Sollaino MC, Borgna-Pignatti C, *et al*. α-globin gene quadruplication and heterozygous β-thalassemia: a not so rare cause of thalassemia intermedia. Acta Haematol. 2014;131:162–164.

[4] Rivella S. beta-thalassemias: paradigmatic diseases for scientific discoveries and development of innovative therapies. Haematologica. 2015;100:418–430.

[5] Rivella S. The role of ineffective erythropoiesis in non-transfusion-dependent thalassemia. Blood Rev. 2012;26:S12–S15.

[6] Alsayegh K, Cortés-Medina LV, Ramos-Mandujano G, et al. Hematopoietic differentiation of human pluripotent stem cells: HOX and GATA transcription factors as master regulators. Curr Genomics. 2019;20:438–452.

[7] Luo Z, Rhie SK, Farnham PJ. The enigmatic HOX genes: can we crack their code. Cancers (Basel). 2019:323;11:323.

[8] Ramos-Mejía V, Navarro-Montero O, Ayllón V, et al. *HoxA9* promotes hematopoietic commitment of human embryonic stem cells. Blood. 2014;124:3065–3075.

[9] Yang D, Zhang X, Dong Y, *et al*. Enforced expression of *HoxA5* in haematopoietic stem cells leads to aberrant erythropoiesis *in vivo*. Cell Cycle. 2015;14:612–620.

[10] Ozment CP, Turi JL. Iron overload following red blood cell transfusion and its impact on disease severity. Biochim Biophys Acta. - 2009;1790:694–701.

[11] Shah FT, Sayani F, Trompeter S, et al. Challenges of blood transfusions in beta-thalassemia. Blood Rev. 2019;37:100588.

[12] Argiropoulos B, Humphries RK. *HOX* genes in hematopoiesis and leukemogenesis. Oncogene. 2007;26:6766–6776.

[13] Pezzani L, Milani D, Manzoni F, *et al*. HOXA genes cluster: clinical implications of the smallest deletion. Ital J Pediatr. 2015;41:31.

[14] Hess JL, Bittner CB, Zeisig DT, *et al*. c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. Blood. 2006;108:297–304.

[15] Mendes A, Jühlen R, Bousbata S, et al. Disclosing the interactome of leukemogenic NUP98-*HoxA9* and SET-NUP214 fusion proteins using a proteomic approach. Cells. 2020;10;9: 1666.

[16] Thorsteinsdottir U, Mamo A, Kroon E, *et al*. Overexpression of the myeloid leukemia-associated *HoxA9* gene in bone marrow cells induces stem cell expansion. Blood. 2002;99:121–129.

[17] Ordóñez-Morán P, Dafflon C, Imajo M, *et al*. *HoxA5* counteracts stem cell traits by inhibiting WNT signaling in colorectal cancer. Cancer Cell. 2015;28:815–829.

[18] Crooks GM, Fuller J, Petersen D, *et al*. Constitutive *HoxA5* expression inhibits erythropoiesis and increases myelopoiesis from human hematopoietic progenitors. Blood. 1999;94:519–528.