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Expression of zinc finger and homeobox 2 in erythroleukaemic cells and gamma-globin expression

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ABSTRACT: During the last few decades, foetal haemoglobin reactivation has been considered as a promising intervention to treat β-thalassaemia and sickle-cell anaemia. Variable foetal haemoglobin (Hb F, $\alpha_2\gamma_2$) production among individuals is under control of distinct chromosome regions, namely, the quantitative trait loci (QTL). One of the QTL affecting Hb F levels on chromosome 8q has never been explored. The zinc fingers and homeodomains 2 (*ZHX2*) transcription factor located at 8q position is remarkably down-regulated in hereditary persistence of foetal haemoglobin, indicating an inverse correlation between the γ-globin and *ZHX2* expression. Here we studied the effect of ZHX2 over the γ-globin expression in K562 erythroleukaemic cell line by transient transfection with a ZHX2 expression plasmid. At 24 h after transfection, the relative expression of endogenous γ-globin mRNA had been reduced to 0.401 ± 0.08 as assessed by real-time PCR. Our finding suggests that the repressive effect on γ-globin probably results from the presence of ZHX2 and supports its possible involvement in the regulation of γ-globin expression.

KEYWORDS: ZHX2, K562, γ-globin, foetal haemoglobin, QTL

INTRODUCTION

Thalassaemia is a genetic disorder affecting the amount of haemoglobin chain production. In early times it was found mainly in the Mediterranean area, the Middle East, Africa, and SE Asia. However, nowadays it is considered as a world-wide disease[1]. Thalassaemia syndromes can be classified as being either α- and β-thalassaemia according to the affected globin chains. In β-thalassaemia, the absence of β-haemoglobin results in an excess of complementary α-haemoglobin whose precipitation leads to oxidative stress, red cell membrane damage, short red cell life span, and ineffective erythropoiesis. An increase in foetal haemoglobin (Hb F) levels in β-thalassaemia has been shown to ameliorate the disease severity[2]. That fact prompts the idea of foetal haemoglobin reactivation and provides a therapeutic approach for β-thalassaemia and also sickle-cell anaemia[3].

Hb F production is controlled by genetic loci both in *cis* and in *trans* to β-globin cluster genes which account for over 80% of the phenotypic variation. The underlying quantitative trait loci (QTL) have been mapped to chromosome 6q[4], 11p[5], and 8q by linkage studies. *HBS1L-MYB* on chromosome 6q, *Xmn*I polymorphism at −158 of G γ-globin on chromosome 11p, and *BCL11a*[6] on chromosome 2p are successfully identified by genome-wide association studies. Unlike other QTL, no candidate gene has been proposed in the region of chromosome 8q until recently. The gene encoding the zinc fingers and homeobox 2 (*ZHX2*) transcription factor located on 8q24 has emerged as a potential candidate gene for γ-globin regulation[7]. Deletion in β-globin cluster leads to unusually high levels of Hb F transcripts in reticulocytes, termed hereditary persistent of foetal haemoglobin. ZHX2[8] comprise 873 amino acid residues and shares two C2H2-typed zinc finger domains and five homeodomains with other members of the family, i.e., ZHX1 and ZHX3. In previous studies[9,10], ZHX2 expressed ubiquitously in various tissues, and was identified as a transcriptional repressor for foetal-expressed genes, e.g., α-foetoprotein and glypican. The homeobox genes generally encode transcription factors that can either activate or repress target genes in a temporal and spatial manner[11]. Cooperative DNA binding often requires different copartners, usually another transcription factor, to

control the transcription of specific target genes[12]. Loss of ZHX2 function in the liver causes persistent α-foetoprotein production in adult mice. Suppression of α-foetoprotein production in hepatocellular cancer cell line by ZHX2 is dependent on the HNF1 binding site[9]. Taken together, by an unknown mechanism, ZHX2 with or without other transcription factor(s) might be involved in the switching off of the γ-globin gene during adulthood.

To define the role of ZHX2 in the γ-globin expression, we overexpressed ZHX2 in the K562 erythroleukaemic cell line. The K562 cell line contains undifferentiated blast cells expressing glycophorins and responds to exogenous reagents to induce the expression of γ-globin and is also useful in the study of erythroid differentiation[13]. The K562 cells perform homogeneously on their genetic background. This could reduce the effect of the genetic modifier on the ZHX2 expression per se and/or alter the responsiveness of the γ-globin gene to ZHX2 action. Hence in this paper we demonstrate the direct effect of ZHX2 on lowering γ-globin gene expression in K562 cells.

MATERIALS AND METHODS

RNA isolation and generation of plasmids

Human white blood cells collected from 5 ml of peripheral blood were used to isolate total RNA using an RNeasy Mini Kit (QIAgen) and quantified by absorbance at 260 nm. The first strand ZHX2 cDNA was reverse transcribed using eAMV reverse transcriptase (Sigma) beginning with 1 μg of total RNA. The cDNA containing sequences encoding human *ZHX2* with haemagglutinin (*HA*) tag in frame was synthesized using the forward and reverse primers as described in Ref. 9 and cloned to the *Kpn*I/*Not*I site of pcDNA3.1/Hygro (Invitrogen) to create the ZHX2 expression vector pcZHX2-HA.

Cell culture and transient transfection

The K562 cell line was obtained from Prof. Suthat Fucharoen and grown in RPMI 1640 (Gibco) with 10% foetal calf serum, 4 mM glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin supplementation. Cells were incubated at 37 °C in 5% CO_2.

The K562 cells (5×10^5) were incubated in serum free RPMI 1640 and seeded onto 6-well plates for 2 h prior to transfection. For transfection, 5 μl of FuGENE HD (transfection reagent) and 1 μg of pcZHX2-HA or blank pcDNA3.1/Hygro were diluted in 100 μl of Opti-MEM reagent (Invitrogen) to form liposome-DNA complexes by following the manufacturer's protocol. The complexes were dropped onto the culture plate. Fresh culture medium was added after 6 h of transfection. The K562 cells expressing ZHX2 were harvested for RNA extraction.

Quantitative real-time PCR

Total RNA was isolated from the cultured cells, treated with DNAase I (Invitrogen), and reverse transcribed to cDNA using reverse transcriptase (Sigma) starting from 1 μg of total RNA. Primers designed using Primer3 v0.4.0 and synthesized by Bio Basic (Canada) were as follows: ZHX2, CTGAGTTGTCCTGGCTGACA (sense) and CTAGTCTGGCCGAGGATCTG (antisense); γ-globin, GCACGTGGATCCTGAGAACT (sense) and GATGCTCAAGGCCCTTCATA (antisense); GAPDH, CGACCACTTTGTCAAGCTCA (sense) and AGGGGTCTACATGGCAACTG (antisense). Quantitative real-time PCR (qRT-PCR) was carried out in LightCycler 480 (Roche) using 1 μg of cDNA, 1 nM of specific primers, and 2X SYBR Green I Master Mix (Roche). No template control reactions were done in parallel and no positive signal was obtained. All data were normalized to the endogenous *GAPDH* control as validated for γ-globin normalization[14] using the ΔΔCt method[15]. The relative value of 1.0 derived from the blank vector transfected experiment (*GAPDH* normalized) was designed as the calibrator. N-fold relative to calibrator expressed quantified gene expression. All experiments were carried out in duplicate.

Western blot analysis

K562 cells expressing ZHX2-HA were lysed in PBS containing 1 mM EDTA, 0.5% (v/v) Igepal, 0.15 M NaCl, 20 mg BSA, 10 mM Tris-HCl pH 7.5, and protease inhibitor cocktail (Sigma). Cell debris was removed after centrifugation. Cellular proteins were separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS and incubated with monoclonal antibodies against human ZHX2 (Santa Cruz) and β-actin (Santa Cruz) followed by anti-mouse antibody coupled to HRP (Dako). The antibody-bound protein was visualized using chemiluminescence.

RESULTS AND DISCUSSION

ZHX2 and γ-globin gene expression profile in K562

The background expression of ZHX2, γ-globin, and GAPDH genes were quantified by real time PCR. Amplification specificity of each primer pair was assured by melting curve analysis (data not shown). We

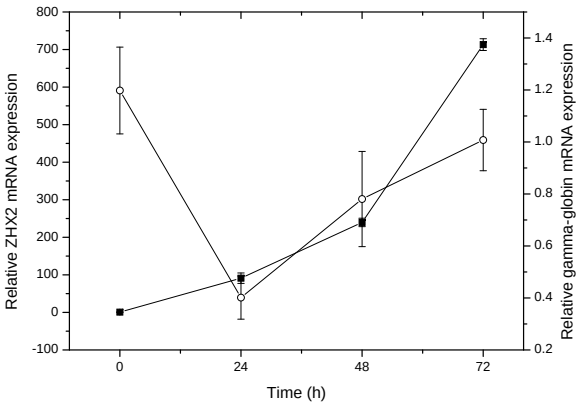

Fig. 1 Overexpression of ZHX2 reduced endogenous γ-globin expression in K562 cells. Quantification of relative mRNA levels by real time PCR at 0, 24, 48, and 72 h after transfection in K562 cells; (■) the relative ZHX2 mRNA of exogenously introduced plasmid, and (○) the relative γ-globin mRNA (each experiment was duplicated).

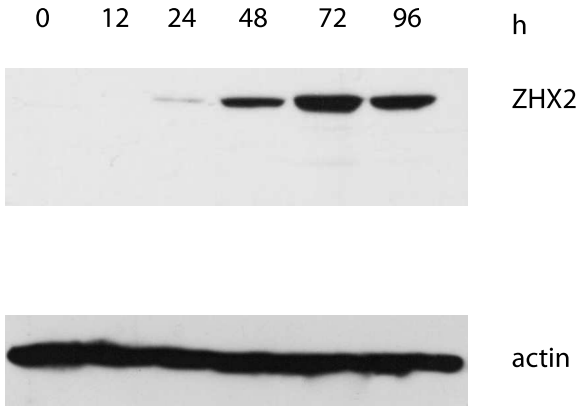

Fig. 2 The immunoblot of ZHX2 protein encoded from pcZHX2-HA. ZHX2 protein (upper panel) upon plasmid transfection at various times in K562 cells determined by monoclonal antibody directed to human ZHX2 as compared to actin (lower panel).

accepted the default settings of the machine software in base line and crossing point selection. The cycle thresholds of ZHX2, γ-globin, and GAPDH genes, which reflect their abundances in K562 cells, were found to be (mean ± SD): 27 ± 0.035, 9.65 ± 0.05, and 12.94 ± 0.03, respectively. There were substantially more transcripts for γ-globin than for the house keeping gene, GAPDH. ZHX2 was endogenously expressed at very low level in K562 cells. This warranted an experimental design using overexpression.

The repression effect of ZHX2 over the γ-globin expression in K562 was dynamic

The relative ZHX2 transcript levels upon the transfection in K562 were determined at various times (Fig. 1). We collected the cultured cells at 24, 48, and 72 h after transfection. ZHX2 mRNA levels increased gradually as seen from their protein levels visualized upon immunoblot against anti-human ZHX2 monoclonal antibody (Fig. 1). Notice that endogenous levels of ZHX2 protein were barely detectable at 0 and 12 h, and this may reflect the undifferentiated characteristics of the K562 cell line. The accumulation of ZHX2 protein began to be detected at 24 h and reached its highest level at 72 h. This implied that pcZHX2-HA is capable of correctly expressing ZHX2 protein in this cell line.

As opposed to an increase in ZHX2 expression, the relative γ-globin mRNA apparently reduced at 24 h but was then relieved from suppression at 48 or 72 h (Fig. 2). The relative γ-globin mRNA expression decreased to 0.401 ± 0.08 at 24 h. It tended to return to basal level at 72 h by the time ZHX2 protein was abundantly present. Thus ZHX2 could reduce the endogenous γ-globin expression as expected albeit this effect was only transient as the γ-globin expression unexpectedly reversed. The data supported the hypothesis that ZHX2 might function as a transcription repressor for γ-globin gene during adult erythroid differentiation.

Here we firstly demonstrated the effect of ZHX2 over-expression and the down-regulation of γ-globin expression in K562 cells. The detailed mechanism of how ZHX2 affects the abundance of γ-globin mRNA is currently unknown. Intriguingly, during the preparation of this manuscript de Andrade et al[16] reported the marked down regulation of ZHX2 in CD34+ cells cultured in a high foetal haemoglobin enriched condition. Given the inverse correlation between ZHX2 and the γ-globin gene in CD34+, our findings not only supports the idea that ZHX2 is involved in the repression of γ-globin gene but also provides evidence that ZHX2 has a direct effect on the γ-globin expression level and might participate in globin gene regulation. ZHX2 is capable of forming homodimers and heterodimers with other transcription factors like ZHX1, ZHX3, or NF-YA. Conceivably, like other homeoproteins, dimerization forms of ZHX2 might be crucial not only to determine its specific target genes but also the outcome of its regulation, i.e., transcriptional repression or activation. However, the effect of ZHX2 on γ-globin certainly needs a supporting study to dissect the mechanism of how ZHX2 acts: by direct action on γ-globin promoters or in cooperation with other proteins in the globin-regulation process.

Further studies on subcellular localization of ZHX2 in parallel with its repression effect on γ-globin gene as well as the identification of its copartner are all important.

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REFERENCES

1. Bianchi N, Zuccato C, Lampronti I, Borgatti M, Gambari R (2009) Fetal hemoglobin inducers from the natural world: a novel approach for identification of drugs for the treatment of β-thalassemia and sickle-cell anemia. *Evid Based Compl Alternat Med* **6**, 141–51.
2. Swank RA, Stamatoyannopoulos G (1998) Fetal gene reactivation. *Curr Opin Genet Dev* **8**, 366–70.
3. Bank A (2006) Regulation of human fetal hemoglobin: new players, new complexities. *Blood* **107**, 435–43.
4. Garner C, Mitchell J, Hatzis T, Reittie J, Farrall M, Thein SL (1998) Haplotype mapping of a major quantitative-trait locus for fetal hemoglobin production, on chromosome 6q23. *Am J Hum Genet* **62**, 1468–74.
5. Garner CP, Tatu T, Best S, Creary L, Thein SL (2002) Evidence of genetic interaction between the beta-globin complex and chromosome 8q in the expression of fetal hemoglobin. *Am J Hum Genet* **70**, 793–9.
6. Menzel S, Garner C, Gut I, Matsuda F, Yamaguchi M, Heath S, et al (2007) A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. *Nat Genet* **39**, 1197–9.
7. de Andrade TG, Peterson KR, Cunha AF, Moreira LS, Fattori A, Saad ST, et al (2006) Identification of novel candidate genes for globin regulation in erythroid cells containing large deletions of the human beta-globin gene cluster. *Blood Cell Mol Dis* **37**, 82–90.
8. Kawata H, Yamada K, Shou Z, Mizutani T, Yazawa T, Yoshino M, et al (2003) Zinc-fingers and homeoboxes (ZHX) 2, a novel member of the ZHX family, functions as a transcriptional repressor. *Biochem J* **373**, 747–57.
9. Shen H, Luan F, Liu H, Gao L, Liang X, Zhang L, et al (2008) ZHX2 is a repressor of alpha-fetoprotein expression in human hepatoma cell lines. *J Cell Mol Med* **12**, 2772–80.
10. Morford LA, Davis C, Jin L, Dobierzewska A, Peterson ML, Spear BT (2007) The oncofetal gene glypican 3 is regulated in the postnatal liver by zinc fingers and homeoboxes 2 and in the regenerating liver by alpha-fetoprotein regulator 2. *Hepatology* **46**, 1541–7.
11. Kim YH, Choi CY, Lee S-J, Conti MA, Kim Y (1998) Homeodomain-interacting Protein Kinases, a Novel Family of Co-repressors for Homeodomain Transcription Factors. *J Biol Chem* **273**, 25875–9.
12. Mann RS, Chan S-K (1996) Extra specificity from extradenticle: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet* **12**, 258–62.
13. Koeffler HP, Golde DW (1980) Human myeloid leukemia cell lines: a review. *Blood* **56**, 344–50.
14. Silver N, Best S, Jiang J, Thein S (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* **7**, 33.
15. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–8.
16. De Andrade T, Moreira L, Duarte A, Lanaro C, De Albuquerque D, Saad S, et al (2010) Expression of new red cell-related genes in erythroid differentiation. *Biochem Genet* **48**, 164–71.