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Review Article

RECENT TRENDS IN FOETAL HAEMOGLOBIN RESEARCH: A REVIEW

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ABSTRACT
The respiratory protein haemoglobin and its different phenotypes are considered to be idle genetic markers and one of the most sensitive and informative molecule seen in primate blood. One of the normal phenotype, Foetal haemoglobin (Hb F) is the principal haemoglobin produced by the fetus but can transport oxygen efficiently even in a low oxygen environment. Contradictory to its expression in foetal stage, the expression is noticed to be drastically high along with some haemoglobin disorders in adulthood. The persistence of foetal haemoglobin in adult life, Hereditary Persistence of Foetal Haemoglobin (HPFH) projected with clinical interest due to disease ameliorating potential against sickle cell anaemia and different forms of thalassemias. The gamma gene expressions are controlled by transcription factors, chromatin modifiers, co-activators, beta gene repressors, regulatory molecules and micro RNAs (miRNAs) which are playing important role in globin gene expression. These regulatory molecules binds with locus control region, enhancers, promoters and different regulatory domains of beta globin gene cluster situated at chromosome 11. Induction of foetal haemoglobin is safe and sometimes it is possible to achieve a complete switching over from faulty adult haemoglobin to normal and competent foetal haemoglobin with an assumption that it can be counteract for therapeutic benefits. Discover pharmacologics that could elevate foetal haemoglobin with minimum toxicity profiles since he genotoxicity of demethylating agents are also a concern.

Keywords: *Haemoglobin, Beta Globin Gene Cluster, Foetal Haemoglobin, Hereditary Persistence of Foetal Haemoglobin, Gene Modulators, Hb F Induction*

INTRODUCTION
Haemoglobin is the oxygen-binding protein found in almost all vertebrates as well as certain invertebrates. This respiratory protein is considered to be an idle genetic marker (Ramesh and Veerraju, 1999) and one of the most sensitive and informative molecule seen in primate blood (Ehabal and Bansal, 2005). This respiratory protein was first observed in the crystalline form by Friedrich Ludwig Hunefeld, in 1840 (Giege, 2013). Haemoglobin carries a prosthetic haeme group (iron proto-porphyrin IX) associated with four globular polypeptide chains (Hori and Kitagawa, 1980). The globin tertiary structure comprises a helical structure joined together by non-helical segments. Four such globin chains are arranged together, giving rise to the spherical quaternary structure of haemoglobin.

The genes responsible for the production polypeptides of haemoglobin are situated in two different chromosomes. The locations are broadly named as α globin gene locus and β globin gene locus. Human alpha globin gene locus is situated at the short arm of 16th chromosome (16p13.3) and having genes for α polypeptides and α like polypeptides. The cluster also consists of zeta (ζ), psi-zeta (ψζ), psi-alpha-1(ψα1), alpha-2(α2) and alpha-1(α1) from 5'end to 3' end respectively (Forget and Hardison, 2009). The human beta globin gene is composed of five genes located on a short region of 11th chromosome (11p15.5), which is responsible for the production of beta polypeptide and beta like polypeptides. In this cluster genes are aligned in the order of epsilon (ε), G-gamma (Gγ), A-gamma (Aγ), Psi-beta-1 (ψβ-1), delta (δ) and beta (β) respectively from 5' end to 3' end (Levings and Bungert, 2002).

Expression of genes present in the beta globin locus is controlled differentially during the various stages of development. This regulation is leaded by remote regulatory sites in the particular gene loci. Such

types of regulatory sequence in the β-globin locus are collectively referred to as locus control region (LCR) (Li and Starnatoyannopoulos, 1994). The LCR of β globin gene locus consists of five hypersensitive sites (HS). They are scattered in between 6 kb and 20 kb 5' to the globin gene. Another hypersensitive site is present approximately 20 kb 3' to the globin gene. It is thought to be spotting the 3' boundary of the globin gene (Stamatoyannopoulos and Grosveld, 2001). The LCR is significant in globin gene expression. By acting as a powerful enhancer it helps to maintain an open chromatin state during transcription of globin gene (Forrester *et al*., 1990). During each stage of development, it controls the production of relevant beta polypeptide by activating corresponding genes in β globin gene cluster. Various genes in β globin cluster are arranged in such a manner that embryonic and foetal globin genes are nearby and adult globin genes are distant from the LCR (Van der Ploegh *et al*, 1980). During globin gene switching over, the LCR changes its binding with embryonic genes to adult β-globin genes in order to ensure their activation during adult life (Palstra *et al*., 2003). In addition to normal genes, there are numerous supplementary genes-like structures are present in both α and β globin gene cluster. Their sequence homology and exon-intron structures are similar to that of actively expressed globin genes. They are commonly known as pseudo-genes (ψ) (Zhang and Gerstein, 2004). They are present in between normal genes and will not express at any stage of development.

On the either side of the coding regions of globin gene, it is interrupted by stretches of non coding DNA sequences called intervening sequences (IVS) or introns (Tilghman *et al*., 1978). In the case of β-like globin genes, introns are spanning between the codons 30 and 31 and codons 104 and 105 at the two ends respectively. In the family of α-globin gene, IVS interrupt exon sequence between the codons 31 and 32 and codons 99 and 100 respectively (Forget and Hardison, 2009). Even though the particular position of codon numbers at which the interruption happens, it vary between the α- and β-like globin genes. The intervening sequence is seen specifically at the same position in the primary sequence of α. In both α- and β-globin genes the first intervening sequence (IVS-1) is shorter than the second intervening sequence (IVS-2). However, the IVS-2 present in human β-globin gene is larger than that of α and γ -globin gene. For proper splicing, the di-nucleotides GT and AG are at the 5' and 3' ends respectively of the intron (Burset *et al*., 2000). Any type of changes in the precise sites of these nucleotide leads to abnormal haemoglobin production (Forget and Hardison, 2009).

Different types of normal haemoglobins are produced at different stages of human development like embryonic, foetal and adult. Each of this haemoglobin consists of any of the two polypeptides produced from β gene locus and two polypeptides produced from the duplicated alpha genes of alpha gene cluster. Production of alpha polypeptide is always constant throughout the development except embryonic haemoglobins. But, the production of beta locus polypeptide will vary from time to time (Bonavetura and Riggs, 1968). Haemoglobin produced during the embryonic stage (first few months of life) is termed as embryonic haemoglobin. There are Hb Portland-1 ($\zeta_2\gamma_2$), Hb Gower-1 ($\zeta_2\varepsilon_2$), and Hb Gower-2 ($\alpha_2\varepsilon_2$) (He and Russell, 2001). After a few weeks of development embryonic haemoglobin production ceases and foetal haemoglobin (Hb F) begins to produce. It is composed of two alpha polypeptides and two gamma polypeptides ($\alpha_2\gamma_2$). Before birth, the foetal haemoglobin level declines and adult haemoglobin (Hb A) starts to produce in low quantity as an adaptation. Adult haemoglobin is made up of alpha polypeptides and beta polypeptides ($\alpha_2\beta_2$). After birth which extends its production to 95-98 %. Hb A2 is a second type of adult haemoglobin produced in smaller quantities, which is composed of alpha and delta polypeptides ($\alpha_2\delta_2$).

All the haemoglobin types are showing some sort of differences between each other. These variations are specifically helpful and advantageous during specific developmental stages. Even though, these haemoglobins have similarity in their structure, they differ in O_2 affinity (Hoffman and Brittain, 1996 ; He and Russell, 2001). Haemoglobin switching over takes place for two times in the life time of man, embryonic haemoglobin to foetal haemoglobin and then to adult haemoglobin. There are a wide variety of haemoglobin variants besides the normal ones, which are synthesized on various reasons. Changes in the genetic makeover of specific genes and the subsequent changes or alterations in the amino acid sequence

are the key factors behind the production of abnormal haemoglobin. Hb S, Hb E, Hb D, Hb Q, Hb H and Hb Lepore etc are the most prevalent and altered Hb variants in childhood as well as in adulthood (Bonavetura and Riggs, 1968). Even though Foetal haemoglobin (Hb F) is a type of normal haemoglobin variant, it is present in some individuals in their adulthood in high percentages (Edoh *et al*., 2006). Hb F seems to be associated with certain haemoglobin disorders (Thein and Craig, 1998) and its elevation is of high clinical significance.

Foetal Haemoglobin

Haemoglobin F (Hb F) is the principal haemoglobin produced by the fetus but can transport oxygen efficiently even in a low oxygen environment. The nearly identical genes of β globin gene cluster, *HBG1 (Aγ)* and *HBG2 (Gγ)* encode the γ globin polypeptide chains of foetal haemoglobin. The only difference between Aγ and Gγ is that, which contain glycine or alanine residue at 136th of γ polypeptide respectively (Adachi *et al*, 1990). On 6 to 12 month of age foetal haemoglobin is almost completely replaced by adult haemoglobin (HbA0), later on it is measured to be less than 1% of total haemoglobin (Weatherall and Clegg, 1981). Hb F may constitute up to 90% of the total haemoglobin in some haemoglobin disorders like beta-thalassemia major or other combinations of beta thalassemia and foetal haemoglobin mutations. Productions of various haemoglobins are transcriptionally regulated according to the necessity. The expressions are controlled by many transcription factors, which are playing important role in globin gene expression by binding with locus control region and promoter regions of the gene. These transcription factors are essential for making interaction and forming stability between LCR and its corresponding gene (Drissen *et al.*, 2004; Vakoc *et al.*, 2005; Song *et al*., 2007).

***Hereditary Persistence of Foetal Haemoglobin* (HPFH)**

Hereditary Persistence of Foetal Haemoglobin (HPFH) is a clinical condition characterized by the persistence of high percentage of foetal haemoglobin in adulthood. HPFH was first recognized by Edington and Leahmann in 1955. In HPFH, it is suggested that the synthesis of γ- globin chain fails to switch over to *β*-chain. Though deletional as well as non-deletional types of HPFH have been identified, the exact regulatory mechanism is unknown.

In case of non-deletional point mutations, majority are in the promoter region of the gamma globin gene. The non-deletional HPFH is considered to be the result of mutations occurring in the promoter region of *HBG* and Single Nucleotide Polymorphisms (SNPs) in the Quantitative Trait Locus (QTLs). Mutations responsible for non-deletion HPFH are point mutations, which are usually formed at three regions surrounding -114, -175, and -200 in the 5' promoter regions of both *HBG1* and *HBG2* (Liu *et al*., 2005). The nucleotide sequences surrounding -200 regions have been revealed to be a binding site for different types of erythroid transcription factors (Steinberg *et al.*, 2001). Similarly, a mutation near -175 position prevents binding of *OCT-1* and *GATA-1*; mutation in −117 (promoter region) affects the regulatory CCAAT box and -114 mutation prevents binding of *NF-E3* (an erythroid specific factor), *CP1* and *CP2* (ubiquitous trans-acting factors) (Liu *et al*., 2005). Genetic studies conducted in patients with sickle cell anaemia and β-thalassemia and in healthy adults have identified three major Quantitative Trait Loci (QTL) such as *Xmn1-HBG2*, *HBS1L-MYB* intergenic region on chromosome 6q23 and *BCL11A* on chromosome 2p16. These three QTL are playing a major role in the variation and the production of 20-50% of foetal haemoglobin.

Deletional mutations are thought to interfere with the interactions between various transcription factors and the promoter region of corresponding genes (Forget, 1998). To date, eight types of HPFH due to deletions have been reported. These deletions are of variable size and are at different positions in the beta globin gene cluster. They are HPFH-1 (Black) (Adams *et al*., 1985; Collins *et al*., 1987; Feingold and Forget, 1989), HPFH-2 (Ghanaian)(Collins *et al*., 1987), HPFH-3 (Asian Indian)(Henthorn *et al.*, 1986; Mayuranathan *et al*., 2014), HPFH-4 (Italian)(Saglio *et al*., 1986; Huisman *et al*., 1997), HPFH-5 (Italian)(Camaschella *et al*., 1990), HPFH-6 (Vietnamese)(Kosteas *et al*., 1997; Panyasai *et al*., 2004), HPFH-7 (Kenyan)(Huisman, 1972) and SEA-HPFH (Southeast Asian) (Bhardwaj and McCabe, 2005;

Changsri *et al*., 2006). In addition Mayuranathan *et al* (2014) identified two novel HPFH mutations, 49.98kb (HPFH-9) and 86.7kb (HPFH-10) from Indian population.

Indian HPFH or HPFH-3 is caused as a result of non-homologous recombination events (Henthorn *et al.,* 1986). In this case, it removes 48.5 kb DNA from the 5' end of the ψβ gene to a region 30 kb downstream of the beta gene. The 5' deletion breakpoint is situated in the Alu family repeat 3' to the Aγ globin gene and 3' break point is situated within a region that contains a portion of l1 (*Kpn I*) repeat. There is a palindrome sequence with 160 bp and a set of 41 bp direct repeats that are found elsewhere in human genome (Mayuranathan *et al*., 2014). An average of 65: 35 % Gγ:Aγ ratio is seen in Indian type of HPFH. The Hb F value is reported to be around 22-30 % in heterozygotes.

HPFH mutations have got clinical interest in recent times, because the resulting elevation of Hb F levels can help to reduce disease severity of SCD or β-thalassemia. Foetal haemoglobin is considered as a strong genetic modulator (Stamatoyannopoulos *et al*., 1975), as it can decrease the disease severity by disrupting the polymerization of deoxy-Hb-S (Goldberg *et al.,* 1990). It is revealed that both the Gγ and Aγ globins have similar depolymerizing effects on Hb S (Adachi *et al.,* 1990). In HPFH, foetal haemoglobin is producing at high percentages in almost all red cells and thereby protecting all red cells from sickling. In the absence of HPFH, heterogeneous distribution of foetal haemoglobin is observed in patients with higher levels of Hb F; thereby half of the cells became sickle shaped and occlude with in microcirculation. Besides that, the deformed cells would block natural flow of normal cells with high Hb F. In such situation the patient may experience clinical manifestations of sickle cell disease. It can be concluded that persistence of foetal haemoglobin offer protection from sickle cell disease or thalassemia.

Regulation of Foetal Haemoglobin

In order to explain the elevated levels of foetal haemoglobin, three feasible mechanisms have been proposed by Steinberg such as mutation occurring at regulatory sequences, juxtaposition of enhancers, interactions between LCR and *HBG* as a consequence (Steinberg *et al*., 2001). The persistent production of foetal haemoglobin and globin gene expression is controlled by different tissue restricted erythroid factors and ubiquitous transcription factors. Peterson (2003) explained that the *cis* acting elements present in the beta globin gene locus interacting with *trans* acting proteins like transcription factors, chromatin modifiers, co-activators and repressors. These interactions play a pubertal role in the globin gene switching during development. Transcriptional factors such as *GATA-1*(Katsumura *et al*., 2017), *FOG* (Tsang *et al.,* 1997), *SP1* (Feng *et al.,* 2005; Hu *et al.,* 2007), *Sox6* (Yi *et al.,* 2006; Xu *et al*., 2010), *NFE2* (Ney *et al.,* 1990; Pace and Zein, 2005; Kim *et al*., 2016), *NFE3* (Filipe *et al*., 1999), *EKLF* , *KLF3*/*BKLF* (Funnell *et al*., 2012) *TR2*, *TR4* (Tanabe *et al.,* 2007) and *TAL1* (Yun *et al*., 2014; Kim *et al*., 2016) are controlling foetal haemoglobin and maintaining haemoglobin homeostasis in blood stream.

The non-coding RNAs like microRNAs (miRNA) are regulating the globin gene expression at the transcriptional level (Azzouzi *et al.,* 2011; Sankaran *et al.,* 2011; Bianchi *et al.,* 2009; Bianchi *et al.,* 2012; Ma *et al.,* 2013). The importance of micro RNA is that it can target more than one mRNA, where as a single mRNA can bind with more than one micro RNA through its specific binding sites at the 3'UTRs.

Micro RNA, miR-210 in differentiating erythroid cells can increase the expression of gamma globin genes. The mode of expression is found to be in a time dependent and dose dependent manner along with elevated foetal haemoglobin in differentiating erythroid cell lines (Bianchi *et al.,* 2009). The kit receptor complex miR-221-222 is reported to be involved in the haemoglobin switching of humans (Gabbianelli *et al.,* 2010) through kit receptors. Decline in miR 221-222 unblocks kit protein production at mRNA level and prevent Hb F synthesis by influencing early embryoblast. Globin gene expression can also be controlled by another micro RNA, miR-96 by binding with the CDS region (Azzouzi *et al.,* 2011). In people with trisomy 13, two micro RNAs miR-15a and miR-16-1 are playing a vital role for the up regulation of foetal haemoglobin (Sankaran *et al.,* 2011).

Two important miRNAs, miR-23 and miR-27 can interact with beta globin negative regulators like *KLF3* and *SP1* and can enhance the gene expression by extending a synergistic role. During erythropoiesis *KLF3* binds with its target site, the CACCC sequence in the promoter region of miR-23a cluster

composed of miR23a, 27a and 24-2. This interaction form a positive feedback loop to regulate the expression of beta like globin genes and the miRNA cluster during erythropoiesis in the K562 cells. Conversely, *KLF3* and *SP1* protein levels were increased after endogenous miR-23a or miR-27a was blocked when using the corresponding miRNA inhibitors in K562 cells (Ma *et al.,* 2013).

Micro RNA-486-3p regulates *BCL11A* expression by interacting with the highly extended isoform of *BCL11A* 3′UTR. Over expression of miR-486-3p located on chromosome 8p11 within the Ankyrin-1 (ANK1) gene in erythroid cells resulted in reduced *BCL11A* protein levels (Lulli *et al.,* 2013). These findings indicate that miR-486-3p contributes to Hb F regulation by post-transcriptional inhibition of *BCL11A* expression during adult erythropoiesis. Micro RNA-34a can be used for the activation of foetal haemoglobin by the gene silencing of *STAT- 3* (Ward *et al*., 2016). In 2009, observed a micro RNA, miR-144 negatively controlling the expression of α globin by influencing an erythroid specific Kruppel-like transcription factor, *KLFD* (Fu *et al.,* 2009; Saki *et al*., 2016).

Foetal haemoglobin mediated therapy

Modest induction of foetal haemoglobin is sufficient to ameliorate the disease severity of a major share of haemoglobin disorders. Induction of foetal haemoglobin is safe and sometimes it is possible to achieve a complete switching over from faulty adult haemoglobin to normal and competent foetal haemoglobin with an assumption that it can be counteract for therapeutic benefit (Bauer *et al.,* 2012).

Until 1970, there was no therapy for sickle cell diseases were reported (Powars *et al.,* 1984; Platt *et al.,* 1991). Induction of Hb F in baboons by demethylation of DNA using 5-azacytadine has got attention in 1980s (DeSimone *et al*., 1982; Ley *et al.,* 1982; Sankaran *et al.,* 2011). Induction of Hb F under altered cell kinetics and stress erythropoiesis may happen while demethylating beta globin promoters (Ley *et al.,* 1984). Experts paved way to identify S phase inhibitors to discover pharmacologics that could elevate foetal haemoglobin with minimum toxicity profiles since he genotoxicity of demethylating agents are also a concern (Letvin *et al.,* 1984; Platt *et al.,* 1984). As a result of the combined efforts of scientific world, a new treatment strategy was documented with potential inducing agent hydroxy urea with substantial benefits in 1984 (Platt *et al.,* 1984; Platt, 2008).

After that many drugs including erythropoietin, butyrate and its analogues have been shown to induce Hb F production. But hydroxyl urea is observed to be more effective than others. Rochette *et al.,* (1994) highlighted the efforts undertaken to find out ideal therapeutic drugs to increase Hb F levels with minimal toxicity in SCD or in thalasseamia patients. Hydroxy urea appeared to be most suited for clinical trials because of its ease of administration and relative safety (Charache *et al.,* 1995). But, not all patients are sensitive in Hb F induction towards hydroxyurea (Saleh and Hillen, 1997). Since hydroxyl urea seems to be unexpressive in one third of population suffering from various haemoglobinopathies (Fathallah and Atweh, 2006). The Multicenter studies on hydroxy urea in sickle cell anemia demonstrated the efficacy of hydroxyurea in reducing the rate of painful crises compared to placebo (Moore *et al.,* 2000). Besides these hydroxy urea has of variable efficacy, requires careful monitoring with dose-limiting myelosuppression, and is of limited utility for beta thalassemia (Bauer *et al.,* 2012). Rees and Brousse (2016) reported that it is widely using in USA, even though its prolonged safety is unknown.

Numerous novel therapeutic approached against sickle cell anaemia and other haemoglobinopathies are under experimentation. Haley *et al.,* (2003) studied the pharmacological elevation of Hb F and inhibition of Hb S polymerization due to elevated Hb F. In order to identify the Hb F inducing agents in adults they performed one screening approach based on induction of γ-globin gene expression in erythroid cells. Through their screening, they successfully identified active and potential therapeutic compounds against for sickle cell anemia from defined chemicals and fungal extracts.

A renewed and novel interest has been put forward by the scientific world to inhibit DNA methylation through various inhibitors to activate gene of interest (Fathallah and Atweh, 2006). Poor comprehension of the molecular mechanisms operative during the hemoglobin switch has limited the development of novel therapeutics. Upsurge of knowledge in the new era has reinvigorated the pursuit of rationally designed Hb F inducers (Bauer *et al.,* 2012).

Robust synthesis of Hb F molecule is seen with down regulation of *BCL11A* in the primary adult erythroid cells. Manipulation of genes targeting *BCL11A* is emerging as a therapeutic model in the treatment of haemoglobinopathies (Sankaran *et al.,* 2008). Wilber *et al.,* (2011) reported that knock down of *BCL11A*, *SOX6* and *FOP* had given elevated production of foetal haemoglobin Hb F and *FOP* silencing was more effective than *BCL11A*. The identification of micro RNAs involved in erythroid differentiation and Hb F production opens new options and ideas for developing therapeutic approaches against β-thalassemia and sickle cell anemia (Finotti *et al.,* 2014).

In 2016, Breda *et al* put forward a concept to activate gamma globin gene expression using a small gene construct of ZF protein and Ldb1 domain. They are arguing for the competence of this lentiviral mediated construct with less toxic effect while comparing with other herapeutic strategies (Breda *et al*., 2016). A study conducted in 2017 revealed that epigenetic modulators like micro RNAs can be used for the induction of foetal haemoglobin by acting at gamma globin gene. these non coding RNAs can replace conventional therapeutics like sodium butyrate without any complications (Tayebi *et al*., 2017). Role of micro RNA in hydroxy urea treated haemoglobinopathic cases also established with studies using erythroid progenitors (Hojjati *et al*., 2017).

CRISER-Cas9 technique is the most advanced one in the manipulation of beta as well as gamma globin genes. The upstream transcriptional start site of gamma globin gene positioned at -115 and -200 bp are the binding of two gamma (γ) repressors, BCL11A and ZBTB7A (LRF) respectively. A few benign HPFH non deletional mutations targeting these specific sites can be incorporated in erythroid cells for the upregulation of gamma globin gene (Martyn *et al.,* 2018).

Indel generation within regulatory elements using TALEN(Transcriptional activator-like effector nucleases) and non homologous end joining (NHEJ) has proven the de-repression of foetal haemoglobin in human peripheral blood stem cells (hPBSCs). This type of gene editing can solve the problem of haemoglobinopathic patients and it sustains too when translated clinically (Lux *et al*., 2019).Induction of Hb F producing genes (γ genes) are upcoming trend in the future to cure various haemoglobin disorders (Mosaca *et al.,* 2009; Ginder, 2015; Guda *et al*., 2015; Ngo *et al.,* 2015; Sankaran and Weiss, 2015; Vinjamur *et al*., 2018).

CONCLUSION

Foetal haemoglobin can be considered as a boon for those who are suffering from haemoglobinopathies. Induction of Hb F producing genes are upcoming trend in the future to cure various haemoglobin disorders since it is sufficient to ameliorate the disease severity of a major share of haemoglobin disorders. Foetal haemoglobin is safe and sometimes it is possible to achieve a complete switching over from faulty adult haemoglobin to normal and competent foetal haemoglobin with an assumption that it can be counteract for therapeutic benefit. Strategies for the activation of *cis* and *trans* regulatory regions responsible for the Hb F elevation using different in vitro and in vivo bio modulators will be promising in the field of erythroid regulation of foetal gene.

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