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Epigenetic Analysis of the Human Alpha- and Beta-Globin Gene Clusters

Hassana Fathallah, Gregory Portnoy, and George F. Atweh

Division of Hematology and Medical Oncology, Mount Sinai School of Medicine, New York, NY 10029

Abstract

K562 erythroleukemia cells have been widely used as a model for the study of globin gene regulation. A number of agents have been shown to activate or suppress globin gene expression in these cells. However, the molecular effects of these agents on the epigenetic configuration of the α - and γ -globin genes that encode HbF are not known. In this report, we investigated the relationship between globin expression and histone acetylation of the human α - and β -globin clusters in the fetal erythroid environment of K562 cells. Our studies suggest that acetylation of histone H3 may be important in regulating developmental stage-specific expression of the different β -like globin genes while acetylation of both histones H3 and H4 may be important for the regulation of tissue-specific expression of these genes. In contrast, acetylation of both histones H3 and H4 at the α -like globin promoters appears to be important for both developmental stage- and tissue specific-expression. Interestingly, butyrate-induced activation of α -globin gene expression in K562 cells is associated with significant increase in histone acetylation levels while TPA-induced inhibition is associated with decreased histone acetylation at its promoters. In contrast, changes in histone acetylation and DNA methylation do not appear to be important in the regulation of γ -globin gene expression by the same agents. These data suggest that the butyrate-mediated induction of the fetal γ -globin genes in K562 cells is not a direct result of its histone deacetylase inhibitor activity of butyrate on the chromatin of the γ -globin promoters, while the induction of the α -globin genes could be a result of a direct effect of butyrate on chromatin at its promoters. This is another example of the important differences in the molecular mechanisms of regulation of the genes of the α - and β -like globin clusters.

Keywords

Erythroid Cells; Globins; Chromatin; Histone acetylation; Gene Expression

INTRODUCTION

Reactivation of fetal hemoglobin (HbF) expression in adult life is an effective therapy for patients with hemoglobin disorders. In sickle cell disease, an increase in HbF interferes with the polymerization of sickle hemoglobin while in β -thalassemia, an increase in γ -globin chain synthesis decreases non- α : α chain imbalance. Hydroxyurea, an anti-metabolite chemotherapeutic agent, is the first and only member of a class of pharmacological inducers

CORRESPONDING AUTHOR: Hassana Fathallah, PhD, Mount Sinai School of Medicine, Division of Hematology/Medical Oncology, Box 1079, One, Gustave L. Levy Place, New York, NY 10029. Phone: 1-212-241 7931, Fax: 1-212-426 4390, e-mail: hassana.fathallah@mssm.edu.

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of HbF that is currently approved by the FDA for the treatment of patients with moderate and/or severe sickle cell disease [1,2]. Several other pharmacological inducers of HbF including 5-azacytidine [3], decitabine [4,5], butyrate and other histone deacetylase (HDAC) inhibitors [6,7] are currently under investigation.

Although pharmacological agents have been shown to induce HbF production *in vivo*, their effects on the epigenetic configuration of the human α - and β -globin clusters remain largely unknown. As a first step towards understanding the mechanism of action of agents that induce HbF, we conducted studies in a simplified model system. The human K562 cell line has been used extensively for the study of erythroid and megakaryocytic differentiation and for the study of the regulation of globin gene expression. The major aims of the experiments described in this report are to characterize the epigenetic patterns at the promoters of the α - and β -like globin genes in the fetal erythroid environment of K562 cells and investigate the effects of pharmacological induction or silencing of globin gene expression on the epigenetic modifications in the human α - and β -globin gene clusters.

MATERIAL AND METHODS

Cell Culture

K562 cells were cultured in RPMI-1640 medium and HeLa cells were cultured in DMEM medium. The media contained 10% FBS, penicillin 100kU/L and streptomycin 100mg/L. Cells were cultured in 5% CO₂ humidified atmosphere at 37°C. For induction, K562 cells at a concentration of 1×10^5 cells per ml were incubated in the absence (control) or presence of butyrate (1mM), hemin (30 μ M) or TPA (20nM) for 3 to 4 days.

Chromatin Immunoprecipitation Assay (ChIP)

Analysis of histone modification was carried out as recommended by the manufacturer (Upstate Biotechnology, Lake Placid, NY). Briefly, protein-DNA cross-linking was performed by incubating 1×10^6 cells at 37°C for 10 min in 1% formaldehyde. After washes, the cells were incubated on ice for 10 min in 200 μ l of lysis buffer. The lysate was sonicated to reduce DNA fragments to a size of 0.2 to 1 Kb. DNA was co-immunoprecipitated with acetylated histones with either a polyclonal antibody against diacetyl-histone H3 (#06-599, Upstate Biotechnology) or antiserum against tetra-acetyl-histone H4 (#06-598, Upstate Biotechnology). Immunoprecipitated (IP) DNA was purified by phenol/chloroform extraction and dissolved in 50 μ l distilled water. On the other hand, 50 μ l aliquots of the DNA solution before immunoprecipitation were directly subjected to DNA extraction and dissolved in 100 μ l distilled water.

Quantitative Real-Time PCR Analysis

3 μ l of IP DNA and 1 μ l of total DNA were analyzed in duplicate by quantitative real-time PCR using 200 nM of TaqMan probes and primers in a 25 μ l iQ-Supremix (Bio-Rad, Hercules, CA) reaction volume. Data were collected relative to a standard curve at the threshold where amplification was linear. To generate a standard curve, serial dilutions of genomic DNA from K562 cells solutions were used. To adjust for the DNA amount used in the amplification, the IP DNA and the total DNA values for each gene were normalized to the constitutively expressed β -actin promoter. The Relative Unit for each primer pair was determined by dividing the level of target sequence in IP DNA by the level of target sequence in total DNA. As a control for the analysis of histone acetylation of non-expressed gene, we selected the promoter of human skeletal α -actin gene, which is not expressed in K562 and HeLa cells. The primers and TaqMan probes (Table 1) were designed using Beacon Designer Software (Bio-Rad).

Bisulfite treatment and methylation analysis

Methylation at the promoters of the γ - and β -globin genes was analyzed by bisulfite treatment of DNA followed by pyrosequence analysis. Genomic DNA was prepared from cells using a DNA purification kit (Gentra Systems). Sodium bisulfite conversion of 1 μ g of DNA was carried out in duplicate using a CpG genome DNA modification kit (Chemicon International). A fragment of the promoter of the γ -globin gene (−107 to 73) that includes the five CpG dinucleotides at positions −54, −51, +5, +16 and +49 was amplified from an aliquot of converted DNA with primer set 1 (Table 2). An aliquot of the first PCR amplification was subjected to a second amplification either with primer set 2 or primer set 3 (Table 2). Methylation of three CpG dinucleotides at positions −306, −265 and −125 of the β -globin gene was assessed by amplifying an aliquot of converted DNA either with primer set 4 or primer set 5 (Table 2). The PCR products were then prepared for pyrosequence analysis in a Vacuum Prep Workstation (Biotage) and the sequencing reactions were performed on a PSQ 96HS system (Biotage). For each CpG site, the mean methylation level of duplicate samples is presented as percent methylation. The mean methylation level under each experimental condition is presented as the average of the methylation level of all CpG sites for each gene. Primer set 1 was designed using Beacon Designer Software and primers set 2, 3, 4 and 5 and the sequencing primers were designed using PSQ Assay Design Software 1.0 (Table 2).

Quantitative Reverse-Transcription Real-Time PCR Analysis

Total RNA was extracted from uninduced and induced cells using TRIzol kit (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Ambion, Austin, TX) for 30 min at 37°C and 5 min at 75°C. cDNA was synthesized with oligo (dT) primers using an Omniscript RT Kit (Qiagen, Valencia, CA) in a 25 μ l reaction volume. Aliquots of cDNA were amplified using 200 nM of TaqMan probes and primers for all globin genes in a 25 μ l iQ-Supremix reaction volume. Aliquots of cDNA were amplified using 200 nM primers for the β -actin gene in a 25 μ l iQ SYBERGreen-Supremix (Bio-Rad) reaction volume. Data were collected relative to a standard curve at the threshold where amplification was linear. The levels of expression of the studied genes were expressed to the level of expression of 18S rRNA (Applied Biosystems, Foster City, CA) and the data expressed as fold change relative to uninduced cells. The primers and TaqMan probes (Table 3) were designed using Beacon Designer Software.

Western blot analysis

Immunodetection of diacetyl-histone H3 was performed on extracts of uninduced and induced K562 cells. The extracts were solubilized in sample buffer (62.5 mM TRIS-HCl, 2% SDS, 5% 2 β -mercaptoethanol, 12.5% glycerol, 0.01% bromophenol blue, pH: 6.8) and denatured by heating at 95°C for 5 min. Proteins were separated on 15% acrylamide gels by SDS-PAGE and transferred to PVDF membrane. The membranes were blocked (5% milk, 150 mM NaCl, 20 mM TRIS-HCl, 0.1% Tween 20, pH: 7.4) at 4°C overnight and divided into two parts. The upper part was probed with mouse monoclonal anti-actin IgM and labeled with peroxidase-conjugated goat anti-mouse IgM (Oncogene Science, Cambridge, MA). The lower part was probed with rabbit polyclonal anti-diacetyl-histone H3 (Upstate Biotechnology) and labeled with peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL). Detection was performed by enhanced chemiluminescence (Pierce). The quantification was done by densitometric analysis and normalized to actin expression and the data are expressed as fold change relative to uninduced cells.

Data analysis

Student's t-test was used to determine statistical significance. $P \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

We used a quantitative real-time PCR-based ChIP assay to determine the pattern of acetylation of histone H3 and H4 at the promoters of the α - and β -like globin genes in K562 cells. The regions of the α - and β -globin clusters that were subjected to PCR amplification are shown in figure 1A. Analysis of histone acetylation at the promoters of the globin gene clusters in uninduced K562 cells showed high levels of acH3 and acH4 at the promoters of the embryonic (ζ - and ϵ -) fetal (γ -) and adult (α - and β -) globin genes (Figure 1B&1C). In marked contrast, the same globin loci that are transcriptionally inactive in non-erythroid cells were hypoacetylated in HeLa cells (Figure 1B&1C). This suggests that histone acetylation may play an important role in the regulation of tissue-specific expression of the genes of the α - and β -globin clusters. Furthermore, the levels of acetylation of histone H3 at the β -globin cluster correlate with the level of expression of the different genes of that cluster in the embryonic-fetal environment of K562 cells (Figure 1B). In other words, the high level of histone H3 acetylation at the promoters of the embryonic ϵ - and fetal γ -globin genes is consistent with the high-level expression of these genes in K562 cells. Similarly, the low level of acetylation at the promoter of the β -globin gene is consistent with its low but detectable (by RT-PCR) level of mRNA expression. In contrast, acH4 levels were high at the promoters of all the β -like genes, regardless of their relative levels of expression (Figure 1C). Previous studies by other investigators had shown that in the adult erythroid environment of mouse erythroleukemia cells, the levels of acH3 but not acH4 correlate with the levels of expression of the different genes of the β -globin cluster [8]. Interestingly, in the α -globin cluster, the relative levels of diacetyl-histone H3 and tetra-acetyl-histone H4 at the promoters of the α -like globin genes correlate with their respective levels of expression (i.e. ζ -globin > α -globin) in K562 cells (Figure 1B&1C). This is consistent with previous observations by Anguita et al. [9] that showed a correlation between the level of mono-acetylated histone H4 and the level of expression of α -like globin genes during normal development. Finally, in both K562 and HeLa cells, the transcriptionally silent α -actin gene showed essentially no H3 and H4 acetylation at its promoter. Thus, it appears that acetylation of H3 might play an important role in the regulation of developmental-stage specific expression of the genes of the β -globin cluster while the acetylation of both histones H3 and H4 might be important for the developmental stage-specific expression of the genes of the α -globin cluster. The reasons for these differences in the roles of histone H3 and H4 acetylation in the regulation of the α - and the β -globin clusters are not clear.

To further investigate the relationship between expression and histone acetylation of the globin genes in K562 cells, we examined the effect of butyrate on histone acetylation in the α - and β -globin clusters. Butyrate, a well-known inhibitor of HDACs, is being used in clinical trials for the induction of HbF in patients with sickle cell disease [6] and β -thalassemia [7]. As expected, exposure of K562 cells to butyrate increased the expression of the genes of the α - and β -globin clusters by 4 to 8 fold (Figure 2A). As a result of its enzymatic activity, butyrate increased the global level of acH3 in K562 cells by more than 3 fold (Figure 2B). A three fold increase in the levels of acH4 were also seen in butyrate-treated cells (data not shown), in agreement with previously published observations [10]. We expected the induction of globin gene expression with butyrate to be associated with increased histone acetylation at the respective promoters. Surprisingly, the levels of acH3 and acH4 at the promoters of the β -like genes did not change significantly in response to butyrate (Figure 2C&2D). We also measured DNA methylation at the promoters of the γ - and β -globin genes using DNA bisulfite treatment followed by pyrosequence analysis. In K562 cells, the methylation levels at the expressed γ -globin promoters were very low, ranging from 0% to 4% (mean 2 ± 1.4). In contrast, the methylation levels at the poised β -globin promoter ranged from 59% to 88% (mean 75 ± 2) (Figure 2E&2F). We did not observe significant changes in the levels of DNA methylation at the promoters of the γ - and β -globin genes following butyrate induction of K562 cells (Figure

2E&2F). In contrast, butyrate increased the levels of acH3 and/or acH4 at the promoters of the ζ - and α -globin genes (Figure 2C&2D). This increased histone acetylation results in a more open chromatin configuration and makes the DNA more accessible to transcription factors. Thus, the mechanisms of butyrate-induced activation of α - and γ -globin expression in K562 cells appear to be different.

We also investigated the effects of hemin, another well known inducer of globin gene expression in K562 cells, on histone acetylation in the α - and β -globin. Hemin is the Fe^{3+} oxidation product of heme, which is essential for all hemoprotein metabolisms. The mechanisms of induction of erythroid differentiation and globin gene expression by hemin are not known. Hemin exposure resulted in the expected increased expression of the ϵ -, γ -, ζ - and α -globin genes between 2 to 3 fold (Figure 3A). As expected, the level of global acH3 did not change significantly following hemin induction (Figure 3B) since hemin does not have a direct effect on histone acetylation. Moreover, hemin induction of K562 cells did not result in increased levels of acH3 and acH4 at the promoters of the ζ -, α - and γ -globin genes (Figure 3C&3D). At the β -globin promoter, hemin induced a relatively small increase in acH4 levels (by 50 percent), with no change in the level of acH3. Moreover, the levels of DNA methylation at the promoters of γ - and β -globin genes were not affected by hemin exposure (Figure 3E&3F). Interestingly, acetylation of histones H3 and H4 increased by more than 200 percent at the ϵ -globin promoter in response to hemin exposure (Figure 3C&3D). Similar observations were recently described in a report by Kim and Dean [11] that showed an increase in histone acetylation at the ϵ -globin promoter following hemin induction of K562 cells. Interestingly, the fold increase in globin gene expression of the ϵ -globin gene was greater than that of the γ -globin genes in hemin-induced K562 cells (Figure 3A). Thus, we speculate that the induction of ϵ -globin gene expression by hemin may be a result of a combination of two different effects. The first may be an effect of increased acetylation of histones at the promoter of the ϵ -globin gene, which makes the promoter more accessible to the transcription machinery. In addition, hemin might have a second non-epigenetic effect that results in increased expression of the ϵ - and γ -globin genes. Interestingly, Ikuta et al. [12] had previously shown that hemin induction of K562 cells results in a change in the pattern of *in vivo* protein-DNA interaction at HS2. They proposed that these changes may be a reflection of changes in the binding of NF-E2, which would lead to the transcriptional activation of all the genes of the β -globin cluster.

In contrast to butyrate and hemin which increase globin gene expression in K562 cells, TPA is known to inhibit globin expression and induce the expression of megakaryocytic genes [13]. Thus, we asked whether TPA induction would make the chromatin of the globin clusters less accessible, as is the case in non-erythroid HeLa cells. Down-regulation of globin gene expression in response to TPA-induction of K562 cells was previously shown to be a result of both transcriptional repression and mRNA destabilization [14]. However, the molecular mechanisms responsible for these activities are still not clear. Our studies showed that exposure to TPA results in the expected decrease in the level of expression of all α - and β -like globin genes (Figure 4A). Thus, the levels of DNA methylation at the γ - and β -globin promoters do not appear to be important for their down-regulation in K562 cells since the basal levels of methylation did not change in response to TPA exposure (Figure 4E&4F). Interestingly, TPA induction resulted in an increase in the global level of acH3 in K562 cells by more than 3 fold (Figure 4B). Furthermore, TPA exposure resulted in a decrease in the levels of acetylation of either histone H3, histone H4 or both at the promoters of the ϵ -, β -, ζ - and α -globin genes, suggesting a chromatin configuration that is less open than that in the control cells. These changes in acetylation may be important for the transcriptional down-regulation of these globin genes in TPA-induced K562 cells. Surprisingly, the level of acH3 did not decrease at the γ -globin promoters in response to TPA-induction of K562 cells, while the level of acH4 paradoxically increased (Figure 4C&4D). The significance of this unexpected observation is unclear. Interestingly, exposure of K562 cells to TPA resulted in an increase in global acH3

and acH4 levels. We believe that the increase in acH4 levels at the promoters of the γ -globin genes is unlikely to be a reflection of a generalized increase in acH4 since the levels of acH4 either decreased or did not change at the promoters of all the other globin genes that we studied.

Our studies summarized above show that the levels of acetylation of histones H3 and H4 correlate with the levels of α -globin gene expression. This suggests an important role for histone acetylation in the regulation of the α -globin expression in K562 cells. In contrast, these epigenetic modifications do not appear to play a role in the regulation of γ -globin expression in K562 cells. A number of previous studies have demonstrated that the genes of the α - and β -globin gene clusters may be regulated by different mechanisms [15–17]. The general context and the chromosomal location of the genes of the two globin clusters are very different. The α -globin cluster exists in a gene-rich region near the telomeric end of chromosome 16. This chromosomal domain is also very GC-rich and constitutively unmethylated. Moreover, the α -globin cluster has a relatively open chromatin configuration and replicates early in most cell types [9,18–20]. In contrast, the β -globin cluster exists in a non-telomeric region of chromosome 11. The genes of this cluster are present in an AT-rich domain that replicates early in erythroid cells and late in non-erythroid cells. Moreover, the chromatin structure of the β -globin cluster is open and accessible in erythroid cells and closed in non-erythroid cells [8, 11,18,21,22]. Our studies described above have identified further differences in developmentally determined and pharmacologically induced histone acetylation in the genes of the α - and β -globin clusters in K562 cells.

Although butyrate induction of γ -globin gene expression in the fetal environment of K562 cells is not associated with changes in histone acetylation or DNA methylation, this observation should not be extrapolated to the butyrate-mediated induction of fetal hemoglobin in patients with hemoglobin disorders. We have recently shown that butyrate induction of γ -globin expression in the adult environment of BFU-e derived erythroid cells of patients with sickle cell disease is associated with increased histone acetylation and decreased DNA methylation at the γ -globin promoters [23,24]. Thus, epigenetic modifications appear to play different roles in the induction of γ -globin gene expression in these two different experimental systems. Induction of K562 cells by butyrate results in the activation of expression of the γ - and β -globin genes and not a switch from γ - to β -globin expression. We speculate that the butyrate-mediated induction of γ -globin gene expression in K562 cells may be a result of the effect of butyrate on erythroid differentiation rather than true hemoglobin switching, as is the case in patients treated with butyrate *in vivo*. This hypothesis will have to be examined in detail in future studies.

In conclusion, the studies described above show that both the α - and β -globin clusters are in an open configuration in erythroid K562 cells. However, the epigenetic mechanisms of activation and silencing of the α - and γ -globin genes are clearly different in K562 cells. We should emphasize that our studies were focused on the potential role of acetylation of histone H3 and H4 in the regulation of globin gene expression in K562 cells. We did not investigate all possible epigenetic modifications, nor did we measure the levels of the different erythroid-specific transcription factors that are believed to be important in the regulation of globin gene expression. Earlier studies suggested the involvement of the erythroid transcription factors GATA-1 and NF-E2 in the induction of erythroid differentiation of K562 cells by butyrate and hemin [25–28]. Thus, it is possible that butyrate exposure might result in changes in the expression of one or more of these transcription factors, resulting in the indirect transcriptional activation of γ -globin gene expression. More recently, butyrate was also shown to increase γ -globin chain synthesis in patients with SCD by increasing the efficiency of translation of γ -globin mRNA [29]. Our studies do not allow us to detect such a mechanism of γ -globin activation. Further studies are clearly needed to fully elucidate the molecular mechanisms of induction of HbF production in K562 cells and in patients with hemoglobin disorders.

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Figure 1. Patterns of histone acetylation in uninduced K562 and HeLa cell lines

(A) Schematic map of the α - and β -globin clusters. The globin genes are represented by solid boxes. The regions analyzed for histone acetylation are indicated by a solid line. (B, C) Levels of diacetyl-histone H3 (acH3) and tetra-acetyl-histone H4 (acH4) at the promoters of different globin and α -actin genes were assessed by quantitative real-time PCR ChIP analysis of the. The data are expressed as relative unit. This experiment is representative of three independent experiments.

Figure 2. Effects of butyrate-induction

K562 cells were induced with 1mM butyrate for four days. (A) Levels of different globin mRNA measured by quantitative real-time RT-PCR and expressed as fold change to the control. (B) Western blot analysis of global diacetyl-histone H3 (acH3) levels in cell lysates and expressed as fold change to the control. (C, D) Levels of acH3 and tetra-acetyl-histone H4 (acH4) at the promoters of different globin and α -actin genes were assessed by quantitative real-time PCR-based ChIP analysis. The data are expressed as relative unit. (E, F) Levels of DNA methylation at 5 CpG dinucleotides at γ -globin promoters and 3 CpG dinucleotides at β -globin promoter were determined by pyrosequencing. The data are expressed as % methylation. The means of 4 experiments are shown, with the standard errors of the means.

Figure 3. Effects of hemin-induction

K562 cells were induced with 30 μ M hemin for three days. (A) Levels of different globin mRNA measured by quantitative real-time RT-PCR and expressed as fold change to the control. (B) Western blot analysis of global diacetyl-histone H3 (acH3) levels in cell lysates and expressed as fold change to the control. (C, D) Levels of acH3 and tetra-acetyl-histone H4 (acH4) at the promoters of different globin and α -actin genes were assessed by quantitative real-time PCR-based ChIP analysis. The data are expressed as relative unit. (E, F) Levels of DNA methylation at 5 CpG dinucleotides at γ -globin promoters and 3 CpG dinucleotides at β -globin promoter were determined by pyrosequencing. The data are expressed as % methylation. The means of 3 experiments are shown, with the standard errors of the means.

Figure 4. Effects of TPA-induction

K562 cells were induced with 20 nM TPA for three days. (A) Levels of different globin mRNA measured by quantitative real-time RT-PCR and expressed as fold change to the control. (B) Western blot analysis of global diacetyl-histone H3 (acH3) levels in cell lysates and expressed as fold change to the control. (C, D) Levels of acH3 and tetra-acetyl-histone H4 (acH4) at the promoters of different globin and α -actin genes were assessed by quantitative real-time PCR-based ChIP analysis. The data are expressed as relative unit. (E, F) Levels of DNA methylation at 5 CpG dinucleotides at γ -globin promoters and 3 CpG dinucleotides at β -globin promoter were determined by pyrosequencing. The data are expressed as % methylation. The means of 4 experiments are shown, with the standard errors of the means.

Table 1

Sequences of primers and TaqMan probes used in quantitative real-time PCR for ChIP assay.

Gene	PCR primers	Sequence 5' → 3'
ζ-globin	Forward	CCTCACCTGACCAATGGCCAC
	Reverse	CAGCTGCACTGGAGTTGGGC
α-globin	Forward	CCGATCCCGCTGGAGTCGAT
	Reverse	AGCGCCACCCTTTCCTTTCG
ε-globin	Probe	TexRd-XN/TCCAGCGCGTGCCAGGCCGG/BHQ2
	Forward	AGAGAGGCAGCAGCACATATCTG
γ-globin	Reverse	CATCTTGCTCCACAGGCTAGTGA
	Probe	FAM/AGCTGCAATCACTAGCAAGCTCTCAGGCC/BHQ1
β-globin	Forward	GGCTGGCTAGGGATGAAGAATAAA
	Reverse	TGGCGTCTGGACTAGGAGCTTA
α-actin	Probe	TexRd-XN/CCTTCAGCAGTTCCACACACTCGCTTCTGG/BHQ2
	Forward	AGGGAGGGCTGAGGGTTTGA
β-actin	Reverse	CAGGGTGAGGTCTAAGTGATGACA
	Probe	FAM/TCCAACCTCCTAAGCCAGTGCCAGAAGAGCC/BHQ1
α-actin	Forward	GTCTCCCTGTCCTTGCAGAAACTA
	Reverse	CCCACGATGGACGGGAACAC
β-actin	Probe	FAM/ACAATGTGCGACGAAGACGAGACCACCG/BHQ1
	Forward	CCCTGGCGGCCTAAGGACTC
β-actin	Reverse	CACATGCCGGAGCCGTTGTC
	Probe	HEX/CGACGAGCGCGCGATATCATCATCCATG/BHQ2

Table 2

PCR and sequencing primers used in the pyrosequence analysis for the DNA methylation assay.

PCR primers	Sequencing primers	CpG	
γ-globin Set 1			
Forward	TTGATAAGGTAAATTTGATTAATAGTTTTA		
Reverse	CCTCCTCTATAAAATAACCCATAAC		
Set 2			
Forward	GAGTATTTAGTGAGGTTAGGGG	ATTTAGTGAGGTTAGGGG	−54 and −51
Reverse	Biotine-ATACTTCCTTTTATTCTTCATCCC		
Set 3			
Forward	Biotine-TTGGTTAGGGATGAAGAATAAAAG	AAAACCTTATTAATAACCTCA	+5 and +16
Reverse	CCTCCTCTATAAAATAACCCATAA	TCCTCTATAAAATAACCCAT	+49
β-globin Set 4			
Forward	GTGTAATAAGAAAATTGGGAAAA	TGTAATAAGAAAATTGGGAA	−306
Reverse	Biotin-ATATCTCTTAACCCCATACCATCA	AGTTGTGATTTTAAATATTA	−265
Set 5			
Forward	GGGTTGAGGTTTGAAGTTTAATT	AGAAGAGTTAAGGATAGGTA	−125
Reverse	Biotin-CCAACCCTAAATATAACTCCACA		

Table 3

Sequences of primers and TaqMan probes used in quantitative reverse transcriptase real-time PCR.

Gene	PCR primers	Sequence 5' → 3'
ζ-globin	Forward	GGCCAAGATCTCCACGCAGG
	Reverse	CAGGTCGAAGTGCGGGAAGT
	Probe	FAM/CGGCACCGAGACTCTGGAGAGGCTCTTC/BHQ1
α-globin	Forward	GGTGGACCCGGTCAACTTCAA
	Reverse	GGTGCTCACAGAAGCCAGGAA
	Probe	TexRd-XN/CTCCTAAGCCACTGCCTGCTGGTGACC/BHQ2
ε-globin	Forward	GCTGACTTCCTTTGGAGATGCTATTAA
	Reverse	GAGTAGCCAGAATAATCACCATCACG
	Probe	FAM/ACATGGACAACCTCAAGCCCGCCTTGC/BHQ1
γ-globin	Forward	AATGTGGAAGATGCTGGAGGAGAA
	Reverse	CTTCTTGCCATGTGCCTTGACTT
	Probe	TexRd-XN/CCCTGGGAAGGCTCCTGGTTGTCTACCC/BHQ2
β-globin	Forward	CCTGAGGAGAAGTCTGCCGTTAC
	Reverse	TAGACCACCAGCAGCCTGCC
	Probe	TexRd-XN/ACCACCAACTTCATCCACGTTACCTTGCC/BHQ2
β-actin	Forward	AAGAGCTACGAGCTACCTGAC
	Reverse	ATGCCACAGGACTCCATGCC