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Association of Heme Oxygenase-1 (Number of GT Repeats) with The Methemoglobin Levels in Recessive Congenital Methemoglobinemia in Indian Population

Das S, Chiddarwar A, Warang P and Kedar P*

Department of Hematogenetics, National Institute of Immunohaematology, Indian Council of Medical Research, K.E.M Hospital Campus, Parel, Mumbai-400012, India

Abstract

Heme oxygenase-1 (HO-1) is important in the defense against oxidative stress. Length polymorphisms in this GT repeat region correlate with levels of *HO-1* expression and associates with several diseases. The aim of this study was to test for possible association of *HO-1* (number of GT repeats) with methemoglobin levels in recessive congenital methemoglobinemia in Indian population. Genotyping of DNA isolated from whole blood of 25 RCM patients due to NADH cytochrome b5 reductase deficiency and 50 healthy controls was performed. Fragment size analysis by sequencing was used for genotype/allele definition by ABI 3130 genetic analyzer and size of PCR product was determined by Gene Mapper software. Significance of findings was tested using $\chi(2)$ test. The *HO-1* (number of GT repeats) polymorphisms was significantly associated with RCM. Results of genotyping analysis indicated that a genotype carrying short alleles (<24 (GT)n repeats was preferentially associated with RCM patients than in control ($P < 0.001$). The short allele (<24 GT repeats) genotype were present in 82% of the methemoglobinemia patients group and long allele ($\geq$31 GT repeats) observed in the normal control group. This study is supporting the association of *HO-1* (number of GT repeats) polymorphism and RCM. . Allele and genotype frequencies for *HO-1* polymorphisms show significant association with disease severity. This data could also be valuable to the clinicians, mainly hematologists, when attempting a definitive diagnosis for the cause of methemoglobinemia that will help clinical decisions for treatment.

Introduction

Heme oxygenase-1 (HO-1) is the rate limiting enzyme that catabolizes free heme into carbon monoxide (CO), ferrous iron, and biliverdin/bilirubin. Heme oxygenase activity is induced by its substrate heme and by various non-heme substances. To date, two functional isoforms (HO-1, HO-2) have been described [1]. *HO-1* and HO-2 belong to the heme oxygenase family. While *HO-1* is constitutively produced by most of the cells, *HO-1* protein is induced by its substrate heme and a broad array of acute stress stimuli, many of which are associated with critical illnesses [2,3]. In humans, polymorphisms in the *HO-1* gene promoter may influence the magnitude of *HO-1* expression. *HO-1* is up regulated by numerous conditions, including oxidative stress, and in these conditions, it is considered to play a protective role [4]. We have previously demonstrated that the role of UGT1A1 and *HO-1* gene promoter polymorphisms increases the risk of hyperbilirubinemia and gallstones in patients with hereditary spherocytosis [5]. In many diseases including murine malaria, *HO-1* induction produces protective anti-inflammatory effects, but observations from patients suggest these may be limited to a narrow range of *HO-1* induction, prompting us to investigate the role of *HO-1* in methemoglobinemia patients [6]. The (GT)n repeat is the most frequent of the simple repeats scattered throughout the human genome, and many of these repeats exhibit length polymorphisms. This purine-pyrimidine–alternating sequence, possessing Z-conformation potential, negatively affects transcriptional activity in the rat prolactin gene [7]. A (GT)n repeat in the 5′-flanking region of the human *HO-1* gene is highly polymorphic and may modulate gene transcription under thermal stress [8].

Methemoglobin can be increased due to two main causes. Some cases of methemoglobinemia are genetic, meaning that NADH-cytochrome b5 reductase deficiency leads to an increased proportion of methemoglobin or hemoglobin variant (Hb-M) [9,10]. Most cases

of methemoglobinemia are, however, acquired rather than inborn. Exposure to certain oxidizing substances may lead to the conversion of hemoglobin to methemoglobin. Severity of symptoms depends upon the percentage of methemoglobin, and symptoms are related to the lack of oxygen delivery to tissues. There may be serious symptoms such as cyanosis, bluish discoloration of the skin, shortness of breath, lethargy, headache, dizziness and deterioration of mental functioning [9,10]. It is already reported in the literature that methemoglobinemia resulting from smoke inhalation also. It has shown that in smokers there is a correlation between the length of the (GT)n repeat and susceptibility to the development of chronic pulmonary emphysema, an oxidative stress–inducing disease. Furthermore, these authors have shown that H_2O_2 exposure, up regulates the transcriptional activity of the *HO-1* promoter/luciferase fusion genes in A549 cells or Hep3B cells transfected with a short (GT)n repeat but not with a long (GT)n repeat. They proposed that the large (GT)n repeat in the *HO-1* gene promoter may reduce the induction of *HO-1* by reactive oxygen species present in cigarette smoke, thereby resulting in the development of chronic pulmonary emphysema [11]. Therefore, we thought that the microsatellite polymorphism of this gene might be associated with the development of cyanotic diseases that are induced by oxidative stress.

*Corresponding author: Dr. Prabhakar S Kedar, Department of Hematogenetics, National Institute of Immunohaematology, Indian Council of Medical Research, 13th Floor, New Multistoried Building, K.E.M Hospital Campus, Parel, Mumbai 400012, India, Tel: 9122 24138518; Fax: 9122 24138521; E-mail: kedarps2002@yahoo.com

In the present study, we have focused on methemoglobinemia cases because there is a strong link between the incidence of hypoxia and cyanosis in patients with the pathogenesis of methemoglobinemia. We screened allelic frequencies of the $(GT)_n$ repeats in the *HO-1* gene promoter in RCM patients and normal control group and examined the association between severity of methemoglobinemia and the length of the $(GT)_n$ repeats.

Methods

Clinical protocol and characteristic of patients

At the National institute of Immunohematology in Mumbai, we have collected blood samples from 25 patients who were diagnosed as cases of recessive congenital methemoglobinemia due to NADH cytochrome b5 reductase deficiency. Written informed consent was obtained from each subject after a full explanation of the study to analyze the *HO-1* gene promoter potentially related to cyanotic disease. The present study was approved by the ethical committee of the National institute of Immunohematology in Mumbai. Clinical, hematological and molecular characterizations of all 25 patients were published elsewhere [12-14].

Analysis of length variability of $(GT)_n$ repeats in *HO-1* gene promoter

Venous blood samples were collected in tubes containing Na_2EDTA. Genomic DNA was extracted with the use of a QIAamp blood kit (Qiagen) according to the manufacturer's protocol. The polymerase chain reaction products were analyzed by ABI 3130 genetic analyzer (ABI PRISM310 Genetic Analyzer, Perkin-Elmer). The *HO-1* gene 5′-flanking region containing a poly $(GT)_n$ repeat was amplified by PCR

with a sense primer (5′-AGAGCCTGCAGCTTCTCAGA-3′) and an antisense primer (5′-ACAAAGTCTGGCCATAGGAC-3′), which were designed on the basis of the published sequence [15]. The PCR cycle of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds was carried out for a total of 30 cycles. The PCR products were subsequently run on a denaturing polyacrylamide gel (6%, acrylamide:bis-acrylamide 19:1) at 2000 V for 2 hours, followed by autoradiography. Blood samples typically have 2 different sizes of $(GT)_n$ repeats from the 2 alleles. The sizes of the PCR products were analyzed with a laser-based automated DNA sequencer and the exact size of the polymerase chain reaction products was determined by Gene Mapper software. Each repeat number was calculated with 2 alleles as size markers.

Statistical analysis

All analyses were conducted using the statistical software package SPSS16.0 (SPSS Inc). Distribution of continuous variables in groups were expressed as means ± SD. Normal distribution of data was analyzed using the Kolmogorov–Smirnov normality test. Data with a normal distribution were compared by student t test or ANOVA and Comparisons of allele frequencies between patients and control groups were determined using a Pearson χ^2 test using SPSS for windows version 16.0 software. All tests were two-tailed and $p < 0.05$ was considered as significant value. In addition, overall subjects were divided into three groups based methemoglobin percentage in the patients group.

Results and Discussion

The age of the patients at the time of investigation varied from 17 days to 52 years. The general characteristics of the study subjects are presented in Table 1. The clinical presentations such as cyanosis, bluish decolorization of nail beds, lips and tongue were significantly noted in

Sl. No	Case ID	AGE/ SEX	Meth-Hb Level (%)	NADH Cyb5r Activity (IU/g Hb)	(GT)n repeat	NADH-CYB5R3 Genotypes
			Methemoglobinemia Type-I			
1	BS	13 Y/M	23	06.21	24, 24	p.Gly154Glu (Homo)
2	SM	20 Y/M	9	14.15	24, 24	p.Ile177Thr (Homo)
3	BD	52 Y/F	48	15.49	24, 24	p.Ala178Thr/Arg49Trp
4	VJ	22 Y/F	38	13.50	24, 24	p.Ala178Thr
5	UV	18 M/F	35	15.33	24, 24	p.Arg57Gln/Gly75 Ser
6	SH	18 Y/M	37	14.15	27, 32	p.Arg49Trp (Homo)
7	AB	14 Y/M	22	15.81	24, 24	p.Ala178Thr (Homo)
8	BK	33 Y/M	9	20.90	31, 31	p.Ala178Thr(Homo)
9	DB	14 Y/F	23	16.15	24, 31	p.Pro144Ser (Homo)
10	VB	45 Y/M	18	23.15	24, 31	p.Val252Met (Homo)
11	SK2	29 D/M	33	15.11	24, 24	p.Ala178Thr/ Arg49Trp
12	DR	49 Y/M	17	16.10	24, 30	p.Arg49Trp (Homo)
13	MM	38 Y/F	7	22.40	24, 31	p.Met176 Ile (Homo)
14	NM	6 Y/M	19	14.35	24, 31	p.Met176 Val (Homo)
15	AT	10 Y/M	20	20.50	29, 25	p.Ala178Thr (Homo)
16	PM	21 Y/M	38	07.30	24, 24	p.Met176Val (Homo)
17	SK3	42 Y/M	46	13.12	24, 24	p.Arg49Trp (Homo)
18	BD	24 Y/M	40	17.21	24, 24	p.Arg49Trp (Homo)
19	MP	35 Y/M	38	15.45	20, 24	p.Arg159Pro (Homo)
			Methemoglobinemia Type-II			
20	SS	5 Y/F	30	6.00	24, 26	p.Arg49Trp (Homo)
21	VS	8 Y/M	25	9.60	26, 28	p.Gly75 Ser(Homo)
22	BP	25 D/M	32	16.50	24, 24	p.Gly75 Ser (Homo)
23	SK	3 Y/F	42	11.07	24, 24	p.Trp235Ter (Homo)
24	AN	4 Y/F	15	12.15	24, 24	p.Trp236Ter (Homo)
25	SV	11 M/M	35	15.05	24, 26	p.Cys204Arg (Homo)

Note: (Normal range: Methemoglobin <2.0% and NADH Cyb5r Activity 35±5IU/g Hb)

Table 1: Showing meth-Hb level, NADH Cytochrome b5 reductase activity, NADH-CYB5R3 genotypes and length of (GT)(n) repeats in the HO-1 gene in 25 RCM patients.

the patients group also, total methemoglobin levels were significantly higher in cases than in controls, which is due to NADH CYB5R deficiency in the patients. Methaemoglobin levels were increased, varying from 11% to 42%. (Normal range: <1.5%) and haemoglobin levels varied from 6.9 g/dl to 19.5 g/dl. On the basis of methemoglobin level, we have classified patients group into three type such as mild (Meth-Hb: ≤ 10%, medium: 11-20% and severe: ≥ 20%). In addition, there was a significant difference in Meth-Hb concentrations in each group ($P < 0.001$). There was a history of consanguinity among the parents of 15 cases. The methemoglobin levels were within normal limits in the parents wherever available. The clinical presentation of five patients of type II RCM had severe methemoglobinemia (Meth-Hb: ≥ 20%) with mental retardation, microcephaly, delayed development, convergent squint, and spasticity of the limbs and dystonic posture of the toes. The NADH-CYB5R activity in all the patients was reduced by 35-65% as compared to normal (Normal range: 35.0 ± 5.0 IU/g Hb).

Allele and genotypic frequencies of *HO-1* microsatellite polymorphism

The allele frequencies of $(GT)_n$ microsatellite polymorphism in the *HO-1* promoter region were highly polymorphic, ranging from 15 to 37 with $(GT)_{24}$ and $(GT)_{31}$ being the two most common alleles in our study population. Mann–Whitney rank sum test showed that the repeat numbers in case and control groups are significantly different ($P = 0.001$), (Figures 1A-1C). The distribution of the (GT)n repeat numbers was bimodal, with one peak at 24 GT repeats and the other at 31 GT repeats. Therefore, we divided the alleles into 2 subclasses according to the number of (GT)n repeats: class SS included alleles with ≤24 GT repeats; class LL included alleles with ≥ 31 GT repeats. The patients were then classified as having an S/S, S/L, or L/L genotype according to each of their *HO-1* alleles. The distribution of genotypes is shown in Table 1. Because the proportion of allele frequencies of either 31 or >31 *GT* repeats was about 95% in control group, whereas allele frequencies of either ≤ 24 or >24 *GT* repeats genotype was present in 82% of the methemoglobinemia patients ($P = 0.001$). Initially, we examined the association between the total methemoglobinemia patients and length of the (GT)n repeats in all 25 patients. Mild disease was found in 1 (7%) of 25 patients with genotype 31.3 ± 3.83(L/L) (P<0.032), medium disease in 4 (13%) of 25 patients with genotype 26.83 ± 3.51(S/L) (P<0.001), and severe disease were found in 20 (80%) of 25 patients with genotype 24.84 ± 2.24 (S/S) (P<0.001) where as healthy 50 control samples has genotype 31.04 ± 2.95 (L/L). We observed a significant correlation between methemoglobinemia and *HO-1* polymorphism. It was found that the individuals who were either homozygotes or compound heterozygotes for the short $(GT)_n$ repeats alleles had a significant higher methemoglobin levels as shown in Figure 1C.

Functional effect of the *HO-1* gene polymorphism

Analysis on the $(GT)_n$ repeats in *HO-1* gene promoter of the 25 cases revealed that there were 3 cases had mild cyanosis (Meth-Hb:0.0-10%), 5 cases had medium cyanosis (Meth-hb:11-20%) and severe cyanosis (Meth-Hb : ≥ 20%). Regarding the *HO-1* $(GT)_n$ polymorphism, The number of repeats ranged from 15 to 37, with the highest frequency for $GT_{(31)}$ repeats in healthy subjects and $GT_{(24)}$ repeats in the patients group. Mann–Whitney comparison for means in each group revealed statistically significant differences (P<0.001). Multivariate regression models determined that the genotypes of (GT)n repeats in the human *HO-1* gene promoter were significantly related to RCM patients (Table 2).

In humans, polymorphisms in the *HO-1* gene promoter may
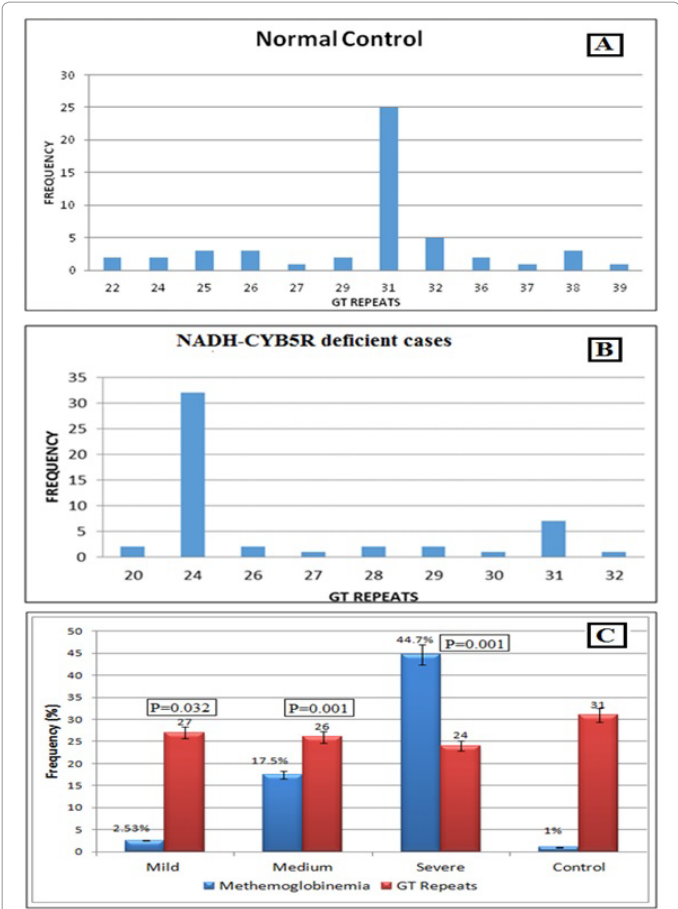
Figure 1: Frequencies of HO-1 gene promoter polymorphism in (A) normal control group and B) NADH-cyb5r deficient cases. (C) Frequency distributions of GT repeat with methemoglobin level.

Variables	Total Genotype	HO-1 Protein level (ng/ml)
Control (meth-Hb ≤1.5%)	$(31.04 \pm 2.95)_{50}$	3.50±0.24 ng/mL (2.71-4.69) (P< 0.022)*
Mild patients (≤10% meth-Hb)	$(27.3 \pm 3.83)_1$ (P< 0.032)*	--nd--
Medium patients (10-25% meth-Hb)	$(26.83 \pm 3.51)_4$ (P< 0.001)*	--nd--
Severe patients (≥ 25% meth-Hb)	$(24.84 \pm 2.24)_{20}$ (P< 0.001)*	4.44±0.27 ng/mL (3.87-5.26) (P< 0.044)*

Note: No of samples are n $(GT)_n$. Variables are mean ± SD; nd=Not done
*P < 0.001 values were calculated using independent-sample t test or Chi-square test

Table 2: Association of dinucleotide (GT)n HO-1 promoter polymorphism with antioxidant markers in RCM patients and normal controls.

influence the magnitude of *HO-1* expression. In many diseases including murine malaria, *HO-1* induction produces protective anti-inflammatory effects, but observations from patients suggest that these may be limited to a narrow range of *HO-1* induction, prompting us to investigate the role of *HO-1* in methemoglobinemia [7,11,16]. The hemeoxygenase system includes the heme catabolic pathway, comprising HO and biliverdin reductase, and the products of heme degradation, carbon monoxide (CO), iron, and biliverdin/bilirubin. A number of polymorphisms in the human *HO-1* locus, including (GT)n microsatellite polymorphism in the *HO-1* promoter that alters the levels of *HO-1* expression, have been associated with the incidence and/or progression of a variety of diseases. Based on the number (n) of GT repeats, ranging from 20 to

32, individuals with short repeats have higher *HO-1* expression, and individuals with long (GT) repeats have lower *HO-1* expression. This microsatellite (GT)n polymorphism might regulate *HO-1* expression by modulating *HO-1* transcription and/or translation. HO-1, an inducible form of HO, provides cellular protection against heme and non-heme mediated oxidant injury. Likewise, an exogenous administration of *HO-1* in the rat lung by gene transfer was shown to protect against injury caused by hyperoxia [17]. These lines of evidence support that *HO-1* is an essential component for the lung to keep a delicate balance between oxidants and antioxidants. A $(GT)_n$ repeat is the most frequent of the simple repeats scattered throughout the human genome, and many of these exhibit length polymorphism. This purine-pyrimidine alternating sequence, possessing Z-conformation potential, negatively affects transcriptional activity in the rat prolactin gene [17]. $(GT)_n$ repeat in the 5′-flanking region of the human *HO-1* gene is indeed highly polymorphic and may modulate gene transcription under thermal stress [7,8]. Therefore, it could be hypothesized that, if the expression of the *HO-1* gene alters according to the number of $(GT)_n$ repeats, the microsatellite polymorphism may be associated with the development of the oxidative stress–inducing diseases.

Oxidative stress in the RBCs causes the oxidation of the ferrous iron state (Fe^{2+}) to the ferric state (Fe^{3+}). When the iron in hemoglobin is oxidized to the ferric state, this is known as methemoglobin. Methemoglobin reduction is dependent on the oxido-reductase activity of cytochrome b5 reductase and required reduces NADH and NADPH substrate. Both the substrates reduce cytochrome b5, which in turn is able to reduce the oxidized ferric ion of haemoglobin when an electron is transfer from reduced cytochrome b5. It is also reported that heme oxygenase expression is induced by oxidative stress, and in animal models increasing this expression seems to be protective. Carbon monoxide released from heme oxygenase reactions can influence vascular tone independently or influence the function of nitric oxide synthase. Carbon monoxide released from the reaction of free heme in the bloodstream of someone with the sickle-cell trait is believed to lessen the effects of cerebral Malaria [4]. It is also reported that biliverdin is responsible for the coloration of blue eggs and is secreted onto the eggshell by the shell gland. Previous studies confirmed that a significant difference exists in biliverdin content between blue eggs and brown eggs, although the reasons are still unknown. Because the pigment is derived from oxidative degradation of heme catalyzed by heme oxygenase (HO), this study compared heme oxygenase (HO-1), the gene encoding HO expression and HO activity, in the shell glands of the Dongxiang blue-shelled chicken and the Dongxiang brown-shelled chicken [18,19].

The distribution of the numbers of $(GT)_n$ repeats in the *HO-1* gene promoter in the patients enrolled in the present study were consistent with the distribution in patients and controls in previous reports [7,8]. In the present study, we focus on severity of disease as a paradigm for elucidation of the contribution of 5′-flanking polymorphisms in the *HO-1* gene, because there is a marked association between reactive oxygen species in cyanosis and pathogenesis of the disease. To assess this hypothesis, we screened allelic frequencies of the $(GT)_n$ repeats in the *HO-1* gene promoter from methemoglobinemia patients and normal control. We also examined the association between the severity of disease and length of the $(GT)_n$ repeats and we have observe significant differences in allelic frequencies of the $(GT)_n$ repeats in the HO-1gene promoter in the methemoglobinemia patients. Our results are consistent with many other reports associated with the incidence and/or progression of a variety of diseases. Similar relationship has been found in the patients with hyperbilirubinemia [6]. This may

given that pharmacological induction of *HO-1* or administration of end products of its activity can afford protection against diseases like methemoglobinemia, this would argue that suboptimal expression of *HO-1* is one of the factors for causing methemoglobinemia.

Conclusion

In conclusion, our present study supports the role of the *HO-1* promoter (GT)n polymorphism in the prognosis of patients with methemoglobinemia and this could be the major contributing factor for cyanosis in NADH-cytochrome b5 reductase deficient patients. This data could also be valuable to the clinicians, mainly hematologists, when attempting a definitive diagnosis for the cause of methemoglobinemia due to NADH-cytochrome b5 reductase deficiency that will help clinical decisions for treatment.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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