Genetic Polymorphisms of Heme-oxygenase 1 (HO-1) may Impact on Acute Kidney Injury, Bronchopulmonary Dysplasia and Mortality in Premature Infants

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Abstract

Background—Heme Oxygenase 1 (HO1) catalyzes heme degradation, and offers protection for several organs, including the kidney. Genetic polymorphisms of HO-1 are associated with poor clinical outcomes in several populations.

Methods—Population: We prospectively enrolled 117 premature infants (birth weight ≤200 gm. or post gestational age ≤31 weeks) and evaluated 2 DNA genetic variants proximal to the promoter region of HO-1 (GT(n) repeats, and–413T>A SNP). We evaluated how these polymorphisms affect 2 clinical outcomes i) AKI - rise in serum creatinine (SCr) ≥0.3 mg/dl or ≥150–200% from lowest previous value, ii) bronchopulmonary dysplasia (BPD) defined as receipt of oxygen at 36 weeks post menstrual age (PMA) / mortality.

Results—AKI occurred in 34/117 (29%) of neonates; 12/117 (10%) died; 29/105 (28%) survivors had BPD. Neonates with TT genotype at –413T>A before the HO-1 promoter) had higher rates of AKI (p<0.05). There was no difference in GT(n) repeats and clinical outcomes.

Conclusions—We did not find an association between the GT(n) tandem repeat of HO-1 and AKI nor BPD/mortality. However, infants with TT genotype of the 413T>A genetic alteration had lower incidence of AKI. Further studies using larger cohorts are needed to better understand these relationships.

Introduction

Advancements in perinatal medicine have improved outcomes in critically ill neonates but many do not survive and more are left with long-term vital organ damage1. Acute kidney injury occurs in up to 29% of extremely low-birth weight (ELBW) infants (birth weight ≤1000g), and is associated with a very high mortality rate2 and poor long-term renal outcomes3,4. Many premature infants develop bronchopulmonary dysplasia (BPD) which
carries significant long-term consequences\textsuperscript{5,6}. Strategies are needed to reduce the short and long-term renal and pulmonary consequences in premature infants.

Heme oxygenase activity, regulated by an inducible isoform heme oxygenase-1 (HO-1) and a constitutive isoform heme oxygenase 2, catalyzes the rate-limiting step of heme degradation liberating iron, carbon monoxide (CO), and biliverdin, which is then converted to bilirubin\textsuperscript{7,8}. HO-1 regulates several important biological processes as its products exert anti-oxidant, anti-inflammatory, and anti-apoptotic effects in renal and pulmonary settings\textsuperscript{9}. In the kidney, induction of HO-1 is adaptive and protective in ischemia-reperfusion\textsuperscript{10}, rhabdomyolysis\textsuperscript{11}, and nephrotoxin-induced\textsuperscript{12,13} animal models of AKI. These encouraging results have prompted intervention trials on drugs which can induce HO-1 in effort to reduce ischemia-reperfusion kidney injury (clinicaltrials.gov NCT01430156). In a murine model of hyperoxia-induced BPD, HO-1 has been shown to preserve vascular growth and barrier function through iron-independent antioxidant and anti-inflammatory pathways\textsuperscript{14}.

Molecular genetics of the HO-1 gene provide additional support that HO-1 protects against AKI. Animals with aberrant HO-1 genetic composition develop higher rates of AKI\textsuperscript{12}. Modulated by several identified functional polymorphisms in the HO-1 gene, humans differ quantitatively in their ability to mount an HO-1 response. Specifically, a tandem (GT)n repeat region between \(-198\) and \(-258\) of the human HO-1 promoter functions as a negative regulator of the gene. In several human diseases, subjects who have longer repeats have lower HO-1 activity and more severe disease compared to those with shorter GT repeats\textsuperscript{15}. Similarly, at 413 kb pairs prior to the HO-1 promoter, a single nucleotide peptide (SNP) alteration (413 T>A) has been shown to affect the transcriptional activity of HO-1, whereby the A allele culminates in higher HO-1 transcription than the T allele.\textsuperscript{16,17} Whether longer GT(n) and genetic alterations in 413T>A affect clinical outcomes remains controversial. We postulated that allelic variation modulate HO-1 expression and more importantly clinical outcomes in premature infants.

In order to test whether genetic variations of HO-1 are associated with AKI and other clinical outcomes in premature infants, we conducted a prospective cohort study on 117 premature infants (BW \(\leq 1200\) grams, and/or gestational age < 31 weeks). We tested the hypothesis that HO-1 genetic polymorphisms [GT(n) repeats and 413 T>A SNP] are associated with i) AKI and ii) bronchopulmonary dysplasia (BPD)/death.

**Results**

AKI was documented in 34/117 (29\%) of the cohort. Baseline differences between those with and without AKI are shown in Table 1. Of the 34 infants with AKI, 29 had Stage 1, 2 had stage 2, and 3 had stage 3. Those with AKI had had lower BW, earlier GA, and lower maternal pre-eclampsia and higher rates of umbilical artery catheters (UAC), higher rates of neonatal indomethacin, and higher rates of positive blood cultures.

A total of 12/117 (10\%) infants died and 29/105 (27\%) survivors had BPD. Baseline differences between those with and without the composite BPD/mortality are shown in Table 2. Those with BPD/mortality had lower GA, lower BW, lower 1 and 5 minute
APGAR scores, and higher rates of UAC, surfactant, Indocin, AKI and positive blood cultures.

We explored differences in outcomes by the number of GT(n) repeats. We did not find differences in neither the average number of GT(n) repeats in both alleles, nor genotypes (SS vs. SL vs. LL) stratified by AKI, BPD, death or the composite BPD/death (all p > 0.05) (Table 3).

For the evaluation of the 413T>A sequence alteration, those with TT genotype were less likely to have AKI than those with AA or AT genotype (p < 0.04). No statistically significant differences were seen for an association between these genotypes and BPD, mortality or the composite of BPD/mortality (Table 4).

We did not find an association between plasma HO-1 RNA at 12 days of life and clinical outcomes or allelic alterations in HO-1 GT(n) or 413T>A. (Table 6).

Discussion

We evaluated whether sequence variations in HO-1 DNA were associated with renal and pulmonary outcomes in premature infants. We did not find any outcome differences based on the number of GT(n) repeats in the promoter region of HO-1; however, we found that those with the TT genotype for the 413T>A variant were less likely to have AKI. To our knowledge, this study represents the first assessment of the potential impact of sequence variations of HO-1 and clinical outcomes in premature infants. It adds to literature which suggests that genetic alterations in HO-1 may predispose humans to poor outcomes.

The association between genetic polymorphisms in the HO-1 promoter with diseases in several organ systems including the kidney have been described in other populations. Studies in critically ill adults shows a positive correlation in subjects with long (GT)n repeats with multiple organ failure and acute respiratory distress syndrome. Whether GT(n) repeats affects clinical outcomes is controversial as some have found no correlation of the (GT)n repeats with renal disease progression, while others have. Kanai et al. showed significant difference in the allele frequencies of each number of (GT)n repeats between Japanese and German populations, but was unable to find a relation between those polymorphisms and neonatal hyperbilirubinemia. Our current study suggests that GT(n) repeats are not associated with AKI in premature infants.

HO-1 is now recognized as a protectant against diverse insults in assorted tissues. Heme-oxygenase activity is cyto-protective against different animal models of AKI. The basis for the cyto-protection include protective properties of the byproducts of HO-1 including bilirubin, ferritin, and carbon monoxide. The role by which HO-1 asserts cyto-protection is ongoing as data is emerging that it plays a key role in mediating the protective effects of specific cytokines, stem cells and therapeutic agents in AKI. HO-1 has recently been shown to be a potential biomarker of AKI.

To our knowledge the role that the 413T>A SNP has on neonatal outcomes has not been explored. In adult critically ill subjects, Saukkonen et al. explored the haplotype of HO-1.
gene including GT(n), −413 T>A and +99 G>C allelic variations and their associations with plasma HO-1 levels and multi-organ failure in subjects with septic shock\textsuperscript{17}. Those with −413T/+99C/long GT(n) had lower plasma HO-1 levels and lower severity of illness scores. Similarly in our study, the T allele in the 413T>A SNP was associated with favorable outcome. On the other hand, Ono et al. noted that adults with the AA genotype has lower incidence of ischemic heart disease compared to those with AT or TT genotypes in a cohort of 597 patients compared to 1972 controls\textsuperscript{16}. The reasons for this discrepancy may come from different clinical scenarios by which higher HO-1 or lower HO-1 expression affects outcomes. The effect of HO-1 in context of neonatal hyperbilirubinemia has yet to be determined. Further studies with bigger populations will be needed to shed light on apparent discrepancy.\textsuperscript{25,26,27}

The strengths of our study include our serial measurements of SCr to determine whether infants had AKI, the use of contemporary neonatal AKI definitions, and the prospective cohort design. Despite these strengths, we acknowledge several limitations to the study. First, we acknowledge that the sample size was small and although we were able to show significant differences between the genotypes and AKI outcomes, we may not have been powered accurately to show differences in other outcomes. In addition, although we can speculate that HO-1 expression affected tissue response, we were unable to show differences in HO-1 expression as measured by plasma HO-1 at 2 weeks of age. In addition, due to the small sample size, we could not control for potential confounders. For example, if the effect of HO-1 affects vascular tone in neonates, AKI could be a manifestation of vascular tone and not the presumed protective cellular processes ascribed to anti-inflammatory/anti-oxidant properties of heme-oxygenase.

In conclusion, we did not find an association between the GT(n) tandem repeat of HO-1 and AKI nor BPD/mortality. However, infants with TT genotype of the 413T>A SNP had lower incidence of AKI. Further studies using larger cohorts are needed to better understand the role by which genetic alterations of SNP in HO-1 gene affects outcomes in premature infants.

**Methods**

**Population**

This prospective cohort study was conducted in the NICU located on the University of Alabama at Birmingham (UAB) campus between February 2012 and June 2013. UAB’s Institutional Review Board approved the study. We followed enrolled infants from the time of birth until 36 weeks post-menstrual age (PMA) or hospital discharge. Criteria for study inclusion were birthweight (BW) ≤ 1200 gm or gestational age (GA) <31 weeks, and parental informed consent. Infants with known congenital abnormality of the kidney or urinary tract were excluded. All available families were asked to consent to all procedures and 117/284 (41%) VLBW eligible for the study were enrolled. The reasons for non-enrolment included non-interested (n=75), not available (n=76), transfer to other hospital (n=8), and refused to allow genetic studies (N=8). Overall, there were no difference between those who agreed to be in the study and those who did not in regards to BW, GA, 5 minute Apgar scores (figure 1).
Outcome definitions (AKI, BPD, and mortality)

In order to ascertain whether a child developed AKI within the first 2 weeks of life, we measured SCr on days 1, 2, 3, 4, and 12 on most infants in addition to any clinically measured values. The mean number of SCr values obtained for each patient during the first 2 weeks of life was 5 (range 2–14). Neonatal AKI was defined according contemporary definition modified for neonates as we have previously described (Table 7)\(^{29}\). Since SCr decreases in neonates after birth dependent on GA, each SCr is compared to the lowest previous SCr value to date. We did not include urine output criteria as it is often difficult to measure urine output in babies and many premature infants with AKI are non-oliguric due to poor tubular function.

BPD was defined as per the National Institute of Health criteria for BPD if an infant was oxygen dependent at 36 weeks PMA\(^{30}\). We report survival if the infant survived until 36 weeks PMA or hospital discharge, whichever occurred first, as commonly done to explore hospital outcomes in VLBW infants. We combined BPD/Mortality as a composite primary outcome as they represent competing outcomes and are the most common method of analysis for chronic lung disease in neonates\(^{5,6,31}\).

Evaluation of genetic variants and measurements of plasma HO-1RNA

DNA was collected using Oragene saliva collection kits (Genotek, Kanata, Ontario Canada) and isolated using the Gentra Puregene kits (Qiagen, Valencia, California) as per manufacturer recommendations. The HO-1 DNA promoter region containing the GT(n) region and the 413T>A SNP (Figure 2) were amplified with the Type-It Mutation Detect PCR Kit (Qiagen). Capillary electrophoresis (Figure 3) was performed to deduce the molecular weight of the PCR product. (Homo sapiens chromosome 22 HMOX1 NCBI Ref Seq NG_023030.1) The number of GT(n) repeats were determined for each infants’ alleles and classified as short (≤27) or long (>27). We explored GT(n) repeats by averaging the number of GT repeats in the two alleles. We also explored the genotype for the allele length (2 short alleles = SS; one short and one long = SL; 2 long alleles = LL) between groups.

We determined the base at position 413 T>A for each allele using LTI TaqMan Assay (rs2071746) Context Sequence [VIC/FAM]. AGTTCCTGATGTGCCCACCAGGCT[AV/ TTTGCTTGAGCCGCTGCCCTCCC] Plasma HO-1 RNA evaluation from day of life 14 was determined by qPCR for HO-1 against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Blood (500uL) was immediately stabilized in Qiagen RNA protect tubes (500uL) (Qiagen), stored at 4 degree Celsius (C), and RNA was extracted within five days with Qiagen RNA Protect Animal Blood Kit (Qiagen)Primescript RT Master Mix (Clontech/ Takara, Mountainview California) was used for cDNA synthesis. Quantitative PCR was performed using Ex Taq for probe qPCR and TaqMan Primer Probe sets for HO-1 (HMOX1=Hs01110250_m1) (Clontech/Takara) against GAPDH Endogenous Control (VIC®/MGB probe, primer limited)=4326317E. (Life Technologies Inc., New York); GAPDH=Human GAPD (GAPDH) Initial denaturation was for 30 seconds at 94 degree C and cycling was 94 degree C for 15 seconds followed by 60 degree C for 30 seconds for 40 cycles.

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Descriptive statistics were performed to determine differences between groups. Normally distributed continuous variables were compared using student t test, and non-normally distributed variables were analyzed using Mann-Whitney U test. Cochran– Mantel-Haenszel chi square statistics was used to analyze stratified categorical data and a p value < 0.05 was considered statistically significant. SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analysis.

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Figure 1.
Enrollment and reasons for non-participation in those who met inclusion and exclusion criteria for the study. Birth weight, Apgar scores, and gestational age were not different between consented and non-consented groups.
Figure 2.
Primers for promoter of HO1 Homo sapiens heme oxygenase (decycling) 1 (HMOX1), RefSeqGene on chromosome 22 NCBI Reference Sequence: NG_023030.1 (primers are shown in italic; GT(n) repeats are shown with bold; −413 location is shown in bold).
The −413 T>A SNP was documented for each allele using aqMan Assay (rs2071746)
AGTTCCCTGATGGCCACCAGGCT[A/T][TTGCTCTGAGCAGCAGCGCTGCTCCCA]
□ = AA Alleles, △ = AT Alleles, ○ = TT Alleles; RFU=relative fluorescent units.

Figure 3.
Table 1
Demographic of infants with and without AKI by postnatal day 14

|                         | AKI (N=34) | No AKI (N=83) | p value |
|-------------------------|------------|---------------|---------|
| **Infant Characteristics** |            |               |         |
| Male (n, %)             | 14 (41%)   | 44 (53%)      | 0.24    |
| Race (n, %)             |            |               | 0.12    |
| Black                   | 19 (56%)   | 45 (54%)      |         |
| White                   | 15 (44%)   | 30 (36%)      |         |
| Hispanic                | 0 (0%)     | 8 (10%)       |         |
| Gestational age (mean±SD; weeks) | 26.5 ± 0.3 | 28.0 ± 0.2 | 0.0002  |
| Birth weight (mean±SD; g)    | 867 ± 336  | 1009 ± 302    | 0.03    |
| 1 Min Apgar (median±SE)    | 3 ± 2      | 4 ± 2         | 0.44    |
| 5 Min Apgar (median±SE)    | 6 ±1       | 6 ± 1         | 0.17    |
| Umbilical Arterial Catheter (n, %) | 19 (56%)   | 30 (36%)      | 0.05    |
| Surfactant Administration (n, %) | 21 (62%)   | 40 (48%)      | 0.18    |
| Indocin Administration (n, %) | 20 (59%)   | 28 (34%)      | 0.01    |
| Positive blood culture day 15 (n, %) | 4 (12%)    | 1 (1%)        | 0.03    |
| **Maternal Characteristics** |            |               |         |
| Prenatal care (n, %)     | 31 (92%)   | 77 (93%)      | 0.76    |
| Diabetes (n, %)          | 93 (8%)    | 19 (10%)      | 0.74    |
| High Blood pressure (n, %) | 11 (32%)   | 23 (28%)      | 0.62    |
| Antenatal Steroids (n, %) | 32 (94%)   | 81 (98%)      | 0.35    |
| Antenatal Indomethacin (n, %) | 6 (18%)    | 7 (8%)        | 0.15    |
| Smoking (n, %)           | 5 (15%)    | 12 (14%)      | 0.83    |
| Pre-eclampsia (n, %)     | 3 (9%)     | 31 (37%)      | 0.002   |
| Multiple birth (n, %)    | 13 (38%)   | 22 (26%)      | 0.21    |
| History of drug abuse (n, %) | 3 (9%)     | 5 (6%)        | 0.59    |
| Clinical Chorioamnionitis (n, %) | 16 (47%)   | 36 (43%)      | 0.72    |
Table 2
Demographic of infants with and without BPD/mortality at 36 weeks post-menstrual age.

| Infant Characteristics                      | BPD/Mortality | P value |
|--------------------------------------------|---------------|---------|
|                                            | Yes (N=41)    | No (N=76) |         |
| Male (n, %)                                | 19 (46%)      | 39 (51%) | 0.60    |
| Race (n, %)                                |               |          | 0.39    |
| Black                                      | 25 (61%)      | 39 (51%) |         |
| White                                      | 13 (32%)      | 32 (42%) |         |
| Hispanic                                   | 3 (7%)        | 5 (7%)   |         |
| Gestational age (median, IQR; weeks)       | 25 (24–27)    | 29 (27–29.5) | <.0001 |
| Birth weight (mean±SD; grams)              | 738 ± 266     | 1093 ± 272 | <.0001 |
| 1 Min Apgar (median, IQR)                  | 2 (1–4)       | 6 (3–7)  | <.0001 |
| 5 Min Apgar (median, IQR)                  | 7 (4–7)       | 8 (7–8)  | <.0001 |
| Umbilical Artery Catheterization (n, %)     | 35 (85%)      | 14 (18%) | <.0001 |
| Surfactant (n, %)                          | 39 (95%)      | 22 (29%) | <.0001 |
| Indocin (n, %)                             | 32 (78%)      | 16 (21%) | <.0001 |
| Max Scr week one (mean±SD; mg/dl)          | 1.2 ± 0.4     | 1.1 ± 0.2 | 0.02   |
| AKI by day 15 of life (n, %)               | 17/41 (41%)   | 17/76 (22%) | 0.03   |
| Positive blood culture day 15 (n, %)       | 12 (29%)      | 6 (8%)   | 0.002   |

| Maternal Characteristics                   |               |         |
|--------------------------------------------|---------------|---------|
| Prenatal care (n, %)                       | 39 (95%)      | 69 (91%) | 0.40   |
| Diabetes (n, %)                            | 4 (10%)       | 8 (10%)  | 0.89   |
| High blood pressure (n, %)                 | 9 (22%)       | 25 (33%) | 0.21   |
| Antenatal Steroids (n, %)                  | 40 (98%)      | 73 (96%) | 0.66   |
| Antenatal Indomethacin (n, %)              | 5 (12%)       | 8 (11%)  | 0.78   |
| History of Smoking (n, %)                  | 4 (10%)       | 12 (16%) | 0.36   |
| Pre-eclampsia (n, %)                       | 16 (39%)      | 18 (24%) | 0.08   |
| Multiple birth (n, %)                      | 14 (34%)      | 21 (28%) | 0.46   |
| History of drug abuse (n, %)               | 2 (5%)        | 6 (8%)   | 0.53   |
| Clinical Chorioamnionitis (n, %)           | 19 (46%)      | 33 (43%) | 0.76   |
### Table 3

Association of GT(n) repeat numbers and Outcomes

|                      | Mean GT(n)      | P   | Short vs. Long | P   |
|----------------------|----------------|-----|----------------|-----|
| AKI Status           |                |     |                |     |
| Yes (N =34)          | 26.1 +/- 0.6   | 0.8 | 22             | 9   |
| No (N =83)           | 26.2 +/- 0.3   | 0.6 | 48             | 28  |
| BPD status at 36 weeks |               |     |                |     |
| Yes (N =29)          | 26.0 +/- 0.3   | 0.9 | 17             | 10  |
| No (N =76)           | 26.0 +/- 0.6   | 0.3 | 48             | 22  |
| Mortality            |                |     |                |     |
| Yes (N =12)          | 27.8 +/- 1.1   | 0.07| 5              | 5   |
| No (N =105)          | 26.0 +/- 0.3   | 0.1 | 65             | 32  |
| BPD / Mortality      |                |     |                |     |
| Yes (N =41)          | 26.3 +/- 0.6   | 0.3 | 22             | 15  |
| No (N =76)           | 25.8 +/- 0.4   | 0.4 | 48             | 22  |
Table 4
Association between genetic variants of HO-1 (−413T>A) and Clinical Outcomes

|                   | AA    | AT    | TT    | p-value |
|-------------------|-------|-------|-------|---------|
| **AKI Status**    |       |       |       | 0.04    |
| Yes (N = 34)      | 10 (29%) | 20 (59%) | 4 (12%) |         |
| No (N = 83)       | 21 (25%) | 34 (41%) | 28 (34%) |         |
| **BPD status**    |       |       |       | 0.1     |
| Yes (N = 29)      | 7 (24%) | 26 (55%) | 6 (21%) |         |
| No (N = 79)       | 19 (25%) | 36 (47%) | 21 (28%) |         |
| **Mortality**     |       |       |       | 0.9     |
| Yes (N = 12)      | 5 (42%) | 2 (17%) | 5 (41%) |         |
| No (N = 105)      | 26 (25%) | 52 (50%) | 27 (25%) |         |
| **BPD / Mortality**|     |       |       | 0.4     |
| Yes (N = 41)      | 12 (29%) | 18 (44%) | 11 (27%) |         |
| No (N = 76)       | 19 (25%) | 46 (47%) | 21 (28%) |         |
Table 5

Association between plasma HO-1 RNA at day 14 and clinical outcomes

|                      | HO1 mRNA     | p-value |
|----------------------|--------------|---------|
| AKI Status           |              |         |
| Yes (N =16)          | 0.03 +/- 0.01| 0.8     |
| No (N =55)           | 0.03 +/- 0.007|        |
| BPD status           |              |         |
| Yes (N =18)          | 0.02 +/- 0.006| 0.09    |
| No (N =39)           | 0.04 +/- 0.009|        |
| Mortality            |              |         |
| Yes (N =3)           | 0.02 +/- 0.01| 0.6     |
| No (N =68)           | 0.03 +/- 0.006|        |
| BPD / Mortality      |              |         |
| Yes (N =20)          | 0.02 +/- 0.005| 0.2     |
| No (N =45)           | 0.04 +/- 0.009|        |
Table 6

Association between plasma HO-1 mRNA at day 14 and genetic variants of HO-1 DNA

|                   | HO-1 mRNA | p-value |
|-------------------|-----------|---------|
| −413 KB           |           | 0.4     |
| AA                | N = 14    | 0.02 +/- 0.009 |
| AT                | N = 34    | 0.04 +/- 0.01  |
| TT                | N = 23    | 0.03 +/- 0.008 |
| GT(\(n\)) repeats|           | 0.7     |
| SS                | N = 43    | 0.03 +/- 0.008 |
| SL                | N = 22    | 0.03 +/- 0.01  |
| LL                | N = 6     | 0.02 +/- 0.01  |
Table 7

KDIGO Classification of AKI Modified for Neonates:

| AKI Stage | Serum Creatinine (SCr)                                      |
|-----------|-------------------------------------------------------------|
| Stage 1   | SCr > 0.3 mg/dl from lowest previous value or               |
|           | SCr > 150–200% from lowest previous value                   |
| Stage 2   | SCr > 200–300% from lowest previous value                   |
| Stage 3   | SCr > 2.5 mg/dl or                                          |
|           | SCr > 300% from lowest previous value                       |

Baseline SCr was defined as the lowest previous SCr value because SCr decreases in neonates after birth, dependent on gestational age.