Longitudinal profile of sHLA-G during pregnancy and its association with small for gestational age births in North Indian pregnant females: A nested case-control study

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Abstract

Abstract Objective: To assess the difference in the trajectories of soluble HLA-G in maternal sera during pregnancy between women delivering small for gestational age (SGA) and appropriate for gestational age (AGA) neonates. Design and Settings: Pilot case-control study nested within a cohort study - Garbh-Ini. Population: North-Indian pregnant females delivering SGA (N=23) or AGA (N=17) neonates. Method: Soluble HLA-G1/G5 was estimated in the maternal sera at different time points in pregnancy using sandwich ELISA. Linear mixed models were built and compared to study the association between sHLA-G levels during pregnancy and SGA births. Main Outcome Measure:Birth of SGA or AGA neonates. Results: No significant difference was observed in the sHLA-G trajectories during pregnancy in mothers delivering SGA as compared to those delivering AGA (p-value = 0.5677). A trend towards higher sHLA-G levels at the first trimester of pregnancy (<14weeks of gestation) was observed in mothers delivering SGA neonates (Median= 41.71, IQR= 21.31 to 71.38) as compared to those delivering AGA neonates (Median=37.58, IQR=19.05 to 73.57). Conclusion: The trajectory of sHLA-G during the course of pregnancy is not different between mothers delivering SGA and those delivering AGA. However, a trend towards higher sHLA-G levels at the first trimester was observed in mothers delivering SGA, which could be explored further in studies with larger sample sizes. Funding: "Department of Biotechnology, Ministry of Science and Technology, Government of India (BT/07/IYBA/2013-12), (grant BT/PR9983/MED/97/194/2013)" and "Grand Challenges India–All Children Thriving Program, Biotechnology Industry Research Assistance Council (grant BIRAC/GCI/0114/03/14-ACT)". Keywords: India, SGA, pregnancy, sHLA-G

Introduction

SGA neonates, defined as "neonates with birthweight less than 10th centile for a specific gestational age and sex"[1] are at a greater risk of death, lung disease, hypotension, necrotizing enterocolitis, poor thermoregulation, polycythemia, insulin resistance, type II diabetes mellitus, cardiovascular diseases (like hypertension), stunted growth, poor neurodevelopment outcomes and cognitive impairments[2,3,4].

One of the various maternal and fetal risk factors associated with SGA births, is the aberrant immunological interactions in the placenta. The precise mechanisms steering the tolerance of semi-allogeneic fetus by mother are not completely elucidated, however, one of them is the expression of Human Leukocyte Antigen–G (HLA-G) protein in extra-villous trophoblasts in the placenta[5]. A difference in the HLA-G expression has been reported as one of the causes of aberrant immunological interactions in the placenta[6].

HLA-G is known to perform a crucial role in establishment and maintenance of pregnancy by abrogating the activation of maternal immune cells[5,6]. HLA-G directly binds to inhibitory receptors (ILT-2, ILT-4, and KIR2DL4) that are expressed by monocytes, B cells, T cells and NK cells; monocytes and dendritic cells;

and CD56 NK cells, respectively[7]. These interactions also trigger the release of cytokines and chemokines which signal placental development and remodeling of maternal uterine spiral arteries[8,9].

HLA-G expressed in its soluble form in the placenta may enter maternal circulation as it has been observed that circulatory sHLA-G levels are 2-5 folds higher in pregnant women versus their non-pregnant counterparts[10]. Aberrant levels of sHLA-G protein in the peripheral circulation and in the placenta (mRNA/protein) of pregnant females is linked to pregnancy complications, like preeclampsia, intrauterine growth restriction (IUGR) and adverse birth outcomes like recurrent pregnancy loss (RPL), and premature birth[11,12,13,14,15]. Moreover, low HLA-G expression in human embryo culture supernatants was found to be correlated to their poor implantation in the in-vitro fertilization process in humans[16, 17].

HLA-G expression in adequate amount at the maternal-fetal interface during pregnancy is essential. However, there is currently no data available on the progression of HLA-G throughout pregnancy in the Indian population. The lack of studies exploring the association of sHLA-G levels with SGA births in India emphasizes the need for its investigation. Hence, we aim to study and compare the trajectories of sHLA-G expression in the sera of pregnant women delivering SGA or AGA neonates.

Methods

Pregnancy cohort

The study population consisted of participants who enrolled in the "Interdisciplinary Group for Advanced Research in BirtH outcomes- DBT India Initiative (Garbh-Ini)". Ethical approval was obtained from "Institutional Ethics committee Gurgaon Civil Hospital and Safdarjung Hospital" and "Institutional Ethics Committee (Human Research), Translational Health Science and Technology Institute". The pregnant mothers visiting the antennal clinic at Gurgaon Civil Hospital were approached for enrolment in the Garbh-Ini cohort. Garbh-Ini is a unique pregnancy cohort, started in 2015 at the "Civil Hospital, Gurugram, Haryana, India" with the ultimate objective to determine the clinical, epidemiological, genetic, epigenetic, proteomic and microbial correlates of preterm birth. Women who give a written informed consent are enrolled at <20 weeks of gestation and are followed up at regular intervals till delivery and once post-partum. Detailed clinical and epidemiological information from pregnant women, growing fetus and from the newborn are being documented. Different biospecimens (maternal blood, urine, saliva, high vaginal fluid, cord blood and placenta) are being collected according to the study protocols. The objectives and methodology of Garbh-Ini are published elsewhere[18]. The stored maternal sera samples of women (delivering SGA or AGA neonate) in the Garbh-Ini program were used to accomplish the objective of this study.

Definitions

SGA was defined as birthweight less than 10^{th} centile for a specific gestational age and sex. The neonates were categorized as SGA or AGA on the basis of their weight at birth, sex and completed gestational age in weeks using Fenton Growth Chart[19].

Sample Size

We conducted this pilot study with a sample size of 50 (25 mothers delivering SGA and 25 mothers delivering AGA) participants. Due to technical challenges and limited amount of sample availability sHLA-G could be detected only in 17 mothers delivering AGA and 23 mothers delivering SGA.

Selection of the study participants

The study population was selected from the enrolled participants of the Garbh-Ini cohort. *Inclusion criteria:* The mothers who had their blood sera stored at enrolment i.e., < 14 weeks, 18-20 weeks, 26-28 weeks of pregnancy and at delivery, and gave birth to live, singleton neonates without any birth defects were included in this study. Exclusion criteria: Mothers who had documented major illness like HIV, autoimmune diseases, asthma or malignancy, bacterial or viral infections like dengue fever, malaria, Torch infection or vaginal bacterial infection at any time during pregnancy and those who took immunomodulatory drugs during pregnancy were excluded. All these factors have been reported to be associated with increased expression

of HLA-G, which could confound the association of sHLA-G protein levels with SGA outcome. The study flow is shown in Figure1.

Estimation of soluble HLA-G blood serum concentrations

sHLA-G1/G5 protein concentration was estimated in the blood sera of pregnant women delivering SGA or AGA infants. Sandwich enzyme immunoassay (ELISA) (Exbio, Praha, Czech Republic) kit was obtained commercially, and sHLA-G was measured as per the manufacturer's protocol. A β2- microglobulin (β2m)-associated form of sHLA-G1/G5 is detected in Units/ml. The limit of detection as per the recommended kit is 0.6U/ml. The samples were evaluated in duplicates, with calibrators and blank samples on each plate. Blood sera samples were diluted 4 times using the dilution Buffer (60 µl samples to 180µl dilution Buffer) provided with the kit. 100µl of diluted sera samples were placed in duplicates onto the microtiter plate pre-coated with MEM-G/9 (monoclonal antibody, anti-HLA-G1/G5). The plate was incubated overnight at 4°C. 100µl of monoclonal antihuman β2-microglobin antibody labeled with horseradish peroxidase (HRP) was added to each well post washing steps with the washing buffer. The plate was then incubated for one hour at room temperature on shaker (300rpm). 100µl of substrate solution with tetramethylbenzidine (TMB) was added to each well post the washing steps. With a last incubation step at room temperature for 25 min without shaking, 100µl of acidic stop solution was added to each well. The plate was then analyzed at 450 nm with reference wavelength 630 nm on a microplate reader.

Statistical Analysis

R statistical software was used to perform the statistical analyses. Due to non-normal distribution of the data an unpaired Wilcoxon test was applied to compare the clinical characteristics of the two study groups. Analysis of this study involved measurement of sHLA-G i.e., a dependent variable over time (independent variable) during the course of pregnancy in the pregnant mothers. It represents a classical design of repeatedly measured longitudinal data. Linear mixed effect model was used to analyze this longitudinal data in order to determine changes in the sHLA-G levels during pregnancy in the mothers delivering SGA and those delivering AGA. This method of analyzing the data was selected as it allowed for the inclusion of multiple measurements (of sHLA-G) per participant (i.e., one measurement at each time point they were assessed), and also allowed the inclusion of pregnant women who did not have all the measurements in the analysis, whether due to nonavailability of the sera sample at postpartum (42 days to 6 months) or because sHLA-G levels were undetectable in some mothers at certain time-points (26 to 28 weeks and delivery) of pregnancy. This model could also account for the subject-specific variability generated in our study as all the mothers were not sampled at exactly the same day of pregnancy.

Analysis of sHLA-G dynamics across pregnancy

Loess regression approach: Loess (locally estimated scatter plot smoothing) curves were constructed to summarize and analyze the relationship between the period of gestation (in days) (treated as independent variable) and sHLA-G levels (treated as dependent variable) in SGA and AGA neonates. Due to the skewed distribution of the data, loess regression was applied using Generalized Additive Models for Location Scale and Shape "GAMLSS" package (available in R programming language).

Analysis of repeated measurements

Linear Mixed Effects model: A Linear mixed effect model was built with sHLA-G as a function of gestational age with the group membership (SGA/AGA) as a fixed effect and participant ID as a random effect using lme4 package under the R statistical language and environment (www.r-project.org). The equation used was as follows: sHLA-G ˜ time (in days) + Group + (time (in days) |Participant ID). Another model devoid of group membership (SGA/AGA) was constructed. The equation used was as follows: sHLA-G ˜ time (in days) + (time (in days) |Participant ID). Both the models were compared using ANOVA function to evaluate whether being in SGA or AGA group was associated with variations in sHLA-G trajectories across pregnancy.

Results

Demographics of the study population

The baseline clinical characteristics of the study groups have been provided in Table1. To compare the clinical characteristics of the study participants in case (small for gestational age, N=23) and control (appropriate for gestational age, N= 17) groups, a univariate analysis was carried out. The analysis revealed that in our study population, the difference in maternal height, maternal BMI and parity was statistically significant between case and control groups, whereas no significant difference was observed in maternal age. Neonate's birthweight, length and head circumference were significantly lower in SGA cases as compared to AGA controls in our study population.

Dynamics of sHLA-G across pregnancy in mothers delivering SGA neonates and those delivering AGA neonates

The levels of sHLA-G1/-G5 proteins in the blood sera of pregnant women (collected at $<$ 14 weeks, 18-20 weeks, 26-28 weeks, at delivery and postpartum i.e., 42 days to 6 months post-delivery) delivering SGA or AGA infants was estimated by sandwich ELISA. sHLA-G concentrations are reported as median and interquartile range in Table2. sHLA-G levels were below detection limit at 26-28 weeks in 3 controls and 1 case, and at delivery in 1 control and 2 cases. Maternal sera were available for sHLA-G detection at postpartum only in 8 controls and 12 cases.

Loess regression analysis revealed that sHLA-G levels were highest at the start of gestation and start decreasing as pregnancy progresses to term in both cases and control pregnancies. The loess curves revealing the trend of sHLA-G in mothers delivering SGA or AGA are shown in Figure 2.

Comparison of trajectories of sHLA-G during pregnancy in mothers delivering AGA neonates with those delivering SGA neonates

The two models, one with period of gestation as fixed effect and participant id as random effect and the other with period of gestation and SGA/AGA group membership as fixed effects and participant id as random effect were compared to evaluate whether being in SGA or AGA group was associated with variations in sHLA-G trajectories across pregnancy. No significant difference was found between the two models (p-value = 0.5677) (Table3). The results suggest that the trajectories of sHLA-G levels across pregnancy are same in the mothers delivering AGA as compared to those delivering SGA.

Discussion

Main findings

In this study we explored the progression of HLA-G during pregnancy and assessed the differences between sHLA-G trajectories in 23 mothers delivering SGA and 17 mothers delivering AGA. sHLA-G levels were measured at $<$ 14 weeks, 18-20 weeks, 26-28 weeks of gestation, at delivery and postpartum i.e., 42 days to 6 months after delivery in our study population. The loess curves generated on the data reveal highest levels of sHLA-G at the start of gestation that start decreasing as pregnancy progresses and finally diminish at parturition in both cases and control pregnancies. No significant difference was observed in the sHLA-G trajectories during pregnancy in mothers delivering SGA as compared to those delivering AGA. A trend towards higher sHLA-G levels at the first trimester of pregnancy ($<$14weeks of gestation) was observed in mothers delivering SGA neonates as compared to those delivering AGA neonates.

Strengths and Limitations

The major strength of this study is its longitudinal design. The current study is possibly the first study to describe the physiological changes in sHLA-G levels during pregnancy to post delivery in the North Indian females. This is a case-control study nested within a unique pregnancy cohort Garbh-ini. The biospecimen from enrolled participants are being collected in a longitudinal manner and well curated data on multiple clinical variables is being documented. We were able to study associations in a homogenous population with a well-defined phenotype of SGA. The clinical data available on multiple variables allowed us to exclude potential confounders in our study. Instead of using traditional generalized linear models, we used linear

mixed model to explore and compare the trajectories of sHLA-G in mothers delivering SGA vs AGA infants. The traditional methods can handle only balanced repeated measure designs. A linear mixed model can handle missingness in the data (unbalanced repeated measure designs), which our study had because of unavailability of the sera sample at postpartum (42 days to 6 months) or because sHLA-G levels were undetectable in some mothers at certain time-points (26 to 28 weeks) of pregnancy and at delivery. This model was robust enough to handle the subject-specific variability which was generated in our study because all the mothers were not sampled at exactly the same day of pregnancy. Inclusion of a post-partum time point in this study reflects the levels of sHLA-G at pre-pregnancy or non-pregnant state. The main limitation of the study is its small sample size and hence the results should be cautiously interpreted.

Interpretation

To explore and compare the levels of sHLA-G during pregnancy in mothers delivering SGA and those delivering AGA, we chose a homogenous population of mothers delivering singleton live term SGA and AGA infants A univariate analysis was carried out to compare the maternal and neonatal characteristics between the two groups. Our analysis revealed a statistically significant difference in maternal height, maternal BMI and parity between cases and controls. Short stature (<145cm)[20], BMI<18kg/m^2 [21] and nulliparity[22] have been reported as risk factors for SGA births in previously published reports. Our results reflect the same with higher frequency of nulliparous and shorter mother with lower BMI in cases as compared to control. As there are no associations reported between aberrant levels of sHLA-G and maternal height, low BMI or parity, these variables were not adjusted for in the analysis.

Soluble HLA-G protein produced in the human placenta has been reported to enter maternal circulation[10] and in corroboration to these studies effect of sHLA-G on cytotoxic T cells and cytokine stimulation has been described to be dose-dependent[23]. In line with these observations, we wanted to explore if the differential levels of sHLA-G protein in the pregnant mothers is associated with SGA births. sHLA-G levels measured in our study population across pregnancy were found to be highest at the start of gestation and revealed a decrease as pregnancy progressed and finally diminished at delivery in both cases and control pregnancies. This finding corroborates with the earlier studies conducted in Spanish and Turkish pregnant females where it was found that in healthy control pregnancies HLA-G levels were high at early gestation and start decreasing as pregnancy progressed to term[24, 25]. A drop in the levels of sHLA-G was observed at 26-28 weeks of gestation because the invasion of HLA-G expressing EVT into the maternal uterine spiral arteries is completed by 18-20 weeks of gestation[26, 27] and hence the need of high levels of sHLA-G. Invasion of EVTs into the maternal decidua is needed for successful establishment of pregnancy and also for remodeling of maternal spiral arteries from high resistance high pressure to low resistance low pressure arteries[28]. The levels of sHLA-G drop further at term for parturition to occur.

No significant difference was observed in the sHLA-G trajectories during pregnancy in mothers delivering SGA as compared to those delivering AGA. A trend towards a higher sHLA-G levels at the first trimester of pregnancy (< 14weeks of gestation) was observed in mothers delivering SGA infants as compared to those delivering AGA infants. A higher level of sHLA-G at the first trimester of pregnancy in mothers delivering SGA as compared to those delivering AGA could be due to interplay of several immune mechanisms that operate in the placenta and help in establishment and maintenance of a healthy pregnancy. A perturbation in the immune mechanisms could have triggered the increase in expression of sHLA-G in the placenta as a compensatory mechanism in SGA fetuses so as to protect the fetus from being an adverse event at the initial stage in pregnancy. This is being reflected by the higher sHLA-G levels in the first trimester of pregnancy in the peripheral circulation of the mother. This mechanism is not sturdy enough to counter-balance the disturbed immune status and ultimately results in the delivery of an SGA infant. It has been demonstrated that HLA-G plays an important role in shifting Th1/Th2 balance towards Th2 polarization in the decidual tissues and this shift is crucial for establishment and maintenance of pregnancy[29]. It is also known that IL-10, a crucial anti-inflammatory cytokine of pregnancy, is released by extra villous trophoblast cells and induces HLA-G expression in EVTs. IL-10 also induces differentiation of CD4+ T cells into Th2 cells[30]. In line with this literature, we could speculate that in case of pregnancies complicated by SGA, there is a

disruption in Th2 polarization and in order to achieve Th2-skewing state, more of IL-10 is released by the Tregs, EVTs and other decidual cells. Increased levels of IL-10 may upregulate the expression of HLA-G in these pregnancies. A concurrent upregulation of IL-10 and sHLA-G has been also been observed in cervical cancer patients, suggesting that IL-10 induces an immunosuppressive environment in the cancer cells by up regulating HLA-G expression[31]. In case of pregnancies with SGA fetuses, these compensatory mechanisms are not sufficient to restore the immune balance and ultimately results in the birth of an SGA neonate, but albeit protecting it from other mortal adverse outcomes such as recurrent pregnancy loss. The reduced levels of sHLA-G have been associated with recurrent pregnancy loss[32].

Another explanation for higher sHLA-G levels at the first trimester in mothers delivering SGA could be that apart from the fetal derived sHLA-G, maternal-derived sHLA-G is also being expressed at the maternal-fetal interface to support implantation of SGA fetuses and hence the sHLA-G that is being detected in the maternal circulation in the first trimester is a mix of fetal-derived and maternal derived sHLA-G. This speculation could be supported by the study in which it has been reported that those women who had successful pregnancies after IVF expressed higher sHLA-G in the pre-ovulatory phase as compared to those with failed IVF[33]. Maternal sHLA-G was able to protect the fetus from rejection but could not save it from being born SGA.

Conclusion

The objective of this study was to determine the association between circulatory levels of sHLA-G in pregnant mothers and SGA births. No difference was observed in the trajectory of sHLA-G during the period of pregnancy in mothers delivering SGA as compared to those delivering AGA. However, a trend towards higher sHLA-G levels at first trimester was observed in mothers delivering SGA, which can be explored further in studies with a larger sample size. sHLA-G levels combined with clinical and/or imaging (as seen in the ultrasound) risk factors could be used to build predictive models for early prediction of SGA births. Early prediction, proper management, and timely intervention of pregnancies that could result in SGA, may help in decreasing the mortality and morbidity in SGA neonates.

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Disclosure of interests

The authors have no conflicts of interest to declare.

Contribution to authorship

This study was conceived and supervised by PK. SS, carried out experiments in the lab, processed and analyzed the experimental data and wrote the manuscript. SS and SV implemented statistical analysis on the data. PK and SS made substantial contributions to interpretation of the results. PK and SB revised the manuscript for intellectual content. SB and PK obtained the funding and contributed to designing this study. All the authors have approved the final version of this manuscript.

Details of ethics approval

The study was approved by "Institutional Ethics committee Gurgaon Civil Hospital (Letter No. ETHICS/GHG/2016/2.3/24thSept 2016)" and "Institutional Ethics Committee (Human Research), Translational Health Science and Technology Institute (Letter No. THS. 1.8.1/ (50) dated 15th Dec 2016)".

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Table 1: Baseline clinical characteristics of the two study groups

Clinical Characteristics	AGA Median (IQR) (N=25)	SGA Median (IQR) (N=25)	p-value
Maternal age	22(20-25)	21(20-23.5)	0.3764
Maternal height	154(150.1-157.1)	152(149.5-154.2)	0.0007
Maternal BMI at enrolment	19.59(18.47-21.15)	19.45 (18.42-21.11)	<0.0001
Parity Nulliparous Primiparous and Multiparous	11 14	19 6	0.043
Birthweight	2.86 (2.72-3.04)	2.56 (2.46-2.75)	<0.0001
Gestational age	38 (38-39)	39 (39-40)	<0.0001
Gender Males Females	14 9	15 10	1.000
Infant Length	48.90 (48.50-50.00	47.00(46.35-48.05)	0.0007
Head circumference	34(33.5-34.8)	32.80 (31.80-33.55)	0.0017

AGA, appropriate for gestational age; SGA, small for gestational age; BMI, body mass index; IQR, interquartile range**Table 2:** Serum concentrations of sHLA-G in mothers delivering SGA infants and mothers delivering AGA infants

Time points	Concentration of sHLA-G(U/mL)	Concentration of sHLA-G(U/mL)
	AGA Median (IQR) (N)	SGA Median (IQR) (N)
<14 weeks	37.58 (19.05 to 73.57) (N=17)	41.71 (21.31 to 71.38) (N=23)
18-20 weeks	34.02 (22.27 to 58.48 (N=17)	34.06 (22.14 to 59.16) (N=23)
26 to 28 weeks	3.48 (2.47 to 10.34 (N=14)	10.13 (2.28 to 16.81) (N=22)
At Delivery	5.14 (3.30 to 15.57) (N=16)	4.85 (2.70 to 23.90) (N=20)
Postpartum *42 days to 6 months*	5.20 (3.66 to 9.11) (N=8)	3.30 (3.06 to 8.95) (N=12)

AGA, appropriate for gestational age; SGA, small for gestational age;IQR, interquartile range

Table 3: Regression coefficients (β) of period of gestation and/or SGA/AGA groupmembership for predicting log sHLA-G levels according to linear mixed modeling (Model1, Model2 and comparison of Model 1 and Model 2).

Models	Fixed Effect/Random Effect	Estimate (β coefficient)	Standard error	p-value
Model 1	Fixed effect= period of gestation (days) Random effect= Participant Id	-1.0639	0.1347	<0.0001
Model 2	a)Fixed effect= period of gestation(days) b) Fixed effect=Group (SGA/AGA) Random effect= Participant Id	-1.0760 -0.1028	0.1365 0.1796	<0.0001 0.5701

Models	Fixed Effect/Random Effect	Estimate (β coefficient)	Standard error	p-value
Comparison of Model 1 with Model 2	Comparison of Model 1 with Model 2	Comparison of Model 1 with Model 2	Comparison of Model 1 with Model 2	0.5677

AGA, appropriate for gestational age; SGA, small for gestational age

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`Figure 2.docx` available at https://authorea.com/users/726009/articles/708796-longitudinal-profile-of-shla-g-during-pregnancy-and-its-association-with-small-for-gestational-age-births-in-north-indian-pregnant-females-a-nested-case-control-study