Reproductive Sciences
2014, Vol 21(2) 215-220

DOI: 10.1177/1933719113492209
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Increased Levels of Cell-Free Human Placental Lactogen mRNA at 28-32 Gestational Weeks in Plasma of Pregnant Women With Placenta Previa and Invasive Placenta

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Abstract

We compared the levels of cell-free human placental lactogen (hPL) messenger RNA (mRNA) in maternal plasma at 28 to 32 weeks of gestation between women with diagnosis of placenta previa or invasive placenta and women with an uneventful pregnancy. Sensitivity and specificity of hPL mRNA for the prediction of invasive placenta were further explored. Plasma hPL mRNA were quantified by real-time reverse-transcriptase polymerase chain reaction in women with placenta previa (n = 13), invasive placenta (n = 5), and normal pregnancies (n = 92). Median (range) hPL mRNA was significantly higher in women with placenta previa, 782 (10-2301) copies/mL of plasma, and in those with invasive placenta, 615 (522-2102) copies/mL of plasma, when compared to normal pregnancies, 90 (4-4407) copies/mL of plasma, $P < .01$ and $P < .05$, respectively. We found a sensitivity of 100% and a specificity of 61.5% for the prediction of invasive placenta among women with placenta previa. In conclusion, expression of hPL mRNA is increased in plasma of women with placenta previa and invasive placenta at 28 to 32 weeks of gestation.

Keywords

hPL, cell-free RNA, placenta previa, placenta accreta

Introduction

Invasive placenta is the clinical manifestation of a spectrum of an exaggerated placental adhesion into the uterine wall, varying from an invasion beyond the inner third of the myometrium (placenta accreta) through deeper structures such as the entire myometrium (placenta increta) and adjacent organs such as bladder or bowel (placenta percreta).[1] Each case of invasive placenta represents a real challenge for the obstetricians. Invasive placenta is clinically characterized by massive hemorrhage that potentially leads to disseminated intravascular coagulopathy, renal failure, and death of the mother.[2] Thus, an early and accurate prenatal diagnosis is crucial for proper management in order to minimize serious maternal and neonatal complications. The diagnosis is currently performed by ultrasonography with a sensitivity of 77% to 87% and specificity of 96% to 98%.[3,4] Although magnetic resonance imaging (MRI) may contribute to the diagnosis, its use is reserved for patients with the ambiguous ultrasound findings or a suspicious of a posterior invasive placenta.[2]

Some biomarkers such as maternal serum creatinine kinase and α-fetoprotein, have been previously tested in the diagnosis of invasive placenta with no satisfactory results.[5,6] Circulating fetal nucleic acids in maternal plasma have been investigated as potential markers for placental function and placental-related disorders. In this instance, we have previously communicated the increased levels of cell-free fetal DNA in maternal plasma of women with placenta increta.[7] Additionally, cell-free human placental lactogen (hPL) messenger RNA (mRNA), which is entirely produced by trophoblast cells, is detectable in maternal plasma form early stages of pregnancy and undetectable

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postdelivery.[8-10] Interestingly, cell-free hPL mRNA has been reported to be increased in pregnancies complicated by placenta previa and invasive placenta.[11-13] Yet, the mechanisms of increasing hPL observed in patients with invasive placenta are not clear; it is interesting to note that hPL has also been reported to be decreased in pregnancies complicated by preeclampsia,[14] where there is an abnormally shallow invasion of the trophoblast into the myometrium.

In this study, we aim to compare the expression levels of cell-free hPL mRNA in maternal plasma at 28 to 32 weeks of gestation between women with diagnosis of placenta previa or invasive placenta and women with an uneventful pregnancy. Additionally, we aim to predict bleeding at the time of cesarean section using the expression levels of hPL mRNA in maternal plasma.

Material and Methods

Study Population

This was a case–control study at the National Defense Medical College Hospital (Saitama, Japan) between November 2005 and March 2010. Blood samples were collected at 28 to 32 weeks of gestation from 13 women with the diagnosis of placenta previa (case group 1), 5 women with invasive placenta (case group 2), and 92 women with uneventful pregnancies (control group). The local ethics committee approved the study, and each patient gave written informed consent before participation. Gestational age was determined by last menstrual period or crown-rump length when appropriate. Exclusion criteria included multiple gestation, intrauterine growth restriction, gestational diabetes, preeclampsia, illicit drug use, and preexisting medical conditions such as diabetes, chronic hypertension, and renal disease.

Placenta previa was diagnosed by ultrasound at 28 to 32 weeks of gestation. Patients with suspicious invasive placenta were further explored at 32 to 34 weeks by both ultrasound and MRI. Sonographic signs of invasive placenta included loss of the anechoic retroplacental space, prominent placental lacunae, and increased vasculatrity at the interface of the uterus and the bladder. The MRI signs of invasive placenta included uterine bulging, heterogeneous signal intensity, and the presence of dark intraplacental bands related to lacunae on T2-weighted images. All cases of invasive placenta had undergone hysterectomy, and a detailed pathological examination confirmed the diagnosis.

During cesarean section, the amount of blood loss was collected through a suction device and soaked gauze swabs. The sum of blood in suction device and gauzes was considered as bleeding. In vaginal delivery cases, the amount of blood loss was measured with soaked gauze and pad for 2 hours postpartum.

Blood Sample Collection

Maternal blood samples were collected at 28 to 32 gestational weeks in EDTA-containing tubes and centrifuged at 1600*g* for 10 minutes at 4°C. The resulting plasma was carefully transferred into plain propylene tubes and stored at −20°C until taken to the Laboratory of the Department of Obstetrics and Gynecology at Showa University School of Medicine (Tokyo, Japan) for analysis.

RNA Extraction

Total RNA was extracted from 0.8 mL of harvested plasma. Plasma was mixed with 2.4 mL of Trizol LS reagent (Invitrogen, Carlsbad, California) and 0.2 mL of chloroform. This mixture was then centrifuged at 17 300*g* for 15 minutes at 4°C, and the aqueous layer was transferred to new tubes. One volume of 700 mL ethanol was added to 1 volume of the aqueous layer. Then, the mixture was applied to an RNeasy minicolumn using RNeasy mini kit (Qiagen, Valencia, California) and processed according to the manufacturer's recommendations. Total RNA was eluted with 20 μL of RNase-free water.

Reverse Transcription and Quantitative Real-time PCR

Extracted RNA (12 μL) was immediately reverse transcribed into complementary DNA (cDNA) using Omiscript RT kit (Qiagen) in a total reaction volume of 20 μL according to the manufacturer's instructions. Briefly, we use 2 μL 10 × RT buffer, 2 μL 25 × deoxyribonucleotide triphosphates, 1 μL 10 × RT random primer, 1 μL oligo (dT) primer, 1 μL RNase inhibitor, and 1 μL Omniscript RT. The process was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, California) with the following thermal conditions: 60 minutes at 37°C, followed by 5 minutes at 95°C.

Transcripts levels of hPL mRNA (primer sense: 5′-CATGACTCCCAGACCTCCTTC-3′ and antisense 5′-TGCGGAGCAGCTCTAGATTG-3′) and ribosomal protein large P1 (RPLP1, catalogue # Hs_01653088_g1) as control gene were analyzed simultaneously by quantitative polymerase chain reaction (PCR) using 2 μL aliquot of cDNA in a reaction volume of 20 μL. TaqMan gene expression assays and the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California) were used with the following thermal conditions: 95°C for 15 minutes followed by 40 cycles at 94°C for 15 seconds and 60°C for 1 minute. Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. Standard curves were generated using 10 to 10^6 copies of cDNA plasmids, and the transcript numbers were determined from linear regression of these standard curves. Levels of hPL mRNA were expressed in terms of copies/mL of maternal plasma by the method described elsewhere.[15] Additionally, we calculated the relative expression ratios normalized by the housekeeping gene, RPLP1. Before our experiment, we validated RPLP1 as a suitable reference gene in maternal plasma in the third trimester (data not shown).

Statistical Analysis

Comparison between each case group and the control group was carried out by Fisher exact test or Wilcoxon rank-sum test where appropriate. Comparison between the levels of hPL

Table 1. Clinical Characteristics and Measurements of the Study Population.[a]

Variables	Control n = 92	Placenta Previa n = 13	P1 Value	Invasive Placenta n = 5	P2 Value
Maternal age, years, median (range)	30 (20-42)	34 (23-41)	.014[b]	31 (25-40)	.600
Multiparity	53 (56.5%)	12 (61.5%)	.728	4 (80%)	.393
Previous cesarean section	14 (15.2%)	4 (44.4%)	.227	4 (80%)	.004[c]
Bleeding, mL (range)	342 (95-1887)	1419 (304-3240)	.004[c]	1649 (1323-2582)	<.001[c]
Gestational age at delivery, weeks, median (range)	39 (37-41)	37 (32-37)	<.001[c]	36 (32-38)	.006[c]
Neonatal weight, g, median (range)	3073 (2445-4350)	2880 (1618-3230)	<.001[c]	2620 (1842-2970)	.018[b]
Gestational age at blood sampling, weeks, median (range)	30 (28-32)	31 (28-32)	.146	31 (28-32)	.2328
Storage duration, months, median (range)	58 (35-73)	60 (30-80)	.684	57 (47-79)	.665

[a]Data are presented as median (range) or number (%): comparison between each case group and control was carried out by Fisher exact test or Wilcoxon rank-sum test where appropriate. *P*1: control versus placenta previa. *P*2: control versus invasive placenta.
[b]$P < .05$.
[c]$P < .01$.

Table 2. RNA Expression Levels at 28 to 32 Gestational Weeks.[a]

Variables	Control n = 92	Placenta Previa n = 13	P1 Value	Invasive Placenta n = 5	P2 Value
hPL mRNA, copies/mL, median (range)	87 (4-4407)	782 (10-2301)	.002[b]	615 (522-2102)	.022[c]
RPLP1 mRNA, copies/mL, median (range)	3523 (152-41044)	5919 (812-28327)	.157	3722 (368-8691)	.788
hPL mRNA relative expression, ×1/100, median (range)	2.99 (0.02-27.67)	13.20 (0.54-21.60)	<.001[b]	20.75 (14.17-141.88)	<.001[b]

Abbreviations: hPL, human placental lactogen; mRNA, messenger RNA; RPLP1, ribosomal protein large P1.
[a]Comparison between each case group and control was carried out by Wilcoxon rank-sum test. *P*1: control versus placenta previa. *P*2: control versus invasive placenta.
[b]$P < .01$.
[c]$P < .05$.

mRNA relative expression between each groups were also carried out using Steel-Dwass test. A receiver–operator characteristic (ROC) curve was also constructed to assess the potential hPL mRNA in the prediction of invasive placenta among women with placenta previa. Additionally, we correlate the amount of bleeding in women undergoing cesarean section with the levels of hPL mRNA relative expression. All analyses were carried out using the software JMP version 10.0.2 (SAS Institute, Cary, North Carolina). A value of $P < .05$ was considered statistically significant.

Result

Table 1 shows the demographic and clinical characteristics of the study population. Women with placenta previa and invasive placenta were more likely to have a prior cesarean section compared with normal pregnancies (44.4% and 80.0% vs 15.2%, $P < .001$). Additionally, women with placenta previa and invasive placenta were more likely to deliver earlier than normal pregnancies (37 and 36 vs. 39 weeks, $P < .001$) and consequently to deliver infants with lower birth weight than normal pregnancies (2880 and 2620 vs 3063 g, $P < .001$). There was no significant difference in gestational age in weeks at blood sampling and storage duration between groups.

Median (range) maternal plasma cell-free hPL mRNA was significantly higher in women with placenta previa, 782 (10–2301) copies/mL of plasma, and in those with invasive placenta, 615 (522–2102) copies/mL of plasma, than normal pregnancies, 90 (4-4407) copies/mL of plasma, $P < .01$ and <.05, respectively (Table 2). Similarly, median (range) hPL mRNA relative expression (×1/100) was significantly higher in women with placenta previa, 13.2 (0.5-21.6), and in those with invasive placenta, 20.8 (14.2-141.9), when compared to normal pregnancies, 0.3 (0.02-2.8), $P < .001$ (Table 2). In the control group, hPL mRNA expression and hPL mRNA relative expression had no correlation with storage duration of blood samples (Figure 1). Additionally, hPL mRNA relative expression was significantly higher in women with invasive placenta than in those with placenta previa ($P < .001$; Figure 2).

The ROC analysis showed that a cutoff of 14.2 of hPL mRNA relative expression yields a sensitivity of 100% and a specificity of 61.5% for the prediction of invasive placenta among women with placenta previa. The same cutoff value yields a sensitivity of 100% and a specificity of 88.6% for the prediction of invasive placenta in the total population (Figure 3).

Women with placenta previa and women with invasive placenta underwent cesarean section in all cases. In the control group, 22 women underwent cesarean section. As shown in Figure 4, there was a strong positive correlation between bleeding at the time of cesarean section and hPL mRNA relative expression ($\rho = .357$, $P = .024$).

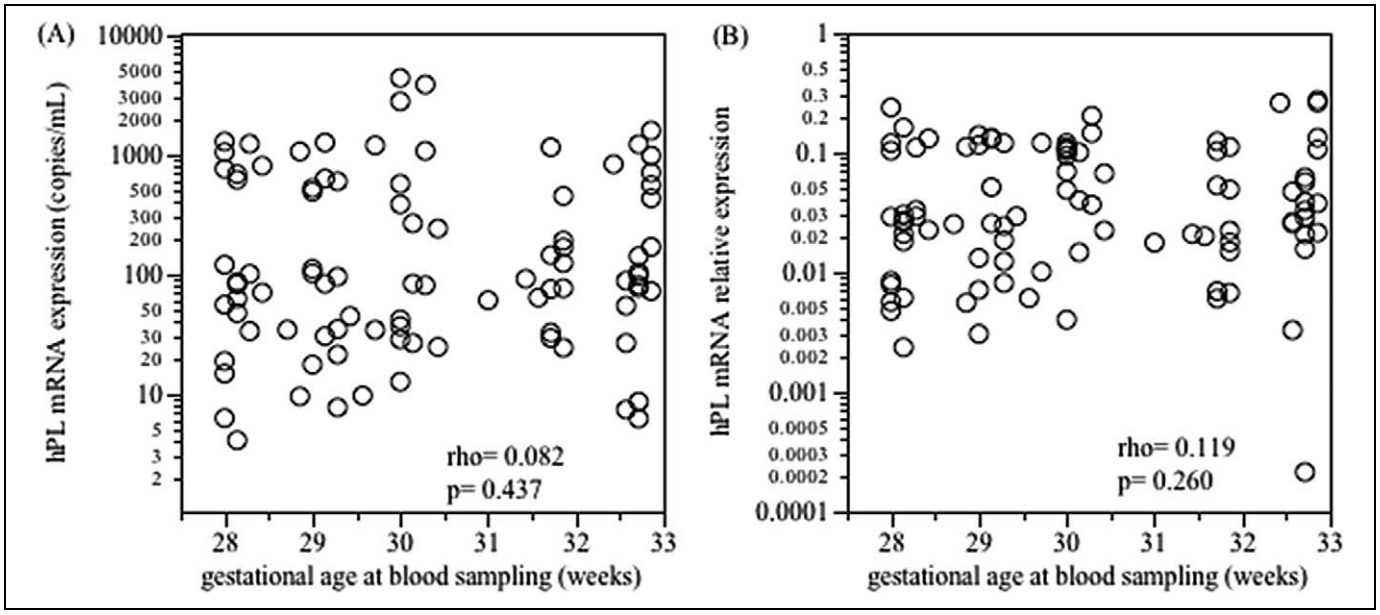

Figure 1. Levels of human placental lactogen (hPL) messenger RNA (mRNA) in copies/mL (A) and in relative expression (B) according to the gestational age at the time of blood sampling (ρ: Spearman rank correlation coefficient and P = value of significance).

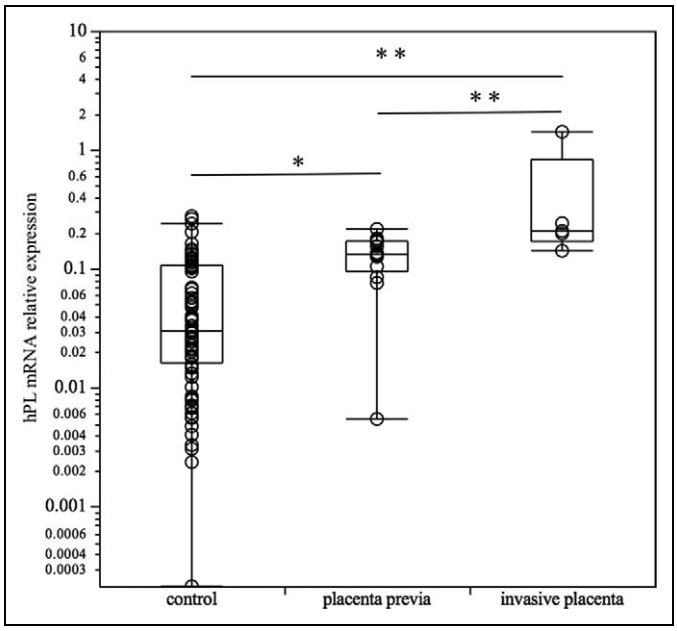

Figure 2. Box plots of human placental lactogen (hPL) messenger RNA (mRNA) relative expression in maternal plasma between groups. Comparison between the groups was carried out by Steel-Dwass test. *P < .05 and ** P < .01.

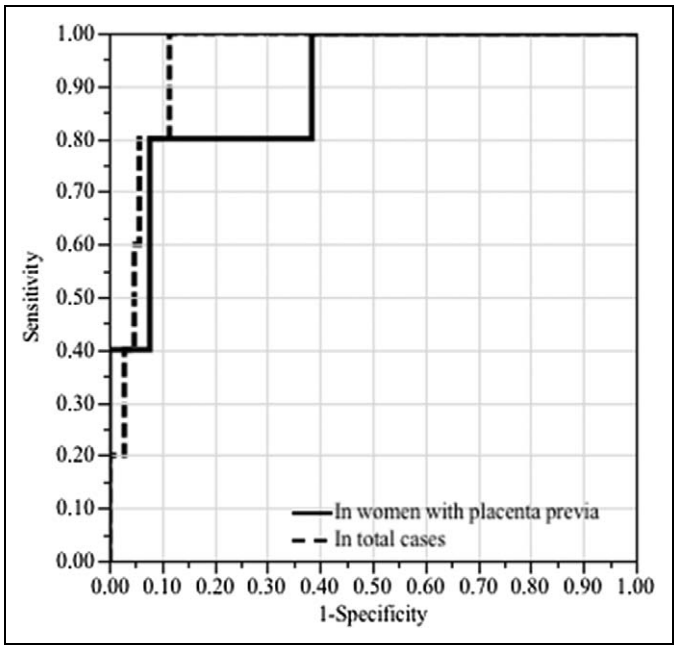

Figure 3. Receiver–operator characteristic (ROC) curve for the prediction of invasive placenta among women with placenta previa (solid line) and total population (dotted line). AUC (area under the ROC curve): 0.86 and 0.97, respectively.

Discussion

This study reports the increased expression levels of hPL mRNA in maternal circulation at 28 to 32 weeks of gestation in women with placenta previa and particularly in those with invasive placenta. Additionally, we show that the amount of bleeding in women undergoing cesarean section correlates positively with the levels of hPL mRNA. Our results are consistent with previous reports. Miura et al reported increased maternal plasma levels of hPL mRNA in women with placenta previa undergoing hysterectomy due to placenta accreta.[12] Similarly, El Behery et al reported increasing levels of hPL mRNA in women with placenta accreta according to the placental invasion.[11] Furthermore, we report for the first time that

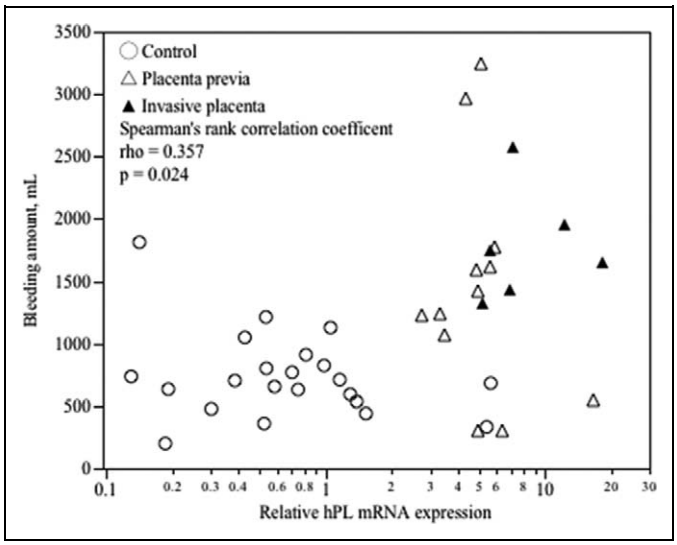

Figure 4. Plot graph showing the correlation between the amount of bleeding and human placental lactogen (hPL) messenger RNA (mRNA) relative expression (log-transformed). ρ= correlation coefficient, *P*= value of significance.

expression of cell-free hPL mRNA correlates with the amount of bleeding in women undergoing cesarean section.

Interestingly, previous studies have reported decreased levels of serum hPL in women with severe preeclampsia, a pregnancy complication characterized by a shallow placental invasion.[14] During placental formation, interstitial extravillous trophoblast cells, referred as ''intermediate'' trophoblast cells, are crucial for invasion of the placenta into the utero, and it is thought that these cells produce high levels of hPL.[16] Thus, we hypothesize that increasing levels of hPL in women with placenta previa or invasive placenta observed in our study may reflect the activity of interstitial extravillous trophoblast.

In this study, cell-free hPL mRNA is presented as copies/mL as suggested by Ng et al,[17] and also normalized values of hPL mRNA as ratio (hPL mRNA/RPLP1 mRNA). Noticeable, both gave rise to similar results. Here, we did not adjust hPL mRNA according to the gestational age, as the concentration of hPL and RPLP1 were stable during the period of 28 to 32 weeks of gestation. Additionally, main maternal characteristics were nonsignificantly different between the groups;hence, no further adjustment was necessary. Although the numbers of patients included in each group is small, we provide important data regarding the potential of hPL as a predictive biomarker of these infrequent obstetric conditions. In this regard, it is also important to note that the data distribution of hPL mRNA relative expression between the groups was still significant even after removing the outliers. These findings must be confirmed before further considerations. Although circulating RNA in plasma is remarkably stable,[18] a strict protocol has been followed from blood collection until the PCR assay to minimize any effect of storage time over RNA integrity. As shown in Table 1, storage time was similar between the groups and furthermore, RPLP1 was confirmed to be homogenously expressed in all the samples.

In conclusion, we report increasing levels of hPL mRNA in plasma of women with invasive placenta. Further large prospective studies are warranted to establish the role of hPL in predicting placenta previa, invasive placenta, and severe complications in these patients.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Nos. 23592417, 24659737, and 24791723).

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