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Increased Plasma mRNAs of Placenta-specific 1 (*PLAC1*) and Glial Cells-missing 1 (*GCM1*) in Mothers with Pre-eclampsia

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ABSTRACT

In this study we have investigated whether quantitative analysis of placental mRNAs in maternal plasma provides a way to monitor placental status. We measured plasma concentrations of human chorionic gonadotropin β-subunit (*βhCG*) and human placental lactogen (*hPL*) mRNAs as previously reported mRNAs and pregnancy associated plasma protein A (*PAPP-A*), placenta-specific 1 (*PLAC1*) and glial cells-missing 1 (*GCM1*) mRNAs, which have not been measured during the course of normal pregnancy. Firstly, peripheral blood was obtained at various times from healthy pregnant women to clarify the time course of placental mRNAs. Secondly, blood was obtained from women with pre-eclampsia and gestational age-matched controls to examine whether placental mRNAs change in pre-eclampsia. Plasma was separated from these samples for extraction of RNA, followed by reverse transcription polymerse chain reaction analysis. Median concentrations of *PLAC1* and *GCM1* mRNA in plasma of pre-eclamptic subjects respectively were 1625 and 2141 copies/ml, significantly higher than 195 and 881 copies/ml, the values for controls (Mann-Whitney test, $p<0.001$). No significant difference was seen in *hPL*, *βhCG*, or *PAPP-A* mRNA concentration between pre-eclamptic and control groups. Plasma *PLAC1* and *GCM1* mRNAs appear promising as noninvasively measurable molecular markers for pre-eclampsia.

Key words: *Quantitative analysis of placental mRNA, Noninvasive prediction markers for pre-eclampsia, Placenta-specific 1 (PLAC1), Glial cells-missing 1 (GCM1)*

RNAs of interest present in the circulation independently of cells have been assayed recently as a noninvasive means of profiling gene expression[6]. Fetal or placental mRNA detected in maternal plasma during pregnancy has been found to be relatively resistant to degradation *in vivo*[10]. Unlike fetal DNA analysis, circulating mRNA determinations are applicable to all pregnancies irrespective of differences in gender or genetic polymorphisms between mother and fetus[10]. Recent refinements of methods have enhanced our ability to use plasma mRNA for molecular diagnosis. A quantitative study of plasma mRNA encoding γ-globin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs showed significantly higher concentrations in pregnant than in nonpregnant women[15], suggesting a major placental contribution. Quantitative analysis of placental mRNAs in maternal plasma therefore may be useful in monitoring placental status.

Pre-eclampsia, a serious complication of pregnancy characterized by elevation of blood pressure, proteinuria, and complex biochemical disturbances, occurs in 6% to 8% of pregnancies and contributes significantly to stillbirths and to neonatal morbidity and mortality[14]. Pre-eclampsia is characterized by impaired placental function. Pathologic processes involving abnormal trophoblast invasion, deficient physiologic maternal spiral artery modification[13], increased apoptosis of trophoblastic cells[5], and placental ischemia are all associated with the release of specific molecules[2].

In this study, we investigated the kinetics of human chorionic gonadotropin β-subunit (*βhCG*), human placental lactogen (*hPL*), pregnancy associated plasma protein A (*PAPP-A*), placenta-specific 1 (*PLAC1*) and glial cells-missing 1 (*GCM1*) mRNAs in plasma during the course of normal pregnancy. Additionally, we examined the concentrations of these mRNAs in pre-eclampsia at 36 weeks of ges-

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tation and investigated whether maternal plasma placental mRNAs differed in pre-eclampsia from the values seen in normal pregnancy.

PATIENTS & METHODS

Patients

Peripheral blood samples were collected from pregnant women receiving ambulatory prenatal care in the Department of Obstetrics and Gynecology at Hiroshima University Hospital who had provided written informed consent to study participation. This investigation was approved by the Research Ethics Committee of Hiroshima University.

In the first phase of the study, blood samples were obtained from healthy women during pregnancy. Peripheral blood samples were taken from 21 subjects at 11 and 24 weeks, from 38 subjects at 15 weeks, from 26 subjects at 36 weeks, from 22 subjects just before delivery, 30 min after delivery, 1 day postpartum, and from 10 subjects 1 month after delivery. At the time of sampling, none of the women had manifested complications of pregnancy, such as hypertension or threatened preterm labor. As negative controls, 10 nonpregnant women (mean age: 32.6) were studied.

In the second phase, 36 patients participated: 10 pre-eclamptic women, and 26 pregnant women without pre-eclampsia. Median gestational ages in the pre-eclamptic and control groups respectively were 35.8 weeks (interquartile range, 35.1 to 36.8) and 36.5 weeks (interquartile range, 35.3 to 36.9). Clinical features in the pre-eclamptic patients and the pregnant control group are presented in Table 1.

Pre-eclampsia was defined in this study as gestational blood pressure elevation with proteinuria, usually occurring after 20 weeks of gestation[14]. Because of its poor specificity, edema is no longer included in the diagnosis of pre-eclampsia. Hypertension was defined as a blood pressure ≥140 mmHg systolic or ≥90 mmHg diastolic in at least two separate determinations in a woman who was normotensive before 20 weeks of gestation. Proteinuria was defined as excretion of ≥0.3 g of protein in a 24-hr urine specimen, which correlated with a 1+ or higher protein dipstick reading in two random urine samples. The control group included pregnant women with no preexisting medical diseases or antenatal complications.

Processing of blood samples

Plasma was separated within 6 hr of venipuncture. Blood samples were processed based on a previously reported protocol[9]. In brief, 10 ml blood were collected into EDTA-containing tubes and centrifuged at 1,600 *g* for 10 min at 4°C. Plasma was then carefully transferred into polypropylene tubes with no additives and recentrifuged at 16,000 *g* for 10 min at 4°C. Supernatants were then placed in new polypropylene tubes.

RNA extraction

We mixed 1.6 ml of the plasma harvested after the centrifugation steps described above with 2 ml of Trizol LS reagent (Invitrogen, Carlsbad, CA) and 0.4 ml of chloroform[10]. The mixture was centrifuged at 12,000 *g* for 15 min at 4°C, and the aqueous layer was transferred to new tubes. One volume of 700 ml/liter ethanol was added to one volume of aqueous layer. The mixture was then processed with a MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Total RNA was eluted with 20 μl of RNase-free water and stored at –80°C before measurement.

Reverse Transcription

Reverse transcription (RT) was performed using an Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The entire sample of total RNA (12 μl) from peripheral blood was reverse-transcribed in a final volume of 20 μl. Incubation in a GeneAmp PCR System 9700

Table 1. Features of pre-eclamptic patients and controls

	Control (n=26)	Pre-eclampsia (n=10)
Maternal age (years)	29.7 ± 5.2	33.0 ± 7.9
Gravidity	1.5 ± 1.4	1.0 ± 0.7
Parity	0.6 ± 0.6	0.6 ± 0.8
Systolic blood pressure at diagnosis (mmHg)	109.3 ± 10.9	155.8 ± 11.3*
Diastolic blood pressure at diagnosis (mmHg)	66.9 ± 8.4	95.6 ± 9.4*
Proteinuria (%)	11.5	100*
Gestational age at blood sampling (weeks)	36.1 ± 0.80	35.7 ± 1.7
Gestational age at delivery (weeks)	39.8 ± 1.2	37.5 ± 0.9
cesarean section (%)	0	20
Birth weight (g)	3114.4 ± 375.5	2508.8 ± 543.2
placental eight (g)	606.7 ± 100.4	530.3 ± 139.9

*$p<0.05$

Each value represents mean ± SD

Table 2. Nucleotide sequences of primers and probes

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Probe (5'–3')[a]
βhCG	CTA CTG CCC CAC CAT GAC CC	TGG ACT CGA AGC GCA CAT C	CCT GCC TCA GGT GGT GTG CAA CTA
hPL	CAT GAC TCC CAG ACC TCC TTC	TGC GGA GCA GCT CTA GAT TG	TTC TGT TGC GTT TCC TCC ATG TTG G
PAPP-A	TCG TCA TTT CAT TCC TTC AGT CAT C	TTT TAG GGC CTT TGA GCT GCT G	TCT GTG TAG GTG ACC GGA GCA CTG AGA GG
PLAC1	ATT GGC TGC AGG GAT GAA AG	TGC ACT GTG ACC ATG AAC CA	CTG CGT TTT CAG CCG GTT CAG GA
GCM1	GGA GAA GCT CTT GTG CAA TTA GC	GTT TAT TGC ATG GTT CTC TCC AGA T	CCG GCC ATG TTT GAA TGC CAG TTT C
GAPDH	GAA GGT GAA GGT CGG AGT	GAA GAT GGT GAT GGG ATT TC	CAA GCT TCC CGT TCT CAG CC

[a]Dual-labeled fluorescent probes contained a reporter (FAM, 6-carboxyfluorescein) at the 5' end and a quencher (TAMRA, 6-carboxytetramethylrhodamine) at the 3' end.

thermal cycler (Applied Biosystems, Foster City, CA) was performed in two steps: 60 min at 37°C, followed by 5 min at 93°C.

Real-time quantitative PCR

Real-time PCR analysis was performed by use of a PE 7700 Sequence Detection System (Applied Biosystems). Each primer set and TaqMan probes for *βhCG*, *hPL*, *PAPP-A*, *PLAC1*, *GCM1* and *GAPDH* genes are listed in Table 2. Primers and probes for *βhCG*, *hPL*, and *GAPDH* were prepared as described previously[10]. Calibration curves for quantitation of each mRNA were prepared by assaying serial dilutions of high performance liquid chromatography (HPLC)-purified single-strand synthetic DNA oligonucleotides from each PCR amplicon with concentrations ranging from 10^7 to 10 copies/ml. Concentrations are expressed as cDNA copies/ml.

For PCR analysis, 20 μl of reaction volume contained 2 μl of cDNA. The reaction mixture contained amplification primer (200 nM for each mRNA), dual-labeled probe (100 nM for each gene), and the necessary components provided in the TaqMan Universal PCR Master Mix (Applied Biosystems). We analyzed all samples in duplicate and determined the mean concentrations. Several negative water blanks were included in each real-time quantitative PCR analysis. No amplification was observed for this control analysis, indicating specificity of the assays for the respective mRNAs. The thermal profile of incubation was 2 min of initial incubation at 50°C, followed by a 10 min initial denaturation step at 95°C and then by 40 cycles of 1 min each at 60°C plus 15 sec at 95°C.

Statistical analysis

Statistical analysis was performed with Stat View software (ver. 5.0; SAS Institute, Cary, NC). The Mann-Whitney test was used for comparison of maternal plasma mRNA concentrations between pre-eclamptic and control groups.

RESULTS

Occurrence of placental mRNAs in plasma during normal pregnancy and clearance after delivery.

We first examined the time course of quantitative changes in five placental mRNAs during and after normal pregnancy. Concentrations of *hPL* mRNA gradually increased during pregnancy to peak (respective medians: 2346.2 and 2397.9 copies/ml) at 36 weeks and just before delivery, while mostly disappearing by the first day of the puerperium (Fig. 1A). *βhCG* mRNA showed its highest concentration (median: 2819.6 copies/ml) at 11 weeks of pregnancy; although it then declined, it slightly increased just before delivery, then largely disappeared by the first day of the puerperium (Fig. 1B). *PAPP-A* mRNA increased gradually during pregnancy, attaining highest concentrations just before and after delivery (respective medians: 5410.0 and 6498.4 copies/ml), and then decreasing on the first day of the puerperium (Fig. 1C). Some *PAPP-A* mRNA also was detected in nonpregnant women; concentrations in subjects 1 month after delivery had fallen to approximately those in nonpregnant subjects. *PLAC1* mRNA concentrations increased gradually during pregnancy, being highest just before and after delivery (respective medians: 880.5 and 1672.4 copies/ml), then showing a decrease on the first day of the puerperium, followed by disappearance at 1 month (Fig. 1D). *GCM1* mRNA increased gradually during pregnancy, with highest concentrations before and after delivery (respective medians: 1966.3 and 1857.8 copies/ml). A decrease was evident by the first day of the puerperium, and *GCM1* mRNA had disappeared at 1 month after delivery (Fig. 1E).

Apart from *PAPP-A*, no placentally associated mRNAs were detected in nonpregnant subjects. On the other hand, in subjects 1 month after delivery only *PAPP-A* mRNA was detectable. A transcript from a house keeping gene, *GAPDH* mRNA, was detectable and showed a constant level in all samples including those where no other mRNA could be detected.

Placentally related mRNA concentrations in pre-eclampsia vs. normal pregnancy.

Median concentrations of each mRNA measured in plasma at 36 weeks of gestation from pre-

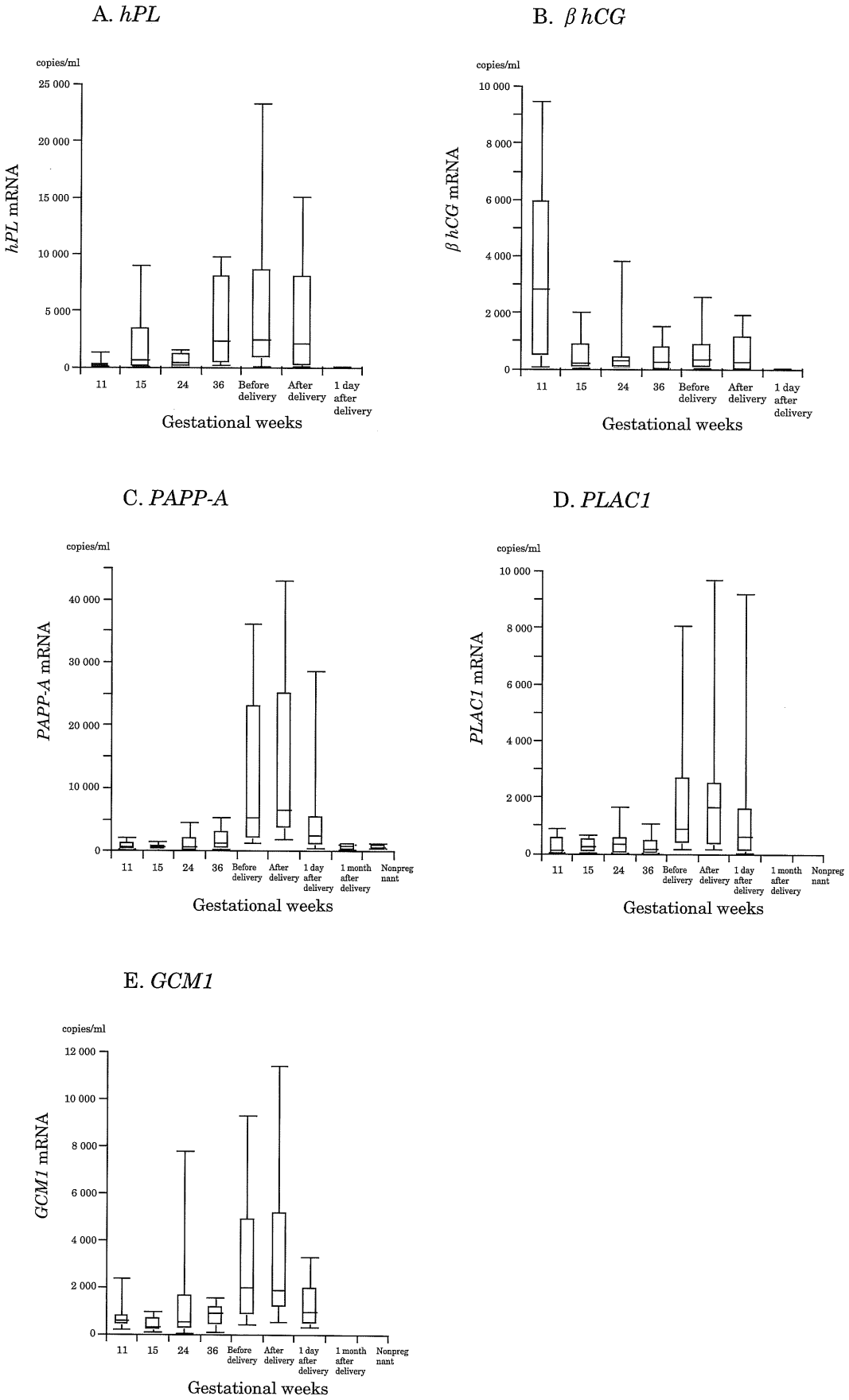

Fig. 1. Serum concentrations of various placental mRNAs.
A. *hPL*, B. *βhCG*, C. *PAPP-A*, D. *PLAC1*, E. *GCM1*
Horizontal lines within the boxes denote medians. Boxes indicate intervals between the 25th and 75th percentiles. Bars denote intervals between the 10th and 90th percentiles.

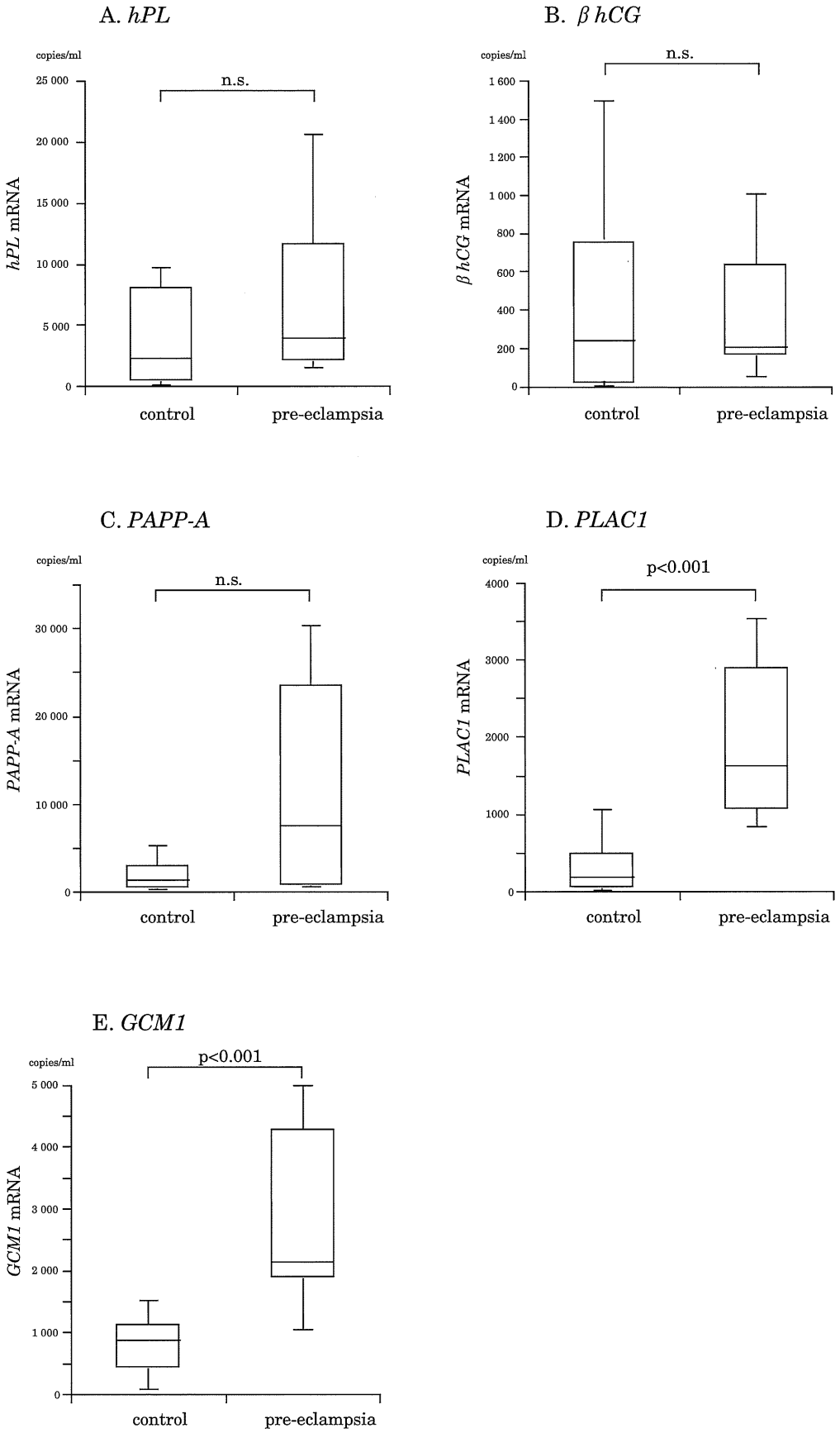

Fig. 2. Comparison of placental mRNAs in plasma between patients with pre-eclampsia and others with uncomplicated pregnancies.
A. *hPL*, B. *βhCG*, C. *PAPP-A*, D. *PLAC1*, E. *GCM1*
Horizontal lines within the boxes denote medians. Boxes indicate intervals between the 25th and 75th percentiles. Bars denote intervals between the 10th and 90th percentiles.

eclamptic women and healthy pregnant women, respectively, were *hPL*, 788 copies/ml (interquartile range, 233 to 3562) and 375 copies/ml (interquartile range, 16.6 to 2162) (Fig. 2A); *βhCG*, 386 copies/ml (interquartile range, 165 to 1080) and 244 copies/ml (interquartile range, 0 to 2598) (Fig. 2B); and *PAPP-A*, 6470 copies/ml (interquartile range, 4891 to 27731) and 1322 copies/ml (interquartile range, 269 to 9781) (Fig. 2C). The concentrations of these mRNAs showed no significant difference between pre-eclampsia and normal pregnancy.

The concentration of *PLAC1* mRNA was significantly higher in the pre-eclampsia than in the normal group ($p<0.001$). Median *PLAC1* mRNA concentrations in plasma from pre-eclampsia and normal pregnancies were, respectively, 1625 copies/mL (interquartile range, 804 to 3311) and 195 copies/ml (interquartile range, 1.9 to 2301) (Fig. 2D). Thus, the median plasma *PLAC1* mRNA concentration was 8.3 times higher in pre-eclampsia than in normal pregnancies. *GCM1* mRNA concentrations were also significantly higher in pre-eclampsia than in controls ($p<0.001$). Median *GCM1* mRNA concentrations in plasma from pre-eclampsia and normal pregnancies, respectively, were 2141 copies/ml (interquartile range, 856 to 5053) and 881 copies/ml (interquartile range, 0 to 1401) (Fig. 2E). Accordingly, the median plasma *GCM1* mRNA concentration was 2.4 times higher in pre-eclampsia than in normal pregnancies.

DISCUSSION

The present study is the first to report the effects of pregnancy and pre-eclampsia on several placental mRNAs assayed in maternal plasma samples. The mRNA for *hPL* showed a time course in agreement with previous reports, disappearing rapidly following delivery, after having peaked at 36 weeks[10]. The sensitivity of our assay was high in comparison to that used in a previous report[10]: where Ng et al. could no longer detect *βhCG* mRNA in the third trimester, we could detect it throughout gestation.

As mRNAs not previously reported, we examined *PAPP-A*, *PLAC1*, and *GCM1* mRNA concentrations in maternal plasma. Initially, we assumed that *PAPP-A* mRNA was placenta-specific, and therefore undetectable in the nonpregnant state. In fact, mRNA for *PAPP-A* was detected in our nonpregnant subjects, consistent with a report of *PAPP-A* protein being detected in ovarian follicles in surprisingly high concentrations[12], and even with *PAPP-A* mRNA detection in men[11]. Recently, placental expression of the *GCM1* gene was reported to decline significantly after 37 weeks of gestation[2]. In contrast, *GCM1* mRNA in our subjects peaked in the perinatal period. The *GCM1* mRNA concentration in maternal plasma appeared to be unrelated to the degree of *GCM1* expression in the placenta. Although *PLAC1* and *GCM1* mRNA were both reported to be undetectable 1 day after delivery[3,4], we could detect them on day 1 of the puerperium. The relatively long time required for clearance of *PLAC1* and *GCM1* mRNAs probably reflected the higher efficiency of the extraction kit, the reverse transcription kit using the present techniques.

Considering the relations of the concentration of mRNAs and their proteins, *hPL* and *βhCG* mRNAs were reported to have time courses similar to proteins[10]. *PAPP-A* mRNA had a different time course: where mRNA reached its highest concentration just after delivery and then rapidly decreased, the concentration of protein was reported to rise steadily during the course of pregnancy[16]. No report has described *PLAC1* and *GCM1* protein in the maternal circulation. Post-transcriptional modification could be considered as a cause which does not have any relation to mRNA and protein concentrations. It is also possible that there are some differences between the clearance of mRNAs and that of proteins in maternal plasma.

We next compared placental mRNA concentrations in pre-eclampsia with those in normal pregnancies to examine whether these mRNAs might serve as markers of trophoblast damage when detected in maternal plasma. At 36 weeks of gestation, the concentrations of *PLAC1* and *GCM1* mRNAs were significantly higher in pre-eclampsia than in normal pregnancies, although the concentrations of *hPL*, *βhCG* and *PAPP-A* mRNAs showed no significant difference. Thus *PLAC1* and *GCM1* mRNAs in maternal plasma might serve as new molecular markers for pre-eclampsia. One mechanism that might elevate *PLAC1* mRNA concentration in pre-eclamptic patient plasma could be leakage from damaged chorionic villus cell injured by pre-eclampsia. An alternative or complementary possibility might be the increased placental *PLAC1* gene expression involved in repairing the damaged trophoblast; this would resemble earlier increases in *PLAC1* mRNA expression during trophoblast differentiation[7]. *PLAC1* mRNA concentrations in maternal plasma might increase as a consequence. Although decreased placental *GCM1* mRNA expression has been reported in pre-eclampsia[2], mRNA concentrations in maternal plasma and expression in the placenta would not necessarily correlate. Previously Ng et al reported that the concentration of the mRNA encoding corticotropin-releasing hormone (*CRH*) in maternal plasma was significantly higher in pre-eclampsia than in uncomplicated pregnancy[8]. They suggested, as a possible mechanism for the increased *CRH* mRNA concentration in pre-eclampsia, that the increase in cell death within the placenta might contribute to the

increased release of mRNA into maternal plasma.

The expression of *PAPP-A* gene has been demonstrated in the placenta, including the syncytiotrophoblast, cytotrophoblast, and decidua[11]. If the trophoblast were damaged, we reasoned that *PAPP-A* mRNA concentrations in maternal plasma would increase. Although pre-eclamptic patients have been reported to have increased *PAPP-A* protein in serum compared with controls[1], our examination of circulating *PAPP-A* mRNA in pre-eclampsia did not show a significant difference from uncomplicated pregnancy. Any demonstration that this mRNA could be a marker of pre-eclampsia still awaits further investigation. No report has described a relationship between *hPL* or *βhCG* protein concentrations and the severity of pre-eclampsia. To the present we have found no consistent pattern of the corresponding mRNA concentrations that differentiated uncomplicated pregnancy from pre-eclampsia. If we had examined the concentrations of mRNAs at the first or second trimester, possibly other mRNAs would have shown differences between pre-eclamptic and control groups.

In conclusion, our results suggested that quantitative analysis of several placental mRNAs (e.g. *PLAC1* and *GCM1*) in maternal plasma might represent a useful way to monitor placental status. As a future view, if there is a mRNA which shows a significant difference from normal pregnancies before the pre-eclampsia onset, it would become a very useful precognition marker of pre-eclampsia.

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