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ORIGINAL PAPER

Pregnancy-specific beta-1-glycoprotein 6 is a potential novel diagnostic biomarker of placenta accreta spectrum

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Abstract

Early diagnosis is essential for the safer perinatal management of placenta accreta spectrum (PAS). We used transcriptome analysis to investigate diagnostic maternal serum biomarkers and the mechanisms of PAS development. We analyzed eight formalin-fixed paraffin-embedded placental specimens from two placenta increta and three placenta percreta cases who underwent cesarean hysterectomy at Sapporo Medical University Hospital between 2013 and 2019. Invaded placental regions were isolated from the uterine myometrium and RNA was extracted. The transcriptome difference between normal placenta and PAS was analyzed by microarray analysis. The PAS group showed markedly decreased expression of placenta-specific genes such as *LGALS13* and the pregnancy-specific beta-1-glycoprotein (PSG) family. Term enrichment analysis revealed changes in genes related to cellular protein catabolic process, female pregnancy, autophagy, and metabolism of lipids. From the highly dysregulated genes in the PAS group, we investigated the expression of PSG family members, which are secreted into the intervillous space and can be detected in maternal serum from the early stage of pregnancy. The gene expression level of *PSG6* in particular was progressively decreased from placenta increta to percreta. The PSG family, especially PSG6, is a potential biomarker for PAS diagnosis.

Keywords Placenta accreta spectrum · Diagnostic marker · Transcriptome analysis · Pregnancy-specific beta-1-glycoprotein

Introduction

Placenta accreta spectrum (PAS) is a disease that not only causes massive hemorrhage during delivery, but also may require hysterectomy when dissection of the placenta from the uterus is impossible [1]. PAS can damage adjacent organs such as the bladder and rectum through surgery or direct invasion and even result in maternal death [1]. PAS is classified into three major groups according to adhesion severity: placenta accreta, increta, and percreta. In the latter, the most severe PAS, the average blood loss without any prophylactic method is 4800 ± 9950 g [2]. It has been suggested that blood loss can be dramatically reduced by interrupting blood flow with interventional radiology [3]. Although characteristic complications were reported with prophylactic interventional radiology [4], it can significantly reduce the risk of massive hemorrhage and maternal death.

Repeated cesarean section and placental previa are the most important risk factors for the development of PAS [5]. Recently, IVF has also been shown to be a risk factor for the development of PAS [6]. Therefore, the frequency of PAS may continue to increase not only because of the increasing age of pregnant women, but also because of the expansion of IVF technology. Accordingly, a reliable method for the early diagnosis of PAS is an essential issue in perinatal care, now and in the future. However, the diagnosis of PAS is limited to ultrasound or MRI imaging. According to guidelines and expert opinion, ultrasonography is the most useful tool for PAS diagnosis [7]. The ultrasonographic diagnosis of PAS principally relies on disappearance of the normal uteroplacental interface (clear zone), extreme thinning of the underlying myometrium, and vascular changes within the placenta

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(lacunae) and placental bed (hypervascularity) [8]. However, the detection sensitivity is limited to about 90%. MRI can be used as a secondary examination if necessary [7], but some controversial reports suggest that MRI is not useful because it can be misleading [9]. Thus, there is currently no reliable method for the preliminary diagnosis of PAS.

Due to the above situation, various attempts have been made to establish biomarkers. Although several studies have suggested that placenta-specific proteins such as plasma protein-A (PAPP-A) and alpha-fetoprotein (AFP) may be useful for the detection of PAS [10], their lack of disease specificity and diagnostic accuracy has undermined their practical application. In recent years, the ability of omics analysis to detect biomarkers has been explored. A study using plasma proteomics by SOMAscan analysis (SomaLogic, Inc., Boulder, CO) detected the presence of antithrombin III and medial plasminogen activator inhibitor [11]. However, no placenta-specific marker was identified due to the limited library used in the SOMAscan analysis. In another investigation, microarray transcriptome analysis of fresh cytotrophoblasts from PAS showed increased expression of the *DOCK4* gene, which is thought to be involved in cancer invasion. The association of trophoblast invasion with *DOCK4* was only confirmed in this work, and no diagnostic biomarker has been established [12]. Although not an omics analysis, a new technology has been reported to capture circulating trophoblast (cTB) cells from maternal blood using a NanoVelcro chip [13]. This novel method is able to distinguish PAS from non-PAS with very high accuracy (area under the curve, 0.943) by detecting cTB cell clusters [13].

In the present study, we aimed to establish a biomarker for the diagnosis of PAS by harvesting placenta from the junction of the myometrium and placenta in patients with severe PAS for microarray transcriptome analysis. We found that the expression of pregnancy-specific beta-1-glycoprotein (*PSG*) family genes, especially *PSG6*, decreased with the severity of placental invasion. The PSG family can be identified in maternal blood as protein [14, 15] or cell-free mRNA [16], suggesting that PSG6 may be useful as a new biomarker for PAS diagnosis.

Materials and methods

Patients and specimens

Surgical specimens from placenta increta and percreta patients requiring hysterectomy at the time of cesarean delivery were harvested. In these patients, the uterus and placenta were removed at the same time because of the difficulty in releasing the adhesions. We also harvested normal placenta for a negative control of placental invasion from patients who underwent repeated cesarean sections, at around 37 weeks of gestation. All patients had been treated at Sapporo Medical University Hospital from 2013 to 2019. We analyzed formalin-fixed paraffin-embedded (FFPE) samples as described below and used two samples from different sites of the invasion for placenta percreta cases because of their severe placental invasion into the myometrium. The patients are summarized in Table 1.

Total RNA isolation from FFPE placenta specimens and microarray analysis

FFPE blocks of placental specimens were sliced into 5-µm-thick sections, and the placental region was manually macrodissected from uterine myometrium using a scalpel for PAS specimens (Fig. 1). Macrodissection was not performed for control FFPE placenta specimens and a whole slice was used for analysis. RNA was isolated from FFPE placenta specimens using an RNeasy FFPE Kit (Qiagen, Valencia, CA) following the manufacturer's protocol and as previously reported [17]. The Agilent 2100 Bioanalyzer microchip electrophoresis system and Agilent RNA 6000 Nano Kit (Agilent Technologies Inc., Santa Clara, CA) were used to determine RNA integrity number (RIN) values (Table S1). Purified

Table 1 Clinical backgrounds of the specimens used in the microarray analysis

Group	Case No	Sample No	Disease	Operative date (year)	Surgical method	Gestational age at delivery	Birth weight (g)	Apgar score (1/5 min)	Blood loss (g)
PAS	1	1, 2	Percreta	2015	Cesarean hysterectomy (subtotal)	34w5d	2436	5/8	7180
	2	3	Increta	2013	Cesarean hysterectomy	37w1d	2146	8/9	720
	3	4	Increta	2019	Cesarean hysterectomy	32w6d	1952	7/8	1410
	4	5, 6	Percreta	2018	Cesarean hysterectomy	34w3d	2112	6/8	2600
	5	7, 8	Percreta	2019	Cesarean hysterectomy	31w1d	1888	6/7	7700
Control	6	9	Repeat C/S	2019	Cesarean section	37w1d	2374	8/9	142
	7	10	Repeat C/S	2019	Cesarean section	36w6d	2504	8/9	645
	8	11	Repeat C/S	2019	Cesarean section	37w1d	3094	8/9	1128

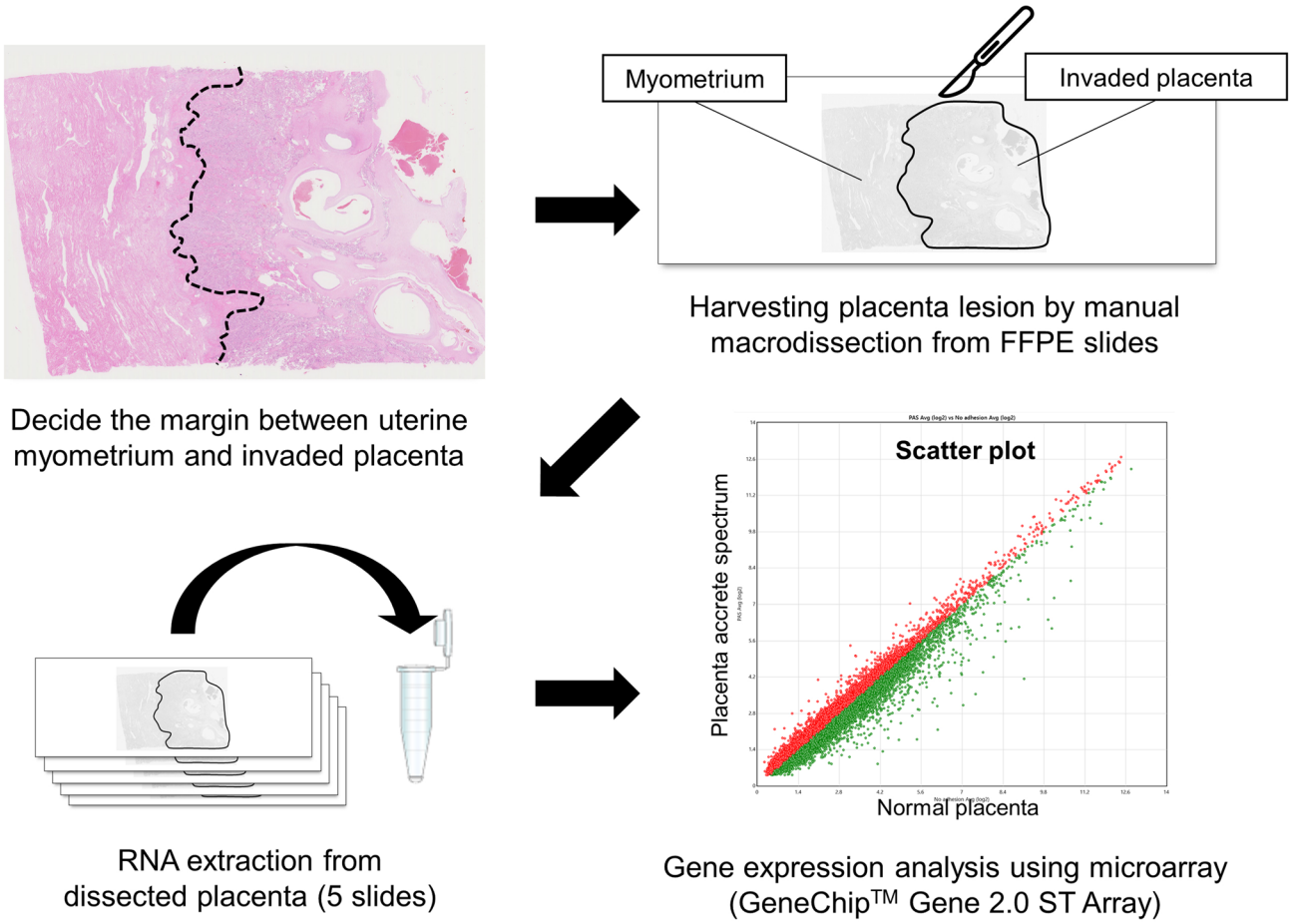

Fig. 1 Outline of the macrodissection of FFPE placenta specimens and microarray preparation. Invaded placenta was separated from FFPE slides by macrodissection. Five slices were used for each specimen in RNA extraction

total RNA was used as input for cDNA preparation using the GeneChip™ WT Pico Reagent Kit (Affymetrix, Thermo Fisher Scientific, Waltham, MA). Fragmented and labeled samples were hybridized to GeneChip™ Human Gene 2.0 ST Arrays (Affymetrix, Thermo Fisher Scientific). Scanning was performed on a GeneChip™ Scanner 3000 7G (Affymetrix, Thermo Fisher Scientific). The resulting CEL files were loaded into the Transcriptome Analysis Console 4.0 software package (TAC 4.0, Affymetrix) for data analysis. Genes were filtered (|fold change: FC|> 1.2, log2 scale; $p < 0.05$) for the identification of differentially expressed genes. In addition, only probes linked to NM RefSeq accession IDs were included. Enrichment analysis was performed using Metascape [18]. The raw microarray data have been deposited in the NCBI GEO database (GSE189267).

Immunohistochemical staining and quantification of intensity

After epitope retrieval using Novocastra Epitope Retrieval Solution (pH 9) and quenching of endogenous peroxidase activity using 3% peroxidase, sections (5-μm-thick) of FFPE placental specimens were immunostained using antibodies. To stain specimens, we used a monoclonal antibody against PSG6 (1:1000; MAB8598, R&D) and a polyclonal antibody against human chorionic gonadotropin (hCG) (1:1000; A0231, DAKO). Subsequent incubation with a biotinylated secondary antibody was performed. Slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohol to a nonaqueous solution, and coverslipped with mounting medium. The intensity of PSG6 staining was assessed as strong (3), moderate (2), weak

(1) or negative (0). And proportions of positively stained trophoblast regions were recorded for each intensity level. Finally, we calculated an Immunoreactivity score (IRS) for each slide by multiplying the intensity level with the proportion and summing them as previously reported (i.e., intensity 1 × proportion 0.2 + intensity 2 × proportion 0.5 + intensity 3 × proportion 0.3 = immunoreactive score 2.1) [19, 20]. The score was statistically analyzed by two-tailed student's *t* test.

Results

Clinical backgrounds of PAS cases

The clinical backgrounds of the patients analyzed in this study are summarized in Table 1. Of the five PAS patients, three had placenta percreta and two had placenta increta, and all required total hysterectomy due to difficult placental removal from the uterus during cesarean section. All PAS cases were finally diagnosed by pathologists by histological examination. The mean number of weeks of gestation was 33.6 weeks, and four of the five cases gave birth in the preterm period. The average amount of blood loss at the time of surgery was 3922 g, but all mothers and babies survived. Placentas from repeat cesarean section patients were used as negative controls. All placentas of control cases detached smoothly from the uterus, and no abnormalities were observed grossly or histologically.

Unique gene expression signatures in PAS groups

Expression variations were examined in 1,010 genes after statistical filtering. Of the 1010 genes, 254 had upregulated expression in PAS and the remaining 756 genes had decreased expression. This bias was highly pronounced in the volcano plot (Fig. 2A). In hierarchical clustering, PAS and normal placenta were clearly classified by gene expression patterns, and the sample number of each specimen is shown in parentheses. However, samples No. 1 and 2 were classified into different clusters within the PAS cluster, even though they were from the same patient. This result suggests site-specific heterogeneity of gene expression even within placenta.

The genes from the highest to lowest fold change were compared under various conditions and are summarized in Table 2 and Supplementary Fig. 1. The upper part of the table compares normal placenta and PAS while the lower part compares placenta increta and percreta. Notably, all of the genes with low expression in PAS were placenta-specific genes, and four of them belonged to the *PSG* family. A comparison of placenta increta and percreta revealed placenta-specific gene changes. Notably, gradual downregulation of

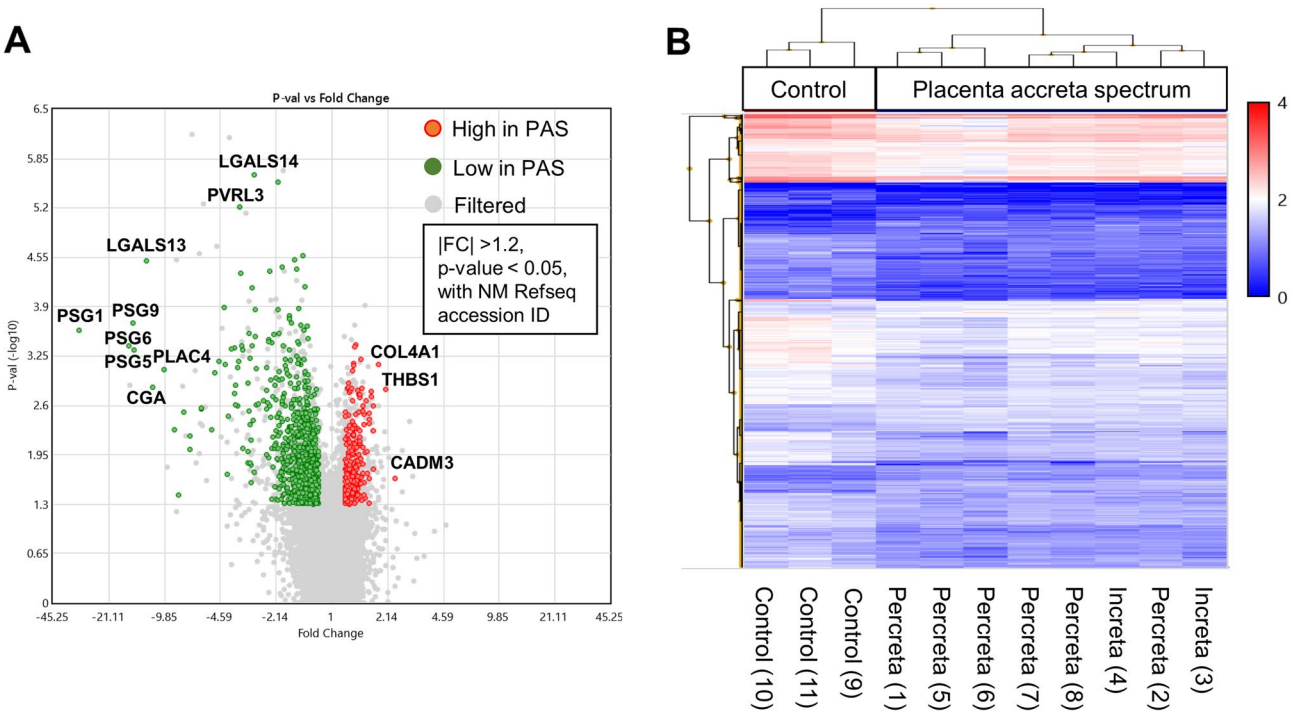

Fig. 2 Profiling of variable genes in PAS from normal placenta. **A** Volcano plot of gene expression analysis. Genes showing significant differences are displayed as a volcano plot (|FC|> 1.2; $p < 0.05$). **B** Hierarchical clustering of genes. Hierarchical clustering clearly separated the two groups, normal placenta and PAS

Table 2 List of genes showing increased variability with placental invasion

Control vs PAS	PAS avg (log2)	Control avg (log2)	Fold change	*p* value	Refseq number	Gene symbol	Description
High expression in PAS	4.11	2.85	2.39	0.0229	NM_001127173	CADM3	Cell adhesion molecule 3
	5.71	4.64	2.1	0.0016	NM_003246	THBS1	Thrombospondin 1
	3.23	2.4	1.77	0.0054	NM_001145346	RBMXL3	RNA-binding motif protein, X-linked-like 3
	6.18	5.36	1.77	1.71E-02	NM_003525	HIST1H2BI	Histone cluster 1, H2bi
Low expression in PAS	4.13	9.12	−31.68	0.0003	NM_001184825	PSG1	Pregnancy-specific beta-1-glycoprotein 1
	6.07	10.07	−16.05	0.0004	NM_001031850	PSG6	Pregnancy-specific beta-1-glycoprotein 6
	3.12	7.04	−15.13	0.0002	NM_001301707	PSG9	Pregnancy-specific beta-1-glycoprotein 9
	3.54	7.45	−15.08	0.0005	NM_001130014	PSG5	Pregnancy-specific beta-1-glycoprotein 5
	1.79	5.44	−12.56	3.11E-05	NM_013268	LGALS13	Lectin, galactoside-binding, soluble, 13
Increta vs percreta	Percreta Avg (log2)	Increta Avg (log2)	Fold change	*p* value	Refseq number	Gene symbol	Description
High expression in percreta	3.46	1.7	3.38	0.0006	NM_001006933	TCEAL3	Transcription elongation factor A (SII)-like 3
	2.84	1.75	2.12	0.0081	NM_005557	KRT16	Keratin 16, type I
	2.03	0.97	2.07	0.0186	NM_005747	CELA3A; CELA3B	Chymotrypsin-like elastase family, member 3A; chymotrypsin-like elastase family, member 3B
	3.43	2.47	1.94	0.0153	NM_001030287	ATF3	Activating transcription factor 3
	2.39	1.54	1.81	0.0223	NM_203400	RPRML	Reprimo-like
Low expression in percreta	5.49	7.68	−4.54	0.0461	NM_001031850	PSG6	Pregnancy-specific beta-1-glycoprotein 6
	3.96	5.78	−3.52	0.0295	NM_182832	PLAC4	Placenta-specific 4
	1.51	3.05	−2.91	0.0005	NM_019111	HLA-DRA	Major histocompatibility complex, class II, DR alpha
	2.24	3.68	−2.7	0.0368	NM_018327	SPTLC3	Serine palmitoyltransferase, long chain base subunit 3
	4.07	5.42	−2.55	0.0159	NM_002775	HTRA1	HtrA serine peptidase 1

PSG6 was observed from placenta increta to percreta, suggesting that its expression changes with disease severity.

Gene expression enrichment of biological processes and related diseases in PAS

As mentioned above, expression changes in placenta-specific genes such as those of the *PSG* family tended to be decreased in the PAS group. Therefore, we performed enrichment analysis of the genes with decreased expression in the PAS group ($FC < -1.2$; $p < 0.05$). The results of term enrichment analysis are shown in Fig. 3A. The top-ranked terms were cellular protein catabolic process, female pregnancy, autophagy, and metabolism of lipids, which are mainly related to response to invasion and stress. Figure 3B shows the results of disease-related genes using the DisGeNET database [21]. The top-ranked diseases were choriocarcinoma and pre-eclampsia and the results indicated that the genes changed in PAS were linked to the regulation of choriogenesis and invasion.

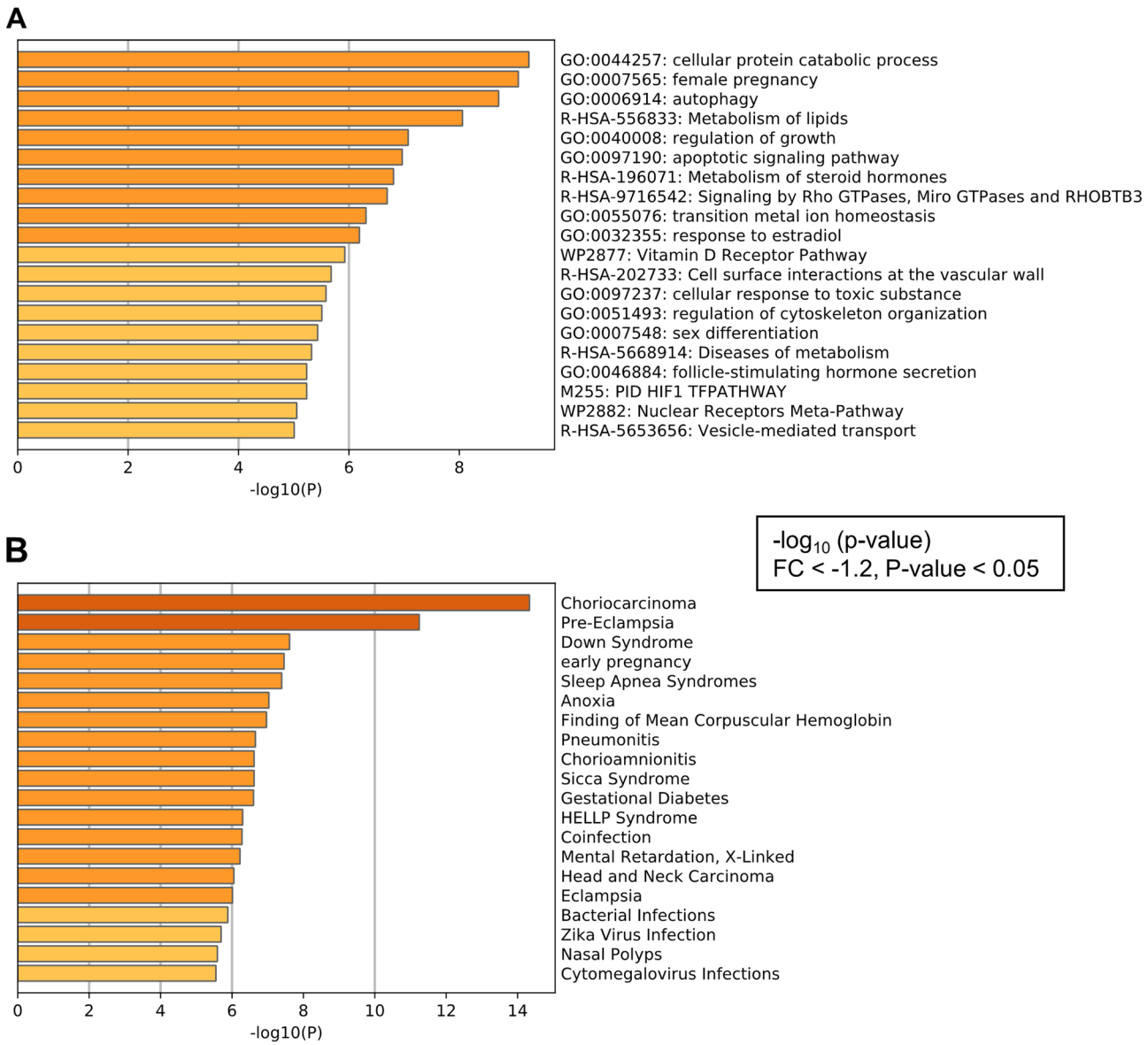

Fig. 3 Functional enrichment analysis of mRNA expression in PAS. **A** Enriched terms across dysregulated gene lists. Result of term enrichment analysis in dysregulated genes in PAS ($FC < -1.2$, $p < 0.05$) using the Metascape database. **B** Summary of enrichment analysis in DisGeNET. Enrichment analysis using the DisGeNET database revealed the related diseases with variable genes in PAS from normal placenta

Different PSG6 expression pattern in the syncytiotrophoblast in PAS

Because PSG6 is secreted from the syncytiotrophoblast, we performed immunohistochemical staining with hCG to check the development of the syncytiotrophoblast in PAS. hCG expression was clearly higher in placenta percreta than in normal placenta, suggesting trophoblast overgrowth in PAS (Fig. 4). Immunostaining for PSG6 in the same specimens showed a marked decrease in PSG6 expression in the placenta percreta specimen, as in the microarray results. In addition, the expression of PSG6 tended to be lower in PAS than in the control group, although there were some cases of high expression in placenta increta (Case 3, Sample 4) and percreta (Case 5, Samples 7 and 8) (Fig. 5A). The immunoreactivity score (IRS) of PSG6 showed a decrease in progress of placental invasion (control: 2.60 ± 0.10, increta: 2.45 ± 0.35, percreta: 1.92 ± 0.26). The IRS was significantly lower in percreta specimens compared to control specimens

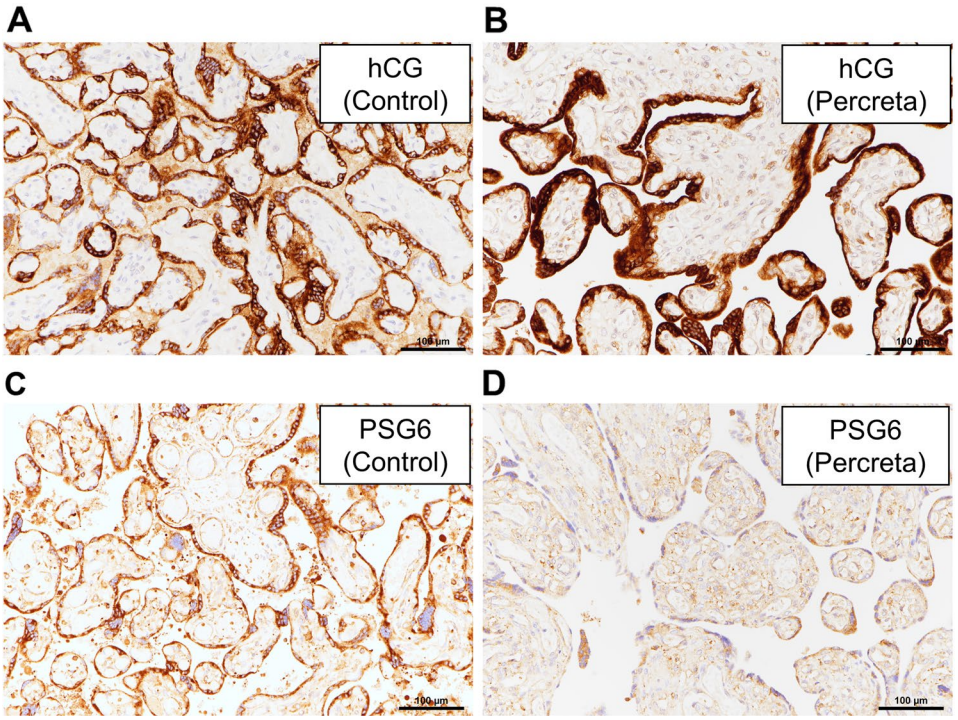

Fig. 4 Development of the syncytiotrophoblast in PAS and PSG6 expression. Immunohistochemical staining of FFPE control placenta (**A**, **C**; Case 6, Sample 9) and placenta percreta (**B**, **D**; Case 1, Sample 1) are shown. Images **A** and **B** were stained for hCG and C and D were stained for PSG6, as labeled. All photomicrographs were taken at × 200 magnification

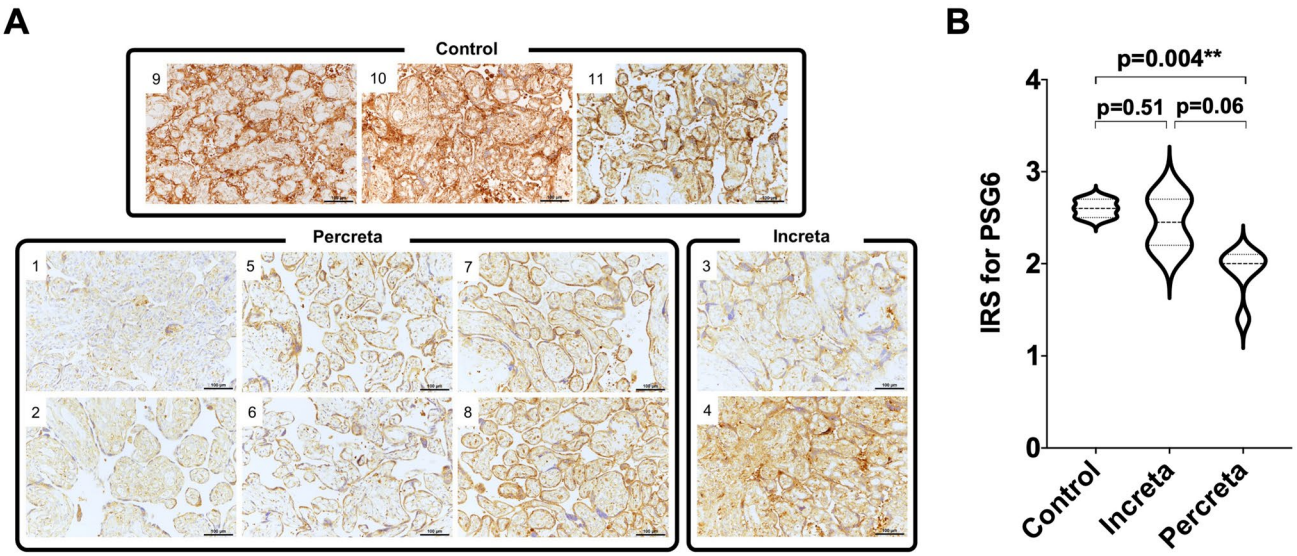

Fig. 5 PSG6 expression in whole placental specimens. **A** Immunohistochemical staining for PSG6 of PAS and control specimens. All FFPE placental specimens analyzed in microarray were immunohistochemically stained with PSG6 monoclonal antibody. The images are labeled with each sample number and grouped into control, percreta, and increta. All photomicrographs were taken at × 200 magnification. **B** Quantitative analysis of PSG6 intensity. Violin plot of immunoreactivity score (IRS) of PSG6 immunohistochemical staining. IRS of PSG6 in each PAS status were statistically analyzed by student's *t* tests

(Fig. 5B). However, there was no significant difference in scores between control specimens and increta specimens.

Discussion

In this study, we performed microarray transcriptome analysis using placenta increta and percreta specimens and

confirmed that there were significant changes in various placenta-specific genes. The main purpose of this study was to investigate diagnostic biomarkers for PAS, and we were able to extract PSG family members, especially PSG6, as candidates that can be detected in maternal blood [14, 16]. In addition to the placenta-specific genes, various other genes related to PAS mechanisms were also identified by transcriptome analysis. The terms cellular protein catabolic process, autophagy, and metabolism of lipids were significantly extracted by enrichment term analysis (Fig. 3A). All of these terms are associated with cell responses to stresses such as hypoxia and invasion. Loss of autophagy in a trophoblast cell line has been reported to markedly reduce cell invasion and vascular remodeling, especially under hypoxic conditions [22, 23]. The results of term enrichment analysis suggested that excessive trophoblast invasion may result in physiological inhibitory control of the advanced invasive region of the adherent placenta.

Although the members of the PSG family are some of the most abundantly expressed proteins in the placenta and have been reported since the 1970s [14], their function remains unclear. A total of 11 PSG family genes have been reported in humans. All of the PSG family are members of the immunoglobulin (Ig) superfamily and are clustered in a very short region on the long arm of chromosome 19 [15, 24]. However, most reports on PSGs are limited to PSG1. For PSG6, which was identified here, reports have suggested an association with the prognoses of stomach adenocarcinoma [25] and gestational diabetes [26], but no individual function has been reported. We also constructed a PSG6 overexpressed choriocarcinoma cell-line and examined changes in cell invasive ability. However, no significant difference was observed in the matrigel invasion assay when compared to the mock control (data not shown). PSG6 does not appear to be a direct regulator of cell invasion, at least in choriocarcinoma cells. The members of the PSG family are highly homologous, and PSG1 has been suggested to have domain-specific functions [27, 28]. Many members of the PSG family, including PSG1 and PSG6, are composed of four domains—N, A1, A2 and B2—and may exhibit similar traits [15].

As one of the pathogenic mechanisms of PAS, extensive neovascularization stands out [10]. Uteroplacental vascular remodeling failure is a contributor to early-onset preeclampsia, which can lead to placental ischemia and various conditions such as intrauterine fetal growth restriction [29]. In contrast, overexpression of angiogenesis-related factors such as VEGF and Ang-2 has been identified in PAS [30]. In addition, sFlt-1, which is upregulated in early onset preeclampsia with inefficient placental invasion [31], is downregulated in placenta increta and percreta [32]. Therefore, preeclampsia and PAS may be considered polar conditions in placental angiogenesis. Although there are only a few reports on the PSG family in relation to placental angiogenesis, administration of PSG1 increases the expression of VEGF-A via increased expression of TGF-β1 [33]. In addition, the B2 domains of PSG1, PSG6, and PSG9 induce angiogenesis in endometrial and trophoblastic cell lines [27]. In the clinical situation, there are reports that PSG1 is decreased in preeclampsia [34] and that it is associated with small-for-gestational-age fetuses [35], indicating the importance of the PSG family in placental vascularization. In the term enrichment analysis in this study, preeclampsia was the second most relevant disease (Fig. 3B), strongly suggesting that the disease mechanism of PAS is partially shared with that of preeclampsia.

We also assume that the PSG family may be related to the pathogenesis of PAS in terms of immunological regulation. Decidual natural killer (dNK) cells are thought to regulate immunological tolerance during trophoblast invasion in early-phase pregnancy [36]. In addition, dNK cells have been found to be significantly decreased in intraoperative decidual biopsy of PAS [37]. Therefore, loss of function of dNK may be one of the important factors in trophoblast overinvasion in PAS. dNK cell activity has been suggested to be decreased by elevated TGF-β1 in preeclampsia [38]. On the other hand, PSG1 forms a complex with TGF-β1 [39] and also activates the latent isoforms of TGF-β1 and -2 [28]. Therefore, we believe that PSG may also regulate trophoblast invasion via TGF-β1-mediated regulation of dNK cell activity in PAS.

In the future, we must consider the expression level of PSG in the serum when applying these results to clinical practice. Although the 11 PSG family members have a high degree of homology [15], it is not clear whether the antibodies [13] used in older studies were able to classify them adequately [14]. The expression level of each *PSG* mRNA depends on the stage of pregnancy [40], but the actual course of the *PSG* level during pregnancy is not yet clear. Although PSG1 can be detected in serum, validation of other PSG families is needed. However, cell-free mRNA of placental origin can be identified in maternal blood [16] and it has been suggested that these placental-specific mRNAs are increased in maternal blood in PAS patients [41]. These mRNAs include PSG1, -2, -3, -5, -6, and -9. If PSG6 is chosen as a diagnostic biomarker, it will be possible to establish a more exact cutoff value using cell-free mRNA of maternal blood when we could not establish it as a serum protein marker. However, there is an important report regarding cTB cells captured using NanoVelcro chips [13]. The authors showed that the gene expression of PSG1, -2, -3, and -11 was upregulated in the PAS group when compared with the normal placental control in cTB cells [13]. Our transcriptome analysis found the opposite results regarding the expression level of PSG1. This indicates that the gene expression of cTB cells and of locally invaded placenta may be different. Because gene

expression is often different between metastasis sites and primary sites of cancers, care is also required when circulating cells and cell-free RNA are being assessed in PAS.

The strength of this study is that the specimens were precisely sampled from the advanced placental invasion area. Because the specimens were dissected by macrodissection from the placental invasion area into the uterine myometrium, we consider our method to be more accurate for detecting biomarkers than previous studies. In addition, the results of gene enrichment analysis were consistent with the characteristics of PAS, suggesting that the targeted gene analysis was successful. However, the most important limitation to consider is that the samples used in this analysis were FFPE specimens. In general, FFPE specimens are not ideal for expression analysis because RNA degradation is more advanced than in frozen specimens. Nonetheless, a previous report compared the microarray results of FFPE and frozen specimens and showed a correlation coefficient of 0.8 or better [42]. Although the RIN value was reduced to about 2, as in the present study (Table S1), the specimens seemed to provide sufficient analytical results. Therefore, we believe that the results of the present study are also valuable for the precise analysis of PAS.

Conclusions

The PSG family is very promising as potential diagnostic biomarkers of PAS. The PSG family is also likely to play a role in the regulation of placental growth, and further functional analysis is warranted regarding therapeutic targets.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00795-023-00371-y.

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Author contributions HK, YF: data collection, manuscript writing. TM, MU, SO, TK, MS, TB: data collection and analysis. TS, TT, YH: project development. SI: manuscript editing.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethics approval and consent to participate Informed consent was obtained in the form of opt-out on the website of Sapporo Medical University. Patients who declined to give consent were excluded. This investigation of PAS was approved by the Ethics Committee of Sapporo Medical University of Medicine.

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