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Original Article

Joint effect of plasma C21orf105 and PLAC4 on diagnosing fetal trisomy 21

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Abstract: This study is proposed to investigate whether the expressions of C21orf105 and PLAC4 could identify trisomy 21 fetus during pregnancy. 38 females with fetal trisomy 21 and 40 healthy controls were admitted in the second-trimester between November 2011 and November 2013. Trisomy 21 was diagnosed based on the karyotype. We conducted RT-PCR with different storage time and temperature in order to evaluate their effects on blood sample stability *in vitro*. RT-PCR and western blot were conducted to evaluate the expressions of C21orf105 and PLAC4. In addition, ROC curve was generated to estimate the sensitivity and specificity of C21orf105, PLAC4 and their combination. There were no significant differences in age, height, weight, BMI index, weeks at blood-taking, nulliparous and family history of trisomy 21 between the case and control group (all $P > 0.05$). The expression levels of both C21orf105 and PLAC4 are up-regulated in trisomy 21 pregnancies compared to the control group (all $P < 0.05$). Importantly, AUC of C21orf105, PLAC4 and their combination were 0.77 (95% CI: 0.65-0.88), 0.90 (95% CI: 0.82-0.97) and 0.93 (95% CI: 0.86-0.99), respectively. The combination of C21orf105 and PLAC4 could be used for diagnosing fetal trisomy 21 during the second-trimester period of pregnancy.

Keywords: Trisomy 21, C21orf105, PLAC4, plasma, diagnosis

Introduction

Down's syndrome (DS), also known as trisomy 21, is characterized by Chromosome 21 abnormity occurred during pregnancy and it could result in congenital defects and mental disorders [1]. Common clinical symptoms of DS include congenital mental retardation, prolonged physical growth and intellectual impairment and eventually disability and even death could occur [1]. The incidence of DS ranges between 1/600 and 1/800, approximately two-thirds of DS cases may result in spontaneous abortion or fetal intrauterine death [2]. Since no effective treatment for DS has been developed, ongoing care for patients with DS imposes huge long-term social and economic burdens. Hence, prevention of DS during prenatal stages is critical to tackle challenges resulted from this disease [3]. Karyotype analysis is the "gold standard" for prenatal diagnosis of DS, but it requires invasive procedures to obtain fetal cells, such as amniotic fluid, umbilical cord blood or chorionic villi [4]. Studies have suggested that free fetal DNA contained in maternal blood has provided feasibility for identifying fetal chromosomal abnormality [5, 6]. Free fetal DNA contained in maternal blood could precisely identify fetal chromosomal abnormality during early pregnancy stages and its concentration is increased with gestational age [7]. Studies have suggested that the concentration of free fetal DNA is increased in pregnant female with fetal DS and it can provide assistance for diagnosing fetal aneuploidy diseases [8]. Since placenta specific gene expression was steadily increased with gestational age, the plasma mRNA of placental genes could be considered as a noninvasive fetal markers for prenatal screening and diagnosis [9].

The current available biochemical markers that are used for diagnosing DS are associated with low sensitivity and a relatively high false-positive rate. Therefore, most pregnancies that are positively diagnosed as fetal trisomy 21 will undergo amniocentesis which may result in

Table 1. The primer sequences of C21orf105 and PLAC4

Gene		Primer sequences
C21orf105	F	5'-CGCTGAGTACGTCGTGGAGTC-3'
	R	5'-GCTGATGATCTTGAGGCTGTTGTC-3'
PLAC4	F	5'-GGAGTATGTGGCAGTCGTAATGG-3'
	R	5'-TTTCAGGGGGAGTTGTTGGTTT-3'
β-actin	F	5'-GTTGCGTTACCCTTTCTTGAC-3'
	R	5'-CTCGGCCACATTGTGAACTTTG-3'

F, forward; R, reverse; β-actin as a loading control.

fetal mortality [10]. Therefore, specific markers that are able to increase the precision of detection and reduce the false-positive rate should be discovered. Since a large number of different endogenous retrovirus families are highly expressed in normal placental tissues, endogenous retroviruses may have important functions in reproduction [11]. C21orf105 gene is part of an endogenous retrovirus provirus and it is encoded by the chromosome 21 and located in the Down syndrome critical region (DSCR) [12]. It has been reported that the expression of C21orf105 was up-regulated in trisomy 21 placentas [10]. Therefore, C21orf105 may be considered as a candidate gene for screening and diagnosing DS.

Placenta-specific 4 (PLACA4) gene is located on chromosome 21 and it is specifically expressed in placenta. A previous study suggested that mRNA level of PLAC4 in maternal plasma could be used to diagnose fetal trisomy 21 [13]. Although the mRNA of PLACA4 can be detected in peripheral blood of pregnant females, it is negatively expressed in non-pregnant females and therefore, the expression of PLACA4 mRNA is pregnancy-specific [14]. The expression level of PLACA4 mRNA is not correlated with gestational age and it significantly varies among different individuals [15]. For instance, Poon *et al.* discovered that the placenta specific gene PLACA4 mRNA obtained from fetus was presented in the maternal plasma [16]. As a result of this, this study explored the feasibility of both C21orf105 and PLACA4 for prenatal diagnosis of fetal trisomy 21 and assessed their diagnostic accuracy.

Materials and methods

Subjects

About 8-10 mL of peripheral blood samples were collected from 38 females with fetal trisomy 21 and 40 females with non-trisomy 21 fetus. All females were singleton pregnancies and they were in the second-trimester of pregnancy between November 2011 and November 2013. Fetal trisomy 21 was diagnosed based on the karyotype. The control group included females with normal fetus karyotype or their infants had no signs of chromosomal or congenital defects during the follow-up period (usually the first few weeks after birth). An extra blood sample was collected for all pregnant females recruited in our study after a fetal karyotype was confirmed. Our study was approved by the Ethics Committee of Linyi People's Hospital and all subjects signed the informed consent prior to blood sample collection.

Treatment of blood samples

Blood samples were further divided into 8 groups by different treatments in order to evaluate the effect of storage time and temperature on the stability of C21orf105 and PLAC4 *in vitro*: group C (control group) were stored at room temperature or 4°C for 0 h; group A1, A2, and A3 were stored at room temperature for 6 h, 12 h, and 24 h, respectively; group B1, B2, and B3 were stored at 4°C for 6 h, 12 h, and 24 h, respectively.

RNA extraction from maternal plasma and RT-PCR analysis

RNA was extracted from plasma samples using Blood RNA Kit (Omega, USA) under the manufacturer's instructions. RT-PCR was performed using SuperScript Vilo cDNA Synthesis Kit (Invitrogen, USA) along with the RT-PCR 7300 detection system (Applied Biosystems, USA). The primers of C21orf105 and PLAC4 were obtained from Shanghai Meiji Biological Co, Ltd. and the primer sequences were listed in **Table 1**.

RT-PCR conditions were set as the followings: 10 min at 95°C; 15 sec at 95°C; 1 min at 60°C; 2 min at 72°C (40 cycles); followed by 10 min at 72°C and cooling. The RT-PCR reaction system used in this experiment included 10 μL 2× Ultra SYBR Mixture, 0.8 μL upstream primers and 0.8 μL downstream primers (both 10 μmol/L), and 2.0 μL cDNA template. The mRNA expression levels of C21orf105 and PLAC4 was normalized to that of the housekeeping gene (β-actin) which was set as the internal control. Both C21orf105 and PLAC4 expressions were

Table 2. Baseline characteristics of the study population

Maternal characteristics	Case (n=38)	Control (n=40)	*P* value
Maternal age in years	26.7±4.5	27.6±4.9	0.402
Weeks at blood-taking	20.8±0.8	20.5±0.9	0.125
Maternal weight (kg)	66.4±4.8	67.2±4.2	0.435
Height (cm)	161.6±6.0	161.3±6.1	0.827
BMI	25.6±3.0	26.0±2.6	0.53
Nulliparous			0.672
Yes, n (%)	21 (55.3)	24 (60.0)	
No, n (%)	17 (44.7)	16 (40.0)	
Hereditary history			0.302
Yes, n (%)	1 (2.6)	0 (0)	
No, n (%)	37 (97.4)	40 (100.0)	

BMI: body mass index (kg/m^2).

Table 3. The mRNA expression levels of C21orf105 and PLAC4 with different treatments of the temperature and time

Group	Time	C21orf105	P^*	P^{**}	PLAC4	$P^{\#}$	$P^{\#\#}$
C	0 h	0.047±0.013			7.19±3.74		
A1	6 h[a]	0.051±0.017	0.101		6.27±2.47	0.072	
A2	24 h[a]	0.044±0.022	0.301		8.12±2.72	0.078	
A3	72 h[a]	0.045±0.024	0.519		7.46±2.97	0.618	
B1	6 h[b]	0.050±0.026	0.363	0.777	6.84±3.43	0.543	0.235
B2	24 h[b]	0.042±0.021	0.076	0.562	7.35±3.01	0.769	0.096
B3	72 h[b]	0.048±0.027	0.769	0.464	6.49±3.42	0.224	0.06

[a]room temperature; [b]4 °C; P^*, compared with the C21orf105 mRNA expression level at 0 h; P^{**}, compared with the C21orf105 mRNA expression level at the same time after treatment; $P^{\#}$, compared with the PLAC4 mRNA expression level at 0 h; $P^{\#\#}$, compared with the PLAC4 mRNA expression level at the same time after treatment.

calculated using the $2^{-\Delta\Delta CT}$ method and the experiments were replicated for at least 3 times.

Western blot analysis

Western blot was conducted to evaluate the protein expression level of PLAC4. Plasma lysates (20-25 μg) were separated using 10% polyacrylamide gels and were transferred to PVDF membranes (BioRad, Hercules, CA), and then were blocked overnight with primary antibodies targeting PLAC4 (1:7500) and GAPDH (1:7500) before blotting. Membranes were then washed with PBST for 3 times, and HRP-conjugaed secondary antibodies were for incubation. After that, membranes were then detected using a chemiluminescence detection system (Santa Cruz Biotechnology, USA). Relative densitometry was evaluated using the Image Lab (BioRad) in which GAPDH was set as the control group.

Statistical analysis

Statistical analysis was undertaken using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences in parametric data and non-parametric data between the case and control group were compared by the unpaired t-test and chi-square test, respectively. Logistic regression was performed to calculate the odds ratios and their corresponding 95% confidence intervals (CI). The Receiver Operating Characteristic (ROC) curve was generated to estimate the sensitivity and specificity of C21orf105, PLAC4, and C21orf-105 combined with PLAC4; the area under the curve (AUC) indicates the overall diagnostic accuracy for DS. All statistical inferences were carried out based two-sided tests and $P < 0.05$ was considered as statistically significant.

Results

Clinical characteristics of pregnancies with or without trisomy 21 fetus

The clinical characteristics of the case and control group are shown in **Table 2**. The average age of the case group (n=38) was 26.7±4.5 years old, while this figure was 27.6±4.9 years old in the control group (n=40). All participants were all in the second trimester and the number of weeks at blood-taking in the case and control group were 20.8±0.8 and 20.5±0.9, respectively. The average weight in the case and control group were 66.4 (52.7-74.1) kg and 67.2 (57.0 to 75.6) kg, respectively. The average heights in the case and control group were 161.6±6.0 cm and 161.3±6.1 cm, respectively. In addition, the average BMI in the case and control group were 25.6±3.0 and 26.0±2.6, respectively. Furthermore, 21 cases in the patient group (55.3%) were the first-time delivery, while the proportion of the first-time deliver in the control group was 60.0%. There was only one case in the patient group who had heredi-

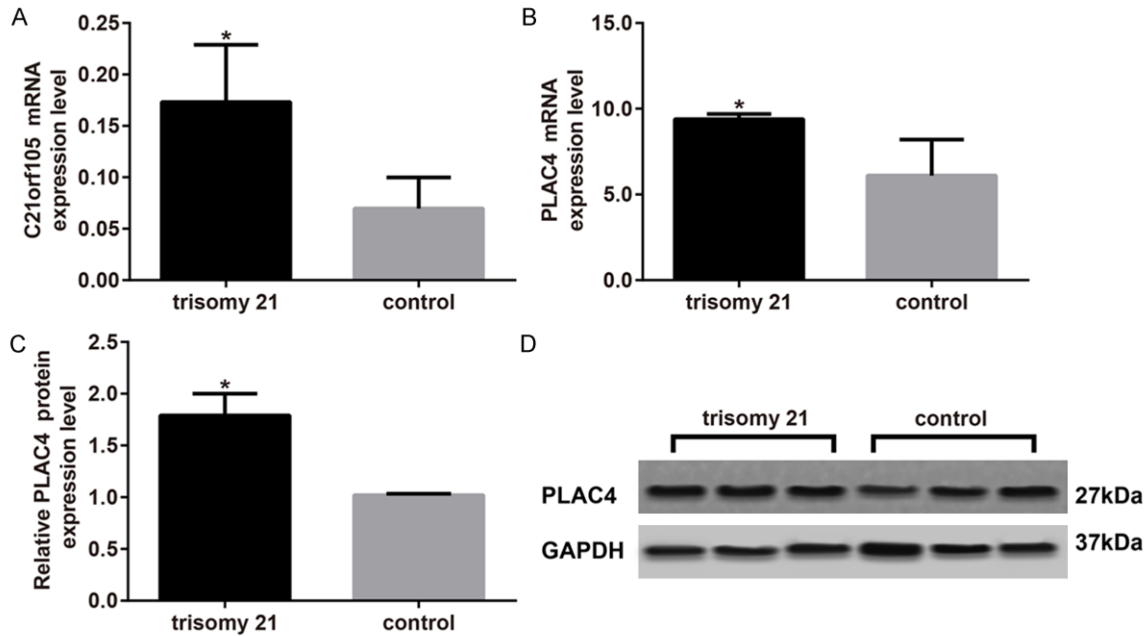

Figure 1. The expression levels of C21orf105 and PLAC4 in plasma samples of 38 trisomy 21 pregnancies and 40 controls. RT-PCR was carried out to assess the mRNA expression levels of (A) C21orf105 and (B) PLAC4, and (C, D) PLAC4 protein expression was evaluated by western blot. The GAPDH was regarded as loading control in western blot analysis. Data expressed as mean ± SEM.

Table 4. Individual and combined performance of C21orf105 and PLAC4 in pregnancies with or without Trisomy 21

Variables	AUC	SE[a]	Sensitivity	Specificity	*P* value	95% CI
C21orf105	0.77	0.06	0.67	0.67	< 0.001	0.65-0.88
PLAC4	0.90	0.04	0.66	0.79	< 0.001	0.82-0.97
Combination*	0.93	0.03	0.74	0.68	< 0.001	0.86-0.99

SE, standard error; [a]under non parametric assumptions; *$P < 0.05$ compared to the data of C21orf105; AUC, areas under the curve; 95% CI, 95% confidence interval.

tary disease history whereas none of the participant in the control group had hereditary disease history. However, there were no significant differences in age, height, weight, BMI index, the number of weeks at blood-taking, nulliparous and family history of trisomy 21 between the case and control group (all > 0.05).

Stability analysis of C21orf105 and PLAC4 mRNA expression levels in maternal plasma samples

As suggested by **Table 3**, the mRNA expression levels of C21orf105 and PLAC4 showed no significant difference among the 8 groups of plasma samples recruited in this study (all > 0.05). Therefore, we concluded that storage time or temperature did not have significant effect on the C21orf105 and PLAC4 mRNA expression levels in maternal plasma samples.

C21orf105 and PLAC4 are up-regulated in pregnancies with trisomy 21 fetus

RT-PCR was conducted to compare the expression levels of C21orf105 and PLAC4 between the patient (*n*=38) and control group (*n*=40). The data revealed that the expression levels of C21orf105 and PLAC4 were significantly up-regulated in the patient group in comparison to the control group (all $P < 0.05$, **Figure 1A**, **1B**). Similarly, results from western blot indicated that females with trisomy 21 fetus exhibited a significant increase in PLAC4 protein production compared to females with non-trisomy 21 fetus ($P < 0.05$, **Figure 1C**, **1D**). Thus, our data provided notion that both of the expression levels of C21orf105 and PLAC4 were increased in females with trisomy 21 fetus.

Diagnostic accuracy of C21orf105, PLAC4 and their combination

The ROC curve under a regression model was established to assess the diagnostic power of

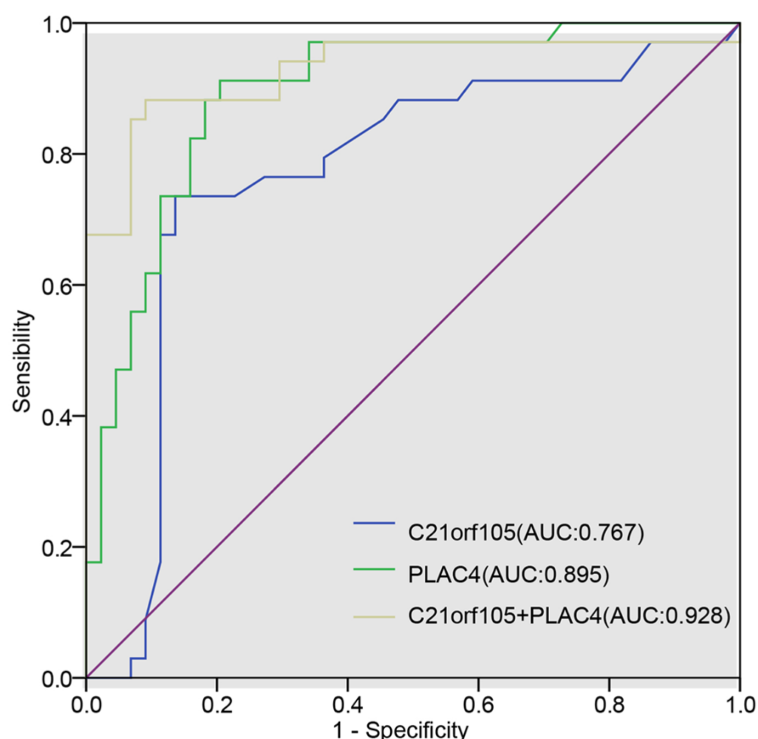

Figure 2. The classifier was evaluated by logistic regression with the individual and combined ROC. Diagnostic accuracy of individual and combined plasma C21orf105 and PLAC4 for all pregnancies with trisomy 21 versus all controls were analyzed by ROC curve. AUC: areas under the curve, ROC: receiver operating characteristic curves.

C21orf105, PLAC4 and their combination (**Table 4**). As suggested by the regression model, AUC of C21orf105, PLAC4 and their combination were 0.77 (95% CI: 0.65-0.88), 0.90 (95% CI: 0.82-0.97) and 0.93 (95% CI: 0.86-0.99), respectively. Therefore, we concluded that both the individual and combined C21orf105 and PLAC4 may exert reliable diagnostic power for trisomy 21 fetus (all $P < 0.001$, **Figure 2**). Furthermore, the combination of C21orf105 and PLAC4 offered a significantly more powerful diagnostic accuracy than C21orf105 alone ($P < 0.05$, **Figure 2**) while such diagnostic superiority was not reflected compared to PLAC4 ($P > 0.05$, **Figure 2**). As a result of this, the combination of C21orf105 and PLAC4 may be considered as a promising diagnostic indicator for trisomy 21 fetus.

Discussion

The gold standards for diagnosing fetal trisomy 21 have always been contentious due to their invasive natures and potential hazards. For instance, amniocentesis used for diagnosing trisomy 21 fetus is often criticized since it is likely to trigger fetal mortality. As a result of this, safe and non-invasive diagnostic techniques that are able to precisely distinguish trisomy 21 fetuses from normal ones may provide numerous benefits for prenatal screening, care and examination.

PLAC4 is a gene which is located in chromosome-21 and it has been considered as a key biochemical marker for screening fetal trisomy 21 because PLAC4 mRNAs or proteins are cell-free and can be detected in maternal circulation [17, 18]. Hence, PLAC4 was selected as one of our targets and its potential diagnostic value for fetal trisomy 21 was assessed. In this study, the expression levels of PLAC4 showed significant difference between females with fetal trisomy 21 and those with non-trisomy 21 fetus. We also discovered that the expression levels of PLAC4 were significantly increased in females with trisomy 21 fetus compared to the control group. Moreover, ROC curve under a regression model suggested that PLAC4 alone is a reliable diagnostic tool for fetal trisomy 21 with an AUC of 0.90.

We discovered that higher concentration of PLAC4 mRNA was observed in the maternal plasma from females with trisomy 21 fetus and this was consistent with the results provided by Lo *et al.* [17] and Tsui *et al.* [14]. As suggested by Banzola *et al.* [19] and Yang *et al.* [20], the expression levels of PLAC4 mRNA in the maternal plasma from females with trisomy 21 fetus were not significantly different from those in normal pregnancies. For one thing, different results may be attributed to differences in individuals and in study designs. Besides that, studies conducted by Banzola *et al.* and Yang *et al.* had relatively small sample sizes which may have significant impacts on the conclusions [19, 20]. Since it has been reported that the

PLAC4 mRNA levels may vary with gestational ages, difference in blood sample collection time may result in difference in PLAC4 mRNA levels [17, 20]. Therefore, the collection time of blood samples is considered as a major confounding factor which may result in biased conclusions. Additionally, the expression of biomarker may contribute to the misidentification of trisomy 21 fetus due to cell heterogeneity observed in different fetal tissues [21]. Finally, storage time and temperature did not appear to have significant effect on the mRNA expression levels in maternal plasma samples and this confirmed that different conclusions.

As suggested by Tsui et al. [14], Zhang et al. [22] and Yang et al. [23], PLAC4 RNA-SNP is another diagnostic method which is believed to be efficient in prenatal diagnosis of trisomy 21 with heterozygous fetuses. Moreover, Tsui et al. proved that the diagnostic accuracy of PLAC4 RNA-SNP for trisomy 21 heterozygous fetuses was higher than the concentration approach as we conducted [14]. However, the PLAC4 RNA-SNP approach was restricted to trisomy 21 with heterozygotes and a larger number of SNPs of PLAC4 mRNA are required to be detected in order to cover more population subsets [22]. In order to address the issues encountered by the PLAC4 RNA-SNP and the concentration approach, we tested another candidate target that is related to trisomy 21 diagnosis, mRNA C21orf105 [24]. Our study also demonstrated that the combination of C21orf105 and PLAC4 improved the diagnostic accuracy for fetal trisomy 21.

C21orf105 is a kind of mRNA encoded by chromosome 21 and it can be detected in maternal plasma [25]. Our results showed that the expression levels of C21orf105 were up-regulated in females with trisomy 21 fetus. Moreover, the AUC of C21orf105 for diagnosing trisomy 21 fetus was 0.77, suggesting its feasibility in prenatal diagnosis of pregnancies with fetal trisomy 21. However, plasma C21orf105 offered a less diagnostic accuracy for pregnancies with fetal trisomy 21 compared to plasma PLAC4.

On the contrary, Go *et al.* showed that the expression levels of C21orf105 in maternal plasma were not significantly higher in pregnancies with fetal trisomy 21 [25]. However, plasma samples in the study conducted by Go *et al.* were obtained from females who were in the first trimester of their pregnancy (9-15 weeks). Apart from that, the expression level of C21orf105 is likely to vary with fetus growth. Therefore, the diagnostic accuracy of C21orf105 for pregnancies with fetal trisomy 21 may be determined by several factors including the timing of sample collections and cell heterogeneity of different fetal tissues [21]. This study also enabled us to assess the joint effect of C21orf105 and PLAC4 on the diagnostic accuracy for pregnancies with trisomy 21 fetus. C21orf105 combined with PLAC4 provided an unexpectedly high AUC of 0.93 which was significantly more favourable than C21orf105 alone. Therefore, we concluded that the combination of C21orf105 and PLAC4 may significantly improve the diagnostic power and it can be considered as a promising diagnostic tool for pregnancies with trisomy 21 fetus.

Overall, this is a pioneer study which evaluated the diagnostic power of C21orf105 in conjunction with PLAC4 for pregnancies with trisomy 21 fetus. Importantly, our experiment revealed that both storage time and temperature did not have substantial effect on C21orf105 and PLAC4 mRNA levels contained in maternal plasma. Nevertheless, several limitations may arise from the current study. Firstly, the association between gestational age and C21orf105 or PLAC4 concentrations in maternal plasma was not assessed and therefore we were not able to determine the optimal timing for collecting plasma samples during gestational period. Secondly, whether our approach is more precise than the PLAC4 mRNA-SNP approach in diagnosing trisomy 21 heterozygous fetuses should be further confirmed.

In conclusion, the expression levels of C21orf105 and PLAC4 in maternal plasma are up-regulated for pregnancies with fetal trisomy 21. Hence, both C21orf105 and PLAC4 can be served as diagnostic biomarkers for fetal trisomy 21. Moreover, our study provided notion that combining C21orf105 with PLAC4 significantly improved the diagnostic power for fetal trisomy 21 and therefore they are highly recommended as prenatal screening and diagnostic tools.

Disclosure of conflict of interest

None.

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