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*Original Article*

# Cell-free fetal mRNAs are stable markers for noninvasive prenatal screening of trisomy 21

Yajuan Xu*, Yingying Zhang*, Xiaohua Luo, Shanshan Zhai, Limin Ran

*Department of Obstetric, The Third Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, China. *Equal contributors.*

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**Abstract:** Recently, the demonstration of the presence of cell-free fetal mRNA in maternal plasma has opened up new possibilities for noninvasive prenatal assessment. This original study aimed to confirm the stability of C21orf105 and PLAC4 mRNA in maternal plasma, and explored whether plasma C21orf105 and PLAC4 mRNA levels are likely to be associated with fetal trisomy 21. Thirty blood samples from singleton pregnancies at 11-$13^{+6}$ weeks of gestation were collected randomly. Subsequently, we obtained thirty-eight blood samples from trisomy 21 pregnancies and forty blood samples from normal pregnancies. Then plasma C21orf105 and PLAC4 mRNA levels between pregnant women with trisomy 21 fetuses were compared, which were measured by real-time RT-PCR. (1) There was no significant difference for plasma C21orf105 and PLAC4 mRNA levels either during the period of 72h at the same temperature ($P > 0.05$) or in different temperature at the same time ($P > 0.05$); (2) There was significant difference of maternal plasma C21orf105 ($P < 0.01$) and PLAC4 mRNA ($P < 0.01$) between trisomy 21 group and normal pregnancies group. C21orf105 and PLAC4 mRNA are stable enough to be amplified at room temperature for up to 72 h, and are discriminative markers for prediction of Down syndrome.

**Keywords:** Cell-free fetal mRNA, C21orf105, PLAC4, real-Time RT-PCR

## Introduction

Trisomy 21, known as Down's syndrome, is the most common chromosome abnormality in infants [1]. At present, the diagnostic gold standards of fetal chromosomal aneuploidies are these invasive tests such as chorionic villus sampling between 11 and 14 weeks of gestation and amniocentesis after 15 weeks of gestation, which may impose approximately 1% risk to miscarriage [2]. Because of the potential risk with such invasive methods, it is a long-sought goal to develop noninvasive methods in prenatal diagnosis research [3]. At present, these methods available to detect aneuploidies involving in biochemistry and ultrasound examination were only developed to detect the associated epiphenomena with a limited sensitivity and specificity [4, 5]. Pregnant women therefore highly demand for a reliable and practical method with direct assessments of fetal chromosome dosage with maternal plasma.

The discovery of cell-free fetal nucleic acids in maternal circulation opens a new window for noninvasive prenatal testing (NIPT) [6], which has been moved into the application of testing fetal genetic disorders [7]. Cell-free fetal nucleic acids in maternal blood include the fragments of cell-free fetal DNA (cffDNA) and cell-free fetal RNA (cffRNA), which can be both detected in all three trimesters of pregnancy [8]. It is believed that the perfect future of NIPT will be sequencing of the entire fetal genome, but it is some way off [9]. Plasma placental RNA allelic ratio is a method of using allelic ratios of placental-specific mRNA in maternal plasma to detect fetal chromosomal aneuploidy [10], which makes use of the genetic single-nucleotide polymorphism (SNP) in certain populations [11].

*Oudejans et al.* [12] have shown that C21orf105 (LOC90625) was a chromosome 21-encoded gene located within the Dowan syndrome critical region (DSCR), and was expressed by early placental tissue. As C21orf105 mRNA which was fetal-specific could be detectable in maternal plasma during all trimesters of pregnancy [4], the overexpression of C21orf105 mRNA

**Table 1.** The information of contributors

| Groups | Number | Age | Gestational Age |
|---|---|---|---|
| Trisomy 21 | 38 | 26.74±4.519 | 20.55±0.92 |
| Control | 40 | 27.63±4.855 | 20.46±0.90 |

was reported in pregnancies with trisomy 21 fetuses [13], which indicated that C21orf105 mRNA in maternal plasma was a promising biological marker for noninvasive prenatal screening or diagnosis for Down's syndrome. Additionally, Lo et al [14] found that PLAC4 mRNA could meet the same criteria as C21orf105 mRNA by microarray-based strategy. Although cffRNA was detectable in maternal plasma and has been identified as potential molecular markers for trisomy 21, the stability of cffRNA should be determined after obtained from maternal plasma to confirm the clinic application of cffRNA. Furthermore, although the expression level of C21orf105 [15] and PLAC4 [16] mRNA was abnormal in trisomy 21 pregnancy compared with normal pregnancy, conflicting results [17, 18] claimed that these ccfRNAs might not be discriminative marker genes for prediction of Down syndrome in maternal plasma.

We aimed to determine the stability of fetal mRNA C21orf105 and PLAC4 mRNA in maternal plasma, which confirmed the possibility for clinical application of cffRNA. Then to examine whether cffRNA was potential marker for prenatal screening of trisomy 21, the expression of the C21orf105 and PLAC4 mRNA in maternal plasma was compared between pregnancies with euploid fetuses and those with trisomy 21 fetuses.

## Materials and methods

*Participants recruit*

We recruited pregnant women who attended to the Clinic of Obstetrics at the Third Affiliated Hospital of Zhengzhou University. 38 pregnant women whose fetuses were trisomy 21 and 40 controls with euploid fetuses were recruited in our study. The mean gestational ages for the euploid and trisomy 21 group were respectively 20.46±0.90 weeks and 20.55±0.92 weeks. The mean maternal age was 27.63±4.855 years for the euploid group and 26.74±4.519 years for the trisomy 21 group (**Table 1**). Peripheral blood from healthy women with singleton uncomplicated pregnancies were collected into EDTA-containing tubes with informed consent. The protocol for the research project was approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University and the informed consent letters were obtained from participants.

*Processing of blood samples*

Blood samples were collected into EDTA tubes and all plasma were obtained by two sequential centrifugation (11) as follows: the blood samples were centrifuged at 1,600 g for 10 min at 4°C, and the supernatants were re-centrifuged for 10 min at 16,000 g at 4°C followed by transferring plasma into fresh RNase free tubes. All plasma samples after processing were kept at -80°C until RNA extraction.

*Total RNA extraction*

The total RNA was isolated by TRI Reagent BD (MRC) from 1.6 ml plasma. For every 2 ml of plasma-TRI Reagent BD mixture, 20 µL 5 N acetic acid were added. The lysed samples were stored for 5 min at room temperature, followed by addition of 200 µL chloroform. After vortex-mixed for 15 s, then the mixture was left at room temperature for 5 min followed by centrifugation at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, and 500 µL of isopropanol was added; samples were stored at room temperature for 5 min. After centrifugation, 1 ml of 75% ethanol was added. Then perform the centrifugation at 16,000 g for 5 min at 4°C. The ethanol was removed and the RNA pellet was air-dried briefly for 5 min. Finally, the RNA was eluted with 15 µL RNase-free water. RNA was stored at -80°C until use.

*Real-time*

The mRNA level were measured using the two-step, one-tube real-time reverse-transcriptase PCR in the 7500 Real-time PCR System (Applied Biosystems) using SYBR GREEN I assay according to the manufacturer's instructions (CWBIO, China) in a reaction volume of 25 ml. In brief, 13 µl RNA was heated for 5 min at 65°C, followed by immediate cooling on ice. Four microliters of 4X DNmaster mix (with gDNA Remover) was subsequently added and incubated for 5

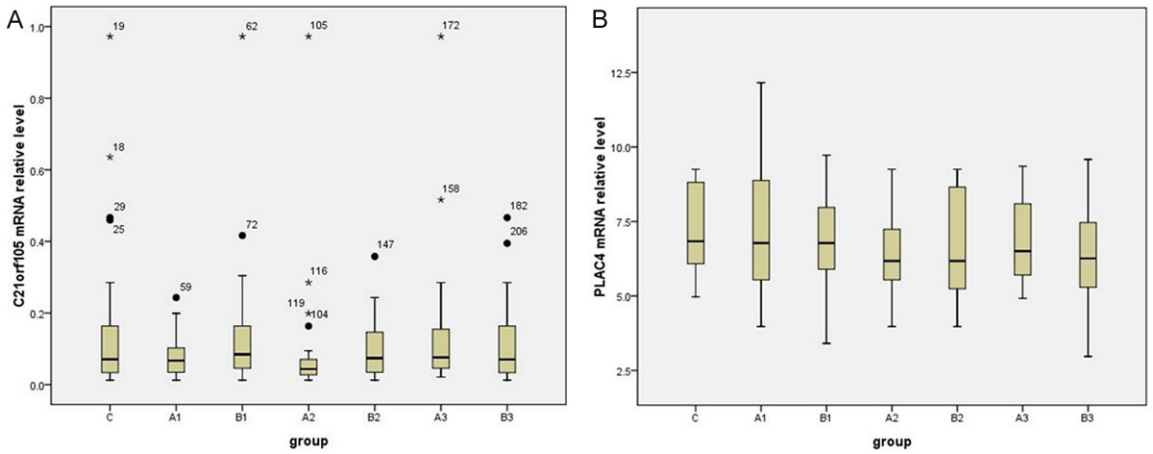


**Figure 1.** The variation of plasma C21orf105 (A) and PLAC4 (B) mRNA relative level in different storage temperatures or processing times. Note: Plasma samples were divided into seven groups, including A1, A2, A3, B1, B2, B3 and C. For Group A1, A2, A3, plasma samples were left at room temperature for 6 h, 24 h and 72 h respectively while group B1, B2, B3 were left at 4°C for 6 h, 24 h and 72 h respectively and then we mixed them with TRI Reagent. Group C were immediately mixed with TRI Reagent after centrifugation. We then extracted mRNA from these samples and measured the levels of C21orf105 and PLAC4 in maternal plasma.

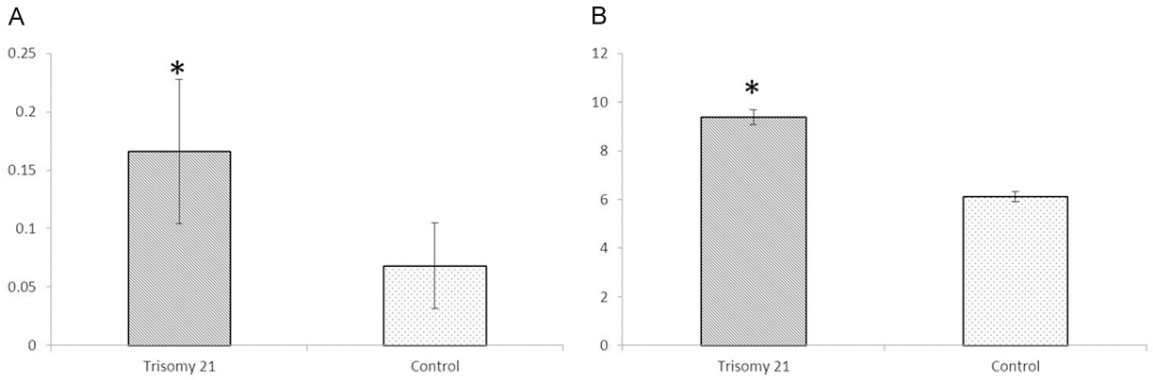


**Figure 2.** Comparison of C21orf105 (A) and PLAC4 (B) mRNA relative level between the trisomy 21 group and the control group. Note: *presented as $P < 0.01$ compared with control group.

min at 37°C. Then, 4 μl of 5X RTmaster mixII was added. The cDNA was stored at -80°C. After reverse transcription for 5 min at 50°C denaturation for 2 min at 95°C, PCR was performed as follows: initial denaturation for 10 min at 95°C, 40 cycles of denaturation for 10 s at 95°C, annealing for 30 s on 60°C, and extension for 1 min on 60°C, followed by a final extension for 10 s at 72°C. Each reaction contained 10 μl 2X UltraSYBR Mixture with ROX, 1 μL each of forward and reverse primers and 2 μl template cDNA in a final volume of 20 μl. All reactions were performed identically. Each cDNA sample was analyzed in triplicate.

The PCR primers were synthesized by Generay Biotech Co., Ltd. (Shanghai, China). The gene-specific primers used were as follows: C21orf105-F (5'-CGCTGAGTACGTCGTGGAGT-C-3') and C21orf105-R (5'-GCTGATGATCTT-GAGGCTGTTGTC-3'); PLAC4-F (5'-GGAGTATG-TGGCAGTCGTAATGG-3') and PLAC4-R (5'-TTT-CAGGGGGAGTTGTTGGTTT-3'); β-actin-F (5'-GT-TGCGTTACCCTTTCTTGAC-3') and β-actin-R (5'-CTCGGCCACATTGTGAACTTTG-3').

*Statistical analysis*

Statistical analysis were performed with SPSS 17.0. The expression level of target genes and housekeeping gene was presented as $2^{-\Delta CT}$. Pairwise multiple comparisons were used to investigate the stability of C21orf105 and PLAC4 mRNAs. Two independent samples t-test was used to investigate whether plasma C21orf105 and PLAC4 mRNA levels are likely to

be associated to fetal trisomy 21. $P < 0.05$ was considered statistically significant.

## Results

*The stability of C21orf105 and PLAC4 mRNA in maternal plasma*

To determine the stability of C21orf105 and PLAC4 mRNA, 30 blood samples from singleton pregnancies at $11\text{-}13^{+6}$ weeks of gestation were collected randomly. C21orf105 and PLAC4 mRNA were indeed detectable in the plasma of 30 pregnant women (**Figure 1**). The housekeeping gene β-actin mRNA was also detectable in all of plasma samples. At the same preserved temperature, there was no significant difference for plasma C21orf105 (**Figure 1A**) and PLAC4 (**Figure 1B**) mRNA levels left for 0, 6, 24, 72 h ($P > 0.05$). Additionally, there was also no significant difference in the levels of C21orf105 and PLAC4 mRNA in samples left for the same time at different temperature ($P > 0.05$, **Figure 1**). These results indicated that C21orf105 and PLAC4 mRNA in plasma was stable for up to 72 h at room temperature.

*The relationship between the expression levels of C21orf105 and PLAC4 mRNA in plasma and trisomy 21 pregnancies*

To investigate whether the expression level of C21orf105 and PLAC4 mRNA in maternal plasma of pregnancies was associated with trisomy 21 fetuses, C21orf105 and PLAC4 mRNA were observed in all samples, as well as the housekeeping gene β-actin mRNA, which confirmed the available application of C21orf105 ($P < 0.01$, **Figure 2A**) and PLAC4 mRNA ($P < 0.01$, **Figure 2B**) in maternal plasma. And for both C21orf105 (**Figure 2A**) and PLAC4 (**Figure 2B**) mRNAs, there was significant difference ($P < 0.01$) between trisomy 21 group and control group.

## Discussion

Our study identified the stability of fetal-specific C21orf105 and PLAC4 mRNA in maternal plasma, which ensured their clinic application. *Ng et al.* [19] found that when placed maternal blood at room temperature for 24 h, there was no significant variation between hPL and hCG mRNA concentrations in maternal plasma. It is not known whether the results can be generalized to all kinds of fetal-specific mRNA in plasma. Although C21orf105 and PLAC4 mRNA was the potential biomarkers for trisomy 21 pregnancy [4], the clinic application of C21orf105 and PLAC4 mRNA should be confirmed based on the storage temperature and processing time. For the storage temperature and processing time of blood samples, studies reported blood samples were left at room temperature for 3 h [20], 6 h [21], 24 h [19] or at 4°C for 72 h [18] before centrifugation. Therefore, we choose different groups at 4°C or room temperature for 6 h, 24 h and 72 h. Our data offered a striking demonstration of the surprising stability of maternal plasma C21orf105 and PLAC4 mRNA, which confirmed that fetal-specific mRNA of C21orf105 and PLAC4 in maternal plasma was stable enough to be amplified after the plasma was left for 72 h at room temperature.

Because of the location on chromosome, the expression of C21orf105 and PLAC4 mRNA are theoretically expressed higher in trisomy maternal plasma than normal maternal plasma. A number of investigators [13, 16] have shown that the plasma concentration of a chromosome 21-transcribed placental C21orf105 and PLAC4 mRNA were increased in trisomy 21 pregnancies whereas other studies [17, 18] suggested that the concentration of C21orf105 and PLAC4 mRNA in maternal plasma was not significantly increased in trisomy 21 pregnancies compared with normal pregnancies. Further studies are required to investigate the relation between the expression level of fetal-specific C21orf105 and PLAC4 mRNA in maternal plasma and trisomy 21 fetuses. The results of the present article showed that C21orf105 and PLAC4 mRNA was detected in plasma either from trisomy 21 pregnancies or euploid pregnancies. Additionally, there was significant difference of C21orf105 mRNA and PLAC4 mRNA between pregnancies with trisomy 21 fetus and normal pregnancies. And the impact factors of ccfRNAs remain to be investigated. Nevertheless, fetal mRNA in maternal plasma is an important step toward the research in the field of prenatal screening, because of its gender-independent and high abundance compared with fetal DNA in maternal blood. And the surprising stability of cffRNA provides researchers more opportunities to develop novel detec-

tion methods of cffRNA for noninvasive prenatal screening of trisomy 21.

In summary, C21orf105 and PLAC4 mRNA are stable enough to be amplified at room temperature for up to 72 h, and are discriminative markers for prediction of Down syndrome.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Yajuan Xu, Department of Obstetric, The Third Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, China. E-mail: xuyajuan1357@163.com

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