Placenta 35 (2014) 900–906

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

# Pregnancy associated plasma protein-A2: A novel biomarker for down syndrome

S. Munnangi [a], S.J. Gross [b], R. Madankumar [c], G. Salcedo [d], S.E. Reznik [a, e, f, *]

[a] Department of Pharmaceutical Sciences, St. John's University, Queens, NY, USA
[b] Natera, San Carlos, CA, USA
[c] Department of Obstetrics and Gynecology, North Shore-LIJ Health System, Glen Cove, NY, USA
[d] Endoclab, Porto, Portugal
[e] Department of Pathology, Albert Einstein College of Medicine, Bronx, NY, USA
[f] Department of Obstetrics and Gynecology and Women's Health, Albert Einstein College of Medicine, Bronx, NY, USA

ARTICLE INFO

*Article history:*
Accepted 5 August 2014

*Keywords:*
Pregnancy associated plasma protein-A2
Biomarker
Down syndrome
Placenta
Maternal serum
Second trimester

ABSTRACT

*Introduction:* In an effort to improve prenatal screening for Trisomy 21, we evaluated pregnancy associated plasma protein-A2 (PAPP-A2) as a potential novel second trimester biomarker for Trisomy 21.
*Methods:* Trisomy 21 and normal control mid-trimester placental samples were subjected to quantitative rt PCR analysis of seven genes we had previously found to be differentially expressed in Trisomy 21 placentae. The localization and differential expression of PAPP-A2 in second trimester placentae from normal and Trisomy 21 pregnancies was determined by immunohistochemistry. PAPP-A2 maternal serum protein levels in ten Trisomy 21 and ten diploid pregnancies were compared by Western blotting. Maternal serum PAPP-A2 levels were measured in 30 Down syndrome cases and 142 normal controls, using ELISA. Regression analysis was used to determine the correlation of PAPP-A2 with other existing markers of Trisomy 21.
*Results:* PAPP-A2 (aka PLAC 3) mRNA and protein expression were both increased in Down syndrome placentae as compared to diploid placentae. PAPP-A2 was also increased in maternal serum from Down syndrome pregnancies as compared to diploid pregnancies. PAPP-A2 expression correlated weakly with established markers.
*Discussion:* This work takes advantage of our previously performed systematic approach to the discovery of novel maternal serum biomarkers for Trisomy 21, using cDNA microarray analysis. Beginning with the validation of the microarray results, we have tracked PAPP-A2 overexpression in Down syndrome from placental mRNA to maternal serum protein.
*Conclusion:* PAPP-A2 could serve as an additional maternal serum marker in prenatal screening for Trisomy 21.



## 1. Introduction

Prenatal screening has evolved significantly in the past two decades with the introduction of novel markers. Merkatz et al. was the first to identify an association between low maternal serum concentrations of alpha-fetoprotein (AFP) and fetal Trisomy 21 [1]. Before this association, maternal age was the only known risk factor for an aneuploid pregnancy [2–4]. Subsequently, maternal serum AFP concentration combined with maternal age was part of generally practiced prenatal screening protocol. Combining maternal age with AFP levels the detection rate achieved was about 40% [1,5]. The quadruple test, which significantly changed the landscape of prenatal screening comprises four maternal serum markers, namely human chorionic gonadotropin (hCG), Inhibin A, AFP and uE3. Levels of hCG and Inhibin A are elevated whereas levels of AFP and uE3 are decreased in Trisomy 21 [6–10].

At a 5% false positive rate, in combination with maternal age, maternal serum markers can detect approximately 70–80% of cases of Trisomy 21 and, by incorporating fetal ultrasound measurements, slightly improved detection rates can be achieved [11,12]. The issue of improved screening remains a priority due to the number of pregnant women affected, residual risk and false positive rates, and the serious ramifications with respect to

* Corresponding author. Department of Pharmaceutical Sciences, St. John's University, Queens, NY, USA.
*E-mail address:* rezniks@stjohns.edu (S.E. Reznik).

http://dx.doi.org/10.1016/j.placenta.2014.08.001
0143-4004/

**Table 1**
Chromosomal location of various placental genes looked at using RT-PCR.[a]

| GenBank Access # | N31645 | AA598517 | H51848 | AA001841 | N93686 | AA464595 | AA156461 |
|---|---|---|---|---|---|---|---|
| Gene | EST 433020 | *KRT8* | *PAPPA2* | EST 611475 | *ALDH7* | *KISS1* | *PTTG1IP* |
| Chromosome | 21q22 | 12q13 | 1q23-25 | 21 | 11q31 | 1q31 | 21 |

[a] Genes named in table were previously found to be differentially expressed in Trisomy 21 placentae by one of us (SJG), using cDNA microarray analysis.

reproductive health care [13–15]. In the current study, we have evaluated the potential of pregnancy associated plasma protein-A2 (PAPP-A2) as a novel biomarker for screening pregnancies for Trisomy 21. PAPP-A2 is expressed abundantly in human placenta and in non pregnant mammary gland and to a lower extent in various other tissues, including the kidney, fetal brain and pancreas [16–18]. It is known to cleave insulin growth factor binding proteins IGFBP-4 and IGFBP-5 and has been found to be up-regulated in hypertensive disorders associated with pregnancy, such as pre-eclampsia [19–21].

The current study builds on previous work that was based on a systematic approach to discover better maternal serum markers of Trisomy 21, using microarray analysis of Trisomy 21 and diploid placentae [22]. Differential expression of placental proteins, because of the highly vascularized nature of the placenta and its proximity to maternal circulation, may be reflected in maternal serum and may be the basis of improved detection in prenatal screening for Trisomy 21.

## 2. Methods

The current study was approved by the St. John's University and Jacobi Medical Center Institutional Review Boards.

### 2.1. Sample collection

Serum samples from the second trimester of normal and Trisomy 21 pregnancies were used in the experiment. All the serum samples from normal pregnant women were collected by a phlebotomist at Jacobi Medical Center, New York with informed consent and double IRB approval (St. John's University and Jacobi Medical Center IRBs) prior to amniocentesis or CVS and concurrently with quad screening. Diagnoses were ultimately confirmed by pregnancy outcomes. Serum samples from Trisomy 21 pregnancies were procured with patient informed consent from the clinical laboratory Endoclab, Porto, Portugal with permission obtained from Endoclab. Placental tissues were obtained from second trimester voluntary terminations or spontaneous abortions at Jacobi Medical Center with appropriate consent and IRB approval.

### 2.2. Quantitative real time reverse transcription polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was performed to compare the expression levels of *PAPPA2*, *ALDH7*, *KISS1*, *PTTG1P*, *KRT8* and EST transcripts (id433020, id611475) in Trisomy 21 and normal placental tissue, all previously found to be differentially expressed in microarray analysis of Trisomy 21 placentae [22]. Total RNA was extracted from the chorionic villi of human placental tissues using an RNAeasy kit (Qiagen, Valencia, CA), after which quantitation and purification assessment was performed using NanoDrop spectrophotometer (Thermo scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies). The relative expression levels of the above mentioned seven genes were assessed using quantitative RT-PCR with an ABI PRISM 1500 Sequence Detection System (Perkin–Elmer, Foster City, CA, USA). Equimolar amounts of total RNA from 7 Trisomy 21 and 7 diploid placentae were reverse transcribed and amplified using a one step RT-PCR kit (Qiagen), according to the manufacturer's instructions. The GenBank access numbers of the genes are listed in Table 1. The expression of the housekeeping gene GAPDH was also measured. Quantitative RT-PCR was performed using 1 cycle for 10 min at 95 °C then 40 cycles at 95 °C for 30 s, 60 °C for 1 min and finally at 72 °C for 30 s. A standard curve was obtained for all 7 genes using various dilutions and samples were measured in triplicate.

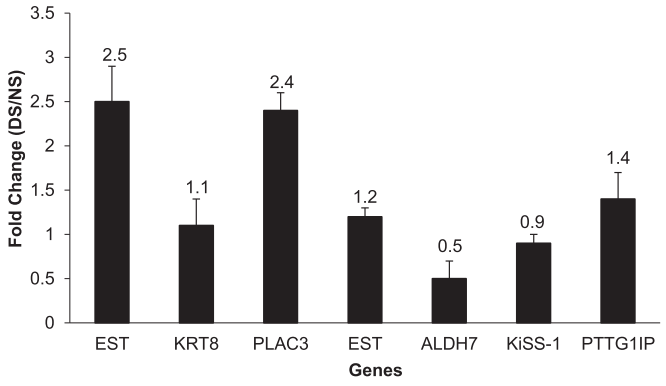


**Fig. 1.** Differential expression of various genes in Trisomy 21 placentae ($n = 7$). Results are expressed as fold changes in Trisomy 21 placentae compared to normal placentae. mRNA levels are normalized to *GAPDH*. Error bars refer to standard deviations. All samples were run in triplicate.

### 2.3. Immunohistochemistry

Placental tissue sections from 7 Trisomy 21 placentae and 7 diploid placentae were deparaffinized with 100 per cent xylene (BDH-VWR) for 4 min, rehydrated with serial alcohol solutions (100–70%) and washed in distilled water. The deparaffinized sections were treated with 3%$H_2O_2$ for 30 min to quench endogenous peroxidase activity. Sections were rinsed in distilled water and then microwaved in Antigen Retrieval Solution (8.2 mM sodium citrate, 1.8 mM citric acid-pH 6.0, containing 0.01 per cent Triton X-100). After subsequent washing with TBS-T (Tris-buffered saline with 0.1% Tween-20, pH 7.8), sections were incubated in a humidified chamber and a 1:15 dilution of the primary monoclonal anti-PAPP-A2 antibody (Sigma–Aldrich Corp. St. Louis, MO, USA) in TBS containing 1%bovine serum albumin and 0.2%Tween 20 overnight at 4 °C. In negative controls, serial sections were incubated with 1% BSA without the primary antibody. After overnight incubation, the sections were washed in TBS containing 0.2%Tween 20 and immunostaining was achieved using an avidin–biotin complex developer kit (Dako, Carpinteria, CA, USA) and 3, 3-diaminobenzidine as a substrate. Sections were counterstained with Mayer's hematoxylin. The grading of immunohistochemistry staining intensity was performed by three blinded observers by choosing the three most intensely staining regions of chorionic villi, following a pre-defined scoring scheme established by a practicing placental pathologist (SER). The inter-observer variability was tested among the three observers and was found to be insignificant.

These were graded based on a scale of 1–4 with 1 being the lightest staining and 4 being the most darkly staining villi seen by each observer.

### 2.4. Western blotting

Protein concentrations of 10 Trisomy 21 maternal serum samples and 10 normal control maternal serum samples were determined by performing a Bradford assay, using bovine serum albumin as the standard. SDS sample buffer 4× (Invitrogen) was added to the samples, which were denatured at 95 °C for 4 min. Gel electrophoresis was carried out and the proteins were separated using NuPAGE® Novex 3–8% Tris-Acetate Gels, 1.5 mm, 10 welled (Invitrogen, Carlsbad, CA). Pure PAPP-A2 protein (R&D systems) was used as a positive control. The proteins were then transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were wetted with TBS-T (Tris-buffered saline with 0.1% Tween-20, pH 7.8) for 10 min, and were blocked with Western blocker solution (Sigma) for 120 min. The membranes were then incubated with anti-PAPP-A2 primary antibody (R&D systems), diluted to

**Table 2**
Maternal characteristics of the study population (placental specimens).

| Maternal characteristic | DS ($n = 7$) | NS ($n = 7$) |
|---|---|---|
| Age range (Years) | 22–40 | 19–35 |
| Median age | 39 | 29 |
| Mean gestational age (Weeks) | 18 ± 0.81 | 19.7 ± 2.21 |
| Ethnic origin | 85.72% White<br>14.28% African American | 42.85% Hispanic<br>42.85% African American<br>14.28% White |