Reference Centile Chart for Fetal Nuchal Translucency, Maternal Serum PAPP-A and Free Beta hCG

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Objective: *To create reference centile chart of fetal nuchal translucency, maternal serum pregnancy associated plasma protein-A (PAPP-A) and maternal serum free beta human chorionic gonadotropin (beta-hCG) in order to predict preliminarily Down syndrome in Thai fetuses during 10-14 weeks of gestation.*
Material and Method: *This was a prospective, descriptive cohort study. From 1 January 2004 to 31 December, 2006, a total of 1,000 pregnant women during 10-14 weeks of gestation were participated in the present study. Pregnancy outcomes were reviewed from the records. The excluded cases were chromosomal and major structural abnormalities, twin pregnancy and cases resulting in miscarriage or intrauterine death. All women had a scan for nuchal translucency (NT) and had blood taken for measurement of maternal serum PAPP-A and free beta-hCG level.*
Results: *The mean NT was 1.6 $\pm$ 0.8 mm (range 0.3-14 mm). The 5th, 50th and 95th centile of PAPP-A and free beta-hCG during 11-14 weeks of gestation were 1.54-69, 14-28, 51-57 and 24.8-17, 78-47, 181.6-126.5 mIU/mL, respectively. The distribution and the 5% and 95%, lower and upper limits of NT, PAPP-A and free beta-hCG was presented.*
Conclusion: *The present study shows that NT measurements increase with increasing gestational age. The mean serum PAPP-A rises and the mean serum-free beta hCG decreases from 10 to 14 weeks of gestation in normal Thai fetuses. These results can be used for reference value to predict fetal Down Syndrome.*

Keywords: *Nuchal translucency, Pregnancy associated plasma protein-A, PAPP-A, Serum free beta human chorionic gonadotropin, Free beta-hCG, Reference centile chart*

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Increased NT during the first trimester of pregnancy is well-established in fetal chromosomal abnormality and fetal abnormalities[1,2]. NT has been widely used as a screening test for fetal Down Syndrome in the first trimester either alone or in combination with serum markers[2]. Many serum markers have been used to evaluate the pregnant women who have the risk of having a Down syndrome fetus[3]. The non-invasive serum markers screening keep reducing unnecessary invasive testing including genetic amniocentesis, chorionic villous sampling or cordocentesis which is correlated with pregnancy loss and high cost of procedures. Many serum markers in the first trimester are used, especially pregnancy associated plasma protein-A (PAPP-A) and free beta human chorionic gonadotropin (beta-hCG) combined with nuchal translucency (NT). The present study presents the centile chart of NT, PAPP-A and free beta-hCG between 10-14 weeks of gestation for a preliminary prediction of the normal Thai fetus.

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Material and Method

The present study was approved by Siriraj Ethics Committee of the Faculty of Medicine Siriraj

Hospital, Mahidol University, based on the Declaration of Helsinki in June 2003, No. 119/2003. During 1 January 2004 to 31 December, 2006, a total of 1,000 pregnant women who attended at the antenatal clinic at Siriraj Hospital during 10-14 weeks of gestation were participated in the present study. Pregnancy outcomes were reviewed from the records. The excluded cases were chromosomal and major structural abnormalities, twin pregnancy and cases resulting in miscarriage or intrauterine death. The gestational age was calculated from the first day of the last menstrual period and confirmed by crown-rump length measurement[4]. If the estimated gestational age by menstrual and ultrasound estimation were different for more than 7 days, the ultrasound estimation was used. Fetal NT was measured using trans-abdominal or transvaginal sonography (ALOKA, Dynaview 2, SSD 1700 with a 3.5 MHz convex transducer). The fetus which was in a good mid-sagittal section occupied at least 75% of the image on the screen (Fig. 1).

All women had blood taken for measurement of serum PAPP-A and free beta-hCG level. The clotted blood was centrifuged as soon as possible, but not greater than 6 hours after collection. Serum samples were kept at 2-8°C for maximum 48 hours. For adequate specimens to analyze, the samples were collected at -20°C for 1 month avoid multiple freeze-thaw cycles for any specimen. The Behring Elisa Processer III (BEP III) machine was used for specimens analysis[5]. (Test principle and methods of measurement were discussed separately). Both tests were performed together in the same period

According to the regression equation, the expected 5^{th}, 50^{th}, and 95^{th} percentile values of fetal NT, maternal serum PAPP-A and free beta-hCG were obtained for gestational age.

Measurement of NT[6]

The NT was defined as the black area between the inner skin outlines echo and the outer border of the soft tissue overlying the cervical spine. The maximal thickness of the black area was measured with a caliper placed on the lines (representing the nuchal skin and the underlying soft tissue) when the sagittal section of the fetuses was obtained (Fig. 1). At the same time, the fetal crown-rump length (CRL) was measured. Cystic hygroma was defined as a sonolucent area consisting of two systemic cavities completely separated by a midline septum, irrespective of size[7]. These cases were excluded from the present study. The measurements could not be performed in cases of hyper-extended fetus and those indistinguishable between the fetal skin and the amniotic membrane[8]. The excluded cases were also fetal malposition. At least three measurements were taken during the scan and the largest was recorded.

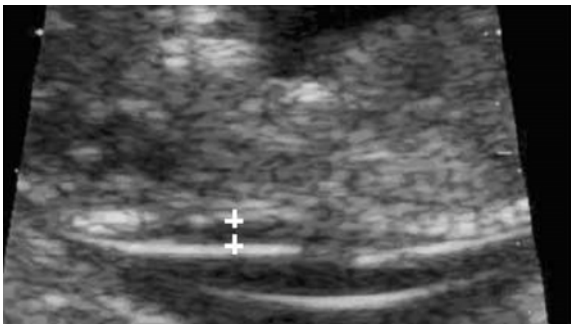

Fig. 1 Nuchal translucency was measured

Measurement of PAPP-A[5]

Reagent used

PAPP-A ELISA

Test principle

The PAPP-A ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a rabbit immunoglobulin anti-PAPP-A.

PAAPP-A presented in the sample binds to the anti-PAPP-A antibody coated onto the microtiter plate. After removal of unreacted material by a washing step, an antibody anti-PAPP-A labeled with horseradish peroxidase (HRP) is added to the wells.

So, the following complex is formed:

Rabbit IgG anti-PAPP-A* PAPP-A*

Antibody Anti-PAPP-A peroxidase conjugate.

After a second incubation followed by a washing step, the immunocomplex is detected by reaction to TMB substrate and the development of a blue color which changes into yellow by stopping the enzymatic reaction to sulfuric acid.

The intensity of this color is directly proportional to the amount of PAPP-A in the sample.

Absorbance at 450 nm is read using an ELISA microtiter plate reader.

Test procedure

Bring all reagents, samples, calibrators and controls to room temperature before use. Prior to starting the assay, the distribution and identification plan for all specimens and calibrators should be

carefully established. Select required number of microtiter strips and place in the strip holder. Allow 1 well for the substrate blank and 6 wells for the calibrators but it is recommended to perform the standard curve in duplicate.

Interpretation of results was automatically selected semi-log Cubic spline graph on the microplate reader.

Precision of the assay

Precision was evaluated upon intra and inter-assay variability, in 3 sera at different PAPP-A concentration (mg/L).

The performance of PAPP-A Elisa test has been assessed by determination of PAPP-A concentration in 51 samples in comparison with another commercially available kit. The correlation was 95%.

Measurement of free beta-hCG[5]

Reagent used

Free beta hCG ELISA

Test principle

The beta hCG test is a sandwich ELISA: the wells of the microplate are coated with a monoclonal anti beta hCG.

If beta hCG is presented in sample, beta hCG is captured by the antibodies immobilized on the microplate. After an incubation and a washing step to remove unbound material, specific monoclonal antibodies and beta hCG conjugated to HRP are added and bind to beta hCG.

So, the following complex is formed:

Mouse anti-beta hCG IgG * beta hCG* Mouse anti-beta hCG peroxidase conjugate.

After a second incubation followed by a washing step, the immunocomplex is detected by reaction with TMB substrate and the development of a blue color which changes into yellow by stopping the enzymatic reaction with sulfuric acid.

The intensity of this color is directly proportional to the amount of PAPP-A in the sample.

Absorbance at 450 nm is read using an ELISA microtiter plate reader.

Kit reagents

Reagents	Quantity	Physical state
Wells	96	Ready for use
Calibrates 0-5	6 x 1.2 ml	Lyophilised
Control Sera	2 x 0.5 ml	Lyophilised
HRP conjugate	1 x 0.25 ml	Concentrated 100x
Washing solution	1 x 100 ml	Concentrated 50x
TMB Substrate solution	1 x 15 ml	Ready for use
Blocking reagent	1 x 15 ml	Ready for use
Dilution buffer	1 x 25 ml	Ready for use

Intra-assay

Samples	Mean (mg/L)	CV %	Replicates
1	8.22	7.56	32
2	23.07	4.62	32
3	40.76	4.07	32

Inter-assay

Samples	Mean (mg/L)	CV %	Replicates
1	2.5	3.00	3
2	5.76	3.22	3
3	28.12	2.40	3

Kit reagents

Reagents	Quantity	Physical state
Wells	96	Ready for use
Calibrators 0-5	1 x 7 ml	Lyophilised
Calibrators 1-5	5 x 0.5 ml	Lyophilised
Control Sera	2 x 0.5 ml	Lyophilised
Enzyme conjugate	1 x 0.25 ml	Concentrated 100x
Washing solution	1 x 55 ml	Concentrated 15x
Chromogen Substrate	1 x 15 ml	Ready for use
Blocking reagent	1 x 11 ml	Ready for use
Dilution buffer	1 x 35 ml	Ready for use

Intra-assay

Samples	Mean $\pm$ SD (mg/L)	CV %	Replicates
1	38.5 $\pm$ 1.57	4.1	32
2	101.4 $\pm$ 6.08	6.0	32
3	193.2 $\pm$ 10.04	5.2	32

Inter-assay

Samples	Mean $\pm$ SD (mg/L)	CV %	Replicates
1	34.8 $\pm$ 1.28	3.7	3
2	100.5 $\pm$ 5.7	5.7	3
3	406.5 $\pm$ 30.4	7.5	3

Test procedure

Results reliability depends on strict adherence to the described procedure. Bring all reagents, samples, calibrators and controls to room temperature before use. All reagents must be mixed without foaming. Prior to starting the assay, the distribution and identification plan for all specimens and calibrators should be carefully established. Select required number of microtiter strips and place in the strip holder. Allow 1 well for the substrate blank and 7 wells for the calibrators but it is recommended to perform the standard curve in duplicate. A standard curve must be included in each assay.

Interpretation of results was automatically selected semi-log Cubic spline graph on the microplate reader.

Precision of the assay

Precision was evaluated upon intra and inter-assay variability, in 3 sera at different beta hCG concentration.

The performance of Free Beta hCG Elisa test has been assessed by determination of free beta hCG concentration in 40 samples in comparison with another commercially available kit. The correlation was > 90%.

Statistical analysis

SPSS version 13 was used to analyze data. Results were reported as means, standard deviations (SD), percentages and 5th, 50th and 95th percentile value.

Results

From 1 January 2004 to 31 December 2006, a total of 1,000 pregnancies which satisfied the above inclusion criteria were included for analysis. One fetus could not be measured NT due to fetal malposition. The mean maternal age was 28.03 $\pm$ 5.8 yr (range 14-47 yr). The mean maternal weight was 52.63 $\pm$ 9.06 kg (range 35-95 kg) The mean NT was 1.6 $\pm$ 0.8 mm (range 0.3-14 mm). The 5th, 50th and 95th centile of PAPP-A and free beta-hCG during 11-14 weeks of gestation were 1.54-69, 14-28, 51-57 and 24.8-17, 78-47, 181.6-126.5 mIU/mL, respectively. The distribution and the 5% and 95%, lower and upper limits of NT, PAPP-A and free beta-hCG according to the simple linear regression was shown (Fig. 2-4). The number of pregnant women at each gestational age is presented in Table 1. Mean maternal NT, PAPP-A, free beta-hCG at each gestational age are shown in Table 2-4.

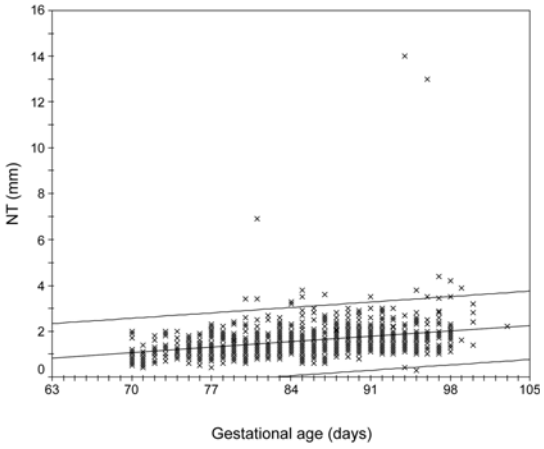

Fig. 2 The correlation of NT and gestational age[5]

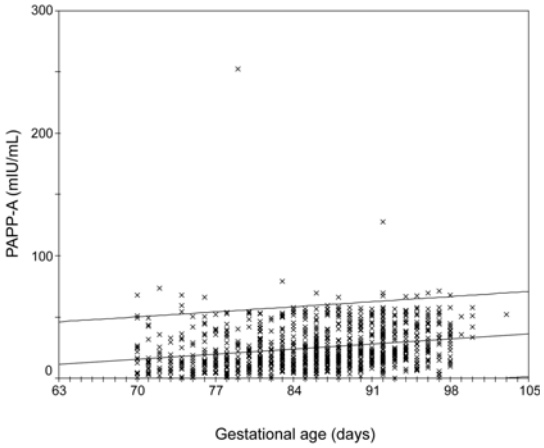

Fig. 3 The correlation of maternal serum PAPP-A and gestational age

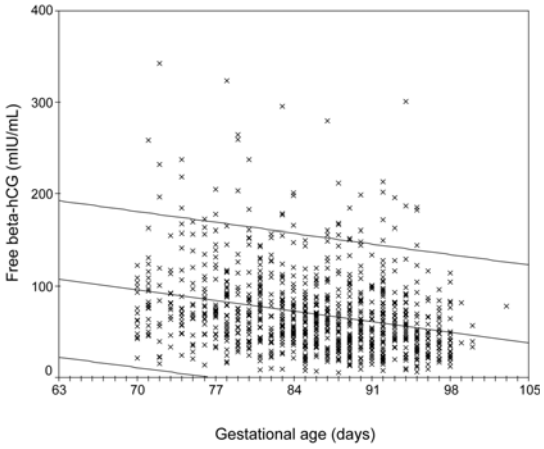

Fig. 4 The correlation of maternal serum free beta-hCG and gestational age

Table 1. Number of pregnant women measured at each week of gestation

GA (weeks)	Number of women	Percentage
10	12	1.2
11	125	12.5
12	237	23.7
13	339	33.9
14	287	28.7
Total	1,000	100

Table 2. Mean NT during 10-14 weeks of gestation is presented

GA (weeks)	Mean (mm)	SD	5th centile	50th centile	95th centile
11	1.2	0.5	0.6	1	2
12	1.4	0.77	0.8	1	2
13	1.6	0.66	0.9	2	3
14	2.0	1.18	1	2	3

Table 3. Mean maternal serum PAPP-A during 10-14 weeks of gestation

GA (weeks)	5th centile	50th centile	95th centile
11	1.54	14	51
12	3.68	16	52
13	5.28	20	55.1
14	69	28	57

Table 4. Mean maternal serum free β-hCG during 10-14 weeks of gestation

GA (weeks)	5th centile	50th centile	95th centile
11	24.8	78	181.6
12	24	73	161.8
13	20	58	148.1
14	17	47	126.5

Discussion

A screening test with NT measurement may identify over 70% of trisomy 21 pregnancies in the first trimester, with a 5% false positive rate[1,2]. Increased NT during 11-14 weeks of gestation is also strongly correlated with fetal structural defects, genetic syndromes, and poor perinatal outcomes[9-11]. NT measurements between fetuses of different ethnic origins showed a significant difference[12]. Many non-invasive tests have been provided for screening Down Syndrome fetuses. Most tests combined both serum and ultrasound screening which increases the sensitivity and specificity of the tests. The tests that were performed in the first trimester involve ultrasound determination of NT combined with serum markers of PAPP-A and free beta-hCG during 10-14 weeks of gestation.

The previous study[10,13,14] showed that NT measurements and a false positive rate increase with increasing gestational age which did not correlate with the present studies. Therefore, a fixed cut-off point through the first trimester was not appropriated and each NT measurement should be examined according to the gestational age. The normative distribution of fetal NT measurement was established.

Jou et al suggested that NT differences, race-specific normative data should be used[15]. Other reports concluded that it is acceptable to use a single standard, because screen positive rates in different NT groups are similar[12,16]. In the present study, the mean NT in normal Thai fetuses was 1.6 mm. Compared to the study in a Taiwanese population by Jou et al[15], mean NT in this result was smaller than those by 0.08 mm. Therefore, race-specific normative data for interpretation of NT should be used.

Serum markers are measured by immunoassay technique after serum taking and analyzed within a week[16]. The present study presents the data of fetal NT, serum PAPP-A and beta-hCG in gestation-specific mean. NT during 10-14 weeks of gestation increases by 10-20% per week. Serum PAPP-A levels in the first trimester increase by 40-50% per week during 10-14 weeks of gestation while serum beta-hCG levels decline by 35-40% per week during those periods.

The present study shows that the mean serum PAPP-A rises from 10 to 14 weeks of gestation, therefore the mean values of the first trimester PAPP-A in Down Syndrome pregnancies decreases as gestation progresses. Mean serum free beta-hCG decreases from 10 to 14 weeks of gestation, therefore the mean values of the first trimester serum beta-hCG in Down Syndrome pregnancies increases as gestation progresses.

Maternal serum PAPP-A and beta-hCG are altered by the placental secretory products such as progesterone[17], human placental lactogen[18], and pregnancy-specific glycoprotein 1[19], and rose in the second trimester of the affected Down fetus. Race is

the factor which effects the concentrations of maternal serum PAPP-A and free beta-hCG. Many studies supported the difference in analyte levels for races[20-22]. Therefore, data of mean levels of maternal Thai serum PAPP-A and free beta-hCG were created to use for analysis of the risk of fetal Down Syndrome.

Conclusion

In conclusion, the present study offers normative data of the fetal NT, maternal serum PAPP-A and free beta-hCG in Thai fetuses, which may be very beneficial in establishing screening for Down Syndrome in the first trimester.

Acknowledgement

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แผนภูมิอ้างอิงของความหนาของผิวหนังบริเวณต้นคอทารกในครรภ์ ระดับ PAPP-A และ free beta hCG ในเลือดมารดา

สายฝน ชวาลไพบูลย์, ประคอง ชื่นวัฒนา

วัตถุประสงค์*: เพื่อจัดทำแผนภูมิอ้างอิงของความหนาของผิวหนังบริเวณต้นคอทารกในครรภ์ ระดับ PAPP-A และ free beta hCG ในเลือดมารดา เพื่อการทำนายกลุ่มอาการดาวน์ในทารกไทยช่วงไตรมาสแรก*

วัสดุและวิธีการ*: ทำการศึกษาแบบไปข้างหน้าในช่วงระหว่าง 1 มกราคม พ.ศ. 2547–31 ธันวาคม พ.ศ. 2549 สตรีตั้งครรภ์ในช่วงอายุครรภ์ 10-14 สัปดาห์ จำนวน 1,000 ราย จะได้รับการตรวจอัลตราซาวด์ เพื่อวัดความหนาของผิวหนังบริเวณต้นคอของทารกในครรภ์ และสตรีตั้งครรภ์จะได้รับการตรวจเลือดเพื่อหาปริมาณของ PAPP-A และ free beta hCG สตรีตั้งครรภ์ที่ทารกมีโครโมโซมผิดปกติ มีโครงสร้างของร่างกายผิดปกติ ครรภ์แฝด มีการแท้งค้าง และทารกเสียชีวิตในครรภ์จะถูกคัดออกจากการศึกษานี้ สตรีตั้งครรภ์ และทารกทุกราย จะได้รับการตรวจติดตามในช่วงหลังคลอด*

ผลการศึกษา*: พบว่าความหนาของผิวหนังบริเวณต้นคอทารกในครรภ์มีค่าเฉลี่ย 1.6 $\pm$ 0.8 มิลลิเมตร (0.3-14 มิลลิเมตร) ส่วนค่า 5, 50 และ 95 เปอร์เซ็นต์ไทล์ของ PAPP-A และ free beta-hCG ในช่วงอายุครรภ์ 11-14 สัปดาห์ มีค่าระหว่าง 1.54-69, 14-28, 51-57 และ 24.8-17, 78-47, 181.6-126.5 mIU/m ตามลำดับ การกระจายของค่าความหนาของผิวหนังบริเวณต้นคอ, PAPP-A และ free beta-hCG ในช่วงระหว่าง 5-95 เปอร์เซ็นต์ไทล์ แสดงดังในแผนภูมิ*

สรุป*: ค่าความหนาของผิวหนังบริเวณต้นคอทารกในครรภ์จะมากขึ้นตามอายุครรภ์ที่มากขึ้น ค่าเฉลี่ยของ PAPP-A และ free beta-hCG ค่าเฉลี่ยของ PAPP-A จะเพิ่มขึ้น และ free beta-hCG จะลดลงในช่วงอายุครรภ์ 10-14 สัปดาห์ ในทารกไทยที่ปกติ ผลที่ได้สามารถใช้เป็นค่าอ้างอิงในการทำนายทารกกลุ่มอาการดาวน์ได้*