

Prenatal Diagnosis of Alpha-Thalassemia of Southeast Asian Deletion with Non-Radioactive Southern Hybridization

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Background: α -thalassemia is a common hereditary disease in Taiwan. Affected patients always carry a heavy burden of morbidity and early death. Prenatal diagnosis has reduced the disease burden on families and the health care system. This study evaluated a new non-radioactive Southern blotting hybridization method for prenatal diagnosis of this disease.

Methods: Seventy two chorionic villi samples (CVS) and 30 amniocyte samples from 102 pregnancies of couples who were both heterozygous for α -thalassemia-1 of the Southeast Asian (SEA) type deletion were studied. A non-radioactive Southern blotting hybridization method using a dig-alkaline phosphate detection system was developed for use in this study.

Results: Non-radioactive Southern blotting hybridization data showed that 19 (26%) CVS and five (17%) amniotic fluid samples had 10Kb and 4Kb fragments, indicating homozygosity of the α -thalassemia-1 SEA type deletion. DNA samples were extracted from most of the aborted tissue of the 24 fetuses with a diagnosis of homozygous for the α -thalassemia-1 SEA type deletion. Homozygosity for α -thalassemia-1 SEA type deletion was reconfirmed by Southern blotting hybridization in all of these samples.

Conclusions: The non-radioactive Southern hybridization protocol used in this study allows efficient and accurate early prenatal diagnosis of α -thalassemia-1 SEA type deletion. It can be routinely used for testing couples who both carry the α -thalassemia-1 SEA type deletion.
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Key words: non-radioactive southern blotting hybridization, α -thalassemia-1, chorionic villus sampling, genetic amniocentesis.

Alpha-thalassemia is a very common hereditary disease in Taiwan. The carrier rate is about 3.5% in the general population.⁽¹⁾ The human α -globin genes consist of two duplicate cis-arranged genes located on the short arms of chromosome 16.⁽²⁻⁴⁾ Deficient α -globin genes affect both fetal

hemoglobin ($\alpha^2\gamma^2$) and adult hemoglobin ($\alpha^2\beta^2$) production. The homozygous genotype of the α -thalassemia-1 has been shown to lead to hydrops fetalis.^(5,6) Lin et al. found 20 hydropic fetuses among 10156 births from six maternity hospitals in Taipei city.⁽⁷⁾ Eighteen of the cases were studied and

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homozygous Hb Bart's hydrops was detected in cord or cardiac blood of six infants, an incidence of 33.3%. In Southeast Asia, Hb Bart's hydrops is mostly due to deletion of four α -globin genes.⁽⁸⁻¹¹⁾ Fetuses with Hb Bart's hydrops fetalis syndrome usually die in utero during the third trimester or shortly after birth because of severe intrauterine anemia. Serious developmental anomalies, including severe retardation of brain growth, have been reported in these fetuses.^(12,13) Moreover, their mothers have an increased risk of serious complications including polyhydramnios, preeclampsia, ante- or postpartum hemorrhage, and difficult vaginal delivery.^(13,14)

Owing to the high carrier rate in Taiwan area, it is strongly recommended that married couples should consider undergoing genetic screening test for α -thalassemia-1 of Southeast Asia (SEA) type before conception. Under the direction of the Department of Health of the Executive Yuan, premarital screening for carriers of α -thalassemia and prenatal diagnosis of Hb Bart's hydrops have become an important public health policy. In this study, we developed a screening test for α -thalassemia for use in premarital and early prenatal examinations. This system is useful for routine prenatal diagnosis of α -thalassemia when both parents have the same α -thalassemia-1 SEA.

METHODS

From December 1995 through March 2001, pregnant women with mean corpuscular volume (MCV) <80fl, hemoglobin (Hb) A_2 <3.5% and normal serum ferritin at Chang Gung Memorial Hospital were transferred to the high risk pregnancy center. They were encouraged to undergo molecular screening tests for α -thalassemia along with the fathers of the fetuses. The carrier status of the couples was verified using results of the α -thalassemia screening program at the Chang Gung Medical Center in Lin-Kou, Taiwan.

When both the father and the mother were found to carry the SEA deletion, chorionic villus sampling or genetic amniocentesis was suggested to test for fetal α -thalassemia.

For the detection of α -thalassemia-1 carriers of the SEA type, 3 ml blood samples of both parents were collected antecubitally. DNA was extracted using a commercial kit (Qiagen, Germany).

Polymerase chain reactions (PCR) were performed to detect the ($--^{SEA}$) genotype using the S1, S2, and S3 primer combination as described by Ko et al.⁽¹⁵⁾ The relative primer positions are shown in Figure 1. Reactions were carried out in a total volume of 50 μ l which contained 300 ng of DNA, 200 μ M each of dATP, dTTP, dCTP, and dGTP, 20 pmol each of primer S1 and S2 or S1 and S3, 1 U hot start Taq polymerase (Qiagen, Germany), and 5 μ l of 10X buffer. Primer sets (L:CTGTCTTGTAACCTTGAT-ACC; R: TGAAGTCCAACCTCCTAAGCCA) specific for human β -globin gene, generating a 338 bp fragment, were used as internal controls for the PCR.

For detection of Hb Bart's hydrops in couples carrying the ($--^{SEA}$) deletion and avoidance of radioactive waster, a non-radioactive Southern blotting hybridization method using a dig-alkaline phosphate detection system was developed. Genomic DNA was extracted from chorionic villi or cultured amniocyte samples using a commercial kit (Qiagen, Germany). Fifteen micrograms of DNA samples were digested in 20 units of Acc 65 and Bgl (Biolab, USA) overnight at 37°C according to the conditions recommended by the manufacturer. The digested DNA was fractionated with a 0.75% agarose gel in 1X TBE buffer (Amersco) and transferred to a Hybond N⁺ membrane (Amersham) using standard Southern blotting procedures.⁽¹⁶⁾ After 2X washing with 2X SSC (1X SSC=0.3 M NaCl, 0.03 M sodium citrate), the DNA was UV-crosslinked to the membrane and subjected to hybridization with the ϕ ζ globin gene probe.⁽¹⁷⁾

The ϕ ζ globin gene probe (Fig. 1) was prepared using PCR. The reaction was carried out in a total volume of 50 μ l containing 20 ng of pBam 6.8 plasmid, 10 pmol of each primer (M13, direct: GTAAAACGACGGCCAGT; reverse: CAG-GAAACAGCTATGAC), 5 μ l of 10X buffer, 5 μ l of Q solution (Qiagen, Germany), 200 nM each of the dNTP, and 2 U of hot start Taq DNA polymerase (Qiagen, Germany). After preheating at 95°C for 15 min, the mixture was cycled 30 times for denaturation (94°C, 1 min), annealing (55°C, 1 min), and elongation (72°C, 2 min). An additional 10 min period at 72°C was added at the end of the last cycle. Fifteen μ l of the PCR products (~500bps) were resolved with a 2% agarose gel. The DNA was retrieved from the agarose block with 600 μ l H₂O and used as a template in the secondary PCR.

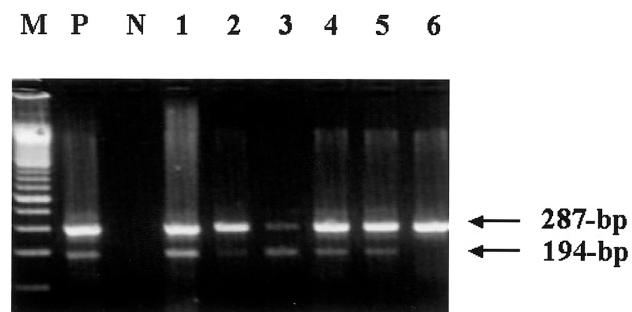


Fig. 1 PCR results with primer combinations of S1 and S3, and S1 and S2. M: 100bp DNA ladder, P: positive control, N: negative control. Lanes 1 to 5: samples heterozygous for α -thalassemia-1 of SEA type deletion (although the 194-bp band in lane 2 was very weak). Lane 6: results from a subject without α -thalassemia-1 SEA type deletion. The 338 bp bands: internal control for PCR (see text).

Reaction conditions of the secondary PCR were the same as above except for the addition of 5 μ l of 10X Dig DNA labeling mixture (Boeringer Mannheim, Germany). The secondary PCR products were subjected to purification using a gel extraction kit (Qiagen, Germany). The prepared probe was stored in -20°C until used.

RESULTS

One hundred and two pregnancies with increased risk of Hb Bart's hydrops because both partners carried the α -thalassemia-1 SEA type deletion were included in the study (Fig. 1, lanes 1 to 5). The median maternal age was 26 years (range, 16-37 years). Of these pregnancies, 72 received CVS at 9 to 12 weeks (median, 11 weeks) of gestation and 30 received genetic amniocentesis at 15 to 18 weeks (median, 16 weeks) of gestation. Among the 72 CVS samples and the 30 amniocyte samples analyzed, non-radioactive Southern blotting hybridization showed that 19 of the CVS samples and five of the amniotic fluid samples had 10 Kb fragments, indicating homozygosity of α -thalassemia-1 SEA type deletion (Fig. 2).

Thirty one of the 72 CVS samples and 17 of the 30 amniotic fluid samples had 12 Kb, 10 Kb, and 4 Kb fragments, indicating their heterozygous genotypic composition for the α -thalassemia-1 SEA type

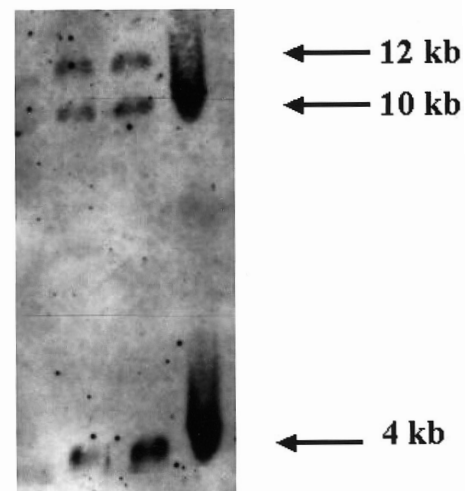


Fig. 2 Non-radioactive Southern blotting hybridization data. Lanes 1 and 2: cases heterozygous for α -thalassemia-1 SEA type deletion. Lane 3: a chorionic villus sample homozygous for α -thalassemia-1 of SEA type deletion.

Table 1. Results of Prenatal Diagnosis of α -thalassemia-1 SEA type from Both Parents with the Same Carriers

	CVS	AF	Total
Normal	22 (31%)	8 (27%)	30
Carrier	31 (43%)	17 (56%)	48
Hydrops	19 (26%)	5 (17%)	24
Total	72	30	102

Abbreviations: CVS: chorionic villi samples; AF: amniotic fluid.

deletion (Fig 2). Twenty-two CVS samples and eight amniotic fluid samples had only the 12 Kb and 4 Kb fragments, revealing a normal α -globin genotype free of the α -thalassemia-1 SEA type deletion (Table1).

Among the 48 heterozygous pregnancies and 30 pregnancies of normal genotype, no complications associated with α -thalassemia-1 were noted at birth or postnatally. All of the 24 fetuses with a diagnosis of homozygous for the α -thalassemia-1 SEA type deletion using the non-radioactive Southern blotting hybridization were terminated at the request of the parents. DNA samples were extracted from all of the aborted fetuses and homozygosity of α -thalassemia-1 SEA type deletion was reconfirmed in all of them using Southern blotting hybridization.

DISCUSSION

In Taiwan, PCR has been used as a diagnostic tool for the rapid detection of α -thalassemia-1 SEA deletion.^(15,18) In 1996, Cheung et al. reported successful prenatal diagnosis of thalassemia using PCR analysis of fetal cells in maternal blood.⁽¹⁹⁾ However, this approach must be simplified to keep costs down and to reduce the level of technical expertise required to obtain reliable results. Although PCR can be used even with trace amounts of DNA, the presence of maternal cell contamination in the CVS or amniotic fluid samples make the results difficult to interpret. Therefore, an alternative protocol using conventional Southern blotting hybridization for prenatal diagnosis of this disease was proposed.^(15,17) This protocol analyzed genomic DNA directly without PCR amplification, thus, the influences of potential maternal cell contamination are avoided.

Because conventional Southern blotting hybridization uses radioactive material such as ^{32}P -labeled probes to detect the fragments, it is not feasible in many clinical laboratories since handling radioactive waste is costly and creates the additional problem of exposure of the laboratory technicians to radiation. The non-radioactive Southern blotting hybridization protocol for the detection of α -thalassemia-1 SEA type developed for this study avoids the problems of the conventional methods. The technique uses digoxigenin-dUTP (Boeringer Mannheim) in the synthesis of the ϕ ζ -globin gene probe.⁽¹⁷⁾ After hybridization, the detection is performed using alkaline phosphatase-conjugated anti-dig antibody and disodium 3-(4-methoxyspiro{1, 2-dioxitane-3, 2'-(5'-chloro) tricyclo [3.3.1.1] decan}-4-y 1) pheny 1 phosphate (Roche) as the substrate. Chemiluminescence is captured on Kodak Biomax film.

In our study, 24 of 102 CVS or amniotic fluid samples were homozygous for the α -thalassemia-1 SEA type deletion and all of these cases were re-confirmed using Southern blotting hybridization performed on DNA extracted from aborted tissue after termination of the pregnancies. However, PCR data for the nine CVS and one amniotic fluid samples with homozygous α -thalassemia-1 SEA type showed that the deletion was present in only one of the α -globin gene alleles. This finding is not concordant

with the results of the Southern blotting hybridization. Thus, maternal cell contamination was likely to be the cause of the false negative PCR results. These results suggest that although the PCR-based protocol is useful in the identification of α -thalassemia-1 SEA type carriers, it might not be appropriate for use in prenatal diagnosis with CVS or amniotic fluid samples.

During the past 25 years, there have been important advances in the management of individuals with α -thalassemia. Available treatment options include prenatal red blood cell transfusion, pharmacologic interventions to increase fetal hemoglobin levels, and stem cell transplantation.⁽²⁰⁾ Improvement in these approaches and the development of means to replace defective genes with normal ones using gene transfer techniques offer hope for the future, but management-associated complications, expense, and a paucity of suitable donors have limited their application.⁽²¹⁾ The lack of a widely available and effective treatment indicates the necessity for disease prevention. Genetic analysis of fetal tissues, amniocytes or blood for early identification of Hb Bart's hydrops fetalis allows elective termination of affected pregnancies. In Hong Kong where is also a high prevalence area of α -thalassemia, a community-based screening program and prenatal diagnosis for high risk couples have reduced the disease burden to families and to the public health care system.⁽²²⁾

In conclusion, we have successfully developed a non-radioactive Southern blotting hybridization protocol for prenatal diagnosing of α -thalassemia-1 SEA type. The technique is a useful routine test for couples when both partners carry the α -thalassemia-1 SEA type deletion.

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利用非放射性南方墨點雜交反應作東南亞缺失型甲型 海洋性貧血之產前診斷

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背景： 在台灣地區甲型海洋性貧血的發生率甚高。提早的產前診斷足以減輕此一疾病對家庭及健康照護體系的負擔。本研究嘗試利用非放射性南方墨點雜交反應來達成此一目標。

方法： 取得夫妻皆為東南亞缺失型甲型海洋性貧血異質基因型者共72個絨毛採樣檢體及30個羊水細胞檢體。利用毛地黃標誌探針及鹼性磷酸酶冷光呈色系統 (dig-alkaline phosphatase detection system) 作非放射性南方墨點雜交反應以為產前遺傳診斷。

結果： 非放射性南方墨點雜交反應顯示絨毛採樣檢體19個案(26%)及羊水細胞檢體5個(17%)出現10kb及4kb片段，表示其為東南亞缺失型甲型海洋性貧血同質配合的基因型。這24個(100%)被診斷為東南亞缺失型甲型海洋性貧血同質配合的基因的個案經引產後取自流產胚胎的DNA檢體，皆經南方墨點雜交反應進一步確認其基因型。

結論： 目前，對於夫妻皆帶有東南亞缺失型甲型海洋性貧血異質基因者，非放射性南方墨點雜交反應法在早期診斷東南亞缺失型甲型海洋性貧血同質基因個案是一個兼具環保與準確性且值得推廣的理想方法。

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關鍵字： 非放射性南方墨點雜交反應，甲型海洋性貧血，絨毛採樣，羊水穿刺。

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