REVIEW ARTICLE

Prenatal Diagnosis of Thalassemia

Sunarto

(Department of Child Health, Medical School, Gadjah Mada University, Yogyakarta)

Abstract. Thalassemia is an individual as well as a community health problem in some countries. It causes a lifelong suffering for the affected individuals. There is no treatment other than supportive, i.e. regular transfusions and removal of iron overload from the body. Only by such continuous and expensive treatment thalassemic patients can generally achieve nearly normal health, but the health burden of such therapy for a large number of thalassemic patients is unaffordable by the affected communities. Prevention of the births of thalassemic babies is the choice for controlling the thalassemia and has been successful in many countries. For this purpose reliable and time accurate prenatal diagnosis is a conditio sine qua non. Blood fetal sampling is safe and can be done after 16 weeks gestation, amniocentesis after 14 weeks, and even chorionic villi sampling as early as 8 weeks gestation. In vitro globin syntesis analysis applied to the fetal blood sample is very reliable to measure the rate of synthesis of the globin chains that make up the hemoglobin. The DNA analysis of the fibroblasts obtained by amniocentesis or of the chorionic villus sample is very sensitive and specific for the diagnosis of the genetic disorder in thalassemias. By involving the prenatal diagnosis, the birth of B-homozygous thalassemia has decreased by up to 90% . **[Paediatr Indones 1933; 33: 191-9].**

Introduction

Thalassemias are genetic disorders that constitute serious health problems in many countries.[1,2] Among the thalassemias, α- and β-thalassemia are of clinical importance. They cause a lifelong suffering for the affected individuals. No treatment other than supportive is up till now available. Bone marrow transplantation results in 2 years disease free in 60% advanced thalassemia cases,[3] but this approach is very expensive and serves only as an individual solution. Especially homozygote β-thalassemia needs routine tranfusions, as frequent as every 2-3 weeks,[4,5] otherwise patients will have poor quality of life and many complications will occur. Most of patients with homozygote beta-thalassemia cannot reach adult life.[6] Hemosiderosis with its unfavorable consequences is a late complication, either due to frequent transfusions or the increased iron absorption as

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the characteristic of the disease. Iron overload finally contributes to the patient's death usually in the second decade of life. Removal of the iron excess from the body by iron chelating agents is then needed. This should given lifelong and many problems will arise both from the side of patients and the health services.[5] By such treatment patients can achieve essentially normal life; their life expectancy is longer, but it also means that the health services should be increased to cope with the increasing survivors, while more babies with thalassemia are born. As the supportive treatment is very expensive, the increasing number of patients makes the health burden unaffordable by the affected community, especially in the developing countries. In Cyprus in 1979 the total cost of iron chelating agent alone represented 6% of the annual budget of the Ministry of Health, while only 77% of 597 known homozygotes received the agent.[7]

For those reasons, besides health service for the patients, prevention of the birth of thalassemic babies is the choice in thalassemia control program. For that purpose a safe and reliable prenatal diagnosis is the most essential issue. The diagnosis should be done as early as possible during pregnancy and the diagnostic test should be extremely sensitive and specific. The problem has now been partly solved; prenatal diagnosis has been included in the thalassemia control in some countries with 50-90% reduction of thalassemia-major birth-rate.[2,8,9]

In this paper the prenatal diagnosis of thalassemia, concerning technical aspects and its effect on the disease control, will be discussed.

Fetal tissue sampling

Prenatal diagnosis has been of great benefit for diagnosing many fetal hematological pathologies. Various techniques have been applied, such as ultrasonography, amniocentesis, chorionic villus sampling (CVS), fetal blood sampling (FBS), and cordocentesis. Ultrasonography provides prenatal diagnosis of Hb Bart's hydrops fetalis, detection of the affected fetuses. Most major structural malformations can be detected in the second trimester of pregnancy. However, the findings need confirmation. Hydrops fetalis can be diagnosed unambiguously on ultrasonography by the presence of hydrothorax, pericardial effusion, and anasarca edema, but it can be made only at 18-20 weeks of gestation which leads to a late termination. Anyhow, ultrasound plays an important role in guiding invasive procedures and in providing important supporting information. All invasive procedures need ultrasonic guidance for their success. Whichever procedure is being used, ultrasound is necessary as a preliminary step to evaluate the number and viability of the fetus, the gestational age, the placental site, and the best approach to the target which could be the chorion frondosum, a pool of amniotic fluid, the umbilical vein, or even the fetal heart.[10] The introduction of FBS during pregnancy has opened a new field of prenatal diagnosis of congenital hematological disorders and the management of some of these.

The first FBS was succesfully performed by Valenti in 1973[10] to obtain blood from the umbilical cord in patient undergoing hysterectomy in the second trimester. Since then different approaches have been established to obtain fetal blood during pregnancy, originally in the second but are increasingly being used in the third trimester as well.[10,11] Fetal blood is obtained by introducing needle into the placenta, the cord insertion, the hepatic part of the umbilical vein, or the fetal heart.

Cordocentesis is done transplacentally. The fetal blood sample is obtained by using a 20-gauge needle, 15 cm length, inserted under ultrasonic guidance into the umbilical vein of about 1 cm from the placental cord insertion. Local anesthesia is used but sedation is not necessary.[10,11] Blood samples obtained from needle puncture at the cord insertion give a better yield of pure fetal blood with minimal (even nil) maternal red cell contamination. By experienced hands serious complications occur in less than 3% . Usually about 0,5 ml of pure fetal blood is enough for in vitro globin chain synthesis.[11,12]

The technique of amniotic fluid sampling (AF) was first reported in 1930 by Menes and in 1950 Bevis demonstrated the clinical importance of AF in the evaluation of fetal erythroblastosis.[10] It has been used for over 20 years for prenatal diagnosis of fetal chromosome abnormalities and metabolic defects. The technique consists of the introduction of needle (generally 21-gauge) transabdominally into the amniotic cavity, with scanning immediately beforehand or with the simultaneuos use of real-time ultrasound with or without a biopsy guide. Local anesthesia is not required. By this technique fetal cells (fibroblast) can be obtained for immediate analysis or after cell culture. Usually 10-15 ml of AF will contain enough DNA for laboratory analysis. The risk of amniocentesis for the mother and the fetus is very low; the risk of miscarriage following the procedure is about 0,5 to 1%.[11] Fine needle amniocentesis at 11 or 12 weeks removes proportionally larger volume of amniotic fluid than amniocentesis at 16 weeks, with the threat of vascular disturbance in the trophoblasts when the amniotic sac contracts down.[13] CVS is a newer procedure whereby a small sample of chorion is removed for analysis. CVS can be performed transcervically with few complications at 8-10 weeks of gestation. Transabdominal CVS can be done at any stage of pregnancy provided that placenta is in appropriate position. The risk of CVS includes spontaneous abortion, perforation of the membrane which leads to amniotic fluid leakage, infection and intrauterine death. However, by experienced hands transabdominal CVS is associated with only 1-2% fetal loss.[11,12] The average abortion rate following CVS in various centers is now estimated to be around 3,5%, with individual rates ranging from 1,8 to 4,6%. Lower rate occurs in those centers with an experience greater than 1000 cases.

Different approaches to CVS are currently used. The most commonly used technique is aspiration of villi through a canule inserted through the cervix and

guided under ultrasonic control to reach the chorion frondosum; at this point vacuum aspiration is applied, which is performed using a 20 ml syringe.[10] The presence of chorionic villi is confirmed microscopically bedside. Normally 20-50 mg of tissue is needed for the conventional DNA analysis, while gene amplification technique needs smaller sample. The early diagnosis by CVS reduces the social and psychological stress and avoids the hazards of second trimester termination if needed. Although other related complications may occur, for example the likely risk of one in 60 of facial and limb deformities following transabdominal CVS, and the rise of maternal a-fetoprotein, the demand for first trimester diagnosis makes CVS a valued option until we have a better alternative.[13]

Laboratory tests

There are two strategies for prenatal diagnosis of thalassemia,[19] i.e. globin chain analysis and DNA analysis. Laboratory analysis of fetal blood sample can give many information, such as the assessment of fetal hemoglobin and hematocrit, globin chain ratios, plasma clotting factors, platelet number, characterization of leucocyte, complement level; cytogenetic and DNA analysis can also be performed.[10] In the suspected case, by hemoglobin electrophoresis of the fetal blood obtained from percutaneus cordocentesis, the presence of 85% Hb Bart's and 15% Hb Portland indicates the diagnosis of Hb Bart's hydrops fetalis.[12]

Homozygous β-thalassemia and β-thalassemia/Hb E are the most prevalent hemoglobinopathy in Southeast Asian countries, including Indonesia.[14] Thalassemia syndrome is caused by reduction or diminition of globin synthesis, and analysis of in vitro globin synthesis can reveal the disorders.[15-18] From 0,5 ml pure fetal blood, in vitro B/G globin synthesis can be measured. In this procedure blood (reticulocyte) is incubated with radioactive leucine for a certain period, washed, lysed and the globin chains are separated by collumn chromatography. The amount of radioactivity incorporated into the globin chains shows the synthesis of the individual chains during the period of experiment.[1] Usually this procedure is carried out only for the prenatal diagnosis of possible β-thalassemia syndrome, as the α-thalassemias are not such a great problem because Bart's hydrops fetalis is stillborn or does not live long after birth, while HbH thalassemia is never as severe as β-thalassemia syndrome.[14] Globin chain analysis is a very reliable method of prenatal diagnosis, up to 99% accuracy;[19] unfortunately FBS can be done only after 16 weeks of pregnancy. The following table shows different synthesis ratio of the B/G globin chain in various β-thalassemia syndrome.[12]

DNA analysis is relatively a new technique. The basic principle underlying the procedure is that all cells of living organisms contain all the genes present in the individual. For example, the human skin cell possesses the gene to produce insulin but it does not do so, while the gene in the β-islet cell of the pancreas does so. Therefore the DNA analysis to detect mutation in the genes coding for globin synthesis can be done on any nucleated cell, like polymorphonuclear neutrophil, fibroblast, buccal epithel, etc; in prenatal diagnosis the fetal cell is obtained by CVS or amniocentesis.[19] Molecular study of hemoglobinopathy has been done extensively and the molecular structure and DNA sequence of normal as well as a vast majority of abnormal hemoglobin have been documented.[20,21] It has made the molecular diagnosis of thalassemia is already in practice. The following methods are now available:[6,20]

1. *Direct detection of gene deletions*, by applying Southern blot analysis, the α-gene deletions in heterozygous α-thalassemia can be detected by using Z-gene probe, and in α^+-thalassemia and homozygous α^o-thalassemia by using an α-gene probe

2. *Direct detection of mutant genes by restriction enzyme analysis*: restriction endonucleases - bacterial origin enzyme-cut DNA at specific sites. The resultant restriction fragments are of different sizes and can be separated by electrophoresis. When a mutation abolishes a restriction site within the gene, a restriction fragment of different size will result and this serves to identify the abnormal gene.

3. *Detection of mutations with synthetic oligonucleotide probes*: when the mutation is known, an oligonucleotide probe, a short strand of DNA complementary to the mutant, can be synthesized that will identify the mutant directly.

4. *Detection of mutations by linkage analysis*: this method can be used when the mutations is not known. As mentioned above, restriction endonucleases cut DNA at specific sites resulting in fragments of different length. Some sites are polymorphic and inherited in a Mendelian

Table 1. B/G globin synthesis ratios detected at 19-22 weeks of gestation in normal fetuses and fetuses with various β-thalassemia syndrome[12]

Phenotype of fetus	Range of B/G	Pattern of chromatography
Normal	>0.10	G,B^A,a
β-Thalassemia heterozygote	0.06-0.10	G,B^A,a
HB E heterozygote	0.06-0.10	G,B^A,B^E,a
β-Thalassemia / Hb E	0.03-0.04	G,B^E,a (no B^A)
Homozygous β-Thalassemia	0.03-0.40	G,a (no B^A)
Homozygous HB E	0.045	G,B^E,a (no B^A)

manner; restriction fragment length polymorphism (RFLP). If a mutant locus is closely linked to polymorphism, DNA analysis of the parents and other family members for the polymorphism may identify whether the fetus has inherited the mutations.

The above mentioned procedures need sufficient amount of cells, that is difficult to obtain by amniocentesis. This amount can be achieved by cloning.[22] Cell cloning needs several weeks and it means a late termination. By CVS it is possible to obtain up to 100 mg of pure fetal DNA at 8-10 weeks of pregnancy. Polymerase chain reaction procedure (PCR) can amplify DNA from a very small sample, so that it overcomesthe problem.[23-25] The principle of this technique is that synthetic oligonucleotide that is complementary with a segment of DNA or a gene that the sequences have been knwn, can act as primer for amplification of the gene by *in vitro* enzymatic process. This procedure is simple, needs only 3 hours to complete the reaction and by the introduction of Taq polymerase it is possible for automatization.[26] This technique is very sensitive, specific, take a short time and much easier to perform. The DNA sequence of interest can be amplified more than 10^6 folds.

A recently developed procedure is direct detection with mutant specific primers which is called amplification refractory mutation system (ARMS). The principle is: only primer that is complementary with the sequence of the studied mutant can amplify the segment of the target DNA.[27,28] If a mutant that is complementary with the specific applied primer exists, the DNA will be amplified and can be visualized directly after electrophoresis under UV light with ethidium bromide staining. No band appears in the electrophoresis means no amplification occurred, and this means that there is no mutant that is complementary with the applied ARMS primer. If genetic mutation pattern in a certain population has been documented, by applying ARMS primers that are specific for common occurred mutants in the population, the mutation in the fetal samples can be detected easily. By PCR technique and then oligonucleotide probing or direct visualizing the DNA pattern under UV light prenatal diagnosis can be obtained within 12-24 hours after FBS.[12,27] This approach can be used to detect Hb Bart's hydrops fetalis and β-thalassemia mutations which either creates or eliminates a particular restriction enzyme cleavage site. During the course of prenatal diagnosis it is possible to reveal a previously unknown mutation.[29]

Implementation

Due to the enormous health burden of thalassemia, some countries implement prevention programs. Earlier attempts to control thalassemia were not effective, since they tried to decrease the birth rate of homozygotes by altering the marriage behaviour of heterozygotes. The introduction of fetal diagnosis provides a new solution because it allows conventional marriage behavior with the possibility of modifying reproductive behavior. The pilot study in European and Mediterranian centers in 1970-ies have shown that this form of control has been highly effective.[9] Antenatal diagnosis has had an increasing influence on the reduction of homozygote births in Cyprus.[7] Table 2 shows the effects of the control program in Ferrara, Northern Italy.[9]

Monitoring of the residual thalassemia-major birth-rate in other Mediterranian countries implementing thalassemia prevention has shown falls of 50% in Greece, 60% in Sardinia, 92% in Cyprus in the past 5 years. However, thalassemia control program introduced in 1977 in Britain showed much lower results. It has proved to be highly acceptable to the at risk couples of Cypriot and East African Asian origins, but less so to couples of Pakistani origin. The thalassemia-major birth rate has reduced by 60% in Cypriots and by 20% in East African Asians, but it has not reduced at all in Pakistanis. Social and cultural barriers have a great effect.[8] After introduction of 1st trimester diagnosis by CVS, 99% of couples in Sardinia accepted prenatal diagnosis as method to detect the presence of an affeced fetus.[30] Almost all prenatal diagnosis is now done by CVS rather than amniocentesis because CVS can be carried out early in gestation.

Prenatal diagnosis which is the necessary "option" for at risk couples must be established at the beginning of thalassemia controlprograms. Trained staffs should include obstetricians to perform the fetal tissue sampling and scientists to analyze the samples by either DNA or protein technics.[31] Future developments will improve sampling procedures and method of analysis:[30]

The possibility of defining mutation by analysis of limited number of cells or

Table 2. Effects of screening and fetal diagnosis on the incidence of thalassemia major in Ferrara [9]

Phase	Thalassemia major birth rate				
	Years	No. of births	Expected	Found	% reduction
Screening and counselling: no contraception	1970-1972	15,035	24	24	0
Screening and contraception: no fetal diagnosis	1973-1976	18,057	29	19	34
Introduction of fetal diagnosis	1977-1978	7,341	12	6	50
Screening, contraception, and established fetal diagnosis service	1979-1980	6,119	10	1	90

. The possibility of defining mutation by analysis of limited number of cells or even a single cell may lead to earlier prenatal diagnosis or to preimplantation diagnosis. Minimal amount of cells by amniocentesis or CVS at 6-7 week gestation will provide enough DNA for analysis. Preimplantation diagnosis may be carried out by biopsy at the morula stage following in vitro fertilization or biopsy of blastula washed from uterine cavity following in vitro fertilization.

Conclusion

Technical development in fetal tissue sampling, in biochemical as well as molecular concepts and diagnostics have made the prenatal diagnosis of thalassemia easier and much more practical to be implemented. Prenatal diagnosis has been introduced into thalassemia control programs and result in much decrease of homozygous thalassemia.

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