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Prenatal Diagnosis of Hemoglobinopathies: A Case Study on Tunisia

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Abstract

Hemoglobinopathies are the most common genetic disease in Tunisia with a total carrier prevalence of 4.48%, reaching 12.5% at certain affected regions. The β-thalassemia trait frequency is 2.21% and the sickle cell trait is 1.89%. A prenatal diagnosis (PND) unit has been progressively installed since 1986 at the biochemistry and molecular biology department of children's hospital of Tunis. The present study tries to explore the prenatal diagnosis of the Hemoglobinopathies in Tunisia for the period of 1994-2012 and tries to share its experiences and the progress achieved in overcoming this challenge.

340 out of 461 fetuses examined for this study were at risk and couples that have agreed for pre-diagnosis have benefited a lot in averting the major health risks like beta-thalassemia (41%), sickle cell anemia (40.3%), S/beta-thal (14.7%) and the remaining fetuses were at risk for a compound heterozygote hemoglobinopathies (S/O, O/beta-thal, S/C). 25.8% of the couple covered in this study have voluntarily asked for PND several times, as they were worried about giving birth to a child with Hemoglobinopathy. Fetal DNA was collected from chorionic villis biopsy of 53 cases, amniotic fluid samples are considered in 397 cases and CVS was followed in 7 cases for lack of results. Out of 461 tested foetuses, 26.2% of them were affected, 50.5% were carriers of the disease and 19.3% were completely healthy. PND fail to detect the problem in 3.9% of the cases. Except 13% of the affected fetuses, the entire number of cases detected with defected fetuses had been aborted. Although abortion is legal in Tunisia, 13% of pregnant women with affected fetus have refuted to abortion due to religious reasons. The total number of fetus aborted remained 1.53%. Although PND was successful in our lab, it was insufficient to cover the entire Tunisian territory. The researchers hence recommend to the health authorities to establish widespread PND services in places that are worst affected with these cases in Tunisia to prevent hemoglobinopathies

Keywords: Prenatal diagnosis; Hemoglobinoparhies; Beta-thalassemia; Sickle cell disease; CVS; Amniocentesis

Introduction

Hemoglobinopathies are autosomal recessive disorders of globin chain synthesis, affecting the expression (Thalassemia) or the structure (Sickle-cell disease SCD) of the globin gene products, leading to homozygous state of chronic and severe anaemia.

Beta thalassemia and SCD constitute the most common monogenic hemoglobin disorders in Tunisia. Beta thalassemia results in reduced (β+) or absence of (β0) synthesis of the beta globin chain. This condition leads to Sickle cell disease as a result of a gene defect, causing substitution of the glutamic acid for valine on the 6th codon of the β globin gene. In addition, there are some other rare hemoglobin variants such as hemoglobin C and hemoglobin O arab. A combination of these variants leads to compound heterozygote forms (e.ghemoglobinose SC, hemoglobinose SO)

Beta thalassemia is a chronic hemolyticanemia. It requires a long term transfusion program leading to severe complications, including premature death in some cases. Sickle cell disease is also a chronic hemolyticanemia with acute and chronic complications like severe anemia, strokes, pneumococcal infections, and painful crisis, which are potentially lethal. The only effective treatment for the hemoglobinopathies is the allogenic bone marrow transplantation, which remains inaccessible due to its high cost and lack of donors.

Previous epidemiologic studies have showed that the total hemoglobinopathies carrier prevalence in Tunisia was 4.48% and it may reach to 12.5% in certain identified risky regions. The β thalassemia trait frequency is 2.21% while the frequency of 'Haemoglobin S' carrier is 1.89% [1].

Several molecular studies have established the complete molecular spectrum of β thalassemia and this is very useful in the effective execution of prenatal diagnosis. 29 β thalassemia mutations have been identified during this study period and cd39 C–T and IVS1-110 G-A [2-5] were the most common mutations in these cases.

The high frequency, morbidity, mortality and lack of curative treatment for hemoglobinopathies made it a major public health issue, justifying the development of preventive programs. These programs can be accomplioshed through genetic counselling, carrier detection, newborn screening and prenatal diagnosis of couples at risk.

The main objective of the prenatal diagnosis is to reduce new cases of hemoglobinopathies that registered a steady raise over the years due to high rate of consanguineous marriages and lack of preventive programs.

Ethical and religious considerations are highly influencing the acceptability of prenatal diagnosis followed by termination of pregnancy in certain cases.

The researchers aim to report their findings on prenatal diagnosis of hemoglobinopathies in Tunisia over a period of 18 years starting from1994-2012 and would like to assess the impact of the preventive program on hemoglobinopathies status in Tunisia.

Material and Methods

Patients and procedure

340 out of 461 cases with defected fetuses have been benefited by the prenatal diagnosis at biochemistry and molecular biology department of Tunisian children's hospital for the past 18 years, starting from 1994 to 2012.

The pregnancies that were at risk for β-thalassemia major, sickle cell disease, and compound heterozygote forms of hemoglobinopathies (S/O, S/C, S/beta-thal, O/beta-thal) have been considered for the study.

Paediatricians, gynaecologists, congenital and hereditary disorders department, Charles Nicolle hospital, and the outpatient division of Tunis children's hospital have referred most of the cases to the department of hemoglobinopathies for treatment, while a section of patients have approached this wing on their own (Figure 1).

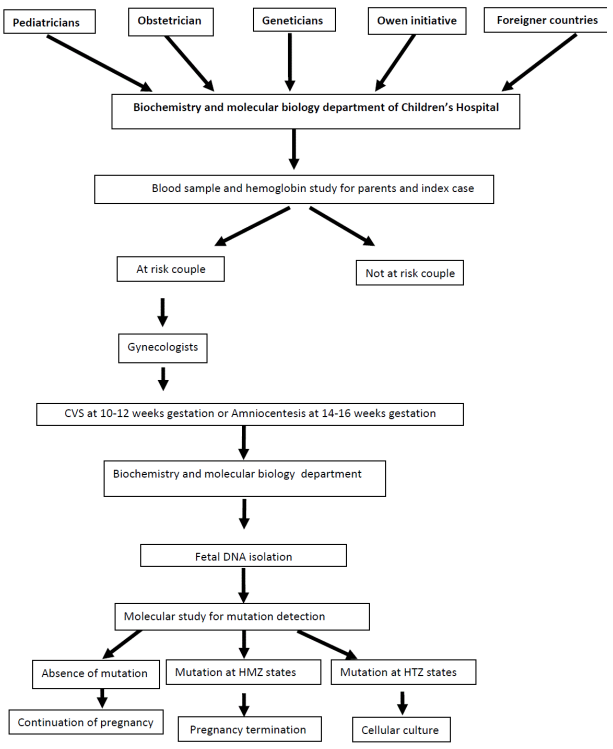

Figure 1: Hemoglobinopathies PND organigramme.

Before prenatal diagnosis, the heterozygosity of both the parents is confirmed by haemoglobin study and complete blood count (CBC). Molecular analysis is performed for both the parents in order to identify the mutation. Sometimes, all these tests are conducted simultaneously along with fetal DNA analysis.

Both the couple at risk are counselled; they are informed about the risks and the complications of the fetal sampling such as fetal loss rate, etc. and about the possibility of a misdiagnosis. They were also informed about the possible fetuses diagnostics. Continuation or termination of pregnancy would be left to the couple's discretion.

Collection of fetal samples

A written informed consent would be obtained from all couples prior to fetal sampling collection.

Tunis National centre for Maternity and neonatology or in the Gyneco-obstetrical department of Charles Nicolle hospital generally collect the fetal and they are immediately sent to our department for the molecular analysis.

Chorionic villus sampling (CVS) is conducted for fetus of 10-12 weeks of gestation or amniotic fluid sampling is followed after a14-16 weeks of gestation. PND is carried out using ultrasound techniques and CVS is perfoormed trans-abdominally.

Twenty milliliters (20 ml) of amniotic fluid would be used for DNA analysis of fetalamniocytes and an additional 20 ml sample is cultured for caryotyping when the mother's age is over 35 years, and to provide the source of DNA if additional analysis is required.

Hematological analysis

Haematological parameters are determined by the flow cytometry on an automated Beckman LH750TM Haematologyanalyser (Beckman Miami, Flo, USA). Haemoglobin (Hb) identification was carried out through High Performance Liquid Chromatography (HPLC), using Variant TM II System (Bio-Rad Laboratories, Hercules, CA, USA).

DNA analysis

DNA is extracted from blood leucocytes of the parent's genomic using salting-out technique. Fetal DNA is obtained using the same procedure either from chorionic Willis after dissection and removal of maternal tissue under a microscope, or from amniotic fluid containing fetal cells.

β-thalassemia mutations are screened for the detection discrepancies using polymerase chain reaction (PCR) based procedures, including the reverse dot-blot technique [6] and the Amplification Refractory Mutation System [6,7]. Further investigations are carried out by means of Denaturing Gradient Gel Electrophoresis (DGGE) analysis and direct sequencing on ABI prism 310 Genetic Analyser using the fluorescent dideoxy-termination method (Big-Dye-Terminator cycle sequencing Ready Applied Biosystems Foster city, CA, USA) [2,4]. BetaS-globin genotype was assessed using MstII digestion of a β-globin gene segment through PCR amplification.

Short Tandem Repeats (STR) technique is used to detect the maternal cells contamination (MCC) in prenatal diagnosis (PND) for hemoglobinopathies. During our PND experience we have used many

STR. For this work we have chosen the five most informative: D14S261, D14S72, D14S990, D14S68, and D18S1147.

The STR were studied by PCR with fluorescence labelled primer (tet, hex, fam) and then analyzed on ABI prism 310 (Applied Biosystems, Warrington, Lancashire, UK) as described earlier [8].

Results

Prenatal diagnosis has been performed on 461 fetuses during the past 18 years (Table 1); 41% of the samples were at risk for beta thalassemia major, 40.3% were at risk for sickle cell anaemia, and 14.7% were at risk for S/beta thal. The remining fetuses were at risk for a compound heterozygote hemoglobinopathies (S/O, O/beta-thal, S/C).

	Homozygous	Heterozygous	Completely healthy	Not identified	Total
TT*	52	89	38	13	192
SS**	49	98	37	2	186
ST	17	37	11	3	68
SO***	1	4	2	0	7
SS or ST	0	1 AS	0	0	1
TT or ST	1 TT	3 AS	0	0	4
SO or ST	1 ST	0	0	0	1
SC****	0	1	0	0	1
OT	0	0	1	0	1
Total	121	233	89	18	461

TT:thalassemia major;SS:sickle cell disease;SO:sickle cell disease and HbOarab association; SC:sickle cell disease and HbOarab association.

Table 1: Number of fetuses tested for the risk of hemoglobinopathies and prenatal diagnosis results.

Concerning the origin of PND requests, 36% of the cases were referred to us by paediatricians, 12.69% by geneticians; 14.44% by gynecologists and 34.35% by their own initiative. 2.4% PND were done for women coming from foreigner countries (Figure 2).

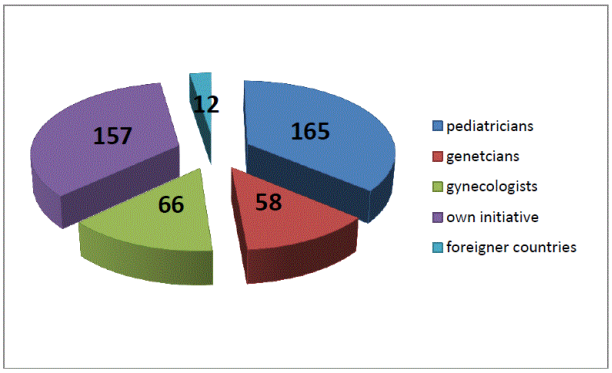

Figure 2: Origin of the PND request.

Mean age of mother was 32 years (20-43 years).

Majority pregnant women had one or more affected children in the past and only 10 cases out of 461 were not reported with an affected child. Among 88 couples that have voluntarily approached the centre and tested for PND, 62 couples have applied twice, 19 of them have applied three times, 6 numbers have applied for four times and a particular couple have applied for five times.

(The researchers should first spell-out the total number of applied couples. They should then proceed for giving details not in terms of numbers but in terms of %.

Fetal DNA was obtained through chorionic willis biopsy samples in 53 cases and amniotic fluid in 397 cases. In 7 cases a chorionic willis biopsy was conducted followed by a second sample obtained by amniocentesis.

The molecular diagnosis showed that 26.2% of the fetuses were homozygous or compound heterozygous for sickle cell anemia and/or beta thalassemia, while 50.5% fetuses were heterozygous, and 19.3% were completely healthy. 3.9% of cases did not provide any result (Table 1). The genotypes of tested fetuses are presented in Table 2.

	cd39 (C→T)	IVSI-110 (G→A)	FSC6 (-A)	IVSI-1 (G→A)	Cd 30 (G→C)	FSC 44 (-C)	FSC 8 (-AA)	IVSI-2 (T→G)	IVSI-6 (T→C)	IVSII-849 (A→C)	IVS-1-5 (G→A)	IVSII-745 (C→G)	Cd25/26 (+T)	βS	βC	βA
cd39 (C→T)	**24**			1	1					1		1		12		42
IVSI-110 (G→A)		**9**	3		2		1	1						4		22
FSC6 (-A)																3
IVSI-1 (G→A)				**2**												10
Cd 30(G→C)					**2**											
FSC 44 (-C)						**1**								1		5
FSC 8 (-AA)																2

IVSI-2 (T→G)									-
IVSI-6 (T→C)									-
IVSII-849 (A→C)									1
IVS-1-5 (G→A)				**2**			1		6
IVSII-745 (C→G)					**1**				5
Cd25/26 +T									1
S							**49**	1	102
C									
A									**89**

Table 2: Genotypes of tested fetuses.

The pregnancies with normal or heterozygous fetuses were continued. With the exception of 13 cases, all the other pregnancy cases with affected fetuses were terminated.

Fetal loss was observed in 7 pregnancy cases, two of which were gemellary (9 fetuses). 2 fetuses were lost within the 2 weeks following the fetal sampling (one amniocentesis and one chorionic wills biopsy). 7 fetuses were lost after amniocentesis during the 20th week (2 fetuses), 24thweek (1 fetus), 28th week (twin fetus) and 32th weeks (twin fetus).

Diagnostic errors were reported in 3 cases (3 false negatives).

Discussion

In Tunisia PND is offered free of cost as a public service and couple are free to decide whether to go for it or not, contrary to countries like Greece, where the prenatal diagnosis is mandatory for all couples that are at risk [9].

Laboratory at Tunisia Children's Hospital is prominent and first of its kind for PND for hemoglobinopathies, since 1986; during this period, several other countries have acquired these methods [10].

From 1986 to 1991, our laboratory received 17 samples from the gyneco-obstetrical department of the Rabta hospital in Tunis and these were sent to Robert-Debré hospital in Paris for PND [11]. In 1991 the first PND was simultaneously performed in Tunisia and in Paris, and then gradually the system was completely set up by 1994 [12].

Concerning the origin of PND request, 36% of pregnant women were referred to us by pediatricians following their affected children from several at risk regions.

Tunisian association for the prevention and the fight against abnormal hemoglobin and thalassemia (Alphatt) generally provides Genetic counselling. However, majority pregnant women (34.35%) observed in this study have approached this centre on their own initiative.

This association has been working very hard for the past several years by organizing two sensitization meetings annually at places that had been identified risky. Professionals of Healthcare industry (nurses and physicians) practicing in the primary care centres are trained to offer genetic counselling. According to Studies, following brief training primary care providers can provide effective genetic counselling for hemoglobinopathies carriers [13]. Gynaecologists also play an important role in the detection of couples at risk through pre marital counselling and by sensitizing pregnant women who have already affected child to benefit from PND. Gynaecologists have referred 14.44% of pregnant women with a family past history of hemoglobinopathies and the department of congenital and hereditary disorders referred 12.29% of cases to our lab. Pregnant women above 35 years that need Karyotyping also approach us for advice.

Despite these efforts, our lab conducts only 30 PND test every year. Many pregnant women at risk with past family history do not approach for prenatal test due to lack of awareness after the third trimester and thus loose the PND benefit.

Although there is a progressive increase in the number of demands for PND from 1994 to 2008, the numbers decreased from 2005-08 onwards. This could be explained as a radical interpretation of the Muslim religion, which forbids the pregnancy termination. Despite these progress related to PND requests, it remains insufficient if we consider the number of registered cases, reaching to 2100 with an estimated annual nativity rate of 16.8% [12].

Twelve PND were conducted for foreign nationals coming from Algeria (10) and Guinea (2). These families approached Tunisia for PND as it is not practiced in their countries due to technical or religious reasons. Cultural, legal and religious restrictions exist in Algeria, prohibiting selective abortion of an affected fetus. Abortion is punished by law unless the mother's life is in danger.

Majority (97.3%) pregnant women had one or more affected children. The remaining women have a family past history of hemoglobinopathies leading them to ask for a genetic counselling during which they were identified as couples at risk for hemoglobinopathies. Facts related to hemoglobinopathies are generally emerging prior to marriage, first pregnancy or during the initial stages of pregnancy. The absence of a nationwide premarital screening for hemoglobinopathies in Tunisia is one of the important reasons explained for the low number of women asking for PND. Premarital tests should be made compulsory, especially when the couple is originating from the affected areas like Cyprus [14] and Turkey [15].

Eighty eight couples that were generally worried about having another affected child had applied several times for PND. The couples that have benefited from 3 or 4 PND have a reasonable socio-economic and cultural level and they live in and around Tunis.

CVS has been proved as providing fast result and it remained as the most widely used procedure at present [16]. However, only 13.8% of the total number of fetal samplings was conducted by CVS due to higher risk of miscarriage and neonatal complication after CVS compared to amniocentesis [17,18], the risk of maternal contamination, technical inconvenience that may delay PND.

The molecular result for β-thalassemia among couples that are at risk is indicated as 13 b-thal mutations (Table 2). The data shows that there were 41 homozygous, 11 double heterozygous and 97 heterozygous states. Among thirteen mutations, the codon 39 (C→T) and IVS1-110 (G→A) were most common [2,3]. Among the fifty two affected fetuses, forty one are true homozygous due to high consanguinity and high endemicity of tested couples. The third most common mutation in our cohort the IVS1-1 (G→A) was subject of controversies between two earlier references [2,3].

Test outcome is delivered quickly in the cases of beta-thalassemia only if mutations are already known. Sickle cell disease results are generally delayed since the researchers have to identify at least one mutation. Generally it takes 3 to 7 days if the fetus is affected or completely healthy. In case where the fetus is heterozygous, confirmation of results is obtained after cell culture that would take 3 to 4 weeks. Molecular analysis of DNA extracted from cultured cells would serve to test maternal contamination. Usage of STR for the past three years however has reduced these delays.

The clinical lab at Tunisia could offer definitive diagnosis in almost all fetuses. Only in 18 cases, the pregnancies were continued although we failed to identify the fetal genotype. In those 18 cases, 13 fetuses were at risk for β thalassemia, 4 for sickle cell/β thalassemia and one for sickle cell anemia. Thus, we note that the main difficulties were found with the detection of β-thalassemia mutations. On the other hand, in the majority (14 of 18) of the cases, PND was carried out by amniocentesis, and the amniotic fluid was poor in amniocytes. In the 4 other cases the CVS was quantitatively insufficient to extract DNA and the women refused to opt for amniocentesis later. Generally in those situations when we find difficulties to provide a result, we recommend a blood cord sampling at the time of delivery to perform a hemoglobin study. We received a blood cord sampling only in 3 cases in which one was affected, one sample was heterozygous and the last one was completely healthy.

Pregnancies with normal or heterozygous fetuses were continued. Except in 13 cases, pregnancies with affected fetuses were terminated. Despite its legality in Tunisia, some women still refuse the abortion because of religious considerations.

The total fetal loss, in our experience, was 1.53% (n=7 pregnancies) this is lower than total fetal loss rates (2% to 2.3%) in the past [14]. This relatively low rate may be explained by the low number of CVS and the technical obstetrician's proficiency. In the case of the 2 gemellary pregnancies, the lost of the 4 fetuses was not related to the sampling procedure. It was a fetal intrauterine death due to both renal micro-cysts and to trisomy 18 in one case, and unknown etiology in the other case.

Concerning the 3 false negative PND result, the error was related to maternal contamination. In these cases STR analysis were not performed due to technical inconveniences.

Conclusion

The high frequency of haemoglobin disorders in Tunisia should incite the Ministry of health to implement effective prevention programs for hemoglobinopathies, especially in the risk areas. This program has to act on several levels: genetic counselling, the heterozygote's screening, and the premarital testing which must be compulsory, and the PND for the couples at risk.

Although PND has been effective at this particular lab for years, it remains insufficient to cover the entire Tunisian territory.

In our procedures, we should promote the use of trophoblaste sampling rather than the amniotic fluid sampling. Thus the PND can be carried at an early stage to allow the termination of pregnancy at a right time, minimizing the risks. Although PND is safety preventive measure, it remains invasive and should be progressively replaced by less invasive methods like PND through fetal DNA in the maternal plasma or fetal cells in maternal circulation and preimplantation genetic diagnosis in case of in vitro fertilization.

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