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The Experiences of Prenatal Diagnosis in China

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1. Introduction

China is situated in the south-eastern part of the Eurasian continent and has a total land area of 9.6 million square kilometers. It is composed of 23 provinces, five autonomous regions (Inner Mongolia, Ningxia Hui, Guangxi Zhuang, Xinjiang Weiwuer, and Tibet), four municipalities directly under the central authorities (Beijing, Shanghai, Tianjin and Chongqing), and two Special Administrative Region(SAR)(Hong Kong and Macao). China is a unified, multinational country (56 nationalities), with a population of about 1.37billion, of which 1.34 billion live in mainland. According to a survey carried out in 1987, the birth defects were of 4% in China. It is a big burden for the population. The government has paid attention to this situation. In order to improve the public health, China government passed the "Maternal and Child Health Law" in 1994.

Prenatal diagnosis for severe birth defects was implemented in China step by steps.

2. The outlines of the development of prenatal diagnosis in China

2.1 Prenatal diagnosis of central neural tube defects

The earliest cases of prenatal diagnosis for central neural tube defects (NTD) were performed with measuring the level of alpa-fetal protein in maternal serum in 1976. This work was awarded by the MOH of China and WHO. Ultrasoundnography was used to diagnose NTD routinely after 1980′.

2.2 Karyotyping

Fetal chromosome karyotyping was performed in 1970′ on cultured amnion fluid cells to detect chromosomal diseases[1,2]. It became the major technique used for prenatal diagnosis in China in 1980′. The scale was once declined in the 1990′. After the prenatal diagnosis spreed in China, cytogenetics is the main field for prevention of hereditary diseases.

2.3 Fetoscopy

Usage of fetoscopy was reported in the late of 1980′. It was used for biopsy of the fetal material and for fetal therapy[3,4].

2.4 Maternal serum screening

Maternal serum screening for chromosomal abnormalities and other abnormalities, such as NTD, started in the early of 1990′[5]. This procedure was adopted nationwide in the 21 century. A standard operation protocol was formulated in 2010 by the National Committee of Exports on Prenatal Diagnosis.

2.5 Biochemical genetics

Electrophoresis analysis was perform on amnion fluid as the first technique in prenatal diagnosis of metabolic disease[6,7]. After the enzyme assay methods were established, prenatal diagnosis of lysosomal diseases was carried out by enzyme assay[8]. More than 20 protocols were developed for diagnosis of lysosomal storage diseases.

2.6 Techniques developed for sampling fetus material

2.6.1 Amniocentesis

Amniocentesis was the first procedure for fetal cell sampling. It was performed as early as in 1970 in China. It became the major procedure in collection fetal material nationwide now.

2.6.2 Chorionic villus sampling (CVS)

At the early stage, chorionic villus sampling via the cervix under was invented by a Chinese obstetrician[9]. Transcervical chorionic villus sampling(TC-CVS) was reintroduced into China in the early of 1980′ and got popular afterward. It was performed blindly at the very beginning, e.g. without the guidance of ultrasonography as does later, for prenatal diagnosis at early pregnancy(first trimester)[10]. It was further improved. Transabdominal chorionic vilus sampling(TA-CVS) was established in 1994[11].

2.6.3 Fetal blood sampling

Fetal blood sampling via abdominal cordocentesis was performed in the late 1980′ for cell culture or hemoglobin analysis[12]. It is used rather routinely in the area where there is a high prevalence of hemoglobinopathies, since the blood component can be analyzed with the fetal blood.

2.7 Gene diagnosis

The first prenatal gene diagnosis was performed in the early of 1980′ on thalassemia[30]. Then prenatal diagnosis was performed on more and more monogenic diseases, when the gene mutations had been characterized for the disease, such as DMD, PKU, and hemophilia A (see details in section 3).

2.8 Regulations on prenatal diagnosis

As the prenatal diagnosis was offered nationwide, there were more and more problems emerged. It was necessary to make a regulation on the practices of prenatal diagnosis. In the second national conference of prenatal diagnosis 2000, this issue was discussed and a proposal on the regulation for prenatal diagnosis was sent to the Ministry of Public Health

after the meeting. A special group was organized to investigate the demands on prenatal diagnosis and the diseases needed to be put on the list for prenatal diagnosis. In the third national conference of prenatal diagnosis 2001, the draft of the regulation was discussed. After revising, "the regulations on prenatal diagnosis and diagnosis of genetic diseases" was distributed nationwide for further discussion and for modification. After one year debating, "The Regulation on Management of Techniques for Prenatal Diagnosis" was published on Dec. 22, 2002 and was implemented after May 1, 2003 [http://www.china.com.cn/policy/txt/2003-02/14/content_5276708.htm]. A National Committee of Exports on Prenatal Diagnosis was established in 2004 to assist the work of the Division of Women's Health and Community Health Care, MOH in prenatal diagnosis.

2.9 The current situation of the organizations for prenatal diagnosis

The techniques applied in the practices of prenatal diagnosis are cytogenetics, maternal serum screening, and ultrasounography. The prenatal diagnosis centers were assigned by the authorities in charge of public health of the local government. Up to year 2009, there were 511 centers carrying out maternal serum screening for chromosomal diseases and neural tube defects; 169 centers performing karyotyping for pregnancy at high risk or with a positive result of serum screening, and ultrasoundography for malformations.

A total of 93905 prenatal diagnoses were carried out for chromosomal diseases and monogenic diseases in 2009. Fetal sampling was mainly carried out via amniocentesis (80%), only a small section was by CVS (1.8%).

There were only 76 centers offering gene diagnosis, of which the most were located in the developed areas, such as Beijing , Shanghai, Hunan, Henan, Guangdong, and Guangxi.

Nineteen diseases were carried out in some of the prenatal diagnosis centers, most of which were α-thalssemia, β-thalassemia, DMD, PKU, SMA, achondroplasia, APKD, and hereditary non-syndromic deafness.

3. Prenatal gene diagnosis, the strategies and experiences in several common diseases

3.1 Thalassemias

Thalassemia is one of the most common monogenic disorders in the world. The incidence for this disease is high in tropical and subtropical areas including southern China.

In China, the carrier rate of α-thalassemia was 17.55% in Guangxi, 8.53% in Guangdong and 1% to 4.2% in other province of southern China[13,14]. The frequencies of α-thalassemic allele in Li Monority was 0.347. Fortunately, most of the thalassemic alleles were α-thal-2 ($-\alpha^{3.7}$/ or $-\alpha^{4.2}$/) , only 0.0058 for α-thal-1 allele ($--^{SEA}$/)[15]. The estimated incidence of carriers of β-thalassemia is as high as 6.43% in Guangxi, and 1% to 2.54% in populations in the endemic areas of southern China[13,16].

3.1.1 Mutation characterization

Researches on thalassemias started in the early of 1980's in China.

The organization of the α globin was analyzed with Southern blotting. Data collected in Guangxi showed the defects of α globin genes in α-thalassemia patients were mostly

deletion types, --^{SEA}/,-$\alpha^{3.7}$/, and -$\alpha^{4.2}$/.[17] Three primers bridging the deletion breakpoints were designed to detect these three deletion mutations [18]. Abnormal hemoglobins of the α globin gene, Hbα^{CS} and Hbα^{QS}, were characterized as thalassemic mutation. After sequencing the non-deletion α thalassemic mutant *HBA*, 12 mutations were revealed responsible for α-thalassemia*[http://globin.cse.psu.edu].* Reverse dot blotting with ASO probes (RDB) for α^{CD30}(-GAG), α^{CD31}(G>A), α^{CD59}(G>A), α^{Westmead} (CD22, C>G), α^{QS}(CD125, T>C) and α^{CS} (CD142, T>C) were developed in detection of the non-deletion α-thalassemia alleles. [19]

The mutations causing β-thalassemia were exclusively point mutations[20]. It used to be the strategy to use the RFLP haplotype to predict mutations since there was linkage disequilibrium between the haplotypes and certain mutations [21]. The -29 A>G mutation was revealed as it was associated with a new haplotype. The mutations were identified via cloning and sequencing, the routine procedure for identifying mutations at that time. It was the first mutation of β-thalassemia characterized by the scientist from mainland China on patients collected in Guangzhou, Guagndong Province[22]. In the 1990', most of the mutations were characterized with direct sequencing the PCR products. Five mutations, CD41-42 (-CTTT), IVS2-654 (C>T), －28 (A>G), CD26 (G>A) and CD17 (A>T), account for 90% of the common mutations in south China. New mutation was revealed with the RDB and characterized by direct sequencing. To date, 46 single-nucleotide mutations and small deletions in *HBB* have been characterized in the Chinese patients with β-thalassemia [*http://globin.cse.psu.edu*]. The recently developed technique is the probe-based melting curve analysis (MeltPro HBB assay). It is a qualitative *in vitro* diagnostic method designed to genotype a panel of 24 single-nuclueotide mutations and small indels in the *HBB* gene that cause β-thalassemia or abnormal hemoglobin. The test kit is based on a proprietary, multicolored, self-quenched, probe-based melting curve analysis performed with a standard real-time PCR instrument, from which genotype information for each mutation is retrieved based on the melting temperature (Tm) or difference in Tm between wild-type and mutant DNA samples[16].

3.1.2 The strategy in thalassemia prevention

In the report on the middle term evaluation on the progress of the National Project on thalassemia during the Seventh Five-Year Developing Plan in 1988, Shangzhi Huang had made a proposal on performing a population prevention program on thalassemia by hematological screening, mutation detection, and prenatal gene diagnosis in certain areas. The trail of population screening carriers with thalassemias was carried out on the Li Minority in Hainan in 1990. It showed that it was feasible to screening thalassemias with hematological parameters [23]. In the Li Minority in Hainan the carrier rate of β-thalassemia was 8.6%, and the mutation c.124-127delTTCT accounted for 94.7% of the mutant alleles in this population [24]. In 1991, carrier screening for thalassemias was carried out for people in marriage registration in Fushan City, Guangdong Province [25]. In 1993, Xiangmin Xu et al. started the hospital-based screening program for pregnant women during their first prenatal checking up in Guangdong and Guangxi [26].

In hematological screening, quantitative test of hemoglobin, erythrocyte osmotic fragility, mean corpuscular volume (MCV), and hemoglobin electrophoresis were performed. When

erythrocyte osmotic fragility <60%, MCH<27pg, and MCV <80 fl, hemoglobin electrophoresis was carried out on the positive subjects. When $HbA_2 \geq 3.5\%$ or HbA_2 normal but HbF >3%, β-thalassemia was suspected, while if $HbA_2 < 3.5\%$, α-thalassemia would considered. Iron deficiency anemia should be ruled out with serum iron test. The DNA were isolated later and Gap-PCR[27] and RDB[28] methods were used for α-thalassemia genotyping. β-thalassemia genotyping were carried out using RDB analysis for 18 common types of point mutation[29]. In the RDB procedure two-step hybridization was designed: the first dot strip contained ASO probes specific for seven common mutations, e.g., -28A>G, CD17A>T, CD26G>A, IVS1 nt5G>C, CD41/42 del CTTT, CD71/72 +A, IVS2 nt654C>T, and IVS2 nt652C>T; the second dot strip contained 11 less common but not rare mutations in Chinese, e.g.,-32C>A. -30T>C, -29A>G, +40_43 del AAAC, initial CD ATG>ACG, CD14/15+G, CD27/28+C, IVS1 nt1G>T, CD31-C, CD43G>T, and CD71/72+T [29]. DNA sequencing was also performed if there was an unknown allele of no-deletion α mutation remained. Four common types of α-thalassemia mutations, $-\alpha^{3.7}/$, $-\alpha^{4.2}/$, $--^{SEA}/$, and $\alpha^{CS}\alpha/$ alleles, were tested for all the subjects with β-thalassemia trait to reveal possible situation of β/α-thalassemia double heterozygote [26]. If the woman was identified as a heterzygote of thalassemia, her spouse would be tested for the same type thalassemia. If the couple were both heterozygous for the same type of thalassemias, either β- or α-thalassemia, prenatal diagnosis will be suggested to the couple.

3.1.3 Prenatal diagnosis

Thalassemia is a severe disease, and its treatment requires life-long transfusion and iron chelating. While many cities in China have facilities for diagnosis and treatment of this disease, free medical services are not provided by the government in most rural areas. Treatment is available to only a potion of patients who can afford it, although this situation is more favorable in some regions with better economical development. So, there is still a demand for prenatal diagnosis of thalassemia.

Prenatal gene diagnosis on α-thalassemia was carried out in the early stage by Southern blotting [30] or with a simple but not accurate method, dot blot hybridization with α globin probe[31]. When polymerase chain reaction (PCR) was established, gap-PCR became a major approach in prenatal diagnosis of α-thalassemia to detect the deletion mutation on fetus [32], or RDB was used to detect the point mutation of α-thalassemia [28].

Haplotypes of the RFLP sites on the β-globin cluster were used for linkage analysis in the early stage for diagnosis of β-thalassemia. Prenatal diagnosis was mainly performed with ASO probes routinely now, also there were several modifications being made, such as labeled with non-radioactive material, RDB, primer extension, and probe-based melting curve analysis (MeltPro HBB assay) [16].

The first prenatal diagnosis of β-thalassemia was carried out in 1985 with RFLP linkage analysis[33,34]. It was PCR technique that overcame the obstacle of the limited amount DNA from fetus available for RFLP analysis. Using PCR technique combined with the radioactive labeled allele specific oligonucleotide (ASO) probes, prenatal gene diagnosis for β-thalassemia become much easier, not only for less DNA was used but also for its simple and quick performance [35] . The non-radioactive labeling ASO probes and RDB made the prenatal diagnosis procedure even easier. This new techniques has been the routine approach for prenatal diagnosis of β thalassemia in China[36].

There were other techniques used in China for prenatal diagnosis, such as allele specific PCR(AS-PCR)[37], multiplex AS-PCR[38]. Another approach is RFLP analysis by introducing an artificial base substitution to create restriction sites. PCR products were cut by certain restrictive enzyme and then visualized the fragments after electrophoresis in agarose gel [39].

3.2 Duchenne muscular dystrophy (DMD)

3.2.1 Clinical aspect

Duchenne and Becker muscular dystrophy (DMD/BMD) (MIM#300377 and 300376) are allelic disorders, caused by mutations in the *DMD* gene coding for dystrophin, which locates on Xp21. The term "pseudohypertrophic muscular dystrophy" was used in the past; however, it is not used currently because pseudohypertrophy is not unique to the DMD or BMD phenotype. DMD is the severe form and usually presents in early childhood with develop delayed, including delays in sitting and standing independently. Progressive symmetrical muscular weakness, proximal severer than distal, before age 5 years, with a waddling gait and difficulty climbing, often with calf hypertrophy. Serum creatine phosphokinase (CK) concentration elevated, generally 10 times the normal range. Electromyography (EMG) is useful in distinguishing a myopathic disease from a neurogenic disorder. The characteristics were demonstrating short-duration, low-amplitude, polyphasic, rapidly recruited motor unit potentials. Muscle histology early in the disease shows nonspecific dystrophic changes, including variation in fiber size, foci of necrosis and regeneration, hyalinization, and, later in the disease, deposition of fat and connective tissue. Immunohistochemistry with antibody against dystrophin showed no signal of the dystrophin. Western blot can be used to distinguish DMD from other muscular dystrophies, such as Limb-girdle muscular dystrophy (LGMD), which are clinically similar to DMD but were autosomal recessive or autosomal dominant inheritance. LGMDs are caused by mutations in genes that encode sarcoglycans and other proteins associated with the muscle cell membrane that interact with dystrophin [40]. Testing for deficiency of proteins from the transmembrane sarcoglycans complex is indicated in individuals with dystrophin-positive dystrophies.

The affected children will loss their walking ability by age 12 years old. Few patients survive beyond the third decade, with respiratory complications and cardiomyopathy, which are the common causes of death. BMD is the mild form characterized by later-onset skeletal muscle weakness. Individuals with BMD remain ambulatory into their 20s and the lifespan is longer. Female carriers have clinical features of *DMD* were resulted in the situations either of X-Auto-chromosome crossing over involving *DMD* locus, or because of Turner syndrome or non-random X-chromosome inactivation (XCI), so called “unfortunate Lyonization”.

3.2.2 Mutation analysis

The size of *DMD* gene is over 2.3 Mb, and composes 79 exons. It is the largest known human gene. The cDNA is 14kb. Large fragment deletion or duplication were the most common mutations of DMD, 60% for deletion and 6% for duplication with some were indel type, e.g., deletion with addition of several base pairs at the same site. There were cases with two deletions or deletion plus duplication scattered in the same allele[41-43]. There are two

hotspots of deletion/duplication, one located on the 5′ portion, harboring exons 2 to 20, the other in the 3′ portion involving exons 44 to 53. The remaining mutations were small deletion, insertion, point mutations and splicing mutations.

There were several methods for deletion/duplication analysis. In the early stage, Southern blotting was used with either genomic probes or cDNA probes, both deletion and duplication mutations were detectable[44]. With multiplex PCR approximately 98% of deletions are detectable[45,46]. Multiplex ligation-dependent probes assay (MLPA) has been employed for deletion/duplication analysis of the *DMD* gene in probands and for carrier detection in recent years[42,43].

Array chips for deletion or duplication mutations were also developed, it had the same power in detection of large deletion/duplication as MLPA, but the strength on detection of alterations with small size is under evaluation[47].

Mutation scanning with denaturing high performance liquid chromatography (DHPLC) or high resolution melting assay (HRM) followed by direct sequence analysis was performed, but the efficiency is low [48]. It might be a good choice to perform RT-PCR and then sequencing. It would much easier to detect the size changes of the mRNA just by agarose gel electrophoresis, which were caused by large deletion/duplication or splicing abnormalities. If there is no or reduced RT-PCR products, mutations occurred on the regulatory elements would be suspected and sequence analysis would be performed then using genomic DNA to detect the sequence change. It also facilitates detection of the point mutations since the size of cDNA is much shorter than the genomic DNA.

A "one-step approach" was developed with multiplex PCR using 9 primer pairs to detect deletions and to perform linkage analysis simultaneously[49]. The primers amplified 3 exons, exon 8, 17 and 19, in the 5′ hotsport[50], and 6 short tandem repeats, 5′CA at the brain specific promoter region [51]and MP1P[52] at 3′UTR, and STR markers in introns 44, 45, 49 and 50[53]. The primers were grouped into three triplex PCR, Group A: 5′CA, MP1P and exon 8; Group B: exon 17, i44 and i49; Group C: exon 19, i45 and i50. The amplified fragments in the group can also serve as the internal control, since deletion is rarely spread all the gene, although one deletion gene had been detected, of which only one fragment was amplified with the primers for 5′CA site [Huang S, unpublished data]. The primers at both ends of the gene, 5′CA and MP1P, were used to detect the possible recombination.

3.2.3 Carrier - Testing

DMD is an X-linked recessive disease. Female carriers were at the risk to give birth to an affected male infant. Performing carrier testing offered a favorable opportunity for the carrier to get prenatal diagnosis at the first pregnancy. For deletion/duplication mutations, dosage analysis can be performed with either real-time PCR or MLPA[42,43], no mater it was a familial case or sporadic case, even the proband deceased. For point mutation, it can be carried out by direct sequencing.

For familial cases, linkage analysis can be performed[54], no mater what kind the mutation was. It would be offered with caution for the sporadic cases, especially the diagnosis of DMD was not confirmed. The markers used for linkage analysis should be highly polymorphic and informative, and lie both within and flanking the *DMD* locus[51-53].

The large size of the *DMD* gene leads to an appreciable risk of recombination. It has been estimated that the genetic distance was 12 centimorgans between the two ends of the gene[55]. Multiple recombination events may occur during meiosis and may not be detected when the marker was homozygous; thus, it might be aware in interpretation of the data generated from a linkage study.

Males with DMD usually die before reproductive age or are too debilitated to reproduce. It would be convincible that there was a plenty of de novo mutations responsible for the sporadic cases. For sporadic cases, the proband may be resulted from: 1) the mutation occurred after conception and is thus present in some but not all cells of the proband's body (somatic mosaicism), the proband's mother is not a carrier and the recurrence risk was very low; 2) germline mutation, passed from the mother. The later situation was completed, since the mutation could be one of the followings: a) a *de novo* mutation occurred in the egg, a meiosis mistake and the mother was normal; b) the mutation is resulted in mitosis in the ovary and partial of the cells carry the mutation, termed "germline mosaicism" or "gonad mosaicism", the recurrence risk depends on the proportion of the mutant cell line; c) somatic mosaicism, the mutation presented in some but not all of the mother's body including the ovary, the recurrence risk is high, up to 50%; d) a germline mutation, passed from one of grand-parents, or passed from ancestor on the grandmother side, the mother is the carrier. Put all these considerations together, carrier testing on the mother may be not informative, since the mutation could only be detectable in the last two situations. Since it is hard to tell at which level the mutations occurred, to provide carrier testing on the proband's mother in the sporadic case is controversial. There might be a misleading for the family, since the negative result of the testing may be misunderstood even misinterpreted as the mother was not a carrier at all, and the prenatal diagnosis was not necessary. It would be dangerous. For these reasons, prenatal diagnosis must be suggested to all cases especially the mutation has been identified; just in case the mother had the mutant cell line. The carrier status of the patient's sister can be performed if there was a detectable mutation in the proband. It should be aware that if the germline mutation is of the grandfather's origin, the maternal aunts would be at risk for a carrier, since there was a possibility that the grandfather is of germline mosaicism. The origin of the mutation can be revealed with linkage analysis[54,56]. This information is important in genetic counseling for determining which branches of the family are at risk for the disease. The carrier detection can be performed with either mutation detection if the proband's mutation was identified, or with linkage analysis. There is an ethics issue for carrier detection: the optimal time for determination of genetic risk, clarification of carrier status, and discussion of availability of prenatal testing, is before the person's pregnancy. It is appropriate to offer genetic counseling to young adults female relatives who are at risk of being carriers[56].

3.2.4 Prenatal diagnosis

In preventing birth of DMD fetus, prenatal test was carried out by sex selection in the early era, e.g., if the fetus was 46,XY, abortion would be induced.

The first case of prenatal diagnosis was performed in 1987 in China, via pathology analysis of the fetus muscle biopsy taken at 20 week gestation[57]. CK measurement on amnion fluid was used for prenatal diagnosis of DMD before gene analysis was available[58]. This

approach is still used as the complementary or "rescue" procedure in prenatal diagnosis, when gene analysis is uninformative or linkage analysis has revealed that the fetus inherited the same haplotype as the proband in sporadic case.

Prenatal diagnosis for at-risk pregnancies requires prior identification of the disease-causing mutation in the family. It can be offered with 50% exclusive detection if the small mutation detection can not be carried out. The fetal material is obtained either by chorionic villus sampling (CVS) at approximately ten to 12 weeks' gestation or by amniocentesis usually performed at approximately 15-18 weeks' gestation.

The usual procedure in prenatal diagnosis is to determine fetal sex by PCR amplification of the male specific fragment, such as the *ZFY* gene. If the fetus is male, DNA from the fetus will be analyzed for the known causal mutation or tracking linkage established previously. If the fetus is a female, test will be stopped at this point, unless the mutation of the proband was identified. In this circumstance the carrier status of the female fetus might be tested.

Prenatal testing is possible for the first pregnancy of woman in the family whose carrier status has been recognized by mutation detection or linkage analysis.

The first gene diagnosis on DMD was carried out with restriction fragment length polymorphism (RFLP)[59]. With PCR method available, it is much easier to perform prenatal diagnosis for deletion cases using multiplex PCR[60,61], or with linkage analysis using STR markers[56,62,63], and recently MLPA method was used[56, 64].

In our center, prenatal gene diagnosis on DMD was performed mainly with "one-step approach", a linkage analysis protocol[49]. In reviewing the data of the cases analyzed with one-step approach since 1995, it was informative in almost all the families. After MLPA analysis on the remaining"non-deletion" cases screened by "one-step approach" , it revealed that 80% of the deletions were detected with linkage analysis [43,47]. For prenatal diagnosis, it was required that differentiation diagnosis and gene analysis should be performed prior to prenatal diagnosis with one-step approach. Since this approach can detect deletion and establish linkage phase simultaneously. Prenatal diagnosis was performed with the information collected from the pre-analysis. If it was uninformative with the linkage analysis, or the male fetus shared the same haplotype with the proband, or there was a chromosome recombination observed, MLPA analysis would be performed as a rescue procedure to see whether there was a deletion/duplication existed for the proband or not[56].

As for all X-linked genetic diseases, it should be aware that contamination with maternal DNA is a critical risk causing error in prenatal diagnosis. It should be incorporated the personal identification procedure in prenatal diagnosis. It was carried out with a set of multiplex PCR of unrelated markers, such as the STR markers on chromosome 21(please refer to the section of quality control, QC). We had the experiences that the material of CVS was from the mother and the amniocentesis cell grown up in the cell culture was of maternal origin. Fortunately, all these contamination events were revealed with the QC procedure.

3.3 Phenylketonuria (PKU)

3.3.1 Heterogeneity and prevalence of HPA

Phenylketonuria (PKU) (MIM 261600), an autosomal recessive inherited metabolic disease, is caused by phenylalanine hydroxylase (PAH) deficiency. It was called classical PKU in

China before, for distinguishing it from BH4 deficiency, which was called un-classic PKU or malignant PKU. In China the newborn screening data showed that the incidence of hyperphenylalaninemia (HPA) is around 1 in 11000[65], with the highest prevalence of 1 in 1666 in Gansu province[66]. Differentiated diagnosis was made by HPLC pterin analysis of the urine, measurement of RBC DHPR activity, and BH4 loading test. Classic PKU is diagnosed in individuals with plasma phenylalanine (Phe) concentrations higher than 1000 μmol/L in the untreated state. PKU accounts for 80% to 94% of HPA cases in Chinese, with the remaining as BH4 deficiency[67]. Patients with PKU are intolerance to the dietary intake of phenylalanine, one of the essential amino acids. Without dietary restriction of phenylalanine in the early life, most children with PKU will develop irreversible profound intellectual disability. Newborn screening has been launched in the early of 1980's in China [68]and free dietary treatment for the patients detected through the newborn screening program started in the early of 21 century, although the coverage of the newborn screening program varied from 100% in the developed area to 20% in developing areas, and no screening program at all in the remote areas, such as Tibetan and Qinghai province.

3.3.2 Mutation spectrum of *PAH* in China

PAH gene, located on 12q23.2, contains 13 exons and spans 90 kb, coding for a 2.4-kb mature mRNA. Mutation detection of the *PAH* gene in the PKU patients started in the late of 1987 on Chinese patients[69]. More than 100 different mutations have been identified up to date in Chinese PKU patients, accounting for 94.3% of the mutant alleles [70,71]. Mutations observed in the *PAH* gene include missense, splice site, and nonsense mutations, small deletions, and insertions. Eight mutations, c.728G>A(p.R243Q), IVS4-1G>A(rs62514907), c.611A>G (p.Y204C), c.1238G>C(p.R413P), c.331C>T(p.R111X), c.1068C>A(p.Y356X), and c.1197A>T(pV399V), accounting for 66.2% of the mutant alleles, with the proportions of 18.8%, 10%, 10%, 9.4%, 7%, 6%, and 5% of mutant alleles respectively [71].

When a panel of samples was collected in a certain area, mutation scanning was carried out with either ASO hybridization, denaturing high-performance liquid chromatography, or multiple AS-PCR. In the clinical service, we use a two-step procedure to detect the mutations of the *PAH* gene. In the first step, six exons, exon 3, 5, 6, 7, 11 and 12, and their flanking intronic sequences were amplified by PCR and then sequenced. In a panel of the patients, mutations were identified in 83% of the mutant alleles in the first step. If there was unknown allele remained in an individual, the second step of mutation scanning was carried out by sequencing the other seven exons. The total detection rate was 92.3%[71].

3.3.3 The strategy of prenatal diagnosis of PKU

Although there has been a good newborn screening program in China for decades and efficient early dietary treatment was available in the country[72], there is still a demand for prenatal diagnosis on the second pregnancy in the family. Prenatal diagnosis of PKU may be controversial if the testing is for the termination of the affected fetus. But it is the alternative choice of the families. The reasons for the parents seeking for prenatal diagnosis are: the first, the cost of the dietary treatment is high, beyond the economic capacity for the families with an average income, although there is a free treatment in some province, and the second is that in the families the parents had the bit experience already in managing the first child

and would not want to risk the further baby bearing with the condition. It was not convenience in bring up the affected child and it is hard for them to make the child adhered to the dietary.

The first prenatal diagnosis on PKU was performed with RFLP linkage analysis in 1987[73]. There were other centers offering prenatal diagnosis, of which the centers at Peking Union Medical College[74,75] and Shanghai Xinhua Children's Hospital[76] played the main roles now.

In the beginning, linkage analysis with RFLP was tried. But unfortunately it was not as informative with RFLP markers as in the Caucasians, since 80% of the chromosomes were with haplotype 4 in Chinese [77]. Prenatal diagnosis got into service until mutation detection was carried out by PCR amplification and hybridized with ASO probes labeled with isotope [78]. The mutations of PAH gene could also be detected by methods of AS-PCR[79], single strand conformation polymorphism(SSCP)[80], or RFLP[81]

The limitation for all these procedures was that the two mutant alleles in the family must be characterized. But not in all of the cases the two mutant alleles could be identified, especially there was no time for detecting the mutations as the pregnant women came to clinic asking for prenatal diagnosis without any previous gene analysis. In addition, as more and more mutations being characterized in Chinese, it was hard and inconvenient for any diagnostic center to have all the probes ready for use. The quick procedure needed to be deveoped to perform prenatal diagnosis. The linkage analysis with short tandem repeats (STR), (ACAT)n in the intron 3 of *PAH* gene, emerged in 1992[82]. This marker was introduced into China in the same year [83]and a "quick approach" for prenatal diagnosis of PKU was proposed by combining this STR maker with a novel SNP (IVS3 nt-11A/C)in the *PAH* gene[84]. It was further expanded with additional STR markers incorporated. Three markers, PAH-STR, PAH-26 and PAH32, were used for haplotype analysis. They are highly informative and are both intragenic and flanking to the *PAH* locus. Linkage phase of mutant alleles and the haplotype of the markers could be established in almost all the cases[85]. For families in which only one *PAH* mutation was identified, or there is a urgent case for prenatal diagnosis, linkage analysis would be an option for prenatal diagnosis. But it should be kept in mind that the diagnosis of phenylketonuria should be confirmed (e.g. it is caused by mutation of phenylalanine hydroxylase gene). The shortage for using linkage strategy is that there is genetic heterogeneity of the HPA and not all the HPA were caused by mutation in *PAH* gene. If the differentiation diagnosis could not be made, there would be a miss management to treat all the cases as that caused by *PAH* mutation. Linkage studies are based on accurate clinical diagnosis of PAH deficiency in the affected family and accurate understanding of the genetic relationships in the family. Prenatal diagnosis should be applied with a caution and linkage analysis should be performed with combined with mutation detection.

Different families have different story: the mutation details, the linkage phase for the STR markers. So, it was required for cases asking for prenatal diagnosis, that gene analysis should be carried out prior to being pregnant, e.g., the differentiation diagnosis, especially when linkage analysis was employed, and DNA typing, either mutation detection or STR typing. In cases that probands deceased, it was still possible to perform prenatal diagnosis

if the Guthrie card bloodspot was available, which might be kept in the newborn screening center. If differentiation diagnosis had been confirmed on the patient before death, linkage analysis can be done utilizing the remained genetic material. It would be better if mutations can be characterized using the DNA isolated from the bloodspots. The mutations in the family could be also identified by sequencing the parents' DNAs if the proband's DNA was not available, although the procedure was rather complicated in confirming the causal mutation.

3.4 Spinal muscular atrophy (SMA)

3.4.1 Molecular genetics of SMA

Spinal muscular atrophy (SMA) is characterized by progressive muscle weakness resulting from degeneration and loss of the anterior horn cells in the spinal cord and the brain stem nuclei. Onset ranges from before birth to adolescence or young adulthood. Electromyography (EMG) reveals denervation and diminished motor action potential amplitude.

SMA was classified into clinical subtypes according to the onset age and maximum function achieved. Infants of SMA 0, with prenatal onset and severe joint contractures, facial diplegia, and respiratory failure, died soon after birth. Children with SMA I (Werdnig-Hoffmann disease) manifest weakness before age six months and never be able to sit independently. The life expectancy for SMA I patients is less than two years. For SMA II (Dubowitz disease), with onset between age six and 12 months, the life expectancy is not known with certainty, some live into adolescence or as late as the third or fourth decade. The individuals with SMA III (Kugelberg-Welander disease**)** clinically manifest their weakness after age 12 months and are able to walk independently. SMA IV is the adult onset type.

SMA is inherited in an autosomal recessive manner. Two genes were considered related to SMA, *SMN1* (survival motor neuron 1) and *SMN2*, which locate on 5q12.2-q13.3 head to head with *SMN1*. *SMN1* is the primary disease-causing gene. Most people have one copy of *SMN1* on each chromosome, and there are about 4% of the chromosomes have two copies of *SMN1*. The number of *SMN2* copies ranges from zero to five in normal individuals, while there is at least one copy of *SMN2* remains in SMA patients. *SMN1* and *SMN2* differ by five single base-pair, locate in introns 6(g/a) and 7(a/g and a/g), and exons 7(C/T) and 8(G/A). The C/T SNP at position 6 of exon 7 is critical, since this change causes skipping of exon 7 in the mRNA of *SMN2* resulted in loss the function of *SMN2*, while the difference in exon 8 is a SNP in the 3'UTR of the genes.

About 95%-98% of individuals with SMA are homozygous deletion and about 2%-5% are compound heterozygotes for an *SMN1* deletion and an intragenic *SMN1* mutation. Some of the deletion of *SMN1* is caused by conversion between *SMN1* and *SMN2*, with the 3' portion of *SMN2*, not actually a real deletion.

3.4.2 Advantages in genetic testing

Prenatal gene diagnosis was carried out in China mainly on homozygous deletion cases, typically determined by lack of exon 7 of *SMN1*. Since there is at least on copy of *SMN2* remains in the SMA patients, Methods developed for deletion detection attempting to avoid

amplification of the *SMN2* gene. In the beginning diagnosis of homozygous deletion of *SMN1* was carried out by SSCP analysis [86]. Since the pattern of the SSCP bands was rather complicated, a PCR-RFLP protocol was then adapted to replace SSCP procedure[87]. The difference at position 6 of exons 7 between *SMN1* and *SMN2* was considered for primer design. An artificial restriction site of Dra I (T*T*TAAA) was created by introducing a mismatched *T* in the primer. The exons 7 of both *SMN* genes were amplified and the PCR products were cut with Dra I. Since the products of *SMN2* has the restriction site while *SMN1* fragment doesn't, there would be no band corresponding to *SMN1* and only a smaller band for *SMN2* remained in homozygous deletion patients. For the PCR-RFLP procedure, there is a shortage of uncompleted digestion of the SMN2 fragment, which would be misinterpreted as the present of normal *SMN1* copy. It was dangerous when it occurred in prenatal diagnosis. Other methods were investigated to overcome this limitation, such as DHPLC and MLPA to detect the copy numbers of *SMN*s[88]. For the purpose for detection of homozygous deletion, a more simplified approach was developed. *SMNl* gene was amplified specifically with double allele-specific PCR(AS-PCR)(e. g. both primers for *SMN1* amplification were *SMN1* specific, utilizing SNPs at exon 7 and intron 7), coupled with a pair of primers for an irrelevant gene as internal control[89]. The products were separated by agarose gel electrophoresis or PAGE to determine whether the patients were of homozygous deletion of SMN1 gene. Comparing to PCR-RFLP and DHPLC used in the past, this approach can diagnose homozygous deletion of *SMN1* much more accurate, easier and more convenient without the interference of *SMN2*.This approach could be further modified by keeping the PCR cycles by 25 and quantitatively determining the density of the bands by densitometer, or using fluorescent labeling primer for quantitative PCR.

3.4.3 The feasibility of prenatal diagnosis for first pregnancy at risk for SMA

With all the progress, it is possible to perform prenatal diagnosis for the family in which the proband deceased, the same story as in PKU. Deletion detection could be performed by dosage analysis if there were tissue samples available from the proband, such as bloodspots from newborn screening. If there is no DNA available, *SMN1* dosage analysis may be performed on both parents to see if they were both with only one copy of *SMN1*. It is much easier to confirm the carrier status for the parents with real-time PCR or MLPA. But there might be a blind corner when the patient was a compound heterozygote or one of the parents has two *SMN1* copies on one chromosome when dosage analysis was performed. It is reasonable to offer prenatal diagnosis for these family as a 50% exclusive diagnosis, in hope that the deceased proband was really homozygous deletion no mater that one of his/her parents had two copies of *SMN1*on one chromosome or he/she was resulted in a de novo mutation of deletion. In case of intragenic mutation, the mutation might be revealed by sequencing the *SMN1* gene of the parent with two copies of *SMN1*. It is also feasible to detect carrier of deletion mutation in the population using this approach, at least for the relatives of the patient and his/her spouse. It was proposed to carry out population screening with the double AS-PCR procedure, since the rate of the carrier with deletion type was as high as 1 in 50[89]. It will make the prenatal diagnosis possible on the first pregnancy for the couples both with a single copy of *SMN1*, as doing on thalassemias in Southern China.

It may be further increase the chance of prenatal diagnosis for the patients of non-homozygous deletion by screening the possible intragenic mutation by sequence *SMN1*

gene of the patient. But it should be offered with caution since the heterozygote frequency of SMN1 deletion is as high as 1 in 50, the diagnosis of SMA must be confirmed. For sequencing the *SMN1* gene, it can be achieved by a method that facilitates *SMN1*-specific amplification. Since there were more copies of *SMN2* than *SMN1* at DNA level, and only 15% of the SMN2 mRNA was full length (fl-mRNA), it may be easier to reveal the mutation by cDNA sequencing approach. Sequencing the full length mRNA of *SMN*s will increase the signal of the mutant allele since the ratio of fl-mRNA of *SMN2* to that of *SMN1* was reduced at the mRNA level. It has additional strength with cDNA sequencing strategy. The mutations affecting mRNA processing would be revealed much easier, no mater how deep it was hided within the intron. It will be able to detect the mutation responsible for transcription regulation if quantitative analysis of *SMN1* mRNA was performed. There is also an alternative approach for characterizing the intragenic mutation, e.g., by long-range PCR protocol and subcloning.

3.4.4 Be ware of contamination

Prenatal diagnosis of SMA was relied on deletion detection. If there was no amplification the fetus would be considered as affected. It would be dangerous when contamination of maternal material occurred. The amplification of SMN1 exon 7 might be from the maternal DNA, since the mother had a normal *SMN1* allele. To rule out the contamination of maternal material linkage analysis was performed routinely with STR markers on chromosome 21(section of quality control for prenatal diagnosis).

3.5 Personalized service in rare disease

Prenatal diagnosis was also offered to families with other genetic diseases as personalized services. In this case, mutation detection should be carried out in advance. There were more than 40 diseases available for this service in our center and more than 70 diseases were offered in other prenatal diagnosis center or department of medical genetics.

4. Quality control on the prenatal diagnosis

4.1 Be ware of heterogeneity

In practice of prenatal diagnosis the accuracy is very important. Since there is heterogeneity for genetic diseases, it was critical to confirm the diagnosis of the disease in the family seeking prenatal diagnosis.

4.2 Using different procedures to confirmed the output

It would be helpful to use two different protocols to carry out prenatal diagnosis, mutation detection and linkage analysis. For deletion analysis, in addition to use internal control linkage analysis should be used, which can tell if there was a contamination occurred.

4.3 Emphasis on pre-analysis in advance to prenatal diagnosis

It is always happened that the pregnant women come for prenatal diagnosis at high gestation weeks without confirmation diagnosis of the proband or with no previous gene

analysis. Without genetic analysis in advance, it would be not sure if the prenatal diagnosis can be performed, since it might be uninformative with the commonly used polymorphic markers, or the mutations in the family can not be identified.

4.4 Ensure the fetal material is really from fetus

The maternal contamination was a serious risk, esprcially for deletion mutation. The mother always carried a normal allele which will give a positive amplification. We put an emphasis on sampling and sample processing: 1)CVS would be the first choice to get fetus material in our practice. When the sample received the chorionic villus would be carefully selected. 2)If amnion fluid had to be used the sample should be check for sure there was no blood contaminated. It can be told by sitting the fluid for a half hour to see if there was red cells precipitated. If it is so, cell culture will be set up. To avoid omission of the contamination, linkage analysis should be performed paralleling with mutation detection.

4.4.1 Get three birds with one stone

In our practice we performed linkage analysis first using two STRs on chromosome 21. By this procedure, we can get three answers: 1) whether the fetus was at risk for trisomy 21. If there is three alleles for the fetus or there were just two alleles but the density of one allele was nearly doubled, the fetus was affected with trisomy 21. Since we detected 2 markers, the chance for there was only one band, e. g. the parents were homozygous for two markers, was rare; 2) whether the fetal sample was taken from the fetus tissue. If the alleles were the same as the maternal ones and there is no paternal allele, it implied that the sample was not from the fetus but of maternal origin. It also showed whether there was a contamination with the maternal material, if there was an extra allele passed form the mother, and the density of the extra band was much weaker; 3) was the sample misplaced, if the genotypes of the parents and fetus were not matched.

In our practice we have confronted with the situation 2, there were maternal contamination and even worse that the sample was taken from the mother, not from the fetus. To confirm the later situation, more marker, other than markers on chromosome 21, were used for personal identification as doing in paternity testing. We called this procedure as “get three birds with one stone”. If it implied the possibility for trisomy 21, markers on other chromosome would be tested. If there was no extra allele for the new markers, trisomy 21 was suspected, and AF sampling would be suggested in order to rule out the confined trisomy 21 in the CVS. If there was maternal contamination, fetus sample should be collected again. If there was a trouble in sample preparation, DNA would be extracted from the back up material, which was reserved in the original sample tube. If the polymorphic marker didn’t give sufficient information, dosage analysis could be help. In our practice the contaminating band was always with a lower density, much less than 1 copy.

4.4.2 Be aware of the maternal origin of the grown cells from AF culture

When contamination of the maternal blood was suspected on AF sample, the routine way to get ride of the maternal white blood cells is to culture the amnion fluid cells. After several changes of the medium the blood cells would be washed away, leave the fetal cells grow up as fibroblast cells adhering on the culture flask. But in our practice in one case it turned to be

that the grown cells were maternal origin since all the markers tested gave the same genotype as the mother's DNA did. Considering this situation, we added the STR testing procedure to all the prenatal diagnosis for monogenic diseases, such as for enzyme assay.

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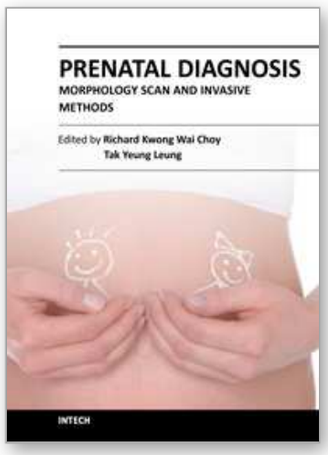
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This book provides detailed and comprehensive coverage on various aspects of prenatal diagnosis-with particular emphasis on sonographic and molecular diagnostic issues. It features sections dedicated to fundamentals of clinical, ultrasound and genetics diagnosis of human diseases, as well as current and future health strategies related to prenatal diagnosis. This book highlights the importance of utilizing fetal ultrasound/clinical/genetics knowledge to promote and achieve optimal health in fetal medicine. It will be a very useful resource to practitioners and scientists in fetal medicine.

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