

The Contribution of Molecular Techniques in Prenatal Diagnosis and *Post mortem* Fetus with Multiple Malformation

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1. Introduction

The development of conventional cytogenetic techniques in the 50's led to a rapid increase of the knowledge on the etiology of malformation syndromes, being chromosomal anomalies reported as the most common genetic condition in humans (Pena, 1998). Around 2-3% of newborns may have congenital malformations, and from those, just 20% have an established etiology (genetic or environmental), being 80% of these multifactorial or unknown (Stevenson & Hall, 2006). But this is only the tip of the iceberg, as probably half of the human concepts may have some kind of chromosomal defect (A. Boué & J. Boué, 1973), indicating that cytogenetic analysis is fundamental for the investigation of these cases. Since the 70's, prenatal diagnosis for detecting cytogenetic abnormalities has become a routine procedure in many countries, and an important tool for the prevention of birth of handicapped children (A. Milunsky & J. Milunsky, 1998).

Cytogenetic analysis is an important component of invasive prenatal diagnosis as chromosomal abnormalities are detected in about 1 in 200 newborns and constitute a major cause of mental retardation and congenital malformations (Shaffer & Lupski, 2000). Microscopic chromosome analysis of cultured cells has been regarded as the gold standard method for prenatal diagnosis, since its first application to prenatal testing in 1966 by Steele and Breg (Steele & Breg, 1966) and the routine use of chromosome banding analysis in 1970s. Karyotyping has proved to be highly reliable for diagnosis of numerical chromosome abnormalities and structural rearrangements in fetal cells obtained invasively by either amniocentesis in the second trimester of pregnancy, or chorionic villus sampling (CVS) in the first trimester, since the early 1980s. The diagnostic accuracy of karyotyping fetal cells from cultured amniotic fluid (AF) has been found to be 99.4%-99.8%, and that of CVS 97.5-99.6%. However, the main limitation of karyotyping is the requirement of a cell culture, resulting in a period of 10-14 days for obtaining the final results (Bui, 2007). Furthermore, the success of cell culture depends on many factors: very good laboratory conditions for tissue culture, technician's experience, and satisfactory cell growth with good quality of metaphases. Unfortunately, due to failure in one of these steps the whole process becomes jeopardized.

In the early 1980s, as better ultrasonographic imaging became available, the access to fetal blood was improved, and it could be obtained at about 18-20 weeks' gestation from umbilical cord (cordocentesis) (Daffos, et al., 1983). Although blood sample allows rapid karyotyping within 72 hours, the gestational age at collection is already advanced, and in positive cases it would be too late for interruption. Besides, this procedure is associated with higher risk of complications than other prenatal diagnostic and, hence, has been performed only in selected cases (Daffos & Hobbins, 2002).

When a fetus with multiple malformations is detected by ultrasound, the list of possible etiologies is very broad, and the possibility of a chromosomal anomaly is high. However, the result of a karyotype, so important for the clinical evaluation, is not always achieved. In some cases this is caused by the factors explained above, in others, due to death of the fetus before the initiation of any diagnostic investigation. In any case, it is very difficult to provide an appropriate genetic counseling without a karyotype result, and the family remains without information on the fetus condition and the risk for future pregnancies. This is a very unpleasant situation, and trying to find an alternative to decrease the anxiety of those families is the main goal of the strategy described below which involves the application of molecular techniques in different fetal materials to overcome this situation.

2. Methodology

We obtained different tissues from 50 multiple malformation fetuses distributed as: umbilical cord (15), lung (7), amniocytes (14) and paraffin embedded tissues (14). For traditional karyotypes, we used AF, UC and alternative materials from 115 fetuses, also with multiple malformations. The criteria for including the fetus as multiple malformations with indication for chromosomal aberrations were based on the "Catalogue of Unbalanced Chromosome Aberration in Man" (Schinzel, 2001). They are summarized in Table 1.

Cleft palate, cleft lip, or both
Esophageal atresia, TE fistula; anal atresia with fistula
Malrotation of the gut, common mesentery; omphalocele
Malformation of the heart and the great vessels
Malformation of the kidney and urinary tract
Certain brain malformation, particularly holoprosencephaly and agenesis of corpus callosum
Absence or hypoplasia of radius and thumb
Postaxial hexadactyly
Microphthalmia, ocular coloboma
Spina bifida (occipital or lumbar)

Table 1. Common malformations in autosomal chromosomal aberrations

2.1 DNA extraction

The DNA from fresh tissues was extracted as described by Miller (Miller et al., 1988) with slight modifications. Tissue specimens were grinded before addition of the nuclei lysis buffer

and 1/10 of the reagent's volume used for blood extraction was used. In a few cases (n=8) a commercial kit was used for DNA extraction. (NucleoSpin®Tissue from Macherey-Nagel).

2.2 DNA extraction from paraffin embedded tissue

This technique was adapted from Andreassen (Andreassen et al., 2004) and Coura (Coura et al., 2005) as follows: Paraffin block was sliced in small pieces between 5-10 μ and 5 slices were placed into an ependorff. Xylol (1,5ml) was added and incubated for 30 minutes at 37°C. Tubes were centrifuged at 14.000 rpm for 3 minutes. The supernatant was removed and all steps were repeated once. After the supernatant removal, samples were washed with 70% Ethanol and centrifuged for 3 minutes at 7.200 rpm. This step was repeated twice and samples were left at room temperature for at least 30 minutes. After completely removal of the paraffin, 300 μ l of Nuclei Lysis buffer, 20 μ l of SDS and 20 μ l of proteinase K were added. Samples were incubated for 3 days at 60°C and on the third day an extra volume of 5 μ l of proteinase K was added. The remaining steps for DNA precipitation were the same ones as described above for tissue DNA extraction.

2.3 Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) is a semiquantitative analysis based on polymerase chain reaction (PCR). It possesses many advantages such as high efficiency, simple operation, low cost and has been widely applied in researches of diseases associated with copy number variation, point mutation and methylation (Zhou & Ren, 2009).

This new multiplex method is able to detect abnormal copy numbers of genomic DNA sequences requiring a minimum of 20ng of human DNA (Schouten et al., 2002). In this technique, it is not the nucleic acid, but the probes added to the samples that are amplified. MLPA allows discrimination of sequences that differ only in a single nucleotide, therefore MLPA can be used for detection of known mutation. It is basically a method to make a nuclei acid sample suitable for multiplex polymerase chain reaction (PCR) with the use of only one pair of primers. In the currently available kits, the products generated by PCR are separated by sequence-type electrophoresis. The thermocycler and sequencing-type electrophoresis equipment that are required, are present in most DNA diagnostic laboratories. Up to 96 samples can be handled simultaneously, 45 DNA sequences, and results can be obtained within 24 hours. One of the currently MLPA kits (P095, MRC-Holland, Amsterdam) is commercially available and contains eight independent probes for each of the chromosomes involved in almost frequent aneuploidies: 13, 18, 21, and X, and four Y-specific probes; and it is used as a rapid prenatal test by several medical centers on a large scale (Schouten et al., 2002). MLPA profiles must be compared with a similar profile obtained from a control DNA sample. Compared with a control reaction, the relative peak area of each amplification product reflects the relative copy number of the target sequence of that probe in the analyzed sample. An aberrant copy number of one or more of the sequences detected by MLPA probes can therefore be detected by a decrease or increase in relative peak area of the amplification products of the probes detecting those sequences.

The length of the amplification product of each probe is different, and ranges in size between 130 and 480 nucleotides. This provides an optimal separation and low background on sequencing type electrophoresis gels. Although performing an MLPA reaction is easy,

the development of new MLPA assays is complex and time-consuming, and the success of the results depends basically on the quality of the DNA extraction.

Briefly the protocol consists of: denaturing 20-500 ng of DNA by heating to 98°C in a thermocycler; adding the MLPA probes and leaving overnight at 60°C for hybridization. For the next step add the ligase and ligase buffer at 54°C for 15 min. (ligation of the two probe parts); inactivate the ligase by heating to 98°C; add PCR primers, dNTPs, and polymerase and start the PCR (amplification of probes). The amplification products are separated by capillary electrophoresis.

2.4 Quantitative Fluorescent-Polymerase Chain Reaction (QF-PCR)

This method uses PCR amplification and fluorescent dye labeled primers targeting highly polymorphic regions of DNA sequence called short tandem repeats (STRs) that are located on the chromosomes of interest (Mansfield, 1993). Each target marker is specific to the chromosome on which it is located, thus the copy number of the STR marker reflects the copy number of the chromosome. Informative STR markers which exhibit a high heterogeneity have been selected so that copy number can be easily determined. A normal diploid sample has the normal complement of two of each of the somatic chromosomes, thus two alleles of a chromosome specific STR are determined by the QF-PCR technique as two peaks in a 1:1 ratio. The observation of an extra STR allele as either a three peak pattern in a 1:1:1 ratio or two peak pattern in a 2:1 ratio is diagnostic of a presence of an additional sequence which in turn may represent an additional chromosome, as in the case of a trisomy.

Amplified products of the QF-PCR technique are analyzed quantitatively on a capillary Genetic Analyzer (ABI 3100) to determine the copy number of the analyzed STRs markers.

The kit used in the study was from ELUCIGENE. The ELUCIGENE QST*^R™ range of products are DNA based multiplexed assays for the rapid prenatal determination of aneuploidy status for the three most common autosomal trisomies and the sex chromosomes X and Y. PCR products are observed as a 5 dye labelled system using filter set G5. Filter set G5 detects the 6-FAM (blue), VIC (green), NED (yellow) and PET (red) labelled fragments plus the Size Standard marker labelled with LIZ (orange) on an electrophoretogram in the Genotyper program.

The markers used are described in Tables 2 and 3.

Marker	Location	Observed Heterozygosity	Allele Size Range (bp)	Marker Dye Colour
DXS981	Xq13.1	0.86	225-260	blue
DXS1187	Xq26.2	0.72	122-170	green
HPRT	Xq26.2	0.78	265-300	green
DXS7423	Xq28	0.74	372-388	green
DXYS267	Xq21.3/Yp11.2	0.87	240-280	red
AMEL	Xp22.22/Yp11.2	-	104-110	yellow
DXS6807	Xp22.32	0.70	331-351	blue
DXS1283E	Xp22.31	0.89	292-340	yellow
SRY	Yp11.31	-	244-251	yellow
DYS448	Yq11.223	-	323-381	red

Table 2. X and Y markers in ELUCIGENE QSTRs

Marker	Location	Observed Heterozygosity	Allele Size Range (bp)	Marker Dye Colour
D13S252	13q12.2	0.85	260-330	red
D13S305	13q13.3	0.75	418-470	green
D13S628	13q31.1	0.69	425-472	yellow
D13S634	13q21.33	0.81	355-440	blue
D13S325	13q14.11	0.86	235-320	green
D18S386	18q22.1	0.88	320-407	green
D18S390	18q22.3	0.75	345-400	yellow
D18S391	18q11.31	0.75	196-230	green
D18S535	18q12.3	0.92	450-500	blue
D18S819	18q11.2	0.70	370-450	red
D18S978	18q12.3	0.67	180-230	yellow
D21S11	21q21.1	0.90	220-283	blue
D21S1437	21q21.1	0.84	283-350	blue
D21S1409	21q21.2	0.81	160-220	red
D21S1411	21q22.3	0.93	256-345	yellow
D21S1435	21q21.3	0.75	152-210	blue

Table 3. 13, 18 and 21 markers in ELUCIGENE QSTRs

2.5 PCR Set Up

PCR was performed according to the manufacturer's instruction as described briefly: the thermal cycler is programmed for a single step cycle to activate the DNA polymerase at 95°C, for 15 minutes, linked to an amplification cycling program of 30 seconds at 95°C (denaturation), 1 minute and 30 seconds at 59°C (annealing) and 1 minute and 30 seconds at 72°C (extension) for 26 cycles. This should be linked to a 30 minutes time-delay at 72°C (extension) on the final cycle; sufficient vials should be separated to pre-aliquoted QSTR reaction mix for a number of samples and controls to be run. The vials are centrifuged at 12,000g for 10 seconds; 2.5µl of test DNA is added to a sample vial containing QSTR reaction mix; the 95°C activation program is initiated (step 1). On completion of the amplification program the samples may be stored at room temperature overnight or at 2-8°C up to 7 days before analysis by capillary electrophoresis.

Optimal results can be obtained using an ABI 3100 Genetic Analyzer

We also obtained 115 samples from pregnant women with multiple malformations fetus, for traditional karyotype analysis. The biological samples obtained were: AF, UC and alternative materials, such as urine, cystic hygroma fluid, intraperitoneal or cerebrospinal fluids. AF and alternative materials were cultivated as long term culture, with Amniomax medium, at 37°C, in CO₂ incubator. The blood culture (UC) was processed as short term culture (72hs) following standard cytogenetic procedures.

3. Results

We obtained 50 samples from different fetal materials for molecular techniques analysis, and 115 for traditional karyotyping (AF, UC, or alternative materials). All samples were from multiple malformations fetus without diagnosis.

For the molecular techniques, we first tested all the samples with the MLPA kit P095 and we observed that obtaining genetic profiles from samples containing minimal amounts of DNA can be difficult. Unfortunately, the quantity and quality of DNA was not adequate for this analysis and it was quite difficult to interpret the data obtained (the peak areas tend to be too variable when the DNA quality is not good enough). We then ran all samples again using QF-PCR. The XY test is very sensitive so that even when the quality of DNA was poor we could still determine the presence or absence of a Y chromosome. Thus, for some probands (09 cases), we could only give results for XY and not for autosomes. At times it was not possible to say whether the proband was 45,X or 46,XX since insufficient probes amplified, and the peaks were very weak (Table 4).

Case number	Chr. 21	Chr. 18	Chr. 13	X/Y	Interpretation/comments	Traditional Karyotype
01	2	2	2	XX	No T13, 18, 21 detected	
02	2	2	3	XY	Trisomy 13, meiotic error	-
03	2	2	2	XY	No T13, 18, 21 detected	
04	2	2	2	XY	No T13, 18, 21 detected	
05	-	-	-	-	Not enough DNA	
06	-	-	-	XY	Very weak peaks	
07	-	-	-	XX	No DNA left	
08	2	2	2	XX	No T13, 18, 21 detected	
09	2	2	2	XY	No T13, 18, 21 detected	
10	2	2	2	XX	No T13, 18, 21 detected	
11	2	2	2	XY	No T13, 18, 21 detected	
12	2	2	2	XY	No T13, 18, 21 detected	
13	-	-	-	XY	Very weak peaks	
14	2	2	2	XX	No T13, 18, 21 detected	
15	2	2	2	XY	No T13, 18, 21 detected	
16	-	-	-	XX	Very weak peaks	
17	-	-	-	XX	Very weak peaks	46,XX
18	2	2	2	XY	No T13, 18, 21 detected	
19	2	2	2	XX	No T13, 18, 21 detected	
20	-	-	-	X/XX?	Very weak peaks	
21	-	-	-	XX	Very weak peaks	
22	-	-	-	XX	Very weak peaks	
23	-	-	-	-	No peaks	
24	-	-	-	XY?	Very weak peaks	
25	-	-	-	X/XX?	Very weak peaks	
26	-	-	-	-	No peaks	
28	-	-	-	-	No peaks	
29	-	-	-	-	No peaks	46,XX
30	-	-	-	-	No peaks	46,XY
31	-	-	-	-	No peaks	47,XX+21
32	-	-	-	-	No peaks	
33	-	-	-	-	No peaks	

Case number	Chr. 21	Chr. 18	Chr. 13	X/Y	Interpretation/comments	Traditional Karyotype
37	-	-	-	-	No peaks	
38	-	-	-	-	No peaks	46,XX
39	-	-	-	-	No peaks	45,X
40	-	-	-	-	No peaks	46,XY
41	-	-	-	-	No peaks	
42	-	-	-	-	No peaks	
43	-	-	-	-	No peaks	
44	-	-	-	-	No peaks	
45	-	-	-	-	No peaks	
46	-	-	-	-	No peaks	
47	2	3	2	XY	Trisomy 18, meiotic error	47,XY,+18
48	2	2	2	XX	No T13, 18, 21 detected	46,XX
49	-	-	-	-	No peaks	46,XX
50	-	-	-	XY	Very weak peaks	47,XY+21
51	-	-	-	XY	Very weak peaks	47,XY,+18
52	2	3	2	XY	Trisomy 18, meiotic error	47,XY,+18
53	2	2	2	XX	No T13, 18, 21 detected	47,XX,+mar
54	2	2	2	XX	No T13, 18, 21 detected	47,XX

Table 4. Results of 50 samples from different tissues analyzes by Q-F PCR

From the 50 cases, we could get partial results in 30. Although we had a very good technique for extracting DNA from paraffin (we've got enough DNA concentrations and relative good quality of DNA), this material showed to be inappropriate for molecular techniques, probably due to the formalin buffer used to embed the tissue at the time of collection. In all those cases we got no detectable peaks.

In one case, the physician was quite sure about the clinical diagnosis of trisomy 13, and when the fetus died, his lung was collected for culture and karyotyping, and also umbilical cord for posterior DNA analysis. The lung culture failed, but we were able to apply QF-PCR in DNA extracted from UC and confirmed the clinical indication. In this case, three peaks were detected with markers D13S252, D13S305, D13S634 and D13S325. For marker D13S628 we observed two peaks being one higher than the other indicating an extra allele.

In two cases, trisomy 18 was detected, confirming the previous karyotyping. In one of the cases, three peaks were detected with marker D18S386 and D18S390, and two peaks, one being higher than the other, with markers D18S535 and D18S391. In the other case, three peaks were detected with marker D18S386, and two peaks detected, again one being higher than the other, with markers D18S391 and D18S978.

From the floating amniocytes that did not adhere to the flask, and were collected at the first medium change (14 cases), we succeeded in extracting DNA and performed QF-PCR. This material would be normally discarded. From those, we were able to obtain eight molecular results from which two did not have a previous successful karyotype.

The best results obtained for molecular analysis were from DNA extracted using a commercial kit as described before (Material and Methods).

From 6 cases of aneuploidies that had previous karyotype, we were able to obtain two confirmations (cases number 47 and 52). On the other hand, for one case (case number 2)

which remained without karyotype, we obtained a positive result through QF-PCR. Case number 53 (table 4) could not be confirmed by molecular analysis because the origin of the extra chromosome marker was unknown and could not hybridize with the probes used. We had no discordant results between the molecular and traditional techniques for those cases where both analyses were performed.

In Table 5, we describe the results from different sources of fetal material for karyotyping in 115 multiple malformation fetuses. When, for some anatomical reason, AF CVS or UC could not be collected, the obstetrician strategy, sometimes for therapeutic reasons, was the collection of alternative fetal material such as bladder drainage. Those biological materials could be also used for karyotyping, and although there were few cases available, we had 100% of culture success and karyotyping.

Fetus sample	Number of cases (n)	Culture success (n)	Success rate (%)
AF	87	83	95.5
UC	15	14	93.3
Alternative fluids ^a	13	13	100
TOTAL	115	110	95.6

^aBladder (6), cystic hygroma (2), intraperitoneal (2), displastic kidney (1), cystic lung (1), cerebrospinal (1) fluids.

Table 5. Different materials from multiple malformations fetus and success rate of cell cultures

4. Discussion

Cytogenetic analysis is an important tool for detecting chromosome abnormalities, once this is the cause of most common genetic disease in man (Pena, 1998). It has been very useful for prenatal diagnosis, and also in clinical genetics. However the traditional technique has some limitations, and in order to overcome these problems some new molecular and rapid techniques have been developed, such as QF-PCR and MLPA. In our study, we tried to use all possible biological sources obtained from the fetuses to be able to reach a diagnosis for a multiple malformation that could evolve to death, or had already died without any laboratory findings. The main limitation we wanted to overcome was to avoid the situation of leaving a multiple malformation fetus without a final diagnosis or karyotype which is a vital information for genetic counseling and for the family. Thus we tried to apply molecular techniques in postmortem or paraffin-embedded fetal tissues, or even non adhered amniocytes that would be normally discarded after culture.

Karyotyping unconventional fetal samples, when obtaining traditional biological material is difficult, is not a very common approach in most laboratories (Donnenfeld et al., 2001; Gole et al., 1997). Nevertheless, we used this alternative whenever necessary, and achieved 100% success rate on a limited sample of 13 cases (Kessler et al., 2008), being this rate much higher when compared to other studies (Teoh et al., 1996; Donnenfeld, 2001).

There is an ongoing debate whether Rapid Aneuploidy Diagnosis (RAD) should be employed as an adjunct to karyotyping or whether it could be used as a stand-alone test in selected groups of women (Leung et al., 2003; Cirigliano et al., 2006). The controversy is due to residual probability of a chromosome abnormality (both balanced and unbalanced) when RAD demonstrates a normal result. Few studies have estimated the residual risk of a

clinically significant chromosome aberration for different indications when RAD results are normal. In a meta-analysis of 12 studies involving invasive tests, the risk of having a chromosome aberration that was not expected to be detected by RAD methods, was estimated to be 0.9% (Leung & Lao, 2005). In our results, from 6 aneuploidies already diagnosed by karyotyping, only two cases (33.3%) were detected by QF-PCR, probably because of poor quality of DNA (mainly in paraffin). On the other hand, a third case detected by QF-PCR, had failed for tissue culture at the time of karyotyping analysis, the result was obtained only using molecular approaches. Little is known about the patients' preference regarding the type of analysis to be performed. However in Sweden a research was made including 6000 women and 70% chose QF-PCR analysis alone (Bui, 2007). In our opinion, the combination of both techniques is safer, in order to overcome the particular limitations of each one. .

MLPA has the same inherent limitations as those of QF-PCR in that it will not detect most structural chromosome aberrations, or balanced rearrangements such as translocations and inversions. Moreover, maternal cell contamination and 69,XXX triploidy will not be diagnosed by MLPA (Bui, 2007). In our study, for example, when a marker chromosome was detected by the traditional karyotype, the molecular techniques were not able to detect it, because the origin of the marker was unknown, so no specific probe could be applied.

Although we developed a good protocol for extracting DNA from paraffin block wax, this material showed to be inappropriate for the molecular techniques used, as demonstrated in other studies which also needed smaller fragments of DNA (Halvarsson et al. 2004). Another study in postmortem tissues embedded in paraffin succeeded to obtain longer amplification fragments of around 300 bp using a specific treatment called pre-PCR restoration (Bonin et al., 2003), thus achieving better results.

Whenever AF was set up for culture, not all amniocytes adhered to the bottom of the flask. After few days, by the first medium change, we observed that there were still a considerable number of floating cells, which could be used to obtain a considerable amount of DNA. We proposed and developed a protocol to extract DNA from those cells in order to guarantee a result independently of the cell culture success, and also to abbreviate the result with molecular techniques. Although we did not succeed as expected, we could obtain results from two cases when cell culture had failed. We could probably improve the success rate of these analyses by extracting DNA with commercial kits rather than in-house techniques in order to obtain better quality DNA, which is essential for the molecular analysis used here. The need of faster testing methods which do not require cell culture has been recognized by the scientific community to improve pregnancy management and alleviate parental anxiety (Nicolini et al., 2004).

The best results obtained were from the last 8 cases, in which the DNA was extracted using a commercial kit. This is very important information and corroborates the fact that high quality DNA is necessary, from which we can obtain results even with degraded DNA (Roeder et al., 2009).

5. Conclusion

In conclusion, for follow up diagnostic testing, karyotyping has proved to be the gold standard method. This technology has remained essentially unchanged over 30 years, as no new technology has proven to be superior in terms of being able to detect such a wide range of abnormalities with the necessary precision (Slater et al., 2009). Nevertheless, molecular

testing, such as QF-PCR or MLPA, are becoming important alternatives in order to give a rapid result with low cost. Although these molecular techniques have some limitations, we did not find any discordant result, in comparison to traditional karyotype. Nevertheless, the appropriate approach is always performing simultaneously both techniques.

The importance of this study remains in the alternatives we proposed to give a final diagnosis to a multiple malformation fetus. We suggested some approaches to achieve a final laboratory result and deliver to the family the information they need to rebuild their lives, and make plans for their future, with the help of more rapid and efficient technology (RAD) and appropriate genetic counseling.

6. Acknowledgements

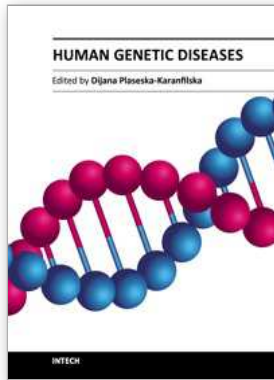
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The genetics science is less than 150 years old, but its accomplishments have been astonishing. Genetics has become an indispensable component of almost all research in modern biology and medicine. Human genetic variation is associated with many, if not all, human diseases and disabilities. Nowadays, studies investigating any biological process, from the molecular level to the population level, use the "genetic approach" to gain understanding of that process. This book contains many diverse chapters, dealing with human genetic diseases, methods to diagnose them, novel approaches to treat them and molecular approaches and concepts to understand them. Although this book does not give a comprehensive overview of human genetic diseases, I believe that the sixteen book chapters will be a valuable resource for researchers and students in different life and medical sciences.

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