# Special Article

# Role of Molecular Biology in Obstetrics – Modern Single Gene Disorders Diagnosis Techniques

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*Single gene mutations may lead to an inherited disorder with Mendelian inheritance patterns, of which over 8,000 disorders have been catalogued. The strategy of population screening, offering genetic counseling, prenatal diagnosis and termination of affected pregnancy has been successfully applied worldwide to reduce the number of new patients. Common fetal sampling techniques in utero include chorionic villous sampling, amniocentesis, and fetal blood sampling. Then appropriate analysis is applied for diagnosis, where karyotyping is mainly for chromosome abnormalities and PCR is for single gene disorders. Several modern molecular techniques are useful for identification of defects in single genes. Preimplantation genetic diagnosis is an advanced alternative giving the couple the chance to start a pregnancy ensuring that the baby is free from the genetic disease. It is the role of obstetricians to make most use of the advance molecular biology knowledge to have a healthy community.*

***Keywords:*** *Chromosome abnormalities, Down's syndrome, Embryo selection, Molecular biology, Polymerase chain reaction (PCR), Preimplantation genetic diagnosis (PGD), Prenatal diagnosis (PND)*

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Mutations in single genes can lead to inherited disorders with Mendelian inheritance patterns. Individual single gene disorders are rare, but together affect around 1% of the general population. More than 8,000 different single gene disorders have been catalogued to date (www.ncbi.nlm.nih.gov/omim/, 2006) and can be categorized by their inheritance patterns as autosomal dominant (over 3,400 disorders), autosomal recessive (over 3,200 disorders), and sex-linked disorders (over 1,300 disorders). Common fetal sample collection techniques and the role of molecular biology in Obstetrics, in particular the aspect of modern techniques for single gene disorders diagnosis, are discussed in this article.

**Diagnosis of genetic diseases**

Couples who are affected or who carry a genetic defect possess a high risk of passing on the abnormality to their children. Prior to the era of prenatal diagnosis (PND), such subjects had to decide whether to be childless or risk having children who might inherit the genetic disorder. With the evolution of molecular genetic technologies, the testing of the growing number of inherited genetic diseases has become feasible. In addition to the correlation of the particular gene defect to each disorder, such tests are notably useful in identifying asymptomatic carriers who are at risk of transmitting the genetic abnormality to their offspring. This knowledge, when incorporated with genetic counseling and appropriate prenatal diagnosis techniques, helps in reducing the number of the births of new affected cases[1,2].

Mutation analysis can be performed directly if the responsible genes in the family have been identified and cloned. However, in cases where the causative mutations are still unspecified or direct analysis techniques are unavailable, the examination of polymorphic loci adjacent to the causative mutation can be employed for linkage analysis in order to track down the transmission of the mutant gene in the family. The polymorphic linked markers with point variation can be identified using restriction fragment length polymorphisms (RFLP), while those with hypervariable micro-

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satellite loci, which give more alleles with varied size, are more useful.

**Prenatal diagnosis**

The combination of molecular techniques for diagnoses of genetic diseases and procedures to collect fetal cells in utero enables prenatal diagnosis of many genetic disorders at an early stage of pregnancy. Normal testing results reassure the parents that their fetus is free from the disease; while abnormal results give them a chance of deciding to discontinue the pregnancy, to have fetal therapy where possible, or to continue the pregnancy.

***Amniocentesis***

Amniocentesis is the commonest invasive procedure for prenatal diagnosis. Amniocentesis is usually performed at 15 weeks of pregnancy or over. Karyotyping is standard method for diagnosis of chromosome abnormalities, i.e. Down syndrome. However, modern analysis methods, including FISH[3] and polymerase chain reaction (PCR)[4] on uncultured amniocytes have become popular alternatives to accelerate diagnosis reports. The application of amniocentesis for single gene disorders is less common due to high risk of maternal cells contamination.

***Chorionic villus sampling (CVS)***

Chorionic villus sampling (CVS) is a technique to retrieve fetal tissue for analysis in the first trimester. The major advantage of this technique includes the opportunity to obtain sufficient fetal cells for the tests and early fetal diagnosis results that enable first trimester abortion in cases of affected fetuses. Moreover, direct DNA analysis, fast karyotyping following a rapid culture technique and biochemical analysis from the biopsied chorionic villus are also possible. The event called confined placental mosaicism (CPM) can be found in approximately 1% of pregnancies and can lead to discordant results between the CVS and the fetus[5]. Therefore, confirmatory amniocentesis or fetal blood sampling are required in some cases to confirm CVS results.

***Fetal blood sampling (FBS)***

Fetal blood sampling from the umbilical cord or cordocentesis was preliminary performed using a fetoscopic technique[6]. However, this technique has been superseded by the percutaneous ultrasound guided approach[7] when high resolution ultrasound was developed. Fetal blood samples are useful for diagnosis of thalassemias, hematologic disorders, quick karyotyping, fetal isoimmunisation, hypoxia, infection, metabolic disorders, and single gene defects.

**Preimplantation genetic diagnosis (PGD)**

At present the strategy of population screening, genetic screening and offering appropriate prenatal diagnosis procedures is the best tool to control the incidence of new cases with genetic disorders in many countries. Preimplantation genetic diagnosis (PGD) is the genetic testing of preimplantation stage embryos for inheritable chromosome abnormalities or single gene defects[8]. This allows the selection of unaffected embryos prior to the establishment of a pregnancy, and therefore gives the couple the chance to start a pregnancy with a disease free baby. Consequently, the need for termination of an affected pregnancy can be eliminated. The first PGD baby in Thailand was born in June 2005 at Chiang Mai University Hospital[9].

**Modern molecular biology techniques**

***Fluorescent PCR (F-PCR)***

The traditional methods of visualizing the PCR products following electrophoresis include ethidium bromide or silver staining or the use of radioactive labeled primers. The introduction of F PCR[10] has been useful for DNA analysis, increasing the sensitivity and specificity. The application of oligonucleotide primers attached to fluorescent molecules gives rise to amplified products labeled with fluorescent dye. When these F PCR products migrate under electrophoresis to the position where the laser bisects the gel, the fluorescent molecules are activated by the laser and give a signal with a specific wavelength that can be detected by a CCD (charged couple device) detector and analyzed by computer software. This technique allows the size standards to run in the same lane, consequently, the size analysis is as precise as a single base pair difference.

***Multiplex PCR***

Multiplex PCR is a technique allowing more than one locus to be amplified by using a combination of unrelated sets of primers in a PCR tube[11]. The PCR products of different loci can be distinguished by designing the primers to generate amplified fragments with different sizes. This strategy benefits the analysis of multiple loci, i.e. the detection of more than one disease or the combination of mutation detection and a linked or unlinked polymorphic locus without increasing time and cost. The additional information of an

informative polymorphic marker is useful for a back up linkage analysis result (for a linked marker only) and contamination identification[12].

**Modern mutation analysis methods**

The amplified products with size differences can be analyzed using traditional agarose gel, polyacrylamide gel electrophoresis or F PCR. However, base pair substitutions without size alteration, or small deletions or insertions are more difficult to detect. For substitution mutations, major detection systems include those for a particular mutation and those that can identify several mutations at a time. Scanning methods are helpful for searching for uncharacterized mutations and for diseases that are caused by a variety of mutations, i.e. beta thalassemia. Popular techniques of this group include heteroduplex analysis (HA), single strand conformational polymorphism (SSCP) and denaturant gradient gel electrophoresis (DGGE). Mutation specific analysis techniques are useful for detecting common mutations and provide definite results. These techniques include restriction fragment length polymorphism (RFLP) and amplification refractory mutation system (ARMS).

***Restriction fragment length polymorphism (RFLP)***

RFLP is a mutation specific analysis technique. The analysis is based on the identification of the difference of the DNA sequence, i.e. mutation, by digestion of the DNA using restriction endonucleases. The bacterial enzymes can recognize a particular DNA sequence and cleave the DNA strand at the recognition site. By knowing the DNA sequence, a specific restriction enzyme can be used to digest either the normal or the mutant sites giving a size difference between both alleles[13]. An artificial restriction site can be created using a primer of modified sequence as site specific mutagenesis (SSM) method if there is no natural one[14].

***Dot-blot and reverse dot-blot hybridization***

Allele specific oligonucleotide probes can be used for specific mutation detection from uncultured amniocytes or chorionic villous samples as dot-blot and reverse dot-blot hybridization[15]. The disadvantage with these methods is that a particular allele-specific oligonucleotide (ASO) probe can hybridize with only one mutation in the analysis.

***Amplification refractory mutation system (ARMS)***

ARMS is another mutation specific analysis method. This technique involves the annealing of allele-specific oligonucleotides and the use of three oligonucleotides: one for the common upstream sequences and two for the normal and mutant sequences. The allele-specific oligonucleotides in this method function as primers for PCR. Successful amplification of the DNA being tested indicates that the specific normal/mutant primer has annealed, and thus confirms the presence of the specific normal/mutant alleles. Primers for the normal and mutant alleles can be included in the same PCR tube and designed to have size difference or be labeled with different fluorescent dyes in order to differentiate both alleles[16].

***Heteroduplex analysis (HA)***

When denatured and allowed to reanneal, the complementary strands of the normal and mutant alleles of a heterozygote sample will form hybrid molecules (heteroduplex) with an area of mismatch of the different sequence. The heteroduplex molecules will give extra bands during the electrophoresis due to their different migration pattern from the homoduplex molecules. Samples with homozygous genotypes do not generate an extra heteroduplex band, unless the PCR product of the different homozygous genotype is added[17].

***Single strand conformational polymorphism (SSCP)***

SSCP analysis is a scanning technique relying on the electrophoretic resolution of the sequence specific conformations of single stranded DNA fragments[18]. This strategy is useful in detecting small deletions and insertions and single base pair substitutions of the fragment size from 100 to 500bp. A single base pair mutation can give rise to a markedly different single strand conformation, which results in a different migration rate during the electrophoresis, and therefore the different alleles can be identified. By this method, multiple mutations that lie within the same amplified fragment can be identified using a single protocol. This can benefit the diagnosis of compound heterozygous genotypes.

***Denaturant gradient gel electrophoresis (DGGE)***

The related strategy to SSCP, DGGE is based on the altered melting characteristics due to sequence difference that influence migration rates during the electrophoresis through a polyacrylamide gel with an increasing concentration of denaturant. The test fragments in this method are usually amplified using specially designed primers with a stretch of approximately 40 guanine or cytosine residues (GC clamp)[19].

**Advanced molecular analysis techniques**

***Sequencing***

The use of a modern automated laser fluorescence DNA sequencer that can visualize up to four fluorescent dyes allows the sequencing reaction to be accomplished within a single tube and the sequencing product to be analyzed on a single lane. These methods allow the detection of virtually any mutation, with little or no adjustment of methodology. Theoretically, sequencing should be particularly useful in cases of disease caused by a heterogeneous spectrum of mutations, as it will often be possible to design a single set of primers that allows the detection of multiple affected genotypes, in particular beta-thalassemias[20].

***Mini-sequencing***

The minisequencing technique employs the same strategy as the standard fluorescent sequencing, but extends only one nucleotide after the minisequencing primer. This gives rise to a shorter fragment size and consequently a faster analysis time for the electrophoresis. Both techniques are useful for the detection of single nucleotide substitutions, deletions, and insertions. A particular minisequencing primer, which locates just before the mutation, is used for each specific mutation. By changing the primers used in the PCR step and minisequencing primers, this protocol can be used as a versatile analysis protocol[20].

**Contamination problem**

In PND, contamination is a major problem. PCR set up in a DNA-free environment away from the analysis area can help in reducing the chance of getting 'carry over' PCR products from previous amplifications. All media and reagents should be tested regularly. Despite all efforts, contamination can still occasionally take place and cause misdiagnosis. Maternal DNA contamination is most crucial and difficult to prevent. For a dominant disease, if the mother carries the causative gene, maternal DNA contamination would lead to an affected result and consequently termination of a normal baby. In case of a recessive disease, the contamination of maternal heterozygote DNA in a homozygote affected sample would lead to a heterozygote result, and continuing the affected pregnancy. In order to reduce the risk of misdiagnosis caused by contamination, the concept of DNA fingerprinting has been introduced to track down the presence of contamination by amplifying a highly polymorphic marker together with the test gene. The genotype in a fetus that deviates from the four possible combinations of parental alleles indicates the possibility of contamination[21].

**Conclusion**

Of all the genetic disorders, thalassemia is prevalent and causes a huge health and financial burden in Thailand. Carrier screening, providing genetic counseling and PND for couples at risk and termination of affected pregnancy is the most effective strategy to combat serious genetic diseases. The combination of choices of fetal sample retrieval methods and advanced molecular biology techniques enables PND of a growing number of genetic diseases. Future molecular analysis techniques include real-time PCR and microarray (microchip) are being developed and tested and will be very useful in the near future in term of reducing testing time and analyzing several mutations simultaneously. In the era of the Molecular Biology with the success of the Human Genome Project, it is the role of the obstetricians to make most use of the genetic knowledge to make sure that every family will have a new healthy member and consequently a healthy population for the country.

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## บทบาทของชีววิทยาระดับโมเลกุลในสูติศาสตร์ะ เทคนิคสมัยใหม่ในการวินิจฉัยโรคพันธุกรรมชนิดจีนเดี่ยว

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*การกลายพันธุ์ของจีนเดี่ยวอาจทำให้เกิดความผิดปกติที่ถ่ายทอดทางพันธุกรรมที่มีรูปแบบการสืบทอดแบบเมนเดล ซึ่งมีรายงานไว้กว่า 8,000 ชนิด การใช้กลยุทธ์ในการคัดกรองพาหะในประชากร ให้คำปรึกษาทางพันธุศาสตร์ และบริการการตรวจวินิจฉัยก่อนคลอด และทางเลือกในการทำแท้งในครรภ์ที่ตรวจพบว่าทารกเป็นโรคสามารถลดจำนวนผู้ป่วยใหม่ได้อย่างมีประสิทธิภาพทั่วโลก เทคนิคในการเอาเซลล์ทารกในครรภ์มาตรวจที่ใช้บ่อยได้แก่ การตัดตรวจเนื้อรก การเจาะตรวจน้ำคร่ำ และการเจาะตรวจเลือดจากสายสะดือทารกในครรภ์ จากนั้นจึงนำเซลล์ดังกล่าวไปตรวจวิเคราะห์ด้วยเทคนิคที่เหมาะสม โดยมักใช้เทคนิค karyotyping ในการตรวจหาความผิดปกติของโครโมโซมและเทคนิคปฏิกิริยาลูกโซ่ PCR ใช้ในการตรวจความผิดปกติของจีนเดี่ยว มีเทคนิคทางชีววิทยาระดับโมเลกุลสมัยใหม่หลายวิธีที่เป็นประโยชน์ในการตรวจวิเคราะห์ความผิดปกติในจีนเดี่ยว การวินิจฉัยระยะก่อนการฝังตัวของตัวอ่อนเป็นทางเลือกขั้นสูงที่ช่วยให้คู่สมรสมีโอกาสเริ่มการตั้งครรภ์โดยแน่ใจว่าทารกปราศจากโรคทางพันธุกรรม จึงเป็นบทบาทของสูติแพทย์ที่จะใช้ประโยชน์ให้มากที่สุดจากความรู้ขั้นสูงทางด้านชีววิทยาระดับโมเลกุลมาใช้เพื่อให้ประชาคมมีสุขภาพดี*