The Molecular Pathology Laboratory of the 21st Century*

FREDERICK L. KIECHLE, M.D., Ph.D.,
XINBO ZHANG, M.D., Ph.D.,
and TADEUSZ MALINSKI, Ph.D.†

Department of Clinical Pathology,
William Beaumont Hospital,
Royal Oak, MI 48073

and

†Department of Chemistry,
Oakland University,
Rochester, MI 48309

ABSTRACT

Human cells contain deoxyribonucleic acid in mitochondria and nuclei. Human diseases may be caused by mutations in mitochondrial DNA, nuclear DNA or both. The volume of work performed in the diagnostic molecular pathology laboratory will continue to grow as more disease-related mutations are discovered. Many factors will influence the diagnostic molecular pathology laboratory in the 21st century, such as future clinical laboratory organization, amplification methods, specimen integrity, ethical guidelines and opportunities to expand service. In the evaluation of a patient suspected of a mitochondrial DNA mutation, care must be exercised in the selection of a primer for amplification and of the specimen to be examined for the mutation. The uneven distribution of normal and abnormal mitochondrial DNA within the various tissues (heteroplasmy) may result in a normal mitochondrial DNA sequence if the wrong tissue is examined. The presence of mitochondrial-like sequences (pseudogenes) within nuclear DNA may result in amplification of nuclear genes if generic primers are used to duplicate a mitochondrial DNA gene. Diabetes mellitus is a heterogeneous disease with mutations occurring in a variety of proteins leading to either prereceptor, receptor or postreceptor defects. In this example, the diagnostic molecular pathology laboratory may be asked to define the specific genotype a specific patient with this common phenotype may possess.

Introduction

Deoxyribonucleic acid (DNA) is located in the nuclei and mitochondria of mammalian cells.1,2 Human diseases may be caused by mutations in mitochondrial and/or nuclear DNA.1-3 There is growing interest in providing diagnostic assays based on identifying specific DNA sequences.1-4 The Human Genome Project has focused attention on the association of mutations in nuclear DNA (nDNA) with human diseases.5 Mitochondrial DNA (mtDNA) has been completely sequenced and each strand possesses 16,569 base pairs.1,3
The field of diagnostic molecular pathology is expanding rapidly. As new genes are sequenced, the search for a disease-related mutation or rearrangement of the gene rapidly follows. Applications of diagnostic molecular pathology include genetics, inborn errors of metabolism, forensic pathology, hematopathology, coagulation, neoplasia, virology, microbiology, histocompatibility and mtDNA disorders.

This article will review the workload of a diagnostic molecular pathology laboratory from 1991 to 1997; consider factors which will shape the diagnostic molecular pathology in the 21st century, such as future clinical laboratory organization, amplification methods, specimen integrity, ethical guidelines and expansion of service opportunities; review specific issues related to defining mtDNA disorders; use diabetes mellitus as a model to illustrate how diagnostic molecular pathology may aid in classification and treatment.

**Diagnostic Molecular Pathology Tests at William Beaumont Hospital**

Under the direction of D. Crisan, M.D., Ph.D., and D.H. Farkas, Ph.D., the Molecular Probe Laboratory in the Department of Clinical Pathology at William Beaumont Hospital was opened in November, 1991. The total number of billable procedures has increased each year (figure 1). A royalty agreement for clinical use of polymerase chain reaction (PCR) was signed with Roche in late 1992. In 1993, PCR assays were introduced and now exceed the number of Southern blot procedures performed in the laboratory (figure 1). In 1996, ligand chain reaction (LCR) assays for *Chlamydia trachomatis* and *Neisseria gonnorhoea* were introduced. These changes reflect the benefits of nucleic acid amplification tests, including faster turnaround time, decreased labor intensity and increased efficiency. Today, the laboratory offers four Southern blot assays (B/T cell, *bcr, bcl-2* gene rearrangements, fragile X syndrome detection), five PCR assays (paternity by histocompatibility loci, Factor V, hereditary hemochromatosis, 5,10 methylene-tetrahydrofolate reductase gene, angiotensin converting enzyme I gene), and two reverse transcriptase PCR procedures (qualitative and quantitative hepatitis C virus detection). There has been a rapid increase in test volume from 0.04 percent (84 assays) of total laboratory test volume in January, 1994, to 0.35 percent

![Figure 1](image-url). Annual volume of tests based on Southern blot (—♦—), polymerase chain reaction (—■—), ligase chain reaction (—▲—) and total tests performed from 1991 to 1994 at William Beaumont Hospital.
(1,093 assays) in July, 1997. The laboratory no longer operates with a deficit. To achieve the prediction of 5 percent of total laboratory test volume in the early 21st century,17 a total of 15,500 molecular pathology procedures per month would need to be performed. Factors which will influence the growth in diagnostic molecular pathology will be described.

Clinical Laboratory of the Future

Benge, et al,18 reviewed the costs of delivering clinical chemistry services at Vanderbilt University (Nashville, TN) over ten years. Five primary conclusions were derived: 1) increases in direct costs were entirely attributed to salary increases; 2) there was no change in inflation-corrected direct costs per test; 3) the profit margin was directly influenced by the hospital’s accounting method for calculating indirect costs; 4) the workload performed by each employee increased with time; and 5) labor costs were approximately 50 percent of the laboratory’s operating budget. As the number of capitated commercial contracts for laboratory services increases, cost reduction efforts in purchased supplies, consumables and capital equipment will reach a maximum. Attention will then turn to staff reduction. This reduction in labor costs may be achieved by consolidation of laboratories within a hospital or two or more hospitals, outsourcing specific laboratory services, or introduction of laboratory automation in isolated work cells or in a core laboratory model.19–23

Therefore, the future hospital laboratory will consist of a core laboratory featuring total laboratory automation performing non-time dependent, high-volume tests in hematology, coagulation, clinical chemistry and immunology. The automated line will also contain a single module or separate units capable of preanalytical processing like centrifugation, aliquotting, decapping and recapping.22 Adjacent to this total laboratory automation unit will be manual or semi-automated laboratory functions which support the core lab. For example, manual differential counts will be performed from slides generated by the algorithm in the hematology analytical unit defining when a slide should be prepared by the slide maker/stainer.24 Laboratories which are labor intensive, like transfusion medicine, histocompatibility, microbiology, virology, diagnostic molecular pathology and flow cytometry, will remain in separate locations or may be sent out to reference laboratories. If the core laboratory is located one or more miles from the hospital(s) it serves, the hospital(s) will need to have an immediate response laboratory to handle time-critical specimens. This rapid response lab will contain equipment which duplicates some of the services offered in the core lab, like electrolytes, glucose, etc. The automated line in the core laboratory may be fast enough to handle STAT specimens, but it may be located too far away to provide timely specimen delivery. One method to reduce the test volume in the immediate response lab is to offer those assays that improve patient outcome as a part of the point-of-care testing program.

Assessment of Technology

New technology must be assessed. For example, bioelectronic chips provide a method for lysing bacteria on one chip and hybridization for identification on a second chip.25 This technology comes currently with a high price.26 However, in the future, photolithographed 1 cm² chips may provide the possibility for DNA mutation analysis and infectious microbe identification at the bedside. The time required to identify a viral or microbiological organism will be significantly reduced by molecular detection. Chip technology has been used for continuous-flow PCR with a total reaction time for 20 cycles of 90 seconds27 and a variety of other applications.28,29

Amplification methods for increasing the number of gene or transcript copies are often used to increase the sensitivity of molecular biologic assays. Sensitivity may be increased further by using specific gene probes in hybridization assays of the amplified nucleic acid targets.30 Careful evaluation of each assay is required. For example, the thermal stable
DNA polymerase may be inhibited during PCR by heparin,31 heme from lysed red cells,32 urine,33 vitreous or aqueous fluid34 and RNA.35 Polyamines36 interfere with or formamide37 promotes the specificity of this PCR-based amplification. PCR is influenced by other factors, including annealing temperature, magnesium concentration and primer selection. A mutation in a target molecule's primer binding site may lead to no generation of amplicons (DNA) during PCR.38 Careful primer selection improves PCR sensitivity and specificity.39,40

Alternate methods for target or signal amplification have been reported including self-sustained sequence replication, strand displacement activation, repair chain reaction, ligase chain reaction, Q-beta replicase system, ligation activated transcription and nucleic acid sequence-based amplification.41,42 Each of these alternatives to PCR are being evaluated for potential clinical applications. The availability of standardized clinical applications of these amplification methods will provide a wider selection of kits for diagnostic molecular pathology detection challenges.

**SPECIMEN INTEGRITY**

DNA is a very stable macromolecular, while RNA species are likely to be rapidly degraded by RNases. There is limited data available on the stability of nucleic acids in medical specimens. The Reference Guide for Diagnostic Molecular Pathology/Flow Cytometry: Fascicle VII43 and other references describe issues related to nucleic acid stability in a variety of medical specimen types.44–46 For example, for Southern blot-based assays, table I outlines DNA stability when tissue is stored at a variety of conventional temperatures.

Blood collected for inborn error of metabolism screening on Guthrie cards contains DNA which is stable for three years if stored from 4–25°C.47 The whole blood spots contain PCR inhibitors47 which may be removed by water wash or commercial DNA purification kit.48 The purified DNA may be used for the detection of genetic diseases by PCR including cystic fibrosis.47,48

RNA-based tests like RT-PCR-based hepatitis C virus or HIV I detection may be collected in a physician's office or clinic some distance from the laboratory. Since RNA is quite labile, the RNA target must be processed rapidly and the RNA removed from contaminating RNases released from neutrophils in the peripheral blood sample.43,45 The need for rapid specimen processing will require the collection site to process the specimen. Aso, et al.,49 report that mononuclear cells collected by conventional density gradient methods may be

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
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<tbody>
<tr>
<td><strong>Southern Blot–based Assays – Tissue</strong></td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th><strong>NCCLS</strong></th>
<th><strong>PPSH – CAP</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Room Temperature (25°C)</strong></td>
<td>NR, freeze or fix immediately</td>
</tr>
<tr>
<td><strong>Refrigerator (2–8°C)</strong></td>
<td>Transport on wet ice; cold phosphate–buffered saline (2–6°C) 24 hours – slices (&lt; 0.5 cm)</td>
</tr>
<tr>
<td><strong>Freezer (–20°C)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Freezer (–70°C)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Fixed (25°C)</strong></td>
<td>Minced in ethanol &gt; 1 year</td>
</tr>
</tbody>
</table>

*NR = Not recommended.*
filtered through glass fiber membranes. The RNA on the dried membranes was stable at room temperature for one week. This method provides a specimen that is easy to transport to the Diagnostic Molecular Pathology Laboratory. Improved methods for the collection of stable nucleic acid preparations from medical specimens, especially for RNA species, are still needed. Their availability will aid in the acquisition of good quality specimens for RT-PCR or other RNA-based clinical assays.

**Ethical Guidelines**

The resolution of ethical and procedural issues will facilitate the timely acceptance and introduction of new diagnostic molecular procedures. The discovery of a candidate gene for hereditary hemochromatosis and two disease-associated mutations has been reported. An expert panel reviewed the ethical and health policy implications of genetic testing for this disorder. They concluded that population-based screening not be implemented at the present time secondary to uncertainties related to disease prevalence, penetrance and care of asymptomatic people with a hemochromatosis gene mutation. These guidelines will be reviewed in one year. Other policy statements and guidelines have been published for specific diseases and their detection methods or specific molecular biological methods and their current limitations. This area requires continuous attention and updating as new information becomes available.

An additional impediment to progress in the development of the diagnostic molecular pathology laboratory rests in the current practice of patenting sequences in DNA. If the medical use of a specific gene and its mutated forms are patented for evaluation of patients or relatives for a specific disease, then a royalty would need to be paid to the patent holder. A variety of companies have applied for a patent for the hereditary hemochromatosis gene. One company (Progenitor, Menlo Park, CA) has assigned exclusive rights to SmithKline Beecham Clinical Laboratories (Collegeville, PA) to perform laboratory testing under their patent. A similar situation occurs for the medical use of PCR for diagnostic use held by Roche (Nutley, NJ). The use of PCR to generate a billable test result requires royalty payment to Roche.

**Expansion of Service Opportunities**

The service provided by the hospital-based Diagnostic Molecular Pathology Laboratory is limited primarily to diagnostic screening or monitoring of disease processes. However, in the future, this laboratory will be asked to assist in evaluating and monitoring a variety of treatment strategies like bone marrow transplantation, somatic gene therapy and antisense oligonucleotides. One example may be the selection of specific chemotherapeutic agents that induce the greatest amount of apoptosis in a patient's leukemic cells. Chemotherapeutic agents exert their antitumor effects by inducing programmed cell death or apoptosis. Kravtsov, et al, found that purified leukemic cells from patients with the same diagnosis demonstrated significant variability in chemosensitivities among three chemotherapeutic agents tested for the ability to induce apoptosis.

We have used a model cell line, BC3H-1 myocytes, known to undergo apoptosis in the presence of the antitumor fluorescence dye, Hoechst 33342. The short-lived radical nitric oxide (NO) has recently emerged as a novel inhibitor or inducer of apoptosis. NO can induce apoptosis in different types of cells. To determine if Hoechst 33342-induced apoptosis may be enhanced or reduced by NO, we treated BC3H-1 myocytes with 7.5 μg/mL Hoechst 33342 and increasing concentrations of the NO donor, sodium nitroprusside (SNP), from 1–10 mmol/L (figure 2). Incubation of BC3H-1 myocytes for six hours with different concentrations of SNP plus 7.5 μg/mL Hoechst 33342 significantly enhance BC3H-1 myocyte death when compared to the SNP-treated group or 7.5 μg/mL Hoechst 33342-treated group, suggesting that NO and Hoechst 33342 interact synergistically in inducing cell death (figure 2). This synergy is
Figure 2. Effect of sodium nitroprusside (SNP) and Hoechst 33342 (H33342) in inducing cell death. BC3H-1 myocytes cultured in MEME/10 percent fetal bovine serum were treated for 6 hours with different concentrations of SNP from 1–10 mmol/L in the presence of 7.5 μg/mL Hoechst 33342. Each bar indicates the mean ± standard deviation from three separate experiments. *, p < 0.02; **, p < 0.001.

illustrated by the more marked nuclear morphologic changes (chromatin condensation, half moon configurations) induced in BC3H-1 cells by 10 mmol/L SNP plus 7.5 μg/mL Hoechst 33342 (figure 3D) compared to SNP (figure 3C) or Hoechst 33342 (figure 3B) treatment alone. To confirm further that Hoechst 33342 and SNP treatment induced cell death via apoptosis in the BC3H-1 myocytes, genomic DNA was extracted from BC3H-1 myocytes after treatment with 10 mmol/L SNP, 7.5 μg/mL Hoechst 33342 or the combination of both and analyzed by 1.5% agarose gel electrophoresis (figure 4). No DNA fragmentation ladder of multiples of 180 base pairs was observed in control or Hoechst 33342-treated cells (figure 4A, B). However, both 10 mmol/L SNP and SNP plus Hoechst-induced DNA fragmentation in the characteristic ladder of multiples of 180 base pairs observed in apoptotic cells (figure 4C, D). The greatest degree of DNA fragmentation occurred in the BC3H-1 myocytes treated with both SNP and Hoechst 33342 (figure 4D), suggesting synergistic action. These data demonstrate how techniques to evaluate apoptosis using cells, such as the patient’s leukemic cells, in tissue culture may be used to evaluate effective combinations of chemotherapeutic agents to treat specific malignant processes.

Evaluation of mtDNA Disorders

Although mtDNA disorders have a low prevalence among specific populations, there are an increasing number of disease-associated mutations reported and specific pitfalls which may lead to an underestimation of mtDNA disease. Most human cells contain between two to ten copies of mtDNA per mitochondrion. The genes encoded in mtDNA include two ribosomal ribonucleic acids (rRNA), 22 transfer ribonucleic acids (tRNA) and 13 polypeptides utilized to
assemble proteins required for oxidative phosphorylation. Other proteins required for mitochondrial assembly and mtDNA replication are encoded in nDNA, are synthesized in the cytosol and imported into the mitochondria by a specific process. Mitochondrial disorders may result from mutations in mtDNA, nDNA or both. The first mtDNA disorder was described at the molecular level by Wallace, et al. in 1988. They reported a specific point mutation in the mtDNA gene for nicotinamide adenine dinucleotide (reduced form) dehydrogenase which is associated with Leber’s hereditary optic neuropathy. Since that time, numerous other mtDNA mutations have been described which are associated with specific diseases. These mutations include base substitutions in protein subunit genes, base substitutions in tRNA or rRNA, deletions in mtDNA, duplications of mtDNA or depletion of mtDNA (table II). These mutations may lead to altered mitochondrial function, including impaired respiratory function and decreased mitochondrial membrane potential.

There are several potential pitfalls to avoid during the molecular biologic evaluation of a patient with a mtDNA disorder: polymorphisms of mtDNA, primer selection for amplification of mtDNA genes and the importance of heteroplasmy (normal and abnormal mtDNA in the same mitochondria or tissue) on the selection of the specimen to be analyzed and on the molecular biologic method to be used.

**Polymorphism of mtDNA**

At least 130 normal silent polymorphic variations have been discovered in human mtDNA using restriction enzyme analysis, DNA sequence analysis, denaturing gradient gel electrophoresis and polymerase chain reaction combined with single-strand conformation analysis. These polymorphic variants may result in amino acid substitution in protein which does not affect the protein’s function. The presence of these sequence polymorphisms does increase the complexity of establishing an association between mtDNA point mutations and disease. The most variable region of the mtDNA is the 1,122 base pair noncoding control region which contains two hypervariable regions. The development of an integrated and comprehensive human mtDNA database is funded by the Eu Biotech Program and is called MitBASE. Polymorphisms are stored in the sequence variant section of the database which can be found on the internet at http://www.ebi.ac.uk/htbin/Mitbase/mitbase.pl.

**Amplification of mtDNA**

If the frequency of a mtDNA mutation is very low (0.001 to 0.1% of total mtDNA), the mtDNA gene with the mutated sequence may need to be amplified to simplify detection. For example, polymerase chain reaction amplification is required to detect mtDNA deletions which are reported to increase with age. However, Southern blot analysis is adequate for detection of the higher frequency of mtDNA deletions (20 to 50% of total mtDNA) observed in Pearson syndrome and in muscle but not other tissues in Kearns-Sayre syndrome.

Usually, mtDNA regions are amplified in preparations of total cellular DNA containing both nDNA and mtDNA. During evolution, mtDNA fragments have been inserted into nDNA. This process has been so extensive that nDNA sequences homologous to the majority of the human mtDNA genome have been reported. Mitochondrial sequences or pseudogenes may be present in nDNA as single or multiple copies. In three cases of MELAS (A3243G mutation), the variations between the respective level of normal and mutated mtDNA species due to the co-amplification of nuclear-embedded mtDNA sequences was never greater than 10 percent. Therefore, nuclear-encoded mitochondrial pseudogenes did not make a major contribution in the determination of the level of heteroplasmy in these patients. Primers designed to amplify nDNA may generate mtDNA or synthesize single-stranded mtDNA.
by mismatch priming. Primers designed to amplify mtDNA may generate the pseudogene and adjacent nDNA sequences. These problems should be suspected if polymerase chain reaction amplification produces more than one band or different bands, nucleotide sequence is unusual and/or unexpected deletions/insertions or frameshifts are observed. Therefore, primer selection is critical when using crude total cellular DNA to avoid binding of nDNA primers to mtDNA or binding of mtDNA primers to nDNA. Lists of primer sequences not found in mtDNA for nDNA primer construction and frequent sequences in mtDNA for mtDNA primer assembly have been reported.

**Heteroplasmcy**

Normal and mutated mtDNA are inherited maternally. Diseases of mitochondrial dysfunc-
tion with Mendelian inheritance pattern suggest a mutation in a nDNA gene required for mitochondrial function. All children receive the maternal mtDNA but only daughters pass it on to their progeny.

If a tissue possesses only normal or abnormal mtDNA, it is said to be homoplastic for one or the other type of mtDNA. There are very few examples where a homoplastic distribution of abnormal mtDNA has been reported in mtDNA diseases. More frequently, there is a mixture of normal and abnormal mtDNA per mitochondrion and/or per tissue studied. This condition is called heteroplasm. The frequency of abnormal mtDNA in a specific tissue dictates the method selected for detecting the mutation (see amplification above) and which specimens should be selected for investigation.

This situation is illustrated by a mtDNA disorder called mtDNA depletion, which is characterized by very low levels of normal mtDNA in affected tissues. This disorder is usually detected within the first two years of life and may occur with an autosomal recessive inheritance pattern or sporadically in two clinical forms. The first clinical presentation is a fatal congenital form or early-onset form with liver failure. In these patients, the liver has the greatest reduction in mtDNA as measured as a ratio to a nDNA gene. The liver may contain 7 percent mtDNA compared to
control tissue, the muscle 50 percent and kidney, brain and heart have normal mtDNA content. The second late-onset form presents with weakness, hypotonia or developmental delay in the first two years of life. Muscle biopsies of these patients yield 2 to 34 percent of mtDNA content compared to controls. Other tissues possess a minor reduction or normal number of mtDNA copies. Therefore, it is important to investigate enzyme deficiencies and mtDNA depletion in the appropriate tissue. A reduction in the nuclear encoded gene product, transcription factor A, required for mtDNA replication (table III) has been reported as a potential explanation for the reduction in DNA observed in these patients. However, a causal explanation is still lacking and this finding may be a secondary phenomenon.

A reversible reduction in mtDNA in skeletal muscle has been reported after long-term Zidovudine (Azidothymidine) therapy. Azidothymidine triphosphate inhibits mtDNA polymerase gamma, thus reducing the mtDNA content in skeletal muscle. (table III)

**Summary**

Care should be exercised in selection of primers used for amplifying mtDNA genes to reduce the possibility of making multiple copies of a nDNA encoded pseudogene with mtDNA sequence homology. Also, the degree of heteroplasmacy among tissues must be considered prior to selecting specimens for investigation of mtDNA mutations. Therapy for mtDNA disorders is primarily supportive, however, gene therapy using transfected mitochondria may provide hope for the future.

**Diabetes Mellitus: Mutations in mtDNA and/or nDNA**

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. Mutations have been reported attributing to diabetes mellitus at all three major sites of insulin action (table IV). Errors may occur prior to insulin binding to its membrane bound receptor (prereceptor defects), at the structure or function of the insulin receptor (receptor defects) or at the transmembrane signaling mechanism initiated after insulin bind to its receptor (postreceptor defects). Table IV demonstrates that the etiology of diabetes mellitus is very heterogeneous. A brief description of mutations noted in table IV will follow and illustrates the power of diagnostic molecular pathology in defining the specific genotypic cause of a common phenotype.

**Genetic Defects Altering B-cell Function**

Insulin secretion from the beta cell involves several steps. First, glucose travels into the beta cell using a specific glucose transporter (GLUT 2) in the plasma membrane. Glucose is then phosphorylated to glucose-6-phosphate by glucokinase. This reaction is followed by a complex sequence of biochemical reactions which results in increased intracellular ATP produced by mitochondria. ATP-dependent potassium channels are closed and voltage-activated calcium channels open to increase intracellular calcium which triggers the secretion of insulin.
TABLE II
Examples of Mitochondrial DNA Disorders

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>Associated Disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depletion</td>
<td>&gt; Fatal congenital form with liver failure, autosomal recessive or sporadic.</td>
</tr>
<tr>
<td></td>
<td>&gt; Infantile-onset myopathy, autosomal recessive or sporadic.</td>
</tr>
<tr>
<td></td>
<td>&gt; Zidovudine (Azidothymidine) therapy.</td>
</tr>
<tr>
<td>Deletion (with or without duplication)</td>
<td>&gt; Pearson syndrome.</td>
</tr>
<tr>
<td></td>
<td>&gt; Kearns–Sayre syndrome.</td>
</tr>
<tr>
<td></td>
<td>&gt; Noninsulin–dependent diabetes mellitus with deafness.</td>
</tr>
<tr>
<td></td>
<td>&gt; Wolfram syndrome.</td>
</tr>
<tr>
<td></td>
<td>&gt; Hypokalemic periodic paralysis.</td>
</tr>
<tr>
<td></td>
<td>&gt; Inherited idiopathic dilated cardiomyopathy.</td>
</tr>
<tr>
<td></td>
<td>&gt; Aging</td>
</tr>
<tr>
<td>Base substitute in protein subunit genes</td>
<td>Mitochondrial ATPase 6:</td>
</tr>
<tr>
<td></td>
<td>&gt; Mild retinitis pigmentosa and migranes.</td>
</tr>
<tr>
<td></td>
<td>&gt; Neurogenic muscle weakness, ataxia and retinitis pigmentosa.</td>
</tr>
<tr>
<td></td>
<td>&gt; Leigh disease.</td>
</tr>
<tr>
<td></td>
<td>Variable mitochondrial proteins:</td>
</tr>
<tr>
<td></td>
<td>&gt; Leber's hereditary optic neuropathy.</td>
</tr>
<tr>
<td>Base substitution in mt tRNA</td>
<td>tRNA^Leu(UUR) &gt; MELAS/PEO/diabetes with hearing loss.</td>
</tr>
<tr>
<td></td>
<td>&gt; MELAS/multisystem disease.</td>
</tr>
<tr>
<td></td>
<td>&gt; MERRF/PEO/diabetes.</td>
</tr>
<tr>
<td></td>
<td>&gt; MELAS.</td>
</tr>
<tr>
<td></td>
<td>&gt; Cardiomyopathy.</td>
</tr>
<tr>
<td></td>
<td>&gt; Cardiomyopathy/myopathy.</td>
</tr>
<tr>
<td></td>
<td>tRNA^Acn: &gt; Encephalocardiomyopathy.</td>
</tr>
<tr>
<td></td>
<td>&gt; Cardiomyopathy.</td>
</tr>
<tr>
<td></td>
<td>&gt; PEO.</td>
</tr>
<tr>
<td></td>
<td>tRNA^Lys: &gt; Myopathy.</td>
</tr>
<tr>
<td></td>
<td>tRNA^Pro: &gt; MERRF.</td>
</tr>
<tr>
<td></td>
<td>&gt; MERRF/MELAS.</td>
</tr>
<tr>
<td></td>
<td>tRNA^Gly: &gt; Chronic intestinal pseudo-obstruction with myopathy and ophthalmoplegia.</td>
</tr>
<tr>
<td></td>
<td>tRNA^Pro: &gt; Prominal myopathy.</td>
</tr>
<tr>
<td>Base substitution in mt rRNA</td>
<td>&gt; Maternally inherited aminoglycoside-induced deafness.</td>
</tr>
<tr>
<td></td>
<td>&gt; Familial deafness.</td>
</tr>
</tbody>
</table>

MT = Mitochondrial.  
tRNA = Transfer ribonucleic acid.  
rRNA = Ribosomal ribonucleic acid.  
ATPase = Adenosine triphosphatase.  
MELAS = Mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes.  
MERRF = Myoclonic epilepsy and ragged red fiber disease.  
PEO = Progressive external ophthalmoplegia.
TABLE III
Mitochondrial DNA Depletion – Mechanism

tmtDNA requires:
1. DNA polymerase gamma (inhibited by AZT)
2. Short RNA primer from transcription of one mtDNA strand which requires:
   >RNA polymerase
   >mt transcription factor A (decreased in autosomal recessive forms)

mt = mitochondrial.
AZT = Azidothymidine.
RNA = ribonucleic acid.

Autosomal recessive hyperinsulinemic hypoglycemia of infancy is associated with a mutation in β-cell ATP-sensitive K+ channel which is inactive. Therefore, the β-cell membranes are always depolarized, leaving the voltage-dependent Ca2+ channels open, which results in a continuous release of insulin.

GLUCOKINASE (MODY2)

Glucokinase catalyzes the phosphorylation of glucose and represents the rate limiting step in glycolysis. The glucokinase gene is expressed only in insulin-secreting pancreatic β-cells and in hepatocytes. The enzyme regulates insulin secretion in β-cells by sensing the concentration of glucose and regulates hepatic glucose disposal by a similar mechanism.

The glucokinase gene consists of 12 exons. Mutations have been detected using genomic DNA from peripheral white blood cells and PCR primers chosen to amplify each of the 12 exons of the glucokinase gene and adjacent flanking sequences, like exon/intron junctions or single-strand conformational polymorphism analysis with sequencing of bands with abnormal mobility. Forty-two glucokinase mutations have been detected. The majority are missence mutations which lead to decreased glucokinase activity. Diabetic patients with these mutations have one normal and one abnormal gene. The severity of their disease may be related to the degree of dysfunction exhibited by the mutant glucokinase.

Mutations in the glucokinase gene may represent the most common cause of type 2 diabetes identified to date. Mutations have been identified in a variety of racial and ethnic groups. In France, 57 percent (20/35) families studied with maturity-onset diabetes of the young type 2 have a mutation in the glucokine gene. By extrapolation, a mutation in this gene may be present in 6 percent of patients with type 2 diabetes in France.

GLUT 2

The glucose transporter (GLUT 2) has been implicated, along with glucokinase, in glucose regulation of insulin secretion in the pancreatic β-cell. Mutations in the GLUT 2 gene leading to a decrease in glucose transport activity contribute to the development of gestational diabetes mellitus in a patient with a valine 197 to isoleucine mutation. Three different homozygous GLUT 2 mutations have been reported in patients with the Fanconi-Bickel syndrome. In each case, the mutated GLUT 2 molecule is predicted to lack function as a glucose transporter. The Fanconi-Bickel syndrome is characterized by hepatorenal glycogen accumulation, Fanconi nephropathy and impaired utilization of glucose and galactose.

MODY1, MODY3 AND MODY4

The activation of transcription of a variety of genes known to be important in glucose metabolism in liver, pancreatic islets and other tissues are dysfunctional in patients with MODY1, MODY3 and MODY4. MODY1 is associated with mutations in hepatic nuclear factor-4α located on chromosome 20q. It is known to activate transcription of the hepatic nuclear factor-1α (mutated in MODY3). Only 30 percent of patients with MODY1 became insulin-requiring, which may lead to the development of associated microvascular complications.

MODY3 is associated with mutations in at least three functional domains of the hepatic nuclear factor-1α gene located on chromo-
TABLE IV
Some Mutations Associated with Diabetes Mellitus

<table>
<thead>
<tr>
<th>Prereceptor Defects:</th>
<th>Glucokinase (MODY2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic defects altering β-cell function</td>
<td>Glucose transporter 2 (Fanconi–Beckel syndrome)</td>
</tr>
<tr>
<td>Hepatic nuclear factor–4α (MODY1)</td>
<td></td>
</tr>
<tr>
<td>Hepatic nuclear factor–1α (MODY3)</td>
<td></td>
</tr>
<tr>
<td>Insulin promoter factor–1 (MODY4)</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial DNA (maternally inherited diabetes mellitus)</td>
<td></td>
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<table>
<thead>
<tr>
<th>Receptor Defects</th>
<th>Insulin receptor mutations</th>
</tr>
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<table>
<thead>
<tr>
<th>Postreceptor Defects</th>
<th>Insulin receptor substrate–1 mutations</th>
</tr>
</thead>
</table>

MODY = Maturity-onset diabetes of the young.

This transcription factor directly regulates the expression of glucose metabolism genes, like the insulin gene, the glucokinase gene, the pyruvate kinase gene, and others. These patients exhibit a severe form of diabetes requiring insulin and is associated with microvascular complications.

The transcription factor called insulin promoter factor-1 (IPF-1) is required for pancreatic development and insulin gene transcription in response to glucose. Homozygous inheritance of IPF-1 mutation leads to pancreatic agenesis and heterozygous carriers of the mutant IPF-1 allele develop autosomal dominant early onset type 2 diabetes mellitus (MODY4). The mutant IPF-1 also inhibits the transcription of insulin and other β-cell specific genes in these heterozygotes.

MITOCHONDRIAL DNA

The inhibition of mitochondrial oxidative phosphorylation in pancreatic beta cells impairs insulin secretion. A mutation in a mtDNA gene for specific tRNA species may lead to abnormal truncated protein or kinetically dysfunctional secondary to modification of the enzyme’s tertiary structure. The oxidative phosphorylation sequence requires some proteins produced in the mitochondria from the transcription and translation of mtDNA.

This process would be dysfunctional, resulting in lower levels of mitochondrial ATP production and, therefore, less insulin secretion.

Maternally inherited type 1 and type 2 diabetes mellitus and deafness have been associated with mutations with tRNA^{Leu(UUR)}; base pair 3243 (A → G) or 3264 (T → C) and in tRNA^{Lys}; base pair 8296 (A → G). Diabetics with a tRNA^{Leu(UUR)} mutation are estimated to be approximately 1.5 percent of total patients and tRNA^{Lys} mutation at 1.0 percent.

STRUCTURALLY ABNORMAL INSULIN OR PROINSULIN

Defects in the insulin gene have been reported to be inherited as an autosomal dominant phenotype with patients presenting with either hyperproinsulinemia or hyperinsulinemia. The patients present with characteristic clinical and laboratory findings are outlined in table V.

Insulin was the first protein to be purified, crystallized and sequenced. The insulin gene located on the short arm of chromosome 11, was the first gene to be cloned, and human insulin was the first pharmacologic product manufactured with the aid of recombinant-DNA technology.

The insulin gene is found in all nucleated cells but only expressed in beta cells of the
TABLE V
Abnormal Insulin Syndrome

<table>
<thead>
<tr>
<th>Autosomal dominant phenotype.</th>
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</thead>
<tbody>
<tr>
<td>Hyperinsulinemia or hyperproinsulinemia.</td>
</tr>
<tr>
<td>Hyperglycemia/diabetes in some cases.</td>
</tr>
<tr>
<td>Abnormal and normal insulin by HPLC.</td>
</tr>
<tr>
<td>Increased insulin:C-peptide ratio.</td>
</tr>
<tr>
<td>Increased abnormal insulin half-life.</td>
</tr>
<tr>
<td>No increase in insulin–antagonists.</td>
</tr>
<tr>
<td>No antibodies against insulin or insulin receptor.</td>
</tr>
<tr>
<td>Normal response to exogenous insulin.</td>
</tr>
<tr>
<td>Decreased response to endogenous insulin.</td>
</tr>
</tbody>
</table>

Islets of Langerhans in the pancreas. The insulin gene is composed of three exons and two introns. A single polypeptide precursor, preproinsulin, is translated from the mRNA transcribed from the insulin gene. Preproinsulin is converted to proinsulin which contains 86 amino acids. Following the loss of four amino acids, equimolar amounts of C-peptide and insulin (20 amino acid A-chain; 31 amino acid B-chain connected by two disulfide bridges) are produced. Substrate recognition by the protease or convertase that changes proinsulin to insulin appears to be complex.

Defects in the insulin gene have been reported to be inherited as an autosomal dominant phenotype, with patients presenting with either hyperproinsulinemia or hyperinsulinemia. Three specific gene mutations result in reduced biological activity for the mutant insulin (Insulin Chicago, PheB25Leu; Insulin Los Angeles, PheB24Ser; Insulin Wakayama ValA3Leu). The patients present with abnormal insulin syndrome characteristics. The B24, B25 and A3 amino acids represent contact sites between insulin and its receptor. Hyperproinsulinemia may be the consequence of a mutation in the connecting sequence between the C-peptide and A-peptide (ArgC65His and ArgC65Leu) or in the B-chain (HisB10Asp), resulting in a proinsulin species that is not completely converted to insulin. Proinsulin or its intermediates account for more than 80 percent of circulating “insulin” immunoreactivity (normal <20 percent). The glucose tolerance of affected subjects deteriorates with age.

Mutations in the insulin gene were initially detected in genomic DNA extracted from leukocytes by Southern blot analysis using the $^{32}$P-labeled insulin gene. Screening for structurally abnormal insulin or proinsulin using restriction endonucleases demonstrated a frequency of mutant insulin (B24 or B25) of 4.69 per 1,000 patients with NIDDM.

HUMAN INSULIN RECEPTOR MUTATIONS

The human insulin receptor (HIR) is a transmembrane glycoprotein composed of two $\alpha$ subunits of 135 kilodaltons (kD) each and two $\beta$ subunits of 95 kD each held together by inter- and intrasubunit disulfide bonds. There are approximately 100 to 250,000 cell surface receptors per mammalian cell. Insulin-responsive tissues, like adipose, muscle and liver cells, contain a higher number of receptors. Two insulin receptor variants (HIR-A, HIR-B) have been described that differ in the C-terminal end of the $\alpha$ subunit. HIR-A lacks 12 amino acids at position 716 in the COOH-terminus of the extracellular $\alpha$ subunit. The HIR-B variant is increased in the skeletal muscle from patients with non-insulin dependent diabetes mellitus compared with control. Insulin binding and endocytotic rates differ for the two HIR variants. It is not known whether the two variants transmit different intracellular signals after insulin binding.

At least 50 HIR mutations have been identified, including point mutations and deletions, leading to a variety of consequences (table VI). For example, one receptor variant contains a silent mutation at amino acid 1046 and a point mutation (TGG → TCG) at position 1188 converting tryptophan to serine. This latter mutation occurs within the tyrosine kinase domain and may explain the impaired beta subunit activity observed in this patient with severe insulin resistance, acanthosis nigricans and polycystic ovary syndrome. NIDDM
is associated with a missense mutation (Thr831 → Ala) in the HIR of three of 51 NIDDM Japanese patients screened. Patients with HIR mutations seldom present with common form of NIDDM but rather with syndromes of severe insulin resistance like Type A insulin resistance, leprechunism or Robson-Mendenhall syndrome.

IRS-1 AND TYPE 2 DIABETES MELLITUS

Insulin binds to the extracellular α-subunit of the HIR and activates an intrinsic tyrosine kinase within the intracellular β-subunit of HIR. This enzyme autophosphorylates specific tyrosine residues within the β-subunit. Insulin receptor substrate-1 (IRS-1) and IRS-2 are peptide substrates phosphorylated by the activated insulin receptor tyrosine kinase. Both IRS-1 and IRS-2 undergo phosphorylation at multiple tyrosine residues. The phosphorylated IRS-1/2 then binds with proteins that contain a src-homology-2 (SH2) domain. IRS-1/2 act as adaptor or docking molecules which link HIR to various cellular reactions regulated by insulin. For example, IRS-1/2 bind to the p85 subunit of phosphatidylinositol 3'-kinase and activate it, resulting in insulin-stimulated GLUT4 translocation. IRS-2 is dephosphorylated much more rapidly and activates PI 3'-kinase more transiently than IRS-1 in skeletal muscle cells. Other signal transduction pathways result in the activation of serine/threonine kinases.

Efforts to identify IRS-1 mutants in patients with severe insulin resistance have uncovered two mutants that demonstrate dysfunctional signal transduction, a postreceptor defect. One mutant IRS-1 failed to bind normally to PI 3'-kinase p85 and the second demonstrated a decreased mitogenic response secondary to reduced binding to the HIR. Additional studies to uncover new IRS-1 mutations continue.

SUMMARY

Dissecting insulin action into potential defects at the prereceptor, receptor or post-receptor level will demonstrate subsets of patients with heterogeneous pathogenetic pathways and potential new therapeutic methods. Diagnostic molecular pathology laboratory support will be required for the identification of mutations in specific genes associated with diabetes mellitus.

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References


83. Alonso A, Martin P, Albarran C, Garcia O, Sancho M. Rapid detection of sequence polymorphisms in the human mitochondrial DNA control region by polymerase chain reaction and single-strand confor-
84. Attimonelli M, Culo D, Cooper JM, et al. The mito-
26:116–119.
85. Fromenty B, Manfredi G, Sadlock J, Zhang L, King
MP, Schon EA. Efficient and specific amplification of
identified partial duplications of human mitochon-
drial DNA by long PCR. Biochim Biophys Acta 1996;
1308:222–230.
86. Hu G, Thilly WG. Multi-copy nuclear pseudogenes
of mitochondrial DNA reveal recent acute genetic
changes in the human genome. Curr Genet 1995;28:
410–414.
87. Avctander P. Comparison of a mitochondrial gene
and a corresponding nuclear pseudogene. Proc R Soc
88. Zhang D-X, Hewitt GM. Nuclear integrations: chal-
enges for mitochondrial DNA markers. Trends Ecol
89. Parfait B, Rustin P, Munnich A, Rötig A. Coampli-
fication of nuclear pseudogenes and assessment of
heteroplasy of mitochondrial DNA mutations. Bio-
AK, George M. Isolation and characterization of ani-
mal mitochondrial DNA. Biochim Biophys Acta 1993;
Identification of 14 new glucokinase mutations and des-
cription of the clinical profile of 42 MODY-2 families. Di-
Halban PA, Polonsky KS. Insulin production: from gene to granule. Dia-
93. Bakker HD, Schölte HR, Dingemans KP, Spelbrink
JN, Wijburg FA, Vanden Bogert C. Depletion of
mtDNA depletion. Biochem Biophys Res
the hepatocyte nuclear factor-4a gene. Arch Biochem Biophys
in the Glut 2 glucose transporter gene of a diabetic
patient abolishes transport activity. J Biol Chem
mtDNA and a corresponding nuclear pseudogene. Proc R
97. Lewis W, Simpson JF, Meyer RR. Cardiac mitochon-
drial polymerase-γ is inhibited competitively and
noncompetitively by phosphorylated Zidovudine.
98. Fromenty B, Manfredi G, Sadlock J, Zhang L, King
MP, Schon EA. Efficient and specific amplification of
identified partial duplications of human mitochon-
drial DNA by long PCR. Biochim Biophys Acta 1996;
1308:222–230.
99. Kiechle FL, Malinski T, Moore KH. Insulin action:
imPLICATIONS for the clinical laboratory. Lab Med
100. Gerbitz K-D, van den Ouweland JW, Maassen JA,
Jaksch M. Mitochondrial diabetes mellitus: a review.
101. Report of the expert committee on the diagnosis
and classification of diabetes mellitus. Diabetes Care
102. Matschinsky FM, Glaser B, Magnuson MA. Pancre-
atic β-cell glucokinase. Closing the gap between theo-
retical concepts and experimental realities. Dia-
of 14 new glucokinase mutations and description of
the clinical profile of 42 MODY-2 families. Diab-
in the Glut 2 glucose transporter gene of a diabetic
patient abolishes transport activity. J Biol Chem
H, Steinman B, Schauba J. Mutations in GLUT2, the
gene for the liver-type glucose transporter, in patients
with Fanconi-Bickel syndrome. Nature Genet
1997;17:324–328.
H, Steinman B, Schauba J. Fanconi-Bickel syn-
drome—a congenital defect of the liver-type facilita-
tive glucose transporter. J Inher Metab Dis 1998;21:
191–194.
mutations and a mutational hotspot in the MODY 3
complications in NIDDM kindreds linked to the
MODY 3 locus on chromosome 12q. Diabetes Care
mutation in hepatocyte nuclear factor-4α, resulting in a
reduced transactivation activity, in human late-onset
non-insulin-dependent diabetes mellitus. J Clin Invest
mutations and a mutational hotspot in the MODY 3
complications in NIDDM kindreds linked to the
MODY 3 locus on chromosome 12q. Diabetes Care
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