Feature Story

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The role of cardiovascular pathology in the diagnosis and management of cardiomyopathy is continuously evolving, just as the concept of cardiomyopathies or heart muscle diseases continues to change when new disease entities are recognized.

Advances in the investigation of the molecular basis of heart failure have revealed several mutations in genes encoding protein components of the sarcomere, cytoskeleton, cell junction and ion channels. Consequently, it is recognized that the traditional classification of cardiomyopathies into dilated, hypertrophic and restrictive type has its limitations. Patients harboring mutations in the same gene can present with a hypertrophic or dilated phenotype. In other cases, a patient can progress from an initial hypertrophic phenotype to a dilated phenotype. Recently, the American Heart Association issued a scientific statement with a proposal for a revised classification of cardiomyopathies (1). In this classification, the primary cardiomyopathies are divided into genetic, mixed and acquired. This proposed classification relies heavily on the molecular genetics of cardiomyopathy.

Glycogen Storage Disease Affects Adults, Too

Evaluation of endomyocardial biopsies and explanted hearts in heart failure patients oftentimes reveals myocyte hypertrophy and degeneration and interstitial fibrosis. In some cases however, the myocytes are enlarged with a vacuolated appearance. The cytoplasm may appear granular. Actual inclusions may be seen in some. Ultrastructural examination may reveal displacement of myofibrils either by accumulation of glycogen, intralysosomal deposits or proliferation of mitochondria; myofibrillar loss also occurs in varying degrees.

Mutations in two genes resulting in a late-onset glycogen storage disease predominantly involving the heart and mimicking hypertrophic cardiomyopathy have been described. The first gene encodes the γ regulatory subunit of the adenosine monophosphate-activated protein kinase (PRKAG2). AMP-activated protein kinase is involved in the regulation of glucose uptake and glycolysis in muscle. Clinically, affected patients present with a triad of ventricular pre-excitation (Wolff-Parkinson-White), progressive conduction system disease and cardiac hypertrophy (2,3). Most of these patients by the fourth decade of life present with a hypertrophic type of cardiomyopathy. This observed phenotype morphologically has been shown to be caused by accumulation of glycogen in the heart.

The second gene encodes the lysosome-associated membrane protein 2 (LAMP2). Mutations in LAMP2 result in a deficiency of the protein and clinically is recognized as Danon disease with a triad of cardiomyopathy, skeletal myopathy and mental retardation (4). It is an X-linked dominant lysosomal disease that typically affects young male patients. Cardiomyopathy is severe with onset of heart failure before age 20. Most patients do not survive beyond the third decade of life. Skeletal myopathy is mild with proximal limb and neck muscle weakness. Mental retardation is also mild. Affected females present later in adulthood with mean age at onset of 38 years.

Between January 2005 and June 2006, a series of seven cardiac transplant cases with light microscopic findings suggestive of metabolic disorder were studied to screen for mutations in the PRKAG2 and LAMP2 genes. An eighth case was identified retrospectively because she was the mother of one of the patients in the series and she also underwent cardiac transplantation. The PRKAG2 mRNA (GenBank accession number NM_016203) was blasted against the DNA sequence of chromosome 7 (AC093583, AC006358 and AC006966) to locate the exons and to design primers for amplification. Likewise, LAMP2 mRNA (GenBank accession number NM_002294) was blasted against the genomic sequence of chromosome Xq23 (AC002476). Genomic DNA was extracted from frozen tissue of the explanted hearts. Entire coding regions including 16 exons of the PRKAG2 and nine exons of LAMP2 genes were sequenced and both strands of the PCR-
amplified fragments were directly sequenced using ABI Prism 3100 Genetic Analyzer. (Applied Biosystems, Foster City, Calif.)

Three of the eight patients were found to have mutations in the coding regions of either of these two genes. The first patient was a 12-year-old male who presented with dilated cardiomyopathy. Skeletal myopathy with proximal weakness was subtle and there was no mental retardation. His family history revealed that his mother had undergone cardiac transplantation seven years earlier at age 32. She also presented with dilated cardiomyopathy, the explanted heart showed severe myocyte vacuolization and fibrosis, but no further work-up was done at that time. DNA sequencing revealed a G274A mutation in the LAMP2 gene resulting in a premature stop codon at Trp46X in the mother and son. (Figure 1)

A third patient was a 48-year-old woman who had history of WPW, atrial fibrillation and progressive biventricular heart failure over 6 years of follow-up. No systemic involvement was evident. Explanted heart showed marked myocyte hypertrophy with vacuolated cytoplasm and severe fibrosis. A C1810A missense mutation in the PRKAG2 gene leading to a Thr400Asn substitution was identified.

Mitochondrial Proliferation as a Manifestation of Cardiomyopathy

Cardiomyopathies can be secondary to a number of alterations in mitochondrial function. One needs to remember that mitochondrial function depends on the interaction of two genomes. The mendelian genome in our chromosomes and the mitochondrial genome encoded by DNA present only in the mitochondria. Mitochondrial cardiomyopathies can result from mutations in the mitochondrial DNA (mtDNA), which encodes two rRNAs, 22 tRNAs, and 13 polypeptides. Specific diseases can result from mutations in the mtDNA. Defects in the regulation of division of mtDNA also can produce disease. The regulatory elements are present in a large segment of the mtDNA called the control region or D-loop. (Figure 2) Other mechanisms that affect mitochondrial function are due to genetic alterations of proteins associated with ATP electron transport chain enzyme. Metabolic defects that affect fatty acid oxidation such as acyl-CoA deficiencies and alterations in pathways that affect carnitine metabolism also produce dysfunction of the mitochondria.

Figure 1. Light micrograph of myocardium showing basophilic cytoplasmic inclusions that are stained positive with PAS indicative of abnormal glycogen storage. Direct sequencing of genomic DNA in a dilated cardiomyopathy female patient shows a heterozygous mutation in base pair 294 of mRNA (exon 2) of the LAMP2 gene in both the forward and reverse sequence.

Figure 2. The mitochondrial DNA is composed of 16,569 bases in a closed circular molecule that codes for two ribosomal RNAs, 22 transfer RNAs used in mitochondrial protein synthesis and 13 polypeptides of the oxidative phosphorylation complexes. Mutations in mtDNA can produce disease by altering individual polypeptides, the tRNAs, the rRNAs or the control region (D-loop).

The challenge for cardiovascular pathology is that the morphologic alterations seen in endomyocardial biopsies and explanted hearts are limited and can range from very subtle to marked. Moreover, they are not very specific to point out the source of the alteration. In fact, in some instances there may be no morphologic

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alteration recognizable in the specimen. Of the alterations noted, the most common is the increase in number of mitochondria in the cardiac myocyte and alterations in the size and morphology of these mitochondria.

To address mitochondrial diseases produced by alterations in the mtDNA, we have developed a molecular test that allows direct sequencing of the mtDNA. Since there are many polymorphisms in the human mtDNA, it is important that the actual sequence of the mtDNA be ascertained to evaluate possible pathologic mutations that correlate with the phenotype. This requires a thorough approach that allows determination of the sequence of both strands of the mtDNA.

Our assay divides the 16 kilobases of the mtDNA into 43 fragments that are individually amplified and then sequenced. The image in Figure 3 is an example of a heart with mitochondrial proliferation with a pathologic mutation in one of the two tRNAs that transfer the amino acid leucine (Figure 4) to the nascent polypeptide chains within the mitochondria during protein synthesis. This particular mutation is well known to produce the syndrome of mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (5).

With this approach, one can study the incidence and prevalence of mtDNA mutations in human cardiomyopathy. Furthermore, the complexity of interactions of the mtDNA with other cellular functions in the human body leads to applications of these assays in the fields of neurodegenerative diseases, endocrinology and cancer (6).

Molecular Diagnostics of Cardiomyopathy at Cleveland Clinic
A diagnostic strategy coupled with increased awareness of these diseases will improve the identification of patients with familial types of cardiomyopathy. Variable expression of disease is well known in mitochondrial diseases and is increasingly recognized in glycogen storage disease. While most studies report a hypertrophic phenotype for these diseases, our patients at the time of clinical presentation already had a dilated cardiomyopathy. In addition, pediatric cardiomyopathy patients may benefit from clinical muscular testing and serum CK determination, which will be abnormal in patients with Danon disease.

Careful histologic and ultrastructural evaluation of heart muscle tissue often provides a clue to the diagnosis. The presence of cardiomyopathy in combination with skeletal myopathy or conduction system abnormalities should prompt direct molecular screening for heritable genetic disorders involving mutations in mtDNA, LAMP2 and PRKAG2 among others. Further support is needed to continue to increase the repertoire of molecular diagnostic tests that we can offer to our patients at Cleveland Clinic, not only in the field of cardiomyopathies but also in valvular diseases, aortic diseases and heart transplantation.

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When physicians order clinical tests, they presume that the results are precise and accurate. This requirement demands that the clinical chemists use the most accurate methodologies and follow protocols with vigorous quality assurance.

High-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) is a versatile, highly specific and sensitive technology that has broad applications in clinical testing to provide the quality that is impossible or too cumbersome for other current technologies to achieve (1).

Mass spectrometry measures ion abundance at specified mass-to-charge (m/z) ratios. There are currently four basic types of mass spectrometers: time-of-flight (TOF), quadrupole and derivatives, quadrupole ion trap, and Fourier transform ion cyclotron resonance. All the mass spectrometers require a high vacuum system for ions to travel.

The most popularly used technology for small molecule quantification in clinical laboratories is quadrupole tandem mass spectrometry. This type of instrument works on the basis of electric fields generated by voltages applied to four axial rods consisting of direct current and radiofrequency components. To improve specificity, two mass analyzers can be connected in series with a collision cell in between to form a “tandem” mass spectrometer. While ions with specified m/z are selected by the first quadrupole and fragmented in the collision cell by colliding with an inert gas, particular fragments formed may be selected and detected by the second mass analyzer.

### References

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This tandem mass spectrometer has been applied primarily to small molecule (generally < 2,000 Daltons) quantification through interfacing with an HPLC analyzer. HPLC technologies were developed in the late 1970s and early 1980s. While HPLC is a versatile chromatography technique, conventional detectors (UV/Visible, fluorescence, electrochemical, etc) are not extremely specific or sensitive. Therefore, long chromatographic analytical time and cumbersome sample preparation are usually necessary to obtain reliable results.

HPLC and MS/MS can be interfaced with soft ionization technologies, including the Nobel prize-winning electrospray ionization. This combination provides highly specific and sensitive assays with significantly higher throughput and less demand for sample cleanup compared with all other chromatography-based technologies.

The HPLC-MS/MS technology finds a niche in clinical applications that is superior to conventional methods including immunoassays. Especially with the availability of high-throughput Turboflow online extraction technology, HPLC-MS/MS has become cutting-edge in specialty clinical testing and has been utilized by many academic medical centers and leading reference laboratories (Mayo Clinic, ARUP, Quest Nichols Institute, LabCorp Esoterix, etc.) for both clinical specialty testing and clinical research. Application of HPLC-MS/MS in clinical services is increasing. Several of the broad clinical applications will be discussed here.

**Inborn Errors of Metabolism**

Tandem mass spectrometry offers a new vision for newborn screening to detect more than 30 metabolic disorders in a single assay with one small disc of dry blood, compared with classic screening techniques with the philosophy of one analysis, one metabolite (2). Some of the metabolites measured are amino acids, acylcarnitines and organic acids (3). As a followup to the initial screening and monitoring of disease treatment or management, clinicians may order quantitative assays in serum. These screening programs have proven highly cost-effective since severe lifelong disability can be avoided by early initiation of specific diets or treatments.

**Steroids**

Immunoassays are the most commonly used techniques for serum testosterone analysis. However, at lower concentrations (<1.7 nmol/L or <49 ng/dL) immunoassays cannot reliably measure the testosterone level that is typically seen in women and children (4). HPLC-MS/MS offers very sensitive and specific methods for serum testosterone measurements that are suitable for diagnosis of androgen disorders in women and children (5).
transplantation patient care. The current immunoassays not only overestimate the parent drugs by cross-reacting with many metabolites formed by P450 isoenzymes but also produce significantly variable results among different methods. HPLC-MS/MS offers highly specific testing with the capability of measuring cyclosporine A, tacrolimus, sirolimus and everolimus in a single analytical run (8). With a multiplexing online extraction system, the new technology may offer high-throughput testing to meet the needs of the transplantation team with the most reliable results. For mycophenolic acid, the state-of-the-art system will eliminate much sample preparation and produce undisputable testing results. This technology is also readily used for monitoring new drugs (e.g., everolimus) for which no commercial assays are available.

Toxicology
Wide-spectrum drug screening is an obvious application of HPLC-MS/MS. It offers not only simultaneous measurement of numerous drugs but also requires much less tedious sample cleaning. HPLC-MS/MS has been used in detecting drug doping in athletes and drugs of abuse in patients. It is the method of choice for establishing a comprehensive toxicology screening program.

Novel Biomarkers for Cardiovascular and Renal Diseases
Measurement of novel markers for medical research, especially cardiovascular and renal diseases, may be accomplished using HPLC-MS/MS. Asymmetric dimethylarginine (ADMA), an arginine derivative, has been shown in many clinical studies as a biomarker for atherosclerosis, cardiovascular events and renal function deterioration. ADMA is a highly promising risk marker for cardiovascular and renal diseases. HPLC-MS/MS is the most reliable technology for quantification of serum or urine arginine derivatives.

Proteomics
Proteomics is a promising means for biomarker development and/or identification in the diagnosis and followup of cancer, stroke, cardiovascular and renal diseases. HPLC-MS/MS is the most versatile and powerful technology in this area for protein screening and identification. In addition, HPLC-MS/MS may also be used to detect post-translational modification of key proteins. In the next five to 10 years, we may see clinical biomarkers developed using HPLC-MS/MS and implementation of the clinical application using this technology.

With the advance of the technology, HPLC-MS/MS has become more affordable, compact and user-friendly. Front-end automation is also made possible by introducing online solid-phase extraction to reduce laborious sample cleanup and to provide high quality results.

In conclusion, HPLC-MS/MS is a versatile technology that offers high sensitivity and specificity for clinical testing. HPLC-MS/MS has been successfully applied in clinical services for inborn errors of metabolism screening, therapeutic drug monitoring, ultra-sensitive steroid testing, vitamin D metabolites, toxicology, new biomarker development and proteomics. This technology enables clinical chemists to offer clinical testing with superior quality and simplicity compared with the current technologies used in clinical laboratories.

References

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