Mutation detection, interpretation, and applications in the clinical laboratory setting

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Abstract

Mutation detection plays an increasingly significant role in clinical diagnostic testing, posing formidable challenges for laboratories. The expanding indications for clinical molecular testing and the nuances of interpreting test results are discussed. Methods for screening mutation detection platforms and monitoring assay reliability are presented, and results of platform comparisons are described. The potential for irrevocable medical interventions following a positive mutation analysis is highlighted to stress the imperative for accuracy in mutation detection and vigilance in the clinical arena.

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Keywords: Laboratory; Mutation detection; Positive mutation analysis

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1. Introduction

Molecular diagnostics plays an increasingly significant role in routine clinical medicine. Our laboratory currently offers more than 30 different molecular tests, and volume continues to increase at a pace of >20% per year. Some mutations we assay are not technically SNPs, as they do not involve single nucleotide polymorphisms. These mutations include the triplet repeat expansions in Fragile X Syndrome, Huntington disease (HD), and Kennedy disease, large deletions in the alpha globin gene responsible for alpha thalassemia, and testing for the complex rearrangement known as BLMAsh which causes >99% of cases of Bloom Syndrome in Ashkenazi Jews. Therefore, I use the term mutation rather than SNP in this paper. For the purposes of this discussion, I consider only those assays for mutations in the germ line. The detection of somatic mutations, gene amplifications and deletions, and gene expression analysis is beyond the scope of this article.

This article addresses the demands for scrupulous accuracy of detection platforms in the high-throughput clinical laboratory and outlines the benchmark validation system we have developed prior to introducing a test into the clinical repertoire. Our laboratory’s current approach to molecular analyses (Fig. 1) and the medical indications for each form of testing are presented (Table 1).

2. Clinical indications for molecular testing

There are several possible reasons for a physician or genetic professional to order a molecular test. These include pre-natal diagnosis, confirmation of a suspected diagnosis of genetic disease, mutation identification in a patient with a known genetic disease for the purposes of carrier detection and pre-natal diagnosis for family members, pre-disposition testing for cancer, cancer risk assessment, pre-disposition testing for thrombophilia, pre-symptomatic testing in an at-risk patient for a genetic disease, pharmacogenetics, follow-up of abnormal newborn screening results, primary population based carrier screening, and follow-up of positive carrier screens by other testing methods. Table 1 is partial of list of popular molecular tests divided by categories. This list is meant to be representative and not...
Table 1
Listing of popular molecular tests by category

<table>
<thead>
<tr>
<th>Category</th>
<th>Disease</th>
<th>No. of mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmation of suspected diagnosis</td>
<td>Hemochromotosis (HFE)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fragile X Syndrome</td>
<td>Triplet repeat expansion</td>
</tr>
<tr>
<td></td>
<td>Huntington disease</td>
<td>Triplet repeat expansion</td>
</tr>
<tr>
<td></td>
<td>Cystic Fibrosis</td>
<td>25 (initial screen), sequencing</td>
</tr>
<tr>
<td></td>
<td>Myotonic Dystrophy</td>
<td>Triplet repeat expansion</td>
</tr>
<tr>
<td></td>
<td>Prader-Willi Syndrome</td>
<td>Methylation studies</td>
</tr>
<tr>
<td>Mutation identification in known patients</td>
<td>Cystic Fibrosis</td>
<td>25 (initial screen), sequencing</td>
</tr>
<tr>
<td></td>
<td>Duchenne Muscular Dystrophy</td>
<td>Deletion analysis</td>
</tr>
<tr>
<td></td>
<td>Beta thalasemia</td>
<td>Sequecing test</td>
</tr>
<tr>
<td>Cancer pre-disposition testing</td>
<td>Breast cancer</td>
<td>3 (AJ), sequencing</td>
</tr>
<tr>
<td></td>
<td>Hereditary nonpolyposis colon cancer (HNPCC)</td>
<td>Sequencing test and rearrangement analysis</td>
</tr>
<tr>
<td></td>
<td>Multiple endocrine neoplasia (Ret protooncogene)</td>
<td>Sequencing test</td>
</tr>
<tr>
<td>Thrombophilia</td>
<td>Factor V Leiden</td>
<td>1 (with reflex to HR2)</td>
</tr>
<tr>
<td></td>
<td>Factor II prothrombin</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Methylene tetrahydrofolate reductase</td>
<td>2</td>
</tr>
<tr>
<td>Pre-symptomatic Testing</td>
<td>Huntington disease</td>
<td>Triplet repeat expansion</td>
</tr>
<tr>
<td></td>
<td>Myotonic Dystrophy</td>
<td>Triplet repeat expansion</td>
</tr>
<tr>
<td>Follow-up of abnormal newborn screens</td>
<td>Cystic Fibrosis</td>
<td>25 (initial screen) followed by sequencing test</td>
</tr>
<tr>
<td></td>
<td>Congenital hearing loss (connexin 26)</td>
<td>2 (initial screen) followed by sequencing test</td>
</tr>
<tr>
<td>Population based carrier screening</td>
<td>Cystic Fibrosis</td>
<td>25</td>
</tr>
<tr>
<td>Caucasians</td>
<td>Tay Sachs</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Canavan disease</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Gaucher disease</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Bloom Syndrome</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fanconi Anemia Type C</td>
<td>1</td>
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<tr>
<td></td>
<td>Familial Dysautonomia</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Niemann-Pick disease</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>mucopolysaccharides IV</td>
<td>2</td>
</tr>
<tr>
<td>Follow-up of abnormal carrier screen</td>
<td>Beta thalasemia</td>
<td>Sequencing test</td>
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<tr>
<td></td>
<td>Spheroidosaemia</td>
<td>Deletion and rearrangement</td>
</tr>
<tr>
<td>Pharmacogenomics</td>
<td>CYP2D6 (psychotropic)</td>
<td>10–30</td>
</tr>
<tr>
<td></td>
<td>CYP2C9 (Coumadin)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>TPMT (mercaptopurinase)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DPD (5FU)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CYP2C19 (analgetics)</td>
<td>2</td>
</tr>
<tr>
<td>Pre-natal diagnosis</td>
<td>All of the above</td>
<td></td>
</tr>
</tbody>
</table>

all-inclusive. Some illnesses such as Huntington disease appear in more than one category as the indication for Huntington disease testing may be a symptomatic patient without a family history of HD or an asymptomatic patient with a parent or sibling with the diagnosis of HD who requests pre-symptomatic testing.

3. Interpretation of molecular testing

Clearly, the interpretation of genetic testing varies with the test performed, but also varies within the same test according to the indication for testing. For example, a heterozygous result for the delta F508 Cystic
Fibrosis (CF) mutation has different implications for an individual whose indication for testing is population-based carrier screening versus a fetus whose indication for testing is that both parents are carriers of delta F508 versus a fetus whose parents have had a previous child with CF but only one parent’s mutation has been identified. In the first case, the critical result is that the patient is a CF carrier; in the second case, the critical result is that the fetus is unaffected; in the third case, the critical result is that the fetus has a 50% risk of being affected.

A common example of changing interpretations with indication for testing is mutation analysis for the C282Y and H63D mutations in the hemochromatosis (HFE) gene associated with hereditary hemochromatosis (HH). A homozygous patient for C282Y who also has increased ferritin saturations and arthritis is almost certainly affected with HH. However, the same genotype in an asymptomatic patient tested because of a family history of HH indicates that the patient is at <1% risk of developing HH in his or her lifetime[1,2].

Another example of the crucial importance of clinical context is in cancer pre-disposition testing using BrCa1 and BrCa2. There are founder mutations present in nearly 10% of Ashkenazi Jewish women[3,4]. A founder BrCa1 mutation detected in a woman with breast cancer alerts her physician that she is also at risk for ovarian cancer and may alter the choice of chemotherapy. The same result in a young woman without breast or ovarian cancer means she is at a significant risk of developing breast or ovarian cancer. This result may also be an indication for prophylactic mastectomies (surgical removal of both breasts without evidence of cancer) and prophylactic oopherectomies (removal of both ovaries without evidence of ovarian cancer), and is certainly an indication for intensive monitoring [5]. Since the clinical decisions made as the result of genetic testing may be significant and irreversible (i.e., mastectomy), it is imperative that such testing is performed with the highest possible level of accuracy.

Even when clinical molecular testing is performed flawlessly, it is possible that misdiagnoses may occur due to biologic variations in individual patients. For example, when DNA sequencing is performed in the clinical arena, either both strands are sequenced or the same strand is sequenced in different directions. This is done because DNA sequencing has an error rate and this assures that mutations are only reported when found by two sequencing reactions.

In an actual case, one laboratory analyzed a sample submitted for sequencing analysis for the Ret protooncogene and reported a negative result (no mutation found). Mutations in the Ret protooncogene are associated with multiple endocrine neoplasia type 2. Because of the high risk of developing thyroid cancer, a positive result usually results in a prophylactic thyroidectomy (removal of the thyroid without evidence of disease) [6]. The test was repeated in a second laboratory and a mutation was discovered. Review of data from the first laboratory analysis demonstrated that the mutation was seen in only one of the two sequencing reactions. Further sequencing analysis was necessary to discover that this patient had a novel polymorphism in the primer sequence of the reverse sequencing reaction on the mutant allele. Therefore, this false negative result was due to a previously undescribed polymorphism. Fortunately, the clinician had ordered two tests to be performed by separate laboratories because he or she understood that although molecular testing can be extremely accurate, biological variation in terms of polymorphisms might cause false negative results. If the first laboratory had performed both tests, a false negative result would have been reported twice, leading to inappropriate reassurance.

Both false positive and false negative results can have a devastating impact on the lives of patients. A false positive result can lead to an inappropriate removal of an organ (breast, ovary or thyroid). A false negative result can lead to false reassurance and even death if a malignancy occurs.

4. Specific areas of interpretative difficulties

Interpretation of any result of a molecular test is dependent on its clinical context. It is vital that the physicians who order these tests become familiar with the significance of the results in various clinical contexts.

Interpretation can be challenging in sequence-based assays when novel changes or extremely rare mutations are detected. Under these circumstances, it may not be possible to predict the effect of a given mutation on the function of the protein. The biological significance of alterations near splice sites is difficult
to assess. Functional assays must be developed in order to accurately predict the effects of novel mis-
sense mutations, splice site mutations, and in-frame
deletions and insertions.

Pharmacogenomic testing analyzes sequence varia-
tions in genes responsible for drug metabolism to pre-
dict and explain the range of patient responses to drugs
and the variable occurrence of side effects. Some sys-
tems are relatively straightforward. For example, the
enzyme cytochrome p450 2C9 (CYP2C9) metabolizes
the anti-coagulant Warfarin (Coumadin). The wild type
allele is designated *1 and there are two variant alleles
designated *2 and *3 that demonstrate lower levels of
drug metabolism and are designated poor metabolizer
alleles (PM). Patients with 1 PM allele, either *2 or *3,
are more likely to have bleeding complications with
standard doses of Warfarin. Patients with 2 PM alleles
were at even higher risk for adverse events [7].

Interpretation for other pharmacogenetic tests is
more complicated. There are more than 20 described
variants in the CYP2D6 gene. The CYP2D6 partici-
pates in the metabolism of several different neurolep-
tic drugs but many of these drugs are also metabolized
by other enzymes. Most of these alleles are PM alleles
but there is also an allele consisting of a gene duplica-
tion that results in an ultra-rapid metabolizer. Because
there are such large numbers of potential PM alleles
and the metabolism of each individual drug is affected
in varying degrees by CYP2D6, there is an insufficient
amount of data to predict the effect of any single allele
on the metabolism of any particular drug. Interpreta-
tion of a CYP2D6 genotype can only predict the pa-
tient to be an ultra-rapid metabolizer, rapid metabolizer
(RM) (wild type), intermediate metabolizer (heterozy-
gous PM/RM), or poor metabolizer (homozygous PM)
[7,8]. Many clinicians find the concept of an interme-
diate metabolizer elusive and have not learned to apply
this information to select dosages and medications for
their patients. This may be one reason why pharmaco-
genetic tests have not yet become routinely ordered by
clinicians.

5. Demands of a high through-put clinical
molecular laboratory

There are myriad detection systems available for
mutation analysis. Unlike the research setting, clinical
mutation detection requires near perfect performance
in terms of sensitivity and specificity of a mutation de-
tection platform. Since individuals will usually have
only a single test in their entire lives, it is impera-
tive that molecular testing be accurate to at least an
order of magnitude greater than when SNP analysis
is performed for genomic scans. For example, a plat-
form capable of performing mutation analysis with a
99% sensitivity and specificity would be perfectly ap-
propriate for a genome scan, but in a laboratory per-
forming 10,000 genotypes a year, 200 patients would
be genotyped incorrectly and these inaccurate results
could result in morbidity and mortality for the patient
or flawed reproductive choices. Even at 99.9% sensi-
tivity and 99.9% specificity, 20 misdiagnoses would be
made per year. This is why we have established a 1000
sample benchmark for mutation detection platforms,
described below.

Another imperative to increase accuracy in a high
through-put clinical genotyping laboratory is automa-
tion. People performing repetitive tasks are prone to er-
or, so whenever possible automation is implemented.
In our laboratory, bar coding is used to assure speci-
men integrity. Samples are placed into a 96-well microtiter
plate that includes rotating positive controls, negative
controls and a “quality control” blank that allows a re-
viewer to detect if a plate rotation has occurred. The
laboratory information system (LIS) stores the “plate
map” that contains the locations of all patient speci-
mens and controls on the bar coded microtiter plate.
The samples remain in this orientation from before
DNA extraction, through PCR, mutation detection, and
genotype assignment. This avoids potential pipetting
errors. The initial genotype assignment or sequencing
result is performed by computer software and then re-
viewed by laboratory personnel and, when appropriate,
laboratory directors. Direct interfacing of laboratory
instrumentation with the LIS avoids potential errors of
result entry. Error trapping procedures are implemented
to allow directors to determine if any errors have oc-
curred.

The exception to these automated procedures is the
Southern blot analysis performed for Fragile X Syn-
drome. Because of the inability to reliably amplify large
CGG repeats by PCR and the appreciable incidence of
mosaicism, Southern blots must be performed. This
assay still requires loading of gels and manual inter-
pretation and data entry.
6. The 1000 sample benchmark

Because of the potentially devastating impact of an incorrect genotyping result, we have established a benchmark for evaluating new platforms prior to their introduction into the clinical laboratory. Initially, the platform is tested with various genomic DNA samples containing all the mutations purported to be detected by the platform. The samples are anonymized and randomized so the laboratory is blinded to the genotype of the sample. Whenever possible, both homozygous and heterozygous samples are tested. For some rare mutations, homozygous samples may not be available. The importance of using genomic rather than synthetic or cloned controls cannot be stressed enough. Our laboratory has had multiple experiences of an assay appearing to perform well with synthetic or cloned controls that failed to detect the same mutation in a genomic sample [9].

This preliminary screening determines whether the platform is capable of detecting the genotypes required of the assay. Once we have optimized the platform for our conditions (i.e., DNA isolation procedures, DNA concentrations) and established that all requisite mutations can be detected, the platform is subjected to a 1000 consecutive sample benchmark test. Samples submitted for testing are divided into two aliquots. The first aliquot proceeds through our standard testing protocol. The second undergoes testing by the platform under evaluation. After 1000 samples have been completed by both platforms, the results are compared and any discrepancies resolved.

7. Results of benchmark testing

Since the initiation of the benchmark-testing program, we have performed more than 10 benchmark evaluations. Of interest is that several platforms sold as analyte specific reagents (ASR) for particular mutation detection have failed the benchmark test. The definition of ASR has been determined by the Food and Drug Administration as reagents developed for detection of particular analytes and can be sold without formal FDA approval as an in vitro diagnostic kit.

We recognized the need for a rigorous benchmark test due to our initial attempts to discover a platform capable of accurate determination of the C282Y and H63D mutations in the HFE gene associated with hereditary hemochromatosis [2]. In all, six platforms were evaluated including three laboratory developed tests and three tests developed by vendors. In all, three of the five tests failed the benchmark test, including two of the three commercially available platforms. We eventually chose a laboratory developed fluorescent RFLP test for implementation because of cost and automation considerations. The identity of the commercial platforms evaluated in this series cannot be revealed secondary to confidentiality agreements.

We performed two benchmark tests for the Factor V Leiden mutation. The initial test compared a laboratory developed RFLP test with the Promega ReadIT system [10]. No discrepancies were found. Subsequently we began using the ReadIT system as our primary assay method for single mutations. Subsequently, we performed a second benchmark comparison of the ReadIT platform with a non-PCR based system developed by Naxcor. There were three discrepant genotypes, and in each instance the ReadIT system had determined the correct genotype. This led us to have even more confidence in the ReadIT system and to reject the Naxcor system for the clinical laboratory [11].
In 2001, the American College of Medical Genetics (ACMG) [12] and the American College of Obstetrics and Gynecology (ACOG) [13] published guidelines for population based carrier detection for CF. Prior to instituting a testing program, we performed a benchmark study of two commercially available CF testing reagents, the Linear Array CF Gold 1.0 ASR from Roche Molecular Biochemicals and the ABI CF Genotyper V 2.0 from Applied Biosystems Incorporated. There was one discrepant result. The Roche ASR, a reverse dot blot hybridization assay, identified an individual as a compound heterozygote for delta F508 and 1898 + 1 G → A. The ABI oligonucleotide ligation based assay identified the patient as a heterozygote for delta F508 only. Sequencing revealed the patient actually carried a different mutation, 1898 + 1 G → C, that is not on the ACMG panel. It was difficult to fault either system for this situation[14].

Subsequently, we developed a proprietary chip-based assay for the ACMG/ACOG CF mutation panel. We subjected this chip to two separate benchmark tests, one compared to the Roche ASR and another to the CF Genotyper V 3.0 ASR from ABI. There were no discrepant results in either series [7]. We therefore demonstrated that there are three platforms capable of performing error-free analysis for 25 CF mutations for 2000 patients each or an impressive total of 50,000 mutation analyses.

8. Quality assurance monitoring

One of the vital principles of laboratory testing is a continuous monitoring of processes and results to identify developing problems before errors are made. Elaborate software has been developed to monitor high volume laboratory testing in order to detect disturbing trends, such as a drifting upward of the median results for a quantitative assay. This allows laboratorians to identify a developing problem such as a failing detector or deteriorating reagents.

Molecular diagnostics requires a unique approach to Quality Assurance monitoring. Because most of the assays performed are qualitative, it is not possible to apply the same minute-to-minute monitoring that can be accomplished, for example, with a serum sodium measurement. However, molecular assays are not immune to reagent problems and equipment malfunctioning.

For example, if an assay requires a specific oligonucleotide for a wild type allele and a second specific oligonucleotide for a mutant allele, partial degradation of the oligonucleotide specific for the mutation could result in the failure to detect a mutation in a proportion of patients. If the deterioration is progressive, eventually the positive controls will begin to test negative and the problem will be identified. There may be a period of time when the assay is intermittently failing to detect mutations when present either randomly or in samples with a lower DNA concentration than the positive controls or when inhibitory substances are present. In this time period, undetected genotyping errors may occur. We have developed software capable of monitoring the proportion of mutant alleles detected in any given assay over any given period of time in order to detect such events.

For example, if the percentage of individuals testing positive for a mutation were to decrease over a period of weeks, this might be evidence that a reagent specific to the mutation is beginning to fail. Such an observation could ideally lead to early intervention and retesting of samples so that any previously reported erroneous results could be discovered and corrected. The program is still early in its deployment and no examples of such an occurrence have been detected thus far.

9. Conclusions

Mutation detection has become an important part of routine clinical diagnostics. Gene based testing represents one the highest growth areas in our laboratory with consistent growth of 20% per year. The demands of clinical mutation detection are enormous because of the nature of the testing and the potential catastrophic consequences of a genotyping error. Platforms exist that are capable of consistently identifying mutations in a clinical setting with error rates of <1:1000, but several commercially available reagents fail to perform at that level.

It is essential that clinical laboratories adhere to rigorous standards in order to maximize the accuracy of their testing results. Despite all best efforts, it is possible that biological variation in the form of polymorphisms will cause genotyping errors. For this reason, it is important to confirm all positive testing results, preferably in a different laboratory, or by a different
technique in the same laboratory whenever possible prior to performing an irreversible intervention such as surgical removal of organs. The laboratory must remain vigilant for any variations that might affect assay performance.

References


