

A Policy Context for the Case Studies

These four case studies have been selected out of a much larger number we considered because they seemed to best raise the key questions we are addressing at the Workshop. Each case poses questions relevant to the specific test (we list some of them) as well as to the overall inquiry.

In reviewing the cases, we suggest that there are at least six dimensions to consider in discussing both the types and levels of evidence to support clinical utility:

1. *Prevalence*: number of affected individuals;
2. *Penetrance*: degree of predictive value;
3. *Potential value as a health care service*. Hypothetically, assume a definitive test exists for a condition (for example, road rage) that reaches both prevalence and penetrance thresholds, but is largely beyond the scope of the services the health care system provides. A test of lesser prevalence and penetrance (for example, for a particular type of heart disease), might nonetheless be more favorably assessed because it may enhance the effectiveness of treatments deliverable through the health care system and, hence, have a greater impact on public health;
4. *Potential value to individuals and families*. Hypothetically, if a susceptibility test for Alzheimer's Disease were determined to have predictive value, but there is no established therapeutic intervention, different questions arise. With the rise of a "personal genomics" market (e.g., companies such as 23 & Me and Navigenics), these questions then pose issues of *information utility*, rather than clinical utility;
5. *Social policy factors*. Two tests might be "equal" in all relevant clinical utility measures, and both could be effectively delivered through our health care system, but one (for example, addressing obesity) might have arguably greater social policy value than another (for example, male pattern baldness);
6. *Specific bioethical issues*. Though genetic testing generally poses a number of bioethical issues, there may be some tests that raise distinctive bioethical issues, such as the complexity of informed consent issues associated with gene expression profiles.

Case Examples

The cases summarized here are intended to provide basis for discussion of two related questions:

- Clinical utility - what evidence is needed to inform decisions about clinical use?
- Funding decisions - what factors inform decisions about test coverage and reimbursement?

Case	Purpose of testing	Usual location(s) for testing	Testing technology	Other potential or associated uses
CYP2C9 and VKORC1 testing for patients being placed on warfarin	Identify individuals requiring lower doses, to avoid adverse events (bleeding)	Inpatient; Primary care; Anti-coagulation clinic	DNA-based	
Gene expression profile to predict breast cancer recurrence risk (OncoDx, MammaPrint)	Determine which patients are most likely to benefit from adjuvant chemotherapy	Oncology	RNA-based	
Test for susceptibility to type 2 diabetes mellitus (deCODE T2™)	Identify individuals at increased risk for Type 2 diabetes mellitus (T2DM)	Primary care	DNA-based	Identify genetic contributor in patient with T2DM
Duchenne/Becker Muscular Dystrophy	Provide definitive diagnosis for child or adult with suggestive symptoms	Medical genetics; Neurology	DNA-based	Carrier testing for female relative; Prenatal diagnosis; Selection of candidates for clinical trials

***CYP2C9* and *VKORC1* testing for patients being placed on warfarin**

Background

Several studies have confirmed that variants of the *CYP2C9* and *VKORC1* genes influence response to the anti-coagulant warfarin. Specific variants of each gene are associated with lower dose requirements, and variants of *CYP2C9* with a higher risk of bleeding complications. Tests for these variants might help to identify patients who would benefit from lower loading doses or more careful monitoring of warfarin therapy. The FDA has recently changed the warfarin label to provide information in the "Precautions" section about pharmacogenetic testing; however, the new labeling does not require testing or make specific recommendations about changes in dosing. The incremental cost of testing is estimated at \$250-\$500 (but is likely to drop).

There are currently no prospective randomized data to assess the outcome of genetic testing for *CYP2C9* and *VKORC1* variants in patients receiving warfarin therapy. A recent cost-effectiveness analysis estimated that testing for *CYP2C9* variants prior to warfarin therapy could result in more than \$1,000,000,000 in reduction in health care spending annually, with further savings expected with the addition of *VKORC1* variants (McWilliam et al 2006); however, this study has been criticized for using overly optimistic assumptions about baseline risk and effectiveness (Veenstra 2007).

Prevalence

Variants in the *CYP2C9* and *VKORC1* genes that are associated with lower warfarin dose requirements occur in about a third of the US population. Significant differences occur across racial/ethnic groups: *CYP2C9* variants are less common in people of African or Asian ancestry, while *VKORC1* variants are more common in people of Asian ancestry and less common in people of African ancestry.

Penetrance (predictive value)

Variants in the *CYP2C9* and *VKORC1* genes are estimated to account for 35% to 50% of the individual variation in warfarin dose requirements. *CYP2C9* variants have been associated with a 2-4x higher risk of major bleeding events in the first year of therapy; the incidence of major bleeds is approximately 4-10% in the first year. To date, variants of *VKORC1* have not been associated with increased bleeding risk, and no variants of either gene have been associated with thromboembolic risk.

Purpose of testing

To identify individuals requiring lower doses of warfarin or more careful surveillance of warfarin therapy, in order to prevent adverse events.

Key Questions

1. What incremental benefit over current standard of care is needed to justify use of this test?
2. What is level of evidence for benefit, and magnitude of benefit?
3. What is the incremental cost—increasing or cost-saving?

4. How should a drug interaction that has a similar phenotypic effect be considered?
5. What are the relative merits of directing use via regulatory mechanisms vs. clinical guidelines vs. reimbursement?

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Gene expression profile to predict breast cancer recurrence risk (Examples: OncotypeDx, Mammaprint)

Background

Gene expression profiling (GEP) of tumor tissue (that is, the measurement of patterns of RNA production in tumor tissue) has attracted interest as a means to improve the accuracy of cancer prognosis. Several studies suggest that GEP results can be used to identify cancer patients with a higher likelihood of cancer recurrence, and that these profiles may perform better than other prognostic strategies currently in use. In the case of breast cancer, two gene expression profile tests (OncotypeDxTM and Mammaprint) have been developed as tests to identify women most likely to benefit from adjuvant chemotherapy. For women with early breast cancer (EBC), chemotherapy offers a 5 to 10% absolute 5 year survival advantage. Although this is a significant improvement, many women must be treated with uncomfortable and risky chemotherapy for a relatively few to achieve a survival benefit. GEP is proposed as a means to determine which women with EBC should receive chemotherapy.

The two tests currently available

OncotypeDx (Genomic Health): Cost is \$3650.

Information from the Genomic Health website

(<http://www.genomichealth.com/oncotype/about/hcp.aspx>):

Oncotype DXTM is a clinically validated, multi-gene assay that provides a quantitative assessment of the likelihood of distant breast cancer recurrence and also assesses the benefit from chemotherapy; the data on chemotherapy benefit is derived from the NSABP Study B-20 which compared hormonal therapy alone versus CMF based chemotherapy and hormonal therapy. The assay — performed using formalin-fixed, paraffin-embedded tumor tissue — analyzes the expression of a panel of 21 genes and the results are provided as a Recurrence ScoreTM (0-100). The gene panel was selected and the Recurrence Score calculation was derived through extensive laboratory testing and multiple independent clinical development studies.

Candidates for testing are women with newly diagnosed breast cancer that is Stage I or II, node-negative and estrogen receptor positive, who will be treated with tamoxifen.

Mammaprint[®] (Agendia): Cost is ~\$3200

Information from the Agendia website: Cost is similar to Oncotype Dz

(<http://www.agendia.com/en/Agendia/Press-Releases/Press-Release> Accessed October 1, 2007):

Agendia's MammaPrint[®] breast cancer prognosis test is the world's first In Vitro Diagnostic Multivariate Index Assay (IVDMIA) to acquire market clearance from the U.S. Food and Drug Administration (FDA). MammaPrint[®] is a DNA micro array-based in vitro diagnostic laboratory service that measures the activity of 70 genes, providing information about the likelihood of tumour recurrence. The MammaPrint[®] test measures the level of expression of each of these genes in a sample of a woman's surgically-removed breast cancer tumour and then uses a specific formula or algorithm to produce a score that determines whether the patient is deemed low risk or high risk for spread of the cancer to another site. The result may help a doctor in planning appropriate follow-up for a patient when used with other clinical information and laboratory tests.

Information from FDA press release

(<http://www.fda.gov/bbs/topics/NEWS/2007/NEW01555.html> Accessed Oct 1, 2007)

Agendia compared the genetic profiles of a large number of women suffering from breast cancer and identified a set of 70 genes whose activity confers information about the likelihood of tumor recurrence. The MammaPrint test measures the level of activity of each of these genes in a sample of a woman's surgically removed breast cancer tumor, then uses a specific formula, known as an algorithm, to produce a score that determines whether the patient is deemed low risk or high risk for spread of the cancer to another site. The result may help a doctor in planning appropriate follow-up for a patient when used with other clinical information and laboratory tests. The MammaPrint is the first cleared in vitro diagnostic multivariate index assay (IVDMIA) device. Several months ago, FDA issued a draft guidance document concerning the need for these complex molecular tests to meet pre-market review and post-market device requirements even when the tests are developed and used by a single laboratory. Although FDA regulates diagnostic tests sold to laboratories, hospitals and physicians, it uses discretion when regulating tests developed and performed by single laboratories.

Issues related to evidence and regulatory oversight

The scientific challenges in establishing that a particular GEP predictive test works well are obvious. First, measurement of response of treatment takes at least three years of follow-up, and many more years would be preferred. Second, it would be difficult ethically to withhold chemotherapy treatment without a very good possibility that the predictive test is likely to work well. Third, chemotherapy regimens could be improving over time, so the measured outcome could be a moving target. Fourth, women and their physicians may be reluctant to forgo chemotherapy even given a prediction that the probability of recurrence is very low. In February 2005, the BlueCross BlueShield Association Technology Evaluation Center (TEC) conducted a technology assessment review of four GEP tests proposed for this purpose, and concluded that sufficient evidence, especially peer-reviewed published analyses—was lacking to warrant coverage (http://www.bcbs.com/betterknowledge/tec/vols/20/20_03.html Accessed June 20, 2007). Thereafter, in June, 2007, TEC re-considered its earlier decision and found that the test, “meets the.....criteria for women with estrogen receptor-positive, node-negative, tamoxifen-treated breast cancer.”

To accelerate the development process, the inventors of *Oncotype DX*TM recognized that samples from earlier NIH-sponsored trials could be used to validate the test, if they could be genotyped. They developed and patented a technique for extracting genetic information from these old paraffin-embedded samples. This strategy was also contingent on the fact that basic chemotherapy for EBC had changed little in the past 20 years. They first analyzed samples from the tamoxifen-treated arm of studies where women had not received any other chemotherapy. Based on their hypotheses about candidate genes that might be related to disease progression, they identified a 21-gene panel that was predictive of whether women eventually progressing to metastatic breast cancer. The prediction utilized a scoring algorithm to provide a “recurrence score” based on the gene panel. Using samples from a

later NIH trial involving treatment, they then attempted to validate their prediction equation by seeing whether they could predict whether or not the women had responded to chemotherapy. Their algorithm was sufficiently persuasive that the NIH agreed to undertake a prospective trial—called TailorRx--based on their recurrence score, randomizing some women to treatment or not based on their score. This trial is in process.

Genomic Health provides the test as a laboratory developed test (LTD) at their California laboratory. This approach had two important potential effects: 1) it appeared to obviate the requirement for FDA premarket approval, and 2) it allowed the test to be reimbursed nationally through the California Medicare carrier. The latter aspect supported a “premium” pricing and reimbursement strategy, as the test was priced initially at \$3460, now at \$3650 due to increasing demand (an uncommonly high figure for a diagnostic test) and not linked via reimbursement coding to previous tests. Thus when the Medicare carrier approved it for reimbursement, it set a precedent. Genomic Health also conducted economic studies that projected that *Oncotype DX*[™] would be cost-saving from a health system perspective and not just cost-effective. Their strategy seemed to be working well until September of 2006 when the FDA issued draft guidance on in vitro diagnostic multivariate index assay (IVDMIA) devices. The FDA was thereby asserting its prerogative to review the evidence in support of this test.

In contrast to Genomic Health’s strategy, in February of 2007, the FDA approved MammaPrint®, a competitor to *Oncotype DX*[™], developed by researchers in the Netherlands and the Molecular Profiling Institute, and marketed in the US by Agendia, Inc. MammaPrint® followed a different evidentiary and regulatory pathway including submission of its data to the FDA. Based on fresh frozen samples from recent clinical trial data on 302 patients at five European centers, they developed a prediction algorithm based on a 70-gene array. They developed their algorithm on historical (1983-1996) free-frozen samples from tumors of women who did and did not experience recurrence, and validated it on retrospective evidence from 295 patients. FDA clearance occurred through the premarket notification 510(k) mechanisms, resulting in approval for estimation of recurrence risk. Agendia has started a prospective multicenter trial---called MINDACT—in Europe.

In May of 2007, the FDA issued a guidance that the GEP systems for breast cancer diagnosis would be treated as Class II Special Controls. A Class III designation would have required pre-market approval, increasing the development costs. Instead, Class II status requires the developer to file for 510(k) premarket submission that complies with the FDA’s special controls. For example, this includes a “validation strategy,” that provides the details of the needed studies and statistical analyses.

Penetrance (predictive value)

Still under study (see above)

Prevalence of condition

The American Cancer Society estimates that in 2007 there will be approximately 178,500 women diagnosed with invasive breast cancer and that over 40,000 women will die from the disease. Most of these newly diagnosed patients will have early stage cancers and would be candidates for GEP testing.

Purpose of testing

To determine which breast cancer patients are most likely to benefit from adjuvant chemotherapy due both to their risk of recurrence and in the case of OncoTypeDx, their likelihood of response to chemotherapy.

Key Questions

6. How do patients and physicians respond to test results?
7. Does a test for acquired genetic change have different implications than a test for inherited change?
8. What kind of evidence is needed to establish clinical utility?

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Test for susceptibility to type 2 diabetes mellitus (deCODE T2™)

Background

Several studies have confirmed an association between variants in the *TCF7L2 gene* and type 2 diabetes mellitus (T2DM). This finding is one of many recent reports of associations between gene variants and T2DM (see References)

The test currently available

The test currently available (deCODE T2™) identifies a variant that is associated with an approximately 1.5-fold higher risk for T2DM in heterozygotes (people with one copy of the variant) and an approximately 2-fold higher risk in homozygotes (people with two copies of the variant).

From the deCode web site

(<http://www.decodediagnostics.com/T2.php>):

deCODE T2™ is a reference laboratory test to detect a version of a single SNP (single nucleotide polymorphism) – a one letter variation in the sequence of the genome – in a gene called TCF7L2 that deCODE has linked to increased risk of T2D. By understanding inherited risk, it may be possible to take actions that reduce or minimize the likelihood of an individual ever developing a disease.

In deCODE's findings, published in 2006 and which have since been validated in studies by independent researchers in tens of thousands of people from populations around the globe, between 8-11% of the general population have been shown to carry two copies of the risk variant, where as two copies are approximately two times more frequent among type 2 diabetics. In other words, having two copies of the risk variant – a positive result for the deCODE T2™ test – has been shown to correspond to an approximate doubling of the likelihood of developing T2D. The risk variant is the "T" allele of SNP rs7903146, located within the transcription factor 7-like 2 (TCF7L2) gene.

A published analysis of data from a U.S. government supported clinical trial involving prediabetics, the Diabetes Prevention Program, also showed that those with two copies of the risk variant were at a 1.8 times greater risk of progressing to type 2 diabetes within the next four years as were prediabetics with one or no copies of the risk variant. Approximately 20% of prediabetics who went on to develop T2D within the four year period studied carried two copies of the risk variant – which would yield a positive result for the deCODE T2™ test – compared to 11% of study participants overall.

Importantly, this study also showed that this increased risk could be effectively reduced through weight loss and treatment with certain insulin response-boosting medications such as metformin. The deCODE T2™ test may therefore offer a new means to help physicians decide which prediabetic patients they wish to treat more aggressively either through lifestyle modification or drug treatment.

Issues related to evidence and regulatory oversight

No prospective studies of clinical testing have been undertaken. Because the test is provided as a laboratory service, it is not subject to pre-market review by FDA.

The test is currently being offered direct to consumers via DNA Direct (www.dnadirect.com). This approach to testing has generated some professional concerns (eg, see statement on direct to consumer testing by the American Society of Human Genetics at http://genetics.faseb.org/genetics/ashg/news/dtc_statement.pdf)

Prevalence

The prevalence of the variant has varied in different populations studies, but is generally high. In populations of European descent, about 30-40% have a single copy of the variant and 5-10% have two copies.

Penetrance (predictive value)

If lifetime risk of T2DM is assumed to be 33%, the positive predictive value of the test for heterozygotes would be about 40-45% and the negative predictive value about 70-75%. The comparable estimates for homozygotes would be a positive predictive value of about 60% and a negative predictive value of about 70%. Overall, the test provides risk prediction similar to that obtained with a positive family history of T2DM in a parent (Janssens et al 2006b, Edwards et al 2003).

Purpose of testing

Identify individuals at increased risk for Type 2 diabetes mellitus (T2DM), in order to promote lifestyle measures to reduce risk.

Key questions

- What benefits are provided by identification of genetic susceptibility to T2DM?
- What interventions or services can (or should) be offered to individuals who test positive?

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Array based molecular testing for Duchenne/Becker Muscular Dystrophies

Background

Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD) are caused by mutations in the *DMD* gene, which codes for the muscle protein dystrophin. DMD is characterized by progressive symmetrical muscular weakness, proximal greater than distal, often with calf hypertrophy; CPK (creatine kinase) is elevated to 10x normal levels. Approximately 20% of DMD patients may have some degree of mental retardation. *BMD* is a milder disease that was historically considered a separate clinical entity. This disorder differs from DMD by its later onset and milder course. CPK levels are elevated to 5x normal. DMD and BMD are X-linked recessive disorders. In DMD symptoms typically present before age five, wheelchair dependency is reached by age 12, and death often occurs in the late teens or early twenties, while for BMD, symptoms have a much later age of onset, wheelchair dependency, if present, typically occurs after age 16, and more than 90% of BMD patients are still alive in their twenties.

The *DMD* mutations that result in DMD cause total or near total loss of the dystrophin protein from skeletal muscle. DMD and BMD can be further distinguished by muscle biopsy results. Individuals with DMD have complete or almost complete absence of dystrophin protein by immunohistochemistry, while individuals with BMD have either normal or slightly reduced dystrophin protein.

With the understanding that these two clinically distinct disorders involved the same gene, a family of clinical disorders known as the 'dystrophinopathies' was defined. Rare individuals (intermediate patients or outliers) have manifestations intermediate between DMD and BMD. A third dystrophinopathy, *DMD*-related X-linked dilated cardiomyopathy, has also been defined. This disorder is caused by *DMD* mutations that lead to selective loss of dystrophin from cardiac muscle, while dystrophin levels in skeletal muscles remain normal or near-normal. Some carrier females have also been shown to have cardiomyopathy.

Dystrophin is the largest known human gene, and spans 79 exons making comprehensive testing difficult. DMD/BMD is observed in all ethnic groups. Molecular genetic testing of the *DMD* gene is currently available using targeted mutation analysis, mutation scanning, and sequence analysis (See Table 1). Current methods of clinical mutation detection in the *DMD* gene each detect only a portion of possible mutations and the methods are labor intensive. Many large deletions in males can be detected using a simple multiplex PCR test that screens the exons most commonly deleted and allows accurate genetic counseling in affected families via DNA-based diagnostics. Duplications are harder to detect and require southern blot analysis. More recently Multiplex Ligation Dependent Probe Amplification (MLPA) has been used. Point mutations can be detected by using mutation scanning techniques followed by sequencing to characterize the mutations. Multiplex PCR detect deletions in 32 (79) exons of the *DMD* gene and point mutations detection is a very laborious process. MLPA detects only deletions and duplications but is not a very robust assay. Mutations in introns are rarely detected by any of these assays and assessing the carrier status in females is very

difficult. Currently up to 15% of males may still require muscle biopsy with its inherent risks for diagnosis. Testing of carrier females without a known familial mutation can be difficult with the current technologies.

Table 1
Current testing for DMD/Becker
 (based on Prior & Bridgeman 2005 and laboratory websites)

Method	% detection in males	Detection in females	TAT – Turn Around Time	Advantages/Disadvantages*
Southern	~60%	Low sensitivity	2-4 weeks	Low detection rate for females Low sensitivity for duplications Cannot identify point mutations
Multiplex PCR	~60%	Low sensitivity	5 weeks	Low sensitivity for duplications Low detection rate for females Deletion boundaries are not identified Reading frame is not established Cannot identify point mutations
MAPH	??			Low detection for large del/dup Cannot identify point mutations
Sequencing	~90%	X	6-8 weeks	Long TAT/cost
SCAIP	~97%	No	8-12 weeks	Females cannot be tested Cannot detect duplications Must be confirmed with another method
MLPA	~60%	Low sensitivity	8-12 weeks	Low sensitivity for duplications Low detection rate for females Long TAT

New testing methods

The mutational spectrum of dystrophin is complex, involving deletions, duplications, and point mutations. Mutation detection in carrier females can be especially problematic. New testing methods, utilizing array comparative genomic hybridization (CGH) and resequencing arrays offer an opportunity for increased mutation detection (Table 2). This approach offers advantages over currently used approaches, including better resolution of breakpoints, easier carrier identification, and fast turnaround time for the re-sequencing array. A two tiered approach was proposed with the first tier analyzing for deletions and duplications using a custom 385 K CGH array from NimbleGen, and the second tier analyzing the entire coding region and flanking intronic sequences, eight promoters and five previously described intronic mutations with a custom 385 K DMD re-sequencing array. A manuscript has been submitted to *Genetics in Medicine* detailing the validation of the methods (See below).

Table 2
Array based technology for identification of DMD/BMD mutations

Proposed test method	Type of mutation detected	Proportion of this disease attributed to mutations in this gene ¹	Mutation detection frequency for this method ²
DMD			
Array CGH	Del/dup	71-75%	>98-99%
Resequencing Array	Point mutation	25-29%	>98-99%
BMD			
Array CGH	Del/dup	91-95%	>98-99%
Resequencing Array	Point mutation	5-9%	>98-99%

Prevalence

DMD is the most common X-linked recessive lethal disease with an incidence of approximately 1 in 3500 newborns. Approximately one third of the cases result from new mutations. BMD occurs approximately one-tenth as frequently with an incidence of approximately 3 per 100,000 newborns.

Penetrance (predictive value)

Penetrance is complete in males. About 15% to 25% of carrier females manifest symptoms, usually mild.

Purpose of testing

Testing for DMD/BMD has several potential purposes:

- Molecular testing is currently the practice standard for confirming the diagnosis in patients with suggestive symptoms.

- Approximately 15% of patients currently require muscle biopsy due to limited detection in testing methodologies
- Carrier testing can be offered to female relatives if the familial mutation is known. If a woman is identified as a carrier, she can be offered prenatal diagnosis.
- Several pharmaceutical therapies are in clinical trials (PTC 124). Currently these proposed treatments target specific types of mutations, making mutation detection for all patients critical.
- Extended turn-around-times with current testing methodologies may lead to muscle biopsy for prognosis

Key questions

- Does definitive molecular diagnosis improve quality of care or patient quality of life?
- Should sequential testing using current technologies be considered first and array technology reserved for difficult cases?
- What counseling services should be provided with testing? Should counseling differ for different test purposes?
- How often does testing yield indeterminate results?
- What are the gains from array technology?
- Should individuals with a clear clinical diagnosis in which no mutation was found by current testing technologies be re-tested with array technology?

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ADDENDUM: VALIDATION OF ARRAY BASED TESTING FOR DMD/BMD

Validation Samples: Fifty nine samples with previously characterized DMD mutations were obtained from OHSU DNA Diagnostic Laboratory, LabPLUS of New Zealand, and the Emory Genetics Laboratory. These samples had been analyzed using a combination of the standard DMD multiplex PCR, Southern blot analysis and sequencing. A total of 29 patients had structural alterations: 11 males with deletions, 4 males with duplications, 12 females with deletions, and 2 females with duplications. These 29 samples were used to validate the CGH array. The remaining 30 patients comprised of 14 males and 16 females had point

mutations, including nonsense and missense alterations. These samples were used to validate the re-sequencing array.

CGH Array:

Fifteen male positive control samples (11 deletions, 4 duplications) were analyzed. One patient was previously found to have a single exon deletion and ten were found to have multiple exons deleted. All deletions and duplications were detected by the CGH array. Two patients had breakpoints reassigned based upon the CGH array. The first patient had been previously found to have a deletion of exons 45-52. The second patient had been found to have a deletion of exons 48-52. It should be noted that the currently used multiplex PCR assays (Briggs and Chamberlain) only include one exon beyond exon 52. The CGH array demonstrated the deletion in the first patient actually included exons 53 and 54 and that the deletion in the second patient included exon 53. The results in these two male controls were verified by a commercially available DMD assay, Multiplex Ligation-dependent Probe Amplification (MLPA), available from MRC-Holland.

A total of 14 female positive control samples were also analyzed using the CGH array. All deletions and duplications were detected by the CGH array. As with the male controls, two female controls had breakpoints reassigned based upon the CGH arrays. One female control was found to have a deletion of exons 45-55, expanded from the previous finding of exons 45-52. The second female control was determined previously to have a deletion of exons 3-4. The CGH array indicated a deletion of exons 4-7. MLPA was used to verify the results for all 14 female positive control samples.

Re-sequencing Array:

Fourteen male samples were available to validate the resequencing array. The mutations previously identified included 4 nonsense, 2 splice site mutations, 4 small deletions, and 4 small insertions. Sixteen female samples comprising 6 nonsense, 2 splice mutations, 6 small deletions, and 2 small insertions were available for analysis. The resequencing array had 100% concordance with all previously determined mutations. A total of 32 SNPs were detected in these 30 control samples.

Patient Sample: Following validation of the methods, a sample was received on a 4 year old male patient. The array CGH revealed a deletion of exons 8-13, which was verified using MLPA. CGH array analysis of the mother did not detect the deletion. MLPA was used to confirm the mother's results. MLPA indicated 2 copies of exons 8-12 but 1 copy of exon 13. Exon 13 was then sequenced in the mother. The mother was found to be a heterozygous carrier of a SNP (c.1554T>A; p.D518E) that is the 3' end of the MLPA forward probe for exon 13. Thus, this allele failed to amplify with the MLPA system, mimicking a deletion of exon 13.

TECHNICAL DETAILS

CGH Array: The entire 2 Mb *DMD* gene is covered by 385,474 probes with average spacing between the probes of 5 bases. The array also contains 4115 internal controls. Briefly, the procedure is to sonicate patient and control DNA and then label with Cy3 and Cy5, respectively. Labeled DNAs are purified, combined, desiccated, resuspended in hybridization buffer along with control probes, and hybridized to the array for 16-20 hours. After washing, arrays are scanned. Data are analyzed using SegMNT and/or GLAD. Results are presented graphically in a bar graph with the y-axis demonstrating loss or gain of material. Use of this array should allow more accurate determination of breakpoints involved in deletions.

Re-sequencing Array: If no gain or loss of material is found with the CGH array, samples will then be analyzed using a custom 385 K DMD resequencing array. This array contains the full 14 kb transcript, 100 bp upstream and downstream of each exon, eight promoters and five previously identified intronic mutations. Briefly, each sample is amplified as 85 fragments, which are then pooled and labeled with Cy3. The purified labeled DNA is then desiccated, mixed with control oligonucleotides, and hybridized to the array for 16-20 hours. After washing and scanning, grid alignment and data extraction are performed by RATools followed by base calling with the ABACUS algorithm. Sequence analysis is conducted with Mutation Surveyor software.