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NEWS AND COMMENTARY

Cystic fibrosis diagnostic testing

When good CF tests go bad

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Two papers in this issue of *Eur J Hum Genet*[1,2] serve to remind us that, even in the well-established world of cystic fibrosis (CF) diagnostics (2009 sees the twentieth anniversary of the identification of the CF gene), results may not always be as they seem.

The report by Stuhrmann *et al*[1] describes two cases in which, following genetic testing using a commercial kit, the most common CFTR genotype of homozygosity for p.Phe508del (HGVS nomenclature, www.hgvs.org) was initially assigned to the index case in both families. However, testing of the parents of the index cases revealed that neither index case was, in fact, homozygous for p.Phe508del. In one case, the index case genotype observed was due to the presence of p.Phe508del on one parental chromosome and the presence of c.1545_1546delTA (the second most common mutation in the Turkish population) on the other parental chromosome. Owing to its close proximity to p.Phe508del (c.1521_1523DelCTT) in exon 10, the presence of this mutation most likely compromised the amplification of the wild-type allele. In the second family, genotyping revealed heterozygous p.Phe508del in one parent and heterozygosity for a large deletion in the other.

The cases described raise several issues within the area of CF genetic testing. The two examples represent scenarios that could arise in any testing laboratory. Experienced laboratories are likely to have come across similar scenarios and are likely to have systems in place to address the issues raised, whereas laboratories that are relatively new to CF genetic testing will benefit from the careful study of these cases. The two cases also serve to validate the relevant recommendations in recently published CF genetic testing guidelines[3] and in earlier guidelines.[4,5]

Highlighted by the cases presented by Stuhrmann *et al* is the importance of confirming the genotype of an index case by identifying the mutation(s) on the parental alleles before prenatal testing is offered to the parents and before carrier testing is offered to other family members. As shown by the authors, testing parental alleles in these cases revealed an unexpected result, thus prompting further investigation. Failure to test the parents in these cases could have resulted in misdiagnosis of an affected foetus as healthy (case 1) and in case 2, in misdiagnosis of a carrier as a non-carrier. However, although the importance of testing parental samples is apparent from these examples, the authors refer to difficulties they had in obtaining these parental samples and difficulties that are also likely to be experienced by other testing laboratories. Parental samples had only been received for approximately 9% of their cases with a diagnosis of CF. Routinely incorporating a request for parental samples in the report to the clinician of the CFTR genotype for the index case, together with the recommendation that parental alleles should be assigned before further testing for the family proceeds, may help address this problem.

The two cases described illustrate the need for testing laboratories to be alert to the two common causes of erroneous apparent homozygosity, even when it involves the identification of homozygosity for the most common CF mutation in a patient with a clinical diagnosis of CF (p.Phe508del represents approximately 22–75% of CFTR mutations worldwide).[6] The first case demonstrates the effect of the presence of an interfering sequence variant, in this case the common Turkish mutation c.1545_1546delTA. It is not surprising that this mutation was not included in the panel of any of the commercial kits used by the authors, because these panels are optimised for American and Western European populations. Of greater concern is the false-positive result obtained with one of the commercial kits. A homozygous p.Phe508-del genotype was obtained following testing of case 1 with two versions of the Elucigene kit (Tepnel Diagnostics, Manchester, England; CF29 v2003 and v.2, 03/2007). Mutation c.1545_1546delTA (commonly used nomenclature: 1677del-TA) is not listed in the cross-reactivity data of v.2 or the most current version (CF-HT v.3, 09/2007).[7] The authors also mention other well-known examples of interference caused by mutations that were not included in the mutation panel and not accounted for in the design of the commercial kit. In our own experience with a different assay, we have come across similar examples of interference to those described in case 1. During validation studies, we observed genotype miscalls for two cases with compound genotypes, comprising p.Phe508del and p.Val520Phe in one case and p.Phe508del and p.Gln493X in the other. In both cases, an erroneous homozygous p.Phe508del genotype was obtained. These compound genotypes have been identified by us in nine and two apparently unrelated CF families, respectively, representing approximately 1.0 and 0.2% of CFTR mutations in our population. It is not possible for all potential interferences to be taken into account by companies during the design stages of commercial kit development. However, particular attention should be directed towards identifying those interferences that could potentially disrupt binding of primers and probes targeted to the wild-type p.Phe508del position, resulting in a 'dropout' of the wild-type allele. Testing laboratories are less likely to be alerted to initiate further investigations when homozygosity for the most common CFTR mutation is the genotype obtained for a patient with a diagnosis of CF.

Even when interferences are listed in the manufacturer's instructions for use, kit

users do not always pay attention to them. A recent paper[9] described a field trial of a synthetic CF reference material containing six homozygous mutations and one polymorphism. The results revealed multiple instances of misreporting of genotypes due to interferences that were documented in the manufacturers' literature.

The importance of being aware of population-specific mutation spectra is addressed in CFTR testing guidelines,[3–5] which state that knowledge of the ethnic or geographic origins of patients and their parents and grandparents is important to determine the analysis to be performed. The guidelines also state that a complementary panel of mutations may be required to test population-specific mutations at a frequency above 1%. Also deemed critical is referral by testing laboratories to a collaborative network of testing labs, which will have particular expertise in testing for mutations prevalent in their own population. The aim should be to make every effort to provide testing at as high a level of sensitivity as possible.

The second case described by Stuhrmann *et al* provides an example of erroneous homozygosity in a CF index case caused by the presence of a large deletion within the CFTR gene on one of the parental alleles. Large intragenic CFTR gene deletions may account for approximately 1–3% of all CFTR mutations,[8] and this figure is likely to increase as more laboratories incorporate screening for large rearrangements into their mutation screening strategy. A case has even been described of a CF patient compound heterozygous for two deletions.[10] Case 2 demonstrates to testing laboratories that the possibility of a large CFTR deletion in one parental allele being the underlying cause of apparent homozygosity should not be ruled out, even in the case of p.Phe508del.

CF guidelines that address the issue of erroneous homozygosity recommend that parental testing is performed to confirm the result. Although this may be widely accepted for rarer mutations, these two cases demonstrate that the possibility of erroneous homozygosity should also be considered for common mutations.

Many laboratories now use commercial kits for their CFTR testing. The 25-mutation panel that forms the basis of most commercial kits is based upon a worldwide mutation frequency of more than 0.1% (ACMG/ACOG recommended mutation panel). Case 1 serves to reiterate the importance of laboratories and companies to be aware of the existence of mutations that may be not be included in this panel but yet are present in a relatively high frequency in a specific population. Awareness of the position of these mutations relative to the primers and probes in the kit is also important for the likelihood of interference to be assessed. As IVD manufacturers rarely reveal the positions of the primers and probes in their kits, diagnostic laboratories cannot themselves check for mutations or SNPS that might compromise the assay. This places full responsibility of this role on the assay manufacturers. Users of approved devices (kits) have a responsibility to report adverse incidents and device malfunctions to the manufacturer and to the approving authority.

No matter how much validation the manufacturer has done, the end user has a responsibility to validate or at least verify the performance of any new test to ensure that it performs according to the laboratory's requirements. EuroGentest has established a diagnostic validation workgroup to develop and promote the use of centralised procedures and guidelines to facilitate the implementation of technologies, methods and kits into diagnostic routine activities. Specific objectives within this address the implementation of commercial kits/technologies for CFTR testing and the development of guidelines for the laboratory validation of in-house assays and commercial kits (http://www.eurogentest.org/web/info/unit1/validation.xhtml).

This topic of the validation of commercial assays by end-user laboratories is highlighted by the second paper.[2] In a survey of 125 participants of the European CF External Quality Assessment (EQA) scheme – therefore, laboratories who have enough interest in quality to join an EQA scheme – fewer than half of them had validated their assays in-house before implementation, and more than half of them had modified the manufacturer's recommended protocol. Manufacturers carry out extensive validation of the accuracy, reproducibility and robustness of their assays before placing them on the market. Even so, it is incumbent upon each end user to verify that the assay performs to the stated specifications before bringing it into service. When an end user deviates from the manufacturer's recommended protocol – typically by diluting or dividing the reagents to make them go further – they must then perform a full validation to ensure that they have not compromised the assay.

While focused on CF testing, these papers have relevance for genetic testing for any disorder. They serve as a useful reminder that most of the time, we are not looking at the patient's DNA, but at PCR products, which may or may not accurately represent the patient's underlying genotype. Any primer-based or probe-based technology (and that is pretty much all genetic testing) is susceptible to interference from SNPs, mutations and deletions. A careful assay design and validation can minimise the occurrence of such interferences, but we must still be vigilant, even when testing reveals results that are entirely expected. In recessive disorders, requesting parental samples to confirm the familial mutations must continue to be considered the best practice, despite the additional costs involved. When the genotype in the index case has not been confirmed by testing the parents, appropriate caution should be exercised when interpreting prenatal or carrier testing results ■

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