Pitfalls of automated comparative sequence analysis as a single platform for routine clinical testing for NF1

Mattocks et al have used direct DNA sequencing and comparative sequence analysis to study patients with neurofibromatosis type 1 (NF1) and claim this study “achieved the highest recorded mutation detection rate using a single technique for this gene.” As a key point, the paper states that they studied 91 subjects fulfilling the NIH NF1 diagnostic criteria and achieved a mutation detection rate of 89% using automated comparative sequence analysis. They continue by saying “This detection rate is the highest for a single technique and is therefore appropriate for routine clinical practice.”

When developing genetic tests, especially for large and complex genes such as NF1, a large cohort of patients needs to be studied in a comprehensive way in order to fully understand the spectrum of mutations present in that gene. From our experience, it is of utmost importance to analyse the complete gene for the presence of all possible alterations that may result in a premature stop codon or affect splicing. A significant fraction of the mutations in the NF1 gene cause aberrant splicing, and many of them are caused by alterations outside the canonically conserved AG/GT acceptor and donor sequences and even reside deep in the large introns. Also, various exonic mutations mimicking nonsense, missense, and even silent mutations at the genomic level have been described that are splicing mutations and exert their effect by creating a novel splice donor or acceptor or affect the function of an exonic splicing enhancer (ESE) or exonic splicing silencer (ESS). Although we have now studied over 600 patients fulfilling the NIH criteria using multiple complementary techniques, we are still challenged and surprised by the diversity of mutations leading to this disorder.

There have, unfortunately, been some examples of published reports where an alteration is claimed to be a pathogenic mutation, and where later on this statement needs to be revoked as the alteration is proven to be an innocent polymorphism. For thousands of hereditary disorders for which the genes have been cloned, patients await the availability of a reliable and sensitive diagnostic test and clinical molecular genetic laboratories worldwide rely on published reports to help distinguish a polymorphism or rare benign variant from a deleterious mutation.

This distinction is of the utmost importance and has major ethical implications with respect to the genetic counseling of patients seeking diagnostic, pre-/oligosymptomatic, and prenatal testing.

There is a need for a reliable and sensitive genetic test for the NF1 gene, to help resolve diagnostic dilemmas in patients not fulfilling the NIH diagnostic criteria, especially young children but also atypical patients, to determine the affection status of family members of an affected person and to carry out prenatal or preimplantation diagnosis, if desired.

NF1 is a progressive disorder and prediction of disease risk, one year of age and 5% still will not fulfil these criteria by the age of eight.’ Waiting for more symptoms to appear with time in order to ascertain the diagnosis on a clinical basis, is causing much stress for affected families. Earlier diagnosis of NF1 allows one to offer genetic counselling to parents and relatives earlier, as well as to initiate interventions for learning or developmental problems sooner. Later on this alteration will become even more important once more therapeutic options are available. A direct genetic test may help to establish the diagnosis earlier, especially in sporadic patients, but only when the testing has a high sensitivity—that is, finds the mutation in (almost) all patients who will eventually fulfil the NIH criteria (false negative results) and, equally important, does not confuse a benign variant with a pathogenic mutation (false positive results).

We have identified multiple sequence changes in the paper by Mattocks et al that are classified and hence need rectification to address potential misdiagnoses based on the latter information.

Whereas the title of the paper states that automated comparative sequence analysis identifies mutations in 89% of NF1 patients, table 2 describes those sequence alterations as “potentially significant” sequence alterations. There is also a table 3 summarising polymorphisms found in the study, which adds to the confusion and further suggests that alterations in table 2 are pathogenic mutations, to which the title also alludes. We think we need to make a clear distinction between a deleterious mutation, an unclassified variant, a rare benign variant, and a polymorphism.

Table 2 contains four silent nucleotide changes: Q282Q, C680C, K1724K, and R1180R. The authors predict that these changes lead to a truncated peptide, but no experimental evidence is given to prove this. Two of these sequence changes have been observed by us and others and are definitely rare benign variants. c.5172G→A (K1724K) was first described by Peters et al as a polymorphism in NF1. There were two other silent nucleotide changes: 0.99 for c5127G and 0.01 for c5172A. Peters et al report on a patient who carries this polymorphism in exon 28, as well as a frameshift mutation in exon 28. Fahsold et al also describe this sequence change as a pathogenic frameshift mutation in exon 37: c.6789delE4. One of us (LM) has observed this sequence change in 2/570 NF1 patients in whom a clearly pathogenic mutation—c.3326delE6C and c.1756_1759delACTA—was found as well. Thus this sequence variant represents an infrequent benign variant that should not be confused with a bona fide pathogenic mutation. Mattocks et al demonstrated that the silent nucleotide alteration K1724K was also found in the affected mother and thus segregated with the disease. However, the authors did not show that this segregation with the disease is not sufficient to provide final evidence of whether a variation is pathogenic. An effect on splicing must be shown before a silent change can be classified as a pathogenic.

A second silent sequence change Q282Q (c.846G→A) predicted by Mattocks et al to result in a truncated protein has been reported by Luca et al as a polymorphism with a frequency of 2%. One of us (LM) also found Q282Q in two of 190 control samples, which was reported previously to needs to be considered with great caution, especially as these changes fulfil none of the classic criteria for pathogenic mutations: neither of these silent changes has been reported previously, they have not been demonstrated to occur de novo in sporadic patients, neither were they shown to segregate with the disease in a given family, and most importantly they were not proven to affect splicing. Hence, these changes cannot be considered pathogenic unless data are produced showing that these changes have an effect on the correct splicing of the NF1 gene. Through the study of over 600 unrelated NF1 patients, we identified 29 patients carrying a pathogenic truncating mutation as well as a silent mutation, the latter without an observed effect on splicing (Messaia et al, unpublished results).

Apart from the silent sequence changes, the classification of NF1 missense mutations is also particularly challenging. Table 2 contains at least one missense alteration (D176E) which was reported previously to be a polymorphism. One of us (LM) also identified D176E in one NF1 patient carrying another clearly pathogenic alteration and in one of 190 normal control samples, confirming that D176E is indeed a rare benign variant. We do not understand why the authors list a patient carrying this sequence change in table 2, as in their table 3 they state that this alteration is a polymorphism also found in unaffected individuals. Moreover, they list a patient carrying the missense alteration c.2617C→T (R873C) in table 2, while at the same time state in table 3 that this also is a benign variant they found in a patient who carries a clearly pathogenic mutation c.1-14_74del122p.

Nevertheless, the authors mention both alterations in table 2 and these data, as well as the formerly mentioned misclassified silent change, make it difficult to come to the conclusion that the technique has an 89% detection rate.

Y489C (c.1466A→G) is one of the most frequent recurrent mutations in NF1 patients, and was the first well understood genetic truncating mutation as well as a silent mutation that could be misclassified as a missense mutation if only genomic DNA was studied. This mutation results in the creation of a perfect novel splice donor instead of the wild-type exon 10b donor, and leads to skipping of the last 62 nucleotides of exon 10b. Y489C has since been reported in many families and was the first clearly pathogenic mutation that could be identified. It cannot be considered a purely missense mutation either: Ars et al reported a splice effect in five patients. We observed this splice effect in three unrelated
patients as well (Messiaen LM et al, unpublished results): the observed splicing error is readily understood by the creation of a novel splice acceptor site by this mutation, leading to skipping of the first 41 bp of exon 12b. Recalculating the number of putative missense mutations after subtraction of the above mentioned misclassifications, table 2 of the Mattocks et al paper still contains 12 different missense mutations or small deletions of one or two amino acids that affect 15 patients fulfilling the NIH diagnostic criteria—that is, H31R, L143P, E337V, C324R, L352P, S574R, L844P, R1276G, R1276Q, AE1438, MI1658-9, and ANF2366-7. Hence as many as 16.5% of the patients fulfilling the NIH diagnostic criteria (15 of 91) harbour putative missense mutations and small deletions as deletions of one or two amino acids that affect 15 patients fulfilling the NIH diagnostic criteria (15 of 91) harbour putative missense mutations and small deletions as described. This number appears quite high in comparison with previous reports with high mutation detection rates.1–4 Thus it may very well be that a portion of the novel missense mutations found in the study by Mattocks et al are splicing mutations. The authors are aware of this possibility and have developed tools such as a functional splicing assay using a mini-gene system to test for the effect on splicing. We do not understand why they did not apply these tools in this study to achieve a conclusion on the effect of silent and missense mutations. Furthermore, some of the novel missense mutations—such as D176E, R873C, and A2058D—may turn out to be non-pathogenic rare sequence variants. Indeed, even after analysing a large number of unrelated control samples, the putative missense mutations and small dele-
tions found in the study by Mattocks et al do not withstand a critical review of the data provided. We believe it is not justified to draw conclusions on detection rates of the assay presented here unless the pathogenicity of the novel sil-
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