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► **To cite this version:**

Sally Halsall, Gemma Thornton, Adeline Nicholas, Howard Martin, Christopher Woods. Critical consequences of finding three pathogenic mutations in an individual with recessive disease. *Journal of Medical Genetics*, 2010, 47 (11), pp.769. 10.1136/jmg.2010.079277 . hal-00557401

HAL Id: hal-00557401

<https://hal.science/hal-00557401>

Submitted on 19 Jan 2011

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**Communication to Journal of Medical Genetics:
Critical consequences of finding three pathogenic mutations in an individual
with recessive disease**

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We report the unexpected finding of three different nonsense mutations in two unrelated individuals with a classical autosomal recessive disease; that is one in which two pathogenic mutations in trans are expected. This finding of 'triple pathogenic mutations' appears to be a rare phenomena¹, for which we can find no guiding literature for incidence, mechanism or consequence; however they have been reported previously in the recessive disorders Insensitivity to Pain with Anhidrosis due to TRKA mutations (OMIM 256800) and Gaucher disease due to GDI mutations (OMIM 230800). For mutation analysis in research or clinical settings such findings can have significant implications, whilst for genetic counselling the consequences can be profound.

The two individuals we report both had a clinical diagnosis of autosomal recessive primary microcephaly (MCPH), a disorder characterised by congenital microcephaly and mental retardation^{2,3}. MCPH is genetically heterogeneous and can be caused by mutations in at least five genes⁴. Fortunately for clinical practice 2/5ths of cases are explained by mutations in the gene *ASPM*⁵. To date all definitive mutations found in *ASPM* have been nonsense and predicted to lead to nonsense mediated decay of the

ASPM message – the exception being a large intragenic deletion in a Sicilian family^{3,5-8}.

During 2009 thirty-six possible MCPH cases were referred to us and analysed in an NHS Molecular Genetics laboratory. We found *ASPM* mutations in six; a detection rate of 16%. However, in two cases we found three different nonsense mutations. In the first: c.3796G>T (p.Glu1266X), c.7569_7570delAA (p.Gln2523fs) and c.10060C>T (p.Arg3354X), and in the second: c.3977G>A (p.Trp1326X), c.4184G>A (p.Trp1395X) and c.6994C>T (p.Arg2332X). We subsequently assessed the parents and found that in each case one parent carried two of the nonsense mutations. For case 1 a parent carried in cis c.3796G>T in exon 16 and c.10060C>T in exon 26. And for case 2 it was c.3977G>A in exon 17 and c.6994C>T in exon 18. From this we concluded that *ASPM* haplotypes exist bearing two separate nonsense mutations in cis.

Having found these two instances of triple pathogenic *ASPM* mutations, we were surprised this had not been reported previously. Our own research strategy for *ASPM* mutation detection was guided by reporting of few recurrent mutations, and no mutation hot spots. Based on this, every exon of *ASPM* would be sequenced and the data analysed sequentially, starting from the first exon and proceeding until two mutations were found. This means that any potential third nonsense mutation may have evaded detection if it lay in an unanalysed exon. On retrospective review, we had sequenced *ASPM* in 84 research index cases with a clinical diagnosis of MCPH. In 31 cases all exons were sequenced and no mutations found. In 53 cases two mutations in *ASPM* were identified, and in 12 of these not all exons had been analysed. However, upon analysis of the remaining exons no additional mutations were found.

To calculate the incidence of triple mutations in *ASPM* in MCPH, we amalgamated our service and research data. There were a total of 59 confirmed cases of MCPH with *ASPM* mutations, 17 in non-consanguineous families, 42 in consanguineous (10 of which were recurrent mutations on the same haplotype). Therefore there were 66 (17*2 + 32*1) *ASPM* mutation bearing haplotypes, of which two carried double nonsense mutations, so giving an incidence of 3%.

We considered how two separate pathological mutations might occur on the same allele. Upon examination, all four double nonsense mutations would clearly be regarded as pathogenic and were entirely typical of other reported *ASPM* mutations in MCPH. Positional analysis of all mutations showed that they were spread throughout the gene and whilst four were C>T transitions, *ASPM* has a cytosine/guanidine content typical of coding genes ($4,037/10,887 = 37\%$) suggesting excess cytosine methylation misrepair was unlikely to be contributory. In addition, like most *ASPM* mutations, all four were novel, previously unreported mutations. This is of importance as we had considered that the size of the *ASPM* gene, exceeding 10.8Kb of coding sequence, may have contributed to the occurrence of double mutations. A haplotype of a large gene should be more likely to accrue a second mutation than that of a small gene. Additionally, older mutations might be expected to be more widespread in populations and hence should be more likely to be multiply inherited. However, such second mutations would be expected to arise on older haplotypes, and as all of the disease mutations are novel, we have no evidence supporting this. The presence of an untranslated *ASPM* pseudogene would allow two further possible mechanisms for the presence of multiple mutations. As pseudogenes are not subject to evolutionary pressure they can accumulate multiple mutations. Sequencing primers which are not specific for the translated gene may return sequence from a pseudogene which contains identical or similar primer binding sites. This has been well described for the *NF1* gene where the presence of at least 3 pseudogenes considerably complicates the molecular diagnosis⁹. Alternatively recombination with the pseudogene may introduce multiple mutations from the pseudogene into the *ASPM* gene. The molecular diagnosis of Gaucher disease and Congenital Adrenal Hyperplasia is affected by the presence of pseudogenes for the *GBA* gene and *CYP21* respectively^{10,11}. However, we have no evidence to support the presence of an *ASPM* pseudogene.

For genetic counselling the identification (or overlooking) of 'triple pathogenic mutations' has important consequences. Where all three mutations are found in an index case, individuals carrying a haplotype with two of the three pathogenic mutations could be wrongly assigned as 'affected'. This could apply to a parent, but

more likely a sibling. Prenatal diagnosis requires the identification of all three mutations.

However, a much more dangerous situation could occur where incomplete sequencing means only two of three mutations are found in the index case. Consequently, if one parent has both “discovered” mutations, then the affected child may be falsely designated as having uniparental heterodisomy. Alternatively, if one parent is unavailable for testing, and the remaining parent carries neither of the detected mutations, non-paternity may be invoked and an erroneous low recurrence risk given. More importantly, unaffected siblings may be wrongly ascribed as ‘affected’ if they have both detected mutations, or falsely ascribed a ‘non-carrier’ if they have neither detected mutation but do carry the undetected mutation. This would be a particular problem in prenatal diagnosis.

It is unclear how common triple pathogenic mutations are, but even if they only comprise 1% of cases it is essential that they are not overlooked. We suggest that research teams should ensure that a report that states ‘all exons sequenced’ also means ‘all exons analysed’. Moreover, clinical teams should not only continue to confirm research findings in a proband but should always check that segregation is correct, meaning that each parent carries one pathogenic mutation. This simple approach will ensure that the finding of ‘triple pathogenic mutations’ in a recessive disease does not cause clinical problems of misdiagnosis.

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Competing Interest: None declared.

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