

**Title: Diagnosis of uncertain significance: can next generation sequencing replace the clinician?**

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## Abstract

New sequencing technologies are revolutionising disease gene discovery and testing with tremendous benefits for the diagnosis of rare diseases. Yet, the more we sequence, the more we discover and the challenge is to carefully assess the numerous variants in the clinical and genetic context to establish the correct diagnosis. Clinician and geneticist must work together for this, as failure to do so can result in incorrect advice with potentially serious consequences.

Advances in genetic sequencing technology dramatically facilitate large scale diagnostic testing and can thereby transform medicine. Previously, diagnosis of rare inherited diseases was the domain of the expert clinician, who with intimate knowledge of the suspected disorders could perform the relevant clinical assessments and thereby establish -or refute- a diagnosis. While these experts are still most likely to request genetic testing, in principle anybody, including the patient, can send off a blood sample for unbiased testing by next generation sequencing (NGS), such as whole exome (WES) or even whole genome sequencing (WGS). This obviously has enormous advantages, especially for patients with ultrarare or previously unrecognised disorders, in whom a clinical diagnosis may have been elusive. In a cohort of adults with chronic kidney disease, WES established the molecular cause for a previously undiagnosed condition in 23% and changed the clinical diagnosis in 11%.<sup>1</sup> Not surprisingly, NGS has also dramatically accelerated the discovery of new disease genes.

But, as with any “disruptive” technology, there are also downsides and it is important that clinicians, faced with an increasing number of genetic results, are aware of the need to critically interpret identified variants, before linking them to a patient’s phenotype. A report by Jobst-Schwan *et al.* in this issue of *Kidney International* on genetic diagnosis in distal renal tubular acidosis (dRTA) is a perfect example of both the advantages, as well as the problems encountered with NGS.<sup>2</sup> Currently, in approximately 60-70% of paediatric dRTA cases, the clinical diagnosis can be genetically confirmed, suggesting the existence of yet undiscovered disease genes.<sup>3</sup> Jobst-Schwan *et al.* investigated patients from 17 families with paediatric onset dRTA and identified variants in the known disease genes *ATP6V0A4*, *ATP6V1B1*, *SLC4A1* and *WDR72* in 12 of these (71%). In addition, they identify a homozygous variant in *ATP6V1C2* in one patient. This gene, like *ATP6V0A4* and *ATP6V1B1*, encodes one of the subunits of the proton pump in the intercalated cells of the collecting duct and had been previously

investigated in patients with dRTA.<sup>4</sup> Although no mutations were found at the time, the association of *ATP6V1C2* with dRTA makes perfect sense. So, why do Jobst-Schwan *et al.* call it a “likely candidate” instead of “disease gene”?

With NGS, the main problems are related to the sheer number of variants: every individual has an estimated 4-5 million variants, of which about 100,000 are rare (<0.5%).<sup>5</sup> The challenge is to sift through this enormous number of variants and identify any that are likely to be relevant for the patient’s phenotype. Previous studies have shown that over a quarter of published “disease-causing” variants were either common polymorphisms or lacked direct evidence for pathogenicity.<sup>6</sup> This, obviously, is an enormous problem, as a false genetic diagnosis may result not only in erroneous prognostic and therapeutic advice, but also confound reproductive counselling, such as with regards to termination of pregnancy or pre-implantation genetic diagnosis. Consequently, a reasonable level of certainty is needed to establish a genetic diagnosis and strict guidelines for the interpretation of genetic variants have been developed. The American College of Medical Genetics (ACMG) identified a number of criteria to facilitate variant classification as either benign, likely benign, uncertain significance, likely pathogenic or pathogenic.<sup>7</sup> The criteria themselves are graded as either supportive, moderate, strong or very strong evidence and a combination of these must be met, before a variant can be classified as (likely) pathogenic and therefore be considered diagnostic. Importantly, the ACMG specifically cautions against using these criteria for variants identified in candidate genes, or, as they call it “genes of uncertain significance” (GUS).<sup>7</sup> For acceptance of a new disease gene, several patients with matching phenotype and deleterious variants should be identified. Let us examine these issues with respect to *ATP6V1C2* as reported by Jobst-Schwan *et al.*:

*Number of patients:* Current guidelines consider a minimum of 3 unrelated patients as moderate evidence for gene pathogenicity<sup>8</sup>, yet only a single patient with a homozygous variant in *ATP6V1C2* is presented. Virtually everybody carries biallelic protein-altering variants.<sup>9</sup> Consequently, the identification of a homozygous variant does not constitute proof of pathogenicity, even if found in a gene with potential relevance to the phenotype. Indeed, Jobst-Schwan *et al.* demonstrate this with the finding of a homozygous variant in *SLC4A2*, which they appropriately dismiss as non-pathogenic.<sup>2</sup>

*Phenotype:* For this, clinical input is absolutely essential. In research studies, such as the 100,000 genome project (<https://www.genomicsengland.co.uk/about-genomics-england/the-100000-genomes-project/>), multidisciplinary conferences involving genetic scientists and clinicians are mandatory to interpret identified variants in the clinical context. Unfortunately, clinical details provided by Jobst-Schwan *et al.* are limited. The patient reportedly had hypokalaemic acidosis with elevated urine pH, which is typical for dRTA, but can also be seen in proximal RTA once treatment with alkali has been started. More concerning, untreated dRTA results in hypercalciuria, which in turn is associated with nephrocalcinosis in more than 90% of cases.<sup>10</sup> Surprisingly, the reported patient had neither! Another characteristic feature of dRTA is the ability to normalise all biochemical abnormalities by alkali supplementation, which, if achieved, provides further diagnostic evidence. Unfortunately, no data on treatment response are provided. Lastly, while chronic kidney disease is common in dRTA, end-stage kidney disease (ESKD) is not. In a cohort of 340 dRTA patients up to the age of 70 years, not a single one had ESKD.<sup>10</sup> And yet, the reported patient died of “renal failure” at the age of 9 months! Therefore, based on the clinical data provided, it is difficult to confidently ascertain a diagnosis of dRTA. It is of course possible, that

mutations in *ATP6V1C2* cause a previously unrecognised phenotype including RTA and ESKD; or that the patient suffered from two distinct disorders, of which only one is explained by *ATP6V1C2*. But it is also possible that the variant is completely incidental to the patient's phenotype.

*Deleteriousness of the variant in ATP6V1C2.* ACMG criteria are typically used for variant interpretation.<sup>7</sup> As *ATP6V1C2* is a GUS, only few can be applied, summarised in Table 1. Jobst-Schwan *et al.* state that the variant is likely pathogenic, but do not list the criteria for it. In our analysis, the evidence is conflicting: *in silico* tools and the structural modelling all predict the change to be deleterious, therefore the PP3 criteria can be applied at a supporting level. Yet, there are 205 missense variants observed in the Genome Aggregation database (gnomAD; <https://gnomad.broadinstitute.org/about>) versus the expected 241.2 (from gene size) therefore the gene is not constrained against missense variants ( $z=0.25$ ) and the PP2 criteria does not apply.

The reported functional studies in a yeast model also support pathogenicity of the variant. However, judgement needs to be applied as to what level of evidence this supplies. As this is not a validated assay which has been reproduced in a clinical diagnostic lab setting and it was not performed using patient samples, PS3 at a supporting level is appropriate.

Conversely, when analysing population data, we find evidence against pathogenicity, as the variant has been reported in 232 heterozygotes in the gnomAD database, with an overall frequency of 0.00082. While this is a rare variant, the frequency is higher than expected: assuming Hardy-Weinberg equilibrium, the frequency of individuals homozygous for the variant is  $0.0008^2$  or 0.00000064. Therefore, in the US alone (population of >300 million), we expect more than 200 homozygous individuals. Even more concerningly, the variant is ten

times more frequent in the African population (the reported patient is from Egypt). Thus, if this variant was pathogenic, we would expect the frequency of dRTA in Africa *from this variant alone* to be ~1:15.000, or about six times higher than the usually assumed incidence of dRTA from all genetic causes. Consequently, population data strongly argue against pathogenicity of the variant and are consistent with benign evidence criteria BS1 (allele frequency is greater than expected for disorder). It is theoretically possible, that Hardy-Weinberg does not apply and that no other homozygote individuals have so far been identified because of an association with infantile lethality, as observed by Jobst-Schwan *et al.*. In that case, investigations into infantile renal failure might be more likely to identify further cases rather than dRTA, highlighting the uncertainty over a potentially associated phenotype.

None of the other 28 ACMG criteria can be applied as this is the first report of a possible pathogenic variant in this gene and the variant was inherited from both parents. Therefore, the criteria for likely pathogenic or likely benign are not met and it must be classified as a variant of uncertain significance (VUS).

In summary, Jobst-Schwan *et al.* harness the power of NGS to confirm the diagnostic yield of genetic testing of around 70% in paediatric onset dRTA and further confirm the importance of *WDR72* as a dRTA disease gene. But, with respect to *ATP6V1C2*, they report a variant of uncertain significance in a gene of uncertain significance in a patient with uncertain phenotype. This is why *ATP6V1C2* remains a “likely candidate gene”. Arguably, it is less likely a dRTA disease gene now than when it was proposed as such in 2002<sup>4</sup>, considering that in all these years neither convincing mutations have been reported, nor even an animal model, such as a knock-out mouse. Consequently, no clinical decisions should be based on the discovery of variants in *ATP6V1C2*. The report confirms the strength of NGS in identifying

molecular causes of rare diseases, but also the challenges in variant interpretation. It highlights the critical importance of multidisciplinary teams involving the expert clinician and genetic scientist to carefully interpret genetic variants in light of the clinical phenotype. While NGS facilitates making a diagnosis of an inherited disease, it is unlikely to replace the clinician in the foreseeable future.



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**Table 1: ACMG criteria applicable for the variant c.503T>C, p.(Ile168Thr) in *ATP6V1C2***

Code	Strength	Category	Criteria met?
<b>Evidence of pathogenic</b>			
PS3	Strong Moderate Supporting	Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product. Note: Functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established	Yes, at supporting level
PP3	Supporting	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)	Yes
PP2	Supporting	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease	No
<b>Evidence of benign</b>			
BS1	Strong	Allele frequency is greater than expected for disorder	Yes

Shown are the applicable ACMG criteria for the variant in *ATP6V1C2* reported by Jobst-Schwan *et al.*. Since *ATP6V1C2* is not an established disease gene, only the listed criteria can be applied. There are two pieces of evidence for pathogenicity at supporting level and one piece of evidence at a strong level for benign. For classification as “likely pathogenic” at least one additional “strong” or two “moderate” criteria would be required. Conversely, for classification as likely benign, at least one additional supporting criterion is needed.<sup>7</sup> Whether a specific piece of evidence is judged as “supportive”, “moderate” or “strong” can be subjective to a degree. For instance, the functional data from the yeast model some analysts may have scored as “moderate” rather than “supportive”. Yet, while this still would not be sufficient for classification as “likely pathogenic”, key is that the evidence here is

contradictory, with the allele frequency providing strong evidence for the variant being benign. Therefore, this variant is of uncertain significance and *ATP6V1C2* is a gene of uncertain significance.