Title: Beyond NGS: Detailed Analysis of Sanger, MLPA and RNA Confirms Tuberous Sclerosis Complex in Clinically Diagnosed Patients

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Introduction:

Tuberous sclerosis complex (TSC) is a well-known genetic condition, characterized by growth of non-cancerous tumors in various organs. Despite established diagnostic criteria and molecular testing, 10-25% of clinically diagnosed TSC patients have negative genetic testing. This is attributed to deep intronic or low frequency mosaic variants that standard sequencing and deletion/duplication analysis may miss.

We report two adult female patients with clinical diagnoses of TSC but negative conventional NGS testing, who sought further evaluation to inform reproductive decision making. Utilizing MLPA, Sanger sequencing, and RNA studies, both patients had novel low-frequency mosaic variants in *TSC2,* confirming their molecular diagnoses.

Case Presentation:

Patient 1 was 18 years old and 11 weeks pregnant, with a known clinical diagnosis of TSC including rhabdomyomas, cortical tubers, renal angiomyolipomas and cysts, facial angiofibromas and learning difficulties. Microarray, TSC panel and exome sequencing were non-diagnostic. She also had congenital lymphedema and neuronal intestinal dysplasia.

Patient 2 was 30 years old and undergoing an infertility evaluation, with a known clinical diagnosis of TSC including subependymal giant cell astrocytoma, cortical tubers, renal angiomyolipomas and cysts, hypomelanotic macules, and facial angiofibromas. A TSC panel yielded non-diagnostic results.

Diagnostic Workup:

A conclusive result was identified for both patients by manually reviewing RNA, Sanger, and MLPA data following a custom cancer panel that included *TSC1* and *TSC2* plus RNA.

Patient 1 had a pathogenic variant in *TSC2,* specifically a tandem duplication of exons 32-37, detected at levels indicative of a low frequency mosaic variant. This copy gain was first detected by MLPA, and confirmed in the RNA data, where junctional reads supported tandem duplication of these exons.

Patient 2 had a pathogenic variant in *TSC2*, specifically c.690C>A p.C230*, with a low variant allele frequency of <10% in DNA-based reads. This was detected through more sensitive analysis of the NGS reads, followed by confirmation by Sanger sequencing and identification of the variant in the RNA data as well.

Treatment and Management:

Both patients were counseled about the reproductive implications of the molecular diagnosis and the low frequency variants. There were no changes in the clinical management of their TSC related symptoms.

Outcome and Follow Up:

Patient 1 declined prenatal diagnostic testing and chose to pursue postnatal genetic testing. Patient 2 brought the results to her fertility team to determine how it may impact their recommendations. Both patients were glad to have found a genetic cause after so long.

Discussion:

We present two adult, female patients with clinical diagnoses of TSC without molecular confirmation following DNA-only analysis. Additional scrutiny of MLPA, Sanger, and RNA data successfully identified low frequency pathogenic variants in *TSC2*. These data suggests that additional testing modalities beyond conventional NGS should be considered for patients with clinically diagnosed TSC who have negative DNA testing results.

An important caveat is that these low frequency variants may not be causal and there are other disease-causing alterations that were not detected due to technical limitations.

Challenges include that RNA testing requires a blood sample, whereas most DNA analyses can be performed on buccal or saliva-based samples. In addition, current RNA testing for *TSC1* and *TSC2* is performed as part of a cancer panel, and associated risk estimates for those reports are cancer-focused, which may not be the primary concern for individuals with TSC.

Conclusions:

These cases highlight the importance of including additional testing modalities, such as MLPA, Sanger and RNA analysis in the molecular diagnosis of TSC, particularly when no significant variants are identified through conventional NGS testing. By including additional testing methodologies, a greater

chance of molecular diagnosis is achieved. Successfully identifying a causative variant enhances patient care, enabling TSC patients and their families to make informed decisions regarding preventive screening, healthcare decision-making, and family-planning.