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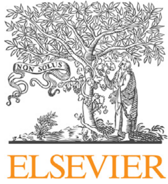

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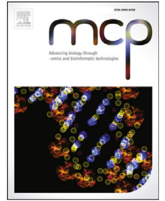

Taking the next step forward – Diagnosing inherited infantile cholestatic disorders with next generation sequencing

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Identifying rare genetic forms of infantile cholestasis is challenging due to their similar clinical presentation and their diverse etiology. After exclusion of common non-genetic causes a huge list of rare differential diagnosis remains to be solved. More than 90 genes are associated with monogenic forms of infantile cholestasis, thus preventing routine genetic workup by Sanger sequencing.

Here we demonstrate a next generation sequencing approach to discover the underlying cause in clinically well characterized patients in whom common causes of infantile cholestasis have been excluded. After validation of the analytical sensitivity massive parallel sequencing was performed for 93 genes in six prospectively studied patients. Six novel mutations (*PKHD1*: p.Thr777Met, p.Tyr2260Cys; *ABCB11*: p.Val1112Phe, c.611+1G > A, p.Gly628Trp*fs**3 and *NPC1*: p.Glu391Lys) and two known pathogenic mutations were detected proving our multi gene panel for infantile cholestasis to be a sensitive and specific method overcoming the complexity of the phenotype-based, candidate gene approach.

Three exemplary clinical cases of infants with cholestasis are presented and discussed in the context of their genetic and histopathological findings (autosomal recessive polycystic kidney disease, atypical PFIC and Niemann–Pick syndrome type C1). These case reports highlight the critical impact of integrating clinical, histopathological and genetic data during the process of multi gene panel testing to ultimately pinpoint rare genetic diagnoses.

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1. Introduction

Infantile cholestasis affects about 1:2500–5000 children. The etiology is diverse and includes obstructive (i.e. biliary atresia), metabolic, infectious, toxic (i.e. total parenteral nutrition) as well as other rare causes [1]. A relatively large proportion of infantile cholestasis [2] has always been considered to be idiopathic. However, due to the advancement in genetic testing more and more patients were retrospectively found to be highly suspicious of defined genetic or metabolic disorders [3]. Diagnosing inherited cholestatic syndromes is challenging. The differential diagnoses are numerous and range from the mild to severe manifestations requiring liver transplantation within the first years of life. Additionally many multisystemic disorders such as mitochondrial disorders, storage diseases and ciliopathies can primarily manifest as a hepatic disease in infancy with or without cholestasis. Deoxyguanosine kinase (DGUOK) deficiency for example can manifest as an isolated hepatic disease in infancy or later in childhood with progressive intrahepatic cholestasis, some cases may even emerge as a virally induced liver failure [4]. Other specific genetic causes of cholestatic liver disease in infancy include: Alagille syndrome, cystic fibrosis, galactosemia, tyrosinemia type 1, citrin deficiency, inborn errors of bile acid synthesis, alpha-1 antitrypsin deficiency, lysosomal storage diseases, acid lipase deficiency (Wolman disease), Zellweger spectrum syndrome and progressive familial intrahepatic cholestasis (PFIC) [5,6].

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Traditionally Sanger sequencing of the PFIC genes (*ATP8B1, ABCB11*) is available for infants presenting with itching, intrahepatic cholestasis and normal GGT levels. However, the percentage of underlying PFIC diagnoses in this clinically well characterized cohort is currently unknown.

Diagnosing inherited cholestasis syndromes with elevated GGT or unclear liver histology is clinically and genetically even more challenging due to the large genetic heterogeneity and the complex clinical presentation of these cases. Therefore studies have begun to try to identify the underlying genetic cause in unclear clinical presentation using DNA-chip based technology [7]. More recently one study was able to identify *TJP2* as a novel candidate gene for low GGT intrahepatic cholestasis using massive parallel sequencing [8].

In the current study we established and validated multi gene panel based diagnostics for inherited infantile cholestasis disorders, which comprises 93 associated genes. Advantages and limitations of this novel method are discussed. Three clinical cases are presented to demonstrate the clinical and genetic diversity of inherited cholestatic disorders.

2. Material & methods

2.1. Patient cohort

To validate the multi gene panel for infantile cholestasis we selected 15 samples of patients who were previously referred to our laboratory for PFIC diagnostics. All patients had already been screened by conventional Sanger sequencing for mutations in *ATP8B1*, *ABCB4* or *ABCB11* and were now re-evaluated by massive parallel sequencing. The cohort represented a total of 20 different mutations including 7 mutations in the *ATP8B1* gene, 10 in the *ABCB11* gene and 3 mutations in the *ABCB4* gene.

Prospective genetic testing with massive parallel sequencing of the established multi gene panel was performed for six children with infantile cholestasis and atypical liver histopathology or atypical clinical presentation. The discussed patients (case reports) were all seen and personally followed up by at least one of the authors. The study was approved by the ethics committee of the University of Regensburg, Germany. Informed consent was obtained from all patients/families included in this study.

2.2. Massive parallel sequencing

The multi gene panel includes 93 genes with partially overlapping phenotypes associated with inherited cholestatic disease in infancy and childhood (Supplementary material Table 1a). Included are genes associated with cholestasis in the neonatal period and infancy, hepatopathies with and without associated malformations, ciliopathies, congenital bile acid synthesis defects, peroxisome biogenesis disorders, lysosomal storage disorders, mitochondrial disease and congenital hepatic fibrosis/hepatopathies with ciliary dyskinesia. For massive parallel sequencing genomic DNA of each patient was processed according to the Nextera Enrichment protocol (Illumina, Inc., San Diego, CA, USA). Library quantification was carried out with the High Sensitivity DNA Kit on a Bioanalyzer (Agilent Technologies, Böblingen, Germany) and the Qubit™dsDNA HS Assay Kit (Life Technologies, Darmstadt, Germany). The Library was sequenced as a 150bp paired-end run on a MiSeq™ system (Illumina, Inc., San Diego, CA). All reads were aligned to the human reference genome (UCSC hg 19, NCBI build 37.1) and variant detection was performed with DNASTAR ArrayStar® (DNASTAR Inc., Madison, WI, USA) and Illumina VariantStudio (Illumina, Inc., San Diego, CA, USA).

2.3. In silico characterization of variants

The Illumina Variant Studio software was used to filter and categorize all variants that were found in the multi gene panel for infantile cholestasis. Only variants that were covered more than five times were taken into account and out of these only variants with a frequency less than 5% in the general population were analyzed further.

All mutations were screened and categorized as either previously described pathogenic mutations (VUS5), or novel sequence variants. Novel sequence variants were further evaluated by various bioinformatic programs such as dbSNP database, Exome Variant Server, Mutation Taster-2 [9], Polyphen-2, SIFT and Alamut-2 (version 2.1; Interactive Biosoftware, Rouen, France). Nucleotide and protein conservation, protein domains and position close to other known mutations were also taken into account. Splice Mutations were calculated with Alamut Splicing (including flybase and ESE finder) and BDGP (Flybase; NNSPLICE). After *in silico* characterization the sequence variants were then classified as VUS 1-5 adapted according to the algorithm suggested by Plon et al. [10] and Bauer [11]: VUS1: variant with no clinical significance (error probability <0.1%), VUS2: probably not clinically significant variant (error probability <5%), VUS3: variant of unknown clinical significance, VUS4: probably pathogenic variant (error probability <5%), VUS5: pathogenic mutation (error probability <0.1%).

2.4. Sanger sequencing

In the retrospective cohort genomic DNA of each patient was tested for potential sequence variations in the coding region and flanking splice sites of the *ATP8B1*, *ABCB11* and *ABCB4* gene by direct sequencing. To confirm sequence variations found in our massive parallel sequencing approach for infantile cholestasis Sanger sequencing of the respective variations was performed. Conventional Sanger sequencing was carried out on an ABI 3100Dx XL Avant Sequencer using the ABI Prism Big-Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacture's recommendations.

2.5. Histological analysis and transmission electron microscopy (TEM)

For histological analysis of the biopsies routine staining methods (Hematoxylin–eosin, Azan) were performed. To highlight the bile ducts a cytokeratin immunohistochemical staining was performed using a cytokeratin antibody according to the manufactures recommendations (clone OV-TL12/30; dilution 1.220; Dako, Glostrup, Denmark). In order to highlight the vascular architecture a CD34 staining was performed (clone QBEnd 10; dilution 1:100, Dako).

For electron microscopy examinations small (approx. 1 mm^3) liver, bone marrow and peripheral blood buffy coat samples were fixed in 0.1 M cacodylate-buffered Karnovsky solution (2.5% glutaraldehyde and 1% paraformaldehyde; overnight, room temperature) and postfixed in 1% osmium tetroxide (2 h) at pH 7.3; dehydrated in graded ethanols, and embedded in the EmBed-812 epoxy resin (all reagents from Science Services, Munich, Germany). After 48 h heat polymerization at 60 °C, semithin (0.8 μm) sections were cut, stained with toluidine blue/basic fuchsine and after selection of appropriate areas of interest the Epon block was trimmed for ultrathin sectioning. Ultrathin (80 nm) sections were double contrasted with aqueous 2% uranyl acetate and lead citrate solutions for 10min each. The sections were examined in a LEO912AB electron microscope operating at 80 kV in the zero-loss mode and the observations documented with a 1kx1k pixel digital

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