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Research Paper

Rapid Diagnosis of 83 Patients with Niemann Pick Type C Disease and Related Cholesterol Transport Disorders by Cholestantriol Screening☆

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ABSTRACT

Niemann Pick type C (NP-C) is a rare neurodegenerative disorder caused by an impairment of intracellular lipid transport. Due to the heterogeneous clinical phenotype and the lack of a reliable blood test, diagnosis and therapy are often delayed for years. In the cell, accumulating cholesterol leads to increased formation of oxysterols that can be used as a powerful screening parameter for NP-C. In a large scale study, we evaluated the oxysterol cholestane-3β,5α,6β-triol (c-triol) as potential biomarker for a rapid diagnosis of NP-C. Using GC/MS, c-triol has been analyzed in 1902 plasma samples of patients with the suspicion for NP-C. Diagnosis in patients with elevated oxysterols was confirmed by genetic analysis. 71 new NP-C patients (69 NP-C1 and two NP-C2) and 12 Niemann Pick type A/B patients were identified. 24 new mutations in *NPC1*, one new mutation in *NPC2* and three new mutations in the *SMPD1* gene were found. Cholestane-3β,5α,6β-triol was elevated in Niemann Pick type C1, type C2, type A/B and in CESD disease. No other study has ever identified so many NP-C patients, proving that c-triol is a rapid and reliable biomarker to detect patients with NP-C disease and related cholesterol transport disorders. It should replace the filipin test as the first-line diagnostic assay.

1. Introduction

Niemann Pick type C (NP-C) is a neurovisceral disease that is caused by an impaired intracellular transport of cholesterol and glycolipids based on mutations in the *NPC1* or *NPC2* gene (Carstea et al., 1997; Naureckiene et al., 2000). NP-C is underdiagnosed and not readily identifiable due to a variable age of onset and a variety of age-dependent symptoms. Whereas younger patients present primarily with visceral symptoms such as hepatosplenomegaly followed by progressive intellectual and neurological deterioration, adults often develop psychiatric problems, including depression and psychosis (reviewed by Patterson et al., 2012; Mengel et al., 2013). Due to the heterogeneous clinical phenotype, diagnosis is often delayed for many years or missed altogether. Since a disease modifying therapy is available (Patterson et al., 2007) and more are being developed, there is an urgent need for a reliable and robust biomarker.

Interruption of cholesterol transport leads to an increased non-enzymatic oxidation of a very small fraction of the accumulated cholesterol in NP-C cells. The oxidation products of cholesterol, called oxysterols, can be measured by GC–MS or LC–MS/MS in human plasma. It has been shown that 7-ketocholesterol (7-KC) and cholestane-3β,5α,6β-triol (c-triol), are elevated in the plasma of NP-C1 and NP-C2 patients (Porter et al., 2010; Boenzi et al., 2014; Reunert et al., 2015; Jiang et al., 2011).

In a large scale investigator-initiated study, we evaluated c-triol as a potential biomarker for the diagnosis of Niemann Pick type C disease. Using GC–MS, 1902 plasma samples of patients with the suspicion of NP-C disease, carriers of a heterozygous mutation in the *NPC1* gene and confirmed NP-C patients were analyzed.

Our data demonstrate that analysis of plasma cholestane-3β,5α,6β-triol fulfills the need for a rapid and reliable biomarker for NP-C disease and related cholesterol transport disorders, making diagnosis and early therapy of these severe neurodegenerative disorders much easier.

2. Material and Methods

2.1. Patient Consents

Informed consent according to local laws was obtained from either the patient or their legal guardians by the physician in charge.

Abbreviations: NPC, Niemann Pick type C; c-triol/cholestantriol, cholestane-3β,5α,6β-triol; 7-KC, 7-ketocholesterol; CESD, cholesterol ester storage disease; EVS, exome variant server; HGMD, Human Gene Mutation Database; ROC, receiver-operating-characteristic.

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2.2. Sampling/Collection of Plasma Samples

2 ml EDTA-blood samples from patients suspected of having NP-C disease were collected. Samples of five confirmed NP-C1 patients, treated in our hospital, served as internal quality control. In order to include heterozygote carriers of NP-C mutations in the study, samples of parents and siblings of known NP-C patients were collected as well. If arrival in the laboratory within 48 h after drawing the blood was guaranteed, the sample was sent at room temperature. Otherwise, the plasma was separated from the cell pellet and both samples were sent on dry ice. Process of sample handling after receipt of the c-triol result can be seen in a flow chart in the supplementary material (Fig. S1).

2.3. Chitotriosidase Activity

The chitotriosidase activity was measured as previously described (Reunert et al., 2015; Hollak et al., 1994).

2.4. Mutation Analysis

In all samples with elevated plasma cholestane-3β,5α,6β-triol concentration, as well as in cases with strong clinical suspicion of NP-C but normal oxysterols, confirmatory molecular genetic analysis of the *NPC1* and *NPC2* genes was performed. The coding region of NPC1 (NM_000271) and NPC2 (NM_006432) and flanking intronic sequences were amplified by PCR and analyzed by Sanger sequencing. Putative mutations were confirmed by sequencing independent PCR products. In four cases, additional sequencing of the NPC1 or NPC2 transcript was necessary to confirm splicing defects. In positive samples that did not contain more than one mutated allele for *NPC1* or *NPC2*, a genetic analysis of the *SMPD1*, *GBA* and/or *LIPA* genes was performed. Primer sequences for *NPC1*, *NPC2*, and *SMPD1*, can be found in the supplement (Supplementary Table S4), and primer sequences for *GBA* and *LIPA* are available upon request. If available, parental samples were analyzed, confirming the segregation of the mutations.

2.5. GC–MS-analysis

100 μl plasma was used to quantify the cholestane-3β,5α,6β-triol concentration. As internal standard, 10 ng of d7-cholestane-3β,5α,6β-triol (Santa Cruz) was added. Measurement of cholestane-3β,5α,6β-triol concentrations was done as described (Reunert et al., 2015). The cut-off value was 50 ng/ml. In rare cases where only serum was available, serum instead of plasma was analyzed.

2.6. Role of the Funding Source

The funding organization did not play a role in design of the study, interpretation of data, preparation and submission of the manuscript. All authors had full access to all data in the study and the corresponding author had final responsibility for the decision to submit for publication.

3. Results

Within three years (2012–2014), cholestane-3β,5α,6β-triol was measured in 1902 plasma samples of patients with suspected NP-C disease (Fig. 1). 1704 samples had a normal c-triol concentration.

3.1. Heterozygotes

Six out of 24 confirmed carriers for a heterozygous mutation in NPC1 showed an increased c-triol concentration (s. Fig. 1). Three of these samples underwent sequencing of the *NPC1* and *NPC2* genes, but only the previously confirmed heterozygous mutation was found.

3.2. Previously Identified Patients

41 blood samples were drawn from five previously identified, genetically confirmed NP-C patients. Consecutive samples from patients were drawn upon routine follow-up visits in the hospital. All patients were on miglustat treatment. The c-triol concentration in these patients was in the range of 60 to 300 ng/ml, except for the first patient (s. Fig. 1). This patient presented for the first time at our hospital at the age of ten weeks, with cholestatic jaundice and a profound hepatosplenomegaly. The initial c-triol concentrations were massively increased up to 840 ng/ml. Although organomegaly did not improve with time, c-triol concentrations decreased reaching the same range as the other confirmed NP-C1 patients.

3.3. Newly Identified NP-C Patients

80 samples of 72 different patients showed an increased c-triol concentration (s. Fig. 1). Subsequent genetic analysis in these 72 patients revealed either a homozygous mutation or two compound heterozygous mutations in NPC1 (n = 69) or NPC2 (n = 3) (s. Supplementary Table S1). Three NP-C2 patients presented similar c-triol concentrations as 69 NP-C1 patients and could not be distinguished from the NP-C1 patients by c-triol analysis. One of these NP-C2 samples was a frozen serum sample, preserved for several years. It belonged to a deceased NP-C2 patient that had already been described by Griese et al. (2010). NPC-2 details have been published (Reunert et al., 2015).

C-triol concentrations, chitotriosidase activities, filipin staining results (if available) and results of genetic analysis of all patients are summarized in the Supplementary material (Table S1).

3.4. Specificity

33 patients have been false positive for NP-C. An elevated amount of plasma c-triol was detected in all of these patients (range 53–782 ng/ml), but in 30 patients no mutation in either NPC1 or NPC2 has been found; three patients were heterozygous for one mutation in NPC1. In only two patients, a second sample was available which also revealed a c-triol concentration >50 ng/ml.

In 12 patients, additional genetic analyses revealed compound heterozygosity or a homozygous mutation in the *SMPD1* gene, identifying them as Niemann Pick type A/B patients (including two of the patients with one heterozygous mutation in NPC1). The diagnosis of the remaining patients is still unknown. In two patients with confirmed cholesterol ester storage disease (CESD) c-triol was also elevated (121 ng/ml–181 ng/ml). Analysis of the false positives for *LIPA* mutations did not reveal additional CESD patients. Further analysis of the *GBA* gene was done in two patients with a massively elevated chitotriosidase, as typically seen in Gaucher's disease, but did also not identify any mutations (see Supplement Table S3). In addition to the 33 false positive samples, there were 11 patients with an elevated c-triol concentration, where the second sample revealed normal values.

3.5. Sensitivity

In seven patients with normal c-triol concentrations, mutations in *NPC1* (n = 6) or *NPC2* (n = 1) were detected, confirming NPC disease. The chitotriosidase activity was elevated in three of these patients. Despite normal c-triol concentrations, a genetic analysis of *NPC1* or *NPC2* was performed, as these patients presented typical clinical symptoms, including ataxia, dysarthria, vertical supranuclear gaze palsy, dysphagia and psychosis (see Supplementary Table S2). In three of the seven patients, a second sample was available, which also revealed a c-triol concentration below 50 ng/ml. Two of the false negative NP-C patients showed a classical and three