Presence of Lipid Vacuoles in Kupffer Cells as a Marker for Early Detection of Niemann-Pick Disease Type C in Neonatal Cholestasis

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Abstract

Background

Early diagnosis of Niemann-Pick disease type C (NP-C) is the key for reduction of organ damage since a medical treatment is available now. However, early detection of patients with a high clinical suspicion of NP-C in neonatal cholestasis is still challenging. Plasma oxysterol studies demonstrate a relatively low specificity for NP-C in neonatal cholestatic patients. This study explores the significance of lipid vacuoles in Kupffer cells for early detection of NP-C in neonatal cholestasis.

Results

Between January 2018 and December 2020, 168 neonatal cholestatic patients with unexplained causes were underwent both liver biopsy and genetic tests, and 26 detected lipid vacuoles in Kupffer cells by CD68 staining. NP-C was diagnosed in 6 out of the 26 patients (23.1%), comparing to none of the remaining 142 neonatal cholestatic patients without vacuolar Kupffer cells ($P < 0.001$). In neonatal cholestatic patients with vacuolar Kupffer cells plus splenomegaly, the ratio of positive diagnosis of NP-C was 31.6% (6/19). Between January 2015 and December 2017, 3 additional NP-C patients were diagnosed. Of them, 1 patient underwent liver histologic studies also revealing lipid vacuoles in Kupffer cells. The 7 NP-C patients underwent liver biopsy at ages ranging from 35 to 112 days. Lipid vacuoles were only detected in a few Kupffer cells in the early disease course (about 1 month of age). The amount of vacuolar Kupffer cells increased with age, and the size became enlarged. Typical foam cells were only observed in HE sections of liver tissues obtained beyond 3 months of age when enlarged Kupffer cells with lipid vacuoles became obvious. In addition, 4 out of the 9 NP-C patients had fasting hypoglycemia.

Conclusions

Lipid vacuoles in Kupffer cells from liver biopsy can serve as a screening marker for early detection of NP-C in neonatal cholestasis.

Introduction

Niemann-Pick disease type C (NP-C) is a rare progressive and life limiting lysosomal storage disorder. It results from compound heterozygous or homozygous pathogenic variants in either of the two genes: *NPC1* or *NPC2*^[1–2]. Nearly 95% patients are caused by *NPC1* deficiency, with approximately 5% caused by *NPC2* deficiency^[3]. According to the onset age of neurological manifestations, NP-C is usually classified as visceral-neurodegenerative form (early-infantile), neurodegenerative form (late- infantile and juvenile), and psychiatric-neurodegenerative form (adult)^[4–5]. Primary manifestations of NP-C are age dependent. In early infancy, clinical manifestations are predominantly visceral, with cholestasis, hepatosplenomegaly, and (in some instances) pulmonary infiltrates^[5]. Cholestasis in majority of NP-C

patients spontaneously resolves after 3 ~ 4 months of age, while splenomegaly persists and neurological symptoms develop with age^[4, 6]. The early diagnosis is the key for reduction of organ damage since a medical treatment is available now^[7].

Newborn screening for NP-C has not been developed. Hepatosplenomegaly in an infant with neonatal cholestasis (NC) raises a suspicion of NP-C^[4]. Two plasma oxysterols, 7-ketocholesterol (7KC) and cholestane-3 β ,5 α ,6 β -triol (C-triol), are biomarkers for aiding diagnosis of NP-C^[8-9]. A diagnosis of NP-C is established if biallelic pathogenic variants are identified in either *NPC1* or *NPC2*. However, early detection of NP-C is still challenging as hepatosplenomegaly is non-specific in early infancy^[8-9]. Plasma oxysterol profiling studies also demonstrate a relatively low specificity for NP-C in NC patients^[10-11], and the method of oxysterols screening is unavailable in many centers. Sequencing of *NPC1* and *NPC2* by Sanger sequencing or next generation sequencing (NGS) is not readily available and time-consuming^[12]. Therefore, it is necessary to explore an alternative marker for NP-C which identifies patients with a high clinical suspicion of NP-C and facilitates the early diagnosis of NP-C in infants with NC.

This study explored the significance of lipid vacuoles in Kupffer cells from liver biopsy tissues for early detection of NP-C in NC and summarized the clinical manifestations of genetically confirmed NP-C patients presenting as NC.

Results

Diagnostic value of the lipid vacuoles in Kupffer cells

Between January 2018 and December 2020, 168 NC patients with unexplained causes underwent both liver biopsy and genetic tests. Kupffer cells with lipid vacuoles were detected in 26 patients, and 6 of whom were diagnosed as NP-C for harboring biallelic pathogenic or likely pathogenic variants in *NPC1* (Table 1). No patient was found to harbor biallelic pathogenic variants in *NPC2*. The ratio of positive diagnosis of NP-C was 23.1% (6/26) in NC patients with vacuolar Kupffer cells. NP-C was not diagnosed among the remaining 142 NC patients who did not detect lipid vacuoles in Kupffer cells (6/26 vs. 0/142, $\chi^2 = 33.983$, $P < 0.001$). The sensitivity and specificity were 100.0% (6/6) and 87.7% (142/162) respectively.

Of the 26 NC patients with vacuolar Kupffer cells, 19 who had splenomegaly included the 6 NP-C patients. The ratio of positive diagnosis of NP-C was 31.6% (6/19).

Table 1
Basic information of the 168 infants with neonatal cholestasis

	With vacuolar Kupffer cells (n = 26)	Without vacuolar Kupffer cells (n = 142)
Gender (male/female)	14/12	68/35
Hepatomegaly	26 (100%)	142 (100%)
Splenomegaly	19 (73.1%)	47 (33.1%) *
Age at liver biopsy (Days)	68 [46, 90]	73 [59, 100]
NP-C (<i>NPC1</i>)	6 (23.1%)	0 (0.0%) *
NP-C, Niemann-Pick disease type C.		
* $P < 0.05$.		

Vacuolar Kupffer Cells Evolve Into Foam Cells

Between January 2015 and December 2017, 3 additional NC patients (P6, P8, and P9) were diagnosed as NP-C (Table 2). Of them, 1 patient (P6) underwent liver biopsy. Hence, a total of 7 NP-C patients (P1 ~ P7) underwent liver biopsy. P1 and P2 underwent liver biopsy at the age of 35 days and 36 days respectively. Lipid vacuoles were detected in a few Kupffer cells by CD68 staining (Fig. 1). Lipid vacuoles were detected in more Kupffer cells of P3 and P4 who underwent liver biopsy at the age of 49 and 63 days respectively. P5 underwent liver biopsy at 89 days of age. Lipid vacuoles were detected in almost all Kupffer cells, and a few vacuolar Kupffer cells became enlarged. Enlarged Kupffer cells with lipid vacuoles became obvious in both P6 and P7 who underwent liver biopsy at the age of 110 days and 112 days respectively.

Foam cells were not detected in hematoxylin and eosin (HE) staining sections in 5 patients (P1 ~ P5), but were typical in P6 and P7. Characteristic foam cells resided within the liver sinusoid, and were negative by periodic acid-schiff (PAS) staining.

Table 2
Molecular findings in *NPC1* (NM_000271) of 9 patients with neonatal cholestasis

Patient	Gender	Variant 1	Variant 2	Origin
1	Male	c.1757 + 3_1757 + 6delGAGT	c.3254_3255delAT	M/F
2	Female	c.10delC	c.1211G > A (p.R404Q)	ND
3	Male	c.1024T > C (p.W342R)	c.2970_2971insTCCT	M/F
4	Male	c.3254A > C (p.Y1085S)	c.3254A > C (p.Y1085S)	F/M
5	Female	c.1138C > T (p.L380F)	c.1211G > A (p.R404Q)	F/M
6	Female	c.352_353delAG	c.2000C > T (p.S667L)	ND
7	Male	c.2207_2208dupTC	c.2972_2973delAG	M/F
8	Female	c.1421C > T (p.P474L)	c.2728G > A (p.G910S)	ND
9	Female	c.1301C > T (p.P434L)	c.3425T > C (p.M1142T)	ND
ND, not done; F, father; M, mother.				

Novel pathogenic variants are shown in bold font.

Molecular Findings And Clinical Manifestations

Of the 9 NP-C patients (Table 2), 16 distinct variants were identified in *NPC1*, including 10 known pathogenic variants and 6 novel variants (4 frameshift indels and 2 missense variants) absented from both 1000 Genomes Project (TGP) and Exome Aggregation Consortium (ExAC). Novel missense variants, c.1024T > C (p.W342R) and c.3254A > C (p.Y1085S), were predicted to be disease causing or damaging by MutationTaster, Polyphen-2 and SIFT. According to the American College of Medical Genetics (ACMG) guideline, the 4 novel frameshift indels were rated as pathogenic variants, while the 2 novel missense variants as likely pathogenic variants.

The 9 confirmed NP-C patients came from 9 nonconsanguineous families. Jaundice and hepatosplenomegaly were identified in all 9 patients (Table 3). Six exhibited acholic stools. Cholestasis was confirmed by liver function tests (LFTs). Seven patients, but not patient (P) 3 and P7, could be classified into cholestasis with high serum γ -glutamyl transpeptidase (GGT) (GGT > 100U/L). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were elevated, and the ratios of AST/ALT ranged from 2.1 to 7.5. Blood glucose levels were lower than 3.0mmol/L in 4 patients (P5, P6, P8 and P9) after fasting for 3 hours. Bone marrow aspiration was performed in 4 patients (P1, P3, P5 and P6) at ages ranging from 42 days to 113 days (Fig. 1). No foam cell was observed in all 4 bone marrow samples.

Ursodesoxycholic acid (UDCA) and fat-soluble vitamins were given in all 9 patients. P4 and P6 lost to follow-up. P3 died at 8 months of age. For the remaining 6 NP-C patients, jaundice resolved at a median

age of 4.5 months (ranging from 3 months to 9 months), but splenomegaly persisted. UDCA was stopped after jaundice resolved. At last follow-up at a median age of 15 months (ranging from 9 months to 34 months), none of the 6 patients had jaundice recurrence, but AST are still elevated in 6 patients. LFTs normalized in P9 at 17 months of age.

Table 3
Clinical information of the 9 NPC patients with neonatal cholestasis

	P1	P2	P3	P4	P5	P6	P7	P8	P9
First symptoms	J	J	J	J	J	J	J	J	J
Age at first symptoms (Days)	4	2	1	7	2	28	5	3	4
Other symptoms and signs									
Acholic stools	+	+	+	-	+	+	+	-	-
Hepatomegaly	+	+	+	+	+	+	+	+	+
Splenomegaly	+	+	+	+	+	+	+	+	+
Liver function tests (LFTs)									
Age at tests (Days)	35	33	44	62	89	53	79	97	60
TB (μmol/L)	164	168	211	141	125	287	56	158	104
DB (μmol/L)	136	102	119	108	104	229	46	117	58
ALT (U/L)	55	59	41	110	152	117	63	72	58
AST (U/L)	206	199	309	338	371	250	174	283	177
GGT (U/L)	144	254	54	147	222	126	49	259	167
TBA (μmol/L)	69	96	96	84	66	114	59	158	106
Alb (g/L)	34.6	35.0	38.4	41.8	32.2	40.2	36.3	39.7	43.3
Glu (mmol/L)	4.0	ND	6.0	4.2	2.9	2.5	4.1	2.0	1.9
P, patient; J, jaundice; ND, not done; TB, total bilirubin; DB, direct bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; Alb, albumin; Glu, Glucose.									

-, negative; +, positive.

Discussion

The diagnosis of NP-C is often delayed in NC patients because genetic tests are not readily available and time-consuming. Data from adult patients indicates that plasma oxysterol has an ideal sensitivity and specificity for NP-C screening ^[13]. However, it does not work well to identify patients with a high clinical

suspicion of NP-C in NC patients. To increase the chances of early treatment, it is vital to develop a new marker that can be applied to NC patients. This study explored the significance of lipid vacuoles in Kupffer cells for early detection of NP-C in NC. We found that lipid vacuoles were detected in Kupffer cells by CD68 staining in all NP-C patients, even as early as at 35 days of age. The ratio of positive diagnosis of NP-C was 23.1% in NC patients with vacuolar Kupfer cells. In fact, other disorders also contribute to the formation of lipid vacuoles in Kupffer cells, such as acid sphingomyelinase deficiency ^[14]. Although the presence of lipid vacuoles in Kupffer cells is not specific for NP-C, it may serve as a marker for early detection of NP-C in NC with a high sensitivity.

Differentiated from vacuolar Kupffer cells, foam cells were only detected in 2 (28.6%) of the 7 liver tissues obtained at ages ranging from 35 days to 112 days. According to the published date, about 37%-50% pediatric patients with NP-C can have detectable foam cells in liver tissues ^[15-16]. It is believed that liver foam cells develop with age and can only be detected in older infants ^[15]. In current study, we found that lipid vacuoles were only detected in a few Kupffer cells in the early disease course (about 1 month of age). The amount of vacuolar Kupffer cells increased with age, and the size became enlarged. Typical foam cells were only observed in HE sections of liver tissues obtained beyond 3 months of age when enlarged Kupffer cells with lipid vacuoles became obvious. These indicate that Kupffer cells with lipid vacuoles evolve into characteristic foam cells. It is a reasonable explanation why the presence of lipid vacuoles in liver Kupffer cells can serve as an early marker for NP-C.

Demonstration of foam cells in bone marrow adds to clinical suspicion of NP-C ^[4, 16], but it can be negative in early infancy ^[15]. It is believed that foam cells may become apparent in bone marrow as the disease evolves. In our study, foam cells were not identified in all 4 bone marrow samples obtained within 4 months of age. The diagnosis of NP-C may be missed if early bone marrow aspiration is relied on. It challenges the importance of bone marrow aspiration for the diagnosis of NP-C in early infancy.

NP-C patients presenting as NC usually have high GGT ^[17-18], but in some instances NP-C patients can present as cholestasis with low GGT (GGT < 100U/L) ^[6]. Here, we reported 2 NP-C patients manifested cholestasis with low GGT. It might due to decreased expression of P450 enzymes in NP-C patients which play an important role in the synthesis of bile acids ^[19]. Furthermore, 4 NP-C patients were found to have fasting hypoglycemia. The reasons of hypoglycemia are unclear. It may be related to mitochondrial dysfunction which has been demonstrated in fibroblasts derived from patients with NP-C ^[20]. Therefore, the levels of blood glucose should be routinely monitored in NP-C patients presenting as NC.

Conclusion

Identifying individual patients with a high clinical suspicion of NP-C is a key for early diagnosis. Lipid vacuoles are detected in Kupffer cells from liver tissues obtained from NP-C patients, even as early as at 35 days of age. Lipid vacuoles in Kupffer cells can serve as a screening marker for early detection of NP-C in NC.

Methods

Patients and definitions

This study enrolled NC patients with unexplained causes (onset < 3 months of age), who were referred to the children's hospital of Fudan University and underwent both genetic testing and liver biopsy, between January 2018 and December 2020. Cholestasis was defined as follow ^[21]: serum direct bilirubin (DB) > 20.0% of total bilirubin (TB) if TB > 85.5mg/dL; or DB > 17.1mg/dL if TB < 85.5mg/dL. Following a comprehensive work-up as described previously ^[22], surgical, infectious, endocrinological, parenteral nutrition, and drug-induced causes were excluded. Confirmed NP-C patients was defined as NC patients harboring biallelic pathogenic / likely pathogenic variants in *NPC1* or *NPC2*. Clinical data were collected by reviewing their medical records. Hepatomegaly and splenomegaly were diagnosed by ultrasonography.

For summarized clinical manifestations of NP-C patients, the sample size was expanded by also including confirmed NP-C patients presenting as NC between January 2015 and December 2017.

Genetic Testing

Genomic DNA was extracted from the peripheral blood. *NPC1* variants (NM_000271) and *NPC2* variants (NM_006432) were screened by NGS, including panel sequencing, medical exome sequencing, and / or whole exome sequencing. The procedures of sequencing, data analyses, and variation classification were described previously ^[12,23-24]. Novel missense variants were predicted by MutationTaster, SIFT and Polyphen-2. Variant pathogenicity was assessed according to the standards for interpretation of sequence variants ^[25].

Histologic Studies

Liver tissues were obtained by needle biopsy or intraoperative wedge biopsy, and were formalin-fixed and paraffin-processed for routine sections. Sections at 4 μm were routinely stained with HE, PAS, and anti-CD68 (GENE, Shanghai, China). Bone marrow aspirations were stained with Wright's stain.

Statistical analysis

Statistical analysis was performed using SPSS Inc. version 17.0 software (University of Chicago, Chicago). Difference among ratios was tested by Chi-square test using Fisher's exact value. Comparison of medians between two groups was done by nonparametric Mann-Whitney test. $P < 0.05$ was considered significant.

Abbreviations

NP-C: Niemann-Pick disease type C

NC: neonatal cholestasis

NGS: next generation sequencing

TGP: 1000 Genomes Project

ExAC: Exome Aggregation Consortium

ACMG: American College of Medical Genetics

LFTs: liver function tests

GGT: γ -glutamyl transpeptidase

ALT: alanine aminotransferase

AST: aspartate aminotransferase

UDCA: Ursodesoxycholic acid

DB: direct bilirubin

TB: total bilirubin

HE: hematoxylin and eosin

PAS: periodic acid-schiff

Declarations

Ethics approval and consent to participate

The ethics committees of the Children's Hospital of Fudan University approved this study. Informed consent was obtained from the parents.

Consent for publication

The parents signed the consent form.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NLW, LC, JL, and JSW: designed the work, drafted the manuscript and revised it. XBX, YL, and KA: acquisition and interpretation of data. All authors read and approved the final manuscript.

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Figures

Figure 1

Histologic studies of bone marrow aspirations and liver tissues in NP-C patients presenting as neonatal cholestasis. Kupffer cells with lipid vacuoles (black arrow) are observed in CD68 staining sections of all 7 liver tissues. Foam cells (red arrow) are observed in HE sections when enlarged Kupffer cells with lipid vacuoles become obvious. BM, bone marrow aspiration; LS, liver specimens; P, patient.