Identification and Evaluation of Biomarkers for Niemann-Pick Disease Type C Based on Chemical Analysis Techniques

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Abstract
Niemann–Pick disease type C (NPC) is an autosomal recessive disorder with an incidence of approximately 1/80,000 [1-3]. Niemann–Pick as a group were first defined by Crocker in 1961 and can be classified as types A to D [4]. The classification is based on the time of onset, clinical symptoms, and sphingomyelin accumulation levels in tissues. Later, Niemann–Pick disease types A and B (NP-A/B) were shown to be mutation of the acid sphingomyelinase gene [5]. However, the mutation was not found in NPC. Subsequently, cholesterol esterification dysfunction was observed in both NPC model mice and patients [6-8]. Afterwards, cholesterol esterification was shown to be normal and the transporting function of exogenous cholesterol was the abnormal factor in NPC pathology. The NPC1 gene of a model mouse with intracellular cholesterol accumulation exists on chromosome 18 [9-11]. Insertion of the wild-type NPC1 gene alleviated cholesterol accumulation in NPC cells [12,13]. Later, two types of genes in NPC patients were definitively characterized [14,15]. The NPC2 gene is not coded on chromosome 18 and the HE1 gene on chromosome 14 was identified as NPC2 [16]. Currently, the ratio of NPC1:NPC2 prevalence in NPC patients is approximately 95:5 [3]. In normal cells, cholesterol is taken

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1. General background of Niemann–Pick disease type C
Niemann–Pick type C (NPC) is an autosomal recessive disorder with an incidence of approximately 1/80,000 [1-3]. Niemann–Pick as a group were first defined by Crocker in 1961 and can be classified as types A to D [4]. The classification is based on the time of onset, clinical symptoms, and sphingomyelin accumulation levels in tissues. Later, Niemann–Pick disease types A and B (NP-A/B) were shown to be mutation of the acid sphingomyelinase gene [5]. However, the mutation was not found in NPC. Subsequently, cholesterol esterification dysfunction was observed in both NPC model mice and patients [6-8]. Afterwards, cholesterol esterification was shown to be normal and the transporting function of exogenous cholesterol was the abnormal factor in NPC pathology. The NPC1 gene of a model mouse with intracellular cholesterol accumulation exists on chromosome 18 [9-11]. Insertion of the wild-type NPC1 gene alleviated cholesterol accumulation in NPC cells [12,13]. Later, two types of genes in NPC patients were

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up through the LDL receptor on the cellular membrane and transported to each organelle via lysosomes (Fig. 1). However, deficiencies in the function of lysosomal cholesterol transporting proteins in NPC cells impair the transport of free cholesterol, resulting in cholesterol accumulation [17]. The NPC1 protein is a transmembrane transporter comprising 1278 amino acid residues [13]. The characteristic N-terminus sequence contains the cholesterol binding site and the protein is localized in late endosomes and lysosomal membranes. NPC1 is regulated by ubiquitination by cholesterol levels [18]. In contrast, NPC2 is a water-soluble secretory protein 151 amino acid long [16] with a hydrophobic pocket for cholesterol binding.

2. Clinical features of NPC

NPC induces lipid accumulation as described above; however, various symptoms in NPC patients can be observed that affect the central nervous system, peripheral nerves, and systemic organs (Fig. 2). In addition, the age of onset varies in NPC patients [1], from perinatal (Birth–2 months), early infantile (2 months–2 years), late childhood (2–6 years), young (6–15 years), and adolescent/adulthood (≥15 years). The degree of lipid accumulation is often high in young-onset cases, resulting in clinical symptoms. The most common systemic, visceral organ symptoms include hepatomegaly and splenomegaly, with fetal hydrops often present. In addition, 55% of NPC patients have dysphagia [19] and the associated lung lesions are the most common cause of NPC death, requiring tracheostomy and pharyngeal isolation treatments. These lesions form most often during the infantile period [20]. A typical central nervous system symptom is cataplexy, also referred to as acute seizures [21]. Narcolepsy may occur at the same patients [22] and 70% suffer from vertical supranuclear gaze palsy [23]. In addition, cerebellar ataxia, dystonia, dysarthria, and epilepsy can be observed in NPC patients [1]. Psychiatric symptoms are commonly observed as additional cortical symptoms of epilepsy. Patients ≥6 years old are at highest risk of developing these symptoms and 45% of patients >15 years old show these symptoms [24]. In a study regarding the psychiatric features of NPC patients, genome sequencing analysis of 250 schizophrenia patients found a total 3 NPC patients within the group [25].

After diagnosis, two gold standards are available in terms of diagnostic methods for NPC. The first is Filipin staining and the other is DNA sequencing. Filipin staining is a pathological method for diagnosing NPC by staining accumulated cholesterol using the fluorescent antibiotic Filipin in cultured fibroblasts or bone marrow foam cells [6-8,26,27]. This method requires a highly invasive skin biopsy, long cell culturing times, and complicated procedures. In some cases, this test often provides weak intensity results, producing false-negatives [27,28]. In the

DNA sequencing method, mutation analysis of the NPC1 (MIM # 607623) or NPC2 (MIM # 601015) genes are performed. Conventionally, PCR [29-31] and denaturing HPLC [32] have been used, but more recently next-generation sequencers have also been applied [33]. Hundreds of mutations have been reported to date, and novel mutations continue to be characterized [34]. However, a significant disadvantage of this method is its high cost.

In terms of NPC treatment, the only approved therapeutic drug is miglustat, which inhibits central nervous system progression [35]. After animal studies for the efficacy were reported [36,37], clinical trials showed success and the drug was approved. Improvements in neurological symptoms in children [38] and adult patients were observed, particularly the suppression of aspiration pneumonia [19,39] that contributes to the risk of death from NPC. In addition, 2-hydroxypropyl-β-cyclodextrin (HPBCD), a cyclodextrin derivative with cholesterol inclusion activity, was reported to be effective in NPC model animals [40-43]. In Japan, intravenous infusion [44] and intracerebroventricular administration [45] have been performed in several cases of NPC. A recent phase 2 clinical trial in the USA has shown effective results as well [46].

3. Introduction of diagnostic biomarkers for NPC

As mentioned above, because of the importance of early treatment and the shortcomings of conventional diagnostic methods, significant attention has been focused on biomarkers. These have the potential to be used more easily and rapidly for chemical diagnosis and screening. Many biomarkers for NPC were reported in the 2000s. All biomarkers were identified and evaluated based on chemical analyses via chromatography (LC) and mass spectrometry (MS) or tandem mass spectrometry (MS/MS), which will be outlined in this review.

4. Plasma oxysterols

The first reported biomarkers for NPC were oxysterols, which are a class of oxidized cholesterol molecules (Fig. 3). Oxysterols in NPC cells were initially observed in model rats [47], as increased oxidative stress in NPC pathology has been reported, and oxysterols are thought to be a product of this condition [48,49]. In fact, free radicals are
increased in NPC cells [50] and their antioxidant capacity is reduced [51]. Various oxysterols exist with a representative enzymatically produced example being 27-hydroxycholesterol (27-OHC). Non-enzymatically produced oxysterols include 7β-OHC, 7-ketocholesterol (7-KC), and 5β-cholestan-3β,5α,6β-triol (C-triol). 7α-OHC, 4β-OHC, and 25-OHC can be produced from two pathways, while 7α-OHC and 27-OHC are subsequently metabolized to bile acids as major cholesterol metabolites. In particular, 24S-OHC is an oxysterol that can be used as a biomarker for neurological pathophysiology [46]. In the initial clinical studies of NPC patients wherein oxysterols were measured, various oxysterols were identified and analyzed (Fig. 3). In particular, 7-KC and C-triol showed good diagnostic performance for NPC [52] as plasma 7-KC and C-triol concentrations in NPC patients were 804.1±148.5 and 358.0±164.07 ng/mL, respectively, representing 10.6 times and 9.9 times the respective concentrations of healthy subjects. From the diagnostic performance evaluation using receiver operating characteristic (ROC) analysis, the areas under the curve (AUCs) for 7-KC and C-triol were 0.9984 and 1.0, respectively, indicating that they are extremely accurate diagnostic markers. Heterozygous NPC carrier also showed significant 1.8- or 1.9-fold increases in 7-KC and C-triol, respectively. Gas chromatography mass spectrometry (GC/MS) was used as the analytical method to identify these metabolites. After extracting the lipids using the Bligh and Dyer method, saponification was performed to hydrolyze oxysterol-fatty acid esters. After adding a stable isotope labeled compound as an internal standard (IS), the analytes and IS were derivatized to their respective trimethylsilyl ethers at 60 °C for 1 h and an aliquot was taken for GC/MS analysis. The total analysis time was approximately 40 min and allowed for identification of the analytes and IS were derivatized to their respective isotope labeled compound as an internal standard (IS), the derivatives provide characteristic ions of 79 Da from the hydroxyl groups in C-triol other than the 5α-hydroxy group. For 7-KC, DMG reacted in a 1:1 ratio prior to LC/MS/MS analysis. Using a C18 column, a 8.5 min/run gradient analysis was performed using water containing trichloroacetic acid (TCA) and acetic acid as mobile phase A and acetonitrile containing TCA and acetic acid as mobile phase B with atmospheric pressure chemical ionization (APCI). As selective reaction monitoring (SRM) transitions, singly charged protonated molecules were selected as precursor ions, and DMG-derived ions at m/z 104 were selected as a product ions. After analytical method validation, plasma samples from NPC patients and controls were analyzed. At a cut-off concentration of 24.5 ng/mL for C-triol, the AUC, sensitivity, and specificity were 0.9958, 97.3%, and 100%, respectively. For 7-KC, the cutoff was set to 47.5 ng/mL, and the AUC, sensitivity, and specificity were 0.9907, 96.4%, and 100%, respectively. Other methods for analyzing oxysterols, including combinations of derivatization by picolinic acid and LC/MS/MS can be used as well [56-59]. Enzyme-assisted derivatization (EADSA), a derivatization method using an enzyme, has also been reported [60,61]. In this method, partial structures of 3β-hydroxy-5-cholestene in oxysterols are converted to a 4-cholesten-3-one via cholesterol oxidase and subsequently changed to hydrazine derivatives using Girard’s P reagent. The derivatives provide characteristic ions of 79 Da from neutral loss in MS/MS. Lin et al. succeeded in analyzing 7-KC by LC/MS/MS without derivatization [62] using ESI instead of APCI, and an ammonium adduct ion as a precursor ion. In this paper, C-triol could not be analyzed due to low ionization efficiency. This paper also reported that 7-KC was high in NP-A/B patients as well.

### 5. Abnormal chenoicoic acids

Other cholesterol metabolites biomarkers for NPC classified include chenoicoic acids and their conjugates. Alvelius et al. discovered multiply conjugated cholesterol metabolites with unusual skeletons in NPC patient urine [63]. ESI-MS and GC/MS analysis for the de-conjugates suggested that 3β-, 7β-dihydroxy-5-chenoic acid was present in the skeleton and sulfuric acid and N-acetylglucosamine (GlcNAc) as conjugate groups. Iida et al. succeeded in their total synthesis by chemical methods [64]. In addition, Kakiyama et al. synthesized 3β-sulfoxy-7β-OH-24-nor-5-chenoic acid, a nor-type compound, as a possible IS for these potential biomarkers.
[65]. Building on these studies, Maekawa et al. developed a simultaneous analytical method for the determination of 3β-sulfooxy-7β-GlcNAc-5-cholenoic acid and its glycine, taurine conjugate (SNAG-Δ5-CA, SNAG-Δ5-CG, and SNAG-Δ5-CT; Fig. 4 (A)–(C)) using LC/ESI-MS/MS [66]. For LC, a column switching system was adopted and a Shim-pack MAYI-C8 (Shimadzu GLC, Kyoto, Japan) was used as a trapping column. This column can be classified as an internal reversed phase column that is capable of removing protein and extract low weight molecules. As an analytical column, YMC Pack-Pro C18 (YMC Co. Ltd., Kyoto, Japan) was used with a 20 mmol/L ammonium acetate buffer (pH 5.5): methanol (9:1, v/v) as a mobile phase for trapping and 20 mmol/L ammonium acetate buffer (pH 5.5): methanol (1:1, v/v) as a mobile phase for analysis. The column oven was set to 40 °C and the flow rate for analysis and trapping were set to 0.2 and 1.0 mL/min, respectively. For urinalysis, the IS was added at a 1:1 (v/v) ratio to the urine specimen. Afterwards, a 50 μL aliquot was injected for LC/MS/MS analysis. The mobile phase for trapping was used for a 3 min wash. After the urine samples were washed and concentrated on the trapping column, switching valve rotation was initiated. The mobile phase for analysis was pumped from the opposite side of the trapping column with back flushing flow, and the concentrated sample at the edge of the trapping column was eluted and sent to the analytical column. Subsequently, the analytes and IS were separated on the analytical column and elutes were subjected to ESI-MS/MS analysis (Fig. 5). SRM analysis mode was used for separation and detection and SNAG-Δ5-CA and the IS were ionized as singly charged deprotonated molecules. SNAG-Δ5-CG and SNAG-Δ5-CT were ionized as doubly charged deprotonated molecules and used as precursor ions. Each characteristic fragment ion was selected as a product ion and the SRM conditions were set as: m/z 672>97, 364>433, 389>460, 455>97 for SNAG-Δ5-CA, SNAG-Δ5-CG, SNAG-Δ5-CT and the IS, respectively. The analytical method was validated and applied to urinalysis. Only two NPC patient specimens were analyzed in that study, showing higher metabolite concentrations than the healthy subjects and controls with other metabolic abnormalities. Maekawa et al. continued to collect urine samples, analyzing samples from 28 healthy subjects, 23 NPC patients, 1 cerebrotendinous xanthomatosis (CTX), 1 biliary atresia [79]. Therefore, the breakdown of cholesterol-related metabolic disorders and hepatobiliary diseases [73,74]. In 3β-hydroxysteroid-Δ5-C27-steroid dehydrogenase (HSD3B7) deficiency, a significant amount of 3-oxo-4-cholenoic acid conjugates are found in urine [75,76]. In addition, it is well-known that abnormal conjugated cholesterol metabolites can be identified by LC/ESI-MS/MS analysis. The breakdown of cholesterol-related metabolic disorders and hepatobiliary diseases [73,74]. In 3β-hydroxysteroid-Δ5-C27-steroid dehydrogenase (HSD3B7) deficiency, a significant amount of 3-oxo-4-cholenoic acid conjugates are found in urine [75,76]. In 3β-hydroxysteroid-Δ5-C27-steroid dehydrogenase (HSD3B7) deficiency, a significant amount of 3-oxo-4-cholenoic acid conjugates are found in urine [75,76]. In 3β-hydroxysteroid-Δ5-C27-steroid dehydrogenase (HSD3B7) deficiency, a significant amount of 3-oxo-4-cholenoic acid conjugates are found in urine [75,76]. In 3β-hydroxysteroid-Δ5-C27-sterol dehydrogenase (HSD3B7) deficiency, a significant amount of 3-oxo-4-cholenoic acid conjugates are found in urine [75,76]. In 3β-hydroxysteroid-Δ5-C27-sterol dehydrogenase (HSD3B7) deficiency, a significant amount of 3-oxo-4-cholenoic acid conjugates are found in urine [75,76].
homeostasis was hypothesized to cause abnormal cholesterol metabolism with abnormal metabolites acting as biomarkers in bodily fluids such as urine and plasma. Maekawa et al. investigated the MS/MS behaviors of conjugated cholesterol metabolites [80], and the MS/MS patterns of 64 cholesterol metabolites including five non-conjugates were examined. Each conjugate provided product and neutral loss ions based on the characteristics of the conjugated group. Using these patterns, the MS/MS precursor ion and neutral loss scans enabled the comprehensive analyses of target molecules. A focused metabolome analysis method for the conjugates was developed by combining HPLC and MS/MS [81]. When a mixed standard solution containing 80 cholesterol metabolites was analyzed using this method, only the targeted conjugates corresponding to every MS/MS condition were comprehensively detected. This method was subsequently applied to urine samples, where few peaks were observed in the urine of healthy subjects, whereas many peaks were observed in the urine of NPC and HSD3B7 patients. A total of 140 peaks were observed and in the urine of NPC patients, SNAG-Δ^2-CA (peak number 45), SNAG-Δ^2-CG (peak number 27), and SNAG-Δ^2-CT (peak number 28) were particularly intense peaks. As described above, 3β,7α-dihydroxy-5-cholenoic acid 3-sulfate (m/z 469.6, retention time 33.5 min, peak number 95) and 3β,7α,12α-trihydroxy-5-cholenoic acid sulfate as well as their glycine conjugates were detected in urine of a HSD3B7 patient. In the urine of the NPC patients, an isomeric peak (m/z 469.6, retention time 21.0 min, peak number 69) to peak number 95 was detected at a faster elution time than peak number 95. Two other peaks were observed with high intensity (m/z 467.6, retention time 23.4 min, peak number 73 and m/z 453.5, retention time 16.1 min, peak number 51). Further studies were required for identification of these characteristic peaks.

The chemical formula was determined by high resolution mass spectrometry, and the accurate mass of peak number 51 indicated that it was not a sulfate conjugate of a cholesterol metabolite. In contrast, C_{24}H_{38}O_{7}S and C_{24}H_{36}O_{7}S were proposed from the accurate masses of peak numbers 73 and 69, respectively, and the formulas were consistent with those of sulfate cholesterol metabolite. Considering their retention times and proposed formula, the structures of peak numbers 73 and 69 were proposed to be 3β, 7β-dihydroxy-5-cholenoic acid 3-sulfate (S7B-Δ^2-CA) and 3β-hydroxy-7-oxo-5-cholenoic acid 3-sulfate (S7O-Δ^2-CA), respectively. Next, their chemical syntheses were performed and the retention time and m/z value of the peaks of the NPC urine with the synthesized standards were compared. The two peaks were identified as S7B-Δ^2-CA and S7O-Δ^2-CA with complete matching. In summary, the two urine peaks were successfully identified as S7B-Δ^2-CA and S7O-Δ^2-CA as novel biomarker candidates for the diagnosis of NPC (Fig. 4. (D) and (E)) [82].

A simultaneous analytical method for SNAG-Δ^2-CA, SNAG-Δ^2-CG, SNAG-Δ^2-CT, S7B-Δ^2-CA, and S7O-Δ^2-CA in urine was developed using LC/MS/MS, and its diagnostic performance for NPC was evaluated [83]. Along with other previous reports [66,67,81,82], a LC/ESI-MS/MS system equipped with a column switching system was used. A C18 Capcell pak C18 BB-H (Osaka soda, Osaka, Japan) was used as an analytical column and OASIS HLB (Waters) was used as a trapping column for online pretreatment with 20 mM ammonium acetate buffer (pH 5.5) as mobile phase A and methanol as mobile phase B. The flow rates were 0.3 and 1.0 mL/min for analysis and trapping, respectively. Because the structures of S7B-Δ^2-CA and S7O-Δ^2-CA are more simple than those of SNAG-Δ^2-CA, SNAG-Δ^2-CG, and SNAG-Δ^2-CT, many interference peaks in urine were observed. Therefore, chromatographic separation with a gradient elution was essential for their accurate separation. After optimization of the LC conditions, a 60 min/run gradient elution with B (%) 35% to 55% over 50 min provided complete separation the five analytes and IS from all interference peaks (Fig. 6). Analytical method validation was also successfully achieved. Next, the urine samples of healthy subjects (N=38), NPC patients (N=28), and 10 other control disease patients, (cerebrotendinous xanthomatosis; Gaucher disease, Hunter disease, and Smith-Lemli-Opitz syndrome) were analyzed using the newly developed method. The concentrations of the five metabolites in the urine of NPC patients were compared with those of healthy

![Chromatography](image-url)
subjects, with SNAG-Δ^5-CA and S7B-Δ^5-CA showing significantly increased levels (\(P = 0.0281\) and 0.0099, respectively). In addition, all metabolites showed excellent diagnostic performance with AUC values of >0.9. In particular, S7B-Δ^5-CA exhibited an AUC of 1.0 and 100% sensitivity and specificity. Thus, it was suggested that these molecules are useful as urinary laboratory tests for NPC.

Mazzacuva et al. and Jiang et al. reported that some non-sulfate conjugated cholenoic acids in plasma could be useful for NPC diagnosis [84,85]. 3β-Hydroxy-7β-GlcNAc-5-cholenoic acid is a de-sulfated metabolite of SNAG-Δ^5-CA, while 3β,5α, 6β-trihydroxycholanoyl-glycine and its de-glycine metabolite reported by both groups are C-triol metabolites. Accordingly, these cholenoic acids and associated conjugates can be metabolized from oxysterols. However, cholenoic acid and its conjugates are superior in terms of analysis simplicity (no derivatization requirements) and sensitivity of LC/MS/MS analysis. From a biological perspective, oxysterols also exhibit various physiological actions via the liver X nuclear receptor [86-88], but its relationship with the pathophysiological mechanism in NPC remains largely unknown. Sulfate conjugations of bile acids are generally considered to be part of a detoxification system for accumulated cholesterol [89].

6. Sphingosylphosphorylcholine and glucosylsphingosine

Unlike NP-A/B, which are sphingomyelinase deficiencies, NPC is a cholesterol storage disease caused by NPC1 or NPC2 protein deficiency. However, it is well-known that sphingomyelin also accumulates in NPC [4,5,17,90,91] along with glycosphingolipids [92]. Welford et al. reported the biomarker performance of sphingosylphosphorylcholine (SPC; Fig. 7. (A)) and glucosylsphingosine (GlcSph) for NPC. These molecules are deacylated metabolites of sphingomyelin and glucosylceramide, respectively [93]. The analysis of human plasma SPC and GlcSph were performed using LC/MS/MS. For the IS, lysosphingomyelin (d17:1) (nor-SPC) and 1-b-D-glucosylphosphorylcholine (D-GlcSph), were used as they are not endogenous human metabolites. SRM transitions were monitored at m/z 465>184, 451>184, 462>282, and 460>280 for SPC, nor-SPC, GlcSph, and D-GlcSph, respectively. The analytical column stationary phase was C8 and the oven temperature was set to 55 °C. Mobile phase A was water containing 0.1% formic acid and mobile phase B was a mixture of acetone:acetonitrile (1:1) containing 0.1% formic acid pumped at a flow rate of 0.9 mL/min. The gradient program was set as follows; (B) 10% to 66% over 4 min, washing for 1 min at (B) 100%, and equilibration for 0.5 min at (B) 10%. Pretreatment was performed by solid phase extraction (SPE) using OASIS HLB (Waters). The plasma samples from 57 NPC patients and 70 healthy subjects were analyzed and the average plasma concentration of SPC and GlcSph in NPC patients was 2.8- and 1.4-fold higher than those of healthy controls. The increased levels of SPC and GlcSph in NPC patients were less dramatic than those of oxysterols and cholenoic acids. In terms of ROC analysis, SPC showed and AUC of 0.999, whereas the GlcSph AUC was 0.776.

7. N-Palmitoyl-O-phosphocholine-serine, previously referred to as lysosphingomyelin-509

A year after the report by Welford et al. [93], Giese et al. reported lysosphingomyelin-509 (Lyso-SM-509) as a SPC-related metabolite [94]. The authors analyzed plasma samples from 10 healthy individuals and 10 NPC patients to identify biomarker peaks via LC/MS/MS. Using an accurate mass spectrometer (Orbitrap XL, Thermo Fisher Scientific, Waltham, USA), Lyso-SM-509 was identified as a biomarker specific peak in NPC patient samples. However, the chemical structure of this peak was not determined and its formula was speculated to be C_{24}H_{49}N_{2}O_{7}P from the accurate mass data. The peak was named Lyso-SM-509 because the difference from SPC was the mass carbon dioxide (CO₂) and the product ion detected at m/z 184 indicated the presence of a phosphorylcholine moiety. Afterwards, quantitative analysis was performed by SRM analysis using a triple quadrupole mass spectrometer coupled to a UHPLC system. Similar LC and SRM conditions as those used by Welford et al. [93] were adopted and the SRM transition for Lyso-SM-509 was monitored at m/z 509>184. Lyso-globodiaosylsphingosine (Lyso-Gb2) was used as the IS (detected at m/z 624>282). The retention times of SPC, Lyso-SM-509, and the IS were 3.2, 3.6, and 3.2 min, respectively. Quantification was performed based on SPC and the accurate concentration of Lyso-SM-509 was not determined because of the lack of proper standard. Plasma samples from 110 NPC patients, 63 NPC carriers, 21 NP-A/B patients, 5 NP-A/B carriers, and 43 healthy patients were analyzed in the paper. The median plasma Lyso-SM-509 concentration in the NPC patients was 6.7 ng/mL (IQR, 3.4–10.3 ng/mL). For NP-A/B patients, the concentration was approximately 4 times that of the NPC patients at 29.4 ng/mL (IQR, 14.8–40 ng/mL). In healthy subjects, the determined concentration was 0.04 ng/mL (IQR, 0.03–0.06 ng/mL). At a cut-off concentration of 1.4 ng/mL, the AUC, sensitivity, and specificity values were 0.99, 91%, and 100%, respectively. In addition a correlation was observed between the C-triol and Lyso-SM-509 concentrations (\(R=0.675, \ P<0.001\)). Furthermore, a correlation between the annual severity increment score (ASIS), which is an indicator of severity, and Lyso-SM-509 (\(P=0.039\)) was observed, suggesting that Lyso-SM-509 was generated from NPC pathology. However, the structure was not characterized in the paper.
Later, the unknown peak was reported to be useful for NPC diagnostic screening [95] and immediately cited in many guidelines [3,96,97]. Multiplex analyses of Lyso-SM-509 were useful for differentiation from other lysosomal diseases [98]. The differentiation between NPC and NP-A/B [99,100] and correlations between cholenoic acids and Lyso-SM-509 have also been reported [101]. However, these reports did not use authentic standards, and are semi-quantitative studies that cannot be applied as chemical diagnostics. The presence of the unknown metabolite suggests the existence of an undiscovered pathological mechanism. Accordingly, the identification of Lyso-SM-509 remains significant and difficult issue for NPC diagnostics.

Maekawa et al. aimed to characterize the structure of Lyso-SM-509. Various methods are available for determining the structures of unknown metabolites, including mass spectrometry and NMR. However, these methods can only be performed after that the metabolites are isolated. However, this metabolite is found in relative low quantities in Npc1 (-/-) gene trap Chinese hamster ovary cells [102], an NPC model cell. Accordingly, extraction and isolation using the cell culturing method was difficult. Therefore, structural analysis was performed using various MS/MS analyses to enable the qualitative and quantitative determination of the target compound. Using normal low-energy CID conditions, product ions derived only from a phosphorylcholine group were obtained for both positive and negative ions, confounding the chemical structure determination. Therefore, a combination of derivatization reactions and LC/MS/MS analyses were performed for functional group determination. Acetylation, 7-nitro-2,1,3-benzoxadiazole (NBD) derivatization [103-105], and methylation were used for identification of hydroxyl, amino, and carboxylic acid groups, respectively. The theoretical masses of the reacted products were set as precursor ions, and all product ions were observed at m/z 184. After the reaction, the solutions were subjected to SRM analysis. In SPC that contains single hydroxy and amino groups and no carboxylic acids, acetylation and NBD derivatization proceeded, while methyl esterification did not proceed. These results agreed with the known functional groups of SPC. In contrast, when the same derivatizations were performed on Lyso-SM-509 in the serum of NPC patients, acetylation and NBD derivatization did not proceed, but methylation proceeded. In summary, the results from Lyso-SM-509 were completely opposite to those of SPC, which was considered to be a structural analog [94]. To obtain further structural information, hydrogen abstraction/attachment dissociation (HAD)-MS/MS analysis was performed [106]. Unlike normal CID conditions, this method can cleave the double bonds in lipids [107]. As a result, product ions at m/z 255.086, 271.113, and 299.100 were obtained, suggesting the presence of the partial structure of N-acyl-phosphocholine-serine. Therefore, Lyso-SM-509 was assigned as N-palmitoyl-O-phosphocholine-serine (PPCS; Fig. 7 (B)). PPCS was chemically synthesized and an authentic standard was obtained. When the HPLC retention times were compared, the peak retention times in the serum of NPC patients and the synthesized standard exactly matched in both the reversed phase and hydrophilic interaction chromatography (Fig. 8). In addition, the exact mass, HAD-MS/MS patterns, and derivatization reaction results matched completely. Thus, it was concluded that Lyso-SM-509 was PPCS [108]. From the simultaneous analysis of PPCS and SPC in the serum/plasma of 20 healthy subjects, 15 NPC, 6 other lysosomal disease, and 43 patients suspected for NPC without the NPC1 or NPC2 mutations, the metabolites in the NPC patient samples were significantly higher than the other subjects. Compared to SPC, the degree of increase in PPCS was significantly greater and a correlation between SPC and PPCS was observed (R²=0.8177, P<0.0001). This suggested that an
unknown metabolic association was operative. In addition, the in silico SRM conditions were set based on the theoretical masses, and target lipidomic analysis was performed. All peaks with fatty acids other than palmitic acid on PPCS showed high intensity in NPC patients compared to the healthy controls. Therefore, it was suggested that N-acyl-O-phosphocholine-serine groups increased significantly due to the molecular mechanism of NPC pathology. However, the metabolic pathway(s) that produce these lipids have not been elucidated, but a pathway involving N-acyl-phosphatidylserine has been proposed [109-112]. Sidhu et al. also reported the existence of these molecules around the same time [113]. In the future, it is expected that the physiological significance of these lipids and their relationship with NPC pathophysiology will be elucidated.

8. Conclusion

Herein, biomarkers for NPC were reviewed in the context of clinical diagnoses of NPC due to problems in conventional diagnostic methods and practical complications. In NPC, the lack of functional lysosomal cholesterol transporting proteins results in cholesterol accumulation. Oxyestersols, cholenic acids, and associated conjugates are abnormal metabolites that are derived from the accumulated cholesterol. These metabolites are products of the combined oxidative stress and accumulated cholesterol. As sphingolipid accumulation was originally characterized, SPC and GlcSph, which are lyso-metabolites of sphingolipids, were reported as potential biomarkers. In addition, an unknown lipid, Lyso-SM-509, identified in 2015, represented a novel class lipid “PPCS”. Its formation mechanism remains unknown and it is likely produced by an unknown molecular mechanism, as suggested by its structural determination. In the proceeding qualitative analysis to determine its structure and quantitative analysis in biological samples, analytical chemistry techniques such as chromatography and mass spectrometry, as well as organic synthesis and derivatization assisted in the characterization. In addition, to assess these biomarker molecules, it is important to consider the fundamental biochemistry and pathophysiology. Low molecular weight biomarkers are important parts of the metabolome, the lowest layer of the central dogma of biology, and sensitively reflect disease specific pathology. Thus, metabolomics may be useful for the elucidation of unknown pathological mechanisms. In the future, biomarker research for various diseases based on chromatography and mass spectrometry is expected.

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Conflict of interest

The authors declare no conflicts of interest, financial, or otherwise.

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