MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

LIMITATION OF FILIPIN STAINING TEST FOR NIEMANN-PICK DISEASE TYPE C

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Abstract: Niemann-Pick disease type C (NPC) is a progressive neurovisceral lysosomal cholesterol transportation and lipid storage disorder with a wide range of clinical symptoms. A disease such as NPC often remains unrecognized and diagnosis is usually delayed. Recently there has been some progress in diagnosing this rare disease. When clinical suspicion is present, diagnosis can be confirmed by some plasma biomarkers, molecular analysis of *NPC1* and *NPC2* genes and complemented by filipin cholesterol staining test of cultured skin fibroblasts. All of these laboratory methods have some limitations: specificity of the biomarkers is currently under investigation, genetic tests revealed variants of unknown significance, while laboratory tests, including cultured fibroblasts, are complex cell biology analyses. In this article we want to show the complexity of diagnosis of NPC and our experience with carrying out the Filipin test on cultured fibroblasts.

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INTRODUCTION

Lysosomes are membrane-enclosed cellular organelles that contain about 50 different hydrolytic enzymes with the purpose of degrading proteins, DNA, RNA, polysaccharides and lipids.[1] The main role of lysosomes is digestion of input material by endocytosis. Most lysosomal enzymes are acid hydrolases, such as glycosidases, sulphatases, proteases and esterases, which are only active in acidic pH range. Mutations of genes encoding these enzymes result in their reduced activity. There are about 50 lysosomal storage diseases known to date. Their incidence is estimated to be from 1:5,000 to 1:8,000 live-born children.[2] These diseases are characterized by progressive clinical course, while the progression rate varies depending on specific disease and can occur anytime throughout life. Clinical picture is heterogeneous and can range from mild to severe forms within one type of lysosomal storage disease.[3, 4]

Niemann-Pick disease

Niemann-Pick disease (NPD) was described for the first time by German pediatrician Albert Niemann in 1914 in a young child with hepatosplenomegaly and progressive neurological deterioration.[5] Ludwig Pick continued histopathological studies in the 1920s after death of a child with similar clinical symptoms and he suggested a new lipid storage disorder. In 1958, Crocker and Farber published a case series on patients who presented with foamy cells (lipid-laden macrophages) and increased tissue sphingomyelin.[6] Crocker later classified these disorders as Niemann-Pick diseases type A, B, and C.[7] Niemann-Pick disease type A, NPA (OMIM # 257200) is caused primarily by acid sphingomyelinase deficiency and includes patients with neurodegenerative disease

with very poor prognosis. Niemann-Pick disease type B, NPB (OMIM # 607616) is also caused by primary acid sphingomyelinase deficiency and includes patients with lung involvement, but without nervous system symptoms. NPA and NPB are caused by mutations in the *SMPD1* gene which causes acid sphingomyelinase deficiency. Niemann-Pick disease type C, NPC (OMIM # 257220) is an autosomal recessive lipidosis characterized by specific mutations in *NPC1* and *NPC2* genes with consequences in disturbed cellular transportation of cholesterol that is associated with lysosomal accumulation of unesterified cholesterol (Figure 1).[8, 14] Cholesterol belongs to the family of steroids and is produced in the human body by the liver.[9] Cholesterol is present in tissues and plasma in two forms, as free cholesterol and as cholesterol esters.[10] Free cholesterol cannot be transported into the blood stream without specific transporters,[11] low density lipoprotein (LDL) and high density lipoprotein (HDL). LDL transports cholesterol from the liver to the rest of the body and can be incorporated into the cell membrane, while HDL transports cholesterol back to the liver.[12, 13]

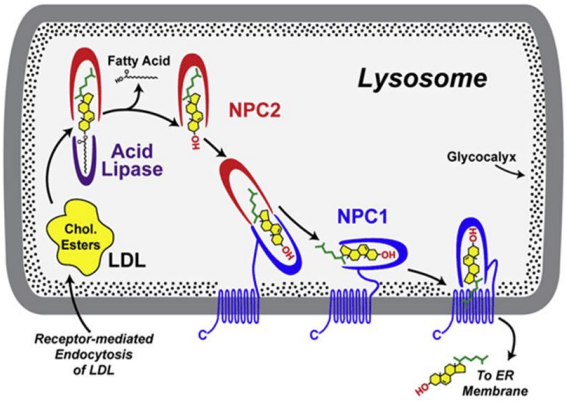

Figure 1. The NPC1 and NPC2 proteins mediate intracellular cholesterol transport from lysosomes.[14]

NPC genes

NPC1 gene was discovered in 1997. It is located on chromosome 18 (18q11-q12), contains 25 exons and consists of 1278 amino acids.[15, 16] NPC1 protein is located in the membrane of late endosomes in cells.[17] *NPC1* gene mutations cause the majority of NPC disease (~95%): so far, 104 pathogenic mutations, 65 likely pathogenic, 104 variants of uncertain significance, 16 of conflicting interpretation, 42 likely benign and 44 benign mutations have been identified in this gene.[18] A second gene, *NPC2*, was described in year 2000. It is located on chromosome 14 (14q24.3), has only five exons and consists of 132 amino acids.[19, 20] This gene is responsible only for a small group of patients with NPC (~5%). Approximately 20 mutations have been found in the *NPC2* gene to date.[21]

DISCUSSION

NPC is a progressive lysosomal storage disorder with an estimated incidence of 1:120,000 live births.[22, 23] Clinical manifestations of NPC are heterogeneous. The disease is characterized by accumulation of cholesterol and lipids in late endosomes, lysosomes and tissues such as liver, spleen and brain.[24] Due to that specific accumulation, it is necessary to examine in patients visceral (hepatosplenomegaly, isolated splenomegaly), neurological (ataxia, dystonia, cataplexia, myoclonus, seizures), ophthalmologic (problems with eye movements) and psychiatric symptoms (intellectual disability and developmental delay) in detail.[23, 25, 26]

Laboratory diagnostic strategies

Diagnosis of NPC is an integral part of a selective metabolic screening after clinical suspicion. The first step in laboratory diagnostic procedure for NPC is to use some of the available biomarkers. The most established and widely used biomarkers in plasma are oxysterols (cholesterol oxidation products) and chitotriosidase activity.[27] When the initially selected plasma biomarkers do not show a profile consistent with that of NPC, additional biomarkers should be considered, such as Lyso-SM-509.[27] If a patient under clinical suspicion of NPC disease shows abnormal results of biomarkers, genetic analysis or filipin staining test is necessary to confirm the diagnosis. However, in many countries, a confirmatory molecular genetic analysis in NPC patients is predominantly used rather than initial diagnostic procedure with some of the biomarkers.[27] Sequencing methods for *NPC1* and *NPC2* genes are becoming increasingly common as part of the routine diagnostic procedure.[23, 27] Sanger sequencing of DNA is currently the most widely used routine molecular analysis in NPC patients, but next-generation sequencing (NGS) is becoming increasingly accessible method for many genetic analysis as well.[27, 28]

Laboratory diagnosis with biomarkers, NGS and filipin cholesterol staining test in clinically suspected NPC patients has been limited in Croatia so far. Patients (brother and sister) who had NPC in Croatia were described for the first time with two disease-causing mutations of NPC1 protein (N1156S and Q922X).[29] Molecular genetic tests were made in the Department of Biomedical Sciences, Metabolic and Neuroscience Department of Biomedical Sciences, Modena, Italy. [29] The skin biopsy to determine lipid inclusions was performed at the University Medical Center, New York, while filipin cholesterol staining in cultured fibroblasts was performed at the National Institute of La Santé and the Recherché Médicale of Lyon.[29] It is important to know that one part of the NPC diagnostic test can be established in Croatia, while other confirmation tests can be requested from the co-operative laboratories.

The aim of this paperwork was to present possibility to establishing the culture of human skin fibroblasts from

a patient with NPC and implementation of filipin cholesterol staining test for the first time in Croatia.

Establishing the culture of human skin fibroblasts

The cell culture of skin fibroblasts is optimal biological material for carrying out diagnostic tests in patients suspected of hereditary metabolic diseases. Fibroblasts are cultivated in sterile conditions using plastic tissue culture flasks 1x75 cm^2 (Greiner bio-one, Kremsmünster, Austria) in RPMI-1640 Medium supplemented with 20% Fetal Bovine Serum, 1% L-glutamine and 1% antibiotic/antimycotic (Sigma-Aldrich, Gillingham, UK). Isolation and cultivation of human skin fibroblasts are performed by a routine method of skin exploration in areas that provide sterile conditions (Figure 2). During fibroblast cell culture establishment, incubation at 37°C with a 5% CO2 and 95% air atmosphere is performed. When cells reach a density of about 80%, it is necessary to trypsinize them with 5 mL of 0.25% Trypsin / 1 mM EDTA (Sigma, St Louis, USA). Before trypsinization, the rinsing the cells with Dulbecco's Phosphate Buffer Saline Ca^{+2} and Mg^{+2} Free-PBS (EuroClone S. p.A., Milano, Italy) is repeated twice by removing the rest of the medium from the bottom of flask. Subsequently, the flask with trypsin has to be placed in a CO_2 incubator (Kambič, Semič, Slovenia) for five minutes. Equal amount of cultured media is added to the flask and centrifuged at 1500 rpm / 5 min at +4°C (Eppendorf 5810R, Hamburg, Germany). After centrifugation, the supernatant is carefully removed from the cell pellet at the bottom of the tube. Next, 5 to 7 mL media are added to the tube and the content is well resuspended in order to obtain individual cells. If fibroblasts are not used in this step, they can be immediately stored in labeled cryogenic vials with a freezing medium and put in a deep freezer at -70°C overnight. For a long-term storage, cryovials with fibroblasts can be transferred into liquid nitrogen at -196°C.

Filipin staining protocol

Filipin staining in fibroblasts is the historical gold standard assay for NPC diagnosis and one of the confirmatory tests, especially in cases when clinical suspicion of NPC is present and molecular diagnosis is unavailable. The fibroblast culture is seeded in four 35x10 mm Petri dishes (BD Falcon™, New Jersey, USA). Two Petri dishes contain skin fibroblasts from a patient with confirmed diagnosis and another two control fibroblast culture from a healthy subject. In each Petri dish with non confluent fibroblasts, 2 mL of medium supplemented with 10% Lipoprotein-deficient serum, LPDS (Merck KGaA, Darmstadt, Germany) are added. Fibroblast cultures are incubated in CO_2 incubator for three days. On the last day, two media should be prepared: Medium A which contains 10% LPDS with 40 µg/mL Lipoprotein, Low density, LDL (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to maximize LDL-receptors, and Medium B which contains RPMI medium supplement with 10% human unfrozen serum. In two Petri dishes (patient and healthy control), medium A is added while medium B is added into another two Petri dishes (also patient and healthy control). All of the Petri dishes are incubated for 24 h because staining must be done on fixed fibroblasts.

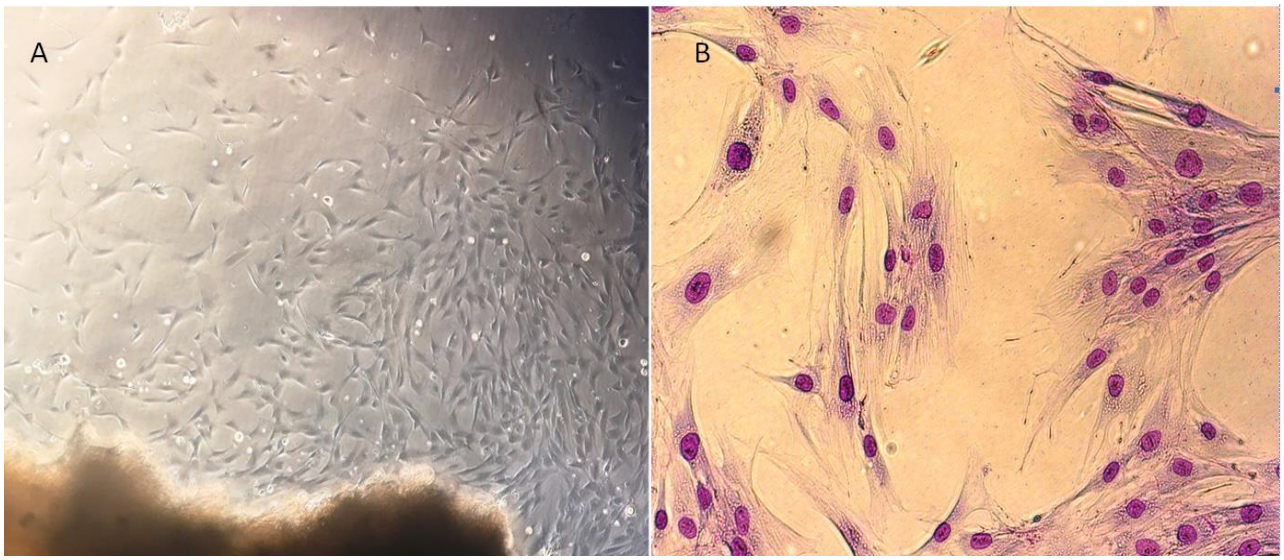

Figure 2. Establishing the culture of human skin fibroblasts from the tissue culture flasks with skin biopsy (A). The fibroblast culture visualized by an inverted microscope (Olympus IX 50, Hamburg, Germany) with 10% Giemsa stain, objective 20X (B).

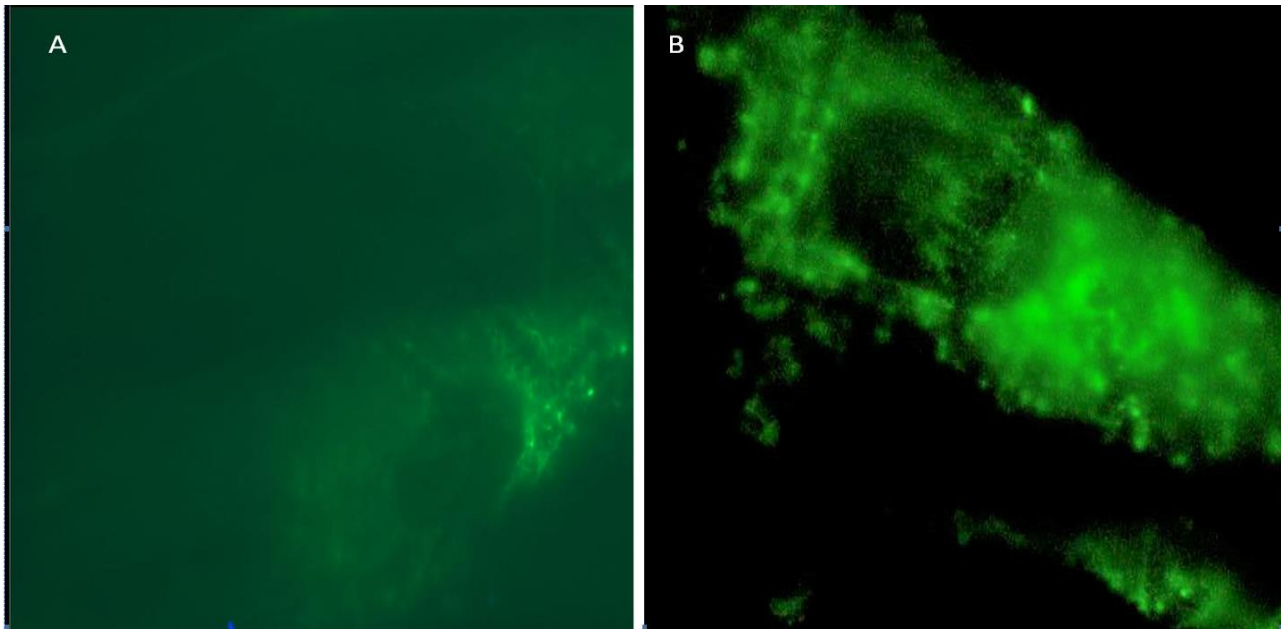

Figure 3. Filipin staining of human skin fibroblasts from a healthy individual (A) demonstrated little staining. Human skin fibroblasts from a patient with NPC (B) showed intense perinuclear cholesterol accumulation. The staining was visualized on Carl Zeiss Imager Z2.

After incubation, medium should be removed and cells rinsed twice with PBS. Before filipin staining with Filipin complex (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and fluorescence microscopy examination, fibroblasts should be fixed in 10% buffered formalin and left for 1 h in a dark place to prevent filipin from fading. Cells should be washed twice with PBS and examined under fluorescence microscope with suitable filters (Carl Zeiss, Gottingen, Germany).

More than 80% of NPC cases have a typical staining profile (*NPC1*, *NPC2* gene) which is easy to interpret and supports the final diagnosis.[27] Unfortunately, the method has its disadvantages since it is invasive (the cell culture of fibroblasts need skin biopsy), time-consuming (up to five weeks are needed for establishment of the culture and one additional week for testing), complex (laboratory technique including cell culture is demanding) and can be difficult to interpret (approximately 15% of NPC cases have variant staining profile with fewer and varied number of positive cells). Previous results show that up to 10% of cells in fibroblast cultures from healthy individuals show positive staining, while intense perinuclear cholesterol accumulation is expected in NPC patients.[27]

However, methodological variations in implementation of filipin staining and variability of patterns often make the interpretation of results difficult. Therefore, after the filipin test in the first two patients in Croatia (a brother and a sister), the diagnosis had to be confirmed by molecular analysis that showed two disease-causing mutations of NPC1 protein (N1156S and Q922X).[29]

CONCLUSION

Laboratory diagnosis of NPC patients is complex and therefore should be performed in specialized centers with experience in lysosomal storage disorders. Tandem mass spectrometry and molecular genetics technology have greatly improved laboratory diagnostics in NPC patients. Diagnostic value of biomarkers should be further investigated.

In Division for Laboratory Diagnostics of Inborn Errors of Metabolism and Newborn Screening, Zagreb, Croatia, the diagnosis of NPC is performed by filipin cholesterol staining in cultured fibroblasts. However, the results were as we expected because the fibroblasts from a patient with NPC showed intense perinuclear cholesterol accumulation while the fibroblast from a healthy individual demonstrated little staining (Figure 3). Therefore, filipin staining test was implemented in the laboratory as routine diagnostic method. The future steps regarding this method will be after implementation of filipin cholesterol staining test in routine laboratory work, the final diagnosis of NPC should be confirmed by DNA sequencing.

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