ISSN 0233–7657. Biopolymers and Cell. 2014. Vol. 30. N 5. P. 388–393 doi: http://dx.doi.org/10.7124/bc.0008B6

UDC 616-056.7-07

Specificities of Sanfilippo A syndrome laboratory diagnostics

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Mucopolysaccharidosis type IIIA (MPS IIIA) occurs due to the deficiency of lysosomal enzyme heparan-N-sulfatase (sulfamidase), which is caused by mutations in the SGSH gene. ***Aim****. To identify the characteristics of biochemical diagnosis of MPS IIIA and determine the prevalence of major mutations R74C and R245H in the SGSH gene in Ukrainian patients.* ***Methods****. After all the required phases of laboratory diagnosis of this disease, the diagnosis of MPS IIIA was confirmed in 12 patients from 12 families. The level of sulfamidase activity in leukocytes did not exceed 36 % of the control in all patients. Due to very low sulfamidase activity in leukocytes it is sometimes difficult to interpret the results of the analysis which may lead to false-negative or false-positive diagnosis of MPS IIIA.* ***Results****. The analysis of the results of molecular investigation of the patients' samples showed that the incidence of mutation R74C was 17/24 (70.8 %), the incidence of mutation R245H was 2/24 (8.3 %). The total incidence of major mutations R74S and R245H among the patients with MPS IIIA in Ukraine was the highest among European countries – 11 out of 12 patients, these mutations were found in at least one allele.* ***Conclusions****. Given very high prevalence of major mutations R74S and R245H in the SGSH gene among the patients with MPS IIIA in Ukraine – 79.1 % – it is appropriate to have the screening of mutations in the diagnostic algorithm along with the definition of biochemical sulfamidase activity to prevent false-negative or false-positive diagnosis of Sanfilippo syndrome A.*

Keywords: mucopolysaccharidosis, glycosaminoglycans, heparan-N-sulfatase, gene, genotype, allele.

Introduction. Mucopolysaccharidosis type III (MPS III) or Sanfilippo syndrome is a heterogeneous group of hereditary diseases with autosomal recessive mode of inheritance, occurring due to the decreased activity of one out of four enzymes, involved in successive breakdown of heparan sulfate [1], which leads to the accumulation of the latter in the lysosomes of cells with subsequent excretion of excessive amounts of this polysaccharide or its fragments into biological liquids of the organism. There are four subtypes of MPS III, depending on the biochemical deficiency – A, B, C, D.

According to the data of different authors, the total incidence of MPS III type is in the range of 0.28–4.1 per 100,000 live births [2–4]. The majority of patients with MPS III in European countries (about 70 %) have a biochemical deficiency, corresponding to MPS IIIA [4].

MPS IIIA is conditioned by the deficiency in the lysosomal enzyme of heparan-N-sulfatase (sulfamidase) (EU 3.10.1.1). This deficiency causes the pathological intracellular accumulation of heparan sulfate, which results in the disorder in the functioning of different organs, the nervous system, most notably. The study, involving a large group of patients with MPS III, demonstrated that the clinical course of Sanfilippo syndrome A was more aggravated compared to other subtypes, with the manifestations in earlier age, severe progression of symptoms and shorter lifespan [1].

The heparan-N-sulfatase gene – *SGSH* – is localized on the long arm of human chromosome 17 in locus 17q25.3 [5, 6]; it is 11 kbp long and has 8 exons, separated by 7 introns. The enzyme consists of 502 amino-acids and has 5 N-glycosylated sites. At present there are over 68 defined and characterized mutations in the *SGSH* gene [6–9], including 48 missence-mutations, 5 nonsense-mutations, 1 splicing mutation, 8 deletions and 7 insertions. The most frequent mutations in European populations are mutations R74C and R245H – the total incidence of these mutations varies from 56 % in Germany, Poland and the Netherlands to 33 % in Italy.

The aim of the current work was to determine the specificities of biochemical diagnostics of Sanfilippo A syndrome (MPS IIIA) and to define the incidence of major mutations R74C and R245H in the *SGSH* gene in Ukrainian patients.

Materials and methods. The material of the research was the biological material (urine and blood samples) of patients from different regions of Ukraine, who came to the Center of Metabolic Diseases of the National Children's Specialized Hospital Okhmatdyt with the preliminary diagnosis of Sanfilippo syndrome. The work was approved by the Ethics Committee of the State Institute of Genetic and Regenerative Medicine of National Academy of Medical Sciences of Ukraine. All patients have signed the informed consent form for the study. The confirmation of the diagnosis required determining the level of glycosaminoglycans (GAG) excretion in the 24-h collection urine, the availability of heparan sulfate fraction while conducting the thin-layer chromatography of glycosaminoglycans and the activity of sulfamidase in leukocytes.

The level of GAG excretion in the 24-h urine was defined using the nephelometric test with cetylpyridinium chloride (CPC-test) in equivalent to a gram of creatinine [10].

The thin-layer chromatography (TLC) of urine glycosaminoglycans was performed on Silica Gel plates of 100 μm and 8–12 μm granulosity in the system of *p*-propanol solvents: ammonium hydroxide: water (4:6:1). The alcian blue was used to stain the plates [11].

The enzymatic activity of sulfamidase was determined in periphery blood leukocytes. The leukocytes were isolated from the whole blood, received with the anticoagulant (EDTA) using the standard method [11]. The amount of protein in leukocytes was determined using the standard Lowry method [12]. The sulfamidase activity in the lysate of leukocytes was evaluated by the degradation of fluorogenic substrate of 4-methylumbelliferyl-α-D-N-sulfoglucosaminide («Sigma», USA) [11]. The activity of sulfamidase was calculated in nmol/h/mg of protein.

DNAs were extracted from whole heparinized blood using the commercial kit DNA-sorb B (CSRI of Epidemiology of the Russian Federal Service for Consumer Rights Protection and Human Welfare). The quality of DNA preparations was evaluated by the method of optical density ratio at 260 and 280 nm which was defined using Specord-40 spectrophotometer («Analytik Jena AG», Germany) [12]. DNA preparations were kept at 4 °C.

The molecular and genetic research was conducted using PCR method [13]. Oligonucleotide primers, described in the work of Bunge *et al*. 1997 [14], were used to detect the mutations R245H and R74C.

The identification of the mentioned mutations was conducted by the RFLP method (using restriction fragment length polymorphism) with the standard application of restriction endonucleases Eco52I and BstFNI, respectively [14]. The analysis of restriction products was performed by the electrophoresis method in 3 % agarose gel with subsequent staining with ethidium bromide solution in the concentration of 10 g/l.

The visualization and registration of electrophoresis results was conducted using the ultraviolet transilluminator and the videosystem with the software for image analysis Biotest-A («Biocom», Russia).

Results and discussion. In 1995–2013 386 patients with the suspected mucopolysaccharidosis were examined in the laboratory of the Center of Metabolic Diseases, NCSH Okhmatdyt.

The diagnostics of this disease at the current level requires both selective screening methods (the evaluation of the level of excretion of total GAG with urine using the CPC-test with further GAG fractioning) and the methods of confirmatory diagnostics with the determination of primary biochemical (the analysis of activity of the corresponding lysosomal enzyme) and genetic (the determination of mutations in the corresponding gene) deficiency [10, 11].

After all the required stages of laboratory diagnostics of mucopolysaccharidoses, the diagnosis of Sanfi-

Table 1
The biochemical characteristic of patients with Sanfilippo A syndrome

Patients	Age at the moment of examination, y. o.	GAG excretion with urine		Activity of heparan-N-sulfatase in leukocytes	
		Total GAG, units of CPC/g of creatinine		nmol/h/mg of protein	% of the control
		Proband	Control		
1	5	611	≤198	0.18	23
2	6	239	≤185	0.20	26
3	7	246	≤173	0.20	26
4	11	473	≤131	0.12	16
5	4	839	≤213	0.28	36
6	10	452	≤140	0.22	28
7	11 months old	747	≤280	0	0
8	4	415	≤213	0.26	33
9	4	605	≤213	0	0
10	6	280	≤185	0	0
11	4	299	≤213	0	0
12	3	343	≤228	0.20	26
Control	–	–	–	0.46 –1.1	100

N o t e. GAG fractions – heparan sulfate + chondroitin sulfate; control – only chondroitin sulfate.

Table 2
The genotype of patients with Sanfilippo syndrome A

Genotype	Number of patients
R74C/ R74C	6
R74C/ n. d.	3
R74C/R245H	2
n. d./n. d.	1

N o t e. n. d. – mutations R74C and R245H in the *SGSH* gene were not determined.

lippo syndrome was confirmed by us in 19 patients, among whom 12 had MPS IIIA, 4 had MPS IIIB and 3 had MPS IIIC.

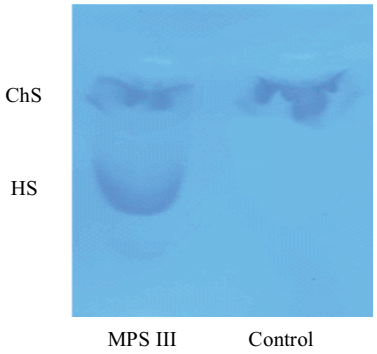
The age of patients with MPS IIIA at the moment of determining the diagnosis ranged from 11 months to 11 years, including 6 male and 6 female patients (Table 1). On average the final diagnosis of patients was set in the age of 4–6 years. All the patients were determined to have high excretion of total GAG with urine and the availability of fractions of heparan sulfate and chondroitin sulfate while conducting the thin layer chromatography of glycosaminoglycans. The level of sulfamidase activity in blood leukocytes of all the examined patients did not exceed 36 % of the control.

After the final confirmation of the diagnosis the DNA of all the patients was analyzed for the availability of the most frequent mutations R74C and R245H in the *SGSH* gene.

The analysis of the results of our molecular study of the patients with MPS IIIA demonstrated that the share of alleles-carriers of mutation R74C was the highest – 17/24 (70.8v %) (Table 2). Five patients were the heterozygous carriers of this allele and six – the homozygous ones.

The share of the alleles-carriers of mutation R245H in the patients with MPS IIIA, examined by us, was 2/24 (8.3 %). There were no patients in our group, homozygous by mutation R245H. Among our patients with genotype R74C/R245H there were two patients – compound heterozygotes.

One patient did not have any allele with mutations R74C and R245H.



GAG fractioning by the thin layer chromatography at Sanfilippo syndrome. ChS – chondroitin sulfate; HS – heparan sulfate

The specificity of laboratory diagnostics of Sanfilippo syndrome is the availability of specific changes in biochemical indices during the analysis of the level of GAG excretion with urine. This is the only type of mucopolysaccharidosis, where the isolated hyperexcretion of heparan sulfate is observed along with chondroitin sulfate, which is a normal constituent of urine GAG [1]. Due to this fact the determination of such excessive excretion allows defining the type of mucopolysaccharidosis at the stage of selective screening, but the final confirmation of the diagnosis with the determination of the primary biochemical deficiency and, as a consequence, the determination of the subtype of MPS III, is possible only after the enzymatic study.

The analysis of biochemical data, obtained by us, demonstrated that the level of the total GAG excretion with urine for all the examined patients was on the average 2.5-fold higher compared to the norm (from 1.4 to 3.9-fold). In all the cases the hyperexcretion of GAG was characterized by the presence of a considerable share of heparan sulfate which is abnormal and should not be present in the urine of a healthy individual (Figure). It allowed us to confirm the presence of Sanfilippo syndrome in 12 patients.

The determination of the subtype of MPS IIIA in all the cases was based on the reduction in activity of sulfamidase compared to the control values (Table 1). The control was defined as the blood of healthy individuals who did not have any consanguinity with the proband and were studied together with the proband and his family. It should be noted that rather low activity of sulfamidase in leukocytes at norm often complicates the interpretation of the analysis results and may lead to both false negative and false positive diagnostics of MPS IIIA.

While analyzing the level of activity of sulfamidase we did not find any age-related dependence of falling ill and the disease severity on the residual activity of sulfamidase, which is in good agreement with the results of other authors [1].

The study on genetic specificities of a certain disease and the incidence of the most common mutations among different Eastern-European populations is of great scientific and practical significance. The elaboration of a maximally efficient diagnostic program of selective screening of major mutations in patients, which allows both identifying the patients and determining the heterozygous carriers among their family members, is possible only under the condition of defining the abovementioned specificities [13].

The spectrum of mutations in the *SGSH* gene among the patients with MPS IIIA in European populations is considerably different. There are also some ethnic specificities regarding the incidence of major mutations. As seen from the data in Table 3 the mutation R74C is the most frequent in the majority of eastern European countries (Poland, Russia) whereas the mutation R245H is more frequent among the patients of south-western Europe (the Netherlands, Germany). There is a notable gradient increase in the incidence of mutation R74C from west to east while the incidence of mutation R245H, on the contrary, increases gradient-wise from east to west. It was remarkable that the total incidence of major mutations R74C and R245H among Ukrainian patients with MPS IIIA was the highest in European countries – 11 out of 12 patients had these mutations at least in one allele.

Therefore, taking into consideration a very high incidence of major mutations R74C and R245H in the *SGSH* gene among the Ukrainian patients with MPS IIIA – 79.1 % – it is reasonable to use the screening of these mutations in the diagnostic algorithm along with the biochemical determination of the sulfamidase activity in order to prevent false negative or false positive diagnostics of MPS IIIA.

Conclusions. Rather low activity of sulfamidase in leukocytes sometimes complicates an interpretation of the analysis results and may lead to both false negative and false positive diagnostics of MPS IIIA.

The incidence of major mutations R74C and R245H in the *SGSH* gene among the examined patients with

Table 3
The incidence of mutations R74C and R245C in the SGSH gene in different populations

Country	Incidence of mutant alleles of R74C, % (*)	Incidence of mutant alleles of R245H, % (*)	Total incidence, %	Reference source
Ukraine	70.8 (17/24)	8.3 (2/24)	79.1	Separate investigations
Poland	56.3 (18/32)	3.1 (1/32)	59.4	[14]
Russia	47.5 (9/21)	7.5 (2/21)	55	[15]
Germany	20.8 (10/48)	35.4 (17/48)	56.2	[14]
Austria	18.2 (8/44)	11.4 (5/44)	29.6	[16]
Great Britain	13.3 (4/30)	20 (6/30)	33.3	[7]
Italy	1.8 (1/56)	0 (0/56)	1.8	[17]
Spain	0 (0/52)	0 (0/52)	0	[18]
The Netherlands	0 (0/90)	57.8 (52/90)	57.8	[9]

*The ratio of the mutant alleles to the studied ones.

MPS IIIA in Ukraine is in good agreement with the incidence indices of these mutations among the countries of Eastern Europe (Poland, Russia) and is the highest for the European countries.

High total incidence of mutations R74C and R245H in the *SGSH* gene for the patients with Sanfilippo syndrome A in Ukraine allows using RFLP analysis of these mutations as an alternative method of differential diagnostics of MPS IIIA.

Особливості лабораторної діагностики синдрому Санфіліппо А

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Резюме

Мукополісахаридоз типу ІІІ А (МПС ІІІ А) обумовлений дефіцитом лізосомного ферменту гепаран-N-сульфатази (сульфамідази), який виникає внаслідок мутацій в гені SGSH. ***Мета****. Встановлення особливостей біохімічної діагностики МПС ІІІ А та визначення розповсюдженості мажорних мутацій R74C і R245H в гені SGSH у пацієнтів з України.* ***Методи****. Біохімічні та молекулярно-генетичні.* ***Результати****. Діагноз МПС ІІІ А підтверджено у 12 пацієнтів. У всіх обстеженних осіб рівень активності сульфамідази в лейкоцитах крові не перевищував 36 % від контролю. Аналіз результатів проведеного молекулярного обстеження пацієнтів з МПС ІІІ А показав, що частка алелів, які несуть мутацію R74C, виявилася найбільшою і становить 17/24 (70,8 %). Частка алелів з мутацією R245H у обстежених пацієнтів із синдромом Санфіліппо А дорівнює 2/24 (8,3 %). Сумарна частота мажорних мутацій R74C і R245H серед пацієнтів з МПС ІІІ А з України була найвищою з-поміж європейських країн – у 11 з 12 пацієнтів ці мутації зареєстровано хоча б в одному алелі.* ***Висновки****. Зважаючи на дуже високу частоту мажорних мутацій R74C і R245H в гені SGSH серед пацієнтів з МПС ІІІ А з України – 79,1 % – доцільно використовувати скринінг таких мутацій в діагностичному алгоритмі паралельно з біохімічним визначенням активності сульфамідази для запобігання хибнонегативної або хибнопозитивної діагностики синдрому Санфіліппо А.*

Ключові слова: мукополісахаридоз, глікозаміноглікани, гепаран-N-сульфатаза, ген, генотип, алель.

Особенности лабораторной диагностики синдрома Санфилиппо А

Н. С. Трофимова, Н. В. Ольхович, Н. Г. Горовенко

Резюме

Мукополисахаридоз типа III А (МПС III А) обусловлен дефицитом лизосомного фермента гепаран-N-сульфатазы (сульфамидазы), возникающего в результате мутаций в гене SGSH. ***Цель****. Установление особенностей биохимической диагностики МПС III А и определение распространенности мажорных мутаций R74C и R245H в гене SGSH у пациентов с Украины.* ***Методы****. Биохимические и молекулярно-генетические.* ***Результаты****. Диагноз МПС III А подтвержден у 12 пациентов. У всех обследованных лиц уровень активности сульфамидазы в лейкоцитах крови не превышал 36 % от контроля. Анализ результатов проведенного молекулярного обследования пациентов с МПС III А показал, что доля аллелей, несущих мутацию R74C, оказалась наибольшей и составляет 17/24 (70,8 %). Доля аллелей, несущих мутацию R245H, равна 2/24 (8,3 %). Суммарная частота мажорных мутаций R74C и R245H среди пациентов с МПС III А из Украины была самой высокой среди европейских стран – у 11 из 12 пациентов эти мутации обнаружены хотя бы в одном аллеле.* ***Выводы****. Учитывая очень высокую частоту мажорных мутаций R74C и R245H в гене SGSH среди пациентов с МПС III А из Украины – 79,1 % – целесообразно использовать скрининг таких мутаций в диагностическом алгоритме параллельно с биохимическим определением активности*

сульфамидазы для предотвращения ложноотрицательной или ложноположительной диагностики синдрома Санфилиппо А.

Ключевые слова: мукополисахаридоз, гликозаминогликаны, гепаран-N-сульфатаза, ген, генотип, аллель.

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Received 31.03.14