Petritsa N. A., Lukyanenko N. S., Kens K. A., Tretyak B. I. Effect of undifferentiated connective tissue dysplasia on the development of vesicoureteral reflux in infants. Journal of Education, Health and Sport. 2017;7(10):186-197. eISSN 2391-8306. DOI http://dx.doi.org/10.5281/zenodo.1101092
http://ojs.ukw.edu.pl/index.php/johs/article/view/5110

The journal has had 7 points in Ministry of Science and Higher Education parametric evaluation. Part B item 1223 (26.01.2017).
1223 Journal of Education, Health and Sport eISSN 2391-8306 7

The authors declare that there is no conflict of interests regarding the publication of this paper.
Received: 10.10.2017. Revised: 27.10.2017. Accepted: 30.10.2017.

UDC: 616.62-009.1-018.2-007.17-053.4

EFFECT OF UNDIFFERENTIATED CONNECTIVE TISSUE DYSPLASIA ON THE DEVELOPMENT OF VESICOURETERAL REFLUX IN INFANTS

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Abstract

Introduction. Taking into consideration annual increase in the number of patients, the problem of congenital malformations of urinary organs, especially in infants diagnosed with vesicoureteral reflux, is becoming increasingly relevant.

One of the main underlying causes of congenital malformations is the microanomalies of the internal organs, which can develop and enlarge depending on the peculiarities of connective tissue structure so they are regarded as undifferentiated connective tissue dysplasia. Polymorphism or mutations of a large number of genes in different combinations play the role in the occurrence of this pathology, as well as the effect of various environmental factors.

The objective of the study was to establish the possible associations between the peculiarities of the distribution of polymorphic variants of *GSTM1* and *GSTT1* genes and

rs565470 of a type IV collagen *COL4A1* gene polymorphism with undifferentiated connective tissue dysplasia and vesicoureteral reflux in children.

Materials and methods. We examined 49 children with II-IV stage vesicoureteral reflux, 15 children with pyelonephritis and 42 children who were systemically well. Oxiprolin was detected in the urine and the determination of the genotypes/alleles of the polymorphic locus rs565470 of *COL4A1* gene was carried out, as well as the presence of the deletion alleles of *GSTM1 0/0* and *GSTT1 0/0* genes in the genotype of the child by Restriction Fragment Length Polymorphism method from the blood.

Discussion. The carrier rate of *GSTM1 0/0* zero-allele in the genotype was determined in 51.4% of the examined children, which is associated with its predisposition to the inflammatory process of the kidneys. The carriage of the combination of *GSTT1 0/0 + GSTM1 0/0* zero alleles was found in 22.6% of children and it is associated with the presence of undifferentiated connective tissue dysplasia and the tendency to vesicoureteral reflux. Following the genotyping of rs565470 of COL4A1 gene polymorphism in children with vesicoureteral reflux, compared with healthy controls, it was found that the CT genotype with the given polymorphism increases the risk of vesicoureteral reflux in infants by a factor of three (OR = 3.00, CI 95%: 1.08–8.37), and in the presence of this genotype in a child, the risk of developing undifferentiated connective tissue dysplasia, as well as vesicoureteral reflux, increases sevenfold (OR = 6.75; CI 95%: 1.40–32.55).

Conclusions The obtained results allow assuming the association of rs565470 of COL4A1 gene polymorphism with the formation of undifferentiated connective tissue dysplasia and the development of vesicoureteral reflux in infants.

Keywords: **infants, vesicoureteral reflux, undifferentiated connective tissue dysplasia.**

Introduction

One of the most important social problems among the population of Ukrainian society is the issue of children's disability. According to Ukrainian Institute of Strategic Studies of the Ministry of Health of Ukraine "Annual report on the population’s health, sanitary and epidemiological situation and the results of activities of health care system of Ukraine. 2015"

more than 14 thousand children have been registered with congenital malformations of urinary organs (CM UO), and 3.5% of them are already supported by social aid.

The vesicoureteral reflux (VUR) is one of the most common CM UO in infants and it is diagnosed in 1.0-2.0% of the pediatric population, and its rate reaches 70.0% in combination with infections of the urinary system [1].

According to T.I. Kadurina (2016), one of the main underlying causes of congenital malformations is the microanomalies of internal organs, which can be formed and enlarged depending on peculiarities of connective tissue structure.

One of the main components of the connective tissue is collagen, which is responsible for its versatility. Because of collagen degradation, peptides are broken diwn by specific enzymes to amino acids. Oxiprolin (OP), which is released from these peptides, and is the main amino acid of the connective tissue, is found in blood and urine and is observed in the diseases manifested by a phenomenon of connective tissue weakness [2].

The study of the issue of connective tissue and its pathology was commenced in the early twentieth century by E. Ehlers and A.A. Danlos, who in detail described the symptom complexes indicating generalized weakness, or dysplasia of connective tissue. During the long study of this pathology, the term "dysplasia" was divided into differentiated connective tissue dysplasia (DCTD) and undifferentiated (UCTD) [2, 3].

Undifferentiated connective tissue dysplasia is diagnosed when a set of patient’s clinical signs does not fit any hereditary monogenic disease. According to various authors, UCTD rate ranges from 26.0% to 80.0%, depending on age, place of residence, race and other factors [4].

According to modern data, UCTD leads to homeostasis disorders on the tissue, organ and organism levels in the form of various morphofunctional disturbances of visceral and locomotor organs with deteriorative course [5]. In particular, UCTD can be manifested in infants by multifactorial diseases, including vesicoureteral reflux [3, 5].

The basis of UCTD diagnosis are external and internal phenotypic features evaluated by Milkovskaya-Dimitrova and Karkashev criteria. However, when examining a patient, it is necessary to consider not only the number of phenotypic manifestations, but also the degree of their severity and clinical significance [2, 3].

The presence of 5 or more phenotypic signs of UCTD is observed in 39.0% of children with urinary tract disorders, and in the case of confounding family history of urinary pathology

with high stigmatization, 90.0% of children will have kidney pathology [2, 3, 5]. Manifestations of phenotypic markers of connective tissue can be clearly marked in preschool and primary school age, and the diagnosis of UCTD in newborns and infants based only on phenotypic markers is rather complicated [2].

Moreover, one of the causes of undifferentiated connective tissue dysplasia is considered to be multifactorial effects on the fetus during its intrauterine development.

However, connective tissue dysplasia often occurs not so much because of genetic defects in collagen, but due to defects in dozens of genes affecting biosynthesis, posttranslational modifications, secretion, self-assembly and remodeling of collagen fibers [6].

The genetic marker that determines dysplasia is currently unknown, but it is assumed that the primary deviation is observed in one or more collagen genes, while molecular genetic studies have revealed the genetic heterogeneity of congenital connective tissue dysplasia [7].

The pathogenetic basis of morphological changes in UCTD in the kidneys is the deficiency or weakness of type IV collagen in the basal membranes of the glomeruli and tubules, which is encoded by the genes *COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6.*

Undifferentiated forms of CTD have polygenic-multifactorial nature. Both polymorphism or mutation of a large number of genes in different combinations and the influence of various environmental factors play a role in their occurrence [3].

The complex pathogenesis, as well as the variability of clinical manifestations of secondary pyelonephritis in the settings of vesicoureteral reflux, suggests that there is an effect of many candidate genes on the development of these diseases [8].

Most of the toxins that enter the body do not have direct biological effects, but they are subject to various transformations, so-called biotransformation. The genetically programmed system of biotransformation, degradation and excretion of xenobiotics, including toxins of infectious agents, is a unique difference of each person due to the polymorphism of the respective genes, the genes of " the external environment" [8].

One of the potential gene modifiers for VUR and secondary pyelonephritis is the biotransformation genes of xenobiotics. The system of biotransformation of xenobiotics, including toxins, takes part both in protecting the body against the consequences of inflammatory reactions, and can act as the genes of propensity to the effects of external environmental factors,

which may result in congenital malformations of the urinary organs, namely, vesicoureteral reflux.

Therefore, **the objective of the study** was to establish the possible association between the peculiarities of the distribution of polymorphic variants of *GSTM1* and *GSTT1* genes and rs565470 of a type IV collagen *COL4A1* gene polymorphism with undifferentiated connective tissue dysplasia and vesicoureteral reflux in children.

Materials and methods. The study included 49 children with vesicoureteral reflux and 15 children with pyelonephritis who underwent in-patient examination and treatment in the 2nd paediatric department of the Regional Children's Clinical Hospital (RCCH) "OHMATDYT" in Lviv in 2015-2017. The data were compared with 42 children who were systemically well, representative by age. All children underwent a comprehensive clinical and laboratory examination.

In addition, all children were given a number of special biochemical and molecular genetic studies. The following parameters were determined:

- excretion of oxyproline with urine as a marker of undifferentiated connective tissue dysplasia [9];

- the presence of the deletion alleles of the genes *GSTM1 0/0* and *GSTT1 0/0* in the genotype of the child, the products of which, the glutathione-S-transferase of M and T class, are responsible for the biotransformation of the toxins, and therefore the detoxification capacity of the organism, using the multiplex polymerase chain reaction method (multiplex PCR) [10];

- genotypes/alleles of the polymorphic locus rs565470 of the gene *COL4A1* by RFLP (Restriction Fragment Length Polymorphism) method [10, 11].

Discussion of the study results. This study was conducted in two stages. In the first stage included the estimation of the rate of zero-alleles *GSTM1 0/0* and *GSTT1 0/0* in 35 infants with VUR, the presence of which leads to a decrease in the activity of glutathione-S-transferase of M and T class providing detoxification of toxins in children with pyelonephritis in the settings of VUR and without it (Table 1).

Table 1

Peculiarities of the distribution of deletion alleles GSTT1 0/0 and GSTM1 0/0 of the GST gene among children with secondary pyelonephritis in the settings of vesicoureteral reflux as compared to control data

Distribution of alleles:	Groups of children:					
	All patients with VUR		PN-Control		Healthy Controls	
	n= 35	%	*n*=15	%	*n* = 29	%
GSTT1 0/0 genotype	12	34.3	4	26.7	9	31.0
GSTM1 0/0 genotype	18	51.4*	9	60.0*	9	31.0
GSTT1 0/0 + GSTM1 0/0 genotype	8	22.9*,**	0	-	3	10.3

* - the probable difference of the indicator compared with the data of healthy controls; $p<0.05$

** - the probable difference of the indicator compared with the data of children with PN; $p_1<0.05$

Carriage of deletion alleles *GSTT1 0/0* and *GSTM1 0/0* in healthy children of the control group was the same - 31.0% in each group, and the combination of these alleles was detected in 10.0% of children (Table 1). These results correspond to the rate of zero-alleles established for the Caucasian race for *GSTM1 0/0* - 30.0-45.0% and for *GSTT1 0/0* - 15.0-30.0% [12].

The carriage of the zero-allele *GSTT1 0/0* was established in 34.3% of children with secondary pyelonephritis in the settings of VUR, and 26.7% - in children of the control group with pyelonephritis without VUR, which did not differ statistically from the data of healthy controls (Table 1). However, the carrier rate of the deletion allele *GSTM1 0/0* in the children of the study group (51.4%) and the control group with pyelonephritis (60.0%) significantly differed from that of healthy controls (31.0%) (Table 1). Consequently, we can conclude from the obtained results that there is the association of pyelonephritis in the settings of VUR with the presence of the deletion allele *GSTM1 0/0* in the genotype of the child.

The carrier rate of the combination of zero-alleles *GSTM1 0/0* and *GSTT1 0/0* in VUR children significantly differed from that of healthy controls. At the same time, such a combination in the genotype was generally not noticed in children with primary pyelonephritis without VUR (Table 1). The obtained results allow to confirm the association between the combination of functionally inferior alleles of genes *GSTM1 0/0* and *GSTT1 0/0* and VUR.

The rate and distribution of genotypes of rs565470 of the *COL4A1* gene polymorphism in 49 infants with VUR compared to control data were also determined. The results obtained are presented in Table 2.

Table 2

Distribution of genotypes of rs565470 of the COL4A1 gene polymorphism in infants with vesicoureteral reflux compared with control data

Genotypes	Study group (*n* = 49)			Control group (*n* = 42)			χ^2	p	OR(CI)
	n	%	HWE p=2.0E-7	*n*	%	HWE p=0.03			
TT	7	14.3	0.327	9	21.4	0.300	0.796	>0.05	0.61(0.21 – 1.81)
CT	42	85.7	0.490	28	66.7	0.495	**4.622**	<0.05	3.00(1.08 – 8.37)
CC	0	0	0.184	5	11.9	0.205	**6.172**	<0.05	0.07(0.00 – 1.28)

Children with VUR with the highest rate (85.7%) had heterozygous genotype (*CT*) for rs565470 of *COL4A1* gene polymorphism, while its rate in the control group was 66.7%. No child with VUR had the homozygous genotype for the allele (C) of rs565470 of COL4A1 gene polymorphism, the incidence of which in the control group was 11.9%. The carrier rate of the homozygous genotype for allele (T) of rs565470 of *COL4A1* gene polymorphism in children with VUR was significantly lower (14.3%) as compared to healthy children (21.4%) (Table 2).

Statistical analysis of the obtained results using the Pearson's test ($\chi 2$) showed a significantly higher rate of CT genotype ($\chi 2 = 4.6$; $p<0.05$) and a significantly lower rate of CC genotype ($\chi 2 = 6.18$; $p<0.05$) for rs565470 of COL4A1 gene polymorphism in children with VUR compared to controls.

The calculation of the odds ratio (OR) with 95% confidence interval (CI) showed that the risk of developing VUR increases by a factor of three in the presence of CT genotype of rs565470 of *COL4A1* gene polymorphism (OR = 3.00, CI 95%: 1.08 - 8.37).

The second stage of the study was to determine the distribution of deletion alleles of *GSTT1 0/0* and *GSTM1 0/0* genes in children with secondary pyelonephritis in the settings of vesicoureteral reflux, depending on the presence of UCTD syndrome. All children with VUR

were divided into two subgroups with the presence or absence of excretion of oxyproline with daily urine, the main UCTD marker.

Estimation of the carrier rate of *GSTM1 0/0* and *GSTT1 0/0* zero-alleles in children with pyelonephritis in the settings of VUR and without it depending on the test result for oxyproline is presented in Table 3.

Table 3

Peculiarities of the distribution of deletion alleles GSTT1 0/0 and GSTM1 0/0 of the GST gene among children with secondary pyelonephritis in the settings of vesicoureteral reflux as compared to control data, depending on the presence of oxyproline in their urine

Distribution of alleles:	Groups of children:									
	VUR, *n*= 35				PN-Control, *n*=15				Healthy Controls, *n* = 29	
	OP (+)		OP (-)		OP (+)		OP (-)			
	n=23	%	*n*=15	%	*n* =7	%	*n*=6	%	*n* = 29	%
GSTT1 0/0 genotype	7	30.4	5	33.3	2	28.6	2	33.3	9	31.0
GSTM1 0/0 genotype	10	53.5*	8	43.3*	5	71.4 *	4	66.7 *	9	31.0
GSTT1 0/0 + *GSTM1 0/0* genotype	6	26.1*,** ,***	2	13.3**	0	-	0	-	3	10.3

* - the probable difference of the indicator compared with the data of healthy controls; $p<0.05$
** - the probable difference of the indicator compared with the data of children with PN; $p_1<0.05$
*** - the probable difference between the data of children with OP (+) and OP (-) in a group of children with VUR and in a group of children with PN; $p_2<0.05$

There was no significant difference in the rate of *GSTT1 0/0* and *GSTM1 0/0* deletion alleles in the subgroups of OP (+) and OP (-) with VUR and PN (Table 3).

In children of VUR subgroup OP (+) a significant difference in the registration of a combination of *GSTM1 0/0* and *GSTT1 0/0* zero-alleles (26.1%) was revealed, compared with the data of children of the subgroup of VUR OP (-) (13.3%) and compared with the data of healthy controls (10.3%). These results suggest the presence of a carrier association of the combination of functionally defective *GSTM1 0/0* and *GSTT1 0/0* alleles in children with VUR, which subsequently leads to the progression of connective tissue dysplasia, which complicates the course of the disease, especially if it has arisen as a result of such a birth defect of UO as VUR.

Moreover, the distribution of polymorphism genotypes of rs565470 of *COL4A1* gene in infants with vesicoureteral reflux was determined, depending on the presence of oxiprolin in their urine compared with the data of controls. The results are presented in Table 4.

Table 4

Distribution of polymorphism genotypes of rs565470 of *COL4A1* gene in infants with vesicoureteral reflux with UCTD manifestations compared with the data of the children of the general population control group

Genotypes	Study group (*n* = 29)			Control group (*n* = 42)			χ^2	p	OR(CI)
	n	%	HWE	n	%	HWE			
TT	2	6.9	0.286	9	21.4	0.300	2.767	>0.05	0.27(0.05 – 1.36)
CT	27	93.1	0.498	28	66.7	0.495	**6.868**	**<0.01**	**6.75(1.40 – 32.55)**
CC	0	0.0	0.217	5	11.9	0.205	3.714	>0.05	0.12(0.01 – 2.18)

The heterozygous genotype (CT) of rs565470 of *COL4A1* gene polymorphism was found with the highest rate (93.1%) in children with VUR OP (+). The rate of the homozygous genotype for the allele (T) was 6.9%. No child from the VUR group OP (+) had the presence of the *CC* genotype for the given polymorphism. While the rate of genotypes of rs565470 of *COL4A1* gene polymorphism was distributed in the control group as follows: *TT* - 21.4%, *CT* - 66.7%, and *CC* - 11.9%.

Statistical analysis of the obtained results using Pearson's test ($\chi 2$) showed a significantly higher rate of *CT* genotype of rs565470 of *COL4A1* gene polymorphism in children with VUR OP (+) compared with the control group ($\chi 2 = 6.87$; $p < 0.01$).

The calculation of the odds ratio (OR) with 95% confidence interval (CI) showed that the risk of developing UCTD and VUR increases by a factor of 6.75 in the presence of CT genotype of rs565470 of *COL4A1* gene polymorphism (OR = 6.75, CI 95%: 1.40 - 32.55).

The results of the distribution of polymorphism genotypes of rs565470 of *COL4A1* gene in infants with vesicoureteral reflux without manifestations of UCTD are presented in Table 5.

The rate of CT genotype of *rs565470* of *COL4A1* gene polymorphism was higher in infants with vesicoureteral reflux without manifestations of UCTD compared with the data of the control group of children (75.0% versus 66.7%). The rate of TT genotype with the same polymorphism did not differ in children with VUR without UCTD compared to the controls; while the rate of CC genotype was significantly lower (0.0% vs. 11.9% in the controls).

Table 5

Distribution of polymorphism genotypes of rs565470 of *COL4A1* gene in infants with vesicoureteral reflux without manifestations of UCTD compared with the data of children of the general population control group

Genotypes	Study group (n = 20)			Control group (n = 42)			χ^2	p	OR(CI)
	n	%	HWE	n	%	HWE			
TT	5	25.0	0.391	9	21.4	0.300	0.099	>0.05	1.22(0.35 – 4.27)
CT	15	75.0	0.469	28	66.7	0.495	0.443	>0.05	1.50(0.45 – 4.97)
CC	0	0.0	0.141	5	11.9	0.205	2.59	>0.05	0.17(0.01 – 3.16)

Statistical analysis of the results using the Pearson's test ($\chi 2$) did not show any probable difference in the rates of polymorphism genotypes of rs565470 of *COL4A1* gene in children with VUR without UCTD compared with the controls.

The calculation of the odds ratio (OR) with 95% confidence interval (CI) showed no significant increase in the risk of the development of VUR without UCTD manifestations in the presence of genotypes of rs565470 locus of COL4A1 gene in infants.

Conclusions

1. Carriage of *GSTM1 0/0* zero-allele in the genotype of the child is associated with its propensity to the inflammatory renal process, and the carriage of the combination of *GSTT1 0/0 + GSTM1 0/0* zero-alleles in the child's genotype is associated with the presence of undifferentiated connective tissue dysplasia and the tendency to vesicoureteral reflux.

2. Analysis of the results of polymorphism genotyping of *rs565470* of *COL4A1* gene in children with vesicoureteral reflux in comparison with healthy controls found that *CT* genotype with the given polymorphism increases the risk of vesicoureteral reflux in infants by a factor of three (OR = 3.00; CI 95%: 1.08 – 8.37), and in the presence of the given genotype, the risk of undifferentiated connective tissue dysplasia, including vesicoureteral reflux, increases sevenfold (OR = 6.75; CI 95%: 1.40 - 32.55). The obtained results suggest there is association between rs565470 of *COL4A1* gene polymorphism with the development of undifferentiated connective tissue dysplasia and vesicoureteral reflux in infants.

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