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Correlation of polymorphous variants (ApaI, TagI, BsmI) of the VDR receptor gene with the vitamin D level and liver fibrosis in children with autoimmune hepatitis

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The aim. To study the correlation of the frequency distribution of alleles, genotypes and their combinations for the allelic variants of ApaI, TagI, BsmI of the Vitamin D receptor gene (VDR) with the vitamin D levels and the stage of fibrosis in children with autoimmune hepatitis.

Materials and methods. 51 children with autoimmune hepatitis were examined between 2016 and 2018. In all children, the diagnosis was confirmed in accordance with the International Guidelines for the study of liver diseases (European Association for the Study of Liver (EASL) Clinical Practice Guidelines: Autoimmune Hepatitis, 2015). In the examined children, the elastography of the liver parenchyma was performed by the shear wave method. Needle biopsy of the liver with histological examination of the specimens was performed in 42 children. The disease activity was determined using the histological activity index (HAI) by Knodell based on the results of a morphological study of the liver biopsy and biochemical parameters. The stage of the disease was evaluated by the histological index of fibrosis using the METAVIR scoring system and semi-quantitatively using the share wave elastography of liver parenchyma. A level of 25(OH)D was determined in the blood serum. The determination of the ApaI, TagI, BsmI polymorphic loci of vitamin D receptor (VDR) was carried out using the molecular-genetic method. The association of polymorphic variants of the VDR gene with the level of vitamin D and the stage of liver fibrosis in children with autoimmune hepatitis has been evaluated.

Results. 72.0 % of the examined children had advanced severe liver fibrosis (F3-4 by the METAVIR score), among them 34.0 % of children had signs of liver cirrhosis. Children with advanced severe fibrosis (F3–4 by the METAVIR score) had the CC genotype of the polymorphic version of the TagI of the VDR gene significantly more often (χ^2 = 3.953; P < 0.05), and the genotype AA/CC/AA according to the investigated allelic variants of the VDR gene (χ^2 = 3.953; P < 0.05). In 72.5 % of the examined children, there was a deficiency of vitamin D. In children with severe fibrosis (F3–4 by the METAVIR score) vitamin D deficiency was significantly more frequent as compared to children with less severe fibrosis (F1–2 by the METAVIR score) (χ^2 = 5,207; P = 0.023). The genotype GA of the BsmI polymorphic variant was associated with a decrease in serum vitamin D levels (P < 0.05). At the AC/TC/GA combination of the ApaI, TagI and BsmI allelic variants of the VDR gene, vitamin D deficiency was registered significantly more frequently (P < 0.05).

Conclusions. The level of vitamin D in children with autoimmune hepatitis was dependent on the stage of fibrosis. Children with severe fibrosis had a genetically determined vitamin D deficiency significantly more often. Vitamin D deficiency was associated with the CC genotype presence of the TagI polymorphic variant of the VDR gene in patients as well as the GA genotype of the BsmI polymorphic variant and the AC/TC/GA genotype of the three allelic variants of the gene studied.

Ключові слова: діти, автоімунний гепатит, фіброз, вітамін D, поліморфізм генів, ген VDR.

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Зв'язок поліморфних варіантів (ApaI, TagI, BsmI) гена рецептора VDR із рівнем вітаміну D і фіброзом печінки в дітей з автоімунним гепатитом

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Мета роботи – вивчити зв'язок розподілу частот алелей, генотипів та їх комбінацій за алельними варіантами ApaI, TagI, BsmI гена рецептора вітаміну D (VDR) із рівнем вітаміну D і стадією фіброзу в дітей, які хворі на автоімунний гепатит.

Матеріали та методи. Обстежили 51 дитину з автоімунним гепатитом у 2016–2018 рр. Усім дітям діагноз встановили відповідно до міжнародних рекомендацій із вивчення захворювань печінки (European Association for the Study of Liver (EASL) Clinical Practice Guidelines: Autoimmune hepatitis, 2015). У дітей, яких обстежили, виконали еластографію паренхіми печінки методом зсувної хвилі. У 42 дітей виконали пункційну біопсію печінки з гістологічним дослідженням біоптату. Активність захворювання визначали за допомогою гістологічного індексу активності (ІГА) за Knodell за результатами морфологічного дослідження біоптату печінки та біохімічними показниками. Стадію захворювання оцінювали за гістологічним індексом фіброзу METAVIR і напівкількісно за допомогою еластографії зсувної хвилі паренхіми печінки. У сироватці крові визначили рівень 25(OH)D. Молекулярно-генетичним методом дослідили поліморфні локуси ApaI, TagI, BsmI гена рецептора вітаміну D (VDR). Оцінили асоціацію поліморфних варіантів гена VDR із рівнем забезпеченості вітаміном D і стадією фіброзу в дітей, які хворі на автоімунний гепатит.

Результати. 72,0 % дітей, яких обстежили, мали виразний фіброз печінки F3–4 METAVIR, із них у 34,0 % виявили ознаки цирозу печінки. Діти з фіброзом F3–4 METAVIR вірогідно частіше мали CC генотип за поліморфним варіантом TagI гена рецептора VDR (χ^2 = 3,953; p < 0,05), генотип AA/CC/AA за дослідженими алельними варіантами гена рецептора VDR (χ^2 = 3,953; p < 0,05). У 72,5 % обстежених дітей встановили дефіцит вітаміну D. У дітей із фіброзом F3–4 METAVIR віро-

гідно частіше визначали дефіцит вітаміну D порівняно з дітьми зі стадією фіброзу F1–2 METAVIR (χ^2 = 5,207; p = 0,023). Генотип GA за поліморфним варіантом BsmI був пов'язаний зі зниженням рівня вітаміну D у сироватці крові (p < 0,05). При комбінації AC/TC/GA за алельними варіантами ApaI, TagI, BsmI гена рецептораVDR вірогідно частіше виявляли дефіцит вітаміну D (p < 0,05).

Висновки. Забезпеченість вітаміном D у дітей з автоімунним гепатитом залежала від стадії фіброзу. Діти з виразним фіброзом вірогідно частіше мали генетично зумовлений дефіцит вітаміну D. Дефіцит вітаміну D асоційований із наявністю у хворих CC генотипу за поліморфним варіантом TagI гена VDR, а також генотипу GA за поліморфним варіантом BsmI і генотипу AC/TC/GA за трьома алельними варіантами гена, який дослідили.

Связь полиморфных вариантов (ApaI, TagI, BsmI) гена рецептора VDR с уровнем витамина D и фиброзом печени у детей с аутоиммунным гепатитом

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Ключевые слова: дети, аутоиммунный гепатит, фиброз, витамин D, полиморфизм генов, ген VDR.

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Цель работы – изучить связь распределения частот аллелей, генотипов и их комбинаций с аллельными вариантами ApaI, TagI, BsmI гена рецептора витамина D (VDR) с уровнем витамина D и стадией фиброза печени у детей с аутоиммунным гепатитом.

Материалы и методы. Обследовали 51 ребенка с аутоиммунным гепатитом за период 2016–2018 г. Всем детям диагноз установлен в соответствии с международными рекомендациями ассоциации по изучению заболеваний печени (European Association for the Study of Liver (EASL) Clinical Practice Guidelines: Autoimmune hepatitis, 2015). Детям проведена эластография паренхимы печени методом сдвиговой волны. 42 детям выполнена пункционная биопсия печени с гистологическим исследованием биоптата. Активность гепатита определяли с помощью гистологического индекса активности (ИГА) по Knodell по результатам морфологического исследования биоптата печени и биохимическим показателям. Стадию заболевания оценивали по гистологическому индексу фиброза METAVIR и полуколичественного с помощью эластографии сдвиговой волны паренхимы печени. В сыворотке крови определен уровень 25(OH)D. Молекулярно-генетическим методом исследовали полиморфные локусы ApaI, TagI, BsmI гена рецептора витамина D (VDR). Оценены ассоциации полиморфных вариантов гена VDR с уровнем витамина D и стадией фиброза у детей с аутоиммунным гепатитом.

Результаты. 72,0 % обследованных имели фиброз печени F3–4 по METAVIR, из них у 34,0 % детей установлены признаки цирроза печени. Дети с фиброзом F3–4 METAVIR достоверно чаще имели CC генотип по полиморфному варианту TagI гена рецептора VDR (χ^2 = 3,953; p < 0,05), генотип AA/CC/AA по исследованным аллельным вариантам гена рецептора VDR (χ^2 = 3,953; p < 0,05). У 72,5 % детей установлен дефицит витамина D. У детей с фиброзом F3–4 METAVIR достоверно чаще отмечен дефицит витамина D по сравнению с детьми со стадией фиброза F1–2 METAVIR (χ^2 = 5,207; p = 0,023). Генотип GA по полиморфному вариантуBsmI связан со снижением уровня витамина D в сыворотке крови (p < 0,05). При комбинации AC/TC/GA по аллельным вариантам ApaI, TagI, BsmI гена рецептора VDR достоверно чаще диагностирован дефицит витамина D (p < 0,05).

Выводы. Уровень витамина D у детей с аутоиммунным гепатитом зависел от стадии фиброза. Дети с выразительным фиброзом достоверно чаще имели генетически обусловленный дефицит витамина D. Дефицит витамина D ассоциирован с наличием у больных CC генотипа по полиморфному варианту TagI гена VDR, а также генотипа GA по полиморфному варианту BsmI и генотипа AC/TC/GA по трем аллельными вариантами исследованного гена.

Introduction

Genetically determined predisposition, linkage to the antigens of the human main histocompatibility complex (HLA), alleles 1-B8-DR3, DR4, DR7, DR17, leads to the development of a severe, progressive liver disease such as autoimmune hepatitis in individuals who have lost their immunological tolerance to liver antigens [28]. In recent years, one of the trigger factors in the development and progression of autoimmune hepatitis is recognized as a violation of the vitamin D metabolism in the body as a factor that can accelerate the progression of the pathological process in the liver and negatively affect the effectiveness of therapy [1,2]. Low levels of 25 (OH) D in patients with chronic liver disease (CLD) occur in 90 % of cases, including those in whom the severe deficiency is correlated predominantly with the histological changes, degree of the disease progression and liver fibrosis development as well as the response to treatment [2,3].

Vitamin D has been shown to play a key role in cell differentiation and proliferation, apoptosis, angiogenesis, and immunomodulation [4,5]. This action is regulated by more than 900 genes [6–8]. Biological effects (autocrine, paracrine, immunoregulatory) are due to the hormonally active form of 1 alpha, 25-dihydroxyvitamin D (1 alpha, 25(OH)2D; calcitriol) by binding to vitamin D receptors of (VDR). VDR belongs to a huge family of nuclear receptors of steroid hormones that are present in more than 30 tissues, including liver, pancreatic islet cells, epithelial cells of the gastrointestinal tract, genitourinary system, organs of endocrine system, endothelium, hematopoietic cells, myocardium and striated muscle, neurons, placental cells, as well as in monocytes of blood and activated T and B lymphocytes [9–12]. D-hormone, penetrating into the nucleus of a cell, binds to a nuclear receptor encoded by the vitamin D receptor gene (VDR).The hormone-receptor complex operates through a system of transcription factors or directly binds to functionally active sites of genes. The VDR gene, which is located on the long arm of chromosome 12 (12q 13.11), has a size of 75 KB consisting of 63495 nucleotide pairs with 11 exons [13]. Recently, 1,518 single-nucleotide polymorphisms (SNPs) of the human VDR gene have been described. A large number of polymorphic genetic variants explain the variability and diversity of vitamin D biological influences. The most studied, considering the importance for pathology development, are polymorphisms: FokI

(rs2228570), BsmI (rs1544410), ApaI (rs7975232), TaqI (rs731236), EcoRV (rs4516035), Tru9I, Cdx2 (rs11568820). The association of the VDR gene polymorphism with the development of diabetes mellitus, urolithiasis, psoriasis, malignant neoplasms, cardiovascular, neurodegenerative and autoimmune diseases has been established. It is proved that polymorphic genetic variants are associated with different blood levels of 25-(OH)-D, while decreased levels of the vitamin, in turn, are associated with autoimmune diseases development [1,14–17]. Studies by Vogel and co-authors suggest a possible association of vitamin D receptor gene polymorphism with an increased risk of developing autoimmune hepatitis. Thus, polymorphic variants of the VDR gene, in particular BsmI and TagI, were associated with autoimmune hepatitis development [18]. The role of FokI polymorphism is associated with an increased risk of autoimmune hepatitis in patients from China and Germany [16], and the CC genotype (rs7975232, ApaI polymorphism) was associated with progression of the disease and liver fibrosis development [19,20]. Reduced expression of the VDR gene in autoimmune hepatitis was correlated with progression of the disease and, accordingly, with an increased risk of cirrhosis and liver fibrosis [19].

Today, the causal link between the vitamin D deficiency and the course of autoimmune hepatitis is poorly understood. In the scientific literature, there are no papers relating to the study of the VDR gene allelic polymorphism in association with the progression of autoimmune hepatitis, depending on the level of vitamin D in children; this has defined the direction of our investigation.

The aim

To study the correlation of the frequency distribution of alleles, genotypes and their combinations for the allelic variants of ApaI, TagI, BsmI of the Vitamin D receptor gene (VDR) with the vitamin D levels and the stage of fibrosis in children with autoimmune hepatitis.

Materials and methods

The study was conducted at the Pediatric Hepatology Center, SI "Institute of Pediatrics, Obstetrics and Gynecology named after academician O. M. Lukianova of NAMS of Ukraine". A total of 51 children with autoimmune hepatitis, aged from 1 to 18 years were examined between 2016 and 2018. The study included children who had not received calcium and vitamin D supplements for 6 months.

In all children, the diagnosis was confirmed in accordance with the International Guidelines for the study of liver diseases (European Association for the Study of Liver (EASL)) Clinical Practice Guidelines: Autoimmune Hepatitis, 2015). For verifying the diagnosis, liver biopsy was performed in 42 patients with further morphological and immune-histochemical studies of the biopsy samples. The disease activity was determined using the histological activity index (HAI) by Knodell based on the results of a morphological study of the liver biopsy and biochemical parameters. The stage of the disease was evaluated by the histological index of fibrosis using the METAVIR scoring system and semi-quantitatively using the share wave elastography of liver parenchyma. In 9 patients, for whom the morphological examination of the liver biopsy was performed at the place of residence, the phase of fibrosis was evaluated by the parameters of the liver parenchyma stiffness by the shear wave elastography. Detection of the liver tissue stiffness was carried out in the SI "Institute of Nuclear Medicine of the National Academy of Medical Sciences of Ukraine", Kyiv using the scanner "Radmyr ULTIMA" on an area of the right intercostal spaces with convex (5 MHz) and linear array (10 MHz) abdominal transducers and a high-frequency transducer for the liver surface. The median index of the obtained measurements characterized the elasticity of the liver parenchyma; the result was expressed in kilopascal (kPa). To interpret the obtained parameters and determine the stage of fibrosis, we used the data of L. Castera and co-authors' investigation, according to which the values of elastography below 5.8 kPa, F1 ≥5.8-≤7.2 kPa corresponded to the level of F0 fibrosis (minimal changes), F2 – ≥7.2-≤9.5 kPa (moderate changes), F3 – ≥9.5-<12.5 kPa (significant changes) and F4 – more than 12.5 kPa (liver cirrhosis) [31].

In order to verify the diagnosis of vitamin D deficiency and insufficiency, the classification (2011) adopted by the International Institute of Medicine and the Endocrine Practice Guidelines Committee was used. According to this classification, vitamin D deficiency in children and adults is considered as a clinical syndrome due to low levels of 25(OH)D in blood serum (below 20 ng/ml or 50 nmol/l). The serum level of 25(OH)D from 21 ng/ml to 29 ng/ml (i.e., from 50.1 to 74.9 nmol/l) should be considered as vitamin D insufficiency. A normal level of vitamin D equals the blood serum concentration of 25(OH)D above 30 ng/ml. The serum level of 25(OH)D was measured with an electrochemiluminescent method using the Elecsys 2010 system on the Cobas e 411 analyzer (Roche Diagnostics, Germany). The serum levels of total 25(OH)D that can be determined according to this method are in the range of 7.5–175.0 nmol/l, the variation coefficient is within 3.0 %.

The molecular-genetic study of polymorphous variants of the VDR gene was performed by the polymerase chain reaction (PCR) method. Initially, DNA was extracted from the peripheral blood using the commercial Quick-DNA Miniprep Plus Kit test system (manufactured by Zymo Research, USA). For the determination of the polymorphic variants of the BsmI G/A (rs1544410), TaqI T/C (rs731236) [30] and ApaI A/C (rs7975232) [31] of the VDR gene, the modified protocols with oligo-nucleotide primers and the restriction fragment length polymorphism (RFLP) analysis were used. The investigated genes were amplified using specific primers (produced by Metabion, Germany) and the commercial Dream Taq Green PCR Master Mix (manufactured by Thermo Scientific, USA). The test tubes with the final amplification mixture were transferred to the Flex Cycler BU amplifier (Analytic Jena, Germany) to provide an appropriate temperature regime.

The products of the DNA fragments amplification (amplicons) of the VDR gene were subjected to hydrolytic cleavage by restriction endonuclease BsmI, TaqI and ApaI (produced by Thermo Scientific, USA), respectively. For the restriction analysis, separate mixtures were prepared and transferred into the pre-labeled test tubes, and then amplicons were added. The proportional composition of the components in the template mixture is given in *Table 1*.

Table 1. Composition of the template mixtures for RFLP analysis

Gene (polymorphism)	Reagents	Volume	The size of the restriction fragments
VDRBsmIG/A (rs1544410)	10xBuffer*R*	1 µl	*GG* genotype: 644 and 179 bp *GA* genotype: 823, 644and179 bp *AA* genotype: 823 bp
	Enzyme *BsmI*	1 µl	
	Water	8 µl	
	Amplicon	5 µl	
VDRTaqIT/C (rs731236)	10xBuffer*TaqI*	1 µl	*TT* genotype: 496 and 249 bp *CT* genotype: 496, 295, 249 and 201 bp *CC* genotype: 295, 249 and 201 bp
	Enzyme *TaqI*	1 µl	
	Water	8 µl	
	Amplicon	5 µl	
VDRApaIA/C (rs7975232)	10xBuffer*B*	1 µl	*AA* genotype: 501 bp *AC* genotype: 501, 288 and 213 bp *CC* genotype: 288 and 213 bp
	Enzyme *ApaI*	1 µl	
	Water	8 µl	
	Amplicon	5 µl	

The reaction of the fragments restriction for BsmI G/A (rs1544410) and VDR ApaI A/C (rs7975232) of the VDR gene was carried out according to the manufacturer's recommendations in a solid-state microthermostat at 37 °C for 16 hours. The process was stopped by raising the temperature to 65 °C for 20 minutes. The restriction of TaqI T/C (rs731236) of the VDR gene was incubated at 65 °C for 16 hours without further thermo-inactivation of the enzyme (in accordance with the manufacturer's instructions). The state of the restriction fragments of the VDR gene was analyzed on 3 % agarose gel (agarose produced by Cleaver Scientific, UK) stained with ethidium bromide. Gene Ruler 50 bp DNA Ladder molecular weight marker (manufactured by Thermo Scientific, USA) was added to evaluate the fragments size (*Fig. 1–3*).

As shown in *Fig. 1*, the *VDR* gene BsmI G/A (rs1544410) amplifiers were subjected to hydrolytic cleavage at an existing restriction site 5'-GAATGCN↓-3', resulting in fragmentation having a molecular weight of 644 bp and 179 bp – the *GG* genotype. The restriction site disappeared at the nucleotide substitution from G to A, so if the size of the amplified DNA fragments after interaction with the restriction nuclease remained unchanged (823 bp), the AA genotype was recorded. Accordingly, in the heterozygous genotype (GA), all three types of fragments were observed simultaneously: 823, 644 and 179 bp.

Fig. 2 shows the electrophoregram of the VDR gene TaqI (rs731236) T/C restriction fragments. The amplicons were subjected to hydrolytic digestion by the TaqI restriction endonuclease at the specific restriction site 5'-T↓CGA-3'. On the amplified fragments of the VDR gene, one of these sites was always present that formed fragments of 496 and 249 bp under the action of TaqI endonuclease. By their presence, the TT genotype was determined. In response to the nucleotide substitution from T to C, an additional restriction site appeared. As a result, in the CC genotype, in addition to 249 bp, restriction fragments with a molecular weight of 295 bp and 201 bp were formed. In the heterozygous genotype (TC), fragments 496 bp, 295 bp, 249 bp and 201 bp, respectively, were observed.

The size of the amplified ApaI (rs7975232) A/C fragment of the VDR gene remained the same (501 bp) under the influence of the ApaI restriction endonuclease in the absence of a nucleotide substitution (the AA genotype), (*Fig. 3)*. The presence of a polymorphic variant was determined by the appearance of the restriction site 5'-GGGCC↓C-3', resulting in formation of fragments 288 bp and 213 bp in

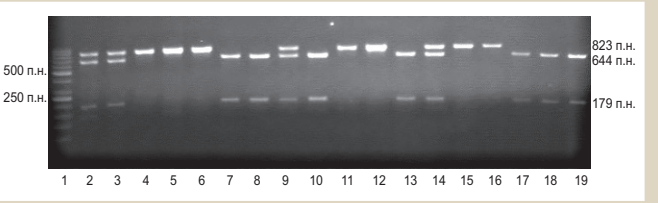

Fig. 1. Electrophoregram of the distribution of VDR BsmI (rs1544410) G/A restriction fragments.

sample 1: molecular weight marker; **samples 7–8, 10, 13, 17–19:** the GG genotype; **samples 2, 3, 9, 14:** the GA genotype; **samples 4–6, 11, 12, 15, 16:** the AA genotype.

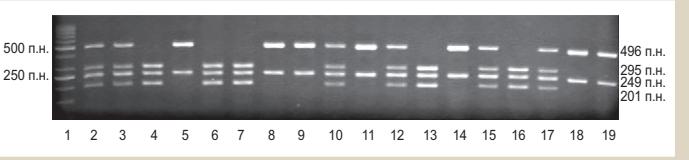

Fig. 2. Electrophoregram of the distribution of VDR TaqI (rs731236) T/C restriction fragments.

sample 1: molecular weight marker; **samples 5, 8, 9, 11, 14, 18, 19:** the TT genotype; **samples 2, 3, 10, 12, 15, 17:** the TC genotype; **samples 4, 6, 7, 13, 16:** the CC genotype.

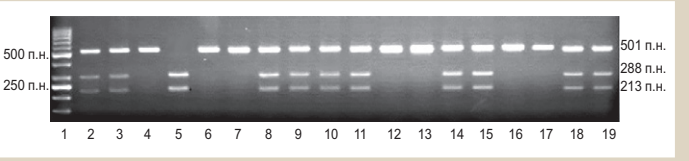

Fig. 3. Electrophoregram of the distribution of VDR ApaI (rs7975232) A/C restriction fragments.

sample 1: molecular weight marker; **samples 4, 6, 7, 12, 13, 16, 17:** the genotype AA; **samples 2, 3, 8–11, 14, 15, 18, 19:** the genotype AC; **sample 5:** the genotype CC.

size (the CC genotype). The heterozygous genotype (AC) was characterized by the all types of fragments presence: 501 bp, 288 bp, and 213 bp.

The obtained data were statistically analyzed using the Statistica 6.1 software package and SPSS17.0 (SPSS, Inc., Chicago, Illinois, USA). The general statistical analysis included median (Me) and interquartile intervals (UQ-LQ) calculations. Laboratory indices were presented in the form of arithmetic data (mean (M ± m), standard error of the mean (SEM)). For nominal variables, the correlation was calculated using the Pearson ($\chi 2$) criterion and Fisher's (two-tailed) criterion; those differences were considered statistically significant, for which a P value was <0.05.

Table 2. Frequency distribution of the alleles and genotypes of the ApaI, TagI and BsmI polymorphisms of the VDR gene in children with autoimmune hepatitis (abs., %)

ApaI (A/C)			TagI (T/C)			BsmI (G/A)		
Genotype	n	%	Genotype	n	%	Genotype	n	%
AA	16	31.0	TT	21	41.0	GG	20	40.0
AC	26	51.0	TC	22	43.0	GA	15	29.0
CC	9	18.0	CC	8	16.0	AA	16	31.0
A Allele	58	57.0	T allele	64	63.0	G allele	55	54.0
C allele	44	43.0	C allele	48	37.0	A allele	47	46.0

Table 3. Vitamin D levels in children with autoimmune hepatitis depending on the stage of fibrosis, abs., %

Stage of fibrosis by the METAVIR score, (n = 51)	Vitamin D levels, abs., %		
	Optimal level (n = 6)	Insufficiency (n = 8)	Deficiency (n = 37)
F1–2 (n = 15)	4 (27.0)	3 (20.0)	8 (53.0)*
F3–4 (n = 36)	2 (5.5)	5 (14.0)	29 (80.5)

*: the difference is significant (P < 0.05) between the groups of children with the stage of fibrosis F1–2 and F3–4.

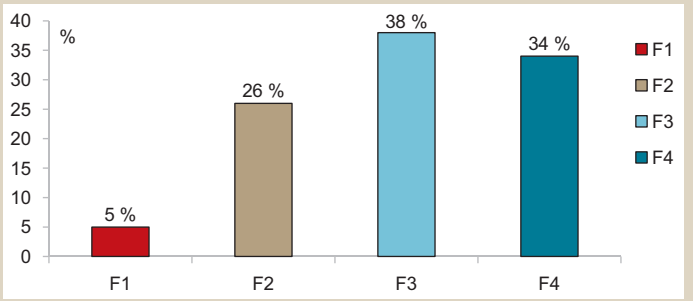

Fig. 4. Distribution of children with AIH by the stage of fibrosis (n = 51), (%).

Results

Among the surveyed children (n = 51), girls dominated and accounted for 61.0 %, and boys made up 39.0 %. Such a distribution corresponds to the clinical features of the disease, which is more often recorded among girls. Higher prevalence among females is associated with the HLA antigens system: HLA-1-B8-DR3 or DR4 [24–26]. Autoimmune hepatitis (AIH) belongs to orphan diseases with a small frequency in the general population that has caused a small number of children included in the study. While Verma et al. [26] believe that the prevalence of AIH in the world is between 2 and 17 per 100.000 children, and it may vary within ethnic groups. The disease is more often diagnosed at the age of 10–30 years. The average age of patients at the time of our study was 11 [8; 15] years, corresponding to the literature data [28].The average age for boys was 10 [8; 14] years, for girls 11.5 [9; 15]. 62.7% (n = 32) of patients were in the age group of 11–18 years.

We did not find a significant difference between the frequency distributions of the of alleles and genotypes of the ApaI, TagI and BsmI polymorphic variants of the VDR gene in children with AIH by gender and age (P > 0.05), so further analysis of the studied gene variations effects was performed in the general group of patients. The results of the identified peculiarities of distribution in the general group are presented in *Table 2*. Distribution of genotypes based on the VDR ApaI, TagI, BsmI genetic variants was analyzed according to the Hardy-Weinberg Law. The concordance with the Hardy-Weinberg law was found for the ApaI and TagI genotype variants but not for the BsmI variant, that may be associated with a higher risk of multifactorial disease development such as autoimmune hepatitis.

According to our data, 72.0 % (n = 36) of the subjects had advanced liver fibrosis (F 3-4 METAVIR), and 34.0 % of them (n = 17) had signs of liver cirrhosis. Distribution of children by the stage of fibrosis is shown in *Fig. 4*.

The mean concentration of vitamin D in the examined children was 16.3 [10.9; 22.0] ng/ml corresponding to vitamin D deficiency. The vitamin D levels in children with AIH, depending on the stage of fibrosis, are given in *Table 3*.

12.0 % (n = 6) of children had optimal levels of vitamin D, deficiency was diagnosed in 15.5 % (n = 8), and deficiency was found in 72.5 % (n = 37) of the subjects. For computational convenience, we combined the subgroups of children with the stage of fibrosis F1 and F2, as well as F3 and F4.

According to our data, vitamin D levels in children depended on the stage of liver fibrosis. Patients with advanced fibrosis (F3-4 METAVIR) were significantly more likely to have vitamin D deficiency ($\chi^2 = 5.21$; P = 0.022) compared to children with F1–2 fibrosis by the METAVIR score (*Table 5*).

The evaluation of the AIH genetic risk was not the goal of our research. However we studied the effects of genetic variants on the complicated course of the disease associated with liver cirrhosis development by intra-group comparisons. We calculated genetic risk models including additive, recessive, dominant, multiplicative and co-dominant for complicated AIH development. The study of the association between genotypes of ApaI, BsmI and TagI polymorphic variants of the VDR gene with the stage of fibrosis in children with AIH demonstrated that children with advanced fibrosis (F3–4 METAVIR) were significantly more likely to have the CC genotype of the polymorphic variant TagI of the VDR gene ($\chi^2 = 3.953$; P < 0.05) compared to children with the fibrosis stage F1–2 by the METAVIR score (*Table 4*). The highest predictive ability was shown for recessive inheritance model.

In children of the general group, the association between the serum levels of vitamin D and the VDR gene polymorphism was investigated. There was no significant difference in the levels of vitamin D depending on the geno-

types studied with the exception of the BsmI allelic variant. In children with the GA genotype by the BsmI polymorphic variant, there was a significant decrease in serum vitamin D levels (12.442 ± 5.515) compared to those with the AA (18.540 ± 7.805) and GG (19.230 ± 7.057) genotypes. For the TagI and BsmI of the VDR gene polymorphic variants in children with AIH, there was no significant difference in the levels of vitamin D (*Table 5*).

According to the results of our study, the following combinations of the genotypes in the three allelic variants of the gene were not found among the examined patients: AA/TT/GG, AA/TT/GA, AA/TT/AA, AA/TC/GG, AA/TC/GA, AA/CC/GG, AA/CC/GA, AC/TC/GG, AC/TC/AA, AC/CC/GG, AC/CC/GA, AC/CC/AA, CC/TT/GA, CC/TT/AA,CC/TC/GG, CC/TC/AA, CC/TC/GA, CC/CC/GG, CC/CC/GA, CC/CC/GG. The following six combinations of the genotypes were detected in the examined patients: AA/TC/AA (n = 8), AA/CC/AA (n = 8), AC/TT/GG (n = 11), AC/TT/GA (n = 1), AC/TC/GA (n = 14), CC/TT/GG (n = 9). We analyzed the effects of three allelic variants combinations of the VDR gene (ApaI, TagI, BsmI) on the vitamin D levels and the liver fibrosis severity in this contingent (*Tables 6, 7*).

The results demonstrated that children with the AA/TC/AA, AC/TT/GG and CC/TT/GG genotype combinations were significantly more likely to have higher vitamin D serum levels than children with the AC/TC/GA genotype combination of the polymorphic variants ApaI, TagI, BsmI of the VDR gene ($P < 0.05$). In general, all the examined children had varying degrees of vitamin D deficiency. Children with the AA/TC/AA genotype combination had the highest level of 25(OH)D – 19.84 ± 8.29 ng/ml.

The correlation analysis of the VDR gene allelic variant combinations with the fibrosis stage showed that children with the genotype AA/CC/AA combination were significantly more likely to have advanced fibrosis F 3-4 by the METAVIR score ($\chi^2 = 3.953$; $P < 0.05$) (*Table 7*).

Discussion

While searching for the scientific research results in PubMed, EMBASE and Cochrane Library we did not find any studies on the association between polymorphic variants of the VDR gene with the risk of AIH development or their peculiarities in the pediatric population, only some studies conducted among adults were revealed. Most studies were devoted to polymorphisms of the VDR gene in patients with primary biliary cirrhosis [20–23,29]. Thus, M. Vogel and co-authors, analyzing the effect of the VDR gene polymorphisms in AIH and primary biliary cirrhosis, revealed the association between BsmI and TaqI polymorphisms of the VDR and primary biliary cirrhosis in the German population, as well as the association between FokI and TaqI polymorphisms and AIH. The authors established a significant correlation between the GA ($\chi^2 = 8.33$; $P = 0.004$; OR = 0.44 [0.25; 0.78]) and AA ($\chi^2 = 7.37$; $P = 0.001$; OR = 2.1 [1.22; 3.62]) genotypes in patients with primary biliary cirrhosis compared with the control group. A noticeably weaker association with primary biliary cirrhosis was additionally demonstrated for the TC genotype ($\chi^2 = 4.79$; $P = 0.003$; OR = 0.54 [0.31; 0.94]). The analysis of the FokI polymorphic locus distribution showed that the frequency of the CC genotype was increased significantly ($\chi^2 = 8.09$;

Table 4. Distribution of children by the ApaI, TagI and BsmI polymorphic variants of the VDR gene depending on the stage of liver fibrosis by the METAVIR score, abs.,%

Genotype n	F1–2, n = 15		F3–4, n = 36	
	%	n	%	n
VDR, ApaI				
AA	3	20.0	13	36.0
AC	10	67.0	16	44.5
CC	2	13.0	7	19.5
A allele	16	53.0	42	58.0
C allele	14	47.0	30	42.0
VDR, TagI				
TT	7	47.0	14	39.0
TC	8	53.0	14	39.0
CC	–	–	8	22.0*
T allele	22	73.0	42	58.0
C allele	8	27.0	30	42.0*
VDR, BsmI				
GG	6	40.0	14	39.0
GA	6	40.0	9	25.0
AA	3	20.0	13	36.0
G allele	18	60.0	37	51.0
A allele	12	40.0	35	49.0

*: the difference is significant ($P < 0.05$).

Table 5. Vitamin D levels in children with autoimmune hepatitis depending on the polymorphic variant of the VDR gene, M ± m, ng/ml

ApaI		
AA (n = 16)	AC (n = 26)	CC (n = 9)
18.540 ± 7.805	15.460 ± 7.707	18.750 ± 4.357
TagI		
TT (n = 21)	TC (n = 22)	CC (n = 8)
18.830 ± 7.100	15.193 ± 7.482	17.240 ± 7.618
BsmI		
GG (n = 20)	GA (n = 15)	AA (n = 16)
19.230 ± 7.057*	12.442 ± 5.515	18.530 ± 7.805**

*: the difference is significant ($P < 0.05$) between the GG and GA genotypes of the BsmI polymorphic variant; **: the difference is significant ($P < 0.05$) between the GA and AA genotypes of the BsmI polymorphic variant.

Table 6. Vitamin D levels depending on the genotype combinations of the ApaI, TagI and BsmI variants of the VDR gene, abs., M ± m, ng/ml

AA/TC/AA n = 8 (16.0)	AA/CC/AA n = 8 (16.0)	AC/TT/GG n = 11 (21.0)	AC/TT/GA n = 1 (2.0)	AC/TC/GA n = 14 (27.0)	CC/TT/GG n = 9 (18.0)
19.84 ± 8.29*	17.24 ± 7.61	19.57 ± 8.72*	11.09	12.54 ± 5.71	18.76 ± 4.36*

*: the difference is significant ($P < 0.05$).

Table 7. Distribution of children by the METAVIR fibrosis score depending on combinations of the ApaI, TagI and BsmI allelic variants of the VDR gene, abs.,%

Combinations of the allelic variants	F1–2, n = 15		F3–4, n = 36	
AA/TC/AC	3	20.0	5	14.0
AA/CC/AA	–	–	8	22.0*
AC/TT/GG	1	7.0	–	–
AC/TT/GA	4	27.0	7	19.5
AC/TC/GA	5	33.0	9	25.0
CC/TT/GG	2	13.0	7	19.5

*: the difference is significant ($P < 0.05$).

P = 0.004; OR = 1.94 [1.23; 3.07]), and the frequency of the TT genotype was decreased compared with the control group (χ^2 = 5.13; P = 0.002; OR = 0.50 [0.28; 0.92]) [21].

The information retrieval conducted by us has validated our study on the possibility of the VDR gene analysis application in the pediatric hepatology as a prognostic marker for the unfavorable course of AIH with progression to liver cirrhosis.

Analysis of the FokI polymorphic locus in our AIH patients was not performed. The distribution of allelic and genotype frequencies found in our study were similar to those found in patients from Germany.

Conclusions

Thus, according to our data, deficiency of vitamin D was found in 72.5 % of patients with AIH. The level of vitamin D depended on the stage of liver fibrosis. Children with advanced fibrosis F3–4 by the METAVIR score were significantly more likely to have vitamin D deficiency.

Vitamin D deficiency was associated with the genetic peculiarities of the patients: the GA genotype of the BsmI polymorphic variant presence and the AC/TC/GA genotype combination of the ApaI, TagI and BsmI polymorphic variants of the VDR gene.

The CC genotype of the TagI polymorphic variant and the AA/CC/AA genotype combination of the ApaI, TagI and BsmI polymorphic variants of the VDR gene were associated with advanced fibrosis F3–4 by the METAVIR score in children with AIH.

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