ORIGINAL ARTICLE

ANALYSIS OF THE VITAMIN D RECEPTOR BSMI GENE POLYMORPHISM IN CHILDREN WITH GROWTH HORMONE DEFICIENCY

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ABSTRACT

The aim: The objective of the study was to investigate the polymorphism of the vitamin D receptor (VDR) BsmI gene in children with growth hormone deficiency and the level of their vitamin D supply.

Materials and methods: Sixteen children diagnosed with of growth hormone deficiency who were treated at the State Institution «V.P. Komisarenko Institute of Endocrinology and Metabolism of the National Academy of Medical Sciences of Ukraine» were examined. The patient's gender and age, the anthropometric data, the vitamin D level in the blood, the bone age, the GH level, the IGF-1 levels, the level of calcium in the blood and VDR gene polymorphism were taken into account.

Results: It was shown that in the presence of the G/A genotype, the risk of growth hormone deficiency development was increased OR = 1,096 (95% CI 0.39-3.02; p = 0.86). For BsmI, mean values of height, body mass, height SDS, serum 25(OH)D, in the studied population (16 children) were 123.49 ± 19.62 cm, 26.96 ± 11.11 kg, -2.25 ± 0.85, 48.86 ± 16.71 nmol/l, respectively; total calcium level consisted of 2.40 ± 0.12 mmol/l, serum phosphorus – 1.43 ± 0.11 mmol/l.

Conclusions: The allele frequency of the VDR BsmI polymorphism was 62.5% for the G allele (n = 20) and 37.5% for the allele A (n = 12). The G allele carrier of the polymorphic locus BsmI rs1544410 of the VDR gene (rs11568820) is associated with an increased risk of growth hormone deficiency development OR = 1.31 (95% CI 0.62-2.75; p = 0.47).

KEY WORDS: VDR gene, children, growth hormone deficiency, polymorphism

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INTRODUCTION

As is generally known the vitamin D and its active metabolites play a key role in phosphorus-calcium homeostasis and bone metabolism, regulate the cell growth and differentiation in different target organs [1,2].

The vitamin D is a ligand for the nuclear receptor which is encoded by the VDR gene and is a regulator of the activity of many target genes by interacting with specific DNA sequences in the promoter regions of these genes [3].

The vitamin D levels are generally lower in patients with growth hormone (GH) deficiency than in the control group with different prevalence of insufficiency or deficiency, and this status may make worse the already known cardiovascular and metabolic risks of growth hormone deficiency, although this statement (affirmation) is not generalized for all the studies. In addition, data on the effect of growth hormone treatment on vitamin D levels in patients with GH deficiency are quite controversial. On the contrary, in active acromegaly, a condition characterized by chronic growth hormone excess, both increased and decreased levels of vitamin D have been observed, and the interaction between vitamin D and the growth hormone / IGF-1 axis becomes even more complex when considering acromegaly treatment [4].

Hamza R.T. et al. (2018) evaluated the vitamin D status in prepubertal children with idiopathic growth hormone deficiency and the effect of treatment on the vitamin D level. The vitamin D deficiency was revealed in 40 % of children with idiopathic growth hormone deficiency; a deficiency was found in 44 %, a sufficient level of the vitamin D was only observed in 16 %. A positive correlation between the vitamin D and peak GH levels was noted. The GH peak was a significant predictor of the vitamin D levels. After 1 year growth hormone therapy, the level of vitamin D was significantly increased. Overall, the level of vitamin D remained insufficient in 22 % of cases and the vitamin D deficiency was found in 24 % of children. It was proved that Vitamin D negatively correlated with parathyroid hormone (PTH). Therefore, hypovitaminosis D is common in children with idiopathic growth hormone deficiency and was significantly reduced 1 year after GH therapy [5].

P. Ameri, et al. [6] and F. Bogazzi, et al. [7] show that the vitamin D levels affect the function of the GH / IGF-1 axis. Vitamin D can increase the production and secretion of IGF-1 (insulin-like growth factor-1) and IGFBR-3 (insulin like growth factor binding protein 3) in the liver. On the other hand, both STH and IGF-1 increase renal production of vitamin D by increasing the kidney activity

of 1α-hydroxylase [8]. During the vitamin D therapy the effect of recombinant GH(rGH) on the bone formation in GH treatment [9].

The main function of the vitamin D substances is, first of all, to regulate the bone metabolism. Only 10-15 % of calcium and about 60 % of phosphorus are absorbed without the vitamin D participation. 1.25(OH)2D – a hormone-active form of vitamin D, interacting with the VDR, increases the absorption of intestinal calcium and phosphorus by 30-40 % and 80 %, respectively. At the same time, the process of calcium mobilization from the bone tissue is controlling by vitamin D, which is also necessary to create optimal conditions for its growth. Calcium and phosphate metabolism are regulated not only by vitamin D but also by the level of ionized calcium, PTH and calcitonin [10].

The vitamin D receptor belongs to a family of transactive transcription regulatory factors and has a similarity to the steroid and thyroid hormone receptors, confirming the functioning of vitamin D as a hormone [11,12].

Vitamin D receptors are known to be encoded by the eponymous VDR gene (also known as NR1I1) localized on chromosome 12q12-q14.

This gene is characterized by polymorphism, that is, the existence of its different allelic variants in the population. The most significant are polymorphisms of the VDR gene involved in disease development: BsmI, FokI, TaqI Fokl, ApaI [12]. To date, 1518 single-nucleotide polymorphisms (SNPs) of the human VDR gene have been described.

Among them is BsmI, localized in the eighth intron. The nature of the BsmI polymorphism lies in the fact that guanine is substituted for adenine at position 58980. By themselves, polymorphisms in introns are not functionally significant, since they do not change the sequence of the nitrogenous bases in the content part of the gene, however, being linked to the regulatory regions of the gene, may serve as markers of functional relationships of other polymorphisms with the development of pathological processes and diseases. The association of BsmI polymorphism with different pathological processes and diseases has been studied in many trials of different populations. In some of them, this SNP was associated with osteoporosis [13], pathological fractures [14], type 2 diabetes [15], prostate cancer [16], breast cancer [17], Parkinson's disease [18].

THE AIM

The aim was to investigate the polymorphism of the VDR BsmI gene in children with growth hormone deficiency and the level of their vitamin D supply.

MATERIALS AND METHODS

The serum 25-hydroxycalciferol (25(OH)D) level was determined by immunochemiluminescent method. The GH and IGF-1 levels were studied using radioimmunoassay and enzyme immunoassay methods. The control group consisted of 250 healthy children and adolescents aged 9 to 18 (mean age 8.24 ± 3.83 years) [19]. Sixteen children were examined with diagnosis of GH deficiency who were treated at the State Institution «V.P. Komisarenko Institute of Endocrinology and Metabolism of NAMS of Ukraine». The patient's sex and age, anthropometric data, vitamin D level in the blood (excluded summer months of patient recruitment), bone age, GH level after stimulation tests (clonidine, insulin), IGF-1 levels, blood levels of total and ionized calcium were taken into account. The mean age of children (11 boys, 5 girls) who were included in the study was 10 ± 3.0 years. The average growth delay was minus 2.25 (± 0.85) SDS. At the time of the examination, all the patients were in a euthyroid state. The study included children who had not received the calcium and vitamin D drugs for 6 months. Children with growth hormone deficiency had a significant decrease in IGF-1 levels (from 27.83 to 94.89 ng / ml). Statistical processing of the study results was performed using Microsoft Excel statistical programs.

In order to verify the diagnosis of vitamin D insufficiency and deficiency, the classification (2011) was adopted by the International Institute of Medicine and Endocrine Medicine, *Committee practical guidelines*. According to this classification, the vitamin D deficiency in children and adults is considered to be a clinical syndrome due to low serum 25(OH)D level (below 20 ng/ml or 50 nmol/l). The serum 25(OH)D level from 21 ng / ml to 29 ng/ml (from 50.1 to 74.9 nmol/l) should be considered as the vitamin D deficiency. The normal level of vitamin D is equal to the serum 25(OH)D concentration above 30 ng / ml.

The determination of VDR BsmI gene (rs1544410) polymorphism was performed using the polymerase chain reaction (PCR) method, followed by analysis of the length of the restriction fragments upon their detection by agarose gel electrophoresis.

For genotyping, the venous blood was collected under sterile conditions in 2.7 ml monovets with potassium salt of ethylenediaminetetraacetic acid ("Sarstedt", Germany), which served as an anticoagulant. First, DNA was eliminated from the peripheral blood using a commercial Quick-DNA™ Miniprep Plus Kit (manufactured by Zymo Research, USA).

The genes studied were amplified using specific primers (Metabion, Germany) and commercial Dream Taq Green PCR Master Mix (Thermo Scientific, USA). The tubes with the final amplification mixture were transferred to the Flex Cycler BU amplifier (Analytic Jena, Germany) to provide the appropriate temperature regime.

The amplification products of DNA fragments (amplicons) of the VDR gene were subjected to hydrolytic cleavage by restriction endonuclease BsmI (Thermo Scientific, USA), respectively. Separate mixtures were prepared for restriction analysis and transferred to pre-labeled tubes, and then the amplicons were added.

The fragment limiting reaction for the BsmI G / A (rs1544410) of the VDR gene was performed according to the manufacturer's recommendations in a solid-state micro thermostat at 37 ° C for 16 hours.

The process was stopped by increasing the temperature to 65°C for 20 minutes. The state of the restriction fragments

of the VDR gene was analyzed by a 3% agarose gel (agarose firm Cleaver Scientific, UK), with the addition of ethidium bromide, a marker of molecular weight "GeneRuler 50 bp DNA Ladder" (Thermo Scientific, USA) and subsequent visualization using a transilluminator stained with ethidium bromide by computer program "Vitran".

Amplifiers of the VDR BsmI G / A gene (rs1544410) were hydrolytically cleaved in the presence of a 5'-GAATGCN ↓ -3 'restriction site, resulting in the restriction formations with molecular mass a 644 bp and 179 bp – the GG genotype. The restriction site disappeared with nucleotide replacement from G to A, if the size of the amplified DNA fragments remained unchanged after interaction with the restriction nuclease (823 bp), then the AA genotype was recorded. Accordingly, all three types of fragments: 823, 644 and 179 pp in the heterozygous genotype (GA) were simultaneously observed.

The data obtained were statistically analyzed using Statistica 6.1 and SPSS17.0 software package (SPSS, Inc., Chicago, IL, USA). General statistical analysis included median (Me) and interquartile interval' (UQ-LQ) calculations. Laboratory parameters were presented in the form of arithmetic data (mean (M ± m), standard error of mean) (SEM). For nominal variables, the ratios were calculated using the Pearson test ($\chi 2$) and the Fisher test (two-sided); these differences were considered statistically significant for which the P value was <0.05.

The study was conducted in accordance with the basic principles of bioethics of the Council of Europe Convention on Human Rights and Biomedicine (the 4th of April 1997), the World Health Association Helsinki Declaration on Ethical Principles for Conducting Medical Research with the Participation of People (1964-2013). Commission on Biomedical Ethics of the State Institution «V.P. Komisarenko Institute of Endocrinology and Metabolism of the National Academy of Medical Sciences of Ukraine" did not find violations of moral norms during the study. Informed consent was obtained from the participants and their parents.

RESULTS

Acting through its receptor, the hormone-active form of vitamin D – $1.25(OH)_2D$ can cause many effects that affect various biological processes in the body.

In the target tissues, the vitamin D receptors are functioning in both the cell nuclei (gene regulation level) and plasma membranes (non-gene regulation level). At the gene level, active metabolites of vitamin D bind to specific receptors, forming the hormone receptor complex D3-VDR, which has its own specific DNA-binding domain (a specific DNA sequence), thereby controlling the transcription of the corresponding genes. This process, in one's turn, leads to the biosynthesis of new mRNA molecules and the translation of the corresponding proteins involved in the physiological responses) [11,12].

Analysis of the distribution of allele and genotype frequencies of the polymorphic locus BsmI (rs1544410) gene in the group of patients with GH deficiency and in the control sample [19] is statistically significant (Table 1).

The allele frequency of the VDR BsmI polymorphism was 62.5% for the G allele (n = 20) and 37.5 % for the allele A (n=12). The study found that the *G allele carriers* of polymorphic locus BsmI (rs1544410) of the vitamin D receptor gene (rs11568820) is associated with an increased risk of growth hormone deficiency OR = 1,31 (95 % CI 0.62-2.75; p = 0.47).

It was also shown that the risk of growth hormone deficiency is increased in the presence of the G/A genotype, OR = 1.096 (95% CI 0.39-3.02; p=0.86); also in G/G variant, the risk of growth hormone deficiency was OR = 1.27 (95 % CI 0.44-3.63; p = 0.65); in the A/A genotype variant, the risk of growth hormone deficiency was minimal OR = 0.56 (95 % CI 0.12-2.58; p=0.46).

For BsmI, mean values of height, weight, height SDS, serum 25(OH)D, in the studied population (16 children) were 123.49 ± 19.62 cm, 26.96 ± 11.11 kg, -2.25 ± 0.85, 48,86 ± 16,71 nmol/l, respectively, level of total calcium – 2,40 ± 0,12 mmol/l, serum phosphorus -1,43 ± 0,11 mmol/l (Table 2).

The vitamin D deficiency is occurred in all the children with growth hormone deficiency regardless of the polymorphic locus rs1544410 BsmI of the vitamin D receptor gene. Vitamin D was significantly lower (32.05 ± 11.67 nmol/l) in children with polymorphic variant G/C than that in children with other VDR BsmI polymorphisms, but not significantly.

The GH level after the clonidine stimulation test was significantly lower in group with the VDR BsmI polymorphic variant A / A (0.65 ± 0.05 ng/ml), and significantly higher in the VDR BsmI polymopphic G/G variant (5.59 ± 0.42 ng/ml).

Growth SDS (Standard Deviation Score) was significantly lower in the group of children with polymorphic variant A / A (-3.09 ± 0.12) compared to variants of the polymorphic locus BsmI (rs1544410) of the VDR gene G/A (-2.02 ± 0, 42) and G / G (-2.51 ± 1.35).

IGF-1 in all surveyed was low with polymorphic variant G / G VDR BsmI (94.89 ± 44.34 ng/ml), with polymorphic variants G / A and A / A as well, but not significantly (27.83 ± 12.61 ng/ml and 37.75 ± 18.03 ng/ml, respectively). Normal levels of total and ionized calcium in serum were found in all the children examined.

DISCUSSION

The vitamin D and IGF-1 levels affect each other: on the one hand, the increase in vitamin D increases the level of IGF-1 [20], and on the other hand, IGF-1 stimulates the activity of the enzyme 1α-hydroxylase, which, in one's turn, regulates the renal production of vitamin D : $1,25(OH)_2D$ or calcitriol [21].

In addition, GH itself has a direct stimulating effect on the production of $1.25(OH)_2D$ [22]. Besides, both GH and IGF-1 seem to increase the activity of CYP27A1, a multifunctional cytochrome P450 enzyme that catalyzes 25-hydroxylation of vitamin D in hepatoblastoma cells [23].

Table 1. Distribution of allele and genotype frequencies of the polymorphic locus of vitamin D receptor gene BsmI-rs1544410 in the group of patients with growth hormone deficiency and in the control sample

Groups and number individuals (n)	Allele' frequencies, %		χ 2; df = 1	Genotype frequencies, %			χ 2; df = 2
	G	A		G/A	G/G	A/A	
Population sample (218)*	244 (56)	192 (44)	0,52 p=0,47	104 (47,7)	70 (32.1)	44 (20.2)	0.59 p=0.74
Patients with GH deficiency	20 (62.5)	12 (37.5)		8 (50)	6 (37.5)	2 (12.5)	

Note: Hardy-Weinberg equilibrium for BsmI (P = 0.79)
* – data from source19.

Table 2. Effect of VDR polymorphism on growth values and some serum biochemical parameters in children with growth hormone deficiency

Values	Genotype		
	BsmI (rs1544410), n=16		
	GA	GG	AA
Growth SDS	-2.02±0.42	-2.51±1.35	-3.09±0.12*
GH level after stimulation testwith clonidine, ng / ml	2.12±0.12	5.59±0.42*	0.65±0.05**
25(OH)D, nmol/l	47.63±16.91	32.05±11.67	41.52±4.41
IGF-1, ng / ml	27.83±12.61	94.89±44.34	37.75±18.03
The total calcium, mmol/l	2.44±0.09	2.43±0.11	2.20±0.13
Calcium ionized, mmol/l	1.19±0.06	1.22±0.07	1.11±0.01

Note: * - the significance level between values of the BsmI (rs1544410) GA and GG genotypes (p<0.05);
** - significance level between the values of BsmI (rs1544410) GA and AA genotypes (p<0.05).

Another target, which is rich in VDR, is represented by the pituitary gland. It is likely that $1.25(OH)_2D$ acts on the human pituitary VDR, stimulating GH secretion and modulating the expression of some genes [24].

G. Saggese et al. [25] studied the status of vitamin D in 26 children with GH deficiency and found normal concentrations of 25OHD but low levels of $1.25(OH)_2D$ before growth hormone (treatment and a significant increase in $1.25(OH)_2D$ levels after 12 months of rGH treatment. Data of 80 Sicilian children with growth hormone deficiency were analyzed by A. Ciresi et al.[8] These authors reported of the higher 25OHD values in children with growth hormone deficiency in the solar seasons (31.1 ± 11.1 ng/ml in June – September) than in the cold season (17.3 ± 5.3 ng / ml in November – February), 35% of children have vitamin D insufficiency and 40% – vitamin D deficiency . E. Witkowska-Sedek et al. studied 84 children and adolescents with GH deficiency and found the low concentrations of 25OHD (22.3 ± 6.9 ng / ml) [26], and M.C. Savanelli et al. analyzed 41 adult patients with growth hormone deficiency and found an average 25OHD concentration of 21.3 ± 12.3 ng/ml, the vitamin D deficiency was found in 51% of patients compared with 14.6% of the control group[27]. In addition, P. Ameri et al. found 69 adult patients with GH deficiency and only 6 patients (8.7%) had a normal serum concentration of 25OHD more than 30 ng/ml. They also reported a positive correlation between the vitamin D status and IGF-1, and found a tendency in treatment to increase doses of growth hormone in patients with the vitamin D deficiency (25OHD <15 ng/ml), suggesting that the better vitamin D level may facilitate the achievement of normal IGF-1 level in patients with growth hormone deficiency. In the literature review, these authors suggested that the assessment of the vitamin D levels may be an appropriate method for determining the doses of recombinant GH for the treatment of adult patients with GH deficiency [6].

Taking into account that GH increases $1.25(OH)_2D$ level [20,25,28-29], although very likely indirectly by IGF-1, it can be considered that patients with growth hormone deficiency due to detachment of the pituitary leg have a low concentration of serum $1.25(OH)_2D$. In our study, all the children with GH deficiency had the 25(OH)D deficiency.

The analysis of important VDR polymorphisms in the pathogenesis of various diseases is difficult. Discovering the genetic variants associated with susceptibility to diseases may be the key to their preventing. Thus, VDR regulates the expression of a number of genes in the bone cells, many of which are encoded bone remodeling, have catabolic or anabolic actions, as well as stimulate the secretion of hormones that affect the vitamin D metabolism and mineral metabolism.

The role of VDR gene polymorphisms in the formation of skeletal pathology was actively studied. Thus, a study of the association of BsmI polymorphism with osteoporosis in different parts of the skeleton in postmenopausal women revealed a positive association of the disease with the genotype G/G (p = 0.009) and the G allele (p = 0.016) polymorphism [30]. L. Bao et al. in a meta-analysis showed that genetic BsmI polymorphism correlates with the level of bone mineral density in children, in particular the b(G) allele and the b/b (G/G) genotype are more likely to occur in children with the higher bone mineral density [3].

CONCLUSIONS

1. The allele frequency of the VDR BsmI polymorphism was 62.5% for the G allele (n = 20) and 37.5% for the A allele (n = 12). *G allele carriers* of the BsmI (rs1544410) polymorphic locus of the VDR gene (rs11568820) was

associated with an increased risk of growth hormone deficiency OR = 1.31 (95% CI 0.62-2.75; p = 0.47).
2. It was also shown that in the presence of the G / G genotype, the risk of growth hormone deficiency was OR = 1.27 (95% CI 0.44-3.63; p = 0.65).
3. The vitamin D deficiency occurred in all children with growth hormone deficiency regardless of the BsmI rs1544410 polymorphic locus of the vitamin D receptor gene. In children with the BsmI polymorphic variant of G / G VDR the level of vitamin D was significantly lower (32.05 ± 11.67 nmol / l) than in children with other VDR BsmI polymorphisms, but not significantly.

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"Investigation of Vitamin D3 (VDR3) receptor Bsm1 gene polymorphism, VDR gene TaqI (rs731236) polymorphism, and VDR Gene ApaI polymorphism (rs7975232) and establishing an association of identified disorders with clinical manifestations of short stature and patient phenotype".
The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study.

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