

Common *VDR* polymorphisms and idiopathic short stature in children from northern Greece

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Abstract

Background: A Vitamin D Receptor gene (*VDR*) polymorphism, rs10735810 (*FokI*), has been associated in the past with idiopathic short stature (ISS) in a linkage study. We have investigated the association of the same, as well as a different polymorphism in the same gene [rs731236 (*TaqI*)] with ISS, in an independent study in Greek children.

Methods: The *VDR* rs10735810 (*FokI*) and rs731236 (*TaqI*) polymorphisms were genotyped in a group of ISS children (n= 47) and an age and sex-matched group of normal height children (n= 60) from northern Greece. Genotyping was accomplished through established PCR-RFLP methods.

Results: An association trend of rs10735810 with ISS was observed, with the TT (ff) genotype being apparently under-represented among ISS children compared to controls (p= 0.076; OR= 0.165, 95% CI= 0.025-1.094).

Conclusions: The above results, together with recent evidence related to the functionality of the rs10735810 polymorphism, cannot exclude an involvement of *VDR* in the pathogenesis of ISS. Hippokratia 2015, 19 (1): 25-29.

Keywords: Vitamin D receptor gene, rs10735810, *FokI* polymorphism, idiopathic short stature, Greek children

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Introduction

Short stature constitutes a serious cause of concern and anxiety for affected children and their parents, who seek professional consultation and - if possible - therapeutic intervention¹. In the absence of an identifiable disorder, Idiopathic Short Stature (ISS) is – somewhat arbitrarily defined – as having an age-adjusted height more than 2 standard deviations (SD) below the mean, for a given population^{2,3}. ISS is usually diagnosed in childhood, with an average age of referral at 10 years of age⁴. ISS diagnosis takes into account known genetic, hormonal, systemic and environmental factors, the children's family history of stature, and age-related bone and sexual development. Diagnostic subcategories of ISS include “constitutional delay of growth and puberty” (CDGP), “familial short stature” (FSS) or “ISS in its narrow meaning” although it is often hard to distinguish between them in clinical practice³.

Previous studies, mostly focused on the growth hormone (GH) - insulin-like growth factor (IGF-1) axis, have tried to shed light into the aetiology of this highly heritable trait^{5,6}. However, identifiable abnormalities along this axis account for only a minority of ISS cases, raising questions as to the wisdom of using GH therapy for treating children with ISS,

especially given the high cost, inconvenient dosing and largely unknown long term adverse effects⁷⁻⁹. Deletions and mutations in and around the homeobox transcription factor SHOX gene, have also been causally associated with ISS, yet they characterize only about 5% of tested cases¹⁰, while another 10% of ISS cases may be explained by rare copy number variations (CNVs)¹¹. On the other hand, a C to T substitution in the translation initiation site, in exon 2 of the vitamin D receptor (*VDR*) gene, the so-called *VDR FokI* polymorphism (rs10735810/rs2228570), has also been associated with ISS, in a genome-wide linkage study with ISS children from Germany¹². According to that study, the C (F) allele is over-transmitted to children and adolescents with ISS, leading to a population attributable risk as high as 34%¹². Curiously enough, no attempt to independently replicate this finding has been published since, to the best of our knowledge.

The aim of this study was to examine the association of the *VDR* rs10735810 polymorphism with ISS, in an independent study group that included children from northern Greece (regions of Macedonia and Thrace). The association of another common *VDR* polymorphism, *TaqI*

(rs731236), with ISS was also tested for comparison.

Patients and methods

Patients

Recruitment of children with ISS was accomplished from referrals to the endocrinology unit of the 4th department of paediatrics, of the medical school of Aristotle University of Thessaloniki, at Papageorgiou general hospital, for short stature evaluation, from May 2008 to December 2010. Perinatal, family and personal history was taken from all families and each child underwent a thorough physical examination and extensive laboratory testing to exclude all identifiable causes of symmetric short stature. In all children with ISS two provocation tests were performed in order to exclude growth hormone deficiency. Furthermore, in all female participants a karyotype analysis was performed in order to exclude Turner syndrome. There was no genetic testing for other causes. Hypothyroidism and celiac disease were also excluded by measurement of

thyroid hormones/TSH and specific antitransglutaminase antibodies, respectively.

Forty seven Greek children were finally included in the ISS study group. The control group included 60 Greek children of similar age and gender distribution to the ISS group, selected from among outpatients who presented at our Department during the study period, for a variety of reasons. Exclusion criteria for the enrolment in the control group were short stature and chronic or endocrine disease, including obesity. Demographics of the control and study groups are shown in Table 1. The study was approved by the Ethics Committee of the Medical School of the Aristotle University of Thessaloniki, and informed consent was acquired from all participants' parents/guardians. The study conformed fully to the Declaration of Helsinki for biomedical research on human subjects.

Anthropometric measurements

Height measurements were performed using a Harp-

Table 1. Demographic characteristics of the 60 children with normal stature (control) and 47 ISS children included in the study.

	Controls (n= 60)	ISS (n= 47)
Boys/Girls (%)	30/30 (50.0/50.0)	21/26 (44.7/55.3)
Age (years, mean \pm SD)	9.28 \pm 3.24	10.42 \pm 3.20
Height (cm, mean \pm SD)	137.3 \pm 17.11	127.2 \pm 16.32
Height (HAZ, mean \pm SD)	0.84 \pm 0.91	-2.23 \pm 0.53
Weight (kg, mean \pm SD)	33.93 \pm 12.78	27.73 \pm 9.92
BMI (kg/m ² , mean \pm SD)	17.39 \pm 3.30	16.57 \pm 2.86

ISS: idiopathic short stature, SD: standard deviation, HAZ: height for age z-score according to the world health organization, BMI: body mass index.

Table 2. Genotype frequencies of VDR gene polymorphisms the 60 children with normal stature (control) and 47 ISS children included in the study.

rs10735810 (FokI) genotype	ISS n (%)	Controls n (%)	p	Caucasians ¹⁵ (%)	Greek children ¹³ (%)
CC (FF)	21 (44.7)	28 (46.7)	0.137	(36)	(44.9)
CT (Ff)	25 (53.2)	25 (41.7)		(48)	(43.5)
TT (ff)	1 (2.1)	7 (11.7)		(16)	(11.6)
Hardy-Weinberg, p	0.074	0.935			
rs731236 (TaqI) genotype	ISS n (%)	Controls n (%)	p	Caucasians ¹⁵ (%)	Postmenopausal Greek women ¹⁶ (%)
TT (TT)	18 (39.1)	21 (36.8)	0.923	(36)	(34.5)
TC (Tt)	20 (43.5)	27 (47.4)		(47)	(49.7)
CC (tt)	8 (17.4)	9 (15.8)		(17)	(15.7)
Hardy-Weinberg, p	0.837	0.999			

VDR: Vitamin D Receptor, ISS: idiopathic short stature. Traditional genotype terminology is included (in parentheses) to allow comparison with older references.

Table 3. Distribution of HAZ values following stratification according to the rs10735810 polymorphism.

Genotype	HAZ (mean \pm SD)		
	ISS (n)	Controls (n)	Total (n)
CC	-2.17 \pm 0.54 (21)	0.62 \pm 0.89 (28)	-0.74 \pm 1.59 (49)
CT	-2.11 \pm 0.51 (26)	0.84 \pm 0.85 (25)	-0.50 \pm 1.64 (50)
TT		0.47 \pm 0.98 (7)	0.14 \pm 1.30 (8)
p	0.993	0.558	0.408

HAZ: height for age z-score according to the world health organization, SD: standard deviation, ISS: idiopathic Short Stature. In the ISS group the HAZ distribution was computed for the T carriers, since there was only a single TT genotype present.

enden stadiometer (Holtain, Crymych, UK). Height-for-age z-scores (HAZ) were calculated for each participant in accordance with World Health Organization (WHO) anthropometric standards. The Z-score system expresses anthropometric values as several standard deviations (SDs) below or above the reference mean or median value. Because the Z-score scale is linear, summary statistics such as means, SDs and standard errors can be computed from Z-score values. Z-score summary statistics are also helpful for grouping growth data by age and sex. The summary statistics can be compared with the reference, which has an expected mean Z-score of 0 and a SD of 1.0 for all normalized growth indices. In order to classify children into an ISS subtype, target height, pubertal stage and bone age were determined. The subjects were divided into different pubertal stages, according to Tanner. A child target height was calculated, correcting for secular trend (4.5 cm per 30 years), from the formula: the target height of a boy = [(father's height + mother's height + 13)/2] + 4.5 cm, and the target height of a girl = [(father's height + mother's height - 13)/2] + 4.5 cm. The target range spanned between 1.3 SD (approximately 9 cm). The Greulich and Pyle method was used to calculate bone age.

Genotyping

Genomic DNA from the study participants was isolated from peripheral blood, using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). Genotyping for the VDR rs10735810 and rs731236 polymorphisms was conducted with previously established PCR-RFLP methods, with small modifications^{13,14}. More specifically, a 273 bp region containing the FokI site (rs10735810) was amplified using the following primers: forward, AGC TGG CCC TGG CAC TGA CTC TGC TCT; reverse, ATG GAA ACA CCT TGC TTC TTC TCC CTC, with 35 cycles of 15 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C. Amplicons were digested with the FokI restriction endonuclease (New England Biolabs, Ipswich, MA, USA), at 37°C, overnight, to yield a 206 and a 67 bp fragment characteristic of the T (f) allele. A single, undigested 273 bp fragment is indicative of the C (F) allele (Figure 1A).

A 745 bp region, containing the TaqI site (rs731236) was amplified using the primers: forward, CAG AGC ATG GAC AGG GAG CAA; reverse, GCA ACT CCT CAT GGC TGA GGT C, with 33 cycles of 30 sec at 94°C, 45 sec at 68°C, and 60 sec at 72°C.

Digestion of the PCR product with the TaqI enzyme (Promega Corp., Madison, WI, USA), at 65°C, for 4 hrs, produces two fragments of 494 and 251 bp for the T (T) allele, and three fragments of 293, 201 and 251 bp, for the C (t) allele (Figure 1B).

Statistical Analysis

The χ^2 goodness-of-fit test was used to examine possible deviation of genotype distributions from the Hardy-Weinberg equilibrium. The χ^2 test of independence was used to compare genotype and allele frequencies in the

ISS and control groups.

The association of the rs10735810 polymorphism with HAZ was accomplished with a general linear model with type III sum of square statistics. $p=0.05$ was used as the limit of statistical significance. The Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA) was used for all calculations.

Results

All participants were successfully genotyped for the rs10735810 polymorphism, whereas genotyping for the rs731236 polymorphism failed to produce unequivocal results in the case of three controls and one ISS individual. There was no statistically significant deviation from the Hardy-Weinberg equilibrium with respect to any of the two VDR polymorphisms, in either group of children (Table 2). In the control group, the distributions of genotype frequencies for both polymorphisms were very similar to those previously published for Caucasian and, more specifically, Greek populations (Table 2).

When comparing the genotype frequency distributions for the two polymorphisms, between control and ISS children, we became aware of an apparent underrepresentation of the rs10735810 TT (ff) genotype among the latter. While this difference did not reach statistical significance, it does register as a strong trend when the rs10735810 C (F) carriers are pooled together ($p=0.076$; Odds ratio=0.165, 95% CI=0.025 - 1.094). No significant differences were found regarding the rs731236 polymorphism (Table 2).

Given the trend for the underrepresentation of the rs10735810 TT genotypes in the ISS group, we have examined the association of the rs10735810 polymorphism with HAZ, in the total sample as well as in each individual group of participants, with univariate analysis, using sex as a covariate. Results are shown in Table 3; no statistically significant association was detected.

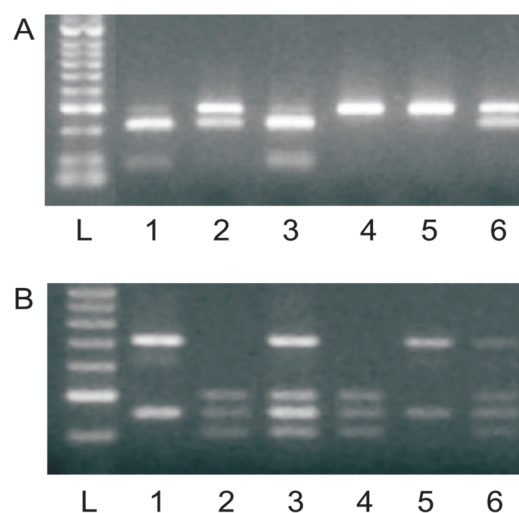


Figure 1. Agarose gel electrophoresis of restriction-digested, PCR-amplified DNA harbouring the rs10735810 (A) and the rs731236 polymorphisms (B). A: L, DNA ladder; 1,3, TT; 2,6, CT; 4,5, CC. B: L, DNA ladder; 1,5, TT; 2,4, CC; 3,6, TC.

Discussion

The vitamin D receptor (VDR) binds 1,25(OH)₂D₃ and, following heterodimerization with the retinoid X receptor (RXR), interacts with vitamin D response elements (VDREs) in the promoter and enhancer regions of genes involved in calcium and phosphorus homeostasis, and in cell growth and differentiation, among others¹⁶⁻¹⁸. Hence, over the last two decades, the association of VDR to height was examined in numerous studies, the majority of which - as well as related meta-analyses - pointed to a very small, if any, effect however^{19,20}. Moreover, VDR was not included among the many genes identified in genome wide association (GWA) studies²¹⁻²³. While, on the other hand, adult height in general appears to be an extremely polygenic trait - with as many as 180 loci accounting for as little as 10% of its phenotypic variation - short stature is considered an extreme phenotype wherein the effect of a relatively limited number of genes/ polymorphisms may be amplified to the extent that they become more easily identified²⁴. In our study, we fell short of detecting a statistically significant association between the VDR rs10735810 (FokI) polymorphism and short stature, but we did notice a considerable underrepresentation of the TT (ff) homozygotes among ISS children. The distributions of the VDR rs731236 (TaqI) genotypes on the other hand, were almost identical between ISS children and children of normal height. While our results do not represent an independent confirmation of the study by Dempfle *et al*¹², their high attributable risk was by no means detected, it is encouraging that they point essentially to the same direction, as that study reported an over-transmission of the C (F) allele and no association of the rs731236 polymorphism with ISS.

Recently published evidence supports the functional significance of the VDR rs10735810 (FokI) polymorphism, in terms of altered transactivation of vitamin D target genes^{25,26}. Vitamin D receptor response elements (VDREs) have been identified in the promoter regions of the genes encoding GH and IGF-binding protein 3 (IGFBP-3)^{27,28} while vitamin D is known to regulate circulating IGF-1 and the gene expression of its receptor and of various binding proteins²⁹. Short children with GH deficiency were excluded from our study, but IGF-1 levels were unfortunately not recorded for the majority of the participants, due to technical difficulties, and a study of the effect of rs10735810 on serum IGF-1 could not take place. On the other hand, most ISS children tend to have normal serum levels of IGF-1 and the effect of the polymorphism -if any- could be exerted on IGFBP-3 rather than on IGF-1 itself³⁰. Moreover, the directionality of the effect rs10735810 exerts on VDR-driven transactivation appears to depend on both race and target gene²⁶, making a prediction of the effect of the polymorphism on the gene expression of GH or IGFBP-3 impossible at this point.

A serious limitation of our study is, admittedly, its small sample size. As it turns out, for the recorded difference of the rs10735810 genotype distributions between ISS and control children to be statistically significant with

an 80% power of detection, we would need to have twice as many participants. Our results can only be characterized then as preliminary. Another difficulty is the possible confounding effect of non-genetic factors, acting either in utero or post-partum³¹. This may be particularly true of vitamin D, where the optimal levels for VDR function may be individual-specific²⁶. Even so, our tentative conclusion is that VDR is an ISS gene which most likely exerts its effect through the GH – IGF-1 axis. Additional research should help resolve this issue.

Conflict of interest

Authors declare no conflict of interest.

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