

Polymorphisms in the P1 promoter of the *IGF-1* gene in children with growth disorders

Polimorfizmy w promotorze P1 genu *IGF-1* u dzieci z zaburzeniami wzrastania

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Abstract

Background/Aims. The aim of this study was to associate children's growth disorders with polymorphisms detected in the P1 promoter region of *IGF1* (including SNP and (CA)_n microsatellite repeat polymorphism) and IGF1 and IGFBP3 levels. **Methods.** *IGF-1* gene P1 promoter polymorphism was analyzed in DNA obtained from the blood of 51 children with growth disorders and 50 healthy children without growth disorders by means of PCR-SSCP and sequencing. **Results.** Among children with growth disorders and the control group we found previously described polymorphisms in the P1 promoter of the *IGF-1* gene (rs35767, rs5742612) and different genotypes. The frequency of both detected polymorphisms was no significantly different in the study and the control groups. The CA repeat sequence within the group of children in the study ranged from 11 to 21. The most common were homozygote 19/19 (49.02%) and heterozygote 19/20 (27.45%). Our results did not show any association between polymorphisms in the P1 promoter and IGF-1 levels in the serum of children with growth disorders. **Conclusions.** This study demonstrated that SNP and (CA)_n microsatellite repeat polymorphisms by themselves are not the primary regulatory elements of IGF-1 expression. However, our bioinformatics analysis has shown that the (CA)_n microsatellite region in the P1 promoter of IGF-1 is able to form DNA loop structures which can modulate transcription.

Key words:

IGF1, promoter, polymorphism, growth disorders, SNP

Streszczenie

Cel. Celem tego projektu była analiza korelacji polimorfizmów (pojedynczych nukleotydów SNP i powtórzeń mikrosatelitarnych) w promotorze P1 genu *IGF-1* z występowaniem zaburzeń wzrastania u dzieci. **Metody.** W analizie wykorzystano DNA wyizolowane z krwi 51 dzieci z niskorosłością i 50 dzieci niemających zaburzeń wzrastania (jako kontrola). Polimorfizmy w rejonie promotora genu *IGF-1* były analizowane przy użyciu metody PCR-SSCP i sekwencjonowania. **Wyniki.** W grupie dzieci z zaburzeniami wzrastania jak i kontrolnej zidentyfikowaliśmy wcześniej opisane polimorfizmy w promotorze P1 genu *IGF-1* (rs35767, rs5742612) i ich różne genotypy. Częstotliwość zidentyfikowanych polimorfizmów nie różniła się statystycznie w grupy badanej i kontrolnej. W przypadku powtórzeń CA najbardziej popularna okazała się homozygota 19/19 (49.02%) i heterozygota 19/20 (27.45%). Nie wykazano korelacji pomiędzy polimorfizmami w rejonie promotora P1 a poziomem IGF-1 w surowicy dzieci z zaburzeniami wzrastania. **Podsumowanie.** Nasze badania wykazały, że polimorfizmy SNP i powtórzeń mikrosatelitarnych CA nie wpływają na ekspresję *IGF-1*. Analiza bioinformatyczna wskazuje jednak, że powtórzenia mikrosatelitarne CA w promotorze genu *IGF-1* mają zdolność tworzenia pętli w DNA, która może wpływać na wiązanie czynników transkrypcyjnych.

Słowa kluczowe:

IGF1, promotor, polimorfizm, zaburzenia wzrastania, SNP

Introduction

Insulin-like growth factor 1 (IGF-1) is the major mediator in the growth and development of many tissues in the body. IGF-1 has also been implicated in cancer, diabetic and cardiovascular diseases, and aging. The predominant source of IGF-1 is

the liver, which supplies approximately 75% of total circulating IGF-1 for the body; however, tissue-specific expression is also noticeable (1). Thus, IGF-1 reveals both endocrine and auto/paracrine activity. The actions of IGF1 are modulated by bin-

ding proteins (IGFBP1-6), proteases and receptors. Most circulating IGF-1 is bound to IGFBP-3 (2).

The *IGF-1* gene is present as a single copy on chromosome 12q22-q24. *IGF-1* transcription is controlled by two promoters: P1 and P2 that are located upstream of exon 1 and 2, respectively. The main promoter P1 consists of 1630 nucleotides of regulatory region and 322 nucleotides of 5'UTR of exon 1, which is thought to be necessary for correct and efficient transcription from P1 promoter. The sequence of exon 1 is highly conserved among mammalian species and contains multiple initiation sites and binding sites or regulatory elements for such factors as HNF hepatocyte nuclear factor (HNF1, HNF3), CCAAT/enhancer binding protein (C/EBP), GATA element, muscle regulator factors (MRFs), cAMP response element (CRE) and prostaglandin (3-8). However, several polymorphisms have been identified in its 5' flanking promoter region expanding up to 1630 bp upstream on the transcription initiation site of promoter P1 (9-12). (CA)_n microsatellite repeat polymorphism, comprising a variable length of a CA repeat sequence, is located up to around 600-700 bp upstream of the transcription initiation site. The number of CA repeats ranges between 10 to 25 times. The most common allele (in Caucasian populations) contains 19 repeats (12). Polymorphism of promoter CA di-nucleotide repeats has been associated with IGF-1 serum level, birthweight and body height and with such diseases as diabetes, cancer and cardiovascular diseases (13-22). On the other hand, the relation between the CA repeat polymorphism and IGF-1 level depends on race and ethnicity (10-12). Currently nothing is known about the potential role of these sequences as additional regulators of *IGF-1* transcription.

The levels of IGF-1 in tissues and serum vary dramatically and are regulated ontogenetically as well as by multiple hormones and nutritional factors. GH is the most prominent post-natal stimulus for IGF-1 production. IGF-1 transport in serum is mediated mostly by IGFBP3 and the acid labile subunit (ALS), which extend half-life, and may modulate its bioavailability and action. In addition to regulating IGF-1 bioavailability and action, IGFBP-3 also possesses IGF-independent actions, including inhibition of cell growth and induction of apoptosis. It has been proved clinically that measurements of circulating IGFBP-3 and IGF-1 might be useful for the investigation of growth disorders in children (1, 23).

The aim of this study was to associate children's growth disorders with polymorphisms detected in the P1 promoter region of *IGF-1* (including SNP and (CA)_n microsatellite repeat polymorphism) and IGF-1 and IGFBP-3 levels.

Materials and Methods

Patients

We examined a group of 51 children (aged 6-13 years) with significant growth retardation (height standard deviation score [HtSDS]-5.4 to -3.6). Parental heights were normal and did not affect growth prognosis. Growth velocity ranged from 0.3 to 2.6 cm/year. Bone age was delayed on average 3.2 years (range

2.6-4.3 years); according to Grelich and Pyle. As a control group, we examined 50 healthy children without growth disorders. In both groups of children, growth retarded and control groups, we excluded GH deficiency, thyroid deficiency and GHR and IGF1R disorders. Blood samples were collected from healthy controls and growth disorder patients to measure the levels of IGF-1, IGFBP-3 and to isolate genomic DNA. This study was approved by the University Ethics Committee (Medical University of Poznan, Poland).

Plasma IGF-1 and IGFBP-3 analysis

Plasma IGF-1 and IGFBP-3 were measured via the radioimmunoassay technique with the use of commercial IGF1 ELISA kits (IDS Ltd, Immunodiagnostic Systems Limited, Boldon, UK) and IGFBP-3: DIAsource IGFBP-3ELISA Kit (DIAsource ImmunoAssays S.A., Nivelles, Belgium). Plasma IGF1 and IGFBP3 levels were on average 110.3 ng/ml and 1385 ng/ml respectively for children with growth retardation and 201.3 and 1506.5 ng/ml for control group. This study was approved by the University Ethics Committee, Medical University of Poznan, Poland.

DNA isolation

Genomic DNA was isolated from the leukocytes (after separation of blood cells from serum) using a Qiamp DNA Blood Mini Kit (Qiagen, Cat. No. 51104) according to the manufacturer's instructions.

Detection of Polymorphism by SSCP and by Sequencing

Genomic DNA was used for in vitro amplification by PCR with a specific set of primers complementary to the examined region of the *IGF-1* gene. Primers were selected from *IGF-1* gene promoter sequences taken from the Entrez Gene database as described previously (24).

Four fragments of IGF-1 promoter sequence (region a: between -1404 to -1134nt, region b: between -1115 to -784 nt, region c: between -633 to -305 nt and region d: between -250 to +49 nt) were amplified by means of a polymerase chain reaction (PCR).

PCR products were analyzed by means of a single strand conformation polymorphism (SSCP) and by sequencing.

Analysis of the CA repeat polymorphism of the IGF-1 promoter genotyping

PCR, using primers designed to amplify the polymorphic (CA)_n repeat region of human *IGF-1* gene, was performed and analyzed as described previously (25).

Bioinformatics analysis

Transcription factor binding site localization and frequency in the *IGF-1* promoter sequence were analyzed using Alibaba 2.1 software <http://www.gene-regulation.com/pub/programs/alibaba2/index.html> and the TRANSFAC database <http://www.gene-regulation.com/pub/databases.html> (26, 27).

Secondary structures of DNA were predicted using Mfold software <http://www.bioinfo.rpi.edu/applications/mfold> (28).

Statistical analysis

For statistical analysis CA-repeats genotyping data as the mean value of the two alleles (biallelic mean) were applied to Student's t-test. Analyses were carried out by means of the Microsoft Office Excel 2007. Mann Whitney U test was used to assess the difference between SNP genotype groups.

Results

IGF-1 gene P1 promoter polymorphism

IGF-1 gene P1 promoter polymorphism was analyzed in the DNA obtained from the blood cells of 51 children with growth disorders by means of PCR-SSCP and sequencing. Analysis of three regions of the IGF-1 gene P1 promoter from 51 samples showed 2 polymorphisms: RS35767 and RS5742612, previously described in NCBI data. We found 8 genotypes C/T (15.68%), 1 T/T (1.96 %), and 42 C/C (82.35%) for polymorphism RS35767 with localization at -1245nt of the P1 promoter (Figure 1). For polymorphism RS5742612, two heterozygote C/T (3.92%) and 49 (96.08%) homozygote T/T were found. The frequency of both detected polymorphisms was similar in the study and control groups and not significantly different ($P > 0.05$, Mann Whitney U test) (Table 1).

A coexistence of genotype C/T RS35767 and genotype C/T RS 5742612 was found in one subject. One case of coexisting homozygote T/T for RS35767 and heterozygote C/T for RS 5742612 was also observed (Table 1).

The analysis of the CA repeats situated in the P1 promoter region (-1.115 and -784 nt) located at 1kb upstream from the first transcription start site of the IGF-1 gene was also per-

med. The CA repeat sequences in the study group of children ranged from 11 (1.96%), 17 (0.98%), 18 (4.9%), 19 (72.54%), 20(14.7%), 21 (4.9%) (Table 2). Based on repeated CA nucleotides, several genotypes were distinguished (Table 2). The most common was homozygote 19/19 (49.02%) and heterozygote 19/20 (27.45%) (Table 2). The mean biallelic average lengths were $19,02 \pm 0,85$ and $19,06 \pm 0,75$ for children with growth disorders and control group, respectively. These values were no significantly different ($P = 0.8$, Student's t test)

Association analysis of IGF-1 and IGFBP-3 levels and detected polymorphisms among children with growth disorders

Among a group of 51 children with growth disorders, we found 9 children with several genetic polymorphisms (Table 4). Our results did not show any associations between IGF-1 and IGFBP-3 levels and detected SNP and CA repeat sequence polymorphisms.

Bioinformatics analysis

Our bioinformatics studies demonstrated that CA repeat regions of the IGF-1 promoter are able to form DNA secondary structures which can serve as modulators of transcription. The CA regions formed DNA loops which may probably serve as a recognition scaffold for transcription factors or other molecules (Fig. 1)

According to the Alibaba 2.1 software, there is one binding site for WT1 in the context of 11 CA repeats in the IGF-1 promoter, two WT1 binding sites for 19 CA repeats and three for 21 CA repeats.

The hypothesis that the microsatellite CA repeat region creates a loop is supported by the DNA secondary structure prediction program Mfold (Figure 1).

Table 1. Frequency of different genotypes

Tabela 1. Częstość występowania różnych genotypów polimorfizmów RS35767 i RS5742612 wśród dzieci z niskorosłością i w grupie kontrolnej

Polymorphism RS35767			Polymorphism RS5742612			Various polymorphisms among studied group
Type of genotype	Frequency among patients	Frequency among control group	Type of genotype	Frequency among patients	Frequency among control group	
C/T	8/51 (15.68%)	10/50 (20%)	C/T	2/51 (3.92%)	2/50 (4 %)	1 subject of A*
T/T	1/51 (1.96%)	0/50 (0%)	T/T	49/51 (96.08%)	48/50 (96%)	1 subject of B*
C/C	42/51 (82.35%)	40/50 (80%)	-	-	-	42 subjects of D*

A* (Genotyp C/T of RS 35767, Genotyp C/T of RS5742612)

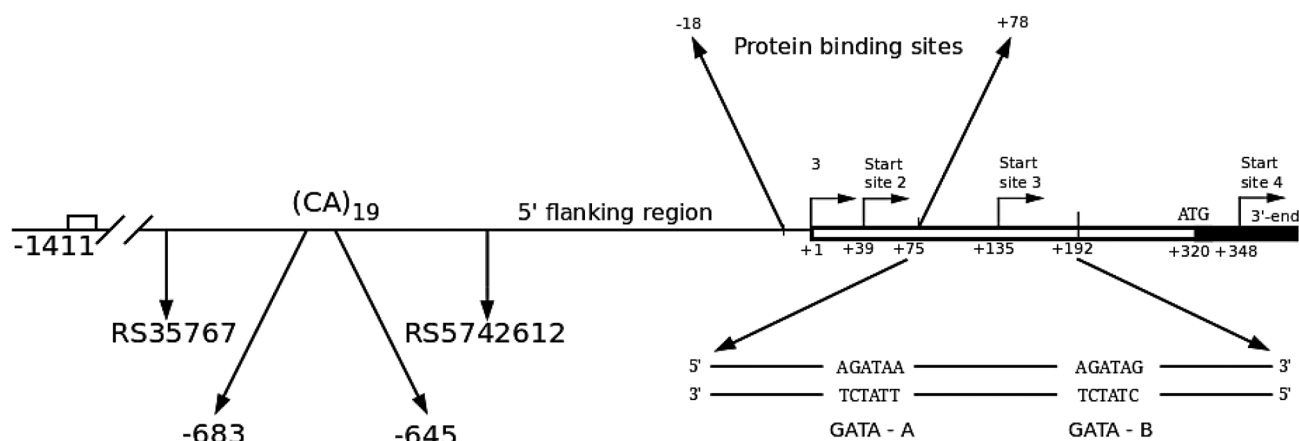
B* (Genotyp T/T of RS 35767, Genotyp C/T of RS5742612)

C* (Genotyp C/T of RS 35767, Genotyp T/T of RS5742612)

D* (Genotyp C/C of RS 35767, Genotyp T/T of RS5742612)

Table 2. Frequency of genotypes based on repeated CA nucleotides situated in the P1 promoter region (-1,115 and -784 nt) in population of children with growth disorders**Tabela 2.** Częstość genotypów opartych na powtórzeniach CA w rejonie promotora P1 (-1,115 ; -784 nt) w populacji dzieci z niskorosłością i w grupie kontrolnej

Genotypes based on repeated CA nucleotides situated in the P1 promoter region (-1,115 and -784 nt)	Frequency of genotypes in examined population	Frequency of genotypes in examined control group
19/19	25/51 (49.02%)	27/50 (54%)
19/20	14/51 (27.45%)	9/50 (18%)
19/21	3/51 (5.88%)	5/50 (10%)
11/19	1/51 (1.96%)	1/50 (2%)
17/19	1/51 (1.96%)	1/50 (2%)
18/19	5/51 (9.8%)	6/50 (12%)
20/21	1/51 (1.96%)	1/50 (2%)
11/21	1/51 (1.96%)	-

**Fig. 1.** Schematic diagram of the human IGF1 promoter showing localization of analyzed RS35767, RS5742612 and CA repeat sequence polymorphism. The pictures also show transcription start sites, the translation start site (ATG), the location and sequences of putative GATA elements and the ERE-like (EREL) sequence. Numbers are relative to the translation start site (ATG). The white bar represents 59-UTR and the black bar represents a coding sequence. Based on Kang 2012 and Wang L 2000, but modified
Ryc. 1. Schemat promotora ludzkiego genu IGF-1, pokazujący lokalizację analizowanych polimorfizmów: RS35767, RS5742612 oraz powtórzeń CA (na podstawie pracy Kang 2012 and Wang L 2000 ale zmodyfikowany)

Discussion

Transcriptional control of the *IGF-1* gene is complex and depends on: two promoter sites: P1 and P2, multiple transcription factor binding sites, as well as a CA repeat sequence, which is probably involved in transcription modulation. The majority of the control of *IGF-1* gene expression occurs at the P1 site. Deregulation of *IGF-1* expression may lead to a wide variety of human disorders (1). Many studies have also reported the association between P1 promoter polymorphisms, IGF-1 blood level and such diseases as diabetes, cancers and cardiova-

scular diseases (13-22). In the present study, we examined the association between *IGF-1* P1 promoter polymorphisms, IGF-1 levels and growth disorders in children.

Among the studied children with growth disorders we found two previously described polymorphisms, RS35767 and RS5742612, (22) and 8 different genotypes. *IGF-1* gene polymorphism genotype distribution between the study group and the control group of children was not statistically different.

In the study group, the most frequent allele of the CA repeat sequence polymorphism contains 19 CA repeats and homozygote carriers. 19 CA repeats were found in 49% of the studied

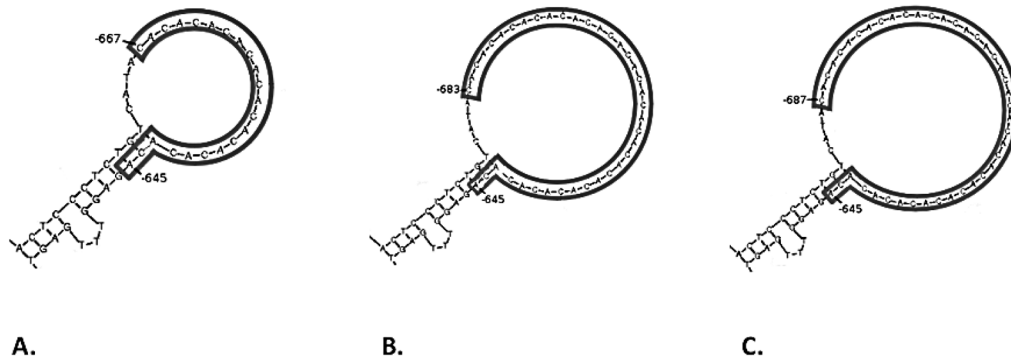


Fig. 2. DNA secondary structure prediction of the IGF1 promoter microsatellite region by means of M fold software. DNA secondary structure for 11 CA repeats (A), 19 CA (B) and 21CA repeats (C)

Ryc. 2. Model przedstawiający drugorzędową strukturę DNA w promotorze genu *IGF-1* w miejscu występowania 11 powtórzeń CA (A), 19 powtórzeń CA (B) oraz 21 powtórzeń CA (C)

children. The length of the repeat sequence in children with growth disorders ranged from 11 to 21. A similar distribution of CA polymorphism was observed among the control group. The 19 CA repeats allele is most frequent in both Caucasians and African-Americans (12). Akin et al. indicated that alleles with 20 CA of the *IGF-1* promoter have higher circulating IGF-1 levels than others in patients with acromegaly (14). Other studies have indicated that carriers of the allele with 19CA repeats and/or 20 CA repeats of the *IGF-1* promoter have higher, lower or similar circulating IGF-1 levels than non-carriers of these alleles (29). No effect on stature of *IGF-1* gene polymorphisms was demonstrated by Tekcanet al (30). This study has reported conflicting results on the associations between P1 polymorphisms and IGF-1 levels in blood serum. We observed that some of the children with growth disorders also had significantly higher levels of IGF-1 and IGFBP-3. IGFBP-3 regulates IGF-1 availability and function.

IGFBP-3 also possesses IGF-independent actions, including inhibition of cell growth and induction of apoptosis (31). All this data suggest that detected *IGF-1* P1 promoter polymorphisms by themselves are not the primary regulatory element of IGF-1 expression. Probably, additional not well known factors, such as the combination of genetic, nutritional, and environmental factors, may be involved in *IGF-1* expression regulation.

Our bioinformatics studies showed that the CA repeat region of the P1 promoter of *IGF-1* is able to form DNA loop structures which can probably serve as a transcription modulator. Such DNA loop structures are formed for example in the promoter region of *Bcl2* (32) or in RNA of protonated adenine-cytosine base pairs that contribute to structural stabilization and are important protein recognition sites (33-35). Therefore, we suggest that the CA repeat region of IGF-1 may probably serve as a recognition site for the WT1 transcription factor or other molecules and might have an influence on *IGF-1* promoter activity and the blood levels of IGF-1. However, further studies are needed to prove this hypothesis. WT1 is a transcription factor acting as a tumor suppressor protein regulating growth and differentiation. Vasileiou et al. indicated that WT1 gene expression is implicated in

altered IGF-1 and IGF-2 activity and mutations of the gene may lead to overgrowth during fetal life (35). On the other hand, most biological actions of IGF-1 are mediated by the IGF-1 receptor which is associated with ras/raf MAPK and phosphatidylinositol 3 kinase signaling cascades. In vitro studies confirmed the unequivocal correlation between the IGF-1 receptor and WT1, suggesting that expression of the WT1 protein likely suppresses IGF-1 receptor activity to a different extent (36).

Changes in the numbers of CA repeats as well as aberration in WT1 expression might have an influence on *IGF-1* promoter activity; however, further studies are needed to prove this hypothesis. Hewitt et al., by means of a chromatin immunoprecipitation assay, indicated binding of the estrogen receptor alpha at the human *IGF-1* promoter, where there are no estrogen response element (ERE) sequences present (37). Telgmann et al (8) described, in the distal region of the P1 IGF-1: -1411 C>T site, a transcriptionally active enhancer containing a cluster of several partly overlapping TFBSs(AP1, HNF3, C/EBP) capable of integrating transcriptionally active stimuli from different signal transduction pathways in a cell type-specific manner. *IGF-1* promoters also respond to a diet and caloric intake (38). Maternal nutrient deprivation results in intrauterine growth restriction of the offspring, and neonates from such deprivation have deficiencies in postnatal circulating IGF-1. Additionally, CpG islands are methylated (39). These epigenetic modifications of the IGF-1 promoter repress transcription. Further studies on a large population are required to confirm these data.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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