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Screening of *GHRHR* and *Npr2* genes as a genetic contributors of dwarfism in familial dwarfism of Khyber Pakhtunkhwa, Pakistan

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Abstract

Dwarfism is a condition characterized by abnormal growth of body. In this condition, the parts of the body which have direct role in height like cartilages, bones and tendons, remain under developed. On the basis of tissues or cell suffering, dwarfism has been identified in large number of people have prevalence up to 200 such as achondroplasia, hypoachondroplasia, skeletal dysplasia, and simple dysplasia. Several factors for example hormones, genes involved in body growth, and even an environment have been studied as a cause of dwarfism. Some of the genes that are identified in individuals with dwarfism are *GH, FGHR3, PNCT* and *GHRHR*. This study was designed to investigate some of the important genes in three consanguineous families A, B and C. Different information such as pedigree construction, clinical features and reports of the patients were obtained. All the families were having autosomal recessive mode of inheritance of dwarfism. Techniques of Thermal Cycler and 2D gel electrophoresis were used for this analysis. The genotyping results of the families A, B and C, showed no linkage for the specific loci of the two genes. From the above study it may be concluded that this was the first report on the analysis of GHRHR gene as well as the subject samples of the District Karak, Khyber Pakhtunkhwa, Pakistan.

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Introduction

Dwarfism is a condition of a short stature characterized by constriction of certain body parts including bones, cartilages, tendons and other muscles which have a dominant role in body skeleton or stature (Rayan,2014). Squeezing of vertebral column and low length of the dorsal portion of the body are some of the additional features of dwarfism [Jabs *et al*, 1994]. It has autosomal recessive mode of inheritance with occurring ratio of 1/1,000,000 [Kruse II, 2003]. The growth of the body of dwarf individual retards directly after birth, and causes abnormal growth of skeletal muscles, cartilages, bones and other muscles [Peng *et al*, 20034]. A number of genes such as GHRHR, Npr2 and FGFR3 play role in the body growth. Previous researches investigated that mutation in the genes, causes unusual body growth. For example, FGFR3 gene mutation, disabling this gene function, was reported in the individuals who were suffering with achondroplasia [Wilkin *et al*, 1998, Kimura *et al*, 2001]. Furthermore, another mutation named as G380R inactivates and makes the truncated protein product after alteration [Bellus *et al*, 1999]. Also, Npr2 known as natriuretic peptides is another important gene which belongs to a receptor family [*Kishimoto I* 2001, Wang *et al*, 2014, *Tamura et al,2004,Langenickel TH,2006,* Thompson IR,2009].

This gene is involved in number of biologically important processes that occurs in body regulation of blood pressure, ossification and axonal path through binding to the cell surface receptors which are involved in the process of ossification [Wang *et al*, 2014]. The gene has many subunits like A, B, C for specific function expressed in different cells. For example, NPR-B in heart, vessels, brain, uterus and chondrocytes (Potter *et al.*, 2006, Silberbach *et al.*, 2001, Pagel-Langenickel *et al.*, 2007). Another form of a short stature known as acromesomelic dysplasia identified in some of the families in Pakistan is considered to be due to alteration in Npr2 gene located on chromosome number 9 with cytogenetic location 9p13-q12 (Khan *et al.*, 2012). Some other researchers identified other mutations in the Npr2 (Bartels *et al.*, 2004; Yoder *et al.*, 2010). Likewise, also, in GHRHR gene, several mutations causing dwarfism have been identified (Salvatori *et al.*, 2002; Alatzoglou *et al.*, 2012). Previously, only one investigator from India analyzed the GHRHR gene in the dwarf individuals from Sindh (province of Pakistan) in the US research laboratories (Maheshwari *et al.*, 1998). In the current study, three families belonging to District Karak of Province Khyber Pakhtunkhwa, Pakistan, were investigated. All the families' samples were having all the features of dwarfism with minimum of two effected individuals. All the subject samples were screened for GHRHR and Npr2 genes which were proposed to be the cause of dwarfism. This is the first study on the dwarf individuals of the District Karak and also GHRHR gene was screened for the first time in Pakistani dwarf individuals in Pakistan's research laboratories.

Methods and materials

Ethical Approval of Study and Investigation of the Families

Different genetic contributors of dwarfism in three consanguineous families (A, B, C) of district Karak, Khyber Pakhtunkhwa, Pakistan, were investigated. All the families were having autosomal recessive mode of inheritance of the dwarfism. The proposed research was preceded after obtaining informed consent from the patients and final approval was given by Board of study (BOS) of International Islamic University Islamabad (IIUI), Islamabad, Pakistan.

Pedigree Construction

Pedigree chart contains certain specific symbols including circles, squares, filled squares, circles, double lines, single line and straight lines. Each and every symbol has its own genetic meaning. Male and female are represented by squares and circles, respectively. Those who were abnormal are shown by filled circles and squares for both male and females, respectively. Deceased male and female individuals are represented by filled circles or squares, respectively. Roman numbers indicate generation of the respective families. Family or cousin marriages are shown by double lines.

DNA Isolation and Confirmation

Blood samples from the two families including both affected and unaffected individuals were collected in EDTA vacationers (BD, Franklin NG, USA). DNA from the blood was extracted through phenol – chloroform method which is also known as organic method (Chomczynski& Sacchi 1987; Kramvis *et al*., 1996; Walsh *et al*., 1991). Confirmation of DNA was done by agarose gel electrophoresis (Casse *et al*., 1979; Orita *et al*., 1989).

Micro-satellite Markers Designing

Markers were synthesized using bioinformatics tool, The University of California Santa Cruz (UCSC) genome browser and ordered for suspected selected genes; GHRH-R and Npr2. Markers were diluted by centrifugation for 13000 rpm for 1 minute and Tris EDTA (TE) buffer was added to markers in a ratio of 1:3 for dilutions.

PCR Amplifications

Amplification conditions for both the genes microsatellite markers were kept according to the method described by Carakushansky *et al.* (2003). For Npr2, method of Khan *et al.* (2010) was used. Linkage analysis for both the candidate genes, GHRHR and NPr2 for both the families was carried out by using their respective microsatellite markers; D7S2491, D7S2496, D7S435, D7S2252 for GHRHR gene mapped on chromosome 7 (7p13-p21), and D9S1118, D9S1845, D9S1817, D9S50, D9S1874 for *NPR2* mapped on chromosome 9 (9p13-q12).

Screening GHRHR and Npr2 genes

Amplification of both the genes exon-intron borders were carried out by using polymerase chain reaction through Veriti Thermal Cycler (ThermoFisher Scientific, USA) with specific primers which were designed for the candidate genes. Conditions for PCR amplifications were optimized as; 95 °C for 1 min denaturation process which was then followed by other 30 cycles of 95 °C for the duration of 35 s, 60 °C for 35 s, and 70 °C for 3.5 min, followed by a single incubation at 70 °C for 10 min.

Results

Clinical features

Three different families namely A, B and C were collected from three different villages including Ganderikhattak, Ahmadabad and ZarkiNasrati, respectively of Distract Karak, Khyber Pakhtunkhwa, Pakistan. The pedigrees of all these collected families (Figs 1-3) were constructed up to four generation using the information given by the elders of the concerned families. Pedigree analysis suggests and clearly shows that the dwarfism is autosomal recessive in its mode of inheritance. The number of family's members was six, seven and six, respectively. Blood was collected from all individuals.

Table 1. Age, height, medical condition and associated disorder of normal and dwarf individuals.

S. No.	Pedigree No.	Individual		Father's height/Inches	Mother's height/Inches	Individual's clinical feature	Individual's associated disorder
		Age	Height/Inches				
1	VI-1	38	46	68	61	Achondroplasia	Sunken eyes
2	VI-2	33	43	68	61	Achondroplasia	N/A
3	VI-4	27	41	68	61	Achondroplasia	N/A
4	VI-5	27	37	65	59	Hypopituitarism	Irregular teeth
5	VI-2	28	37	65	59	Hypopituitarism	Irregular teeth
6	IV-3	10	29	N/A	63	N/A	N/A
7	IV-4	28	42	N/A	63	N/A	N/A

All the affected individuals in the collected families were clinically examined and their clinical reports were compared and studied which were clearly declared by orthopedic surgeons as hypopituitarised. Table 1 lists age, height of individuals and their parents, and individual's clinical features and associated disorders. Other differences such as irregular teeth, and sunken eyes were also observed

(Fig. 4). The clinical report of the individuals of family A were looking normal in all other respect except of short stature while that of family B individual was found with relatively large head size as compared to the main trunk. Family C individual was identified with deep and sunken eyes and relatively large face (Fig. 4).

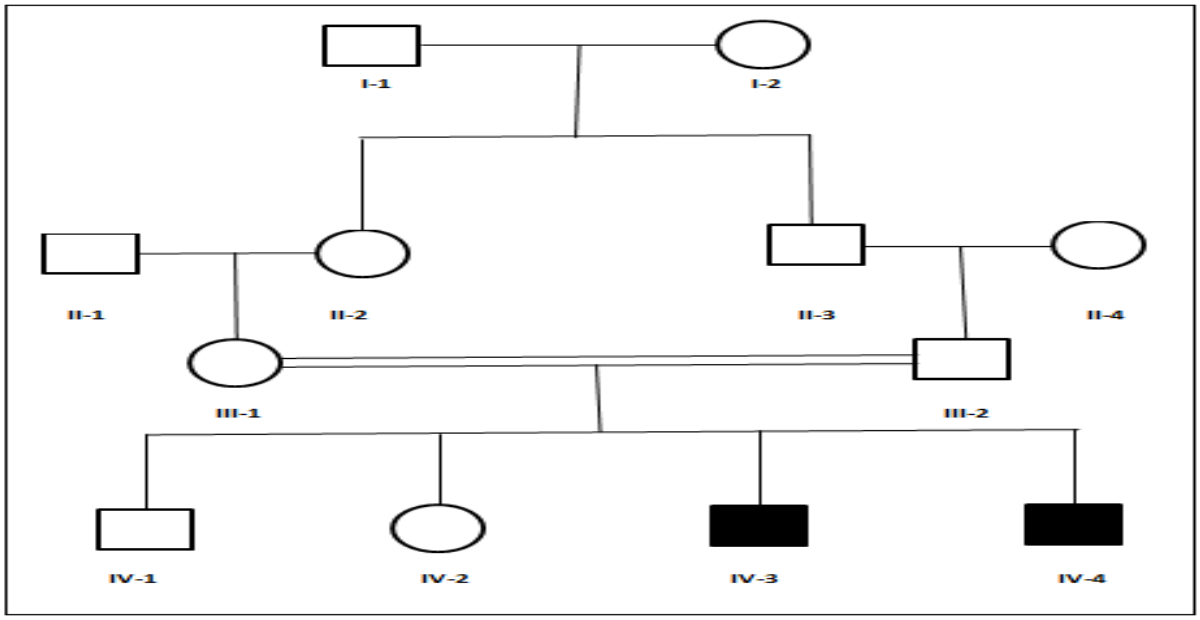

Fig. 1. Pedigree of family A (Village GanderiKhattak) having two dwarf individuals.

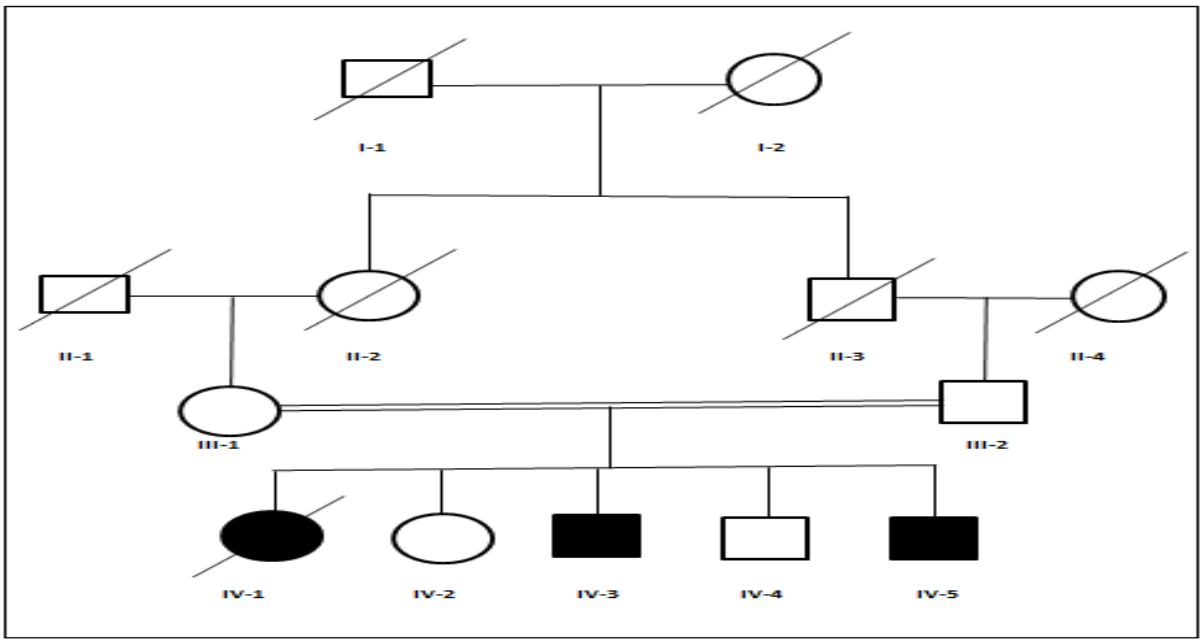

Fig. 2. Pedigree of family B (Village Ahmad Abad) having three dwarf individuals.

Linkage and mutation analysis of GHRHR and Npr2 genes

The families A, B and C were investigated for the proposed alteration or mutation in the GHRHR and Npr2 genes and linkage screening. Both genes were genotyped by using microsatellite markers at optimized condition of temperature and time for polymerase chain reaction through Thermal Cycler.

The PCR product was then investigated by using 8% polyacrylamide gel and all the markers were stained using ethidium bromide.

Fig. 3A-D. A and B show similar stature, C showing the bigger and deep eyes, and D showing the bigger head size of a dwarf individuals.

Fig. 4. DNA bands of all the individuals of the family.

These markers were visualized under UV documentation system and photos were taken through computerized camera attached to UV gel documentation system (Fig.5). On the basis of studying the linkage phenomena, banding pattern of the families for the suspected gene loci of GHRHR and Npr2 indicated the same banding pattern of all markers.

Therefore, no linkage was established. Results of some of the micro-satellite markers of banding pattern are given in Figs6-8.

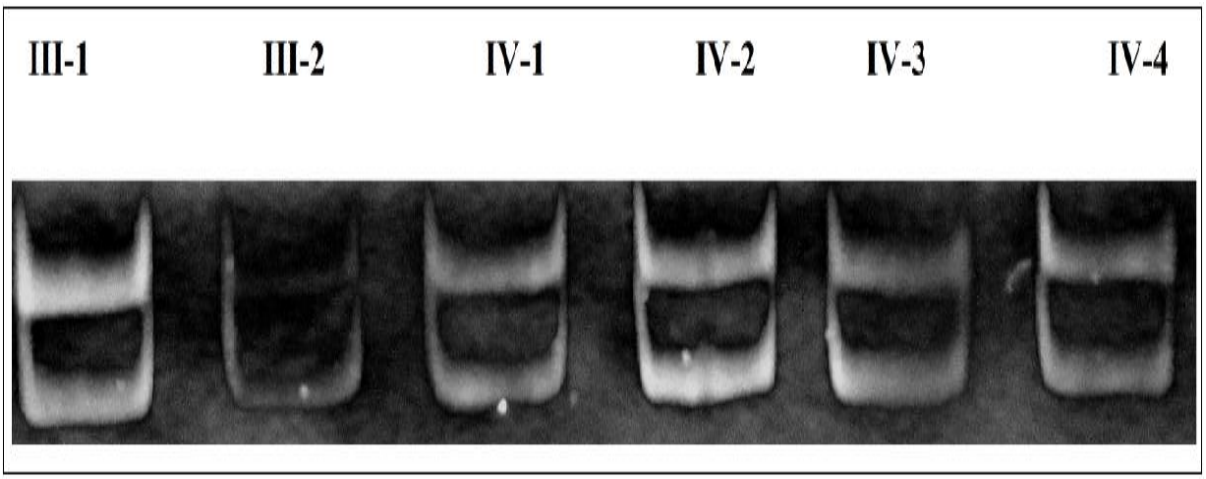

Fig. 5. Banding patterns of normal and affected individuals on micro-satellite marker D7S2252 on GHRHR gene locus of family B. IV-3 and IV-4 pedigree number shows the dwarf individuals of the Family B.

Discussion

The diagnoses of the three families having dwarf individuals were made upon the clinical record and medical reports issued to their family members. The clinical reports of short stature, large head and face size, and deep and sunken eyes were also previously reported in Japanese patients (Tamura *et al.*, 2004).Miglianoet *al.* (2013) reported both the Npr2 and GHRHR genes mutation in little mice. GHRHR gene mutation in dwarf individual of province Sindh (Pakistan) was reported by Baumann *et al.* (1997).

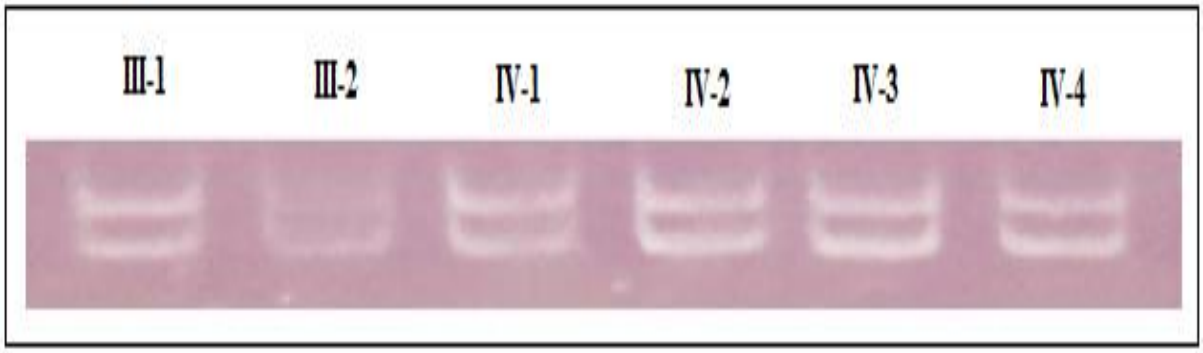

Fig. 6. Banding pattern of D7S2496 micro-satellite marker having cytogenetic location 47.04 cM of Family B.

The mutation detected in these cases was a nonsense mutation in the exon no 3 which altered codon (GAG-->TAG, Glu50-->Stop) as well as function of GHRHR gene causing under developed growth in the investigated individuals. Other researchers reported inactivating mutation in GHRHR gene that converts a functional codon to a stop codon (Maheshwari*et al.*, 1998).

Furthermore, mutation in GHRHR gene in some other form of dwarfism such as isolated growth hormone deficiency was also reported (Hilal*et al.*, 2008). This mutation was of transversion type in which alteration of base pair of pyrimidine or purine group are replaced by their group base pairs such as guanine-adenine (G-A), cytosine-thiamine (C-T) (Salvatori *et al.*, 1999). Another stop or inactivating mutation was identified at position 72 which converts Glu72–stop codon previously identified in little mouse (Wajnarijch*et al.*, 1996).

In some other individuals suffering with isolated growth hormone deficiency, were identified with missense mutation at position number 176 (Carakushansky*et al.*, 2003).

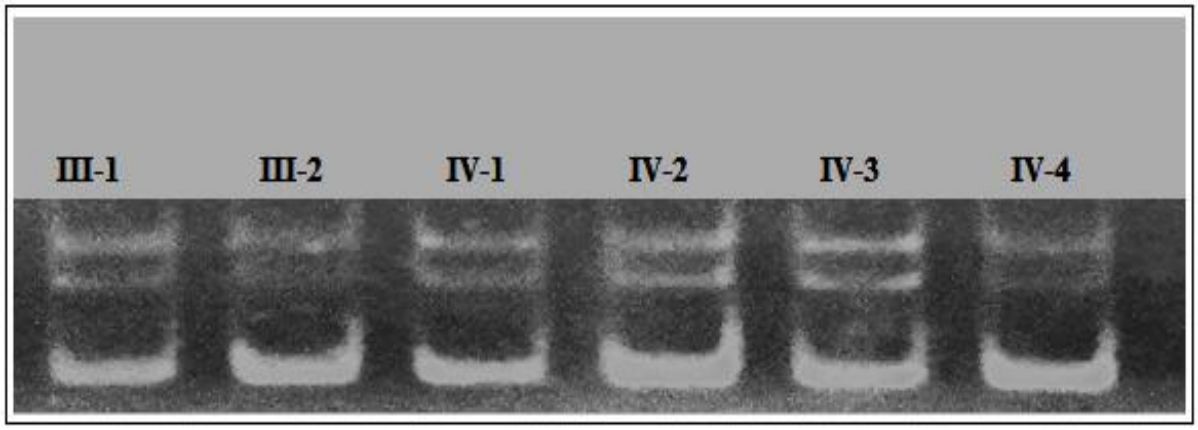

Fig. 7. Banding pattern of D7S2252 marker having location 50.85 cM on chromosome 7 for GHRHR gene locus of normal and dwarf individuals.

Conclusion

This research was designed to find out the biological and genetic changes which affect body growth or height. The effect of the two genes GHRHR and Npr2 genes on the body growth was studied in three different families of distract Karak who were having individuals with dwarfism. Both the genes Npr2 and GHRHR having the key roles in the process of ossification were screened for their role in bones, cartilages, other body muscles elongation, and development.

The current research did not show any linkage on both the loci for GHRHR and Npr2 genes. Therefore, this study recommends investigating some other genes such as FGFR3 and GH genes for dwarfism disorders.

Acknowledgement

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Conflict of Interest

The Authors have no conflict of interest.

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