Chapter 08

Utility of Adoption a Molecular Method for Diagnosis of Short Limb Dwarfism

Abdel-Rahman Eid[1]*, Mohammad Al-haggar[1] and Islam Nour[2]

[1]Genetics Unit, Pediatrics Department, Mansoura University Children's Hospital, Mansoura University, Egypt
[2]Neonatal Intensive Care Unit, Pediatrics Department, Mansoura University Children's Hospital, Mansoura University, Egypt

***Corresponding Author:** Abdel-Rahman Eid, MD, Ph.D., Pediatrics Department, Mansoura University, Children's Hospital, Mansoura University, 35516, Egypt, Tel: 00966545466780; Email: Abdelrahmaneid_pedia@yahoo.com

Abstract

Background and Objectives: Mutation analysis in Egyptian children with clinical diagnosis of short limb dwarfism (Achondroplasia). In addition, to adopt a molecular method for both prenatal and postnatal diagnosis.

Materials and Methods: Eight sporadic cases of short limb dwarfism were studied. A mutation analysis was done by PCR/RFLP (Polymerase chain reaction/Restriction fragment length polymorphism). Results of RFLP were confirmed using sequencing of PCR products.

Results: The G380R mutation was positive in all eight probands (100%) and negative in all parents (0%). Results of RFLP were confirmed using sequencing that revealed substitution of guanine by adenine at the nucleotide (nt. 1138).

Interpretation and Conclusions: All cases had the G-A transition at nt. 1138 of the FGFR3 gene. Our conclusion is that vast majority of Egyptian achondroplasia patients have the same mutation. Mutation analysis is useful for both prenatal and postnatal diagnosis.

Keywords

Short Limb Dwarfism; Achondroplasia; Molecular Diagnosis

Abbreviations

Bp: Base Pair; FGFR: Fibroblast Growth Factor Receptor; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism

Introduction

Achondroplasia is a form of short limb dwarfism that has captured man's imagination since ancient times. It is the most common skeletal dysplasia in humans which caused by defect in cartilage-derived bone. The prevalence is estimated to be 1 in 12,000, and it is usually recognized at birth [1].

Achondroplasia is inherited as an autosomal dominant disorder with complete penetrance. The gene for achondroplasia was localized to 4p16.3 [2,3]. DNA analysis has revealed that the vast majority of the achondroplasia patients have the same mutation at DNA nucleotide 1138 in the gene encoding fibroblast growth factor receptor 3 "FGFR3". It was mapped to the distal short arm of chromosome 4 in 1994. Essentially all patients with the classical features of achondroplasia have the same glycine to arginine substitution at position 380"G380R". Additional FGFR3 mutations were subsequently detected in thanatophoric dysplasia, hypochondroplasia and other disorders whose clinical phenotypes resemble achondroplasia. More than 97% of achondroplasia have a G to A point mutation at nucleotide 1138 of the FGFR3 "c.1138G>A" while approximately 1% have a G to C point mutation at the same site "c.1138G>C" [4].

Most analyses were performed on individuals with heterozygous achondroplasia, but the G380R mutation was also detected in several individuals with homozygous achondroplasia, in which both parents of the proband had achondroplasia [5]. Most of the heterozygous achondroplasia cases are sporadic, as 80% to 90% of the cases are due to new mutation [6].

Achondroplasia clinical diagnosis is based definite on physical features. In heterozygous state, it is non-lethal, with average intelligence unless hydrocephalus or other central nervous system com-

plications arise. They are at risk of medical complications like cervicomedullary compression, spinal stenosis, obstructive sleep apnea, frequent sinusitis and otitis media. In homozygous state, achondroplasia is a lethal disorder [7].

The present study was carried out to screen the Egyptian children with clinical diagnosis of achondroplasia, for the worldwide prevalent mutation "c.1138G>A" in FGFR gene. In addition, our aim is to adopt a molecular method for the solid diagnosis of this relatively common disease of hereditary short stature as an initial step for genetic counselling giving these families prenatal diagnostic options and even therapeutic options through preimplantation genetic diagnosis (PGD).

Materials and Methods

The study was conducted in the outpatient Genetics Clinic in Mansoura University Children's Hospital and National research Centre. Eight cases of clinically screened achondroplasia were studied. Diagnosis was based on characteristic clinical features and radiological findings. All patients were subjected to full history taking, pedigree construction, clinical examination, complete bone survey, and molecular analysis for all probands and their parents as well. Characteristic clinical features are rizhomelic limb shortening, face showing frontal bossing and mid-face hypoplasia, large head, trident hand and exaggerated lumbar lardosis. Radiological findings are progressive narrowing of interpedicular distance between L1-L5, smaller greater sciatic notch, short tubular bones, square shape iliac, contracted base of skull and long fibula.

Molecular Diagnosis

DNA was extracted from peripheral leukocytes. PCR was performed on the extracted DNA using both outer and inner primers according to the following protocol: an initial denaturation step at 94°C for 5 min, followed by 33 cycles of 25 s at 94°C, 35 s at 65°C, 25 s at 72°C, and a final extension step of 10 min at 72°C. These steps were automated on the Invitrogen Platinum PCR supermix (Invitro-

gen, Life Technologies, Carlsbad, CA, USA), 50 ng genomic DNA, and 1.5 pmol of forward and reverse primers. The PCR product was electrophoresed on 2% standard agarose gels. The fragments were visualized by ethidium bromide on a UV transilluminator. The primers used to amplify the codon 380 of FGFR3 are as follows: outer G380R-fC-sense-: 5′-GTGCTGAGGTTCTGAGCCCCCTTCC- 3′; outer G380R-rC –antisense-: 5′-ACTTTCTGTTACCTGTCGCTT-GAGCGGG-3′; inner G380R-G -antisense-: 5′-CAGGATGAACA-GGAAGAAGCCCACACC-3′; inner degenerate G380R-R –sense-: 5′GTGTGTATGCAGGCATCCTCAGCTCC -A/C- 3′. PCR product of 164 base pair (bp) was subjected to restriction enzyme digestion. PCR product was digested with Sfc1 (New England Biolabs) to identify G→A mutation and Msp1 (Fermantas Inc USA) to identify G→C mutation at 25°C and 37°C overnight respectively. In presence of G→A mutation, PCR product of 164 bp is cut into two fragments of size 109 bp and 55 bp. These fragments were separated on 15% polyacrylamide gel electrophoresis. If GC mutation is present then the Msp1 would cut PCR product into 107 bp and 57 bp.

Sequencing for PCR products were cycle-sequenced and analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequence and mutation analyses were performed using mutation Surveyor v2.51 (Soft Genetics, Pennsylvania, USA), Alamut v1.31e (Interactive Biosoftware, Rouen, 76000 France). Sequencing was done to confirm results of PCR for RFLP.

Results

This study included eight sporadic cases (3 males and 5 females) with clinically suspected achondroplasia. All cases were of pediatric age group and none of the parents were pathologically short. All patients and their parents were subjected for molecular analysis with the exception of two fathers who were abroad. All cases had negative consanguinity except one family (case No. 2). History of prenatal exposure to heat and chemicals was negative in all cases (Table 1). Informed consents were obtained from legal representatives of all children included in the study.

Table 1: Demographic data of studied probands.

Case number	Age at time of enrollment	Sex	Consangui-nity	Paternal age at child birth	Maternal age at child birth	Family history of pathological short stature
AA-A1	6 months	male	negative	30 years	24 years	negative
AA-B1	9 months	female	positive	30 years	23 years	negative
AA-C1	19 months	male	negative	25 years	21 years	negative
AA-D1	11 days	male	negative	43 years	31 years	negative
AA-E1	5 years	female	negative	45 years	35 years	negative
AA-F1	4 years	female	negative	43 years	39 years	negative
AA-G1	2 years	female	negative	43 years	18 years	negative
AA-H1	3 years	female	negative	34 years	31 years	negative

All cases have the characteristic facial stigmata of achondroplasia in the form of frontal bossing, mid face hypoplasia and depressed nasal bridge. General examination revealed lumber lordosis which was marked in proband AA-E1. Examination of upper and lower limbs in the selected cases revealed that all cases have apparent rhizomelic shortening of all limbs (Figure 1), bowing of legs or genu varum and multiple skin creases over the limbs. Abdomen, heart and chest were clinically free in all cases. Neurological examination revealed hypotonia in all cases.

Bone survey: Midface hypoplasias, frontal bossing and large with relative small skull base were common findings in bone survey for the selected cases. Anteroposterior view of the spine revealed caudal narrowing of the interpediculate distance, while lateral view of the spine revealed shortening of the pedicles with significant posterior scalloping (Figure 2 and 3). The pelvis has small square iliac wings, small short sacrosciatic notch, flat horizontal acetabular roof and short femoral neck with elongated trochanters (Figure 4). Long bones are short and thick with metaphyseal flaring. The humerus is markedly shortened and femur is shortened as well. The fibula is typically longer than the tibia. The hands are broad with short metacarpals and phalanges (brachydactyly) and a trident configuration.

Figure 1: Clinico-radiological criteria of achondroplasia.
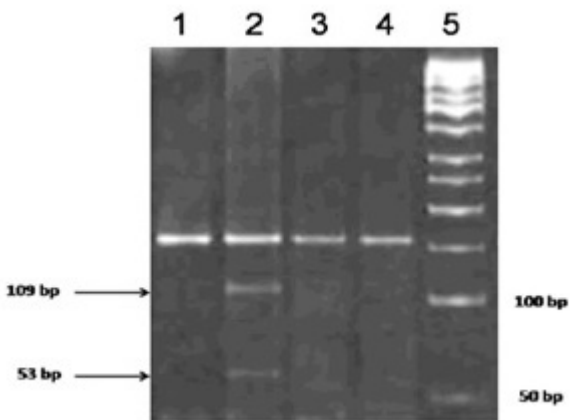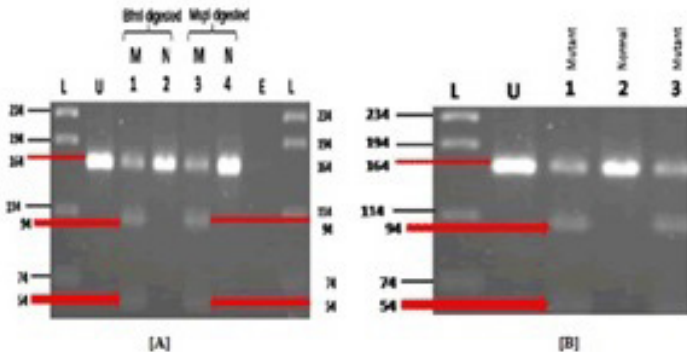

Figure 2: Gel electrophoresis for mutation analysis, lane 1 and 2 represent undigested and digested PCR product of a patient where mutation was found. Lane 3 and 4 represent undigested and digested PCR product of a patient where no mutation was found. Lane 5 is 50 bp DNA ladder.

Figure 3: (A) Gel electrophoresis of BfmI and MspI digested PCR products: lane L→DNA ladder; lane U→ undigested PCR product (control); lane 1→BfmI digested PCR product of a 1138G›A heterozygous; lane 2→ BmfI digested amplification product of a normal individual; lane 3→ MspI digested product of a 1138G›A heterozygous; lane 4→ MspI digested amplification product of a normal individual. (B) Gel electrophoresis of BfmI digested PCR products: lane L→DNA ladder; lane U→ undigested PCR product (control); lane 1, 3→ digested PCR product of patients heterozygous for 1138G›A mutation; lane 2→ digested PCR product of a normal individual.
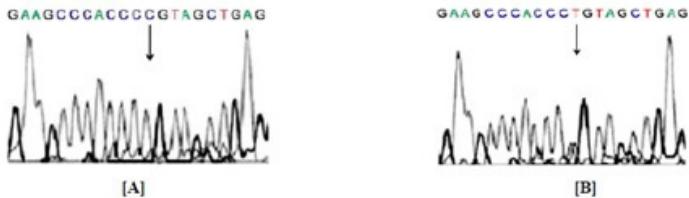
Figure 4: (A) Normal sequence of DNA stretch of FGFR3 gene embracing the position 1138. (B) Arrow showing heterozygous G→A mutation at position 1138 of transmembrane domain of FGFR3 gene in patient.

Molecular studies: Molecular evaluation of extracted and amplified DNA was done firstly using the RFLP for nt. 380 of FGFR3 for all probands and parents. The G380R mutation was positive in all 8 probands (100%) and negative in all parents (0%). Results of RFLP were confirmed using sequencing that revealed substitution of guanine by adenine.

Discussion

Achondroplasia is by far the most common form of dwarfism in humans. Hence, very few mutations are responsible for achondroplasia, it is phenotypically and genetically homogenous. It is inherited in an autosomal dominant pattern [1,8]. Eighty eight percent of cases in the present study were the offspring of non-consanguineous parents. Consanguinity was noticed only in 1 out of 8 cases (12%) supporting the autosomal dominant inheritance of the disease where consanguinity has no role in its inheritance.

All studied cases were sporadic. A paternal age at birth of cases was above 35 years in 4 cases (50% of cases). Achondroplasia was the first genetic disorder that was hypothesized to have a paternal age component. Sporadic cases of achondroplasia and other dominant genetic disorders have been associated with advanced paternal age, suggesting that these mutations occur preferentially during spermatogenesis [9,10].

Diagnosis of achondroplasia is based on clinical findings, radiographic features and genetic test results [11]. In our study, all cases were disproportionately short with short limbs mainly proximal (rhizomelic); they had relatively long narrow trunk. It was noted that short stature becomes more apparent with age. Upper Segment/Lower Segment ratio was above the 97th centile in all cases indicating that disproportionate short stature is caused primarily due to short limbs.

All included cases had the typical facial features described in Achondroplasia including large head with frontal bossing, mid face hypoplasia, flat nasal bridge and short neck. The hands were short and broad with fingers exhibiting a trident appearance. Multiple skin creases were evident in most of the cases. They also had hyperextensibility of all joints especially the knees and hands, but full extension and rotation of the elbow was usually restricted in all cases. No dislocation of radial head was present.

Skeletal radiographs can be used to confirm the diagnosis in cases with achondroplasia with specific age-related criteria [12]. Skeletal survey was carried out for all of our studied cases to explore the characteristic radiological features of achondroplasia. Lateral view of skull X-ray revealed midface hypoplasia, frontal bossing, large with relative small skull base and narrow foramen magnum. Anteroposterior view of the spine revealed caudal narrowing of the interpediculate distance in all cases. Lateral view of the spine revealed shortening of the pedicles with significant posterior scalloping that becomes more apparent with age while Anterior vertebral wedging becomes less evident. Exaggerated lumbar lordosis was noticed in most cases and kyphosis was seen in the first 5 cases. The pelvis has small square iliac wings, small short sacrosciatic notch, flat horizontal acetabular roof and short femoral neck with elongated trochanters. Long bones were short and thick with metaphyseal flaring. The humerus is markedly shortened. The distal femoral physis have an inverted-V (chevron) shaped configuration best noted with age. The fibula was longer than the tibia. The hands were broad with short metacarpals and phalanges (brachydactyly) and a trident configuration.

Although clinical manifestations and radiological investigations are crucial for the diagnosis of achondroplasia, definitive diagnosis and prenatal diagnosis in high risk pregnancies are carried by molecular analysis [13,14]. Ultrasonographic examination can detect shortening of long bones in heterozygote achondroplasia cases but the shortening of long bones become appreciable only in late pregnancy (third trimester). Prenatal diagnosis can be provided early in pregnancy by DNA based methods on chorionic villi. Prenatal diagnosis of homozygous achondroplasia can be done by mutation detection at 10 to 12 weeks of gestation as against 16 to 20 weeks by ultrasonographic examination [15].

In the present study, all cases (100%) had the G-A transition at nucleotide 1138 of the FGFR3 gene; concluding that the vast majority of Egyptian achondroplasia patients have the same mutation that has been most often defined in patients with achondroplasia from other

countries. The common mutations detected in our cases were in the heterozygous state, no homozygous gene mutation was identified in our sample of achondroplasia patients. Homozygosity in achondroplasia gene results in a severe lethal skeletal, survival beyond infancy is rare.

The molecular results in this study will enable us to provide better prenatal molecular diagnosis of achondroplasia for families at high risk and try to provide perfect molecular diagnosis for achondroplastic fetuses that are found on prenatal ultrasound examinations to have short extremities but no family history of bone dysplasias. If the specific mutation is found, it will help to differentiate between achondroplasia and other possible types of skeletal dysplasias, some of which might be lethal such as thanatophoric dysplasia, and provide accurate genetic counselling to the families in the future.

Acknowledgments

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