Research Article

Mutational analysis of Sonic Hedgehog (*SHH*) gene in Polydactyly families from Balochistan, Pakistan

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Abstract

Polydactyly (PD) is the most frequent inherited limb abnormal development, featuring extra digits in feet and/or hand. Polydactyly is omnipresent and has been found in other species of kingdom animalia and others including, horses, dogs & pigs. The current study was conducted with main aim to analyze and sequence the causative gene responsible for autosomal recessive malformation, namely Polydactyly in affected individuals from Balochistan, Dermatology and Venerology Department, Sandemn Provincial Hospital (SPH) Quetta, from 1st of May 2018 to 30th of November 2018. The samples (blood) were obtained from affected and normal individuals from four families and extracted Genomic DNA from blood samples by Inorganic method followed by DNA dilution, Gel Electrophoresis, Polymerase Chain Reaction (PCR) and DNA sequencing. The sequencing data failed to identify the disease causing mutation in the coding region and splice site junctions of *SHH* gene. Our results suggest that the disease-causing mutation in the studied families with polydactyly may be present in the other known gene, i.e. *GLI3*. It is also possible that the polydactyly in these four families is caused by mutation in a novel gene. An additional study is required to explore mutations in other genes (*FGF4*, *FGF8*, and *WNT7a*) which will additionally strengthen our knowledge about characteristics of polydactyly on the level of its molecular genetics especially in Balochistan.

Keywords: Polydactyly; Quetta; Sandemn Provincial Hospital; *SHH* gene

Introduction

Polydactyly (PD) is one of the most common inherited malformation and a non-traumatic hereditary condition with additional digits in hands and/or feet. Polydactyly, Greek, polys meaning “many” and daktylos “fingers” and is credited, in the 17th century to a Dutch

surgeon Theodor Kerckring [1]. The Polydactyly incidence in the world population shows quite broad deviation amongst diverse ethnic groups variety commencing from 0.37 to 1.2/1000 live births [2]. It's the most often seen inherited limb abnormality observed instantly at birth.

The frequency is likely to exist 0.3–3.5/1,000 in alive-deliveries and 1.7–10.6/1,000 in universal inhabitants. Females are less affected as males, i.e. twice [3]. In nature, Polydactyly is omnipresent and has been found in other species of kingdom animalia and others including, dogs & pigs [4]. Typically, Polydactyly classified into three main types that have been documented globally that are preaxial polydactyly, mesoaxial/central polydactyly and postaxial polydactyly. Polydactyly can take place spontaneously, characteristically as a sign of autosomal dominant mutations, or in coincidence with a syndrome of inherited defects [5]. Castilla et al. conducted detailed epidemiological and Genetic studies of polydactyly and remarked considerable variations in its prevalence, phenotype, expressivity, transmission, and associated anomalies, suggesting a high order of heterogeneity in polydactyly. Unfortunately Medical literature is devoid of such data for the Asian populations, Particularly for Pakistan, no overall information is available regarding epidemiology, phenotypic variability, and genetic attributes of polydactyly [3]. The role and signaling of *SHH* gene is crucial in individuality and deciding digit number [6]. By the cells of the Zone of Polarizing Activity (ZPA), the signaling molecule Sonic Hedgehog (SHH) is produced particularly and perform a crucial task in the development of template formation. As to template formation, the limited appearance of *SHH* is responsible, it is obligatory to unravel the machinery that controls *SHH* gene appearance is vital to realizing limb growth. Eventually sonic hedgehog (SHH) was recognized as the nominated morphogen [3, 7]. The fundamental values and principles essential to categorize SHH as the morphogen were met. 1st of all and may be the most important one is that, it is a secreted molecule and 2nd, in the posterior of the limb, it is expressed in

a controlled focus inside the Zone of the Polarizing Activity (ZPA), 3rd, and may be more significantly, SHH independently transfer the action of the ZPA in organ transplantation. Actually, cells expressing SHH when relocated to the inner edge of the limb, persuade symmetrically duplications of the digits. The long preferred morphogen that identifies front-to-back pattern in growing vertebrate limbs encodes by sonic hedgehog [3]. The reliable genetic mechanisms for duplication disorders, exploring other causes of polydactyly and further exact loci of SHH and other genes will be described with time, as molecular research proceeds [8]. In view of the above facts the present work was conducted with the aims to analysis *SSH* gene and to investigate the exact causes/factors of inherited malformation (polydactyly) and to observe the various risk aspects particularly consanguine marriages which is most common in the population of Balochistan.

Materials and Methods

Enrolment of affected families

This prospective/descriptive study was accomplished in Dermatology and Venerology Department of Sandemn Provincial Hospital Quetta, Pakistan from 1st of May 2018 to 30th of November 2018. Total of four families (PD1, PD2, PD3 and PD4) affected with Polydactyly were located in various regions of Baluchistan Province. Families with additional toes and fingers were chosen. Comprehensive record and history was obtained as of every family to reduce the existence of other anomalies and to ensure congenital limb malformation.

Collecting of blood and lineage (Pedigree) exploration

The samples of blood of affected and normal individuals were assembled as of the families with polydactyly. By means of conventional pattern, Pedigrees of the nominated families were sketched (Fig. 1) which explained by Bennet *et al.* [9]. Through interrogating different members of the families, all the

collected data and information were verified and reviewed.

The extraction of genomic DNA

Blood samples (3ml) kept at -20°C for about 24 hours proceeding to DNA extraction. Frozen blood samples were melted at room temperature. Approx 11 to 12 milligram (ml) of Tris Ethylenediamine Tetraacetic Acid (Tris EDTA) lysis buffer (EDTA 0.5 Mm, Tris HCL 10 Mm) added with 3ml of blood and then the solution was vortexed for four minutes. The falcon tubes were centrifuged at 3400/3500 rpm for 7 to 10 min. Supernatant was discarded and the pellet was tapped to split it and to undergo in 12 ml Tris EDTA (TE) lysis buffer. as supernatant was disposed off and 70 µl of 10% Sodium Dodecyl Sulfate (SDS) alongside with 120 µl of proteinase k and 3 ml Tris-EDTA Chloride (TNE) buffer (Tris HCL 1 Molar, NaCl 6 Molar, EDTA 0.5 Molar) was summed as to assimilate cellular protein, The falcon tubes were incubated at 40°C for whole night in water bath as to digest protein completely. The samples were took out from water bath, 300 µl supersaturated sodium hydrochloride (NaCl) 6 mol, was added in the samples and remained tubes in ice cold water for 25 min. To part the content into two layers, the samples were centrifuged for 15 minutes at 4,500 rpm. Higher level restrained DNA whereas lower level restrained salt and protein. The upper layer was splitted in new tube and isopropanol was added equivalent quantity after that centrifuged for 7 min. to precipitate DNA, the tubes were overturned for numerous times, after that samples were centrifuged at 4000 Revolutions Per Minutes (rpm) for 5 min as that DNA pellet fixed to wall of falcon tubes and cautiously isopropanol were removed. DNA pellet which was rinsed with 70% ethanol and allowed them to dry. As ethanol was dispersed, the DNA liquefying TE buffer was added to liquefy the DNA. The 15 µl DNA was liquefied in 85 µl of purified water to

make a total of 100 µl DNA working solution.

Gel electrophoresis

The genomic DNA samples were scrutinized via agarose gel. 1% agarose gel was arranged by measuring 1g of agarose and merged with 100 ml 1X Tris-Borate EDTA (TBE) in microwavable flask .To dissolve agarose completely, the solution was micro waved for 1-4 min. allowed the solution become cold approx 50° C for 6 min. Added ethidium bromide, mixed well and transfer the gel into gel tray with well-placed combs. Permit at room temp for 25 to 30 minutes, until coagulate completely. The gel placed into electrophoresis tank and captiously loaded the samples. Electrophoresis process was beard out for 30 min in 1X TBE running buffer at 110 volts. After electrophoresis, the amount and worth of extorted DNA was checked by Ultra violet Transilluminator.

The PCR reaction was carried out by arranging Master Mix through adding PCR buffer, Magnesium Chloride (Mgcl₂), Taq DNA polymerase and Deoxyribonucleotide triphosphate (dNTPs). For 10 µl reaction, 0.5µl sample DNA dilution, 5 µl PCR water, reaction mixture 3.7 µl and each one forward and reverse primer (0.5 µl) were added. DNA gel was prepared as to analyze the amplified DNA samples. 2gm of agarose was melted in 100 ml of TBE buffer 1X (1M Tris base, 0.5 M EDTA and 55 g Boric Acid) in micro oven, agarose gel was prepared. In gel 9 µl ethidium bromide solutions (0.5 µl /ml) was added as to stain DNA. The Solution was transferred in the gel caster and left for an hour as to solidify. The combs were detached. PCR yield were merged with DNA loading dye (Bromophenol blue 0.25%, prepared in sucrose solution 40%) After loading samples, gel electrophoresis was performed for 30 min in 1X TBE running buffer at 110 volts. For analysis of amplified products, ultraviolet Transilluminator was used.

Figure 1. Pedigrees of the four polydactyly families with affected and normal individuals

Polymerase chain reaction (PCR)

Primers were planned manually with reference sequence gained from University of California, Santa Cruz (UCSC) genome browser (Table 1). The PCR yield was scrutinized on agarose gel 2% (Fig. 2). DNA Ladder was used, 100 Base Pair (100bp), (O Range Ruler™, MBI Fermentas, and UK). The augmented PCR yields were clarified by means of speedy PCR refinement stuff (Marligen, USA). 300 µl of requisite solution (concentrated Tris-HCl, EDTA, Guanidine HCl, and Isopropanol) was summed in augmentation reaction. For adsorption of double stranded DNA, the blend was applied on silica-based membranes to rotate and it manipulates the temperature and buffer composition. With the help of buffer

containing alcohol, the EDTA, Tris-HCl, NaCl, Polymerase (DNA) dNTPs and primers were eliminated. The Clarified and refined PCR yields were submissive to cycle sequencing which was carried out with the help of sequencing buffer (USA PE Applied Biosystem) and reaction mixture (Dye terminator V 3.1). To denture DNA template, temperature was set at 95°C for 5 minutes, pursued via 30 cycles of amplification and every one comprising of three steps, 30 seconds at 95°C for DNA denaturation into solitary strands; for annealing of primers, 30 sec and 4 minutes for extension at 72°C and single cycle at 72°C for 10 minutes to synthesize un extended strands which are left over by the help of Taq DNA Polymerase. The sequencing yields were clarified by

ethanol precipitation protocol (POP6 Protocol). Sequencing yields, comprising 16 µl of purified H₂O and 64 µl 100% ethanol, were shifted to 1.5 ml micro centrifuge tube. These tubes reserved at 25°C for 15 min and centrifuge for 20 min at 14,000 rpm. Floating was detached and 70% ethanol (250 µl)

augmented to tubes for centrifugation, for 10 min at 14,000 rpm. Later than using 95°C temps for denaturation for 2 minutes, the samples were ordered (sequenced) via ABI Prism 310®, automatic Genetic Analyzer (Applied Biosystem, USA).

Table 1. Primer used for sequencing of *SHH* gene

Exon	Primer	Sequence	Product size
Exon 1	Forward	GGAAGGGAAAGCGCAAG	577 bp
	Reverse	GTCTGGAAGTGTTCGGCTTC	
Exon 2	Forward	TCGGCTGTTTCAGGACCTGG	599 bp
	Reverse	TCTTGAATCAAGCCGAGGTGC	
Exon 3a	Forward	GCCTCCCTCTCGGAACTC	517 bp
	Reverse	CTCCTCGCTTAGGGTCACG	
Exon 3b	Forward	CGCACCTGCTCTTTGTGG	751 bp
	Reverse	GGGTCCTTGTTTCCTTAGAGTC

Figure 2. The four exons of sonic hedgehog (*SSH*) gene

Results

Total eight blood specimens were collected from the individuals of polydactyly families of different ages from Balochistan, Pakistan. Out of the total eight individuals, 4 were males and 4 were females. DNA extracted from blood of these affected and normal individuals then was screen the coding exon

and splice site junctions of *SHH* gene. After sequencing, the data was analyzed carefully by using different software including Chromas and SeqMan and compared with the reference sequence obtained from UCSC genome browser. Analysis of the sequencing data failed to identify the disease causing mutation in the coding region and splice site

Figure 4. Sequencing chromatogram of exon-2 of SSH gene

Figure 5. Sequencing chromatogram of exon-3a of SSH gene

Figure 6. Sequencing chromatogram of exon-3b of SSH gene

Discussion

Polydactyly can either be hereditary in autosomal dominant mode or autosomal recessive. It appears to be a single definable entity but genetic heterogeneity has been observed. Genetic heterogeneity is well recognized in Polydactyly and thus far various genes (GLI3, FGF4, FGF8, WNT7 and others) have been revealed. Mutations in these genes are thought to possess a significant job to take part in causing Polydactyly.

Genes involved in causing polydactyly

The zone of polarizing activity (ZPA)

The ZPA is a region of mesoderm that holds indicators which educate the growing limb bud to shape alongside the frontal/subsequent (anterior/posterior) axis. This mesoderm at the subsequent (posterior) edge in the early hours appendage shoot when relocated to the opposed, frontal (anterior) edge of a succeeding appendage shoot gave rise to additional digits demonstrated as parallel image replications. It was referred as the ZPA

because these areas of the limb shoot holding the polarizing tissue. The ZPA is a reduction of mesodermal tissue beside the subsequent (posterior) aspect of the appendage, educating the frontal (anterior) to subsequent (posterior) direction of the appendage. The characteristics of the ZPA guided to the theory that the polarizing action was turned on by an extracted molecule performing as a morphogen [10]. The remarkable aggregation of the ZPA among Sonic hedgehog appearance in the appendage shoot recommended that sonic hedgehog may be component of the mechanism throughout which the ZPA applies its pressure.

Apical ectodermal ridge (AER)

AER has been recognized as the key constituent of the AER signaling and acts through transmitting signals to the nearby mesoderm by fibroblast growth factor (FGF) [11]. Both AER-FGF signaling assure that there are adequate precursor cells essential for the ordinary appendage growth [12]. The signals of FGF from the AER regulate

mesodermal existence (directly or indirectly), propagation and individualization throughout growth. The apical ectodermal ridge (AER) is a morphological distinctive epithelium that goes from frontal to subsequent (anterior to posterior) at the distant edge of the shoot. removal of the AER outcomes in a malfunction of successive PD development demonstrating that signals from the edge are constantly essential for development of the appendage [13, 14]. The initial and the 1st noticeable expression of edge morphogenesis in the mouse appendage shoot is an alteration in the form (from plane to cuboidal) of the presumed AER ancestor all through the distal-ventral epidermis (ectoderm). Investigation by Saunders and Gasseling in 1948 recognized the AER and its succeeding contribution in proximal distal development [15]. The AER articulates FGF8 which tempts *Shh* appearance in the subsequent mesoderm. The *Shh* then excites FGF4 to be articulate in the posterior part of the AER. After these proceedings, there is an interdependency between FGF-4 and *SHH* for their successive appearance and sustenance.

GLI3 gene

The *GLI3* gene is particularly important in embryonic patterning of human [16]. *GLI3* expresses at the anterior end (i.e. opposite to *Shh*-end) and its protein that is antagonist of *Shh*. In the *Shh* passageway, *GLI3* itself is a downstream mediator and this passageway contains many genes that provoke anomalous human phenotypes when altered. It has double roles: the entire *GLI3* performs as a stimulator (*GLI3A*) of *Shh* passageway whereas its reduced version *GLI3R* performs as *Shh* repressor [17]. An appropriate balance between the *GLI3A* and the *GLI3R* denotes appendage figure number and individuality. Therefore *GLI3* is a constituent of an evolutionary preserved developmental cartridge or module that is utilizes to complete different jobs all over the growing

organism. For instance, the *SHH/GLI* passageway is utilized to identify the limb, neural tube, and some others. This developmental module is commonly utilized to identify situational or polarity information inside part of the growing embryo. A renowned instance of this is in the growing appendage bud where *SHH* jobs as a morphogen in the zone of polarizing activity to set up anterior-posterior polarity [8].

FGF4 and FGF8 genes

In the ventral limb ectoderm, *Fgf8* is expressed when the appendage bud can first be noticed, appearance of *Fgf8* has also been noticed in the intermediary mesoderm (IM) of both mouse and chick embryos before limb bud commencement [18]. The job of *Fgf8* expressed in the AER in the continuance of appendage bud outgrowth has been well recognized [3]. *Fgf8* is articulated earlier to *Shh* appearance during the growth of both usual and ectopic appendage buds [19]. Fibroblast growth factor 4 is a protein that in human beings is coded via the *FGF4* gene. Throughout embryonic growth, the 21-kD protein FGF4 acts as an indicating molecule. Throughout propagation, FGF-4 is available in the subsequent area of the apical ectodermal ridge (AER). *Fgf8* and *fgf4* in the AER are necessary for the sustenance of appendage shoot development. As earlier revealed for the hind appendage in the nonexistence case of both *FGF* family elements, broad programmed cell death in proximal appendage shoot mesenchyme eliminate the creation of some skeletal elements [20]. Because a programmed cell death goes on and appendage shoot development is entirely eliminated in the lack of both *fgf4* and *fgf8*, it shows that *Fgf4* is capable to moderately reimburse for the nonexistence of *fgf8* in the sustenance of appendage bud development/limb mesenchyme existence. It will also be vital to decide what functions are performed by other (non-FGF) genes in the AER [21]. The *fgf8*

is articulated in the AER of *Shh* altered appendage shoots, sustaining the theory that Fgf8 roles upstream of Shh. An optimistic response loop between fgf4 and Shh gives one procedure through which development and modeling of the appendage could be controlled by a coordinated fashion [22, 23]. It has been found through genetic interpretation that inactivating of both fgf4 and fgf8 in the AER created more harsh phenotypes than inactivating of only/alone Fgf8.

WNT7a gene

In the regulation of SHH appearance and anteroposterior pattern shape, WNT7a can alternate for the role of dorsal ectoderm. The new research and Studies suggest that *SHH* appearance is reliant on collaboration between WNT7a and signals from the ridge or FGF4. Wnt7a is expressed solely in the dorsal ectoderm, in the vertebrate limb [24] and thus is a good contestant for the dorsal ectoderm signal essential for SHH appearance. Though, throughout usual limb growth, WNT7a, FGF4 and SHH are reliant on the activity of each other. Wnt7a manipulate two aspects of limb growth: it provides the dorsalizing signal essential for dorsal pattern formation, and it sustains expression of Shh, which is essential for anteroposterior pattern formation [25].

There are a great number of genetic causes involved in limb development and only a few genes are known to cause additional digits. This brief compilation could be a helpful resource for future molecular mapping and mutation studies. The present analyses shed light on the genotypic and phenotypic aspects of polydactyly [26-28]. This study though, does not offer the true prevalence and incidence rates of polydactylies which could be useful in estimating the frequencies of mutation(s) and heterozygote subjects in the populations. Therefore, it would be advisable to identify Balochistani subpopulations that might be suitable for further molecular

studies on specific polydactyly types. Many fundamental facts about the genetics of polydactyly remain unsolved primarily because of the etiological heterogeneity which is not taken into consideration when large-scale studies, based on hospital/health record are performed [28]. Molecular and embryological studies have shown that there are several routes of developmental disruption during digit morphogenesis which may lead to additional digits [7]. Additionally, environmental, extra-genic and random factors contribute in the etiology of a substantial proportion of polydactyly cases [28]. However, in the present, no pathogenic variation is seen in any of the exons of *SHH* gene and There could be believable explanations behind not discovering mutations in the selected exons of *SHH*: Firstly, every one of the exons of *SHH* gene were sequenced in view of previously reported alterations from various world population whereas reports on molecular genetics of polydactyly in Pakistani nationals (especially in Balochistan) have rarely been reported. The probability is that there might be inclusion of some other genes whose mutation is prompting polydactyly in these families.

Secondly, there is also a greater chance that some alteration might be available in the promoter regions which have not been tried in the course of the study.

Conclusion

studies on diverse genetic disorders reveal that inherited Polydactyly is a disorder for which a versatile pond of genes are implicated in causing this disorder, Moreover; Pakistan especially Balochistan has been believed among the mainly unstudied division of the globe on the analysis of the genetics concerned in causing Polydactyly. Failure of detecting of disease causing mutation in *SHH* gene recommends an additional study which will additionally strengthen our knowledge about

characteristics of polydactyly on the level of its molecular genetics. The new supplementary study may reveal the involvement of another identified or novel gene in the development of PD phenotype in these four families.

Authors' contributions

Conceived and designed the experiments: IA Rasool, Performed the experiments: JK Bashardost, Analyzed the data: S Khan & S Jamali, Contributed materials/ analysis/ tools: IA Rasool, Wrote the paper: JK Bashardost.

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