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Novel Mutation in the EXT-1 Gene in an Iranian Family Affected with Hereditary Multiple Exostoses

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Abstract: Identification of casual mutations in Hereditary Multiple Exostoses (HME) is important because of similar conditions in which multiple exostoses occur. Therefore mutation analysis can help to confirm the clinical diagnosis and to improve the management of therapy. HME is an inherited disorder of bone growth. HME can be referred to by various names such as Heredity Multiple Exostoses, Hereditary Multiple Osteochondromata, Multiple Carthaginous Exostoses, etc. People who have HME grow exostoses, or bony bumps, on their bones which can vary in size, location and number depending on the individual. HME is inherited in an autosomal dominant manner with an estimated prevalence of 1/50,000 in western countries. At least three loci (EXT1, EXT2 and EXT3) thought to be involved in this skeletal disease. Approximately 90% of affected families possess mutations in the coding regions of EXT1 and EXT2 genes and the majority of these mutations cause loss of function. EXT1 and EXT2 genes encode related members of a putative tumor suppressor family. In this first report from Iran we identified a frame shift mutation (1100-1101 insA) in exon 3 of EXT1 gene in a family being suspicious of HME. This mutation leads to a premature stop codon and previously not described. Additionally, we have found an unreported silent mutation in the exon six of EXT1 gene with uncertain significance.

Key words: Exostoses, EXT1, EXT2, HME, Iranian family

INTRODUCTION

Hereditary Multiple Exostoses (HME) is an inherited disease due to an autosomal dominant predisposing gene mutation with a penetrance of 96%. Ten percent of affected individuals have hereditary multiple exostoses as the result of a new gene mutation (Schmale *et al*., 1994). About 2% of HME patients show malignant degeneration (Rambeloarisoa *et al*., 2002), but most affected individuals indicate multiple benign bony outgrowths (exostoses), typically located at the juxtaepiphysed regions of bones that develop by encondral bone formation (Hudson *et al*., 1984; Hennekam, 1991). However, there are similar conditions with multiple exostoses (Bakker *et al*., 1993; Hall *et al*., 2001). The risk for malignant degeneration to osteochondrosarcoma increases with age, otherwise the lifespan is not reduced and the majority of individuals with HME lead active, healthy lives (Schmale *et al*., 1994; Wicklund *et al*., 1995). Exostoses can be associated with a reduction in skeletal growth, bony deformity and inequality in limb, bowing of the forearm, deformity of the ankle, restricted motion of joints, shortened stature and premature osteoarthritis (Stieber and Dormans, 2005). Approximately 70% of individuals have clinically apparent exostoses about the knee (Schmale *et al*., 1994; Wicklund *et al*., 1995). Compression or stretching of peripheral nerves usually causes pain but may also cause sensory or motor deficits. Nerves and vessels may be displaced from their normal anatomic course (Pacifici *et al*., 2005). Research studies have found that approximately 64 to 76% of families with HME possess mutations in the EXT1 gene on chromosome 8 and 21-30% mutations in the EXT2 gene on chromosome 11 (Dobson-Stone *et al*., 2000; Francannet *et al*., 2001; Wuyts *et al*., 2002). Even with sequencing of the entire coding regions, fewer than 90% of the mutations are identified (Philippe *et al*., 1997; Raskind *et al*., 1998). Although the EXT1 genes are ubiquitously expressed in many tissues, the only known effect of mutated or inactivated EXT1 appears to be specific to actively growing bone. The development of exostoses is mainly due to loss of function of EXT genes, consistent with the

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hypothesis that the EXT genes have a tumor suppressor function (Francannet *et al*., 2001; Le Merrer *et al*., 1992; Blanton *et al*., 1996). EXT genes products are involved in the biosynthesis of heparan sulphate. EXT1 and EXT2 encode glycosyltransferases that interact as hetero-oligomeric complexes (McCormick *et al*., 2000). Therefore, loss of heparan sulphate polymerase activity by EXT1 or EXT2 mutations is expected to reduce the amount of heparan sulphate on the cell surface as result of haploinsufficiency (McCormick *et al*., 1998). The extra cellular heparan sulphate chains of cell-surface proteoglycans in normal cells function as co-receptors of growth factors, thereby increasing the binding affinity of growth factor receptors (Bernfield and Hooper, 1991). Therefore mutations in the EXT1 or EXT2 genes could perturb normal chondrocyte differentiation and as well as bone development. Recently a third locus (EXT3) is identified that probably involved in the HME, but neither the EXT3 gene nor any additional EXT genes have been cloned.

MATERIALS AND METHODS

Clinical assessment: The project was approved by the Medical University of Joundi Shapour's Ethics Board, in last year (2006). After informed consent, all participants were questioned on their personal medical history and a family tree was drawn.

Sample collection and DNA isolation: Diagnosis of affected individuals, mother and her two daughters was ascertained through familial history (Fig. 1). Primary prognosis of HME disease was according to the radiography information of involved bones and clinical examinations. The 57 years old mother with the mild HME has had exostoses about the knee and shorten toe. Her daughters, 33 and 27 years (Fig. 1, 2), showed many osteochondroma contributed in ulnae, elbow, hip and ileu. No other family members have shown exostoses. The patients have had a short status with the height from 150 to 157 cm. Mild short stature is also a characteristic feature of the condition with the mean height being 170±7.9 cm for males and 155±6.9 cm for females (Wicklund *et al*., 1995). From affected and healthy individuals were EDTA-treated whole blood collected and DNA was extracted by salting out technique.

Polymerase chain reaction: PCRs were performed in an Eppendorf mastercycler for 35 cycles with genomic DNA containing 50 ng DNA, 10 pmol of each primer, 200 μM of dNTPs, 2.5 μL of 10 x PCR buffer and 2 units of *Taq*. polymerase (Roche, Swiss) in a volume of 25 μL. The full

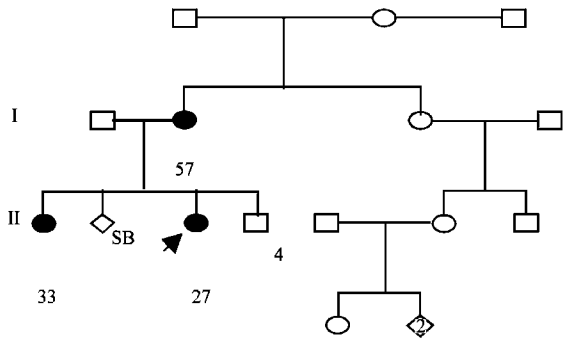

Fig. 1: Pedigree of the affected family

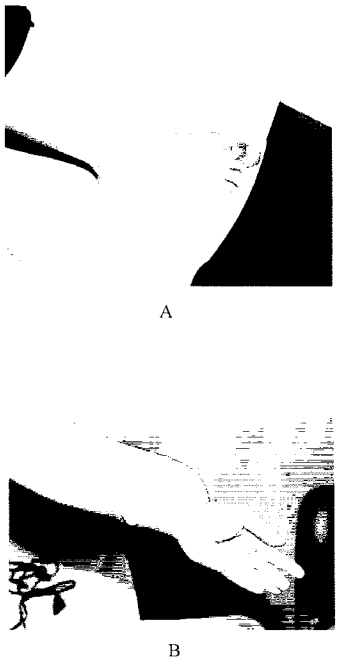

Fig. 2: The affected mother with shorten fourth toe (A) and her 27 years old daughter with severe form of HME (B)

length of exons and a part of the flanking intron sequences were amplified with designed primer pairs and annealing temperatures as shown in Table 1. The EXT1 gene includes eleven exons. Each exon was amplified by PCR, separately. To amplify the full length of each exon, primer positions were chosen on the flanking intron close to the splice sites. All fragments were completely sequenced.

Direct sequencing: The PCR products were direct sequenced with ABI-PRISM 3700 DNA analyzer (Applied Biosystems). The same PCR primers were used for

Table 1: Summary of PCR conditions and designed primers

Exon	Primer sequences	Product length (bp)	Annealing temperature (°C)
1	F:5-AATCCTCTTGACCCAGGCA-3		
	R:5-CCAGCCCAGACACTTACTTCT-3	1006	52
2	F:5-TCAATATCCCCACATTCGCA-3		
	R:5-GAGGTGATAATGTTAAACCCA-3	235	54
3	F:5-TCACGAAGTCCCCTTTTTTG-3		
	R:5-AGAGCTGACCTTTTGGATTCA-3	236	50
4	F:5-TGGGTTATTTTGATCAAGTGC-3		
	R:5-TGGACCAATCACACATCCCTA-3	293	55
5	F:5-CCACCATCTTTCGAATTTGG-3		
	R:5-GCCTTTAGTTCTGTATGACAT-3	287	52
6	F:5-AGCAAGGAGGAGTAATTTTCT-3		
	R:5-TCTGTAACCCATCCCTTCCCT-3	411	54
7	F:5-TTGGGTTGGAGGCATACATA-3		
	R:5-AAAGTGCCCCATGGAGAAA-3	238	52
8	F:5-TTACCTCTTTCCCTCATTCCC-3		
	R:5-AGCATTAGCATCGTGCAACA-3	228	52
9	F:5-GAAGGTAATGTTTTGTTGACA-3		
	R:5-TCCTATTTATGCAGCAGCCA-3	299	55
10	F:5-CCTGTGAAACCCATCTTTGA-3		
	R:5-AAGGGAAGAGGGCTCTTCTAT-3	232	56
11	F:5-CACCTTGCACTTCTCTCATCA-3		
	R:5-TGGGAAGAGAGAGCAGCTTGA-3	300	54

F: Forward primer; R: Reverse primer

sequence reactions. Sequence reactions were performed with forward and reverse primers, (Table 1). Finally, the sequences were compared to the reported gene sequence (NT 008046) using the BLASTN program.

RESULTS AND DISCUSSION

Sequence analysis of the EXT1 gene revealed an insertion mutation in exon 3 (1100-1101 insA) causing framshift in all three individuals being diagnosed with HME (Fig. 3). Furthermore, it was identified a silent mutation in the third base of codon 477(C to T) from exon six of EXT1 gene.

Mutation screening of entire coding region from EXT1 gene in this family revealed a same frame shift in exon 3 in all affected individuals. According to the published data, present finding represents an unreported mutation in the EXT1 gene (Table 2, 3), known as a rare condition (Wuyts and Van Hul, 2000).

It seems that this novel mutation represents an isolated case. Therefore, we propose to screen a larger sample of Iranian HME patients for EXT1 genes in order to define the proportion of such families whose predisposition is attributable to mutations in this gene. The most mutations reported are distributed over the first six exons of the EXT1 gene compared with the last five exons, which contain a conserved carboxyterminal region, having significantly fewer mutations (Wuyts and Van Hul, 2000). The mutations in this gene are of all type (missense, framshift, in frame deletion, nonsense and splice site). But most HME cases arise from missense or

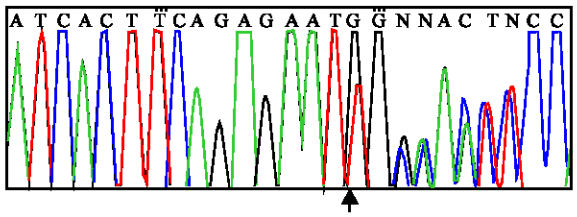

Fig. 3: Direct sequencing profile, which revealed a 1 bp insertion in exon 3 of EXT1 gene. The sequence reaction was performed with reverse primer as shown in Table 1. The arrow indicates the position of inserted base causing subsequent overlapped sequences in the case of heterozygosity

Table 2: Number and type of mutation according to HGMD (Human Gene Mutation Database) site for EXT1 gene

Mutation type	Total No. of mutations
Nucleotide substitutions (missense/nonsense)	33
Nucleotide substitutions (splicing)	8
Nucleotide substitutions (regulatory)	0
Small deletions	46
Small insertions	15 listed in Table 2
Small indels	2
Gross deletions	3
Gross insertions and duplications	0
Complex rearrangements (including inversions)	2
Repeat variations	0
Total	109

Table 3: Insertion mutations for EXT1 gene in HGMD (Human Gene Mutation Database) site

Accession No.	Nucleotide	Codon	Insertion	Phenotype
CI972594	890	83	C	Multiple exostoses 1
CI012286	352	118	C	Multiple exostoses
CI972595	1071	140	AGGG	Multiple exostoses
CI983088		209	TGGGG	Multiple exostoses
CI000619	742	248	TT	Multiple exostoses
CI000620	876	292	T	Multiple exostoses
CI983909	1093	365	GG	Multiple exostoses
CI010701	1199	400	A	Multiple exostoses
CI983910	1204	402	C	Multiple exostoses
CI014682	1320	441	T	Multiple exostoses
CI010702	1334	445	G	Multiple exostoses
CI983089	2077	476	C	Multiple exostoses
CI972596	2082	478	T	Multiple exostoses 1
CI010703	2080	489	C	Chondrosarcoma
CI983090	1679	560	C	Multiple exostoses

framshift mutations in EXT genes (Zak *et al*., 2002). Inactivating mutation represent the vast majority of mutation in both the EXT1 (77%) and EXT2 (92%) genes, supporting the hypothesis that both EXT1 and EXT2 have tumor suppressor function that is lost during the development of exostoses (Wuyts *et al*., 1998). Previously has been reported that the sites of mutation affected the severity of disease in patients with EXT1 mutations having a significantly worse condition than those with EXT2 mutations (Porter *et al*., 2004). Phenotypic variability has also been noted in HME patients. In recent study, 2/3 of families with an EXT1 mutation were noted to exhibit phenotypic variability, while in all but two

families with EXT2 mutations members showed a homogeneous phenotype (Francannet *et al*., 2001). In other genotype-phenotype study has been shown that the severity of the disease is not differing significantly with gender and can be calculated by stature, number of exostoses, number of surgical procedures, deformity and functional parameters (Porter *et al*., 2004). Because HME is inherited in an autosomal dominant manner, therefore the offspring of affected persons have nearly 50% risk of inheriting the mutant allele. Nevertheless, there is possibility of having a healthy offspring by detection of parental mutation type and prenatal testing.

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