Hereditary multiple osteochondromas in Jordanian patients: Mutational and immunohistochemical analysis of *EXT1* and *EXT2* genes

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Abstract. The aim of the present study was to investigate the molecular characteristics of hereditary multiple osteochondromas (HMO) in a subset of Jordanian patients with a focus on the genetic variants of exostosin (*EXT1*)/(*EXT2*) and their protein expression. Patients with HMO and their family members were included. Recorded clinical characteristics included age, sex, tumors number and location, joint deformities and associated functional limitations. Mutational analysis of *EXT1* and *EXT2* exonic regions was performed. Immunohistochemical staining for EXT1 and EXT2 was performed manually using two different commercially available rabbit anti-human EXT1 and EXT2 antibodies. A total of 16 patients with HMO from nine unrelated families were included, with a mean age of 13.9 years. A total of 75% (12/16) of the patients were male and (69%) (11/16) had a mild disease (class I). *EXT* mutation analysis revealed only *EXT1* gene mutations in 13 patients. Seven variants were detected, among which three were novel: c.1019G>A, p. (Arg340His), c.962+1G>A and c.1469del, p. (Leu490Argfs*9). Of the 16 patients, 3 did not harbor any mutations for either *EXT1* or *EXT2*. Immunohistochemical examination revealed decreased expression of EXT1 protein in all patients with *EXT1* mutation. Surprisingly, EXT2 protein was not detected in these patients, although none had *EXT2* mutations. The majority of Jordanian patients with HMO, who may represent an ethnic group that is infrequently investigated, were males and had a mild clinical disease course; whereas most patients with *EXT1* gene mutations were not necessarily associated with a severe clinical disease course. The role of *EXT2* gene remains a subject of debate, since patients with *EXT1* mutations alone did not express the non-mutated *EXT2* gene.

Introduction

Hereditary multiple osteochondromas (HMO) is characterized by multiple cartilage-capped bony projections (exostoses) that usually arise from the metaphysis of long bones (1). It is also known as hereditary multiple exostoses, multiple cartilaginous exostoses, osteochondromas and diaphyseal aclasis (2). The prevalence of HMO is ~1:50,000, and the male: Female ratio is 1.5:1 (3,4). Although HMO can be asymptomatic and diagnosed incidentally, it can disrupt bone growth and cause short stature, unequal limb lengths and joint deformities with significant morbidity (5,6). The most serious complication of HMO is the malignant transformation into chondrosarcoma, occurring in 0.5-5% of the patients (6). Therefore, clinical and radiological follow up is crucial for the management of patients with HMO. However, there is currently no standard follow-up protocol for HMO. Genetic analysis of *EXT* genes to identify patients with HMO at higher risk of developing severe disease or malignant transformation may contribute to the future management of such patients.

HMO is an autosomal dominant inherited disease with a penetrance of 100% (5). Genetic analysis of HMOs in different populations identified two main causative genes, namely exostosis 1 (*EXT1*) and exostosis 2 (*EXT2*) (5,7,8). Mutations in the *EXT1* and *EXT2* genes account for >90% of all HMO cases (7,9). *EXT1* and *EXT2* both encode for a glycosyltransferase required for heparan sulfate (HS) synthesis and polymerization as HS proteoglycans (HSPGs) (8). The role of EXT1 and EXT2 proteins in HS synthesis involves the

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formation of heterocomplex of both proteins (1). HSPGs play a key role in the regulation of different signaling pathways involved in chondrocyte proliferation and differentiation in the growth plate (8).

The present study investigated 16 Jordanian index cases from nine different unrelated families with confirmed diagnosis of HMO. The different clinical characteristics in addition to the mutational spectrum of the *EXT* genes and the expression of their corresponding proteins were evaluated in this group of patients.

Materials and methods

Patients with HMO. The present study was conducted at several Orthopedic Surgery clinics over a 2-year period between January 2018 and December 2019. A total of 42 individuals were included in this study, among which 16 were diagnosed with HMO. Of the patients, 12 were men and 4 were women who had a mean age of 13.9 years (age range, 6-27 years). HMO diagnosis was confirmed by either histopathological or radiological examinations. These patients were evaluated clinically, and their available radiological examinations were reviewed. Disease severity was determined according to the severity score described by Mordenti *et al* (10) (Table I). Blood samples were collected from all participants. Paraffin-embedded tissues of patients with HMO who underwent surgical excision were available from the archives of Pathology Department. Informed consent was obtained from all individuals who participated in this study. The study protocol was approved by the Human Research Ethics Committee.

Molecular analysis. Genomic DNA from the patients and their available family members was extracted from peripheral blood samples using a Gentra Puregene kit (Qiagen GmbH) following the manufacturer's instructions. The quality and concentration of the DNA was determined by NanoDrop 2000 V7.3.1 (Thermo Fisher Scientific, Inc.). All exons and exon-flanking intron sequences of *EXT1* (NM_000127) and *EXT2* (NM_000401) genes were amplified by PCR (polymerase chain reaction). PCR was performed in a final volume of 25 μl containing 40 ng genomic DNA, 1X Master Mix (GoTaq® Green Master Mix; Promega Corporation), and 5 pmol of forward and reverse primers (Table II). The following thermocycling conditions were used: Initial denaturing step (95˚C for 7 min) followed by 40 cycles of 95˚C for 1 min, annealing at 60˚C for 90 sec, extension step at 72˚C for 90 sec and final extension at 72˚C for 7 min. PCR was performed using an iCycler (Bio-Rad Laboratories, Inc.). The PCR products were separated by 2% agarose gel electrophoresis, visualized by ethidium bromide and the products were purified using the Norgen's PCR Purification kit (cat. no. 45700; Norgen, Bioteck Corp.). GAPDH was used as the loading control and for normalization. Sanger sequencing was performed in both sense and antisense directions by using the BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Sequencing reactions were purified using the NucleoSEQ kit (Macherey-Nagel GmbH) and final analysis performed using

Table I. HMO severity score described by Mordenti *et al* (10).

Class	Subclass
I: No deformities-no functional limitations	
IA	≤5 sites with exostosis
IB	>5 sites with exostosis
II: Deformities-no functional limitations	
IA	≤5 sites with deformities
IB	>5 sites with deformities
III: Deformities-functional limitations	
IIIA	1 site with functional limitations
IIIB	>1 site with functional limitations

an ABI 310 DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

The obtained sequences were compared with the normal *EXT1* (NM_000127) and *EXT2* (NM_000401) genes reference sequences and chromatograms were visualized by using the ChromasPro 1.34 (Technelysium Pty Ltd.) software package or Mutation Surveyor (V4.07; SoftGenetics, LLC). Sequence nomenclatures for the coding and noncoding variants were described in accordance with the Human Genome Variation Society Nomenclature standards (http://www.hgvs.org/mutnomen). To assess and predict the impact of newly identified missense variants, the Mutation taster (http://www.mutationtaster.org/) and Polyphen2 programs (http://genetics.bwh.harvard.edu/pph2/index.shtml) were used.

Immunohistochemistry. Formalin fixed, paraffin embedded HMO tissues were used in this study for immunohistochemical staining of EXT1 and EXT2. Tissues were processed following the manufacturer's instructions. Immunostaining for EXT1 and EXT2 was performed manually on the sample sections using two different commercially available rabbit anti-human EXT1(dilution 1:200; cat. no. abx100786; Abbexa Ltd.; and cat. no. HPA044394; Sigma-Aldrich; Merck KGaA) and rabbit anti-human EXT2 (dilution 1:200; cat. no. abx03435; Abbexa Ltd.; and cat. no. SAB2108124; Sigma-Aldrich; Merck KGaA) antibodies. Tissue sections were observed under a light microscopy at a magnification of x40. Tissues known to express EXT1 and EXT2 (normal femoral head cartilage) were used as positive control, and negative controls were created by omitting the primary antibody step. The scoring criteria for EXT1 and EXT2 were as follow: 0, 0-10%; 1, 10-30%; 2, 30-85%; and 3, >85%. The intensity of the reaction was scored as: 0, negative; 1, weak; 2, moderate; and 3, strong. The samples that were scored as 1 or more were considered as positive.

Results

Patients. A total of nine families (A-I) with 42 members were included in the present study. These families included a total of 16 patients with HMO, and three families (B, E and F) only had one affected member (Table III).

Table II. Primer sequences used for PCR amplification and Sanger sequencing.

Gene	Exon	Forward (5'-3')	Reverse (5'-3')
EXT1	Exon 1	CGAGCGCAGGAGTAAACACC	CGTTTTTTGGCCTGCATGTG
	Exon 1	GAGCTGAAAGTGTTGATTGG	GAGACTCTGCACCTTTGGATC
	Exon 1	CCTCTTTGTCCTGAGTCTGG	CCATCCCCCAACTTCACACC
	Exon 2	CCCCACATTCGCAATGAGTC	GAGAGGTGATAATGTTAAACCC
	Exon 3	CTGATTGGAACAGCTTCTGCTG	TGAAAGTTTGGACGGGGGCAGC
	Exon 4	GTGCATCTCTTTGTTTTACAG	GCTGAGAGAAGTGTATAAAGG
	Exon 5	CCTTTCCAAATATCATCAGG	GGCCTTTAGTTCTGTATGAC
	Exon 6	GAGCAAGGAGGAGTAATTTTC	ATAACAGGTAAGGAGGGCGG
	Exon 7	AAGAGGCTTTGGGTTGGAGG	AAGTGCCCCATGGAGAAAC
	Exon 8	GGGAGAATTGTCCTGAAAAC	ATCGTGCAACATGAGGTGAC
	Exon 9	TTAGTGGGGAGAAGGTAATG	TTCCTATTTATGCAGCAGCC
	Exon 10	GTCTCAGAAGTCCACTTGTC	ACGTGAGTCCTCATTACCTG
	Exon 11	CCTTGCACTTCTCTCATCATTATCC	GAAGAGAGAGCAGCTTGACC
EXT2	Exon 1	GCCTGAATATAAGCACCTAC	AAAAGCGGGCAGTCATTGTC
	Exon 2	TCAAGTGTCATTTGCCATCC	CCCTTCCCTTTAGTTCCCTG
	Exon 3	GGCTTGGGGATCCTTGATAG	ACTTCTAAATCTTCAGGAGG
	Exon 4	ACTCTGTAAACGTTAGCTGG	AGGACCCTACCCTGTAACTG
	Exon 5	TCAGTGGAGGTGAAGACTGG	CATAGGCCAAGCAGCTTTGC
	Exon 6	GTATTGCTTGGCGTCAACCC	GTAGTAGTTCTTGAACCAGG
	Exon 7	GGATGTTGTTTCTGCTTGTG	ACTCAGGCATTCAGCTCCTG
	Exon 8	CCTGGAGTTGACTATGATAG	TTATGCTGCCCTTATCAGGC
	Exon 9	CATGTTTGGGTTTGCTGACG	AAATGGAGGCATGCTGTCTC
	Exon 10	GGATACAAGCTGATTCTCCC	GCACACCTTTTGGACTCTAC
	Exon 11	TGGAACATCTCCAGAATCCC	AAGCCCTCTTGGCAGGTATG
	Exon 12	TATGAGAGAAAGCTTGTCCC	CCAATGTGACCGCATCAATC
	Exon 13	CATGCAACATCTCAGCTTAC	ACTATGGCTACCAGCTGCTG
	Exon 14	CAGACTGTGGCTACTTGAGC	AGTAGGTCAACCTTCCACCC

These patients had a mean age of 13.9 years at their initial diagnosis (range, 6-27 years). A total of 75% (12/16) of the patients were males. The total number of tumors was 135, with over half (58%) being located around the knee. According to HMO severity classification by Mordenti et al (10) (Table I), most (69%) (11/16) of these patients had a mild disease (class I) (Table III). Moderate (class II) and severe (class III) disease forms were recorded in 2 (13%) and 3 (19%) patients, respectively (Table III).

EXT1 and EXT2 genes mutational analysis. Mutational analysis of the 16 patients from nine families (A-I) and their family members for both *EXT1* and *EXT2* genes revealed different heterozygous mutations in only *EXT1* gene. While 13 patients (77%) from seven unrelated families harbored these *EXT1* mutations, the remaining 3 patients (23%) from two families were negative for both *EXT1* and *EXT2* genetic variation in the targeted sequenced regions. Seven different genetic variants were identified in the *EXT1* gene. These variants consisted of; two missense variants [c.1019G>A, p. (Arg340His); and c.82T>A, p. (Phe28Ile)], two deletions variants [c.918del, p.(Lys306Asnfs*53); and c.1469del, p.(Leu490Argfs*9)], one insertion variant [c.1065_1066ins26, p.(Val356Cysfs*12)], one duplication

variant [c.96dup, p.(Ser33Glufs*11)] and one splice site variant (c.962+1G>A) (11,12). Three of the seven variants: c.1019G>A, p.(Arg340His); c.962+1G>A; and c.1469del, p.(Leu490Argfs*9) were novel since they were not found in known databases such as ExAC (http://exac.broadinstitute.org/), GenomeAD (https://gnomad.broadinstitute.org) and dbSNP (https://www.ncbi.nlm.nih.gov/snp). The [c.1019G>A, p. (Arg340His] variant was identified in two unrelated families (B-II.1, C-II.2, C-III.1 and C-III.2; Table III).

In family A, a novel heterozygous variant c.918del, p. (Lys306Asnfs*53) was identified in three affected patients (A-I.1, A-II.1 and A-II.3; Table III and Fig. 1). This deletion variant creates a frameshift starting at codon Lys306 and a new reading frame ends in a new stop codon at position 53 downstream of the mutation (Table III and Fig. 2A). Unaffected individuals in this family were wild type for this variant.

A second novel variant [c.96dup; p. (Ser33Glufs*11)] was detected in family D (D-I.1 and D-II.1; Table III and Fig. 2B). This one-base-pair duplication variant in exon 1 creates a frameshift starting at codon Ser33 and the new reading frame is predicated to end in a stop codon at position 11 (Table III and Fig. 2B).

A third novel heterozygous variant was identified in family F (F-II.3) which was an insertion of 26 base pairs (bps) in

Table III. Clinical severity class and pathogenic *EXT1* gene variants identified.

Family	Patient	Sex	Age at diagnosis (years)	Clinical severity class	Nucleotide change (*EXT1* gene)	Genomic position	Protein level	Clinical significance	Novel mutation	EXT1 protein IHC staining	EXT2 protein IHC staining
A	I.1	M	20	IB	c.918del	g.119122368del (Exon 1)	p. (Lys306Asnfs*53)	Likely pathogenic	Yes	RE	No
	II.1	F	7	IB							
	II.3	F	7	IB							
B	II.1	M	10	IB	c.1019G>A	g.118849384C>T (Exon 2)	p. (Arg340His) VUS	Pathogenic	No[a]	RE	No
					c.82T>A	g.119123204A>T (Exon 1)	p. (Phe28Ile)		Yes		
C	II.2	M	18	IIIB	c.1019G>A	g.118849384C>T (Exon 2)	p. (Arg340His)	Pathogenic	No[a]	RE	No
	III.1	M	12	IA							
	III.2	M	15	IA							
D	I.1	M	6	IA	c.96dup	g.119123190dup (Exon 1)	p. (Ser33Glufs*11)	Likely pathogenic	Yes	RE	No
	II.1	M	27	IB							
E	II.1	M	6	IIA	c.1469del	g.118831982del (Exon 6)	p. (Leu490Argfs*9)	Pathogenic	No[b]	RE	No
F	II.3	M	7	IB	c.1065_1066ins26	g.118847781_118847782ins26 (Exon 3)	p. (Val356Cysfs*12)	Likely pathogenic	Yes	RE	No
G	I.1	F	10	IIIA	c.962+1G>A	g.119122323C>T (Donor splice site of intron 1)	p.?	Pathogenic	No[c]	RE	No
	II.4	M	25	IA							
H	I.1	M	24	IB	None						
I	I.1	F	20	IIIA	None						
	II.1	M	9	IIA	None						

[a]Ref. (11). [b]Ref. (12). [c]https://www.ncbi.nlm.nih.gov/clinvar/variation/642916/. HMO, hereditary multiple osteochondromas; M, male; F, female; RE, reduced expression; VUS, variant of undetermined significance; IHC, immunohistochemical.
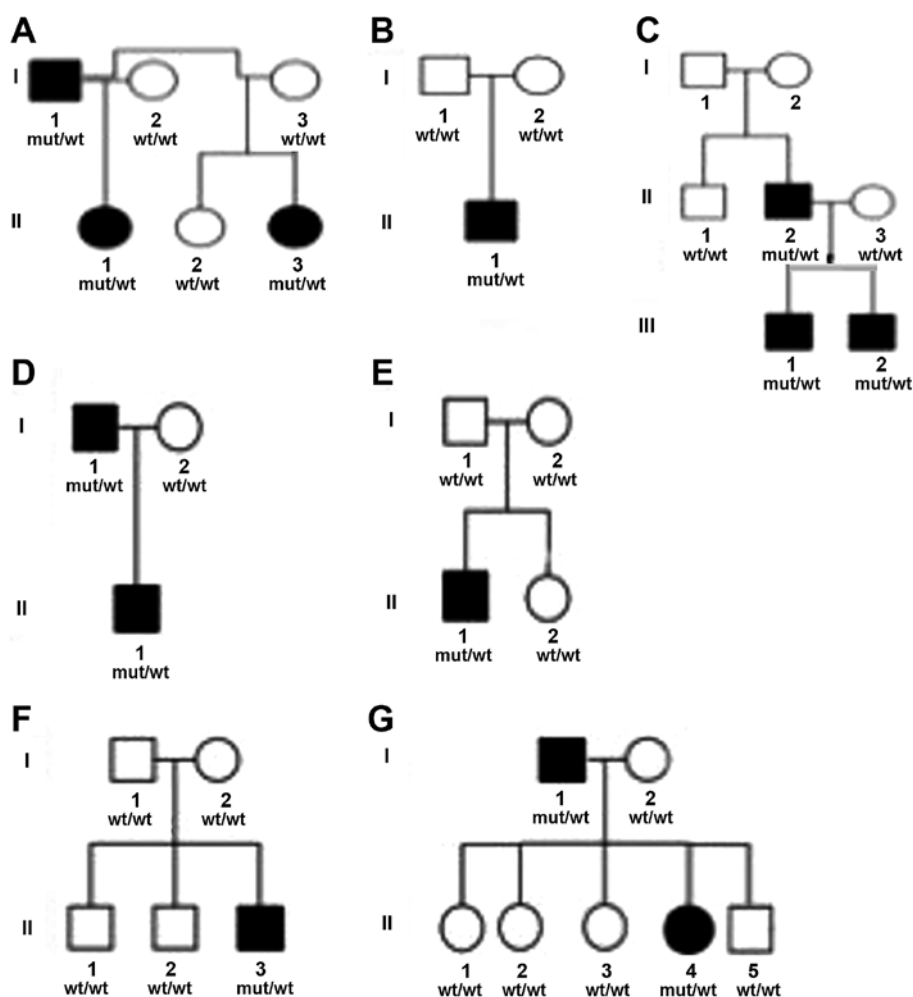
Figure 1. Pedigrees of families (A-G) with *EXT1* genetic variants leading to HMO. (A) Family A, (B) Family B, (C) Family C, (D) Family D, (E) Family E, (F) Family F, (G) Family G. Squares, males; circles, females; solid symbols, affected individuals with heterozygous mutations; open symbols, unaffected individuals. *EXT1* genotypes: Mu, mutant allele; wt, wild-type allele. EXT, exostosin; HMO; hereditary multiple osteochondromas.

exon 3 [c.(1065_1066ins26); p. (Val356Cysfs*12)] (Table III and Fig. 2C). This variant was predicted to create a frame shift mutation starting at codon Val356 and a new reading frame ends in a new stop codon at downstream position 12.

Careful medical evaluation of all family members (affected and unaffected individuals) of families B, E and F, who only had one affected member each (Table III and Fig. 1) was performed. The tested mother and father of these families were clinically normal with no signs of HMO. Furthermore, genetic testing for the presence of a causative genetic variant of the parents showed negative mutation results. The affected individuals (B-II.1, E-II.1 and F-II.3) in these families carry a *de novo* mutation and were the first ones who gained the mutant allele.

Immunohistochemical staining of EXT1 and EXT2. Osteochondroma tissue was available for immunohisto-chemical staining from 12 of the 13 patients with *EXT1* gene mutations. EXT1 protein expression was found to be significantly decreased (weak staining pattern; Fig. 3B) in all examined tissues, apart from tissues obtained from the 2 proband patients of family D (D-I.1 and D-II.1). Their tissues exhibited moderate staining pattern when compared to EXT1 protein expression in normal chondrocytes (Fig. 3A). A novel

variant [c.96dup; p. (Ser33Glufs*11)] was detected in this family. With regards to the *EXT2* gene, no protein expression was detected in any osteochondroma tissues from the 12 tested patients (Fig. 3B).

Discussion

Several HMO studies investigated the associations of the different clinical characteristics with the genetic findings in different populations. The clinical characteristics of patients with HMO in the present study, including the mean age at diagnosis of 13.9 years, as well as male predilection, were similar to what was reported in the literature (1,13). The knee joint was the most common tumor location, which is also consistent with the findings of other studies (14-16).

HMO severity was reported using various clinical classifications (10). These classifications were based on the clinical parameters of HMO patients, including, age, tumor number, joint deformities, limb length discrepancy, in addition to the morbidity associated with HMO tumors. The clinical classification, used in the present study, revealed that the majority of Jordanian patients with HMO have a mild disease form (class 1). Since different HMO studies (14,17,18) used different scoring systems to assess
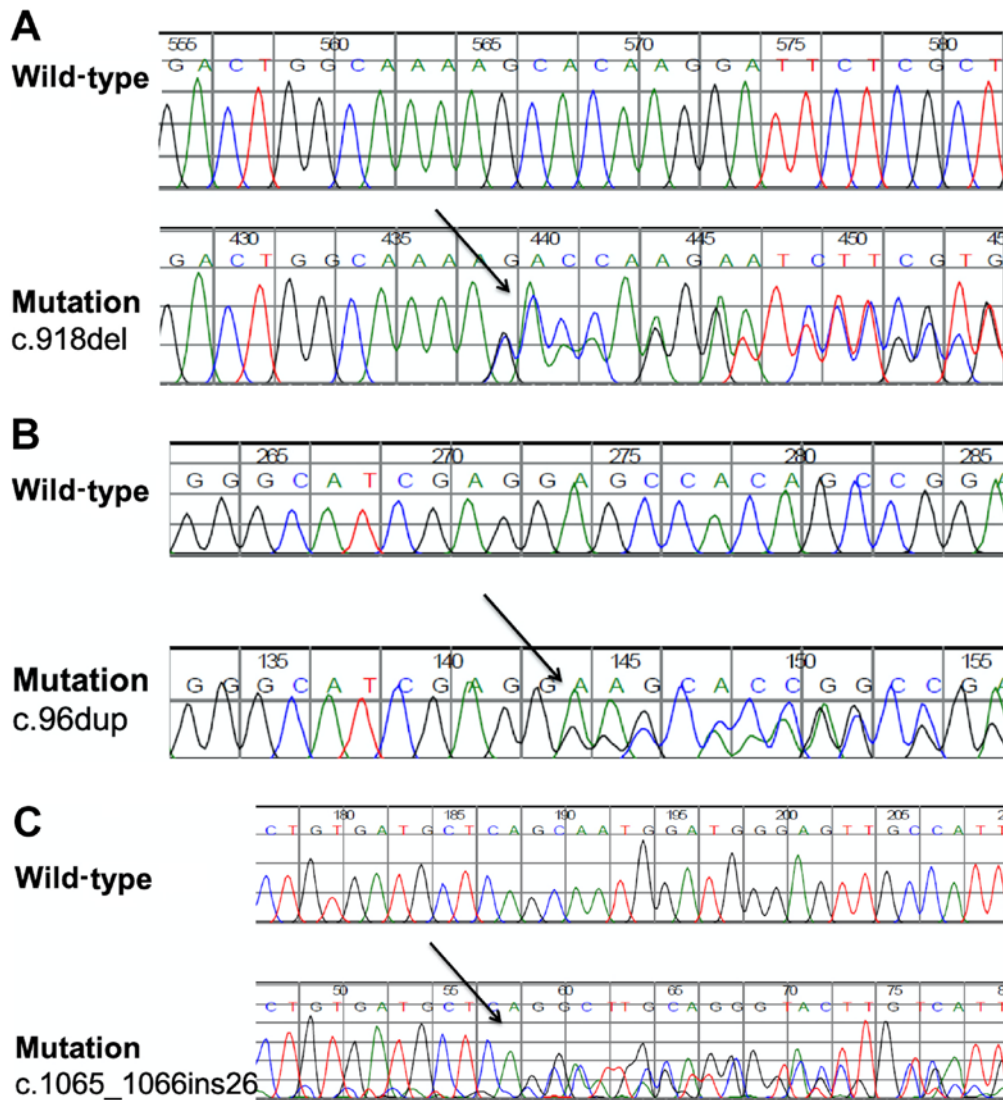
Figure 2. Sanger sequencing data of (A-C) the three novel variants identified in the present study, patients (mutation) and controls (wild-type). (A) Novel Mutation c.918del, (B) Novel Mutation c.96dup, (C) Novel Mutation c.1065_1066ins26. EXT, exostosin; HMO; hereditary multiple osteochondromas.

HMO severity, comparing HMO severity among different populations can be difficult.

HMO is not only clinically heterogenous, but it is also genetically heterogenous (6,17,18). Mutational analysis studies reported variable frequencies of *EXT1* and *EXT2* mutations in different ethnic groups (13). Several authors reported *EXT1* mutations to be more common, particularly in Caucasians (7,19-21). Although the present study investigated a different ethnic group, it revealed a similar predominance of *EXT1* mutations in this group of Jordanian patients with HMO. On the other hand, no potential pathogenic genetic variants of *EXT2* gene were identified in the present study. This was inconsistent with other studies which reported that *EXT2* mutations to be present in 20-45% of the patients (7). This inconsistency may be explained by the presence of mutations in noncoding parts of the genes. In addition, the small number of the included patients can be a contributing factor.

Several phenotype-genotype studies of HMO reported that *EXT1* gene mutations were more likely than *EXT2* gene mutations to be associated with a more severe form of the disease (14,15,18,20-23). Other studies reported no difference

in disease severity between these two gene mutations (19,24). In the present study, only 2 of the 13 patients with *EXT1* mutations exhibited a severe form of the disease (class III). This can be attributed to the variability of HMO severity, even among patients with the same EXT gene mutations particularly *EXT1* gene (18,25). In addition, ethnicity can be considered as another influential factor. This is also consistent with the findings of a previous study investigating osteochondroma in Jordanian patients in whom a milder form of HMO was observed compared with that of other populations (4).

With regards to the mutational analysis of the patients in the present study, *EXT1* allelic heterogeneity was observed and the identified mutations were shown to be dispersed throughout the coding regions of the gene (Fig. 4). Furthermore, the truncated mutations occurred in 66.6% of the tested families. These results are similar to those reported from different studies in the literature (9). A mutational database from these studies is assembled in the Multiple Osteochondromas Mutation Database (MOdb) (http://medgen.ua.ac.be/LOVDv.2.0/), with >600 and 200 different mutations in *EXT1* and *EXT2*, respectively. The majority of these mutations (80%) are nonsense,
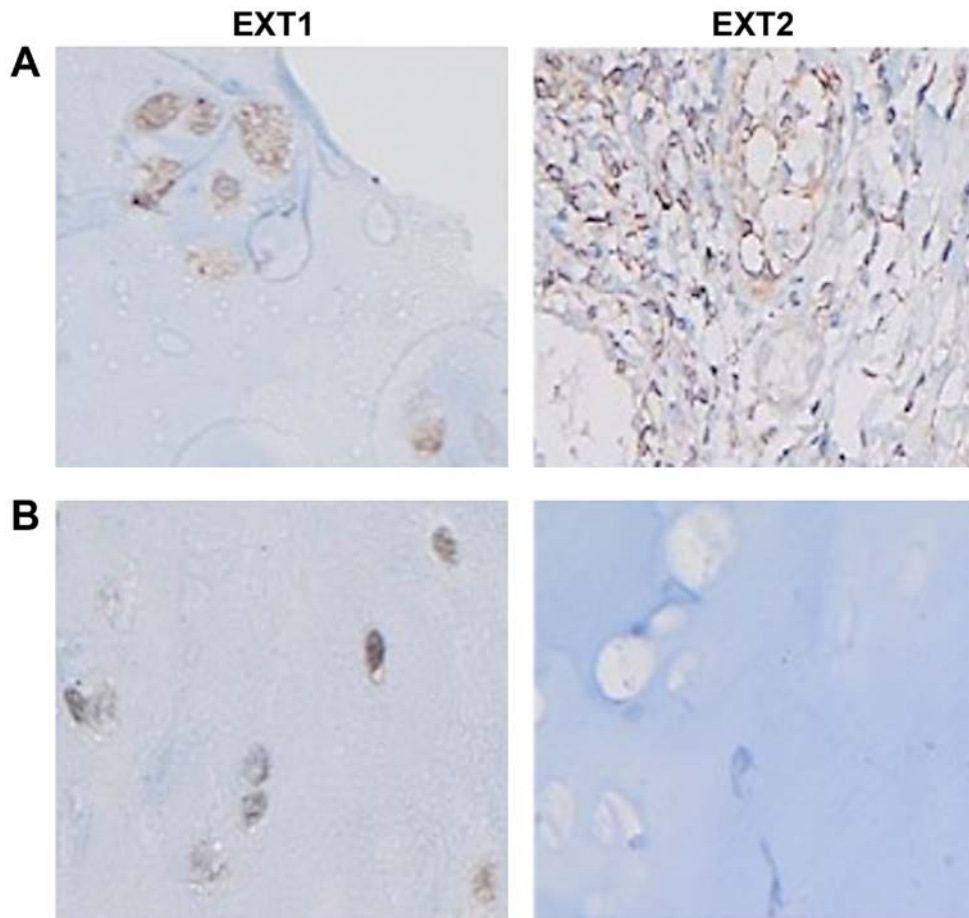
Figure 3. Immunohistochemical staining of chondrocytes. (A) Immunohistochemical staining of EXT1 and EXT2 from control unaffected tissues (magnification, x40). (B) Immunohistochemical staining of EXT1 and EXT2 from a patient with hereditary multiple osteochondromas (magnification, x40). EXT, exostosin.
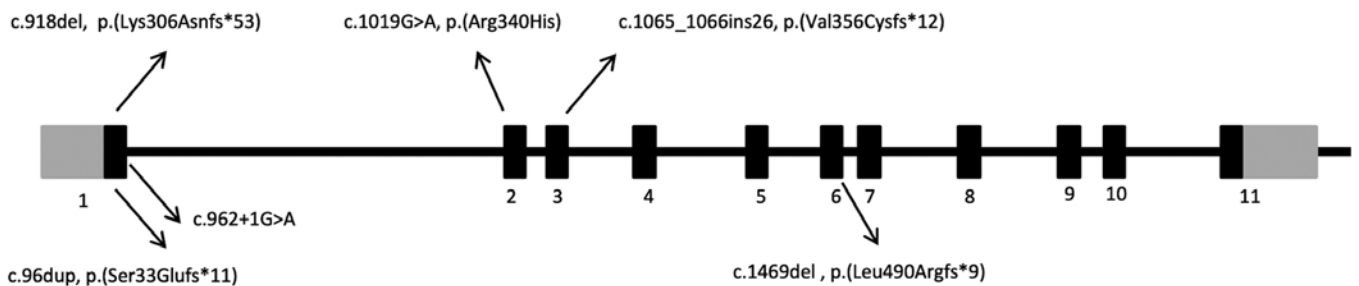

Figure 4. Structure of the *EXT1* gene, with the positions of the identified genetic variants indicated. Gray boxes indicate 5' and 3' untranslated region, and black boxes indicate *EXT1* exonic region. *EXT*, exostosin.

whereas the remaining 20% are frameshift mutations and splice-junction mutations, causing an early termination of translation or partial/complete deletion of the gene and loss of protein function (7).

In the present study, 19% (3/16) of patients with HMO had no point mutations in the coding regions for either *EXT1* or *EXT2* genes. This may be explained by variants involving large rearrangements such as deletions, duplications, inversions, translocations or somatic mosaicism, that include the *EXT1* and *EXT2* genes. Deletion of a single or multiple exons were previously detected in ~10% of all HMO cases (7,26,27). Another study reported a complex rearrangement as causative

mechanism of the disease, which involved a 80.7 kb intronic deletion of *EXT1* gene and a 68.9-kb duplication proximal of *EXT 1* (28). Furthermore, genetic variants in the 5' and 3'UTRs, deep intronic causing variants or in the promoter regions were not determined in the present study. In addition, several studies have reported that 10-15% of patients with HMO have no mutations in either *EXT1* or *EXT2* genes (29-31), suggesting that other genes may be involved in the pathogenesis of the disease. Therefore, testing this subgroup of patients by whole exome sequencing or whole genome next generation sequencing will be an attractive approach to identify other possible disease-causing gene(s) (32,33).
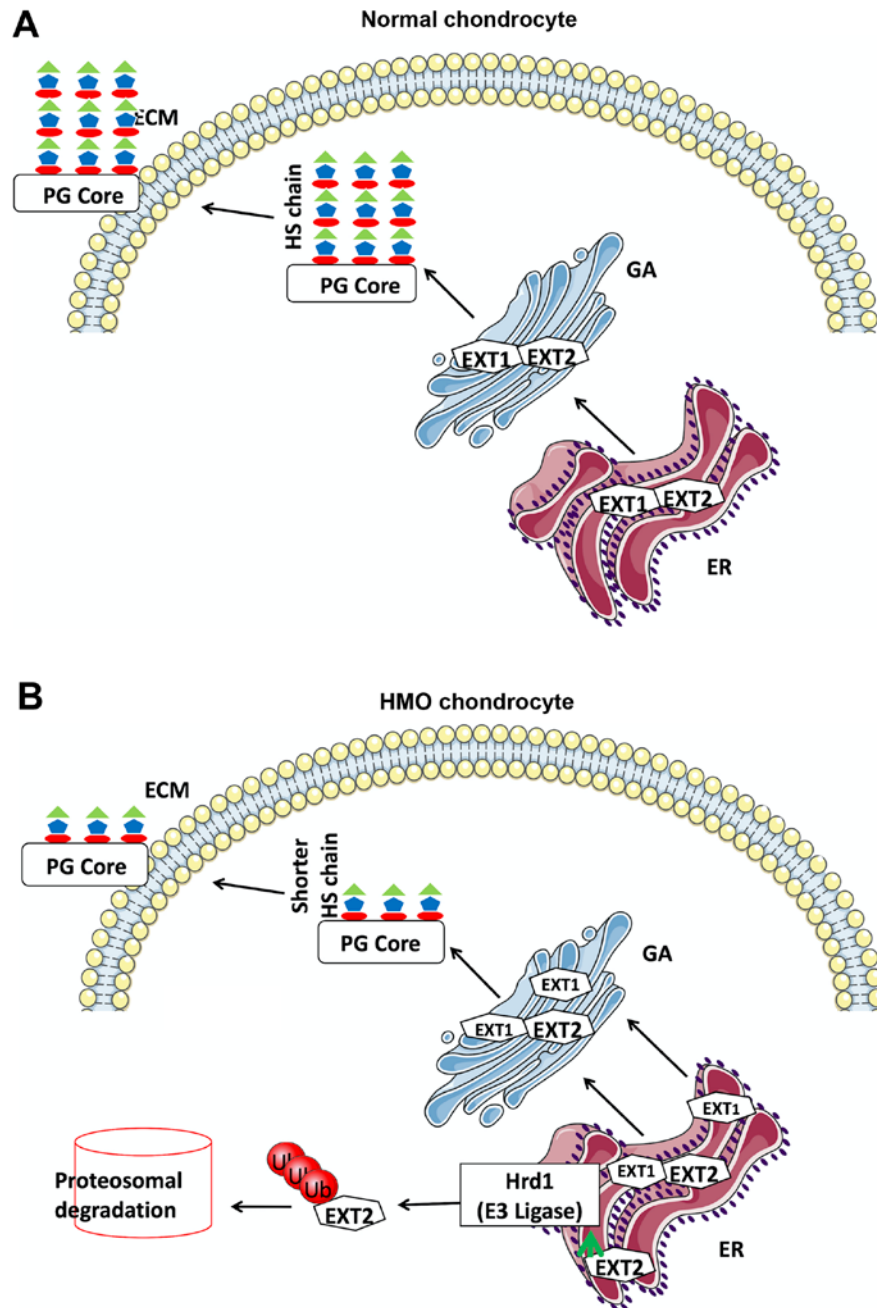
Figure 5. Model of the function of the EXT1/EXT2 complex in HS synthesis in (A) normal chondrocytes and (B) HMO chondrocytes. (A) In normal chondrocytes, EXT1 and EXT2 form a complex, which is involved in the HS synthesis in the Golgi apparatus. The EXT1/EXT2 complex, through its galactosyltransferase activity, aids in the formation of HS proteoglycans HSPGs, which are next exported to the cell exterior. (B) In HMO chondrocytes, decreased levels of EXT1 disrupts the stoichiometry of the EXT1/EXT2 complex, resulting in low HS synthesis and, thus, diminished HSPGs on the cell exterior. Low levels of EXT1 render EXT2 unable to be transported into the Golgi complex, which is instead retained in the ER and thereby targeted for ER-associated protein degradation. EXT2 is shown to be ubiquitinated at Lys 245 and is degraded though ubiquitin-proteasome system. HS, heparan sulfate; HSPGs, HS proteoglycans; EXT, exostosin; HMO; hereditary multiple osteochondromas; ER, endoplasmic reticulum; ECM, extracellular matrix; GA, Golgi apparatus; PG, proteoglycans.

To the best of our knowledge, the majority of phenotype-genotype research studies on HMO did not test for the expression of *EXT* genes in the resected tumor tissues. Immunohistochemistry studies revealed a decreased expression of EXT1 and/or EXT2 corresponding to the *EXT* gene mutations status (3,34,35). In the present study, the patients with *EXT1* mutations exhibited a decreased expression of EXT1 protein. Surprisingly, the same patients exhibited no expression of EXT2 proteins, although none of them harbored

EXT2 mutations. Previous studies demonstrated that the presence of fully functional EXT1 and EXT2 proteins is required for their correct localization in the Golgi complex (36,37). In addition, the present study proposes a model (Fig. 5), in which mutations in *EXT1* result in a truncated product and/or inactive form of the protein that can no longer bind to its EXT2 partner. The very low levels of EXT1, EXT2 and EXT1/2 alter the stoichiometry of the complex and greatly diminish its glycosyltransferase activity. Low levels of EXT1 can no longer

associate with its requisite partner EXT2 and, thus, EXT2 is retained in the endoplasmic reticulum (ER) and is targeted through the ER-associated protein degradation pathway for degradation. The identification of EXT2 protein as a substrate for the Hrd1 E3 ligase and the identification of the lysine involved in ubiquitin attachment to the protein (38,39) is consistent with the aforementioned model, as explained in Fig. 5.

Mutations in *EXT1* and *EXT2* are associated with the pathogenesis of HMO; however, the mechanism through which HS synthesis alteration leads to exostoses has yet to be elucidated. Heterozygous *EXT1* or *EXT2* mutations are common molecular changes identified in >80% of the investigated exostoses (40). There remains the question of whether osteochondromas arise via loss of heterozygosity or haploinsufficiency mechanism (40,41). The results of the present study and early biochemical studies (36,37,42) of the EXT1/2 complex suggest that haploinsufficiency for either EXT1 or EXT2 affects the ability of chondrocytes to synthesize HS, as explained in Fig. 5. Although EXT1 and EXT2 are ubiquitously expressed, mutations in these genes are only manifested in chondrocytes, suggesting that chondrocytes require two fully active EXT1 and EXT2 proteins. However, Reijnders *et al* (41) refuted the haploinsufficiency theory and demonstrated that osteochondromas arise via loss of heterozygosity and inactivation of both alleles (Knudson's two-hit model) (43). Further genetic analysis studies of large cohorts of patients with HMO are required to determine the contribution of LOH and haploinsufficiency in the molecular pathogenesis of the disease.

The main limitation of the present study was the lack of DNA samples from some members of family C (unaffected individuals I.1 and I.2), and so genetic testing or further investigations for *mosaic* mutations (pyrosequencing or cloning of the suspected PCR products) could not be conducted.

In conclusion, the present study conducted a phenotype-genotype study of HMO in 16 Jordanian patients from nine families. These patients are representative of an ethnic group in which the genetic background of HMO is infrequently investigated. The majority of these patients were males, diagnosed the age of ~14 years, and exhibited a mild clinical disease form. Genetic analysis revealed mutations exclusively involving *EXT1* gene and none involved *EXT2* gene. These mutations were not necessarily associated with a severe clinical disease. Three of the identified mutations were novel. Three patients did not show any mutations for either *EXT1* or *EXT2* genes. Upon immunohistochemical testing, osteochondroma tissue resected form all patients with *EXT1* mutations exhibited decreased expression of EXT1 protein. Surprisingly, EXT2 protein was not detected in these patients although none had *EXT2* mutations. Therefore, a model may be suggested that questions the role of *EXT2* gene in HMO pathogenies. HMO continues to represent a clinically and genetically heterogenous disease among different ethnic groups. Therefore, further genetic and immunohistochemical studies are required to further elucidate the pathogenesis of HMO. In addition, mutational analysis studies can be helpful in screening for patients with HMO, particularly those who may be at a risk of developing a severe form of the disease, which will have a significant impact on the clinical management and follow up of patients with HMO.

Availability of data and materials

Sanger sequencing data are available at https://www.ebi.ac.uk/eva with the following accessions: Project, PRJEB41290; analyses, ERZ1673834. The following links can also be used: https://www.ebi.ac.uk/ena/data/view/PRJEB41290, https://www.ebi.ac.uk/eva/?eva-study=PRJEB41290 and https://wwwdev.ebi.ac.uk/eva/?eva-study=PRJEB41290.

Authors' contributions

ZM performed the collection and analysis of data, study design, writing and editing of the manuscript. KB performed the data analysis, study design, writing and editing of the manuscript. MA was responsible for the study design and writing of the manuscript. RA performed the data collection, study design and writing of the manuscript. MAA performed the data collection, study design and writing of the manuscript. ABK cotributed to the data collection, study design and writing of the manuscript. KAB performed the data analysis and writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval for this research was obtained from the Human Research Ethics Committee and IRB at Jordan University of Science and Technology (approval no. 22/116/2018). All individuals included in this study and/or their legal guardians provided written informed consent for participating in this study.

Patient consent for publication

All participants and/or their legal guardians provided a written informed consent regarding the publication of case details and any associated images.

Competing interests

The authors declare that they have no competing interests.

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