RESEARCH NOTE

Genetic screening of *EXT1* and *EXT2* in Cypriot families with hereditary multiple osteochondromas

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Introduction

The purpose of this study was to perform genetic screening of the exostosin 1 (*EXT1*) and exostosin 2 (*EXT2*) genes in Cypriot patients with a clinical diagnosis of hereditary multiple osteochondromas (HMO). Initially, mutation analysis of the *EXT1* gene was performed by Sanger sequencing. When no point mutation was identified in *EXT1*, *EXT2* analysis was performed. When no sequence variant was identified in either of the candidate genes, array-comparative genomic hybridization (CGH) was implemented to detect any large copy number changes (CNCs). In total, three point mutations and a large deletion were identified in *EXT1*: the c.2101C>T(p.Arg701*) mutation and the ~0.16-Mb deletion removing exons 2–11, which were previously reported whereas the c.486_489delCAGA(p.Asp162Glyfs*12) and the c.868G>T(p.Glu290*) mutations which were novel. It is the first report on genetic screening of five HMO patients of Cypriot descent. The observations presented provide additional evidence for the variability in phenotypic expression and the mutational spectrum of this disorder.

The term HMO, or hereditary multiple exostoses (HME), MIM: 133700 and MIM: 133701 respectively is used to describe a genetic disorder characterized by the development of multiple, circumscript, occasionally painful and usually symmetric bony protuberances called osteochondromas (benign cartilaginous tumours which most frequently grow outward from the juxtaphyseal region of the long bones or the surface of flat bones). With advancing age, osteochondromas grow in size and number until the completion of skeletal maturity by which time no new ones tend to be observed. HMO can, beside other problems, lead to a reduction in skeletal growth, secondary bony deformities, restricted joint motion, short stature and compression of peripheral nerves (reviewed extensively in Hennekam 1991; Wuyts *et al.* 1993; updated 21 Nov. 2013). Major radiographic features, include irregular expansion of the metaphyses of the long bones with outgrowths projecting from the sides of the expanded areas, multiple exostoses of the flat bones or jutting from relatively normal diaphysis and secondary bony deformities. The risk for malignant transformation (typically osteochondrosarcoma) increases with age, although the lifetime risk in most studies seems to be low (Legeai-Mallet *et al.* 1997). However, in certain families (Porter *et al.* 2004; Vujic *et al.* 2004), and a large study in 2011 by Pedrini *et al.* (2011), the rate of malignant transformation was calculated to be as high as 5–6%. HMO is inherited in an autosomal dominant manner, having an estimated penetrance of ~96% in females and 100% in males, and displays great interfamilial and intrafamilial variability in phenotypic expression. Mutations in either the *EXT1* gene on chromosome 8q24.11 or the *EXT2* gene on 11p11.2 have been shown to cause HMO accounting for ~60–70% and 20–30% of cases, respectively.

The purpose of this study was to identify mutations in either of the *EXT* gene loci (*EXT1* initially and subsequently *EXT2* in those having no *EXT1* pathogenic variants) in Cypriot patients with a clinical diagnosis of HMO. Five patients, representing the first report of genetic screening of HMO affected individuals in the Cypriot population are described.

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Material and methods

Patients

This study included five patients (from four families) who were enrolled at our outpatient clinic and reviewed by a clinical geneticist. The diagnosis of HMO was suspected on the basis of clinical examination and presence of multiple osteochondromas (at least three or more) confirmed by x-rays. Peripheral blood samples were collected and patient's genomic DNA was sent to our laboratories initially for *EXT1* sequencing. If no *EXT1* mutations were identified, the *EXT2* gene was subsequently sequenced. Where required, array-CGH analysis was also performed to assess for copy number changes. Whenever possible, analysis was also carried out on samples from the HMO patient's parents in an attempt to establish whether the identified mutations were inherited or had occurred as *de novo* events. All study participants tested provided an informed written consent.

DNA amplification

Genomic DNA was extracted from peripheral blood using the QiagenQIAmp DNA Blood Midi Kit (Qiagen, Valencia, USA). Mutation analysis of all the coding regions of the human *EXT1* and *EXT2* genes was performed using intronic primers and optimized PCR conditions (available upon request).

DNA analysis

Direct sequencing of the *EXT* genes was performed on an ABI 3130*xl* apparatus (Applied Biosystems, Foster City, USA) according to manufacturer's procedures. Where no *EXT1/EXT2* sequence variants were identified, array-CGH analysis was performed using Cytochip ISCA array (BlueGnome ver. 1.0) with 180,000 oligos according to manufacturer's recommendations. The array was scanned in a 5 μm resolution using the Agilent DNA microarray scanner (Agilent Technologies, Santa Clara, USA) and fluorescent ratios were calculated using the BlueFuse Multi software (BlueGnome, Cambridge, UK).

Results

Five patients with clinical and radiological evidence of multiple exostoses were selected for genetic screening as described earlier in materials and methods. Patient 1 presented to Clinical Genetics at the age of 32 years. She reported an history of metaphyseal widening apparently having been identified following an injury at the age of 14 years. She was initially diagnosed with Pyle disease. She had a long-standing history of troublesome joint aches and pains, and unilateral hearing loss (figure 1, a and b). Her mother (patient 2), was initially assessed at the age of 60 years. She had an history of multiple bony exostoses and radiological evidence suggestive of HMO. Patient 3 presented to Clinical Genetics at the age of 3 years and 4 months, history of ankle swelling which prompted further evaluation by x-rays which revealed multiple exostoses involving both upper and lower limbs and widening of the metaphyses (figure 1c). In addition to the exostoses she had lumbar lordosis. Patient 4 was clinically diagnosed with HMO at the age of 2 years and 6 months after the identification of bony lumps around the knees. He went on to develop numerous exostoses at several sites and underwent various operations for removal of large osteochondromas. He had significant bony deformities, scoliosis and restricted height. Patient 5 had a family and a personal history of HMO with radiological evidence of multiple exostoses. He had minor symptoms, but his son (not available for testing) was described as being severely affected. A summary of the patients' clinical and molecular characteristics is provided in table 1. Point mutations or deletions involving the *EXT1* gene were detected in all five patients (table 1). No *EXT2* mutations were identified in this cohort.

EXT1 point mutations

An *EXT1* point mutation (c.2101C>T) was identified in two related female patients (patients 1 and 2; daughter and mother respectively, figure 2a). This is a nonsense mutation, reported previously by Seki and colleagues (Seki *et al.* 2001), which results in a premature stop codon in exon 11 (p.Arg701*) of this gene.

The novel mutation c.868G>T(p.Glu290*) in exon 1 of the *EXT1* gene introducing a stop codon was identified in patient 3. Mutation analysis of the parents did not reveal the same mutation identified in their daughter (figure 2b).

EXT1 deletions

Sequence analysis of the *EXT1* gene in patient 4 identified no genetic alterations. Consequently, the *EXT2* gene (the second most common disease-causing locus for HMO) was analysed but revealed no mutations either. To rule out any copy number changes, array-CGH analysis involving either *EXT1* or *EXT2* locus was performed identifying a previously reported (Seki *et al.* 2001; Szuhai *et al.* 2011) intragenic EXT1 deletion of exons 2 to 11 (figure 2c).

In addition, a novel 4-bp deletion, c.486_489delCAGA (p.Asp162Glyfs*12) was identified in a male patient (patient 5) within the coding region of exon 1 of the *EXT1* gene (figure 2d).

Discussion

Both *EXT1* and *EXT2* code for glycosyltransferases involved in the polymerization of heparan sulphate (McCormick *et al*. 1998, 2000) which in turn affects downstream signalling, important in the regulation of chondrocyte proliferation (Bellaiche *et al.* 1998). There is evidence pointing

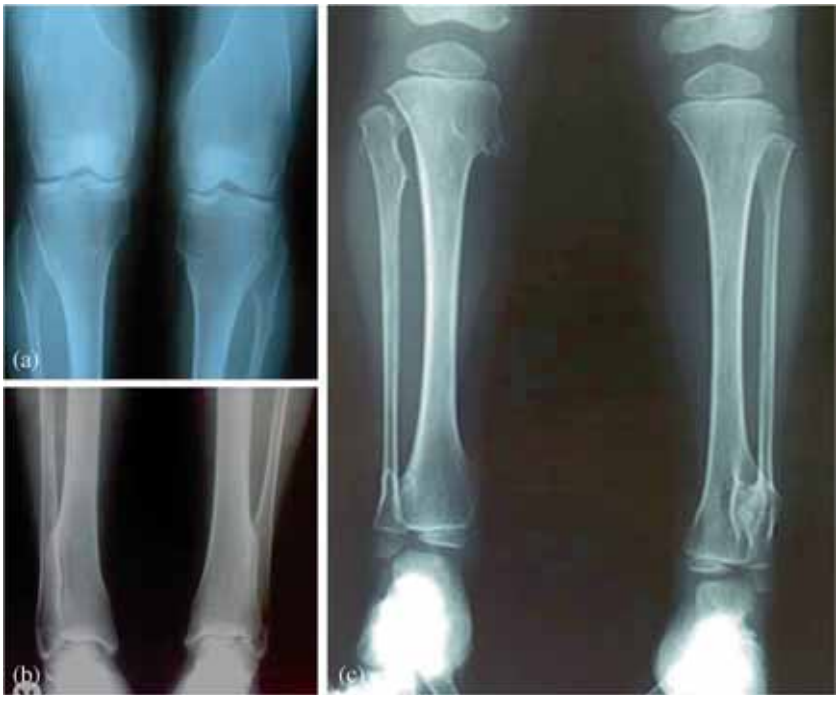

Figure 1. (a&b) Anteroposterior x-ray of the knees and ankles of patient 1, a 32-year-old female. Note the expansion of the distal femoral, proximal tibial and fibular metaphyses with outgrowths projecting from the sides of the expanded areas. There is an abrupt transition from an expanded metaphysis to a normal diaphysis of both distal femora and proximal tibiae. (c) Anteroposterior x-ray of the tibiae of patient 3, a 4-year-old girl, showing similar abnormalities.

Table 1. Clinical and molecular characteristics of Greek-Cypriot HMO patients reported.

Patient	1[a]	2	3	4	5
Gender	F	F	F	M	M
Age at analysis	32 y.o.	60 y.o.	40 m.o.	18 y.o.	56 y.o.
Multiple exostoses	+	+	+	+	+
Clinical course	Moderate	Mild	Mild-moderate	Severe	Mild
Malignancy	–	–	–	–	–
Pathogenic *EXT1* sequence variant	c.2101C>T (p.Arg701*)	c.2101C>T (p.Arg701*)	c.868G>T (p.Glu290*)	N	c.486_489delCAGA (p.Asp162Glyfs*12)
Pathogenic *EXT2* sequence variant	NP	NP	NP	N	NP
CNC on array-CGH	NP	NP	NP	~0.16-Mb *EXT1* deletion (exons 2-11)	NP
De novo mutation	N	ND	Y	ND	ND
Novel mutation	N	N	Y	N	Y

DNA mutation nomenclature is based on mRNA reference sequence (GenBank accession number NM_000127.2) considering nucleotide +1 as the A of the ATG translation initiation codon.

CNC, copy number change; ND, not determined; NP, not performed; +, presence of symptom; −, absence of symptom; Y, yes; N, no; M, male; F, female; y.o, years old; m.o, months old.

[a]Daughter of patient 2.

towards an *EXT1* and *EXT2* tumour suppressor activity and a 'two-hit' hypothesis model has been proposed for the development of osteochondromas, due to the observed loss of heterozygosity in chondrosarcomas (Hecht *et al.* 1997; Bovee *et al.* 1999) and the presence of homozygous *EXT1* deletions in solitary osteochondromas (Hameetman *et al.* 2007). More than 400 pathogenic variants have been described in *EXT1* according to MOdb (http://medgen.ua.ac.be/LOVDv.2.0/home.php), Multiple Osteochondromas Mutation Database (Jennes *et al.* 2009). Most of the reported

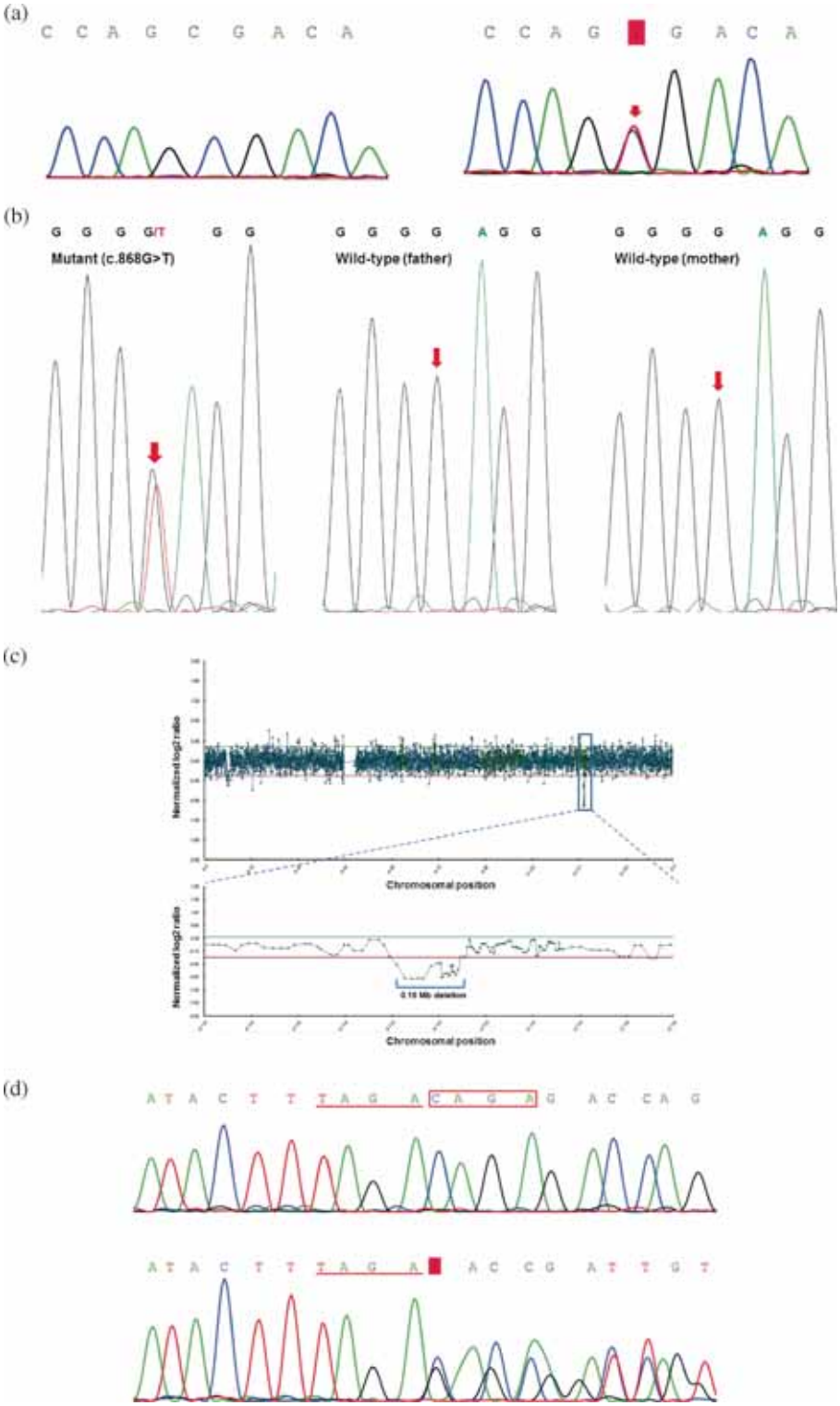

Figure 2. (a) Electropherograms of part of exon 11 of the *EXT1* gene of a healthy individual (left panel) and an affected female (right panel), patient 1. The C>T substitution resulting in a premature stop codon (p.Arg701*) is indicated by an arrow. This mutation was also identified in the patient's mother, patient 2. (b) Sequencing of genomic DNA identified a heterozygous G>T transition in exon 1 of the EXT1 gene (c.868G>T) in patient 3 (left panel). Both parents were wild-type at the given locus (middle and right panels). The mutation introduces a premature termination signal in the EXT1 polypeptide (p.Glu290*). (c) Array-CGH (BlueFuse, BlueGnome) profile of an affected male (patient 4) showing a deletion on chromosome 8 (*EXT1* locus). An enlarged view of the deleted region (~0.16 Mb) is also shown. Normalized $\log_2$ ratios of the chromosome and reference values plotted against the genomic position. (d) Electropherograms of part of exon 1 of the *EXT1* gene of a healthy individual (upper panel) and an affected male (lower panel, patient 5) showing a 4-bp deletion c.486_ 489delCAGA(p.Asp162Glyfs*12).

mutations seem to be 'private' and are dispersed throughout the entire gene.

Most *EXT1/EXT2* mutations are inactivating (nonsense, frameshift or splice-site) and are predicted to result in premature termination of translation. The remaining mutations are usually missense (Wuyts *et al.* 1993 updated 21 Nov. 2013; Park *et al.* 1999; Xu *et al.* 1999; Jennes *et al.* 2009). In the present study, a nonsense mutation in exon 11 (p.Arg701*) of the *EXT1* gene was detected in patients 1 and 2 (figure 2a). This mutation has previously been reported in other HMO patients (Seki *et al.* 2001). According to MOdb, an additional eight, exon 11, *EXT1* mutations have been reported. Four of them involve deletions leading to frameshift and an additional four are nonsense mutations. This suggests that exon 11 of the *EXT1* might be a mutational hotspot.

In patient 3, sequence analysis of the *EXT1* gene identified the c.868G>T (p.Glu290*) mutation (figure 2B). This alteration is predicted to result in premature termination of the gene product in exon 1. The c.868G>T mutation has not been reported previously on MOdb. Mutation analysis of both parents revealed that the mutation had occurred as a *de novo* event in the proband. Approximately 10% of HMO cases reported have a *de novo* mutation in either of the *EXT* genes (Wuyts *et al.* 1993 updated Nov. 21 2013).

Several mechanisms have been implicated in the generation of recurrent or nonrecurrent, disease-associated genomic rearrangements such as nonallelic homologous recombination (NAHR), nonhomologous endjoining (NHEJ) and fork stalling and template switching (FosTeS) (Gu *et al.* 2008). More specifically, it has been shown that in HMO patients, both NAHR which is mediated by *Alu*-sequences as well as NHEJ are causal and that these deletions seem to be nonrecurring (Jennes *et al.* 2011).

Intragenic deletions involving single or multiple *EXT1* or *EXT2* exons are found in ~10% of tested patients (Vink *et al.* 2005; Jennes *et al.* 2009). In the current study, patient 4 was found to have an intragenic *EXT1* deletion of ~0.16 Mb removing exons 2 to 11. Similar deletions have previously been reported in several patients either in constitutional or mosaic forms (Jennes *et al.* 2008, 2011; Szuhai *et al.* 2011; Sarrion *et al.* 2013). In 2011, a study by Jennes *et al*. demonstrated that the *EXT1* breakpoints of a deletion involving exons 2 to 11 were located within a 5-bp homologous sequence. Multiple long interspersed element–1 (LINE1) and *Alu* elements were identified at the distal breakpoint, while at the proximal breakpoint the complement of a deletion hotspot consensus sequence was present (Jennes *et al.* 2011). In addition, there are reports of patients with similar deletions manifesting signs and symptoms even if the deletion was present in a mosaic form (Szuhai *et al.* 2011; Sarrion *et al.* 2013) leading to the suggestion that deletion of a single allele even in a relatively small number of cells as in the case of somatic mosaicism is sufficient enough to trigger the phenotype.

In the case reported in the current study, the large *EXT1* deletion of exons 2 to 11 was identified by array-CGH. We were unable to obtain parental samples to confirm whether this deletion was inherited or was the result of a *de novo* event, but there were reports of the patient's mother also being affected (not evaluated in clinic). It remains unclear whether on a DNA sequence level the breakpoints in our patient are similar to the ones previously reported. Exon 1 is the largest *EXT1* exon and codes for more than 40% of the EXT1 protein (Vink *et al.* 2005). The 4-bp deletion in exon 1 of the *EXT1* gene presented here has not, to our knowledge, been previously reported and can be considered pathogenic as it most probably leads to protein truncation.

Several reports have shown a relation between *EXT1* or *EXT2* mutations and the severity of HMO, with the most severe forms of the disease being associated with *EXT1* mutations (Francannet *et al.* 2001; Porter *et al.* 2004; Alvarez *et al.* 2006; Pedrini *et al.* 2011). The development of HMO was associated with *EXT1* mutations in all patients presented in our study. There was clear evidence of variability in phenotypic expression. Limitations due to a small sample size which do not allow us to draw definitive conclusions but should further enrich available data on genotype–phenotype correlations.

This is the first report of molecularly confirmed HMO patients of Cypriot descent. The observations presented in this report provide additional evidence for the rich variability in phenotypic expression, the mutational heterogeneity and ethnic diversity associated with this condition.

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