Novel mutation of *EXT2* identified in a large family with multiple osteochondromas

XIAO-JUN CHEN[1], HONG ZHANG[2], ZHI-PING TAN[2], WEN HU[2] and YI-FENG YANG[2]

Departments of [1]Nephrology and [2]Cardiovascular Surgery, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, P.R. China

Received October 17, 2015; Accepted September 20, 2016

DOI: 10.3892/mmr.2016.5814

Abstract. Multiple osteochondromas (MO), also known as hereditary multiple exostoses, is an autosomal dominant bone disorder. Mutations in exostosin glycosyl transferase-1 *(EXT1)* and exostosin glycosyl transferase-2 *(EXT2),* including missense, nonsense, frameshift and splice-site mutations, account for up to 80% of reported cases. The proteins EXT1 and EXT2 form a hetero-oligomeric complex that functions in heparan sulfate proteoglycan biosynthesis. A heterozygous *EXT2* mutation, c.939+1G>T, was identified in a five-generation 33-member MO family, and was present in all 13 affected members. The mutation results in deletion of exon 5 in the mRNA, producing a frameshift that leads to a premature termination codon. The present study extends the mutational spectrum of *EXT2*.

Introduction

Hereditary multiple exostoses (HME), also known as multiple osteochondroma (MO), is an autosomal dominant bone disorder with an incidence of 1 in 50,000 live births in western populations (1). The morbidity rate is greater in males than females, with a ratio of 1.5:1, due to the mutations exerting a weaker phenotypic effect in females (2). HME is characterized by the presence of multiple benign cartilage-capped tumors, localized primarily in the long tubular bones, particularly in the humerus (10-50%), forearm (39-60%), knee (33%) and ankle (25%). The majority of patients with HME (~70%) have a family history of the condition (1,3,4).

HME is a genetically heterogeneous disorder with two disease-causing genes identified, exostosin glycosyl transferase-1 (*EXT1)* and exostosin glycosyl transferase-2 (*EXT2*) located at chromosomes 8q24 and 11p11-p12, respectively (5,6).

Correspondence to: Dr Hong Zhang, Department of Cardiovascular Surgery, The Second Xiangya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011, P.R. China
E-mail: xyzhnghong@hotmail.com

Key words: hereditary multiple exostoses, exostosin glycosyl transferase 2, splice mutation

The proteins encoded by human *EXT1* and *EXT2* are type II transmembrane glycoproteins, localized in the endoplasmic reticulum. The EXT1/EXT2 complex is involved in the biosynthesis of heparin sulphate (HS) proteoglycan (HSPG) (7). Prior to deacetylation, the EXT1/EXT2 complex catalyzes the elongation of the HS chain. EXT1 and EXT2 are ubiquitously expressed in developing limb buds, and in osteochondromas their expression was decreased in correlation with mutation status (8,9). To date, ~422 separate pathogenic mutations in *EXT1* and ~221 mutations in *EXT2* have been identified. Mutations in *EXT1* account for 56-78% of cases in MO families, whereas *EXT2* mutations have been identified in 21-44% of cases (4,10-15). However, in China, *EXT2* mutations are identified more frequently than *EXT1* mutations (16). The majority of patients have been identified in a single family or as sporadic cases. Nonsense, frameshift and splice-site mutations, which represent the majority of MO-causing mutations (80%), have been predicted to lead to the premature translational termination of the associated amino acids, and the subsequent production of a truncated protein (17). Mutations in *EXT1* are dispersed along the gene, and may occur in various exons (14,18); however, *EXT2* mutations do not appear to occur in the final third of the gene-coding region (17).

Other than *EXT1* and *EXT2*, the *EXT3* gene has been mapped to chromosome 19p (19). It appears to be a minor locus in HME families and no causative mutations in *EXT3* have been identified (20). Three additional *EXT*-like genes, designated *EXTL1, EXTL2* and *EXTL3* have been identified and mapped to chromosomes 1 (1p36, 1p11-p12) and 8 (8p12) (21-23). Although the *EXTL* genes are considered strong candidate genes for MO, to date no HME family has been associated with these loci.

The present study investigated a rare large family with MO, and identified a novel splice-site mutation in *EXT2*.

Materials and methods

Subjects. Written consent was obtained from all study participants, and the present study was approved by the ethics committee of The Second Xiangya Hospital (Changsha, China). The proband was admitted to The Second Xiangya Hospital in 2012, and presented with a large osteochondroma. The five-generation Chinese family of the proband was subsequently investigated and a pedigree constructed based on

clinical and radiographical evaluations of all family members (Fig. 1). Of the 33 family members, there were 13 affected individuals (11 males and 2 females) aged 8-80 years (average age, 52 years). The incidence rate was therefore 40% within the family, and MO occurred in each generation. The affected individuals had 6-16 exostoses, typically located at the juxta-epiphyseal regions of long bones; however, these were not as large as those present in the proband. There were no other lesions that were atypical of MO and no evidence of short stature (average height of adult male and female was 165 and 156 cm, respectively). All affected individuals had lesions detected prior to age 10, but had never received surgery, with the exception of the proband.

The proband, family member IV-2, a 42-year-old male, presented with 41-year history of multiple osteochondroma. Physical examination revealed a large mass on the left side of the back (Fig. 2A), (39x33x19 cm), and >14 osseous nodules of varying sizes located on the prothorax wall, left scapula, bilateral forearms, knees and left ankle. The patient had had an operation at age 22 due to a rapidly enlarging lumbar spinal osteochondroma.

X-ray analysis revealed a diffuse flocculent shadow with high density in the left lung field and multiple bony protrusions on limbs, as presented in Fig. 2B. Computed tomography (CT) angiography did not reveal any imaging of large arteries, indicating a potential chondroma (Fig. 2C). CT revealed the left back mass was of mixed density and multiple flecked calcifications, which spread into the left side of the chest (Fig. 2D). Histopathological analysis revealed an osteochondroma with focal malignant transformation, as presented in Fig. 2E. Surgery was conducted at The Second Xiangya hospital to remove the osteochondroma. The patient was subsequently followed up once every three months in the first year, and once every year thereafter. No recurrence has been detected as of October 2015.

DNA analysis. DNA of all affected family members was extracted from peripheral blood as previously described (24). The coding regions of the *EXT1* and *EXT2* genes were amplified by polymerase chain reaction (PCR) using primer sets, as previously described (25). The PCR products were purified by BigDye® Terminator version 1.1 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) followed by direct DNA sequencing with an ABI 3100 Automatic Sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.), using forward and reverse primers.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted from the tumor tissue of the proband and healthy lung tissue of an unrelated individual, using the QIAgen RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. cDNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.), using the following primers: Forward, 5'-AACCAGAAC ACACTGCGCATCAAG and reverse, 5'-AGCTCCACG AAGAACCACACAGAA for exons 2-5. Amplification of cDNA was performed and products were purified by BigDye Terminator version 1.1 followed by direct DNA sequencing with an ABI 3100 Automatic Sequencer.

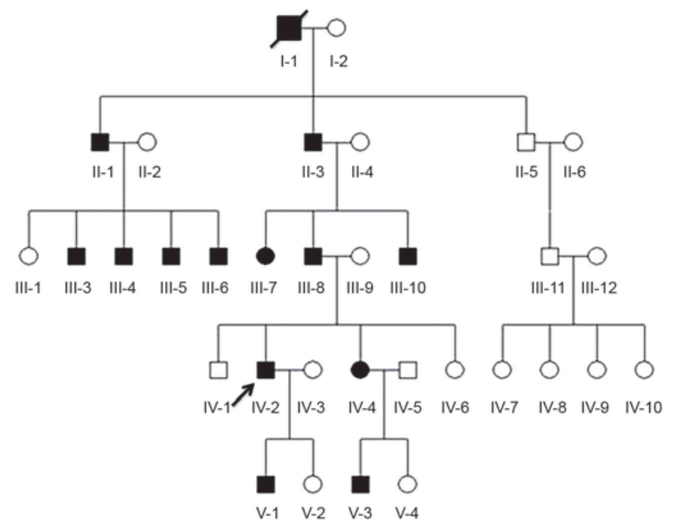

Figure 1. Pedigree analysis of a family with hereditary multiple exostoses. Affected individuals are present in every generation. Solid symbols, affected individuals; open symbols, unaffected individuals; squares, males; circles, females; arrow, proband; oblique lines, deceased.

Results

DNA was extracted from family members with HME (Fig. 1A, solid squares), and the possible mutations residing in the *EXT* genes were scanned in the exon and intron junctions. DNA sequence analysis revealed a heterozygous mutation, c.939+1 G>T in *EXT2* (Fig. 3) in all family members with HME; none of the unaffected family members carried this mutation. This indicated the association of this inheritable mutation with HME. No other mutations were reported in any of the analyzed samples.

The result of PCR gel electrophoresis is presented in Fig. 4. Following RT-PCR at exons 2-5, two bands were identified in the proband; one band of normal size (~500 bp) and one smaller band (~300 bp). The PCR products were purified and sequenced. The size of one band was revealed to be 429 bp, and the other, 296 bp. Compared with cDNA of *EXT2*, sequence analysis confirmed exon 5 skipping in the aberrant allele, resulting in an in-frame deletion of the *EXT2* protein.

Discussion

The MO family investigated in the present study exhibited a ratio of male and female patients at 5.5:1, which differs from the ratio of 1.5:1 reported previously (26). Therefore, the present study performed a detailed physical examination of all healthy females in the 3rd to 5th generation, to rule out misdiagnosis due to a weak phenotype. It was concluded that the difference may be due to a unique family structure.

Linkage analysis has confirmed that HME is genetically heterogeneous, and the genes that have indicated the greatest levels of association are *EXT1*, *EXT2* and *EXT3*. It has been revealed that mutations in *EXT1* or *EXT2* are responsible for the majority of HME cases. The proteins EXT1 and EXT2 form a hetero-oligomeric complex that functions in HSPG biosynthesis. This complex has a substantially greater glycosyltransferase activity than homo-oligomers of EXT1 or

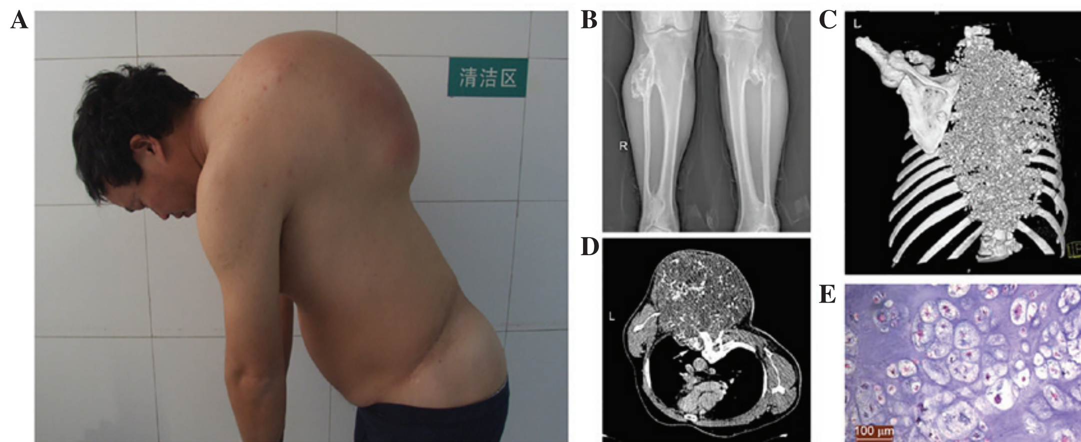

Figure 2. Clinical assessment of family member IV-2. (A) Osteochondroma present on back. (B) X-ray revealed multiple bony protrusions on limbs. (C) CT angiography did not reveal large artery imaging, indicating the potential presence of a chondroma. (D) CT revealed the left back mass had mixed density and multiple flecked calcifications, which broke into the left chest. (E) Histopathological analysis revealed an osteochondroma with focal malignant transformation. Scale bar=100 μm. CT, computed tomography.

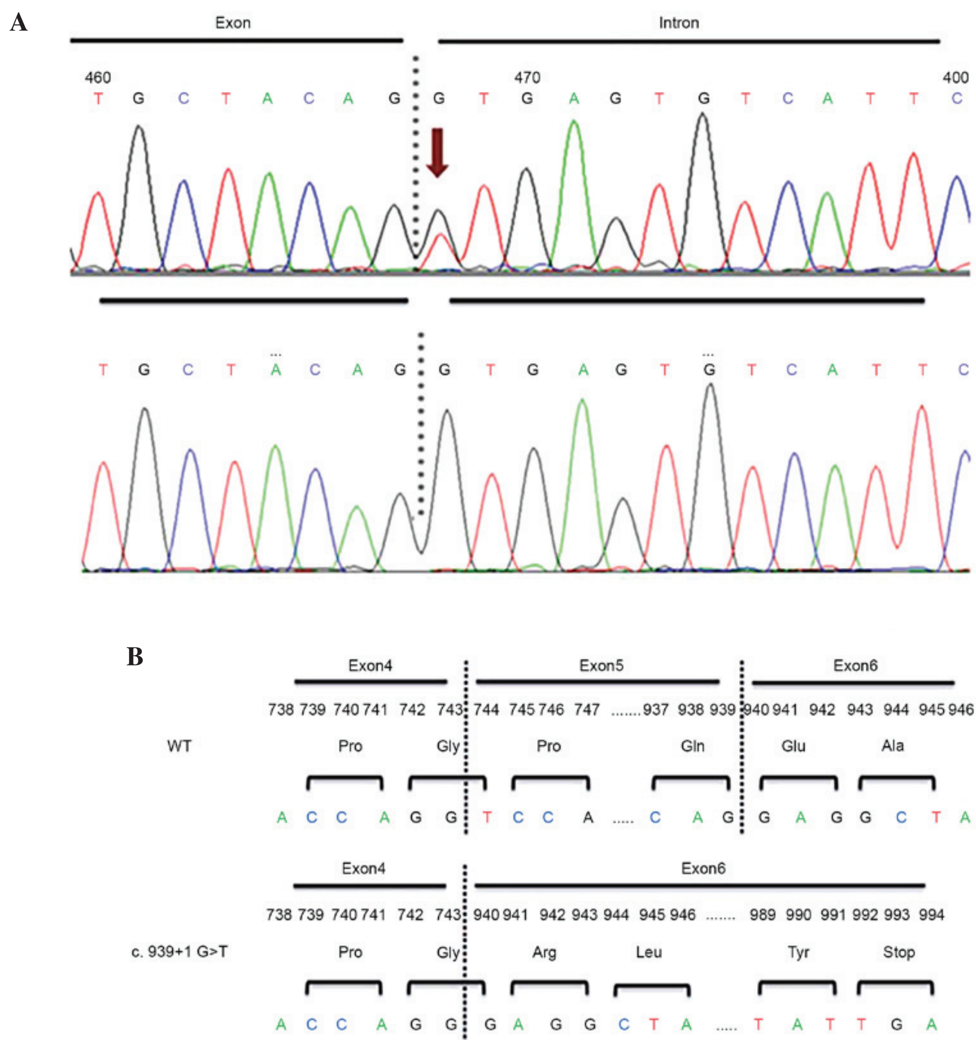

Figure 3. Mutation in exostosin glycosyl transferase-2. (A) DNA sequencing of the polymerase chain reaction product. The position of the mutation in exon 5 is indicated by the red arrow. (B) Resulting alterations to the amino acid sequence in the mutated protein, due to the splicing out of exon 5. WT, wildtype.

EXT2 (27); therefore, a mutation present in *EXT1* or *EXT2* may result in a critical reduction in HSPG (9,28,29). This may subsequently alter the balance of fibroblast growth factor and Indian hedgehog homolog signals (30-32). Thus, the normal signaling pathway involved in bone development may be affected, leading to premature differentiation of cartilage,

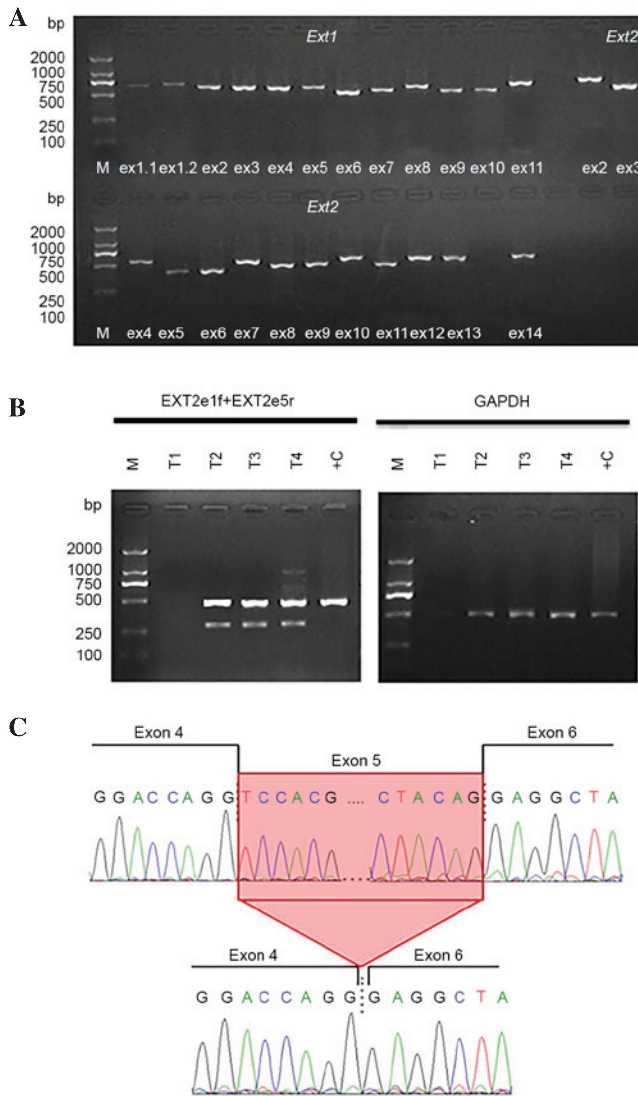

Figure 4. PCR analysis of proband. (A) PCR gel electrophoresis of *EXT1* and *EXT2* genes of the proband. (B) Reverse transcription-PCR of *EXT2* mRNA from the tumor tissue of the proband (T1-T4) and healthy lung tissue from an unrelated individual (+C). There was no band present in the T1 column, indicating no mRNA was present in the first test tube, which may have occurred from RNA degradation due to length of storage. (C) DNA sequencing of the 196 bp fragment revealed the precise excision of exon 5 sequence from the transcript. The genomic DNA sequence of *EXT2* between exons 4 and 6 is presented and aligned for reference. PCR, polymerase chain reaction; *EXT1*, exostosin glycosyl transferase 1; *EXT2*, exostosin glycosyl transferase 2.

cartilage cell proliferation and abnormal bone growth in the adjacent areas (33), resulting in HME.

According to the MO Mutation Database (medgen.ua.ac.be/LOVDv.2.0/home.php), 713 mutations of the *EXT1* gene and 386 mutations of the *EXT2* gene have been identified; however, no mutations in *EXT3* have been reported. Among the 386 *EXT2* mutations, the majority are nonsense mutations, followed by frameshift and substitution mutations. Mutations occur primarily in exons 2-8, seldom occurring downstream. The present study revealed a novel splicing mutation (C.939+1 G>T) leading to deletion of 196 bp in exon 5 of *EXT2*, which may result in a truncated and subsequently pathogenic protein. The *EXT2* gene encodes a protein 718 amino acids in length. This mutation resulted in deletion from codon 744 to 939 of exon 5 of the mRNA, causing a shift in the codon-reading frame, followed by the synthesis of 266 novel amino acids that terminate with a stop codon at position 994. Certain studies have suggested that no mutations exist downstream of exon 8; however, according to the Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac/index.php), 3 patients with mutations in exon 10 and 4 patients with mutations in exon 11 have been identified. Therefore, mutations in the last 6 exons of *EXT2* are very rare. The truncated protein that arises due to the C.939+1 G>T mutation in the family investigated in the present study did not contain the amino acids encoded for by exon 7 to 14. This alteration may cause disease; however, whether it is associated with the rare large osteochondroma that occurred on proband remains to be elucidated. The present study suggested that Knudson's two hit hypothesis or potential mutations in *EXT3* or *EXTL* may explain the occurrence of the osteochondroma.

In conclusion, the present study demonstrated that the C.939+1 G>T (*EXT2*) mutation, present in a five-generation 33-member MO family, resulted in the splicing out of exon 5. These results have extended the mutational spectrum of *EXT2*.

References

1. Schmale GA, Conrad EU III and Raskind WH: The natural history of hereditary multiple exostoses. J Bone Joint Surg Am 76: 986-992, 1994.
2. Legeai-Mallet L, Munnich A, Maroteaux P and Le Merrer M: Incomplete penetrance and expressivity skewing in hereditary multiple exostoses. Clin Genet 52: 12-16, 1997.
3. Saglik Y, Altay M, Unal VS, Basarir K and Yildiz Y: Manifestations and management of osteochondromas: A retrospective analysis of 382 patients. Acta Orthop Belg 72: 748-755, 2006.
4. Porter DE, Lonie L, Fraser M, Dobson-Stone C, Porter JR, Monaco AP and Simpson AH: Severity of disease and risk of malignant change in hereditary multiple exostoses. A genotype-phenotype study. J Bone Joint Surg Br 86: 1041-1046, 2004.
5. Wu YQ, Heutink P, de Vries BB, Sandkuijl LA, van den Ouweland AM, Niermeijer MF, Galjaard H, Reyniers E, Willems PJ and Halley DJ: Assignment of a second locus for multiple exostoses to the pericentromeric region of chromosome 11. Hum Mol Genet 3: 167-171, 1994.
6. Wuyts W, Van Hul W, Wauters J, Nemtsova M, Reyniers E, Van Hul EV, De Boulle K, de Vries BB, Hendrickx J, Herrygers I, *et al*: Positional cloning of a gene involved in hereditary multiple exostoses. Hum Mol Genet 5: 1547-1557, 1996.
7. Esko JD and Selleck SB: Order out of chaos: Assembly of ligand binding sites in heparan sulfate. Annu Rev Biochem 71: 435-471, 2002.
8. Lind T, Tufaro F, McCormick C, Lindahl U and Lidholt K: The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. J Biol Chem 273: 26265-26268, 1998.
9. Hameetman L, David G, Yavas A, White SJ, Taminiau AH, Cleton-Jansen AM, Hogendoorn PC and Bovée JV: Decreased EXT expression and intracellular accumulation of heparan sulphate proteoglycan in osteochondromas and peripheral chondrosarcomas. J Pathol 211: 399-409, 2007.
10. Hall CR, Cole WG, Haynes R and Hecht JT: Reevaluation of a genetic model for the development of exostosis in hereditary multiple exostosis. Am J Med Genet 112: 1-5, 2002.
11. Signori E, Massi E, Matera MG, Poscente M, Gravina C, Falcone G, Rosa MA, Rinaldi M, Wuyts W, Seripa D, *et al*: A combined analytical approach reveals novel EXT1/2 gene mutations in a large cohort of Italian multiple osteochondromas patients. Genes Chromosomes Cancer 46: 470-477, 2007.
12. White SJ, Vink GR, Kriek M, Wuyts W, Schouten J, Bakker B, Breuning MH and den Dunnen JT: Two-color multiplex ligation-dependent probe amplification: Detecting genomic rearrangements in hereditary multiple exostoses. Hum Mutat 24: 86-92, 2004.

13. Pedrini E, De Luca A, Valente EM, Maini V, Capponcelli S, Mordenti M, Mingarelli R, Sangiorgi L and Dallapiccola B: Novel EXT1 and EXT2 mutations identified by DHPLC in Italian patients with multiple osteochondromas. Hum Mutat 26: 280, 2005.
14. Lonie L, Porter DE, Fraser M, Cole T, Wise C, Yates L, Wakeling E, Blair E, Morava E, Monaco AP and Ragoussis J: Determination of the mutation spectrum of the EXT1/EXT2 genes in British Caucasian patients with multiple osteochondromas, and exclusion of six candidate genes in EXT negative cases. Hum Mutat 27: 1160, 2006.
15. Jennes I, Entius MM, Van Hul E, Parra A, Sangiorgi L and Wuyts W: Mutation screening of EXT1 and EXT2 by denaturing high-performance liquid chromatography, direct sequencing analysis, fluorescence in situ hybridization, and a new multiplex ligation-dependent probe amplification probe set in patients with multiple osteochondromas. J Mol Diagn 10: 85-92, 2008.
16. Xu L, Xia J, Jiang H, Zhou J, Li H, Wang D, Pan Q, Long Z, Fan C and Deng HX: Mutation analysis of hereditary multiple exostoses in the Chinese. Hum Genet 105: 45-50, 1999.
17. Wuyts W and Van Hul W: Molecular basis of multiple exostoses: Mutations in the EXT1 and EXT2 genes. Hum Mutat 15: 220-227, 2000.
18. Raskind WH, Conrad EU III, Matsushita M, Wijsman EM, Wells DE, Chapman N, Sandell LJ, Wagner M and Houck J: Evaluation of locus heterogeneity and EXT1 mutations in 34 families with hereditary multiple exostoses. Hum Mutat 11: 231-239, 1998.
19. Le Merrer M, Legeai-Mallet L, Jeannin P, Horsthemke B, Schinzel A, Plauchu H, Toutain A, Achard F, Munnich A and Maroteaux P: A gene for hereditary multiple exostosesmaps to chromosome 19 p. Hum Mol Genet 5: 717-722, 1994.
20. Francannet C, Cohen-Tanugi A, Le Merrer M, Munnich A, Bonaventure J and Legeai-Mallet L: Genotype-phenotype correlation in hereditary multiple exostoses. J Med Genet 7: 430-434, 2001.
21. Wise CA, Clines GA, Massa H, Trask BJ and Lovett M: Identification and localization of the gene for EXTL, a third member of the multiple exostoses gene family. Genome Res 7: 10-16, 1997.
22. Wuyts W, Van Hul W, Hendrickx J, Speleman F, Wauters J, De Boulle K, Van Roy N, Van Agtmael T, Bossuyt P and Willems PJ: Identification and characterization of a novel member of the EXT gene family, EXTL2. Eur J Hum Genet 5: 382-389, 1997.
23. Van Hul W, Wuyts W, Hendrickx J, Speleman F, Wauters J, De Boulle K, Van Roy N, Bossuyt P and Willems PJ: Identification of the third EXT-like gene (EXTL 3) belonging to the EXT gene family. Genomics 47: 230-237, 1998.
24. Miller SA and James RH: Variables associated with ultraviolet transmittance measurements of intraocular lenses. Am J Ophthalmol 106: 256-260, 1988.
25. Wuyts W, Van Hul W, De Boulle K, Hendrickx J, Bakker E, Vanhoenacker F, Mollica F, Lüdecke HJ, Sayli BS, Pazzaglia UE, *et al*: Mutations in the EXT1 and EXT2 genes in hereditary multiple exostoses. Am J Hum Genet 62: 346-354, 1998.
26. Wicklund CL, Pauli RM, Johnston D and Hecht JT: Natural history study of hereditary multiple exostoses. Am J Med Genet 55: 43-46, 1995.
27. Kitagawa H, Shimakawa H and Sugahara K: The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan-protein linkage region. The key enzyme for the chain initiation of heparan sulfate. J Biol Chem 274: 13933-13937, 1999.
28. Romeo S, Oosting J, Rozeman LB, Hameetman L, Taminiau AH, Cleton-Jansen AM, Bovée JV and Hogendoorn PC: The role of noncartilage-specific molecules in differentiation of cartilaginous tumors: Lessons from chondroblastoma and chondromyxoid fibroma. Cancer 110: 385-394, 2007.
29. Bornemann DJ, Duncan JE, Staatz W, Selleck S and Warrior R: Abrogation of heparan sulfate synthesis in drosophila disrupts the wingless, hedgehog and decapentaplegic signaling pathways. Development 131: 1927-1938, 2004.
30. Bellaiche Y, The I and Perrimon N: Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. Nature 394: 85-88, 1998.
31. Lai LP and Mitchell J: Indian hedgehog: Its roles and regulation in endochondral bone development. J Cell Biochem 96: 1163-1173, 2005.
32. Duncan G, McCormick C and Tufaro F: The link between heparan sulfate and hereditary bone disease: Finding a function for the EXT family of putative tumor suppressor proteins. J Clin Invest 108: 511-516, 2001.
33. Wuyts W, Radersma R, Storm K and Vits L: An optimized DHPLC protocol for molecular testing of the EXT1 and EXT2 genes in hereditary multiple osteochondromas. Clin Genet 68: 542-547, 2005.