J Hum Genet (1999) 44:230–234

ORIGINAL ARTICLE

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Germline mutations in the *EXT1* and *EXT2* genes in Korean patients with hereditary multiple exostoses

Received: December 4, 1998 / Accepted: March 26, 1999

Abstract Hereditary multiple exostoses (EXT) is an autosomal dominantly inherited disease characterized by the formation of cartilage-capped prominences (exostoses) that develop from the juxtaepiphyseal regions of the long bones. Recently, *EXT1* and *EXT2* genes were cloned and germline mutations of *EXT1* and *EXT2* were identified in EXT families. In this study, we performed a mutational analysis of *EXT1* and *EXT2* genes in eight unrelated Korean EXT families by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis followed by direct DNA sequencing. As a result, we were able to identify one family (SNU-OC3) with the *EXT1* mutation and another family (SNU-OC15) with the *EXT2* mutation. The *EXT1* mutation was a 10-bp deletion at the 3′ end of exon 5 (CTAATTTAGg) including the splice site of this exon. The *EXT2* mutation identified in the SNU-OC15 family was a missense mutation at codon 85 of exon 2 (TGC → CGC), resulting in an amino acid change from cysteine to arginine. This missense mutation cosegregated with the disease phenotype in this family, suggesting that it is the disease-causing mutation. These two mutations identified in *EXT1* and *EXT2* are novel ones.

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Key words *EXT1* · *EXT2* · Germline mutation

Introduction

Hereditary multiple exostoses (EXT) is an autosomal dominantly inherited disease characterized by the development of cartilage-capped exostoses (osteochondromas) from the juxtaepiphyseal regions of the long bones. Exostoses may be apparent at birth, and they are usually evident by the end of the first decade of life in 80% of individuals (Solomon, 1963). Approximately two-thirds of the affected individuals with EXT have orthopedic complications (Wicklund et al. 1995). But the most serious complication of EXT is malignant degeneration of benign exostoses to chondrosarcoma or to some other sarcomas (Schmale et al. 1994).

EXT is a genetically heterogeneous disease. Linkage analysis has demonstrated that EXT is caused by at least three different genes, *EXT1* on chromosome 8q23–q24 (Cooke et al. 1993), *EXT2* on chromosome 11p11–p12 (Wu et al. 1994; Wuyts et al. 1995), and *EXT3* on chromosome 19p (Le Merrer et al. 1994). However, it now appears that most EXT families are linked to *EXT1* and *EXT2* loci (Halloran-Blanton et al. 1996; Raskind et al. 1998). Recently, the *EXT1* and *EXT2* genes have been cloned and germline mutations of these genes confirmed in EXT families (Ahn et al. 1995; Stickens et al. 1996; Wuyts et al. 1996). *EXT1* and *EXT2* show homology to each other and define a new family of related genes with putative tumor suppressor activity, as demonstrated by loss of heterozygosity in both the *EXT1* region on chromosome 8 and the *EXT2* region on chromosome 11 in chondrosarcomas (Raskind et al. 1995; Hecht et al. 1997). Recent lines of evidence suggest that *EXT1* and *EXT2* are glycosyltransferases required for the biosynthesis of heparan sulfate (McCormick et al. 1998; Lind et al. 1998).

In addition to the *EXT1* and *EXT2* genes, three other homologous genes belonging to the EXT gene family, *EXTL1* (Wise et al. 1997), *EXTL2* (Wuyts et al. 1997), and *EXTL3* (Van Hul et al. 1998) have been cloned through

homology searches with expressed sequence tags (ESTs). So far, germline mutation in *EXTL* genes has not been identified in EXT patients.

Since the cloning of the *EXT1* and *EXT2* genes, there have been a few reports describing the germline mutation screening of these genes in EXT families (Ahn et al. 1995; Hecht et al. 1997; Philippe et al. 1997; Raskind et al. 1998; Stickens et al. 1996; Wells et al. 1997; Wuyts et al. 1996; Wuyts et al. 1998).

In this article, we report the mutation screening of *EXT1* and *EXT2* in eight unrelated Korean EXT families by polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) analysis followed by direct DNA sequencing.

Subjects, materials, and methods

DNA isolation from blood samples

Blood samples and clinical information on eight unrelated Korean EXT families were obtained from the Korean Hereditary Tumor Registry, at the Seoul National University College of Medicine. All families had two or more members affected with EXT. Informed consent was obtained from all patients prior to blood collection. Genomic DNA was prepared from peripheral blood, as described previously (Blin and Stafford 1976).

Mutation screening of *EXT1* and *EXT2*

PCR and SSCP analysis was performed for all coding sequences of the *EXT1* and *EXT2* genes. The primer sequences used for PCR of *EXT1* and *EXT2* have been described previously (Philippe et al. 1997; Wells et al. 1997). DNA samples were PCR amplified prior to SSCP analysis under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C–58°C for 30 s (depending on the annealing temperature for each primer pair), and 72°C for 30 s, and a final elongation step at 72°C for 5 min. Reaction mixtures in a total volume of 25 μl contained the following: 50 ng of DNA template, 0.4 M each primer, 120 M each dNTP, PCR buffer (10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) (Perkin Elmer Cetus, Foster City, CA, USA) 0.1 μCi/μl ^{32}P dCTP (3000 Ci/mmol) (Amersham, Arlington Heights, IL, USA), and Taq DNA polymerase 0.01 U/μl (Perkin Elmer Cetus). After amplification, 15 μl of the reaction mixture was diluted with 15 μl of formamide dye (deionized formamide 95%, 10 mM ethylene diamine tetraacetic acid [EDTA], 0.05% xylene cyanol, and 0.05% bromphenolblue). Samples were denatured at 95°C for 4 min and placed on ice prior to loading on SSCP gel. Four μl of each reaction mixture was loaded into a 6% (48:1 acrylamide:bis) non-denaturing polyacrylamide gel. Electrophoresis was performed at 400 V constant voltage at 4°C for 13–16 h. Gels were dried and exposed to Fuji RX film (Fuji Photo Film, Tokyo, Japan) for 12–24 h.

DNA sequencing

When abnormal band patterns were detected by PCR-SSCP analysis, the PCR products were purified with a QIAquick PCR purification kit (Quiagen, Chatsworth, CA, USA) and sequenced directly with a Taq dideoxy terminator cyclic kit on an ABI 377 automatic DNA sequencer (Perkin-Elmer).

For mutation analysis in the *EXT2* gene by direct sequencing, exons 2 and 4 of *EXT2* were amplified, and the resulting PCR products were ligated into the PCRTM-II vector and subcloned, using the TA cloning system (InVitrogen, San Diego, CA, USA). Several clones were sequenced directly with a Taq dideoxy terminator cyclic kit on an ABI 377 automatic DNA sequencer (Perkin-Elmer).

The sequences were analyzed visually by comparison of the mutated sequence with a sequence from a normal control.

Results and discussion

EXT1 is composed of 11 exons and *EXT2* is composed of 16 exons and the flanking intronic sequences. In this study, we searched for germline mutations of the entire coding sequences of *EXT1* and *EXT2*, excluding exons 1a and 1b of *EXT2* encoding the 5′-untranslated region (UTR).

In one EXT family (SNU-OC3) with EXT-affected members through four generations (Fig. 1a), abnormal bands were detected after SSCP analysis of exon 5 of the *EXT1* gene (Fig. 1b). The proband in this family was a 15-year-old male with multiple exostoses involving the hip, knee, ankle, foot, elbow, scapula, shoulder, wrist, and hand. His father also had exostoses involving both knees. Sequencing of the exon 5 PCR product derived from the proband revealed a 10-base pair deletion at the 3′ end of exon 5 (CTAATTTAGg), including the donor splice site of intron 5 (Fig. 1c). The same mutation was also identified in the proband's father, confirming that this mutation cosegregated with the EXT phenotype in this family. We did not perform a detailed analysis of the consequences of this mutation. But since the mutation involves the conserved GT splicing junction at the 5′ end of intron 5, the mutation would result in improper splicing of this exon. The consequences of this mutation could possibly be intron 5 retention or exon 5 skipping, joining exon 4 to exon 6, or use of the cryptic 5′ splice site. In either case, the most likely result would be premature translation termination, producing a truncated protein.

In the *EXT2* gene, PCR-SSCP analysis did not reveal any abnormal band. This could be due to the limited sensitivity of SSCP, considering the fact that the sizes of the PCR products of *EXT2* varied from 253 bp to 442 bp. So, we directly sequenced exons 2 and 4 of *EXT2*, because *EXT2* mutations are more fequent in these exons (Wuyts et al. 1998). As a result, we were able to identify a missense mutation in the SNU-OC15 family (Fig. 2a,b). In this family, the proband was a 5-year-old boy with exostoses on both

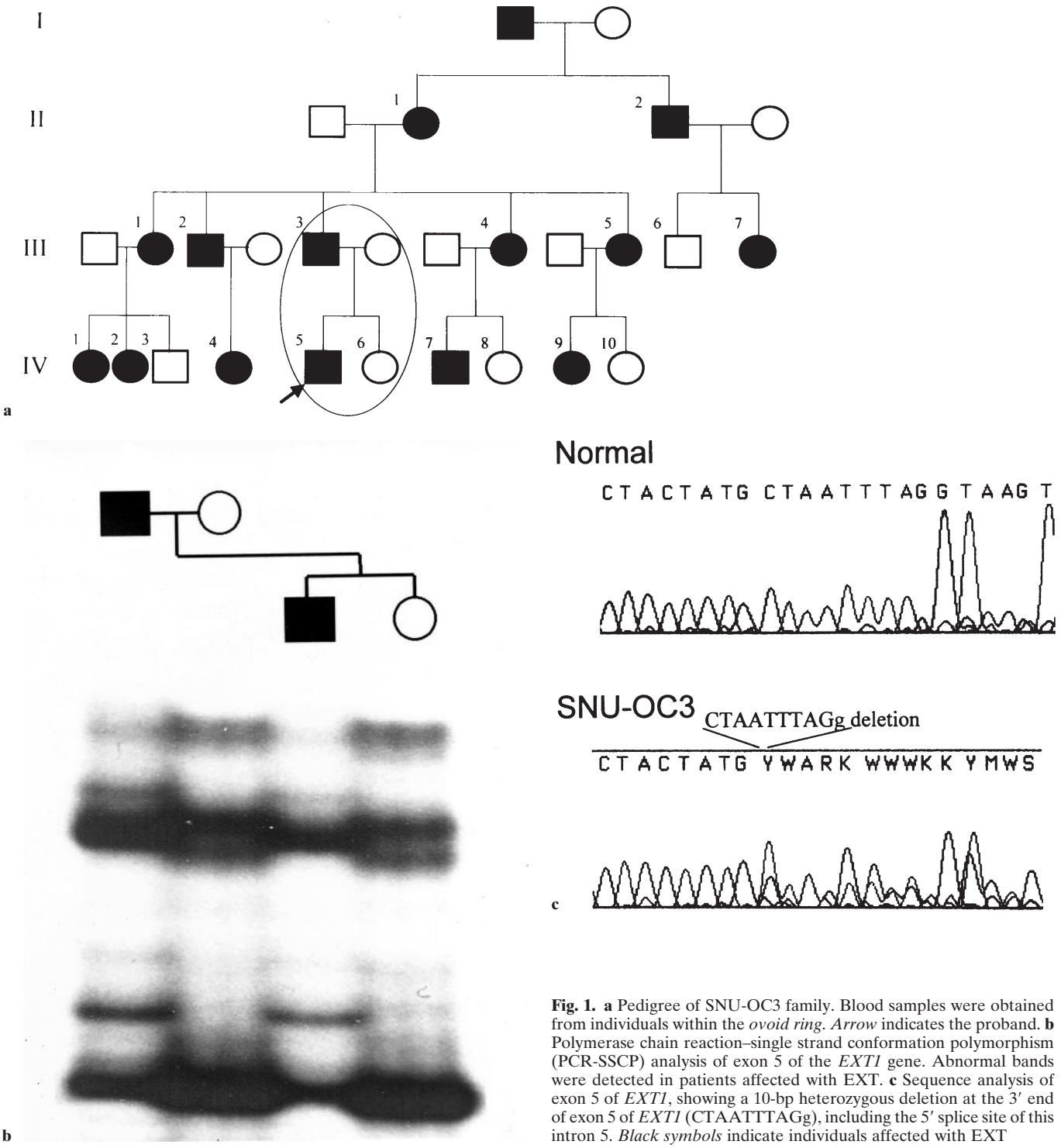
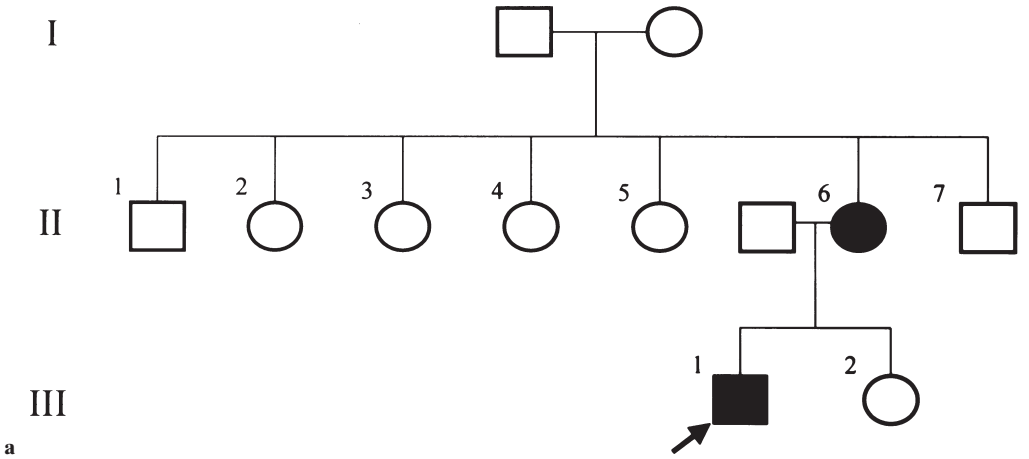
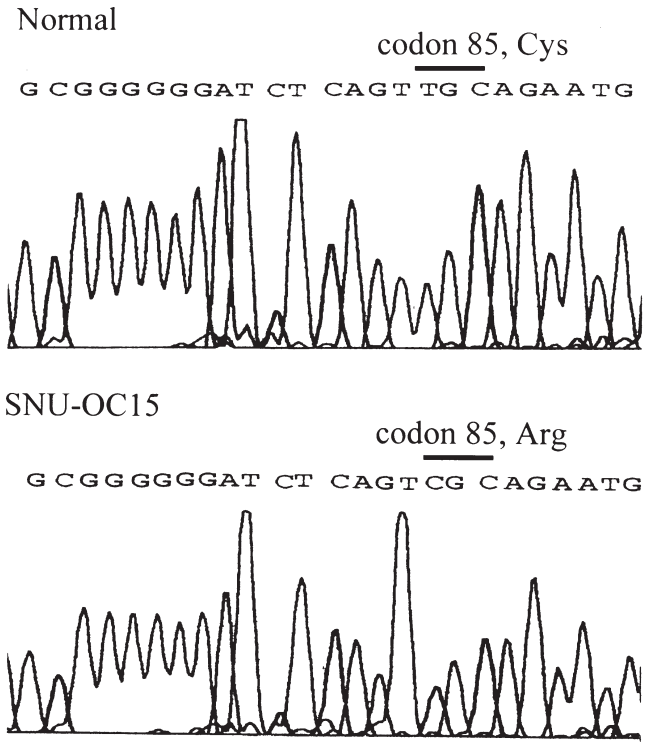


Fig. 1. **a** Pedigree of SNU-OC3 family. Blood samples were obtained from individuals within the *ovoid ring*. *Arrow* indicates the proband. **b** Polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) analysis of exon 5 of the *EXT1* gene. Abnormal bands were detected in patients affected with EXT. **c** Sequence analysis of exon 5 of *EXT1*, showing a 10-bp heterozygous deletion at the 3′ end of exon 5 of *EXT1* (CTAATTTAGg), including the 5′ splice site of this intron 5. *Black symbols* indicate individuals affected with EXT

legs (femur, tibia), both arms (radius, ulna, humerus), and both feet (proximal phalanges). His mother also had exostoses in both knees. Sequence analysis showed that the proband had a germline missense mutation (TGC → CGC) at codon 85 of the *EXT2* gene, leading to a cysteine-to-arginine substitution. The same missense mutation was identified in the proband's mother. To confirm that the identified mutation was the disease-causing one, we sequenced exon 2 of *EXT2* from six normal (without exostoses phenotype) family members, including the younger sister of the proband and three maternal aunts and two uncles. In the rest of the family members, the mutation



a



b

Fig. 2. **a** Pedigree of SNU-OC 15 family. There were only two individuals affected with EXT in this family. *Arrow* indicates the proband. **b** Sequence analysis of exon 2 of *EXT2* revealed a missense mutation at codon 85 (TGC → CGC), resulting in a cysteine-to-arginine substitution. This missense mutation cosegregated with the disease phenotype in this family, suggesting that this mutation is the cause of EXT

identified in the proband and his mother could not be identified. Thus, the missense mutation identified cosegregated with the exostoses phenotype, suggesting that the mutation is the disease-causing one. Direct sequencing of 100 control chromosomes from unrelated and unaffected normal individuals did not reveal this missense mutation, thus excluding the possibility of a rare polymorphism,

Analysis of EXT pedigrees has revealed that most are associated with *EXT1* and *EXT2* (Halloran-Blanton et al. 1996; Raskind et al. 1998), with *EXT3* probably playing only a minor role. Our results provide further support for *EXT1* and *EXT2* genes being involved in EXT.

There have been 35 different *EXT1* mutations described in 43 families, and 12 different *EXT2* mutations described in 16 families (Wuyts et al. 1998; Raskind et al. 1998). All of these mutations have been found in families from Western countries. Seventy-seven percent of the mutations identified in the *EXT1* gene are nonsense mutations, splice-site mutations, or frameshift mutations, which cause premature termination of the EXT1 protein (Wuyts et al. 1998). The consequence of the mutation identified in our SNU-OC15 family (10-base pair deletion at the 3′ end of exon 5, including the splice donor site of intron 5) is unknown. But since the deletion involves the conserved GT splicing junction at the 5′ end of intron 5, it is likely that the mutation would result in improper splicing of this exon with production of a truncated *EXT1* protein. However, there is also a possibility that the mutation may result in a frameshift.

Of the 12 *EXT2* mutations reported thus far (Wuyts et al. 1998), 11 are nonsense mutations, splice-site mutations, or frameshift mutations, which cause premature termination of EXT2 protein (Wuyts et al. 1998). There has only been one missense mutation, described in two families at exon 4 of the *EXT2* gene (Philippe et al. 1997). The

missense mutation identified in our family (SNU-OC15) was at codon 85 of exon 2 of the *EXT2* gene, with a resultant amino acid change from cysteine to arginine. Since this mutation cosegregated with the EXT phenotype in this family and since the same mutation was not identified in 100 chromosomes from an unrelated unaffected normal population, there is a high likelihood that this mutation is the disease-causing one. If this is the case, codon 85 of *EXT2* may be an important domain for the proper function of the EXT2 protein.

The mutations identified in the *EXT1* and *EXT2* genes from our EXT families are novel ones, and extend the germline mutation spectrum identified in EXT.

Acknowledgments This work was supported by grants from the 1995–1997 Good Health R and D Project, the Ministry of Health and Welfare of the Republic of Korea, and from the Korea Science and Engineering Foundation (KOSEF) through the Cancer Research Center at the Seoul National University (KOSEF-CRC-97-8).

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