行政院國家科學委員會補助專題研究計畫成果報告

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與 EXT1、EXT2 相關聯的遺傳性多發外生骨贅的分子醫學研究

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計畫主持人：蔡長海

共同主持人：蔡輔仁、鄔哲源

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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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行政院國家科學委員會專題研究計畫成果報告

Mutation Screening of the EXT Genes in Patients with Hereditary Multiple Exostoses in Taiwan

計畫編號：NSC 90-2314-B-039-001
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一、中文摘要

遺傳性多發外生骨贅症(hereditary multiple exostoses, HME)是一種常見的骨骼發育障礙疾病，屬常染色體顯性遺傳疾病，其主要症狀為長骨末端不正常增生。多發外生骨贅症致病基因具遺傳雜異性(heterogeneity)，目前在基因圖譜中已確認出三個相關的基因:包括第一型(EXT1)，致病基因突變位置位於染色體 8q23-q24;第二型(EXT2)，致病基因突變位置位於染色體 11p11-p13;以及第三型(EXT3)，致病基因突變位置在染色體 19p。其中 EXT1 和 EXT2 基因，近年來已被選殖出來，而且兩者的基因結構具有高度相似性(homologous)。然而針對台灣人的族群無相關研究發表。 在本研究中，我們分析五個台灣籍的多發外生骨贅症病人，其中一例為偶發性的由 EXT1 基因的突變造成，其餘四個個案皆具家族病史。由基因連鎖分析結果顯示，一個家族遺傳病例屬於 EXT1 基因的突變，三個家族遺傳病例屬於 EXT2 基因的突變。由基因突變分析結果，我們發現四個全新的突變點。其中兩個位於 EXT1 基因一為架構轉移突變(frameshift mutation)(K218fsX247) 與無意突變(nonsense mutation)(Y468X)，在 EXT2 基因座上也發現兩個突變點一為誤意突變(missense mutations)(R223P) 與無意突變(nonsense mutation)(Y396X)，同時經由本研究結果發現，針對家族遺傳性疾病，基因連鎖分析可以作為一快速有效之基因診斷方式。

關鍵詞：遺傳性多發外生骨贅症、基因連鎖分析

Abstract

Hereditary multiple exostoses (HME) is an autosomal dominant disorder characterized by growth of being bone tumors. This genetically heterozygous disease comprised three chromosomal loci: *EXT1* gene on chromosome 8q23-q24, *EXT2* on 11p11-p13, and *EXT3* on 19p. Both *EXT1* and *EXT2* genes had been cloned and defined as a new family of potential tumor suppressor genes in previous work. However, no studies have been conducted in the Taiwanese population. To determine if previous results can also be applied to the Taiwanese, we analyzed five Taiwanese probands with clinical features of HME: one of them is a sporadic case, and the others are familial cases. Linkage studies were performed in the familial cases before the mutation analysis to determine to which of the three *EXT* chromosomes these cases could be assigned. Our results showed that one proband is linked to the *EXT1* locus and three are linked to the *EXT2* locus; the sporadic case was subsequently found to involve *EXT1*. We then identified four new mutations which have not been found in other races: two in *EXT1*- frameshift (K218fsX247) and nonsense (Y468X) mutations; and two in *EXT2*- missense (R223P) and nonsense (Y394X) mutations. Our results indicate that in familial cases, linkage analysis can prove useful for

pre-implantation genetic diagnosis.

Keywords: hereditary multiple exostoses, HME, Gene, Linkage studies

二、INTRODUCTION

Hereditary multiple exostoses (HME; MIM 133700), the most frequently found skeletal dysplasia, is an autosomal dominant disorder. It is characterized by the presence of multiple exostoses / osteochondromas localized mainly in the juxta-epiphyseal region of long bones (Solomon, 1963).

Three genetic loci for HME have been identified to date: *EXT1* (MIM 133700) on chromosome 8q23-q24 (Cook *et al*., 1993), *EXT2* (MIM 133701) on 11p11-p13 (Wu *et al*., 1994), and *EXT3* (MIM 600209) on 19p (Le Merrer *et al.*, 1994). The *EXT1* gene is composed of 11 exons and codes for a 2238 bp cDNA that specifies a protein of 746 amino acids (Ahn *et al.*, 1995). *EXT2* is composed of 14 exons that encode protein of 718 amino acids (Stickens *et al*., 1996; Wuyts *et al*., 1996). The *EX1* and *EXT2* genes are ubiquitously expressed and show homology to each other, especially at the carboxyl terminus (Stickens *et al*., 1996; Wuyts *et al*., 1996). Between 50%-70% of the HME cases are linked to the *EXT1* and *EXT2* loci (Cook *et al.*, 1993; Blanton *et al*., 1996; Legeai-Mallet *et al*., 1997). *EXT3* appears to be a minor locus, which is responsible for some 28% of cases (Le Merrer *et al*., 1994; Legeal-Mallet *et al*., 1997). Through mutation detection studies, loss of function of either *EXT1* or *EXT2* appears to be sufficient for tumor formation; it has been suggested that these genes are tumor suppressors (Hecht *et al*., 1995; Bovee *et al.*, 1999).

In this study, we performed a systematic mutation analysis of *EXT* genes, including linkage analysis, single-strand conformation polymorphism (SSCP) and DNA sequencing, to determine the *EXT* chromosomal location and specific mutations responsible for HME cases in Taiwan.

三、Materials and methods

In this study, multigenerational families with hereditary multiple exostoses were obtained from China Medical College Hospital, Taichung, Taiwan. Family members were evaluated by clinical diagnosis or by review of medical records to document the presence or absence of exostoses. Genomic DNA was prepared from peripheral blood lymphocytes by DNA extractor Genomaker DNA extraction kit (Blossom, Taiwan).

Genetic markers and linkage analysis

The microsatellite markers, (D8S556, D8S284, D8S514, D8S272) on chromosome 8q, (D11S904, D11S935, D11S905, D11S1313) on 11p, and (D19S209, D19S216, D19S221, D19S226) on 19p were designed for human genome linkage analysis and were purchased from the ABI Prism Linkage Mapping Set. The fluorescent labeled PCR products were analyzed on the automated DNA sequencer (ABI Prism 377) and Genescan 672 software (PE Applied Biosystems, USA).

Mutation analysis

Primer pairs for exon 1to 12 of *EXT1* and for exon 2 to 14 of *EXT2* were designed as previously described (Plilippe *et al.*, 1997; Wuyts *et al.*, 1998). The PCR products of these exons were subjected to mutation analysis by single strand conformation polymorphism (SSCP), using a GenePhor DNA Electrophoresis System (Pharmacia, Sweden). Exons that exhibited an irregular shift by SSCP were subjected to direct sequencing for mutation identification. Direct sequencing was performed using a Taq DyeDeoxy Terminator sequencing kit (PE Applied Biosystems, USA) with an ABI Prism 377 DNA sequencer. Mutations were confirmed by means of restriction analysis. If necessary, modified primers were designed to create restriction site. The modified primers used in this study were: EXT2-e8M1 (+): 5’-GCATTATTTTTTTATAGGCCCGCT-3’, reverse primer EXT2-e8.2 (-): 5’-ACTGGAATTCACTTACCACA-3’; EXT2-e6M2 (+): 5’-TACTTTCTGTGTGGTTCTACG-3’. The modified nucleotides in modified primers are underlined. Restriction digestions were

performed on PCR amplifications and the fragments were separated on 3% agarose gels.

四、RESULTS

We have examined five probands with the HME phenotype and their relatives. The results of linkage analysis showed that one family is an isolated case, another is linked to the *EXT1* locus (Figure 1), and the others are linked to the *EXT2* locus (Figure 2). The four different mutations identified in these families are summarized in Table 1. The missense mutation (R223P) in *EXT2* (Family 1) and the frameshift mutation (K218fsX247) in *EXT1* (Family2), had been reported before (Shi *et al*., 2000; Shi *et al*., 2001). However, the nonsense mutations (Y394X in *EXT2*-linked Families 3, 4 and Y468X in *EXT1*-linked Family 5) had not yet been reported.

The fist new mutation was found in three affected members of Family 3 and two affected members of Family 4. It originated from a substitution of G for A at position 1181 in exon 8 of the *EXT2* gene (Figure 2B, C). The result was confirmed by *Bfa I* restriction digestion of a 166-bp PCR-amplification product with primers EXT2-e8M1 (+) and EXT2-e8.2 (-). The modified primer EXT2-e8M1 creates a *Bfa I* restriction site (CTAG) that results in digestion to produce 25 bp and 141 bp bands in the mutant allele; whereas the wild type is not digested (results not shown).

The second nonsense mutation is a C to G transversion at position 1404 in exon 5 of the *EXT1* gene, which was found in the isolated case, Family 5 (Figure 1B). This mutation results in a premature stop codon at amino acid 468 and it also introduces a *Bfa I* restriction site. The normal band is predicted to have a length of 237 bp and would be expected to be digested to produce bands of 175 bp and 62 bp, in the presence of the C1404G mutation (results not shown).

Through sequencing analysis, we also found two polymorphisms in *EXT2* (Table 2). The first is a T to G substitution at position 966 (exon6) and the other is an A-to-C at 79 bp upstream of intron 4 (IVS4 (-79) A>C) (Figure 3A, B). Although the T to G transition at nucleotide position 966 in *EXT2* would not be expected to cause any protein change (R322R), we designed a primer-EXT2-e6M2 to generate a recognition site for *HpyCH4 IV* restriction digestion in order to understand the distribution of this polymorphism across the Taiwanese population. The 205- bp amplification fragment is digested into fragments of 185 bp and 20 bp in the presence of the “T” allele (results not shown). Fifty normal individuals, including 25 healthy male and 25 healthy female, were tested. The frequencies of the alleles (T and G at position 966) were 0.90 and 0.10, respectively. The frequencies of the T/T and T/G genotypes were 0.79 and 0.21, respectively.

The second polymorphism (IVS4 (-79) A>C) was detected in five out of nine affected individuals of the three *EXT2*-linked families. The frequency of the A allele was 0.72 and of the C allele, 0.28, in these three families. Since this ploymorphism is not in the coding region, we did not investigate the allelic frequencies in the healthy population.
Through direct sequencing, we also found that six nucleotides of the *EXT2* gene were different from the sequences obtained through GeneBank (accession numbers: U67357, U67359, U67362) (Table 2). All nine affected ones of the three *EXT2*-linked families were homozygous at the indicated positions: C/C at IVS4 (-8), T/T at IVS4 (-22), T/T at IVS5 (+14), T/T at IVS6 (-52), C/C at IVS9 (-8), and A/A at IVS10 (+75) (Table 2, Figure3 C~H). The distribution of above findings is outlined in Figure 4.

五、DISCUSSIONS

Hereditary multiple exostoses (HME) is genetically heterogeneous. From previous studies, it may either be clearly familial or occur sporadically. Our linkage and mutation analyses also showed similar results: we report on one sporadic case, on one *EXT1*-linked family, and on three *EXT2*-linked families. This finding also provides additional support for the major roles of *EXT1* and *EXT2* in HME. However, the most important discovery in our study is the identification of four new mutations in *EXT1* and *EXT2*: a frameshift (K218fsX247)

and a nonsense (Y468X) in *EXT1*; a missense (R223P) and a nonsense (Y394X) in *EXT2*.

For *EXT1*, we found an insertion-deletion (651-665delins TT) in exon 1 resulting in a frameshift (K218fsX247) at position 218 and a translational stop 30 codons downstream in the *EXT1* protein. According to the functional study by Cheung (2001), 323 amino acid residues of the human *EXT1* protein have been evolutionarily conserved among both vertebrate and invertebrate species. These conserved amino acids are essential for *EXT1* to function in the polymerization of heparan sulfate. Therefore, we expect that the observed frameshift, which would be expected to result in the loss of 255 amino acids from this conserved region of the *EXT1* protein, would result in a drastically truncated protein with probable complete loss of function.

We also found a C-to-G transition at position 468 of *EXT1*. It changes a TAC codon to TAG and produces a premature stop codon. This mutation would result in the loss of 278 amino acids from the C-terminus of the *EXT1* protein. Since the conserved carboxy-terminal region of the *EXT1* protein contains a putatively catalytic domain for the synthesis of heparan sulfate (McCormick *et al*., 2000), the truncated *EXT1* protein is probably inactive or degraded rapidly, resulting in a nearly complete loss of function. We therefore suspect that the premature stop codon is very likely to be the disease-causing mutation.

In a previous study, mutations found in *EXT2* were variable and randomly distributed over the first two-thirds of the coding region, without any real mutation hotspots (Wuyts and Van Hul, 2000). However, several EXT2 mutations, including missense, nonsense and frameshift mutations, had been detected in exon 4. They are located at position 211 (FS L211), 218 (FS S218), 222 (Y222X) and 227 (D227N) (Philippe *et al*., 1997; Wuyts *et al*., 1998; Xu *et al*., 1999). In our study, we also found an adjacent aa substitution at position 223 (Arg to Pro) in exon 4. This missense mutation did not seem to cause obviously milder phenotype as compared with mutations leading to premature termination of translation. Therefore, we suspect that amino acid 211 to 230 might be mutation hotspots for *EXT2* and the missense (R223P) mutation might be in a crucial domain of *EXT2*, which must remain intact for proper function of the *EXT2* protein.

Another newly identified nonsense mutation, Y394X, in exon 8 of *EXT2*, was found in two different families (Families 3 and 4). The premature stop codon appearing at Y394, the middle of the *EXT2* protein, leads to a truncated protein missing 324 amino acids from the C-terminus. As observed for *EXT1*, the protein will be severely truncated when it looses C-terminal end, which is associated with glycosyltransferase activities that catalyze the polymerization of heparan sulfate (Philippe *et al*., 1997; McCormick *et al*., 2000; Francannet *et al*., 2001). Therefore, we suspect this mutation might be the genetic defect responsible for the development of exostoses in these two families.

Besides the above four new mutations, we also found two polymorphisms in *EXT2* by direct sequencing; we also noted six nucleotides that are different from the *EXT2* sequences obtained from the white population reported in GeneBank. The first polymorphism was an A-to-G substitution at upstream of intron 4 (IVS4 (-79)). The second one was a c966T-to-G detected in three out of nine affected members: the frequencies of these alleles (T and G at position 966) were 0.833 and 0.167, respectively. However, results obtained from healthy (non-HME) individuals were 0.9 and 0.1 respectively. In other words, the frequency of G allele was higher in HME vs. non-HME individuals in this study. This result suggests that c966T-to-G might be utilized as a single nucleotide ploymorphic marker for *EXT2* and the marker may be associated with the deficiency of the *EXT2* protein. Since there are only three *EXT2* families in this study, larger scale mutation screening should be necessary to prove our inference.

The six nucleotides found in *EXT2* were different from the data obtained through GeneBank. All of the twelve members of our *EXT2*-linked families, were homozygous at

these six positions. Hence, we presumed that the sequences in Taiwanese Chinese are different from those in the white population (the subjects included in the GeneBank database).

Although combining results of mutation detection and linkage analysis can exactly determine the disease-causing mutations/alleles in inherited cases, the whole procedure is costly and time-consuming. On the other hand, the linkage analysis used in our study can provide a reliable clue for prenatal test without mutation detection in such families. The linkage method is relatively low-cost and efficient. The microsatellite markers we chose were in the region of chromosomal 8q22.3 to 8q24.3 for *EXT1*, 11p11.1 to 11p14.3 for *EXT2*, and 19p13.3 to19p13.2 for *EXT3*.

The validity of the linkage method was tested in Family 3 (Figure 2): the baby (III-1) was predicted not to have inherited the disease-causing allele from his father (II-3) and this was confirmed by a subsequent direct sequencing analysis demonstrating the absence of the familial mutation. In the future, in families in which the chromosomal location of the disease-causing mutation/allele is known, the less costly linkage approach can be used to predict disease status in a pre-implantation genetic diagnosis (amniocentesis or chorionic villus sampling).

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Table 1. Mutations identified in the EXT1 and EXT2 genes.

Family	Location of mutation	cDNA sequence change	Protein change	Type of mutation
Familial cases				
1	EXT2/ exon4	c668G>C	R223P*	Missense
2	EXT1/ exon1	651-665 delins TT	K218fsX247*	Frameshift
3	EXT2/ exon8	c1181G>A	Y394X	Nonsense
4	EXT2/ exon8	c1181G>A	Y394X	Nonsense
Isolated case				
5	EXT1/ exon5	c1404C>G	Y468X	Nonsense

*reference: (R223P) Shi *et al*., 2000.

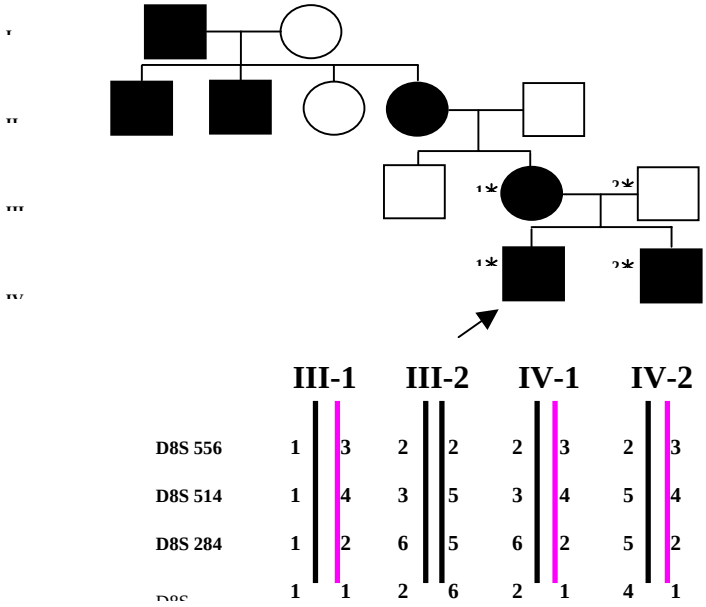
(K218fsX247) Shi *et al.*, 2001.

Table 2. Two plymorphisms and six nucleotide changes found in the EXT2 gene.

EXT2 gene	Location	DNA sequence change	Protein change	Accession ID	Position
Polymorphism	exon6	c966T>G	R322R		
	IVS4 (–79)	A>C		GDB: U67357	328
Nucleotide change	IVS4 (-8)	T>C		GDB: U67357	399
	IVS4 (-22)	C>T		GDB: U67357	385
	IVS5 (+14)	G>T		GDB: U67357	510
	IVS6 (-52)	G>T		GDB: U67359	72
	IVS9 (-8)	T>C		GDB: U67362	384
	IVS10 (+75)	C>A		GDB: U67362	598

Figure 1

(A) Family 2



(B) Family 5

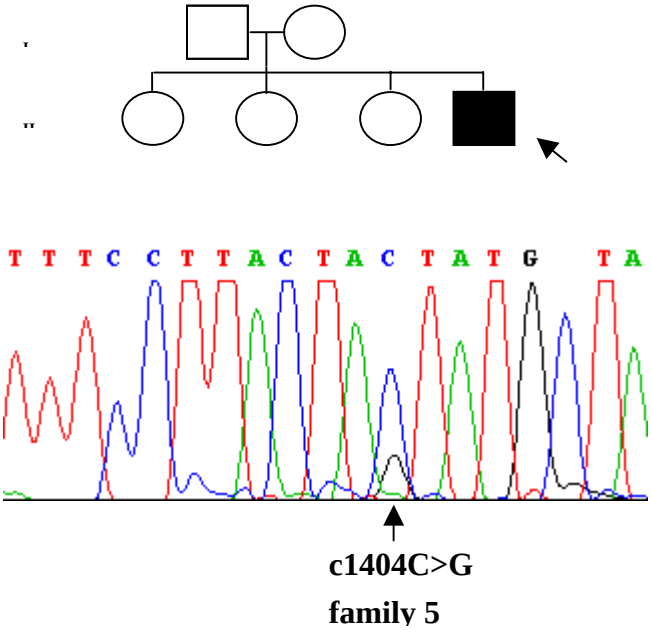


Figure 2

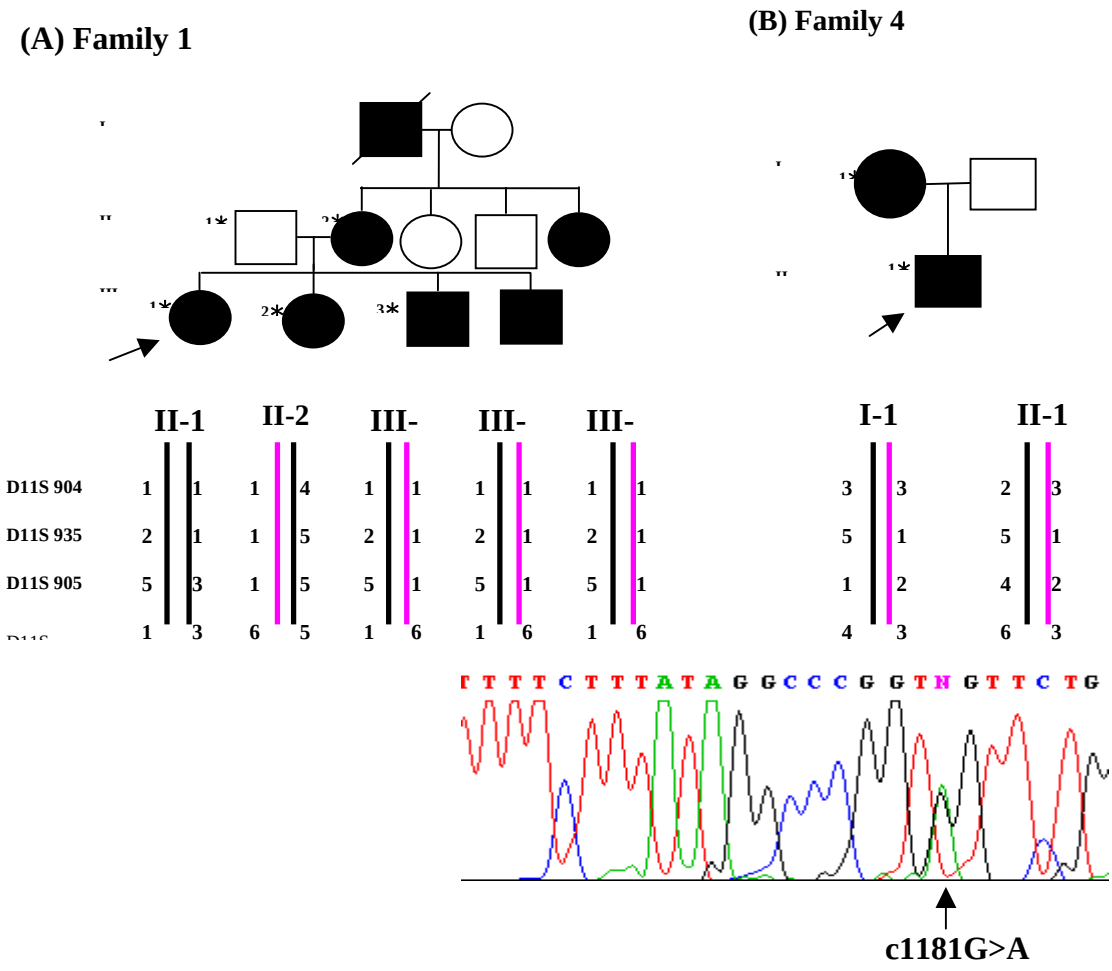
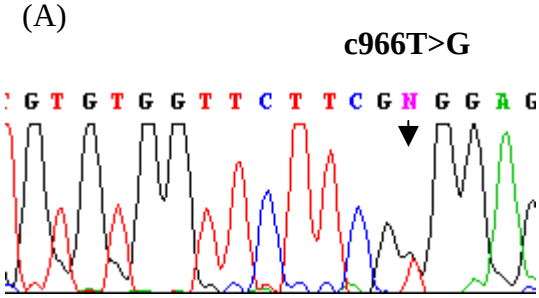
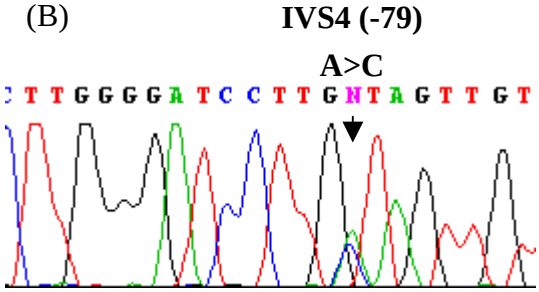

(A) Family 1
(B) Family 4
II-1
II-2
III-
III-
III-
I-1
II-1
D11S 904
D11S 935
D11S 905
c1181G>A
Family 4
(C) Family 3
I-1
I-2
II-1
II-2
II-3
III-1
D11S 904
D11S 935
D11S 905
D11S
c1181G>A
Family 3

Figure 3

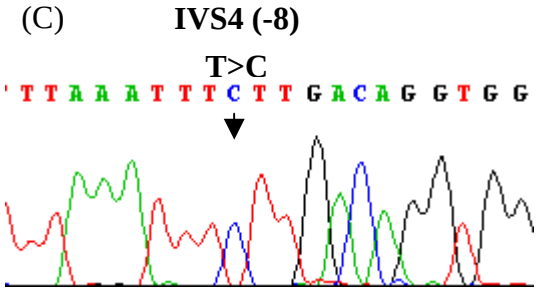
(A)



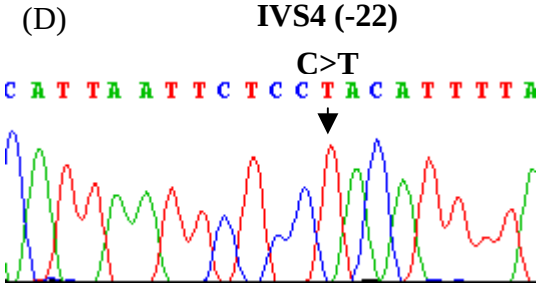
(B)



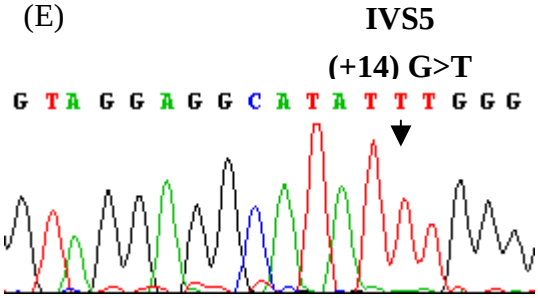
(C)



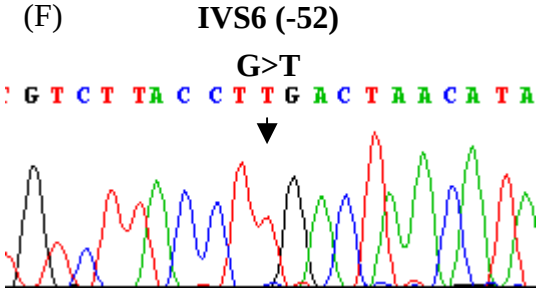
(D)



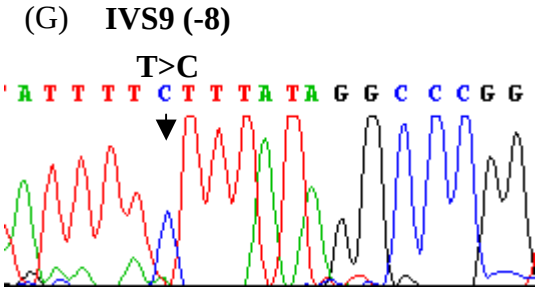
(E)



(F)



(G)



(H)

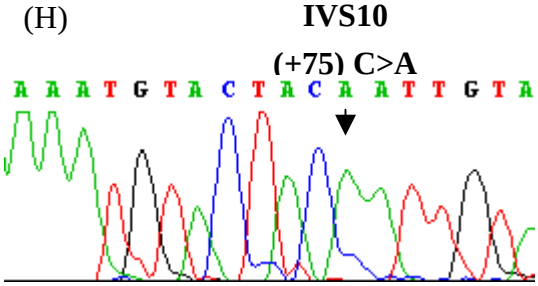


Figure 4

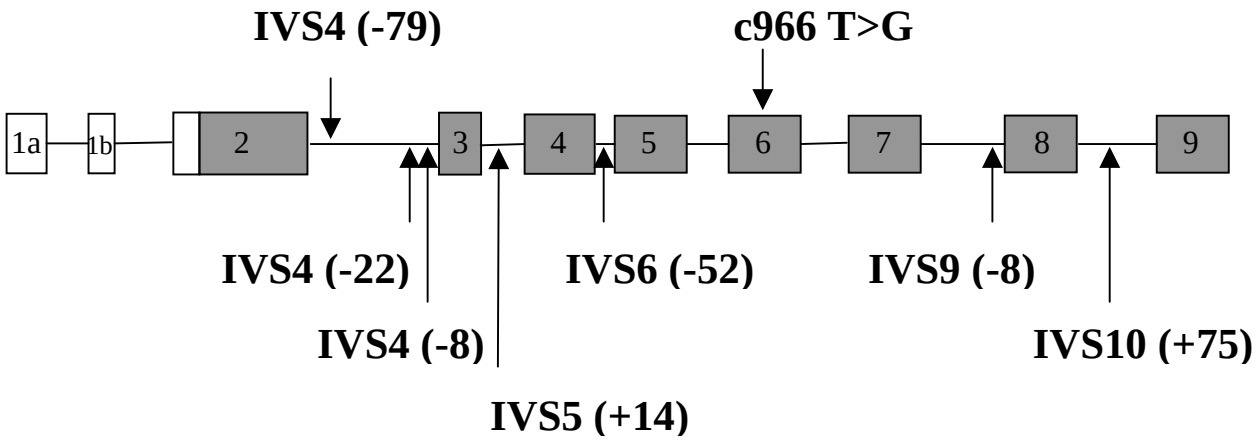

IVS4 (-79)
c966 T>G
1a
1b
2
3
4
5
6
7
8
9
IVS4 (-22)
IVS6 (-52)
IVS9 (-8)
IVS4 (-8)
IVS10 (+75)
IVS5 (+14)

Figure legends

Figure 1. Pedigrees of EXT1-linked families. All blacked circles and squares indicate confirmed affected status. The arrows indicate the probands of these families. (A) Linkage results of family 2 were shown below the pedigree. The red-labeled allele represents the affected-allele in this family. (B) An isolated case (family 5) of EXT1 and a base substitution (c1404 C-to –G) found in the proband.

Figure 2. Pedigrees of EXT2-linked families and the linkage analysis. The affected allele was labeled with red-color. (A) family 1, a EXT2- linked case. (B) A base substitution (c1181G-to- A) was found in the affected members of family 4. (C) The affected members of family 3 had the same mutation (c1181G-to- A) as family 4 did.

Figure 3. Sequence analysis of EXT2 gene in three EXT2-linked families that were studied. (A) The polymorphism in nucleotide 966 showed a heterozygous status (TG) at this position. (B) A (AC) polymorphism was found at upstream (-79) position of intron 4 (IVS4 (-79)). (C) A homozygous status (CC) was found at upstream of intron 4 (IVS4 (-8)). (D) TT was found at IVS4 (-22). (E) TT was found at downstream of intron 5 (IVS5 (+14)). (F) TT was found at IVS6 (-52). (G) CC was found at IVS9 (-8). (H) AA was found at IVS10 (+75).

Figure 4. The distribution of polymorphisms and nucleotide differences of the EXT2 gene reported in the present study. Black boxes represent coding regions. Two polymorphisms are drawn above the schematic representation of the EXT2 gene, and the six nucleotide positions are draw below.