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厚生労働科学研究費補助金（難治性疾患克服研究事業）
研究分担報告書
致死性骨異形成症の診断と予後に関する研究

致死性骨異形成症患者の皮膚線維芽細胞からの軟骨細胞誘導の試み

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研究要旨
致死性骨異形成症の病態解析と治療薬の探索に向けて、致死性骨異形成症患者の皮膚線維芽細胞から軟骨細胞を誘導することを試みる。致死性骨異形成症に特異的な iPS 細胞を誘導した。今後、軟骨細胞を誘導し、病態の解析を試みる。

共同研究者
無し

A. 研究目的

致死性骨異形成症は、気管あるいは肋骨の軟骨の脆弱性による呼吸不全が重篤な症状である。、FGFR3 遺伝子の変異によって起こることがわかっているが、軟骨の脆弱性を引き起こす仕組みはわかっておらず、治療薬は無い。患者の軟骨を採取して調べることが困難なことが、その原因の一つである。Induced pluripotent stem cell の開発を契機に、細胞リプログラミングの技術が進展し、比較的入手しやすい患者の皮膚細胞から軟骨細胞を試験管内で作る、疾患モデリングが可能になってきた。本研究の最終的な目的は、致死性骨異形成症患者の皮膚細胞から軟骨細胞を誘導し、病態解析と薬剤探索の材料に資することである。

B. 研究方法

致死性骨異形成症患者の皮膚細胞培養に、リプログラミング因子を導入して iPS 細胞を作成したのちに、軟骨細胞へと分化させる。健常人由来の細胞からも同様に軟骨細胞を誘導・分化させ、疾患由来細胞と比較する材料に供する。致死性骨異形成症患者の皮膚細胞は、米国の細胞バンクから入手する他、骨系統疾患コンソーシアムを通じて国内の患者からの採取を可能であれば行う。そのための研究計画の倫理審査を受ける。

（倫理面への配慮）

京都大学の倫理委員会に「ヒト患者由来 iPS 細胞および直接誘導軟骨細胞を用いた軟骨形成異常症の病態解明」の研究計画が承認された。この計画の下に、国内の致死性骨異形成患者の皮膚細胞から軟骨細胞を誘導し、病態の解析と薬剤の探索を行う。

C. 研究結果

致死性骨異形成症患者1症例の皮膚細胞培養から、直接誘導による軟骨細胞様細胞の誘導と、iPS 細胞の作製を行った。直接誘導のために、

患者由来の線維芽細胞に因子を導入すると、健常人由来の線維芽細胞に比べて、軟骨特異的レポーター遺伝子発現上昇は抑えられ、軟骨分化能が低下していることが考えられた。患者 iPS 細胞は、健常者由来の iPS 細胞と比較して形態は正常であった。

D. 考察

致死性骨異形成患者の皮膚細胞から、軟骨細胞の直接誘導とiPS細胞誘導のためのセットアップを行った。軟骨細胞を誘導して病態を調べていくための環境が整ったと考える。今後、症例数を増やし、データを蓄積していくことが必要である。倫理審査が承認され、国内患者由来の細胞から誘導することが可能になった。

E. 結論

致死性骨異形成患者の皮膚細胞から、軟骨細胞の直接誘導とiPS細胞誘導のための技術的セットアップを行い、倫理上の配慮にむけて倫理審査の承認を得た。

F. 健康危険情報

無し。

G. 研究発表

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（発表誌名巻号・頁・発行年等も記入）

H. 知的財産権の出願・登録状況

無し

厚生労働科学研究費補助金（難治性疾患克服研究事業）
研究分担報告書
致死性骨異形成症の診断と予後に関する研究

骨系統疾患の疾患遺伝子及び解析可能施設の情報収集について

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周産期委員会骨系統疾患小委員会のプロジェクトの一つとして「疾患遺伝子及び解析可能施設の情報収集」を行いました．この不安定な情報を網羅したデータベースは国内では存在せずその収集は実現すれば非常に有用なものとなります．そこで骨系統疾患コンソーシウムの池川先生と連携してデータベースとその運用を確立することといたしました．情報収集は以下のような方針で行いました．第一にはオーファンネットジャパン(ONJ)，GENDIA，GeneTestsといったウェブサイトで公開されている情報を海外国内問わず収集致しました．第二にコマーシャルベースの情報（BML,SRL,三菱化学メディエンスに問い合わせました）を収集致しました．第三には研究ベースで行っている大学や研究所の情報を収集致しました．しかしながら研究ベースの場合，それぞれの研究室の負担が大きくなる可能性があるために委員会として次のような条件をつけることといたしました．原則として個々の研究室情報は公開せず「可能（応相談）」との情報のみを公開する．遺伝子診断の問い合わせ／依頼があった場合にはまず当委員会（担当：北海道大学山田崇弘）として臨床医との対応を行い，協議の上で池川先生にアドバイスをいただき，適切と判断した場合のみ各ラボにお願いするような対応としようということに致しました．また，解釈が困難な場合にも池川先生に相談に乗っていただけるような体制としました．研究ベースの遺伝子診断は体制が非常に不安定であるため半年から１年ごとの情報更新が必須であります．情報の管理運用が最も重要な課題となります．今後はホームページなどを通じて問い合わせ／依頼に対応して行く事が考慮されます．

一覧表を巻末に添付

Ⅲ．研究成果の刊行に関する一覧表

書籍

無し

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ．研究成果の刊行物・別冊

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ORIGINAL INVESTIGATION

Hyperuricemia cosegregating with osteogenesis imperfecta is associated with a mutation in *GPATCH8*

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Abstract Autosomal dominant osteogenesis imperfecta (OI) is caused by mutations in *COL1A1* or *COL1A2*. We identified a dominant missense mutation, c.3235G>A in *COL1A1* exon 45 predicting p.G1079S, in a Japanese family with mild OI. As mutations in exon 45 exhibit mild to lethal phenotypes, we tested if disruption of an exonic splicing *cis*-element determines the clinical phenotype, but detected no such mutations. In the Japanese family, juvenile-onset hyperuricemia cosegregated with OI, but not in the previously reported Italian and Canadian families with c.3235G>A. After confirming lack of a founder haplotype in three families, we analyzed *PRPSAP1* and *PRPSAP2* as candidate genes for hyperuricemia on chr 17 where *COL1A1* is located, but found no mutation. We next resequenced the whole exomes of two siblings in the Japanese family and identified variable numbers of previously reported hyperuricemia-associated SNPs in *ABCG2* and *SLC22A12*. The same SNPs, however, were also detected in normouricemic individuals in three families. We then identified two missense SNVs in *ZPBP2* and *GPATCH8* on chromosome 17 that cosegregated with hyperuricemia in the Japanese family. *ZPBP2* p.T69I was at the non-conserved region and was predicted to be benign by *in silico* analysis, whereas *GPATCH8* p.A979P was at a highly conserved region and was predicted to be deleterious, which made p.A979P a conceivable candidate for juvenile-onset hyperuricemia. *GPATCH8* is only 5.8 Mbp distant from *COL1A1* and encodes a protein harboring an RNA-processing domain and a zinc finger domain, but the molecular functions have not been elucidated to date.

Electronic supplementary material The online version of this article (doi:10.1007/s00439-011-1006-9) contains supplementary material, which is available to authorized users.

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Introduction

Osteogenesis imperfecta (OI) is a heritable connective tissue disorder characterized by bone fragility and low bone mass. Clinical severities are widely variable ranging from intrauterine fractures and perinatal lethality to very mild forms without fractures. Patients also exhibit associated features including blue sclera, dentinogenesis imperfecta, hyperlaxity of ligaments and skin, and hearing loss (Rauch and Glorieux 2004). The widely used classification initially described by Sillence et al. (1979) distinguishes types I, II, III and IV (MIM# 166200, 166210, 259420, and 166220, respectively) on the basis of clinical and radiographic findings. Recently, five additional types of V, VI, VII, VIII and IX (MIM# 610967, 610968, 610682, 610915, and 259440, respectively) have been reported (Cabral et al. 2007; Glorieux et al. 2000, 2002; van Dijk et al. 2009; Ward et al. 2002). OI type I is the mildest form characterized by fractures with little or no limb deformity and

normal or mildly short stature, whereas type II is a perinatal lethal form, mostly due to respiratory failure resulting from multiple rib fractures. Type III is characterized by progressive deformities and fractures that are often present at birth. Severities of types IV, V, VI and VII are between those of types I and III. Type VIII and IX carry features of both types II and III.

Type I collagen is the most abundant bone protein. Most patients (>90%) with OI types I–IV have dominant or recessive mutation(s) in either of two genes, *COL1A1* (MIM# 120150) on chromosome (chr) 17q21.31–q22 and *COL1A2* (MIM# 120160) on chr 7q22.1 that encode the α1 and α2 chains of type I procollagen, respectively (Rauch and Glorieux 2004). A genetic cause of type V remains undetermined to date. Types VI to IX are caused by recessive mutations. Type VI is caused by mutations in *FKBP10* (MIM# 607063) encoding FK506-binding protein 65 (FKBP65) that is a chaperone in type I procollagen folding (Alanay et al. 2010). Type VII is caused by mutations in *CRTAP* (MIM# 605497) encoding cartilage-associated protein (CRTAP) (Morello et al. 2006). Type VIII is caused by mutations in *LEPRE1* (MIM# 610339) encoding prolyl 3-hydroxylase 1 (P3H1) (Cabral et al. 2007). Type IX is caused by mutations in *PPIB* (MIM# 123841) encoding cyclophilin B (CYPB) (van Dijk et al. 2009). CRTAP, P3H1 and CYPB form an intracellular collagen-modifying complex that 3-hydroxylates proline at position 986 in the α1 chain of type I collagen, which is essential for correct folding and stability of the collagen triple helix. Mutations in *CRTAP* and *LEPRE1* are also identified in severe OI phenotypes including type II (Baldridge et al. 2008; Morello et al. 2006). Recently, recessive mutations in *SERPINH1* (MIM# 600943) encoding a chaperone-like protein for collagens, heat shock protein 47 and in *SP7/Osterix* (MIM# 606633) encoding an osteoblast-specific transcription factor have been identified in patients with types III and IV, respectively (Christiansen et al. 2010; Lapunzina et al. 2010).

Two copies of the α1 chain and one copy of the α2 chain form a core triple helix comprising 338 uninterrupted Gly-X–Y triplet repeats, where X is often proline and Y is often hydroxyproline. Gly repeats at every third position are essential for the stability of collagen because larger amino acids cannot be accommodated in the tightly packed core without disruption of the triple helix (Bodian et al. 2008). The most common mutations (>80%) affect one of the repeated Gly residues in the triple helix. More than 800 mutations in *COL1A1* and *COL1A2* are currently deposited in the human type I collagen mutation database (http://www.le.ac.uk/genetics/collagen/) (Dalgleish 1997; Marini et al. 2007). Clinical phenotypes may be determined by the chain in which the Gly substitution occurs, the position of the mutation within the chain and/or the nature of the mutant amino acids (Bodian et al. 2008; Marini et al. 2007; Rauch and Glorieux 2004), but we still cannot predict a clinical phenotype of a given mutation. On the other hand, mutations that create a premature stop codon within *COL1A1* mostly exhibit a milder OI type I. This is because a truncation mutation is unlikely to have a dominant negative effect, but the abundance of type I collagen chain is half of the normal (Marini et al. 2007; Rauch and Glorieux 2004).

Pre-mRNA splicing is regulated by intronic and exonic splicing *cis*-elements. Both constitutively and alternatively spliced exons harbor exonic splicing enhancers (ESEs) and silencers (ESSs). Splicing *trans*-factors are expressed in a developmental stage-specific and tissue-specific manner, and their expressions tightly regulate alternative splicing of an exon carrying ESEs/ESSs. A mutation in the coding region is predicted to exert its pathogenicity by compromising a functional amino acid, but nonsense, missense and even silent mutations potentially disrupt an ESE/ESS, thereby resulting in aberrant splicing (Cartegni et al. 2002, 2003). Indeed, more than 16–20% of exonic mutations are predicted to disrupt an ESE (Gorlov et al. 2003).

Exome resequencing is a powerful and efficient method to identify a novel gene associated with a rare monogenic disorder, especially when the number of unrelated patients or the number of family members of a patient are too small to apply linkage studies. Filtering against existing SNP database and the exomes of unaffected individuals can remove common variants to identify a causal gene. Ng et al. (2009) sequenced exomes of 12 humans, including four unrelated individuals with autosomal dominant Freeman-Sheldon syndrome (MIM# 193700) and eight HapMap individuals. They successfully identified mutations in *MYH3* (MIM# 160720) in all the affected individuals. Ng et al. (2010b) also sequenced exomes of four patients in three families with autosomal recessive Miller syndrome (MIM# 263750) and de novo identified compound heterozygous mutations in *DHODH* (MIM# 126064). Ng et al. (2010a) additionally sequenced exomes of ten unrelated patients with autosomal dominant Kabuki syndrome (MIM# 147920) and de novo identified nonsense or frameshift mutations in *MLL2* (MIM# 602113). Similarly, Lalonde et al. (2010) sequenced two unrelated fetuses with autosomal recessive Fowler syndrome (MIM# 225790) and de novo identified compound heterozygous mutations in *FLVCR2* (MIM# 610865).

In a Japanese family with OI type I, hyperuricemia co-segregated with OI. To our knowledge, association of hyperuricemia with OI has been reported in two families, in which two of three OI patients had gouty arthritis and hyperuricemia at young ages (Allen et al. 1955). Under-excretion of urate is causally associated with mutations in *UMOD* (MIM# 191845) encoding uromodulin (Hart et al.

2002) as well as with SNPs in three genes: *SLC2A9* (MIM# 606142) encoding glucose transporter 9 (Doring et al. 2008; Vitart et al. 2008), *ABCG2* (MIM# 603756) encoding ATP-binding cassette subfamily G member 2, a urate transporter (Dehghan et al. 2008; Kolz et al. 2009; Stark et al. 2009; Woodward et al. 2009), and *SLC22A12* (MIM# 607096) encoding URAT1, a renal urate-anion exchanger (Graessler et al. 2006; Tabara et al. 2010). On the other hand, overproduction of urate is caused by mutations in *PRPS1* (MIM# 311850) encoding PRPP synthetase I (Roessler et al. 1993) and *HPRT1* (MIM# 308000) encoding hypoxanthine guanine phosphoribosyltransferase 1 (Gibbs and Caskey 1987).

We here identified a dominant missense mutation, c.3235G>A in *COL1A1* exon 45 predicting p.G1079S, in a Japanese family with OI type I and hyperuricemia, and analyzed the molecular bases of two clinical features. First, we tested if mild to lethal phenotypes of mutations in exon 45 of *COL1A1* were accounted for by preservation or disruption of an ESE/ESS element, and found that ESE/ESS elements were not involved in disease severities. Second, we traced a cause of the hyperuricemia by exome resequencing of two siblings and found that a missense mutation in *GPATCH8* encoding the G patch domain-containing protein 8 close to *COL1A1* cosegregated with hyperuricemia.

Patients and methods

Samples and ethical considerations

We obtained blood from each family member and isolated genomic DNA (gDNA) from 2 ml of peripheral blood using the QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's instructions. We also obtained skin biopsy of an affected individual (II-3) of a Japanese family (F1) and cultured non-transformed fibroblasts for splicing and sequencing analysis. Informed consent was obtained from all family members. The studies have been approved by the Institutional Review Boards of the Nagoya University, the University of Verona and the McGill University. Clinical features and mutational analyses have been previously reported in the Italian and Canadian families (Mottes et al. 1992; Roschger et al. 2008).

Microsatellite analysis of *COL1A1* and *COL1A2* to identify a mutation in the Japanese family

We genotyped all family members for three microsatellite markers flanking *COL1A1* (D17S1293, −16 Mbp; D17S1319, −14 kbp; and D17S788, 2 Mbp). As no annotated microsatellite markers were available close to *COL1A2*, we posted three new microsatellite markers to DDBJ (AB499843, −17 kbp; AB499844, 29 kbp; and AB499845, 123 kbp) and analyzed them in the family. We fluorescently labeled the 5′ end of each forward primer with 5FAM (Sigma-Aldrich), and amplified microsatellite markers with the HotStarTaq Plus Master Mix (Qiagen) using gDNA and primers indicated in Supplementary Table 1.

We mixed 1.5 μl of 20-times diluted PCR product with 0.5 μl of GeneScan-500 ROX Size Standard (Applied Biosystems) and 24.5 μl of formamide, and incubated the mixture at 95°C for 3 min. The mixture was run by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer and was analyzed with the GeneScan and GeneMapper software (Applied Biosystems).

Sequence analysis of *COL1A1*

After the microsatellite analysis suggested that *COL1A1* was more likely to be a causative gene, we amplified all exons and flanking intronic regions of ~40 bp, as well as 5′ and 3′ UTRs of *COL1A1* from gDNA of II-3 by PCR. We performed the dye terminator cycle sequencing reaction with the GenomeLab DTCS Quick Start Kit (Beckman Coulter) and ran on the CEQ 8000 Genetic Analysis System (Beckman Coulter) according to the manufacturer's instructions. We compared the chromatograms with the GenBank reference sequences of *COL1A1* gDNA using the Mutation Surveyor software version 2.61 (SoftGenetics). We numbered *COL1A1* mutations with the translation initiator methionine as amino acid +1, and the A of the ATG codon as nucleotide +1 according to the Human Genome Variation Society (http://www.hgvs.org/mutnomen/recs.html). We numbered exons according to the human type I collagen mutation database (http://www.le.ac.uk/genetics/collagen/), in which *COL1A1* exon 33 is named *COL1A1* exon 33–34 to match the exonic annotations of *COL1A2*.

Allele-specific primer (ASP)-PCR to trace the *COL1A1* mutation

We traced the mutation in family members using ASP-PCR. The wild-type ASP was 5′-TCCCGCCGGTCCT GTAGG-3′, and the mutant ASP was 5′-TCCCGCCGG TCCTGTAAG-3′, where the mutated nucleotide is underlined, and an artificially introduced mismatch is shown in bold. The reverse primer was 5′-GCCACGGTGAC CCTTTATGC-3′.

Prediction of effects of mutations on pre-mRNA splicing

Five missense mutations in exon 45 of *COL1A1* cause mild to lethal OI phenotypes (Fig. 2a) (Constantinou et al. 1989;

Hartikka et al. 2004; Lund et al. 1997; Marini et al. 2007; Mottes et al. 1992; Roschger et al. 2008). We predicted the effects on pre-mRNA splicing of 18 sequence variants with or without each mutation in the presence or absence of each of two SNPs (rs1800215 and rs1800217) in exon 45 of *COL1A1* (Fig. 2b) using five Web-based programs: ESEfinder 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) (Cartegni et al. 2003), ESR-search (http://ast.bioinfo.tau.ac.il/) (Goren et al. 2006), FAS-ESS (http://genes.mit.edu/fas-ess/) (Wang et al. 2004), PESXs (http://cubweb.biology.columbia.edu/pesx/) (Zhang and Chasin 2004; Zhang et al. 2005), and RESCUE-ESE (http://genes.mit.edu/burgelab/rescue-ese/) (Fairbrother et al. 2002). We also predicted the effects of the mutations on splice site strength of the 18 sequence variants using two Web-based programs: the NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/) (Brunak et al. 1991; Hebsgaard et al. 1996) and the Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al. 1997).

Splicing analysis of fibroblasts of the OI patient (II-3)

We first examined splicing of *COL1A1* exon 45 in the patient's fibroblasts. We extracted total RNA from cultured fibroblasts of II-3 using Trizol reagent (Invitrogen) and synthesized cDNA using the oligo(dT)$_{12-18}$ primer (Invitrogen) and the ReverTra Ace reverse transcriptase (Toyobo). We examined skipping of *COL1A1* exon 45 using 5′-GGTTCCCCTGGACGAGAC-3′ on exon 43 and 5′-TCCAGAGGGACCTTGTTCAC-3′ on exon 47. We also sequenced RT-PCR products as described above to scrutinize splicing consequences. As skipping of exon 45 results in an in-frame deletion of 54 nucleotides, we did not downregulate the nonsense-mediated mRNA decay (NMD) before harvesting cells.

COL1A1 minigene constructs

We amplified exons 44–46 and the intervening introns of *COL1A1* (Fig. 2b) by PCR. The PCR primers introduced a *Hin*dIII site and the Kozak consensus sequence of 5′-CCACCATG-3′ to the 5′ end, as well as a TAA stop codon and a *Bam*HI site at the 3′ end of the PCR product, so that the minigene transcript is tolerant to NMD (Ohno et al. 2003). We inserted the PCR product into the pcDNA3.1(+) mammalian expression vector (Invitrogen) and confirmed the lack of PCR artifacts by sequencing the entire insert. We next constructed 17 variant minigenes using the QuikChange site-directed mutagenesis kit (Stratagene) (Fig. 2b). We again confirmed presence of the introduced mutations and absence of artifacts by sequencing the entire inserts.

Splicing assays of *COL1A1* exon 45

We transfected 500 ng of a minigene construct into 50% confluent HEK293 cells in a 12-well plate using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. After 48 h, we extracted total RNA from HEK293 cells and synthesized cDNA as described above. To prevent amplification of the endogenous *COL1A1*, we used generic primers of 5′-TTAATACGACTCACTATAGGGAGACC-3′ and 5′-TTAAGGAGGGCCAGGGGG-3′ located on pcDNA3.1. Untransfected cells were used as a negative control.

Founder analysis of three families with *COL1A1* c.3235G>A

To examine if the *COL1A1* c.3235G>A mutation arose from a common founder in the Japanese, Italian (Mottes et al. 1992) and Canadian (Roschger et al. 2008) families, we genotyped three microsatellite markers flanking *COL1A1* described above and sequenced an intragenic SNP (rs2075554) of *COL1A1* in the Italian and Canadian families (Fig. 1).

Sequence analysis of *PRPSAP1* and *PRPSAP2*

To seek for a responsible gene for hyperuricemia, we sequenced the entire coding regions of *PRPSAP1* and *PRPSAP2* on chr 17 using cDNA synthesized from cultured fibroblasts of II-3.

Resequencing of exome-enriched DNA

As we found no mutation in *PRPSAP1* and *PRPSAP2*, we enriched exonic regions of genomic DNA of II-2, II-3 and VIII-2 using the SureSelect human all exon kit v1 (Agilent Technologies) that covers 1.22% (34 Mbp) of the human genome. We sequenced 50 base pairs of each tag in a single direction using a quarter of a cell of the SOLiD 3 Plus system (Life Technologies) for each sample.

For II-2, II-3 and VIII-2, we obtained 79.1, 68.6 and 109.9×10^6 tags of 50-bp SOLiD reads and mapped 2.18 (69.6%), 1.87 (68.5%) and 3.37 (76.5%) Gbp to the human genome hg19/GRCh37, which yielded a mean coverage of 64.1, 55.1 and 99.0, respectively. Among the mapped tags, 70.1, 72.0 and 73.1% were located on the SureSelect exome probes. Among the 34-Mbp regions where the exome probes were designed, 3.4, 3.6 and 3.5% of nucleotides

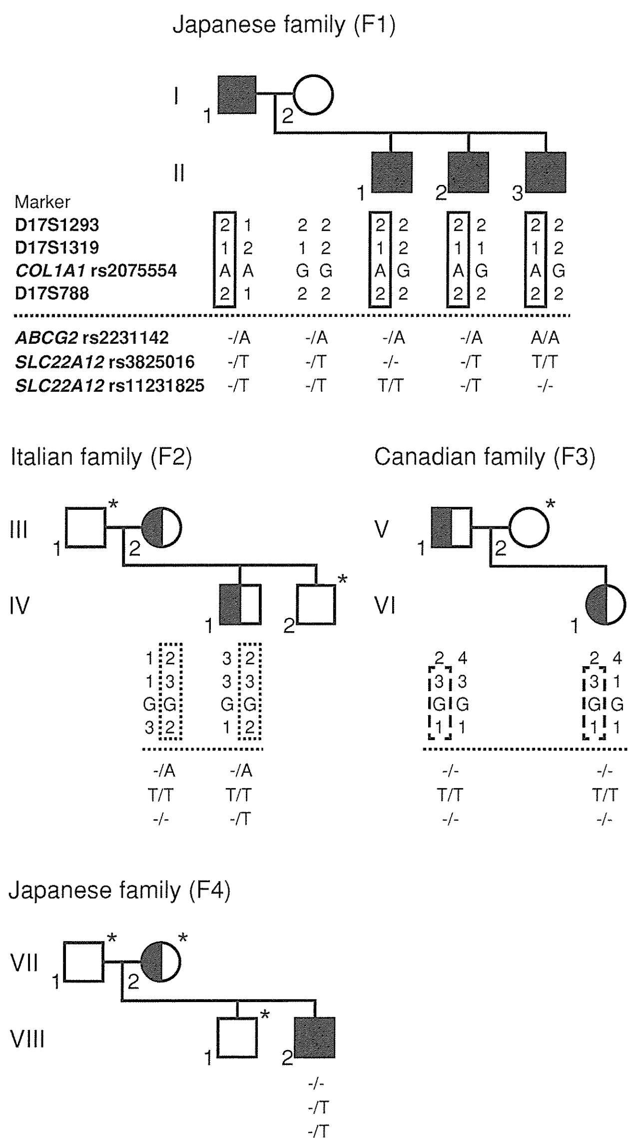

Fig. 1 Pedigrees, haplotypes and genotypes of the Japanese (F1 and F4), Italian (F2) and Canadian (F3) families. Patients in F1, F2 and F3 have c.3235G>A predicting p.G1079S in *COL1A1*. A patient in F4 has c.577G>T predicting p.G193C in *COL1A2*. *Closed symbols* indicate patients with OI and with hyperuricemia. *Half-shaded symbols* represent OI without hyperuricemia. *Asterisks* indicate that DNA sample is not available for our studies. Clinical subtypes are all type I except for the Canadian father (V-1), who exhibits type IV. In F1, hyperuricemia cosegregates with OI. Genotypes of three microsatellite markers (D17S1293, −16 Mbp; D17S1319, −14 kbp; and D17S788, 2 Mbp) flanking *COL1A1* and an SNP (rs2075554) in intron 11 of *COL1A1* are indicated for all the available members in F1, F2, and F3. F1, F2 and F3 carry their unique haplotypes (shown by *solid*, *dotted* and *broken boxes*, respectively). The D17S1293 genotype in F3 is not informative and is not boxed. Genotypes of hyperuricemia-associated SNPs (rs2231142, rs3825016 and rs11231825) are indicated at the bottom of each pedigree tree

were not sequenced at all. Search for single nucleotide variants (SNVs) and indels with Bioscope 1.2.1 (Life Technologies) detected 52,436, 56,941 and 60,303 SNVs/indels. SNVs and indels were compared to dbSNP Build 132.

Analysis of variants in *KRBA2, ZPBP2* and *GPATCH8*

To trace if variants in *KRBA2*, *ZPBP2* and *GPATCH8* cosegregated with hyperuricemia, we analyzed all family members in the Japanese family (F1) by capillary sequencing.

We traced two variants, *ZPBP2* c.206C>T and *GPATCH8* c.2935G>C, in the Italian and Canadian families and 100 normal human genomes using ASP-PCR. The forward primers of *ZPBP2* and *GPATCH8* were 5′-CGTGTCTTCAGCACAAAATGG-3′ and 5′-AGAAGCCGTAGCACCACTCC-3′, respectively. The reverse primers were 5′-GGCCCAATCCATAAGTACAT-3′ and 5′-CCCATGATCTCTTCCTGGAG-3′, respectively. The mutated nucleotide is underlined, and an artificially introduced mismatch is shown in bold.

To search for the identified variants in *ZPBP2* and *GPATCH8* in normal controls, we mapped 50 Tibetan exome reads (SRA accession number SRP002446) (Yi et al. 2010) to a 200-bp region spanning c.206C>T in exon 3 of *ZPBP2* and a 200-bp region spanning c.2935G>C in exon 8 of *GPATCH8* with the bowtie alignment tool version 0.12.7 (Langmead et al. 2009) using default parameters.

We analyzed amino acid conservations of *ZPBP2* and *GPATCH8* using the evolutionary annotation database, Evola, at the H-Inv DB (http://www.h-invitational.jp/evola/). We also predicted functional effects of amino acid substitutions using two Web-based programs: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al. 2010) and SIFT (http://sift.jcvi.org/) (Kumar et al. 2009).

Results

Hyperuricemia cosegregated with OI type I in the Japanese family (F1)

In a Japanese family (F1), a father (age 56 years) and his three sons (ages 29, 26 and 23 years) had OI type I with blue sclera, dentinogenesis imperfecta and joint laxity (Fig. 1). Two sons (II-1 and II-3) had histories of more than ten fractures before age 13 years, but the father (I-1) and another son (II-2) experienced no bone fracture. One son (II-1) had hearing loss from age 10 years likely due to fractures or deformities of small bones in the middle ear and had hip joint deformities due to repeated femoral fractures. Interestingly, all the affected members had hyperuricemia of ~8 mg/dl that was diagnosed at ages 15–30 years. One son (II-3) had a gout attack, and the other two (I-1 and II-1) had urinary stones. Hyperuricemia is currently well controlled by medication in all the members.

Heteroallelic c.3235G>A mutation in *COL1A1* in the Japanese family (F1)

Genotypes of three microsatellite markers flanking *COL1A1* cosegregated with the OI phenotype in F1 (Fig. 1), whereas genotypes of three markers flanking *COL1A2* did not (data not shown). We thus sequenced the entire exons and the flanking noncoding regions and identified a heteroallelic c.3235G>A mutation in exon 45 and a heteroallelic G/A SNP (rs2075554) in intron 11 of *COL1A1*. The c.3235G>A mutation predicts p.G1079S. We genotyped c.3235G>A in family members by ASP-PCR, and found that all affected members were heterozygous for c.3235G>A (Fig. 1).

Phenotypic variability of osteogenesis imperfecta is not accounted for by disruption of splicing *cis*-elements

In addition to c.3235G>A, four more mutations and two SNPs have been reported in *COL1A1* exon 45 with variable phenotypes ranging from mild type I to perinatal lethal type II (Fig. 2a) (Constantinou et al. 1989; Hartikka et al. 2004; Lund et al. 1997; Marini et al. 2007; Mottes et al. 1992; Roschger et al. 2008). We thus hypothesized that disruption or de novo generation of a splicing *cis*-element determines the clinical phenotype. The five mutations and two SNPs in *COL1A1* exon 45 were predicted to affect 16 putative splicing *cis*-elements by ESEfinder, ESRsearch and PESXs (Table 1). FAS-ESS and RESCUE-ESE predicted no splicing *cis*-elements. All the five mutations with or without two SNPs in *COL1A1* exon 45 variably but slightly weaken acceptor and/or donor splice site strengths according to the NetGene2 (Table 2). The splice site prediction by neural network also predicted that c.3235G>A generates a weak cryptic splice acceptor site in *COL1A1* exon 45 (Table 2). We first examined cultured fibroblasts of II-3 by RT-PCR and found that the *COL1A1* c.3235G>A mutation did not induce aberrant splicing of *COL1A1* (data not shown). NMD was unlikely to have masked aberrant splicing, because we observed heterozygous peaks at c.3235G>A in sequencing the RT-PCR product. We next constructed 18 *COL1A1* minigenes with or without each of the five mutations in the presence or absence of each of the two SNPs (Fig. 2b). RT-PCR analysis of transfected HEK293 cells showed that all minigene constructs gave rise to a single fragment of 336 bp, indicating that splicing was not affected in any mutations or SNPs (Fig. 2c).

Japanese (F1), Italian (F2) and Canadian (F3) families with *COL1A1* c.3235G>A share no founder haplotype

We previously reported *COL1A1* c.3235G>A in the Italian and Canadian families (Marini et al. 2007; Roschger et al. 2008). Although we have not measured serum urate concentrations in these families, gout or urinary stone has not been documented in either family, which suggests that hyperuricemia is not simply due to c.3235G>A.

To pursue if a gene responsible for hyperuricemia is on the same chr as *COL1A1*, we looked for a founder haplotype for c.3235G>A in three families by genotyping three microsatellite markers flanking *COL1A1* (D17S1293, −16 Mbp; D17S1319, −14 kbp; and D17S788, 2 Mbp) and an SNP (rs2075554) in intron 11 of *COL1A1*. The analysis revealed that each family carried a unique haplotype and shared no founder haplotype (Fig. 1). Thus, the mutation is likely to have occurred independently in three ethnic groups. Alternatively, c.3235G>A is an ancient founder mutation, and subsequent multiple recombinations and divergence of microsatellite repeats have obscured a founder effect. In either case, lack of a found haplotype supports the notion that a gene responsible for hyperuricemia is potentially but not necessarily linked to *COL1A1*.

Hyperuricemia is not caused by mutations in *PRPSAP1* or *PRPSAP2*

We thus first looked into candidate genes for hyperuricemia on chr 17 where *COL1A1* (17q21.31–q22) is located. Two candidate genes for hyperuricemia are on chr 17: *PRPSAP1* (MIM# 601249) at 17q24–q25 encoding PAP39 (Ishizuka et al. 1996) and *PRPSAP2* (MIM# 603762) at 17p12-p11.2 encoding PAP41 (Katashima et al. 1998). PAP39 and PAP41 are subunits of phosphoribosylpyrophosphate (PRPP) synthetase that leads to urate production. No mutation has been reported in either gene in any diseases. We sequenced cDNAs of *PRPSAP1* and *PRPSAP2* in II-3, but found no mutation in either gene. As we detected no heterozygous SNPs in *PRPSAP1* and *PRPSAP2*, a mutant allele carrying a premature stop codon might have been missed due to mRNA degradation by NMD.

Resequencing of exomes reveals hyperuricemia-associated SNPs

We next traced a causative gene for hyperuricemia in two siblings (II-2 and II-3) by exome resequencing with the SureSelect human all exon kit v1 (Agilent) and with the SOLiD 3 Plus sequencer (Life Technologies).

We similarly analyzed an unrelated Japanese male (22-year-old) with OI type I and hyperuricemia (VIII-2 in F4 in Fig. 1). His hyperuricemia was by chance detected at age 14 years when he had fractures. His hyperuricemia has been well controlled by medication since then. Exome resequencing of VIII-2 disclosed a novel heteroallelic c.577G>T mutation in *COL1A2* exon 12 predicting

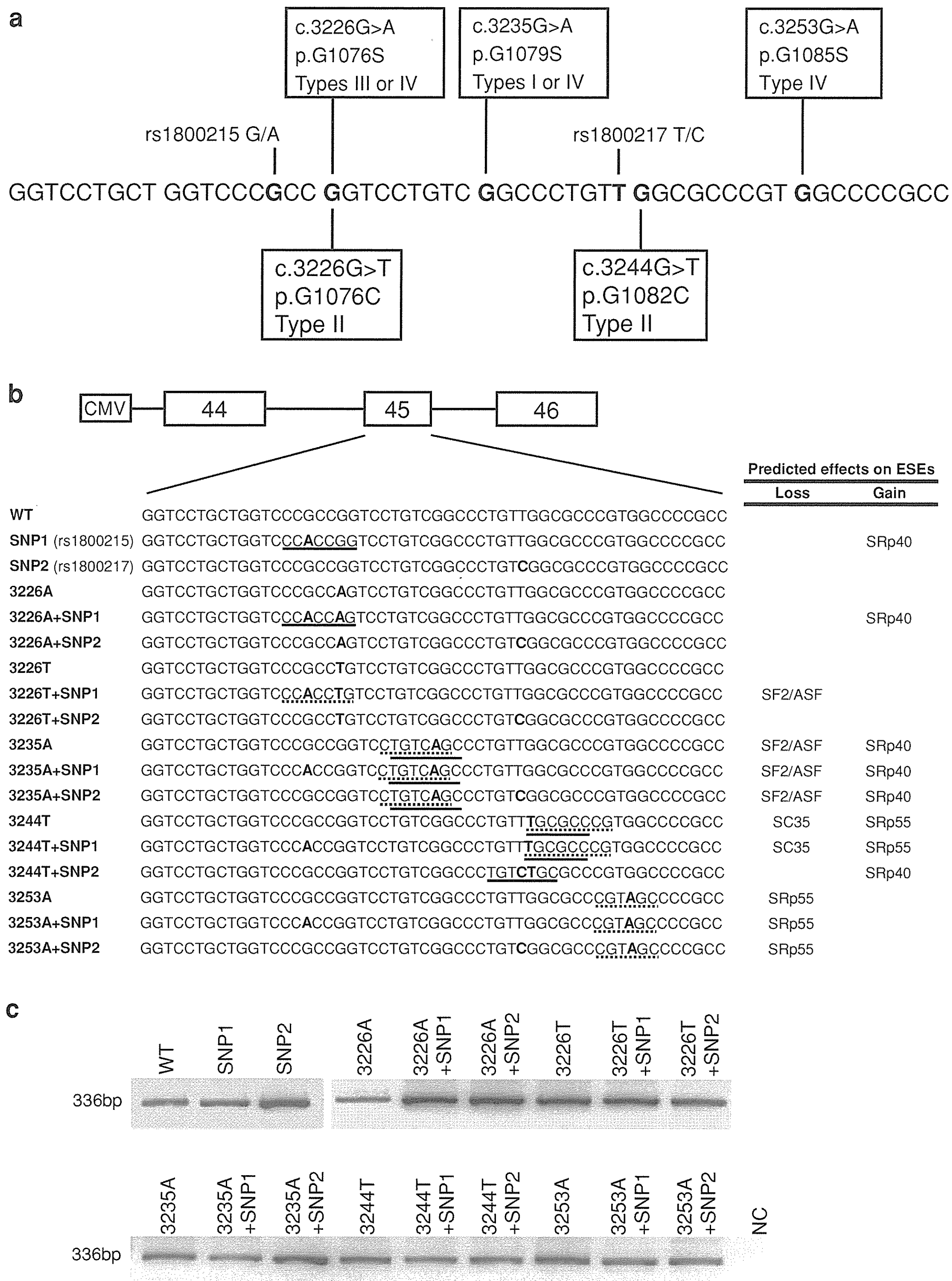

Fig. 2 Splicing assays of *COL1A1* minigenes in HEK293 cells. **a** Positions and phenotypes of OI mutations in *COL1A1* exon 45. Mutations above the wild-type sequence exhibit non-lethal phenotypes, whereas those below the sequence cause a lethal phenotype. **b** Schematic representation of *COL1A1* minigenes. Sequences of mutant minigenes are indicated *below*. Substituted nucleotides are shown in *bold*. Predicted gain and loss of splicing *cis*-elements by ESEfinder (Cartegni et al. 2003) are indicated by *solid* and *dotted underlines*, respectively. **c** RT-PCR of minigenes introduced into HEK293 cells. All constructs show a single fragment of 336 bp, indicating that *COL1A1* exon 45 is not skipped in any constructs. Untransfected cells are used as a negative control (*NC*)

p.G193C. We confirmed the mutation by capillary sequencing. Family samples were not available for our analysis.

In F1, a probability that three siblings inherited an identical allele from the father is $(1/2)^3 = 12.5\%$, which indicates that the causative gene is anywhere in the