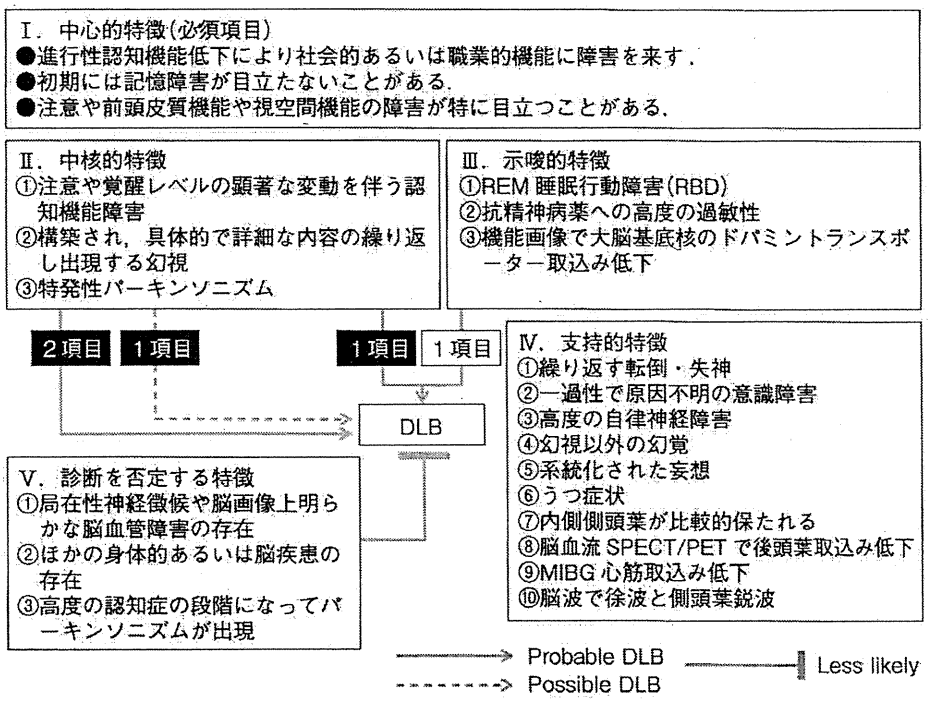

図1　DLB の診断基準(文献2より改変引用)

外路症状に先行して発症している場合はDLBといい，確固としたPDの経過中に認知症症状が起こってきた場合にPDDという用語が用いられている．

2006年に開かれたPDDとDLBとの境界の問題に関するカンファレンスでは，診断，治療，病理，バイオマーカーなどの観点から「DLBとPDDの臨床症状と経過の違いから両者を区別することは正当化されるが，両者はαシヌクレイン封入体という共通の病変を有することから病因研究のためには単一のレビー小体病モデルがより有用と考えられる」と結論づけられている[3]．

2．DLB・PDDの疫学

1）DLBの疫学

系統的レビューによると，すべての認知症におけるDLBの割合は0～30.5%であり，有病率は0～5%と報告されている[4]．ADと同様に年齢とともに増え，一般には60歳代以上に発症しその平均年齢は75歳前後である．ADと異なり男性に多いとされる．ほとんどの症例は弧発性であるが，家族性の症例も報告されている．

2）PDDの疫学

PDにおける認知症はよくある非運動症状であるが，系統的レビューでは横断的に31%のPD患者に認知症を有すると報告され，これは全認知症の3.6%に当たる[5]．PD患者における認知症発症率は健常者の4～6倍であり，10年間の認知症の発症率は少なくとも75%にのぼると報告されている[6]．

DLB・PDDの診断

1．DLBの診断

DLBの臨床診断には国際ワークショップ診断基準改訂版(図1)が使用される[2]．改訂前の診断基準では，①中心的特徴(進行性認知機能低下，必須項目)，②中核的特徴(認知機能の変動，幻視，パーキンソニズムの3項目のうち2項目以上あればprobable DLBとし，1項目だけあればpossible DLBとする)を診断基準の骨子としていた．第1回ワークショップ診断基準

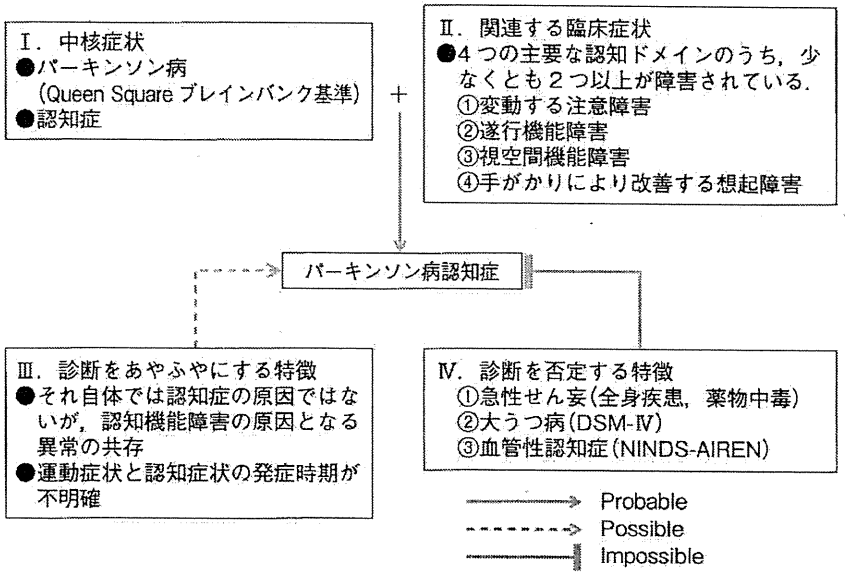

図2　PDDの診断基準(文献7より改変引用)

は，一般に特異度は高いが，感度が低いことが問題となっていた．そこで，DLB臨床診断基準改訂版においては，新たに「示唆的特徴」の項目が加わり，中核的特徴が1つしかなくても示唆的特徴が1つ以上あればprobable DLBとし，中核的特徴がなくても示唆的特徴が1つ以上あればpossible DLBとする判定基準の変更や否定的特徴が付記された．PDDとDLBを鑑別する基準である“1年ルール”が存続したが，改定基準にはPDD自体の診断基準は示されていない．

2．PDDの診断

これまでにDSM-Ⅳにおいて，「そのほかの一般疾患に伴う認知症として」PDDの診断基準があったが，Movement Disorder Society特別委員会からPDD診断基準が提唱された(図2)[7]．これに続いて，同委員会よりPDD診断のための診断手順が示され，診断のための簡易検査(レベルⅠ検査，表1)や，さらに詳細な検査(レベルⅡ検査)が提唱されていて，後者は薬剤効果判定などに使用されるべきとされている[8]．レベルⅠ検査の診断精度が検証されており，感度65.9%，特異度94.6%[9]や，感度78.0%特異度95.5%[10]と報告されていて，後者の検討ではこの検査を用いた場合，過大評価の可能性が指摘されている．

治療エビデンス

1．コリンエステラーゼ阻害薬(ChEI)

1）DLBにおけるエビデンス

DLBでは，アセチルコリン(ACh)系神経細胞の起始核であるマイネルト核の脱落がADより強く，大脳皮質のACh合成酵素(ChAT)が低下している報告や，海馬に神経線維を投射している中隔核のACh系神経細胞の脱落もADより強いことが知られている．第一世代のChEIであるタクリンがAD患者に臨床応用され，タクリンが著効したAD症例が報告された．この症例は後に剖検によりDLBであることが判明し，ChEIとDLBの関連性が注目されるようになった．

a）ドネペジル

4つのオープン試験が報告されているが，いずれも少数例での検討である．Samuelらは，4例のDLB患者と12例のアルツハイマー病(AD)患者に対してドネペジル5 mg/日を6カ月間投与し，ドネペジルの認知機能の改善効果

表1　PDD診断のためのシート

	はい	いいえ
1．パーキンソン病	□	□
2．認知症の前にパーキンソン病を発症	□	□
3．MMSE＜26	□	□
4．認知症は日常生活活動に影響を与える	□	□
5．認知機能障害	□	□
（次の4つのうち少なくとも2つが異常である）		
□月の逆唱　あるいは　シリアル7		
□言語流暢性課題　あるいは　時計描画		
□MMSEの五角形模写		
□3単語の遅延再生		
6．大うつ病ではない	□	□
7．せん妄ではない	□	□
8．そのほかの診断をあやふやにする異常はない	□	□
PDD　確実例　（1～8のすべてが「はい」）	□	□

＜カットオフ＞
月の逆唱：2つ以上の省略
言語流暢性課題：1分間に9個以下
五角形模写：五角形が描けない
シリアル7：2つ以上の誤答
時計描画：指示された時計が描けない
遅延再生：少なくとも1つ誤答

はADに比べDLBにおいて有意に大きかったと報告している[11]．Thomasらは，30例のDLB患者に対してドネペジル5～10 mg/日・20週間投与の試験結果を報告し，治療によりMMSEはDLB群で平均3.9点，増加がみられた．NPIサブスコア(幻覚，妄想，無感情)の改善がみられ，治療前後での運動機能(UPDRS-Ⅲ)の有意な悪化は認めていない[12]．Rowanらは，22例のDLB患者にコンピュータを用いて評価し，治療により注意力，注意の持続性および反応時間のばらつきの改善を示したと報告している[13]．Moriらは，12例のDLB患者に対してドネペジル5 mg/日を12週間投与した結果を報告している[14]．4週時点でドネペジル治療によるADAS-Jcogの有意な改善を認め，NPIスコアはベースラインに比べ投与8週および12週時点で有意な改善を認めた．特に，認知機能の変動項目や妄想，幻覚，無感情，うつ状態に効果を認めた．

b）リバスチグミン

120例のDLB患者を対象としたリバスチグミン6～12 mg/日・20週間投与のRCTがあり[15]，実薬群18例とプラセボ群10例が治験途中で脱落し，92例が治験を完了した．実薬群において一次評価項目の妄想，幻覚，無感情，うつ状態を評価したNPI-4スコアの有意な改善が認められている．二次評価項目のMMSEや臨床全般評価には有意な改善は認めていないが，コンピュータ化認知機能評価システムよる反応時間の有意な改善が認められている．実薬群で消化器症状(悪心，嘔吐，食欲不振)が多かったが，それらは許容範囲のものであるとされる．また，治療による運動症状の悪化は示されていない．

c）ガランタミン

ガランタミンはDLB患者50例を対象とした24週間のオープン試験[16]では，治療により幻覚や夜間行動などの行動・心理症状(BPSD)の改善や臨床全般評価，認知機能(ADAS-cog)，睡眠障害の有意な改善が示されている．有害事象は一般的に軽症で一時的であったと報告されている．

2）PDDにおけるエビデンス

van Laarらのレビューによると，これまでにPDDに対するChEI治療の報告は，リバスチグミン6研究，ドネペジル11研究，ドネペジ

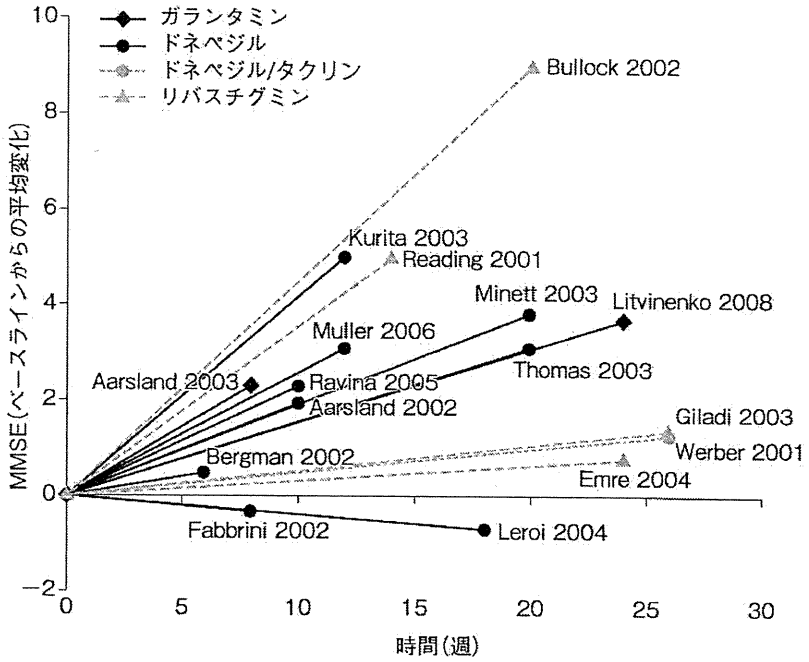

図3　PDD に対する ChEI 治療における MMSE の変化(文献 15 より改変引用)

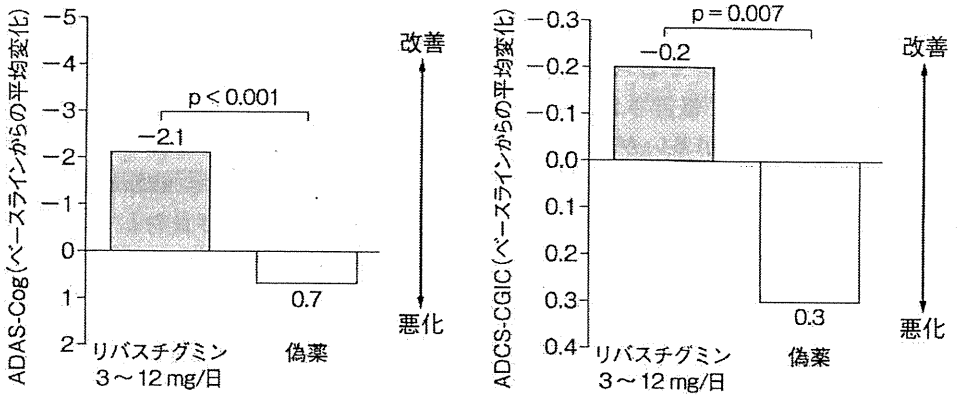

図4　リバスチグミンの大規模治験
24 週後の ADAS-Cog(認知症状)と ADCS-CGIC(臨床全般評価)の変化

ル＋タクリン 1 研究，ガランタミン 2 研究がある[17]．対象患者については，平均年齢は 70 歳前後，Hoehn&Yahr ステージ 3，Mini-Mental State Examination(MMSE)が 20 点前後の軽度〜中等度の PDD 患者である．多くの報告が 40 例未満の小規模オープン試験である．500 例を超える大規模のランダム化プラセボ対照二重盲検試験はリバスチグミンとドネペジルの 2 つがあるが，後者は学会での報告にとどまっている．

図3は治療による MMSE の変化量を示したものであるが，治療効果は報告によりばらつきがあり，治療後に MMSE が減少している報告もあるが，多くの研究では MMSE の改善を認めており，平均 2 点程度の改善がある．また，ChEI による幻覚・妄想の改善も報告されている．

541 例のリバスチグミンの大規模 RCT では，24 週間の治療により ADAS-Cog や臨床全般評

価(ADCS-CGIC)の改善が報告されている(図4)．最も多い有害事象は消化器症状であった．運動症状については，治療群では振戦の増悪が10.2%あり，プラセボ群(3.9%)より有意に頻度が高かったが，UPDRS Ⅲへの影響はない程度である[18]．以上のエビデンスを基に米国食品医薬品局(FDA)は，PDDに対するリバスチグミンを承認している．

2．メマンチン

72例のPDDおよびDLB患者を対象としたRCT[19]はChEIの併用を可能としており，治療による臨床全般評価の有意な改善が示されている．その後に行われたオープン試験の結果では，メマンチン投与を中断した際に，メマンチン群では全般評価の有意な悪化が示されている[21]．

199例のPDDおよびDLB患者を対象としたRCT[20]ではChEIは併用されておらず，メマンチン単独治療の結果である．DLB患者では，臨床全般評価の有意な改善やNPIによるBPSDの改善が示されている．一方，PDD患者ではプラセボ群と有意な差は見出せておらず，DLBとPDDの治療効果の差異が報告されている．その原因ついては不明な点が多いが，PDDと比べてDLBでは発症起点にアミロイドβの関与が強く，その相違が治療成績の差異として現れた可能性があると考察されている．

3．抑肝散

少なくとも6カ月間のドネペジル5 mg/日の投与にもかかわらず，幻視や精神症状が持続した14例のDLB患者対して抑肝散を投与した小規模オープン試験があり，抑肝散7.5 g/日・1カ月間の投与によりNPI総スコアやNPI幻覚サブスコアの有意な改善がみられ，Barthel indexの改善も示されている．治療によるMMSEの有意な変化はなく，認知機能の悪化はなかったと報告されている[22]．多施設オープン試験では，抑肝散7.5 g/日・4週間投与により，NPI，Zarit介護負担尺度，MMSE，Behave-AD睡眠項目の有意な改善が示されている[23]．

日常診療におけるDLB・PDD治療の留意点

1．抗認知症薬

DLB患者およびPDD患者の認知機能の改善を目的に，抗認知症薬であるChEIが使用されている．わが国においては保険適用外である．複数のChEIが使用可能となったが，ChEIの効果を直接比較した試験はないため，明確な差異は示されていない．よって治療薬の選択は治療医師に委ねられる．副作用としては，悪心，嘔吐，食思不振などの消化器症状が共通して認められる．パーキンソニズムの増悪する症例もあるため，認知機能のみならず運動機能にも着目しフォローアップする．特にPDD患者では振戦の増悪に気をつける．幻覚・妄想が増悪し，焦燥性興奮を伴うなどBPSDが増悪する症例がある．ChEIに対しても薬剤過敏を呈する症例があるため，AD患者に通常使用する薬剤量よりも少量で開始し，症状の推移をみながらゆっくりと漸増を図ることを考慮してもよい．実際に，薬剤を微調整(ドネペジルでは0.5～1 mg単位)することで，BPSDの増悪なく認知機能改善を導けた症例も経験するため治療量の工夫が大切である．また，認知機能のみならず幻覚・妄想などのBPSDに対してもChEI治療で改善がみられることがある．BPSDに対して抑制系薬剤(抗精神病薬や抑肝散)を用いる前に，ChEIの治療効果を試してみる．ChEIを使用するに当たり患者および家族には，保険適応外であること，消化器症状などの副作用の発現やBPSDの増悪などの可能性を説明し同意のもとで使用する．

PDD患者とDLB患者に対するメマンチン治療効果に差異が存在する．症例報告のレベルではあるが，メマンチン治療によるBPSDが増悪したDLB症例も報告されており，DLB・PDD患者に対するメマンチン治療については，投与量や患者選択を含めた使用法の検討が必要であり今後の症例の蓄積が待たれる．

2. そのほかの薬剤

DLB・PDDのBPSDに対して抗精神病薬や抑肝散が使用されている．錐体外路症状の発現が少ないなどの理由より非定型抗精神病薬が使用される場合が多い．海外においてはクロザピンの有効性を示すエビデンスが報告されているが[24]，わが国においては無顆粒球症などの副作用から使用制限があるため，クエチアピンが使用されることが多い．PDDを対象とした比較試験でクエチアピンは，クロザピンと同等の効果が報告されている[25,26]．非定型抗精神病薬の中には糖尿病が禁忌である薬剤があり，使用中は血糖の推移を観察する必要がある．ハロペリドール，クロールプロマジン，オランザピン，リスペリドンはD_2遮断作用があり運動機能を悪化させるため，使用を避けるべきである．また，ドパミンの部分アゴニスト/アンタゴニストのアリピプラゾールは一般的に運動機能を悪化させる．

抗精神病薬を使用する際には，保険適応外であること，死亡率の増加が報告されFDAから警告が出されているなどを説明した上で，有効性や副作用などについて十分に説明し，使用の同意を得ておく必要がある．また，DLBやPDDには抗精神病薬過敏性があるため，症状をコントロールし，できる最低限の薬剤投与で加療することが大切で，短期間で治療効果を判断し漫然とした使用は避けることが望ましい．

DLB・PDDには睡眠障害が多く合併し，特にレム期睡眠行動異常症(RBD)がみられる．RBDに対してクロナゼパム，ChEI，ミルタザピン，抑肝散，レボドパなどの使用症例が報告されている．最近では，メマンチンのRBDに対する効果が24週のRCTで検証されている[27]．

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Number of CGG repeats in the *FMR1* gene of Japanese patients with primary ovarian insufficiency

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Objective: To define the number of CGG repeats in the *FMR1* gene of Japanese patients with primary ovarian insufficiency (POI) and normal controls.
Design: Retrospective, controlled cohort study.
Setting: Outpatient department of an academic tertiary center.
Patient(s): One hundred twenty-eight consecutive Japanese patients with sporadic, nonsyndromic POI and 98 controls with normal menstruation.
Intervention(s): Deoxyribonucleic acid was obtained from the plasma of each subject.
Main Outcome Measure(s): Differences in the distribution of CGG repeat numbers between patients with POI and controls.
Result(s): Six alleles in the intermediate range and two in the premutation range were found in five and two patients with POI, respectively, but none were identified in normal controls. The prevalence of *FMR1* premutation among Japanese POI patients was 1.56% (2 of 128). The prevalence of having >36 CGG repeats in the *FMR1* gene was significantly higher in patients with POI than in controls, and age at the onset of amenorrhea was significantly lower in patients with >38 repeats.
Conclusion(s): More than 36 CGG repeats in the *FMR1* might intensify the etiology of POI, at least up to the premutation range. (Fertil Steril® 2011;96:1170–4. ©2011 by American Society for Reproductive Medicine.)

Key Words: Primary ovarian insufficiency, *FMR1*, CGG repeat, premutation allele

Primary ovarian insufficiency (POI) refers to the development of hypergonadotropic hypogonadism before the age of 40 years (1). The reported prevalence of POI among women is 1% (2), and the etiology of the spontaneous type is essentially unknown.

The fragile X mental retardation 1 (*FMR1*) gene, which contains a polymorphic CGG trinucleotide repeat in its 5′ untranslated region, is associated with POI. The fully expanded form, which contains >200 CGG repeats, causes the loss of the RNA-binding FMR1 protein and results in the fragile X syndrome phenotype (3, 4). Premutation alleles that expand to >200 repeats over several generations have been defined in families with FMR syndrome. The premutation range is defined as being between 50 and 199 repeats. Premutation carriers have an increased prevalence of POI (5–7), which ranges from 13% to 26% (6, 8). Recent reports indicate that, in addition to POI, the distribution of age at menopause onset among premutation carriers is shifted approximately 5 years earlier compared with that of noncarriers (8–11).

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Intermediate alleles are alleles with fewer repeats than the premutation range, but they have the potential to become unstable when transmitted from generation to generation. They have been defined by different groups as containing from 41 to 60 (8), 45 to 54 (12), and 46 to 60 (13) repeats.

Recent studies have indicated that infertile women tend to have more *FMR1* CGG repeats than normal controls, even if they are considered to be within the normal range (14). Bretherick et al. (15) and Bodega et al. (16) reported that increased risk of POI is not restricted to the premature range of CGG repeats but also to the high ends of the normal and intermediate ranges. Some studies have suggested that a deviation in CGG repeat number from the narrow normal range also causes occult ovarian insufficiency and early ovarian senescence (14). Studies of infertile patients have indicated that the presence of >30 triplet CGG repeats confers increased risk and severity of occult ovarian insufficiency in parallel with increasing expansion (14, 17–19). With regard to ovarian reserve, Gleicher et al. (17) indicated that the normal range of CGG repeats is 26–34 with a median of 30 (18, 20), which encompasses the distribution peaks described herein and by Chen et al. (21). These findings suggest that the range of CGG repeats associated with ovarian reserve differs from that associated with neuro/psychiatric risk (22, 23) and that a repeat number of >30 is associated with decreased ovarian reserve.

The number of CGG repeats in Asians has a characteristic secondary peak of 34–36 repeats in addition to the most frequent peak of 29 to 30 (9). Studies have indicated numbers of <40 CGG repeats in normal Japanese populations, with a minor population showing a peak at 36 repeats in addition to peaks at 29 and 30 repeats (24, 25). However, the distribution of CGG repeats in *FMR1* among

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TABLE 1

Characteristics of patients with POI (n = 128) and control women (n = 98).

Characteristic	Patients		Controls	
	Mean ± SE	Range	Mean ± SE	Range
Age (y)				
At examination	37.82 ± 0.50	20–54	39.12 ± 1.09	21–59
At onset of amenorrhea	29.5 ± 0.61	12–39		
CGG count				
Short allele	28.88 ± 0.23	12–43	28.90 ± 0.23	20–36
Long allele	32.91 ± 0.51	26–68	31.90 ± 0.33	23–40

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East Asian POI patients, including Japanese, has never been reported. Furthermore, the association between CGG repeat size and age at the onset of amenorrhea, which might reflect disease severity in patients with POI, has never been examined. The present study compares the number of CGG repeats in the *FMR1* gene between Japanese patients with POI and normal controls and investigates the relationship between the CGG repeat numbers and the age at onset of amenorrhea in these patients.

MATERIALS AND METHODS

We investigated 128 Japanese female patients aged 37.8 ± 0.50 (mean ± SE; range, 20–54) years with normo-karyotypic (46,XX), sporadic, and nonsyndromic POI, who were aged 29.5 ± 0.61 (range, 12–39) years at the onset of amenorrhea (Table 1). The patients provided written, informed consent to participate in the study, which was approved by the Ethics Committee for Human Genome/Gene Research at St. Marianna University School of Medicine. They were also treated at the Center of Reproductive Medicine, Department of Obstetrics and Gynecology at the same institution. The diagnosis of POI was based on criteria comprising ≥3 months of amenorrhea, <40 years of age, and having serum FSH levels of ≥40 IU/L on two consecutive occasions. None of the patients had a family history of early menopause or mental retardation. They were phenotypically normal and had no history of pelvic surgery or chemo/radiotherapy. We also analyzed blood samples from 98 normal Japanese women aged 39.1 ± 1.09 (range, 21–59) years with proven fertility and normal menstruation or who had undergone normal menopause. These samples were obtained from the Japanese Collection of Research Bioresources.

White blood cells were isolated from these blood samples by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare), and genomic DNA extracted from the cells using a PUREGENE DNA isolation kit (Gentra Systems) served as polymerase chain reaction templates. The sense and antisense sequences of the primer set for *FMR1* were 5′-gctcagctccgtttcggtttcacttccggt-3′ and 5′-agccccgcacttccaccaccagctcctcca-3′, respectively. Partial sequences containing CGG repeats of the *FMR1* gene were specifically amplified as described by Fu et al. (26). Polymerase chain reaction products were sequenced using sense or antisense primers and an ABI 3100 Avant sequencer (Applied Biosystems).

Correlations between *FMR1* CGG repeat size and age at the onset of amenorrhea in the patients were analyzed by linear regression. Other data were statistically analyzed using the Mann-Whitney *U* and Fisher exact tests. A *P* value of .05 was considered to indicate statistical significance.

RESULTS

We estimated the numbers of CGG repeats in the *FMR1* gene of 128 Japanese patients with POI and in 98 controls. Table 1 shows the characteristics of the patients and controls.

Figure 1 shows the distribution of long and short alleles (Fig. 1A), long alleles (Fig. 1B), and short alleles (Fig. 1C) in the patients and controls. The number of repeats in all alleles was ≤40, except for six in the intermediate range defined by Sullivan et al. (8) from five patients, and two in the premutation range from two patients (Table 2). We found common peaks at 29 and 30 repeats (Fig. 1), as well as at 36 repeats in approximately 10% of all alleles and in approximately 20% of long alleles in both the patients and controls (Fig. 1).

Alleles with >40 CGG repeats (the lower limit of the intermediate zone of reported definitions) (8) were identified only in the POI patients (Table 2), and the prevalence of alleles with >36 repeats (secondary modal frequency) was significantly higher in the POI patients than in the controls (Table 2).

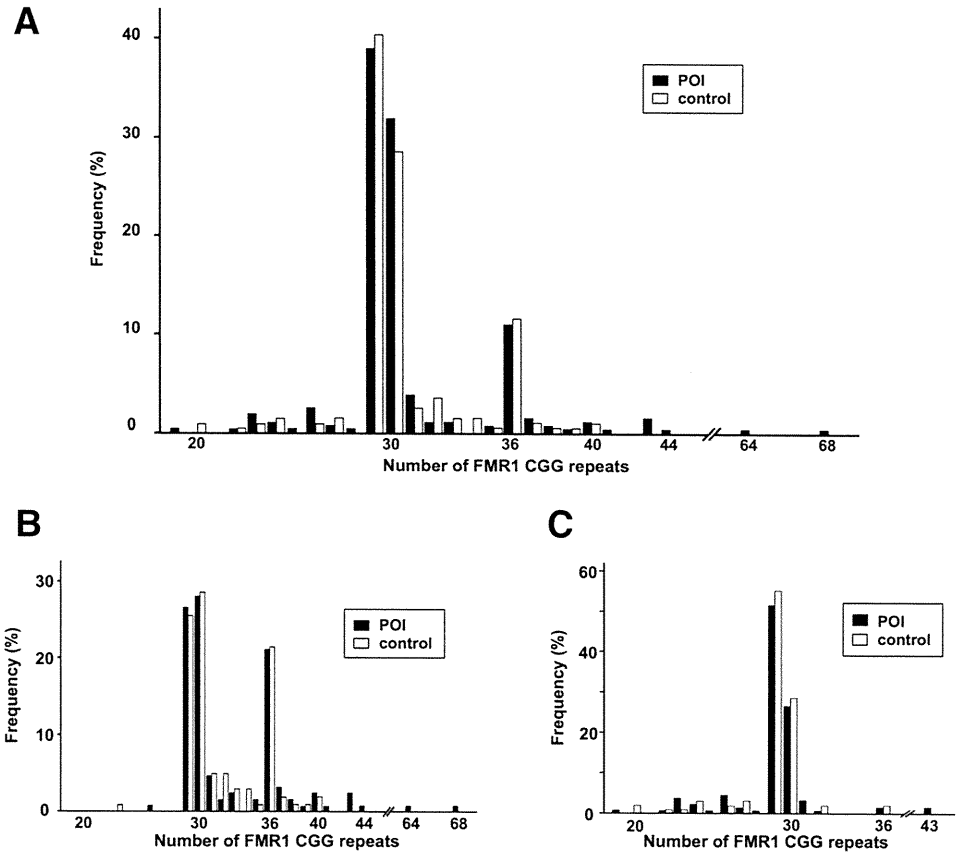
The onset of amenorrhea occurred significantly earlier in the patients with >38 CGG repeats than in the patients with ≤38 CGG repeats (29.9 ± 0.62 vs. 25.1 ± 2.50 years, $P<.05$; Fig. 2).

DISCUSSION

The present study demonstrated that the distribution of CGG repeats in the *FMR1* gene of Japanese patients with POI was identical to that of normal controls, except for five and two patients with repeat numbers in the intermediate (8) and premutation ranges, respectively. The distribution of CGG repeats in our study participants was similar to that reported for Japanese in studies of 370 unrelated, non–mentally retarded males (24) and 576 normal males and 370 normal females (25), all of whom had a primary modal peak at 29 to 30 or 28 to 29 repeats (24, 25) and a secondary modal peak at 36 or 33 to 34 (24, 25). These secondary peaks were not identified in studies of Western populations (9, 24–26). The secondary peak found in the present study was at 36 repeats but was reported at 33 and 34 repeats in the studies of Arinami et al. (24) and Otsuka et al. (25), respectively. These differences might be associated with the automated sequencer methodology and whether AGG interruptions in the sequence were included in the count. Chen et al. (9) reported that the most common pattern of triplets is $(CGG)_9$ AGG $(CGG)_9$ AGG $(CGG)_9$. The modal peaks in Japanese females described by Otsuka et al. (25) were at 27 and 33 or 34 repeats, suggesting that the difference in peaks is due to the inclusion or exclusion of the triplet AGG within the sequence. Chen et al. (9) also showed that the minor modal frequency with 36 repeats among Asian populations typically has the sequence $(CGG)_9$ AGG $(CGG)_9$ AGG $(CGG)_6$ AGG $(CGG)_9$. We demonstrated here that 20% of long alleles and 10% of total alleles showed a peak at 36 repeats in both patients and normal controls, suggesting that this ratio of Japanese women have the same pattern of triplets described by Chen et al. (9).

FIGURE 1

Distribution of *FMR1* CGG repeat numbers in Japanese patients with POI (*filled columns*) and controls (*open columns*). (**A**) Data from both alleles (n = 256 and n = 196 for patients and controls, respectively). (**B**, **C**) Data from the long (**B**) and short (**C**) alleles (n = 128 and n = 98 for patients and controls, respectively).



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The present study found a significant increase in the number of *FMR1* alleles with >36 CGG repeats among Japanese POI patients as compared with normal controls. Repeats of >40 were observed only in POI patients. This is in accordance with the findings of Bretherik et al. (15) that demonstrated a significantly increased number of *FMR1* alleles with between 35 and 54 CGG repeats in the POI patient population as compared with the general population. Furthermore, this study is the first to demonstrate a 1.56% (2 of 128)

TABLE 2

Distribution of CGG repeat numbers in each category of patients with POI and controls.

Category	Range of CGG repeat number	Patients		Controls	
		Patients, n (%)	Alleles, n (%)	Women, n (%)	Alleles, n (%)
Normal	≤30	71 (55.5)	191 (74.6)	54 (55.1)	148 (75.5)
	31–36	40 (31.3)	47 (18.4)	38 (38.8)	42 (21.4)
	37–40	10 (7.8)	10 (3.9)	6 (6.1)	6 (3.1)
Intermediate	41–54	5 (3.9)	6 (2.3)	0	0
Premutation	55–200	2 (1.6)	2 (0.8)	0	0
Total		128 (100)	256 (100)	98 (100)	196 (100)

Note: Prevalence of >36 repeats is higher in patients with POI than in controls (Fisher's exact test).

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frequency of *FMR1* premutations among Japanese patients with POI. The frequency of premutation carriers among women with the sporadic form of POI ranges between 1.6% and 3.3% (27, 28–31) in Caucasian populations, and one study has described a frequency of 4.8% in a group of screened Slovenian patients (32). A study of 80 Indian patients with POI revealed that none of them had CGG repeats within the premutation range (33). The data from these studies and the present study indicate that fewer Asian than Caucasian patients with POI have CGG repeats in the premutation range (11, 29, 31, 32).

The American College of Obstetricians and Gynecologists Committee on Genetics (12) describes a prevalence of 1 in 250 for premutation alleles among the general population, whereas these alleles were not found in the studies of Arinami et al. (24) (370 unrelated Japanese males), Otsuka et al. (25) (946 normal Japanese males and females), and in the present study that included 98 normal females. These findings suggest that the prevalence of premutation alleles in the general population is subject to race/ethnic differences that also affect the prevalence of premutation alleles among patients with POI.

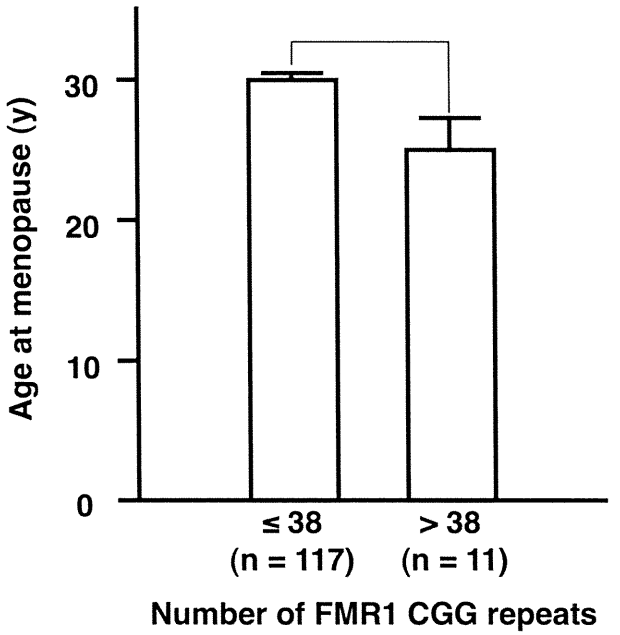
As mentioned in the introduction, Gleicher et al. (17) indicated that, with regard to ovarian reserve, the normal range of CGG repeats is 26–34. In accordance with their data, the present study showed a significantly higher prevalence of CGG repeat numbers >36 in POI patients as compared with controls. This indicates that with regard to ovarian reserve, the secondary peak at 36 repeats seen in the present study on Japanese may mark the higher limit of the normal range.

Gleicher et al. (34) also reported recently on ovarian function-specific genotypes defined according to *FMR1* CGG repeat numbers, namely, norm: both alleles are within the normal range of 26–30 repeats; hom: both alleles are outside of the normal range; het-norm/high: one allele is above, and one is within, the normal range; and het-norm/low: one allele is below, and one is within, the normal range. However, we found no difference in the prevalence of these genotypes between POI patients and controls. Additionally, there was no significant difference in the age at onset of amenorrhea in POI patients according to the differences in these genotypes. However, this should be studied further with larger populations of patients.

The present study demonstrated that the prevalence of having >36 CGG repeats within the intermediate range was higher in patients with POI than in controls. We also showed that the onset of amenorrhea occurred significantly earlier in patients with >38 CGG repeats than in those with ≤38 CGG repeats. These data may indicate that aside from the various direct etiologies of POI, *FMR1* containing >36 CGG repeats, which is the secondary peak of CGG repeat size in Asians, might additionally damage the preservation of the ovarian reserve.

FIGURE 2

Relationship between *FMR1* CGG repeat number on the long allele and age at amenorrhea onset in patients with POI. Comparison of average age at amenorrhea onset between POI patients with ≤38 CGG repeats (29.9 ± 0.62 years; n = 117) and with >38 repeats (25.1 ± 2.5 years; n = 11). Significant difference was found at the 5% level (Mann-Whitney *U* test).



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In conclusion, we examined the number of CGG repeats in the *FMR1* gene of 128 Japanese patients with POI and in 98 normal controls and found similar distribution between the patients and controls when the number of repeats was ≤36. In contrast, the prevalence of having >36 repeats within the intermediate and premutation ranges is higher in patients with POI than in controls. Among POI patients, age at the onset of amenorrhea was significantly lower when the number of repeats was >38, suggesting that the expansion of CGG repeats in the *FMR1* gene over the common, secondary peak frequency of 36 repeats intensifies the etiology of POI in Japanese.

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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.

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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 cosegregated 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). Underexcretion 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 $\times$ 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

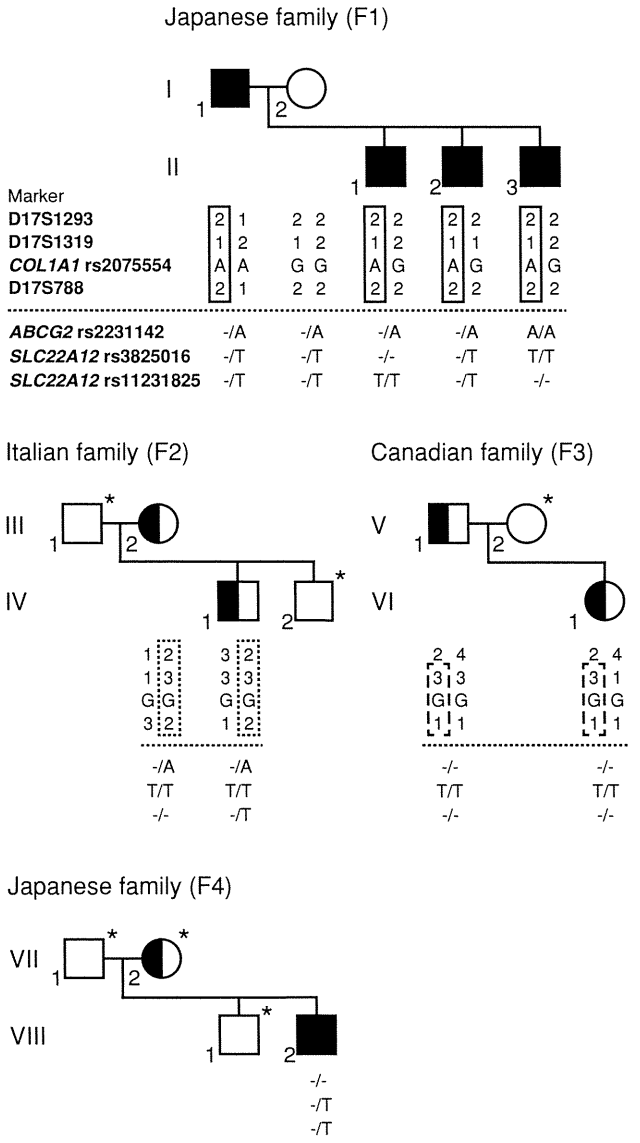

Fig. 1 Pedigrees, haplotypes and genotypes of the Japanese (F1 and F4), Italian (F2) and Canadian (F3) families. Patients in F1, F2 and F3 have c.3235G>A predicting p.G1079S in *COL1A1*. A patient in F4 has c.577G>T predicting p.G193C in *COL1A2*. *Closed symbols* indicate patients with OI and with hyperuricemia. *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′-CGT GTCTTCAGCACAAAATGG-3′ and 5′-AGAAGCCGTA GCACCACTCC-3′, respectively. The reverse primers were 5′-GGCCCAATCCATAAGTACAT-3′ and 5′-CCCA TGATCTCTTCCTGGAG-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

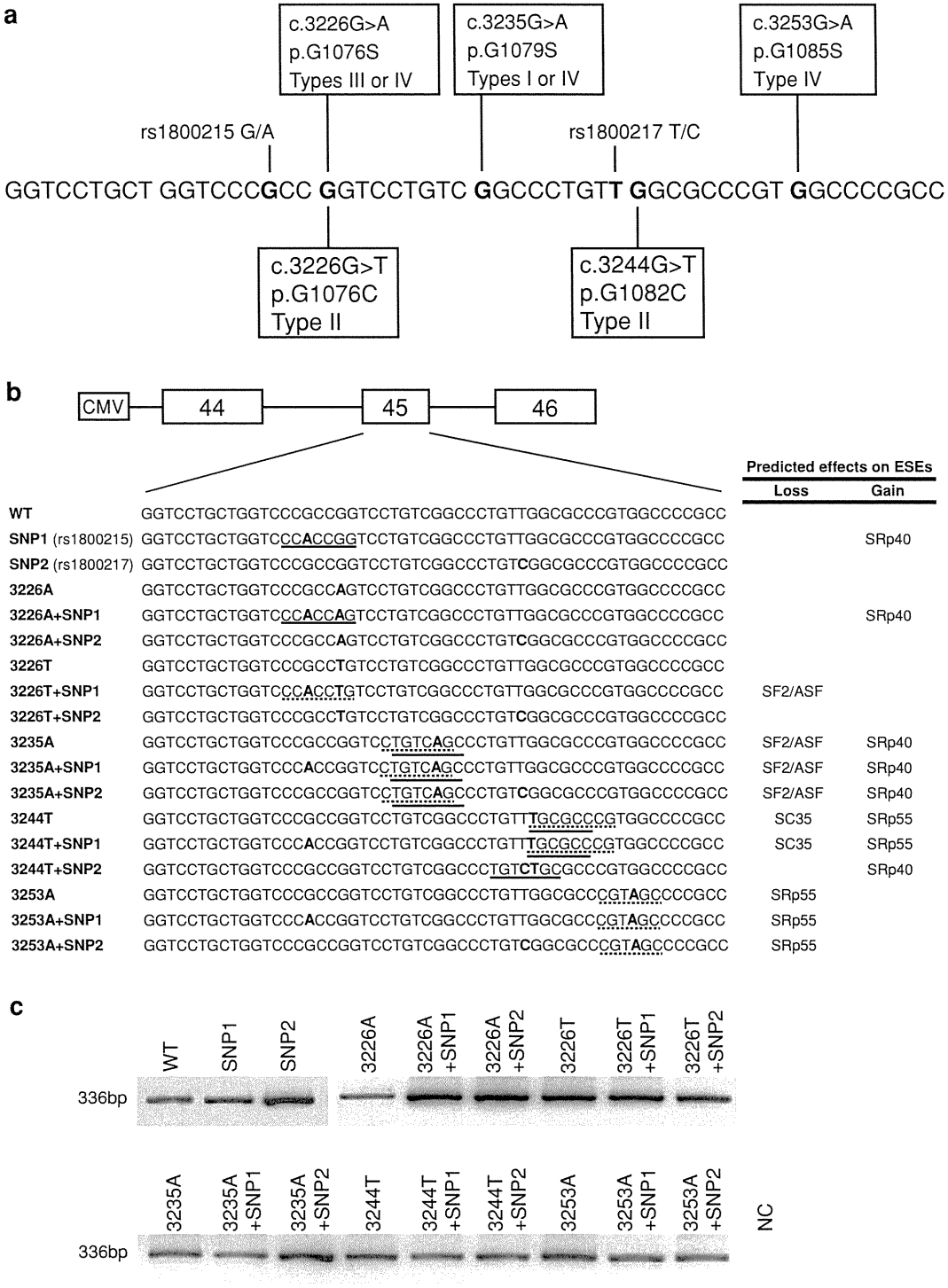

Fig. 2 Splicing assays of *COL1A1* minigenes in HEK293 cells. **a** Positions and phenotypes of OI mutations in *COL1A1* exon 45. Mutations above the wild-type sequence exhibit non-lethal phenotypes, whereas those below the sequence cause a lethal phenotype. **b** Schematic representation of *COL1A1* 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

Table 1 Affected putative splicing *cis*-elements in *COL1A1* exon 45 predicted by ESEfinder, ESRsearch and PESXs

Sequence variation	Normal allele	Variant allele	Predicted effect	Score[d]		Putative *trans*-factor
				Normal	Variant	
rs1800215	CC**G**CCGG	CC**A**CCGG[a]	Gain	1.622	4.231	SRp40
rs1800217	CTGT**T**GGC	CTGT**C**GGC[c]	Gain		n.a.	
c.3226G>A	CGCC**G**G	CGCC**A**G[b]	Gain		n.a.	
	CC**G**GTCCT	CC**A**GTCCT[c]	Gain		n.a.	
c.3226G>A+rs1800215	CC**G**CC**G**G	CC**A**CC**A**G[a]	Gain	1.622	3.663	SRp40
c.3226G>T	CGCC**G**G	CGCC**T**G[b]	Gain		n.a.	
c.3226G>T+rs1800215	CC**G**CC**G**G	CC**A**CC**T**G[a]	Loss	3.498	0.571	SF2/ASF
c.3235G>A	CTGTC**G**G	CTGTC**A**G[a]	Loss	2.492	0.713	SF2/ASF
	CTGTC**G**GC	CTGTC**A**GC[c]	Loss		n.a.	
	TGTC**G**GC	TGTC**A**GC[a]	Gain	1.058	3.613	SRp40
	GTC**G**GC	GTC**A**GC[b]	Gain		n.a.	
	C**G**GCCCTG	C**A**GCCCTG[c]	Gain		n.a.	
c.3244G>T	**G**GCGCCCG	**T**GCGCCCG[a]	Loss	3.109	1.059	SC35
	GGCGCC	**T**GCGCC[a]	Gain	0.721	3.531	SRp55
c.3244G>T+rs1800217	TGT**T**GGC	TGT**C**TGC[a]	Gain	−1.326	3.367	SRp40
c.3253G>A	CGT**G**GC	CGT**A**GC[a]	Loss	2.940	2.331	SRp55

Variant nucleotides are shown in bold and underlined

n.a. not applicable

[a] ESEfinder

[b] ESRsearch

[c] PESXs

[d] Default threshold values employed by ESEfinder are SRp40 = 2.67, SF2/ASF = 1.867, SC35 = 2.383 and SRp55 = 2.676

12.5% regions of the entire genome. We first sought for a mutation responsible for hyperuricemia in 24 genes (see Table 3 and Suppl. Table 2) that are involved in urate metabolisms and excretion, but found none in either patient. We then scrutinized SNPs in ten genes that are known to be associated with hyperuricemia and identified 12 SNPs (Table 3). In this analysis, we excluded SNPs with a minor allelic frequency of 0.01 or less. Among the 12 SNPs, rs2231142 in *ABCG2* as well as rs3825016 and rs11231825 in *SLC22A12* are previously reported and will be addressed in the discussion. We traced the three SNPs in F1, F2 and F3 by capillary sequencing and found that variable dosages of these SNPs were observed in hyperuricemic as well as in normouricemic individuals (Fig. 1).

A missense mutation in *GPATCH8* is likely to lead to hyperuricemia in the Japanese family (F1)

After eliminating SNPs in the dbSNP132 database, only three non-synonymous variants remained shared between II-2 and II-3 on chr 17; c.602A>G in exon 2 of *KRBA2* at 17p13.1, c.206C>T in exon 3 of *ZPBP2* (MIM# 608499) at 17q12 (Fig. 3a) and c.2935G>C in exon 8 of *GPATCH8* at 17q21.31 (Fig. 3b). Capillary sequencing revealed that variants in *ZPBP2* and *GPATCH8* cosegregated with hyperuricemia in F1, but the *KRBA2* variant did not. These two variants were not detected in F2, F3, F4, 100 normal human individuals or exomes of 50 Tibetans (Yi et al. 2010). In addition, exome-capture resequencing of VIII-2 detected no mutations in *ZPBP2* and *GPATCH8*.

ZPBP2 c.206C>T and *GPATCH8* c.2935G>C predict amino acid substitutions of p.T69I and p.A979P, respectively. Threonine 69 and the flanking amino acids of *ZPBP2* are not conserved across mammalian species. Additionally, an SNP rs35591738 mutates the N-terminal proline at codon 68, and an SNP rs34272593 causes a frameshift at codon 70 (Fig. 3a). In contrast, alanine 979 and the flanking amino acids of *GPATCH8* are in the serine-rich region and are highly conserved across mammalian species (Fig. 3b). PolyPhen-2 (Adzhubei et al. 2010) predicted that *ZPBP2* p.T69I was benign with a score of 0.025 and *GPATCH8* p.A979P was damaging with a score of 0.988, where 1.0 was the worst score. Similarly, SIFT (Kumar et al. 2009) predicted that *ZPBP2* p.T69I was tolerated with a score of 0.38 and *GPATCH8* p.A979P was damaging with a score of 0.00, where a score < 0.05 was predicted to be deleterious.