厚生労働科学研究費補助金

難治性疾患等政策研究事業

# 染色体微細欠失重複症候群の包括的診療体制の構築

平成３０年度　総括・分担研究報告書

研究代表者　倉橋　浩樹

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研究代表者　倉橋　浩樹
藤田医科大学・総合医科学研究所・分子遺伝学・教授

研究要旨
本研究では、マイクロアレイ染色体検査により診断される、多発奇形・発達遅滞を主症状とする染色体微細欠失重複症候群の包括的診療体制の構築を目的として、国内の多施設共同研究により、代表的な 31 疾患に関して、全国調査による国内患者の把握や、診療情報の収集と分析、診断基準、診療ガイドラインの策定を実施する。本年度は、実臨床の中での新規患者の掘り起こしに向けたマイクロアレイ染色体検査をおこなった。また、すでに指定難病になっている代表的な 5 疾患に関して、とくに成人期移行という観点からの分析を行った。その結果、エマヌエル症候群（11/22 混合トリソミー）に関して、10 名ほどの成人期の患者を把握し、情報収集を行うことができた。地方の中心都市２箇所で小規模の患者会を開催し、成人期患者と小児期患者の家族を繋ぐことができた。今後、地域の医療機関の内科系医療従事者に疾患の認知度を高めていく啓発活動が重要であると思われた。

研究分担者
大橋博文　埼玉県立小児医療センター・遺伝科・科長兼部長
黒澤健司　地方独立行政法人神奈川県立病院機構神奈川県立こども医療センター・遺伝科・部長
山本俊至　東京女子医科大学・遺伝子医療センター・教授
涌井敬子　信州大学医学部・遺伝医学教室・講師

## A. 研究目的

染色体の欠失や重複のような微細構造異常によるコピー数の変化（copy number variation: CNV）は、量的効果により遺伝子機能に直接影響するため、先天性疾患や知的障害の原因となることが多い。CNV のある染色体領域に依存して含まれる遺伝子の種類が異なるので、症状にはバリエーションがある。ただ、CNV に含まれる数多くの遺伝子量が同時に変化するため、症状は複数の遺伝子の量的変化の効果の合算として現れ（隣接遺伝子症候群）、単一遺伝子病よりも重症となることが多い。従来は G 分染法による染色体検査によるスクリーニングや、特定の疾患に関しては FISH 法での診断が行われてきたが、近年、マイクロアレイ染色体検査が臨床応用され、検出感度が飛躍的に向上した。欧米では 2005 年頃から臨床応用され、多発奇形・発達遅滞の患者で G 分染法では３%であった異常検出率が、マイクロアレイ染色体検査の導入により、15-20%の患者で責任変異を同定できるとされ、すでに欧米では多発奇形・発達遅滞の原因の精査としては従来の染色体検査にかわる第 1 選択の診断ツールとされている。日本でも、マイクロアレイ染色体検査が診断に必須な疾患が指定難病や小児慢性特定疾患に認定されはじめ、その臨床的有用性は高いという認識は拡大しているものの、一方で、高コストという問題があり、まだ保険収載されていない。

研究代表者を含む本研究班員はこれまで、厚労省難治性疾患克服研究事業の支援も受け、マイクロアレイ染色体検査で診断されるような多発奇形・発達遅滞の患者の診療をおこなう中で、個々の疾患の診断基準や重症度基準、診療ガイドライン作成を行ってきた。一部の代表的な疾患に関してはすでに先行研究班で臨床的実態調査がなされており、小児期の疾患の自然歴に関しては十分な情報が集まった。一方で、患者さんの多くは小児期の医療管理の充実化により疾患の予後が改善し長期生存が可能となっており、成人期治療へのトランジッションが重要となってきたが、これら稀少疾患の成人期の臨床情報は皆無に等しい。直面している患者さんやご家族は移行期や成人期の疾患の臨床情報を必要としており、また、小児期の患者さんのご家族も安心材料としての長期的な情報を欲している。そこで、本研究では先行研究を継続する形で、3 年間を通じて、現在すでに作成している患者レジストリーを利用して、長期生存例の直近の情報を入手し、疫学的調査を行うことを第一の目的とする。1 年目には、代表的な 5 疾患（1p36 欠失症候群、4p 欠失症候群、5p 欠失症候群、スミスマゲニス症候群、エマヌエル症候群）に関して、成人期患者の情報収集を行う。2 年目は、収集した情報を分析して、当該疾患の症状の特徴を抽出し、その対処法や合併症の予防法をリスト化する。そして、3 年目には、代表的な 5 疾患に関して、成人期移行も踏まえた新たな診療ガイドラインの作成を行う。そして、対象疾患の残りの 26 疾患に関しては、頻度の低い疾患群なので、引き続き患者サンプルの収集とマイクロアレイ染色体検査を行い、ある程度の情報が集積した疾患から、順次、新規に診断基準の策定などを行うことを目標とする。

B. 研究方法

代表的な 5 疾患に関しては、先行研究ですでに作成している患者レジストリーを利用して、患者情報を、とくに長期生存例の直近の情報を入手し、疫学的調査を行う。研究代表者倉橋浩樹がエマヌエル症候群（指定難病２０４）、大橋博文（以下、敬称略）が 5p 欠失症候群（指定難病１０９）、黒澤健司がスミスマゲニス症候群（指定難病２０２）、山本俊至が 1p36 欠失症候群（指定難病１９７）、涌井敬子が 4p 欠失症候群（指定難病１９８）を担当する。また、リストの 32 疾患（研究代表者を含む研究分担者 5 名が 6 疾患ずつを担当）（資料１）に関しては、患者数の少ない稀少疾患であるので、さらに多くの診断未確定患者の発掘のために、日本全国の主な診療施設の小児科もしくは遺伝診療科に連絡を取り、染色体微細構造異常が疑われるような多発奇形・発達遅滞の患者のサーベイランス、患者登録を行う。この調査は、日本小児遺伝学会（黒澤健司理事長、本研究の研究分担者）との連携のもとに行う。集まった患者情報に基づいて、詳細な臨床情報と末梢血サンプルの収集を行う。末梢血サンプルに対しては、研究代表者を含む各研究分担者が個々の施設でマイクロアレイ染色体検査、必要に応じて FISH 解析にて診断を確定させる。最終的には、診断につながる臨床診断基準を策定し公開する。

（倫理面への配慮）

本研究は、ヒトゲノム・遺伝子解析研究に関する倫理指針、人を対象とする医学系研究に関する倫理指針を遵守して行った。解析試料の取得は書面でのインフォームドコンセントの上でおこない、研究対象者に対するプライバシーの保護など、人権擁護上の問題については十分に配慮したうえで行った。各関連施設から送付される試料は、試料提供機関において連結可能匿名化が行われ、研究代表者や研究分担者の所属機関には匿名化された試料と、予めチェックリストとして作成した臨床データのみが送付されることとした。試料は研究代表者や研究分担者の所属機関にて保管

し、研究期間終了後に同意書に基づき破棄を行う予定である。データは研究代表者や研究分担者の所属機関内の鍵のかかるキャビネットに研究期間内、保管する。報告又は発表に際しては、被験者のプライバシー保護に十分配慮する。偶発的所見を含めた、発生しうる諸問題には、各施設の遺伝カウンセリング部門が対応する。マイクロアレイ染色体検査や、アンケートによる疾患情報収集に関する研究は、すでに研究代表者や研究分担者の所属機関の倫理審査委員会の承認を得ている(「染色体コピー数異常症に関する研究」藤田医科大学・ヒトゲノム・遺伝子解析研究倫理審査委員会、HG13-003。「日本における t(11;22)染色体転座保因者およびエマヌエル症候群患者の疫学調査」藤田医科大学・医学研究倫理審査委員会、HM15-157。)。

C. 研究結果

（1）マイクロアレイ染色体検査について

研究代表者を含め各班員が、所属施設における実臨床の中での新規患者の発見に向けたマイクロアレイ染色体検査、ならびに診療情報のチェックを行った。本研究の対象疾患は、当研究班の前身である「マイクロアレイ染色体検査でみつかる染色体微細構造異常症候群の診療ガイドラインの確立研究班」の対象疾患 32 疾患から、*MECP2* 重複症候群を対象から外した 31 疾患となっている。これら染色体微細欠失重複症候群 31 疾患の掘り起こしを行った。

（2）染色体微細構造異常症 31 疾患の自然歴把握について

2018 年 8 月 26 日に、第 1 回「染色体微細欠失重複症候群の包括的診療体制の構築研究班」班会議を行った。研究代表者が研究計画の概要、班員の役割、予算の概要などを説明した。対象疾患が多すぎるとの意見があり、当面は、それぞれの担当する 5 つの代表的疾患（1p36 欠失症候群、4p 欠失症候群、5p 欠失症候群、スミスマゲニス症候群、11/22 混合トリソミー）に関する情報収集を行い、その他の疾患に関しては徐々に新規診断例を増やして、臨床情報を集めていくこととした。

（3）エマヌエル症候群について

エマヌエル症候群については、7 年前に完了した先行研究で国内 35 名の患者登録を行った。20 名にアンケート調査ができ、年長者は 15 歳以上が 5 名であった。その内訳は、15 歳、20 歳 2 名、27 歳、31 歳であった。身長は男性が-3SD 以下、女性は-1.5SD 以下、体重は男性、約-2.5SD 以下、女性は約−0.5SD 以下となり、成長障害がみられた。15 歳以下に比べるとミキサー食等の無形食から有形食への変化はあるものの、完全な普通食の摂取は難しく、成長期の栄養不足が原因であると考えられた。歩行は、補助者、補助具を用いて可能であったが、トイレの自立は難しい。聴覚は中耳炎に起因する軽度から中等度の問題が残っていたが、概ね聞こえており、家族の言葉を理解しているようであった。有意語は難しいが、指によるサイン等を獲得し、家族とコミュニケーションをとっている。患者が描いた絵や粘土細工による個展を開いた家族もある。エマヌエル症候群の患者は、様々な臨床症状を呈するが、成長とともに安定し、生命予後はよいと考えられた。また、重度の精神発達遅滞、運動発達遅滞を呈するが、個々にあったコミュニケーション法を獲得することで、患者、家族の QOL が向上すると考えられた。

先行研究の終了後、徐々に把握している患者は加齢し成人期に達する患者が増えている。また、追加で把握している患者の数も増加している。本研究においては、把握している成人患者に対するアンケート調査において成人期の情報を集めるために、成人例に焦点を当てた質問表作成に取り組んでいる。まずは、若年患者の家族が成人例の何を知りたいのかを事前に調査することとした。

藤田医科大学病院・臨床遺伝科（遺伝カウンセリング室）には、全国から新規に診断されたエマヌエル症候群の患者が来談される。その面談の中で、成人期の患者の家族に何を聞きたいのか、質問リストを作成してもらっている。また、本年度は福岡（2018 年 4 月 21 日）と仙台（2018 年 5 月 12 日）で数組みの患者家族が集まる小規模な患者会を開催し、そこで成人期患者の情報や、小児期患者の家族が何を知りたがっているのかの情報を収集した（資料２）。また、今後もこのような小規模の集まりができるよう、患者の居住地がわかるような「友だちマップ」というシステムを作成し、研究代表者が運営するエマヌエル症候群の患者と家族の支援サイトの上で運用している。患者の家族も、近隣で同じ疾患の家族がお互いの存在を認識できるようにするためのポスターを作成した。

D. 考察

染色体微細欠失重複症候群のような稀少難病は小児期に診断されるため、診断時に症例報告がなされ、小児期の臨床情報は容易に入手可能であるが、予後の改善により長期生存例が増え、シームレスな移行期・成人期治療へのトランジッションが重要となってきている。本研究では、代表的疾患の成人期情報を収集し、診療ガイドラインに反映させる形で公開することを目指している。

代表的疾患の解析から進めているが､患者会のようなフェイストゥーフェイスの機会が、患者の家族は先輩方から直接に情報を聞くことができるし、じかに成人期の患者と接触することでイメージが湧きやすい。また、底に参加した医療従事者も効率的に情報収集ができる。エマヌエル症候群の場合、長時間の移動が難しい患者が多いため、大都市で行われるような大規模な患者会では参加しにくいという問題がある。そこで、小規模ながらも各地方の主要都市で患者会を開催することができれば、短時間の移動で目的地に着くことができ、いろんな年齢層の患者の家族が患者の家族が集まる場ができ、そこで種々の情報を得ることができる。

本研究で成果が上がれば､小児期に患者登録をして、そのフォローアップ調査をしながら移行期・成人期の情報を充実化して行くというスキームができあがる。また、社会福祉としても、これまでは小児科が診療の中心であり、もともと公費負担が充実している中に、小児慢性特定疾患もあり、患者さんのご家族の負担は大きくなかったが、移行期・成人期となると障害者認定や難病指定が重要となる。個々の疾患単位での難病指定にも限界があるが、本研究のように類縁疾患を一括りにする方法により、小児慢性特定疾患の「常染色体異常」や、指定難病の「先天異常症候群」というような枠組みで対象疾患をまとめて分析して行く方法論ができ、他の疾患群にも応用可能となる。遺伝学的検査の保険収載に関しても、現行の枠組みは個々の疾患単位での FISH などのよる診断法に適用されているが､診断の時点では診断名が付いていないので実際的ではない。類縁疾患を一括りにして染色体微細欠失重複症候群の遺伝学的診断という名目の下に、マイクロアレイ染色体検査のような網羅的解析法を保険適応にして行くひとつのきっかけになれば良いと考えている。

E. 結論

本研究では、多発奇形・発達遅滞を主症状とする染色体微細欠失重複症候群の成人期移行を見据えた診療ガイドラインの確立を目的として、国内の多施設共同研究により、代表的な 5 疾患に関して、とくにエマヌエル症候群について全国調査による国内成人患者の実態調査を開始した。地域ごとの小規模患者会が充実すれば、医療サイドの対応に先行して、患者の家族が自ら対応して行ける体制が整うことが期待され、平行して進めてゆく。

F. **健康危険情報**

**特になし。**

G. **研究発表**

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(66) 河村理恵、加藤武馬、宮井俊輔、鈴木史彦、成悠希、田中敬子、長坂美和子、池田真理子、倉橋浩樹．Parthenogenetic maternal と double paternal allele を有するキメラ症例の解析．第 41 回日本小児遺伝学会学術集会、名古屋、Jan 11-12, 2019.

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H. 知的財産権の出願・登録状況

特になし

（資料１）

表1

## 染色体微細欠失重複症候群の包括的診療体制の構築

| | 対象疾患リスト | 担当 |
|---|---|---|
| 1 | 1p36欠失症候群 | 山本俊至 |
| 2 | 1q44欠失症候群 | 大橋博文 |
| 3 | 1q重複症候群 | 涌井敬子 |
| 4 | 2p15-p16.1欠失症候群 | 大橋博文 |
| 5 | Feingold症候群(2p24.3欠失) | 涌井敬子 |
| 6 | 2q23.1欠失症候群(*MBD5*) | 涌井敬子 |
| 7 | 2q24.2-q24.3欠失/重複症候群(*SCN1A*) | 山本俊至 |
| 8 | 2q32.1-q33.3欠失/重複症候群(*SATB2*) | 黒澤健司 |
| 9 | 2q37欠失症候群 | 大橋博文 |
| 10 | 3p21.31欠失症候群 | 大橋博文 |
| 11 | 4p16欠失(Wolf-Hirschhorn症候群) | 涌井敬子 |
| 12 | Cri-du-chat症候群(5pサブテロメア欠失) | 大橋博文 |
| 13 | 5q14.3欠失症候群(*MEF2C*) | 山本俊至 |
| 14 | 5q31欠失症候群 | 山本俊至 |
| 15 | 8pサブテロメア欠失 | 黒澤健司 |
| 16 | Langer-Giedion症候群(8q24.11欠失) | 倉橋浩樹 |
| 17 | 9q34欠失症候群 | 黒澤健司 |
| 18 | 11p12-p14欠失症候群 | 山本俊至 |
| 19 | Jacobsen症候群(11qサブテロメア欠失) | 倉橋浩樹 |
| 20 | 16p11.2欠失/重複症候群 | 山本俊至 |
| 21 | Miller-Dieker症候群(17pサブテロメア欠失) | 黒澤健司 |
| 22 | 17p13.1欠失症候群(*GABARAP*) | 黒澤健司 |
| 23 | Smith-Magenis症候群(17p11.2欠失) | 黒澤健司 |
| 24 | Potocki-Lupski症候群(17p11.2重複) | 涌井敬子 |
| 25 | 21qサブテロメア欠失症候群 | 黒澤健司 |
| 26 | Emanuel症候群(11/22混合トリソミー) | 倉橋浩樹 |
| 27 | 22q11.2重複症候群 | 倉橋浩樹 |
| 28 | Cat eye症候群(22q11テトラソミー) | 倉橋浩樹 |
| 29 | Phelan-McDermid症候群(22q13欠失) | 大橋博文 |
| 30 | Xp11.3-p11.4欠失(*MAOA*, *MAOB*, *CASK*) | 涌井敬子 |
| 31 | Xq11.1欠失症候群(*ARHGEF9*) | 山本俊至 |

（資料２）

# 福岡の患者会のことを記したブログ

## エマヌエル症候群 うちの子の場合

2017-08-21 10:28:34
テーマ：ブログ

日本で唯一、名古屋にある藤田保健衛生大学の倉橋先生がエマヌエル症候群の研究をされてます。

息子が生まれてから、一度カウンセリングに伺い、倉橋先生、大江先生に色んなお話をして頂きました。

そしてこの度、福岡に倉橋先生と大江先生が来てくださるという事で、家族会を開催する事になりました！！

日程 4月21日(土)
開催場所は福岡です。

もし、このブログを読んでるエマヌエル家族の方がいらっしゃいましたら、是非ご参加下さい！

コメント、もしくはメッセージ頂けましたら、詳細をお伝えします！

楽しみです♩

地域活動支援センター
サンサンはかた

施設には駐車場はありませんので、お越しの際は公共交通機関をご利用ください。
博多駅より徒歩１４分、東光二丁目バス停より徒歩２分です。

## エマヌエル症候群 うちの子の場合

2017-08-21 10:28:34
テーマ：ブログ

待ちに待った家族会に行ってきました！

新幹線に乗り、息子を連れて会場へ。

初めて会うご家族もいれば、息子と共に何度か会った事のあるご家族も。

初めは少し緊張しましたが、同じ子を持つ母として、想いは同じ。

悩んでる事もやっぱり似てる。

そして、同じエマヌエルの子ども達が集まってるのを見て、とても嬉しく思いました。

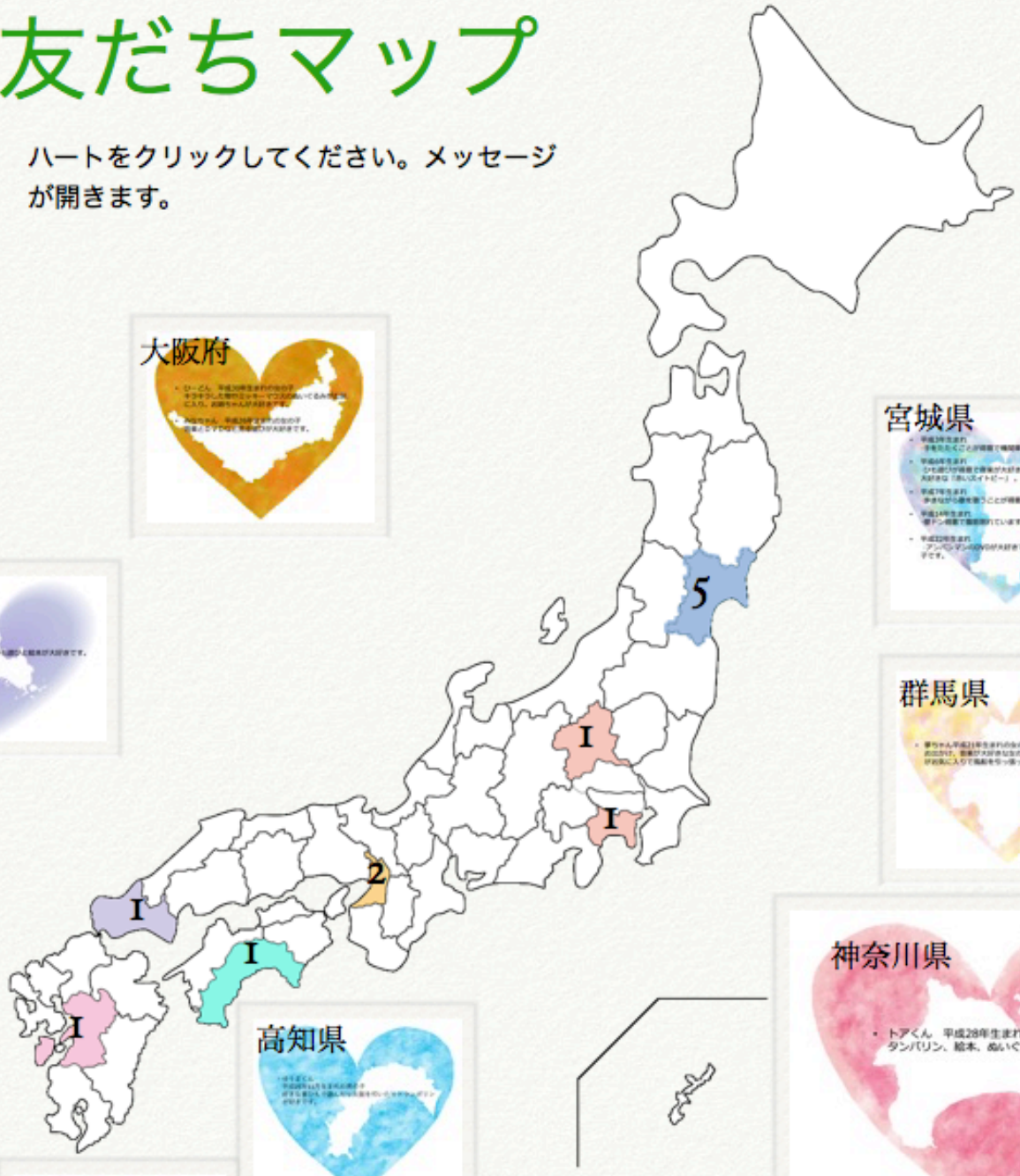
みんな、ゆっくりだけれど、成長してる姿に本当に嬉しく思いました。

息子は最年少だったから、みんなの成長は、息子の少し未来の姿と重なり、今後の成長がとても楽しみになりました。

またすぐに集まるのは難しいですが、年に1回でも、近況報告をかねて、集まれるといいな。

# 友だちマップ

ハートをクリックしてください。メッセージが開きます。



















## <メッセージ>

アーカイブを押すとすべての地域が表示されます。

t(11;22)

≪指定難病２０４≫

# エマヌエル症候群

## 家族会のご案内

エマヌエル症候群は古くは、
トリソミー22、
22 部分トリソミー、
11/22 混合トリソミー、
混合トリソミー22、
11/22 不均衡型転座、
22 番過剰派生染色体症候群、
などと呼ばれていましたが、
長年この転座の研究を行ってきた
米国のエマヌエル博士にちなんで、
2004 年にエマヌエル症候群と
名づけられました。

### もし、ご家族がエマヌエル症候群と診断されたら･･･

どうぞ下記へご連絡下さい。　ともに向き合っていきましょう。

### 藤田保健衛生大学病院

藤田保健衛生大学病院 遺伝カウンセリング室教授の倉橋先生はアメリカ留学中、フィラデルフィア小児病院のエマヌエル博士のもとで染色体疾患について学び帰国後 t(11;22)について研究され、国内の患者家族のカウンセリングをおこなっています。

### 宮城県家族会

2017 年６月、宮城県内の５家族による家族会が誕生し、藤田保健衛生大学病院の倉橋先生との交流がはじまりました。

＊＊＊ 宮城県家族会へのお問合せは ＊＊＊
生活介護事業「仙台つどいの家」（山口）
mail o-yamaguchi@tsudoinoie.or.jp
TEL022-293-3751（受付時間 平日９～６時）
仙台市宮城野区幸町３丁目 12-16

**エマヌエル症候群に関すること、家族会のお問い合わせは･･･**


藤田保健衛生大学病院 遺伝カウンセリング室
mail genome@fujita-hu.ac.jp
〒470-1192　愛知県豊明市沓掛町田楽ヶ窪１番地 98　TEL: 0562-93-2111


◆t(11;22)とエマヌエル症候群のHP　http://www.fujita-hu.ac.jp/~genome/11&22

2018.07

≪指定難病 204≫

# エマヌエル症候群

と、いう名の病気があります。

トリソミー22

22 部分トリソミー

11/22 混合トリソミー

混合トリソミー22

11/22 不均衡型転座

22 番過剰派生染色体症候群・・・

と、過去に診断された方、ご家族の方へ

お心当たりのある方、
まずはご相談ください。
連絡お待ちしています！

長年この転座の研究をされた米国のエマヌエル博士にちなんで

2004 年エマヌエル症候群と名づけられました。

＊＊＊　ともに考えていきましょう！これからのこと　＊＊＊

★相談・カウンセリング★

## ◎藤田保健衛生大学病院◎

藤田保健衛生大学病院 遺伝カウンセリング室　教授の倉橋先生はアメリカ留学中、フィラデルフィア小児病院のエマヌエル博士のもとで染色体疾患について学び帰国後 t(11;22)についての研究及び、国内の患者家族のカウンセリングをおこなっています。

## ◎宮城県家族会◎

2017 年６月、宮城県内の５家族による家族会が誕生し、藤田保健衛生大学病院の倉橋先生との交流がはじまりました。

＊＊＊　家族会に関するお問い合わせ　＊＊＊

生活介護事業「仙台つどいの家」（山口）
仙台市宮城野区幸町３丁目 12-16
TEL022-293-3751（受付時間 平日９時～6時）

＊＊＊ エマヌエル症候群に関するお問い合わせは ＊＊＊


藤田保健衛生大学病院　遺伝カウンセリング室
〒470-1192 愛知県豊明市沓掛町田楽ヶ窪 1 番地 98　TEL: 0562-93-2111
連絡先アドレス：genome@fujita-hu.ac.jp


t(11;22)とエマヌエル症候群の HP　http://www.fujita-hu.ac.jp/~genome/11&22

2018.06

厚生労働科学研究費補助金（難治性疾患等政策研究事業）

分担研究報告書

# 染色体微細欠失重複症候の包括的ケアの検討

研究分担者　大橋博文・埼玉県立小児医療センター遺伝科科長

**研究要旨**

本研究班の目標は、主としてマイクロアレイ染色体検査で診断される微細欠失重複症候群の包括的診療体制構築を目指すことであり、特に成人期への移行が大きなテーマである。本分担研究者は、包括的ケアの一環として微細欠失重複症候群を含む先天異常症候群の集団外来を活用して、医療サイドからの情報提供とともに患者・家族間での交流（年長者が経験したことなどの生活に根ざした情報の共有とピアカウンセリング）を進めてきた。本年度は微細欠失重複を原因としてもちうる多くの疾患を含んだ13疾患（ルビンシュタイン・タイビ症候群、ピット・ホプキンス症候群、カブキ症候群、アンジェルマン症候群、22q11.2欠失症候群、9p重複・9トリソミーモザイク症候群、プラダー・ウィリー症候群、ラッセル・シルバー症候群、コフィン・ローリー症候群、ウィリアムズ症候群、スミス・マゲニス症候群、ソトス症候群、ヌーナン症候群）の集団外来を開催した。参加家族総数は152家族、そのうち県外からの参加者が58家族あった。さらに、これらの集団外来を軸の１つとした先天異常症候群（染色体微細欠失重複症候群）の診療の質の向上のために、希少疾患の包括的支援の先進的取り組みがなされているノルウェー国の希少疾患センターの視察を行った。視察施設は、ノルウェーの希少疾患センターのうちの最大で唯一宿泊施設をもつ施設である「Frambu」とオスロ大学希少疾患リソースセンター、ならびに成人期の教育施設として、オスロ成人教育センターニーダレン校にも立ち寄った。日本には成人期にはこのような特別支援教育機関は存在しないが、その必要性を痛感した。情報の中央化と実践の脱中央化、すなわち、センター機能として、必要十分な情報を保持しつつ（中央化）、それを地域で実践できるように支援する（脱中央化）ことである。そのためには、情報を専門に担当するスタッフの存在が極めて重要と考えられた。

**研究協力者**

清水　健司　（埼玉県立小児医療センター遺伝科）

大場　大樹　（埼玉県立小児医療センター遺伝科）

渡辺　基子　（埼玉県立小児医療センター遺伝科）

金子実基子　（埼玉県立小児医療センター遺伝科）

## A．研究目的

染色体微細欠失重複症候群を含む先天異常症候群は基本的に希少疾患である。希少疾患をもつ患者と家族は、疾患情報に乏しくまた同じ疾患をもつ家族と交流することも難しいこともあり、深刻な不安と孤独を感じていることが指摘されている。また、成人期移行にあたっては成人期での生活の情報を知ることも極めて重要であるが、その情報を得る機会には乏しい。当センターでは様々な先天異常症候群（染色体微細欠失重複症候群を多く含む）を対象とした集団外来に取り組んできた。そこでは、医療サイドからの情報提供とともに、まだ限られた年齢帯までではあるが年長者が経験してきた生活に根ざした情報も得られる場となっている。本年度の分担研究として、先天異常症候群の集団外来の開催の推進とともに、先進的な取り組みのあるノルウェーの希少疾患センターの実際の現地視察も行い、今後の我が国での患者家族の包括的支援に資することを目的とした。

## B．研究方法

### １．先天異常症候群集団外来の推進

平成 30 年 4 月～同年 12 月までの間に、13 疾患（ルビンシュタイン・タイビ症候群、ピット・ホプキンス症候群、カブキ症候群、アンジェルマン症候群、22q11.2 欠失症候群、9p 重複・9 トリソミーモザイク症候群、プラダー・ウィリー症候群、ラッセル・シルバー症候群、コフィン・ローリー症候群、ウィリアムズ症候群、スミス・マゲニス症候群、ソトス症候群、ヌーナン症候群）の集団外来を開催した。

### ２．ノルウェー希少疾患センター視察

平成 31 年 2 月下旬に 7 日（現地 5 日）間のノルウェー希少疾患センター視察を行った。視察施設は、Frambu 希少疾患センター、オスロ大学希少疾患センターである。さらに、成人期の教育施設として、オスロ成人教育センターニーダレン校にも立ち寄った。視察にあたっては、前述の当分担研究者の所属施設で推進している先天異常症候群集団外来の質の向上に資するために、事前に質問事項を整理して視察に臨んだ。

## C．研究結果

### 1．先天異常症候群集団外来の推進

参加家族総数は 152 家族、そのうち県外からの参加者が 58 家族あった。最多参加家族数は 20 家族（カブキ症候群）、最小は 2 家族（コフィン・ローリー症候群）であり、もっとも遠方からの参加者は、北海道（カブキ症候群）と九州（スミス・マゲニス症候群）からであった。情報提供のテーマとしては、疾患概要と健康管理 7 回、疾患特異的合併症関係 2 回、社会福祉制度 1 回、栄養 1 回、作業療法 1 回、家族会（年長者の患者様を持つお母様）からのお話し 1 回、であった。

集団外来後のアンケート調査（回収率 54%）では、研修会に参加してよかった 98%、交流会に参加してよかった 97%であった。情報（医療ならびに生活に関する）が得られ見通しが持てたこと、安心感、希望、子育てに自信

表1．2018年度　先天異常症候群集団外来

| 疾患 | テーマ | 参加家族 | うち県外 |
|---|---|---|---|
| ルビンシュタイン･テイビ症候群 | 疾患概要と健康管理 | 10 | 3 |
| ピット･ホプキンス症候群 | 疾患概要と健康管理 | 6 | 4 |
| カブキ症候群 | 社会福祉制度について（MSW） | 20 | 14 |
| アンジェルマン症候群 | 疾患概要と健康管理 | 10 | 1 |
| 22q11.2欠失症候群 | 22 Hurt Clubの活動について（家族会） | 12 | 0 |
| 9p重複/9トリソミーモザイク | 疾患概要と健康管理 | 12 | 4 |
| プラダー･ウィリー症候群 | 味覚体験～変化するうま味を感じてみよう～（栄養部） | 13 | 6 |
| ラッセル･シルバー症候群 | 疾患概要と健康管理 | 10 | 5 |
| コフィン･ローリー症候群 | 疾患概要と健康管理 | 2 | 1 |
| ウィリアムズ症候群 | ウィリアムズ症候群の心血管疾患について（循環器） | 19 | 7 |
| スミス･マゲニス症候群 | 疾患概要と健康管理 | 8 | 4 |
| ソトス症候群 | 作業療法の視点からみたソトス症候群の発達（幼少期を中心に）（作業療法） | 13 | 6 |
| ヌーナン症候群 | 低身長と成長ホルモン治療について（代謝内分泌科） | 17 | 3 |
| | 合計 | 152 | 58 |

が持てたこと、の感想が多かった。一方、課題としては現実を直視することとなり不安・衝撃があった、個人差が大きいと感じた、などもあった。

## ２.ノルウェー希少疾患センター視察

１）視察施設

a) Frambu希少疾患センター

Frambuはノルウェーに存在する希少疾患センターのうち最大のもので唯一宿泊施設をもつ施設である。Frambuでは100を超える希少疾患についての情報センターとして機能していた。支援のスタイルとしては、疾患情報の提供（面談、電話、e-mail、テレビ会議等）、家族同士の交流、地域医療・福祉のスタッフとの連携推進、“コース”の実施（宿泊コース、夏季キャンプ）があった。15q13.3欠失症候群の宿泊型患者家族研修コース見学を含め、希少疾患に関する（医学、心理、教育、療育専門家チームによる）包括的支援の実践を見学した。特に情報センターとして機能をする上で、ジャーナリスト、司書、メディア（Web、ビデオ）担当のスタッフがいることは特筆すべきであった。

b）オスロ大学稀少疾患センター

遺伝科医師、看護師、心理士、カウンセラー、ソーシャルワーカー、（博士課程）研究者からのレクチャーを受け、センターが果たしている機能（情報リソース、遠隔支援を含めて）に関する情報を得た。血友病患者の現地支援、頭部顔面奇形の専門チームの活動、ハンチントン舞踏病患者（発症リスクのある者を含め）への支援、フェニールケトン尿症のチーム医療などである。このセンターでは治療などの直接介入は担当せず、情報センターとしてそれぞれの地域での稀少疾患患者・家族の支援をサポートしている（情報センターとしての中央化、診療実践としての脱中央化）。センターでは電話による相談も受け付けてお

り、年間4000件に及ぶ相談があるという。また、地域支援として患者さん100人には訪問による支援も行なっている。

c）オスロ成人教育センターニーダレン校

日本には存在しない成人期の教育施設であり、知的障害や脳性麻痺などに対応した教科学習や職業訓練、また視覚障害や言語障害に対応した訓練、パソコンや福祉機器の利用についての学習や、移民のためのノルウェー語学習などに対しての教育支援がなされていた。支援の必要性について当局に申請を行い承認を得ることで３年間の教育が受けられる。継続支援の必要性があれば、新たに継続申請が可能とのことであった。

２）視察での質問事項と回答

a) “コース”対象者のリクルートについて

【質問】コースはどのように案内しているか。ホームページか、連携医療機関への通知か。

《回答》ホームページ上で案内している。申込書もホームページからダウンロード可能である。連携のある他施設の医師や心理士等から紹介されることもある。

【質問】申し込みの手続きはどのような方法か。ホームページか、郵送か。

《回答》コースの2週間前までに申込書を郵送する。メールや電話で質問をすることもできる。

【質問】対象者の要件にはどのようなものがあるか。診断の担保は医師からの紹介状によって行うか。事前にどのような情報を集めているのか。

《回答》参加にあたり、医師の診断がなされていることは必須である。申込書にて、本人や家族の下記を含む基本となる情報を集めている。

・個人情報（名前、住所、性別、診断、診断の時期）

・日常生活の状況（身体面、栄養面、他健康関連、学習面、集中力、眼、耳、情動、社会性）

・必要な援助と参加にあたり用意できるもの（移動手段、コミュニケーション、呼吸など）

・コースへの期待と取り上げてほしい課題

・特記事項（食事、通訳など）

・過去の参加（無／有：有の場合はコースやキャンプ名）

・過去の参加非承認歴（無／有：有の場合は詳細）

・関係者情報（母親、父親、同胞、他）

・主治医と連絡先（住所、電話番号）

・病院と診療科、連絡先氏名

・情報を上記施設から得ることの同意

・専門家などの配置希望（希望の場合には別途様式必要）

・初回登録時には診断を確定する診断書

・遺伝学的検査結果があれば添付

b）今回開催のコース（15q13.3欠失症候群）について

【質問】５日間のプログラムはどのような構成になっているのか。

《回答》コースは、診断と関連する情報の提供とともに、同じ診断を受けた患者家族同士の交流を促すことで、患者家族の専門知識を強化することを目指している。15q13.3欠失症候群のコース期間に我々が受けることができたプログラムは、オスロ大学の臨床遺伝専門医と遺伝カウンセラーによる、疾患や遺伝についての講義と、Frambuの教育担当の専門家による講義であった。それらは、親を主な対象としたプログラムであった。

[注：視察したコースの疾患は近年概念が確立された染色体微細欠失症候群であり、浸透率が必ずしも高くない（染色体異常があっても無症状の場合もある。すなわち、患者さんの親が同じ異常をもつことも稀ではない）など、理解が難しい疾患でもある。この疾患を取り上げたこと自体Fraubuのコースとしてもチャレンジであったと思われる。オスロ大学の遺伝カウンセラーと遺伝科医師からは遺伝学の基礎からかなり専門的な内容（欠失領域に含まれるどの遺伝子が疾患の主症状の原因であるかの解明など）も提供され、その後教育専門家からの具体的に患者支援法などの講義がなされていた。このコースを通して作成されたコンテンツならびに患者・家族情報を広く情報資源としての活用につなげえていく意図があることが理解された]

【質問】プログラム構成にあたってのポイントは何か。
《回答》コースのプログラムで講義を担当する専門家を割り当てるコーディネーターは、人数が少ない疾患に対して、そのためにコースを開催するということ、資源をどう割り当てるのかというバランスを大切にしている。

【質問】本人や同胞も参加しているのか。参加している場合、コースの間どのように過ごしているのか。保育があるのか。ある場合、保育における取り組みはあるのか。
《回答》学校教育をコース参加の間に受けることができるようになっているため、本人や同胞もコースに参加していた。本人のみ、あるいは同胞のみ、また本人と同胞との合同のアクティビティなどが行われる。年齢が離れないように5～7人のグループとしている。施設内には厨房もあり、宿泊もできるようになっている。

【質問】コース最初のアイスブレーキングではどのようなことが行われているか。
《回答》例えば、本人を対象としたプログラムの際は、エクササイズでアイスブレーキングを行うなどしている。

【質問】イベント的な特別なグッズを用いたりしているか。
《回答》施設には、プールや体育館、ロッククライミングなどが設備されていた。また、電子ゲームの部屋もあった。また、イベントで使用すると思われる衣裳部屋もあった。

【質問】患者家族のコミュニケーションを促進する技術としてどのような方法を用いているのか。

《回答》疾患の重症度、年齢、診断後の時期の違いに対する配慮はどのようにしているか。[注：ある程度年齢グループを分けたプログラムを組んでいた。告知については、年齢が低い子はまだ伝えられておらず、15～23歳グループは全員伝えられていた。]

c）コース全般について

【質問】対象疾患とその内容はどのように選んでいるのか。

これまでは大きなグループだったものが、新しい疾患がどんどん見つかることで、別々の疾患に分かれて行ってしまうことが、今後のFrambuの課題である。[注：希少疾患：ノルウェーで500人以下の患者数の疾患]

【質問】両親などの養育者を対象にしたプログラムにはどのようなものがあるか。

《回答》子どもの母親を対象としたコースは、1960年代から提供されている。例えば、プログラムの中の、遺伝についての講義は、親のみで子どもは参加しない。親は、大学病院の遺伝診療科で情報提供を受けてから参加している。教育担当者が行うグループの面談は、事前にテーマは決めないで行い、その時にでた話題について話し合っている。[注：コース2日目のScientific Dayでは、後半の特別支援教育の教師の講義で親御さんからの質問が極めて多くなった。教育については親の関心が高いと思われた]

【質問】本人が主体的に参加できるプログラムにはどのようなものがあるか。

《回答》例えば、教育担当者は、疾患をもつ子どもに自分の疾患のことを知ってもらう試みを行っている。今回のコースでは、15歳から23歳までの本人4名に対し、面談が行われた。グループで実施したが、希望があれば個別面談も可能である。遺伝子や染色体などの基本的なことについて、何がこれから生じるのかなどについて、伝えている。

子どもには、悩みを自由に話せる場を提供する。親には、そこで話題になったテーマについては伝えるが、子どもが語った内容は伝えない。危険な状態がある場合に、子どもの了解を得た上で親に伝える。面談で疾患名を初めて知るようなことがないように、第一には親から診断が伝えられるように配慮している。面談には様々な専門家が関わる。「どうしてここにお母さんやお父さんは君を連れてきたんだと思う？」といった質問を皮切りに、診断の話につなげていくこともある。

小学生年代のグループでは、診断をめぐる考え、気持ちなどを文章や絵でまとめさせ、それを持ち帰ってもらって、家族と話し合ってもらっている。

【質問】同胞が主体的に参加できるプログラムにはどのようなものがあるか。

《回答》同胞にとって、疾患のある兄弟がいることは心を豊かにするが、チャレンジもある。同胞は、精神疾患になる確率が高まるという報告もあり、疾患についての知識をもつことは重要である。心理士でオスロ大学の研究者でもある専門家が、同胞のためのプログラムを開発し、現在実践研究中である。親子

のコミュニケーションを促進したり同胞の心理的ストレスを軽減したりすることを目的としたプログラムとなっている。
[注：2018年には同胞の支援に関しての法律が制定されている]

【質問】祖父母などの養育者を対象にしたプログラムにはどのようなものがあるか。
《回答》今年は祖父母を対象としたコースが開催される。祖父母の支援も一部の家庭にとっては重要となるため、祖父母の理解を促す試みが行われている。

【質問】教育などの支援者を対象にしたプログラムにはどのようなものがあるか。
《回答》年間約50～60の専門家のためのコースがある。ネット上のコースもあり、対象者は専門職（学校、幼稚園、医療関係）である。受講者の要求に応じて、どのプログラムから開始するのかを選ぶことができる。特に遠隔地に住居がある患者、その関係者に対して、動画やポッドキャストなど、インターネットを最大限駆使したプログラムを提供しようという積極的な取り組みがなされていた。

【質問】疾患を越えたプログラムはあるか（年齢、就学、就労、本人告知など）。
《回答》疾患を越えたプログラムを提供している。例えば、早い年齢で診断がつく疾患の年少さんが集まるコースがある。また、症状に関連したコースなどが開催される場合がある。サマーキャンプも実施しており、これは子どもだけの参加となる。サマーキャンプには、疾患を越えた違う年齢の子ども同士が参加する。毎年人気があり、定員以上の申込みがある。同じ疾患を持つ人と出会う貴重な場となっている。

【質問】どのような職種のスタッフが携わっているのか。それぞれの役割、関わり方は。
《回答》コースに携わるスタッフは、全員専門家であった。医師、看護師、心理士、作業療法士、理学療法士、栄養士、ソーシャルワーカー、教育者、保育者などから構成される。他にFrambuのスタッフとして、研究職やセンター管理（清掃など）の職員もいるが、どの職員も対等に働いている。また、マルチメディアの教材を作成する資格をもつ、アニメーションを作成することができるスタッフもいる。現在は75名が勤務しており、その約半数が医療関係である。今回の視察では、施設長、教育長、専門家のコーディネーター（資源の割り当てを行う）、小児科医、心理士、コミュニケーションアドバイサー（メディア関係を専門に行う）との面談を行った。

【質問】スタッフの研修として、どのようなことが行われているのか。
《回答》Frambuではスタッフのための研修を行うというよりは、スタッフは専門家として配属されており、各々の専門性が尊重されていた。

【質問】人的リソースの経済的背景は。
《回答》人件費は、厚生省の希少疾患に関する国の予算が充てられている。

【質問】心理士はノルウェーではどのような背景で、施設内でどのような仕事をしているか。
《回答》Clinical Psychologystは6年間の教育課程の後、分野ごとの5年の専門課程が別にある。活動の現場としては、病院、自治体、教育、また、広告関係など絵で働くものもいる。

【質問】遺伝カウンセラーはノルウェーではどのような背景で、施設内でどのような仕事をしているか。
《回答》遺伝カウンセラーは、修士課程のコースで学ぶ。現在ノルウェー内には50名ほどの遺伝カウンセラーがいる。Frambuでは遺伝カウンセラーは勤務していない。遺伝カウンセラーは、通常、遺伝学的検査を実施する病院で遺伝カウンセリングを提供している。今回のコースでは、オスロ大学病院の遺伝カウンセラーが、患者家族に対して遺伝についての講義を行っていた。

d）その他の取組み
【質問】研究の取組について。
《回答》プロジェクトや個人の助成金を獲得し、研究を行っている。例えば、心理士は、オスロ大学の教員でもあり、他の組織と共同して、同胞のためのプログラム開発に関する研究を実施している。

【質問】コース開催を通じて疾患情報（患児の医療、療育、生活）収集などがあるか。それをどのようにフィードバックしているのか。ホームページにアップする場合、注意点はあるか。
《回答》ネット上には、一般に公開しても問題を起こさない内容、役に立つ情報を提供している。利用者が自分の話を伝える内容もある。その場合、語りたい内容を語ってもらうが、第三者に関係することは削除している。プライバシーに注意し、必ず書面による同意をとる。撤回もできるようにしているが、これまでに撤回をした人はいない。

【質問】コース実践はイベントであるが、どのように継続的支援に活かしているのか。
《回答》登録システムがあり、生涯にわたって、赤ちゃんから高齢者まで、サービスを受けることができる。講習会に来られない人にも情報を提供する。

【質問】個別対応についてはどのように考え、どのようなことを行っているのか。
《回答》個別対応も希望があれば実施している。コース中にも、個別に面談することもできる。面談で個人的な内容がでた場合、専門職と一緒に話すことを勧めることもある。

【質問】他の機関との連携はどのようにしているか。
《回答》ノルウェー国内に、家族支援を行う施設が9施設ある。また、遺伝学的検査を提供する病院が5施設ある。それらの医療機関との連携を行っている。また、教育機関に対する支援も行っている。教育者に対して情報提供を行うなど、訪問支援を行うなどもしている。

訪問支援としては、授業内容が分かるように子どものアフターケアを行うことも含まれる。家族会に関しては、ノルウェーには、家族会が複数存在するが、Frambu自体は、コース開催にあたって特に家族会と連携はしていない。

【質問】希少疾患センターとしての機能として、どのような情報を活用提供しているのか。
《回答》ノルウェーのどこに住んでいても同じ支援を受けられるように、訪問支援を行ったり、ネット上に情報を提供したりしている。ネットに関しては、専門のスタッフが配属されている。コースはビデオを撮影していて、参加者にパスワードを送り、見ることができるようになっている。公開の許可が得られた場合には公開もしている。インターネットサービスは、YouTubeやSoundCloudなどの、既存のサービスを使うことで、経費を削減している。講習会に来られない人にも情報を提供する。重視しているのは、問題を起こさないようなもの、役に立つようなもの。当事者が自分の話を伝える動画もある。当事者は、既にメディアで発言したことがある人にコンタクトを取る。

e）ノルウェーについて
【質問】日本では青年期になるとサポートがなくなっていくが、北欧ではどうなっているのか。ぶつぎりではなく、継続的な支援があるのか。ノルウェーでは、就労は障害者として働いているのか。親亡き後の生活は。年金は。
《回答》ノルウェーでは、18歳を過ぎると親ではなく社会が対応することになる。

【質問】ノルウェーにおける父親と母親の子育ての分担についてはどうなっているのか。
《回答》ノルウェーでは、1970年代に社会体制として男女平等が取り入れられたため、ノルウェーでは、父親も育休を取り、母親と同様に育児に参加することになる。きょうだいのメンタルヘルスと父親のメンタルヘルスとの相関を示唆する調査結果もある。

【質問】コースに参加していない人はどうしているのか。例えば、家から出さないような家族はいないか。そのような家族への参加の促しやフォローはどうしているのか。
《回答》ノルウェーでは、6歳から16歳までは必ず親が学校に行かせ教育を受けさせることになっている。教育機関や心理、リハビリなど複数の機関が各子どもの状況に合わせて話し合う機会も設けており、周囲とつながらないということは考えにくい。コースについては、距離等を理由に参加できないことがあるが、ビデオ会議で参加したり、ネットの講習会を受けたり、訪問カウンセリングを実施したりしている。

【質問】ノルウェーの人は、診断された時、最初どのような気持ちで子どもの違いを受け容れていくのか。社会的な対応が良いと、不安が少ないのか。
《回答》前述したように、ノルウェーでは、18歳を過ぎると親ではなく社会が対応するこ

とになるが、施設ごとによって対応の良し悪しもあり、親が子どもを抱えることもある。出生前診断を受けて診断されたら諦める選択をする人も少なくない。ノルウェーでも、疾患に起因する様々な問題が、親のせいにされることは未だにあり、教育機関も、うまく本人に対応できないことを周りに知られたくない、と隠してしまう場合もあるとのことであった。

【質問】本人への告知についてどのように考えているのか。
《回答》個人的な状況に応じてで、無理に本人に疾患のことを伝えることはない。第一に親が伝えることを大切にしている。20年前から比べると、伝えてほしいと希望する親が増えてきた。

**D．考察**

染色体微細欠失・重複症候群が属する先天異常症候群における包括的診療体制構築に関連する事項として、当研究分担者が所属する小児医療施設を基盤として取り組んでいる先天異常症候群集団外来の仕組みも含めた機能向上に資するために、ノルウェー希少疾患センター視察も踏まえた検討を行った。今後このような希少疾患の包括的診療体制の構築のための重要なキーワードとして、情報の中央化と実践の脱中央化が挙げられると考えた。すなわち、センター機能としては、必要十分な情報を保持しつつ（中央化）、それを地域で実践できるように支援する（脱中央化）ことである。そのためには、情報を専門に担当するスタッフの存在が極めて重要と考えられた。

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**（発表誌名巻号・頁・発行年等も記入）**

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**Ｇ．知的財産権の出願・登録状況（予定を含む。）**

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

特になし

厚生労働科学研究費補助金（難治性疾患等政策研究事業）
分担研究報告書

# Smith-Magenis症候群の成人期における医療管理

研究分担者　黒澤健司
地方独立行政法人神奈川県立病院機構神奈川県立こども医療センター遺伝科部長

**研究要旨**
Smith-Magenis症候群は、染色体17p11.2上にマップされるRAI1のハプロ不全により発症する先天奇形症候群である。特徴的な行動特性やてんかん、腫瘍発生などはあるものの、生命予後は比較的良好である。成人期では、衝動的な行動や自傷行動は持続することもあるが、多くの場合は、成人期になると落ち着きを認める。成人移行を視野に入れたSmith-Magenis症候群の医療管理の検討が、症例を中心とした調査により明らかにされることが期待される。

## A．研究目的

Smith-Magenis症候群は、染色体17p11.2上にマップされるRAI1のハプロ不全により発症する先天奇形症候群である。約95%が同領域のde novoの微細欠失を原因とし、5%がシーケンスで明らかにできる変異に由来する。欠失範囲はLow copy repeats (LCR) に挟まれた標準3.7Mbに及ぶ。ときに欠失範囲が17pter側に位置するPMP22を含む例もある（圧迫麻痺性遺伝性ニューロパチー：HNPPを合併）。特徴的な所見は、発達遅滞、特異顔貌、メラトニンのサーカディアンリズム障害による睡眠障害、自傷や特異行動（polyembolokoilamania）などであり、他に低身長、難聴、虹彩などの眼科的異常、側彎、疾患などを合併する。生命予後は比較的良好である。Smith-Magenis症候群に腫瘍発生はほとんど報告例がないが、医療的管理として重要な課題であると考え、当研究グループでは、Smith-Magenis症候群に腫瘍（白血病）を合併した2症例を報告してきた。

Smith-Megenis症候群は、上述のように特徴的な行動特性やてんかん、腫瘍発生などはあるものの、生命予後は比較的良好であることから、成人期の管理も重要である。しかし、成人期の論文記載は極めて限られている。今回、成人移行を視野に入れたSmith-Magenis症候群の医療管理についてまとめた。

## B．研究方法

「Smith-Magenis syndrome」、「Young-adult」などをキーワードとして文献検索を行い、さらにGeneReviews（https://www.ncbi.nlm.nih.gov/books/NBK1310/）など、成人期記載のある文献を参考とした。

(倫理面への配慮)

すべての個人情報は潜在化させた。

## C．研究結果

青年期：

顔貌の変化が認められる。やや下顎や前額が目立つようになり、眉毛は濃くなり癒合傾向が出てくる。全体としてやや粗な印象が強まることもある。二次性徴は、一般集団と同じタイミング発来はあるが、思春期早発や逆に遅れもある。行動特性としては、思春期とともにややエスカレートすることもあり、睡眠障害は依然として課題の一つである。衝動性は、女性でやや強くなる傾向がある。急激な感情の変化や不安感の高まりは、青年期から成人期にかけての大きな課題の一つである。攻撃的行動も目立つことがある。月経に伴うてんかんもしばしばみられる。異物挿入癖（Polyembolokoilamania）や爪甲損傷癖（onychotillomania）も年齢とともにやや目立つ傾向にある。耳などへの挿入癖は、年齢に関係なくみられるが、鼻、膣、直腸への挿入癖は、成人期になって初めて見られることもある。

成人期：

生命予後に関する情報は限られているが、大きな内臓合併症のない例では、一般集団と違いはない。側彎は増悪することがある。衝動的な行動や自傷行動は持続することもあるが、多くの場合は、成人期になると落ち着きを認める。

## D．考察

今回、成人期の問題や医療管理について文献的検討を行ったが、情報は極めて限られていて、大規模調査はなされていなかった。成人期での落着きは記述あるが、具体的な内容は乏しかった。

成人期での「落着き」は、推測としては、感情と衝動的行動・自傷行動などの抑制・沈静化を含むが、具体的なグループホームや家庭での対応については、ほとんど情報がなかった。特徴的な血清中のメラトニンの逆転パターンの正常化が、どれくらいの年齢からあるのかも調査された記録はなかった。

こうした背景より、今後は小児例で調査された行動特性や生理学的計測の成人例での検討が課題であると思われた。

## E．結論

Smith-Magenis症候群の成人期移行を前提として、その行動特性や医療官について文献的考察を加えた。衝動行動や異物挿入癖は改善が期待できるが、パターンが異なり現れる場合もあることが確認された。行動を中心とした症例調査などが今後必要である。

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## G．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

## 健康危険情報

該当なし。

平成 30 年度厚生労働科学研究費補助金(難治性疾患政策研究事業)
分担研究報告書

# 染色体微細欠失・重複症候群の診断システムについて

研究分担者　山本　俊至　東京女子医科大学遺伝子医療センターゲノム診療科・教授

研究要旨

**研究目的:**
染色体の微細欠失や重複は、いわゆるゲノムコピー数変化(copy number variation; CNV)としてよく知られている。微細な染色体欠失や重複などの CNV を効率的に調べる方法として、欧米ではマイクロアレイ染色体検査が普及している。この方法によって、multiple congenital anomalies/ intellectual disability (MCA/ID)患者のうち、およそ 17%程度で何らかの疾患関連 CNV が認められる。ただ、MCA/ID の原因の内訳としては、CNV より、一塩基変化(single nucleotide variant; SNV)が占める割合の方が高く、次世代シーケンサーが普及してきた現在、マイクロアレイ染色体検査による CNV 解析より、次世代シーケンサーによる SNV 解析を優先させる傾向がある。次世代シーケンサーを用いた CNV 解析も一部では行われているため、次世代シーケンサーfirst の解析による CNV 同定について検討した。
**研究方法:**
次世代シーケンサーによる SNV 解析で得られた BAM file を eXome Hidden Markov Model (XHMM)によって解析し、得られたデータをマイクロアレイ染色体検査で確認した。
**結果と考察:**
発達の遅れと自閉症症状を示す患者において、次世代シーケンサーによる SNV 解析を行ったが有力な病的バリアントを見出すことができなかった。そこで XHMM 解析を行ったところ、15q14 領域の欠失を示唆する所見が得られた。この所見はマイクロアレイ染色体検査で確認できたが、両親には認められず、de novo 変異であった。欠失範囲には *MEIS* 遺伝子が含まれており、この遺伝子が発達遅滞と自閉症の原因となったことが明らかとなった。
**結論:**
次世代シーケンサーfirst による解析によっても CNV を着実に検出できるようになった。そのため、費用を別に考慮すれば、マイクロアレイ染色体検査による CNV 解析より、次世代シーケンサーfirst による解析の方が効率的と考えられる。ただし、XHMM では CNV 範囲の正確な同定や、コピー数の同定を確実に行うことができないため、XHMM で CNV を検出した場合、マイクロアレイ染色体検査による確認が必要である。

## A. 研究目的

染色体の微細欠失や重複は、いわゆるゲノムコピー数変化(copy number variation; CNV)としてよく知られている。微細な染色体欠失や重複などの CNV を効率的に調べる方法として、欧米ではマイクロアレイ染色体検査が普及している。この方法によって、multiple congenital anomalies/ intellectual disability (MCA/ID)患者のうち、およそ 17%程度で何らかの疾患関連 CNV が認められる。ただ、MCA/ID の原因の内訳としては、CNV より、一塩基変化(single nucleotide variant; SNV)が占める割合の方が高く、次

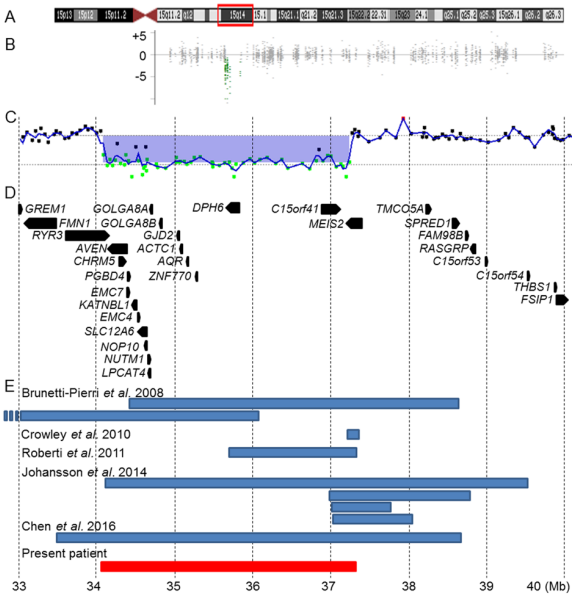
世代シーケンサーが普及してきた現在、マイクロアレイ染色体検査による CNV 解析より、次世代シーケンサーによる SNV 解析を優先させる傾向がある。次世代シーケンサーを用いた CNV 解析も一部では行われているため、次世代シーケンサーfirst の解析による CNV 同定について検討した。

## B. 研究方法

次世代シーケンサーによる SNV 解析で得られた BAM file を eXome Hidden Markov Model (XHMM)によって解析し、得られたデータをマイクロアレイ染色体検査で確認した。

なお、本研究は東京女子医科大学における「遺伝子解析研究に関する倫理審査委員会」で認められた研究の一部として行い、患者あるいはその家族から書面による同意を得て行った。

## C. 研究結果




症例は 9 歳男児。心室中隔欠損による心雑音を示したが、自然閉鎖した。浸出性中耳炎を繰り返している。20 ヵ月で始歩が見られるなど、発達の遅れが認められた。発達指数は 63 と軽度の知的障害を認める。粘膜下口蓋裂のため構音障害を示す。オーム返しが多く、読字困難あり。思い通りにならない場合、しばしば癇癪を起す。コミュニケーションに問題があり、友人関係を構築できない。これらの症状は自閉症を示唆するものであった。

発達の遅れと自閉症症状の原因を明らかにするために、TruSight One (Illumina)を用いた次世代シーケンスによる SNV 解析を行ったが有力な病的バリアントを見出すことができなかった。そこで得られた BAM ファイルを用いて XHMM 解析を行ったところ、15q14 領域の欠失を示唆する所見が得られた。この所見はマイクロアレイ染色体検査で確認できたが、両親には認められず、de novo 変異であった。欠失範囲には *MEIS* 遺伝子が含まれており、この遺伝子が発達遅滞と自閉症の原因となったことが明らかとなった。

## D. 考察

15q14 微細欠失を示す症例は過去に数例報告があり、共通して欠失する領域に MEIS2 が存在している。近年の次世代シーケンスによる解析で、MEIS2 の SNV によって発症した自閉症患者の報告があり、当該遺伝子は原因であることが明らかになっており、本症例は報告と矛盾しない。

## E. 結論

次世代シーケンサーfirst による解析によっても 15q14 微細欠失を同定することができた。このことは、次世代シーケンスデータ

の応用によってCNVを着実に検出できることを示唆している。解析費用を別に考慮すれば、マイクロアレイ染色体検査によるCNV解析より、次世代シーケンサーfirstによる解析の方が効率的と考えられる。ただし、XHMMではCNV範囲の正確な同定や、コピー数の同定を確実に行うことができないため、XHMMでCNVを検出した場合、マイクロアレイ染色体検査による確認が必要であると考える。

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## H. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

厚生労働科学研究費補助金（難治性疾患政策研究事業）
分担研究報告書

# 染色体微細欠失重複症候群の包括的診療体制の構築

研究分担者　涌井 敬子　信州大学医学部遺伝医学教室 講師

研究要旨：マイクロアレイ染色体検査でみつかる染色体微細欠失重複症候群を含む稀少疾患のひとつであるWolf-Hirschhorn症候群を中心に，成人期の臨床症状等に関する情報収集を行った.
日本の稀少疾患患者登録システムを充実するために，諸外国の患者登録のひとつである，米国Sanford Researchが推進する“Coordination of Rare Diseases at Sanford (CoRDS) Registry”の取り組みが非常に参考になると考えられた.

## A．研究目的

マイクロアレイ染色体検査でみつかる染色体微細欠失重複症候群の医療水準の向上や患者のQOL向上をめざし，特に成人期治療へのトランジッションを充実させるために成人期の臨床情報を収集する.

## B．研究方法

１．Wolf-Hirschhorn症候群患者の成人期の臨床情報の文献検索

Wolf-Hirschhorn症候群患者の成人症例についての文献検索を実施した.

２．“Coordination of Rare Diseases at Sanford (CoRDS) Registry”

稀少疾患患者の登録システムとして先行実施されている，米国のシステム“Rare Disease Patient Registry & Natural History Study”についてwebにて情報収集した.

（倫理面への配慮）
本研究の実施に際しては，倫理指針等を遵守し、関係する多発奇形・発達遅滞を有する患者やその家族が不利益を被ることの無いよう、個人情報の保護に留意する.

## C．研究結果

１．Wolf-Hirschhorn症候群患者の成人期の臨床情報（文献検索）

Wolf-Hirschhorn症候群については，Battaglia（イタリア）とCarey（米国）らにより2015年に更新された, GeneReviews® [Internet]. < https://www.ncbi.nlm.nih.gov/books/NBK1183/ > の情報が現時点で最も参考になるreviewと考えられる．2008年に発表された両研究者らによる87名の患者についての情報（Am J Med Genet Part C Semin Med Genet. 2008;148C:246–51）が主となっている.

その後，2017年にBlanco-Lago（スペイン）らにより，51名のコホート研究（Rev Neurol. 2017 May 1;64(9):393-400）が報告された.

医療上, 本症患者で留意が必要なけいれんについてはかなりの情報が蓄積されその研究成果が示されている．またフォローアップとして推奨される事項として，血算，腎機能検査，肝超音波検査が挙げられた．肝腫瘍に関して，BattagliaとCareyらが2018年に，成人移行した患者に留意すべき症状としての注意喚起として，改めて７症例のreviewを報告している（Am J Med Genet A. 2018 Nov;176(11):2389-2394).

わが国においては, 本研究班の研究分担者でもある山本らにより，2012年に34名のWolf-Hirschhorn症候群患者の成長に関するデータが，（J Pediatr Genet. 2012 Mar;1(1):33-7），清水，涌井らにより，2014年に22名のWolf-Hirschhorn症候群患者の欠失範囲と症状の比較が（Am J Med Genet A. 2014 Mar;164A(3):597-609），さらに2018年に山本らにより，2名の成人を含む10名の患者の成長に関するデータ（J Pediatr Genet. 2012 Mar;1(1):33-7）が報告されている.

しかしながら, 患者家族が求めているであろ

う成人期の自然歴情報はまだ十分に明らかになっているとはいえない.

２．“Coordination of Rare Diseases at Sanford (CoRDS) Registry”について

CoRDS は，Sanford Research が取り組んでいる，7000以上の希少疾患を登録対象とた，患者・家族（未診断患者も含む，保因者も含む）と研究者をつなぐためのnatural history studyで，基本的には患者（あるいは保護者）個人が申請する方式で，2010年から100年計画で開始した登録事業である．2013年からは，Sanford Healthがスポンサーとなって米国NIHのClinical Trialsのひとつにもなっている < https://clinicaltrials.gov/ct2/show/NCT01793168?cond=NONDISJUNCTION&rank=1 > ，“Rare Disease Patient Registry & Natural History Study - Coordination of Rare Diseases at Sanford (CoRDS)”にも組み込まれた.

診断がついていない場合は，lifespan, quality of life, and health risks などの状態で申請できるようにしていて，適切かどうかを審査して登録している．協力者に患者団体自身も含まれており，申請項目には英語が話せるか，という質問と母国語を入力する項目も設け，世界中の患者をリクルートしている < https://cordsconnect.sanfordresearch.org/BayaPES/sf/screeningForm?id=SFSFL# >.

2019年4月時点で以下の患者家族支援団体が参加協力している.

***National Ataxia Foundation***
***International WAGR Syndrome Association***
***4p- Support Group***
***ML4 Foundation***
***Cornelia de Lange Syndrome Foundation***
***Stickler Involved People***
***Kawasaki Disease Foundation***
***Klippel-Feil Syndrome Alliance***
***Klippel-Feil Syndrome Freedom***
***Hyperacusis Research Limited***
***Hypersomnia Foundation***
***Kabuki Syndrome Network***
***Kleine-Levin Syndrome Foundation***
***Leiomyosarcoma Direct Research Foundation***
***Marinesco-Sjogren Syndrome Support Group***
***Mucolipidosis Type IV (ML4) Foundation***
***People with Narcolepsy 4 People with Narcolepsy (PWN4PWN)***
***Soft Bones Incorporated***
***American Multiple Endocrine Neoplasia Support***
***Atypical Hemolytic Uremic Syndrome Foundation***
***All Things Kabuki***
***Wiedemann-Steiner Syndrome Foundation***
***Breast Implant Victim Advocates***
***PROS Foundation***
***American Behcet's Disease Association***

そのなかかでも，Wolf-Hirschhorn症候群患者の支援団体 : 4p- Support Groupは，Careyらの尽力により非常に精力的な活動をしていることが確認された.

## Ｄ．考察

わが国で染色体微細欠失重複症候群を含む稀少疾患患者登録の取り組みが推進されようとしているが，自然歴の情報を構築するにはまだ多くの課題があると考えられた.

多くの疾患について遺伝学的検査による診断法が確立してきたが，それは最近のことであり，Wolf-Hirschhorn症候群はある程度臨床的に診断可能であることもあり古くから疾患単位として確立しており，臨床的診断のみの患者や，従来のG分染法のみで解析精度の低い検査法しか実施できていない患者も含まれている．正確なgenotype-phenotypeの関連については，追加解析も含め情報蓄積が必要である.

また，Wolf-Hirschhorn症候群患者の主な臨床症状は欠失範囲と関係すると考えられるが，同一家系内の３姉妹で大きく異なる転帰をとっている例などからも，欠失範囲だけでは自然歴を説明できない場合があることも示されている.

多くの患者は新生児期～小児期に，診断されているが，検査の実施は小児病院や小児科が主である．各病院内で各患者の情報は蓄積されていても，各患者の臨床症状の推移，転帰，死亡した場合の年齢・原因等を含め，詳細は必ずしもまとまった情報となっていないと考えられる．また，合併症が軽く成人した患者は，医療機関のフォローが途切れている患者もいることが推測され，さらにフォローされていても元々の小児科から内科あるいは福祉施設等へフォローの主体となる機関が変わっている可能性も高く，その移行に伴う情報を含め，データを入手し統合することは容易でないと考えられる.

国際的には計100名以上のWolf-Hirschhorn症候群患者について報告があるとはいえ十分とはいえず，まだまだ患者数を増やした，長期にわたる調査研究が必要であることは明白である．実施体制の構築と長期にわたる運用が必要である.

Ｅ．結論

多くが小児期に診断される稀少疾患について，成人期以降の正確な情報収集は容易でない．今後のわが国の稀少疾患患者登録の推進には，100年計画として企画されている“Rare Disease Patient Registry & Natural History Study - Coordination of Rare Diseases at Sanford (CoRDS)”の取り組みなどを参考にすることが必要と考えた．あるいはそこに参加する取り組みなども考慮してもよいのではないかと考えた．

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11p13バンドに染色体転座切断点と微細欠失を伴う複雑構造異常を有するが PAX6 遺伝子に病的ゲノムバリアントを認めない無虹彩症例（口頭発表）涌井敬子, 内山由理, 羽田 明, 朽方豊夢, 山口智美, 古庄知己, 三宅紀子, 福嶋義光, 松本直通．日本人類遺伝学会第 63 回大会，2018.10.10−13，横浜

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Ｈ．知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

**別紙４**

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# Two siblings with 11qter deletion syndrome that had been rescued in their mother by uniparental disomy

Miki Kawai[a], Makiko Tsutsumi[a], Fumihiko Suzuki[a,b], Kiyoko Sameshima[c], Yuri Dowa[d], Takuji Kyoya[e], Hidehito Inagaki[a,f], Hiroki Kurahashi[a,b,f,*]

[a] Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan
[b] Center for Collaboration in Research and Education, Fujita Health University, Toyoake, Japan
[c] Department of Pediatrics, Minamikyushu National Hospital, Aira, Japan
[d] Department of Neurology, Gunma Children's Medical Center, Shibukawa, Japan
[e] Department of Obstetrics, Gunma Children's Medical Center, Shibukawa, Japan
[f] Genome and Transcriptome Analysis Center, Fujita Health University, Toyoake, Japan

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ABSTRACT

Jacobsen syndrome refers to a congenital anomaly caused by deletion at 11q23.3-qter. We here describe two siblings with the same 11q23.3-qter deletion. Both parents were healthy with a normal karyotype. Cytogenetic microarray analysis revealed no mosaicism in either parent but the mother showed uniparental disomy encompassing the deleted region found in the two siblings. The pattern of X chromosome inactivation was almost completely skewed in the mother. These data suggested that the mother was a carrier of the 11q23.3-qter deletion but that this had been rescued by disomy formation during early embryogenesis except for her germinal cells.

## 1. Introduction

Jacobsen syndrome (MIM#147791) is a contiguous gene deletion syndrome caused by deletion of the 11qter region. The typical clinical features of Jacobsen syndrome include pre- and postnatal physical growth and psychomotor retardation, facial dysmorphic features, and thrombocytopenia. Some patients with this syndrome also have malformations of the heart, kidney, gastrointestinal tract, and central nervous system. Ocular and hearing problems can be also present. The estimated occurrence of Jacobsen syndrome is about 1/100,000 births (Mattina et al., 2009).

About 85% of Jacobsen syndrome cases are caused by a simple *de novo* terminal deletion. Other cases result from a variety of chromosomal abnormalities including segregation of a familial reciprocal balanced translocation, *de novo* unbalanced translocations, recombination of a parental pericentric inversion, or other rearrangements such as ring chromosomes. An 11q deletion has also been reported in the mosaic form of this condition. The breakpoints in these deletions occur within or distal to 11q23.3, and the deletions usually extend to the telomere (Grossfeld et al., 2004). The deletion size ranges from 7 to 20 Mb. The chromosomal region conferring specificity for the Jacobsen syndrome phenotype is the 11q24.2 band, but the gene responsible for this phenotype is still unknown.

We here report on two siblings with the same 11q23.3-qter deletion, one with Jacobsen syndrome and the other detected by amniocentesis and terminated. The parents however showed a normal karyotype. Cytogenetic microarray analyses revealed that the healthy mother had uniparental disomy (UPD) encompassing the 11q22.3-ter region deleted in the siblings. A possible mechanism for the recurrence of this deletion is discussed.

## 2. Clinical report

A 4-year old Japanese male subject was the first child of a non-consanguineous healthy 36-year old father and 28-year old mother after having three miscarriages with no notable family history of disease (Fig. 1A). At 22 weeks of pregnancy, a congenital heart defect, mitral valve stenosis and aortic valve stenosis were suspected. He had been born after a 41 week gestation by an induced labor with a birth weight of 2644 g (−0.9SD), height of 47.5 cm (−0.7SD), head circumference of 34.5 cm (+0.8SD), and chest circumference of 30 cm. He showed a hypoplastic left heart, conductive auditory impairment in the left ear,

* Corresponding author. Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi, 470-1192, Japan.
*E-mail address:* kura@fujita-hu.ac.jp (H. Kurahashi).

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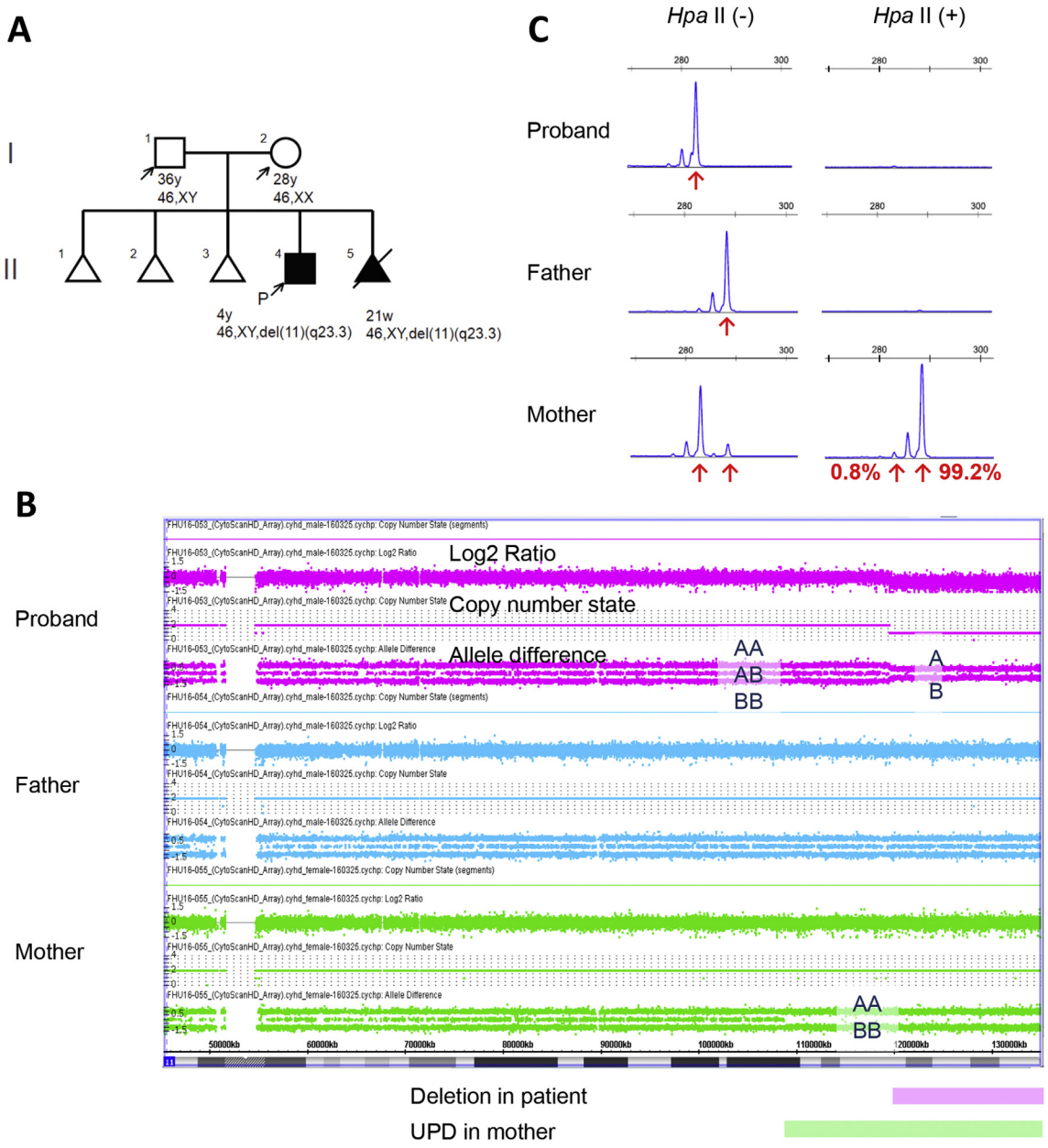


**Fig. 1.** Deletion rescue on 11qter. **(A)** Pedigree of the study family. **(B)** Cytogenetic microarray results for the 11q region for the proband (pink), father (blue), and mother (green). The copy number log2 ratio (top), copy number state (middle), and allele difference (bottom) are shown for each sample. The pink bar at the bottom indicates the deleted region in the proband. The green bar indicates the UPD region in the mother. **(C)** HUMARA assay results. The electropherogram shows the fragment analysis of the amplicons from undigested (left) and digested (right) genomic DNA from the proband (top), father (middle), and mother (bottom). The major peaks depicted by the red arrows indicate the sizes of the PCR products with different numbers of short tandem repeats at the HUMARA locus. The two major peaks found in the mother represent two X chromosome alleles (282bp, 288bp). Digestion of the DNA from the proband (son)'s and father's sample showed complete loss of one allele, whereas a preferential loss of the short alleles was evident in the mother (282bp). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

widely spaced eyes, a short nose, a small ear lobe, thin vermilion of upper lip and lower lip, shortness in both fifth fingers, and a bifid scrotum. He also had an old cerebral hemorrhage in the nucleus basalis.

The subject also showed severely retarded psychomotor development. The ability to hold up his head, roll over, and speak, were recognized at 2 years and 7 months, 2 years and 7 months, and 3 years and 3 months. Serial complete blood counts revealed transient thrombocytopenia. At the age of 4 years and 2 months, he showed a height of −3.5SD, weight of −2.2SD, and head circumference of −1.2SD. His karyotype revealed 46,XY,del (11) (q23.3), which is known as Jacobsen syndrome.

At 2 years after the birth of this first child, the mother again became pregnant with a boy. The fetus was diagnosed with the same karyotype as the brother by amniocentesis at 15 weeks and 4 days of gestation. At 20 weeks and 4 days, a hypoplastic left heart with severe atrioventricular regulation was evident on ultrasound examination. At 21 weeks, the pregnancy was terminated and the fetus was found to weigh 344 g. Karyotype analysis of the parental peripheral blood lymphocytes revealed 46, XY [20] and 46, XX [20].

## 3. Materials and methods

### 3.1. Subjects

Peripheral blood samples were obtained from the study subject and the parents. The research protocol for this study was approved by the local ethics committee of Fujita Health University, Japan. Written informed consent to participate in the study was obtained from the parents.

### 3.2. DNA extraction

Genomic DNA was extracted from whole blood using QuickGene 610 L (Fuji film, Tokyo, Japan). The concentration of the DNA was measured using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and the quality was determined by gel electrophoresis.

### 3.3. Cytogenetic microarray

High-resolution chromosomal microarray analysis using the CytoScan HD array (Affymetrix, Santa Clara, CA) was performed. DNA samples of 50 ng were used in this analysis in accordance with the manufacturer's instructions. The genomic coordinates were based upon genome build 37/hg19 (2009). Hybridization, data extraction and analysis were performed as per the manufacturer's protocols. Chromosome Analysis Suite software 3.0 (ChAS, Affymetrix Santa Clara, CA) was used for raw data analysis, review and reporting. Regions of copy-number changes were extracted with 20 probes of 50 kb. All of the extracted regions containing a copy-number change were confirmed by visual comparisons with the normal control data from Database of Genomic Variants (http://dgv.tcag.ca). UPD regions were extracted with 5 Mb. Regions with a sparse SNP density were carefully evaluated to exclude false calls.

### 3.4. FISH analysis

Peripheral blood lymphocytes and buccal samples were obtained by standard methods. FISH analysis was performed using standard techniques. The probes used for the FISH analysis were TelVysion 11p SpectrumGreen (D11S2071), TelVysion 11q SpectrumOrange (D11S1037) (Abbott Molecular, IL, USA). A hundred interphases nuclei were analyzed for pter/qter of chromosome 11.

### 3.5. HUMARA assay

To assess skewing of the X chromosome inactivation, we performed HUMARA assay according to the protocol described elsewhere (Beever et al., 2003). Briefly, we digested the genomic DNA with methylation-sensitive restriction enzyme *Hpa*II. PCR primers, one of which was labeled with FAM, were designed across the polymorphic CAG repeat as well as two *Hpa*II sites in the androgen receptor gene on the X chromosome. PCR amplification would be achieved only from the inactivated allele having the *Hpa*II sites methylated. PCR products were analyzed by capillary electrophoresis (ABI3730 Genetic Analyzer) and quantified the area under the curve using GeneMapper software.

## 4. Results

We performed cytogenetic microarray analysis to demarcate the deleted region in our current case subject. A 15.4-Mb region was found to have been deleted at 11q23.3q25-qter in this patient (arr [hg19] 11q23.3q25 (119, 484, 933_134, 938, 470) × 1), which is consistent with the typically deleted region in Jacobsen syndrome (Fig. 1B). The deleted region was found to contain 128 Refseq genes, and 70 OMIM genes. Single nucleotide polymorphism (SNP) genotyping indicated that the deleted chromosome was derived from the mother (data not shown).

A possible explanation for the abnormal 46,XY,del (11) (q23.3) karyotype in two siblings from parents with a normal karyotype was that one of the parents harbored FRA11B, a (CCG)n repeat expansion in the 5′untranslated region of the *CBL2* gene. In more than 70% of normal individuals, this repeat is present in 11 copies but can be expanded to several hundred copies and lead to genomic instability and a susceptibility for terminal deletion (Mattina et al., 2009). However, the deletion breakpoint of our current patient was at chr11:119, 484, 933 (hg19), which is approximately 400 kb distal from FRA11B.

Neither of the parents showed deletion mosaicism at the 11q23.3-qter region. Interphase FISH on 100 peripheral blood lymphocytes and 100 buccal cells revealed no deletion for the 11q subtelomere-specific probe (data not shown). It was notable however that SNP array analysis of the patient's mother detected a 26.2-Mb region with a loss of heterozygosity at 11q22.3-qter consistent with uniparental disomy (UPD) (arr [hg19]11q22.3q25 (108, 657, 506_134, 942, 626) × 2 hmz) (Fig. 1B). The deletion breakpoint in the son was 10-Mb distal from the UPD boundary in the mother.

A HUMARA assay was performed to determine when the UPD was generated in the mother. The patterns of X chromosome inactivation (XCI) showed 99.2% skewing in the mother (Fig. 1C), suggesting that she originally had the same deletion as her son and the chromosome copy number loss was corrected by UPD after XCI occurred in the early embryogenesis.

## 5. Discussion

Our analysis by cytogenetic microarray of our current case subject with 11qter deletion syndrome and his family suggests that segmental UPD corrected the chromosomal copy number of the deleted region and thereby rescued the phenotype in his healthy mother. To our knowledge, there have only been two previous reports of siblings showing a deletion of 11q23.3-qter despite a normal parental karyotype (Afifi et al., 2008; Johnson et al., 2014). One of those reports also provided detailed molecular analyses showing a maternal UPD at the 11qter region (Johnson et al., 2014). A 22q13 deletion rescued by paternal UPD has also been reported (Bonaglia et al., 2009). Such deletion rescue event has not been reported for other terminal deletions. Our current case is therefore the third report to describe a deletion rescued by post-zygotic UPD generation.

It is likely that the mother of our current case subject originally carried the 11q23-qter deletion that had been transmitted from a gamete of a maternal grandfather or grandmother. After fertilization, this deletion was likely rescued during the post-zygotic stage via a DNA repair pathway for coincidental double-strand-breaks (DSBs) at the proximal region of the deletion breakpoint, thereby generating the segmental UPD. The UPD boundary in the mother is located 10 Mb more proximal than the breakpoint of deleted region of the patient, which is a strong evidence that the UPD developed after the deletion. The principal molecular mechanisms that have been postulated to explain segmental UPD are mitotic recombination or break-induced replication (BIR) (Costantino et al., 2014; Carvalho et al., 2015).

We observed an almost completely skewed XCI in the mother's DNA. Generally, an XCI pattern increases the extent of skewing with age as a consequence of hematopoietic stem cell senescence. At 20–39 years old, mean skewing level is reported to be 70.6% (Hatakeyama et al., 2004). Our current patient's mother was 30 years old at the time of genetic testing and showed very high skewing at 99.2%. This indicated that her blood cells were derived from a single clone after XCI (Kurahashi et al., 1991). We speculated that the mother originally harbored the 11q23-qter deletion as a zygote which was subsequently repaired in one of the somatic cells by mitotic recombination or BIR after XCI has been completed. The repaired cell likely obtained selective advantage during embryonic development and unrepaired cells were eliminated (Fig. 2). This resulted in a normal phenotype at birth and no evidence of

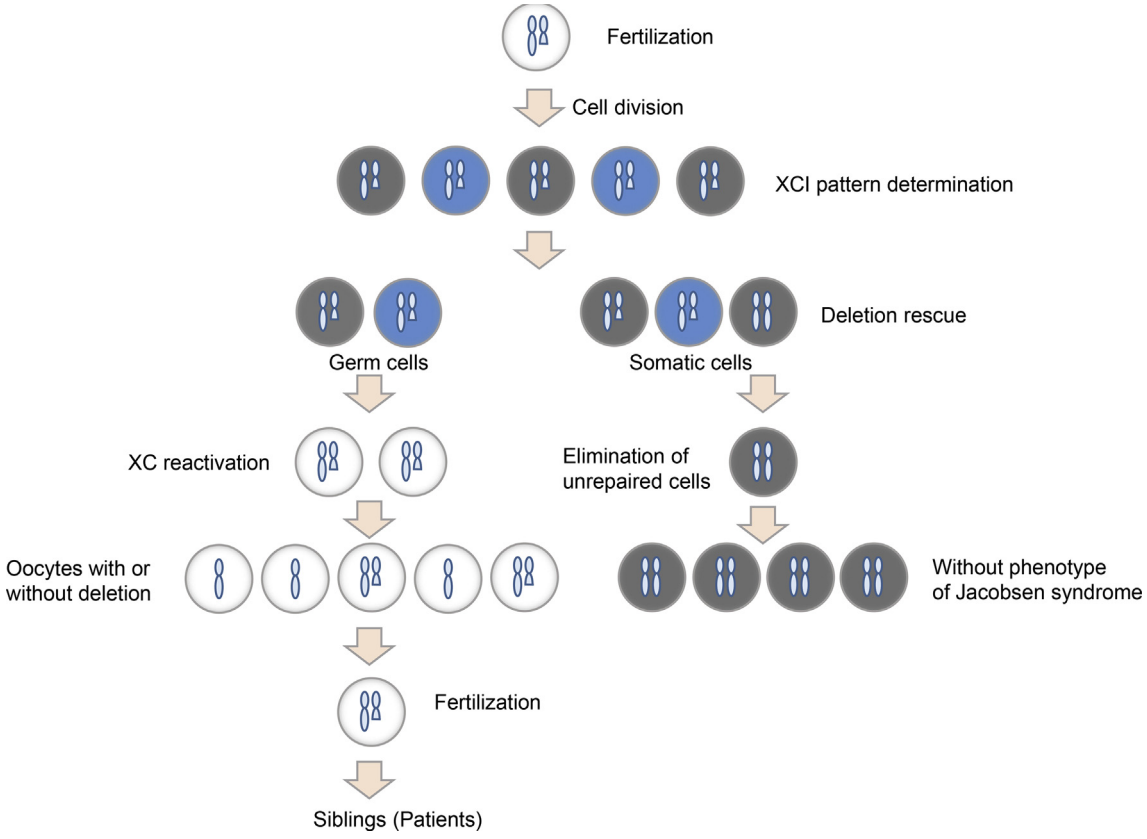


**Fig. 2.** An illustration for the status of the mother and the two siblings.

Jacobsen syndrome. It appears however that although her somatic cells had all been rescued by UPD her germ cells retained the 11q23-qter deletion. Such monoclonality has been described previously in trisomy rescue of chromosome 15 (Butler et al., 2007). Thus, this mother had no Jacobsen syndrome phenotype but transmitted the causative deletion to her two sons.

As far as we are aware, there have been only two other case reports of 'deletion rescue' (Bonaglia et al., 2009; Johnson et al., 2014). It would be intriguing if 11q23.3-qter was found to be a hotspot for deletion rescue. One possible explanation for this phenomenon is a strong negative selection process as a result of gene loss. An alternative possibility is that there might be a DSB hotspot that induces mitotic recombination or BIR at the region proximal to the 11q23.3 breakpoint. If this is indeed the case, the recurrence risk in the affected siblings would be slightly higher than in the general population. SNP array analysis of the parents might be advisable even in an apparent *de novo* case of Jacobsen syndrome.

In conclusion, we speculate that the maternal 11q23.3-qter deletion was repaired in our current study family via mitotic recombination or BIR leading to UPD generation. As a consequence of this DNA repair, the chromosomal copy number was corrected in the mother resulting in a normal phenotype. On the other hand, some of maternal germline cells retained 11q23-ter deletion, leading to a recurrence of Jacobsen syndrome in her offspring. Careful genetic counseling is therefore warranted regarding the recurrence of Jacobsen syndrome.

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# Frequent intragenic microdeletions of elastin in familial supravalvular aortic stenosis

Satoshi Hayano [a,1], Yusuke Okuno [b,1], Makiko Tsutsumi [c,1], Hidehito Inagaki [c,1], Yoshie Fukasawa [a,1], Hiroki Kurahashi [c,1], Seiji Kojima [a,1], Yoshiyuki Takahashi [a,1], Taichi Kato [a,*,1]

[a] Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Japan
[b] Center for Advanced Medicine and Clinical Research, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya, Japan
[c] Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Japan

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ABSTRACT

*Background:* Supravalvular aortic stenosis (SVAS) is a congenital heart disease affecting approximately 1:25,000 live births. SVAS may occur sporadically, be inherited in an autosomal dominant manner, or be associated with Williams-Beuren syndrome, a complex developmental disorder caused by a microdeletion of chromosome 7q11.23. *ELN* on 7q11.23, which encodes elastin, is the only known gene to be recurrently mutated in less than half of SVAS patients.
*Methods:* Whole-exome sequencing (WES) was performed for seven familial SVAS families to identify other causative gene mutations of SVAS.
*Results:* Three truncating mutations and three intragenic deletions affecting *ELN* were identified, yielding a diagnostic efficiency of 6/7 (85%). The deletions, which explained 3/7 of the present cohort, spanned 1–29 exons, which might be missed in the course of mutational analysis targeting point mutations. The presence of such deletions was validated by both WES-based copy number estimation and multiplex ligation-dependent probe amplification analyses, and their pathogenicity was reinforced by co-segregation with clinical presentations.
*Conclusions:* The majority of familial SVAS patients appear to carry *ELN* mutations, which strongly indicates that elastin is the most important causative gene for SVAS. The frequency of intragenic deletions highlights the need for quantitative tests to analyze *ELN* for efficient genetic diagnosis of SVAS.


## 1. Introduction

Supravalvular aortic stenosis (SVAS; MIM #185500) is a congenital heart disease affecting approximately 1:25,000 live births [1]. Congenital narrowing of the lumen of the ascending aorta or peripheral pulmonary arteries provokes increased resistance to blood flow and causes elevated ventricular pressure and hypertrophy resulting in heart failure. Peripheral pulmonary stenosis (PPS) is known to occasionally coexist with SVAS [2]. Approximately 30% to 50% of patients with SVAS have Williams-Beuren Syndrome (WBS; MIM #194050) [2–4], which is a complex genetic disorder caused by 7q11.23 microdeletion and characterized by growth failure, a characteristic facial appearance (so-called "Elfin face"), mental retardation, and SVAS [5].

On the other hand, Eisenberg et al. first reported non-syndromic "familial SVAS" with autosomal dominant inheritance in 1964 [3], accounting for 20% of SVAS cases (approximately 1:125,000 live births) [6]. These patients showed normal intelligence and lacked the dysmorphic features of WBS. Genetic analysis including linkage analysis identified *ELN*, which encodes elastin, as a causative gene of non-syndromic familial SVAS [1,7–17]. In harmony with the genetic findings, luminal obstruction of the aorta was shown in a transgenic mouse model carrying homozygous or heterozygous elastin gene deletion [18,19].

Metcalfe et al. sequenced *ELN* exons of patients with non-syndromic SVAS, which showed truncating mutations in 35 cases, but no causative variants were found in the remaining 64 patients (of which 8 were familial cases) [20]. Micale et al. also investigated *ELN* gene mutations in 14 familial and 10 sporadic cases of SVAS, resulting in 7 novel mutations, including 5 frameshift and 2 donor splice site mutations, but found no *ELN* gene abnormality in the remaining 17 cases [21]. Therefore, less than half of the cases could be explained by *ELN* mutations, whereas it still remains unclear whether *ELN* could explain the remaining cases

* Corresponding author.
*E-mail addresses:* javauma@gmail.com (S. Hayano), yusukeokuno@gmail.com (Y. Okuno), makiko@fujita-hu.ac.jp (M. Tsutsumi), hinagaki@fujita-hu.ac.jp (H. Inagaki), love.rodin.love@gmail.com (Y. Fukasawa), kura@fujita-hu.ac.jp (H. Kurahashi), kojimas@med.nagoya-u.ac.jp (S. Kojima), ytakaha@med.nagoya-u.ac.jp (Y. Takahashi), ktaichi@med.nagoya-u.ac.jp (T. Kato).
[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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0167-5273/

**Abbreviations**

| | |
|---|---|
| SVAS | supravalvular aortic stenosis |
| WES | whole-exome sequencing |
| PPS | peripheral pulmonary stenosis |
| WBS | Williams-Beuren syndrome |
| FISH | fluorescence in situ hybridization |
| MLPA | Multiplex ligation-dependent probe amplification |

with SVAS, or there are unidentified causative genes. In this study, whole-exome sequencing (WES) was performed with careful assessment of *ELN* mutations, including copy number analysis, to elucidate the genetic background of SVAS.

## 2. Methods

### 2.1. Sample collection

This study included seven families of Japanese ancestry with autosomal dominant inheritance of SVAS. There was no developmental delay or dysmorphic features suggestive of WBS or positive fluorescence in situ hybridization (FISH) on 7q11.23 in any family members. The vascular malformation (SVAS and PPS) was diagnosed if the sinotubular junction of the aorta was smaller than the diameter of the aortic annulus and significant pressure gradients were measurable by echocardiogram and/or angiographically [6]. Written, informed consent was obtained from patients or their parents, and whole blood or saliva was collected. Saliva samples were collected using an Oragene DNA self-collection kit (DNA Genotek, Ottawa, Canada). Genomic DNA was extracted from whole blood or saliva using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine (approval number 2015–0032).

### 2.2. Whole-exome sequencing analysis

Exome capture was performed on each proband using SureSelect Human All Exon V5 (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. Generated libraries were sequenced on a HiSeq 2500 platform (Illumina, San Diego, CA). Sequence data were analyzed using an in-house pipeline [22]. Briefly, reads were aligned to UCSC build hg19 reference genome using the Burrows-Wheeler Aligner [23]. Picard tools (http://broadinstitute.github.io/picard) were utilized to remove PCR duplicates. Variants were called using VarScan2, where a variant allele frequency of >0.20 was used as a cutoff [24]. ANNOVAR was used together with in-house scripts to annotate genetic variants [25]. The average depth of coverage across the whole exome for each sample achieved was 111.14 (range 90.68 to 127.66), and the number of mutations found per sample ranged from 25,534 to 25,920.

### 2.3. Mutational analysis

Mutational analysis to define each variant's pathogenicity was essentially based on the latest release of the American College of Medical Genetics (ACMG) guideline [26]. Briefly, variants outside of coding regions and common variants with >1% minor allele frequency in the National Heart, Lung, and Blood Institute ESP (Exome Sequencing Project) 6500 [27], 1000 Genomes Project [28], ExAC (Exome Aggregation Consortium) [29], HGVD (Human Genetic Variation Database) [30], or the in-house database were excluded. Variants expected to cause the disorders (eg, missense variants with reported pathogenicity and nonsense, frameshift insertion/deletion, and splice-site variants on genes known to cause a disease by inactivation) were validated by Sanger sequencing using PrimeSTAR GXL DNA polymerase (Takara, Shiga, Japan) and the Big Dye Terminator 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Waltham, MA) with ABI PRISM 3130xL (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table S1.

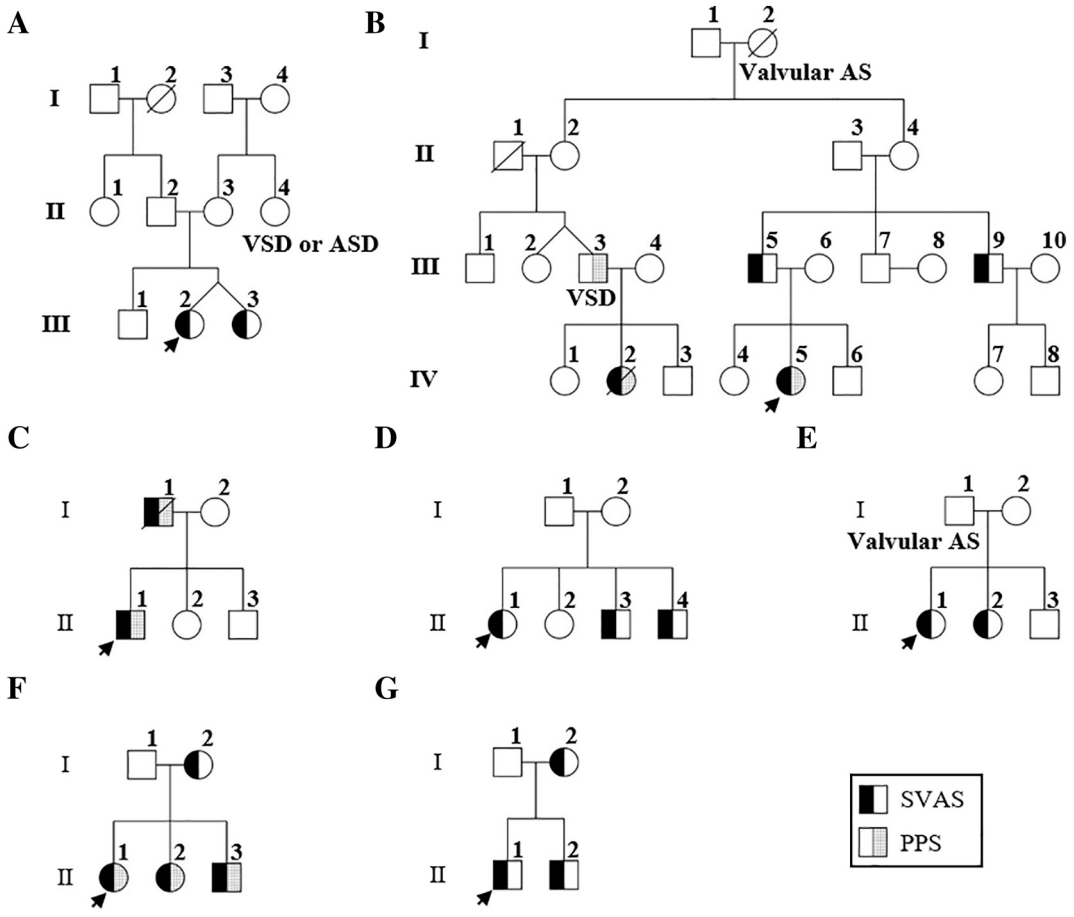


**Fig. 1.** Pedigree chart of families with familial supravalvular aortic stenosis Arrows indicate probands for whom whole-exome sequencing was performed. SVAS, supravalvular aortic stenosis; PPS, peripheral pulmonary stenosis; VSD, ventricular septal defect; ASD, atrial septal defect; AS, aortic stenosis.

*2.4. Copy number analysis*

Copy number analysis was performed by comparing the number of reads conveying each exon normalized by the mean depth of the entire sample with that of unrelated normal DNA samples, as we have previously shown [22]. Exons of normalized coverage >3 standard deviations (SDs) or less than −3 SDs from the mean coverage of reference samples were considered to be candidates for copy number variants. Multiplex ligation-dependent probe amplification (MLPA) according to the manufacturer's protocol with the SALSA MLPA P029-WBS probemix (MRC Holland, Amsterdam, Netherlands), which includes 10 exons of the *ELN* gene (Exon 1, 3, 4, 6, 9, 16, 20, 26, 27 and 33), was performed to validate candidate exonic deletions detected in the *ELN* gene by WES analysis. MLPA analysis software Coffalyser (MRC Holland) was used to identify CNVs.

## 3. Results

WES-based detection of point mutations and copy number alterations was performed in seven families of Japanese ancestry with SVAS showing an autosomal dominant mode of inheritance (Fig. 1). Three heterozygous pathogenic mutations in *ELN* (c.370delT, p.Ser124Leufs*13 in family A, c.572-1G > A splice site mutation affecting the acceptor of exon 12 in family B, and c.218_219insTG, p.Gly74Valfs*49 in family C) were identified. All of these mutations were novel, and they were validated on all available family members by Sanger sequencing (Fig. S1). Mutations were present

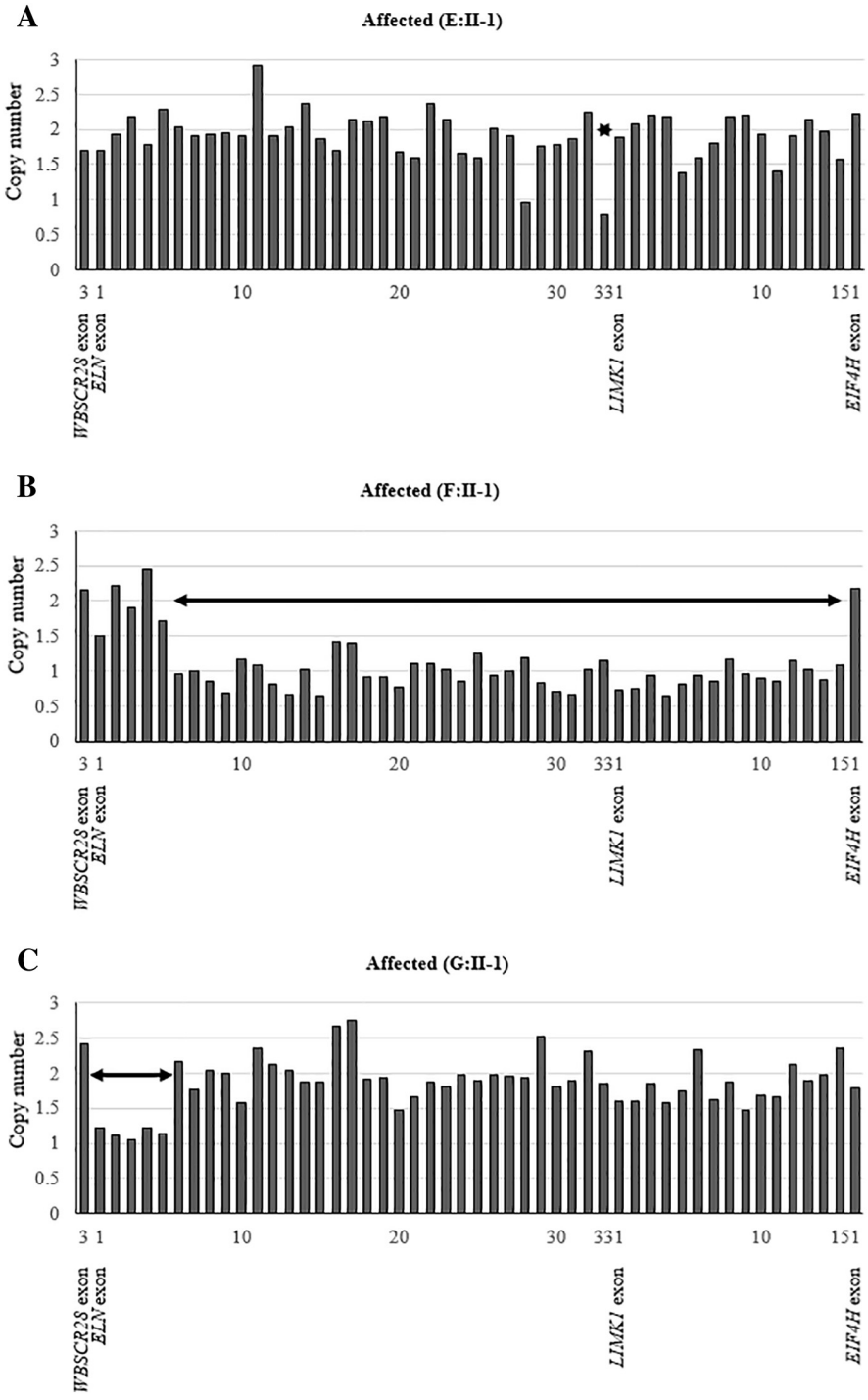


**Fig. 2.** Copy-number analysis The estimated copy number of each exon based on the number of reads in each exon in whole-exome sequencing. Each bar represents an exon, and the vertical axis represents the estimated copy number. Arrows indicate estimated deleted regions.

**Table 1**
Phenotype and segregation of *ELN* mutations.

| Family | Subject | Phenotype | *ELN* mutation |
|---|---|---|---|
| A | II-2 | – | – |
| | II-3 | – | c.370delT, p.Ser124Leufs*13 |
| | III-1 | – | – |
| | III-2 | SVAS | c.370delT, p.Ser124Leufs*13 |
| | III-3 | SVAS | c.370delT, p.Ser124Leufs*13 |
| B | III-3 | PPS, VSD | c.572-1G > A splice site |
| | III-5 | SVAS | c.572-1G > A splice site |
| | III-6 | – | – |
| | IV-4 | – | – |
| | IV-5 | SVAS, PPS | c.572-1G > A splice site |
| | IV-6 | – | – |
| C | I-2 | – | – |
| | II-1 | SVAS, PPS | c.218_219insTG, p.Gly74Valfs*49 |
| | II-2 | – | – |
| | II-3 | – | c.218_219insTG, p.Gly74Valfs*49 |
| D | II-1 | SVAS | – |
| E | I-1 | Valvular AS | Microdeletion (exon 33) |
| | I-2 | – | – |
| | II-1 | SVAS | Microdeletion (exon 33) |
| | II-2 | SVAS | Microdeletion (exon 33) |
| | II-3 | – | – |
| F | I-1 | – | – |
| | I-2 | SVAS | Microdeletion (exon 5–33) |
| | II-1 | SVAS, PPS | Microdeletion (exon 5–33) |
| | II-2 | SVAS, PPS | Microdeletion (exon 5–33) |
| | II-3 | SVAS, PPS | Microdeletion (exon 5–33) |
| G | I-1 | – | – |
| | I-2 | SVAS | Microdeletion (exon 1–5) |
| | II-1 | SVAS | Microdeletion (exon 1–5) |
| | II-2 | SVAS | Microdeletion (exon 1–5) |

All mutations were heterozygous.
SVAS, supravalvular aortic stenosis; PPS, peripheral pulmonary stenosis; VSD, ventricular septal defect; AS, aortic stenosis.

in all patients and several family members without SVAS, indicating incomplete penetrance (Table 1).

Copy number aberrations in *ELN* were identified in three other families, all of which were deletions (exon 33 in family E, exons 5–33 in family F, and exons 1–5 in family G, Fig. 2). Such deletions were validated by MLPA (Fig. S2). All microdeletions showed complete co-segregation with clinical symptoms (Table 1).

In the remaining family D, no diagnostic mutations associated with SVAS could be identified in the proband (D:II-1) either by WES or MLPA (Table S2, Fig. S3).

## 4. Discussion

Pathogenic mutations or deletions in *ELN* gene were identified in six of seven families with autosomal dominant inheritance of SVAS, including three novel point mutations and three intragenic deletions. These findings suggest that intragenic deletions in *ELN* gene could explain the genetic cause in half of so-far unexplained cases with familial SVAS in Japan. Updated by these findings, a comprehensive list of reported pathogenic SVAS mutations is provided (Fig. 3) [7–9,12,17,20,21,31–38].

*ELN* encodes elastin, which is expressed in various tissues and organs, including smooth muscle cells of the great arteries, and contributes to tissue elasticity [39,40]. The molecular mechanism of the pathogenesis of SVAS is not fully elucidated. However, considering accumulating knowledge from patients and transgenic mice [18,19,21,41], it seems likely that mutations of *ELN* impair vascular elasticity, and increased shear stress in the vascular wall could result in SVAS [39,40].

The microdeletions of *ELN* gene shown in the present study were not identified with existing FISH probes for WBS. There are a few case reports showing that microdeletions of *ELN* gene are the cause of SVAS [8,9]. The present finding raises the necessity to investigate the

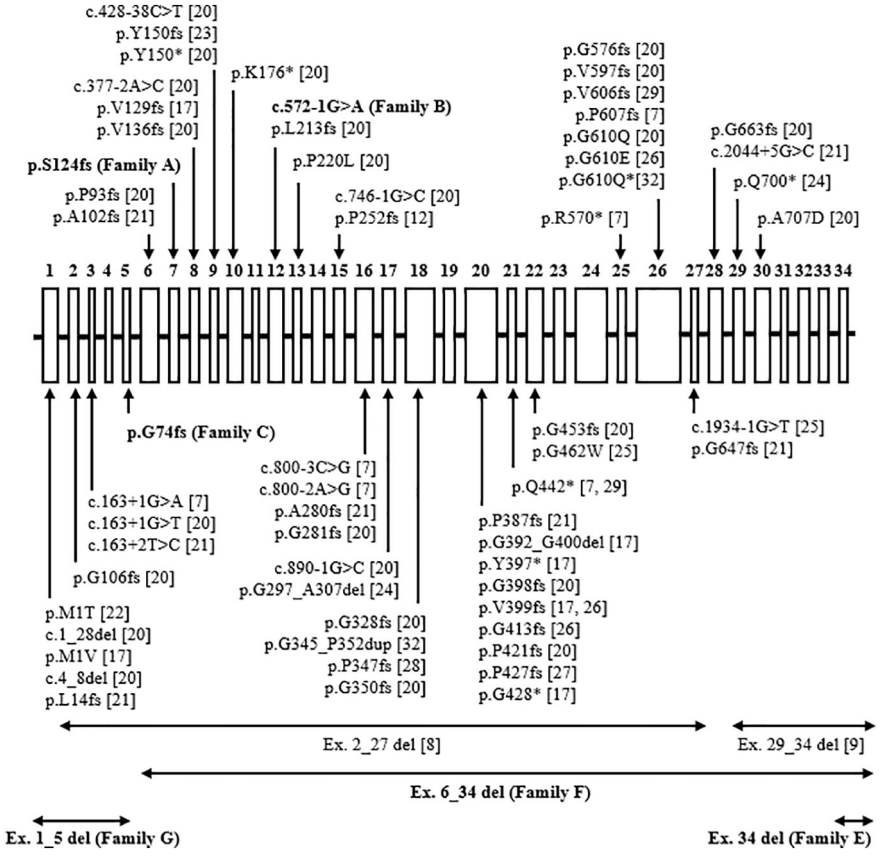


**Fig. 3.** *ELN* cDNA showing the exons and mutations detected This figure summarizes previously reported and newly identified mutations for familial and sporadic SVAS. The numbers above open boxes indicate the exon numbers. The present findings are shown in bold letters.

exon-spanning deletions affecting *ELN* using MLPA, array-CGH, or other methods to establish sufficient coverage for its mutations.

The present analysis showed co-segregation of symptoms and *ELN* mutations in the majority of analyzed individuals. The three mutations identified in this study (c.370delT in family A, c.572-1G > A splice site in family B, and c.218_219insTG in family C) were highly pathogenic truncating mutations that result in premature stop codons (PTCs). A number of PTC mutations have actually been shown to be substrates of *ELN* mRNA insufficiency through nonsense-mediated decay in previous studies [17,21,35].

A highly variable phenotype within families with SVAS has been reported for large studies with many families with point mutations [20,21], and even for a family with apparently damaging 30 kb deletion involving multiple exons [7], ranging from asymptomatic mutation carrier to severe stenosis with multiple arteries. There were also two asymptomatic persons carrying *ELN* mutations (A:II-3 and C:II-3) in the present study. Factors affecting the variability of cardiovascular phenotypes in patients with *ELN* mutation are not yet fully understood, and there has as yet been no clear genotype-phenotype correlation reported for SVAS. Our comprehensive list of reported pathogenic SVAS mutations and deletions showed a universal distribution of variants over the entire *ELN* gene with no significant hotspot.

The primary mechanism for the pathogenesis of SVAS is proposed to be haploinsufficiency of *ELN*, as hemizygosity of *ELN* is established as the mechanism of SVAS in WBS [5]. Incomplete penetrance and a broad range in severity of cardiovascular phenotype are also seen in patients with WBS in whom one copy of *ELN* gene is totally lost [42,43]. Among possible causes affecting the severity of symptoms, the effect of the mutations in the remaining allele of *ELN* is very limited, as only two rare missense changes were identified through exon sequencing of 49 patients with WBS [44]. Currently, there is no definite explanation for the phenotypic variability associated with *ELN* mutations.

The present study showed that microdeletions of *ELN* gene could account for additional cases, around a half of previously unexplained cases, of familial SVAS, which would strengthen the causative role of *ELN* mutations in this disease entity. Therefore, quantitative genetic tests such as MLPA or array-CGH of *ELN* gene should be performed to genetically diagnose patients with familial SVAS to obtain satisfactory sensitivity. Further investigations of a larger cohort and so-far unexplained cases will be needed to elucidate the remaining molecular pathogenetic mechanisms of SVAS.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.09.032.

## Authors' contributions

All authors developed the concept and designed the research; S.H., Y.O., and M.T. performed the experiments; S.H., Y.O., M.T., H.I., H.K., and T.K. analyzed the data; all authors interpreted the results of the experiments; S.H., Y.O., and T.K. prepared the figures; S.H., Y.O., M.T., H.I., Y.F., H.K., S.K., Y.T., and T.K. drafted the manuscript; S.H., Y.O., and T.K. edited the manuscript; all authors approved the final version of the manuscript.

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## Competing interests

The authors report no relationships that could be construed as a conflict of interest.

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CASE REPORT Open Access

# Exonic duplication of the *OTC* gene by a complex rearrangement that likely occurred via a replication-based mechanism: a case report

Katsuyuki Yokoi[1,2], Yoko Nakajima[1], Hidehito Inagaki[2], Makiko Tsutsumi[2], Tetsuya Ito[1] and Hiroki Kurahashi[2*]

## Abstract

**Background:** Ornithine transcarbamylase deficiency (OTCD) is an X-linked recessive disorder involving a defect in the urea cycle caused by *OTC* gene mutations. Although a total of 417 disease-causing mutations in *OTC* have been reported, structural abnormalities in this gene are rare. We here describe a female OTCD case caused by an exonic duplication of the *OTC* gene (exons 1–6).

**Case presentation:** A 23-year-old woman with late-onset OTCD diagnosed by biochemical testing was subjected to subsequent genetic testing. Sanger sequencing revealed no pathogenic mutation throughout the coding exons of the *OTC* gene, but multiplex ligation-dependent probe amplification (MLPA) revealed duplication of exons 1–6. Further genetic analyses revealed an inversion of duplicated exon 1 and a tandem duplication of exons 2–6. Each of the junctions of the inversion harbored a microhomology and non-templated microinsertion, respectively, suggesting a replication-based mechanism. The duplication was also of de novo origin but segregation analysis indicated that it took place in the paternal chromosome.

**Conclusion:** We report the first OTCD case harboring an exonic duplication in the *OTC* gene. The functional defects caused by this anomaly were determined via structural analysis of its complex rearrangements.

**Keywords:** Ornithine transcarbamylase deficiency, Exonic duplication, Complex rearrangement, Fork stalling and template switching (FoSTeS), Non-homologous end joining (NHEJ)

## Background

Ornithine transcarbamylase (OTC) is a mitochondrial urea cycle enzyme that catalyzes the reaction between carbamyl phosphate and ornithine to form citrulline and phosphate [1]. Ornithine transcarbamylase deficiency (OTCD) is one of the most common urea cycle disorders [2] with an estimated prevalence of 1 in 14,000–77,000 [1]. The human *OTC* gene, located on the short arm of the X chromosome (Xp11.4), is 73 kb with10 exons and 1062 bp of coding sequence [3–5]. Because OTCD is inherited in an X-linked manner, deficient hemizygous males usually develop this disorder. However, a remarkable feature of OTCD is that a substantial subset of heterozygous females also develop this condition. The symptoms of carrier females vary in terms of onset and severity. Since the *OTC* gene is subject to X-inactivation, it is believed that this phenotypic variability depends on a skewed degree of this in the livers of carrier females [5].

In 85–90% of patients with a biochemical phenotype of OTCD, a mutation can be identified through sequencing or deletion/duplication testing [6]. A total of 417 disease-causing mutations in the *OTC* gene have been reported to date [1]. Exonic deletions have also been described but no prior case of OTCD caused by exonic duplication has previously been reported [7]. In our current case report, we describe a female patient with OTCD caused by a partial duplication of *OTC* exons 1–6.

* Correspondence: kura@fujita-hu.ac.jp
[2]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
Full list of author information is available at the end of the article

## Case presentation

### Patient

The current study patient was a 23-year-old woman with normal psychomotor development and healthy nonconsanguineous parents. She had frequent episodes of nausea, vomiting, stomachache and temporary elevated transaminase from about 4 years of age. Ammonia and plasma amino acid levels were measured when she was 5 years old. Her serum ammonia was 220 $\mu$g/dl (normal range 12 ~ 60 $\mu$g/dl) and she showed high levels of glutamine (1212 nmol/ml; normal value, 420–700), lower normal limits of citrulline (18.4 nmol/ml; normal value, 17–43), and lower plasma levels of arginine (32.2 nmol/ml; normal value, 54–130). A urine metabolic screen indicated a gross elevation in orotate (orotate/creatinine ratio 234.3 $\mu$mol/g creatinine; normal value, 4.7 ~ 15.9 $\mu$mol/g creatinine). These findings were consistent with OTC deficiency. She was therefore biochemically diagnosed with OTCD and her blood ammonia level has been well controlled since by a protein-restricted diet and by oral sodium phenylbutyrate and arginine. Recently, we performed genetic analysis to identify the genetic alterations of the *OTC* gene in this patient. However, Sanger sequencing revealed no pathogenic mutation.

### Genetic analysis

#### *Mutational analyses*

Sanger sequencing was performed to screen for genetic variations at the nucleotide level throughout all coding exons of the *OTC* gene (Additional file 1). We used UCSC genome browser (http://genome-asia.ucsc.edu/) as human genome assembly. To screen for exonic deletions or duplications, multiplex ligation-dependent probe amplification (MLPA) was performed using the SALSA P079-A3 *OTC* MLPA kit (MRC Holland, Amsterdam, The Netherlands), in accordance with the manufacturer's recommendations. MLPA products were separated by capillary electrophoresis on an ABI3730 genetic analyzer and then processed using GeneMapper software. The peak heights of the samples were compared with control probes and the ratios of these peaks were calculated for all exons. If the dosage quotient was 1.0, the results were considered normal. Thresholds for deletions and duplications were set at 0.5 and 1.5, respectively.

#### *Quantitative real time PCR*

To demarcate the duplicated region, quantitative real-time PCR was conducted on blood DNA from the patient and a male control subject using the Applied Biosystems 7300 real time PCR system (Thermo Fisher Scientific). Several primer pairs were designed for *OTC* (upstream of exon 1 and intron 6) and *RPP30* that was used as an autosomal single copy gene reference to generate amplicons suitable for real-time PCR (Fig. 1b, Additional file 1). The PCR reaction was performed in a 15 μL reaction system, containing 2 μL of template DNA (5 ng/μL), 0.6 μL of each primer set (10 μmol/L), 0.3 μL ROX Reference Dye, 4 μL distilled water, and 7.5 μL of 2xTB Green Premix Ex TaqII (Tli RNaseH Plus, TaKaRa). Two parallel PCR reactions were prepared for each sample. The amplification cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C 5 s and 60 °C for 1 min. Data evaluation was carried out using the 7300 system SDS software and Microsoft Excel. The threshold cycle number (Ct) was determined for all PCR reactions and the same threshold and baseline were set for all samples. The starting copy number of the samples was determined using theΔΔCt-Method. ΔΔCt method was a modification of the method described in Livak et al. for quantifying mRNA [8]. ΔCt represents the mean Ct value of each sample and was calculated for *OTC* and *RPP30*. The starting copy number of the unknown samples was determined relative to the known copy number of the control sample using the following formula:

ΔΔCt = [ΔCt OTC(patient)-ΔCt RPP30(patient)] - [ΔCt OTC(female)-ΔCt RPP30(female)]. The relative gene copy number was calculated by the expression $2^{-\Delta(\Delta Ct)}$. The starting copy number of male control was also determined as a reference value.

#### *Inverse PCR*

Inverse PCR were performed using restriction enzyme *Taq*I (TaKaRa, Shiga, Japan) to isolate the unknown sequences adjacent to the duplicated region of the *OTC* gene in the study patient. ApE – A plasmid Editor software was used to identify the recognition sites for the restriction enzyme. The restriction enzyme was chosen based on the following criteria: (1) no cutting of the expected breakpoint area; and (2) endonuclease activity would be unaffected by CpG methylation of the target sequence. A 100 ng aliquot of genomic DNA from both our patient and a control female was digested with the selected restriction enzyme in a total volume of 30 μl at 65 °C for 90 min. The reaction was inactivated using the QiaQuick PCR Purification Kit. A 20 μL sample of digested DNA was then mixed with 23 μL of DW, 5 μL of 10 × T4 ligase buffer (TaKaRa, Shiga, Japan) and 2 μL of T4 DNA ligase to make a final volume of 50 μL. Ligation reactions were incubated at 16 °C for 16 h. For subsequent PCR, 1 μL of digested and re-ligated DNA template was used in a total reaction volume of 25 μL with Tks Gflex DNA Polymerase (TaKaRa, Shiga, Japan). Primers were designed to avoid repetitive sequences (Additional file 1). The PCR conditions were as follows: 30 cycles of 10 s at 98 °C, 15 s at 60 °C, and 1 min at 68 °C. Amplified products were analyzed by gel

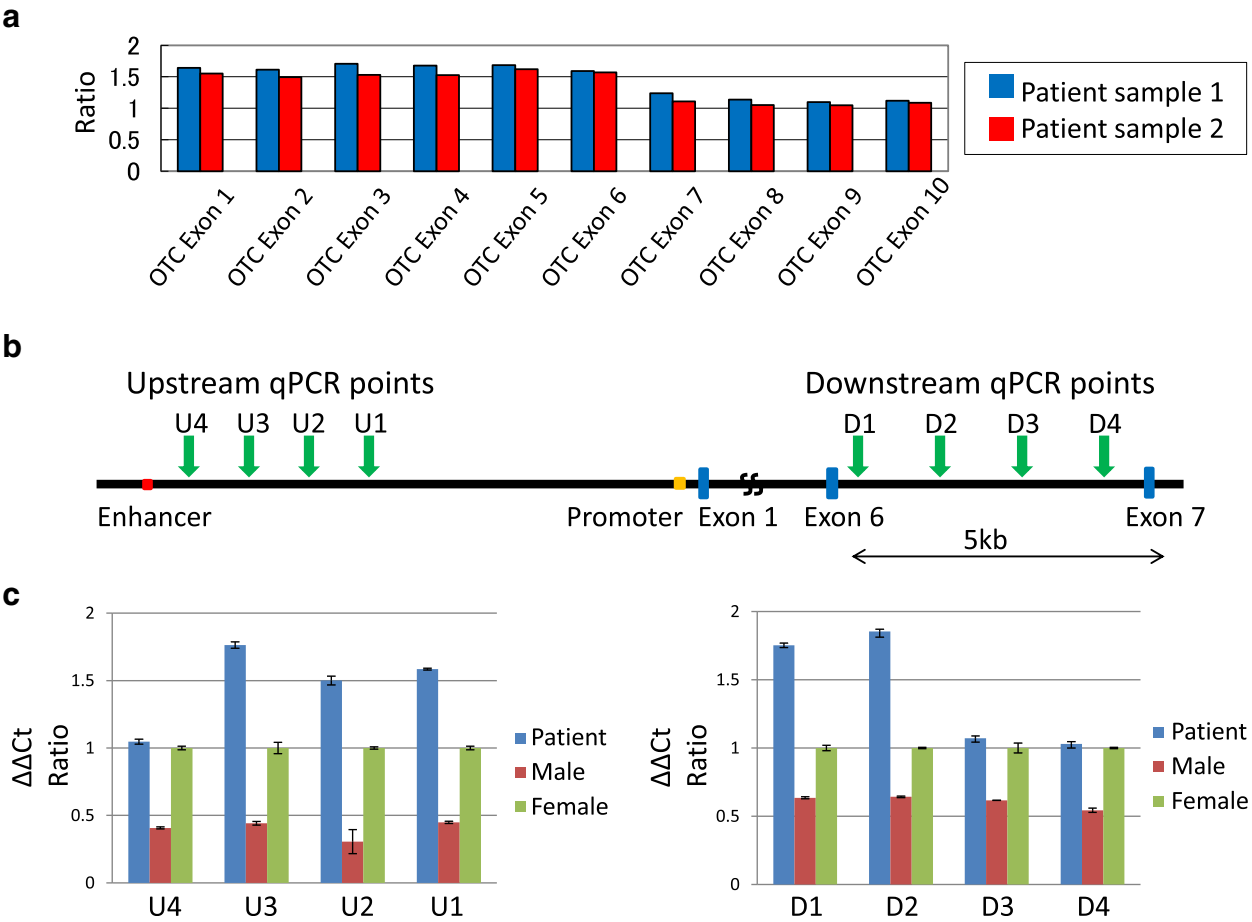


**Fig. 1** Exonic duplication of the *OTC* gene in a female OTCD patient. **a** MLPA results for the *OTC* gene in this patient indicting duplication of exons 1–6. **b** Structure of the *OTC* gene. The blue boxes denote exons. The enhancer and promoter regions are shown in red and orange, respectively. The positions of the qPCR units are indicated by green arrows. Upstream qPCR units: U1 - U4. Downstream qPCR units: D1 - D4. **c** qPCR results. The left and right panels show the qPCR findings for the upstream and downstream regions, respectively. The blue bars indicate the patient and the red and green bars denote the male and female controls, respectively. The Y- axis indicates the **ΔΔ** Ct ratio

electrophoresis and were purified following nested PCR (Additional file 1). The purified PCR products were sequenced via the standard Sanger method.

### *Breakpoint analysis on the other side*

PCR was performed using Tks Gflex (TaKaRa, Shiga, Japan) to confirm the other side of the breakpoint sequence. Primer R which was previously designed for real-time PCR analysis of *OTC* upstream of exon 1 (i.e. *OTC* intron 1) was used as primer F in this reaction (Additional file 1). The PCR conditions and Sanger methodology were similar to those described above.

MLPA revealed the duplication of exons 1–6 of the *OTC* gene in our current study patient (Fig. 1a). We determined the range of the duplication using quantitative real-time PCR (Fig. 1b). We designed four qPCR experiments (U1-U4) between the promoter and enhancer regions to identify the upstream breakpoint. Likewise, we designed four qPCR assays (D1-D4) within intron 6 to identify the downstream breakpoint. In contrast to the male or female controls that showed ΔΔCt ratios of 0.5 or 1.0, respectively, the patient's samples showed aΔΔCt ratio > 1.5 in some of these qPCR assays, suggesting that these regions were duplicated in this patient (Fig. 1c). The results indicated that the putative upstream breakpoints were located between PCR U3 and U4, and that the downstream breakpoints were between PCR D2 and D3.

We next performed inverse PCR to analyze the genomic structure of the duplicated region. *Taq*I-digested DNA was used as a template to produce a 3.5 kb PCR product when amplified with inversely oriented intron 6 primers (Fig. 2a, c). However, an additional small PCR product was detected by agarose gel electrophoresis in the patient sample (Fig. 2a). The amplified products were sequenced after nested PCR (Fig. 2a). As expected,

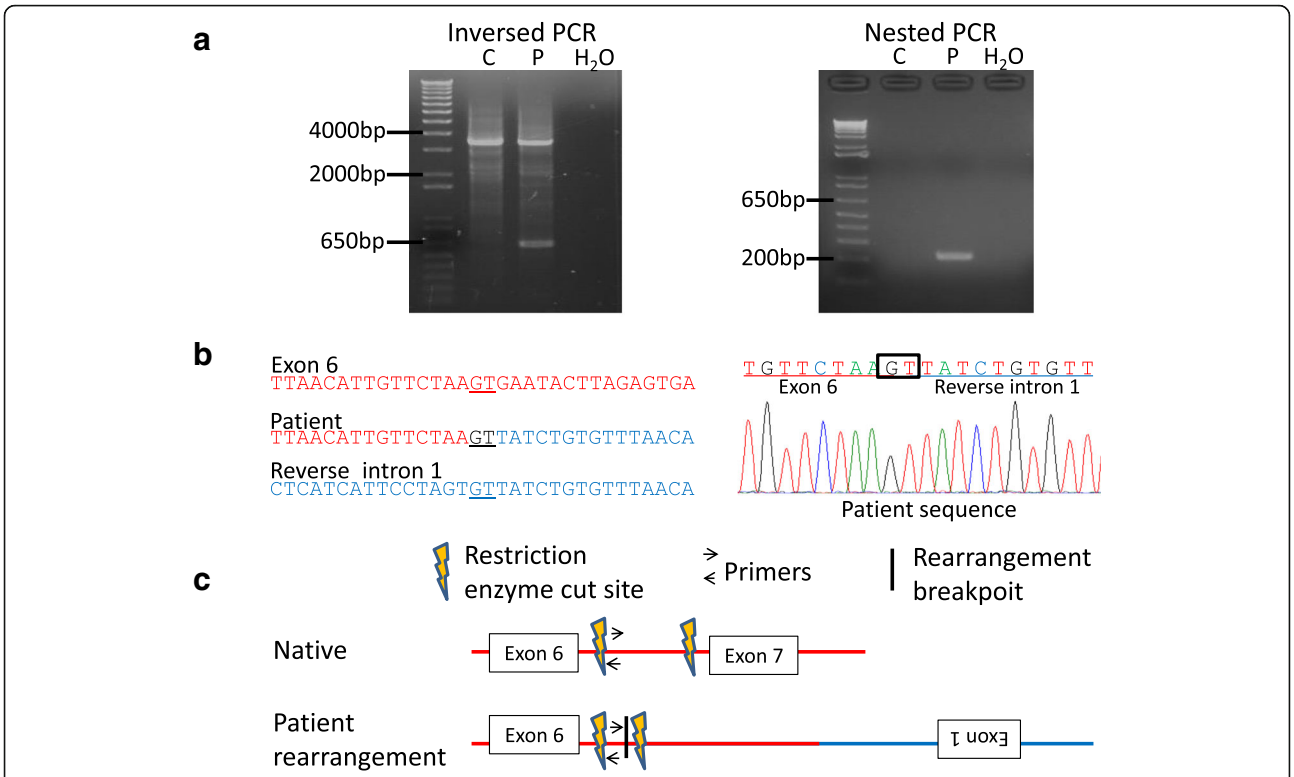


**Fig. 2** Identification of the duplication junction via inverse PCR. **a** Isolation of the junction fragment. Two distinct inverse PCR products were observed following agarose gel electrophoresis. The larger product was derived from a normal allele and the small product from a rearranged allele (left). The amplified products were purified following nested PCR (right). P, patient; C, control; H, $H_2O$. **b** Sanger sequencing of the PCR products including the junction. The unknown sequence next to the junction was identified as intron 1 of the *OTC* gene in the reverse orientation. The normal exon 6 and intron 1 sequences are aligned in red and blue typeface, respectively. Underlined nucleotides indicate microhomology at the breakpoint junction. **c** Predicted structure of the junction. Horizontal arrows indicate the recognition sites of the primers used for inverse PCR and the vertical arrows denote the *Taq*I restriction sites

the breakpoint was located within intron 6 (Fig. 2b, c). Unexpectedly however, this breakpoint was found to be connected with intron 1 of the *OTC* gene in the reverse orientation. The breakpoint junction contained 2 nucleotides of microhomology at the fusion junction (Fig. 2b).

The other side breakpoint was analyzed using standard PCR with primers for the upstream breakpoint region and the breakpoint region in intron 1. The primer pair amplified only products from the patient's DNA (Fig. 3a). By Sanger sequencing, the upstream region of the *OTC* gene was found to make an inverted connection with 1 (Fig. 3b, c). This breakpoint junction contained an additional 4 nucleotides (ACTA) of unknown origin (Fig. 3b). The positions of the two breakpoints in intron 1 were found to be chrX: 38365292 and chrX: 38366694, which were 1402 bp apart (Fig. 3c). We performed the same PCR amplification of both junctions in the patient's parents but detected no products, suggesting that this complex rearrangement arose de novo. The patient's duplicated region included a common single nucleotide variant (rs752750694, NM_000531.5:c.-844C > T). The patient's father carries an A whereas the mother carries a G/G at this site (Fig. 3d). The patient was found to be an A/G heterozygote, but the peak of the A nucleotide was two-fold greater than the G-peak, suggesting that the patient carries two copies of A. These data suggest that the de novo duplication was of paternal origin.

## Discussion and conclusions

We here report the first documented case of OTCD caused by an exonic duplication of the *OTC* gene. Although the MLPA results for this case indicated a simple duplication of exons 1–6, further analysis indicated that it resulted from complex rearrangements. Two possible mechanisms have been proposed for such rearrangements: one is chromothripsis that is caused by chromosome shuttering followed by reunion, and the other is chromoanasynthesis that is a replication-based mechanism also known as fork stalling and template switching (FoSTeS)/microhomology-mediated break-induced replication (MMBIR). According to the replication-based model, the active replication fork can stall and switch templates using complementary template microhomology to anneal and prime DNA replication. This

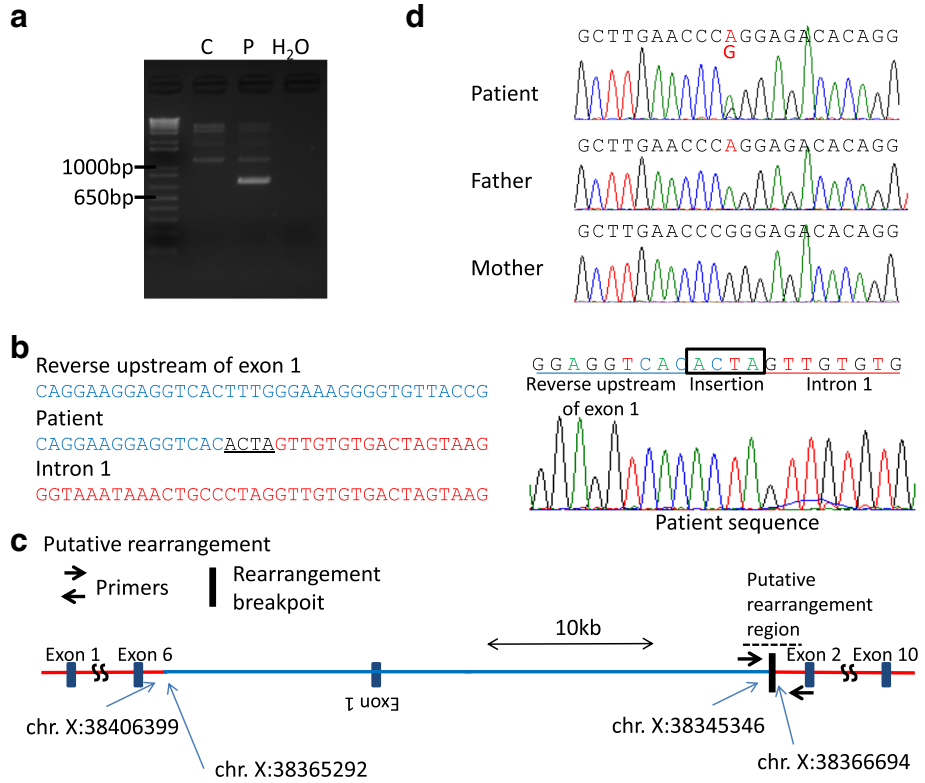


**Fig. 3** The complex rearrangement in the *OTC* gene of the study patient likely occurred via a replication-based mechanism. **a** Identification of the other junction by PCR. The PCR primer pair successfully amplified the junction product only from the OTCD study patient DNA. P, patient; C, control; H, $H_2O$. **b** Sanger sequencing results for the PCR products including the junction. The normal sequence of the *OTC* gene upstream region and intron 1 are aligned in blue and red typeface, respectively. Underlined bases denote a non-templated microinsertion at the junction. **c** Predicted structure of the complex rearrangement leading to the *OTC* gene duplication. The positions of the PCR primers are indicated by black arrows. The first junction is indicated by a blue arrow. The nucleotide position of the breakpoints on the X-chromosome are also indicated. The position of the two breakpoints in intron 1 were found to be chr. X: 38365292 and chr. X: 38366694, which were 1402 bp apart. **d** Trio-genotyping of the common single nucleotide variant (rs752750694, NM 000531.5:c.-844C > T). The patient's father carries A and her mother carries G/G. The patient was found to be an A/G heterozygote, but her A peak was two-fold higher than the G-peak. The areas under the curve (AUC) were 900 (A) and 328 (G) (Image J)

mechanism enables the joining or template-driven juxtaposition of different sequences from discrete genomic positions and can result in complex rearrangements [9].

In our current OTCD case, one junction presented 2 nucleotides of microhomology (GT), and the other junction manifested 4 nucleotides as a microinsertion (ACTA). Copy number variation with complex rearrangements and the presence of microhomology is indicative of a replication-based mechanism but the evidence for a non-templated microinsertion is noteworthy. Microinsertions are often observed in non-proofing DNA repair processes such as non-homologous end joining (NHEJ), which is activated by double-strand breaks [10]. However, a considerable body of evidence now suggests that microinsertions can be identified at junctions mediated by DNA replication-based mechanisms [11, 12]. A recent study has also suggested that an NHEJ-like pathway mediated by Polθ, which is an alternative NHEJ mechanism, may be induced by replication stress [13]. Taken together, an alternative NHEJ pathway might be activated during aberrant replication to restore DNA integrity, thus leading to chromoanasynthesis.

The evidence to date also suggests that de novo mutations occur more frequently in paternal alleles [14]. This bias is attributed to the higher number of DNA replication events in spermatogenesis than in oogenesis. Likewise, chromosomal structural variations are more frequently derived from the father [15]. The complex genomic rearrangements in our present patient were found to be of de novo origin but genotyping of a single nucleotide variant in the *OTC* gene demonstrated that the rearrangement allele originated from her father. Given the higher chance of the DNA replication errors during spermatogenesis, it might also reflect the replication-based mechanism.

MLPA can be used in the molecular diagnosis of several genetic diseases whose pathogenesis is related to the presence of deletions or duplications of specific genes [16]. Although deletions are clearly pathogenic, this is less certain in the case of duplications. In case of the

*OTC* gene for example, duplications of the entire gene are innocuous and present as a normal variant in the general population [7]. In cases of partial duplication as seen in our current patient, gene function may not be necessarily be affected when the additional sequence is inserted into another genomic locus. Even in cases of a tandem duplication, it is feasible that one copy of the *OTC* gene may maintain an intact structure. In the current OTCD case, the inversion of exon 1 occurred together with its duplication. We predicted in this instance that this complex rearrangement would generate a tandem duplication of exons 2–6 and the production of truncated OTC proteins with defective function due to a frameshift or null protein expression due to nonsense-mediated mRNA decay. The functional defects caused by this mutant allele were therefore the cause of the OTCD in this woman.

In conclusion, we report the first case of OTCD caused by a complex rearrangement resulting in exonic duplication of the *OTC* gene. Our present report also emphasizes the necessity of fully investigating whether pathogenicity has resulted from a genomic duplication.

## Additional file

**Additional file 1:** PCR primers and genomic coordinates. (a) Primers for Sanger sequences of *OTC* exons. (b) Primers for qRT-PCR. (c) Other PCR. (XLSX 13 kb)

### Abbreviations

FoSTeS: Fork stalling and template switching; MLPA: Multiplex ligation-dependent probe amplification; MMBIR: Microhomology-mediated break-induced replication; NHEJ: Non-homologous end joining; OTC: Ornithine transcarbamylase; OTCD: Ornithine transcarbamylase deficiency

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### Availability of data and materials

All data generated or analysed during this study are included in this published article [and its Additional files].

### Authors' contributions

KY did most of the experiments, retrieved the data, drafted and revised the manuscript. YN and TI discovered the patients and provided many data. HI and MT supported and supervised experiments. HK have contributed equally to the manuscript. All authors contributed to and reviewed the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2005(5). The study protocol was approved by the Ethical Review Board for Human Genome Studies at Fujita Health University.

### Consent for publication

Written informed consent to publish medical information and images was obtained from all patients reported in this publication.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



### Author details

[1]Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan. [2]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan.

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RESEARCH REPORT

# Disruption of the Responsible Gene in a Phosphoglucomutase 1 Deficiency Patient by Homozygous Chromosomal Inversion

**Katsuyuki Yokoi • Yoko Nakajima • Tamae Ohye • Hidehito Inagaki • Yoshinao Wada • Tokiko Fukuda • Hideo Sugie • Isao Yuasa • Tetsuya Ito • Hiroki Kurahashi**

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**Abstract** Phosphoglucomutase 1 (PGM1) deficiency is a recently defined disease characterized by glycogenosis and a congenital glycosylation disorder caused by recessive mutations in the *PGM1* gene. We report a case of a 12-year-old boy with first-cousin parents who was diagnosed with a PGM1 deficiency due to significantly decreased PGM1 activity in his muscle. However, Sanger sequencing revealed no pathogenic mutation in the *PGM1* gene in this patient. As this case presented with a cleft palate in addition to hypoglycemia and elevated transaminases and creatine kinase, karyotyping was performed and identified homozygous inv(1)(p31.1p32.3). Based on the chromosomal location of the *PGM1* gene at 1p31, we analyzed the breakpoint of the inversion. Fluorescence in situ hybridization (FISH) combined with long PCR analysis revealed that the inversion disrupts the *PGM1* gene within intron 1. Since the initiation codon in the *PGM1* gene is located within exon 1, we speculated that this inversion inactivates the *PGM1* gene and was therefore responsible for the patient's phenotype. When standard molecular testing fails to reveal a mutation despite a positive clinical and biochemical diagnosis, the presence of a gross structural variant that requires karyotypic examination must be considered.

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K. Yokoi · Y. Nakajima · T. Ito
Department of Pediatrics, Fujita Health University School of Medicine, Toyoake, Japan

K. Yokoi · T. Ohye · H. Inagaki · H. Kurahashi (✉)
Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan
e-mail: kura@fujita-hu.ac.jp

Y. Wada
Department of Obstetric Medicine, Osaka Women's and Children's Hospital, Osaka, Japan

T. Fukuda
Department of Pediatrics, Hamamatsu University School of Medicine, Hamamatsu, Japan

H. Sugie
Faculty of Health and Medical Sciences, Tokoha University, Hamamatsu, Japan

I. Yuasa
Division of Legal Medicine, Tottori University Faculty of Medicine, Yonago, Japan

H. Kurahashi
Genome and Transcriptome Analysis Center, Fujita Health University, Toyoake, Japan

H. Kurahashi
Center for Collaboration in Research and Education, Fujita Health University, Toyoake, Japan

## Introduction

Phosphoglucomutase 1 (PGM1) deficiency is a recently defined disease, characterized by glycogenosis and a congenital disorder of glycosylation (CDG) (Tagtmeyer et al. 2014). ς PGM1 deficiency is rare with only 38 patients from 29 families with different ethnic backgrounds described in the literature so far (Perez et al. 2013; Ondruskova et al. 2014; Tagtmeyer et al. 2014; Loewenthal et al. 2015; Zeevaert et al. 2016; Wong et al. 2016; Preisler et al. 2017; Nolting et al. 2017; Voermans et al. 2017). PGM1 is an essential enzyme in carbohydrate biosynthesis and metabolism and functions both in glycogen synthesis and breakdown through a reversible conversion of glucose

1-phosphate to glucose 6-phosphate (Morava 2014). Since glucose 1-phosphate is a precursor of the nucleotide sugars used for glycan biosynthesis, PGM1 activity is also required for protein *N*-glycosylation (Beamer 2015). Hence, PGM1 deficiency has considerably diverse phenotypes. Most of the affected patients develop a congenital anomaly syndrome showing a bifid uvula, cleft palate, and Pierre Robin sequence as clinical manifestations from the time of birth. Hepatopathy, dilated cardiomyopathy (DCM), hypoglycemia, muscle weakness, exercise intolerance, growth retardation, and endocrine abnormalities emerge in these cases over time (Scott et al. 2014). Many of these manifestations can be linked to the role of PGM1 in glucose metabolism and glycosylation (Beamer 2015).

PGM1 deficiency is caused by homozygous or compound heterozygous nucleotide alterations in the *PGM1* gene (Herbich et al. 1985). Several types of mutations have been reported to date including missense mutations, frameshifts, and splicing mutations (Tagtmeyer et al. 2014; Lee et al. 2014; Perez et al. 2013; Timal et al. 2012; Stojkovic et al. 2009; Ondruskova et al. 2014). In our current report, we describe a case of PGM1 deficiency caused by a homozygous chromosomal inversion that disrupts the *PGM1* gene at chromosome 1p31.

## Materials and Methods

### Cytogenetic Analysis

Fluorescence in situ hybridization (FISH) analysis of the patient and his parents was performed using standard methods to detect the breakpoint region at the chromosome level. Briefly, phytohemagglutinin-stimulated lymphocytes or Epstein-Barr virus-transformed lymphoblastoid cell lines derived from the subjects were arrested by exposure to colcemid. Metaphase preparations were then obtained by hypotonic treatment with 0.075 M KCl followed by methanol/acetate fixation. A bacterial artificial clone (BAC) containing 1p31.1, RP4-534K7 (chr1:63,525,021-63,677,603), was used as the test probe, and a chromosome 1 centromere probe (CEN1 SpectrumOrange Probe; Abbott Laboratories, Abbott Park, IL) was used as a reference. The probes were labeled by nick translation with digoxigenin-11-dUTP. After hybridization, the probes were detected with DyLight 488 Anti-Digoxigenin/Digoxin. Chromosomes were visualized by counterstaining with 4,6-diamino-2-phenylindole.

### Sequence Analysis

To isolate the breakpoint, long-range PCR with several sets of primers for the *PGM1* gene was performed using LA Taq (TaKaRa, Shiga, Japan) (Fig. 3c). The PCR conditions were 35 cycles of 10 s at 98°C and 15 min at 60°C. PCR primers were designed using sequence data from the human genome database. PCR products were separated on 0.8% (w/v) agarose gels and visualized with ethidium bromide. The homology between the obtained sequence around the breakpoint within the *PGM1* gene and the 1p32.3 sequence obtained from the database was examined using the BLAT in UCSC genome browser (http://genome-asia.ucsc.edu/human GRCh38/hg38).

### Patient

The current study patient was a 12-year-old boy from consanguineous parents who are first cousins without a family history of congenital metabolic disease (Fig. 1). The patient's height was 137 cm (*z*-score −2.3), and he had a normal body weight of 39 kg (*z*-score −0.6). He was born at term with a normal body weight and length. A cleft palate was noted at birth and closure surgery was performed at 12 months. Persistently elevated transaminases (AST 50–400 U/L [normal value <33 U/L] and ALT 40–300 U/L [normal value <30 U/L]) had been observed since that surgery. In addition, mild hypoglycemia after overnight fasting and an occasionally elevated serum creatine kinase (100–2,600 U/L [normal value <287 U/L]) were evident from 2 years of age. The echocardiogram and electrocardiogram readings showed no abnormalities, and his psychomotor development was normal. Oral administration of uncooked corn starch prior to bedtime was commenced to prevent morning hypoglycemia.

At 2 years of age, the patient was referred to our department for further examination. Intravenous glucose loading at 2 g/kg led to an elevated lactate level (from 7 to 37 mg/dL at 120 min) with a normal lactate/pyruvate ratio. Intramuscular glucagon loading at 0.03 mg/kg caused no increase of blood sugar either during fasting or at 2 h after a meal, indicating a deficiency in the generation of hepatic glucose from glycogen. However, the activity of the debrancher enzyme responsible for glycogen storage disease (GSD) type III, phosphorylase involved in GSD type VI, and phosphorylase kinase enzyme associated with GSD type IX in the peripheral blood was normal. A forearm nonischemic exercise test was performed when the patient was 8 years old. No increase in venous lactate with a large elevation in his ammonia levels (297 μg/dL) was observed, suggesting inadequate glycogen utilization in the muscle. A muscle biopsy was therefore performed, and a significant decrease in PGM activity was identified (62.1 nmol/min/mg [controls 351.1 ± 81.1]). Isoelectric focusing (IEF) of serum transferrin was performed as previously described (Okanishi et al. 2008) and revealed a mixed type I and type II pattern, typical features of CDG-I and CDG-II (Fig. 2) (Tagtmeyer et al. 2014).

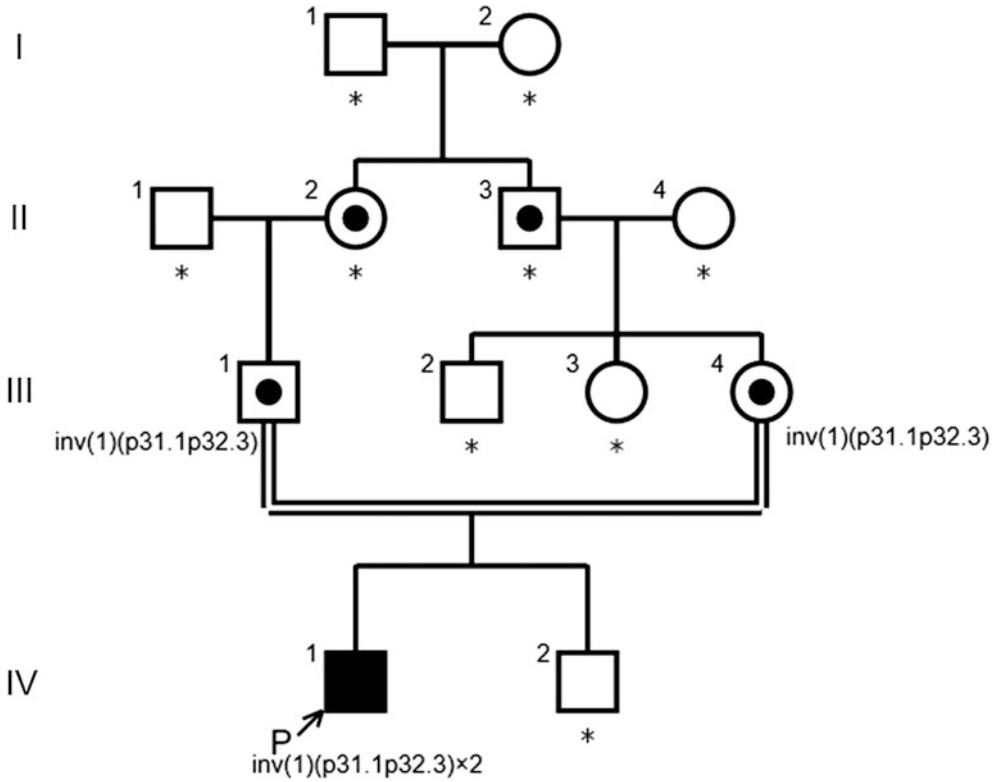


**Fig. 1** Pedigree of the family. Arrow indicates proband. Carriers are represented by a dot in the middle of circles or squares. Asterisks indicate the family members who have not been tested

Mass spectrometry to characterize the molecular abnormality of transferrin was performed as previously described (Wada 2016) and further revealed the presence of a variety of transferrin glycoforms, including forms lacking one or both glycans as well as forms with truncated glycan (Fig. 2). These findings were consistent with a PGM1 deficiency (Tagtmeyer et al. 2014), and genetic analysis was performed to confirm this. Sanger sequencing revealed only c.1258T>C, a common polymorphism in the database. The karyotype of the patient was determined to be 46,XY, inv(1)(p31.1p32.3)x2, of which inv(1) was homozygous (Fig. 3a). Since the *PGM1* gene is localized at 1p31, we hypothesized that the inversion disrupts this gene in our patient, and we thus analyzed its distal breakpoint.

## Results

FISH signals for the BAC RP4-534K7 probe that incorporates the entire *PGM1* gene are observed on the short arm of chromosome 1 in an individual with a normal karyotype. In our current study patient however, two distinct signals were detected on the short arm of both chromosome 1 homologues (Fig. 3b). This result indicated that the inversion breakpoint in the patient had disrupted the *PGM1* genomic region. Karyotype analysis of both parents showed 46,XY,inv(1)(p31.1p32.3). Both parents carried the inv(1) in a heterozygous state, suggesting that the two inv(1) homologues of the patient had been transmitted from each parent, respectively (data not shown).

Long PCR revealed that one of the PCR primer pairs (4F-4R) within intron 1 failed to amplify the products in the patient DNA, indicating that the breakpoint of the inversion was located in intron 1 (Fig. 3d). To analyze the breakpoint region in more detail, we performed additional long PCR. The 4F4-4R but not the 4F3-4R primer pair successfully yielded a PCR product. This indicated that the breakpoint was located between primer 4F3 and 4F4. We did not obtain the sequence of the other breakpoint region at 1p32.3. To ascertain the mechanism leading to the inversion, we obtained the sequence information of the 1p32.3 from the database and analyzed the homology with the 4F3-4F4 sequence. However, we did not find any sequence similarity between the 4F3-4F4 sequence and the genomic sequence at 1p32.3.

## Discussion

PGM1 deficiency is a newly identified metabolic disorder which manifests features of both CDG and glycogenosis (Tagtmeyer et al. 2014). Our present case report describes a young male patient with PGM1 deficiency caused by a homozygous inv(1) inherited from his first-cousin parents that disrupts each of the two *PGM1* alleles. To date, 38 PGM1 deficiency patients have been reported, and patho-

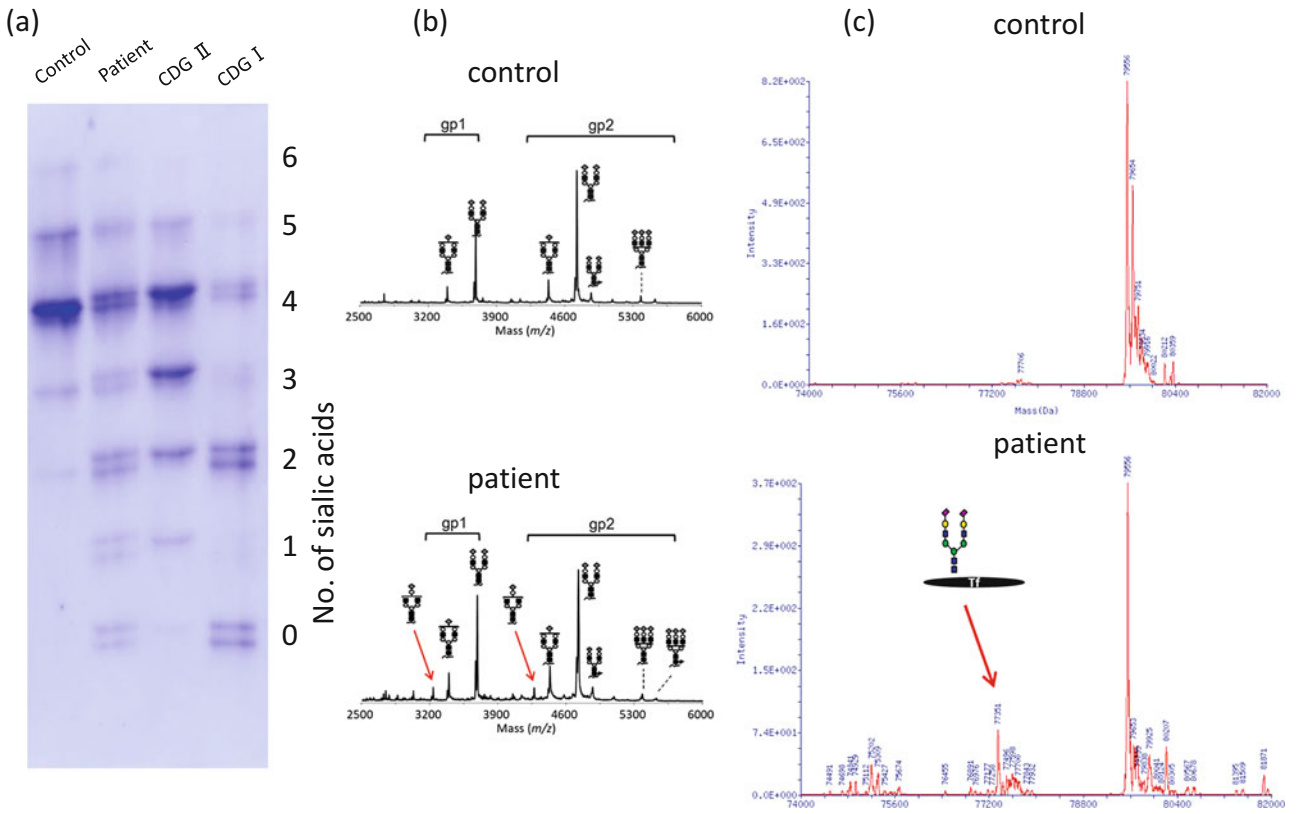


**Fig. 2** Serum transferrin isoelectric focusing (IEF) and mass spectrometry (MS) of serum glycoproteins. (**a**) IEF patterns of serum transferrin. The number of negatively charged sialic acids of transferrin is indicated on the right. Reduced glycosylation of transferrin including an unusual mixture of CDG-I and CDG-II patterns (increased tri-, di-, mono-, and asialotransferrin) is shown. (**b**) Matrix-assisted laser desorption/ionization (MALDI) mass spectrum of (glycol) tryptic peptides of transferrin. A biantennary glycan lacking galactose and sialic acid are observed in patient's transferrin (arrows). (**c**) Electrospray ionization (ESI) mass spectrum of transferrin. An abnormal transferrin isoform having a single glycan is present in the patient (arrow)

genic mutations in the *PGM1* gene were identified and genetically confirmed in most of these cases (Perez et al. 2013; Ondruskova et al. 2014; Tagtmeyer et al. 2014; Loewenthal et al. 2015; Zeevaert et al. 2016; Wong et al. 2016; Preisler et al. 2017; Nolting et al. 2017; Voermans et al. 2017). However, a small subset of patients exists without mutations in the *PGM1* gene. In our present case, Sanger sequencing did not identify any pathogenic mutation in the *PGM1* gene initially. However, subsequent chromosome karyotyping of our patient detected the presence of multiple congenital malformations and led to the identification of the aforementioned chromosomal inversion as the responsible mutation for his condition. Hence, when standard molecular testing does not reveal any abnormalities in patients who have been clinically and biochemically diagnosed with a known congenital disorder, chromosome testing may be a fruitful approach for identifying the responsible mutation in the candidate gene.

In mutational screening for single-gene disorders involving an autosomal recessive inheritance of a known causative gene, it is often the case that only one of the recessive mutations is identified. If standard PCR and Sanger methods fail to identify two pathogenic mutations within the exons or flanking intronic regions of the responsible gene, a subsequent approach can be MLPA (multiplex ligation-dependent probe amplification) analysis of structural variant copy number variations or repeat PCR/Sanger analysis to identify possible mutations in noncoding regions such as the promoter or enhancer. In addition to these methods, standard chromosomal karyotyping is important for identifying large-scale chromosomal abnormalities that may disrupt the causative gene.

A possible mechanism of inversion formation is interspersed repeat sequences that may induce chromosomal aberrations. Direct repeats can induce deletions or duplications via recombination between them, whereas inverted repeats sometimes cause pericentric or paracentric inversion (Lakich et al. 1993). In our present case, we didn't find any specific segmental duplication sequences at the breakpoint region within the intron of the *PGM1* gene. Likewise, there was no evidence of segmental duplication sequences that were common to the proximal and distal breakpoint regions. Our patient harbored a rare homozygous pericentric inversion of chromosome 1 inherited from first-cousin parents. We assume

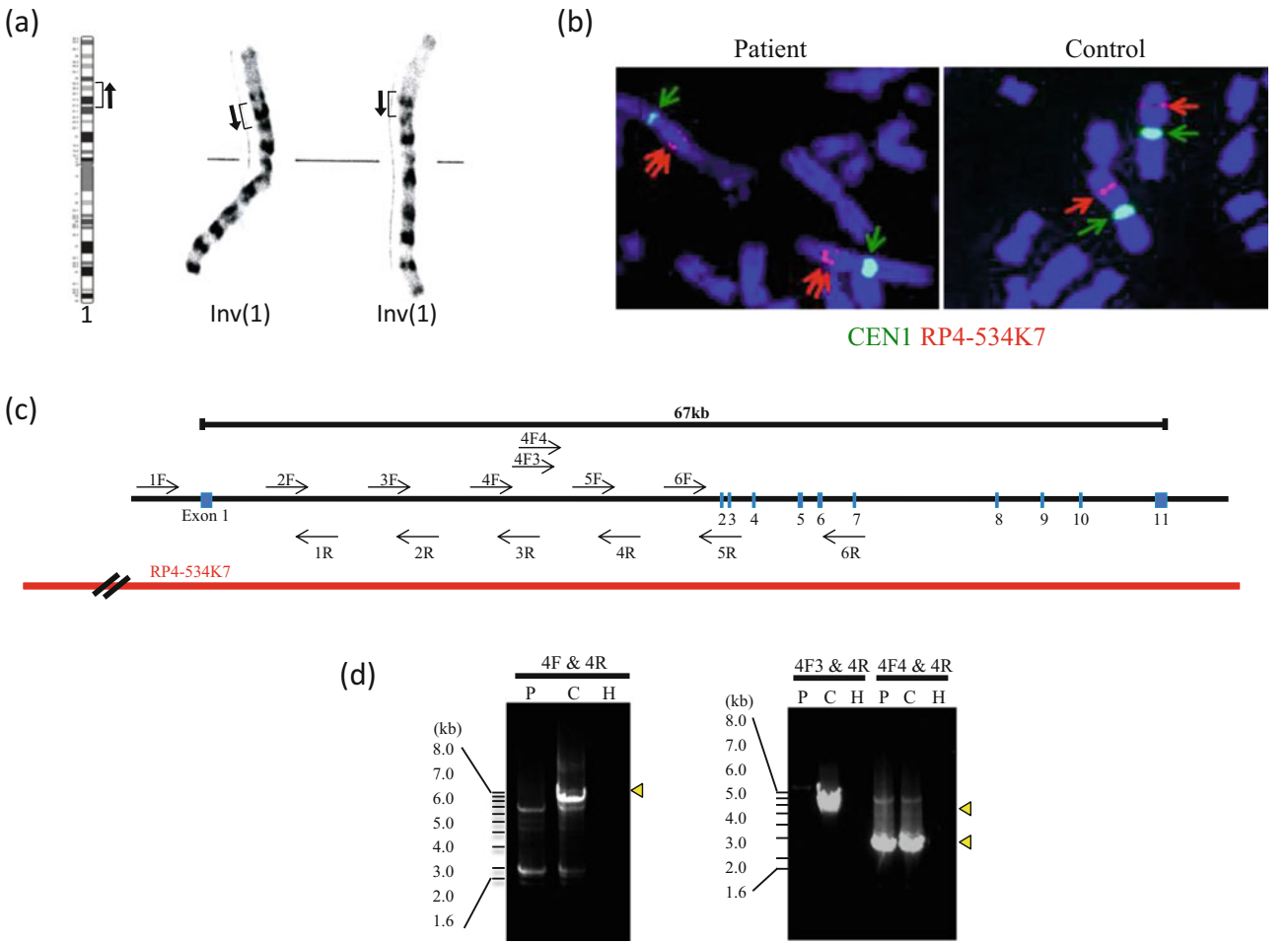


**Fig. 3** Disruption of the *PGM1* gene in the study patient by a chromosomal inversion. (**a**) G-banding of the patient's karyotype which was determined to be 46,XY,inv(1)(p31.1p32.3)x2, in which inv(1) was homozygous. (**b**) FISH signals for *PGM1* (red arrow) are typically observed on the short arm of chromosome 1 in a normal karyotype. In contrast, the two distinctive signals were detected on the chromosome 1 arm in the study patient. (**c**) Schematic representation of the *PGM1* gene structure. The blue boxes denote exons. The positions of the PCR primers are indicated by arrows. The position of the BAC probe is also indicated. (**d**) Agarose gel electrophoresis of long PCR products. 4F-4R and 4F3-4R primer pairs failed to amplify the PCR products in the study patient. *P* patient, *C* control, *H* $H_2O$

therefore that the inversion chromosome in this patient is rare in the general population and is not a recurrent type variation.

Since the initiation codon in the *PGM1* gene is located within exon 1, the inversion in our patient that disrupts intron 1 produces a truncated protein containing only the amino acids encoded by exon 1 or no protein product at all due to nonsense-mediated mRNA decay. The crystal structure of human PGM1 has not been characterized, but the structure of the analogous PGM from rabbit has been described (Liu et al. 1997). Because of the high amino acid sequence identity (97%) between these two proteins, the rabbit PGM structure provides a highly accurate model for the human enzyme. PGM1 is a monomeric protein of 562 amino acids and 4 structural domains (Beamer 2015). The active site is located in a large, centrally located cleft and can be segregated into four highly conserved regions which are located behind exon 2. In our present case therefore, even if a truncated protein was produced, it would have no active site, and PGM1 deficiency would still arise. Further, we performed RT-PCR using the patient's peripheral blood. The exon 1 transcript was found to be present, but we did not find any transcripts distal to the exon 2 (data not shown). Some residual enzymatic activity might be possibly due to other members of phosphoglucomutase family, PGM2 and PGM3, that could compensate the PGM1 activity (Maliekal et al. 2007; Wong et al. 2016).

In conclusion, we have identified and analyzed an inverted chromosome from a PGM1 deficiency patient. Our present report also emphasizes the potential benefits of karyotype analysis in congenital cases in which molecular genetic testing fails to identify the responsible mutations.

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## Synopsis Sentence

Karyotypic examination must be considered when standard molecular testing fails to reveal a mutation despite a positive clinical and biochemical diagnosis.

## Conflict of Interest

Katsuyuki Yokoi, Yoko Nakajima, Ohye Tamae, Hidehito Inagaki, Yoshinao Wada, Tokiko Fukuda, Hideo Sugie, Isao Yuasa, Tetsuya Ito, and Hiroki Kurahashi declare that they have no conflict of interest.

## Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2005(5). Informed consent was obtained from all patients for inclusion in the study.

## Author Contributions

Katsuyuki Yokoi retrieved the data and drafted and revised the manuscript.

Yoko Nakajima and Tetsuya Ito discovered the patients and provided many data.

Tamae Ohye did cytogenetic analysis and sequence analysis.

Hidehito Inagaki supported and supervised experiments.

Yoshinao Wada did mass spectrometry.

Tokiko Fukuda and Hideo Sugie estimated enzyme activity.

Isao Yuasa did IEF of serum transferrin.

Hiroki Kurahashi: conception and design, analysis and interpretation, and revising the article critically for important intellectual content.

All authors contributed to and reviewed the manuscript.

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LETTER TO THE EDITOR

Male Health

# A constitutional jumping translocation involving the Y and acrocentric chromosomes

**Makiko Tsutsumi[1], Naoko Fujita[1,2], Fumihiko Suzuki[3], Takashi Mishima[4], Satoko Fujieda[4], Michiko Watari[4], Nobuhiro Takahashi[5], Hidefumi Tonoki[5], Osamu Moriwaka[6], Toshiaki Endo[7], Hiroki Kurahashi[1,2,3]**

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Dear Editor,

Translocations of the same chromosomal fragments to two or more different chromosomes in different somatic cell lineages are referred to as jumping translocations (JTs).[1] JTs have been mainly reported in hematological malignancies but have been observed in rare instances also as constitutional chromosomal aberrations.[2] The underlying JT mechanism remains unclear, however.

The frequency of Y-autosome translocations is 1 in 2000 and carriers are often identified through spermatogenic defects or by an incidental finding in the absence of clinical symptoms.[3] Notably, translocations of the heterochromatin region of the Y long arm to the short arm of chromosome 15 or 22 are commonly observed as normal variants.[4] We here present an intriguing case of a constitutional Y;13 translocation in a male with oligozoospermia but a Y;15 translocation in his son, who was born with the assistance of reproductive technologies. To uncover the origin of this inconsistency, we examined the Y-autosome translocated chromosomes and concluded that this represented an instance of JT.

Our study family included a 40-year-old male partner of a Japanese infertile couple who was healthy except for a severe oligozoospermia identified during a prior examination for causes of the couple's infertility. As detailed below, he was found to carry a Y;13 translocation. The female partner was 38-year-old with a 46,XX karyotype. This couple had three prior miscarriages before becoming pregnant via intracytoplasmic sperm injection, which produced a healthy boy with normal external genitalia. The genetic testing used in our current analyses was approved by the ethics committee of Fujita Health University (Toyoake, Japan). Blood samples from the participants were obtained following written informed consent in accordance with Local Institutional Review Board guidelines.

G-banding analysis of the father revealed a 45,X,add(13)(p11) karyotype (**Figure 1a**). Fluorescent *in situ* hybridization (FISH) analyses with Y chromosome probes were conducted to determine the origin of the additional chromosomal material. A Yp telomere probe produced a positive signal at the add(13) chromosome (**Figure 2a**). *SRY* (sex-determining region Y), DYZ3 (alphoid satellite DNA) and *DAZ* (deleted in azoospermia) probes were also positive at add(13) (data not shown). No signal for DYZ1, a Y-specific heterochromatin repeat (Yqh), was evident on add(13) or on other chromosomes (data not shown). These results indicated that the additional chromosome in the father was a Y chromosome lacking the distal part of the long arm that had fused with the short arm of chromosome 13 (**Figure 1c** and **2a**). The breakpoint on the Y chromosome was located in the proximity of the boundary region between *DAZ* and the heterochromatin region, and that on chromosome 13 was located in its short arm (**Figure 1c**). Thus, the add(13) chromosome was dicentric, lacking the 13p region. Consequently, the father's karyotype was 45,X,add(13)(p11).ish dic(Y;13)(q11.2 or q12;p11)(SRY+,DYZ3+,DAZ+).

Interestingly, both an amniocentesis and analysis of peripheral blood from the son obtained after birth indicated a karyotype of 45,X,add(15)(p11.2).ish psu dic(15;Y)(p11.2;q11.2)(SRY+,DYZ3+,DAZ+) (**Figure 1b** and **2b–2d**). Unlike father, the Y chromosome material of the son was joined to chromosome 15 and not chromosome 13 (**Figure 1c**).

The haplotypes of the Y chromosomal regions in both the father and son were analyzed by evaluating common STR markers using the AmpFLSTR Yfiler PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The amplification of 17 Y-chromosomal STR loci in both father and son indicated a perfect match (**Table 1**). Furthermore, as the frequency of this haplotype would be expected to be approximately 1 in 6135 in Japan, as calculated using the Kappa method (release R54; https://yhrd.org),[5,6] this finding was very unlikely to have been coincidental. Our analysis thus indicated that the son inherited his Y chromosomal material from his father and that a second translocation event involving the Y chromosomal region occurred during paternal spermatogenesis.

We therefore here report a rare event in which a translocated Y chromosome changed partner chromosomes during its transmission to the next generation. We refer to this as JT. To the best of our knowledge, only one other family has been previously described in which a constitutional JT involving the Y chromosome occurred between a father, tas(Y;19), and son, tas(Y;15).[7] Telomeric associations (TAS) refer to a fusion between telomeres of different chromosomes. As defective

[1]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake 470-1192, Japan; [2]Genome and Transcriptome Analysis Center, Fujita Health University, Toyoake 470-1192, Japan; [3]Center for Collaboration in Research and Education, Fujita Health University, Toyoake 470-1192, Japan; [4]Department of Obstetrics and Gynecology, Tenshi Hospital, Sapporo 065-8611, Japan; [5]Department of Pediatrics, Tenshi Hospital, Sapporo 065-8611, Japan; [6]Kamiya Ladies Clinic, Sapporo 060-0003, Japan; [7]Department of Obstetrics and Gynecology, Sapporo Medical University, Sapporo 060-8556, Japan.
Correspondence: Dr H Kurahashi (kura@fujita-hu.ac.jp)
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**Table 1: Haplotypes of the Y-STR loci in the father and son**

| | *DYS456* | *DYS389I* | *DYS390* | *DYS389II* | *DYS458* | *DYS19* | *DYS385* | *DYS393* | *DYS391* | *DYS439* | *DYS635* | *DYS392* | *YGATAH4* | *DYS437* | *DYS438* | *DYS448* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Father | 16 | 14 | 23 | 29 | 18 | 16 | 10, 19 | 14 | 10 | 11 | 20 | 13 | 12 | 14 | 13 | 18 |
| Son | 16 | 14 | 23 | 29 | 18 | 16 | 10, 19 | 14 | 10 | 11 | 20 | 13 | 12 | 14 | 13 | 18 |

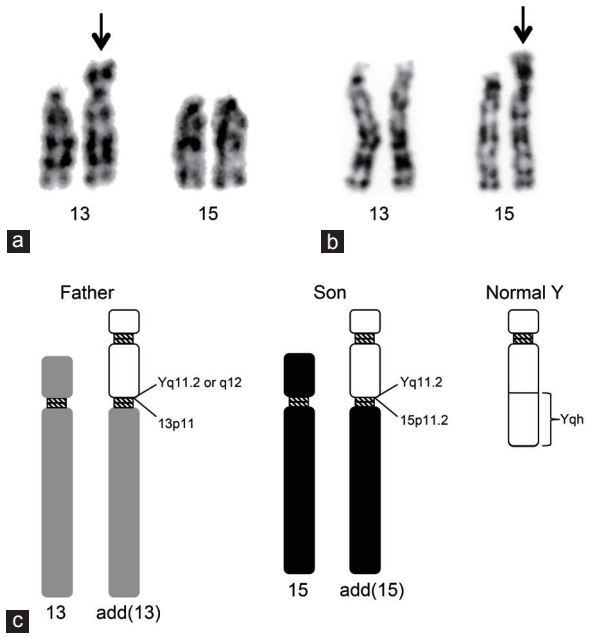


**Figure 1:** G-banded partial karyotypes of the father and son. (**a**) The arrow denotes the add(13) in the father. (**b**) The arrow indicates the add(15) in the son. (**c**) Schematic diagram of the translocated chromosomes. The breakpoints of each translocation are indicated. The Yqh regions are lost in both the father and son.

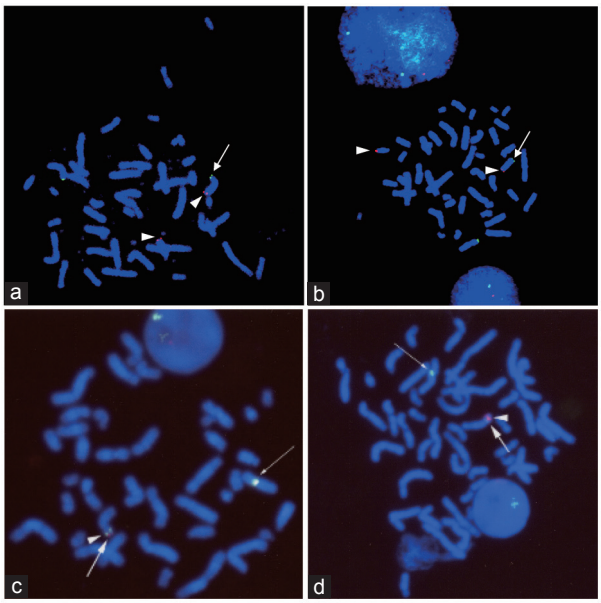


**Figure 2:** FISH analyses of the translocated chromosomes in the father and son. (**a**) Xp/Yp telomere (green) and 13q telomeres (red) in the father. The arrow and arrowheads denote the Yp and 13q probes, respectively. (**b**) Xp/Yp telomere (green) and 15q telomeres (red) in the son. The arrow and the arrowheads indicate the Yp and 15q probes, respectively. (**c**) *SRY* (red, large arrow), DYZ3 (green, arrowhead) and DXZ1 (green, small arrow) in the son. (**d**) *DAZ* (green, large arrow), DYZ3 (red, arrowhead) and DXZ1 (green, small arrow) in the son. Chromosomes were counterstained with DAPI (blue).

telomeric repeats at the junction can elicit conformational instability, it is quite possible that one TAS event could induce another and thereby lead to JT. Our present case was not the result of a TAS event however because the Yqter was lacking at the junction, although it is possible that the junction of the first translocation was unstable due to an unknown sequence-specific mechanism leading to a susceptibility for a second translocation.

Like most constitutional JTs thus far described, our present case family involved acrocentric chromosomes in both translocations.[2] JT breakpoints mostly reside in centromeric/pericentromeric regions or within telomeric sequences, which involve heterochromatin and are rich in repetitive DNA.[8] Chromosome breakages associated with JTs frequently occur at repetitive DNA regions because of their genomic instability.[9] In our current subject family, it is likely that the dic(Y;13) junction comprising a fusion of repetitive DNA of both chromosomal regions produced increased instability as a result of the first translocation. It has been shown that the short arms of the acrocentric bivalents associate with the nucleoli during prophase of meiosis I.[10] The dic (Y;13) paired with the normal chromosome 13 should, therefore, be involved in the nucleolus and come close to the short arm of the bivalent chromosome 15. It is possible that this proximity and the unstable junction might have induced the second translocation in a spermatocyte.

In conclusion, a rare JT event occurred during spermatogenesis in a Japanese man with oligozoospermia and was transmitted to his son. Although it is rare, a JT should be considered in cases where there is an inconsistency between the translocated chromosomes in a parent and child.

## AUTHOR CONTRIBUTIONS

MT carried out the cytogenetic analysis, STR analysis and drafting of the manuscript. NF and FS performed the cytogenetic analysis. TM and SF carried out the gynecological check and provided genetic counseling. MW provided the genetic counseling for the prenatal test. NT carried out the clinical management of the son. HT was responsible for the chromosomal analysis and the clinical genetics. OM conducted the fertility treatment. TE and HK conceived the study and participated in its design. HK drafted the manuscript. All authors read and approved the final manuscript and agreed with the order of presentation of the authors.

## COMPETING INTERESTS

The authors declare no competing interests.

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CASE REPORT



# Twin pregnancy with chromosomal abnormalities mimicking a gestational trophoblastic disorder and coexistent foetus on ultrasound

Akiko Ohwaki[a,b], Haruki Nishizawa[a], Noriko Aida[a], Takema Kato[b], Asuka Kambayashi[a], Jun Miyazaki[a,b], Mayuko Ito[a,b], Makoto Urano[c], Yuka Kiriyama[c], Makoto Kuroda[c], Masahiro Nakayama[d], Shin-Ichi Sonta[e], Kaoru Suzumori[e], Takao Sekiya[a], Hiroki Kurahashi[b] and Takuma Fujii[a]

[a]Department of Obstetrics and Gynecology, Fujita Health University School of Medicine, Toyoake, Japan; [b]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan; [c]Department of Diagnostic Pathology, Fujita Health University School of Medicine, Toyoake, Japan; [d]Department of Pathology and Laboratory Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan; [e]Fetal Life Science Center, Ltd, Nagoya, Japan

## Case report

Gestational trophoblastic disorder with a coexistent foetus occurs in 1 in 20,000–100,000 pregnancies (Wee and Jauniaux 2005) and mostly involves a partial hydatidiform mole with a live foetus and rarely a twin pregnancy with a complete hydatidiform mole and co-twin foetus (Gupta et al. 2015). Most cases of partial hydatidiform mole have triploidy with multiple structural anomalies and result in first trimester miscarriage (Toufaily et al. 2016). However, their management is complicated because the coexistent foetus is occasionally a normal healthy diploid foetus. Furthermore, this condition is often accompanied by severe complications such as hyperemesis, preeclampsia or thromboembolic disease (Matsui et al. 2000; Sebire et al. 2002). Thus, the diagnosis and management of gestational trophoblastic diseases with coexistent foetus are clinically important.

A gravid 33-year-old woman (gravid 4, para 3) was referred to our hospital with vaginal bleeding from 9 weeks of gestation. She was noted on prenatal ultrasound to have a normal foetus with an abnormally thickened space in the placental region. At 11 gestational weeks, a snowstorm pattern was observed on ultrasound examination, but it was slightly different from the typical pattern for hydatidiform mole. Multivesicular areas were prominent, but the other areas appeared relatively normal (Figure 1(A)). At 13 gestational weeks, the snowstorm pattern persisted with a foetal growth retardation of a biparietal diameter of 22.3 mm (−1.9 SD). The serum β-human chorionic gonadotropin (β-hCG) level was alarmingly elevated at 369,065 mIU/ml at 14 gestational weeks, whereas alpha-fetoprotein (AFP) showed a normal level of 109.5 ng/ml. β-hCG was persistently high at 207,336 mIU/ml at 16 gestational weeks, whereas AFP was 159.8 ng/ml.

The couple decided to terminate the pregnancy after considering the risks because the possibility of hydatidiform mole and coexistent foetus could not be excluded. After the curettage, the woman was in good condition and the β-hCG level decreased to 4 mIU/ml. The delivered foetus had a median cleft lip and palate (Figure 1(B)). The placenta appeared to have patchy villous hydropic changes (Figure 1(C)). Histological examination revealed focal villous oedema. Trophoblast hyperplasia was not observed (Figure 1(D)). After receiving approval from the Ethical Review Board and obtaining written informed consent from the couple, we obtained samples from the foetal skin and from the oedematous and normal-seeming areas of the placenta.

Initial cytogenetic analysis by Giemsa staining indicated a normal karyotype (data not shown). Cytogenetic microarray of the foetus revealed three copies of an 8-Mb region at the terminus of 9p, but monosomy 2q and trisomy 4q in the placenta (Figure 1(E–G)). Although hydatidiform moles generally result from dispermic triploidy or diandric diploidy with the paternal genome only, there was no evidence of triploidy or uniparental disomy. The foetus was found to carry arr[hg19] 9p24.3p24.1(326,927–8,441,863)x3, which appeared to be mosaic with normal cells because the copy number (CN) state was 2.80. On the other hand, the placental tissue was found to carry arr[hg19] 2q37.3(237,337,625–242,408,074)x1, 4q25q35.2(113,816,349–190,957,.473)x3. These appeared to be in mosaicism because the CN state was 1.35 and 2.67, respectively. Approximately 65–67% of cells showed monosomy 2q and trisomy 4q, and it is likely that the same cells had monosomy 2q and trisomy 4q simultaneously. The placental tissue also showed 9p trisomy at CN state 2.33, suggesting that 33% of cells carried the 9p trisomy identified in the foetus. On the other hand, we did not detect monosomy 2q and trisomy 4q in foetal tissue at all.

Microsatellite analysis of the DXS0767 locus revealed that there was only a small level of maternal tissue contamination in placental tissue (2–3%, data not shown) and none in foetal tissue. The pattern of whole-genome SNP genotyping also excluded the chimeric pattern but indicated a single zygote origin, suggesting that all of the foetus and placenta were derived from a monozygotic twin or somatic

CONTACT Haruki Nishizawa nharuki@fujita-hu.ac.jp Department of Obstetrics and Gynecology, Fujita Health University, 1-98 Dengakugakubo, Kutsukake, Toyoake, Aichi, 470-1192 Japan

Supplemental data for this article can be accessed here.

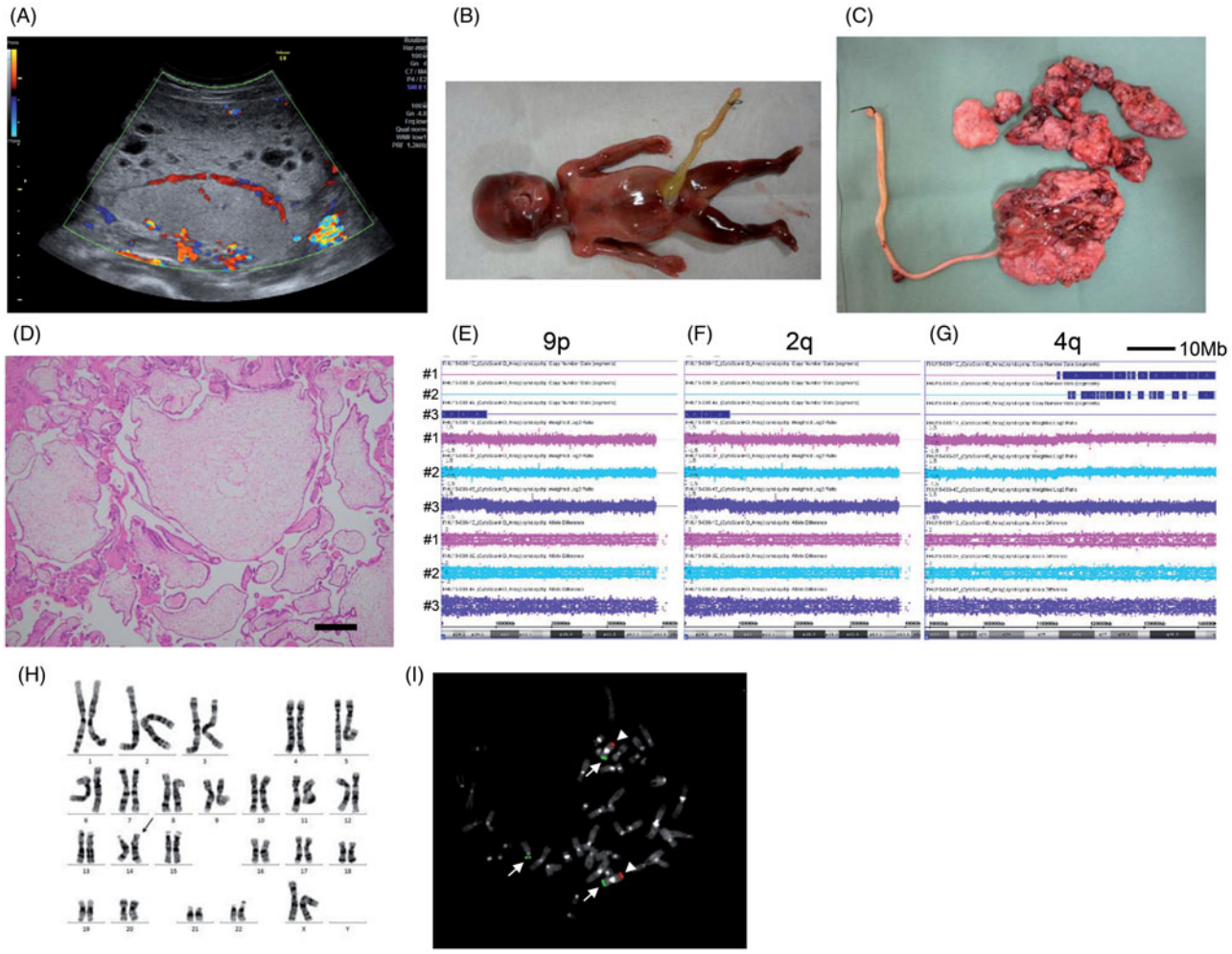


Figure 1. Clinical phenotypes and cytogenetic analysis of the foetus and placenta. Cytogenetic microarray was performed using CytoScan HD Array (Affymetrix). #1: placenta that appeared relatively normal; #2: placenta that included villous hydrops lesions; and #3: foetus. (A) Ultrasound examination at 11 weeks of gestation. Two separate areas – a vesicular area (upper area) and relatively normal area (lower area) – were observed, which are atypical for gestational trophoblastic disease. (B) Foetus. A median cleft lip and palate were observed. (C) Macroscopic analysis of the placenta. Patchy villous hydropic changes were observed. (D) Histological specimen for chorionic villi. Focal villous oedema was observed. Scale bars, 100 m. (E) 9p and 9q. (F), 2q. (G), 4q. Scale bars, 10 Mb. (H), Giemsa staining. Additional material was observed at the terminal region of 14p. (I) FISH. Subtelomeric probes (Vysis ToTelVision, Abbott Molecular) revealed the presence of der(14)t(9;14)(p24;p11.2) (arrow). White arrows: 9p; and white arrow heads: 9q.

mosaicism of a single zygote. As the CN state showed that the cell population with 9p and that with monosomy 2q and trisomy 4q were mutually exclusive, we concluded that they were likely from monozygotic twins (Supplementary Figure).

Reexamination of Giemsa staining of the foetal fibroblasts showed additional material at the terminal of 14p. Subtelomeric FISH was performed to further characterise the CN abnormalities. Trisomy 9p was found to originate from der(14)t(9;14)(p24; p11.2) in all of the 20 metaphases examined (Figure 1(H,I)). As the CN states of monosomy 2q and trisomy 4q are reciprocal, the monosomy 2q and trisomy 4q found in the placenta were likely to have originated from unbalanced t(2;4)(q37.3; q25) translocation. However, subtelomeric FISH did not detect the t(2;4) translocation in any of the foetal cells. We did not study the karyotype of the couple because they did not want to undergo the required examinations.

We recommend careful performance of the differential diagnosis of abnormal placenta with snowstorm pattern, particularly in cases with a coexistent foetus. A molecular cytogenetic study including zygosity test is necessary for differential diagnosis because it is possible that a chromosomal disorder might underlie placental abnormalities. The severities of the clinical symptoms in the foetus with such disorders vary widely. These disorders often result in lethality from multiple congenital anomalies, whereas cases with milder cytogenetic abnormalities can occasionally survive and live to a good age. Furthermore, confined placental mosaicism might affect the foetus to a lesser degree (Johnson and Wapner 1997; Lestou and Kalousek 1998). Thus, the results of the cytogenetic test might seriously affect the choice of treatment for the ultrasound findings.

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## Disclosure statement

The authors report no conflicts of interest.

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RESEARCH ARTICLE

WILEY HGVS HUMAN GENOME VARIATION SOCIETY

# Severe neurocognitive and growth disorders due to variation in *THOC2*, an essential component of nuclear mRNA export machinery

Raman Kumar[1] | Alison Gardner[1] | Claire C. Homan[1] | Evelyn Douglas[2] | Heather Mefford[3] | Dagmar Wieczorek[4,5] | Hermann-Josef Lüdecke[4,5] | Zornitza Stark[6,7] | Simon Sadedin[6,8] | The Broad CMG[9] | Catherine Bearce Nowak[10] | Jessica Douglas[10] | Gretchen Parsons[11] | Paul Mark[11] | Lourdes Loidi[12] | Gail E. Herman[13] | Theresa Mihalic Mosher[13] | Meredith K. Gillespie[14] | Lauren Brady[15] | Mark Tarnopolsky[15] | Irene Madrigal[16,17] | Jesús Eiris[18] | Laura Domènech Salgado[19] | Raquel Rabionet[19] | Tim M. Strom[20] | Naoko Ishihara[21] | Hidehito Inagaki[22] | Hiroki Kurahashi[22] | Tracy Dudding-Byth[23,24] | Elizabeth E. Palmer[23,25] | Michael Field[23] | Jozef Gecz[1,26]

[1]Adelaide Medical School and the Robinson Research Institute, The University of Adelaide, Adelaide, Australia

[2]Genetics and Molecular Pathology, SA Pathology, Adelaide, Australia

[3]Division of Genetic Medicine, Department of Pediatrics, University of Washington & Seattle Children's Hospital, Seattle, Washington

[4]Heinrich-Heine-University, Medical Faculty, Institute of Human Genetics, Düsseldorf, Germany

[5]Institut für Humangenetik, Universitätsklinikum Essen, Universität Duisburg-Essen, Essen, Germany

[6]Murdoch Children's Research Institute, Melbourne, Australia

[7]Department of Pediatrics, University of Melbourne, Melbourne, Australia

[8]Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, Massachusetts

[9]Broad's Center for Mendelian Genomics, Cambridge, Massachusetts

[10]The Feingold Center for Children at the Department of Genetics and Genomics, Boston Children's Hospital, Boston, Massachusetts

[11]Spectrum Health Medical Genetics, Grand Rapids, Michigan

[12]Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain

[13]Nationwide Children's Hospital and The Ohio State University, Columbus, Ohio

[14]Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, Canada

[15]Department of Pediatrics, McMaster University Medical Centre, Hamilton, Canada

[16]Biochemistry and Molecular Genetics Department, Hospital Clínic, IDIBAPS, Barcelona, Spain

[17]Centre for Biomedical Research on Rare Diseases (ISCIII), Barcelona, Spain

[18]Unidad de Neurología Pediátrica, Departamento de Pediatría, Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain

[19]Centre for Genomic Regulation (CRG), Universitat Pompeu Fabra and CIBERESP, Barcelona Institute for Science and Technology, Barcelona, Spain

[20]Institut für Humangenetik, Universitätsklinikum Essen, Universität Duisburg-Essen, Essen, Germany

[21]Department of Pediatrics, Fujita Health University School of Medicine, Aichi, Japan

[22]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan

[23]Genetics of Learning Disability Service, Hunter Genetics, Waratah, NSW, Australia

[24]University of Newcastle, Australia Grow-Up-Well Priority Research Center, Callaghan, Australia

[25]School of Women's and Children's Health, University of New South Wales, Randwick, NSW, Australia

[26]Healthy Mothers, Babies and Children, South Australian Health and Medical Research Institute, Adelaide, SA, Australia

  

**Correspondence**
Jozef Gecz, School of Medicine, The Robinson Research Institute, The University of Adelaide, North Adelaide, SA 5005, Australia.
Email: jozef.gecz@adelaide.edu.au

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## Abstract

Highly conserved TREX-mediated mRNA export is emerging as a key pathway in neuronal development and differentiation. TREX subunit variants cause neurodevelopmental disorders (NDDs) by interfering with mRNA export from the cell nucleus to the cytoplasm. Previously we implicated four missense variants in the X-linked *THOC2* gene in intellectual disability (ID). We now report an additional six affected individuals from five unrelated families with two *de novo* and three maternally inherited pathogenic or likely pathogenic variants in *THOC2* extending the genotypic and phenotypic spectrum. These comprise three rare missense *THOC2* variants that affect evolutionarily conserved amino acid residues and reduce protein stability and two with canonical splice-site *THOC2* variants that result in C-terminally truncated THOC2 proteins. We present detailed clinical assessment and functional studies on a *de novo* variant in a female with an epileptic encephalopathy and discuss an additional four families with rare variants in *THOC2* with supportive evidence for pathogenicity. Severe neurocognitive features, including movement and seizure disorders, were observed in this cohort. Taken together our data show that even subtle alterations to the canonical molecular pathways such as mRNA export, otherwise essential for cellular life, can be compatible with life, but lead to NDDs in humans.

**KEYWORDS**
mRNA export, partial loss-of-function variants, protein stability, THOC2, XLID

## 1 | INTRODUCTION

Intellectual disability (ID), characterized by substantial limitations in both intellectual functioning and adaptive behavior, affects 1%–3% of the population starting before the age of 18 years and has significant impact on individuals, families, and communities (Vissers, Gilissen, & Veltman, 2016). Individuals with ID are more likely than members of the general population to experience poor physical and mental health, have a lower life expectancy, experience inequalities accessing health care, and frequently have limited or no specific therapies for their core symptoms (Bittles et al., 2002; Hosking et al., 2016). Both genetic and environmental factors contribute to the development of ID (Milani, Ronzoni, & Esposito, 2015). Over 120 of the identified >800 ID genes are located on the X-chromosome (Chiurazzi & Pirozzi, 2016; Schwartz, 2015), and diagnosis of X-linked causes of ID remain critically important for accurate genetic counseling of families (Ropers & Hamel, 2005). Dramatic improvements in high-throughput DNA sequencing technologies and analyses software has led to identification of new ID genes and additional variants in the known ID genes (Dickinson et al., 2016; Vissers, et al., 2016). A systematic review of clinical data suggests that ID affected individuals frequently have comorbid neurological, psychiatric, and behavioral disorders (Oeseburg, Dijkstra, Groothoff, Reijneveld, & Jansen, 2011; Vissers et al., 2016), and disease variants in different parts of a gene can lead to a broad range of complex neurocognitive disorders (Palmer et al., 2017; Zhu, Need, Petrovski, & Goldstein, 2014). This complexity contributes to heterogeneity in clinical symptoms and indistinct boundaries between syndromic and nonsyndromic forms of neurodevelopmental disorder (NDD).

In 2015, we reported genetic, molecular, and protein structural data on four missense variants in an X-linked essential gene *THOC2* (MIM# 300957; NM_001081550.1; c.937C>T (p.Leu313Phe), c.1313T>C (p.Leu438Pro), c.2399T>C (p.Ile800Thr), and c.3034T>C (p.Ser1012Pro), RNA not analyzed) (Kumar et al., 2015). The affected individuals had a syndromic NDD, characterized by borderline to severe ID, speech delay, short stature, and adult onset truncal obesity (Kumar et al., 2015). *THOC2* encodes for the THOC2 protein—the largest subunit of the highly conserved TREX (Transcription-Export) mRNA export complex essential for exporting mRNA from the cell nucleus to the cytoplasm (Heath, Viphakone, & Wilson, 2016). The TREX complex is composed of a THO sub-complex (THOC1, THOC2, THOC3, THOC5, THOC6, and THOC7) and accessory proteins (UAP56, UIF, Aly, CIP29, PDIP3, ZC11A, SRRT, Chtop) (Heath et al., 2016). The TREX complex, besides its canonical role in mRNA export in the mammalian cells, has been shown to play critical roles in gene expression, 3′ mRNA processing, stress responses, mitotic progression, and genome stability as well as developmental processes such as pluripotency maintenance and hematopoiesis (Yamazaki et al., 2010). We and others have recently demonstrated that subtle perturbations in mRNA export by gene variants or preferential cytoplasmic aggregation can lead to NDDs (Beaulieu et al., 2013; Coe et al., 2014; Kumar et al., 2015), neurodegeneration (Woerner et al., 2016), or cancer (Chinnam et al., 2014; Hautbergue, 2017; Liu et al., 2015; Viphakone et al., 2015). These alterations can have tissue-specific effects as TREX subunits are shown to have tissue-specific roles; for example, mouse *Thoc5* and *Thoc1* deficiency interferes with the maintenance of hematopoiesis (Guria et al., 2011; Mancini et al., 2010) and testis development (Wang et al., 2009). Taken together, altered TREX function can have diverse molecular and cellular consequences resulting in a range of diseases. Here, we present detailed information on the clinical presentations and functional investigations on an additional eight missense and two splice *THOC2* variants. These data reaffirm and extend our previous findings that *THOC2* variation plays a role in complex neurodevelopmental conditions with the core clinical presentation of ID.

## 2 | MATERIALS AND METHODS

### 2.1 | Molecular and cellular studies

RNA extraction, RT-qPCR (primers listed in Supp. Table S3), cycloheximide chase, and THOC2 immunofluorescence staining were performed as reported previously (Kumar et al., 2015). Molecular studies on the *THOC2* exon35:c.4450-2A>G variant were performed using blood DNA and skin fibroblasts derived from the affected individual and his heterozygous carrier mother. Genomic DNA or cDNA (generated by reverse transcribing the fibroblast total RNAs using Superscript III reverse transcriptase; Life Technologies, Scoresby, Victoria, Australia) was amplified with KAPA HiFi PCR Kit (Kapa Biosystems, Mulgrave, Victoria, Australia) using hTHOC2-4326F/hTHOC2-4519-R (Supp. Table S3) at 95°C for 3 min, 35 cycles of 98°C–15 sec, 59°C–15 sec, 72°C–30 sec, incubation at 72°C for 10 min, gel purified (MinElute Gel Extraction kit; Qiagen, Chadstone Centre, Victoria, Australia) and Sanger sequenced using the same primers. For the *THOC2* exon28:c.3503+4A>C, blood gDNA from unaffected father, carrier mother, and affected son was amplified with TaKaRa ExTaq (Clayton, Victoria, Australia) using THOC2-F/THOC2-R primers (Supp. Table S3) at 94°C for 2 min, 40 cycles of 94°C–30 sec, 60°C–30 sec, 72°C–30 sec, incubation at 72°C for 5 min. The cDNA was generated by reverse transcribing the white blood cell RNAs using Superscript III reverse transcriptase (Life Technologies) and amplified with TaKaRa ExTaq using THOC2-ex27F/THOC2-ex30R (Supp. Table S3) at 94°C for 2 min, 28 cycles of 94°C–30 sec, 60°C–30 sec, 72°C–30 sec, incubation at 72°C for 5 min. The amplified products were analyzed by Sanger sequencing.

### 2.2 | Generation of *THOC2* variant expression constructs

Generation of the wild-type Myc-tagged human THOC2 expression plasmid was reported earlier (Kumar et al., 2015). Briefly, the *THOC2* variants were introduced into the existing pCMV-Myc-THOC2 expression construct by overlap PCR method using the primers listed in Supp. Table S3. The variant plasmid sequences were confirmed by Sanger sequencing. Details relating generation of the *THOC2* variant expression constructs are available on request.

### 2.3 | Transient expression and Western blotting

For transient expression experiments, HEK293T and HeLa cells were transfected with expression constructs (400 ng pCMV-Myc-THOC2 plasmid and 400 ng pEGFP-C1 plasmid/transfection for stability and cycloheximide assays and 4 $\mu$g/transfection for immunofluorescence staining, IF) using Lipofectamine 3000 reagent according to manufacturer's protocol (Life Technologies). Twenty-four hours post-transfection, cells were either fixed with 4% formaldehyde for IF or collected and lysed in buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Triton-X 100, 1 mM EDTA, 50 mM NaF, 1× Protease inhibitor/no EDTA and 0.1 mM $Na_3VO_4$ for Western blot assay as reported previously (Kumar et al., 2015).

### 2.4 | *In silico* pathogenicity prediction

We used CADD v1.3 (includes Phylop, GERP++ & PolyPhen2) (Kircher et al., 2014), Provean (Choi & Chan, 2015), and ACMG (Richards et al., 2015) on-line tools for *in silico* prediction of the pathogenicity of different variants (Table 1 and Supp. Table S1).

## 3 | RESULTS

### 3.1 | Identification of *THOC2* variants

We previously implicated four missense *THOC2* variants in 25 individuals with ID and a range of other clinical features (Table 1 and Supp. Table S1 and 2) (Kumar et al., 2015). We identified an additional five *THOC2* variants (three missense; *de novo* c.2087C>T (p.Thr696Ile), *de novo* c.2138G>A (p.Gly713Asp), maternally inherited c.3559C>T (p.His1187Tyr), and two splicing-defective; maternally inherited chrX:122747561 exon35:c.4450-2A>G and chrX:122757634 exon28:c.3503+4A>C; GenBank: NM_001081550.1) variants in a further six affected individuals, including one pair of monozygotic twins (Table 1 and Figures 1 and 2). Whole exome (WES) or whole genome sequencing (WGS) of probands and parents was used to identify the variants that were confirmed by Sanger sequencing of the PCR amplified variant-carrying region of genomic DNA of the parents and affected individuals. The previously unreported *THOC2* variants affect amino acids that are highly conserved (Supp. Figure S1), are absent in gnomAD database and are predicted to be pathogenic based on a number of *in silico* analyses tools (Table 1). We included in our study a *de novo* missense p.Tyr517Cys variant in a female with moderate-severe ID, speech problems, epileptic encephalopathy, cortical visual impairment, and gait disturbances identified using WES as part of the Epi4K Consortium & Epilepsy Phenome/Genome Project (Epi et al., 2013) (Table 1). We have also collected further, rare and potentially pathogenic variants through international collaboration (Table 1, Supp. Table S1 and 2, and Figures 1 and 3; see Supplementary information for methods used for identifying the variants) and performed functional testing on several of these. The following three variants: c.229C>T (p.Arg77Cys), c.3034T>C (p.Ser1012Pro), and c.3781A>C (p.Asn1261His) showed no clear evidence of altered stability of variant THOC2 proteins in our cell-based assay. The reported variants have been submitted to ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/accession numbers SCV00680065-SCV000680074).

### 3.2 | Clinical presentations

The clinical features of the five previously unreported affected individuals with (likely) pathogenic *THOC2* variants, aged between 3 and 12 years, and the 10 year old female with *de novo* p.Tyr517Cys variant are summarized in Table 2 and photographs, when available, are shown in Figure 2. Detailed clinical information is available in the supplementary data. Each individual clinical center used local diagnostic criteria for determining degree of ID and diagnoses of comorbidities. ID was universal and at least moderate in severity: two out of seven were nonambulatory and three out of seven nonverbal. Behavioral problems

**TABLE 1** Detailed description of the *THOC2* variants with supporting molecular evidence

| | Individual | From | Method of identification | Position hg19 | NM_001081550 | Mode of inheritance | CADD | Provean score | Provean prediction | GERP++ | Phylop | gnomAD frequency | Polyphen2[a] | Variant plasmid tested | Reduced Protein Stability | ACMG pathogenicity classification[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Missense | | Kumar et al. (2015) AJHG | X-Chr Exome sequencing | 122799566 | c.1313T>C:p.Leu438Pro | Maternal inheritance | 28.1 | −6.08 | Deleterious | 5.7 | 1.902 | Absent | D | YES | YES | LP |
| | 1 | Australia | Trio WES | 122767853 | c.2087C>T:p.Thr696Ile | *De novo* | 27.4 | −5.47 | Deleterious | 5.03 | 0.963 | Absent | D | YES | YES | DP |
| | 2 | USA | WES | 122766890 | c.2138G>A:p.Gly713Asp | *De novo* | 31 | −4.69 | Deleterious | 5.73 | 2.412 | Absent | D | YES | YES | DP |
| | | Kumar et al. (2015) AJHG | X-Chr Exome sequencing | 122765621 | c.2399T>C:p.Ile800Thr | Maternal inheritance | 23.9 | −4.01 | Deleterious | 5.97 | 2.016 | Absent | P | YES | YES | LP |
| | 3-4 | Canada/ Germany/ Russia Identical twins | WES | 122757079 | c.3559C>T:p.His1187Tyr | Maternal inheritance; Mother skewed (99.9:0.1%) | 23.1 | −5.07 | Deleterious | 6.07 | 2.571 | Absent | P | YES | YES | LP |
| Splice | 5 | Japan | Trio WES | 122757634 | Exon28:c.3503+4A>C | Maternal inheritance; Mother skewed 98:2% | 10.8 | N/A | N/A | 5.57 | 1.86 | Absent | N/A | N/A | ND | LP |
| | 6 | Canada | WES | 122747561 | Exon35:c.4450-2A>G | Maternal inheritance; Mother skewed 94:6% | 23.7 | N/A | N/A | 5.25 | 1.735 | Absent | N/A | Fibroblasts of the affected male proband and carrier mother | NO | LP |
| Missense | 7 | USA Epi4K Consortium & Epilepsy Phenome/ Genome Project; Nature 501:217–221, 2013 | WES | 122778639 | c.1550A>G:p.Tyr517Cys | *De novo* | 26.6 | −7.87 | Deleterious | 5.84 | 1.955 | Absent | D | YES | YES | DP |

[a] D, probably damaging; P, possibly damaging; N/A, not applicable; ND, not determined.
[b] DP, *de novo* pathogenic; LP, likely pathogenic.

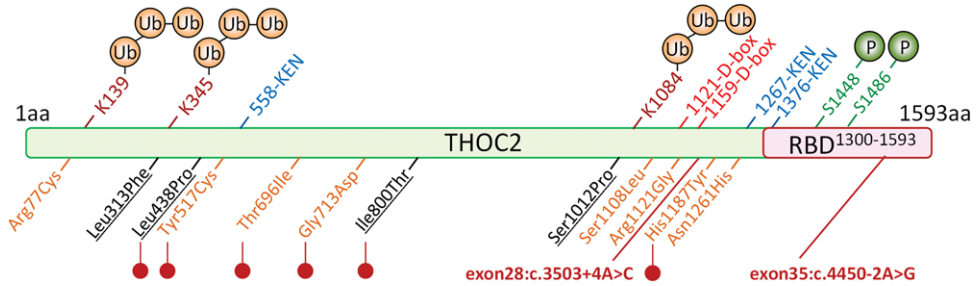


**FIGURE 1** Location of variant amino acids and structural features in THOC2 protein. Ubiquitinated (K139 and K1084 (Kim et al., 2011) and K345 (Lopitz-Otsoa et al., 2012; Wagner et al., 2011)), phosphorylated (S1448 and 1486 (Olsen et al., 2006)) amino acid residues, potential RNA binding domain (RBD) and destruction box (D-box) and KEN box sequences that interact with the Anaphase Promoting Complex/Cyclosome (APC/C) for protein ubiquitination and subsequent destruction by the proteasome (Morgan, 2013) are shown. Unreported (orange) and published (black: (Kumar et al., 2015)) missense variants effecting THOC2 protein stability are marked with red lollipops. The positions of two splice variants are shown in red

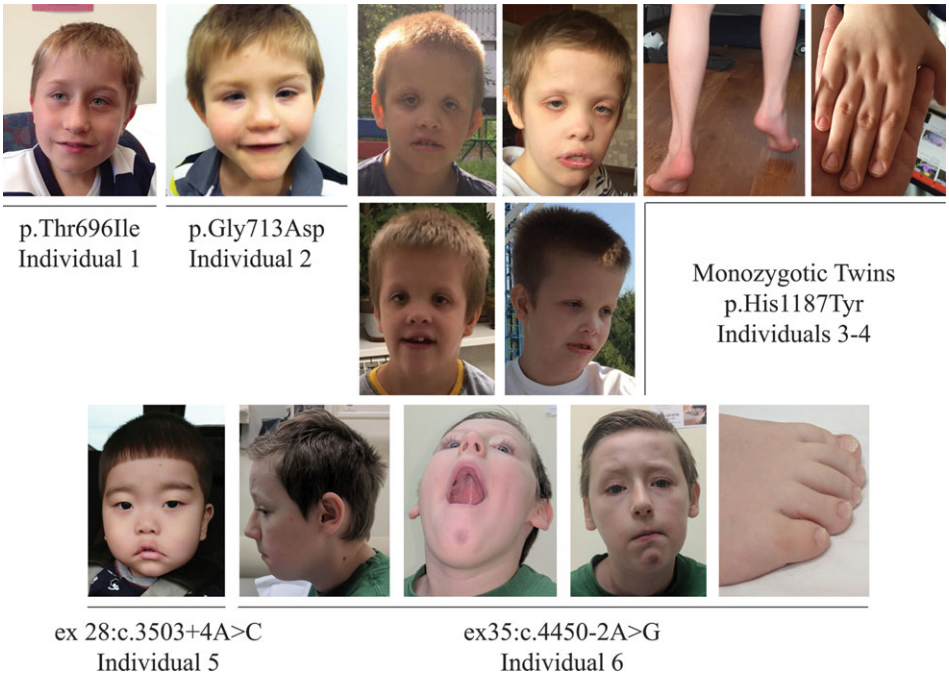


**FIGURE 2** Front and side facial views of the affected individuals with *THOC2* variants

were reported in four individuals, with one meeting diagnostic criteria for autism spectrum disorder. Additional neurological features were common. Four of the seven had infantile hypotonia and two of the seven had tremor. The monozygotic twins (individuals 3 and 4) had a tendency to toe walking, which was considered behavioral as it was not associated with neurological signs of lower limb spasticity. Confirmed seizure disorder was only present in the affected female (individual 7) but suspected in individual 2. Neuroradiological studies were performed in five individuals: this was within normal limits for three individuals, with neuroanatomical differences reported in two. Individual 2 had complex neuroanatomical findings (see supplementary clinical description and Supp. Figure S2A) including changes in cortical gyral morphology, which in the inferior temporal lobes appeared finely nodular, as well as hypoplasia of the corpus callosum and reduced brainstem volume and individual 5 had mild dilatation of the lateral ventricles, mildly delayed myelination and an abnormal white matter lesion in the periventricular area close to the anterior horn (Supp. Figure S2B). Growth abnormalities were common including low birth weight (three out of seven), microcephaly (two out of seven), and short stature (two out of seven). Facial features are shown in Figure 2. Appropriate consent for reporting variants, clinical data and photographs of the affected individuals was obtained from their parents or legal guardians. The research has been approved by the Women's and Children's Health Network Human Research Ethics Committee in Adelaide, Australia.

### 3.3 | THOC2 variant protein localization and stability

Without access to affected individuals' derived cells, we generated Myc-tagged THOC2 missense variant expression constructs to determine protein stability and localization. The THOC2 protein stability was determined in HEK293T cells and localization in both the HEK293T and HeLa cells. Total protein lysates of HEK293T cells ectopically-expressing the wild-type or variant Myc-THOC2 proteins were Western blotted for THOC2, EGFP, and $\beta$-Tubulin. We used HEK293T cells expressing Myc-p.Ile800Thr THOC2 as a control for

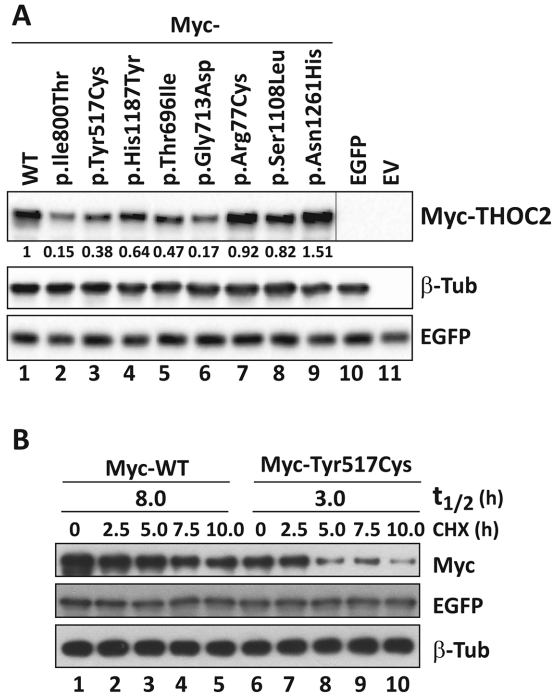


**FIGURE 3** Functional testing of THOC2 missense variants. **A**: THOC2 variant protein stability is reduced in HEK293T cells. pCMV-Myc-THOC2 wild-type or variant expression constructs and pEGFP-C1 plasmid (transfection control) were transfected into HEK293T cells. Total protein lysates of cells 24 hr post-transfection were analyzed by Western blotting with mouse anti-Myc (clone 9E10; Sigma), mouse anti-EGFP (clones 7.1 and 13.1; Roche) and rabbit anti-$\beta$-tubulin (loading control; Abcam) antibodies. pCMV-Myc-THOC2 p.Ile800Thr construct expressing the p.Ile800Thr protein shown to have reduced stability was used as a control (Kumar et al., 2015). Western blot signals were quantified using ImageJ software. Averages of the Myc-THOC2 proteins normalized to the housekeeping $\beta$-tubulin signal from two independent runs are shown. **B**: Myc-p.Tyr517Cys THOC2 protein half-life is substantially reduced in HEK293T cells. pCMV-Myc-THOC2 or pCMV-Myc-THOC2-p.Tyr517Cys expression constructs and pEGFP-C1 plasmid (transfection control) were transfected into HEK293T cells. Next day the cells were cultured in the presence of 100 $\mu$g/ml translation inhibitor cycloheximide and harvested at the time points shown. Total protein lysates were analyzed by Western blotting with mouse anti-Myc, mouse anti-EGFP, and rabbit anti-$\beta$-tubulin (loading control) antibodies

protein stability assay as this variant is shown to cause reduced protein stability (Kumar et al., 2015). The results showed reduced stability of p.Tyr517Cys, p.Thr696Ile, p.Gly713Asp, and p.His1187Tyr THOC2 compared with the wild-type protein (Figure 3A). Presence of comparable levels of EGFP in the cells transfected with different expression constructs indicated that the reduced levels of THOC2 protein were not due to difference in transfection efficiency (Figure 3A). We also determined the turnover rate of Myc-p.Tyr517Cys THOC2 protein by cycloheximide chase. For this assay, the HEK293T cells transfected with pCMV-Myc-WT or pCMV-Myc-p.Tyr517Cys THOC2 and pEGFP-C1 transfection control plasmids were cultured in presence of translation inhibitor cycloheximide for different durations and Western blotted for THOC2, EGFP, and $\beta$-Tubulin. The results showed that p.Tyr517Cys THOC2 turnover rate was 3 hr compared with 8 hr for the wild-type protein (Figure 3B). THOC2 variant proteins, similar to the wild-type, were mainly localized to the nucleus in both the HEK293T and HeLa cells (Supp. Figure S3).

### 3.4 | *THOC2* splice variant: exon35:c.4450-2A>G, p.Arg1483fs52*

Sanger sequencing of amplified target region from affected son and mother's blood genomic DNA showed that the affected boy inherited chrX:122747561 exon35:c.4450-2A>G variant from his unaffected heterozygous carrier mother (Figure 4C). A -2 A>G change in the intron–exon splicing site boundary (acceptor AG) is predicted to abolish splicing (Ohno, Takeda, & Masuda, 2018). To validate this possibility, we generated skin fibroblast cultures from the heterozygous carrier mother and the affected son. We PCR amplified their fibroblast cDNAs using primers with binding sequences located within exon 34 and 35. Amplification of a 194 bp DNA fragment from the mother indicated normal splicing but a 537 bp product from the affected son indicated retention of the intron located between these exons (Figure 4A and B). We confirmed this result by Sanger sequencing of the PCR products generated from genomic DNA that showed presence of A/G nucleotides in the carrier mother but only G (A>G) nucleotide in the affected son (Figure 4C). The cDNA sequence showed presence of normally-spliced mRNA in the mother but retention of intronic sequence upstream of the exon 35 in the affected son indicating defective splicing due to presence of -2 G variant at the intron-exon 35 junction sequence (Figure 4C). The presence of normally spliced mRNA in the unaffected mother is consistent with X-inactivation (94% skewing) of the variant allele in her fibroblasts. We predicted that a retention of intron between exon 34–35 in the affected fibroblasts would result in loss of 110 C-terminal amino acids of the 1,593 wild-type THOC2 protein (that is, 1,483 amino acids); however, overall the variant protein would be 58 amino acid smaller as it would now be a 1,535 amino acid protein comprised of 1,483 amino acids of wild-type THOC2 and 52 translated from intronic sequence in the defective mRNA (Figure 4A). Consistent with our prediction, the Western blot data showed presence of a slightly smaller THOC2 protein band in the affected son's fibroblasts than his unaffected mother. Many independent Western blot runs showed presence of two closely located THOC2 bands—similar to the fruit fly THO2 (Rehwinkel et al., 2004)—in the unaffected mother but a single highly intense band in the affected son's fibroblasts (Figure 4D). The observed difference in levels of THOC2 protein was post-translational as we found comparable amounts of *THOC2* mRNA, as assayed by real time RT-qPCR, in the mother and son (Figure. 4E). Finally, we observed no difference in THOC2 localization in fibroblasts of the affected son and his unaffected mother (Figure 4F).

### 3.5 | *THOC2* splice variant: exon28:c.3503+4A>C, p.Gly1168fs7*, and normal 1,593 aa protein

For the second splice variant chrX:122757634 exon28:c.3503+4A>C, molecular studies were performed on the white blood cells of the unaffected father, carrier mother, and the male proband. Sanger sequencing of target region amplified from the unaffected father and

**TABLE 2** Summary of clinical data of *THOC2* variants with supporting molecular evidence

| | (Likely) pathogenic | | | | | | |
|---|---|---|---|---|---|---|---|
| **Individual** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| **Variant details** | c.2087C>T: p.Thr696Ile | c.2138G>A: p.Gly713Asp | c.3559C>T: p.His1187Tyr Twin 1 | c.3559C>T: p.His1187Tyr Twin 2 | Exon28: c.3503+4A>C | Exon35:c.4450-2A>G | c.1550A>G: p.Tyr517Cys |
| **Gender** | Male | Male | Male | Male | Male | Male | Female |
| **Age (years)** | 12 | 5 | 7 | 7 | 3 | 10 | 10 |
| **Perinatal features** | | | | | | | |
| Gestation (weeks) | 36 | 37 | 37 | 37 | 37 | 41 | NA |
| Low birth weight (<2.5kg) | Yes | No | Yes | Yes | No | No | NA |
| Birth weight (g) | 2,000 | 2,650 | 1,990 | 2,420 | 3,018 | 4,365 | NA |
| **Neurologic features** | | | | | | | |
| Intellectual disability | Severe | Mod+ | Mod+ | Mod+ | Severe | Severe | Profound |
| Speech delay | Yes, single words, signs | Yes, nonverbal | Yes | Yes | Yes, nonverbal | Yes, nasal dysarthria | Yes, non-verbal |
| Hypotonia | No | Yes | NA | NA | Yes | Yes, central hypotonia | Yes |
| Spasticity | No | No | No | No | No | Yes- appendicular spasticity | No |
| Hyperkinesia | No | No | Yes | Yes | Yes | No | No |
| Tremor | No | Yes, intermittent | No | No | No | Yes | No |
| Epilepsy | No | Suspected | No | No | No | No | Yes, epileptic encephalopathy |
| Gait disturbances | No | Yes, gait/balance problems | Yes, toe walking[a] | Yes, toe walking[a] | Non ambulatory | Yes, ataxia/broad based gait | Non ambulatory |
| Behavior problems | No | Yes | Yes | Yes | Yes, ASD | No | NR |
| Anxiety | No | No | No | No | No | No | NR |
| Depression | No | No | No | No | No | No | NR |
| Brain MRI/CT | MRI normal | Thin corpus callosum, low brainstem volume, variability in gyral pattern. | ND | ND | Ventricular dilatation, delayed myelination, periventricular white matter lesion | MRI within normal limits | MRI normal |
| **Growth parameters** | | | | | | | |
| Microcephaly (≤3%) | Yes | Yes, <1% | No | No | No | Yes,2% | No, 5% |
| Short stature (≤3%) | Yes | Yes | No | No | No | No | No |
| Overweight (BMI≥25) | No | No | No | No | No | No | No |
| Broad high forehead | Yes | Yes | Yes | Yes | No | No | NR |
| **Other features** | | Mild joint laxity, subluxed hips, disordered sleep, feeding difficulties (g-tube dependency), laryngomalacia, micrognathia, abnormal palmar creases | Noonan facies, pes planus, hypospadias | Noonan facies, pes planus, hypospadias | | Clinodactyly, nystagmus, abnormality soft palate | Cortical visual impairment |

*Abbreviations*: %, centile; ASD, autism spectrum disorder; CT, computerized tomography scan; g-tube, gastrostomy tube; mod+, at least moderate severity; MRI, magnetic resonance imaging; ND, not done; NA, not available; NR, not reported; NICU, neonatal intensive care unit; VOUS, variant of uncertain significance.

[a]Toe walking in absence of neurological signs of lower limb spasticity, therefore considered a behavioral manifestation.

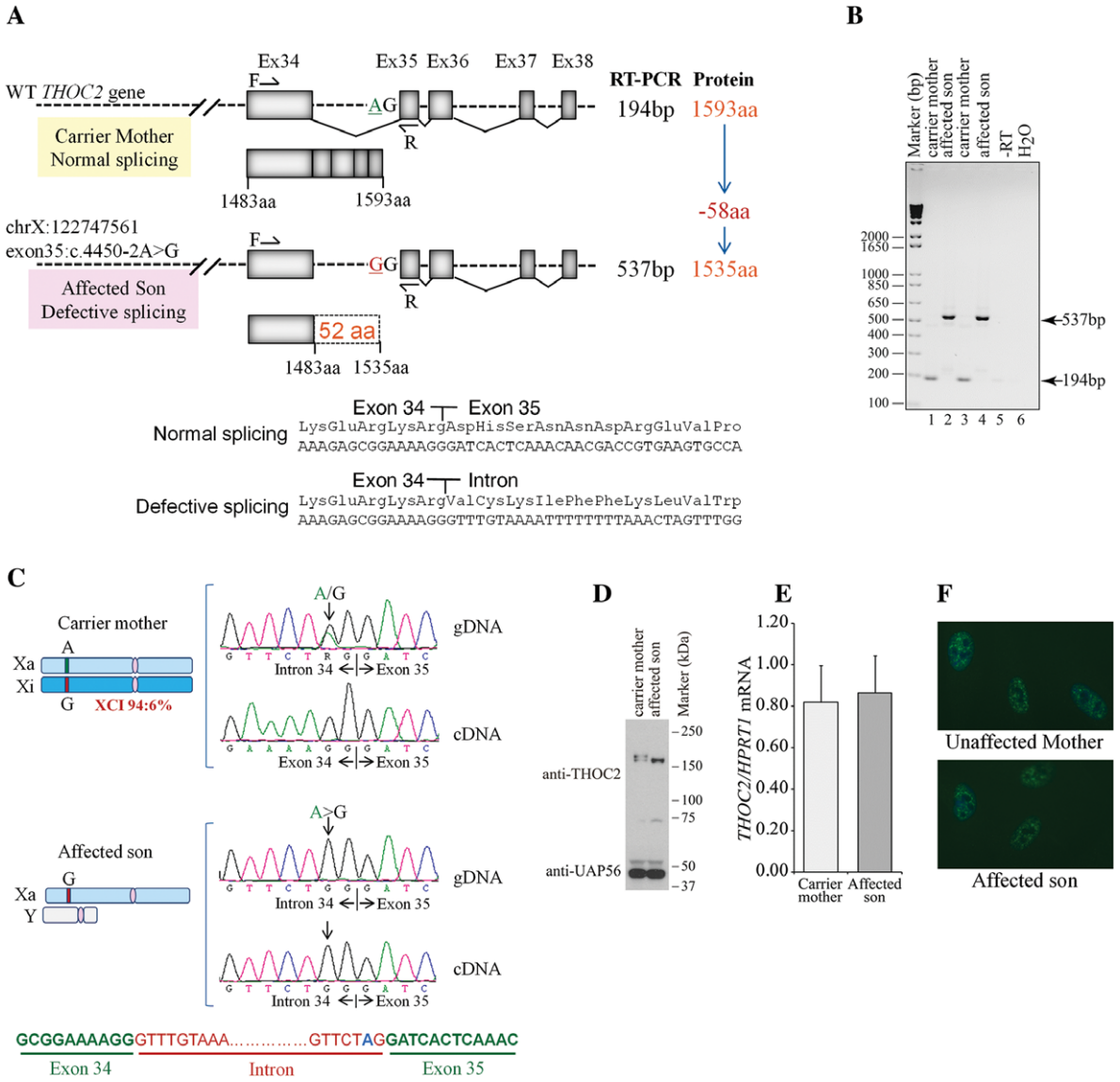


**FIGURE 4** Exon35:c.4450-2A>G variant abolishes splicing of intron between exons 34–35. **A**: Part of the *THOC2* gene showing location of the A/G nucleotide in the heterozygous carrier mother and A>G splice variant in the affected son. The C-terminal part of the 1,593 amino acid wild-type and 1,535 amino acid (that contains 1,483 normal and 52 amino acids coded by the unspliced intron) THOC2 protein in the affected boy are also shown. **B**: Gel showing a 194 bp RT-PCR product from the normally spliced mRNA of the heterozygous carrier mother and a 537 bp product from defective splicing of mRNA causing retention of an intron between exon 34–35 in the affected son. RT-PCR products from total RNA isolated from passage 3 (lanes 1–2) and 5 (lanes 3–4) fibroblasts. Location of the forward and reverse primers within exons 34 and 35 is shown. **C**: Sanger sequencing chromatograms of PCR products amplified from genomic and cDNA of the affected son and his heterozygous carrier mother using primers located within exon 34 and 35. Genomic DNA around the Exon34-Intron-Exon35 region is shown. **D**: Western blot showing THOC2 protein in the affected son and his carrier mother's skin fibroblasts. TREX subunit UAP56 was used as a loading control. **E**: RT-qPCR showing levels of the THOC2 mRNA in the affected son and his carrier mother's skin fibroblasts. **F**: Immunofluorescence detection of THOC2 in skin fibroblasts of the unaffected mother and affected son

mother, and affected son's genomic DNA showed that the affected son inherited the A>C change from his unaffected carrier mother who had A/C nucleotides at this position (Figure 5). The intronic nucleotide change A>C at +4 position of the 5′ exon–intron donor splicing site sequence is predicted to cause aberrant splicing (https://www.med.nagoya-u.ac.jp/neurogenetics/SD_Score/sd_score.html). To confirm this possibility, we amplified cDNA generated by reverse transcribing blood RNA of the father, mother, and the affected son using primers located within exon 27 and exon 30 (Figure 5A and Supp. Table S3). Interestingly, whereas a 491 bp PCR product was observed in highly skewed carrier mother (98:2%) and normal father, 491 and 634 bp PCR products were detected in the affected son. A 491 bp amplified product indicated normal splicing in the mother and father, and 491 bp and 634 bp bands suggested partially defective splicing in the affected son. Amplification of a 634 bp instead of a 994 bp fragment that would have resulted from a complete retention of intron between exon 28–29 indicated aberrant splicing event in the affected son (Figure 5). Sanger sequencing of 491 and 634 bp PCR products from

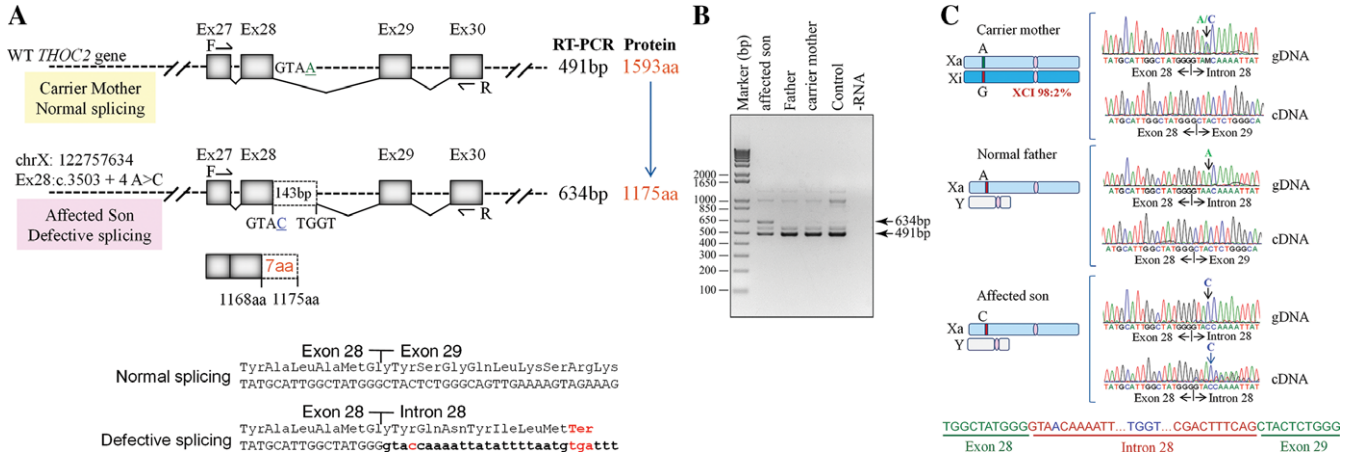


**FIGURE 5** Exon28:c.3503+4A>C variant causes aberrant splicing of intron between exons 28–29. **A**: Part of the *THOC2* gene showing location of the A/C nucleotide in the heterozygous carrier mother and A>C splice variant in the affected son. The C-terminal part of the 1,593 amino acid wild-type and 1,175 amino acid (that contains 1,168 normal and 7 amino acids coded by the unspliced intron) THOC2 protein in the affected son are also shown. **B**: Gel showing a 491 bp RT- PCR product from the normally-spliced heterozygous carrier mother and unaffected father, and 491 and 634 bp (retaining 143 bp of the 503 bp intron between exons 28–29) RT-PCR products derived from the normally and aberrantly spliced mRNAs, respectively, in the affected son. Location of the forward and reverse primers within exons 27 and 30 is shown. **C**: Sanger sequencing chromatograms of PCR products amplified using primers located within exons 27 and 30 from genomic and cDNA of unaffected father and mother and the affected son

the mother, father and son confirmed normal splicing in the mother and father and aberrant splicing in the affected son. The sequence showed retention of a 143 bp instead of complete 503 bp fragment due to activation of a cryptic splice site within the intron between exon 28–29 in the son (Figure 5). Retention of 143 bps from intron between exon 28–29 in the mRNA is predicted to result in a 1175 amino acid truncated THOC2 protein containing 1,168 wild-type amino acids and 7 amino acids from the translation of the intronic sequence retained in the defective mRNA. This aberrant product would be present in addition to the wild-type 1,593 amino acid protein from the normally spliced mRNA in the affected son.

## 4 | DISCUSSION

Here, we present detailed clinical information, and molecular and functional studies, on five previously unreported *THOC2* variants in six affected males (two *de novo* variants and one maternally inherited variant in monozygotic twins) and on one affected female with a previously reported *de novo* p.Tyr517Cys variant. We present evidence that extends the genotypic spectrum beyond the four *THOC2* missense variants that we reported previously (Kumar et al., 2015) by including two intronic variants that affect splicing, and four missense variants that affect protein stability in a cell-based assay system. According to ACMG criteria, they were classified as pathogenic or likely pathogenic (Table 1) (Richards et al., 2015). These findings, along with the four missense variants reported earlier (Kumar et al., 2015), add to the existing evidence that alterations in essential mRNA export pathway cause NDDs (Amos et al., 2017; Beaulieu et al., 2013; Kumar et al., 2015).

We confirm that the core clinical feature of *THOC2*-related disorder in hemizygous males is ID, with several individuals having additional features including behavioral disorders, hypotonia, gait disturbance, tremor, low birth weight, short stature, microcephaly, and variable neuroimaging findings. Although the range of neurodevelopmental features is similar, our original cohort contained males with ID in the mild or borderline range of intellectual functioning (Kumar et al., 2015), whereas all individuals in this cohort have ID which is at least in the moderate range. Individuals 2 and 6 had neurological signs that could be consistent with cerebellar dysfunction including tremor and a broad-based gait for individual 2 and nystagmus, tremor, and an ataxic broad-based gait for individual 6, in the absence of significant cerebellar abnormalities on MRI. This is interesting given the female patient with knockdown of THOC2 function due to a *de novo* X;8 translocation that created a *PTK2-THOC2* fusion had congenital cerebellar hypoplasia and prominent cerebellar signs with mild ID (Di Gregorio et al., 2013). We used computerized face-matching technology to specifically evaluate the cohort to assess if a characteristic facial gestalt was evident across individuals with pathogenic or likely pathogenic variants across our original and this expanded clinical cohort (Supp. Figure S4) (Dudding-Byth et al., 2017). Although a clearly recognizable facial gestalt was not obvious, there are some similarities. The facial gestalt spectrum associated with *THOC2* pathogenic variants will continue to emerge as more individuals are reported.

As was the case in our original cohort, heterozygous mothers were clinically unaffected, and, where available, X-chromosome inactivation (XCI) was highly skewed (Table 1). In contrast individual 7, with a *de novo* missense variant (p.Tyr517Cys) is a female with a particularly severe neurocognitive presentation. This is consistent with other reported severely affected females with *de novo* variants in X linked genes (de Lange et al., 2016; Palmer et al., 2016; Snijders Blok et al., 2015; Zweier et al., 2014). Unfortunately, we did not have access to individual 7′s genomic DNA to test XCI status.

A range of protein–protein interactions are required for mRNA export (Chi et al., 2013). Proteins with altered stability (Hirayama et al.,

2008), localization (Beaulieu et al., 2013) (e.g., THOC6 p.Gly46Arg implicated in syndromic ID), or interaction (Chi et al., 2013) can impact mRNA export and consequently disrupt normal cell function. We did not observe mislocalization of the THOC2 variant proteins in cultured cells and did not test alterations in their interaction with the other known or unknown TREX proteins. However, reduced levels of a number of new (p.Tyr517Cys, p.His1187Tyr, p.Thr696Ile, p.Gly713Asp) and published (p.Leu438Pro, p.Ile800Thr; Kumar et al., 2015) missense THOC2 variant proteins are due to impaired protein stability or reduced levels of normal mRNA due to aberrant splicing (exon28:c.3503+4A>C). We also noted increased stability of p.Asn1261His THOC2 protein. We and others have shown that THOC2 controls TREX function by maintaining the stability of THOC1, 3, 5, and 7 subunits (Chi et al., 2013; Kumar et al., 2015). Reduced levels of THOC2 missense variant proteins are most likely due to enhanced proteasome-mediated degradation as THOC2 is ubiquitinated (Lopitz-Otsoa et al., 2012). THOC2 depletion has been reported to have different consequences in diverse organisms. For example, shRNA-mediated Thoc2 knockdown leads to significant increase in length of neurites in cultured rat primary hippocampal neurons (Di Gregorio et al., 2013) although effects on neurons with persistently reduced THOC2 variant proteins in the affected individuals may be different and *Caenorhabditis elegans thoc2* knockouts, that are completely immobile, slow-growing, sterile, have functional defects in specific sensory neurons and die prematurely (Di Gregorio et al., 2013). *Danio rerio Thoc2* is essential for embryonic development (Amsterdam et al., 2004) and in *Drosophila melanogaster* S2 cells *Thoc2* knockdown inhibits mRNA export and cell proliferation (Rehwinkel et al., 2004). THOC2 depletion also results in chromosome alignment, mitotic progression, and genomic stability in human HeLa cells (Yamazaki et al., 2010). Finally, *Thoc2* and *Thoc5* knockdown experiments have shown their role in regulation of embryonic stem cell (ESC) self-renewal (Wang et al., 2013).

Both the affected individuals carrying the splice-variants presented with severe neurocognitive features. The exon35:c.4450-2A>G and exon28:c.3503+4A>C *THOC2* splice variants present interesting biological scenarios; the former resulting in a 1,535 amino acid truncated protein that is present at higher level and the latter with both normal (albeit potentially much reduced) and a 1,175 amino acid truncated THOC2 protein. We postulate that the clinical outcomes in the exon35:c.4450-2A>G individual are caused by partial loss of function due to loss of 110 amino acid C-terminal region and accumulation of the truncated THOC2 protein. However, pathogenicity in exon28:c.3503+4A>C affected individual is most likely caused by reduced levels of normal and potential dominant-negative effects of the C-terminally truncated THOC2 protein. That reduced THOC2 protein levels are associated with ID and other clinical symptoms is emerging as a frequent theme; for example, due to reduced THOC2 protein stability caused by missense variants (see above) or aberrant splicing. Indeed reduced THOC2 levels are shown to destabilize the TREX complex in humans (Chi et al., 2013; Kumar et al., 2015) and removal of any THO subunit causes destabilization of other TREX components in yeast (Pena et al., 2012).

Systematic functional analysis of the Tho2 C-terminal RNA binding region in yeast provides interesting explanation as to how the truncated THOC2 protein can perturb normal mRNA export function in human cells (Pena et al., 2012) (Figure 6). The data showed that whereas ΔTho2 yeast strain does not grow at 37°C (restrictive temperature), Tho2$\Delta_{1,408-1,597}$ and Tho$\Delta_{1,271-1.597}$ growth is considerably reduced, suggesting that C-terminal 1,271–1,597 amino acids are required for cell survival at restrictive temperature (Pena et al., 2012). If the exon28:c.3503+A>C variant caused complete splicing defect retaining intron between exon 28–29 in all mRNAs, the cells would translate only 1,175 amino acids (with 1,168 normal) THOC2 protein; essentially lacking the C-terminal region encompassing the RNA binding domain (RBD) that when deleted in yeast Tho$\Delta_{1,271-1.597}$ strain restricts its growth at 37°C. However, the affected boy carrying a single allele of the exon28:c.3503+A>C *THOC2* variant, although with severe clinical symptoms, is alive. This could be explained by presence of reduced levels of THOC2 protein produced from translation of about 2/3rd normally spliced mRNA in the affected white blood cells. Taken together, clinical outcomes in the affected boy may be due to perturbed mRNA export caused by reduced levels of THOC2 protein and perhaps also C-terminally truncated THOC2 protein translated from about 1/3rd aberrantly spliced mRNAs that retain a part of intron sequence between exon 28–29.

We also identified a set of previously unreported *THOC2* missense variants that, according to ACMG criteria, are variants of uncertain clinical significance (VOUS) (Supp. Table S1): namely, p.Arg77Cys, p.Ser1108Leu, p.Arg1121Gly, and p.Asn1261His. Nevertheless, these variants have supportive evidence pointing toward potential pathogenicity as they are rare (absent from ExAC/gnomAD databases of reference individuals) (Lek et al., 2016), affect highly evolutionarily conserved amino acid residues, are predicted to be pathogenic by *in silico* analyses and are within the clinical presentations spectrum of those seen in individuals with confirmed *THOC2*-related ID. However, they lack supportive evidence from our existing functional assays. These variants may still have a detrimental effect on THOC2 function due to altered protein structure impacting protein–RNA and/or protein–protein interactions with known or unknown TREX subunits [e.g., Boehringer et al., 2017]. The challenge of proving causality for previously unreported missense variants in NDD genes is well recognized and speaks to the need for ongoing intertwined clinical and research efforts to clarify causality of VOUS (Wright et al., 2018). We therefore report detailed variant and clinical data (see Supp. materials, Supp. Table S1 and S2, and Supp. Figure S4) with the intention of alerting researchers and clinicians to these variants, as future studies, for example identification of their recurrence in affected individuals with overlapping clinical phenotypes or pathophysiological investigations, may help clarify their clinical significance.

*THOC2* is ubiquitously expressed in all human tissues (Thul et al., 2017) and more specifically in the developing and mature human brain (Johnson et al., 2009; Kumar et al., 2015; Uhlen et al., 2015) and mouse brain, with higher abundance in frontal cortex and cerebellum (Di Gregorio et al., 2013; Kumar et al., 2015). THOC2 is an essential mRNA export factor as its siRNA-mediated depletion results in almost complete retention of mRNAs in the cell nucleus (Chi et al., 2013), potentially toxic to the cell. These data are consistent with the findings that *THOC2* is a highly constrained gene (Samocha et al., 2014)

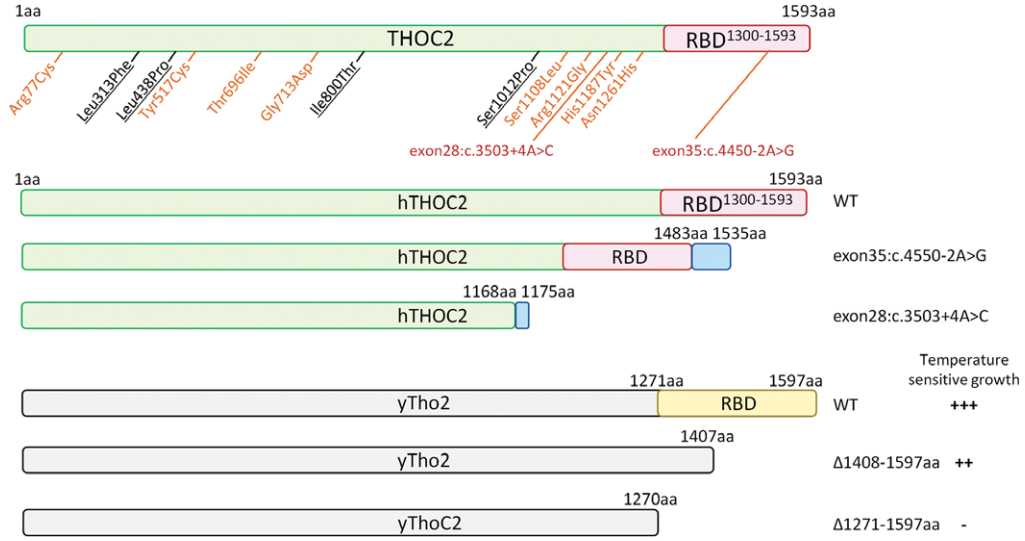


**FIGURE 6** Summary of truncated human THOC2 proteins translated from aberrantly spliced mRNAs and functional outcomes of yeast C-terminal Tho2 deletion strains (Pena et al., 2012). Blue boxes depict the 52 and 7 amino acids coded by unspliced intron sequences of exon35:c.4550-2T>C and exon28:c.3503+4A>C variants, respectively. WT, +++ = normal, Δ1,408–1,597aa, ++ and Δ1,271–1,597aa = reduced growth at restrictive temperature

and *THOC* (e.g., *THOC1*, *3*, *5*, *6*, and *7*) genes are essential for cell survival (Blomen et al., 2015). Taken together, as *THOC2* knockout cells will not survive due to complete mRNA nuclear retention, we predict that the identified *THOC2* variants represent partial loss-of-function that disrupt normal mRNA export in neuronal and possibly other cell types, potentially causing variable clinical presentations.

TREX complex couples transcription and mRNA biogenesis with nuclear mRNA export, and has emerged as an essential pathway in embryogenesis, organogenesis and differentiation (Heath et al., 2016). For example, Thoc2 and Thoc5 selectively bind and regulate export of mRNAs (e.g., *Nanog*, *Sox2*, *Esrrb*, and *Klf4* mRNAs) involved in maintenance of pluripotency of mouse ESCs (Wang et al., 2013) and Thoc5 in maintenance of hematopoiesis and HSP70 mRNA export (Katahira, Inoue, Hurt, & Yoneda, 2009; Mancini et al., 2010). Mouse modeling shows that both *Thoc1* and *Thoc5* knockouts are embryonic lethal (Mancini et al., 2010; Wang, Chang, Li, Zhang, & Goodrich, 2006). However, *Thoc1* and *Thoc5* expression in a range of developing and adult tissues may indicate that the two genes have a more essential role in early embryonic development compared to less stringent requirement during later stages of embryonic or adult development (Mancini et al., 2010; Wang et al., 2006); a functional pattern most likely followed by the *THOC2* gene. Essentiality of *THOC2* gene indicates that *THOC2* knockout will also be lethal. However, reduced levels or perturbed functionality can lead to a range of NDD phenotypes as observed for a cohort of THOC2 variants identified by us. It is now well established that development of brain depends on tightly regulated and complex sequence of events involving neuronal and glial cell proliferation, migration, and maturation (Chiurazzi & Pirozzi, 2016). Therefore, it is not surprising that our *THOC2* variant data and published work (Dickinson et al., 2016) provides strong evidence that even subtle alterations to the canonical molecular pathways such as mRNA export, otherwise essential for cellular life, can be tolerated but at a cost of a NDD.

In summary, we present detailed clinical data on seven individuals with *THOC2*-associated ID caused by both missense and splice variants that meet ACMG criteria for (likely) pathogenicity. They have a core phenotype of ID, and common findings of behavioural disorders, infantile hypotonia, gait disturbance and growth impairment, similar to the affected males with THOC2-associated ID we previously reported (Kumar et al., 2015). Other than the affected female with a *de novo* missense variant, heterozygote carrier females are typically unaffected. We also present data on five individuals with four previously unreported rare missense variants that show clinical overlap with our core group, but where convincing evidence for causality is still required. The significance of these variants may be clarified as additional individuals with *THOC2* variants are reported. We have also "adopted" *THOC2* on the Human Disease Gene (HDG) Website Series (https://humandiseasegenes.nl/thoc2) in an effort to continue to explore the phenotypic-genotypic spectrum for THOC2-related ID.

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## DISCLOSURE STATEMENT

The authors declare no conflict of interest.

## ORCID

*Raman Kumar* http://orcid.org/0000-0001-7976-8386
*Tracy Dudding-Byth* http://orcid.org/0000-0002-9551-1107
*Elizabeth E. Palmer* http://orcid.org/0000-0003-1844-215X
*Jozef Gecz* http://orcid.org/0000-0002-7884-6861

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## SUPPORTING INFORMATION

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# *FOXA2* gene mutation in a patient with congenital complex pituitary hormone deficiency

Hiroko Boda[a], Masafumi Miyata[a], Hidehito Inagaki[b, c], Yasuko Shinkai[c], Takema Kato[b, c], Tetsushi Yoshikawa[a], Hiroki Kurahashi[b, c, *]

[a] Department of Pediatrics, Fujita Health University School of Medicine, Japan
[b] Division of Molecular Genetics, Institute for Comprehensive Medical Science, Japan
[c] Genome and Transcriptome Analysis Center, Fujita Health University, Toyoake, Japan

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ABSTRACT

We report a patient with congenital complex pituitary hormone deficiency (CPHD) with intestinal malrotation and anal atresia. We identified a *de novo* heterozygous mutation, c.664T > G (p.Cys222Gly), in the *FOXA2* gene in this individual. This missense mutation had the potential to affect the DNA binding properties of the FOXA2 protein based on a protein structure prediction. Since a CPHD patient with another missense mutation and one other case with an entire gene deletion have also been reported, we speculated that a haploinsufficiency of the *FOXA2* gene might be a genetic etiology for this disorder. Phenotypic similarities and differences among these three cases are also discussed.

## 1. Introduction

Congenital complex pituitary hormone deficiency (CPHD) is a disorder in which the anterior pituitary hormones (growth hormone, GH; prolactin, PRL; thyroid stimulating hormone, TSH; adrenocorticotropic hormone, ACTH; luteinizing hormone, LH; and follicle stimulating hormone, FSH) are deficient due to pituitary dysplasia [de Graaff, 2000]. The severity and phenotype of CPHD vary due to the combination of defective hormones. The onset of this condition may be noticed early in the newborn period, but can also be diagnosed in later infancy or childhood or adulthood. Occasionally, CPHD manifests as a syndromic disease with some extra-pituitary symptoms.

Since the first report of CPHD with GH, PRL, and TSH deficiencies due to a *POU1F1/PIT1* abnormality, the genes involved in the development of the pituitary-hypothalamus have been clarified consecutively. Abnormalities in many transcription factors such as *POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *PITX2*, *SOX3*, *SOX2*, and *OTX2* have been identified [Tatsumi et al., 1992; Mehta and Dattani, 2008; Giordano, 2016]. The symptoms caused by these abnormalities are diverse, and the mode of inheritance also varies. Although many etiological genes are now known, the proportion of CPHD patients with mutations in these known genes is not high. This suggests that there are many causative genes for this disorder that are yet to be identified.

Recent advances in next generation sequencing have facilitated the identification of novel disease-causing genes. By means of whole exome sequencing, we previously identified a *de novo* missense mutation, c.664T > G (p.Cys222Gly), in *FOXA2* in a girl with CPHD accompanied by gastrointestinal malformations (anal atresia, intestinal malrotation). In the current literature, there are two reports of a *FOXA2* mutation in patients with syndromic CPHD: an entire gene deletion and a c.505T > C mutation (p.Ser169Pro) [Tsai et al., 2015; Giri et al., 2017]. *FOXA2* has thus been identified as another causative gene in CPHD.

## 2. Patient data

A Japanese female infant was born by vaginal delivery at 40 weeks of gestation. Her Apgar score were 8 and 9 at 1 and 5 min after birth, respectively. At birth, her height was 52.5 cm (+1.5 S.D.), weight was 3574g (+0.5 S.D.), and head circumference was 35.0 cm (+1.1 S.D.). She was admitted to our neonatal intensive care unit one day after birth due to hypoglycemia (19 mg/dl at 3 h after birth)

* Corresponding author. Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi, 470-1192, Japan.
*Email address:* kura@fujita-hu.ac.jp (H. Kurahashi)

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which was not improved by breastfeeding. The patient did not have a dysmorphic face. Her mother had hydramnios during the pregnancy. However, her 25-year old mother, 28- year old father and 6-year old sister were all healthy.

After hospitalization, the patient was administered a glucose infusion but she still often had hypoglycemia without hyperinsulinemia. She had no growth hormone (GH) secretion response to an arginine stimulation test (GH < 0.03 ng/ml). She also had a low level of free thyroxine (fT4) (0.75 ng/ml) without thyroid stimulating hormone (TSH) elevation (3.78 μl/ml). There were no fT4 and TSH secretion responses to a thyrotropin-releasing hormone (TRH) stimulation test. Additionally, she had a low level of cortisol (0.1 μg/dl) and adenocorticotrophic hormone (ACTH) was undetectable ( < 1.6 pg/ml). There was no cortisol secretion response to corticotropin releasing hormone (CRH) stimulation. An MRI scan of the patient's brain revealed no sella turcica, and neither the anterior pituitary nor the posterior pituitary was found in its normal position. Additionally, no ectopic posterior pituitary tissue was detected. Based on these results, we made a diagnosis of CPHD and administered thyroxine (10 μg/kg), hydrocortisone (40 mg/m$^2$), and somatropin (0.175 mg/kg/week).

This patient also had low anal atresia with anocutaneous fistula diagnosed by a lower gastrointestinal series. Defecation events were well controlled using an enema. However, as her bilious vomiting continued, we performed an upper gastrointestinal series from which we diagnosed an intestinal malrotation for which a radical operation was performed at 24 days after birth.

She underwent a further radical operation for low anal atresia at 9 months, but developed hypernatremia (158–165 mEq/L), high plasma osmolality (326 mOsm) and polyuria (max 14.9 ml/kg/hour) at five days post-surgery. We diagnosed central diabetes insipidus and commenced desmopressin treatment (0.5 μg/day). At the age of 1 year and 4 months, her growth was inadequate (height 68 cm (−3.3 S.D.), weight 6815 g (−3.2 S.D.), and head circumference 42.8 cm (−1.9 S.D.)) but her psychomotor development was normal.

## 3. Methods

### 3.1. Subjects

Peripheral blood samples were obtained from the patient and her parents. The research protocol for this study was approved by the local ethics committees of Fujita Health University, Japan. Written informed consent for the participation in this study was obtained from the parents.

### 3.2. Cytogenetic microarray

High-resolution chromosomal microarray analysis was conducted using the CytoScan HD array and Chromosome Analysis Suite 3.0 (Affymetrix, Santa Clara, CA) with a threshold level of 20 probes for a 50 kb region.

### 3.3. Whole exome sequencing

Fifty nanograms of the DNA samples were used to generate a whole exome library using SureSelect QXT Reagent and SureSelect Clinical Research Exome (Agilent Technologies, Santa Clara, CA). Sequencing was carried out using a HiSeq 1500 (Illumina, San Diego, CA). After demultiplexing from other sample data, the reads were mapped onto the human reference hg19 using BWA 0.7.15 [Li and Durbin, 2009]. Sorting and recalibration of the mapped reads, and variants were called into a VCF file using Picard tools 2.8.0 [Broad Institute, 2017] and GATK 3.7 [Van der Auwera et al., 2013]. Annotations were added using Variant Studio 2.3 (Illumina). To identify disease causing mutations, we excluded known variants listed in the public databases (dbSNP147, 1000 Genomes Project, NHLBI ESP6500, and Exome Aggregation Consortium [ExAC]) and a control in-house database, except for those also identified as pathogenic mutations in the NCBI ClinVar and HGMD databases. Variants consistent with the phenotype in the pedigree (autosomal recessive or *de novo* dominant variants) were extracted. We focused on non-synonymous single nucleotide variants, insertions and deletions (indels), and splice site variants. The mutation was confirmed by Sanger sequencing of a PCR amplicon of the corresponding region.

### 3.4. Analysis of protein structure

Protein structure predictions were made using UCSF Chimera software [Pettersen et al., 2004].

## 4. Results

Since the current study patient was found to carry multiple congenital anomalies, we initially hypothesized that her phenotype was generated by a contiguous gene deletion syndrome. We performed cytogenetic microarray analysis to identify the responsible variant but no possibly pathogenic copy number variations were identified. We also performed targeted exome sequencing for the capture of 4813 disease-associated genes, including some known CPHD genes. We screened these data using CPHD as a keyword, but no mutation was identified among the candidate genes.

We next obtained parental samples and conducted trio-whole exome sequencing. We identified two *de novo* mutations, one of which was the missense mutation NM_021784.4: c.664T > G; p.(Cys222Gly) in *FOXA2* (Fig. 1A). This was confirmed by Sanger sequencing (Fig. 1B). This variant is not present in databases of normal healthy populations such as ExAC or 3554 Japanese database iJGVD [Yamaguchi-Kabata et al., 2015]. *In silico* functional analysis suggested that the mutation was damaging when using Polyphen2 (1.000) and deleterious with SIFT (0.000). Another missense mutation, NM_002095.5: c.346A > G; p.(Lys116Glu), was also identified in *GTF2E2*, which was not found in the databases either. However, *in silico* analysis predicted it as benign by Polyphen2 (0.153) and tolerated by SIFT (0.123). The classifications of *FOXA2* and *GTF2E2* mutations using the ACMG guideline [Richards et al., 2015] are likely pathogenic and uncertain significance, respectively. Thus, we considered that the c.664T > G; p.(Cys222Gly) mutation in *FOXA2* was the strong candidate as the cause of our patient's phenotype.

The Cys222 residue is located within the forkhead domain of FOXA2 and is highly conserved among various specie (human, mouse, dog, elephant, chicken and zebrafish) (Fig. 1A). Forkhead domains are DNA binding domains that are commonly found in transcription factors. We predicted the effect of *FOXA2* mutation in our current patient by protein structure analysis using Protein Data Bank Japan (PDBj). Recently, the c.505T > C (p.Ser169Pro) mutation in *FOXA2* was reported in a patient with CPHD (Giri et al., 2017). The Ser169 residue in FOXA2 (Fig. 2) is located at a position that is predicted to directly contact the DNA backbone, suggesting that the p.Ser169Pro mutation would affect the DNA binding ability of FOXA2. On the other hand, the Cys222 is located between the α-helix of this protein, at residues Gln206-Phe219, that is predicted to associate with the DNA major groove and the Lys225 that also contacts DNA (Fig. 2), suggesting that the p.Cys222Gly substitution might also affect the DNA binding ability of FOXA2.

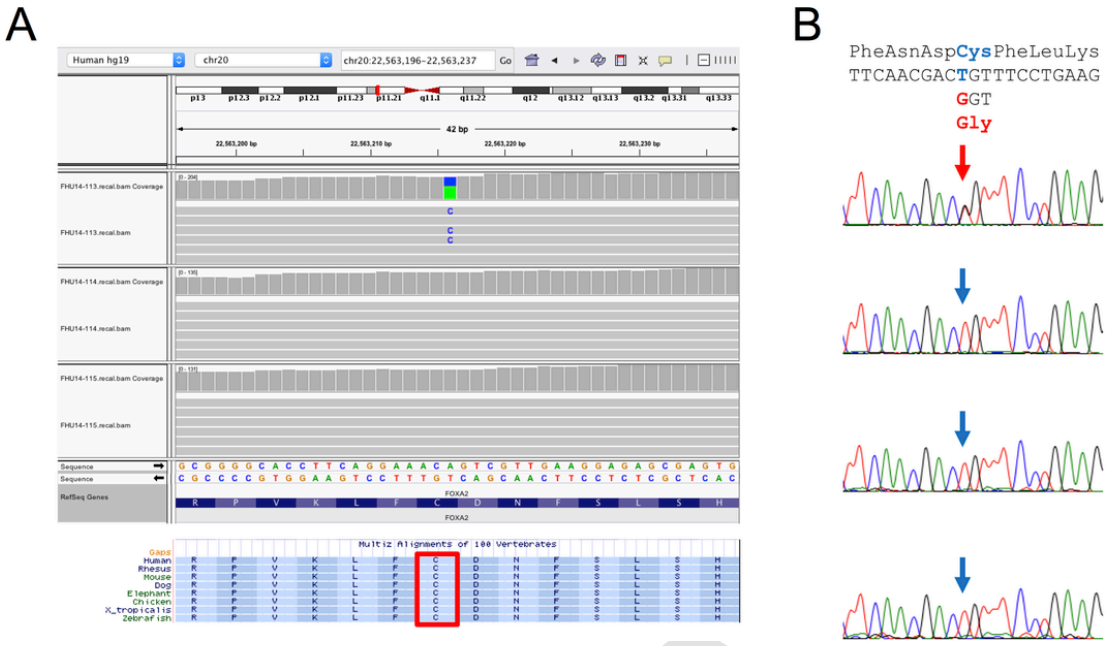


**Fig. 1.** Mutational analysis of the current study patient with CPHD. **A.** Trio analysis of whole exome sequences obtained using a next generation sequencer; patient (top), father (middle), and mother (bottom). A *de novo* heterozygous c.664T > G mutation was identified in *FOXA2*. The complementary strand is shown. Levels of conservation of the forkhead domain in FOXA2 among different species is shown at the bottom. **B.** Confirmation of the c.664T > G mutation by Sanger sequencing. This is a missense mutation that causes a TGT(Cys) to GGT(Gly) substitution.

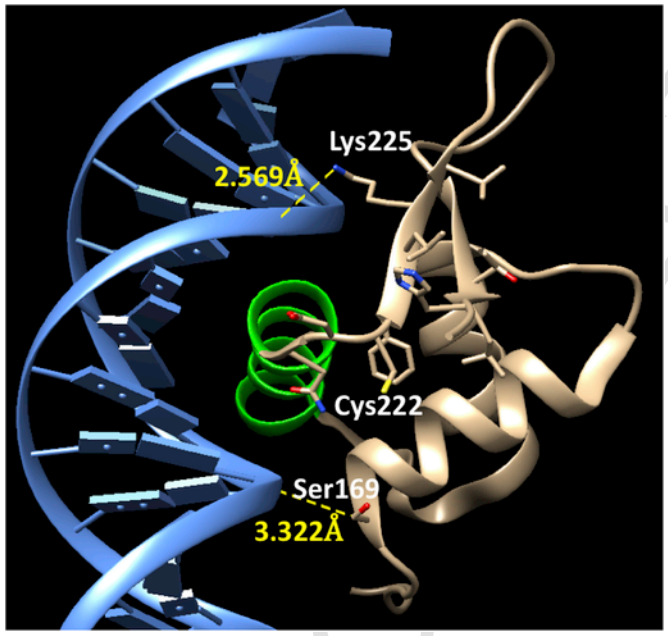


**Fig. 2.** Prediction of FOXA2 structures using PDB ID:5X07. The Ser169, Cys222, and Lys225 residues are indicated. The α-helix of Gln206-Phe219 is colored in green. Blue indicates DNA. The c.505T > C (p.Ser169Pro) mutation may directly affect the DNA binding affinity of the protein. The c.664T > G (p.Cys222Gly) mutation might also impact on the DNA binding ability of FOXA2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 5. Discussion

In this study, we have identified a novel *de novo* missense mutation in *FOXA2* in a CPHD patient. This mutation is not listed in databases for the normal population and was predicted to affect the DNA binding ability of FOXA2. Further, *FOXA2* encoding this transcription factor is abundantly expressed in the hypothalamic-pituitary axis in the developing mouse embryo [Giri et al., 2017]. Two other CPHD patients have been reported in the present literature with a *FOXA2* mutation [Tsai et al., 2015; Giri et al., 2017]. The evidence from these two prior reports and our current analyses thus indicate that *FOXA2* mutations are causative for CPHD.

Our current case and one previous CPHD patient have been found to carry a missense mutation in *FOXA2* [Giri et al., 2017], located within the highly conserved forkhead domain in both instances. Protein structural analysis enabled us to predict the effect of these mutations on the DNA binding propensity of the protein products. Our analysis indicates that these mutations may cause a loss-of-function. Further, as another reported case showing an entire *FOXA2* deletion also manifested CPHD, we speculate that a haploinsufficiency of *FOXA2* might play an etiologic role in this disorder [Tsai et al., 2015]. *FOXA2* is a key transcriptional factor during embryonic development, and *FOXA2* null mice die during the early embryonic stage [Ang and Rossant, 1994; Weinstein et al., 1994]. Individuals with a *FOXA2* haploinsufficiency could possibly survive this stage but might show a combination of congenital disorders of organs whose development is regulated by *FOXA2*.

The disease phenotype of the three CPHD patients reported to date with *FOXA2* mutations, including our current case, are compared in Table 1. The main clinical findings of our present patient were CPHD, intestinal malrotation and anal atresia. The prior case with p.Ser169Pro reported by Giri et al. presented with CPHD but no gastrointestinal malformation other than gastroesophageal reflux [Giri et al., 2017]. Interestingly, a previously reported female proband with an entire *FOXA2* deletion manifested an intestinal malrotation similar to our present patient, but did not have CPHD. However, her father who also carries the deletion suffered from this disorder. Such phenotypic variations, even among cases with the same mutation, are often observed in dominant diseases caused by a haploinsufficiency in a transcriptional regulator that functions in development. Recently, another patient with *de novo FOXA2* mutation, c.770G > T, p.(Arg257Leu) was reported [Vajravelu et al., 2018]. Similar to our case, the mutation was located within the DNA bind-

**Table 1**
Summary of phenotypes of cases with *FOXA2* mutations.

| | Case 1[a] | Case 2[b] | Case 3 (father of Case 2)[b] | Case 4[c] | Present case |
|---|---|---|---|---|---|
| Age | 5 years | 2 months | unknown | 7 h of life | 1 day |
| Sex | Female | Female | Male | Female | Female |
| Genetic findings | *FOXA2*: c.505T > C; p.Ser169Pro | 277 kb deletion of 20p11.21 (*FOXA2* involved) | 277 kb deletion of 20p11.21 (*FOXA2* involved) | *FOXA2*: c.770G > T; p.R257L | *FOXA2*: c.664T > G, p.Cys222Gly |
| Pituitary function | Panhypopituitarism | Normal | Panhypopituitarism | Panhypopituitarism | Panhypopituitarism |
| MRI findings | Thin pituitary stalk, hypoplastic anterior pituitary, thin corpus callosum | N/A | N/A | Small pituitary gland, shallow sella turcica with diminutive pituitary tissue, an ectopic posterior pituitary bright spot along the tuber cinereum, nonvisualization/absence of the infundibulum | Defect of sella turcica, anterior and posterior pituitary |
| Other complications | Hyperinsulinism, single median maxillary central incisor, congenital nasal pyriform aperture stenosis, choroidal coloboma, supra-valvular pulmonary stenosis, feed intolerance, severe gastro-esophageal reflex disease, portal-portal bridging fibrosis in liver, persistent oxygen requirement of unknown etiology, speech and motor developmental delay | Abdominal heterotaxy, biliary atresia, enlarged spleen, intestinal malrotation, interrupted inferior vena cava, cholangitis | Situs inversus, polysplenia, mildly dysmorphic facial features | Hyperinsulinism, coarse facial features, hypertelorism, thin upper lip, low-set ears, widely spaced nipples | Hypoglycemia without hyperinsulinism, anal atresia, intestinal malrotation |

[a] Giri et al., [2017].
[b] Tsai et al., [2015].
[c] Vajravelu et al., [2018].

ing domain. Remarkably, all of the patients with *de novo* FOXA2 mutation exhibited dysfunction of the glucose regulation (Table 1). Further studies are needed to better understand what affects phenotype penetrance among individuals who have a haploinsufficiency in *FOXA2*.

## Competing financial interests

The authors declare no competing financial interests in relation to this study.

## Uncited reference

MacDonald et al., 2014.

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SCIENTIFIC REPORTS

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# Genotype determination of the *OPN1LW/OPN1MW* genes: novel disease-causing mechanisms in Japanese patients with blue cone monochromacy

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Satoshi Katagiri[1], Maki Iwasa[2], Takaaki Hayashi[1,3], Katsuhiro Hosono[4], Takahiro Yamashita[5], Kazuki Kuniyoshi[6], Shinji Ueno[7], Mineo Kondo[8], Hisao Ueyama[9], Hisakazu Ogita[9], Yoshinori Shichida[5], Hidehito Inagaki[10], Hiroki Kurahashi[10], Hiroyuki Kondo[11], Masahito Ohji[2], Yoshihiro Hotta[4] & Tadashi Nakano[1]

**Blue cone monochromacy (BCM) is characterized by loss of function of both *OPN1LW* (the first) and *OPN1MW* (the downstream) genes on the X chromosome. The purpose of this study was to investigate the first and downstream genes in the *OPN1LW/OPN1MW* array in four unrelated Japanese males with BCM. In Case 1, only one gene was present. Abnormalities were found in the promoter, which had a mixed unique profile of first and downstream gene promoters and a −71A > C substitution. As the promoter was active in the reporter assay, the cause of BCM remains unclear. In Case 2, the same novel mutation, M273K, was present in exon 5 of both genes in a two-gene array. The mutant pigments showed no absorbance at any of the wavelengths tested, suggesting that the mutation causes pigment dysfunction. Case 3 had a large deletion including the locus control region and entire first gene. Case 4 also had a large deletion involving exons 2–6 of the first gene. As an intact LCR was present upstream and one apparently normal downstream gene was present, BCM in Case 4 was not ascribed solely to the deletion. The deletions in Cases 3 and 4 were considered to have been caused by non-homologous recombination.**

The human retina contains three types of cone photoreceptors: long-wavelength sensitive cones (L cones), medium-wavelength sensitive cones (M cones), and short-wavelength sensitive cones (S cones). These cone photoreceptors express respective visual pigments, L, M, and S opsins. Among these, the genes encoding L opsin (*OPN1LW*, OMIM; *300822) and M opsin (*OPN1MW*, OMIM; *300821) are present in tandem on the human X chromosome[1,2], forming an L/M pigment gene array. In individuals with normal color vision, the first gene in the array is an L gene, and the downstream (the second and later) gene(s) is/are M gene(s). Abnormalities in the array are reportedly associated with protan and deutan color vision deficiencies[3], blue cone monochromacy (BCM)[4], and Bornholm eye disease[5].

[1]Department of Ophthalmology, The Jikei University School of Medicine, Tokyo, Japan. [2]Department of Ophthalmology, Shiga University of Medical Science, Shiga, Japan. [3]Department of Ophthalmology, Katsushika Medical Center, The Jikei University School of Medicine, Tokyo, Japan. [4]Department of Ophthalmology, Hamamatsu University School of Medicine, Shizuoka, Japan. [5]Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto, Japan. [6]Department of Ophthalmology, Kindai University Faculty of Medicine, Osaka, Japan. [7]Department of Ophthalmology, Nagoya University Graduate School of Medicine, Aichi, Japan. [8]Department of Ophthalmology, Mie University Graduate School of Medicine, Mie, Japan. [9]Department of Biochemistry and Molecular Biology, Shiga University of Medical Science, Shiga, Japan. [10]Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Aichi, Japan. [11]Department of Ophthalmology, University of Occupational and Environmental Health, Fukuoka, Japan. Satoshi Katagiri and Maki Iwasa contributed equally to this work. Correspondence and requests for materials should be addressed to T.H. (email: taka@jikei.ac.jp) or H.U. (email: datt@belle.shiga-med.ac.jp)

BCM (OMIM; #303700) is a rare congenital color vision deficiency with an X-linked inheritance pattern[4,6]. Cases of BCM typically present with severely impaired color discrimination, reduced visual acuity, nystagmus, photophobia, and diminished L/M cone function despite retention of rod and blue cone function[6,7]. The dysfunction in both L and M cones in BCM is reportedly caused by one of the three genotypes. One genotype involves deletion of the locus control region (LCR)[4,8–12], which is located upstream of the L/M gene array (−3,681 to −3,021 from the cap site of the first gene) and believed to be involved in the mutually exclusive expression of L and M genes[13,14]. Therefore, neither gene is expressed in the absence of the LCR. Another genotype involves a deleterious mutation in a single-gene array (either the L or M gene present alone in the array). The derivation of this genotype has two obvious steps: first, non-homologous recombination between the L and M genes to form a single-gene array followed by an inactivating mutation in the single gene (reverse order is also possible). The most common mutation is C203R[4,8,15–17], but other mutations, such as P307L[8], R247X[8], and deletion of exon 2[16], have also been documented. The LIAVA haplotype in exon 3, which affects splicing[18], was also reported in a single L gene[17]. The third genotype involves inactivating mutations in both the L and M genes. Although the C203R mutation has been documented in this genotype[8,16,19], the LIAVA haplotype (or a very similar haplotype) in exon 3 of both genes seems to be frequent[17,20].

Although little is known about the prevalence of BCM in the Japanese population, to date, only two BCM families have been described in the literature, demonstrating the mechanism of deletion of the LCR in both families[11,21].

In the current study, the L/M pigment gene arrays in four unrelated Japanese males with BCM were analyzed. The purpose of this study was to investigate their genotypes in the L/M pigment gene array, which could be categorized into one of the three above-mentioned genotypes, but others were unreported mechanisms and differed from each other.

## Results

**Case 1 (JU#1299).** Long-range polymerase chain reaction (PCR) was successful for the first gene but not for downstream gene(s) (Fig. 1A). Promoter analysis of gene number, by contrast, showed that the first gene promoter was absent (only the downstream gene promoter was detected) (Fig. 1B). From the results of repeated long-range PCR analysis of downstream genes, we concluded that this subject had a single gene (no downstream genes) in the array. The LCR was present upstream of the single gene, and its sequence had no aberrations. The curious result that this subject had only the downstream gene promoter (Fig. 1B) was later found to be due to the unique sequence of the promoter. The first and downstream gene promoters differ by 14 nucleotides, but in the promoter region of the single gene, 8 upstream sites were associated with the first gene, whereas the other 6 sites were a random mixture of these nucleotides (Fig. 2). Moreover, the promoter had a −71 A > C substitution, which has been reported to be associated with deutan color vision deficiency[22] (Fig. 2). The G at the −30 position in the $^{-34}$GCCGGT$^{-29}$ sequence (the number is from the cap site of the first gene) in the promoter analysis indicated that the first gene promoter was absent (Fig. 1B). The −30 (A or G) site discriminates the promoters of the first and downstream genes by *Cfr*10I (recognition sequence = RCCGGY). Our conclusion was that Case 1 had a single gene array because the sequencing of the promoter showed only one curious pattern as shown in Fig. 2. The luciferase activity of the promoter of the single gene was more than twice that of the usual first gene promoter in the reporter assay (Supplementary Fig. S1). No abnormalities were found in exons 1–6 and their adjacent introns in nucleotide sequencing. Exons 2–5 were M type, and the haplotype of exon 3 was MVAIA rather than LIAVA (Table 1). The curious promoter found in Case 1 has not been reported previously.

**Case 2 (JU#1311, KINKI-125-70).** Products of both the first and downstream genes were obtained using long-range PCR (Fig. 1A). Promoter analysis showed that the subject had a 2-gene array (Fig. 1B). Both genes had the same missense mutation (c.818 T > A, M273K) in exon 5 (Fig. 3A,B). The chromosome positions (GRCh38.p7) of the mutation are 154,156,367 (L gene) and 154,193,481 (M gene). The M273K mutation has not been reported previously, and not found in the Single Nucleotide Polymorphism Database (https://www.ncbi.nlm.nih.gov/projects/SNP/), Genome Aggregation Database (http://gnomad.broadinstitute.org/), Exome Aggregation Consortium (http://exac.broadinstitute.org/) and Human Genetic Variation Database (http://www.hgvd.genome.med.kyoto-u.ac.jp/). The analysis of the recombinant proteins of M273K mutants revealed that the opsin with the M273K mutation was significantly detectable in the Western blot and cultured cells (Supplementary Fig. S2) but showed no absorbance at any of the wavelengths tested after reconstitution with 11-*cis*-retinal (Fig. 3C). These results indicated that the M273K mutation in both genes results in dysfunctional opsin protein, probably because of a lack of the ability to bind to 11-*cis*-retinal. We therefore ascribed the BCM phenotype in this subject to the mutation. In the first gene, exons 2, 3, and 4 were L type, exon 5 was M type, and the haplotype of exon 3 was LVAIS. In the downstream gene, exons 2–5 were M type, and the haplotype of exon 3 was MVAIA (Table 1).

**Case 3 (JU#1318, MIE-050-0071).** Long-range PCR was unsuccessful for both the first and downstream genes (Fig. 1A). Promoter analysis for determining gene number showed that the first gene promoter was absent (only the downstream gene promoter was detected) (Fig. 1B). Amplification of the LCR was also unsuccessful, indicating a deletion including both the LCR and first gene. To determine the exact deletion breakpoints, 11 sets of PCR primers were designed to cover the sequence −53,930 to −9,320 (number is from the cap site of the first gene) (NT_025965.12: 707,760 to 752,370). PCR products were obtained when using the UP8F and UP8R pair (Supplementary Table S1) but not the UP9F and UP9R pair (Supplementary Table S1), suggesting that the upstream breakpoint of the deletion was between −32,015 and −28,150 (NT_025965.12: 729,665 to 733,530). Long-range PCR using the primer sets UP8F/E5R and UP8F/E6R was successful in this subject but not in a color-normal control subject (Fig. 1C). According to the human genome database (NT_025965.12), the distance between UP8F- and E5R-corresponding regions and UP8F- and E6R-corresponding regions were 81,131 bp and

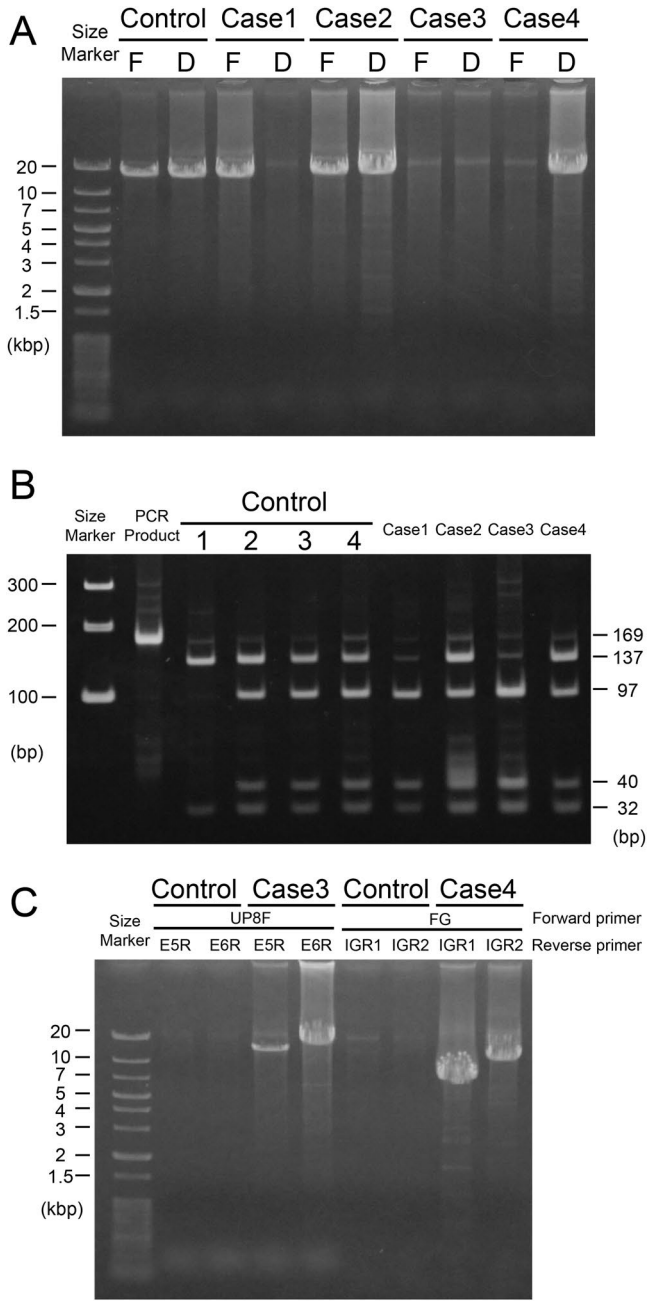


**Figure 1.** Long-range PCR and promoter analysis. (**A**) First and downstream genes in the L/M gene array were amplified separately by long-range PCR. The control was a color-normal subject having both the first and downstream genes. F, first gene; D, downstream gene(s). Thin bands of approximately 20 kb are not amplified products but the templates (genomic DNA, usually approximately 100 ng per reaction). (**B**) Promoter analysis of gene number. Promoters were amplified by PCR using primers common to the first and downstream genes. PCR products (169 bp) digested with *Cfr*10I were loaded onto a polyacrylamide gel. Controls 1–4 have gene numbers 1–4, respectively[28]. (**C**) Long-range PCR beyond the deletion. Combinations of the primers UP8F/E5R and UP8F/E6R were used for long-range PCR in the control and Case 3. Combinations of the primers FG/IGR1 and FG/IGR2 were used for long-range PCR in the control and Case 4.

83,925 bp, respectively (the E5R- and E6R-corresponding regions are those of the downstream gene), which were too far for long-range PCR. In Case 3, however, due to the large deletion including the first gene, the distances between the regions had been reduced to about 12 kbp and 15 kbp, respectively, and therefore, long-range PCR products were obtained in this case (Fig. 1C). The 15-kbp product contained not only exons 1–6 (exons 2, 4, and 5 were L type, exon 3 was M type, with the haplotype of MVAIA) (Table 1) but also the downstream gene promoter. By sequencing the 15-kbp product using the UP12F primer (Supplementary Table S1), the upstream breakpoint of the deletion was determined to be somewhere in the sequence $^{-31,241}$GAACTCCTGACCTCAGG$^{-31,225}$ (the number is from the cap site of the first gene) (NT_025965.12: 730,439 to 730,455), and the downstream breakpoint

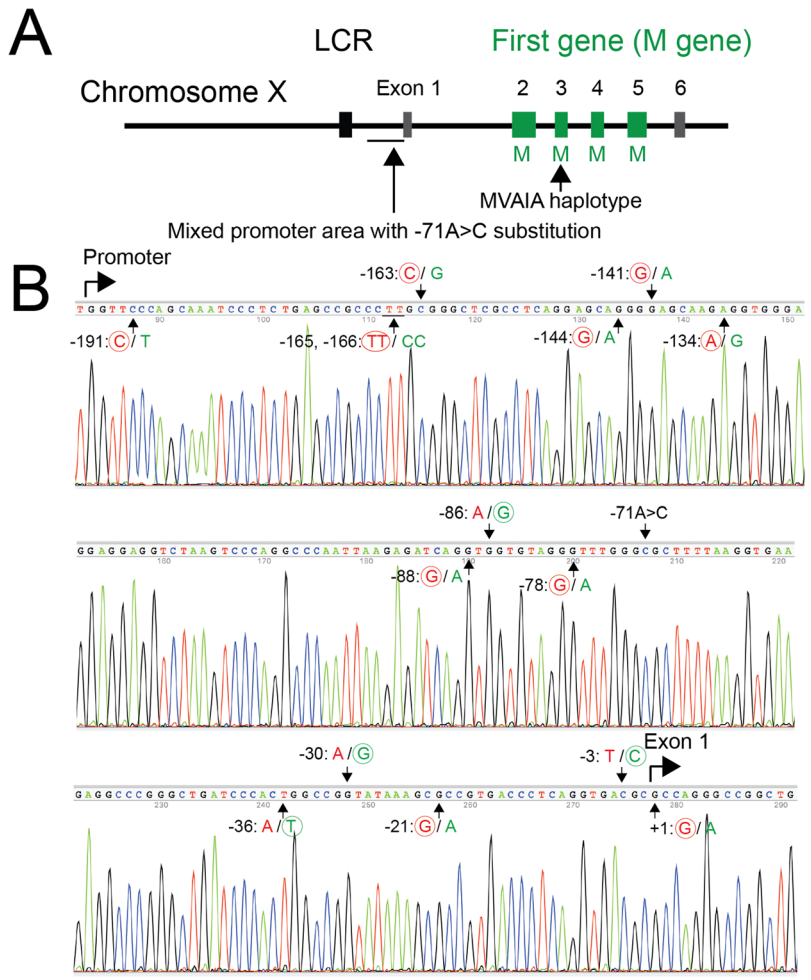


**Figure 2.** Genotype of Case 1. (**A**) Overview of the genotype of Case 1. Case 1 had an intact LCR and a single M gene array in which no aberrations were found. The promoter regions had a unique profile including a −71 A > C substitution. (**B**) The promoter of the single M-gene array. Black arrows indicate the 14 nucleotides differing between first and downstream genes and the −71 A > C substitution. At each position, the usual nucleotide of the first gene promoter is shown on the left side in red and that of the downstream gene promoter is shown on the right side in green. The nucleotides in Case 1 are circled.

was determined to be somewhere in the sequence $^{-407}$GAACTCCTGACCTCAGG$^{-391}$ (the number is from the cap site of the downstream gene) (NT_025965.12: 799,682 to 799,698) (Fig. 4). The reason why long-range PCR was unsuccessful for the downstream gene (Fig. 1A) is that the deletion includes the region corresponding to the forward primer for long-range PCR, DG (−748 to −728 from the cap site of the downstream gene). The estimated size of the deletion was 69,243 bp. The long-range PCR products in Fig. 1C were calculated to be exactly 11,888 bp and 14,682 bp. As a LCR is reportedly necessary for the expression of L/M genes[13,14], the BCM phenotype in this subject was ascribed to the deletion.

**Case 4 (JU#1368, Nagoya-140).** Long-range PCR was successful for downstream gene(s) but not for the first gene (Fig. 1A). Promoter analysis of gene number showed a 1:1 ratio for the first and downstream genes (Fig. 1B). As the first gene promoter was shown to be present, the FG primer–corresponding region (upstream of the promoter) should also be present. PCR analysis using combinations of the FG primer and various intragenic reverse primers revealed that the upstream breakpoint of the deletion was within intron 1 (between the primer I1R1– and primer I1R2–corresponding regions) and that the deletion expanded beyond exon 6. The failure of long-range PCR for the first gene was ascribed to the absence of exon 6 (primer E6R corresponds to exon 6). To determine the downstream breakpoint of the deletion, 15 reverse primers specific to the intergenic region (between the first and downstream genes) were designed for long-range PCR. PCR products were obtained for two primer pairs (FG/IGR1, and FG/IGR2) in this subject but not in the control (Fig. 1C). According to the human genome database (NT_025965.12), the distances between the FG- and IGR1-corresponding regions and between the FG- and IGR2-corresponding regions were 30,137 bp and 34,286 bp, respectively, which were too long for long-range PCR. In Case 4, however, due to the large deletion, the distances had been reduced to approximately 7 kbp and 11 kbp, respectively, and therefore, long-range PCR products were obtained (Fig. 1C).

| | | Exon 2 | | | | Exon 3 | | | | | | | | Exon 4 | | | | | Exon 5 | | | | | | | | | | | | Haplotype in Exon 3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reference | Nucleotide position | 194 | 300 | 331 | 347 | 453 | 457 | 465 | 511 | 513 | 521 | 532 | 538 | 689 | 697 | 698 | 699 | 706 | 820 | 823 | 825 | 828 | 830 | 835 | 849 | 853 | 888 | 892 | 926 | | |
| | L gene | C | A | A | C | G | C | G | G/A | G/T | C/T | A/G | T | T | G | C | T | A | A | T | T | G | A | G | C | A | T | G | A | | |
| | M gene | T | G | G | A | A | A | C | | | | | G | C | A | G | C | G | G | C | G | A | T | T | A | G | C | C | T | | |
| | Amino acid Position | 65 | 100 | 111 | 116 | 151 | 153 | 155 | 171–1 | 171–3 | 174 | 178 | 180 | 230 | 233–1 | 233–2 | 233–3 | 236 | 274 | 275–1 | 275–3 | 276 | 277 | 279 | 283 | 285 | 296 | 298 | 309 | | |
| | L gene | T | L | I | S | R | L | V | V/I | | A/V | I/V | S | I | A | | | M | I | F | | A | Y | V | P | T | G | A | Y | |
| | M gene | I | L | V | Y | R | M | V | | | | | A | T | S | | | V | V | L | | A | F | F | P | A | G | P | F | |
| Case 1 First (single) gene | Nucleotide | T | G | G | A | A | A | C | G | G | C | A | G | C | A | G | C | G | G | C | G | A | T | T | A | G | C | C | T | MVAIA | |
| | Amino acid | I | L | V | Y | R | M | V | V | | A | I | A | T | S | | | V | V | L | | A | F | F | P | A | G | P | F | | |
| Case 2 First gene | Nucleotide | C | A | A | C | G | C | G | G | G | C | A | T | T | G | C | T | A | G | C | G | A | T | T | A | G | C | C | T | LVAIS | |
| | Amino acid | T | L | I | S | R | L | V | V | | A | I | S | I | A | | | M | V | L | | A | F | F | P | A | G | P | F | | |
| Case 2 Downstream gene | Nucleotide | T | G | G | A | A | A | C | G | G | C | A | G | C | A | G | C | G | G | C | G | A | T | T | A | G | C | C | T | MVAIA | |
| | Amino acid | I | L | V | Y | R | M | V | V | | A | I | A | T | S | | | V | V | L | | A | F | F | P | A | G | P | F | | |
| Case 3 Downstream gene (First gene was deleted) | Nucleotide | C | A | A | C | A | A | C | G | G | C | A | G | T | G | C | T | A | A | T | T | G | A | G | C | A | T | G | A | MVAIA | |
| | Amino acid | T | L | I | S | R | M | V | V | | A | I | A | I | A | | | M | I | F | | A | Y | V | P | T | G | A | Y | | |
| Case 4 Downstream gene (First gene was deleted) | Nucleotide | T | G | G | A | G | C | G | G | G | T | G | G | C | A | G | C | G | G | C | G | A | T | T | A | G | C | C | T | LVVVA | |
| | Amino acid | I | L | V | Y | R | L | V | V | | V | V | A | T | S | | | V | V | L | | A | F | F | P | A | G | P | F | | |

**Table 1.** Nucleotides in each gene of the four cases. The positions of nucleotides different between wild-type L and M genes, and polymorphic nucleotide positions 511, 513, 522 and 532 as well, are shown. *Haplotype in Exon 3 was determined by amino acid residues at 153, 171, 174, 178, and 180.

The IGR1 primer corresponds to the region +14,867 to +14,887 (the number is from the stop codon in exon 6) (NT_025965.12: 791,231 to 791,251), and the IGR2 primer corresponds to the region +15,232 to +15,252 (NT_025965.12: 767,324 to 767,329). Using the 11-kbp PCR product and primer I1F (Supplementary Table S1), the upstream breakpoint of the deletion was determined to be somewhere in $^{+5,492}$TGAGCC$^{+5,497}$ (the number is from 5′ splice site of intron 1 of the first gene) (NT_025965.12: 767,324 to 767,329), and the downstream breakpoint was determined to be somewhere in $^{+14,714}$TGAGCC$^{+14,719}$ (the number is from the stop codon in exon 6) (NT_025965.12: 790,713 to 790,718) (Fig. 5). The estimated size of the deletion was 23,389 bp. The long-range PCR products shown in Fig. 1C were calculated to be exactly 6,748 bp and 10,897 bp. PCR confirmed the presence of the LCR, and its sequence had no aberrations. The downstream gene had no abnormalities in the promoter, exons 1–6, or their adjacent introns. Exons 2–5 were M type, and the haplotype of exon 3 was LVVVA (Table 1). As the downstream gene was apparently normal, the cause of BCM in this subject could not be confidently determined.

## Discussion

In this study, we reported the results of gene analyses in four cases of BCM. Their genotypes were unreported and different from each other.

Case 1 had a single-gene array having a curious promoter sequences with a −71 A > C substitution. The −71 A > C substitution was reported to be associated with deutan color vision deficiency due to decreased promoter activity[22]. We hypothesized that the −71 A > C substitution causes dysfunction of the single gene. However, rather than being low, the activity of the promoter in Case 1 was more than twice that of the control in the reporter assay. It is reported that not only the LCR but also normal promoters in the L/M gene array were essential for expression of both L and M genes[13,14]. The LCR was not contained in the constructs for our reporter assay system. Although the dysfunction of the single opsin gene in Case 1 remains unclear, it might be possible that the curious promoter sequences (Fig. 2) might interfere with the LCR binding to the promoter.

Case 2 had a two-gene array, and both genes had a novel missense mutation (M273K) that would cause dysfunction of both gene products (Fig. 3C). The C203R mutation in exon 4 reportedly causes loss of function of the L/M gene products; this mutation causes deutan color vision deficiency when present in the M gene[23,24] and BCM when present in both the L and M genes[8,16,19]. The occurrence of C203R mutation in both the L and M genes was explained by gene conversion[19] (i.e., transfer of the C203R mutation present in the downstream M gene to the first L gene). The deleterious LIAVA haplotype in exon 3 of both the L and M genes was also explained by gene conversion[20]. Also, the occurrence of the M273K mutation in both the first and second genes (Fig. 3A) might be explained by the same mechanism of gene conversion seen in the C203R mutation[19]. Otherwise, because the first gene in this subject had L-type exons 2–4 and M-type exon 5 (with the mutation), we developed the

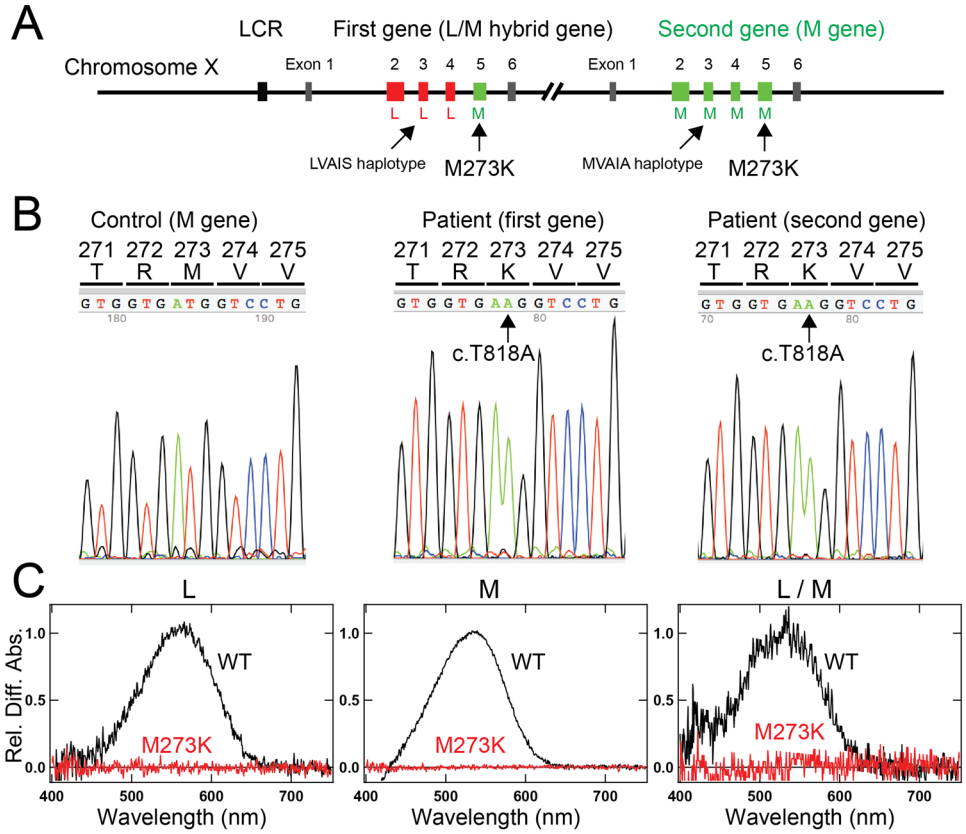


**Figure 3.** Genotype of Case 2. (**A**) Overview of the genotype of Case 2. Case 2 had an intact LCR and two genes. The first gene had exons 2–4 of L type, exon 3 with LVAIS haplotype, and exon 5 of M type. The second gene had exons 2–5 of M type and exon 3 with MVAIA haplotype. Both genes had the same missense mutation (c.818 T > A, M273K) in exon 5. (**B**) Partial sequence data around the missense mutation (c.818 T > A, M273K) in exon 5 in the control and two genes of Case 2. (**C**) Opsin reconstitution experiments. L, L opsin in which exons 2–5–derived amino acid sequences are all L type; M, M opsin in which exons 2–5–derived amino acid sequences are all M type, as in the product of the second gene of Case 2; L/M, M opsin in which exons 2–4–derived amino acid sequences are L type but exon 5–derived amino acid sequence is M type, as in the product of the first gene of Case 2. WT, wild-type opsin; M273K, mutant opsin with the M273K mutation. "Rel. Diff. Abs." indicates relative difference absorption.

following hypothesis as the other alternative possibility. The M273K mutation occurred in the downstream M gene (the array was L-M*; *denotes the mutation), duplication of the M gene occurred (L-M*-M*), followed by non-homologous recombination (L/M* hybrid-M*) as shown in Fig. 3A. Duplication of the second gene was supported by the fact that (i) many color-normal individuals have multiple downstream M genes with the same nucleotide sequence[25] and (ii) the result that three (or more) downstream M genes had the same 11-bp deletion in a protanopia subject[26]. Case 2 had a genotype with two unique profiles; the M273K mutation was novel and present in both L and M genes.

Cases 3 and 4 showed large deletions of 62,934 bp including the LCR and 23,389 bp not including the LCR, respectively. The genotype of Case 3 was consistent with the known genotype of BCM. In Case 4, the first gene was obviously non-functional due to the absence of exons 2–6, but the downstream gene seemed to be functional, as no deleterious mutations were found in the promoter and exons, including their adjacent introns. The clinical phenotype of BCM in Case 4 indicated that the downstream gene was non-functional. The gene array revealed that the promoter of the second gene was directly connected to intron 1 of the first gene with absence of exons 2–6 (23,389 deletion) (Fig. 5). Although we cannot explain reasonable mechanisms underlying BCM in Case 4, the residual sequences (exon 1 and partial intron 1) of the first gene might impact on the promoter activity of the second gene, resulting in suppression of the second gene expression. The breakpoints of the deletion were within *Alu* elements in both cases, as in previously reported BCM cases[11]. Many reports have described deletions in the L/M gene array[4,8–12,17]. However, few studies have determined the exact breakpoints of the deletion at the nucleotide level[4,11,16]; the breakpoints have resulted from simple breakage and fusion[4,16] outside the repetitive sequence and simple breakage and fusion (and insertion) in *Alu* elements[11]. The breakpoints we determined differed from these; non-equal crossing-over occurred between the two *Alu* elements in the region of the same sequence (Figs 4 and 5).

The various haplotypes of five amino acid residues at positions 153 (L/M), 171 (V/I), 174 (A/V), 178 (I/V), and 180 (S/A) in exon 3 have been reported in subjects with normal color vision and subjects with color vision deficiencies[18,20]. The haplotypes have been roughly classified into four groups in terms of the magnitude of the

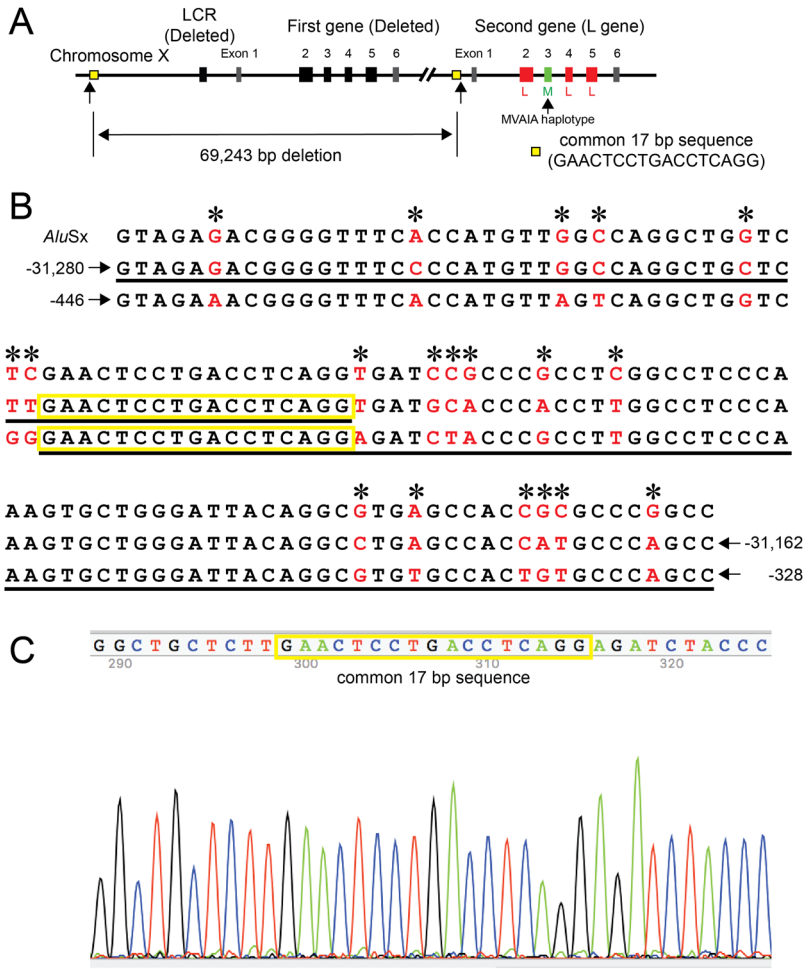


**Figure 4.** Genotype of Case 3. (**A**) Overview of the genotype of Case 3. Case 3 had a large deletion of 69,243 bp including the LCR and first gene. The remaining second gene was an L gene in which exons 2, 4, and 5 were L type. Exon 3 was M type with MVAIA haplotype. (**B**) Upper row shows the consensus sequence for the left monomer of the *Alu* element (complementary sequence of AluSx). Middle row shows a part of the upstream region of the first gene (number is from the cap site of the first gene). Lower row shows a part of the upstream region of the downstream gene (number is from the cap site of the downstream gene). The nucleotides differing among the regions are shown in red and by asterisks. The actual sequence obtained in Case 3 is underlined. (**C**) Partial sequence data around the breakpoint of the deletion. The breakpoint is somewhere in the common 17-bp sequence.

splicing defect[20]; highly deleterious haplotypes include LIAVA, MIAVA, and LVAVA; intermediately deleterious haplotypes include LIAIA, LIAVS, and MVAVA, minor deleterious haplotypes include LVAIA, LVAIS, MVAIA, and MVVVA, and the MVAIS haplotype exhibits no splicing defect. According to our data, the MVAIA and LVAIS haplotypes in Cases 1–3 would be expected to produce essentially correct splicing; however, the LVVVA haplotype in Case 4 could not be classified, as this haplotype was not described in the above-mentioned study[20]. We[18] and other researchers[27] examined the LVVVA haplotype using a mini-gene system and observed that the opsin mRNA retaining exon 3 was in clearly greater abundance than that lacking exon 3. Moreover, we reported one color-normal subject in which the exon 3 haplotype was LVVVA[18]. Based on these results, the BCM phenotype in Case 4 could not be ascribed to the LVVVA haplotype in the downstream gene.

In conclusion, we reported four novel and different genotypes in four unrelated Japanese patients with BCM. In two patients (Case 2 and Case 3), the genotypes were consistent with that of BCM (the same deleterious mutation in both opsin genes and deletion of the LCR), but in the other two cases (Case 1 and Case 4), the cause of BCM could not be clearly determined, although the patients exhibited very unique genotypes.

## Methods

The protocol for this study was approved by the Institutional Review Boards of The Jikei University of Medicine, Shiga University of Medical Science, Hamamatsu University School of Medicine, Kyoto University, Kindai University Faculty of Medicine, Nagoya University Graduate School of Medicine, Mie University Graduate School of Medicine, and Fujita Health University. The protocol adhered to the tenets of the Declaration of Helsinki, and informed consent was obtained from all participants.

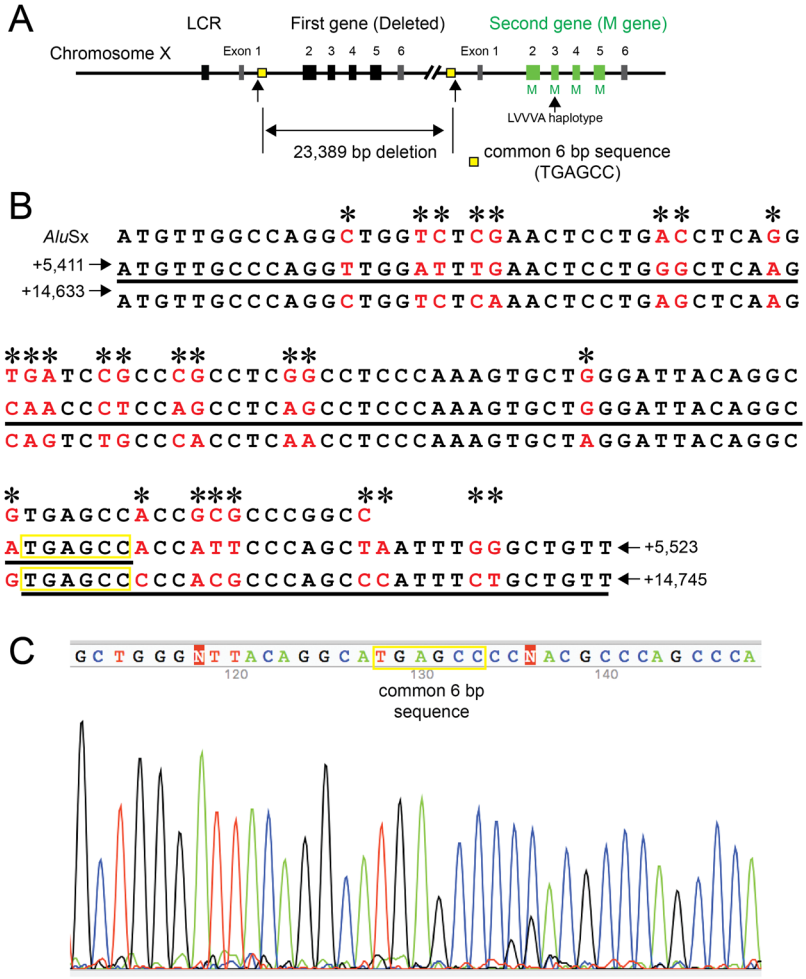


**Figure 5.** Genotype of Case 4. (**A**) Overview of the genotype of Case 4. Case 4 had a large deletion of 23,389 bp including exons 2–6 of the first gene. The intact LCR and second gene were present. The second gene had exons 2–5 of M type and exon 3 with LVVVA haplotype. (**B**) Upper row shows the consensus sequence for the left monomer of the *Alu* element (complementary sequence of AluSx). Middle row shows a part of intron 1 of the first gene (the number is from the 5′ splice site of intron 1). Lower row shows a part of the intergenic region (the number is from the stop codon in exon 6 of the first gene). The nucleotides differing among the regions are shown in red and by asterisks. The actual sequence obtained in Case 4 is underlined. (**C**) Partial sequence data around the breakpoint of the deletion. The breakpoint is somewhere in the common 6-bp sequence.

**Participants.** We recruited four unrelated Japanese male patients with BCM, whose diagnosis of BCM was made according to the findings reported[6,21]. In brief, all participants exhibited clinical findings of BCM, such as decreased visual acuity, severely impaired color discrimination in color vision tests, diminished L and M cone functions but retained S cone function in electroretinography, and an X-linked inheritance pattern in the family history. The detailed clinical findings are summarized in Supplementary Table S2.

**Molecular genetic analysis.** Genomic DNA was extracted from leucocytes in venous blood samples using a Gentra Puregene blood kit (Qiagen, Hilden, Germany). First and downstream genes of the L/M visual pigment gene array were separately amplified by long-range PCR using a QIAGEN LongRange PCR kit (Qiagen). Primers FG and E6R were used for the first gene, and primers DG and E6R were used for downstream gene(s) (Supplementary Table S1). The position of primers used in this study are schematically shown in Supplementary Fig. S3. Primer E6R was common to both genes, but primers FG and DG were designed specifically for the upstream region of each gene. The cycling parameters were: 93 °C for 3 min; 10 cycles of 93 °C for 30 s, 62 °C for 30 s, and 68 °C for 15 min; then 18 cycles of 93 °C for 30 s, 62 °C for 30 s, and 68 °C for 15 min, with a 20-s increment per cycle. The resulting PCR products were used as templates for sequencing the 'promoter + exon 1' and exons 2–6, including their adjacent introns, using a BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and ABI 3130*xl* sequencer (Thermo Fisher Scientific). The primer pairs used for sequencing are listed in Supplementary Table S1.

The LCR, which is located about 3.5 kb upstream of the first gene, was amplified by PCR using primers LCRF and LCRR, and its nucleotide sequence was then determined. When deletion was suspected, multiple sets of primers were designed for PCR to determine the exact deletion breakpoints.

Array gene number was determined by promoter analysis, as previously described[22]. Briefly, the promoters were amplified by PCR using Takara *Taq* DNA polymerase (Takara Bio Inc., Kusatu, Japan) and the primer pair common to both genes (Supplementary Table S1). The 169-bp PCR product was digested with *Cfr*10I (Takara) and analyzed by electrophoresis on a polyacrylamide gel. The first gene promoter was expected to yield two DNA fragments (137 bp and 32 bp), whereas the downstream gene promoter was expected to yield three DNA fragments (97 bp, 40 bp, and 32 bp). Gene number was estimated from the fluorescence ratio ([137 bp +97 bp]/137 bp). Gene Ladder Wide 2 was used as the size marker (Nippon Gene Co., Ltd., Toyama, Japan). Genomic DNAs from four subjects in which the L/M gene number was confirmed to be 1–4 by pulsed-field gel electrophoresis and Southern blotting[28] were used as control templates.

**Promoter assay.** To evaluate the activity of the promoter region, a luciferase reporter assay was performed as previously described[29]. Briefly, the promoter region of interest (−190 to +41 from the cap site of the gene) was amplified by PCR using Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) and restriction site–tagged primers (*Nhe*I site upstream and *Hin*dIII site downstream). The PCR product was cloned between the *Nhe*I and *Hin*dIII sites of a luciferase reporter plasmid, pGL4.17 (Promega Corp., Fitchburg, WI, USA). The resulting plasmid was transfected into WERI Rb1 cells using X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich, St. Louis, MO, USA). Two days after transfection, the cells were collected and lysed using PicaGene Cell Culture Lysis Reagent Luc (Wako Chemicals, Osaka, Japan). Luciferase activity was measured using a luminometer (Lumicounter NU-2500, Microtech Co., Ltd., Funahashi, Japan) and PicaGene Luminescence kit (Wako). Transfection efficiency was monitored in cells co-transfected with a ß-galactosidase–encoding plasmid (Promega).

**Analysis of M273K mutant pigments.** The cDNAs of human L and M pigments and respective hybrid pigment were tagged with the epitope sequence of the anti-bovine rhodopsin monoclonal antibody Rho1D4 (ETSQVAPA) at the C terminus and were inserted into the mammalian expression vector pMT4[30]. cDNAs harboring the mutation M273K were constructed using an In-Fusion cloning kit (Takara). For the spectral analysis, the plasmid DNA was transfected into HEK293 cells using the calcium-phosphate method[31]. After 2 days of incubation, the cells were collected by centrifugation and supplemented with 11-*cis*-retinal in buffer A (50 mM Hepes [pH 6.5] and 140 mM NaCl) to reconstitute the pigments. The reconstituted pigments were extracted using 0.75% CHAPS and 1 mg/mL phosphatidylcholine in buffer A. Absorption spectra of the extracted pigments were recorded at 0 °C using a Shimadzu UV-2450 spectrophotometer. The pigments were irradiated with orange light through an O58 cutoff filter (Toshiba, Tokyo, Japan) for 1 min. Difference spectra were calculated from spectra recorded before and after irradiation. For the western blot analysis, extracts from pigment-transfected or mock-transfected HEK293 cells were subjected to SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with Rho1D4. Immunoreactive proteins were detected by ECL Western Blotting Detection Reagents (GE Healthcare, United Kingdom) and visualized by a luminescent image analyzer (LAS 4000mini, GE Healthcare). For the fluorescence microscopy analysis, pigment-transfected or mock-transfected HEK293 cells were seeded onto poly-L-lysine coated coverslips. After 24 h incubation, cells were fixed in cooled methanol for 5 min. After fixation, cells were washed three times in PBS and were incubated overnight with primary antibody, Rho1D4, in 10% normal goat serum at room temperature. Cells were washed three times in PBS and were incubated for 1 h with secondary antibody, Alexa Fluor 488 anti-mouse IgG, in 10% normal goat serum at room temperature. Cells were washed a final time and were mounted onto slides with home-made aqueous mounting media consisting of glycerol and polyvinyl alcohol.

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## Author Contributions

M.I. and H.U. performed the molecular genetic analyses in all four cases. K.H. and Y.H. performed part of the molecular genetic analysis in case 3. T.Y. and Y.S. performed analysis of M273K mutant pigments. S.K., M.I, T.H., and H.U. interpreted the data and wrote the manuscript. H.I., H. Kurahashi, H. Kondo, H.O., M.O., and T.N. assisted with data interpretation. S.K., T.H., K.K., S.U., and M.K. performed ophthalmic examinations at each institution. T.H. and H.U. designed and supervised the study. All authors have read and approved the final manuscript.

## Additional Information

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# ヒトゲノム・遺伝子解析研究倫理審査委員会承認書

平成 26 年３月 28 日

倉　橋　浩　樹　殿

学長　　黒　澤　良　和　印

下記研究について、以下のとおり決定しましたので通知いたします。

記

1. 審査区分：継続
2. 研究責任者：総合医科学研究所・分子遺伝学・教授・倉橋浩樹
3. 個人情報分担管理者：医療科学部・ﾘﾊﾋﾞﾘﾃｰｼｮﾝ学科・矢野裕章
4. 研究課題名：「染色体ｺﾋﾟｰ数異常症に関する研究」
5. 研究期間：承認後～平成 31 年３月 31 日（予 定）
6. 共同研究機関の名称及び研究責任者氏名等：
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   - ☐ 逸脱に対する承認　　☐ 継続審査
   - ☐ 研究計画の変更　　☐ 新たな情報の入手
9. 審査結果：☑ 承認する（承認日：平成 26 年３月 28 日）
   ☐ 却下する　　☐ 修正の上で承認する
10. 決定理由及び修正の条件：
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次の職員の平成３０年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　難治性疾患等政策研究事業（難治性疾患政策研究事業）

２．研究課題名　染色体微細欠失重複症候群の包括的診療体制の構築

３．研究者名　（所属部局・職名）　藤田医科大学・総合医科学研究所・分子遺伝学・教授

（氏名・フリガナ）　倉橋浩樹・クラハシヒロキ

４．倫理審査の状況

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５．厚生労働分野の研究活動における不正行為への対応について

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| 研究倫理教育の受講状況 | 受講 ■　未受講 □ |

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（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和元年5月8日

国立保健医療科学院長　殿

機関名　埼玉県立小児医療センター

所属研究機関長　職　名　病院長

氏　名　小川　　潔　　印

次の職員の平成３０年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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３．研究者名　（所属部局・職名）　埼玉県立小児医療センター遺伝科・科長兼部長

（氏名・フリガナ）　大橋博文・オオハシヒロフミ

４．倫理審査の状況

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・分担研究者の所属する機関の長も作成すること。

平成３１年４月５日

国立保健医療科学院長　殿

地方独立行政法人神奈川県立病院機構
機関名　神奈川県立こども医療センター
所属研究機関長　職　名　総　長
氏　名　町田　治郎

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３．研究者名　（所属部局・職名）　遺伝科・部長

（氏名・フリガナ）　黒澤　健司（クロサワ　ケンジ）

４．倫理審査の状況

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| 遺伝子治療等臨床研究に関する指針 | □ | ■ | □ | | □ |
| 人を対象とする医学系研究に関する倫理指針（※3） | □ | ■ | □ | | □ |
| 厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針 | □ | ■ | □ | | □ |
| その他、該当する倫理指針があれば記入すること（指針の名称：　　） | □ | ■ | □ | | □ |

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

| | |
|---|---|
| 研究倫理教育の受講状況 | 受講 ■　未受講 □ |

６．利益相反の管理

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|---|---|
| 当研究機関におけるＣＯＩの管理に関する規定の策定 | 有 ■　無 □（無の場合はその理由：　　） |
| 当研究機関におけるＣＯＩ委員会設置の有無 | 有 ■　無 □（無の場合は委託先機関：　　） |
| 当研究に係るＣＯＩについての報告・審査の有無 | 有 ■　無 □（無の場合はその理由：　　） |
| 当研究に係るＣＯＩについての指導・管理の有無 | 有 □　無 ■（有の場合はその内容：　　） |

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

平成 31 年 2 月 15 日

国立保健医療科学院長　　殿

機関名　東京女子医科大学

所属研究機関長　職　名　学長

氏　名　吉岡　俊正　　印

次の職員の平成３０年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　難治性疾患等政策研究事業（難治性疾患政策研究事業）

２．研究課題名　染色体微細欠失重複症候群の包括的診療体制の構築

３．研究者名　（所属部局・職名）　遺伝子医療センター　・　教授

（氏名・フリガナ）　山本　俊至　・　ヤマモト　トシユキ

４．倫理審査の状況

| | 該当性の有無 | | 左記で該当がある場合のみ記入　(※1) | | |
|---|---|---|---|---|---|
| | 有 | 無 | 審査済み | 審査した機関 | 未審査(※2) |
| ヒトゲノム・遺伝子解析研究に関する倫理指針 | ■ | □ | ■ | 東京女子医科大学 | □ |
| 遺伝子治療等臨床研究に関する指針 | □ | ■ | □ | | □ |
| 人を対象とする医学系研究に関する倫理指針（※3） | □ | ■ | □ | | □ |
| 厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針 | □ | ■ | □ | | □ |
| その他、該当する倫理指針があれば記入すること（指針の名称：　　） | □ | ■ | □ | | □ |

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６．利益相反の管理

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| 当研究機関におけるＣＯＩ委員会設置の有無 | 有 ■　無 □（無の場合は委託先機関：　） |
| 当研究に係るＣＯＩについての報告・審査の有無 | 有 ■　無 □（無の場合はその理由　：　） |
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（留意事項）　・該当する□にチェックを入れること。

・分担研究者の所属する機関の長も作成すること。

平成３１年　３月２８日

国立保健医療科学院長　殿

機関名　国立大学法人信州大学

所属研究機関長　職　名　学　長

氏　名　濱　田　州　博　　印

次の職員の平成３０年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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２．研究課題名　染色体微細欠失重複症候群の包括的診療体制の構築

３．研究者名　（所属部局・職名）　医学部　遺伝医学教室・講師

（氏名・フリガナ）　涌井　敬子（ワクイ　ケイコ）

４．倫理審査の状況

| | 該当性の有無 | 左記で該当がある場合のみ記入（※1） | | |
|---|---|---|---|---|
| | 有　無 | 審査済み | 審査した機関 | 未審査（※2） |
| ヒトゲノム・遺伝子解析研究に関する倫理指針 | □　■ | □ | | □ |
| 遺伝子治療等臨床研究に関する指針 | □　■ | □ | | □ |
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| 厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針 | □　■ | □ | | □ |
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