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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

平成２８年度　総括・分担研究報告書

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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

研究代表者　倉橋　浩樹
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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構造異常症候群の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 30 疾患に関して、全国調査による国内患者の把握や、臨床診断基準、重症度判定基準の策定を実施する。昨年度に引き続き、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、ならびに診療情報の収集、チェックなどを行った。一方で、未診断症例に関しては、第一段階のスクリーニング検査としてのエクソーム解析も平行しておこない、その有用性の検討を行った。その結果、XHMM アルゴリズム（エクソーム隠れマルコフモデル法）による定量の有用性が確認されたが、感度は十分ではなく、二次検査の必要もあり、当面はマイクロアレイ染色体検査と併用する必要があることが確認された。

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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、器官発生に関わる転写因子や、ヒストン修飾因子、クロマチン因子などの転写調節因子が遺伝子の量的効果の影響を受けやすいため、先天性疾患の原因となることが多い。従来は G 分染法による染色体検査や FISH 法での診断が行われてきたが、マイクロアレイ染色体検査の普及により、CNV の検出感度が飛躍的に向上した。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。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、数多くの新規疾患も定義された。日本でも、すでに 5000 以上の患者データが蓄積されている。しかし、網羅的検査に特有の意義不明の CNV の解釈（variation of unknown significance: VUS）、偶発的所見（incidental findings: IF）や二次的所見（secondary findings: SF）への対応などの問題点が未解決であり、検査提供体制が整っているとはいえない。昨年、マイクロアレイ染色体検査が診断に必須な疾患が小児慢性特定疾患に追加されるなど、臨床的有用性は高いものの、高コストの問題があり、自費診療の中で一部の患者がその恩恵を被るにとどまる。また、近年は、多発奇形・発達遅滞の患者の原因の精査としては、次世代シーケンサーによるエク

ソーム解析の台頭もあり、十分な検査適応の指針が必要である。

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、多発奇形・発達遅滞の患者の原因の精査としてのマイクロアレイ染色体検査を診療の中でおこなってきた。本研究ではそれを継続する形で、3 年間を通じて、患者サンプルの収集とマイクロアレイ染色体検査を行う。各施設で合計年間 500 例ほどの解析を目標とする。そして、3年目には、代表的な30疾患(1年目に見直し、2 疾患を加え、合計 32 疾患）に関して、新たな臨床診断基準の作成、そして、個々の構造異常の発生メカニズムの解析を行うことを目標とする。これまでにリストの 30 疾患の多くには診療ガイドラインはなく、本研究は極めて有用な成果を創出する。

また、近年は、多発奇形・発達遅滞の患者の原因へのアプローチとしては、次世代シーケンサーによるエクソーム解析の有用性が確立した。このエクソーム解析はリード数を定量することで CNV を同定することが可能であり、マイクロアレイ染色体検査と同等のデータを創出することができる可能性があるので、多発奇形・発達遅滞の責任変異のスクリーニングにおいて第一選択となりうる。本研究では、多発奇形・発達遅滞の責任変異のスクリーニング法としてのマイクロアレイ染色体検査とエクソームの定量の感度や精度を比較し、その有用性を検討する。

B. 研究方法

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。とくに、リストの 32 疾患（資料１）に関しては診断未確定患者の発掘のために、診断につながる臨床情報を公開する。この調査は、日本小児遺伝学会（小崎健次郎理事長、本研究の研究協力者）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 解析を行う。各施設の合計として年間 500 例ほどの解析を目標とする。

研究代表者を含む各研究分担者の研究施設には、すでにマイクロアレイ染色体検査を行う設備が整っており、これまでに臨床検査として行ってきた十分な実績がある。その際、ダウン症候群などの染色体異数性による疾患のような、従来のG分染法が有用である疾患や、22q11 欠失症候群などのように疾患特異的 FISH 解析が第 1 選択になるような疾患を、表現型で除外できるように、染色体微細構造異常の診断のためのマイクロアレイ染色体検査の適応を決めるガイドラインを確立する。

多発奇形・発達遅滞の患者の原因の精査としてマイクロアレイ染色体検査による診断を進め、疾患責任 CNV が確定しない場合にはエクソーム解析へと進めた。一方で、症例によってはエクソーム解析を先行させ、その定量により疾患責任 CNV の候補を推定し、二次検査としてマイクロアレイ染色体検査、MLPA 法、qPCR 法により確認した。エクソームのデータはターゲットエクソーム解析、全エクソーム解析ともに、Log2 変換法や隠れマルコフモデル（exome hidden Markov model: XHMM）によるアルゴリズムなどを用いて観察研究として比較検討を行った。

（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ヒトゲノム・遺伝子解析研究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」藤田保健衛生大学・ヒトゲノム・遺伝子解析研究倫理審査委員会、平成２２年３月１２日承認、5 年後再承認、#86。)。

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。本研究の対象疾患である、染色体微細構造異常症 30 疾患の掘り起こしを行った。

（２）染色体微細構造異常症 30 疾患について

昨年度の第１回「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」班会議で対象疾患の見直しを行い、当初の 30 疾患に 9q34 欠失症候群と 1q 重複症候群の 2 疾患の追加を行い、対象疾患を 32 疾患に拡大した（資料１）。昨年度までに７つの疾患（1p36 欠失症候群、4p16 欠失症候群、5p サブテロメア欠失症候群、11p12-p14 欠失症候群、11/22 混合トリソミー、1q 重複症候群、9q34 欠失症候群）に関しては診断基準、重症度判定基準の作成へと進めることができている。

一方、残りの 25 疾患に関して、順次臨床診断基準の作成をおこなうことに関しては、十分な検討を行った。その結果、疾患によっては難病指定を目指すべき疾患と小児慢性特定疾患を目指すべき疾患があり、それらはすでに「先天異常症候群」や「常染色体異常症」という形で認定されている枠組みに紐付けすることを目指すが、個々の疾患の特性は、疾患によって大きく異なるので、診断基準策定は個別に対応する必要があることが確認された。

（３）エクソーム解析との関連性

定量的エクソーム解析と、マイクロアレイ染色体検査の比較検討を行った。その結果、全エクソーム解析、ターゲットエクソーム解析ともにエクソームのリード数の定量データは、Log2 変換法や XHMM によるアルゴリズムなどを用いることにより、対象疾患が限定されていて候補遺伝子がある場合には十分な CNV 検出感度を示し有効である。実際、エクソン数個の微細欠失重複も高感度に検出できる（資料２）。ただ、他の方法での確認のステップが必要であり、マイクロアレイ染色体検査と MLPA 法は確定検査として有用であった。一方で、エクソームデータの定量は、第一段階のスクリーニング検査としては、十分な検出感度は得られず、見つからなかった症例にはマイクロアレイ染色体検査の併用が必要であると思われた。

D. 考察

昨年度末の本研究班の班会議で、定量的エクソーム解析と、マイクロアレイ染色体検査の位置づけを検討する必要性を確認し、比較検討を開始した。本年度の研究成果により、

定量的エクソームの有用性は確認された。一方で、エクソーム解析での定量は対象疾患が限定されている場合には有効であるが、まだ第一段階のスクリーニング検査としてマイクロアレイ染色体検査が必要であることを確認した。また、定量的エクソームのデータには確認作業が必要であり、エクソームの二次検査としてのマイクロアレイ染色体検査の重要性も再認識された。今後、全ゲノムシーケンスの低コスト化が進むと、完全にエクソーム解析とマイクロアレイ染色体検査の融合検査としての位置づけとなる可能性が高いが、まだ時間がかかると思われる。それまでの期間は、エクソーム解析とマイクロアレイ染色体検査をうまく併用してゆくことで、可能な限り打ち漏らしを減らす工夫が重要となるであろう。

診断基準、重症度判定基準の作成の作業は実質的には小休止となった。「先天異常症候群」や「常染色体異常症」という大きな枠組みの中でのガイドラインの策定なども考慮されたが、個々の疾患の特性は、疾患によって大きく異なるので、最終的には、診断基準策定は個別に対応する必要があることが確認された。「国際標準に立脚した奇形症候群領域の診療指針に関する学際的・網羅的検討研究班（小崎班）」とも連携をとりながら、個別の疾患単位で進める必要がある。今後は、残りの対象疾患に関しても同様の検討を進めてゆく予定である。本年度の班会議で、残りの 25 疾患に関して、まず各班員が 1 疾患ずつを選定し、診断基準策定に向けて準備を開始することとした。倉橋浩樹（cat eye 症候群）、大橋博文（2q37 欠失症候群）、黒澤健司（21q サブテロメア欠失）、山本俊至（16p11.2 欠失/重複）、涌井敬子（Smith-Magenis 症候群）が担当となり、それぞれの疾患の診断基準策定に向けて準備を開始した。これらの疾患に関しては、診療ガイドラインの整備に向けて研究を進めてゆく。診療ガイドラインなどの研究成果は、ウェブ上で公開してゆく。また、指定難病認定に向けての準備、その後、これらの疾患の診断に必要な遺伝学的検査としてのマイクロアレイ染色体検査の保険収載などを視野に入れ、研究を進めていく。本研究の成果は、これらの疾患の患者や家族に対する支援、稀少難病の医療や福祉の向上に貢献することが期待される。

E. 結論

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構造異常症候群の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 32 疾患に関して、全国調査による国内患者の把握や、臨床診断基準、重症度判定基準の策定を開始し、7 つの疾患に関しては診断基準、重症度判定基準の作成を行うことができた。次年度以降も、残りの対象疾患に関して、研究代表者を含め各班員が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、ならびに診療情報の収集、チェックなどを行い、同様の検討を進めてゆく。一方で、エクソーム解析の定量とマイクロアレイ染色体検査との比較検討を行った。エクソーム解析の定量の有用性が明らかとなったが、対象疾患が明らかでない患者のスクリーニング検査や、エクソーム解析の定量の二次検査としてマイクロアレイ染色体検査の重要性が再確認された。

F. 健康危険情報

特になし。

G. 研究発表

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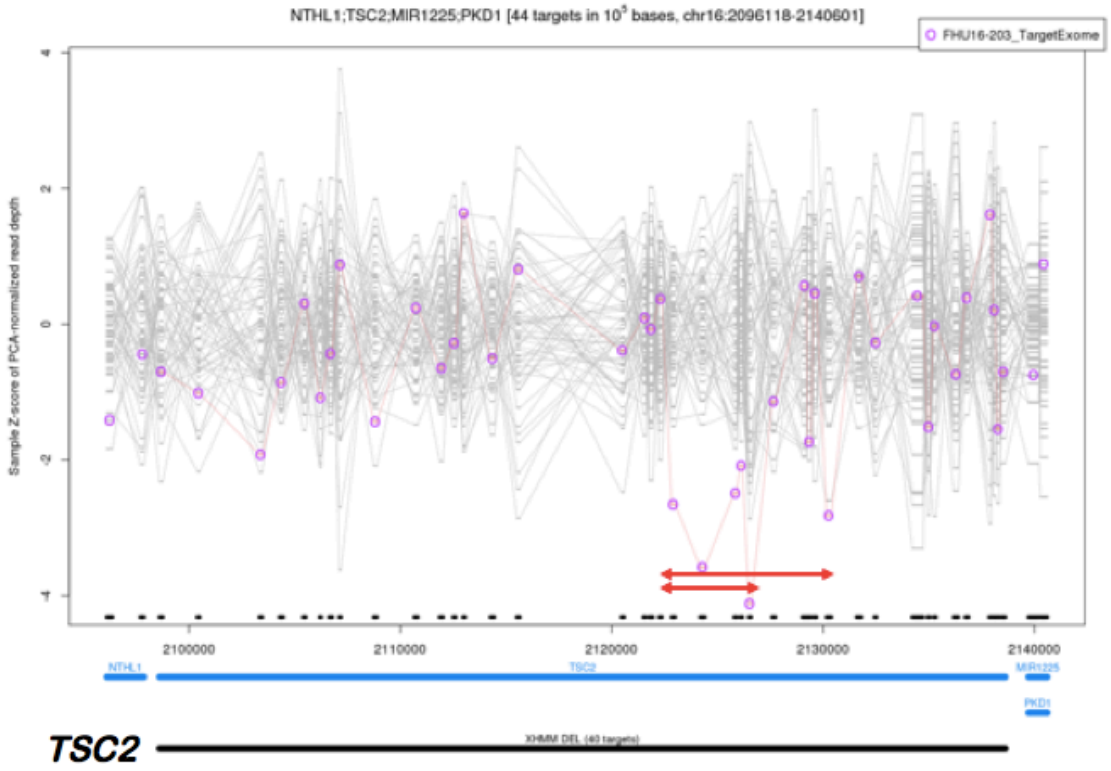
H. 知的財産権の出願・登録状況
特になし。

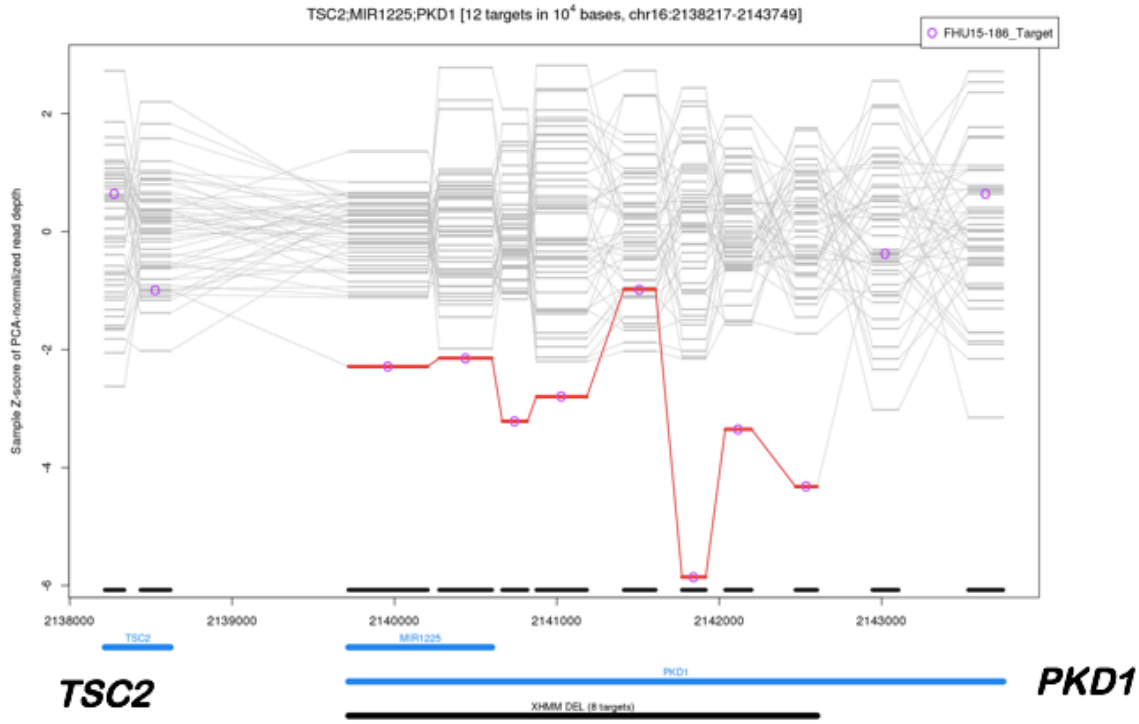
（資料１）

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

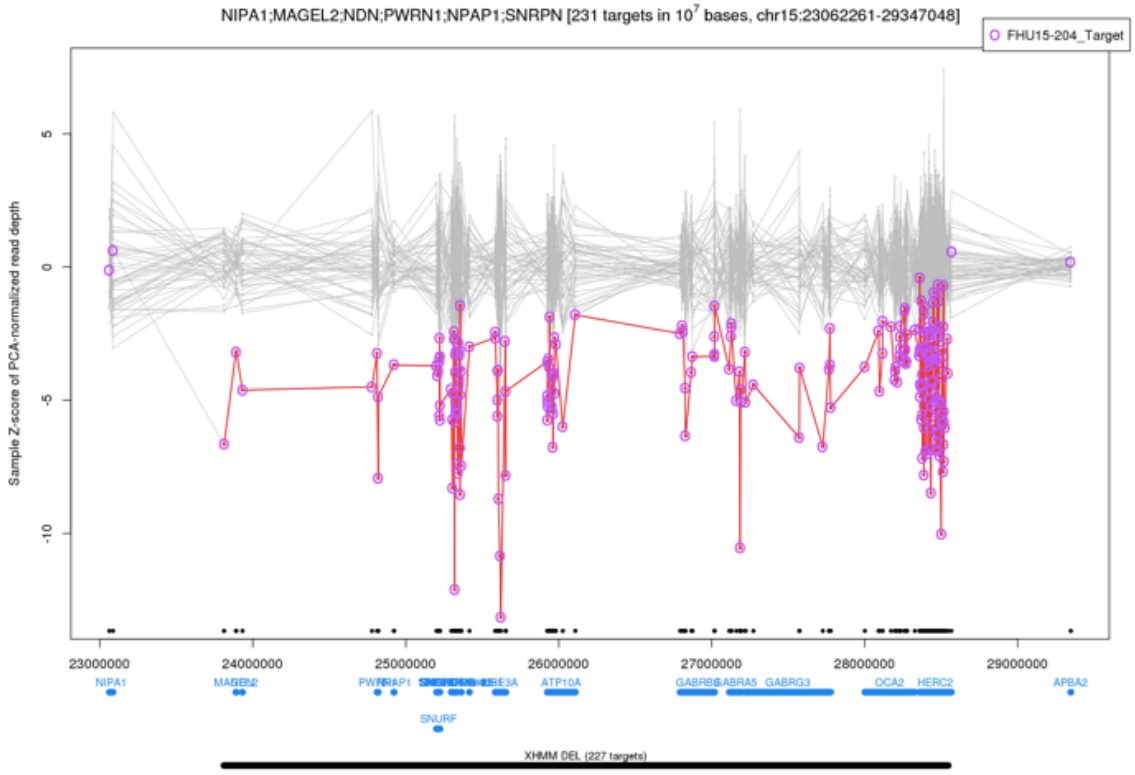
	対象疾患リスト	担当
1	1p36欠失症候群	山本俊至
2	1q44欠失症候群	大橋博文
3	2p15-p16.1欠失症候群	大橋博文
4	Feingold症候群(2p24.3欠失)	涌井敬子
5	2q23.1欠失症候群(*MBD5*)	涌井敬子
6	2q24.2-q24.3欠失/重複症候群(*SCN1A*)	山本俊至
7	2q32.1-q33.3欠失/重複症候群(*SATB2*)	黒澤健司
8	2q37欠失症候群	大橋博文
9	3p21.31欠失症候群	大橋博文
10	4p16欠失(Wolf-Hirschhorn症候群)	涌井敬子
11	Cri-du-chat症候群(5pサブテロメア欠失)	涌井敬子
12	5q14.3欠失症候群(*MEF2C*)	山本俊至
13	5q31欠失症候群	山本俊至
14	8pサブテロメア欠失	黒澤健司
15	Langer-Giedion症候群(8q24.11欠失)	倉橋浩樹
16	11p12-p14欠失症候群	山本俊至
17	Jacobsen症候群(11qサブテロメア欠失)	倉橋浩樹
18	16p11.2欠失/重複症候群	山本俊至
19	Miller-Dieker症候群(17pサブテロメア欠失)	黒澤健司
20	17p13.1欠失症候群(*GABARAP*)	黒澤健司
21	Smith-Magenis症候群(17p11.2欠失)	黒澤健司
22	Potocki-Lupski症候群(17p11.2重複)	涌井敬子
23	21qサブテロメア欠失症候群	黒澤健司
24	Emanuel症候群(11/22混合トリソミー)	倉橋浩樹
25	22q11.2重複症候群	倉橋浩樹
26	Cat eye症候群(22q11テトラソミー)	倉橋浩樹
27	Phelan-McDermid症候群(22q13欠失)	大橋博文
28	Xp11.3-p11.4欠失(*MAOA*, *MAOB*, *CASK*)	涌井敬子
29	Xq11.1欠失症候群(*ARHGEF9*)	山本俊至
30	MECP2重複症候群(Xq28重複)	倉橋浩樹
31	9q34欠失症候群	黒澤健司
32	1q重複症候群	涌井敬子

（資料２）

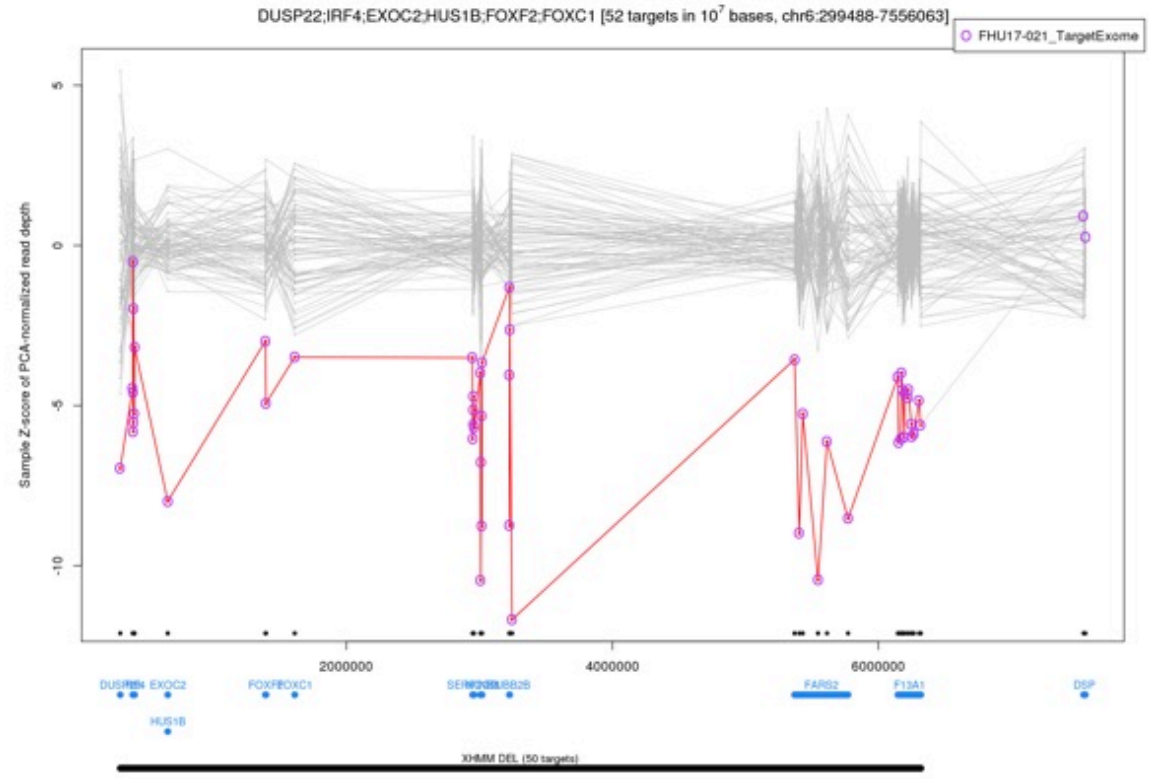




エクソン単位の欠失・重複


NIPA1;MAGEL2;NDN;PWRN1;NPAP1;SNRPN [231 targets in 10^7 bases, chr15:23062261-29347048]
FHU15-204_Target
Sample Z-score of PCA-normalized read depth
5
0
-5
-10
23000000
24000000
25000000
26000000
27000000
28000000
29000000
NIPA1
ATP10A
GABRB3
GABRA5
GABRG3
OCA2
HERC2
APBA2
SNURF
XHMM DEL (227 targets)

UBE3A


DUSP22;IRF4;EXOC2;HUS1B;FOXF2;FOXC1 [52 targets in 10^7 bases, chr6:299488-7556063]
FHU17-021_TargetExome
Sample Z-score of PCA-normalized read depth
5
0
-5
-10
2000000
4000000
6000000
EXOC2
FOXC1
HUS1B
FARS2
F13A1
DSP
XHMM DEL (50 targets)

FOXC1

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

マイクロアレイ染色体検査の臨床運用：常染色体劣性遺伝病の診断経験も含めて

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

１）本研究班での昨年度からの継続研究として、本年度（平成 28 年度）もマイクロアレイ染色体検査の臨床運用を進めた。平成 28 年 1 月～同年 12 月までの期間の症例でマイクロアレイ染色体検査を施行したのは 128 例だった。当センター遺伝科のこの期間の初診患者の 34%がマイクロアレイ解析の対象となっていた。その内分けは、診断不明の先天異常（multiple congenital anomalies; MCA を含む先天異常）をもつ児が 116 例、その他、既に検出した染色体構造異常等の精密診断等が 12 例だった。本研究班がターゲットとする前者の 116 例中、17 例（14.7%）で診断を得た。２）サラ病（Salla disease: MIM#604369）のマイクロアレイ染色体検査による診断例。サラ病は先天性大脳白質形成不全を特徴とする疾患で、6q13 領域に存在する SLC17A5 が責任遺伝子である。症例は３才男児。主訴は発達遅滞と髄鞘化遅延。マイクロアレイ染色体検査で、6q13 領域の約 240Kb のコピー数低下（欠失）を認めた（74,258,424-74,498,173）x1）。この当該 CNV はの原因遺伝子である SLC17A5 遺伝子全長を含んでいた。本遺伝性疾患は劣性遺伝形式であり、この片アレル遺伝子欠失のみでは発症しないが、非欠失アレル遺伝子に変異が存在する可能性を想定した。TruSight One(Illumina)を用いた疾患関連遺伝子（最大 4813）ターゲット領域（hg19）解析の結果、同遺伝子に c.116G>A:p.Arg39His（hemizygote）変異を認め、遺伝学的確定診断に至った。３）先天異常症候群の診断後の患者家族支援としての疾患集団外来の開催も継続して進めた。2016 年５月～2016 年 11 月までの間に、計 10 回の外来を開催した。参加家族数は 3～21 家族（平均 11.5 家族)、合計 115 家族であった。他県からの参加家族も平均 3.8 家族あった。

研究協力者

清水　健司　（埼玉県立小児医療センター遺伝科）

Ａ．研究目的

G 分染による染色体検査の限界を克服するゲノム全体を俯瞰する網羅的検査として、マイクロアレイ染色体検査の運用が期待されている。本研究班における当分担研究者の研究として、地域の小児専門医療施設である当分担研究者の所属する埼玉県立小児医療センターにおけるマイクロアレイ染色体検査の実際

の臨床的な運用に関して、本年度は次の３点について報告する。1）本年度（平成 28 年度）のマイクロアレイ染色体検査の実績解析、2）マイクロアレイ解析で診断し得た常染色体劣性遺伝病の診断例、３）先天異常症候群の診断後の患者家族支援としての疾患集団外来の開催、である。

Ｂ．研究方法

１．マイクロアレイ染色体検査の実施実績

平成 28 年 4 月～同年 12 月までの間に、埼玉県立小児医療センター遺伝科外来を受診した患児に行ったマイクロアレイ染色体検査実績の検討を行った。

２．マイクロアレイ染色体検査による常染色体劣性遺伝病の診断

マイクロアレイ染色体検査によって検出された欠失領域に存在する臨床症状と合致する常染色体劣性遺伝病の原因遺伝子に着目し、非欠失アレル遺伝子の変異同定による遺伝学的確定診断を行う。

3. 先天異常症候群の診断後の患者家族支援としての疾患集団外来の開催

本年度も、比較的頻度が高く受診患者数が多い、新たに診断を受けた患児がいる、集団外来開催を家族が希望している、共有すべき重要な情報や新たな知見がある、臨床研究の推進と関連がある、などを基準に疾患を選定し、集団外来を開催した。

(倫理面への配慮)

マイクロアレイ染色体検査については、関連ガイドラインを遵守して行う。また、マイクロアレイ染色体検査施行に関しては施設の倫理委員会で承認済みである。

Ｃ．研究結果

1．マイクロアレイ染色体検査の実施実績

平成28年１月～同年12月までの期間の症例でマイクロアレイ染色体検査を施行した例は128例だった。当センター遺伝科のこの期間の初診患者数は373例であったので、その34%がマイクロアレイ解析の対象となったことになる。その内分けは、診断不明の先天異常（multiple congenital anomalies; MCAを含む先天異常）をもつ児が116例、その他、既に検出した染色体構造異常等の精密診断等が12例、だった。本研究班がターゲットとする前者の116例中、17例（14.7%）で診断を得た。これら17例のマイクロアレイ解析結果とその解釈を表1に示す。

2. 常染色体劣性遺伝性疾患のマイクロアレイ解析による診断

【症例】

３才男児。主訴は発達遅滞と髄鞘化遅延。経過は、10ヶ月健診で下肢の筋緊張亢進と発達の遅れを指摘され、1才7ヶ月時のMRIで高度の髄鞘化遅延、白質容量減少、脳梁菲薄化、小脳軽度萎縮があった。診察上体幹優位の筋緊張低下があり、深部腱反射は下肢で亢進していた。染色体検査は異常がなかった。

【マイクロアレイ解析】

表1．マイクロアレイ染色体検査診断実績（平成28年1～12月）

①Xp22.33(169,796-595,143)/Yp11.22(119,796-545,143) x3 [Likely pathogenic]	Xp22.33/Yp11.22 の偽常染色体領域における約425Kbの重複を認めました。切断点がSHOX遺伝子領域内に存在しており、SHOX partial duplication が考えられ、確証のためMLPA法施行したところ、SHOX遺伝子内Ex1-3領域の部分重複を認め、アレイ結果との整合性を認めました。この場合低身長やLeri-Weill症候群等との関連が報告されています。[J Clin Endocrinol Metab. 2011 Feb;96(2):E404-12][Genet Med. 2010 Oct;12(10):634-40]
①6p25.3p25.2(919,175-3,501,166)x1 [Pathogenic]	①6p25.3-p25.2 領域における約2.58Mbの中間部微細欠失を認めました。上記は眼や脳などの早期発生に重要な転写因子であるFOXC1遺伝子を包含し、6p25 deletion syndromeとして認識されています。主要所見として、虹彩異常や緑内障、難聴、中枢神経異常（脳室拡大やDandy-Walker variant）、骨格所見、発達遅滞等の報告がありますが、微細欠失で知的障害のない例の報告もあり表現型の幅も認識されています。[Eur J Med Genet 58(2015)310-8]
①22q11.21(18,706,001-21,505,417)x3 [Pathogenic/Clinically associated]	①22q11.21領域の約2.62Mbの重複を認めました。上記重複は22q11.2重複症候群(MIM #608363)として報告がありますが、健康な例から、低身長・学習障害・発達遅滞を呈する例まで表現型の幅が広いことが知られており、児の表現型への寄与については慎重な判断が必要です。また健康な片親に同様の重複を認めることがあります[22q11.2 Duplication. 2009 Feb 17 (Updated 2013 Nov 21). GeneReviews?]
①Xp22.33p11.21(332,683-57,521,192)x1～2, [Pathogenic] ②Xq21.1q28(78,055,922-155,226,944)x1～2 [Pathogenic]	①② Xp22.33 (端部)-p11.21 領域における約57.2Mbのコピー数低下とXq21.1-q28(端部)領域における約77.2Mbのコピー数低下を同時に認めており、これら以外のセントロメア周囲の正常コピー数領域と合わせてr(X)の存在を示唆します。しかしながら、コピー数低下領域のlog2値はそれぞれ-0.52/-0.51と正常コピー数とのモザイク46,X,r(X) /46,XXを示唆します。 G分染法結果との違いは、解析する血液細胞の種類の違いや培養過程の有無に起因する可能性があります。
①17p13.3(751,195-1,732,318)x1[Pathogenic]	①17p13.3領域の約981Kbの中間部微細欠失を認めました。当該欠失はPAFAH1B1遺伝子より遠位に切断点があり、この場合Miller-Dieker症候群で認める滑脳症の合併はありませんが、欠失範囲にYWHAE遺伝子を含むことにより、出生前後の中等度～重度の成長障害や軽度～中等度の発達遅滞、顔貌所見の原因となります。また時に中枢神経形態異常やその他身体合併症の報告も見られます。成長ホルモンの効果があった報告も認めます。[Am J Med Genet 158A:2347-52,2012/ Eur J Med Genet 53(5):303-8,2010]
①Xp22.31(6,552,712-8,097,511)×0 [Pathogenic]	①Xp22.31領域の約1.55Mbの欠失を認めました(Pathogenic)。上記欠失範囲にはSTS遺伝子を含んでおり、X連鎖性魚鱗癬(MIM#308100)の原因となります。また隣接する欠失遺伝子PNPLA4やVCX遺伝子も含んでおり、発達遅滞や注意欠陥多動/自閉傾向に寄与する可能性があります[Gene. 2013 Sep 25;527(2):578-83]。しかしながら本児で認める身体所見すべての原因となっているかどうかは慎重な判断が必要です。
①7p22.2p22.1(2,956,450-5,640,790)x3 [Pathogenic]	①7p22.2-p22.1領域における約2.7Mbのコピー数増加（重複）を認めました。上記は7p22.1微細重複症候群として近年認識されている領域と重なっており、主要候補遺伝子であるACTBを含んでおります。本症候群では、大頭、目立つ前頭部、大泉門開大などの頭蓋顔面所見、発達遅滞（言語遅滞）、骨格所見（脊椎彎曲等）の主徴徴候に加え、心疾患や泌尿器所見の報告も認め、本症例の表現型と矛盾しませんが、本症例で認める出生前後の重度成長障害は非典型的です。軽度所見を有する親からのinheritanceの報告もあり、表現型の幅には注意が必要です。
①18q22.1q23(62,002,702-78,012,829)×1 [Pathogenic]	①18q22.1-q23領域の約16.0Mbの欠失を認めました。当該領域の欠失は18qモノソミー症候群（MIM#601808）のcritical region を含んでおり、髄鞘化遅延、外耳道閉鎖（狭窄）、難聴、足部異常（垂直距骨、内反足、扁平足）、先天性心疾患、口唇口蓋裂、腎臓異常、成長ホルモン分泌不全などの合併症の責任領域もしくは量感受性（ハプロ不全）遺伝子（NETO1,CYB5A,TSHZ1,MBP,NFATC1）を含んでおります[Am J Med Genet 169C:265-280,2015][Hum Genet 133:199-209,2014]
①22q11.21q11.23(21,505,358-23,654,222)×1 [Pathogenic]	①22q11.21-q11.23 領域における約2.1Mbの欠失を認めました。 上記はdistal chromosome 22q11.2 deletion syndrome (MIM#611867) で報告のある欠失であり、LCR22-DとLCR22-Fの間の非アレル間相同組み換えで起こります。この領域の欠失の主要所見として、早産、低出生体重、運動発達遅滞、軽度から中等度の知的障害、行動面の問題、先天性心疾患などの報告があり、本児の表現型に矛盾しません。一方で横紋筋腫瘍のハイリスクとなるLCR22-FとLCR22-G間のSMARCB1遺伝子を含む欠失領域は含んでおりません。[Genet Med. 2014 Jan;16(1):92-100]
①15q11.2(22,765,628-23,146,132)x3 [Pathogenic-Susceptibility Locus]	①15q11.2領域の約381Kbの微細重複を認めました。本欠失範囲はBP1-BP2間のLow Copy Repeatsに惹起された4遺伝子(TUBGCP5,CYFIP1,NIPA1,NIPA2)を含む共通領域の重複であり、15q11.2 microduplicationとして認識されている領域です。臨床所見においては、発達言語遅滞、行動や感情の問題（自閉症スペクトラムや注意欠陥多動）の報告が中心ですが、表現型の幅は広く、また症状がない方も多く不完全浸透と考えられており、健康な片親から受け継いでいる可能性があります。疾患感受性領域としての評価です[Hum Genet；130(4):517-28,2011]。
①3q22.3q23(136,002,607-141,953,305)x1 [Pathogenic]	①3q22.3-q23領域における約5.95Mbの中間部欠失を認めました。上記欠失領域内には瞼裂狭小症候群の原因遺伝子であるFOXL2全長を含んでおり、児の表現型にも合致します。また隣接遺伝子群の欠失も重なるため、低身長、発達遅滞や小頭所見にも寄与していると考えられます。[M.H.de Ru et al. Am J Med Genet 137A:81-7,2005] [S.J. Dean et al.Pediatric Neurology 50:636-9,2014]
①22q13.3q13.33(45,721,068-51,224,252)x1[Pathogenic]	①22q13.31-q13.33(端部)領域における約5.5Mbの欠失を認めました。本欠失は22q13.3欠失症候群のcritical region(SHANK3遺伝子等)を含む44個のOMIM遺伝子を含んでおり、既知の報告と合わせ、病原性と考えられます。主要徴候として、発達遅滞、重度の言語遅滞、自閉症のスペクトラム、時にけいれん等の身体合併症の報告もあります。またリング不安定性としての共通所見である成長障害(22q13.3欠失とは反対所見)、小頭などの原因にもなりえます。 [Am J Med Genet 164A:1659-65,2013]
①1q21.1q21.2(146,507,518-147,824,207)x3 [Pathogenic-Suceptibility Locus]	①1q21.1-q21.2領域における約1.3Mbの微細重複を認めました。疾患感受性領域として認識されており、非アレル間相同組み換えにより、TAR領域を含まない共通領域の重複サイズに一致します。本重複で起こりうる表現型は大頭、筋緊張低下、発達遅滞、知的障害、自閉症巣ペクトラム等で、本症例の表現型に矛盾しないと考えられます。しかしながら表現型の幅は広く、症状がない方も多く不完全浸透と考えられており、健康な片親から受け継いでいる可能性があるため、遺伝カウンセリング上注意が必要です。[Genetics in Medicine2016.18.341-9][Unique: rare chromosome disorder support group,2013]
①4q13.2q21.21(70,303,326-79,209,282)x1 [Pathogenic]	①4q13.2-q21.21領域における約8.9Mbの中間部欠失を認めました。全く同一の欠失領域の報告はありませんが、本領域と重なる複数の4q近位部欠失の病原性報告があります。共通の表現型として、発達遅滞・言語遅滞、成長障害、大頭、時にけいれん（20%程度）が報告されており、児の表現型にも合致しています。内臓奇形や中枢奇形の報告はほとんどないことも矛盾しません。一方、Pibaldismの原因となる4q12領域のKit遺伝子は欠失範囲に含まれていませんが、典型的な色素脱失領域はないものの低色素の薄い毛髪を認めます。[Unique 2006/ Am J Med Genet134A:226-8,2005/ Am J Med Genet 167A:231-7,2015]
①7q31.2q32.3(116,753,232-130,519,488)x1 [Pathogenic]	①7q31.2q32.3領域における約13.8Mbの中間部欠失を認めました。全く同一の欠失領域の報告はありませんが、本領域と重なる複数の7q31-32領域欠失の病原性報告があります。共通の表現型として、発達遅滞や言語遅滞、自閉症スペクトラムがあります。心疾患、けいれん、難聴など身体合併症の報告もありますが、欠失サイズの多様性とともに表現型の幅もあります。また7q32.2のインプリンティング領域（MEST遺伝子）も欠失範囲に含まれており、父由来欠失ではRussell-Silver症候群類似所見の報告があります。本症例はGolderhar症候群の表現型に合致しており、7q31-q32欠失との関連報告は今までありません。[Unique 2011/ Am J Med Genet 172C:102-108,2016/Am J Med Genet 170A:743-749,2015]
①22q13.33(51,107,409-51,224,202)x1 [Pathogenic]	①22q13.33領域における約116.8Kbのコピー数低下を認めました。Log2値や欠失サイズからは、他方法（qPCRやMLPA）による確証が望まれます。確証された場合、本欠失領域は22q13.3末端のSHANK3, ACR,RABL2B遺伝子を含んでおり、Phelan-Macdermid syndrome（22q13.3 deletion syndrome） の責任領域と合致し、主として発達遅滞、言語遅滞の原因となります[GeneReviews]。一方本症例は上記症候群の欠失の中では最も小さな部類で表現型も軽度な傾向が報告されていますが、GERD, けいれん、心臓、腎臓疾患など注意すべき合併症の評価とフォローは大切です。後述する重複領域が表現型の重症度に寄与する可能性はありますが、明らかではありません。
①Xp22.33(477,652-596,208)x3 [Pathogenic-Susceptibility Locus /annotation]	Xp22.33領域（偽常染色体領域:PAR1）における172kbのコピー数上昇を認め、確証のため施行したMLPA法においても同一領域の重複と判断されました。 当該重複はSHOX遺伝子の一部とSHOX遺伝子調節領域の一部を含んでおり、過去の報告においては、低身長、Leri-Weil病、ミュラー管形成不全、自閉症スペクトラムの各疾患で有意にSHOX領域の重複を認めていますが、重複範囲、表現型ともに幅があり、病原性としての議論もあります。本症例の表現型（低身長）からは当該領域の重複が病原性に関与している可能性が考えられます。[Eur J Hum Genet.20:125-7,2011/Genet Med. 12:634-40,2010/ J Med Genet.53:536-47,2016]

Agilent human genome CGH+SNP180K / Agilent CytoGenomics 2.9 / UCSC hg19/GRCh37による解析の結果、6q13領域の約240Kbのコピー数低下（欠失）を認めた（74,258,424-74,498,173）x1）。この当該CNVはサラ病（Salla disease: MIM#604369）の原因遺伝子であるSLC17A5遺伝子全長を含んでいた。本遺伝性疾患は、劣性遺伝形式であり、この片アレル遺伝子欠失のみでは発症しないので、単に本疾患の保因者診断がなされたに過ぎないと判断すべきところである。しかしながら、サラ病は先天性大脳白質形成不全の原因疾患の１つであり、患児の中枢神経所見に合致しているため、この遺伝子欠失のサラ病発症への関与として、非欠失アレルの遺伝子内に変異が存在する可能性を想定した。

TruSight One(Illumina)を用いた疾患関連遺伝子（最大4813）ターゲット領域（hg19）解析の結果、SLC17A5遺伝子に c.116G>A:p.Arg39His（hemizygote）変異を認めた。この変異はデータベース[HGMD,dbSNP138(Common,Flagged)]に報告がないものの、以下の理由から“Likely Pathogenic”と判断した。すなわち、１）一般集団データベースにない (HGVD:0/2,202) か、有意に低い(ExAC:1/121,272) アレル頻度、２）疾患特異的な表現型、３）マイクロアレイ検査にてもう一方のアレルに母由来の遺伝子全長の欠失を認めていること（本変異は父由来）、４）In-Silico解析で病原性との評価（SIFT/Polyphen2/Mutation taster）、５）LOVDデータベースでprobably pathogenicの評価。

3. 先天異常症候群の診断後の患者家族支援としての疾患集団外来の開催

2016 年５月～2016 年 11 月までの間に、計 10 回の外来を開催した。参加家族数は 3～21 家族（平均 11.5 家族）、合計 115 家族であった。他県からの参加家族も平均 3.8 家族あった（表 2）。

Ｄ．考察

マイクロアレイ染色体検査で同定された欠失領域に存在する単一遺伝性疾患の原因遺伝子のうち、通常注目するのはハプロ不全で発症する疾患である。常染色体劣性遺伝性疾患の場合は、一方のアレル（遺伝子）が染色体欠失領域に含まれていても、他方の遺伝子が機能していれば発症しない（保因者）。しかし、非欠失アレル（遺伝子）に変異が存在している場合には、両遺伝子ともに機能を障害され発症する（染色体欠失による劣性遺伝病の顕在化）。このような発症は例外的事象ではあるが、患者の臨床症状から可能性が考えられる場合には劣性遺伝病であっても欠失領域の責任遺伝子に注目して、非欠失アレル遺伝子の変異解析を行うことが重要である。マイクロアレイ染色体検査は優れた網羅的スクリーニング検査であるが、質の高い臨床評価が診断精度に大きく貢献することを示す症例と考えた。

Ｅ．結論

１）マイクロアレイ染色体検査の１年間の検査実施実績検討、２）マイクロアレイ染色体検査による常染色体劣性遺伝病の診断例の

表2. 2016年度開催 先天異常症候群集団外来開催状況

日付	疾患名	テーマ	情報提供担当者	家族数	参加人数	他県よりの家族数
2016/5/10	頭蓋・前額・鼻症候群	疾患の概要	遺伝科医	3	8	0
2016/6/15	5pモノソミー症候群	疾患の概要	遺伝科医	6	14	1
2016/7/20	4pモノソミー症候群	疾患の概要	遺伝科医	3	8	1
2016/7/27	9pトリソミー・テトラソミー症候群	疾患の概要	遺伝科医	7	23	1
2016/8/25	CHARGE症候群	福祉制度と社会資源	ソーシャルワーカー	12	31	1
2016/8/31	22q11.2欠失症候群	ことばのはなし	言語聴覚士	11	25	1
2016/9/21	カブキ症候群	疾患概要	遺伝科医	19	39	12
2016/9/28	ソトス症候群	先輩のご家族の話	母親	21	42	8
2016/10/12	ウィリアムズ症候群	発達	作業療法士	19	36	9
2016/11/24	プラダーウィリー症候群	不適応行動とその対応	精神科医	14	22	4
			2016年度合計	115	248	38
			2016年度平均	11.5	24.8	3.8

経験、３）先天異常症候群の疾患集団外来の開催を行った。

Ｆ．研究発表

（発表誌名巻号・頁・発行年等も記入）

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2. 学会発表

なし

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（難治性疾患政策研究事業））
分担研究報告書

マイクロアレイ染色体検査も含めた診療で用いる包括的遺伝学的検査の説明書・同意書

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研究要旨

保険収載された遺伝学的検査の拡大により、次世代シーケンスの臨床応用が本格化しつつある。網羅的ゲノム解析手法の一つであるエクソーム解析のデータを利用して、CNV 検出を試み、マイクロアレイ染色体検査以上に高い検出感度で疾患特異的 CNV が検出可能であることを確認した。このことから、網羅的ゲノム解析を遺伝学的検査として臨床に導入するためには、マイクロアレイ染色体検査とエクソーム解析を別次元の検査として説明するのではなく、同一次元で説明する方が妥当と考えられた。この事実をもとに、説明同意書の作成を試みた。今後、さらに解析手法が高度化するにしたがって再度の見直しも必要と考えられた。

Ａ．研究目的

平成 28 年度より保険収載された遺伝学的検査は 72 疾患群に増え、遺伝学的検査が本格的に医療に導入されつつある。遺伝学的検査の難しさは、疾患ごとに解析手法や原理が異なり、さらに結果の表記の様式なども大きく異なる点があげられる。解釈に至っては、臨床症状との関連から下すべきであり、検査担当者と検体提出者の間での臨床症状に関する情報の連携がなければ成立しない。次世代シーケンサーの登場により、その技術が臨床に応用され、遺伝医療が大きく進展することが予想されている。上述の 72 疾患群の検査でも、次世代シーケンサーの使用が期待されている疾患が複数含まれている。次世代シーケンサーの利点は、単に網羅的であるだけでなく、deep sequencing によりモザイクを正確に検出することが可能であることや、read depth からゲノムコピー数の変化（CNV）も評価可能であることがあげられる。以上を考慮すると、遺伝性疾患の診断において、網羅的なゲノム解析を用いる場合、その方法としてマイクロアレイ染色体検査と次世代シーケンス解析を別次元で説明し、それぞれに対して同意書を得る従来の方法から、網羅的解析として一元的に説明し、同意を得ることが現実的となりつつある。

こども医療センターでは 2010 年からマイクロアレイ染色体を、2012 年から次世代シーケンス解析を臨床診断に導入したが、従来のような 2 つの解析方法に対して別々の説明を行ってきた。しかし実際には、次世代シーケンスによる臨床エクソーム（メン

デル遺伝病パネル）解析データの CNV 変換で診断に至ったケースを複数経験している。今回、CNV（copy number variant）評価も SNV（single nucleotide variant）評価も可能な網羅的ゲノム解析を診療で用いるための説明同意の在り方を具体例を挙げて検討した。

Ｂ．研究方法

末梢血液リンパ球を用いた通常の染色体分析は、標準的方法によった。次世代シーケンスシステムは、Illumina 社 MiSeq、解析プラットフォームは主に TruSight One Sequencing Panel を用いた。ライブラリー調整およびシーケンス解析は規定のプロトコールに従った。データ解析は、BWA、GATK からなる当センター構築のパイプラインを用い、参照データベースは施設内および公開データベースを利用した。マイクロアレイ CGH は、Agilent 社製マイクロアレイシステムを用い、アレイは SurePrint G3 Human CGHMicroarray kit 8x60K を用いた。解析手順は、Agilent 社による標準プロトコールに準じて進めた。得られたデータの解析は Agilent Workbench ないしは CytoGenomics3.0 ソフトウェアを用いた。データは DLR spread 値< 0.30 を採用した。比較対照 DNA は、Promega 社製 Female および Male genomic DNA を用いた。解析したゲノム DNA は、QIAamp DNA Blood Mini kit を用いて自動抽出機で末梢血液から抽出した。アレイ CGH で検出されたゲノムコピー数異常は、ISCN2016 に準じて記載した。参照ゲノムマップとして UCSC Genome Browser on Human Feb. 2009 (hg19) Assembly を用いた。

（倫理面への配慮）

次世代シーケンシングおよびマイクロアレイ CGH による解析は、こども医療センター倫理審査において、研究課題「原因不明多発奇形精神遅滞症候群のゲノムワイドな病因解析」として平成 22 年 7 月 22 日に承認を得たものである。検査前に十分な説明を行い、文書により同意のもとで解析を行った。解析にあたっては、全ての個人情報を潜在化した。

Ｃ．研究結果

Pitt-Hopkins 症候群 8 歳女児

臨床症状の組み合わせから Pitt-Hopkins 症候群と診断した 8 歳女児例を経験した。Pitt-Hopkins 症候群責任遺伝子 TCF4 の全エクソンならびにエクソン - イントロン境界領域のサンガーシーケンスで変異を認めなかったことから、微細欠失型の Pitt-Hopkins 症候群ないしは症状が共通した他の奇形症候群を想定してマイクロアレイ染色体検査を行った。しかし、明らかな欠失はコールされなかった。そこで read depth を CNV 変換して再評価を行ったところ、TCF4 遺伝子 exon 13 から exon 20 までの欠失を検出した。この欠失範囲は、最少で 33.2kb、最大で 1.88Mb に及んだ。当初のマイクロアレイ染色体のプラットフォームは 60k（オリゴプローブ間隔は平均約 50kb）なので、コールされているとの仮定から、再度マイクロアレイ染色体検査結果を Geneview 画面で確認したところ、確かに該当領域で 1 プローブの低下が確認できた。

Ｄ．考察

次世代シーケンサーによる網羅的ゲノム解析であるエクソームデータを CNV 変換することにより、マイクロアレイ染色体と同等ないしはそれ以上に高い感度で微細な CNV を検出できることが分かった。この結果から、CNV 検出を視野に入れた網羅的なゲノム解析を臨床検査として導入する際には、エクソーム解析とマイクロアレイ染色体検査を別々に行われる遺伝学的検査として説明されるより、全ゲノムを対象とした遺伝学的検査として説明がなされる方が、妥当であると考えられた。この事実を踏まえ、全ゲノムを対象とする遺伝学的検査の説明文書・同意書を検討した。検討を要した点は、１）検査の目的とは別の 2 次的な所見（incidental findings、あるいは secondary findings）の取り扱い、２）解析終了後のデータの取り扱い（有用な in-house データとして遺伝学的検査を実行する上で不可欠な要素）、３）segregation 解析として両親を中心とした家系解析が求められること、４）オプトアウトの設定、などが挙げられた。

今後、本格的にエクソーム解析が遺伝学的検査として導入される場合には、再度の見直しも必要かもしれない。

Ｅ．結論

エクソーム解析データを用いた CNV 検出を試み、マイクロアレイ染色体検査以上に高い検出感度で疾患特異的 CNV を検出することが可能であることを確認した。このことから、網羅的ゲノム解析を前提とした遺伝学的検査として臨床に導入するには、マイクロアレイ染色体検査とエクソーム解析を別次元の検査として説明するのではなく、同一次元で説明する方が妥当とみなし、説明同意書の作成を試みた。今後、さらに解析手法が高度化するにしたがって再度の見直しも必要と考えられた。

Ｆ．研究発表

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Ｇ．知的財産権の出願・登録状況

なし

遺伝学的検査についての説明

（2017 年 4 月 1 日）

ヒトのゲノム・遺伝子解析技術の進歩により、より迅速に、網羅的にゲノム・遺伝子を解析することが可能となりました。このヒトゲノム・遺伝子解析技術を応用して、先天的な病気や健康に関わる体質の原因をゲノムや遺伝子から考えることは医療として重要です。この説明文書では、診療として行われる遺伝学的検査について基本的なことをまとめました。それぞれの疾患に関すること（解析対象遺伝子、得られる結果に基づく治療の可能性など）については、担当医から十分な説明を受け、検査を選択願います。

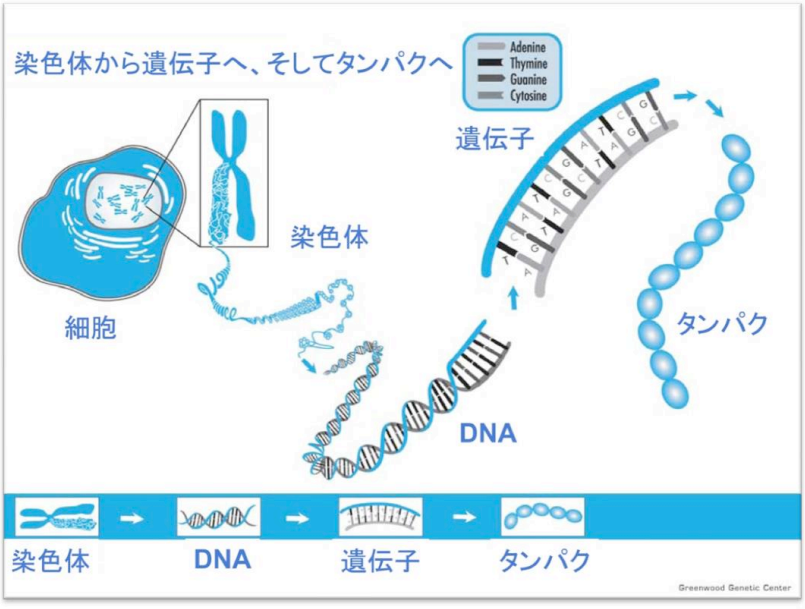
１．検査の目的・意義：

遺伝子やゲノムの変化が原因として発症する疾患を遺伝病と呼びます。ヒトの遺伝子は 20,000 種類以上と推測され、そのうち、疾患発症との関連が明らかなものは約 5,000 種類と言われています。現在さらに研究が進み、遺伝病の原因遺伝子は増えつつあります。原因を遺伝子やゲノムの暗号（塩基配列と呼びます）のなかに見出すことは、診断を確実にし、家系内での再発を推定し、医療管理に役立つこともあります。さらに将来何らかの対策や治療法が生みだされるかもしれません。このように遺伝病の原因をゲノム・遺伝子のレベルで明らかにすることは、極めて重要なことです。

遺伝学的検査とは、現在あなた（患者様）が罹患している遺伝性が疑われる疾患について遺伝子やゲノムを詳細に調べる検査を指します。得られた結果と症状との関連を検討し、医療管理に役立て、遺伝カウンセリング等へ応用をすることを目的にしています。

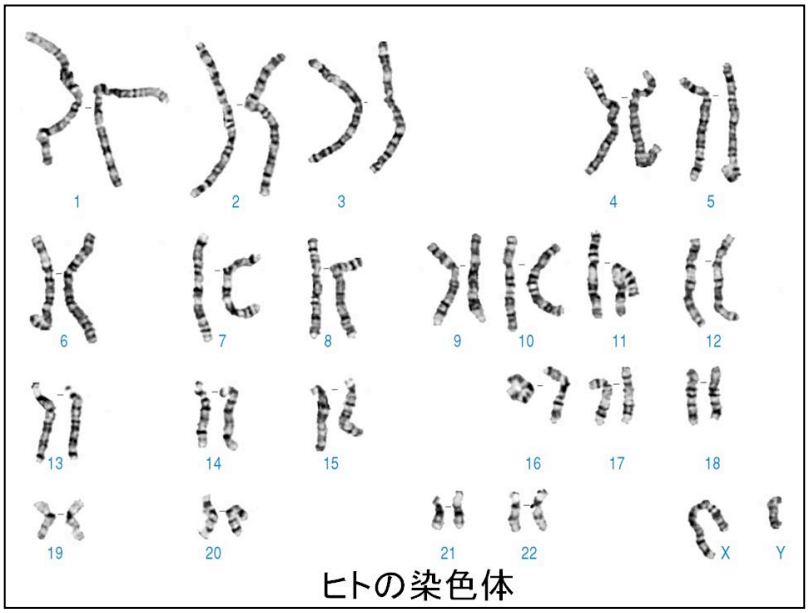
２．遺伝・遺伝子・ゲノム：

《遺伝子とは》



「遺伝」という言葉は、「親の体質が子に伝わること」を言います。ここでいう「体質」の中には、顔かたち、体つきのほか、性格や病気に罹りやすいことなども含まれます。ある人の体の状態は、遺伝とともに、生まれ育った環境によって決まりますが、遺伝は基本的な部分で人の体や性格の形成に重要な役割を果たしています。「遺伝」という言葉に「子」という字が付き「遺伝子」となりますと，「遺伝を決定する小単位」という科学的な言葉になります。人間の場合、2 万種類以上の遺伝子が働いていますが、その本体は「ＤＮＡ」という物質です。「ＤＮＡ」は，Ａ，Ｔ，Ｇ，Ｃという四つの文字（塩基）の連続した鎖です。文字（塩基）は、一つの細胞の中で約 30 億個あり、その文字（塩基）がいくつかつながって遺伝子を構成しています。

このように、一つの細胞の中には 2 万種類以上の遺伝子が散らばって存在しています。この遺伝情報を総称して「ゲノム」という言葉で表現することもあります。人間の体は、数十兆個の細胞から成り立っていますが、細胞の一つ一つにすべての遺伝子が含まれています。遺伝子には二つの重要な働きがあります。第 1 の役割は，遺伝子が精密な「人体の設計図」であるという点です。受精した一つの細胞は，分裂を繰り返してふえ、一個一個の細胞が、「これは目の細胞」、「これは腸の細胞」と決まりながら、最終的には数十兆個まで増えて人体を形作りますが、その設計図はすべて遺伝子に含まれています。第２の重要な役割は「種の保存」です。両親から子供が生まれるのもやはり遺伝子の働きです。人類の先祖ができてから現在まで「人間」という種が保存されてきたのは、遺伝子の働きによっています。遺伝子は祖先から受け継いできた大切な情報です。



ヒトの染色体

《遺伝子・ゲノム解析の特徴》

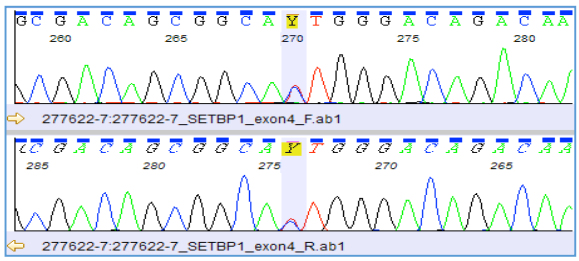
遺伝子には、「人体の設計図」、「種の保存」という二つの重要な役割があることをすでに述べました。ゲノム・遺伝子の変化は、その人の体質を規定する要因にもなりますが、その体質が病的な場合には、遺伝病の原因にもなります。したがって、ゲノム・遺伝子を調べることは、病気の原因を明らかにすることにもなり、さらに、その情報をもとに合併する病気を予防したり、早期発見をすることもできます。また、患者さんの血縁者の中から未発症の患者さんを見つけだし、早期発見、早期治療により病気を治すことも可能かもしれません。もうひとつの有用性としては、得られた結果をもとにした次子再発の可能性に関する遺伝カウンセリングが可能となります。

３．検査の任意性と撤回の自由：

この検査の同意はあなたの自由意志で決めてください。強制いたしません。一旦同意した場合でも、あなたが不利益を受けることなく、いつでも同意を取り消すことができ、その場合は採取した血液やゲノム・遺伝子を調べた結果やゲノム情報などは廃棄され、ほかの診療目的に用いられることはありません。

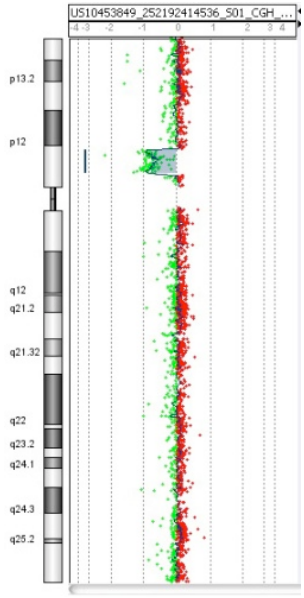
４．検査の原理・方法・精度について：

血液は決められた量を採血します。採血にともなう身体の危険性は一般の採血検査と同じです。疾患や状況によっては、血液以外の組織（唾液や皮膚など）を用いることもあります。これらの組織細胞に含まれるゲノム DNA を抽出し、解析を行います。実際にあなたが受ける遺伝学的検査の基本的な原理については、担当医にご確認ください。代表的なゲノム・遺伝子の解析方法を下記にまとめました。



１）サンガーシーケンス法

対象とする特定の遺伝子を構成する文字（塩基）を直接読む方法です。既に特定の原因遺伝子が疑われている、あるいは家系内で遺伝子の変異が特定されている場合に用いる方法です。解析する範囲には限界があり、また、低頻度のモザイクやゲノムの大きな欠失・重複は検出できません。

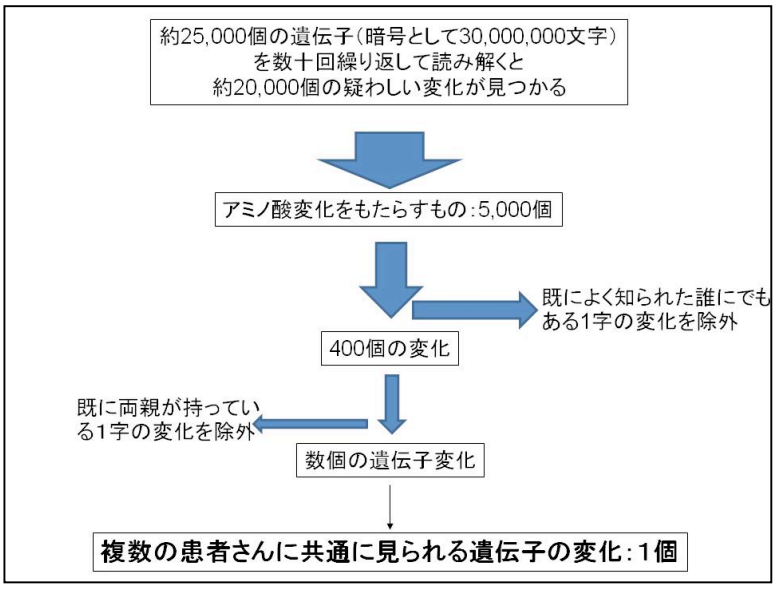


２）マイクロアレイ染色体検査

相対的なゲノム（遺伝情報の総体）のコピー数を比較する手法です。ヒトはそれぞれの遺伝子を父親、母親から１つ（1 コピー）ずつ受け継いで、常染色体では各遺伝子２つ（2 コピー）ずつ持っています。患者 DNA と対照となる正常ヒト DNA 量とを比較し、過剰あるいは不足（欠失）領域を探索します。両親に過剰・欠失がなく、患者 DNA のみにゲノムの量的変化が認められ、かつ、それが複数の同様の疾患患者 DNA で共通する場合、その領域が疾患発症の原因領域と断定できます。普通、人はだれもみな性格も体質も異なり、1 卵性ふたごを除いて同じ人は世の中に存在しません。こうしたそれぞれの違い、つまり個性を規定しているのはゲノム（あるいは遺伝子、DNA の塩基配列、コピー数の変化（copy number variant: CNV））の違いであり、われわれはみな他人と違う膨大なゲノムの変化を有しています。したがって、そのゲノムのコピー数変化（CNV）があなたの病気の原因と考えるためには、患者であるあなた（お子様）にあって、両親にないゲノムの変化を見つけだすことが重要となります。原因の可能性が推測されるゲノムのコピー変化（CNV）が検出された場合には、両親の解析が必要になります。原因が全く不明の遺伝性疾患の検査にこの技術を用いた場合の、診断率は約 10－15％です。マイクロアレイ染色体検査の限界として、この方法では均衡型相互転座を検出することができないこと、染色体再構成の位置的情報が得られないこと、倍数性異常を検出できないこと、低頻度モザイクを検出できないこと、浸透率の低い CNV 効果では判断が難しくなること、などがあげられます。

３）エクソーム（Exome）解析

具体的な原因遺伝子が予想つかない場合には、すべて（約 25000 種類）の遺伝子が検査の対象になります。その 25000 種類の遺伝子の中の暗号 1 文字の違いを見つけ出す方法がエクソーム解析です。この気が遠くなるような作業は、数年前まで、現実的ではありませんでしたが、ごく最近の遺伝子解読機器（次世代シーケンサーと呼ばれます）の開発により、現実的なものとなりました。しかし、この手法もマイクロアレイ染色体検査と同じく、単なる他人との「違い」と疾患発症原因となった「変異」とを区別するのは容易ではありません。なぜなら、膨大な量（数十万）の「他人との違い」の中からたった一つの「固有の違い」を見つけだす作業に匹敵するからです。この方法では、両親とのゲノム情報の比較をして初めて見つけだすことができます。



この解析では次世代シーケンサーのほかに、膨大な量のゲノム情報を処理するバイオインフォマティクスの技術も必要になります。

原因が全く不明の遺伝性疾患の検査にこの技術を用いた場合の、診断率は約 20－25％です。

５．検査後の試料・ゲノムデータ等の保存・使用・廃棄について：

今回の検査に使われるあなたの血液試料（DNA 等）は、再検査等で使用できるように 2 年間保存します。遺伝子・ゲノムの解析技術の進歩によりさらに詳細な解析でそれまで不明であった原因がわかることもあるからです。ただし、この DNA 等の試料は時間とともに劣化する可能性や量的制限もあり、必ずしも将来の検査を保障するものではありません。希望の申し出によって廃棄も可能です。再解析等による保存試料の使用は、改めて担当医と相談願います。

エクソーム解析など網羅的な解析では、血液・DNA 試料だけでなく、疾患以外の個人のゲノム情報も得られます。あなた（患者様）の網羅的なゲノム・遺伝子解析では、比較対象として公開してある数万人以上の人たちのゲノム情報が役立っています。ゲノム情報はあなた自身の診療に役立てることが第 1 の目的ですが、他の人たちの診療に役立つ貴重な情報でもあります。当センターでは個人情報の保護に配慮して院内で保管・利用させていただきます。

６．検査結果の今後の診療等への利用可能性について：

検査結果は、診断を確実にし、家系内での再発を推定し、医療管理に役立つこともあります。さらに将来何らかの対策や治療法が生みだされるかもしれません。遺伝カウンセリング等へ応用をすることも可能です。

７．検査解析期間について：

採血後、結果をお知らせするまでには一定の時間を要します。

複雑な染色体再構成	3－6 か月
マイクロアレイ染色体検査	約 6 か月
特定の遺伝子の塩基配列決定	約 3 か月
次世代シーケンサーによる網羅的解析	約 1 年
その他の遺伝学的検査	担当医に確認

上記は目安であり、詳細は担当にご確認願います。

８．検査に係る費用について：

検査費用は神奈川県立病院機構が定める規定に従います。また、保険の範囲で行われる遺伝学的検査では、結果説明の際に遺伝カウンセリング加算が算定されます。詳細については担当医にご確認願います。

９．検査を受けた方にもたらされる利益、不利益及び負担・予測される結果について：

遺伝学的検査では、あなた（患者様）やあなたの血縁者の方に対して、将来の発病に対する不安や社会的差別などの様々な倫理的・法的・社会的問題が生じる可能性も考えられます。

遺伝子異常が見つかった場合は、両親のいずれかが遺伝子異常を有する場合もあり、両親やご家族の方が心理的・社会的に不安を感じる可能性があります。検査前に、この点について充分な遺伝カウンセリングをおこないます。実際に、両親のいずれかが遺伝子の異常を有している可能性のある検査結果が得られた場合には、遺伝カウンセリングとしてご家族の意向を取り入れながら慎重に検索や結果の説明を進めます。

遺伝学的検査で異常が見つからない場合にも、あなた（患者様）が異常を持っていないと結論づけることは出来ません。結果に関してご家族の方が、就職・結婚などへの影響などの不安を感じたり、さらに詳しい情報を知りたいと思う場合には、遺伝カウンセリングを受けることが出来ます。

10. 検査の限界、あいまいな結果、解釈が困難な結果が生じる可能性について：

遺伝学的検査では、異常が検出されない場合でも臨床診断が否定されたわけではありません。特に低頻度のモザイクでは、原因のゲノムや遺伝子の変異を検出することが困難なことがあります。遺伝学的検査には検査技術に由来するさまざまな限界があります。

遺伝学的検査では、さらに、あなた（患者様）の病気・体質の原因か原因でないかはっきりしない、あいまいな結果がでることもあります。結果により一部の症状は説明がつくものの、必ずしもその変異がすべての症状を説明できない、解釈困難な結果が生じる可能性もあります。

11. 検査の目的とは異なる二次的な検査所見が得られる可能性とその取扱いについて：

全ゲノムを解析範囲とするマイクロアレイ染色体検査やエクソーム解析では、あなた（患者様）の病気や症状とは直接関連性のない膨大なゲノム情報が得らます。しかし、それらは暗号としてのゲノム情報であり、意味づけされて解釈を加えられるのは、あなた（患者様）の病気の発症にかかわることが推定される極めてわずかのゲノム・遺伝子情報です。ゲノム・遺伝子の情報は解釈を加えて初めてその意味がわかります。したがって、膨大なゲノム・遺伝子情報が得られても説明できるのは、意味づけされて症状に直接関連のある遺伝情報のみになります。この膨大なゲノム・遺伝子情報とは下記のような内容を含みます。

将来発症するかもしれないほかの病気のこと
薬に対する反応やアレルギーのこと
身長や体重など体質に関すること
性格や気分などこころの働きに関すること
自分の祖先や血縁者との関連性に関すること

これらのあなた（患者様）の病気や症状とは直接関連性のない、意味づけできない、あるいはされていない膨大なゲノム情報をお伝えすることはしません。

ただし、明らかに試料提供者の健康に支障をもたらすことが予測される場合には、あなた（患者様、同意が得られない場合には代諾者）の同意のもとで結果を開示することもあります。

12. 得られた結果の検証として両親・血縁者のゲノム・遺伝子解析が必要となる可能性について：

こどもの遺伝情報は親に由来することから、得られたゲノム・遺伝子解析結果に関して両親の検査が必要になります。罹患者が家系内に複数いる場合には、家系内罹患者（あるいは非罹患者・非発症者）の検査が必要となることもあります。こうした血縁者の遺伝学的検査は、疾患の発症様式、遺伝形式によって変異の検出方法が異なります。また、結果の解釈も発症者（罹患者）とは異なることがあります。あなた（患者様）の疾患での血縁者検査の意義については、担当医に確認願います。両親や血縁者の遺伝学的検査も診療として扱われます。

13. 再検査・再解析の可能性について：

検査結果があいまい、あるいはデータ量が基準を満たさない場合には、再検査を行うこともあります。また、将来遺伝子解析技術がより進歩した場合やデータ解析プログラムがより高い精度でデータを解析することが可能となった場合には、保存 DNA や保管されたゲノムデータの再解析を行うこともあります。さらに解析の際に参照するデータベースの充実などによっても解析精度が向上することがあります。再検査・再解析であなた（患者様）の診療に有用な結果が得られた場合には、あなた（患者様）に開示されます。

14. 個人情報の保護の方法：

ゲノム・遺伝子の結果は様々な問題を引き起こす可能性があるため、他の人に漏れないように、取扱いを慎重に行う必要があります。あなたの血液や試料 DNA には、新しく符号をつけ、鍵のかかる部屋での保管庫に厳重に保管されることになります。検体試料には、個人の名前など特定できる情報が外されます。このことにより、あなたの個人情報は保護されます。解析の結果についてあなたに説明する場合は、報告書としてあなたにお知らせすることが可能になります。

15. 検査結果の開示について：

あなた（患者様）や両親が説明を望まれる場合に、結果についての説明を診療として行います。たとえあなたの家族に対しても、あなたの承諾または依頼なしに結果を告げることはいたしません。あなたの結果について説明を希望される場合は、血液採取後 5 年以内に申し出て下さい。それ以後はその結果を保管できない場合があります。

方法で述べたとおり、膨大なゲノム情報が得られますが、そのほとんどはあなた（患者様）の健康に直接かかわるか断定できないものです。したがって、原則として対象疾患の原因と考えられるゲノム情報以外のゲノム情報は開示いたしません。しかし、明らかに試料提供者（患者さん）あるいはその血縁者の方々の生命・健康に大きくかかわる問題が生じると予想される場合には、ご本人の同意のもとで開示をすることがあります。これらは、遺伝カウンセリングとして行われます。

16. 検査に伴う遺伝カウンセリングについて：

あなた（患者様）が、病気のことや染色体・遺伝子解析研究に関して、不安に思うことがあったり、相談したいことがある場合に備えて、遺伝カウンセリング外来を設置しています。ここでは、遺伝カウンセリング担当者があなたの相談を受けることが可能です。担当医師・説明者にその旨申し出てください。

17. 将来のより詳細な遺伝学的検査の希望について：

科学の進歩は目覚ましく、全エクソーム解析から全ゲノム解析に向かいつつあります。今回の検査であなた（患者様）の病気の原因がはっきりしない場合でも、将来さらにゲノム・遺伝子を詳しく解析することにより、原因を明らかにすることができるかもしれません。将来のより詳細な遺伝学的検査の希望の有無についてお知らせください。

18. 問い合わせ先：

遺伝学的検査に関するお問い合わせ等は、下記の解析責任者（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）、認定遺伝カウンセラー宛にお寄せください。

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遺伝学的検査に関する同意書

私は、（疾患名）＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿に対する遺伝学的検査について
医師＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿より説明を受け、下記事項を十分理解しましたので、検査実施者に依頼することを同意致します。

□　検査の目的や意義
□　遺伝子やゲノム、疾患の遺伝形式などの基本事項について
□　検査を受けることが任意で、同意の撤回は可能なことについて
□　検査の原理・方法・精度などについて
□　検査後の試料・ゲノムデータ等の保存・使用・廃棄について
□　検査結果の今後の診療等への利用可能性について
□　検査解析期間について
□　検査に係る費用について
□　検査を受けた方にもたらされる利益、不利益及び負担・予測される結果について
□　検査の限界、あいまいな結果、解釈が困難な結果が生じる可能性について
□　検査の目的とは異なる 2 次的な検査所見が得られる可能性とその取扱いについて
□　得られた結果の検証として両親・血縁者のゲノム・遺伝子解析が必要となる可能性について
□　再検査・再解析の可能性
□　個人情報の保護の方法
□　検査結果の開示について
□　検査に伴う遺伝カウンセリングについて

＊将来のより詳細な遺伝学的検査を：　□希望します　□希望しません

患者名（患者本人が同意に関して判断ができないときは代諾者）
平成　　年　　月　　日

氏　名　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

代諾者　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿（続柄）＿＿＿＿＿＿＿

説明を行った医師　　同席者

氏　名　＿＿＿＿＿＿＿＿＿＿＿＿　　氏　名　＿＿＿＿＿＿＿＿＿＿＿＿

病院名　　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
住　所　　〒２３２－８５５５　　横浜市南区六ツ川２－１３８－４
電　話　　０４５－７１１－２３５１

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CASE REPORT

Hearing impairment in a female infant with interstitial deletion of 2q24.1q24.3

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ABSTRACT **Patients with interstitial deletions in 2q24.1q24.3 are rarely reported. These patients manifest a variety of clinical features in addition to intellectual disability, depending on the size and location of the deletion. We report a female patient with interstitial deletion of 5.5 Mb in 2q24.1q24.3, who showed intrauterine growth retardation, hypotonia, global developmental delay, microcephaly, and characteristic facial appearance. In addition, she had hearing impairment, with no auditory brainstem response. Case of 2q24.1q24.3 deletion with hearing impairment is quite rare. We suspect that hearing impairment is caused by bilateral cochlear nerve deficiency due to cochlear nerve canal stenosis. Further studies are necessary to evaluate hearing impairment as a clinical feature in patients with *de novo* heterozygous 2q24.1q24.3 deletion.**

Key Words: 2q24.1q24.3 deletion, cochlear nerve deficiency, hearing impairment

INTRODUCTION

Recently, specific phenotype for 2q24.1q24.3 deletion has been reported, and is characterized by intrauterine growth retardation, hypotonia, severe intellectual disability, microcephaly and autistic spectrum behavior with or without seizures. A total of 17 patients with deletions involving the 2q24.1q24.3 region have been reported in the literature. Although intellectual disability and developmental delay are common in all patients, other symptoms varied, suggesting that some genes are specifically associated with the clinical features of 2q24.1q24.3 deletion. Here we report hearing impairment as a new phenotype of 2q24.1q24.3 deletion and discuss the pathogenesis of this symptom.

CLINICAL REPORT

The patient is the first child from healthy unrelated 29-year-old parents. Her family history was unremarkable. Pregnancy was complicated by poor fetal growth, and she was born at 40 weeks and 5 days of gestation. Her birthweight was 2340 g (below 10th centile), length was 46.8 cm (below 25th centile) and head circumference was 30.5 cm (below 3rd centile), indicating intrauterine growth retardation. Since neonatal hearing screening with automated auditory brainstem response was "refer", she was tested with auditory brainstem response test (ABR), which revealed no response. Neonatal serological testing for TORCH infections was negative, and cytomegalovirus DNA was not detected in her umbilical cord. At 7 months of age she was referred to our hospital because of developmental delay. On examination, the patient had microcephaly with a head circumference of 39.7 cm (below 3rd centile). Height and weight were plotted at the 50th centile for age. She had characteristic facial appearance with hypertelorism, telecanthus, almond shaped palpebral fissures, low-set ear, protruding ears, underdeveloped antihelix, exaggerated cupid's bow, tented mouth, small nose and micrognathia (Fig. 1a,b). Her head and neck were unstable, but she showed eye tracking. Neurological examination showed muscle hypotonia and with normal deep tendon reflexes. Electroencephalography and brain magnetic resonance imaging revealed normal findings. The heart and renal ultrasound findings were unremarkable. Examinations for congenital metabolic diseases, including urine analysis of organic acids, showed normal findings.

At the age of 1 year, audiometric evaluations were performed. The threshold of conditioned orientation reflex was 43.8 dB. ABR and auditory steady-state response were negative (Fig. 2a). Axial images of temporal bone computed tomography (CT) depicted bilateral cochlear nerve canals measuring 1.0 mm in diameter (Fig. 2b). Developmentally, she rolled over at 1 year and 3 months, sat unsupported at 2 years, and walked while holding on to something at 2 years and 9 months. A developmental quotient of Enjoji Scale of Infant Analytical development was 17 at 2 years 8 months. At 5 years of age, her body weight was 14.6 kg (below 10th centile), length was 100 cm (below 10th centile) and head circumference was 46 cm (below 3rd centile). She was able to walk unsupported, but no fine motor skills were found. She still could not use meaningful words, sign language and understand any speech.

CYTOGENETIC ANALYSIS

The G-banded karyotyping identified interstitial deletion of chromosome 2q with the karyotype of 46, XX, del(2)(q24.1q24.3) (Fig. 3a). To identify the precise chromosomal deletion region, we performed array comparative genomic hybridization (Array-CGH) analysis using the Agilent SuperPrint G3 Human CGH Microarray Kit 8 × 60 K (Agilent Technologies, Inc., Santa Clara, CA, USA) and genomic DNA extracted from peripheral blood using Qiagen

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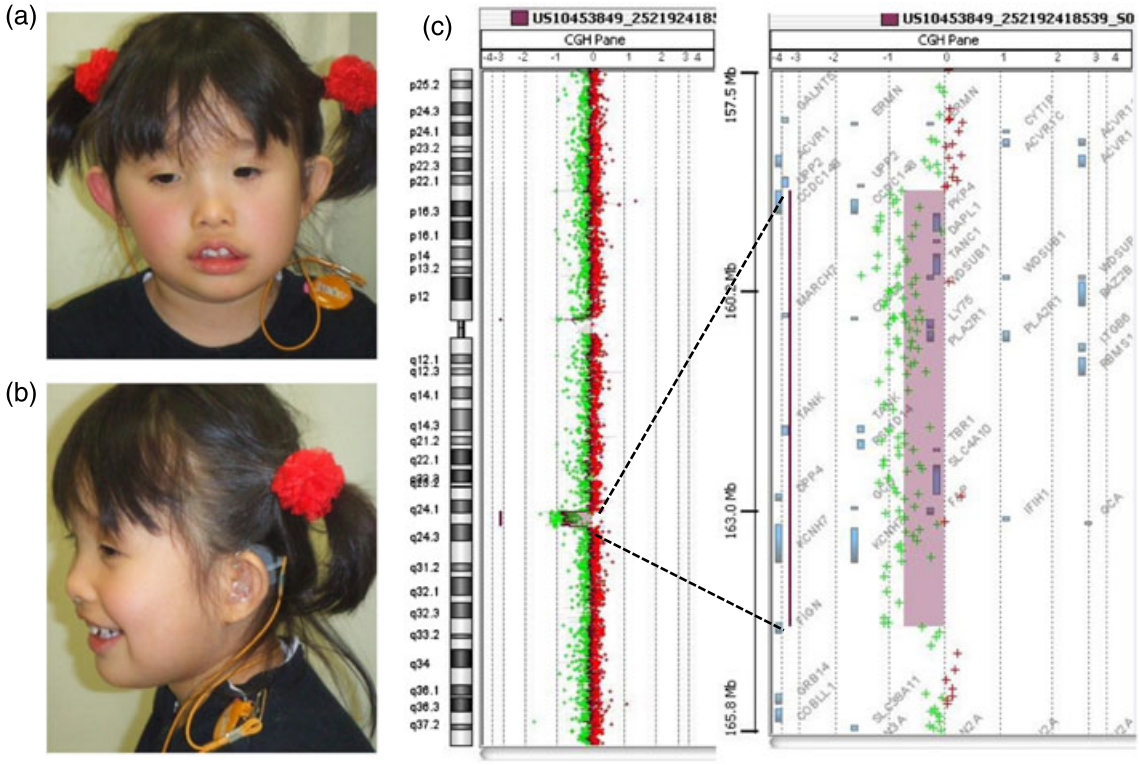

Fig. 1 (a and b): Frontal (a) and lateral (b) view of the proband. Permission was obtained from the parents for presentation. (c) Results of array comparative genomic hybridization analysis. Chromosome view (left) indicates an interstitial deletion in chromosome 2 involving band q24.1 to band q24.3. In the expanded gene view (right) of the deleted 5.5 Mb region (159 028 726–164 512 552), the area shaded in purple contains the genes with imbalance of copy number.

extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Array-CGH identified a 5.5 Mb microdeletion of 2q24.1-q24.3 with proximal base pair coordinates 159 028 726–164 512 552 (Fig. 1c). Genomic positions refer to the human reference sequence (GRCh 37/hg 19) produced by the Genome Reference Consortium. The deletion was confirmed by FISH experiments using BAC clone (RP11-703 K10, 2q24.2 160.2–160.4 Mb) on metaphase cells (Agilent Genomic Workbench Software) (Fig. 3b). Analysis of parental chromosomes showed normal chromosomes, suggesting *de novo* deletion in chromosome 2q in the presented patient.

DISCUSSION

In the presented patient, the deletion spans 5.5 Mb in 2q24.1q24.3, including 34 genes from *PKP4* to *FIGN*. Several genes in the 2q24.1q24.3 region have been reported to be involved in normal brain development and function. Some of these genes have been considered to be candidates for various clinical features. Belengeanu et al. (2014) compared the clinical features of their patient to six previously published patients with a deletion in 2q24.2q24.3 and suggested that six genes (*PSMD14*, *TBR1*, *SLC4A10*, *DPP4*, *KCNH7*, and *FIGN*) could contribute to intellectual disability and/or autistic spectrum behavior. It is noteworthy that the six genes are located in the deleted region of the presented patient.

Cochlear nerve deficiency (CND) has been known as one of the common causes of congenital hearing loss. Cochlear nerve canal stenosis with a diameter of 1.5 mm or less as assessed by CT suggests CND or hypoplasia (Masuda et al. 2013). In the presented patient, temporal CT depicted bilateral cochlear nerve canals measuring only 1.0 mm in diameter. Therefore, we speculated that bilateral CND may be a cause of her sensory hearing loss. The exact causes and mechanisms of CND remain unclear. Previous study demonstrated that TANC1, which is contained in the deleted region, is a scaffold component protein in post-synaptic density regions and strongly binds PDZ domain of SCR1B (Luck et al. 2011). USH1C (also known as harmonin) is a PDZ domain-containing protein expressed in the inner ear sensory hair cells (Verpy et al. 2000). Since the defect in USH1C causes Usher syndrome type 1C associated with profound sensorineural deafness and vestibular dysfunction, we suggest that haplodeficiency of TANC1 may affect the function of USH1C which results in the dysfunction of inner ear sensory hair cell.

Taken together, we report for the first time a patient with 5.5 Mb deletion in 2q24.1q24.3 presenting with hearing impairment possibly due to bilateral CND, in addition to global developmental delay, microcephaly, hypotonia and characteristic facial appearance. Clinical and cytogenetic analyses of more patients with CND and global developmental delay are needed to clarify the relationship between 2q24.1q24.3 deletions and these rare clinical features.

DISCLOSURE

The authors declare no conflict of interests.

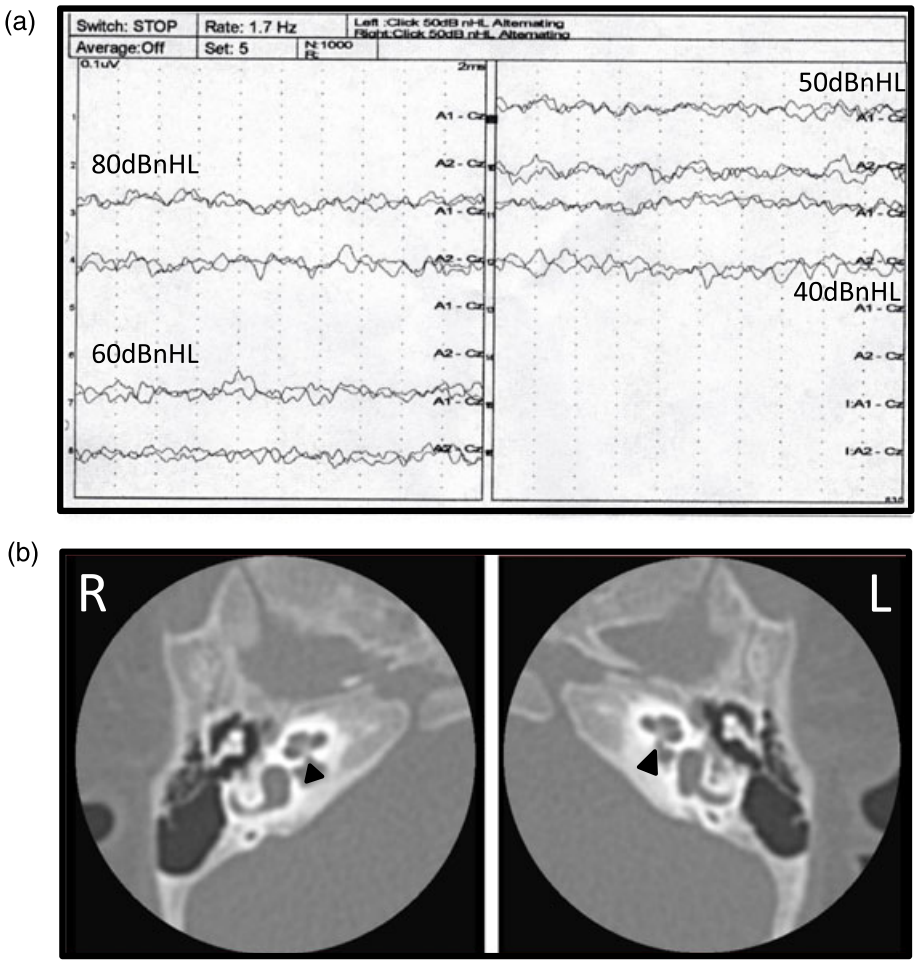

Fig. 2 (a) Auditory brainstem response test shows no response to stimulation ranging from 30 to 80dBnHL. (b) Axial images of temporal bone CT at the level of the cochlear indicate stenoses in bilateral cochlear nerve canals. The left panel shows the right ear (R) and the right panel shows the left ear (L). Arrowhead indicates the stenotic cochlear nerve canal.

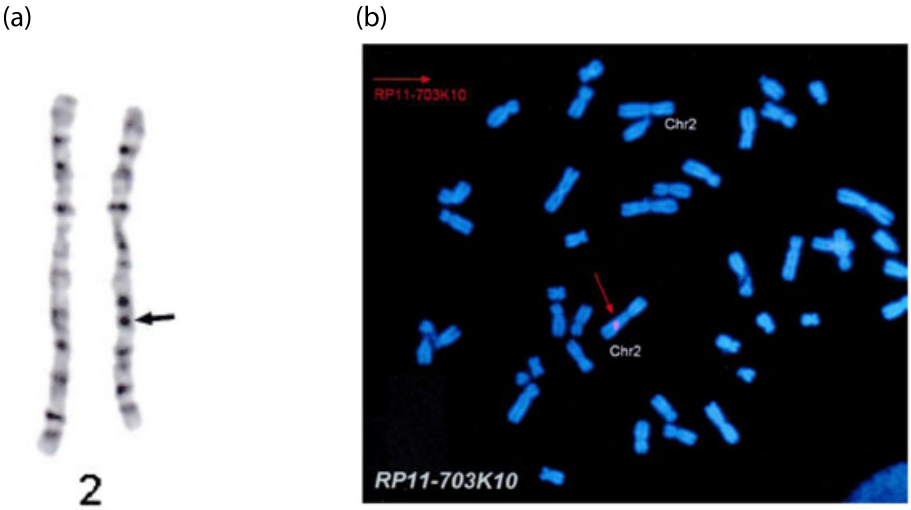

Fig. 3 (a) The G-banded karyotyping showing interstitial deletion of chromosome 2q with the karyotype of 46, XX, del(2)(q24.1q24.3). **b**: FISH image of the patient using a BAC clone RP11-703 K10.

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分担研究報告書

16p11.2 欠失・重複症候群の実態把握について

研究分担者　山本　俊至　東京女子医科大学附属遺伝子医療センター・准教授

研究要旨

研究目的:
16p11.2 欠失・重複症候群は1万人当たり数人の頻度で起こる稀な染色体微構造異常による。2008 年、マイクロアレイ染色体検査によって世界中から発見の報告が相次いだ。その大部分は自閉症患者に認められたというものであった。本邦ではまだほとんど報告がない。そこで診断の一助とするため、本疾患と診断された日本人患者の症状をまとめた。
研究方法:
これまでに診断がついた日本人 16p11.2 欠失・重複症候群患者の情報を収集した。
結果と考察:
分担研究者の単独施設において、これまでに 4 家系の存在を明らかにした。このうち 3 家系の発端者はてんかん症状を認め、残り 1 家系の発端者は自閉症症状を示した。2 家系の患者では軽度知的障害が認められた。3 家系の母親は発端者と同じ遺伝子型を示していたが、神経症状は明らかでなかった。このことは、16p11.2 欠失・重複症候群の浸透率は低く、必ずしも自閉症症状を主な症状とせず、てんかんや軽度知的障害などの広範な神経症状と関連していることを示唆している。
結論:
日本人 16p11.2 欠失・重複症候群の実態は未だに明らかではないが、診断がついた発端者では、広範な神経症状が認められるものの、同じ遺伝子型を示す家族が何ら症状を示さないことが多いことが明らかになった。

A. 研究目的

16p11.2 欠失・重複症候群は、16p11.2 領域のわずか 500 kb 程度の領域が欠失、あるいは重複することによって生じるが、2008 年頃に次々と報告のあった自閉症の大規模コホート研究によって自閉症患者の 1%程度に認められることが示された。欠失を示す場合と重複を示す場合で明らかな症状の違いはない。自閉症以外に患者で認められる症状として、発達遅滞、自閉症を含む発達障害、わずかな身体的特徴などが挙げられる。

16p11.2 欠失・重複症候群で欠失・重複が認められる領域には、両切断端にゲノムの繰り返し構造があり、このため非相同染色体組換による欠失・重複が生じやすく、患者で認められるゲノムコピー数異常の領域は共通している。ゲノム病の1つとして認識される。16p11.2 欠失・重複の領域は遺伝子密度が高く、約 25 個の遺伝子が存在しているが、どの遺伝子がどの症状と関連しているかは明らかになっていない。

本邦における 16p11.2 欠失・重複症候群患者の実態は明らかでない。

B. 研究方法

分担研究者の施設においてこれまでに実施されたマイクロアレイ染色体検査によって明らかになった 16p11.2 欠失・重複症候群患者の臨床情報を収集・調査した。

C. 研究結果

3 家系において 16p11.2 欠失を、1 家系において 16p11.2 重複を認めた。

16p11.2 欠失を示した患者の一人は乳児期早期のてんかん症状で発症したが、それ以外の症状は明らかでない。この患者の両親は検索されておらず、遺伝によるものかどうかは不明である。

16p11.2 欠失を示した残りの 2 家系のうち 1 家系の発端者では自閉症症状が主な症状であった。もう 1 家系の発端者ではてんかん症状と軽度知的障害が認められた。この 2 家系とも母親において同じ 16p11.2 欠失が認められた。

16p11.2 重複を示した 1 家系の発端者はてんかん症状を認めた。同じ重複を持つ母親は無症状であった。

D. 考察

4 家系の 16p11.2 欠失・重複症候群家系の発端者は、必ずしも自閉症症状を主な症状とせず、てんかんや軽度知的障害などの広範な神経症状を示した。このうち 3 家系は無症状の親世代から 16p11.2 欠失あるいは重複を受け継いでいたが、親は無症状であり、16p11.2 欠失あるいは重複の浸透率が低いことが示唆される。

E. 結論

16p11.2 欠失・重複症候群患者の症状は多彩である一方、同じ遺伝子型を示す家族が無症状である場合が多く、実態把握が容易でない可能性が示唆された。

F. 研究発表

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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

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に関する研究

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研究要旨：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構造異常症候群の診療ガイドラインの確立を目的として、代表的な疾患に関して、全国調査による国内患者の把握や、臨床診断基準、重症度判定基準の策定を実施し、これらの疾患の患者や家族に対する支援を通じて、稀少難病の医療や福祉の向上に貢献することをめざす.

Ａ．研究目的

染色体微細構造異常症候群のほとんどは、確定診断に遺伝学的検査としてのマイクロアレイ染色体検査による特定領域のゲノムコピー数バリアント（copy number variants: CNVs）の検出が必須である。マイクロアレイ染色体検査の実施に際しての課題などを、公開データベース／ガイドライン、自験データなどから抽出し、世界で実施されている遺伝学的検査の現状についての認識を深める.

Ｂ．研究方法

１．諸外国で実施されている細胞遺伝学的検査の実態とわが国の課題

‘GeneTests’ < https://www.genetests.org/>は，米国で運用されている先天異常患者の診断目的の遺伝学的検査に関する情報サイトのひとつで，2016年12月8日現在，5,810遺伝子，4,745疾患に関する遺伝学的検査についての情報が掲載されている．ここに掲載されている細胞遺伝学的検査の項目を分析した.

２．マイクロアレイ染色体検査実施に関するガイドラインで指摘されている留意事項

マイクロアレイ染色体検査が原因不明の先天異常患者に実施すべき第一選択の遺伝学的検査として普及している諸外国においては，検査実施に際してのガイドライン等の整備も進んでいる．以下の米国および欧州におけるガイドラインおよび自験データより，マイクロアレイ染色体検査実施の際の留意事項について抽出した.

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（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．諸外国で実施されている細胞遺伝学的検査の実態とわが国の課題

‘GeneTests’に掲載されている情報は，以前は分子遺伝学的手法を用いた遺伝学的検査の情報が主であったが，現在は検査法の分類として，細胞遺伝学的検査も加わっている．それは，染色体微細構造異常症候群を検出するための細胞遺伝学的検査法であるマイクロアレイ染色体検査法が，諸外国では原因不明の先天異常患者に実施すべき第一選択の遺伝学的検査として普及したこと，検出されたCNVsが原因と考えられる疾患が多く認識されるようになってきたこと，そのうちの一部が患者の蓄積により疾患単位として確立してきた，といった背景によると考えられた．そして，検査の項目は，分染法・FISH 法・マイクロアレイ染色体検査法といった解析法の違いだけでなく，出生後・出生前の区別や，検査に用いる試料などによっても分類され，それぞれに費用が決められていた.

一方，神経線維腫症I型やCHARGE症候群といった，シーケンスバリアント (SVs) の検出を目的とした分子遺伝学的解析法が主たる遺伝学的検査である一部の単一遺伝子疾患の遺伝

学的検査法としても，細胞遺伝学的検査（マイクロアレイ染色体検査法やFISH法）を実施している検査施設も少なからず見受けられた．疾患によっては低頻度ながら当該遺伝子に関連するCNVsが病的バリアントとなっていることが知られており，病的バリアントの検索に，SVsのみならずCNVsも対象にする必要性が認識されてきたことを反映していると考えられた．

一方，マイクロアレイ染色体検査で検出しようとする病的バリアントは，優性遺伝形式の疾患がほとんどであるが，劣性遺伝形式の疾患も視野に入れた場合，シーケンス解析やコピー数異常解析では検出できない，一方のアレルの染色体構造異常がもうひとつの病的バリアントとなる場合があることにも留意が必要である（Poot M & Haaf T (Mol Syndromol. 6(3):110-134, 2015)

以上の結果を2016年日本小児遺伝学会にて発表した．

２．マイクロアレイ染色体検査実施に関するガイドラインで指摘されている留意事項

マイクロアレイ染色体検査を臨床検査として実施する際に，留意すべき項目を以下に抽出した．

・アレイデザイン
・検証と妥当性確認
・レファランス DNA
・解析ソフト
・精度管理
・解析および解釈の品質基準
・報告書の品質基準
・CNVs に伴う染色体再構成の確認のための技術利用

解析および解釈の品質基準に関連して，自験データを分析した．既知の染色体微細構造異常症候群として知られている領域以外に検出されたCNVsについては，臨床症状との関連の評価は容易ではない．その際に参照する公開データベースのひとつに，630万人以上の正常対象のCNVsが登録されている，カナダで運用されているDatabase of Genomic Variants (DGV) < http://dgv.tcag.ca/dgv/app/home> がある．自験の先天異常患者に検出されたCNVsの臨床的評価のために実施した，患者の症状を認めない親を対象としたマイクロアレイ染色体検査で検出されたbenignとおもわれるCNVsでも，DGVにも登録がなかったCNVsも少なくなかった．人種差の影響が視され，日本人のCNVsデータベース構築が望まれる．また，特定領域においては関連遺伝子の表現度の多様性による影響も除外できないため，データベースの充実と定期的なデータの見直しが必須と考えられた．症状を認めない親から伝わったCNVsであっても，病的でないことが検証されていない限り，安易にbenignとすべきでないということは，既報告されている諸外国のガイドラインでも指摘されていた．

以上の結果を2016年国際人類遺伝学会にて発表した．

D．考察

今回，‘GeneTests’や諸外国のマイクロアレイ染色体検査実施に関するガイドラインの分析より，世界で実施されている遺伝学的検査の現状についての認識を深めた．わが国に適した遺伝学的検査としてのマイクロアレイ染色体検査実施施設の実施体制の構築とともに，実施施設間で共有する実施ガイドライン等の整備が必要である．

E．結論

国立研究開発法人日本医療研究開発機構（AMED）が立ち上がり，わが国においてもゲノム医療の充実が期待されている．遺伝性疾患の発症原因となる責任遺伝子の病的バリアントは多様であり，次世代シークエンサーを用いた遺伝学的検査法でも特定できない場合がある．わが国においても遅ればせながらクリニカルシークエンスが普及しようとしている今，医療として重要な確定診断に結びつける病的バリアントの検出率向上のためにも，改めてマイクロアレイ染色体検査を含む細胞遺伝学的検査の実施体制の充実が必要と考えた．

G．研究発表

1. 論文発表

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遺伝学的検査情報サイト‘GeneTests’に掲載されている細胞遺伝学的検査についての分

析（ポスター）．涌井敬子，福嶋義光，第39回日本小児遺伝学会学術集会，2016.12.10，東京

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

研究成果の刊行に関する一覧表レイアウト

書籍

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Palindrome-Mediated Translocations in Humans: A New Mechanistic Model for Gross Chromosomal Rearrangements

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Palindromic DNA sequences, which can form secondary structures, are widely distributed in the human genome. Although the nature of the secondary structure—single-stranded "hairpin" or double-stranded "cruciform"—has been extensively investigated *in vitro*, the existence of such unusual non-B DNA *in vivo* remains controversial. Here, we review palindrome-mediated gross chromosomal rearrangements possibly induced by non-B DNA in humans. Recent advances in next-generation sequencing have not yet overcome the difficulty of palindromic sequence analysis. However, a dozen palindromic AT-rich repeat (PATRR) sequences have been identified at the breakpoints of recurrent or non-recurrent chromosomal translocations in humans. The breakages always occur at the center of the palindrome. Analyses of polymorphisms within the palindromes indicate that the symmetry and length of the palindrome affect the frequency of the *de novo* occurrence of these palindrome-mediated translocations, suggesting the involvement of non-B DNA. Indeed, experiments using a plasmid-based model system showed that the formation of non-B DNA is likely the key to palindrome-mediated genomic rearrangements. Some evidence implies a new mechanism that cruciform DNAs may come close together first in nucleus and illegitimately joined. Analysis of PATRR-mediated translocations in humans will provide further understanding of gross chromosomal rearrangements in many organisms.

Keywords: palindrome, inverted repeat, cruciform, chromosomal translocation, gross chromosomal rearrangement

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INTRODUCTION

DNA palindromes consist of two units of identical sequences connected in an inverted position with respect to each other. In palindromes, the sequences on the complementary strands read the same in either direction. In other words, the complementary sequence appears in the same strand in an inverted orientation. Palindromic DNA can consequently form specific tertiary structures,

Abbreviations: PATRR, palindromic AT-rich repeat.

namely, single-stranded "hairpin" or double-stranded "cruciform" DNA. Such unusual DNA tertiary structures are called non-B DNA structures (Sinden, 1994; Wang and Vasquez, 2014). These non-B DNA structures are presumed to be generated in a cell under specific situations, although their *in vivo* existence is still a controversial subject.

Hairpin structures can be formed when the double helix DNA is dissociated into single-stranded DNA molecules at the palindrome. Such single-stranded DNA might occur during DNA or RNA synthesis during replication or transcription. On the other hand, cruciform formation starts from unwinding of the center of the double-stranded palindromic DNA, followed by extrusion at the center of the palindrome to form an intra-strand base-paring of each strand. As the DNA unwinds, the cruciform gets bigger. Cruciform formation requires an under-twisted state, that is, negative superhelicity, of the DNA. Such unusual DNA structure itself could have an impact on DNA replication, repair, transcription, or other important biological pathways (Inagaki and Kurahashi, 2013). The DNA regions that potentially form non-B DNA structures often manifest genomic instability that induces gross chromosomal rearrangements (Pearson et al., 2005; Tanaka et al., 2005; Maizels, 2006; Raghavan and Lieber, 2006; Mirkin, 2007; McMurray, 2010).

PALINDROME-MEDIATED CHROMOSOMAL TRANSLOCATIONS IN HUMAN SPERM

The best-studied palindromic sequences are the breakpoint sequences of the constitutional t(11;22)(q23;q11.2) translocation, a well-known recurrent non-Robertsonian translocation in humans. Balanced carriers are healthy but often have reproductive problems such as infertility, recurrent pregnancy loss, and offspring with Emanuel syndrome (Carter et al., 2009; Ohye et al., 2014; Emanuel et al., 2015). Breakpoint analysis of 11q23 and 22q11 revealed that these regions contain a large palindrome of hundreds of base pairs that is extremely AT-rich (Kurahashi et al., 2000a, 2007; Edelmann et al., 2001; Kurahashi and Emanuel, 2001a; Tapia-Páez et al., 2001). These so-called palindromic AT-rich repeats (PATRRs) have been identified at both breakpoints on chromosomes 11 and 22 and are named PATRR11 and PATRR22, respectively. These PATRRs have several features in common. Both are several hundred base pairs in length and have greater than 90% AT content. They manifest nearly perfect palindromes without spacer regions but share little homology between the two chromosomes.

The most prominent feature of the t(11;22) translocation is that *de novo* translocations frequently arise at a similar breakpoint location. Translocation-specific PCR with primers flanking the breakpoints on chromosomes 11 and 22 can detect all of the t(11;22) junction sequence in the translocation carriers (Kurahashi et al., 2000b). We performed PCR at the single-molecule detection level using sperm DNA from normal healthy men with the 46, XY karyotype as template. Some DNA aliquots tested positive for t(11;22)-specific PCR products while others were negative, suggesting that the PCR detected *de novo* t(11;22) translocations (Kurahashi and Emanuel, 2001b). The frequency was about one in 10,000. However, when the DNA of blood cells or cheek swab cells from the same men was analyzed, no translocation could be found. Furthermore, all of the lymphoblastoid cell lines or cultured fibroblasts examined also tested negative in PCR analysis. These results imply that the t(11;22) translocation arises in a sperm-specific fashion. There is no evidence for the occurrence of the t(11;22) translocation during female gametogenesis because of the limited availability of human oocytes for testing. However, in *de novo* t(11;22) families, analysis of the parental origin of the translocation chromosomes using the polymorphic feature of PATRR11 and PATRR22 revealed that all of the *de novo* t(11;22) translocations were of paternal origin, supporting a hypothesized sperm-specific mechanism of t(11;22) translocation formation (Ohye et al., 2010).

DNA SECONDARY STRUCTURE IN THE PALINDROME: HAIRPIN OR CRUCIFORM

What is behind the sperm-specific occurrence of the PATRR-mediated translocation? It is not unreasonable to discuss the mechanism leading to the t(11;22) translocation in the context of DNA secondary structure. The DNA secondary structure at the PATRR is potentially evidenced by the fact that a polymorphism within the PATRR affects the *de novo* t(11;22) translocation frequency (Kato et al., 2006; Tong et al., 2010). PATRR11 and PATRR22 have size polymorphisms in the general population due to deletion within the palindromic region. Carriers with long symmetric alleles preferably produce *de novo* t(11;22) translocations more frequently than carriers with PATRR asymmetric arms. These data indirectly but strongly implicate the presence of DNA secondary structure during translocation formation.

One hypothesis to explain the sperm specificity of the t(11;22) translocation is that it develops during DNA replication. Sperm production involves many cell divisions, each requiring DNA replication. During DNA replication, single-stranded DNA is generated in the template DNA for the synthesis of not only the lagging strand DNA, but also the leading strand (Azeroglu et al., 2014). When the replication fork comes to the palindromic region, a long single-stranded DNA is formed, inducing the formation of a single-stranded hairpin structure. The stalling of the replication fork produces DNA breakage at the palindromic region that can potentially induce translocations.

Because the germ stem cells in men replicate about 23 times per year, mature sperm from older men have undergone a greater number of replication cycles. The frequency of *de novo* point mutations in sperm cells increases according to the age of the sample donor (Crow, 2000; O'Roak et al., 2012). If the t(11;22) translocation is mediated by replication, the frequency of the *de novo* t(11;22) translocation should be higher in sperm from older men than in younger men for a similar reason. A previous analysis of the t(11;22) translocation

suggested, however, that there is no tendency for an increase in t(11;22) translocation frequency in the sperm of older men (Kato et al., 2007).

To determine the involvement of DNA replication in translocation formation, we established a model system for the t(11;22) translocation in cultured cells by using plasmids harboring PATRR11 or PATRR22 (Inagaki et al., 2009). Both plasmids were transfected into the HEK293 human cell line and we monitored the fusion of the different plasmids at each PATRR using GFP expression or translocation-specific PCR (**Figure 1A**). The results indicated that a translocation-like reaction took place. In this reaction, both PATRRs were cleaved at the center of the palindrome and joined via non-homologous end-joining in a similar manner to the human t(11;22) translocation. Crucially, the plasmids had no replication origin for human cells, which means that the translocation took place without DNA replication.

POST-MEIOSIS HYPOTHESIS FOR PATRR-MEDIATED TRANSLOCATIONS

On the other hand, it is possible that the translocation is mediated by another secondary structure, the DNA cruciform. In our model system, the plasmids were purified from *Escherichia coli* using a standard alkaline lysis method. Plasmid DNA isolated from *E. coli* has a strong negative superhelicity. If the plasmid has a palindromic region, the negative superhelicity facilitates cruciform extrusion (Kurahashi et al., 2004). Under an alkaline condition that induces denaturation of the plasmid DNA during purification, most of the PATRR-harboring plasmids extrude cruciform structures. Via the use of a non-denaturing condition and subsequent topoisomerase treatment, such superhelicity was relieved before cruciform extrusion. In this way, we can prepare different topoisomers of the same plasmid, both cruciform-extruded DNA and not extruded DNA. We tested the effect

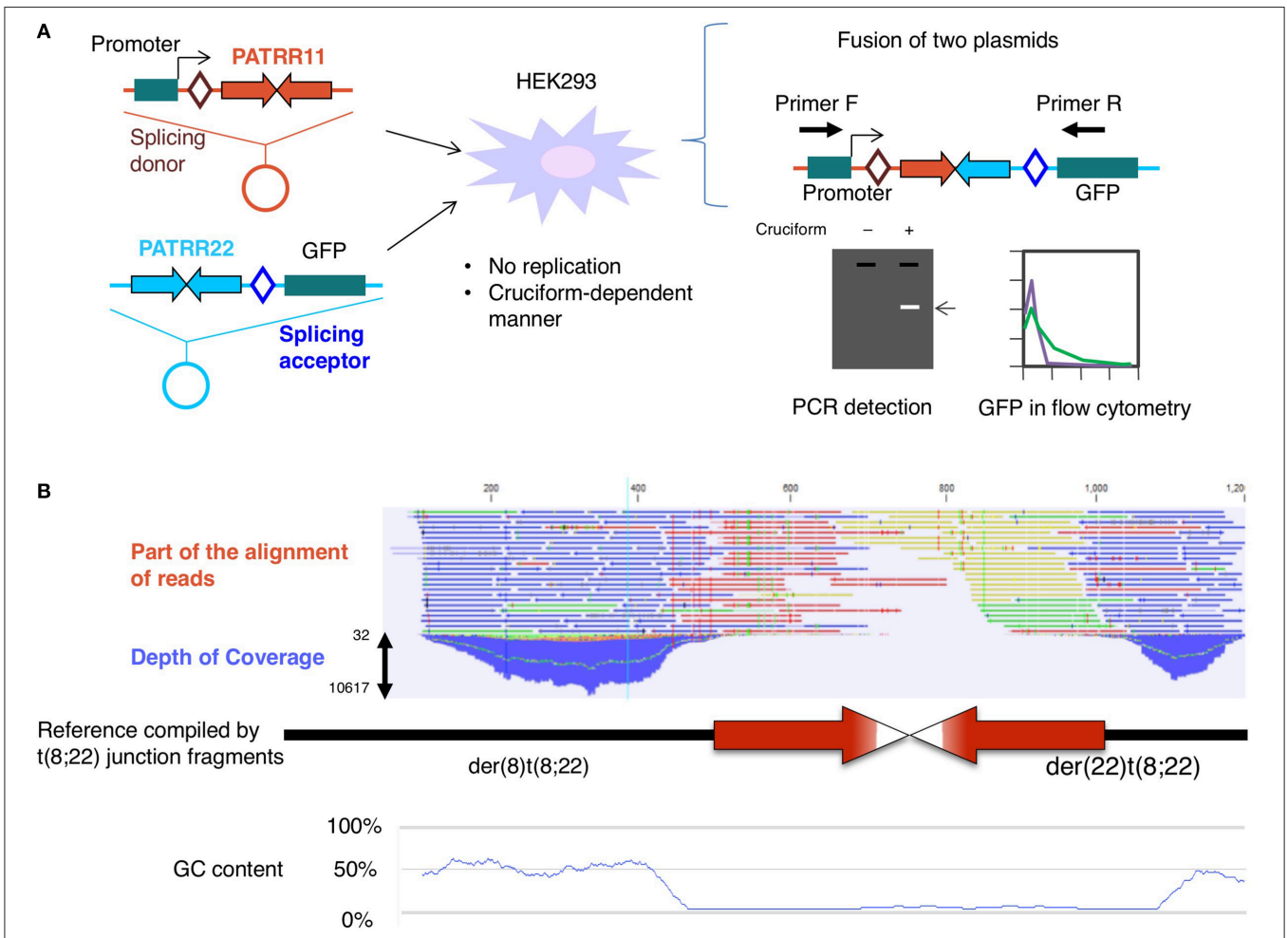

FIGURE 1 | (A) Translocation model system. Two plasmids, one harboring a promoter, splicing donor, and PATRR11, and the other carrying PATRR22, a splicing acceptor, and a coding region of the GFP gene, were simultaneously transfected into HEK293 cells. After 24 h, fusion molecules generated by joining of the PATRR11 and PATRR22 at the center were detected by PCR or GFP-positive cells were monitored by flow cytometry (Inagaki et al., 2013). **(B)** Determination of the PATRR8 sequence by next-generation sequencing. Although the depth of the coverage was low at the center of the palindrome, massive parallel sequencing was able to fill the entire region of the palindrome (Mishra et al., 2014).

of the cruciform on the translocation-like reaction in the cell using a mixture of cruciform and non-cruciform plasmids. The frequency of the translocation-like reaction was found to depend on the proportion of the cruciform-extruded plasmid DNA (**Figure 1A**; Inagaki et al., 2009). These results suggest that cruciform extrusion at the palindromic region induces PATRR-mediated translocation.

Notably however, in living cells the conversion of a DNA structure from that of standard B DNA to cruciform DNA is unlikely to occur under normal physiological conditions from a point of view of thermodynamics. Cruciform extrusion at the palindromic region occurs only when the DNA has strong free negative superhelicity. Theoretically, such superhelicity would potentially occur only at the post-meiosis stage in late spermatogenesis. At this developmental stage, histones are replaced by protamines to reduce the cell size (Gaucher et al., 2010). During histone removal, DNA has a transient excess of negative supercoiling, which might induce cruciform extrusion at the palindromic DNA that leads to translocation formation (Boissonneault, 2002). It is highly possible that PATRR-mediated translocations occur at this developmental stage of spermatogenesis (Kurahashi et al., 2010).

Although the post-meiosis hypothesis is captivating, there is some evidence contradicting this hypothesis. One example is the presence of somatic mosaicism of the t(11;22) translocation and normal cells in humans (Kurahashi et al., 2000b). This indicates that the t(11;22) translocation in this case was generated during the mitotic cell cycles after fertilization. Another example is the existence of *de novo* cases of Emanuel syndrome (Kurahashi et al., 2000b). Emanuel syndrome generally occurs via 3:1 segregation of the translocation chromosomes during meiosis I in a t(11;22) balanced carrier. However, a *de novo* Emanuel syndrome case would have arisen via 3:1 segregation of the t(11;22) chromosomes during the pre-meiotic somatic cell cycles of gametogenesis.

ANALYSIS OF THE PATRR BY NEXT-GENERATION SEQUENCING

In addition to PATRR11 and PATRR22, a dozen PATRRs have been found at other translocation breakpoints. A recurrent t(17;22)(q11.2;q11.2) translocation was found in neurofibromatosis type 1 patients (Kehrer-Sawatzki et al., 2002; Kurahashi et al., 2003). Identification of another recurrent translocation between 8q24.1 and 22q11.2 led to the definition of a new malformation syndrome (Sheridan et al., 2010). Other PATRRs at 4q35.1, 1p21.2, 3p14, and 9p21 were identified at the breakpoints of non-recurrent constitutional translocations (Nimmakayalu et al., 2003; Gotter et al., 2004; Tan et al., 2013; Kato et al., 2014). These PATRRs share little homology but have features of AT-richness and symmetric palindromic structure in common. Intriguingly, all of the palindrome-mediated translocations occur between one PATRR and another PATRR.

We attempted to perform genome-wide screening of *de novo* PATRR-mediated translocations to identify unknown PATRRs using next-generation sequencing. We used the PATRR22 sequence as bait for the detection of any unknown sequences next to the PATRR22 due to *de novo* translocation. However, several difficulties were encountered. We could not confirm the presence of the translocation because most of the PATRR-mediated non-recurrent translocations occurred at a frequency below the detection levels of PCR using sperm from normal healthy donors. Furthermore, we could not analyze the novel translocation junction because the partner sequence could not be mapped to the human reference sequence. None of the translocation-related PATRR sequences identified to date appear in the human genome assembly.

Although the genome projects for many organisms including humans determined their complete nucleotide sequences, difficult-to-sequence regions remain as "gaps." Recent novel sequencing technologies have made it possible to access some of the gaps and provide more precise genomic data (Chaisson et al., 2015). The PATRR sequences do not appear even in such human reference databases. Palindrome sequences are one such type of a difficult-to-sequence region due to a "triple whammy" of factors affecting sequence analysis: the palindromic sequences are generally refractory to cloning to vectors, PCR amplification, and Sanger sequencing (Inagaki et al., 2005; Lewis et al., 2005). These features are due to the nature of the palindromic sequence itself. The longer the palindrome, the more difficult its analysis.

DEEP SEQUENCING OF THE PATRR REGION HAS GENERATED A NOVEL HYPOTHESIS

We applied next-generation sequencing technology to determine the complete sequence of the PATRR on 8q24, which was found at the breakpoint of t(8;22)(q24;q11) (Mishra et al., 2014). Sequencing of a random sheared library of PCR products and reconstruction of the original DNA via the computer-aided alignment of thousands of DNA molecules allowed us to successfully determine the entire PATRR8 (**Figure 1B**). The next-generation sequencing method does not require cloning and can directly analyze numerous DNA molecules at the same time. Although this strategy still requires PCR to amplify the single molecules and improve signal detection, the random digestion of the palindrome increases the chance of generating asymmetric cleavages of the palindromic center, which improves the PCR efficiency.

By means of this system, we determined the entire PATRR8 sequence, even at the center of the symmetry. This PATRR8 sequence allowed us to develop t(8;22)-specific PCR primers to analyze the junction fragments. The breakage always occurred at the center of the PATRR8 and PATRR22. The fusion was accompanied by the deletion of small nucleotides at the breakpoint regions. Interestingly, the nucleotide sequences around the junctions are identical between the der(8) and der(22) (Mishra et al., 2014). This cannot happen if the two breakages at the PATRR8 and PATRR22 occur independently and are followed by random nucleotide deletion at the breakage ends. This implies coordinated processing of PATRR8 and

PATRR22. Similar features of identical junctions in the two derivative chromosomes were also found in t(11;22) and t(17;22) (Kurahashi and Emanuel, 2001a; Kurahashi et al., 2003).

The standard models for gross chromosomal rearrangement include the breakage-first model and the contact-first model (Misteli and Soutoglou, 2009). In the breakage-first model, two DNA breaks located far from each other in the nucleus seek each other out to form a fusion chromosome. The artificial translocation model for the observation of the spatiotemporal chromosomal location in living cells revealed the dynamic movement of chromosomes after their breakage (Roukos et al., 2013). On the other hand, according to the contact-first model, translocation takes place between two closely located sites in the nucleus. Our previous data suggested that PATRR11 and PATRR22 are closer than other control chromosomal regions, indicating that this shorter distance might partly contribute to the recurrent nature of the t(11;22) translocation (Ashley et al., 2006). However, these two models do not explain specific translocations between two PATRRs.

Again, the identical sequences of the two derivative chromosomes imply that the two DNA breakage sites are unlikely to have been processed independently. The two derivative chromosomes were likely to be generated in a coordinated manner. Taken together, in the case of a PATRR-mediate translocation, PATRR appears to extrude cruciform structures at some stage during spermatogenesis. The two cruciform DNA molecules seek each other out and finally join together (**Figure 2**). In our translocation model system in cultured cells described above, the data suggested that two cleavage processes—cleaved diagonal cleavage of the cruciform structure and cleavage of the tip of the hairpin structure—are involved in translocation development (Inagaki et al., 2013). Our data also suggest that the pathway involves the participation of Artemis and ligase IV, which are components of the V(D)J recombination system that act by bringing two chromosomal sites close together

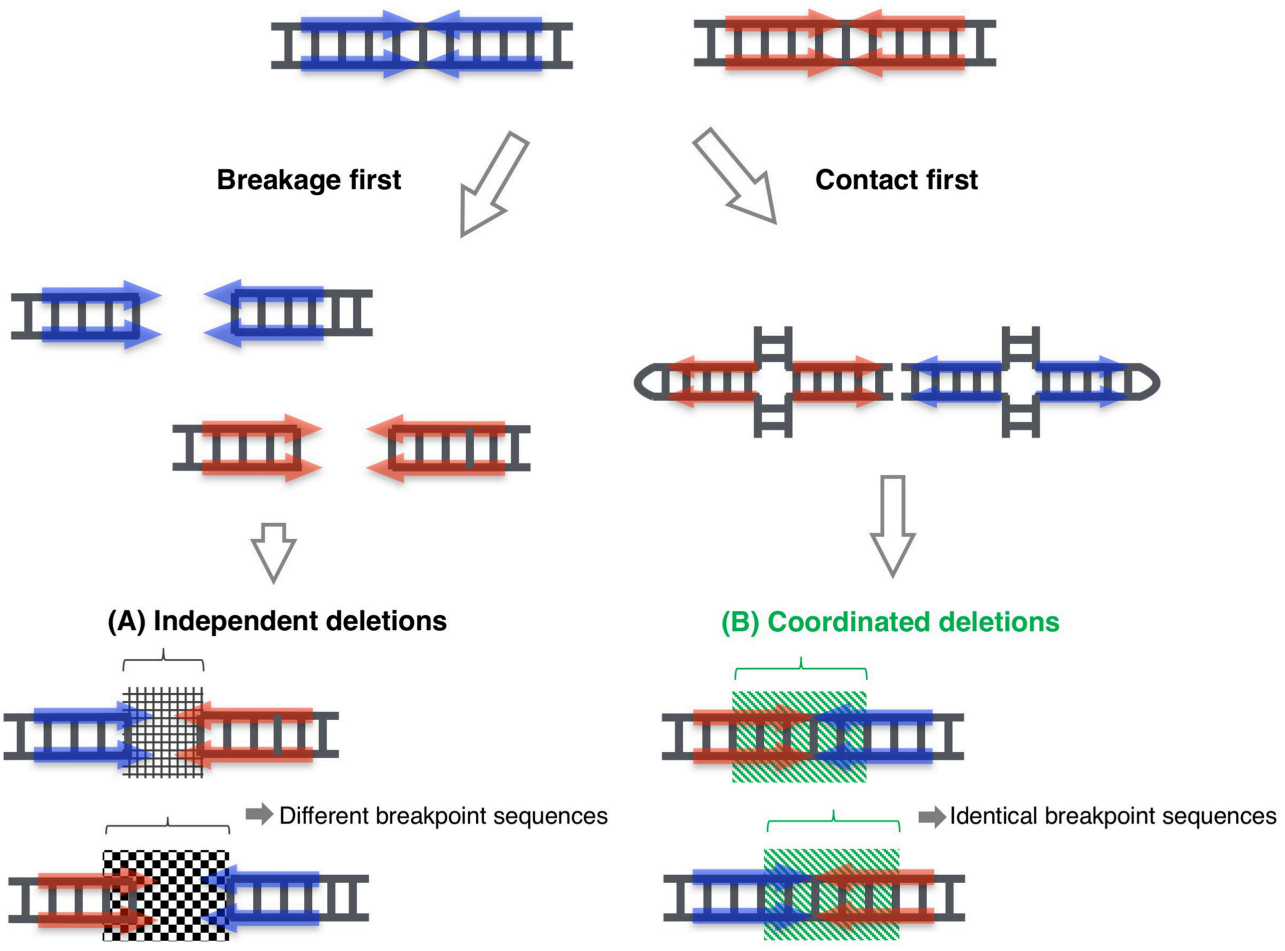

FIGURE 2 | Proposed model for coordinated joining of derivative chromosomes. Two derivative chromosomes have an increased likelihood of having identical junction sequences, indicating that exactly equal-sized deletions occurred in each palindrome center, which then joined to form two junction fragments. This phenomenon cannot be explained by the classical model, where the two double-stranded breakages are processed independently **(A)**. This could happen when the breakpoints of the derivative chromosomes are generated in a coordinated manner **(B)**. (Inagaki et al., 2013; Mishra et al., 2014).

and connecting them. In V(D)J recombination, RAG1 and RAG2 proteins bind the two cleavage sites to hold the resulting ends, both of which are specific for the V(D)J recombination machinery in lymphocytes. Similar mechanism is known in a DNA repair system of non-homologous end joining, in which Ku70/80 holds the two broken end until the subsequent repair machinery associate to process and join the ends (Deriano and Roth, 2013). Artemis and ligase IV as well as DNA-PK and other factors also participate in the joining reactions. It is possible that a part of such systems, or other novel factors might be involved in the contact between the two extruded cruciform structures and in keeping them in position during processing until the two derivative chromosomes are generated. We are now investigating how two cruciform DNA molecules come close together to elucidate the third mechanistic model that leads to recurrent chromosomal translocations in humans. Such investigation of dynamics of the cruciforms in nuclei will shed light on the role of non-B DNAs in gross chromosomal rearrangements in other eukaryotes.

AUTHOR CONTRIBUTIONS

HI and HK wrote the initial manuscript. All authors discussed the text and commented on the manuscript.

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Molecular Cytogenetics

CASE REPORT Open Access

A case with concurrent duplication, triplication, and uniparental isodisomy at 1q42.12-qter supporting microhomology-mediated break-induced replication model for replicative rearrangements

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Abstract

Background: Complex genomic rearrangements (CGRs) consisting of interstitial triplications in conjunction with uniparental isodisomy (isoUPD) have rarely been reported in patients with multiple congenital anomalies (MCA)/intellectual disability (ID). One-ended DNA break repair coupled with microhomology-mediated break-induced replication (MMBIR) has been recently proposed as a possible mechanism giving rise to interstitial copy number gains and distal isoUPD, although only a few cases providing supportive evidence in human congenital diseases with MCA have been documented.

Case presentation: Here, we report on the chromosomal microarray (CMA)-based identification of the first known case with concurrent interstitial duplication at 1q42.12-q42.2 and triplication at 1q42.2-q43 followed by isoUPD for the remainder of chromosome 1q (at 1q43-qter). In distal 1q duplication/triplication overlapping with 1q42.12-q43, variable clinical features have been reported, and our 25-year-old patient with MCA/ID presented with some of these frequently described features. Further analyses including the precise mapping of breakpoint junctions within the CGR in a sequence level suggested that the CGR found in association with isoUPD in our case is a triplication with flanking duplications, characterized as a triplication with a particularly long duplication-inverted triplication-duplication (DUP-TRP/INV-DUP) structure. Because microhomology was observed in both junctions between the triplicated region and the flanking duplicated regions, our case provides supportive evidence for recently proposed replication-based mechanisms, such as MMBIR, underlying the formation of CGRs + isoUPD implicated in chromosomal disorders.

Conclusions: To the best of our knowledge, this is the first case of CGRs + isoUPD observed in 1q and having DUP-TRP/INV-DUP structure with a long proximal duplication, which supports MMBIR-based model for genomic rearrangements. Molecular cytogenetic analyses using CMA containing single-nucleotide polymorphism probes with further analyses of the breakpoint junctions are recommended in cases suspected of having complex chromosomal abnormalities based on discrepancies between clinical and conventional cytogenetic findings.

Keywords: 1q, Complex genomic rearrangement, Uniparental isodisomy, DUP-TRP/INV-DUP structure, Microhomology-mediated break-induced replication model, Template switching, Chromosomal microarray, Breakpoint junction sequence

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Background

Complex genomic rearrangements (CGRs) consisting of two or more breakpoint junctions have been frequently observed during the characterization of nonrecurrent microduplications associated with genomic disorders [1, 2]. The occurrence of CGRs, such as partial tetrasomy induced by an interstitial triplication, contiguous distally with an extended segment uniparental isodisomy (isoUPD), has recently been reported as a rare event [3–7]. The recent establishment of high-resolution chromosomal microarray (CMA) using probes designed to detect copy number variations (CNVs) and genotype single-nucleotide polymorphism (SNP) simultaneously in a genome-wide manner has accelerated the identification of cases with such CGRs + isoUPD observations [8]. Although the cause, mechanism, and phenotypic effect of such CGR + isoUPD remain unclear, Carvalho et al. [5] provided evidence that CGRs generated post-zygotically through microhomology-mediated break-induced replication (MMBIR) can lead to regional isoUPD. In this replication-based mechanism model, a triplicated segment inserted in an inverted orientation between two copies of the duplicated segments (duplication-inverted triplication-duplication, DUP-TRP/INV-DUP) followed by regional isoUPD is generated via template switches between homologs and sister chromatids using MMBIR [5].

Here, we report on a patient with the co-occurrence of interstitial trisomy at 1q42.12-q42.2 and tetrasomy at 1q42.2-q43, followed by a segmental isoUPD for 1q43-qter, as additional evidence for an MMBIR-based model generating DUP-TRP/INV-DUP rearrangement followed by isoUPD. Detailed molecular genetic analyses at the sequence level revealed the presence of microhomology at two breakpoint junctions of the CGR, probably underlying the formation of the complicated genomic alteration (CGR + isoUPD). Notably, this is the first case of CGR + isoUPD detected in the long arm of chromosome 1. In addition, the pattern of flanking duplications experimentally documented in the present case, namely, a long duplicated segment with a size on the order of megabases at the centromeric junction observed by CMA with a short duplication at the telomeric junction only identified by sequencing of the breakpoint, has not been reported previously.

Case presentation

The 25-year-old Japanese male reported on here was the first child of a non-consanguineous healthy mother (G0P0, 24 years of age) and father (details are unclear due to a divorce) with no notable family disease history. After an uncomplicated pregnancy, he had been born at 38 weeks of gestation by a normal delivery. His birth weight was 1958 g (−2.52 SD) and he was introduced into a neonatal incubator to treat intrauterine growth retardation (IUGR) and poor sucking by tube feeding for 20 days, although detailed medical records of his physique are not available. Physical examination at the age of 1 month showed height 46 cm (−3.4 SD), weight 2715 g (−2.6 SD), and head circumference 29.8 cm (−4.6 SD). The abilities to hold up his head, eat solid food, imitate the behaviors of others, and walk alone were recognized at 6 months, 18 months, 2 years and 6 months, and 3 years of age, respectively. The patient had never been able to speak until now, and his comprehension was limited to simple signs, but he recognized various sounds. At 3 years of age, he was diagnosed with the congenital heart defect of tetralogy of Fallot (TOF) but was not treated surgically, although he showed frequent squatting and cyanotic attacks. On physical examination at 24 years and 6 months of age, he showed growth retardation with height 136 cm (−6 SD), weight 28.1 kg (−3.3 SD), and severe mental retardation with a developmental quotient of 5. At 25 years of age, he had TOF, bilateral congenital inguinal hernia, bilateral cryptorchidism, club feet, scoliosis, Chilaiditi's syndrome, and several facial anomalies, such as thinning of the hair, strabismus, widely spaced eyes, a down-slanted palpebral fissure, low-set ears, a prominent forehead, and a coarse face. He has some missing teeth due to having suffered from periodontal disease. Serial complete blood counts showed thrombocytopenia, and magnetic resonance imaging showed cerebral atrophy especially of the frontal lobe, with enlargement of the ventricles. His karyotype at birth was reported to be normal, but repeatedly performed karyotyping revealed 46,XY,dup(1)(q32.1q42.1),inv(9)(p12q13).

Molecular cytogenetic studies

This research protocol for this study was approved by the local ethics committee of Tokushima University. Written informed consent for the participation of the patient in this study was obtained from the patient's mother DNA was extracted from a peripheral blood sample.

A high-resolution CMA using the CytoScan HD array (Affymetrix, Santa Clara, CA) with Chromosome Analysis Suite software (ChAS, Affymetrix) to process the raw data detected a 9.2-Mb trisomy at 1q42.12-q42.2, a 6.7-Mb tetrasomy consisting of the duplication of two haplotypes, each of which probably derives from either the father or the mother, at 1q42.2-q43, and a 8.2-Mb segment with the absence of heterozygosity at 1q43-qter consistent with isoUPD (arr[hg19]1q42.12q42.2(225,101,799_234,324,222)x3,1q42.2q43(234,330,738_240,992,219)x4,1q43qter(240,993,835_249,224,684)x2 hmz, Fig. 1a). Trisomic, tetrasomic, and iUPD regions contain 88, 38, and 94 Refseq genes, and 49, 21, and 24 OMIM genes, respectively. Neither copy number abnormalities nor iUPD around 1q42.2-qter was detected in the DNA of the patient's mother (data not shown). Since the genotyping

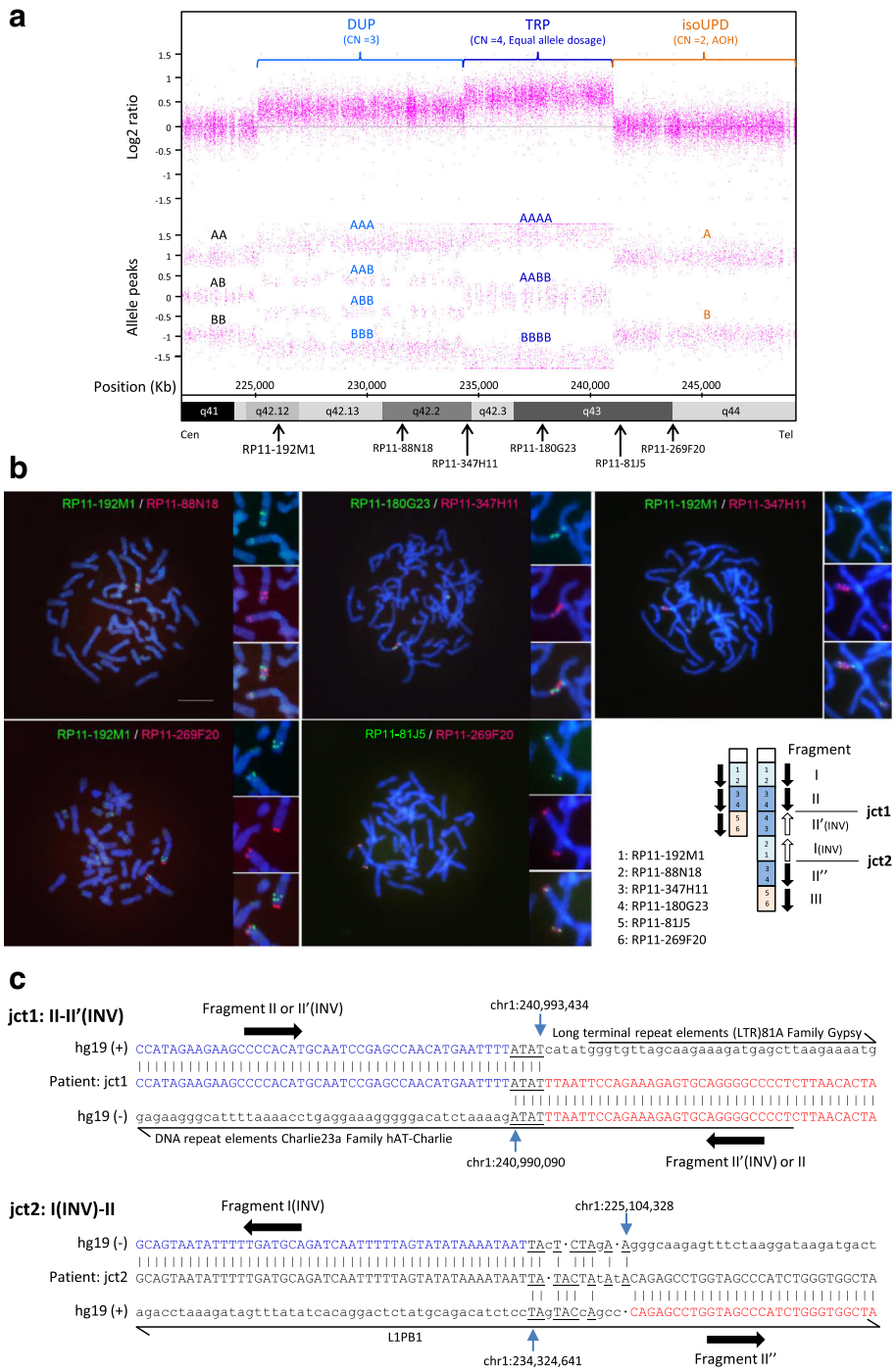

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 a Chromosome Analysis Suite (ChAS) graphic results of Affymetrix CytoScan HD analysis for the 1q region that presented duplication (DUP), triplication (TRP), or isoUPD in the patient. Detection of CGR and isoUPD were performed using an Affymetrix CytoScan HD CMA platform (Affymetrix), which provides 906,600 polymorphic (SNP) and 946,000 non-polymorphic (CNV) markers, according to the manufacturer's recommendations. In addition, we used Chromosome Analysis Suite software (ChAS, Affymetrix) to process the raw data, and the output data were interpreted with the UCSC Genome Browser (http://genome.ucsc.edu; GRCh37/hg19 assembly). *Top*, copy number log2 ratio; bottom, allele peaks. CN, copy number. Possible genotype calls based on the allele dosage normalization algorithm are shown using A and B. The location of each BAC used for FISH analysis is shown. **b** Images of two-color FISH mapping using six BAC clones and the scheme of distal 1q CGR based on FISH data. Metaphase FISH images with high-magnification images of the distal 1q. BAC clones labeled with either FITC (*green*) or rhodamine (*red*) were hybridized to 4′,6-diamidino-2-phenylindole (DAPI)-stained chromosomes of the patient. The location and detailed information of each BAC are shown in Fig. 1a and Additional file 1: Table S1, respectively. In the scheme, arrows indicate the direction of chromosomal fragments I, II (II′, II″), and III, which presented duplication, triplication, and isoUPD, respectively, in CMA. Two junctions (jct 1 and jct2) between fragments II and II′ and between I and II″ are also shown. **c** Color-matched sequence alignment of breakpoint junctions in rearrangements. Top, jct1 (breakpoint junction 1 between segments II and II′); *bottom*, jct2 (breakpoint junction 2 between segments I and II″) (see Fig. 1b). Microhomology at the junctions is represented by underlined letters. Frequent mismatch sequences were only observed near jct2 within long-range PCR products (data not shown). Thick arrows indicate the possible orientation of chromosomal fragments. Various types of repeat elements observed around junctions are shown

results using SNP typing probe within the iUPD region of the patient matched at least one of the maternal alleles, the iUPD segment is likely to have been inherited from his mother (data not shown), although genomic DNA of his father was not available to confirm the inheritance of this region. On the other hand, genotyping results within the trisomic region suggest that the duplicated segment is unlikely to have been inherited from his mother (data not shown). In the tetrasomic region (the triplicated segment), three allele peaks (AA, AB, and BB) with unusually large spaces between them were observed (Fig. 1a), suggesting the presence of AA/AA, AA/BB, and BB/BB tracks, which is only possible if each parent contributed equally with two alleles (either AA or BB).

Next, the location and orientation of each segment within this structurally altered region were determined by a series of dual-color fluorescence *in situ* hybridization (FISH) studies using bacterial artificial chromosome (BAC) clones located around the region (Fig. 1a and b, Additional file 1: Table S1) performed as described elsewhere [9]. Two signals (duplication) with a direct-inverted orientation and three signals (triplication) with a direct-inverted-direct orientation were detected by probes on the trisomic and tetrasomic regions, respectively. The triplicated segment in an inverted orientation was observed between the proximal triplicated segment in a direct orientation (junction 1, jct1) and the distal duplicated segment in an inverse orientation. The distal triplicated segment in a direct orientation is joined with the inversely oriented distal duplicated segment (junction 2, jct2). The isoUPD segment is then joined with this triplicated segment and terminates the abnormal chromosome 1. Taking these findings together, the final karyotype was interpreted as 46,XY,der(1)dup trp(pter → q43::q43 → q42.12::q42.2 → qter).

Genomic investigation

For the precise mapping of breakpoint junctions in the CGR (jct 1 and 2), we first performed mate pair next-generation sequencing using the Nextera Mate Pair Sample Preparation Kit and Illumina HiSeq 1500 with 100 paired-end cycles according to the manufacturer instructions (Illumina, San Diego, CA). Reads were aligned to the human genome sequence using the Burrows-Wheeler Alignment tool 0.7.12. (http://bio-bwa.sourceforge.net). Two recurrent structural variations within 1q42.12-1qter were identified from the discordant read pairs around the estimated boundary areas by the expected number of reads per region and visual inspection using the Integrative Genomics Viewer. Long-range polymerase chain reaction (PCR) using primers designed around the estimated boundaries (Additional file 2: Table S2) and Takara LA Taq (Takara Bio, Otsu, Japan) with the two step protocol according to the manufacturer instructions. The direct sequencing of PCR products defined sequences around two breakpoint junctions, jct1 and jct2 (Fig. 1c). Based on these results, the duplication and the triplication start around chr1:225,104,328 and 234,324,641, respectively, and the triplication stops around 240,990,090. Interestingly, the small telomeric duplication, namely, of approximately 3 Kb, which evaded CMA detection, is located between 240,990,090 and 240,993,434, and isoUPD starts around 240,993,434, although the copy number of the distal flanking duplication was not experimentally validated. Therefore, the CGR observed in our case seems to involve triplication with flanking duplications, which has been characterized as a type II triplication proposed by Liu et al. [10] with a particular DUP-TRP/INV-DUP structure, and isoUPD was also reported to be associated with this type of CGR [5]. Notably, all reported cases with triplication with flanking duplications followed by isoUPD have small flanking duplications (<0.258 Mb and < 0.004 Mb in proximal and distal duplications, respectively) [5], indicating that our case is the first with a large proximal duplication (approximately 9.2 Mb) in this type of CGR. Microhomology (ATAT) was observed at the jct1 breakpoint interval, whereas a microhomologous sequence with some mismatch sequences including insertions, deletion,

and point mutations was observed at the jct2 breakpoint interval (Fig. 1c). Mismatch sequences only near jct2 of CGR, which might occur during the same event as the *de novo* CGR/isoUPD formation, have previously been reported [5]. These mismatch sequences near to the breakpoint junctions of CGR are proposed to be one of the potential signature features of highly error prone replication-based mechanisms using DNA polymerase(s) of low fidelity or a replisome with reduced fidelity [2], although it remains unclear why mismatch sequences have been observed only in jct2 of CGR/isoUPD cases.

Within the isoUPD region, three genes were associated with four autosomal recessive diseases, as determined by a search of the Online Mendelian Inheritance in Man database (OMIM, http://www.omim.org, accessed 1 December, 2016; Additional file 3: Table S3). No phenotypes matching these four diseases were observed in the patient described here, and no pathogenic mutation was found in the three genes by Sanger sequencing. In addition, databases of imprinted genes, such as Geneimprint (http://www.geneimprint.com/site/genes-by-species, accessed 1 December, 2016) and the Catalogue of Parent of Origin Effects (http://igc.otago.ac.nz/home.html, accessed 1 December, 2016), indicated that there are no known imprinting genes within this isoUPD region.

Discussion

In the case presented here, our comprehensive analyses of all of the cytogenetic, microarray, and sequencing data suggest that the MMBIR-based template-switching model (Fig. 2a) recently proposed by Carbalho et al. [5] is one of the most plausible mechanisms underlying the gain of interstitial copy number followed by distal isoUPD to the telomere, which has not previously been described in the long arm of chromosome 1. In this model, two-step template switches triggered by stalled or collapsed replication forks might have occurred. The first template switch is supposed to use a sister chromatid to resume replication. Microhomology at the annealing site (jct1, Fig. 1c) in the complementary strand close to breakpoint is used to prime DNA synthesis, although it is difficult to determine whether this template switching occurred between c and d_c or d and c_c in our sequencing method. Then, unidirectional replication resumes in an inverted orientation and forms an inverted partially duplicated segment. A new event of fork stalling or collapsing might occur and release a free 3' end, which can be resolved by a second template switch to the homologous chromosome using microhomology again, resulting in the formation of a jct2 (Figs. 1c and 2a). This second compensating inversion might contribute to result in a viable cell. A target annealing site was selected between alleles B and C in the present case, and the derivative chromosome results in a DUP-TRP/INV-DUP structure with a unique long proximal duplicated region (b and b_c, Fig. 2b). Because BIR cannot account for the observations of microhomology identified in both jct1 and jct2 (Fig. 1c), MMBIR is probably involved in resolving both the first and the second breaks. In our case and some previously reported cases [5], however, various mismatch sequences including insertions, deletions, and/or point mutations around breakpoint junction sequences were observed only in jct2 of CGR and the size of the proximal duplicated region containing jct2 was commonly larger than that of the distal duplicated region containing jct1. Therefore, the accomplishment of the resolution of the second break might need additional mechanisms. It also remains unknown whether those two events occurred either all at once in a post-zygotic mitotic cell or in two steps: the first step occurring in a pre-meiotic cell was resolved by the second step occurring in a post-zygotic cell. These alternatives cannot be distinguished using the current data. In addition, it also difficult to rule out tissue-specific mosaicism as a post-fertilization mitotic event in this case, although no finding of mosaicism was observed in all data obtained from the peripheral leukocytes/lymphocytes of the patient.

Recently, several cases along with our own with concurrent triplication (tetrasomy) and isoUPD, which may be explained by the MMBIR-based mechanism, detected by CMA containing SNP probes, have been reported [4–7]. However, detailed analyses of centromeric and telomeric junctions of triplicated regions in a tiling array or at the sequence level have only been performed on the cases reported by Carvalho et al. [5] and the present case. In most of those cases with detailed junctional analyses, relatively short flanking duplications were observed. These findings suggest that the small size of flanking duplications might have led to the evasion of array-based detection in three reported cases without detailed junction analyses [4, 6, 7]. Indeed, the concurrent triplication (tetrasomy) and isoUPD were detected by Affymetrix arrays including SNP probes in all cases, but a flanking duplication was observed in this analysis only at the centromeric junction in the present case. In addition, microhomology was observed in breakpoint junctions in most of the cases with the DUP-TRP/INV-DUP rearrangement followed by isoUPD reported by Carvalho et al. [5] and the present case, suggesting that an MMBIR-based mechanism might underline the formation of at least this type of genomic alteration implicated in constitutional disorders. Detailed junction analyses of additional cases showing CGRs + isoUPD will be needed to provide support for an MMBIR-based mechanism inducing complex copy number gains and segmental isoUPD in tandem in subjects with multiple congenital anomalies.

Because partial 1q trisomy is a rare disorder and unbalanced chromosomal translocations are often observed

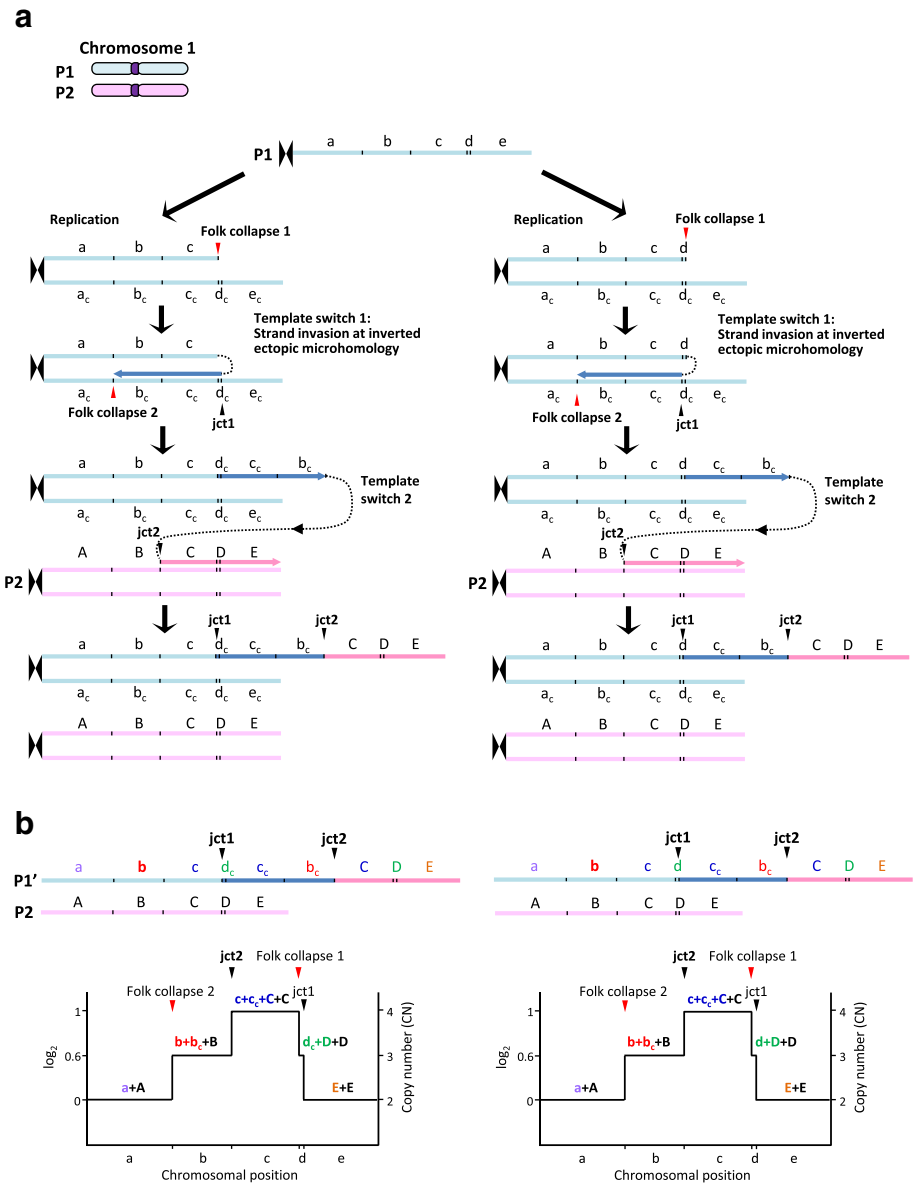

Fig. 2 Replication-based mechanism model for the generation of DUP-TRP/INV-DUP rearrangement followed by isoUPD detected in the present case. **a** The event probably occurred involving parental homolog chromosomes, P1 and P2. The first template switch (template switch 1) have been triggered by a stalled or collapsed replication fork (fork collapse 1), and used a complementary strand to resume replication through using microhomology in the complementary strand at the annealing site (jct1, Fig. 1c) to prime DNA synthesis, resulting in the production of a segment with the inverse orientation compared with the reference genome. Two putative jct1 sites, jct1 between c and d_c (*left*) and jct1 between d and c_c, (*right*) are predicted, because the same sequence result can be obtained in both cases (see Fig. 1c). Then, a new fork stalling or collapsing event (fork collapse 2) have released a free 3' end that can be resolved by the second template switching (template switch 2) through using the microhomology in the homologous chromosome at the annealing site (jct2, Fig. 1c) to prime and resume DNA synthesis, resulting in the generation of jct2 as well as isoUPD. a–d, representative chromosome alleles in P1 chromosome; a_c–e_c, complementary chromosome alleles in P1 chromosome; A–E: corresponding homologous chromosome alleles in the P2 chromosome. **b** *Top*: different genomic structures are predicted to be generated depending on the location of the selected annealing site (jct1) to prime DNA synthesis in the first template switch event. isoUPD will result if the unidirectional replication fork continues until the telomere. *Bottom*: predicted segmental CNV in a simulated CMA. Note that the small size of the telomeric duplication between fork collapse 1 and jct1 led to the evasion of CMA detection (Fig. 1a), because the region was too small to be detected by Affymetrix Cytoscan HD array

with this alteration [11–16], it is difficult to evaluate the contribution of 1q trisomy to the phenotype in cases involving another chromosome. Patients with pure partial distal trisomy 1q are known to demonstrate a wide range of manifestations of variable severity. However, distal 1q duplication syndrome is characterized by the

signs present in many of the previously reported cases [15, 16]. The present case showed some of the symptoms characteristic of distal 1q duplication syndrome, such as psychomotor developmental delay, cardiac defect, widely spaced eyes, a down-slanted palpebral fissure, low-set ear, a prominent forehead, club feet, and scoliosis, although psychomotor developmental delay and cardiac defect were very severe compared with those in previously reported cases and some features commonly found elsewhere were not observed [15, 16]. Because the present patient is the first known case of pure distal partial 1q tetrasomy and trisomy, it is possible that the copy number increase in some of the genes located between 1q42.12 and the middle of 1q43 (approximately 180 RefSeq genes) contributes to these symptoms, although no causal regions responsible for each symptom of distal trisomy/tetrasomy 1 syndrome have been clarified. In addition, the influence of isoUPD on the clinical features of the present case remains unknown because of a lack of reported cases with distal 1q UPD.

Conclusions

We report the first case with concurrent CGR (duplications and triplication) + isoUPD in 1q42.12-qter, from an initial diagnosis of interstitial trisomy 1q by conventional karyotyping. Comprehensive cytogenetic and molecular analyses provide additional evidence that DUP-TRP/INV-DUP rearrangement having a unique long proximal DUP structure followed by isoUPD may be generated by an MMBIR-based mechanism. Because it is almost impossible to quantify precise chromosomal copy numbers and detect UPD by conventional karyotyping, molecular cytogenetic analyses using CMA containing SNP probes with additional detailed analyses of the breakpoint junctions in a sequence level are recommended in cases suspected of having complex chromosomal abnormalities based on clinical and cytogenetic findings.

Additional files

Additional file 1: Table S1. BAC clones used in FISH experiments. (DOCX 14 kb)

Additional file 2: Table S2. List of primer sets used in PCR and sequencing for junctions of the CGR. (DOCX 14 kb)

Additional file 3: Table S3. Autosomal recessive diseases and causative genes aound the isoUPD region. (DOCX 14 kb)

Abbreviations

BAC: Bacterial artificial chromosome; CGR: Complex genomic rearrangements; CMA: Chromosomal microarray; CNV: Copy number variation; DUP-TRP/INV-DUP: Duplication-inverted triplication-duplication; FISH: Fluorescence *in situ* hybridization; isoUPD: Uniparental isodisomy; IUGR: Intrauterine growth retardation; MCA: Multiple congenital anomalie; MMBIR: Microhomology-mediated break-induced replication; PCR: Polymerase chain reaction; SNP: Single-nucleotide polymorphism; TOF: Tetralogy of Fallot

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file. More details are available on request.

Authors' contributions

TK, NO, and TN performed the genetic analysis and drafted the paper. CM performed the FISH experiments. YO, NF, and HI performed the genetic analysis. SS and NO collected the data of the patient. MS, KM, and HK contributed in writing the manuscript. Il performed CMA, contributed in writing the manuscript, and supervised the study. All the authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Mother of the patient has given her informed written consent for publication of the present case report.

Ethics approval and consent to participate

The research protocol for this study was approved by the local ethics committee of Tokushima University. Written informed consent for the participation of the patient in this study was obtained from the patient's mother.

Publisher's Note

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Complex X-Chromosomal Rearrangements in Two Women with Ovarian Dysfunction: Implications of Chromothripsis/Chromoanasynthesis-Dependent and -Independent Origins of Complex Genomic Alterations

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Keywords
Chromothripsis · Genomic rearrangement · Isochromosome · Turner syndrome · X inactivation

Abstract
Our current understanding of the phenotypic consequences and the molecular basis of germline complex chromosomal rearrangements remains fragmentary. Here, we report the clinical and molecular characteristics of 2 women with germline complex X-chromosomal rearrangements. Patient 1 presented with nonsyndromic ovarian dysfunction and hyperthyroidism; patient 2 exhibited various Turner syndrome-associated symptoms including ovarian dysfunction, short stature, and autoimmune hypothyroidism. The genomic abnormalities of the patients were characterized by array-based comparative genomic hybridization, high-resolution karyotyping, microsatellite genotyping, X-inactivation analysis, and bisulfite sequencing. Patient 1 carried a rearrangement of unknown parental origin with a 46,X,der(X)(pter→p22.1::p11.23→q24::q21.3→q24::p11.4→pter) karyotype, indicative of a catastrophic chromosomal reconstruction due to chromothripsis/chromoanasynthesis. Patient 2 had a paternally derived isochromosome with a 46,X,der(X)(pter→p22.31::q22.1→q10::q10→q22.1::p22.31→pter) karyotype, which likely resulted from 2 independent, sequential events. Both patients showed completely skewed X inactivation. CpG sites at Xp22.3 were hypermethylated in patient 2. The results indicate that germline complex X-chromosomal rearrangements underlie nonsyndromic ovarian dysfunction and Turner syndrome. Disease-causative mechanisms of these rearrangements likely include aberrant DNA methylation, in addition to X-chromosomal mispairing and haplo-

E.S., H.S., and M.T. contributed equally to this study.

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insufficiency of genes escaping X inactivation. Notably, our data imply that germline complex X-chromosomal rearrangements are created through both chromothripsis/chromoanasynthesis-dependent and -independent processes.

Complex chromosomal rearrangements are common in cancer genomes and can also appear in the germline [Liu et al., 2011; Kloosterman and Cuppen, 2013]. To date, germline complex rearrangements have been identified in a small number of individuals [Liu et al., 2011; Ochalski et al., 2011; Auger et al., 2013; Kloosterman and Cuppen, 2013; Plaisancié et al., 2014]. Of these, complex autosomal rearrangements were often associated with congenital malformations and mental retardation, which probably reflect dysfunction or dysregulation of multiple genes on the affected chromosome [Liu et al., 2011; Kloosterman and Cuppen, 2013; Plaisancié et al., 2014]. In contrast, complex X-chromosomal rearrangements were detected primarily in women with nonsyndromic ovarian dysfunction and were occasionally associated with other clinical features such as short stature, muscular hypotonia, and an unmasked X-linked recessive disorder [Ochalski et al., 2011; Auger et al., 2013]. The lack of severe developmental defects in women with complex X-chromosomal rearrangements is consistent with prior observations that structurally abnormal X chromosomes, except for X;autosome translocations, frequently undergo selective X inactivation [Heard et al., 1997]. The clinical features of these women, such as ovarian dysfunction and short stature, are ascribable to X-chromosomal mispairing and haploinsufficiency of genes that escape X inactivation [Zhong and Layman, 2012]. Mutations in *BMP15* at Xp11.22, *POF1B* at Xq21.1, *DIAPH2* at Xq21.33, or *PGRMC1* at Xq24 have been shown to lead to ovarian dysfunction, while mutations in *SHOX* at Xp22.33 impair skeletal growth [Bione et al., 1998; Bione and Toniolo, 2000; Mansouri et al., 2008; Zhong and Layman, 2012]. However, considering the limited number of reported cases, further studies are necessary to clarify the phenotypic characteristics of germline complex X-chromosomal rearrangements. Furthermore, it remains uncertain whether such rearrangements perturb DNA methylation of the affected X chromosomes.

Recent studies revealed that complex genomic rearrangements are caused by catastrophic cellular events referred to as chromothripsis and chromoanasynthesis [Liu et al., 2011; Pellestor, 2014; Leibowitz et al., 2015; Zhang et al., 2015]. Chromothripsis is characterized by massive DNA breaks in a single or a few chromosomes followed by random reassembly of the DNA fragments [Liu et al., 2011; Pellestor, 2014; Zhang et al., 2015]. Chromothripsis is predicted to arise from micronucleus-mediated DNA breakage of mis-segregated chromosomes, although several other mechanisms such as telomere erosion, p53 inactivation, and abortive apoptosis have also been implicated [Liu et al., 2011; Pellestor, 2014; Zhang et al., 2015]. Chromothripsis typically results in copy-number-neutral translocations/inversions or rearrangements with copy number loss; however, in some cases, genomic rearrangements with copy number gain have also been linked to chromothripsis [Liu et al., 2011; Pellestor, 2014]. Copy number gains in these cases are ascribed to replication-based errors during chromosomal reassembly [Liu et al., 2011]. Chromoanasynthesis is proposed to arise from serial template switching during DNA replication [Leibowitz et al., 2015]. Chromoanasynthesis has been reported as a cause of complex rearrangements with duplications and triplications [Leibowitz et al., 2015]. To date, the clinical significance of germline chromothripsis/chromoanasynthesis has not been fully determined. In particular, it remains unknown whether these catastrophic events account for all cases of complex rearrangements in the germline. Here, we report the clinical and molecular characteristics of 2 women with complex X-chromosomal rearrangements.

Patients and Methods

Patients

Patients 1 and 2 were unrelated Japanese women. Patient 1 was hitherto unreported, while patient 2 was previously reported as a female with Turner syndrome [Uehara et al., 2001]. Both patients underwent G-banding analysis in endocrine clinics and were found to have X-chromosomal rearrangements. Thus, they were referred to our institute for further investigation.

Molecular Analysis

Copy number alterations in the genomes were analyzed by comparative genomic hybridization using catalog human arrays (2x400K or 4x180K formats; Agilent Technologies, Palo Alto, CA, USA). We referred to the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) to exclude benign copy number polymorphisms. Then, we genotyped 15 microsatellite loci on the X chromosome. Each locus was PCR-amplified using fluorescently labeled forward primers and unlabeled reverse primers. Primer sequences are available from the authors upon request. We also examined the X inactivation status by performing methylation analysis of CpG sites and microsatellite assays of a polymorphic CAG repeat tract in the androgen receptor (*AR*) gene. The methods were described previously [Muroya et al., 1999]. Furthermore, to clarify whether the genomic rearrangements in the patients affect the

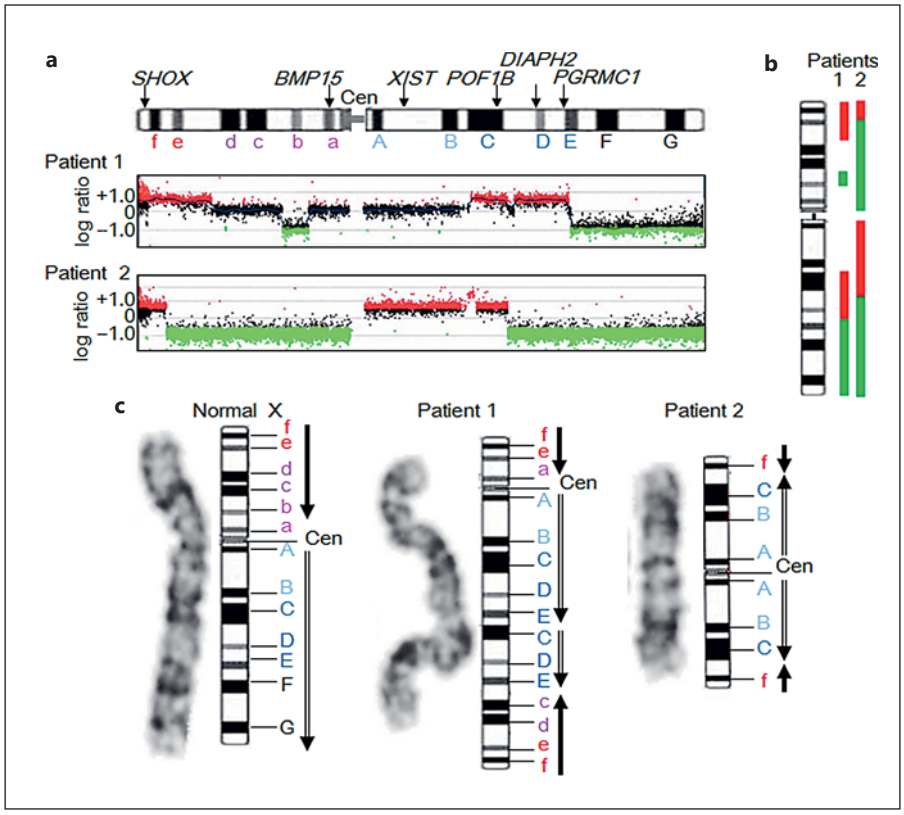

Fig. 1. a Array-based comparative genomic hybridization of the patients' X chromosomes. The black, red, and green dots denote normal, increased (log ratio higher than +0.4), and decreased (log ratio lower than –0.8) copy numbers, respectively. The upper panel shows the structure of the X chromosome and the positions of *SHOX*, *BMP15*, *XIST*, *POF1B*, *DIAPH2*, and *PGRMC1*. Cen, centromere. **b** Summary of copy number alterations in patients 1 and 2. The red and green lines depict duplicated and deleted regions, respectively. **c** High-resolution banding of a normal and the rearranged X chromosomes. The black and double-line arrows indicate the orientation of the X chromosome segments (from pter to the centromere and from the centromere to qter, respectively).

DNA methylation of X-chromosomal genes, we performed bisulfite sequencing for CpG sites in the upstream region of *SHOX*. In this experiment, genomic DNA samples were treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). A DNA fragment (chrX:580,597–580,771, hg19, build 37) containing 12 *SHOX*-flanking CpG sites was PCR-amplified using a primer set that hybridizes with both the methylated and unmethylated clones. The PCR products were subcloned with the TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA, USA) and subjected to direct sequencing.

Results

Clinical Manifestations of Patients 1 and 2

Patient 1 was born to phenotypically normal nonconsanguineous parents. This patient showed normal growth during childhood. At 12 years of age, she developed goiter. She was diagnosed with hyperthyroidism and was treated with propylthiouracil for 13 years. This patient exhibited age-appropriate sexual development and experienced menarche at 12 years of age (mean menarcheal age in the Japanese population: 12.3 years). However, her menstrual cycles were irregular and ceased at 15 years of age. Blood examinations at 26 years of age revealed markedly increased gonadotropin levels. She received estrogen and progesterone supplementation and had periodic withdrawal bleeding. She was otherwise healthy and had no Turner stigmata. Her mental development was normal. Her adult height was within the normal range (151.0 cm, –1.3 SD).

Patient 2 was previously reported as a female with Turner syndrome [Uehara et al., 2001]. At 16 years of age, she presented with short neck, shield chest, and cubitus valgus. She also exhibited hypertension, diabetes mellitus, and autoimmune hypothyroidism. In addition, she showed severe short stature (138 cm, –3.8 SD) despite being treated with growth hormone from 8 years of age. She lacked spontaneous pubertal development and was diagnosed with hypogonadism. Her mental development was normal.

Characterization of Genomic Rearrangements

Patient 1 had a 46,X,der(X)(pter→p22.1::p11.23→q24::q21.3→q24::p11.4→pter) karyotype (Fig. 1). The rearranged X chromosome involved at least 5 breakpoints and showed copy number gain of ~20-Mb and ~27-Mb regions at Xp and Xq, respectively, and copy number loss of ~7-Mb and ~36-Mb regions at Xp and Xq, respective-

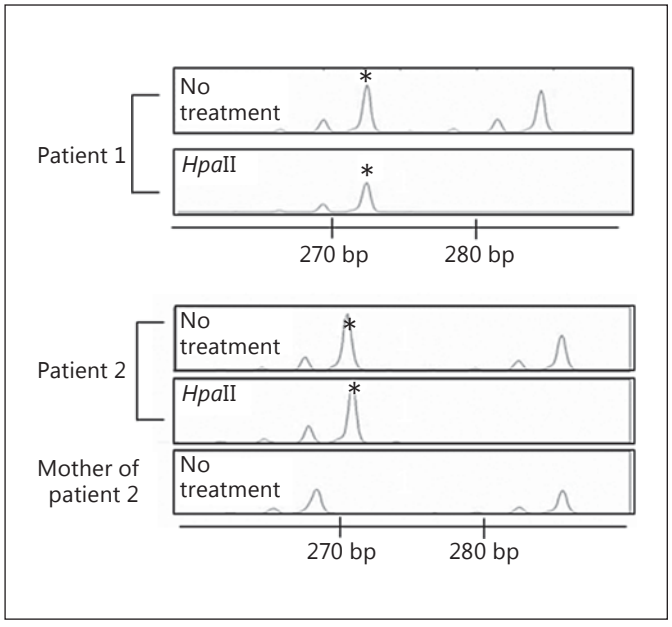

Fig. 2. X-inactivation analysis of *AR*. Microsatellite analysis was performed for polymorphic dinucleotide repeats before and after digestion with the methylation-sensitive enzyme *Hpa*II. In patient 1, the 274-bp peak (indicated by an asterisk) represents the PCR products amplified from the inactive X chromosome, while the 283-bp peak indicates the products amplified from the active X chromosome. In patient 2, the 271-bp peak (asterisk) represents the PCR products amplified from the inactive rearranged X chromosome, while the 286-bp peak depicts the products amplified from the maternally transmitted normal X chromosome. These data suggest that the rearranged X chromosome of patient 2 was of paternal origin.

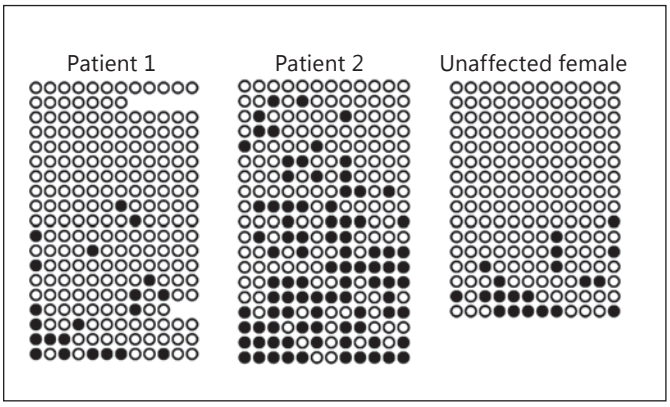

Fig. 3. Methylation analysis of *SHOX*-flanking CpG sites. Each horizontal line indicates the results of 1 clone. Filled and open circles indicate methylated and unmethylated cytosines in the CpG dinucleotides, respectively.

Table 1. Representative results of the microsatellite analysis in patient 2 and her mother

Locus	Chromosomal position[a]	Copy number in the genome of patient 2	PCR products, bp	
			patient 2	mother
SHOX (CA)	Xp22.33	3	142/150	132/142
DXYS233	Xp22.33	3	277	277
DXYS85	Xp22.33	3	200/204	204
DXS1449	Xp22.33	3	116	116
DXS85	Xp22.2	3	174/232	174/232
DXS8025	Xp11.4	1	186	180/186
DXS1069	Xp11.4	1	256	256
DXS1068	Xp11.4	1	254	250/254
ALAS2	Xp11.21	1	155	155/157
AR	Xq12	3	271/286	268/286
DXS8020	Xq22.1	3	194/196	194/196
HPRT1	Xq26.2–26.3	1	290	282/290
DXS8377	Xq28	1	233	229/233
DXS7423	Xq28	1	187	183/187
DXS15	Xq28	1	148	146/148

[a] Based on Ensembl Genome Browser (http://www.ensembl.org).

ly. This rearrangement caused overdosage of *SHOX*, *POF1B*, *DIAPH2*, and *PGRMC1* but did not affect the copy number of *BMP15* or *XIST* (X inactive specific transcript). X-inactivation analysis confirmed completely skewed inactivation (Fig. 2). *SHOX*-flanking CpG sites were barely methylated both in patient 1 and in an unaffected control individual (Fig. 3).

Patient 2 had a 46,X,der(X)(pter→p22.31::q22.1→q10::q10→q22.1::p22.31→pter) karyotype (Fig. 1). The rearranged X chromosome comprised at least 3 breakpoints and showed copy number gain of an ~8-Mb region at Xp and an ~40-Mb region at Xq and copy number loss of an ~53-Mb region at Xp and an ~54-Mb region at Xq. *SHOX*, *XIST*, and *POF1B* were duplicated, while *BMP15*, *DIAPH2*, and *PGRMC1* were deleted. There were no copy-number-neutral regions on this X chromosome. Microsatellite analysis suggested that this chromosome consisted of 2 identical arms ("isochromosome") of paternal origin (Table 1). The rearranged X chromosome was selectively inactivated (Fig. 2). *SHOX*-flanking CpG islands in patient 2 were hypermethylated (Fig. 3).

Discussion

We characterized complex germline X-chromosomal rearrangements in 2 patients. The clinical manifestations of the patients are consistent with the genomic structure. First, both patients manifested ovarian dysfunction. This feature is attributable to X-chromosomal mispairing, as suggested in cases of Turner syndrome due to X mono-

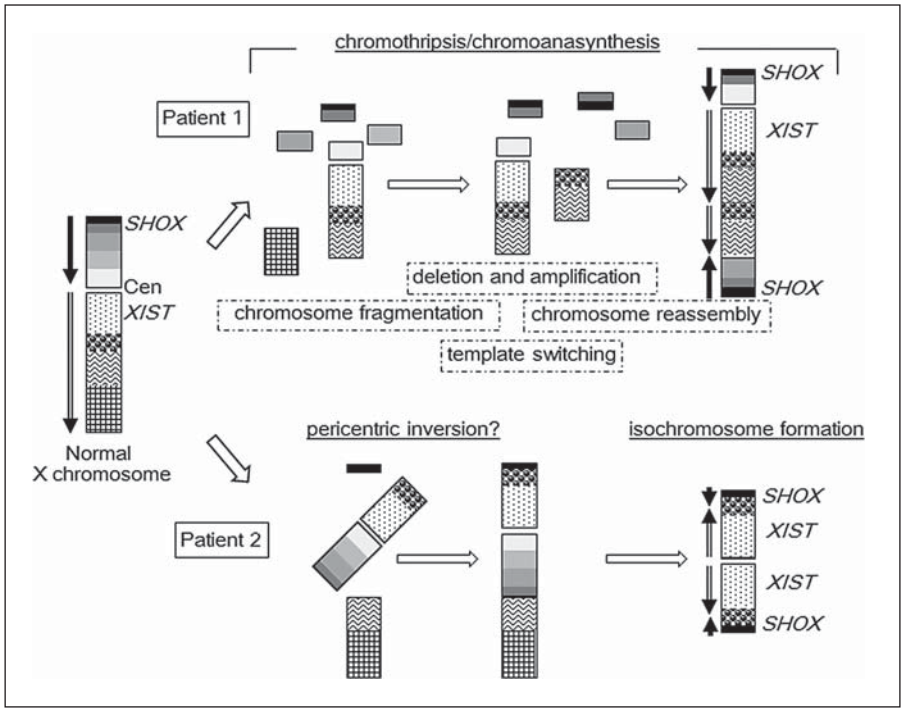

Fig. 4. Predicted mechanisms of the chromosomal rearrangements. The black and double-line arrows indicate the orientation of X chromosome segments (from pter to the centromere and from the centromere to qter, respectively). The rearranged X in patient 1 is consistent with a catastrophic reconstruction due to chromothripsis/chromoanasynthesis, while that in patient 2 likely results from 2 independent sequential events. It remains to be clarified whether the father of patient 2 carries a pericentric inversion.

somy [Ogata and Matsuo, 1995]. Furthermore, patient 2 lacked *BMP15*, *DIAPH2*, and *PGRMC1*, which have been implicated in ovarian function [Bione et al., 1998; Bione and Toniolo, 2000; Mansouri et al., 2008]. Copy number changes of other genes might also have contributed to the ovarian dysfunction in patients 1 and 2, because multiple X-chromosomal loci have been linked to this phenotype [Zhong and Layman, 2012]. Second, Turner stigmata such as short neck, shield chest, and cubitus valgus were observed in patient 2 but not in patient 1. These results support the previously proposed notion that a lymphogenic gene responsible for Turner stigmata resides at Xp11.2 [Ogata et al., 2001a], a genomic region deleted in patient 2 and preserved in patient 1. Third, both patients manifested thyroid disorders. Notably, isochromosome Xq is known to be associated with a high risk of autoimmune thyroid disorders [Elsheikh et al., 2001]. Indeed, the hypothyroidism of patient 2 may have resulted from copy number gain of *GPR174* at Xq21.1, because increased expression of *GPR174* has been linked to the risk of an autoimmune thyroid disorder [Chu et al., 2013]. However, the copy number of *GPR174* remained intact in patient 1. Thus, the genomic interval at Xq21.32q22.1> Xq21.32-q22.1, duplicated in both patients, may contain a hitherto uncharacterized gene associated with autoimmune thyroid disorders. Lastly, patient 1 had a normal stature, and patient 2 showed severe short stature, although both patients carried 3 copies of *SHOX*. This is inconsistent with previous findings that trisomy of the Xp22.3 region encompassing *SHOX* leads to tall stature [Ogata et al., 2001b]. In patients 1 and 2, positive effects of *SHOX* overdosage on skeletal growth may be balanced by negative effects of X-chromosomal mispairing and copy number alterations of minor growth genes on the X chromosome. Furthermore, short stature in patient 2 may be associated with *SHOX* dysregulation, because *SHOX*-flanking CpG islands were hypermethylated in this individual. These sites were barely methylated in the control individual, which is in agreement with the fact that *SHOX* escapes X inactivation [Rao et al., 1997]. It has been shown that in patients with X;autosome translocations, aberrant DNA methylation can spread to regions larger than 1 Mb of the autosomal segments [Cotton et al., 2014]. Hypermethylation of the *SHOX*-flanking CpG sites in patient 2 may reflect decreased physical distance between *SHOX* and *XIST* and/or copy number gain of *XIST*.

The genomic rearrangements in patients 1 and 2 appear to have been formed through different mechanisms (Fig. 4). The rearrangement in patient 1 is consistent with catastrophic reconstruction due to chromothripsis/chromoanasynthesis [Liu et al., 2011; Leibowitz et al., 2015].

This case provides further evidence that X-chromosomal chromothripsis/chromoanasynthesis accounts for a small portion of cases with nonsyndromic ovarian dysfunction. In contrast, the rearrangement in patient 2 is inconsistent with the "all-at-once" nature of chromothripsis/chromoanasynthesis [Liu et al., 2011; Hatch and Hetzer, 2015]. The rearranged chromosome of this patient had 2 identical arms consisting of Xp and Xq material, indicating that this chromosome arose by 2 independent sequential events, namely, a fusion between the Xp22.31 and Xq22.1 segments followed by isochromosome formation. Notably, the rearrangement occurred in the paternally inherited X chromosome. Thus, although the Xp22.31;Xq22.1 translocation is the simplest explanation of this rearrangement, it is implausible in this case, because X;X translocation rarely occurs during male meiosis. The results of patient 2 can be explained by assuming that the phenotypically normal father carried a pericentric inversion, inv(X)(p22.31q22.1), which was subjected to meiotic or postzygotic isochromosome formation (Fig. 4). However, since a paternal DNA sample was not available for genetic testing, we cannot exclude the possibility that this rearrangement was formed via other rare processes.

In conclusion, the results indicate that complex X-chromosomal rearrangements in the germline lead to ovarian dysfunction with and without other Turner syndrome-associated features. Clinical outcomes of such rearrangements likely reflect X-chromosomal mispairing, haploinsufficiency of genes escaping X inactivation, and/or perturbed DNA methylation. Most importantly, our findings imply that germline complex X-chromosomal rearrangements are created through both chromothripsis/chromoanasynthesis-dependent and -independent processes.

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Statement of Ethics

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent.

Disclosure Statement

The authors have no competing interests to declare.

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Intragenic *DOK7* deletion detected by whole-genome sequencing in congenital myasthenic syndromes

OPEN

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ABSTRACT

Objective: To identify the genetic cause in a patient affected by ptosis and exercise-induced muscle weakness and diagnosed with congenital myasthenic syndromes (CMS) using whole-genome sequencing (WGS).

Methods: Candidate gene screening and WGS analysis were performed in the case. Allele-specific PCR was subsequently performed to confirm the copy number variation (CNV) that was suspected from the WGS results.

Results: In addition to the previously reported frameshift mutation c.1124_1127dup, an intragenic 6,261 bp deletion spanning from the 5′ untranslated region to intron 2 of the *DOK7* gene was identified by WGS in the patient with CMS. The heterozygous deletion was suspected based on reduced coverage on WGS and confirmed by allele-specific PCR. The breakpoints had microhomology and an inverted repeat, which may have led to the development of the deletion during DNA replication.

Conclusions: We report a CMS case with identification of the breakpoints of the intragenic *DOK7* deletion using WGS analysis. This case illustrates that CNVs undetected by Sanger sequencing may be identified by WGS and highlights their relevance in the molecular diagnosis of a treatable neurologic condition such as CMS. ***Neurol Genet* 2017;3:e152; doi: 10.1212/NXG.0000000000000152**

GLOSSARY

aCGH = array comparative genomic hybridization; **AChE** = acetylcholinesterase; **CMS** = congenital myasthenic syndromes; **CNV** = copy number variation; **MLPA** = multiplex ligation-dependent probe amplification; **MuSK** = muscle-specific tyrosine kinase; **NMJ** = neuromuscular junction; **WES** = whole-exome sequencing; **WGS** = whole-genome sequencing.

Congenital myasthenic syndromes (CMS) are inherited disorders characterized by fatigable muscle weakness with or without other associated signs or symptoms.[1] They are caused by mutations in genes expressed at the neuromuscular junction (NMJ). DOK7 is one of the components of the NMJ and an activator of the muscle-specific tyrosine kinase (MuSK).[2] Recessive mutations in *DOK7* cause approximately 10% of the genetically diagnosed CMS cases.[1]

CMS are heterogeneous diseases, and to date, more than 25 genes have been reported to be causative. Consecutive single-gene screening has been routinely used as a diagnostic tool; however, next-generation sequencing allows the analysis of all these genes simultaneously to identify the causative variant and obtain a genetic diagnosis. The efficacy of whole-exome sequencing (WES) for the diagnosis of CMS cases has been reported,[3,4] as well as its ability to identify new causal genes.[5,6] However, the limitation is that WES is designed to detect only protein-coding regions and exon-intron boundaries of the genome.

Supplemental data at Neurology.org/ng

From the Institute of Genetic Medicine (Y.A., A.T., T.E., P.J.L., A.R., H.L.), Newcastle University, UK; Division of Neurology (P.J.L.), Federal University of Parana, Brazil; Leibniz-Institut für Analytische Wissenschaften ISAS e.V. (A.R.), Germany; Department of Neurosciences and Mental Health (P.V.), University of Lisbon, Portugal; and Division of Molecular Genetics (H.I., H.K.), Fujita Health University, Japan.

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On the other hand, whole-genome sequencing (WGS) allows the analysis of deep intronic, intergenic, and other noncoding regions. Furthermore, WGS allows to detect copy number variations (CNVs), as coverage is more homogeneous than that of WES.[7]

We present a CMS case in which a large intragenic *DOK7* deletion was identified by WGS compound heterozygous to a known exonic mutation.

METHODS ***DOK7* screening.** DNA from the patient was extracted from whole blood by standard methods. Screening of hot-spot mutations was performed by Sanger sequencing, encompassing a region of ~600 bp covering the previously reported European founder mutation c.1124_1127dup.[2] Subsequently, full screening of coding regions and exon-intron boundaries of the *DOK7* gene was performed. Primer sequences are listed in table e-1 at Neurology.org/ng. Annotation of the human *DOK7* cDNA is according to the GenBank accession number NM_173660.

Mutation analysis by WGS. WGS was performed by the TruSeq PCR–free library preparation kit and HiSeqX v2 SBS kit (Illumina, San Diego, CA) for 30× mean coverage on a HiSeqX sequencer. Reads were mapped against hg19 reference genome using the Burrows-Wheeler transform,[8] and duplicates were removed using Picard tools.[9]

Sequence variants were called using the Genome Analysis Toolkit.[10] WGS data were then analyzed using deCODE's platform (Clinical Sequence Miner; WuXi NextCODE, Cambridge, MA). Rare variants were filtered by threshold of coverage (≥8), variant call (≥2), and ratio of variant (≥0.2) and allele frequency of 1% in 1000 Genomes database.[11]

Sanger sequencing of large deletion. We amplified DNA samples to identify the suspected intragenic deletion with primers 5′-CCCAGATGGTGCGCTTGCTCC-3′and 5′-GCCCACCCCCTCACGCTCAG-3′. The PCR protocol comprised 35 cycles and annealing temperature of 68°C using HotStarTaq DNA polymerase with Q-Solution for the GC rich region (QIAGEN, Düsseldorf, Germany).

Standard protocol approvals, registrations, and patient consents. All human studies including genetic analysis were approved by institutional review boards, and appropriate written informed consent was obtained from all the patients and family members.

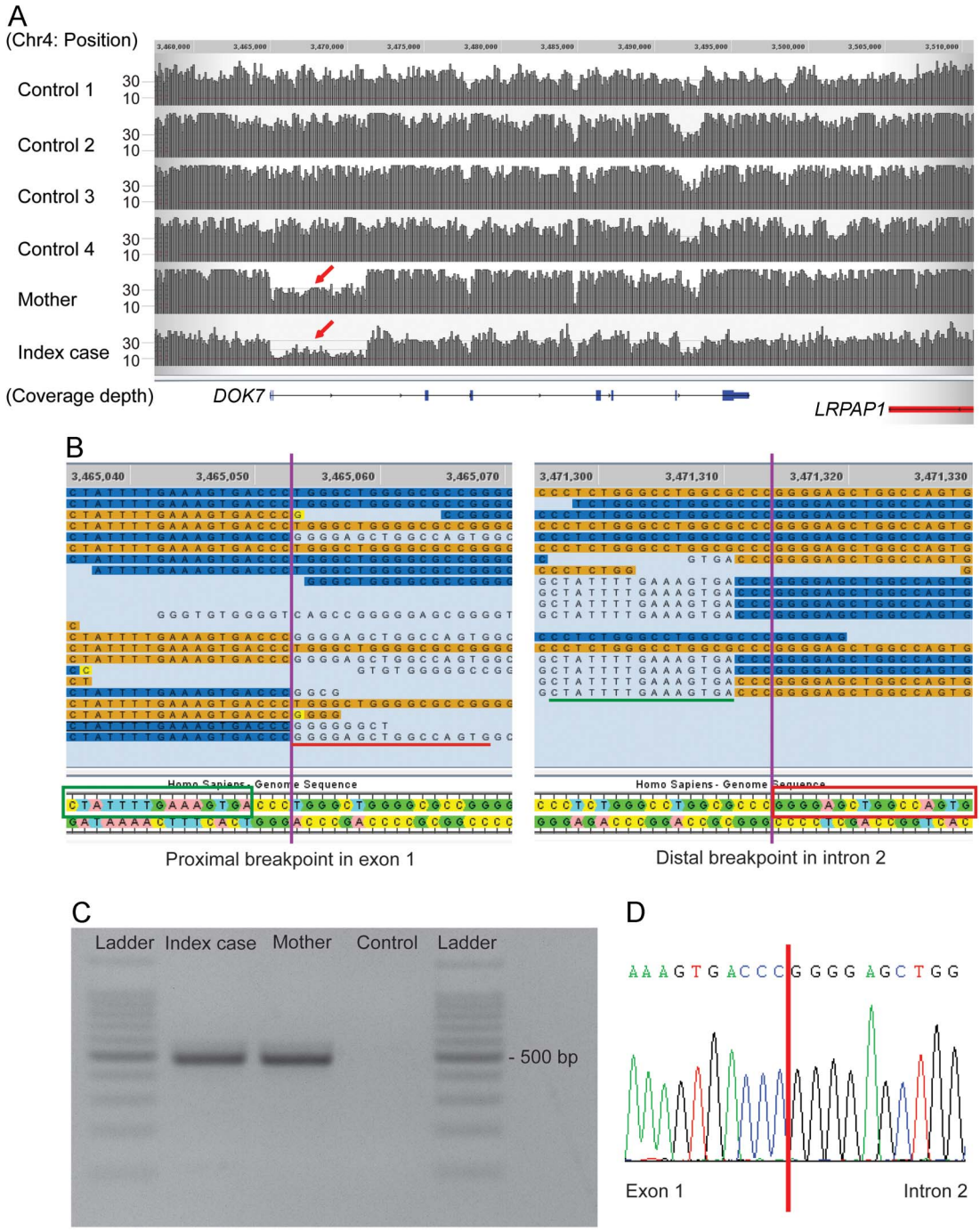
RESULTS **Clinical findings.** The patient is a 39-year-old Portuguese man who presented with bilateral ptosis and exercise-induced muscle weakness. He had no family history of muscle disease, and his motor milestones in childhood were normal. He showed mild ptosis from infancy and noticed mild lower limb weakness at 13 years of age. He was admitted to hospital for a month because of sudden severe generalized muscle weakness and worsening ptosis at 15 years of age. He has bilateral facial weakness and winged scapula, and the clinical diagnosis of a neuromuscular transmission defect was confirmed by neurophysiologic studies. EMG showed myopathic changes on facial muscles. Repetitive nerve stimulation showed a remarkable decremental response of 76% in proximal muscles. Both antiacetylcholine-receptor and anti-MuSK antibodies were negative, and immunosuppressive treatment was unsuccessful. Acetylcholinesterase (AChE) inhibitor of pyridostigmine up to 360 mg/d for 10 years had little effect and was discontinued without clinical deterioration after the trial of oral administration of salbutamol which effected significantly. He has not experienced severe muscle weakness for 5 years since salbutamol was started.

***DOK7* screening.** Based on the limb-girdle clinical presentation of the patient, a hot-spot region of *DOK7* was investigated as a first screening step. Sanger sequencing revealed that the patient carried the heterozygous c.1124_1127dup reported as a founder mutation in European CMS patients.[2] This mutation was not present in the mother (DNA from the father was unavailable). However, this single heterozygous mutation does not explain *DOK7*-CMS, which invariably shows autosomal recessive inheritance. To identify a second heteroallelic *DOK7* variant, the whole coding region and exon-intron boundaries of the *DOK7* gene were Sanger sequenced, but no potentially pathogenic exonic or splice site variants were found. The sample was therefore subjected to WGS to try to identify other mutations within the *DOK7* gene or elsewhere in the genome.

WGS analysis. As expected, applying a standard pipeline for variant filtering (minor allele frequency 1% in coding region), the heterozygous c.1124_1127dup in *DOK7* was detected in the WGS data. This filtering did not identify any other coding variants in known CMS causal genes.

However, visual inspection of the sequencing reads of the *DOK7* gene for this patient revealed that the read depth for exons 1 and 2 was lower than that of neighboring regions and other control samples (figure 1A). Furthermore, there were no heterozygous variants within this region, indicating a run of homozygosity or hemizygosity suggesting a single copy region. Close inspection of the boundaries of this region showed that in some instances, sections of the sequencing reads did not match the reference sequence. These reads were considered chimeric or split reads, as the unmatched sequences did align to a different region of the genome. Split reads are indicative of structural variation. In fact, the 3′ section of the split reads of the proximal boundary aligns to the 3′end of the distal boundary, and vice versa (figure 1B, red underline and red box). The proximal and distal breakpoints lie approximately 6 kb away. These findings suggested that this patient

Figure 1 **Whole-genome sequencing analysis and allele-specific PCR**



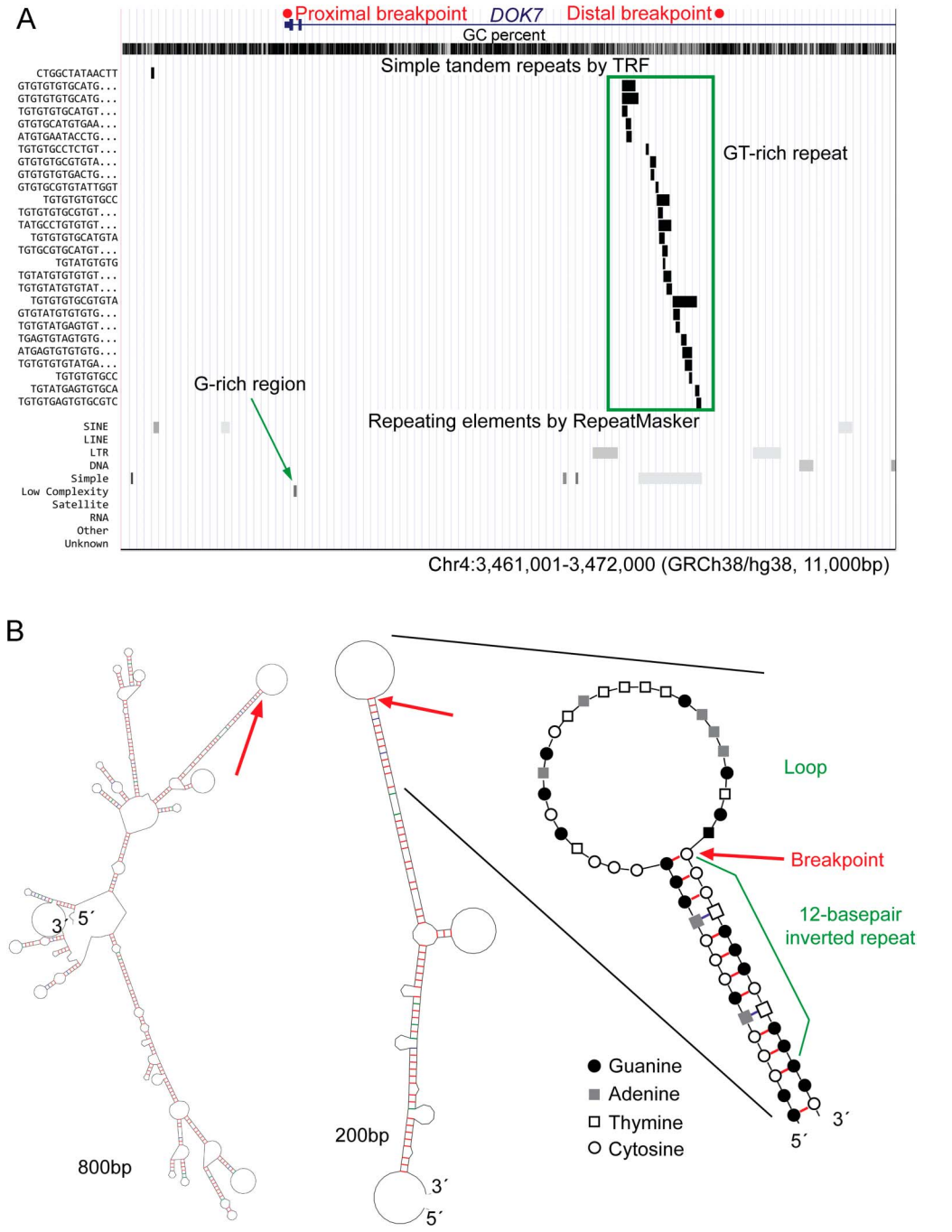
(A) Both index case and his mother show reduced read depth (coverage) from exon 1 to deep intron 2 of the *DOK7* gene (red arrow). Controls 1–4 correspond to samples sequenced and analyzed through the same pipeline and without the diagnosis of congenital myasthenic syndromes. (B) Split reads were observed at both presumed breakpoints. Nucleotides matching the reference sequence of *DOK7* are highlighted in orange/blue. Single unmatched nucleotides are highlighted in yellow, and further unmatched sequences are not highlighted. The unmatched sequence (indicated with red/green underline) of the split reads of the proximal breakpoint aligns to the reference sequence (indicated in green/red boxes) at the distal breakpoint, and vice versa. (C) The expected products amplified by allele-specific PCR were identified in the index case and the mother. (D) The junction of the breakpoint in the allele with the intragenic deletion was confirmed by Sanger sequencing of the PCR product. Coverage and reads were drawn by the graphical user interface of Sequence Miner 5.21.1 (WuXi NextCODE).

has a heterozygous 6-kb deletion in *DOK7* encompassing exons 1 and 2.

Identification and analysis of the intragenic *DOK7* deletion. We performed PCR using a pair of primers designed around 250 bp away from the presumed breakpoints of the deletion, between the 5′ untranslated region and intron 2. The expected product of 488 bp was amplified in the DNA samples of the patient, but not in control DNA

(figure 1C). The junction of the 2 breakpoints was identified by Sanger sequencing of the PCR product (figure 1D). The exact size of the deletion is 6,261 bp. The deletion was also detected by PCR in the mother, who did not carry the c.1124_1127dup mutation. We therefore concluded that the CMS in the patient is caused by the compound heterozygous mutations in *DOK7*.

Figure 2 **Analysis of the breakpoints of the intragenic 6-kb deletion**



(A) University of California Santa Cruz genome browser (genome.ucsc.edu/) view of the deleted region showing the Simple Tandem Repeats track (based on Tandem Repeats Finder, TRF[18]) and the Repeating Elements track (based on RepeatMasker[19]). GT-rich repeat regions (green box) are seen around the distal breakpoint, and a G-rich region (green arrow) is located near the proximal breakpoint. (B) The secondary DNA structure with the lowest delta G value was predicted by the mfold tool (unafold.rna.albany.edu/?q=mfold) for the 800 and 200 bp regions around the proximal breakpoint. An enlarged view of the breakpoint area highlighting the complementary nucleotides is also shown. The proximal breakpoint (indicated by the red arrows) is at the boundary of a loop and a 12-bp inverted repeat that may cause stalling of DNA replication. It is possible that deletion/duplication can occur if stalled replication resumes using an alternate location on the same chromosome. Red/blue/green bars represent hydrogen bonds between G-C/T-A/G-T.

The 2 breakpoints of the deletion have a C-triplet homology region, and the deleted region contains a G-rich region and GT-rich repeat region (figure 2A). In silico secondary structure analysis using the prediction program mfold[12] showed that the proximal breakpoint is at the boundary of a loop and a 12-bp inverted repeat (figure 2B). This may cause stalling of DNA replication and subsequently result in chromosomal structural changes including deletions, if replication resumes using an alternate chromosomal location.

Screening of the intragenic deletion in a CMS cohort. To identify carriers of single heterozygous mutations in *DOK7* (i.e., without a second rare variant within coding regions and exon-intron boundaries), we interrogated our database of clinically diagnosed CMS cases referred to us in the years 1996–2015. The total number of patients with CMS was 577, of which 7 genetically unsolved cases had single frameshift mutations in *DOK7* (c.1124_1127dup in 6 cases and c.1378dup in 1 case). These samples were amplified using the deletion-specific pair of primers used to detect the 6-kb deletion of the index family. All 7 samples were negative using this PCR method. This does not exclude that they carry CNVs in *DOK7* different from the one described in this study.

DISCUSSION We identified an intragenic *DOK7* deletion in a patient with clinically diagnosed CMS. Patients lacking a second heteroallelic mutation in *DOK7* were reported in a previous study.[2] Moreover, multiexon genomic deletions of *RAPSN*[13] and *COLQ*[14] have also been identified as causative of CMS. It is therefore conceivable that CNVs in *DOK7* may explain a proportion of cases assessed as negative or inconclusive by conventional sequencing analysis.

Our study shows the advantage of WGS analysis and detailed interrogation for detecting CNVs, using coverage and visual analysis of split reads. Traditionally, multiplex ligation–dependent probe amplification (MLPA) is considered the method of choice to detect previously described CNVs, where kits are available commercially. To identify new CNVs, however, specific MLPA primers for each gene need to be designed, rendering it expensive and time consuming for testing a genetically heterogeneous syndrome such as CMS. Array comparative genomic hybridization (aCGH) is also a valuable method for CNVs analysis; nevertheless, deletions/duplications are not detectable by aCGH if they are shorter than the spacing of the hybridization probes. In addition, neither MLPA nor aCGH can detect single nucleotide variants. Despite WES being widely used for clinical sequencing, the library preparation step results in uneven coverage, which makes the estimation of CNVs by read depth less reliable. This can be overcome by the homogenous coverage of WGS, allowing both the detection of single nucleotide as well as CNV.

WGS analysis is still more expensive than WES and Sanger sequencing. In addition, computational tools need further improvement in sensitivity and specificity to detect CNVs exhaustively.[15] Taken together, we believe that WGS is advantageous and will become the method of choice for genetic diagnosis in rare, heterogeneous conditions such as CMS. We suggest that previously unsolved cases or the carriers of a single mutation in a causal gene are especially suitable cases of CMS for WGS analysis. The 6-kb deletion was not identified in other cases tested by PCR, although it is inherited from the mother, suggesting this is likely a private mutation. However, it is possible that other CNVs in *DOK7* underlie in CMS cases.

We also determined the breakpoints of the 6-kb deletion, and analysis of the sequence and secondary structure suggested that long inverted repeats might cause the development of the deletion due to a stall of replication, and microhomology might have played a role in the repair process.[16] Further documentation of breakpoints and sequences would help understand the mechanism for the development of CNVs.

Obtaining genetic diagnosis of CMS is very important because the therapy varies depending on the affected gene. Poor response to AChE inhibitors is often observed in patients affected by limb-girdle CMS due to *DOK7* mutations. Salbutamol therapy has now been started for the patient described in this study, which has been reported of good response in *DOK7*-CMS.[17]

AUTHOR CONTRIBUTIONS

Yoshiteru Azuma: drafting the manuscript, acquisition of data, and analysis and interpretation. Ana Töpf: analysis and interpretation and critical revision of the manuscript. Teresinha Evangelista and Paulo José Lorenzoni: acquisition of data. Andreas Roos: analysis and interpretation and study supervision. Pedro Viana: acquisition of data. Hidehito Inagaki and Hiroki Kurahashi: analysis and interpretation. Hanns Lochmüller: study concept and design and study supervision.

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DISCLOSURE

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