ことからやりとりがはじまることである. ETの手の動きはまさにETからおやつを要求していたといえるものであったと考えられる. このエピソードでみられた相互交渉は，単にETがおやつを求めて手を動かしていたものかかわり手が読みとっておやつを渡すというものではなかった．次に，ETの手が，お皿やおやつそのものに向かうというよりは，Kの手に向けられていた. おやつの活動の中で，ETがKの手に触れたり，握ったりすることは以前からあったが，その場合ETはかかわり手の手を口へ運ぼうとする．しかしここでは，Kの手をつかむことなく，なでるようにして触っていた．ここでは，ETが第三の要素に注意を向けつつ，かかわり手に対してその行動を向けたと考えている.

この後主なかかわり手が佐藤に交替して後も，おやつを食べる活動が設定されたが，ETの独力による手での口への運搬操作に注目しすぎてしまい，状況がやや訓練的要素を帯びてしまった．実際ETの自主的なおやつへの接近の様子が減少してしまったため，操作の促進は控えるようにした．すると再び，視線による要求が見られるようになった.

3）もの（教材）の操作活動にみられた三項関係的相互交渉

2010年の時点で，教材としてスポンジ棒（図3）を手渡すと，ほぼ毎回それを口に持っていって噛む，なめるといった活動に専念して，これを介した相互の活動を展開することが難しかった．その後スポンジ棒に細工を凝らして，二人で引っ張れるような教材を作成した．この教材によって「引っ張り合い」を経験したETは，その後，スポンジ棒を出されても，これを口に持っては行くが，すぐに噛むのではなく，しきりに探索するようになり，2011年9月には途中でかかわり手を一瞥したりするようになった

また，毎回のかかわりにおいて，本児の視覚活用の活動として電灯や簡易暗室（リトルルーム）（図2）を使っての光遊びなどを提供してきた．簡易暗室においては，光源そのものへの手伸ばしが当初から明確にみられていたが，ET自身が点灯できるようにスイッチ教材を持ち込んで，ETの手をガイドしながら，自主的能動的な活動展開を目指した．スイッチと電灯との因果関係の理解がまだ不十分な様子が観察された際に，ETがそばにいる係り手に手を伸ばしてくるという行動が観察された．部屋の電灯をつけることも共同活動のメニューとしてきたが，2011年11月に，いったん消灯した電気をETが見上げている時に，かかわり手の手をETの手に添えて，ひもを引っ張って点灯してみると，ETは手のひらを電灯に向けて開いたり，閉じたりした．そして，再度かかわり手が消灯すると，ETはかかわり手の顔を見て，微笑み，そして手を伸ばしてくるということがあった.「楽しさ」の共有と，次の展開への呪術的な意味合いをもつ視線など，ここでは電灯にまつわるイベントをめぐる交渉が展開している.

D．考察

われわれは，これまでの機能評価の結果から，ETの感覚活用は可能ではあっても，相当制限されていることが伺われたので，視覚聴覚以外のもう一つの重要な感覚である触覚に着目し，触覚による外界交渉を積極的に提供した．その際にETの手をガイドすることが多くあったが，そのガイドにおいて単にETの手をこちらの意図通りに動かすのではなく，ET自身の動きが展開するように補助的に行うことが，ETの自主的・能動的な探索を促進することに繋がった.

前回の報告でも記したように，共感的なコミュニケーションが，情報伝達型コミュニケーションも含め，あらゆるコミュニケーションの土台となると考えられる（菅井，2007）. 相互交渉の発達的変化を促進するにおいては，本児の対象への身体の「志向性」に着目して，

注意対象の特定化と共有化を図り，本児に発現した行動が，円滑に展開することをたすけていくという基本方針が有効であった．また，実際のかかわりにおいては，相互作用を重視して可能な限り「やりとり」が展開する活動を工作していくことが，本児の自主的・能動的な外界への働きかけを促進することが示唆された．

E．結論

(1) 感覚障害のある場合の発達促進について

外界への働きかけにおいて，定位反応に注目することの重要性は，重複障害教育において従来から指摘されている．けれども，感覚障害がある場合に，見かけ上の行動から感覚活用を評価するだけでは，活用の潜在的可能性を考慮することができない．したがって，可能な限り感覚活用の実相について，丁寧な評価が必要であり，その具体的内容は前年度の報告で取り上げたとおりである．ここで示された評価に基づいて，係わり合いの場で具体的な配慮が重要になる．本児に活用の可能性が高かった視覚面では対象物の呈示の仕方，その距離，背景，対象物そのものの色やコントラストなどがある．視聴覚という外界からの情報の取り込みにおいて極めて重要な働きをなす感覚が障害される場合，触覚活用に注目することが求められる．振動を利用した教材では，本児からの明確な発信や快の情動の表出がみられた．

(2) 志向性を仮定したコミュニケーション

現象学のテーゼにある，なにかある対象に対して意識を向けることを志向性と捉え，ETの志向性を行動の中に仮定しつつかかわりを行ってきた．それは，ET の行動に特定の対象への方向性がみられたときに可能であった．例えば，おやつの場面で ET の手のひらが係り手の方に向かって開かれたとき，そこに見いだされた手の〈向き〉を捉え仮定した．当初は難しかったこの仮定が，次第に可能になって，それと同時に ET の情動の動きについての把握も可能になってきた．このことは子どもの動きに沿った状況設定や子ども関心に寄り添うこと，子どもの興味・関心を見つけることという従来から言われている重複障害教育の基本であるが，その最初の手がかりは子どもの動きに「向き」を見いだし，そこに志向性を仮定していくことである．この子どもの行動の向きに注意を払い，そこに志向性を仮定していくことが，重度の障害がある子どもの発達促進において一つの有効な手立てではないかと考える．ET においては，そのかかわりの過程で，二項関係的な外界交渉が，三項関係的交渉へと変化した．その経過の整理から，この志向性にもとづく働きかけが変化を促進したと考える．

F. 健康危険情報

特になし．

G. 研究発表

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なし

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H. 知的財産権の出願・登録状況

特になし．

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（資料）

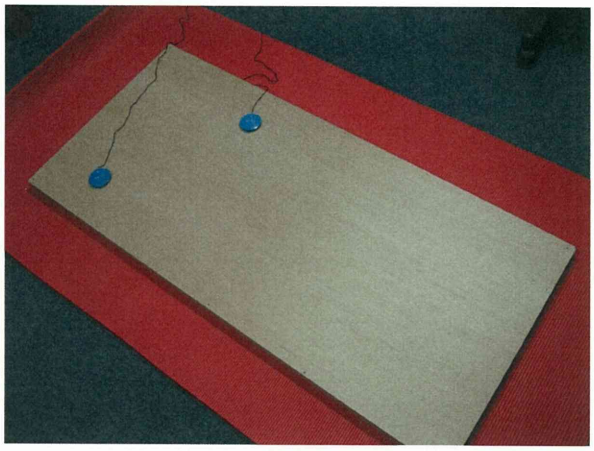

図１．リゾナンスボードと振動スピーカー

高さ 2，３㎝の木枠に厚さ 5～9 ㎜ほどの木板を貼り付けた反響板

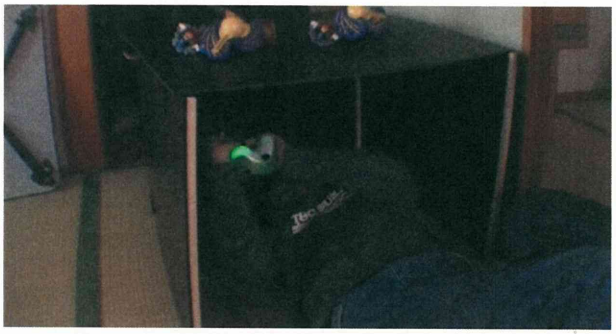

図２．簡易暗室（リトルルーム）

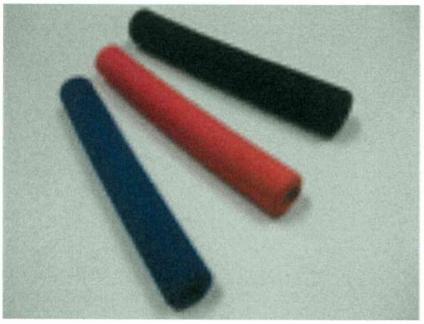

図３．スポンジ棒

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

エマヌエル症候群の疾患頻度とその自然歴の実態調査
（先天異常症候群患者家族支援に関する検討）

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研究要旨

エマヌエル症候群は染色体異常を原因とする先天異常症候群である。その多くは患児の母親が転座保因者であり、母親は自身が保因者としての心理的負担を感じる場合も多いと推察される。平成２２年の研究では、染色体構造異常保因者と診断された者11名を対象に半構造化面接を行い、保因者診断は診断された者に様々な心理社会的な影響を及ぼすことを報告した。このような患者・家族の心理支援としていろいろな患者家族会が活動しているが、本症候群では家族会がまだ設立されていない。本年度の研究として、小児専門の医療施設である埼玉県立小児医療センターに通院する患者家族を対象とした様々な先天異常症候群の疾患集団外来の本年度の活動実績を検討した。開催した集団外来は６疾患（コステロ症候群、4p モノソミー症候群、ベックウィズウィーデマン症候群、コフィンシリス症候群、片側肥大、プラダーウィリー症候群）について計７回であった。平均参加患者数は12家族（参加率は対象者の52%）、平均参加家族総数は 27 人であった。また保育を開催した場合には、２家族あたり１人程度の保育希望者があった。エマニュエル症候群を含めまだ家族会のない稀少疾患においては、地域のセンター的医療機関において集約的にフォロー体制を構築することで、同じ医療施設に通う仲間として集団外来を開催し、疾患の理解とともに患者・家族の心理的支援にもつながるものと期待できる。

A. 研究目的

エマヌエル症候群は染色体異常を原因とする先天異常症候群である。その多くは患児の母親が転座保因者であり、母親は自身が保因者としての心理的負担を感じる場合も多いと推察され、遺伝カウンセリングは殊更重要となる。平成２２年の研究では、染色体構造異常保因者と診断された者 11 名を対象に半構造化面接を行い、保因者診断は診断された者に様々な心理社会的な影響を及ぼすことを見出した。保因者にとっての心理的安定に寄与する要因としては、配偶者や両親など周囲の者の理解が鍵となっていた。また患者・家族会の存在はピアカウンセリングとしての効果が期待されており、様々な遺伝性疾患では患者・家族会が設立されて医療ではなしえない心理社会的支援となっている。しかしながら本邦では本疾患独自の患者・家族会はない。昨年度は本疾患の家族会設立の可能性を考える基礎情報として、他の稀少遺伝性疾患家族会の活動様態についての情報を収集し検討を行った。本年は、小児専門の医療施設としての埼玉県立小児医療センターに通院する様々な疾患について、病院ベースで行なっている患者家族を対象とした集団外来の本年度１年間の開催実績の概要を検討した。

B. 研究方法

埼玉県立小児医療センターで平成２３年度に開催した先天異常症候群の疾患集団外来につ

いて、１）開催した疾患、２）参加患者数(対象患者数、実際に参加した患者数、患者本人と家族を含めたトータルの参加人数)、３)、設定したテーマ（担当者)、４）保育状況について見当した。

（倫理面への配慮）

疾患の診断や病態評価に関わる遺伝学的解析は遺伝カウンセリングと書式によるインフォームドコンセントを得て行なった。

C. 研究結果（表参照）

１）集団外来を開催した疾患

平成２３年度に開催した集団外来は、コステロ症候群、4p モノソミー症候群、ベックウィズウィーデマン症候群、コフィンシリス症候群、片側肥大、プラダーウィリー症候群（乳幼児対象と学童期以降対象の２回開催）の６疾患ついて計７回であった。これらの疾患の中で特に理由があって開催したものとして、厚生労働省難治性疾患克服研究事業研究と連携したものがコステロ症候群と 4p モノソミー症候群、遺伝学的原因解明の研究プロジェクトへの参加に関連したものがコフィンシリス症候群、があった。また今回が初めての集団外来は、コフィンシリス症候群と片側肥大であった。

２）参加患者数

参加家族数は、最少４家族、最多２２家族、平均 11.7 家族であった。集団外来を案内した対象患者全員における参加者の割合は、最少 30%、最多 100%、平均 52%であった。コフィンシリス症候群（4/4；100%）は患者数が４家族と少なく、予め家族の予定を調整して日程を決定したため全員参加が実現している。それ以外ではコステロ症候群（6/7；86%）が最も参加率が高かった。

３）テーマについて

集団外来は２部構成としている。一部が疾患についての情報提供（講義)、二部が患者家族同士の交流である。一部の情報提供として

表．平成２３年度埼玉県立小児医療センター先天異常症候群集団外来

疾患	対象患者数	参加患者数	総数	テーマ	担当	保育	保育希望者	保育ボランティア	備考
コステロ症候群	7	6	10	コステロ症候群と歯科口腔所見について	遺伝科	無			厚生労働省研究と連携
4pモノソミー症候群	12	9	22	マイクロアレイでわかる欠失領域と症状との関連	遺伝科	無			厚生労働省研究と連携
ベックウィズウィーデマン症候群	43	22	58	ベックウィズ症候群の遺伝学的診断について	遺伝科	有	16	3	全国家族会と共同開催
コフィンシリス症候群	4	4	9	コフィンシリス症候群の疾患概念と健康管理について	遺伝科	無			遺伝子解析研究と連携
片側肥大	29	15	28	整形外科的治療について	整形外科	有	4	1	
プラダーウィリー症候群（乳幼児）	46	14	32	プラダーウィリー症候群の運動感覚機能の評価	作業療法士他	無			
プラダーウィリー症候群(学童期以降)	17	12	33	プラダーウィリー症候群の健康管理	遺伝科	有	4	3	

取り上げたテーマでは、７回のうち２回が疾患概念と健康管理という基本事項（コフィンシリス症候群、プラダーウィリー症候群乳幼児対象）で、２回が現在進行中の研究に関係したもの（コステロ症候群、4p モノソミー症候群)、その他に遺伝学的診断について（ベックウィズウィーデマン症候群)、整形外科的治療について（片側肥大)、運動感覚機能評価(プラダーウィリー症候群学童以降)と個別に設定したテーマであった。

４）保育について

保育を設定したのが、７回中３回であった。基本的には保育の開催を前提にしているが、参加者の人数や発育状況から保育の必要性が高くなかった２回（コステロ症候群、4p モノソミー症候群）と、患者本人参加型のプログラム（プラダーウィリー症候群学童期以降対象）では保育を行なわなかった。保育を行なった場合の保育人数は平均８人で、参加家族２家族あたり１人程度であった。また保育は平均２人のボランティアが対応したが、保育人数が多いときには医師や看護のスタッフが応援として加わった。

D. 考察

疾患の根本治療が困難な稀少遺伝性疾患では、その疾患とともに人生を送る同じ立場の当事者が互いの気持ちをわかりあい（ピアカウンセリング)、お互いの生活上の知恵や経験を共有することは、疾患の告知を受けた患者・家族にとって大きな支えとなる。現在規模の大小、組織や運営形態の差異はあるが、多くの疾患の家族会が活動している。ただ、エマヌエル症候群のようにまだ患者家族会が設立されていない疾患も稀ではない。家族会の設立運営は殆どの場合その推進役となる強い意志をもった少数（１人の場合もある）の個人的な負担（多大な時間と労力）のもとに成立していることが多く、先天異常症候群の家族会の設立を公的に支援するシステムは存在しない。そのようななかで、医療機関が場所と時間と専門職の知識を提供することで、患者・家族が集まり、情報を収集し、同じ患者・家族同士で情報を交換・共有し、交流をもつことは現状で実現が可能な一つの患者家族支援の方法と思われる。

人口の多い関東や関西の都市圏にある小児医療専門施設では、稀少疾患であっても一定数の患者をフォローしている場合も多いと考えられるが(実際当センターで本年度開催した疾患はいずれも数万人に１人以下の出生頻度の稀少疾患であるが、集団外来を開催できるだけの患者数のフォローがあった)、小児人口の多くない全国の地域においては、一施設でのフォロー人数には当然限界がある。そのために、複数の医療圏にまたがる広域の地域（人口として数百万人、毎年の出生数として数万人）に１箇所、先天異常症候群の情報センター的な役割をもつ医療機関が存在して、疾患集団外来を含めて包括的なフォローアップ体制を構築することが極めて重要であると考えられた。

E. 結論

先天異常症候群と診断された患者・家族の支援には患者・家族会の役割が重要である。医療機関が中心において疾患の集団外来は、患者・家族が疾患の情報を得て健康管理に生かすとともに、互いの交流によるピアカウンセリング的な心理支援を得るために有意義であると考える。そのために、都市圏では小児医療専門施設が、また地域圏では一定の規模の医療圏にまたがる先天異常症候群の情報センター機能をもつ医療機関の存在が重要と考えた。

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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

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MOLECULAR CYTOGENETICS

RESEARCH Open Access

DNA secondary structure is influenced by genetic variation and alters susceptibility to *de novo* translocation

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Abstract

Background: Cumulative evidence suggests that DNA secondary structures impact DNA replication, transcription and genomic rearrangements. One of the best studied examples is the recurrent constitutional t(11;22) in humans that is mediated by potentially cruciform-forming sequences at the breakpoints, palindromic AT-rich repeats (PATRRs). We previously demonstrated that polymorphisms of PATRR sequences affect the frequency of *de novo* t(11;22)s in sperm samples from normal healthy males. These studies were designed to determine whether PATRR polymorphisms affect DNA secondary structure, thus leading to variation in translocation frequency.

Methods: We studied the potential for DNA cruciform formation for several PATRR11 polymorphic alleles using mobility shift analysis in gel electrophoresis as well as by direct visualization of the DNA by atomic force microscopy. The structural data for various alleles were compared with the frequency of *de novo* t(11;22)s the allele produced.

Results: The data indicate that the propensity for DNA cruciform structure of each polymorphic allele correlates with the frequency of *de novo* t(11;22)s produced ($r = 0.77$, $P = 0.01$).

Conclusions: Although indirect, our results strongly suggest that the PATRR adopts unstable cruciform structures during spermatogenesis that act as translocation hotspots in humans.

Keywords: Polymorphism, Palindrome, Secondary structure, Hairpin structure, Cruciform structure, Breakpoint, Translocation

Background

Accumulating evidence indicates that alternative DNA structures (non-B DNA) cause a diversity of genomic rearrangements [1,2]. It is well known that a subset of repeat sequences such as trinucleotide repeats sustain dynamic mutations via DNA secondary structure intermediates leading to their expansion or contraction [3]. The finding that the t(14;18) translocation observed in follicular lymphoma might result from instability of triplex DNA at the breakpoint implies that gross chromosomal rearrangements can also be mediated by non-canonical DNA structures [4,5]. A large-scale survey demonstrates that translocation breakpoints or deletion endpoints in human genetic diseases are consistently found in proximity to predicted non-B DNA structures [6].

Chromosomal translocations have long been thought to be random events. However, recent findings have highlighted two distinct mechanisms that lead to recurrent translocations in humans [7]. A subset of recurrent translocations arises between two homologous regions located on different chromosomes. Robertsonian translocations are mediated by highly repetitive regions on the short arms of the five acrocentric chromosomes, while t(4;8)(p16;p23) translocations result from exchange between two clusters of olfactory-receptor genes on 4p and 8p presumably via homologous recombination [8,9]. Another mechanism is the so-called palindrome-

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mediated chromosomal translocation [10]. Palindromic AT-rich repeats (PATRRs) were first identified at the breakpoints of the recurrent constitutional t(11;22)(q23; q11) [11-13]. All of the translocation breakpoints are located within the 450 bp PATRR on 11q23 (PATRR11) and the 590 bp PATRR on 22q11 (PATRR22), which do not share sequence homology with one another [14]. The majority of the breakpoints are located at the center of the PATRRs, suggesting that genomic instability of the palindrome center is the etiology of the recurrent translocation [15]. PATRRs also contribute to other recurrent and non-recurrent translocations such as the t (17;22)(q11;q11) [16,17], t(4;22)(q35;q11) [18], t(1;22) (p21.2;q11) [19], and t(8;22)(q24.13;q11.21) [20,21]. Translocation-specific PCR can frequently detect *de novo* t(11;22)s in sperm from normal healthy males [22].

Recently, we also identified *de novo* PATRR-mediated t(8;22)s as well as t(8;11)s by a similar PCR method, suggesting that a considerable proportion of the translocations result from a palindrome-mediated mechanism [21].

Palindromic DNA has the potential to form a secondary structure, an extruded DNA cruciform, through the intra-strand base pairing of adjacent inverted repeat units. A number of palindromic sequences have been identified in the human genome [23], but not all of the palindromes behave as sites for translocation breakpoints. The translocation-associated PATRRs reported so far, share a common structure, 1) a nearly perfect palindrome of several hundred base pairs in length, 2) an AT-rich center and a non-AT-rich region at both ends, 3) another nearby AT-rich region on one side of the PATRR, all of which invoke cruciform structure forming propensity [24]. Indeed, the cloned PATRRs identified at the translocation breakpoints assume a cruciform conformation *in vitro* [25,26]. We propose that the PATRR also adopts a cruciform conformation in living cells, which induces genomic instability leading to translocation formation in humans. In fact, the propensity for secondary structure of the PATRRs on chromosomes 11, 17 and 22 reflects the relative incidence of the relevant chromosomal translocations [27].

In our previous study, we demonstrated that the PATRR11 at the translocation breakpoint often manifests size polymorphisms due to central deletions within the PATRR11, and that this polymorphism affects the frequency of *de novo* t(11;22)s in sperm samples from normal healthy males [28]. Subsequently, we demonstrated that PATRR22 polymorphisms also impact *de novo* translocation frequency [29]. To determine whether PATRR polymorphisms influence secondary structure leading to variation in their translocation frequency, we investigated the secondary structure forming potential of each polymorphic PATRR11 and compared it with its relevant translocation frequency. The results suggest that propensity for secondary structure formation is reflected in the rate of translocations formed.

Results

Size and symmetry of the palindromes affect *de novo* translocation frequency

To better understand how polymorphic variants of the palindromic sequence affects *de novo* translocation frequency in sperm, we classified the polymorphic PATRR11s into three categories based on the size and symmetry of the palindromic sequences (Figure 1A). The most frequent allele is characterized by a nearly perfect palindromic sequence of 442-450 bps (L-PATRR11). We further grouped minor short variants into symmetric short and asymmetric short PATRR11s (SS-PATRR11 and AS-PATRR11). The size of the SS-PATRR11s and AS-PATRR11s were 212-434 bp.

We attempted to estimate the frequency of *de novo* translocations originating from each PATRR allele. To distinguish the allelic origin of translocation products, we selected individuals heterozygous for PATRR11 polymorphisms for analysis. L-PATRR11 produces *de novo* translocations in approximately 10^{-5} gametes (1.30-2.11 $\times$ 10^{-5}). On the other hand, variant PATRR11s generally produce translocations at a lower frequency. For SS-PATRR11, the translocation frequency is about 10-fold lower than that of L-PATRR11 (1.71-1.82 $\times$ 10^{-6}), while AS-PATRR11s rarely produce *de novo* translocation products (≤6.81 $\times$ 10-7) (Table 1, Figure 1B). The differences in translocation frequency were statistically significant between the three groups (P = 0.01).

Thus, having determined that the size and symmetry of the PATRR11 appear to determine the frequency of *de novo* t(11;22)s, it seemed reasonable to hypothesize that polymorphisms of the PATRR11 might dictate translocation frequency through their secondary structure-forming propensity.

Therefore, we analyzed the secondary structure-forming propensity of the PATRR by calculating the free energy required for a transition from standard linear double-stranded DNA to intrastrand annealing, or a so-called hairpin structure [19] (Table 1). We then analyzed the correlation between the calculated secondary structure-forming propensity of a given PATRR11 and its *de novo* translocation frequency. The translocation frequency did not correlate with the free energy for hairpin/cruciform formation (r = 0.37, P = 0.32) (Figure 1C).

In vitro analysis of cruciform extrusion of PATRR plasmids

We then analyzed the *in vitro* cruciform-forming propensity of the PATRRs using plasmids having various PATRR11s as inserts [25,27]. First we cloned each

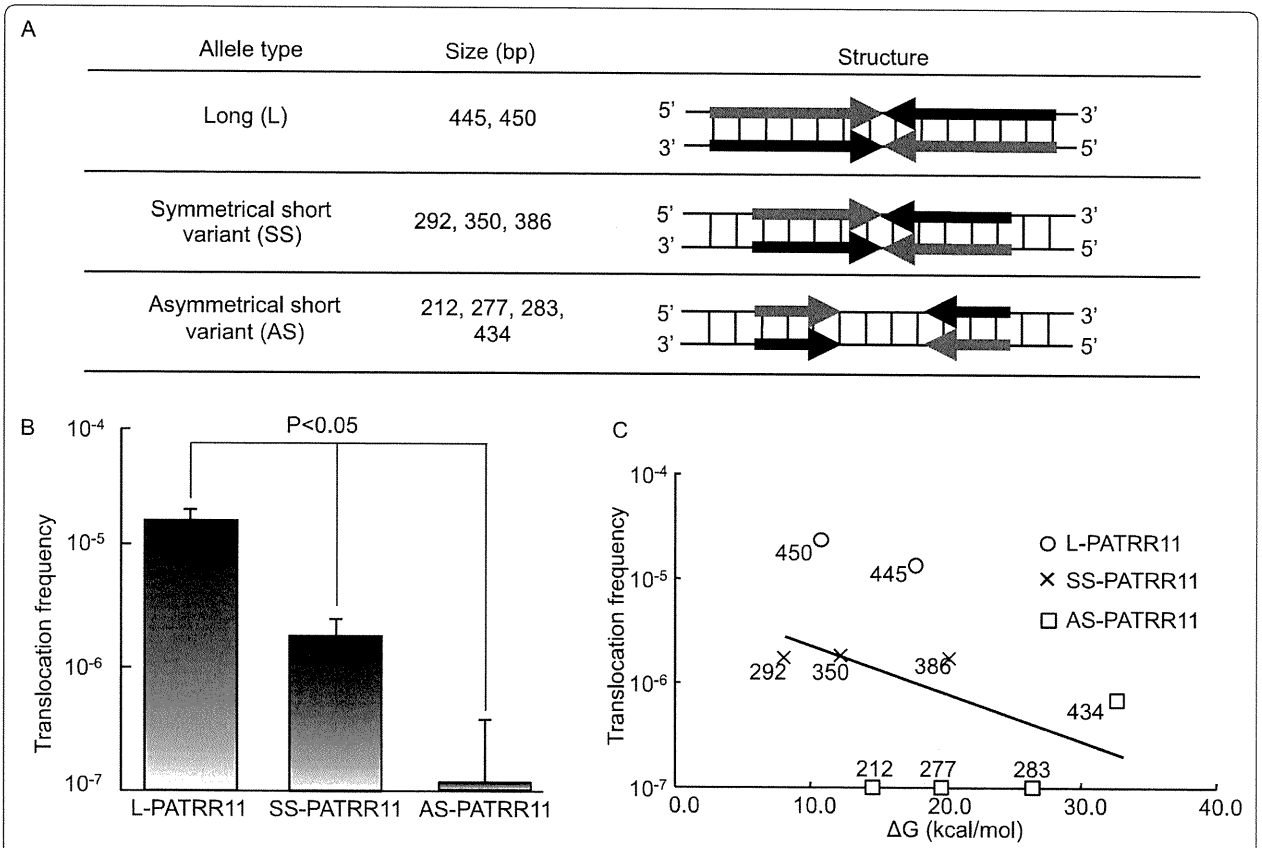


Figure 1 Polymorphism of the PATRR11 affects *de novo* translocation frequency. (**A**) Classification of PATRR11s. Arrows indicate each arm of the palindromic sequences. Grey and black arrows indicate complementary strands. (**B**) Size and symmetry of the palindrome affects the *de novo* translocation frequency. Vertical axis indicates *de novo* translocation frequency in sperm. (**C**) Correlation between the translocation frequency and secondary structure-forming propensity of the PATRR analyzed *in silico*. Horizontal axis indicates the free energy necessary for transition from standard double strand DNA to a hairpin structure, while the vertical axis indicates the *de novo* translocation frequency in sperm. No statistically significant correlation between ΔG and translocation frequency was observed ($r = 0.372$, $P = 0.323$).

polymorphic PATRR11 into a plasmid vector and analyzed its propensity for cruciform formation by an electrophoresis mobility shift assay (EMSA). This assay is based on the fact that mobility is retarded when negative superhelical density is relieved by cruciform extrusion (Figure 2A). We extracted plasmid DNA using the triton-lysis method such that cruciform formation during DNA extraction was minimal. To induce cruciform formation, plasmids were incubated for 30 min at 37°C in 100 mM NaCl. The conformation of plasmid DNA was analyzed by band shift on agarose gel electrophoresis.

Table 1 Potential secondary structure of individual PATRR11 variants by free energy calculation

Type of PATRR11	Nucleotide bp (Accession No.)	G_{ds} Kcal/mole	G_{stru} Kcal/mole	ΔG Kcal/mole	Translocation frequency
L-PATRR11	445(AF391129)	-392.5	-178.4	17.9	1.32×10^{-5}
	450(AB235178)	-397.5	-187.9	10.9	2.11×10^{-5a}
SS-PATRR11	292(AB235183)	-259.8	-121.8	8.1	1.73×10^{-6}
	350(AB235180)	-314	-144.7	12.3	1.82×10^{-6a}
	386(AB235182)	-335.2	-147.3	20.3	1.71×10^{-6}
AS-PATRR11	212(AF391128)	-195.6	-83.1	14.7	$<4.05 \times 10^{-8a}$
	277(AB235187)	-247.9	-104.2	19.8	$<1.67 \times 10^{-7}$
	483(AB235186)	-252.9	-99.95	26.5	$<7.62 \times 10^{-8}$
	434(AB235190)	-380.1	-157.3	32.8	6.81×10^{-7}

[a] These values are the mean of 4 (450 bp L-PATRR11 allele), 2 (350 bp SS-PATRR11 allele), and 3 (212 bp AS-PATRR11 allele)samples.

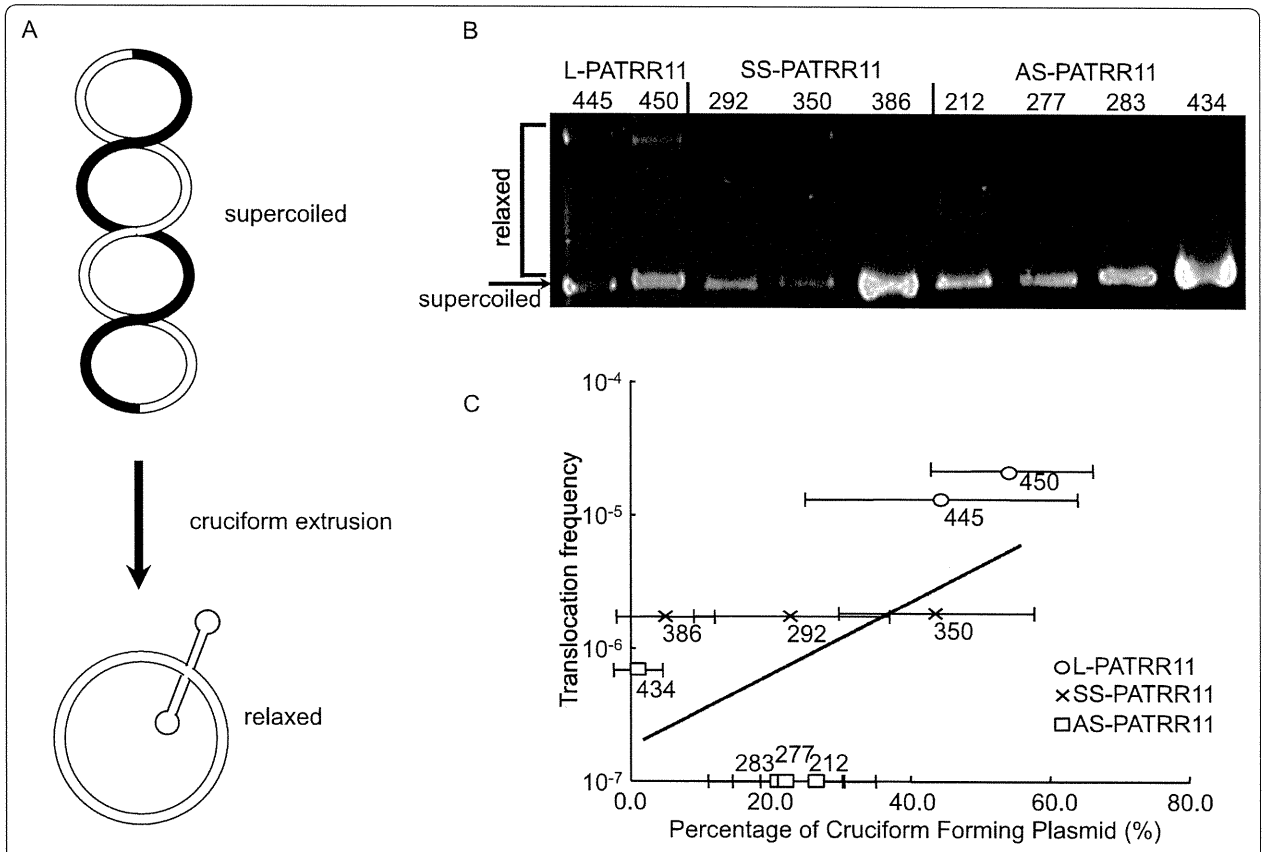


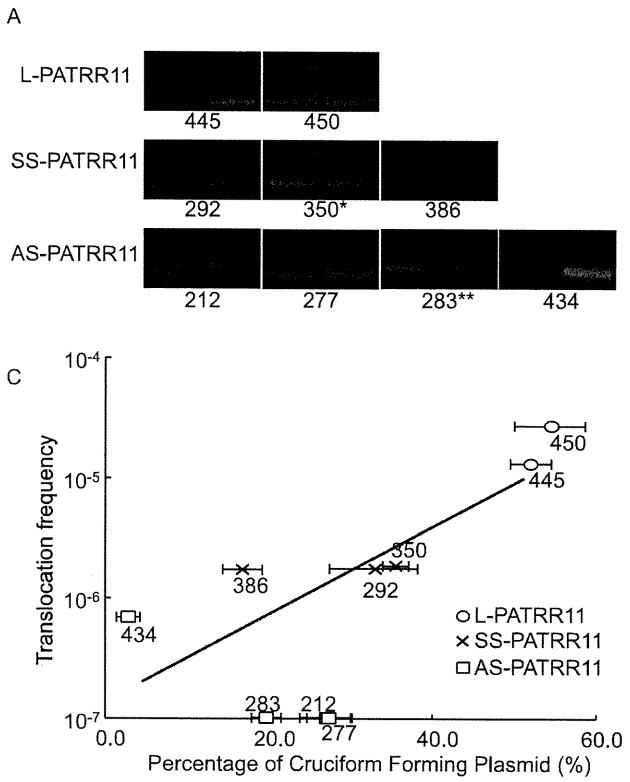
Figure 2 *In vitro* assay for secondary structure formation induced by negative supercoiling. (**A**) The palindromic region can form cruciform structures in the presence of negative torsion of the double-stranded DNA, which is relieved upon cruciform extrusion. (**B**) EMSA for plasmids during agarose gel electrophoresis. The arrow indicates the position for plasmids with a negative supercoil, while the bracket indicates that for a relaxed plasmid that corresponds to the fraction with cruciform extrusion. In general, relaxed plasmids show up as a ladder consisting of various topoisomers with different linking numbers, which appears as a smear due to the low resolution of gel electrophoresis. (**C**) Correlation between translocation frequency and secondary structure-forming propensity of the PATRR estimated by EMSA. The horizontal axis indicates the percentage of plasmid extruding a cruciform, while the vertical axis indicates the *de novo* translocation frequency in sperm. A linear correlation was observed and was statistically significant ($r = 0.71$, $P = 0.03$).

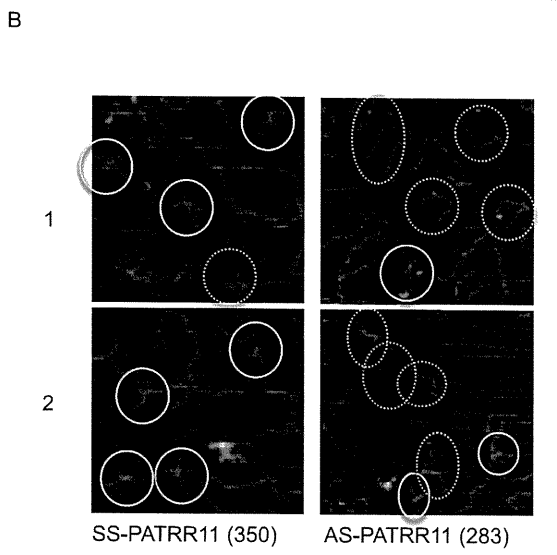
For L-PATRR11, one distinct band with retarded migration was observed accompanied by a ladder of multiple bands (Figure 2B). We confirmed that the plasmids in the retarded band extruded a cruciform by showing that the band disappeared if the plasmid was digested with T7 endonuclease prior to electrophoresis. This enzyme can cut the four-way junction of cruciform DNA (data not shown). Similar results were obtained in the analysis of SS-PATRR11, whereas AS-PATRR11 did not show such retarded bands. To estimate the percentage of cruciform forming plasmids, we summed the intensity of the retarded bands and calculated their ratio to the sum of all of the bands including the band at the standard negative supercoiled position. The ratio correlated to the frequency of *de novo* translocations for each allele ($r = 0.73$, $P = 0.03$) (Figure 2C). However, inter-assay variability was significant due to difficulty in the quantification of multiple bands.

To estimate the prevalence of cruciform extrusion more accurately, EMSA was performed for the plasmid insert only. Since the PATRR cannot maintain a cruciform conformation as short linear DNA, PATRR plasmids were treated with psoralen and ultraviolet light to form covalent cross-links prior to excision of the plasmid insert by restriction enzyme digestion. We detected a clear retarded band derived from the plasmid insert on standard agarose gel electrophoresis (Figure 3A). We confirmed that the DNAs located in the retarded bands originate from the cruciform by cleavage with T7 endonuclease or by direct observation using atomic force microscopy (AFM) (Figure 3B). The intensity of the retarded band on EMSA correlated well with the translocation frequency ($r = 0.77$, $P = 0.01$) (Figure 3C).

Discussion

Our previous study demonstrated that the size and symmetry of polymorphic PATRRs appears to affect the





	SS-PATRR11 (350)		AS-PATRR11 (283)	
	1	2	1	2
Cruciform	5	5	5	3
Linear	5	4	9	6
Percentage	50.0%	55.6%	35.7%	33.3%

Figure 3 ***In vitro*** **assay for secondary structure formation**. (**A**) EMSA for the plasmid insert upon agarose gel electrophoresis. (right) psoralen crosslinking that can retard the mobility of the cruciform-extruding insert. (**B**) Confirmation of cruciform extruding plasmid inserts by AFM. Images were obtained twice for each sample. Linear or cruciform-extruding inserts were counted. The calculated percentages were approximately equal to the values estimated from EMSA. (**C**) Correlation between translocation frequency and cruciform-forming propensity of the PATRR estimated by EMSA. The horizontal axis indicates the percentage of plasmids extruding a cruciform, while the vertical axis indicates the *de novo* translocation frequency in sperm. Linear correlation was observed which was statistically significant (r = 0.77, *P* = 0.01).

frequency of *de novo* t(11;22)s in sperm samples (Kato et al. 2006, Tong et al. 2010). Here we demonstrate that the size and symmetry of PATRRs reflect their secondary structure propensity. It has been suggested that polymorphic variations affect translocation frequency and induce genomic instability leading to translocation susceptibility. We recently established a model system for generating the t(11;22) using human somatic cell lines [30]. In this system, the endogenous PATRR11 and PATRR22 do not generate t(11;22)s, but two co-transfected plasmids containing a PATRR11 and a PATRR22 generate translocation-like rearrangements only when transfected as cruciform-extruding plasmids. This supports the hypothesis that palindrome-mediated recurrent translocations are facilitated through cruciform extrusion of the two PATRRs.

In our data, the percentage of cruciform DNA for the L-PATRR11 was found to be high, while the translocation frequency was relatively low. One possibility is that we performed these experiments under the conditions that favored for cruciform extrusion to see the difference in cruciform propensity clearly among the PATRR11 variants. It is possible that only a small proportion of PATRRs actually extrude a cruciform in living cells. Another possibility is that the longevity of a cruciform might be transient in living cells. Cruciform extrusion requires strong free negative supercoiling, which could be easily resolved by topoisomerase activity prior to translocation formation.

Among the AS-PATRR11s, only the 434 bp PATRR11 produces translocations, although the cruciform forming propensity of the 434 bp PATRR11 is the lowest. The 434 bp PATRR11 was the longest in length among the AS-PATRR11s we examined in this study. In our previous study, size and symmetry of the PATRR are the important determinants for translocation frequency [28,29]. Size might affect the stability of a cruciform once it forms, or, a cruciform-specific nuclease might more readily recognize and digest a larger cruciform leading to translocation formation.

Although a DNA cruciform is likely to be etiologic for palindrome-mediated translocations, the existence of

DNA cruciforms in living cells is still controversial and no direct evidence has yet demonstrated the presence of such a configuration in the context of eukaryotic chromatin [31-34]. Such an energetically unfavorable structure would require sufficient negative superhelicity to stabilize the structure. However, the existence of such a level of negative supercoiling has not yet been proved. Nonetheless, the data in this study indirectly, but strongly imply that PATRRs extrude cruciform structures in living cells. Thus, the question to be answered is when and where such a structure forms and induces a translocation.

We have previously reported sperm-specific occurrence of the t(11;22) translocation in humans [22,35], suggesting that a physiological event during spermatogenesis might be involved in the mechanism of cruciform extrusion and/or structure-dependent instability [36,37]. One way to account for these observations is to postulate that translocations arise during DNA replication. Spermatogenesis engenders a greater number of replications than occur in other somatic tissues or oocytes. The majority of non-recurrent translocations are of paternal origin and *de novo* non-recurrent translocations are often associated with increased paternal age, despite the fact that an age-dependent increase was not observed for the occurrence of the t(11;22) in sperm [38-40]. One possibility is that translocations might occur late in spermatogenesis, when male-specific dynamic changes of chromatin structure take place [41].

In this study, we estimated the ΔG that reflects secondary structures formed by single-stranded DNA. These can be formed within long single-stranded regions of DNA on the lagging-strand template during DNA synthesis. Our data indicate that, similar to the PATRR22, the secondary structure forming propensity of the PATRR11 estimated by its ΔG does not correlate with its translocation frequency [29]. These observations suggest that DNA replication may not significantly contribute to *de novo* translocation formation. This is consistent with the observation that deletions within the PATRR appear to be caused by replication errors, but translocations are not [42,43]. On the other hand, a significant correlate of translocation frequency is observed with the *in vitro* cruciform propensity of PATRR-containing plasmids under torsional constraint. During spermatogenesis standard histones are removed and replaced with protamines at a majority of chromosomal regions. The removal of histones might provide sufficient negative superhelicity to induce cruciform extrusion *in vivo* [44,45]. Such chromatin-remodeling-induced genomic instability deserves further investigation in studies designed to elucidate the mechanism and timing of gross chromosomal rearrangements.

Conclusions

In this study, a significant association between *de novo* translocation frequency and *in vitro* cruciform forming propensity of the polymorphic alleles of the PATRR11 is observed. Our results indirectly but strongly suggest that the PATRR adopts unstable cruciform structures during spermatogenesis that act as a translocation hotspot in humans.

Materials and methods

PCR amplification and cloning of the PATRR

All of the data related to PATRR11 genotype and *de novo* t(11;22) translocation frequency in sperm from normal healthy males have been previously reported [28]. Samples were collected from 2 males who were heterozygous for the L- and SS-PATRR11, while 3 samples were obtained from males heterozygous for L- and AS-PATRR11. Samples from two SS/AS heterozygotes and from one AS/AS heterozygote are also included. All of the donors provided informed consent for further analysis. This study protocol was approved by the Ethical Review Board for Clinical Studies at Fujita Health University.

The PATRR11s were amplified from genomic DNA of the donors by PCR using primers described previously [28]. The plasmids containing the polymorphic PATRR11s were constructed as previously described [26] by TA cloning the PATRR11 PCR products into pT7-blue (Novagen, Madison, WI). The SURE strain (Agilent Technologies, Palo Alto, CA), whose relevant genotype concerning DNA rearrangement and deletion (*recB, recJ, sbcC, umuC::Tn5, uvrC*), is known to show increased stability for palindromic sequences, and was used for cloning and propagation of plasmids.

In silico analysis for secondary structure

Potential secondary structure formed within single-stranded DNA was determined by entering PATRR sequence into the m-fold server http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form). A free energy value (G_{STRUC}) was obtained. Similarly, free energy values for the same sequence annealed to its complementary strand (G_{DS}) were obtained and then halved. Free energy for the formation of secondary structure (ΔG) is calculated as the G_{DS} -G_{STRUC} difference [19].

In vitro cruciform extrusion assay

The cruciform-free plasmids were obtained by a denaturation-free, triton-lysis method as previously described [25]. In brief, the *E. coli* cells from a 50 ml culture were dissolved with 10 ml lysis buffer of 50 mM Tris-HCl (pH7.5), 5% sucrose, 1.5 mg/ml lysozyme, 0.1 M EDTA, 25 μg/ml RNase A and 0.75% Triton X-100. The

plasmids were extracted without the use of phenol, and purified using an ion-exchange column (QIAGEN, Valencia, CA). The plasmid DNA was precipitated in aliquots with 2-propanol and stored at -30°C until used in an experiment. All of the procedures were performed at 4°C in a cold room to avoid spontaneous cruciform formation during the procedure. To induce cruciform formation, the plasmids were incubated for 30 min at 37°C in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and 100 mM NaCl [27]. The plasmids were cooled on ice before electrophoresis at 50V for approximately 4 hours in a 0.9% agarose gel at 4°C. The gel was stained with ethidium bromide and photographed using the Image-Master VDS system (GE Healthcare, Diegem, Belgium). Band intensities were quantified using NIH image 1.62 software.

To examine the cruciform conformation in linear DNA, DNA crosslinking was performed by a method similar to that previously described [25]. In brief, plasmid DNA was dissolved in a solution of 4, 5', 8-trimethylpsoralen (100 μg/ml) and exposed to UV light at 365 nm for 5 min. The DNA was digested with the appropriate restriction enzymes to excise the PATRR-containing fragments, purified, and then divided into two aliquots. One half was used for observation by AFM, and the other half was subjected to 2% agarose gel electrophoresis. To confirm that the shifted bands are the result of cruciform extrusion, the plasmid DNA was treated with 5 units of T7 endonuclease I (New England Biolabs, Beverly, MA). Digestion was performed in 20 μl of NEB2 buffer for 40 min. The reaction was performed on ice so as to minimize additional cruciform extrusion during digestion.

Statistical analyses

Intergroup comparison was performed by one-way analysis of variance, followed by the Mann-Whitney test. Correlations were evaluated with linear straight line regression. In significant difference tests, *P*-values of <0.05 were considered statistically significant.

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Authors' contributions

TK - Participated in the design of the study, carried out the molecular biology work, and drafted the manuscript. HI - Participated in the design of the study, carried out the molecular biology work. MT - Participated in the design of the study, carried out the molecular biology work. HKO - Participated in the design of the study, carried out the molecular biology work. TO - Participated in the design of the study, carried out the molecular biology work. KY - Participated in the design of the study, carried out the molecular biology work. MT - Participated in the design of the study, carried out the molecular biology work. BSE - Coordinated and conceived the study, being involved in the critical revision of the manuscript for important intellectual content. HKU - Coordinated and conceived the study, participated in the design of the study, drafted the manuscript, being involved in the critical revision of the manuscript for important intellectual content.

All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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