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REPORT

TTTCA repeat insertions in an intron of *YEATS2* in benign adult familial myoclonic epilepsy type 4

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Epilepsy is a common neurological disorder and identification of its causes is important for a better understanding of its pathogenesis. We previously studied a Thai family with a type of epilepsy, benign adult familial myoclonic epilepsy type 4 (BAFME4), and localized its gene to chromosome 3q26.32-q28. Here, we used single-molecule real-time sequencing and found expansions of TTTTA and insertions of TTTCA repeats in intron 1 of *YEATS2* in one affected member of the family. Of all the available members in the family—comprising 13 affected and eight unaffected—repeat-primed PCR and long-range PCR revealed the co-segregation of the TTTCA repeat insertions with the TTTTA repeat expansions and the disease status. For 1116 Thai control subjects, none were found to harbour the TTTCA repeats while four had the TTTTA repeat expansions. Therefore, our findings suggest that BAFME4 is caused by the insertions of the intronic TTTCA repeats in *YEATS2*. Interestingly, all four types of BAFMEs for which underlying genes have been found (BAFMEs 1, 4, 6 and 7) are caused by the same molecular pathology, suggesting that the insertions of non-coding TTTCA repeats are involved in their pathogenesis.

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Abbreviation: BAFME = benign adult familial myoclonic epilepsy

Introduction

Benign adult familial myoclonic epilepsy (BAFME) is an autosomal dominant neurological disorder characterized by adult-onset cortical tremor and generalized seizures (Ikeda *et al.*, 1990; Yasuda, 1991). It was first described in Japan (Ikeda *et al.*, 1990) and until now at least 105 families with this disease have been reported in several

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countries (Ikeda *et al.*, 1990; Kuwano *et al.*, 1996; Okino, 1997; Terada *et al.*, 1997; Elia *et al.*, 1998; Mikami *et al.*, 1999; Plaster *et al.*, 1999; Guerrini *et al.*, 2001; Labauge *et al.*, 2002; van Rootselaar *et al.*, 2002; de Falco *et al.*, 2003; Carr *et al.*, 2007; Madia *et al.*, 2008; Depienne *et al.*, 2010; Mori *et al.*, 2011; Yeetong *et al.*, 2013; De Fusco *et al.*, 2014; Cen *et al.*, 2018; Ishiura *et al.*, 2018; Lei *et al.*, 2019; Zeng *et al.*, 2019). Patients with BAFME usually experience tremors, the first symptom, in their second or third decade; they may experience seizures at the same time or develop them in later life. The diagnosis of BAFME is based on clinical and electrophysiological criteria (van den Ende *et al.*, 2018).

BAFME exhibits locus heterogeneity: six chromosomal regions linked to BAFME include 8q23.3-q24.1 (Mikami *et al.*, 1999; Plaster *et al.*, 1999), 2p11.1-q12.2 (Guerrini *et al.*, 2001), 5p (Depienne *et al.*, 2010), 3q26.32-q28 (Yeetong *et al.*, 2013), 16p21.1 (Ishiura *et al.*, 2018) and 4q32.1 (Ishiura *et al.*, 2018) for BAFME1 to BAFME4, BAFME6 and BAFME7, respectively. Since 1999, when the gene for BAFME1 was first found to be localized to chromosome 8q23.3-q24.1 (Mikami *et al.*, 1999; Plaster *et al.*, 1999), tremendous effort has been put in to identify the causative genes. Recently, Ishiura *et al.* used single-molecule real-time sequencing of BAC clones and nanopore sequencing of genomic DNA to successfully identify expansions of intronic pentanucleotide repeats in *SAMD12*, *TNRC6A* and *RAPGEF2* as the causative mutations for BAFME1, BAFME6 and BAFME7, respectively (Cen *et al.*, 2018; Ishiura *et al.*, 2018).

Previously, we identified a large Thai pedigree with BAFME4 and carried out a whole genome linkage study to localize its gene to chromosome 3q26.32-q28 between D3S3730 and D3S1580 with maximum two-point LOD score of 5.419 at D3S1262 (Yeetong *et al.*, 2013). The 10 Mbp region consists of 136 genes. In this study, we used single molecule real-time sequencing and successfully identified expansions of non-coding TTTTA and insertions of TTTCA repeats in the intron 1 of *YEATS2*, as the cause of BAFME4.

Materials and methods

Array comparative genomic hybridization

DNA of an affected female (Patient III-6) and an unaffected female (Case III-5) was sent to Macrogen, Inc to look for copy number variations in their genome. DNA was independently labelled with fluorescent dyes, co-hybridized to a NimbleGen Human CGH 385K chromosome 3 tiling array, and scanned using a 2 µm scanner. Log2-ratio values of the probe signal intensities (Cy3/Cy5) were calculated and plotted versus genomic position using Roche NimbleGen NimbleScan software. The results were displayed in Roche NimbleGen Signal Map software.

Targeted resequencing

The Patient III-6 genomic DNA was used to perform targeted resequencing of the entire 10 Mbp critical region between the D3S2747 and D3S3663 microsatellite markers. With the next-generation sequencing (NGS) service of Macrogen Inc, DNA was captured on customized NimbleGen 2.1 array (Roche NimbleGen) with capturing capacity of 33 Mbp. The targeted region was between nucleotide positions 178 100 000 and 188 700 000 on chromosome 3 according to UCSC hg19 Assembly. The captured library was subsequently sequenced using Illumina platform Genome Analyzer$_{IIx}$ (GAIIX) in a single-end 76 bp configuration. Sequence reads were mapped against UCSC hg19 using BWA software (http://bio-bwa.sourceforge.net/). Single nucleotide polymorphisms and indels were detected using SAMTOOLS (http://samtools.sourceforge.net/) and annotated using SIFT (http://sift.jcvi.org/).

Whole exome sequencing

The genomic DNA of two affected family members (Patients III-2 and III-14) and an unaffected family member (Case II-8) was prepared as an Illumina sequencing library, and the sequencing libraries were then enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced using an Illumina HiSeq 2000 Sequencer.

Whole genome sequencing using short read technologies

The genomic DNA of two affected (Patients III-3 and III-13) and an unaffected (Case III-1) family members was prepared for sequencing using the Illumina platform, while that of an affected family member (Patient III-4) was prepared for sequencing using the BGI platform. Pair-end 100 bp sequencing was performed aiming to get >100Gbp raw data with Q30 >80%. The raw data were aligned to hg19 using Burrows–Wheeler Alignment software (http://bio-bwa.sourceforge.net/). Variant calling was performed using GATK with HaplotypeCaller.

Long-read sequencing

DNA samples from one affected member (Patient III-4) of the BAFME4 family and one unaffected individual were subjected to whole-genome sequencing using single molecule real-time (SMRT) sequencing technology with Sequel Sequencing Kit 2.1 and Sequel sequencer (Pacific Biosciences). Sequencing data (35 Gbp) aligned to the reference genome hg19 by using Structural Variant Calling analysis on SMRT Link 5.1.0.26412.

Based on our previous findings from whole genome linkage analysis on chromosome 3q26.32-q28 (Yeetong *et al.*, 2013), we extracted reads of Patient III-4 from the long-read sequencing aligned BAM that spanned chromosome 3, position 178546601–188543136 (hg19). Then we used Tandem Repeats Finder (TRF) (Benson, 1999) to locate tandem repeats in each read with the following parameters 2 3 3 80 10 2000 6. Results from TRF were kept if the consensus pattern was TTTCA or TTTTA or their reverse complement, or their possible shifts. These two patterns were obtained from Ishiura *et al.*'s (2018) findings.

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Repeated-prime PCR

The pentanucleotide repeat sequence in the intron 1 of *YEATS2* was amplified by TTTTA and TTTCA repeated-prime (RP)-PCR with the sets of primers as shown in Supplementary Table 2. PCR for TTTTA was carried out with 200 ng of genomic DNA, 0.8 µM of each primer, 0.2 mM dNTP mixture, 1× PCR buffer containing 15 mM $MgCl_2$, 1× Q-Solution, 2.5 U HotStarTaq DNA polymerase (Qiagen) in 50 µl. Touchdown PCR was performed with the following conditions: first 15 min at 95°C, then 19 cycles at 94°C for 1 min, 61°C for 1 min and 72°C 1 min with the annealing temperature gradually reduced (0.5°C per cycle), then 19 cycles at 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, and finally 72°C for 10 min. PCR for TTTCA was carried out with 100 ng of genomic DNA, 0.2 µM of each primer, 0.2 mM dNTP mixture, 1× Taq buffer with $(NH_4)_2SO_4$, 0.5 U Taq DNA polymerase (Thermo Scientific) in 20 µl. Touchdown PCR was performed with the following conditions: first 5 min at 96°C, then 19 cycles at 94°C for 30 s, 63°C for 30 s and 72°C 1 min with the annealing temperature gradually reduced (0.5°C per cycle), then 19 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 1 min, and finally 72°C for 10 min. RP-PCR products were detected on an ABI Prism 3100 genetic analyzer (Applied Biosystems) with GeneMarker V2.6.3 software (SoftGenetics, State College, PA, USA).

Long-range PCR

Expanded alleles were amplified by long-range PCR with 100–500 ng of genomic DNA, 0.2 µM of each primer (Supplementary Table 3), 200 µM dNTP mixture, 1× PrimeSTAR GXL buffer, and 2.5 U PrimeSTAR GXL Taq DNA polymerase (Takara Bio Inc) in 50 µl. After 5 min at 94°C, and 30 cycles at 98°C for 30 s, 58°C for 30 s and 68°C 7 min, PCR products were separated by agarose gel electrophoresis in 1% gel, at 100 V for 60 min.

Genomic structures flanking the expanded repeats

The RepeatMasker tool of UCSC table browser was used to identify Alu sequences in the BAFME regions (chr3:183428976–183431010 for BAFME4, chr8:119378055–119380172 for BAFME1, chr16:24623761–24625850 for BAFME6 and chr4:160262679–160264768 for BAFME7).

Data availability

The data that support the findings of this study are openly available in Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/sra/PRJNA547506, SRA accession PRJNA547506.

Results

Several technologies failed to identify the causative gene of BAFME4

Initial attempts to identify the underlying gene of BAFME4 were unsuccessful. Several techniques including targeted resequencing of the 10-Mbp critical region, array comparative genomic hybridization, whole exome sequencing and whole genome sequencing using short read technologies both Illumina HiSeq X (Bridge PCR) and BGISEQ-500 (DNBs, DNA nanoballs) failed to identify any candidate mutations.

Identification of the long expansions of repeat sequences by long-read sequencing

DNA from the affected member Patient III-4 (Fig. 2) and an unaffected control subject underwent whole genome long-read sequencing; total output of each sample was ~35 Gbp and 3 million reads. Based on our previous whole genome linkage analysis, we selected reads from the long-read sequencing aligned bam that spanned the critical region on chromosome 3q26.32-q28 (Yeetong *et al.*, 2013). As the causative mutations for all the three previously identified BAFME loci (BAFME1, BAFME6 and BAFME7) were expansions of TTTCA and TTTTA repeats in introns, we used TRF (Benson, 1999) to detect the selected reads that contain expansions of TTTCA or TTTTA repeats. Three reads with such repeat expansions were found in the genome of the affected member, all located in the intron 1 of the *YEATS2* gene (Fig. 1A and Supplementary Table 1). The expanded repeats are estimated to be $(TTTTA)_{819}(TTTCA)_{221}$ (Supplementary Fig. 1). Long-read sequencing of the control subject showed a genotype of $(TTTTA)_7/(TTTTA)_8$ without TTTCA repeats.

Co-segregation of the TTTCA repeat insertions and disease status in the family

We looked for the presence of the TTTTA and TTTCA repeats in the intron 1 of *YEATS2* by RP-PCR analyses in all 13 affected and eight unaffected family members. TTTCA repeat insertions were found in all affected and were absent in all unaffected family members (Fig. 1B). Moreover, they were not found in 1116 Thai control subjects. For the RP-PCR targeting TTTTA, all 13 affected family members had TTTTA repeat expansions, while all eight unaffected family mambers had the normal short TTTTA repeats.

Determining the repeat expansions and the size of TTTTA repeats by long-range PCR

To confirm the existence of both pentanucleotide repeats, long-range PCR was carried out in all 21 members of the BAFME4 family. All 13 patients showed long allele bands with estimated sizes ranging from 5 to 8 kbp (Figs 2 and 3). The normal alleles of the family had TTTTA repeats ranging from 6 to 59 repeats. Of the 1116 Thai control subjects (2232 chromosomes), 2228 chromosomes had from 4 to 119 repeats, and the remaining four chromosomes had

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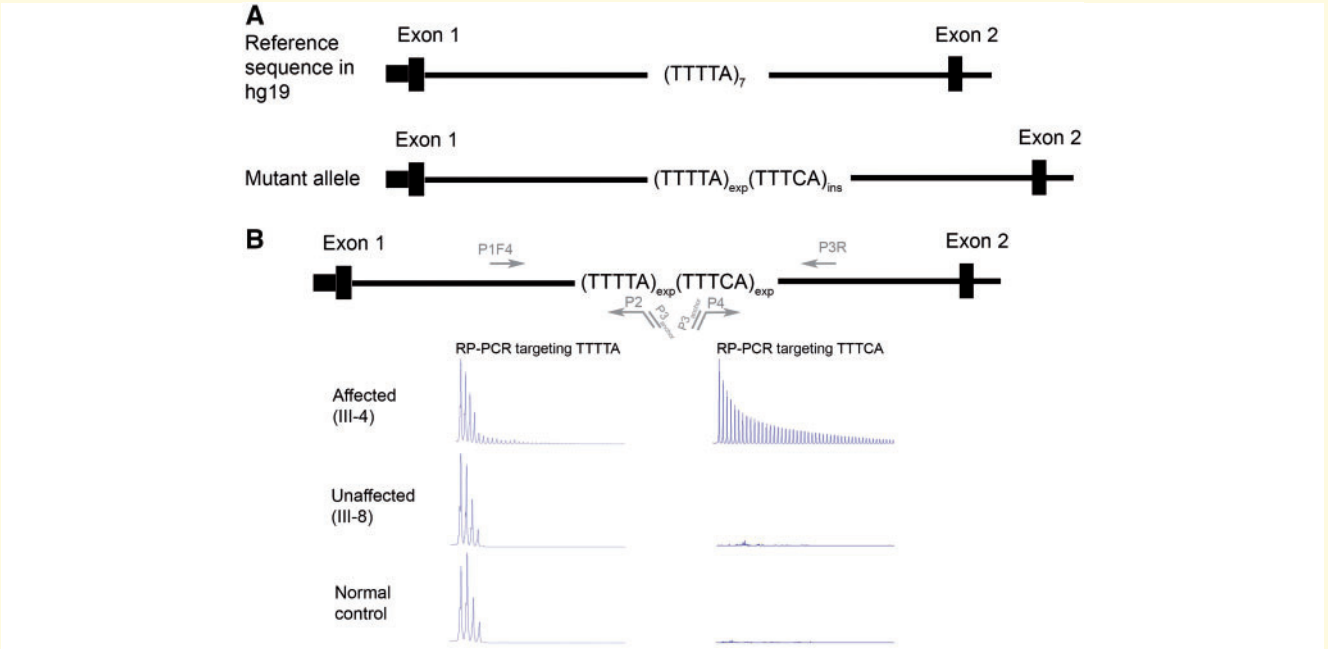

Figure 1 Identification of the molecular pathology. (**A**) Configuration of expanded repeats. The $(TTTTA)_n$ expansion and $(TTTCA)_n$ insertion located in intron 1 of the *YEATS2* gene. (**B**) Repeat-primed PCR for $(TTTTA)_n$ expansions and $(TTTCA)_n$ insertions. The primers P1F4, P2 and $P3_{anchor}$ were designed to detect the TTTTA repeats and P3R, P4 and $P3_{anchor}$ to detect the TTTCA repeats. Patient III-4 had TTTTA repeat expansion and TTTCA repeat insertion (*top*). In unaffected family member (Case III-8) (*middle*) and a normal control subject (*bottom*), short TTTTA repeats were detected without TTTCA repeats.

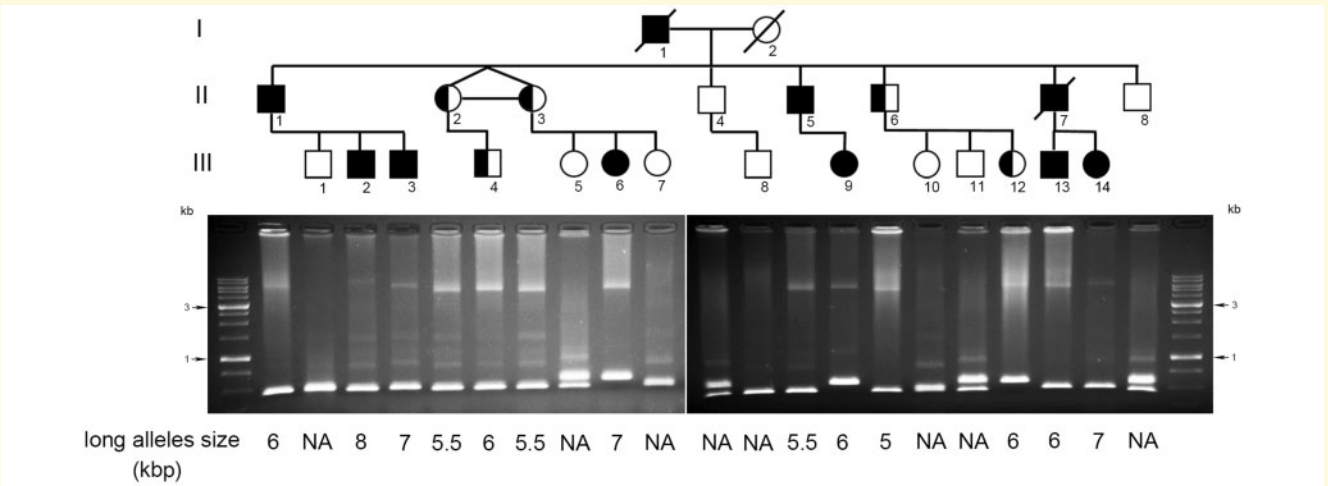

Figure 2 Long-range PCR to determine the size of repeat expansions of each member of the BAFME4 family. Affected members with cortical tremor and seizures are represented by filled symbols; affected members with cortical tremor only are represented by half-filled symbols. The gel revealed 5–8 kbp expanded alleles that segregated with the disease in the family.

719, 919, 919 and 1219 TTTTA repeats, respectively (Supplementary Table 4).

Genomic structures flanking the expanded repeats

We found Alu sequences in the vicinity of the TTTTA repeats of the BAFME1, BAFME4 and BAFME6 regions but not in the BAFME7 region (Fig. 4 and Supplementary Table 5). Notably, the TTTTA repeats of the BAFME4 region are within an AluSx sequence with an AluSg sequence 60 bp centromeric to the AluSx sequence. In addition, we searched for Alu sequences in the read ID 20835 of Patient III-4 obtained from the long-read sequencing and found that the TTTTA repeat expansions and the TTTCA repeat insertion were in the AluSx sequence with

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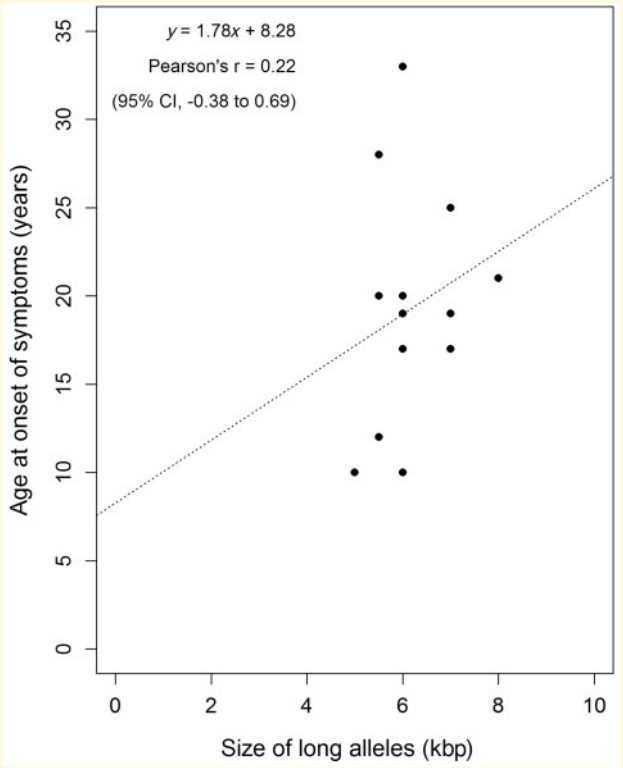

Figure 3 Correlation between repeat lengths and age at onset of symptoms. A linear regression indicated by the dotted line showed no significant inverse correlation between sizes of long alleles that were estimated by long-range PCR and age at onset of symptoms ($P = 0.4679$) of the 13 patients with BAFME4 (black dots).

the AluSg sequence 59 bp centromeric to the AluSx sequence (Fig. 4).

Discussion

We have sought to identify the causative gene for the family with BAFME4 with 21 available members, of whom 13 were affected and eight unaffected. In 2013, we reported the causative mutation for our family to be in the 10-mbp critical region on chromosome 3 (Yeetong *et al.*, 2013). To find the disease-causing genes, we used several available approaches that are suitable for various mutation types. After several experiments failed to identify candidate mutations, we assumed that the molecular pathology causing BAFME4 in our patients might be expansions of repeats (Ardui *et al.*, 2018) and resorted to long-read sequencing using SMRT technology. Our hypothesis was supported by the fact that the three types of BAFME for which genes have been previously found (BAFMEs 1, 6 and 7) are caused by the abnormal expansions of TTTTA repeats and the insertion of TTTCA repeats in gene introns (Ishiura *et al.*, 2018). This has been confirmed by subsequent reports (Cen *et al.*, 2018; Ishiura *et al.*, 2018; Lei *et al.*, 2019; Zeng *et al.*, 2019).

With the aforementioned hypothesis, we carried out long-read sequencing for one affected and one unaffected member and looked for TTTTA repeat expansions and TTTCA repeat insertions in the 10-Mbp critical chromosomal region. We found the TTTTA repeat expansions and the TTTCA repeat insertions in intron 1 of the *YEATS2* gene in the affected but not in the unaffected family member. Subsequently, we used RP-PCR to look for both pentanucleotide repeats in family members and Thai control subjects. The TTTCA repeat insertion was only found in the 13 affected family members but not in the eight unaffected members or in the 1116 healthy control subjects. While the TTTTA repeat expansions were absent in the eight unaffected family members, they were present in four of the 1116 controls. Co-segregation of the TTTCA repeat insertion in the family and its absence in 1116 control subjects strongly suggest that the TTTCA repeat insertion is the cause of BAFME4.

Of the 1116 Thai control subjects, long-range PCR showed that four individuals had TTTTA repeat expansions of 719, 919, 919 and 1219 repeats, respectively (Supplementary Fig. 2). The sizes of the repeats were similar to those of combined TTTTA and TTTCA repeats in our patients with BAFME4. This suggests that TTTTA repeat expansions alone are insufficient but that TTTTA repeat expansions along with TTTCA repeat insertions are necessary for the development of the disease.

Long-range PCR in 13 affected members showed long allele bands with estimated sizes ranging from 5 to 8 kbp and the sizes of affected members in Generation III were always longer than those of their affected parents in Generation II (Fig. 2). However, there was no correlation between repeat length and age at onset, which was different from that found in BAFME1 (Ishiura *et al.*, 2018) (Fig. 3). Moreover, Patients II-2 and II-3, who were identical twins and had the same expansion sizes, manifested a disease at different ages, 20 and 28 years old. This suggests that other factors are involved in age at onset of the disease.

We analysed the genomic structures flanking the TTTTA repeats in the BAFME regions and found that the repeats in the BAFME1, 4 and 6 regions are in or close to the Alu sequences (Fig. 4 and Supplementary Table 5). However, there was no Alu sequence in the BAFME7 region. Whether these Alu sequences play a role in the expansion of the TTTTA repeats requires further investigation.

The *YEATS2* gene encodes a scaffolding subunit of the ATAC complex with acetyltransferase activity on histones H3 and H4 (Nagase *et al.*, 1999). Mutations of this gene have not been associated with any diseases. The fact that all four types of BAFME, of which genes are identified that have the same molecular pathology, TTTCA repeat insertions in non-coding regions, has supported that RNA toxicity plays an important role in the pathogenesis of BAFME. However, involvement of altered functions of the proteins encoded by the genes with the

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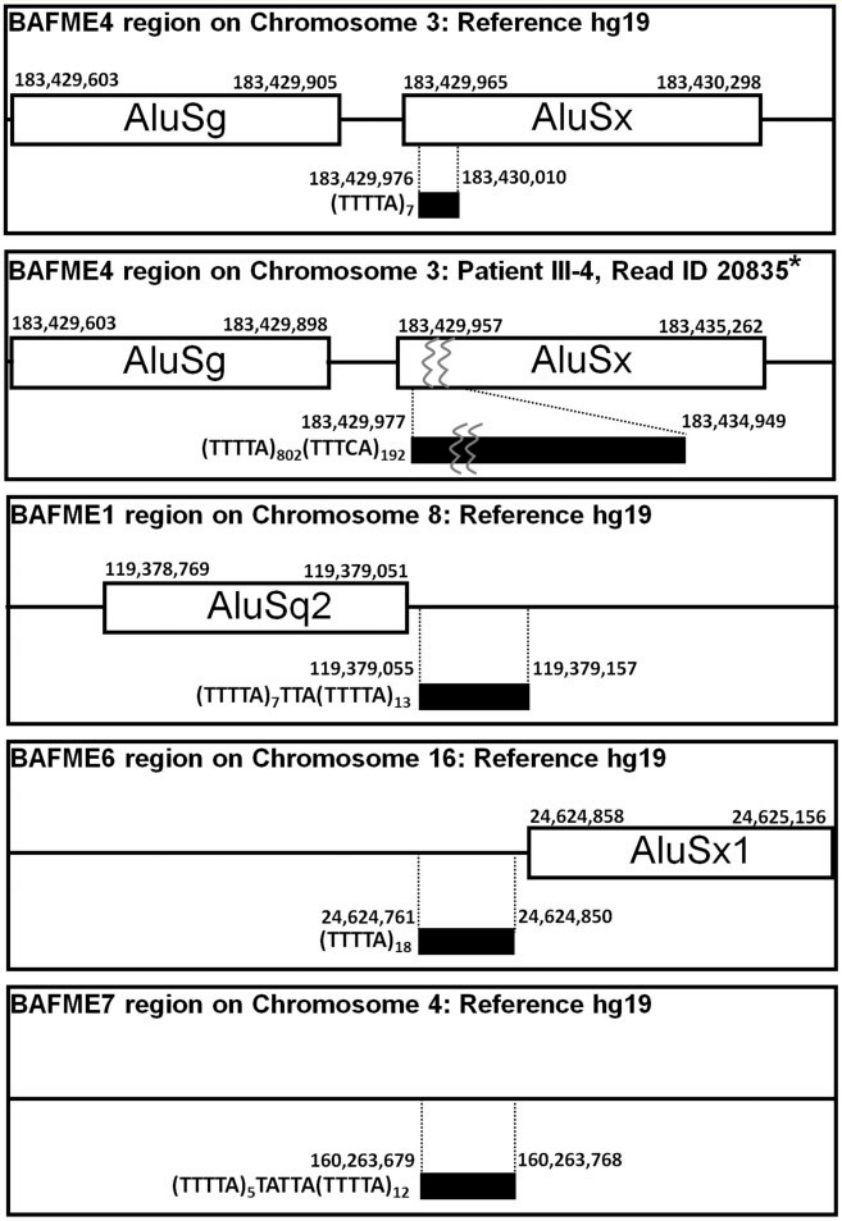

Figure 4 Comparing the genomic structures flanking the expanded repeats of each type of BAFME. For BAFME4, the TTTTA repeat is a part of AluSx and behind AluSg (*first row*) and for Patient III-4, who had both TTTTA expansion and TTTCA repeat insertion, the repeats are also part of AluSx and behind AluSg (*second row*). For BAFME1, the repeat is behind AluSq2 (*third row*). For BAFME6, the repeat is in front of AluSx1 (*fourth row*). No Alu sequence is near the repeat in BAFME7 (*fifth row*).

insertion in the disease's pathogenesis cannot be ruled out. Whether the TTTCA repeat insertions leading to BAFME could be inserted in any genes in the genome or it have to be inserted in specific genes needs further investigation.

In conclusion, we identified the pentanucleotide TTTCA repeat insertion and TTTTA repeat expansion in intron 1 of the *YEATS2* gene as the causative mutation of BAFME4. This will provide further understanding into the molecular basis of the disease, which is caused by the same molecular pathology as with other BAFMEs.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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