

Somatic mosaicism underlies X-linked acrogigantism syndrome in sporadic male subjects

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Abstract

Somatic mosaicism has been implicated as a causative mechanism in a number of genetic and genomic disorders. X-linked acrogigantism (XLAG) syndrome is a recently characterized genomic form of pediatric gigantism due to aggressive pituitary tumors that is caused by submicroscopic chromosome Xq26.3 duplications that include *GPR101*. We studied XLAG syndrome patients ($n = 18$) to determine if somatic mosaicism contributed to the genomic pathophysiology. Eighteen subjects with XLAG syndrome caused by Xq26.3 duplications were identified using high-definition array comparative genomic hybridization (HD-aCGH). We noted that males with XLAG had a decreased log₂ ratio (LR) compared with expected

Key Words

- ▶ X-LAG syndrome
- ▶ mosaicism
- ▶ gigantism
- ▶ pituitary
- ▶ molecular genetics

values, suggesting potential mosaicism, whereas females showed no such decrease. Compared with familial male XLAG cases, sporadic males had more marked evidence for mosaicism, with levels of Xq26.3 duplication between 16.1 and 53.8%. These characteristics were replicated using a novel, personalized breakpoint junction-specific quantification droplet digital polymerase chain reaction (ddPCR) technique. Using a separate ddPCR technique, we studied the feasibility of identifying XLAG syndrome cases in a distinct patient population of 64 unrelated subjects with acromegaly/gigantism, and identified one female gigantism patient who had had increased copy number variation (CNV) threshold for *GPR101* that was subsequently diagnosed as having XLAG syndrome on HD-aCGH. Employing a combination of HD-aCGH and novel ddPCR approaches, we have demonstrated, for the first time, that XLAG syndrome can be caused by variable degrees of somatic mosaicism for duplications at chromosome Xq26.3. Somatic mosaicism was shown to occur in sporadic males but not in females with XLAG syndrome, although the clinical characteristics of the disease were similarly severe in both sexes.

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Introduction

Somatic mosaicism describes a phenomenon in which two or more populations of cells compose one multicellular organism, within which each cell population is represented by its own unique genome (Lupski 2013). Somatic mosaic variants may arise from errors that occur during mitotic DNA replication. Such variants, including single-nucleotide variants (SNVs), small insertions/deletions (indels), absence of heterozygosity, and structural variants (SVs), arise from erroneous, uncorrected mutational events and continually accumulate as cells divide during the development of a mature human individual who consists of $\sim 10^{16}$ cells amassing from a single fertilized cell (Campbell et al. 2015). Somatic mosaic variants accompanying clonal expansion are closely related to aging and cancer in humans (Jacobs et al. 2012, Genovese et al. 2014, Liu et al. 2014b). Identified as low-level mosaicism in blood-derived DNA or in specific tissues, such as those that comprise the nervous system, somatically mosaic variants may also contribute to human genetic or genomic disorders (Lindhurst et al. 2011, Poduri et al. 2013, Jamuar et al. 2014, Pham et al. 2014, Campbell et al. 2015). Alternatively, somatic mosaic variants may not have immediate clinical consequences for the carrier, but contribute to the recurrent risk of genomic disorders in offspring (Campbell et al. 2014a, b).

X-linked acrogigantism syndrome (XLAG, MIM #300942) is a recently characterized genomic disorder of early-onset gigantism caused by a submicroscopic duplication at chromosome Xq26.3 (Trivellin et al. 2014). Affected patients are generally born normal sized following unremarkable pregnancies and develop mixed growth hormone (GH)- and

prolactin-secreting pituitary hyperplasia and/or adenomas within the first 12–36 months of life (Trivellin et al. 2014, Beckers et al. 2015). The XLAG syndrome phenotype of early childhood-onset gigantism is aggressive and difficult to treat; it can be differentiated clinically from other forms of pituitary gigantism due to younger age and more severe hormonal hypersecretion (Rostomyan et al. 2015). In the absence of multimodal neurosurgical and medical therapy, XLAG syndrome is associated with relentless overgrowth due to GH hypersecretion (Naves et al. 2015). The etiology of the pituitary tumor/hyperplasia in XLAG appears to be linked to a central disorder of hypersecretion of GH-releasing hormone (GHRH), which is a unique causative feature for pituitary gigantism in humans (Daly et al. 2016). XLAG syndrome is caused by genomic duplications encompassing *GPR101* (MIM *300393), which encodes an orphan G-protein-coupled receptor; rare, potentially activating point mutations of *GPR101* (e.g., p.E308D) have been identified in some patients with acromegaly, mostly in tumors (Trivellin et al. 2014).

Mosaic variants in genes encoding protein subunits involved in G-protein signaling have a recognized place in the etiology of syndromic conditions in endocrinology. McCune–Albright syndrome (MIM #174800) is caused by mosaic mutations in *GNAS1* and is itself associated with pituitary gigantism and a wide spectrum of disease features (Lumbroso et al. 2004, Vasilev et al. 2014). Moreover, postzygotic, somatic mutational events have also been observed in other classical overgrowth syndromes, such as the AKT paralogs (*AKT1*, *AKT2*, and *AKT3*) that cause Proteus syndrome (MIM #176920), hypoglycemia and asymmetrical

somatic growth (MIM #240900), and hemimegalencephaly (MIM #615937), respectively (Hussain et al. 2011, Lindhurst et al. 2011, Poduri et al. 2012).

Based on these observations of somatic mosaic mutations in several overgrowth syndromes, we hypothesized that potential somatic mosaicism might underlie XLAG syndrome in a proportion of cases. To investigate this possibility, we studied an expanded series of patients with XLAG syndrome, pituitary gigantism, or acromegaly to screen for, detect, and quantify mosaicism for submicroscopic duplications at chromosome Xq26.3 that include *GPR101*.

Methods

Droplet digital polymerase chain reaction

Overview Two droplet digital polymerase chain reaction (ddPCR) experiments were designed: one to assess large numbers of DNA samples for copy number variations (CNVs) at the *GPR101* gene compared to a nearby reference gene on chromosome X not included in duplications causing XLAG syndrome ('screening ddPCR') and a second breakpoint junction (JCT)-specific ddPCR ('quantification ddPCR') to quantify somatic mosaicism at the borders of the Xq26.3 duplication in each affected XLAG syndrome case (described next). In ddPCR, target DNA molecules are distributed in droplets across multiple replicate reactions. The number of positive and negative droplets that contain a target template is used to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence interval (CI) (Hindson et al. 2011). The ddPCR methodology can readily distinguish duplication, triplication, and even quadruplication of a locus (Gu et al. 2016).

Screening ddPCR The screening ddPCR assay was designed to quantify DNA copy number of the *GPR101* gene (ENSG00000165370; X:137030148–137031674) compared to DNA copy number of *ZIC3* exon 1–intron 1/2 (ENSG00000156925; X:137566142–137577691). In previous studies of XLAG syndrome cases, we have found that *ZIC3* is the nearest protein-coding gene that is not included in the microduplications at Xq26.3 (Trivellin et al. 2014, Beckers et al. 2015). These screening analyses were performed on DNA derived from whole blood samples.

Screening ddPCR experiments were performed as follows: each 21- μ L reaction mixture contained 5- μ L of DNA template, 2 \times ddPCR supermix for probes (no dUTP), and *GRP101* and *ZIC3* exon 1 primers and probes assays. The assays were purchased as a 20 \times premix of primers and

probes (Bio-Rad Laboratories) and used at 1 \times concentration. The 1 \times concentration of this assay comprised 900 nM forward primer, 900 nM reverse primer, and 250 nM probe. Primers, hydrolysis probe sequences, and ddPCR conditions are reported in Supplementary Table S1 (see section on supplementary data given at the end of this article). After homogenization, the PCR reaction mixture and droplet generation oil for probes were loaded into an eight-channel droplet generator cartridge (Bio-Rad Laboratories). The PCR reaction mixtures were partitioned into an emulsion of approximately 15,000 droplets (~1 nL per droplet) that were manually transferred to a 96-well PCR plate. The PCR plate was heat sealed and placed in a conventional thermal cycler (PRoFlex PCR systems, Life Technologies), and PCR proceeded according to the manufacturer's protocol. Following the PCR, the 96-well plate was loaded on a QX100 droplet reader (Bio-Rad Laboratories). Analysis of the ddPCR data was performed with QuantaSoft software (Bio-Rad; version 1.7.4.0917), which analyzes each droplet individually using a two-color detection system (set to detect FAM or HEX dyes).

The absolute quantification of DNA is directly dependent on the number of accepted droplets (positive and negative) and the DNA quantity analyzed. The calculation of the 95% CI given by the Poisson law and the distribution of the CNV values according to our cohort of 91 samples and controls led us to consider a sample as duplicated if the CNV value was >2.5 and Poisson CNV minimum value (CNV_{min}) (95% CI) >2.0. The calculations and reporting of each CNV value ratio between *GPR101* and *ZIC3* and for each patient and overall groups account for differences in X chromosome number between males and females.

The population assessed using this screening ddPCR methodology included 36 patients with acromegaly (males/females: 24/12; age range at diagnosis: 22–50 years), six index cases from familial isolated pituitary adenoma (FIPA) kindreds with homogeneous acromegaly, and 22 patients with pituitary gigantism. None of these patients had been reported previously. All patients had pituitary adenomas diagnosed by magnetic resonance imaging and had excess GH/IGF1 secretion established before inclusion. In addition, none of the patients had mutations or deletions in genes known to cause acromegaly–gigantism, such as *AIP*, *CDKN1B*, and *MEN1*, and none had syndromic conditions such as Carney complex or McCune–Albright syndrome (Daly & Beckers 2015). As a positive control, 20 blood, tissue, and pituitary tumor samples from eight previously diagnosed XLAG syndrome cases with established Xq26.3

duplication CNV on HD-CGH were included, whereas seven non-acrogigantism controls without *GPR101* duplication CNV were also studied. All individuals and/or guardians provided informed consent, and the genetic study was approved by the Ethics Committee of the Centre Hospitalier Universitaire de Liège, Liège, Belgium.

High-density array comparative genomic hybridization

We used a custom-designed high-density array comparative genomic hybridization (HD-aCGH) to delineate high-resolution CNVs in the genomic DNA samples derived from the blood of subjects with XLAG. The array design and experimental procedures were reported previously (Trivellin *et al.* 2014, Yuan *et al.* 2015).

JCT sequencing

JCT amplification and sequencing were performed following the protocol described by Yuan *et al.* (2015).

Quantification ddPCR

For the quantification of ddPCR, we developed personalized ddPCR assays for each JCT. JCT-specific primer pairs were designed to amplify duplication JCT in each subject

(Supplementary Table 2). A pair of universal control primers (CTRL-F: 5'-CTCTGCCGCCTCAACTCAACG-3'; CTRL-R: 5'-AAGGTCCGGTTCGAGCTCTTCT-3') targeting exon 1 of *ZIC3* on chromosome X, a nearby region that is apparently copy number neutral in all XLAG syndrome cases identified to date, was designed to amplify a control region in comparison with the JCT amplification. Both JCT-specific and control primers were designed with amplicons sizes of ~500 bp. We performed the JCT-specific ddPCR experiments according to the manufacturer's protocol (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf): 25 µL master-mix containing 25 ng of genomic DNA, forward/reverse primers with final concentration of 1 µM, and 2× QX200 Evagreen supermix was loaded onto QX200 AutoDGddPCR System and followed the procedures of (1) droplet generation; (2) PCR amplification (95 °C for 5 min, (95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min) ×40, 4 °C for 5 min, 90 °C for 5 min, and 4 °C hold); and (3) droplet reading. Data were analyzed with QuantaSoft analysis software (version 1.7.4). ddPCR can readily distinguish duplication, triplication, and even quadruplication of a locus (Gu *et al.* 2016).

Table 1 Mosaicism-level quantification and breakpoint characterization in 18 subjects with XLAG syndrome.

ID	Gender	Inheritance	ddPCR on parental DNA	DLRS	Mosaicism level by aCGH	Mosaicism level by ddPCR	Breakpoint features	Potential mechanism
S1	M	Sporadic	Both NA	0.11	0.538	0.585 ± 0.016	12 bp microhomology	FoStEs/MMBIR, MMEJ
S11	M	Sporadic	Mother negative, father NA	0.18	0.256	0.294 ± 0.014	2 bp microhomology	FoStEs/MMBIR, MMEJ
S15	M	Sporadic	Both NA	0.15	0.161	0.182 ± 0.022	1 bp microhomology	FoStEs/MMBIR, MMEJ
F2A	M	Familial	Both NA	0.27	0.691	NA	2 bp microhomology	FoStEs/MMBIR, MMEJ
F1B	M	Familial	Mother (F1A) positive, father NA	0.14	0.769	0.854 ± 0.052	4 bp insertion	FoStEs/MMBIR, NHEJ
F1C	M	Familial	Mother (F1A) positive, father NA	0.15	0.785	0.845 ± 0.059	4 bp insertion	FoStEs/MMBIR, NHEJ
F1A	F	Familial	Both NA	0.14	1.030	0.839 ± 0.074	4 bp insertion	FoStEs/MMBIR, NHEJ
S2	F	Sporadic	Both negative	0.24	1.377	1.312 ± 0.117	5 bp microhomology	FoStEs/MMBIR, MMEJ
S4	F	Sporadic	Both negative	0.12	1.066	0.957 ± 0.010	CGR with small insertions and microhomology at JCT	FoStEs/MMBIR
S5	F	Sporadic	Both NA	0.13	1.099	1.032 ± 0.026	5 bp insertion	FoStEs/MMBIR, NHEJ
S6	F	Sporadic	Both negative	0.13	0.999	0.944 ± 0.033	2 bp microhomology	FoStEs/MMBIR, MMEJ
S7	F	Sporadic	Both NA	0.14	1.041	0.944 ± 0.051	3 bp microhomology	FoStEs/MMBIR, MMEJ
S8	F	Sporadic	both NA	0.14	0.911	0.968 ± 0.057	3 bp microhomology	FoStEs/MMBIR, MMEJ
S9	F	Sporadic	Both NA	0.24	0.963	1.017 ± 0.034	4 bp microhomology/ 7 bp microhomology	FoStEs/MMBIR, MMEJ
S10	F	Sporadic	Both NA	0.16	0.758	0.986 ± 0.048	4 bp microhomology	FoStEs/MMBIR, MMEJ
S14	F	Sporadic	Both NA	0.16	1.150	0.994 ± 0.007	3 bp microhomology with an 8 bp deletion nearby	FoStEs/MMBIR, MMEJ
S13	F	Sporadic	Both negative	0.11	0.806	0.974 ± 0.040	52 bp insertion	FoStEs/MMBIR, MMEJ
S16	F	Sporadic	Both NA	0.14	0.845	0.915 ± 0.033	291 bp insertion	FoStEs/MMBIR, MMEJ

CGR, complex genomic rearrangement; FoStEs/MMBIR, fork stalling and template switching/microhomology-mediated break-induced replication; MMEJ, microhomology-mediated end joining; NA, sample not available; NHEJ, nonhomologous end joining.

Workflow for mosaicism quantification by HD-aCGH and ddPCR

We utilized a workflow combining HD-aCGH, JCT sequencing, and quantification ddPCR to characterize XLAG duplications and quantify their level of mosaicism (Supplementary Figure 1, see section of supplementary data given at the end of this article).

HD-aCGH The mosaicism level (α_f for female, α_m for male) was calculated based on HD-aCGH LR, which is the mean LR of all probes involved in the genomic segments that are duplicated:

$$\alpha_f = \frac{2^{LR} - 1}{0.5}$$

$$\alpha_m = 2^{LR} - 1$$

JCT-specific ddPCR JCT-specific (JCT) or control (CTRL) ddPCR, as described previously, was performed with equal DNA input (25 ng) in separate reactions for each sample, and the number of positive droplets

was compared between JCT and CTRL to quantify mosaicism. Theoretically, the number of positive droplets indicates the number of chromosomes with positive PCR amplification. CTRL ddPCR provides a positive signal in every droplet sequestering at least one copy of the X chromosome. JCT ddPCR specifically uses the breakpoint as a template and only provides positive signals for the droplets sequestering at least one copy of the X chromosome with the specific XLAG syndrome duplication. Due to the random nature of partitioning in droplet generation, templates are randomly distributed in droplets. As a result, different droplets may contain different numbers of templates. Poisson distribution analysis was subsequently utilized to determine template concentration (M: concentration of JCT; N: concentration of CTRL) (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). Thus, theoretically for males (one copy of X chromosome), a JCT/CTRL ratio of M/N indicates a constitutional XLAG duplication if M=N, whereas a JCT/CTRL ratio of M/N suggests mosaicism if M<N, as M out of N cells harbor hemizygous XLAG syndrome

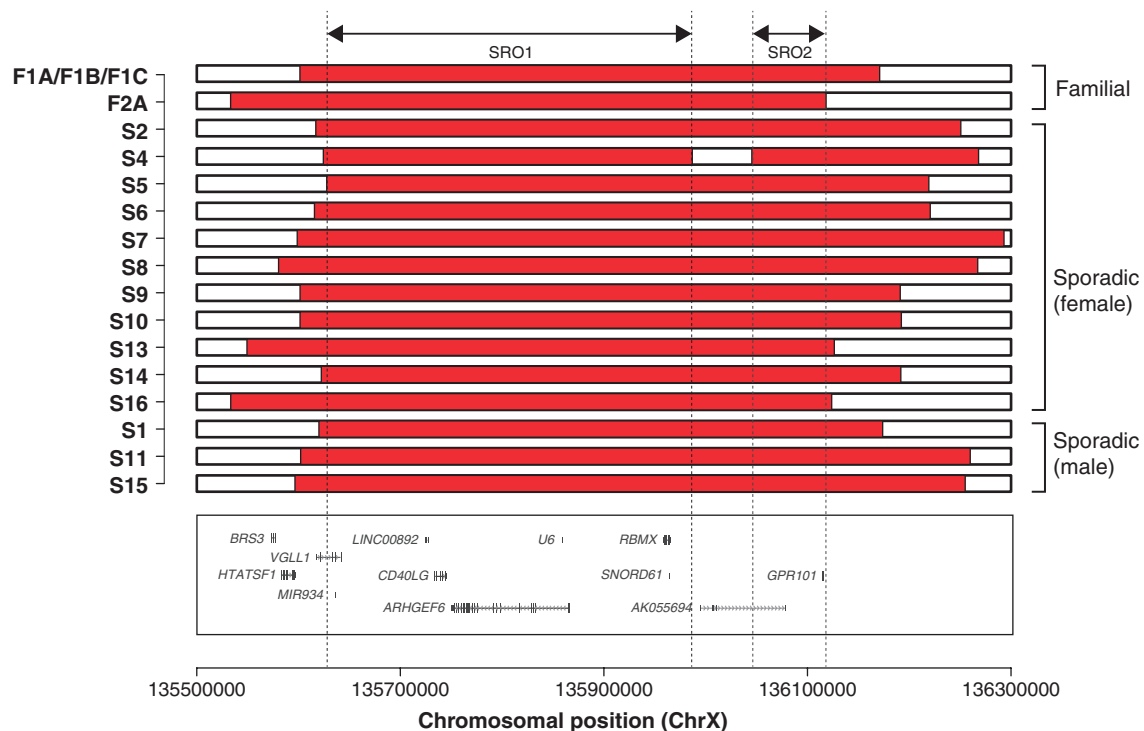


Figure 1

Summary of the XLAG duplications identified in 18 subjects with familial or sporadic XLAG. All duplications are delineated by HD-aCGH, and the boundaries of each gain are demarcated by JCT sequencing. Duplicated genomic segments (red) and nonduplicated segments (white) are shown. The genomic coordinates are shown on the x-axis. The two SROs, SRO1 and SRO2, are demarcated by vertical dashed lines. The box underneath the duplication diagrams contains the genomic contents in the corresponding regions, which are adapted from the UCSC Genes track in the UCSC Genome Browser.

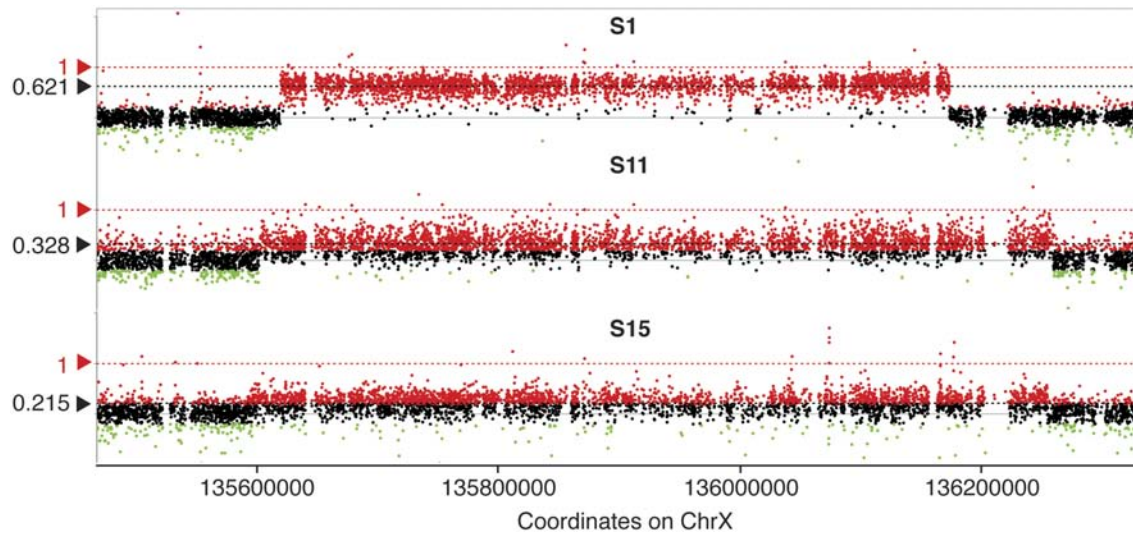


Figure 2

HD-aCGH LR plot showing the genomic gains identified in three sporadic males. The red dashed line in each plot denotes the theoretical LR value of heterozygous duplication on X chromosome in males; the black dashed line in each diagram denotes the LR values of the XLAG duplications identified in each subject. Red probes, $LR > 0.2$; black probes, $-0.2 \leq LR \leq 0.2$; green probes, $LR \leq -0.2$.

duplication (the level of which should be calculated as M/N) (Supplementary Figure 2). However, for females (copy number of X chromosome = 2), a JCT/CTRL ratio of M/N indicates a constitutional XLAG syndrome duplication if $M=N/2$, whereas a JCT/CTRL ratio of M/N is in keeping with mosaicism if $M < N/2$, as M out of N/2 cells harbor heterozygous XLAG duplication (the level of which should be calculated as $2M/N$). Three technical replicates were performed for each ddPCR reaction to determine the mosaicism level.

Results

Eighteen subjects, including 6 males (three sporadic, three familial) and 12 females (11 sporadic, 1 familial), were identified with duplications encompassing *GPR101* (Table 1). Among these, 15 were reported previously (Trivellin et al. 2014, Beckers et al. 2015, Naves et al. 2015), although none was studied previously for somatic mosaicism. The three new XLAG syndrome patients (two females, one male) were adult sporadic pituitary gigantism cases whose disease began at childhood. We now report studies to investigate for potential mosaic duplication in these 18 subjects by orthogonal methods combining HD-aCGH, CNV JCT sequencing, and ddPCR (Campbell et al. 2014b, Gu et al. 2016) to achieve mosaic duplication detection and quantification (Supplementary Figure 1).

On HD-aCGH, all the identified XLAG duplications are unique and have apparently variable boundaries, documenting nonrecurrent duplications (Fig. 1). The new XLAG duplications in this study did not alter the smallest regions of overlap (SROs) reported previously (Trivellin et al. 2014, Beckers et al. 2015). These duplications range in size from 554 to 674kb, and all include the *GPR101* gene that has been functionally demonstrated to be contributing to the disease (Trivellin et al. 2014). Microhomology, small insertions, and one complex genomic rearrangement were identified at the JCT (Table 1, Supplementary Figure 3), and are in keeping with fork stalling and template switching/microhomology-mediated break-induced replication (FoStEs/MMBIR) as the potential mechanism for the duplication (Lee et al. 2007, Hastings et al. 2009, Zhang et al. 2009, Trivellin et al. 2014, Sakofsky et al. 2015).

Using HD-aCGH, we observed that male subjects had a decreased LR level in comparison with the theoretical LR value of constitutional duplication CNV on the X chromosome in males ($LR = 1$). All the male subjects show decreased LR, in keeping with mosaicism (three sporadic males in Fig. 2 and three familial males in Supplementary Figure 4). Taken together, male XLAG syndrome patients, as a group, demonstrated significantly lower LR values compared with female patients as a group (Welch Two Sample *t*-test, $P=0.003$). Moreover, sporadic male XLAG patients had the clearest evidence of mosaicism on

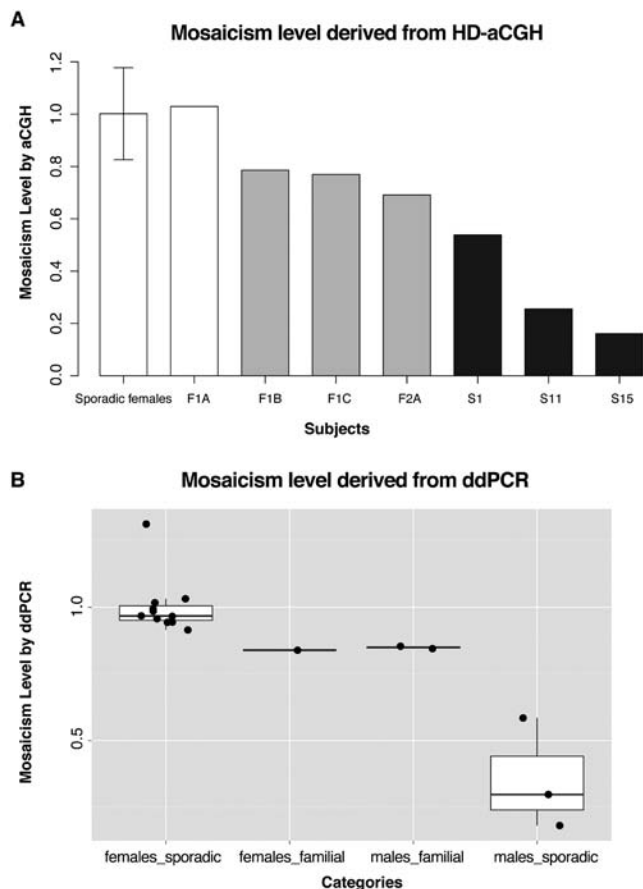


Figure 3 Mosaicism-level quantification. (A) Mosaicism levels quantified by HD-aCGH. White bar indicates the average mosaicism level quantified in 11 sporadic females and 1 familial female (F1A); gray bars indicates mosaicism levels quantified in three familial males; black bars indicates mosaicism levels quantified in three sporadic males. (B) Mosaicism levels quantified by ddPCR. The subjects are divided into four categories (sporadic females, familial females, familial males, and sporadic males), and the mosaicism levels of the subjects in each category are represented by dots in the box plot.

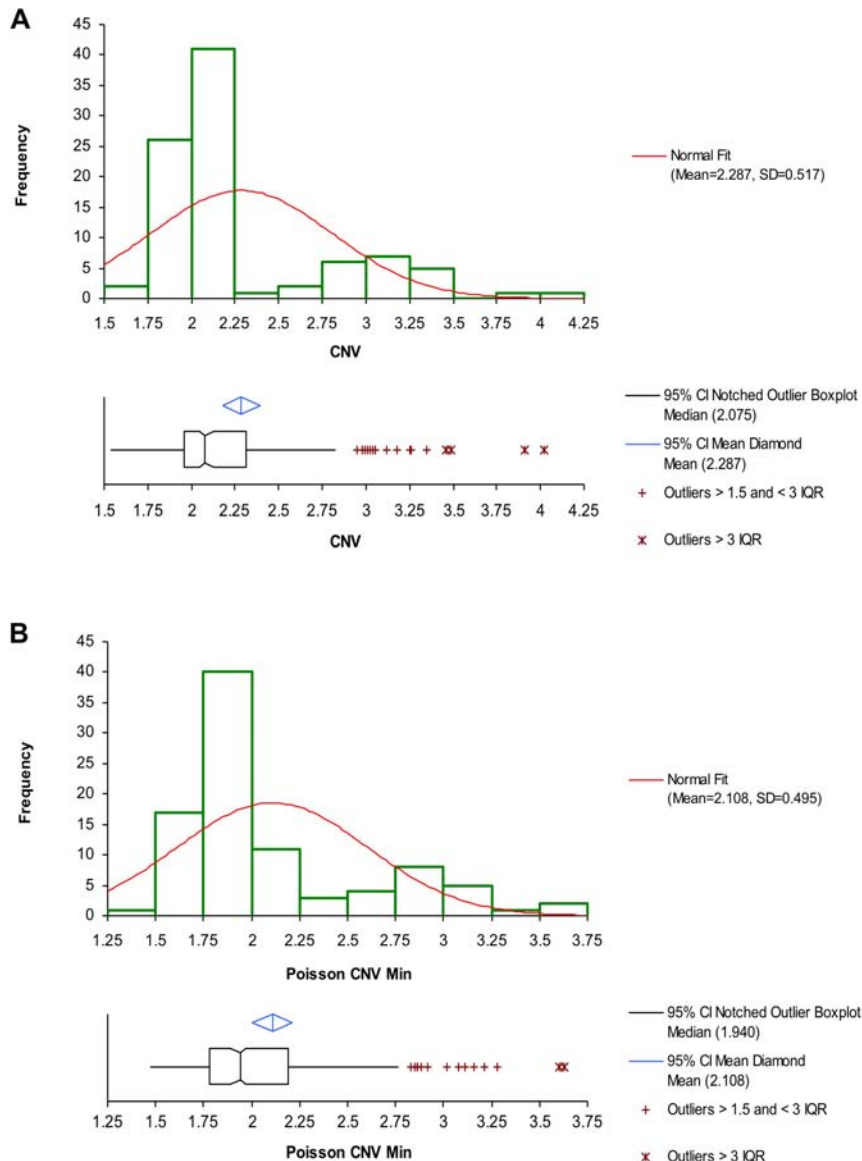
HD-aCGH. The three sporadic males had lower levels of duplication (S1 (53.8%), S11 (32.8%), and S15 (16.1%)) than the three familial XLAG males (F2A (69.1%), F1B (76.9%), and F1C (78.5%)). On HD-aCGH, there was no evidence of mosaicism in female XLAG subjects (Table 1, Fig. 3).

To further examine and precisely quantify the mosaicism level in the cohort, we implemented personalized ddPCR assay targeting the JCT in each subject (Supplementary Figure 1), thus measuring the copy number specifically for the novel JCT (Campbell et al. 2014b, Gu et al. 2016). Using this approach, we confirmed the HD-aCGH finding that female XLAG patients had no evidence of mosaicism (Fig. 3A). On ddPCR, we also confirmed the HD-aCGH finding of low-level mosaicism in blood DNA of three

sporadic male XLAG subjects, S1, S11, and S15. The mosaicism levels in these subjects were $58.5\% \pm 1.6\%$, $29.4\% \pm 1.4\%$, and $18.2\% \pm 2.2\%$, respectively, which are similar in magnitude to those obtained on HD-aCGH. In familial XLAG males, the potential mosaicism level on ddPCR was intermediate between that seen in the sporadic males and the lack of mosaicism in the female XLAG patient group (Fig. 3B, Supplementary Figure 5). Taken together as a group, males with XLAG had significantly lower ddPCR values than females with XLAG (Welch Two Sample *t*-test, $P=0.02677$, Fig. 3), which are again consistent with the HD-aCGH results.

We also developed a ddPCR assay in order to assess the feasibility of screening existing populations with acromegaly and gigantism for abnormalities in copy number at *GPR101* (causative and duplicated in XLAG syndrome) vs *ZIC3* (not duplicated in XLAG syndrome). We studied a total of 91 samples with a median value of 8.77 ng (95.3% CI: 7.13–10.13) for the *ZIC3* gene (exon 1–intron 1/2). The median value of ΔCNV ($\text{CNV}_{\text{max}} - \text{CNV}_{\text{min}}$) was 0.315 (95.3% CI: 0.270–0.350); this difference was calculated according to the Poisson law (95% CI: $\text{CNV value} \pm 0.175$). The CNV distribution showed very few intermediate values, reflecting the capacity of the screening ddPCR to discriminate nonduplicated samples (1.75–2.25) from duplication without identifying intermediate values (Fig. 4). Concordance results between HD-aCGH and screening ddPCR were very good (Pearson's χ^2 statistic=85.78; DF=4, $P<0.0001$). The eight XLAG patients were positive on this screening ddPCR (CNV median: 3.050, 97.3% CI: 2.98–3.26; min 2.73; Supplementary Figure 6A), and the seven normal patients had a normal CNV status (CNV median: 1.93, 98.4% CI: 1.710–2.17; Supplementary Figure 6B).

In the screened population, 60 out of 64 patients had a nonduplicated ddPCR CNV status (ddPCR CNV median: 2.05, 96.0% CI: 2.000–2.070). Of the four patients with CNV values outside of the thresholds empirically established for this screening assay, one female (S16) had a distant history of pediatric-onset acrogigantism (diagnosed >40 years previously) and lifelong active acromegaly; her CNV_{max} and CNV_{min} values were 3.69 and 3.28, respectively. On HD-aCGH, she was found to have a chromosome Xq26.3 duplication. Molecular mechanism studies revealed a 291-base pair insertion at the breakpoint, suggesting FoSTeS/MMBIR for formation of the duplications. A diagnosis of XLAG syndrome was made. A male with adolescent-onset acrogigantism was positive for potential duplication on screening ddPCR, although his values were lower than those seen in XLAG cases (CNV_{max} :

**Figure 4**

Frequency distribution of CNV values at *GPR101* vs *ZIC3* using ddPCR among the total screening population. (A) The frequency distribution of CNVmax values across the screened population, with a normal fit graph outlined in red over the frequency columns. Below this, the 95% CIs for the CNVmax are displayed in a notched median value box plot, with the corresponding 95% CI mean indicated by a blue diamond; outliers outside of these distributions (including control known duplicated cases) are shown by crosses. (B) The corresponding CNVmin frequency distribution in columns, the normal fit curve (red), and the notched median value box plot for the 95% CIs for CNVmin; 95% CI mean (blue diamond) and outliers (crosses) are also shown.

2.89; CNVmin: 2.33); on HD-aCGH, no duplication was discerned. A female pediatric-onset acromegaly patient had inconclusive ddPCR values as her CNVmax (2.36) was below the duplication threshold of 2.5, whereas her CNVmin (2.36) was above the threshold of 2.0. HD-aCGH showed no duplication. A female pediatric-onset gigantism patient had a screening ddPCR result with CNV levels below normal; the HD-aCGH result was normal and no abnormality was seen at the Xq26.3 locus.

Discussion

In this study we demonstrate that somatic mosaicism plays an important role in the pathogenesis of XLAG

syndrome, a newly described, severe form of pediatric-onset gigantism caused by pituitary tumors. In contrast to females who have constitutional submicroscopic duplications at chromosome Xq26.3, we have shown that sporadic male patients with XLAG are somatic mosaics that display a variable degree of mosaicism. Sporadic males can demonstrate quite low levels of mosaicism for the XLAG duplication in the DNA isolated from blood (e.g., 16.1%). Familial males with XLAG are intermediate between sporadic males and the female patients, which indicates that there may be variable mosaicism in males, although too few familial cases are available to make that determination with certainty at this time. This finding was demonstrated first using

an HD-aCGH method specifically focused around chromosome Xq (Trivellin et al. 2014). In addition, we developed a new quantitative method of ddPCR that was specific to each patient and their particular unique duplication characteristics. This ddPCR technique also confirmed the existence of somatic mosaicism in male XLAG patients, with findings that were almost identical to those achieved using HD-aCGH.

Somatic mosaicism arises postzygotically. Mutations that occur at different developmental timings may have diverse tissue distributions and impact distinctly on human genetic or genomic disorders. The number of mitoses (or cell divisions) between generations is estimated to be 400 for males, whereas the number is 30 for females (Drost & Lee 1995, Campbell et al. 2014b). If a mutation occurs in the parental generation and becomes a confined gonadal mosaicism, it may be transmitted to the offspring and appear as a constitutional and apparently *de novo* mutation. Such mosaic mutations are confined to the germ line; thus, they usually do not manifest clinical phenotypes and may evade genetic testing. However, these mutations contribute considerably to the recurrence risk of genetic disorders, a situation in which more than one child from the same family can be born with the same apparently sporadic autosomal dominant condition. However, mutations may occur during early embryonic development of an individual; particularly during the many mitoses that occur with the rapid proliferation accompanying early embryogenesis. As a result, the mutation may be segregated into a limited number of cell lineages. These somatic mosaic mutations may be associated with known genetic or genomic disorders and consequently lead to differential phenotypic consequences. Such mutations are mosaic because they are not uniformly represented by the entire cell population throughout the human organism. If the mutation segregated in hematopoietic stem cells that further develop into blood cells, these may be detected by genetic testing using blood as the specimen, or may not be detectable in blood if the mutation did not segregate in hematopoietic cells. We present here a systematic study, including the detection, molecular investigation, quantification, and clinical correlation of somatic mosaicism underlying XLAG syndrome, a recently defined early childhood-onset form of pituitary gigantism.

XLAG syndrome is a form of gigantism that is likely caused by *GPR101* duplication, whereas potentially activating and inactivating mutations of *GPR101* have been identified in patients with pituitary adenomas and

GH deficiency, respectively (Trivellin et al. 2014, Castinetti et al. 2016). We have identified mosaic XLAG syndrome locus duplications that are likely to be the cause of the disease phenotype. The mosaic XLAG syndrome locus duplication may arise postzygotically as a mitotic event during the early embryonic developmental stage of the sporadic males, affect GH secretion thereafter, and eventually contribute to the XLAG syndrome phenotype. These XLAG locus duplications may also occur before the segregation of blood and pituitary cell lineages, and therefore affect the GH secretion from the pituitary tissue and are detectable by HD-aCGH using blood-derived DNA. Our data reveal that males had significant evidence of mosaicism, whereas females did not (Fig. 3). Moreover, familial males with XLAG syndrome had duplication levels that were intermediate between the constitutional levels seen in females and clear mosaicism seen in sporadic males. However, based on a *a priori* hypothesis, an X-linked mutation is anticipated to be constitutional in these familial males who inherit the same mutation from the mother. The lower level of XLAG locus duplication observed in familial males compared to females may result from the uncertainty of measurements. In spite of this, if mosaicism is eventually confirmed in familial males, it might perhaps be explained by a somatic reversion mechanism mediated by mitotic intrachromosomal non-allelic homologous recombination (NAHR) (Liehr et al. 1996, Steinmann et al. 2007, Liu et al. 2014a), additional experiments could be designed to further test this hypothesis.

A comparison of the clinical phenotype and disease characteristics in the mosaic sporadic males compared with sporadic females with XLAG syndrome reveals some important findings. All three mosaic males had severe early-onset overgrowth due to pituitary adenomas and the disease was diagnosed at a similarly young age as sporadic female XLAG syndrome cases. Furthermore, the severe hormonal hypersecretion and the subsequent overgrowth pattern required complex multimodal surgical and medical therapy in the sporadic mosaic males, again not differing from nonmosaic cases. Final height in pituitary gigantism, irrespective of genetic cause, is determined by a variety of factors, not least early control of hormonal hypersecretion (Rostomyan et al. 2015). Two of the three sporadic mosaic males with XLAG syndrome did not undergo neurosurgery or effective medical therapy during childhood and hence have extreme gigantism (209 cm at 12 years in one case, Z-score >8.7, and >230 cm final height in the other) (Naves et al. 2015). The other patient was controlled by surgery and medical therapy (GH

receptor antagonist, pegvisomant) during childhood and can be expected to have a normal final height (Beckers et al. 2015). Therefore, the clinical profile does not seem to differ between mosaic and nonmosaic XLAG syndrome patients. Relatively low levels of duplication at the XLAG syndrome locus (16.8–32.8% as detected in the blood) can lead to some of the most dramatic pediatric and adult cases of pituitary gigantism in recorded medical history. This suggests that the pathological process is highly sensitive to even minor levels of increased copy number at the XLAG syndrome locus. The pituitary findings in cases of XLAG syndrome are quite uniform (mixed GH–prolactin-secreting pituitary adenomas and/or hyperplasia) irrespective of the level of mosaicism of the patient (Trivellin et al. 2014, Beckers et al. 2015, Naves et al. 2015). Moreover, as males and females are clinically similar, the impact of X-chromosome inactivation in females with XLAG syndrome should be considered, as this could hypothetically alter the level of duplication occurring in specific tissues, such as the hypothalamus.

Daly and coworkers have recently reported that GHRH hypersecretion is implicated in XLAG syndrome and that GHRH antagonism can inhibit GH and prolactin secretion from primary tumor cell culture in XLAG syndrome (Daly et al. 2016). Other rare clinical and experimental instances of chronic GHRH hypersecretion lead to similar pathological effects on the pituitary gland (Mayo et al. 1988, Asa et al. 1992, Borson-Chazot et al. 2012). GHRH is a very potent physiological stimulator of GH, and GHRH secretion by a discreet population of hypothalamic neurons is tightly regulated by integrated central and peripheral signals (Gahete et al. 2009, Veldhuis et al. 2012). *GPR101* is specifically expressed in regions of the hypothalamus and brain that are involved in integration of such signals, the dysregulation of GHRH secretion, and pituitary pathology in XLAG syndrome (Bates et al. 2006, Trivellin et al. 2014, 2016). Taken together, these findings suggest a mechanism by which even modestly increased copy number of *GPR101* could lead to the severe pituitary gigantism observed in XLAG syndrome patients with an Xq26.3 duplication; some of these mosaicism levels may be beyond that which can currently be detected by our techniques. Moreover, mosaicism might occur only in nervous system tissues or only in cells from which the pituitary derives and might not be present in the blood.

Somatic mosaicism, which may introduce false-negative results in genetic testing, is always challenging to detect. A large number of techniques have been described for mosaicism detection (Campbell et al. 2015). Although next-generation sequencing has been successful

in detecting somatically mosaic SNVs in patients with a specific disorder (Huisman et al. 2013, Ansari et al. 2014), difficulty remains for mosaic CNVs (Rahbari et al. 2016). Conventional cytogenetic techniques, such as karyotyping and fluorescence *in situ* hybridization (FISH), provide direct visualization and quantification of mosaic SVs by scoring a sufficiently large number of cells. However, the result may be biased given potential cell culture artifacts (Cheung et al. 2007). Moreover, submicroscopic CNVs with sizes smaller than 50 kb make karyotyping and FISH unrevealing for prospective mosaicism (Pham et al. 2014). In this study, we used aCGH with high-density probes to retrospectively interrogate the known region for XLAG syndrome and provide molecular details of the rearrangement, which allow further quantification by ddPCR. It is suggested that mosaicism may be detected at a level as low as 10–20% under ideal conditions by aCGH (Ballif et al. 2006, Boone et al. 2010, Pham et al. 2014), and potentially to the 5% level utilizing B-allele frequency information from SNP arrays (Conlin et al. 2010). Driven by phenotype, a personalized assay (e.g., targeted deep sequencing or HD-aCGH) may be designed to investigate the known disease-associated loci in detail. Sampling various tissues may also benefit mosaicism detection, as demonstrated in subjects with Cornelia de Lange syndrome (Huisman et al. 2013). In females with XLAG syndrome, we observe roughly equal dosage changes in pituitary tumor-derived DNA compared with blood-derived DNA. At this time, we do not have multiple tissues sampled for the mosaic male subjects, so further analyses of the distribution of mosaicism status in different tissues of new mosaic males will be required in the future.

We used ddPCR to measure *GRP101* duplication because it allows the measurement of low-level mosaicism for CNV (Weaver et al. 2010) and the accurate counting of alleles from DNA isolated from a mixture of heterogeneous cell populations. Previous studies have shown a very high level of concordance between ddPCR and exome sequencing to measure CNV (Handsaker et al. 2015). The HD-aCGH and junction-specific ddPCR techniques provide specific information regarding duplications and mosaicism in individual cases of XLAG syndrome. Neither of these methods is, however, well suited to genetic screening of larger populations of patients with acromegaly and gigantism. To this end, we developed a separate ddPCR assay and validated its use in a population of proven XLAG syndrome cases with known Xq26.3 duplications, normal individuals without Xq26.3 duplications, and a large *de novo* patient population of acromegaly, FIPA kindreds with homogeneous acromegaly, and pituitary gigantism.

Based on the recognition that none of the previously identified cases of XLAG syndrome had duplications that extended telomerically to the *ZIC3* gene, we used this as a reference to compare with *GPR101*, which is a causative gene and is invariably duplicated. By this approach we were able to rapidly 'screen' a sizeable series of target patients with pituitary gigantism and acromegaly (sporadic and familial). Using this screening method, we identified four acromegaly/gigantism cases with results that were abnormal compared to reference controls, of which two were above the threshold, one intermediate, and one that was below the CNVmin threshold; three were normal on subsequent HD-aCGH. The other case was an adult patient with a distant history suggestive of XLAG syndrome, and the ddPCR results were confirmed by HD-aCGH as consisting of a novel duplication at the XLAG syndrome locus. This ddPCR methodology suggested that it could be used as a first step screening assay for studying cohorts of patients with acrogigantism for XLAG syndrome, but would require verification by HD-aCGH in indeterminate and abnormal cases (4.7% of our series).

Using a combination of standard and novel techniques, we have shown that XLAG syndrome, a newly recognized form of severe acrogigantism, has a more diverse genetic pathophysiology than we originally described. Sporadic males in this study all demonstrated evidence of somatic mosaicism for the submicroscopic duplications on Xq26.3 that cause XLAG syndrome. This differs from female XLAG patients who have apparently constitutional duplications. Results obtained using HD-aCGH were validated on CNV assay using a personalized junction-specific ddPCR technique for the unique breakpoints causing Xq26.3 duplication. Moreover, ddPCR screening based on CNV at *GPR101* holds promise for identifying potential XLAG syndrome cases among larger cohorts of acrogigantism patients. Somatic mosaicism is an important pathological mechanism for genetic diseases and genomic disorders, and its contribution to the causation of both new and established disorders should be actively investigated.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-16-0082>

Declaration of interest

J R L has stock ownership in 23andMe; is a paid consultant for Regeneron Pharmaceuticals; has stock options in Lasergen, Inc.; is a member of the Scientific Advisory Board of Baylor Miraca Genetics Laboratories (BMGL); and is a coinventor of multiple US and European patents related to

molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine derives revenue from the chromosomal microarray analysis and clinical exome sequencing offered in the BMGL (<http://www.bmgl.com/BMGL/Default.aspx>). Other authors have nothing to disclose.

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Author contribution statement

A F Daly, B Yuan and F Fina contributed equally to this work. C A Stratakis and A Beckers contributed equally to this work. Adrian F Daly and Frederic Fina designed and performed ddPCR, Bo Yuan and Pengfei Liu designed and implemented HD-aCGH. Bo Yuan performed breakpoint sequencing and junction specific ddPCR. Jean-Hubert Caberg, Emilie Castermans, Giampaolo Trivellin, Isabelle Nanni Metullus, Fabio R Faucz, Leonor Palmeira, Anne Barlier, L'Houcine Ouafik, Vincent Bours and James R Lupski oversaw and performed HD-aCGH, aCGH and sequencing studies. Patient and family data and diagnostic clinical information were analyzed by Adrian F Daly, Liliya Rostomyan, Wouter W de Herder, Luciana A Naves, Daniel Metzger, Thomas Cuny, Wolfgang Rabl, Nalini Shah, Marie-Lise Jaffrain-Rea, Maria Chiara Zatelli, Maya Lodish, Ammar Muhammed, Iulia Potorac, Giovanna Mantovani, Sebastian J Neggers, Marc Klein, Constantine A Stratakis and Albert Beckers. Adrian F Daly, Bo Yuan, James R Lupski, Constantine A Stratakis and Albert Beckers wrote and revised the manuscript.

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