Skewed X-chromosome inactivation in women with idiopathic intellectual disability as indicative of pathogenic variants

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Research Article

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Abstract

Intellectual disability (ID) is an early onset impairment in cognitive functioning and adaptive behavior, affecting approximately 1% of the population worldwide. Extreme deviations of X-chromosome inactivation (XCI) can be related to ID phenotypes caused by pathogenic variants in the X-chromosome. We analyzed the blood pattern of XCI in 194 women with idiopathic ID, using the androgen receptor gene (*AR*) methylation assay. Among the 136 patients who were informative, 11 patients (8%) presented with extreme or total XCI deviation (≥90%), which was significantly higher than the deviation expected by chance. Whole-exome data obtained from these 11 patients revealed the presence of dominant pathogenic variants in eight of them, all sporadic cases, resulting in a molecular diagnosis rate of 73%. All variants were mapped to ID-related genes with dominant phenotypes: four variants in the X-linked genes *DDX3X* (two patients), *WDR45* and *PDHA1*), of which one is an XCI escape gene (*DDX3X*), and four variants in autosomal genes (*KCNB1, CTNNB1, YY1, ANKRD11*). Three of the autosomal genes had no obvious correlation with the observed XCI skewing. However, *YY1* is a known transcriptional repressor that acts in the binding of the XIST long noncoding RNA on the inactive X chromosome, providing a mechanistic link between the pathogenic variant and the detected skewed XCI in the carrier. These data confirm that extreme XCI skewing in females with ID is highly indicative of causative X-linked pathogenic variants and point to the possibility of identifying causative variants in autosomal genes with a role in XCI.

Introduction

Intellectual disability (ID) is a neurodevelopmental condition characterized by impairments in cognitive functioning and adaptive behavior, with onset up to 18 years of age [1], affecting approximately 1% of the population worldwide [2]. It can be an isolated feature or occur in addition to other clinical signs, characterizing a syndromic ID presentation. In many moderate to severe ID cases, the etiology remains idiopathic, even after extensive clinical and genetic investigation. According to Savatt and Myers (2021), the diagnostic yield of ID or global developmental delay is approximately 1% in females and 2% in males for Fragile X syndrome molecular investigation, 15% for chromosome microarray analysis (CMA), and 35% for whole-exome sequencing. Therefore, the cumulative diagnostic yield is only ~50%, and is higher for individuals with lower intelligence quotient scores (IQs) and syndromic presentations [3].

Globally, ID exhibits a sex-biased pattern, affecting 30% more males than females. Several studies have focused on X-linked causes of ID (XLID) in male cohorts [4]. Many hypotheses have been proposed to explain this male bias, such as the fact that males are hemizygous for most of the X-linked genes and will manifest the condition even if it presents a recessive inheritance pattern. However, XL genes cannot be solely responsible for the excess number of males affected by ID, since XLID-related genes correspond to only ~8.2% of the total recognized ID-related genes according to SysNDD data (https://sysndd.dbmr.unibe.ch//; accessed on May 23, 2022) [5]. Alternatively, the functional mosaicism presented by females due to the stochastic nature of X-chromosome inactivation (XCI) minimizes the penetrance and expressivity of phenotypes with dominant patterns [6,7]. Moreover, it was hypothesized

that females with ID would carry an increased burden of genetic variants that are deleterious to cognition compared to affected males. Some studies support this hypothesis, suggesting a protective model for neurodevelopmental disorders (NDDs) in females, in which the threshold for ID would be higher, requiring a larger set of damaging variants to express the deleterious phenotype [8–10].

Consequently, ID manifestation in females is less explored. A causal link between skewed XCI and X-chromosome pathogenic variants has been proposed [7,11–13]. XCI is a natural process that regulates genic dosage between the biological sexes by randomly inactivating one of the X-chromosomes in females, leading to the epigenetic silencing of most of its genes [14–16]. Therefore, due to its stochastic nature, there is a continuous distribution of XCI patterns in the general female population, and skewed XCI is observed in a small proportion of healthy females. In the United States, 1.8% of a cohort of 1,005 healthy females presented extremely skewed XCI (≥90:10) [11], whereas in a Brazilian study, skewed XCI between 80-89% was observed in ~2.6% of a group of 118 healthy females, and no cases of skewed XCI ≥90% were detected [13]. In a group of 53 Brazilian females with idiopathic ID, an extremely skewed XCI (≥90:10) was observed in 11% of the patient cohort [13]. Similarly, a Belgian study found extremely skewed XCI in 7.6% of a cohort of 288 females with idiopathic ID, which was a significantly higher frequency in comparison to the healthy cohort [7].

Hypothetically, clinical conditions in females caused by structural or sequence variants in the X-chromosome, including ID, could be related to deviations from the XCI patterns [7,16,17]. In this study, we investigated whether extremely or completely skewed XCI was increased in a group of females with idiopathic ID that was previously tested by karyotyping/CMA or Fragile X syndrome. Our results provide insights into the role of the ID-related variants found in the X chromosome and autosomal genes in skewed XCI.

Subjects And Methods

X-inactivation studies

We evaluated the pattern of X-chromosome inactivation in 194 unrelated Brazilian females presenting idiopathic ID who were previously referred for genetic evaluation in the Human Genetics Research Laboratory at the Department of Genetics and Evolutionary Biology from the Institute of Biosciences, University of São Paulo, Brazil. Genomic DNA samples from the patients and their parents were extracted from peripheral blood leukocytes using standard procedures. Among the 194 patients, 70 (36%) had nonsyndromic ID and 124 patients (64%) showed other clinical signs (syndromic ID). Most of the cases were sporadic (157 patients, 81%), while 37 patients (19%) reported the occurrence of other cases of ID or global developmental delay in the family. Among the whole cohort, 154 patients (79%) had been previously investigated by karyotype (G-banding) and CMA with normal results, and 68 had undergone molecular testing for Fragile X syndrome, also showing negative results.

XCI analyses were based on the evaluation of the differential methylation of the human androgen receptor (*AR*) gene (HUMARA assay), adapted from Allen et al. (1992) [18]. PCR products were analyzed

by capillary electrophoresis using an ABI 3730 DNA Analyzer (Thermo Fisher Scientific Inc.), and data analysis was performed with GeneMarker™ software (SoftGenetics). Each experiment included a male DNA sample (negative control) and a female DNA sample with a known pattern of extremely skewed XCI (positive control). XCI ratios below 80:20 were considered to represent a random pattern. The XCI ratio calculations followed Bittel et al. (2008) [19]. Three categories were established for patients in which we observed relevant XCI deviations: (1) 80:20 - 89:11 (moderately skewed), (2) 90:10 - 99:01 (extremely skewed), and (3) 100:0 (completely skewed). Extremely/completely skewed XCI (≥90:10) was confirmed in duplicate experiments.

Whole-exome sequencing (WES)

WES was performed for females showing skewed XCI ≥90% using the services of the 3Billion Company (https://3billion.io/). One female (P10) had a previous negative WES report. Sequencing libraries were constructed using xGen Exome Research Panel v2 (Integrated DNA Technologies), and the captured exomic regions were sequenced on the Novaseq 6000 platform (Illumina). The raw data that were generated were then processed, and sequence alignment was based on the reference genome GRCh37/hg19. Analysis of the annotated VCF files was conducted with open-source bioinformatic tools. Allele frequencies were estimated using the gnomAD database (http://gnomad.broadinstitute.org/), and common variants with a minor allele frequency > 5% were filtered out. The automated variant interpretation software EVIDENCE [20] was used to prioritize pathogenic (P) and likely pathogenic (LP) variants based on the American College of Medical Genetics and Genomics (ACMG) guidelines [21] and the patients' phenotypes. The pathogenicity of each variant was determined based on its association with known diseases according to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), UniProt (https://www.uniprot.org/), OMIM (https://omim.org/), and Orphanet (https://www.orpha.net/consor/cgi-bin/index.php).

Sanger sequencing

ID-related P/LP variants identified in the WES analysis were validated by Sanger sequencing. In addition, when parental DNA samples were available, segregation analysis for the variant was also conducted. PCR was performed using standard conditions [95 °C, 5 min (95 °C, 1 min, Tm-5, 1 min, 72 °C, 1 min) x 35 cycles, 72 °C, 4 min], and amplicons were sequenced in both directions using an ABI 3730 DNA sequencer (Thermo Fisher Scientific Inc.).

Statistical analysis

The frequencies of females with skewed XCI among ID patients and healthy females were compared by means of Fisher's exact tests and odds ratio (OR) analyses. The exact and approximate 95% confidence intervals of these frequencies were also estimated. For these calculations, we employed computational programs/scripts developed in R language (Copyright 2018 The R Foundation for Statistical Computing), and in Liberty Basic (Copyright 1992-2010 Shoptalk Systems).

Results

The Figure 1 summarizes the results of this study.

X-inactivation pattern

Fifty-eight (30%) patients were found to be uninformative in the XCI pattern analysis due to homozygosity in the *AR* locus or *AR* alleles with similar sizes, preventing their evaluation (*Supplementary Figure 1).* Most of the 136 informative patients (73%; n = 99) showed XCI ratios <80:20, whereas 26 (19%) presented moderately skewed XCI with ratios between 80:20 - 89:11. In the remaining 11 patients (8%), extremely or completely skewed XCI was observed: seven patients exhibited XCI ratios 90:10 - 99:01, whereas completely skewed XCI (100:00) was detected in four patients. The age at blood collection of the 11 patients with extremely or completely skewed XCI ranged from 1y9m to 16y9m.

Statistical comparison of the frequencies of skewed X-chromosome inactivation in different studies

The frequency of healthy females with XCI deviation observed by Amos Landgraf et al. [11] (1,005 phenotypic normal females with ratios ≥90:10) was $q_1 = x_1/n_1 = 18/1005 = 0.0179$, with an exact 95% confidence interval of 0.0107-0.0281; the corresponding approximate (normal approximation) 95% CI was 0.0097-0.0261, obtained from $q_1 \pm 1.96$ s.e. (q_1). The observed frequency in a Brazilian sample of healthy females [13] was $q_2 = x_2/n_2 = 0/118 = 0.000$, with an exact 95% confidence interval of 0.0000-0.0308. The overlap of both confidence intervals indicates that these two frequencies were not significantly different (Fisher's exact test: P = 0.2447 and a corresponding OR = 0.23, with a 95% confidence interval of 0.03 to 1.70), pointing out that the two samples can be agglutinated for comparison with the frequency of XCI deviation derived from the present study. We then obtained the overall (combined) frequency of healthy females with skewed XCI: $q_3 = (x_1+x_2)/(n_1+n_2) = x_3/n_3 = 18/1123 = 0.0160$ (exact 95% confidence interval of 0.0096-0.0252; the corresponding approximate 95% CI was 0.0087-0.0234). In the present study, eleven females with ID presented XCI deviation: $q_4 = x_4/n_4 = 11/136 = 0.0809$, with an exact 95% confidence interval of 0.0411-0.1401; the corresponding approximate 95% CI was 0.0351-0.1267. The clear nonoverlap of both confidence intervals (in the combined normal sample and among affected women of the present study) strongly indicates that these two frequencies are significantly different. These results were confirmed by Fisher's exact test (P = 0.0001 and a corresponding OR analysis: OR = 5.40, with a 95% confidence interval of 2.49 to 11.70), showing a highly significant difference between the frequencies of the controls and females with ID.

Whole-exome sequencing

Through WES, eight ID-related heterozygous variants classified as pathogenic (P) and validated by Sanger sequencing were detected in eight of the 11 females with extremely or completely skewed XCI (**Figure 1**), resulting in a molecular diagnostic yield of 73%. In six patients for whom parental DNA samples were available, segregation analysis by Sanger sequencing confirmed a *de novo* status of the

variants (**Figure 2**). In one of these patients (P5), a mosaic pattern for the *CTNNB1* pathogenic variant was detected in the WES analysis (18% of the reads) and validated by Sanger sequencing.

Table 1 presents the clinical data of the 11 patients with extremely skewed XCI (≥90%), as well as information regarding the detected variants. The variants detected in P2, P4, P5, P7, and P8 were previously described in ClinVar as P/LP (VCV000545446.2, VCV000212592.19, VCV000265085.10, VCV001031774.1, and VCV000800948.1, respectively), and a splicing variant classified as LP (VCV001251939.1) was mapped to the same position of the variant detected in P1. All variants were deposited in ClinVar, including the novel variants detected in P3 (NM_000284.4:c.1009-16_1010del) and P6 (NM_003403.5:c.1062+1G>A), as well as the variant detected in P1. All patients with positive WES positive findings were sporadic cases.

All variants were mapped to genes associated with conditions with a dominant pattern of inheritance. In four patients, the variants occurred in the X-linked genes *DDX3X, PDHA1* and *WDR45*; two patients carried different P variants in the *DDX3X* gene. Four patients exhibited P/LP variants mapped to autosomal genes (*KCNB1, CTNNB1, YY1, ANKRD11*). Other clinically relevant variants were not identified. We also searched for rare potentially deleterious variants (haploinsufficiency DECIPHER score ≥0.9 for LoF variants or REVEL ≥0.8 for missenses) mapped to the X chromosome, particularly in the *XIST* gene, that could explain the inactivation deviation, with negative results.

Discussion

It is known that neurodevelopmental disorders, including ID, have a sex-biased presentation. Vicoso and Charlesworth (2006) [22] proposed that beneficial variants are better fixed on the X chromosome than on the autosomes because they will always be expressed in hemizygous males under selection. Therefore, the X chromosome would accumulate beneficial variants at a higher rate than the autosomes. Thus, one hypothesis raised was that X-linked genes carrying deleterious variants under negative selection would evolve more slowly than X-linked genes under positive selection [22,23]. This would explain, as discussed by Turner et al. (2019) [24], the discrepancy of ID/NDD between sexes and the enrichment of ID/NDD genes on the X chromosome, stating that it may be caused by the deleterious or lethal status of these genes in a hemizygous state, as well as a higher frequency of variants in escape genes, such as *DDX3X,* in females affected by ID.

Even with the protection of the XCI process, heterozygous females carrying XLID P variants can still manifest ID through several mechanisms, such as the stochastic inactivation process, with a random chance that the X chromosome carrying an alteration will become preferentially active [25–27]; the advancement of age, but in this case a pathogenic variant would only have phenotypic expression in late-onset diseases [28,29]; a positive selection of the mutated X chromosome, due to a growth advantage from the variant; and a negative selection that favors the wild-type chromosome, with escape expression of the mutated allele [26,27,30]. Women usually show milder phenotypes with respect to X-linked variants, even if important inactivation drift occur. A crucial aspect of discussion is the occurrence of disease

phenotype in women with pathogenic variants located on the preferentially inactivated chromosome. Remarkably, this indicates that XCI is not always complete (100:0) and may result in residual expression of the mutated allele.

Amos Landgraf et al. (2006) [11] showed that approximately 1.8% of women in the general population would have extremely or completely skewed XCI deviation (≥90:10) by chance due to the stochastic process of XCI. Additionally, in a sample of 118 Brazilian females, it was demonstrated that moderately skewed XCI (80:20 – 89:11%) was present in 2.6% of the investigated subjects [31]. Our study investigated whether there was an increased frequency of extremely or completely skewed XCI in a cohort of patients with ID. Eleven patients (8%) exhibited skewed XCI ≥90%, of whom four presented with completely skewed XCI (100:0). Considering the ~2% extreme/complete deviation frequency found by Amos Landgraf and colleagues (2006) [11] in the general population, 3/136 women in our study would be expected to have extreme or complete XCI deviation. However, we observed a value that was four times higher with an enrichment of females with ID and skewed XCI.

These findings are supported by previous studies [7; 13], which observed skewed XCI in 7.6-13% of the females with ID. Considering only the 98 informative sporadic cases investigated by our study, eight (8.2%) showed extreme or total XCI skewing. This is smaller than the frequency that was found in the study by Vianna et al. (2020 - 13.2%), which involved only unrelated patients. The smaller frequency observed in our study compared to the study conducted by Vianna et al. (2020) [13] with Brazilian patients could be explained by the fact that we previously excluded patients with a positive CMA result, and chromosomal rearrangements involving the X chromosome can result in extremely skewed XCI.

The most plausible hypothesis to explain the increased frequency of skewed XCI compared to the healthy female population would be the presence of a causative variant on the X chromosome, simultaneously associated with the condition of ID. For all cases of variants in genes with a dominant pattern of inheritance, XCI can be considered a contributing factor to the large heterogeneity of phenotypes and degrees of involvement among those affected.

WES of the 11 patients in our cohort with ID and extremely/completely skewed XCI revealed pathogenic variants in eight of them (~73%), which were all mapped to genes with dominant inheritance patterns, even though only four patients had X-linked variants (*DDX3X*, *WDR45*, *PDHA1*); different pathogenic variants in the *DDX3X* gene were identified in two patients. Of the three X-linked genes identified as mutated, only one of them, *DDX3X* is a gene described to escape XCI, having biallelic expression in females [31,32]. *DDX3X* microduplications were also reported in ID patients, indicating that the disturbance of gene dosage leads to pathological phenotypes [33]. This finding of skewed X-chromosome inactivation in females with *DDX3X* variants was previously described in the study by Fieremans and collaborators (2016) [7] and in our recent work (Fonseca et al., 2021 [30]). In theory, *DDX3X* would have different expression levels in males and females, and skewed expression would not be expected in women carrying mutations. However, the pattern for these genes that escape X inactivation is more complex, as many of them are known to present lower expression in the inactive

chromosome X than in the active X, as well as variable expression among different females and, in some cases, within different tissues of the same woman. These genes would essentially also be "dosage compensated", like genes subjected to inactivation, but with a more heterogeneous expression pattern. *DDX3X* has a homologous paralog on the Y-chromosome – *DDX3Y* gene – and escape genes with retention on the Y chromosome are considered of great evolutionary importance [34]. The conservation of *DDX3X* gene and the high intolerance of *DDX3X* and *DDX3Y* to loss-of-function variants (pLi= 1 and 0.96, respectively, according to gnomAD) indicate great functional relevance: while variants in *DDX3X* are associated with cognitive disease, variants in *DDX3Y* result in infertility [34–36].

Unlike *DDX3X*, *WDR45* and *PDAH1* are subject to XCI. *WDR45* (OMIM *300526) is associated with the regulation of cellular autophagy processes. Therefore, the expression of P/LP variants may result in the accumulation of intracellular debris, affecting the functioning of the affected tissue. Such accumulations tend to primarily impair the central nervous system due to iron assembling in brain tissue and may impact global development, causing ID and cognitive degeneration [37,38]. Skewed X-chromosome inactivation in women carrying *WDR45* variants have been previously reported, in which only the mutated alleles were detected [7,40,41]. The *PDAH1* gene (OMIM *300502) acts in the energy pathway of ATP production, and its mutations have been associated with the accumulation of intracellular pyruvate and lactic acid, resulting in an acidosis state that causes neurological damage [39,40]. Willemsen and colleagues (2006) [41] performed an investigation of the XCI pattern in fibroblasts in four women with *PDAH1* variants who were affected by pyruvate dehydrogenase deficiency, observing a 90% inactivation shift in all patients.

Fieremans et al. (2016) [7] observed that among ID patients who had skewed inactivation ≥90:10, ~45% carried pathogenic variants, nine of which were in X-linked genes, including *DDX3X* and *WDR45* (*DDX3X*, *SMC1A*, *WDR45*, *NHS*, *MECP2*, *MED12*, *HDAC8*, and *TAF9B*), and two on autosomes (*EP300* and *SYNGAP1*). In a Brazilian cohort, Vianna et al. (2020) [13] also identified pathogenic variants in seven patients with skewed XCI, two with structural chromosomal rearrangements (chromosome 3 deletion and unbalanced translocation t(X;2)), and four variants mapped to three X-linked genes were identified (*NLGN4X*, *USP9X,* and *TAF1*), in addition to one variant in an autosomal gene (*DVL1*).

An interesting finding in our study was the detection of variants in four autosomal genes (*KCNB1*, *CTNNB1*, *YY1*, *ANKRD11*), not documented in previous studies using the same approach. In these cases, the skewed XCI may have occurred by chance. However, we cannot exclude the possibility that variants in autosomal ID-related genes may have a crosslink with skewed XCI [7;13]. We did not identify a robust connection between the *CTNNB1*, *ANKRD11,* and *KCNB1* genes and the XCI. Liu and colleagues (2016) [42] observed in hepatocellular carcinoma that the lncRNA for the *FTX* gene (lnc-FTX), which acts in XCI as an activator of Xist in mice [43], interferes with the activity of beta-catenin, which is encoded by *CTNNB1*. However, the reverse effect—that is, a beta-catenin alteration interfering with lnc-FTX levels—was not documented, and it is an interesting hypothesis that could be experimentally evaluated. It would therefore be beneficial to investigate the pattern of XCI in females with *CTNNB1* deleterious variants to assess the hypothesis that this autosomal gene could be related to the whole XCI process.

On the other hand, the autosomal gene *YY1* (OMIM *600013) is directly linked to XCI. *YY1* (OMIM *600013) is a transcription factor involved in embryogenesis, differentiation and cell proliferation and acts as a transcriptional repressor [44]. According to several studies [45–47], *YY1* plays an essential role in XCI, participating via the association of Xist RNA with DNA on the inactive X chromosome and acting as a bridge. The YY1 protein binds to the active Xist RNA, presenting a monoallelic attachment restricted to the inactive X chromosome. In a functional study conducted by Makhlouf et al. (2014) [46], it was reported that *YY1* was also essential in the regulation and maintenance of Xist transcription and expression. *YY1* knockdown decreases Xist levels by 80% in mouse somatic cells after the establishment of X inactivation, as well as the number of cells with the Xist condensation cloud during cell differentiation. This work concluded that YY1 is the first identified factor controlling the monoallelic expression of Xist during the initiation and maintenance of X-inactivation [46]. Therefore, we can hypothesize that a *de novo YY1* pathogenic variant could be the cause of the primary extreme XCI deviation found in P6; only one patient with *YY1* pathogenic variant was previously investigated regarding XCI and she also presented extreme skewing [48]. The investigation of the XCI pattern of other females with pathogenic *YY1* could clarify this issue.

The present study presents limitations, such as the use of a single locus (*AR* gene) to investigate XCI patterns. The use of more than one marker would increase our ability to identify informative females and would also confirm the identified patterns. A second limitation was the use of genomic DNA samples only from peripheral blood, which should not be completely representative of the XCI pattern in the central nervous system; however, a recent study showed that blood results can be used as a proxy for other tissues [32]. Moreover, the *AR* gene methylation study performed to determine the inactivation status of the X chromosome may represent an indirect method, considering that it is not a fully predictive character of the inactivation status of the investigated locus, since does not determine which allele is preferentially inactive in a patient. A more direct and consistent alternative would be to use RNA expression analysis by RTqPCR to study the XCI pattern [27]. Notwithstanding, using this approach, we did identify a representative number of patients with skewed XCI and P/LP variants, in four patients with variants mapped to X-linked genes and in one patient with a variant mapped to an autosomal gene associated with the XCI process itself, supporting our XCI data and the hypothesis that an extremely skewed XCI in females with ID is indicative of the presence of pathogenic variants in X-linked or autosomal genes linked to the XCI process.

Based on these findings, we can conclude that the frequency of XCI deviation in the peripheral blood of females with ID is significantly higher than that in the general population. We also found that the analysis of the XCI pattern in females with ID, a cost-effective molecular procedure, is useful for detecting extreme or complete deviation (≥90%), which was demonstrated as a factor indicative of causative variants located on the X chromosome or even in autosomes. In the scenario of a limited budget, the investigation of XCI patterns would indicate female patients with ID who carry pathogenic monogenic variants to be selected for further genetic tests and genetic counseling.

Declarations

AUTHOR DECLARATIONS

Ethical approval and consent to participate

This study was approved by the ethics committee of our institution (CAAE 80921117.5.0000.5464), and written informed consent was obtained from the patients' parents or legal guardians, in accordance with ethical standards established in the Declaration of Helsinki (1964), its subsequent revisions, and Resolution 466/2012 of the Brazilian National Health Council.

Data availability statement

The pathogenic variants reported here are deposited in the ClinVar database (ClinVar accessions: SCV002506515, SCV002506516, SCV002506517, SCV002506518, SCV002506519, SCV002506520, SCV002506521, SCV002506522). Additional data that support the findings of this study are available upon request from the corresponding author, subject to privacy or ethical restrictions.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

ACVK designed and led the study. Genetic tests were performed especially by LDC but also by SSC, LMLC, GCT, SFP, and GHS. PAO contributed to the statistical analysis. The clinical evaluation of the patients was performed by PAO, LRG, and DRB. Bioinformatic processing was conducted by MOS. The first draft of the manuscript was written by LDC, LMLC, GCT, and SFP. ACVK, CBSR, CR, and AMVM reviewed the manuscript. All authors read and approved the final manuscript. LDC, LMLC, GCT, and SFP shared co-first authorship because contributed equally to this manuscript.

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Table

Table 1 is available in the Supplementary Files section

Figures

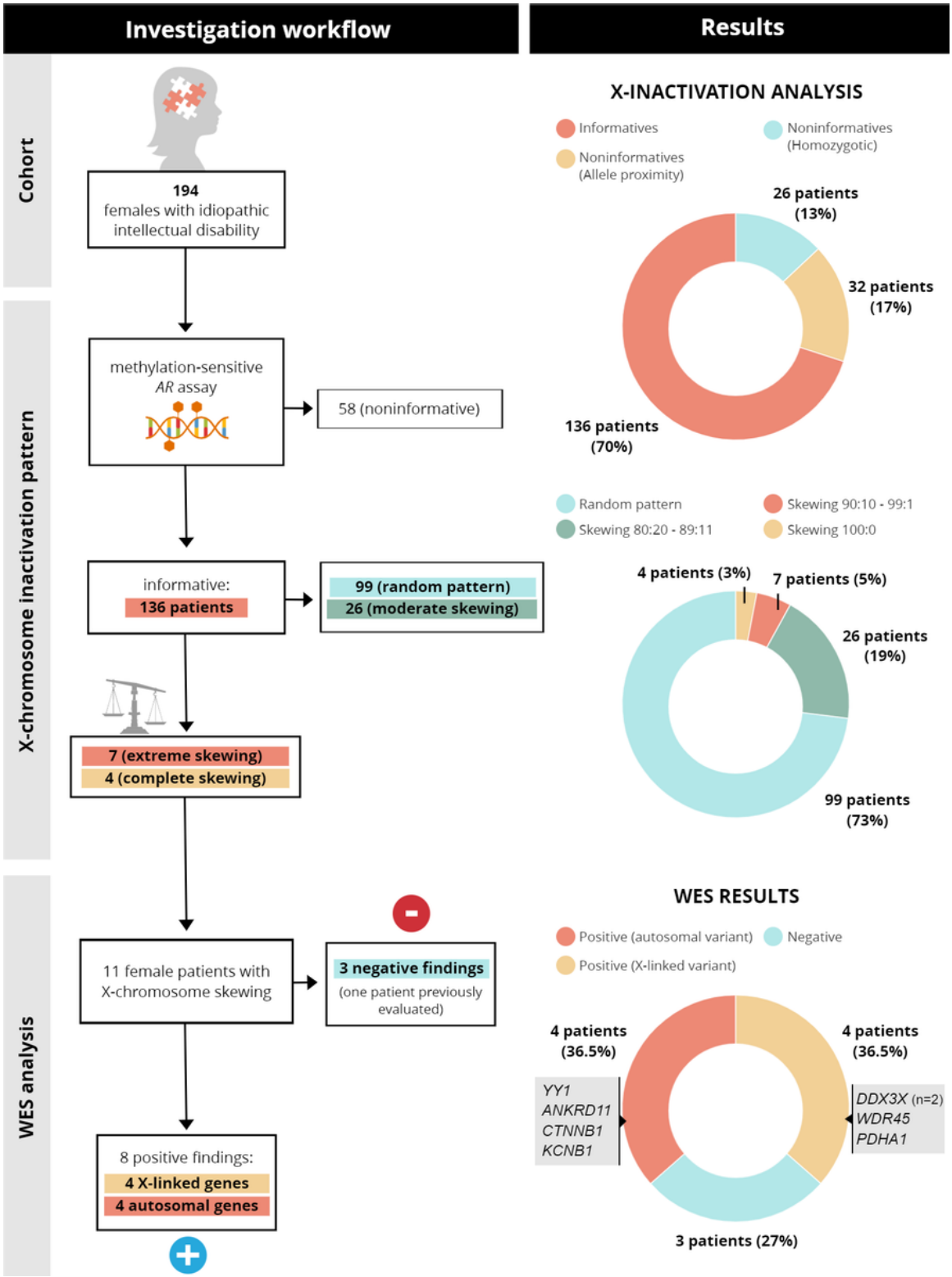

Figure 1

Genetic investigation workflow and description of the results.

We investigated the pattern of X-chromosome inactivation in DNA blood samples of 194 females with ID using the HUMARA assay. After analysis, 136/194 women were informative for the AR locus. Eleven out of the 194 informative females presented >90:10 *X-chromosome inactivation skewing, eight of them with*

positive findings in the WES investigation. In four cases (50%), the detected pathogenic variants were mapped to X-linked genes (DDX3X, WDR45, and PDHA1); in other four cases, mutations were disclosed in autosomal genes (YY1, ANKRD11, CTNNB1, and KCNB1), one of them directly associated with the X-inactivation process (YY1).

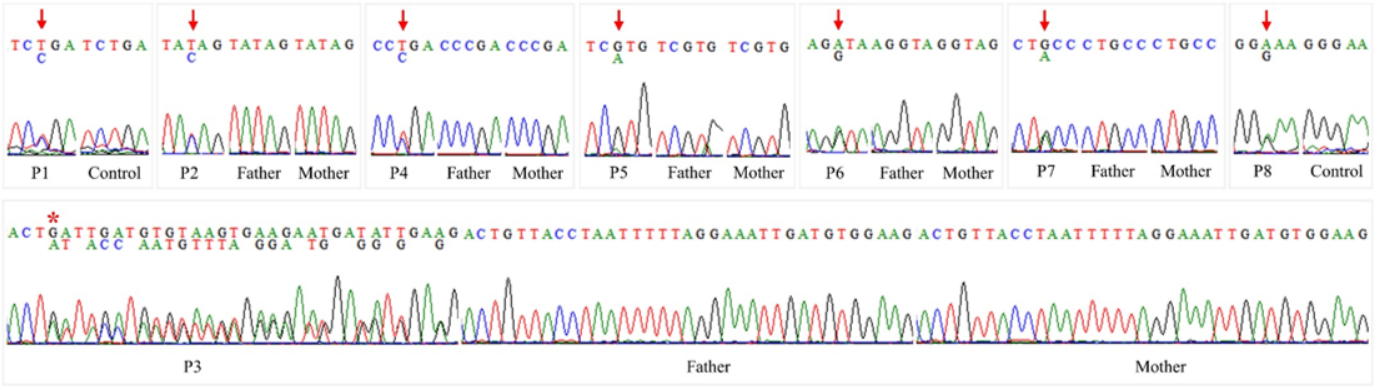

Figure 2

Sanger sequencing for variant validation and segregation analyses. In all patients, the variant was validated in a heterozygous state (P1-P8). In six patients, Sanger sequencing was performed in genomic samples of the trio (proband, father and mother) for segregation analysis, and all variants were identified as de novo.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuplementaryFigure4julho22.docx
- Table1.docx