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Pseudoautosomal abnormalities in terminal AZFb+c deletions are associated with isochromosomes Yp and may lead to abnormal growth and neuropsychiatric function

A. Castro[1,*], F. Rodríguez[1], M. Flórez[1], P. López[1], B. Curotto[2], D. Martínez[1], A. Maturana[3], M.C. Lardone[1], C. Palma[4,5], V. Mericq[1], M. Ebensperger[1], and F. Cassorla[1]

[1]Institute of Maternal and Child Research, School of Medicine, University of Chile, Hospital San Borja Arriarán, Santiago 8360160, Chile [2]Laboratorio de Genética y Enfermedades Metabólicas, Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Santiago 7830490, Chile [3]Psychiatric Unit, Clínica Las Condes, Santiago 7591046, Chile [4]Department of Urology, José Joaquín Aguirre Clinical Hospital, School of Medicine, University of Chile, Santiago 8380453, Chile [5]Department of Urology, Clínica Las Condes, Santiago 7591046, Chile

*Correspondence address. Institute of Maternal and Child Research, School of Medicine, University of Chile, Hospital San Borja Arriarán, Servicio Salud Metropolitano Central, Santiago, Chile. E-mail: acastro@med.uchile.cl

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STUDY QUESTION: Are copy number variations (CNVs) in the pseudoautosomal regions (PARs) frequent in subjects with Y-chromosome microdeletions and can they lead to abnormal stature and/or neuropsychiatric disorders?

SUMMARY ANSWER: Only subjects diagnosed with azoospermia factor (AZF)b+c deletions spanning to the end of the Y chromosome (i.e. terminal deletions) harbor Y isochromosomes and/or cells 45,X that lead to pseudoautosomal gene CNVs, which were associated with abnormal stature and/or neuropsychiatric disorders.

WHAT IS KNOWN ALREADY: The microdeletions in the long arm of the Y chromosome (Yq) that include the loss of one to three AZF regions, referred to as Yq microdeletions, constitute the most important known etiological factor for primary spermatogenic failure. Recently, controversy has arisen about whether Yq microdeletions are associated with gain or loss of PAR genes, which are implicated in skeletal development and neuropsychiatric function.

STUDY DESIGN, SIZE, DURATION: We studied a cohort of 42 Chilean patients with complete AZF deletions (4 AZFa, 4 AZFb, 23 AZFc, 11 AZFb+c) from a university medical center, diagnosed over a period of 15 years. The subjects underwent complete medical examinations with special attention to their stature and neuropsychiatric function.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All subjects were characterized for Yq breakpoints by PCR, and for CNVs in PARs by multiplex ligation-dependent probe amplification (MLPA), followed by qPCR analysis for genes in PAR1 (*SHOX* and *ZBED1*), PAR2 (*IL9R*) and two single copy genes (*SRY* and *DDX3Y*, respectively located in Yp11.3 and AZFa). In addition, karyotypes revision and fluorescence *in situ* hybridization (FISH) for *SRY* and centromeric probes for X (DXZ1) and Y (DYZ3) chromosomes were performed in males affected with CNVs.

MAIN RESULTS AND THE ROLE OF CHANCE: We did not detect CNVs in any of the 35 AZF-deleted men with interstitial deletions (AZFa, AZFb, AZFc or AZFb+c). However, six of the seven patients with terminal AZFb+c deletions showed CNVs: two patients showed a loss and four patients showed a gain of PAR1 genes, with the expected loss of *VAMP-7* in PAR2. In these patients, the Yq breakpoints localized to the palindromes P8, P5 or P4. In the four cases with gain of PAR1, qPCR analysis showed duplicated signals for *SRY* and *DDX3Y* and one copy of *IL9R*, indicating isodicentric Yp chromosomes [idic(Y)] with breakpoint in Yq11.22. The two patients who had loss of PAR1, as shown by MLPA, had an additional reduction for *SRY* and *DDX3Y*, as shown by qPCR, associated with a high proportion of 45,X cells, as determined

by FISH and karyotype. In agreement with the karyotype analysis, we detected DYZ3++ and DYZ3+ cells by FISH in the six patients, confirming idic(Y) and revealing additional monocentric Y chromosome [i(Y)]. Five patients had a history of major depressive disorders or bipolar disorder, and three had language impairment, whereas two patients showed severe short stature (*Z* score: −2.75 and −2.62), while a man with bipolar disorder was very tall (*Z* score: +2.56).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The number of males studied with Y-chromosome microdeletions and normozoospermic controls with normal karyotypes may not be enough to rule out an association between AZF deletions and PAR abnormalities. The prevalence of Y isochromosomes and/or 45,X cells detected in peripheral blood does not necessarily reflect the variations of PAR genes in target tissues.

WIDER IMPLICATIONS OF THE FINDINGS: This study shows that CNVs in PARs were present exclusively in patients with terminal AZFb+c deletions associated with the presence of Y isochromosomes and 45,X cells, and may lead to neuropsychiatric and growth disorders. In contrast, we show that men with interstitial Yq microdeletions with normal karyotypes do not have an increased risk of PAR abnormalities and of phenotypical consequences. Moreover, our results highlight the importance of performing molecular studies, which are not considered in the usual screening for patients with Yq microdeletions.

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Key words: Y chromosome microdeletion / pseudoautosomal region / male infertility / short, tall stature / neuropsychiatric disorders

Introduction

Microdeletions of the Y chromosome (Yq microdeletions) constitute the most important cause for primary spermatogenic failure, with a prevalence of ~10% in subjects with non-obstructive azoospermia or severe oligozoospermia, and involve at least one of three azoospermia factor (AZF) regions (AZFa, AZFb and AZFc) (Vogt, 1998; Krausz *et al.*, 1999; Ma *et al.*, 2000; Foresta, 2001; Silber, 2011). Among these deletions, those of AZFc or AZFb+c are the most frequent (Vogt, 1998; Hopps *et al.*, 2003; Ferlin *et al.*, 2007; Sadeghi-Nejad and Farrokhi, 2007). Paternity is feasible through ICSI thanks to sperm from the ejaculate or from the testis by retrieval in men whose deletions are of AZFc or do not include complete deletions of AZFa or AZFb region (Kleiman *et al.*, 2011, 2012; Krausz *et al.*, 2014). However, infertility may be transmitted through the inheritance of Yq microdeletions to their sons (Kurinczuk, 2003; Krausz *et al.*, 2014). Moreover, due to the mitotic instability of the deleted Y chromosome, their descendants may also be at risk for other chromosomal abnormalities, such as Turner or Klinefelter syndromes and sex-chromosomal mosaicisms (Ferlin *et al.*, 2007; Patrat *et al.*, 2010; Kim *et al.*, 2012b; Krausz *et al.*, 2014). In addition to these outcomes, a recent finding has suggested that infertile men with Yq microdeletions may harbor abnormalities in the pseudoautosomal regions (PARs), including gene duplications or deletions which in turn are associated with other conditions, such as growth and psychiatric disorders, adding new clinical aspects to consider for the management of these subjects (Jorgez *et al.*, 2011). The latter study found that among 74 men with normal karyotype and different AZF deletions, 7 (9.5%) had copy number variations (CNVs) in their PARs. Among the CNVs, they observed gains and losses in PAR1 and only losses in PAR2 (Jorgez *et al.*, 2011). However, whether Yq microdeletions are a cause of PAR abnormalities in men with normal karyotype has been questioned by a subsequent study (Chianese *et al.*, 2013).

Many genes in PARs escape inactivation, requiring diploid expression for an appropriate function (Binder, 2011). Among them, the short stature-homeobox gene (*SHOX*) located in PAR1 is involved in longitudinal bone growth. Although knowledge regarding the effects of CNV on other genes in PARs is still incomplete, some genes appear to be related with psychiatric disorders. *ASMT* (encodes for acetylserotonin o-methyltransferase) located in PAR1 is involved in abnormalities in the sleep/wake cycle and is a candidate gene for bipolar affective (BPAD) and autism spectrum disorders (ASDs) (Cai *et al.*, 2008; Flaquer *et al.*, 2010; Etain *et al.*, 2012). Human IL-9 receptor gene (*IL9R*) located in PAR2 harbors a larger polymorphic CAG repeat allele, which may be involved in the genetic susceptibility for BPAD in males (Vermeesch *et al.*, 1997).

Our aim was to study whether PAR abnormalities are present in subjects with different types of Yq microdeletions, and to investigate their possible impact on longitudinal growth and/or neuropsychiatric function.

Materials and Methods

Subjects

This study was conducted according to the Declaration of Helsinki, was approved by the Institutional Review Boards of the University of Chile, School of Medicine and the San Borja Arriarán Clinical Hospital and the patients and/or parents gave their written consent for this study. We studied 42 Chilean patients (41 adult men and 1 child) previously diagnosed with Yq microdeletions at the Institute of Maternal and Child Research from the University of Chile during a period of 15 years (2000–2015). Men with Yq microdeletions were selected from our population of 326 consecutive infertile patients with azoospermia or severe oligozoospermia (Supplementary Table SI). These infertile patients were referred for molecular diagnosis of Y-chromosome microdeletions. In addition, a diagnosis based on the testicular histology from a biopsy taken at time of TESE (Lardone *et al.*, 2010) was performed in all of the subjects who were non-Yq-microdeleted ($n = 285$) and in 44% (18/41) of Yq-microdeleted infertile patients. There were 90 patients who had normal spermatogenesis on testicular biopsy (obstructive azoospermia controls). The exclusion criteria

for our study were hypogonadotropic hypogonadism, chronic diseases, hyperprolactinemia, clinical varicocele, hormonal treatments, exposure to pesticides and consumption of alcohol or drugs.

Clinical evaluations

Subjects underwent a complete medical examination with special attention to their phenotype and neuropsychiatric function. Their weight and stature were converted into *Z* scores to adjust for chronological age and sex, using the US reference at birth (Kuczmarski *et al.*, 2000) and the National Center for Health Statistics (NCHS) growth reference, which has been shown to be applicable to the Chilean population (Youlton and Valenzuela, 1990). The height measurements could not be performed in 24 and 19% of the non-Yq-microdeleted cases and controls, respectively, and in 4 of the 42 Yq-microdeleted patients.

To establish their current mental health status, psychiatric evaluations were performed by one psychiatrist (A.M.) using the Chilean version of the 12-item General Health Questionnaire (GHQ-12), where poor mental health is suggested by a score of five or higher. In addition, symptoms for mood disorders were evaluated through DSM IV, ICD-10 (Hasin *et al.*, 2006), the Mood Disorder Questionnaire (MDQ) (Hirschfeld *et al.*, 2000) and a 9-item depression scale of the Patient Health Questionnaire (PHQ-9) (Kroenke *et al.*, 2001; Patrat *et al.*, 2010).

Semen analysis

Semen analysis was performed according to the 1999 or 2010 World Health Organization semen analysis guidelines (WHO, 1999, 2010). The diagnosis of azoospermia was based on the absence of sperm in at least two separate semen analyses after centrifugation of the samples at 1000*g* for 5 min.

Yq microdeletions and determination of breakpoints to Yq palindromes

DNA samples were isolated from peripheral blood using the Wizard® genomic DNA purification kit (Promega, Madison, WI, USA). All subjects had been diagnosed with Yq microdeletions as previously described (Castro *et al.*, 2004). Additional screening was performed in order to establish the breakpoint site of Yq microdeletions in all subjects using single PCRs for detection of sequence tagged sites (STSs) (Lardone *et al.*, 2007, 2013; Lange *et al.*, 2008, 2009; Vollrath *et al.*, 1992; Repping *et al.*, 2002). We discriminate for different DAZ genes copies by DAZ-SNVs and Y-DAZ-3, as previously described (Lardone *et al.*, 2007). The STSs used were specific to boundary and spacer-flanking markers on palindromes P1–P6 and P8, proximal to palindrome P5 (DYS199), at boundaries of the IR2 inverted repeat, on both arms of palindromes P1, P3–P4, and markers around the blocks of heterochromatin or at the boundary of PAR2 (Fig. 1).

Multiplex ligation-dependent probe amplification analysis

The multiplex ligation-dependent probe amplification (MLPA) Kit P018-F1 SHOX was used under the conditions specified by the manufacturer (MRC-Holland, Amsterdam, The Netherlands). This kit contains probes for the six exons and Intron 6a of *SHOX*, for sequences upstream and downstream of *SHOX*, for other genes in PAR1 (*CRLF2*, *CSF2RA*, *IL3RA*, *ASMT* and *ZBED1*), for *UTY* in AZFa and for several specific genes on the X-chromosome. In addition, 10 autosomal reference probes were included for normalization.

MLPA data were initially visualized with Peak Scanner™ Software v1.0 (Applied Biosystems™; http://resource.thermofisher.com/page/WE28396_1/) and the peak area data were imported to an Excel spread sheet for simple copy number calculations, as previously described (Sorensen *et al.*, 2010). A value below 0.7 or above 1.3 is regarded as indicative of a heterozygous deletion (copy number change from two to one allele) or duplication (copy number change from two to three or more alleles), respectively. Abnormal results were confirmed using a second independent DNA sample.

Quantitative PCR analysis

Copy number of *SHOX*, *ZBED1* (PAR1), *SRY*, *DDX3Y* (AZFa) and *IL9R* (PAR2) was studied using β2-microglobulin as reference for normalization. All primers, except those for β2-microglobulin (Vaughn *et al.*, 2008), were designed with the PrimerQuest® program (IDT, Coralville, USA; http://www.idtdna.com/scitools), and analyzed with the SNPcheck3 software (https://secure.ngrl.org.uk/SNPCheck) (Supplementary Table SII). Genomic DNA (15 ng) was amplified in triplicates in a reaction containing 2 µl of 5× HOT FIREPol® EvaGreen® HRM Mix (Solis BioDyne, Estonia), 0.5 µM of each primer and nuclease-free water was added until a final volume of 10 µl. Amplification was performed in an Eco Illumina® equipment (Illumina Inc., San Diego, CA, USA) according to the following protocol: preincubation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 20 s. The Ct values were determined by Eco Illumina software. Gene dosage was calculated using the $2^{-[\Delta\Delta Ct]}$ method, using DNA from a healthy normozoospermic man with normal karyotype and normal MLPA analysis as calibrator sample. Additional controls included DNA samples of non-mosaic patients with Turner or Klinefelter syndromes.

Karyotype analysis

Metaphase chromosome spreads were obtained from patients lymphocytes using the conventional methods. CTG and GTG banded chromosomes were analyzed at the 450–550 bands level. At least 50 metaphases were analyzed. Chromosomal abnormalities were reported according to the International System for Human Cytogenetic Nomenclature (ISCN 2013).

Fluorescence *in situ* hybridization analysis

Vysis CEP Y (DYZ3) SpectrumOrange Probe for DNA Alpha Satellite (Yp.11.1-q11.1) was used for visualization of the Y centromere. The Vysis SRY/CEP X Probe Kit, that contains LSI SRY specific to the SRY gene and flanking sequences (~122 Kb, Yp11.31-p11.32) SpectrumOrange Probes and CEP X for DNA alpha satellite (DXZ1; Xp11.1-q11.1) SpectrumGreen, was used for detection of *SRY* and the X centromere. Interphase nuclei and metaphases from lymphocytes were evaluated following the protocol recommended by the manufacturer (Abbott Molecular Inc., Abbot Park, IL, USA).

Statistical analysis

The Statistical Package for the Social Science (SPSS, IBM Corporation, Software Group, Armonk, NY, USA) for Windows, version 21, was used for statistical analysis. The Pearson Chi square was used for testing differences in stature among groups of consecutive infertile men, including men with secretory azo/oligozoospermia men (Yq-microdeleted and non-Yq-microdeleted) and obstructive azoospermia controls. To study possible differences in means or medians, the groups were compared by ANOVA and Student's *t*-test or by the Kruskal–Wallis and Mann–Whitney test, respectively. A *P*-value <0.05 was considered statistically significant.

Results

There were 39 men with Yq microdeletions who had been investigated previously because of secretory azo/oligozoospermia and all of them had a normal karyotype. In addition, three subjects were investigated for Yq microdeletions during infancy or puberty (Cases 1, 3 and

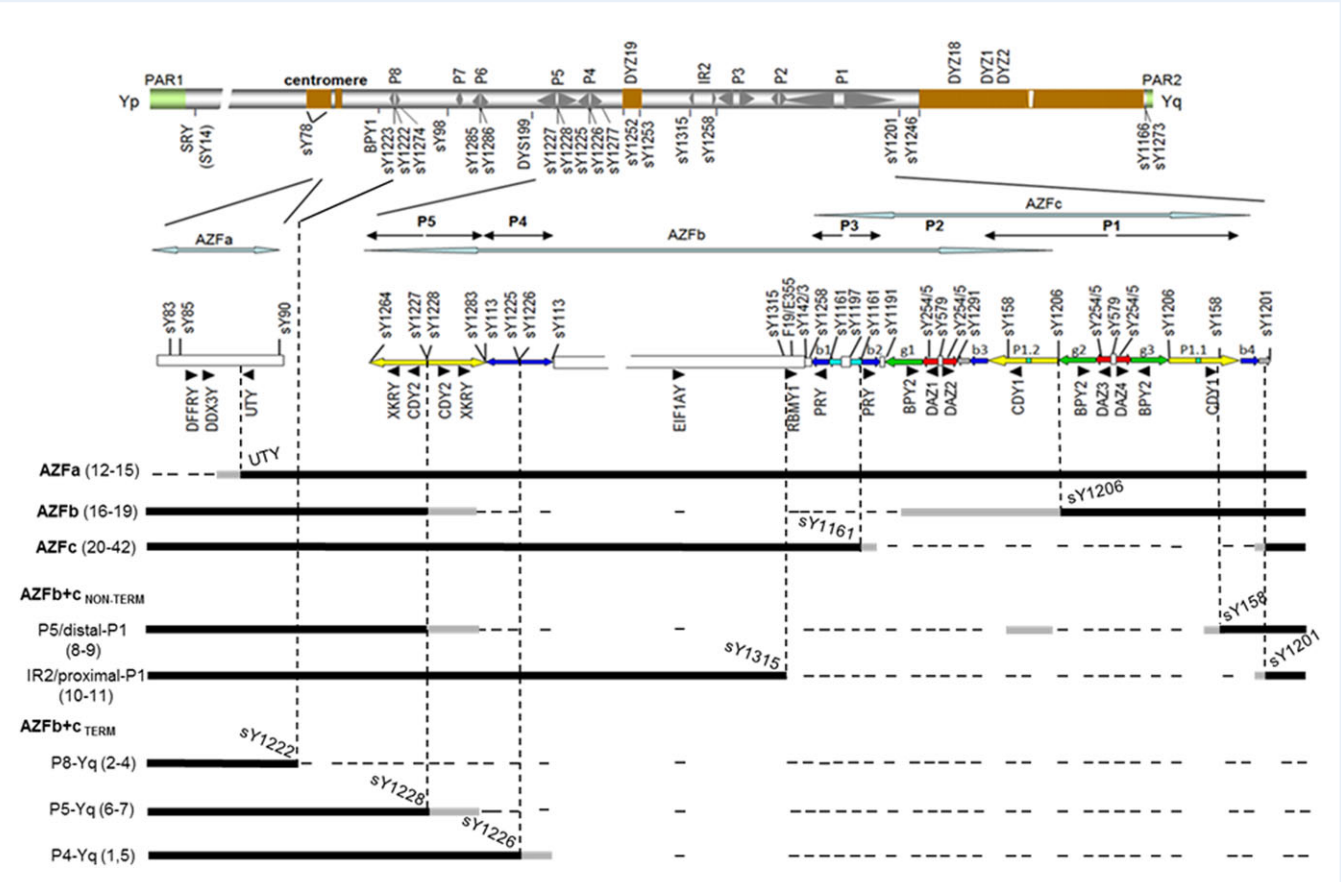

Figure 1 Characterization of Yq microdeletion breakpoints. Schematic representation of Y chromosome. Pseudoautosomal regions PAR1 and PAR2 are depicted with green boxes. Heterochromatic blocks are represented with orange and palindromes, P1 through P8, are indicated with gray arrowheads. Expanded and detailed views of the AZF regions and palindromes, with STSs employed in fine-mapping of breakpoint, are shown in the middle. AZF regions residing genes are indicated with black arrowheads. Results for presence or absence of STSs are shown below. The numbers of cases are shown in brackets. Solid black bars encompass STSs found to be present. Gray bars indicate breakpoint intervals that could not be further narrowed because of cross-amplification at other loci. DAZ-SNVs and Y-DAZ-3 analysis indicated that three patients with AZFb deletions showed absences of *DAZ1-DAZ2* and sY1291 and one showed an absence of *DAZ3-DAZ4*. Minus sign indicates that STS is absent. In contrast to non-terminal deletions, terminal AZFb+c deletions (AZFb+c_{TER}) showed negative amplification for sY1246, sY160 (DYZ1), sY1166 and sY1273.

4) due to a suspicion in their chromosomal analysis (short Y chromosomes showing loss of Yq heterochromatin by Q-banding), indicated by the presence of genetic stigmata and/or short stature. At the time of the study, 41 subjects were adults and 1 was a child.

Mapping of breakpoints to Yq palindromes

The characterization of the Yq-microdeleted subjects showed that similar breakpoints were present in each of AZFa, AZFb or AZFc deletions (Fig. 1). However, in subjects with AZFb+c deletions (n = 11), we observed greater variability with interstitial (P5/distal P1 and IR2 proximal-P1) or terminal AZFb+c deletions with breakpoints in palindromes P8 (Cases 2, 3 and 4), P5 (Cases 6 and 7) or P4 (Cases 1 and 5).

MLPA analysis

Results of CNVs analyzed by MLPA are shown in Fig. 2. We observed that only patients with terminal AZFb+c deletions had PAR abnormalities. In agreement with their terminal deletions, all of these subjects showed loss of signal for one copy of *VAMP-7* (PAR2). The analysis of CNVs in PAR1 showed reduced (Cases 1 and 2) or increased (Cases 3, 4, 6 and 7) signals, whereas one subject did not show any abnormality, except the expected loss of one copy of *VAMP-7* (Case 5). In addition, cases with gains in PAR1 showed an additional gains in *ZFY* (Yp11.3) and *UTY* (AZFa). Subjects with non-terminal AZF deletions (AZFa, AZFb, AZFc or AZFb+c) showed normal results in the MLPA analysis for each one of the probes (Supplementary Fig. S1).

Quantitative PCR analysis

In order to confirm the MLPA results and to analyze genes of the Y chromosome that were not included in the MLPA, we performed qPCR using specific primers for genes in PAR1 (*SHOX, ZBED1*), immediately downstream from PAR1 in Yp11.3 (*SRY*), AZFa (*DDX3Y*) and PAR2 (*IL9R*) (Fig. 3). Subjects who showed gains of one copy in PAR1 by MLPA showed increased relative amplification for *SHOX* (Exons 3 and 5) and *ZBED1* (Xp22.33; Yp11). In addition, they showed a gain of two non-pseudoautosomal genes, *SRY* (Yp11.3) and *DDX3Y* (AZFa), and the expected loss of one copy of *IL9R* in PAR2 (Fig. 3), suggesting

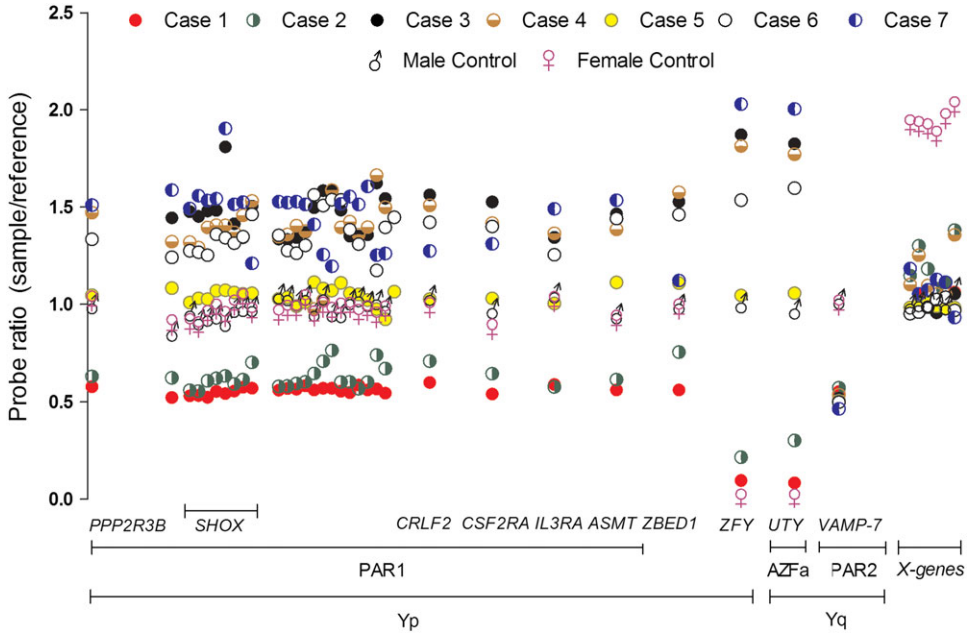

Figure 2 MLPA analysis for sex chromosomes in patients with terminal AZFb+c deletion (Cases 1–7). X-axis shows different probes for genes or DNA regions presents in PAR1, *UTY* in AZFa, *VAMP-7* in PAR2 and specific genes on the X-chromosome (X-genes). Control probes from non-sex chromosome were omitted. Y-axis represents probe fluorescence ratio after intra-normalization with control probe followed by inter-normalization with control (one of three normozoospermic healthy men). For each probe, the ratio <0.7 stands for deletion; and the ratio >1.3 stands for duplication.

the presence of isodicentric Y chromosomes, i.e. duplication of the Y short arm and proximal Y long arm in Cases 3, 4, 6 and 7.

Case 5 showed a normal dose for *SHOX* and no increment for *SRY* and *DDX3Y* by qPCR, in agreement with normal signals for *ZFY* and *UTY* in MLPA. Subjects with AZFa, AZFb, AZFc and non-terminal AZFb+c deletions showed normal dose ranges for *SRY*, *SHOX* (Exon 3 and Exon 5) and *IL9R*, validating the results observed by MLPA (data not shown).

Cytogenetics analysis

In order to confirm the presence of isochromosomes Yp suggested by MLPA and qPCR analysis, we performed new cytogenetic analysis in five subjects diagnosed with terminal Yq microdeletions, finding abnormal karyotypes in all of them: 45,X[45]/46,X,i(Y)(p10)[3]/46,X,idic(Y)(q11.22)[2] in Case 1; 45,X[36]/46,X,i(Y)(p10)[5]/46,XY[9] in Case 2; 46,X,i(Y)(p10) [50] in Case 3; 46,X,i(Y)(p10)[36]/46,XY[14] in Case 4; and 45,X[16]/46,X,i(Y)(p10)[28]/46,XY[6] in Case 5.

These results are in agreement with MLPA and qPCR, and confirm the suspicion of isochromosomes Yp. In addition, this karyotype analysis indicated some normal cells in Cases 2, 4 and 5 and Y nullisomy in Cases 1, 2 and 5. Cases 3, 6 and 7 had a low-resolution karyotype which was normal, but unfortunately it was not possible to obtain another sample to perform a second analysis.

Fluorescence *in situ* hybridization analysis

The presence of idic(Y) in six subjects with terminal AZFb+c deletions was confirmed by two fluorescence *in situ* hybridization (FISH) assays (Supplementary Table SIII) with different mix probes (*SRY*/DXZ1 and DYZ3 probes). In the subjects who showed a gain of PAR1 by MLPA, a double signal for *SRY* (SRY++) was observed in a high proportion (95–97%) of their interphase nuclei and metaphases. In contrast, patients with loss of PAR1 in their MLPA showed an absence of *SRY* signal (SRY−) in 40 and 97% of the nuclei, and a lower proportion of double signal for *SRY* (SRY++). In all of these subjects, we observed nuclei and/or metaphase with normal signal for *SRY* (SRY+), suggesting the presence of a Yq-microdeleted chromosome without the rearrangement of an isochromosome, as was observed in some cells after the revision of the karyotypes in Cases 2, 4 and 5.

DXZ1 and DYZ3 probes allowed us to establish the presence of the X and Y centromeres, respectively, and to determine whether the isochromosomes of Yp in each one of these subjects were monocentric (DYZ3+) and/or dicentric (DYZ3++). Although in a variable proportion, all cases with terminal AZFb+c deletions showed interphase nuclei with two or one signal for DYZ3. Case 1 showed a considerable proportion of interphase Y nullisomic nuclei for DYZ3 (DYZ3−) and *SRY* (SRY^{-}) (94 and 97%, respectively), and one or two signals for DYZ3, as we also detected by C banding in this case. Similarly, FISH analysis of 64 metaphases showed 90 and 98% of negative signals for DYZ3 and *SRY* probes (Supplementary Table SIII). Various Y-chromosome abnormalities are shown in Fig. 4.

All men with non-terminal Yq microdeletions who were available for FISH analysis (10/35) showed normal results in the analysis of 200 lymphocytes interphase nuclei.

Clinical findings

In order to investigate the impact of Yq microdeletions and/or karyotype abnormalities detected in terminal AZFb+c deletions on the

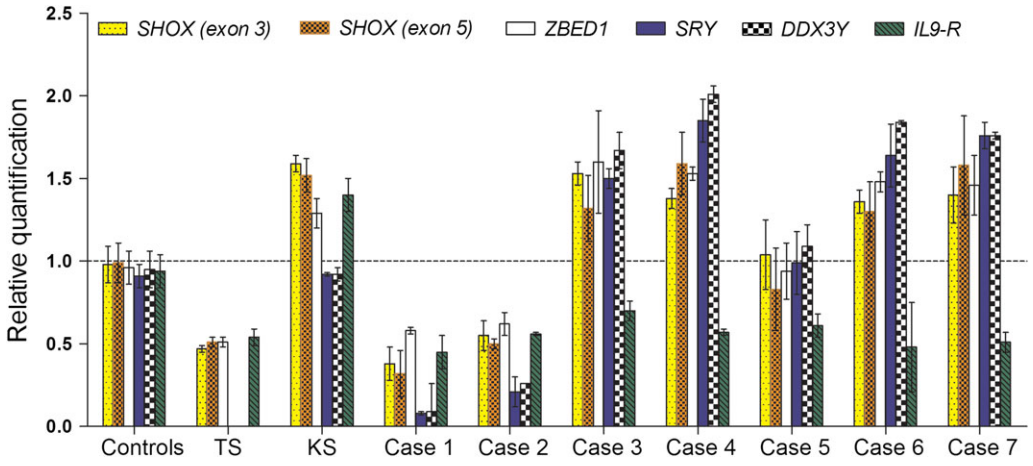

Figure 3 Relative qPCR results for genes on sex chromosomes in men with terminal AZFb+c deletions. Ordinate shows quantification by PCR of *SHOX* (Exon 3 and Exon 5), *ZBED1*, *SRY*, *DDX3Y* and *IL9R* for Cases 1–7 relative to a reference control. TS, non-mosaic Turner syndrome (45, X0); KS, non-mosaic Klinefelter Syndrome (47, XXY). Bars represent mean ± SD of at least two experiments performed in triplicate.

growth and neuropsychiatric features of our patients, we compared our cohort of Yq-microdeleted men with the infertile men without microdeletions analyzed during the same period. We observed a higher proportion of abnormal heights among patients with terminal AZFb+c deletions compared with infertile secretory cases without microdeletions of the Y chromosome (Supplementary Table SIV). Additional nutritional (BMI) and hormonal data of these consecutive infertile men are shown in Supplementary Table SI.

Table I shows a summary of the physical and testicular and/or seminal features, indicating the palindrome breakpoint and the interpretation of cell lines on peripheral blood, based on molecular findings, FISH and karyotype analysis for each of the affected subjects with terminal AZFb+c deletions.

Except for Case 3, who was adopted at 11 months of age so no information regarding his pregnancy and biological parents was available, all patients were born by vaginal delivery, at term from non-consanguineous parents. Growth charts and/or arm X-radiographs were available only for Cases 1–4 (data not shown and Supplementary Figs S2 and S3). Careful anthropometry was possible for Cases 1–5. Case 1 reached a very short adult height (mid-parental target height 181.2 cm), and showed a clear deceleration of growth velocity at ~13 years of age and long bone X-rays showed mild ulnar curvature (Supplementary Fig. S2) and his anthropometry was normal (arm span-height + 3 cm, US/LS 1.1) Case 2 was quite tall (Table I) in concordance with his genetic potential (mid-parental target height 186.5 cm), and his arm X-rays (data not shown) and anthropometry (arm span-height − 4 cm, US/LS 1.13) were normal. Case 3 showed a normal growth velocity, body proportions (arm span-height − 0 cm, US/LS 1.0) and X-radiographs (data not shown). At 13 years and 1 month of age, his bone age was 12 years, so his adult height prediction according to Bayley–Pinneau was 176.3 cm; *Z* score = −0.12. Case 4 was relatively tall compared with his parents (mid-parental target height 1.72 cm). His growth chart for height was normal but from the age of 12 years he developed generalized obesity (Supplementary Fig. S3). His anthropometry showed abnormal proportions (arm span-height + 8 cm, US/LS 1.1). Case 5 showed normal anthropometry (arm span-height – 2.2 cm, US/LS 1.07).

In accordance with the two other cases with complete AZFb+c deletions (P5 distal/P1 of the Cases 8 and 9 in Fig. 1), men with terminal deletions with breakpoints in the palindromes P8 and P5 were azoospermic (Table I). However, Case 6 showed 0.2×10^6 immotile sperm/ml in one seminal analysis and azoospermia in the two others. When the testicular biopsy was available, it showed a severe spermatogenic failure. Table II shows a summary of the main findings regarding the neuropsychiatric development of patients with terminal AZFb+c deletions. Cases 2 and 3 have a bipolar disorder under psychiatric treatment for several years. In three cases (Cases 1, 4 and 5), a psychiatric evaluation excluded active psychopathology, but their clinical histories documented other conditions, such as language delay, attention-deficit hyperactivity disorder (ADHD) and emotional and behavioral problems including anxiety and social disabilities. Patients with non-terminal Yq microdeletions did not have a medical history of neuropsychiatric abnormalities.

Discussion

We studied 42 patients with Yq microdeletions and determined that subjects with terminal AZFb+c deletions, i.e. those that span from a site after the AZFa region until the end of the Y chromosome, may harbor derangements in linear growth and neuropsychiatric function. These appear to be related to PAR abnormalities due to the presence of dicentric and monocentric isochromosomes Yp and mosaicism of 45,X cells. Importantly, we did not find evidence of increased risk of PAR abnormalities in subjects with non-terminal deletions and normal karyotype.

Several authors have indicated that detection of isochromosomes Yp by conventional G-banding karyotype is difficult, due to the relatively low number of cells studied (Takahashi *et al.*, 2006), and especially when they include the significant loss of chromosomal material (Lin *et al.*, 2005), as seen in our cases with terminal AZFb+c deletions.

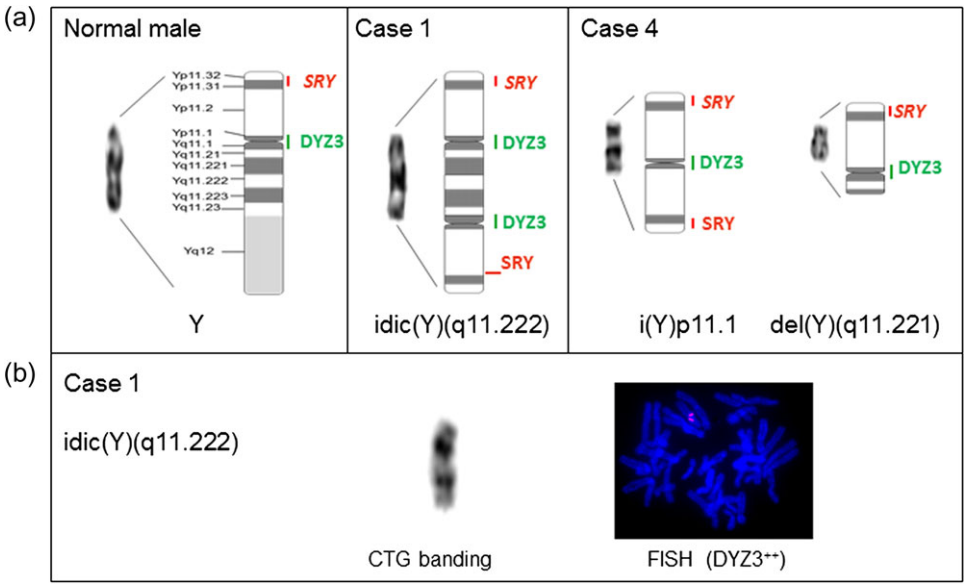

Figure 4 Representative Y-chromosomal abnormalities in men with terminal AZFb+c deletions. (**a**) Partial ideograms of GTG banding karyotypes showing representative normal and isodicentric Y chromosomes [idic(Y)] for Cases 1 and 4 with the corresponding chromosomal bands and position for centromeric (*DYZ3*) and *SRY* (LSI SRY) FISH probes. (**b**) Isodicentric Y chromosome by special CTG banding and Metaphase-FISH for Case 1 showing two centromere bands and signals for *DYZ3* probe showed in red (DYZ3++).

Therefore, considering our results and the current European Academy of Andrology and European Molecular Genetics Quality Network guidelines for the best practice for the molecular diagnosis of Y-chromosomal microdeletions (Krausz *et al.*, 2014), we suggest that the use of terminal markers must be reinforced. We suggest the inclusion of at least one additional marker distal to sY160 (DYZ1), such as sY1273 located at the proximal boundary with PAR2.

In the peripheral lymphocytes of our subjects, we detected by karyotype and/or FISH analysis, the presence of several cell lines with different aberrations of Y chromosome, including monocentric and dicentric isochromosomes Yp. Moreover, a terminal microdeleted Y chromosome without rearrangement was detected in our karyotype analysis and suggested by FISH (SRY+, DYZ3+), which could have precluded the detection of isochromosomes Yp in conventional karyotype analysis.

Similar to our results, some authors (Bettio *et al.*, 2006; Patrat *et al.*, 2010; Kim *et al.*, 2012a) have observed Yq-microdeleted men with mosaicism idic(Y) by karyotype analysis supported by FISH studies, particularly among patients diagnosed with terminal AZFb+c deletions, indicating that both anomalies seem to be related. In fact, Lange *et al.* (2009) have suggested similar mechanisms leading to AZF deletions and isochromosomes Yp, and suggested that cross-over recombination between sister chromatids leads to Y-isodicentric chromosomes with breakpoints in the AZF regions, in the present study idic(Y)(q11.22).

A more frequent detection of isodicentric Yp chromosomes with more proximal breakpoints on Yq has been suggested, because their mitotic stability increases with a shorter intercentromeric distance (Lange *et al.*, 2009). In agreement with this concept, we detected idic (Y) exclusively in those subjects with terminal AZFb+c deletions, and observed greater Y nullisomy in Case 1 who had a breakpoint on a more distal palindrome (P4). The detection of single signal for DYZ3 in a lesser proportion of the nuclei with double signal for *SRY* suggests the additional presence of monocentric Yp isochromosome [i(Y)p], which would result from additional centromeric breakpoints. In fact, in Case 1, we detected the presence of both Y chromosome rearrangements by CTG banding, with one and two centromeric bands, in agreement with interphase-FISH that shows the presence of 7/237 and 8/237 nuclei with single and double signal for DYZ3 (Supplementary Table SIII). Mosaicism manifesting as loss and rearrangement of isodicentric Y chromosome in more stable monocentric isochromosome has been observed in a few subjects (Iourov *et al.*, 2008; Paramayuda *et al.*, 2012). In agreement with previous studies (Valetto *et al.*, 2004; Lange *et al.*, 2009; Reshmi *et al.*, 2011), the presence of lymphocytes with idic(Y) or del(Y) chromosomes in a same subject supports the notion that the mechanisms leading to those abnormalities of the Y chromosomes are associated. The low prevalence of cells with terminal del(Yq) supports a negative selection of the deleted Y chromosome, inherited from the fertilizing sperm, during an early stage of embryogenesis, and leading to subsequent nondisjunction with generation of both 45,X cells and idic(Y). Additional rearrangement of the idic(Y) could generate an i(Y).

On the other hand, the absence of sperm in the seminal analysis and testicular histology of patients with complete AZFb+c deletions is in agreement with the documented low chance of finding spermatozoa in men with complete loss of the AZFb or AZFb+c regions. The infertility in our patients with isochromosome Yp may be mainly attributed to the loss of AZF spermatogenetic genes, but also to the prevention of the critical step of the sex-chromosomal pairing during the meiotic prophase, which seems to occur in isodicentric Yq due to loss of the PAR1 (Lehmann *et al.*, 2012). In fact, it has been observed that idic(Y)(q11.2) detected in lymphocytes of infertile

Table I Physical, spermatic and sex-chromosomal features in patients with terminal AZFb+c deletions.

Case	Age (years)	BMI (*Z* score)	Height (*Z* score (cm))	Dysmorphic features	Semen/testis findings	Palindrome breakpoint	Interpretation of cell lines[&]
1	21	−0.38	−2.75 (156)	Winged ears, high-arched palate, 'genu valgum' and joint hypermobility	AZO	P4	45X/46,XYdel(Y)(q11.222)/ 46,X,i(Y)p11.1/46,X,idic(Y)(q11.222)
2	40	1.34	2.56 (192)	NF	AZO/SCOS	P8	45,X/46,XY,del(Y)(q11.221)/ 46,X,i(Y)p11.1 46,X,idic(Y)(q11.221)
3	13.1	0.31	−1.02 (149)	Low set hair implantation, thick eyebrows, winged ears, thick lips, high-arched palate, widely separated nipples, winged scapulae, hyperlordosis, brachydactily and hypermobility	NA	P8	46,XY,del(Y)(q11.221)/ 46,X,i(Y)p11.1/ 46,X,idic(Y)(q11.221)
4	26	2.32	1.00 (184)	Mild prognatism, thick lips, widely spaced and low set nipples, abnormal palmar creases and long fingers, 'cubitus' and 'genu valgus'	AZO/MA	P8	46,XY,del(Y)(q11.221)/ 46,X,i(Y)p11.1/ 46,X,idic(Y)(q11.221)
5	41	0.82	−2.62 (158)	Widely distanced nipples and brachydactily	AZO	P4	45X/ 46,XY,del(Y)(q11.222)/ 46,X,i(Y)p11.1 46,X,idic(Y)(q11.222)
6	33	1.86	−0.96 (170)	NF	OLZ[#]	P5	46,XY,del(Y)(q11.222)/ 46,X,i(Y)p11.1/ 46,X,idic(Y)(q11.222)
7	32	0.55	−0.96 (170)	NF	AZO/SCOS	P5	46,XY,del(Y)(q11.222)/ 46,X,i(Y)p11.1/ 46,X,idic(Y)(q11.222)

[&]Based on molecular and/or cytogenetic findings.
[#]Patient shows severe oligozoospermia in one seminal analysis but azoospermia in two successive analyses.
P, palindrome; AZO, azoospermia; OLZ, oligozoospermia; SCOS, Sertoli cell-only syndrome; MA, maturation arrest; NF, not found; NA, not applicable.

patients may persist in seminal immature germ cells, arguing against the meiotic impairment in this type of Y-chromosome rearrangement (Kalantari *et al.*, 2014). In addition, there are a few subjects, including our Case 6 with terminal AZFb+c deletions, where it has been possible to detect spermatozoa sporadically, indicating that the meiotic process is not prevented (Kurinczuk, 2003; Bettio *et al.*, 2006; Lange *et al.*, 2009; Kleiman *et al.*, 2011, 2012).

Regarding the controversy of whether Yq microdeletions are a frequent cause of PAR abnormalities (Jorgez *et al.*, 2011; Chianese *et al.*, 2013), we only found PAR abnormalities in patients with isochromosomes Yp and/or Y nullisomy. This is in agreement with Chianese *et al.* (2013) who did not find *SHOX* haploinsufficiency in subjects with Yq-microdeleted and normal karyotype. We detected PAR abnormalities associated with the gain of *SRY* and *DDX3Y*, i.e. idic(Y)(q11.22), by both FISH and qPCR assays, depending on the degree of Y nullisomy. Similar to us, Chianese *et al.* (2013) observed gain of one *SHOX* copy, as was expected in two subjects 46,X,idic(Y)(q11.22). However, in contrast to our results they did not detect any abnormality except infertility. In contrast, Jorgez *et al.* (2011) observed PAR abnormalities, including deletions or duplications of PAR genes, in several subjects with normal karyotype and different types of AZF deletions. We believe that these discrepancies may be related to the presence of 45, X cells or Y isochromosomes that were not detected.

The detection of nuclei and metaphases with single signals for *SRY* (SRY+) by FISH suggests the presence of Y chromosomes harboring true terminal AZFb+c deletions. The presence of i(Y) has been thought to derive from the rearrangement of unstable idic(Y) (Lange *et al.*, 2009).

Although cells with isochromosomes Yp show a gain of PAR1 genes, these cells can lose their abnormal chromosomes due to mitotic instability, as we observed in Cases 1, 2 and 5. We cannot ascertain whether the gain or loss of *SHOX* was responsible for the tall stature observed in Case 2. In addition, the data for Case 3 predict a reasonable final height for the Chilean population because his bone age is delayed (bone age = 12 years in patient at 13.13 years of age), but unfortunately there are no data regarding his biological parental stature. Haploinsufficiency of *SHOX* has been related to short stature, so it seems reasonable to associate the short stature of our patients with the loss of *SHOX* in 45,X cells. Moreover, it has been observed that different tissues from an individual may not harbor the same proportion of Y nullisomic nuclei.

Table II Neuropsychiatric function in patients with terminal AZFb+c deletions.

Case	Age (years)	Specific language impairment	Behavioral/emotional and psychiatric history	Psychiatric evaluation at the age of study
1	21	Mixed receptive-expressive	Depression and episodes of aggressive behavior (psychiatric support during infancy). Stuttering and anxiety on stressful situations	Mild attention impairment and low tolerance to stress, recognizing himself as anxious and referring obsessive compulsive signs. Mild social and behavioral disability
2	40	NR	Mood instability and aggressive behavior starting at age 12 years, followed by four suicide attempts. Diagnosed with bipolar disorder Type II, under treatment from age of 21 years	Bipolar disorder Type II under psychiatric treatment
3	13.1	Expressive (reading and writing)	Under psychiatric treatment since age 7 6/12 years for depressive episodes followed by hypomania. Attention-deficit hyperactivity disorder	Probable diagnosis of bipolar disorder Type I under psychiatric treatment.
4	26	Mixed receptive-expressive	Attention-deficit hyperactivity disorder. Socialization difficulties during childhood and adolescence	Sleep disorder triggered by poor sleep hygiene. Anxious disorder associated with poor eating habits and lack of physical activity. Poor psychosocial adjustment
5	41	NR	Persistent insomnia and major depressive disorder at age 30 years (related to work and marital problems)	Sleep disorder and anxious symptoms related to academic overload during university studies

NR, not reported.

In addition, other investigators have suggested an association between BPAD and lower levels of melatonin or copy variations in *ASMT* (Cai *et al.*, 2008; Flaquer *et al.*, 2010; Etain *et al.*, 2012), and a polymorphism of a larger CAG allele for *IL9R* in males (Hawi *et al.*, 1999). We speculate that a failure in the expression of these two genes may contribute to the higher prevalence of mood disorders in our cases with terminal AZFb+c deletions. In addition, specific language impairment was observed during the early school years in three of our subjects with terminal deletions. Some of these neurodevelopmental disorders may be observed in patients with sex-chromosome aneuploidies such as Turner's syndrome or haplosufficiencies of the Y chromosome, and may be related to Y chromosome nullisomy or i(Y)(p10) (Ross *et al.*, 2009; Temple and Shephard, 2012; Simpson *et al.*, 2014). Some patients with abnormalities in language development harbor deletions in *PCDH11X/Y* (Protocadherin11X/Y), which is located in a non-pseudoautosomal homologous region on Xq21.3/Yp11.2. Therefore, the study of *PCDH11Y* may help to explain the specific language impairment documented in three of our patients with terminal Yq microdeletions (Speevak and Farrell, 2011). In addition, the gene NLGN4Y (neuroligin 4, Y-linked) located in Yq11.221 after the STS sY1222 has been implicated in the brain development of males and its loss in Cases 2, 3 and 4 may also account for their neuropsychiatric conditions (Johansson *et al.*, 2016).

Although our results suggest an association of PAR variations with growth and/or neuropsychiatric disorders in patients with terminal AZFb+c deletions, the small number of subjects studied, and the lack of molecular information in target tissues does not allow us to include or exclude this possibility.

Conclusion

Isochromosomes Yp and chromosomal instability leading to the formation of 45,X cells in subjects with terminal Yq microdeletions may be associated with growth disorders, mild learning disabilities and/or psychiatric dysfunction. Thus, the clinical spectrum of these patients should include the manifestations described in this study. In this sense, standard analysis of Y-chromosome microdeletions should include the detection of DYZ3 and/or terminal sequences boundary at PAR2, especially in cases of AZFb+c deletions. Patients with terminal AZFb+c deletions should be subsequently investigated for the presence of genetic markers located before the breakpoint of the deletion in the long and short arm of the Y chromosome, through molecular assays such as FISH, MLPA and/or qPCR.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

A.C. wrote the paper, conceived the study, directed the experimental protocols, performed PCR assays and interpreted the results. F.R. performed MLPA and contributed with qPCR techniques. M.F. performed PCR and qPCR assays and collaborated in the recruitment of patients and blood samples. P.L. contributed with FISH analysis and karyotypes. B.C. contributed with the analysis and revision of karyotypes. D.M. contributed with the clinical evaluation and follow-up of the pediatric patients. A.M. performed the psychiatric assessments. M.C.L. contributed with PCR experimental protocols, collaborated in the recruitment of patients and reviewed the manuscript. C.P and M.E. recruited the infertile men and performed the andrological assessments. V.M. performed endocrinological evaluations and reviewed the manuscript. F.C. collaborated with the critical analysis and final revision of the manuscript.

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Conflict of interest

None declared.

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