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A case report

Molecular cytogenetic analysis of a ring-Y infertile male patient

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ABSTRACT. In the present study, we report on the case of a 43-year-old male patient seeking for fertility assistance, who showed a seminal analysis and testicular biopsy of complete azoospermia. Peripheral blood culture for chromosome studies revealed a karyotype of 46 chromosomes with a ring-Y-chromosome that lost the long arm heterochromatin. Molecular analysis of genomic DNA from the patient detected the presence of the sex-determining region of the Y-chromosome (SRY) but the complete absence of regions involved in spermatogenesis (AZFa, AZFb, AZFc). Several molecular markers distributed along the Y-chromosome were tested through PCR amplification, and a breakpoint was established close to the centromere, predicting the deletion of the growth control region, in agreement with the short stature observed in this patient. All results obtained through molecular cytogenetic characterization are in accordance with the clinical features observed in this patient.

Key words: Infertility, Ring-Y, Azoospermia, Molecular markers

INTRODUCTION

Approximately 5% of healthy men suffer from apparent infertility for which no clinical explanation has been given (Bhasin et al., 1998, 2000; Hackstein et al., 2000). It has been suggested that up to 60% of undiagnosed male infertility arise from autosomal recessive mutations (Lilford et al., 1994). Furthermore, numerous case reports describing infertile brothers exhibiting similar spermatogenic defects indicate a strong hereditary basis for human male infertility (Escalier, 1999). There are over 50 monogenic disorders associated with male infertility, in a few of which the primary genetic defect has been described (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). During recent years, deletions located in the azoospermia factor region on the long arm of the Y-chromosome have received much attention through their association with azoospermia (Tiepolo and Zuffardi, 1976). However, identifying fertility genes has been difficult, because multiple copies of potential genes are present in the Y-chromosome (McElreavey and Fellous, 1999; McElreavey et al., 2000). Screening for Y-chromosome deletions has become routine practice in the clinical examination of males with idiopathic azoospermia or severe oligospermy. Interestingly, Y-chromosome mutations that cause male infertility must have arisen *de novo* and, in principle, are rarely transmitted to the offspring. Accordingly, Y-chromosome microdeletions contribute only marginally to the totality of human male infertility, but when present, the introduction of intracytoplasmic sperm injection as an artificial reproduction technique may allow for the transmission of such mutations to the next generation (In't Veld et al., 1997; Wallerand et al., 2003; Gazvani and Lewis-Jones, 2004).

It is estimated that approximately 1 in 1000 males have deletions of the Y-chromosome long arm, and these deletions are often associated with a short stature, as well as infertility (Kirsch et al., 2000). Moreover, the critical region responsible for normal male growth located in the Y chromosome has been named growth control region (GCR), and has been located close to the DYS11 marker, but no gene has yet been identified in this region (Kirsch et al., 2002).

In the present study, we describe an infertile patient, whose karyotype analysis showed an XY chromosomal complement, with a ring-Y chromosome that has lost the long arm heterochromatin. Molecular tests were designed to scan different positions of the Y-chromosome and helped define a putative breakpoint, where the ring was formed by end ligation. This analysis showed a positive result for the sex-determining region of the Y-chromosome (SRY) gene, but failed to detect the azoospermia factor regions. A breakpoint was established close to the centromere, deleting the growth control region of the Y-chromosome, thereby justifying the short stature of the patient.

MATERIAL AND METHODS

Case history

Here, we report the case of a 43-year-old married male with a history of infertility. He was 149 cm tall, with an arm span equal to the height and weighed 70 kg. During the physical exam, bilateral gynecomastia (Tanner stage 2) was observed. Facial, axillary and pubic hair had lower than normal density and distribution. The penis measured 8 cm in length, and both testes

were hypotrophic (6.0 and 5.7 mL) but present in the scrotum. He was evaluated after voluntarily seeking medical help in a fertility clinic. Hormone levels were evaluated by private laboratories after being requested by the attending physician.

Cytogenetic analysis

Standard techniques for culturing lymphocytes from peripheral blood were used to analyze the chromosomal constitution of the patient (Moorhead et al., 1960). Samples were treated with trypsin to obtain G-banding (Seabright, 1971).

DNA extraction and analysis by PCR

DNA was extracted using organic solvents (phenol/chloroform) and amplified by PCR using standard procedures. An agarose check gel was used to assess the DNA quality before PCR was performed. After the electrophoretic run, staining was performed with ethidium bromide for agarose gels and silver stain for acrylamide gels. Cycling conditions were optimized for each primer pair, using 2-3°C below the lowest primer melting point. All amplifications were performed as simplex reactions (1 primer pair). A complete description of the primers used, as well as the regions in the Y-chromosome tested by each primer, is given in Table 1.

Deletional analysis and region alignment

Results obtained by PCR amplification were used to determine if a specific region was present or absent in the Y-chromosome. Primer sequences were placed in the Blat engine of the University of California at Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) to predict the exact regions tested by the primers. The Blat output was a graphic representation of the Y-chromosome with the addressed regions highlighted. Images were captured and aligned in order to produce the diagram shown in Figure 3.

RESULTS

Hormonal workup

Serum FSH and LH levels were 2.9 and 1.8 mIU/mL, respectively (normal ranges are 2.5-14.0 and 1.5-12 mIU/mL, respectively). Serum total and free testosterone were 536 and 19.5 ng/dL, respectively (normal ranges are 280-1100 and 12.4-40.0 ng/dL), and prolactin was 3.2 ng/mL (normal range: 0.94-11.94).

Cytogenetic features

RHG-banding and GTG-banding revealed a ring-Y-chromosome in all metaphases analyzed (approximately 100 metaphases analyzed; data not shown), and a complete absence of the Y-chromosome long arm heterochromatin, defining a 46,X,r(Y)(p11q11) karyotype. Figure 1 shows a representative metaphase of the case, with the arrow pointing to the ring-Y-chromosome.

Table 1. Primers used to amplify Y-specific regions.

Primer	Region	Sequence
ZFY	ZFY	F 5' - ACCGCTGTACTGACTGTGATTACAC - 3'
		R 5' - GCACCTCTTTGGTATCCGAGAAAGT - 3'
SY14	SRY	F 5' - GAATATTCCCGCTCTCCGGA - 3'
		R 5' - GCTGGTGCTCCATTCTTGAG - 3'
SRY-F/G*	SRY	F 5' - GTAACAAAGAATCTGGTAGA - 3'
		R 5' – TTTCAGTGCAAAGGAAGGAA - 3'
SRY-D/E*	SRY	F 5' - GGTAAGTGGCCTAGCTGGTG - 3'
		R 5' - GCACAGAGAGAAATACCCGAA - 3'
SY84	AZFa	F 5' - AGAAGGGTCTGAAAGCAGGT - 3'
		R 5' - GCCTACTACCTGGAGGCTTC - 3'
SY86	AZFa	F 5' - GTGACACACAGACTATGCTTC - 3'
		R 5' - ACACACAGAGGGACAACCCT - 3'
SY127	AZFb	F 5' - GGCTCACAAACGAAAAGAAA - 3'
		R 5' - CTGCAGGCAGTAATAAGGGA - 3'
SY134	AZFb	F 5' - GTCTGCCTCACCATAAAACG - 3'
		R 5' - ACCACTGCCAAAACTTTCAA - 3'
SY254	AZFc	F 5' - GGGTGTTACCAGAAGGCAAA - 3'
		R 5' - GAACCGTATCTACCAAAGCAGC - 3'
SY255	AZFc	F 5' - GTTACAGGATTCGGCGTGAT - 3'
		R 5' - CTCGTCATGTGCAGCCAC - 3'
DYS270	Yq11.1	F 5' - TTCCATTTGATGTATTCCCG - 3'
		R 5' - GATTGGACTGGAATGGAATG - 3'
DXYS96	Yp11.2	F 5' - CACATTACTGGTGGGAGGAG - 3'
		R 5' - CATCATCGCCCTACACTACC - 3'
G66153	Yp11.2	F 5' - CAGGGGGAGAAACAGACAAA - 3'
		R 5' - GGGTGAGCCTGTTGATACCT - 3'

*Fuqua et al., 1997.

Molecular analysis

PCR amplification using 3 different primer pairs (Table 1, Figure 2) revealed the presence of the sex-determining region of the Y-chromosome (Figure 2). However, several sets of primers failed to detect the presence of regions involved in spermatogenesis (AZFa, AZFb, AZFc; data not shown). To further extend our understanding about which regions of the Y chromosome were lost or preserved, we used the UCSC Genome Browser to design primers encompassing the short and the long arm of this chromosome (Table 1), scanning to determine which parts of the chromosome were deleted during the ring formation event. These molecular studies (Figure 2) enabled us to draw a diagram of present and absent regions, as well as to determine a breakpoint neighborhood where rupture and end ligation occurred (Figure 3). All PCR reactions were performed at least three times before a final result was established. None of the patient's first-degree relatives showed azoospermia or other signs of infertility, strongly suggesting a *de novo* mutation as the causative agent in this case.

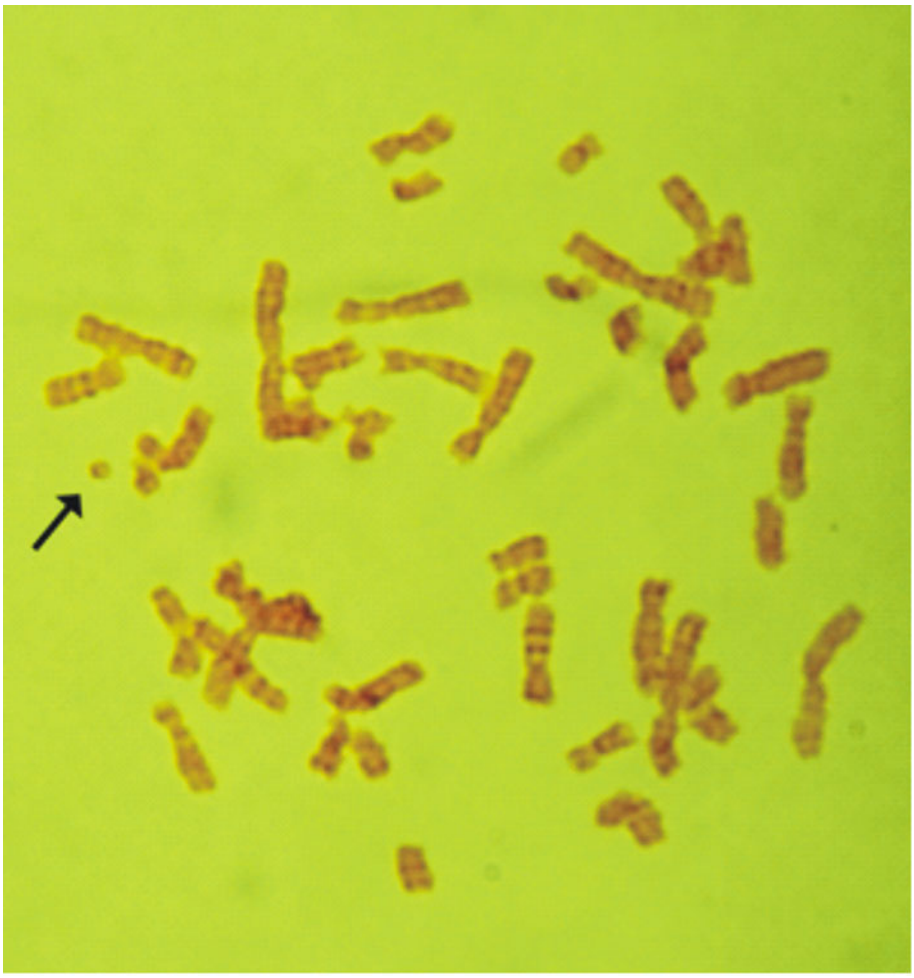

Figure 1. Human male karyotype. Representative metaphase. Arrow points to the ring-Y-chromosome.

DISCUSSION

Because the clinical features of male sex reversal syndrome patients are azoospermia associated with one or more abnormal external genitalia, gynecomastia, short stature, and pelvic cyst, we initially suspected that this was an XX individual with a translocated SRY gene. However, this hypothesis was clearly proven wrong by the karyotypic analysis.

Since this patient had a borderline male phenotype associated with a ring-Y-chromosome structure, we decided to investigate the integrity of diverse regions of the Y-chromosome, in an attempt to explain the observed clinical features. In fact, the ring formation eliminated the AZF region entirely (AZFa, AZFb, AZFc), accounting for the described azoospermia and infertility. Moreover, in agreement with the male phenotype we detected the presence of the SRY gene, using 3 different sets of primers (Table 1 and Figure 2), designed to test for the beginning, middle and end of the gene (Fuqua et al., 1997). Interestingly, marker DYS270, which is proximal to the centromere relative to the GCR, tested negative (no amplification) predicting the

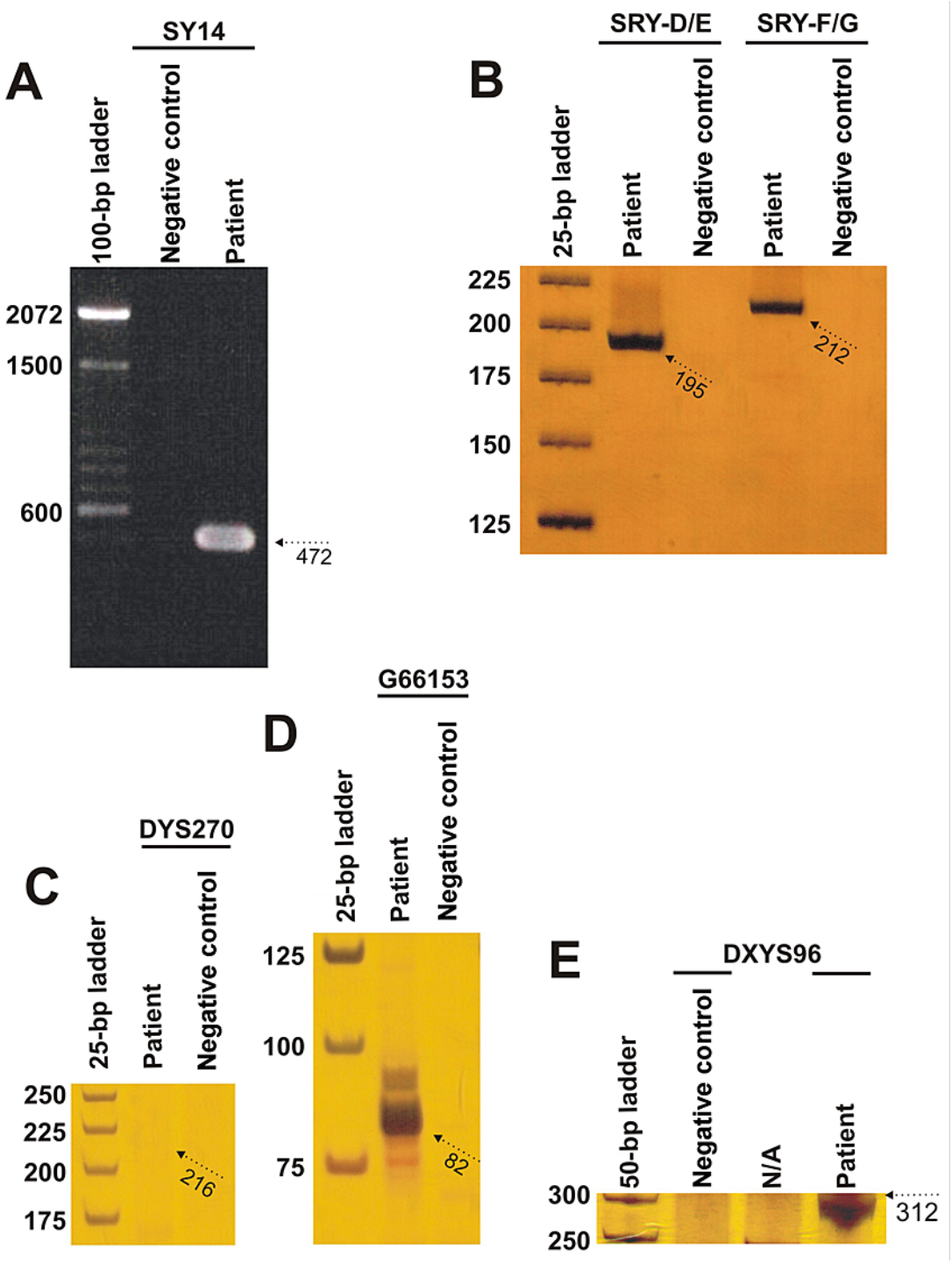

Figure 2. Y-chromosome molecular characterization. **A.** SY14 primer pair for the SRY gene. Agarose gel stained with ethidium bromide. **B.** SRY-D/E and SRY-F/G primer pairs for the SRY gene. Acrylamide gel, silver stained. **C-E.** Markers scanning different regions of the Y-chromosome. N/A = non-applicable (unrelated lane). Arrows with interrupted lines point to the expected position of bands in each PCR reaction.

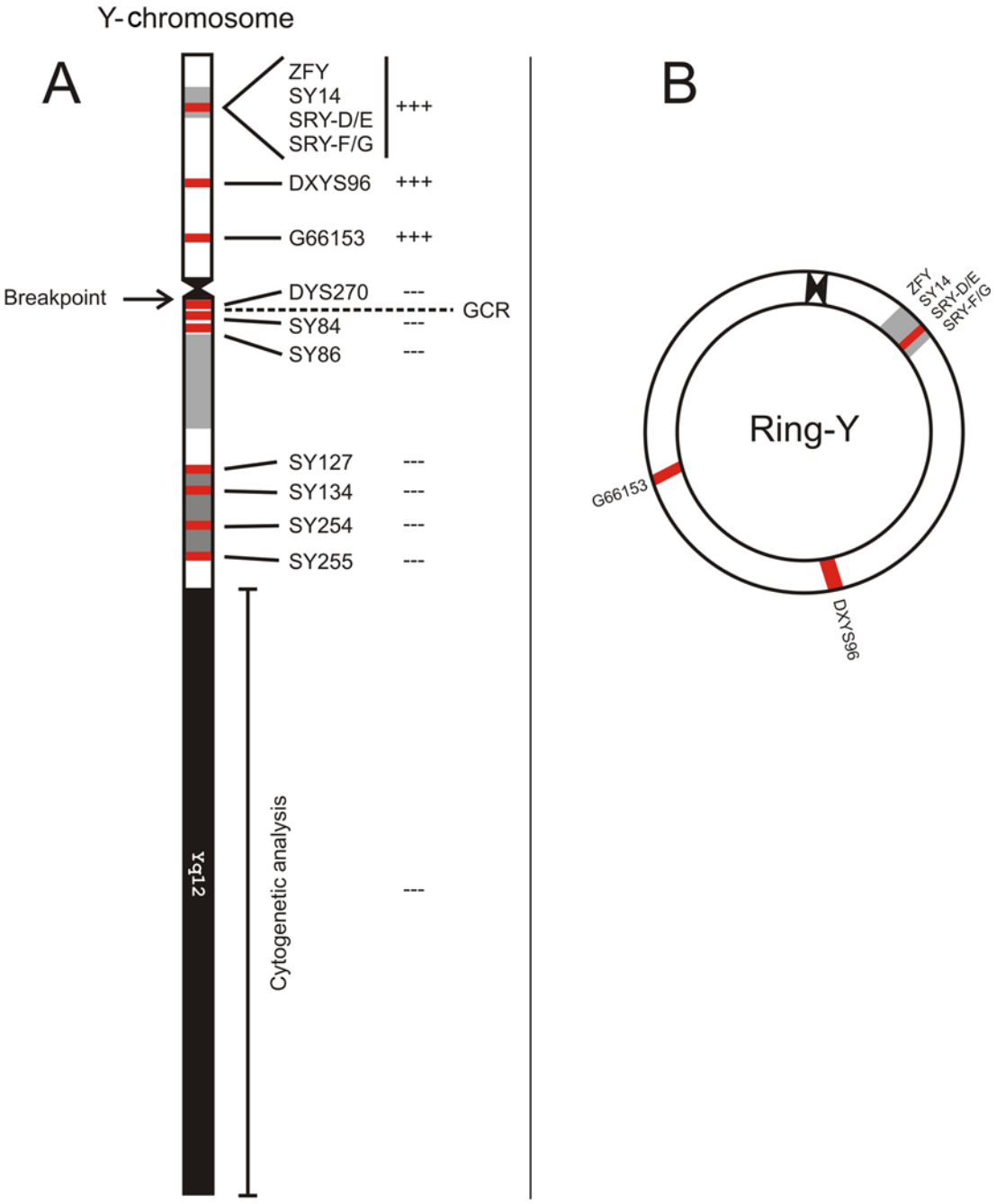

Figure 3. Y-chromosome molecular analysis. **A.** Schematic representation showing all tested regions of the Y-chromosome, according to the primers shown in Table 1. Cytogenetic analysis defined a deletion of the long arm heterochromatin. Positive signs (+++) define PCR amplifiable regions, and negative signs (---) define non-amplifiable regions. A breakpoint was located between the centromere and the DYS270 marker. GCR = growth control region of the Y-chromosome. **B.** Ring-Y-chromosome diagram showing the expected molecular result after breakage and ligation at the breakpoint region. This diagram represents a molecular version of the ring structure observed on the karyotype (Figure 1).

deletion of the GCR on the long arm of the Y-chromosome (Figures 2 and 3), explaining the observed short stature. Because the GCR is defined as a critical region, and is not yet a perfectly established location, we tested for markers on both sides of the critical region.

Finally, aberrant chromosomal structures and the lack of certain gene sequences should be explained to the infertile couple, as part of a comprehensive genetic counseling of the case. This should take into consideration the biological complexities involved in the development of a fertile individual and the social aspects of diagnosing the cause of infertility and establishing the best medical handling.

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