Cytogenetic and molecular study of a premature male infant with 46,XX derived from ICSI: Case report

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We investigated the aetiology of the male phenotype in a premature infant derived from ICSI with a 46,XX karyotype. A karyotypically normal couple underwent ICSI because of obstructive azoospermia in the male partner. Sperm were retrieved by testicular sperm extraction (TESE), cryopreserved, and later used for ICSI. The pregnancy after ICSI ended at 20 weeks. A normal-appearing male was delivered but he did not survive. Umbilical cord blood and placenta were sampled and used for molecular and cytogenetic investigation. The 46,XX karyotype from G-banding in this male infant correlated to a balanced female comparative genomic hybridization (CGH) profile in placental tissue. No PCR amplification of SRY on the p arm of the Y chromosome was observed while fluorescence in-situ hybridization (FISH) with the SRY probe also could not detect the gene in cord blood or placental tissues. CGH and FISH, with X and Y centromeric probes, failed to detect mosaicism in the trophoblast, stroma and amnion. Skewed X-chromosome inactivation (81%) was found in the chorionic villi. The molecular and cytogenetic studies indicated a 46,XX male infant without the SRY gene or 46,XX/XY mosaicism. The possible mechanism in this SRY-negative XX male by ICSI is discussed.

Key words: 46,XX male/comparative genomic hybridization/fluorescence in-situ hybridization/ICSI/SRY gene

Introduction

The Y chromosome determines the male sex, and is linked to the sex-determining region, the SRY gene (Sinclair et al., 1990; Koopman et al., 1991). However, in XX sex-reversed males, testicular differentiation occurs in the absence of the Y chromosome (Kolon et al., 1998; Vernole et al., 2000; Abusheikha et al., 2001). This can be the result of an abnormal X-Y terminal exchange, which transfers the SRY gene from distal Yp to distal Xp during paternal meiotic division. Although Y chromosome sequences can be detected in the majority of male subjects with a 46,XX karyotype, several studies have shown that ~10% of patients lack Y material, including the SRY gene (Palmer et al., 1989; Abbas et al., 1990). A mutation in an autosomal gene or X-linked gene may explain such a phenotype (Vernole et al., 2000). Furthermore, the presence of hidden mosaicism with a Y-bearing cell line could also be responsible for the development of a male phenotype (Fechner et al., 1992). The clinical features of male sex reversal patients are usually hypogonadism, gynecomastia, azoospermia, and hyalinization of seminiferous tubules, with altered hormonal levels at puberty. In a rare case, a 46,XX male may be identified during routine karyotyping done for the investigation of azoospermia (Abusheikha et al., 2001).

ICSI has markedly improved the chances for successful treatment of male factor infertility (Palermo et al., 1992; Ma and Ho Yuen, 2001). However, the issue of a possible genetic risk of this new and powerful procedure has attracted considerable attention. One important aspect in this debate is the possible increased rate of chromosomal abnormalities in the resulting pregnancies. An increase in de-novo chromosomal abnormalities and a higher frequency of transmitted chromosomal aberrations were found in a large cohort of 1586 conceptions through prenatal genetic diagnosis from a single IVF centre (Bonduelle et al., 2002). Our preliminary study, along with others, also showed the same trend (Aboulghar et al., 2001; Lam et al., 2001). Chromosomal abnormalities are mainly involved in numerical and structural abnormalities. So far, 46,XX sex-reversed male infants from ICSI pregnancies have not been reported. We investigated a premature male infant derived from ICSI with a 46,XX karyotype by using molecular and cytogenetic methods to detect the existence of Y-chromosomal material on the X chromosomes, 46,XX/46,XY mosaicism and skewed X-chromosomal inactivation.
Case report

Clinical information

A couple, both with normal karyotypes, underwent ICSI because of obstructive azoospermia in the male partner. Completely immotile sperm were retrieved from the male by testicular sperm extraction (TESE), cryopreserved, and later used for ICSI. In the first ICSI cycle, a pregnancy was established through ICSI with frozen–thawed sperm from TESE, but this pregnancy was spontaneously aborted at 8 weeks when the female partner was 36 years old. Cytogenetic investigation displayed a karyotype of monosomy 21 and the paternal origin of the single chromosome 21 was determined by molecular analysis (Ma et al., 2001). In the second ICSI cycle, immotile sperm were obtained from a frozen–thawed sample from a second TESE and viable sperm were selected based on the phenomenon of sperm tail curling (Ma et al., 2000). Fourteen mature oocytes were used for ICSI of which nine fertilized normally. Four good quality embryos, one at 8-cell stage, three at 4-cell stage, were transferred to the uterus on day 3 of oocyte retrieval. A positive pregnancy test was obtained 14 days after embryo transfer with a serum β-hCG at a concentration of 450 mIU/ml. The pregnancy ended at 20 weeks following premature rupture of membranes. A phenotypically normal male with normal external genitalia was delivered but he did not survive.

Cytogenetic and FISH analysis

A sample of umbilical cord blood from the premature infant was cultured for 72 h in Roswell Park Memorial Institute-1640 medium. Metaphase chromosomes were analysed by the standard G-banding technique. Fluorescence in-situ hybridization (FISH) was performed on stroma, trophoblast and amnion from placenta with X and Y probes to test for sex chromosomal mosaicism. Additionally, FISH was done with an SRY probe for the detection SRY gene locus in the placental tissues. The FISH procedure for the commercially acquired probes was in accordance with the manufacturer’s instructions.

PCR and CGH analyses

The infant’s DNA was extracted from placental tissue according to standard procedure. PCR analyses for the SRY gene were performed using primers as described elsewhere (Vilain et al., 1992).

Comparative genomic hybridization (CGH) was carried out on different placental tissue layers to screen for any other chromosomal aneuploidy missed by conventional cytogenetic and FISH tests and to exclude the existence of confined placental mosaicism (CPM). For CGH, test DNA (infant), labelled with FITC-12-dUTP (green), and the reference DNA (normal 46,XX), labelled with TRITC-6-dUTP (red), at equal amounts were hybridized, along with an excess of unlabelled human Cot-I DNA, on normal male metaphases on a slide. Slides were analysed using a Cytovision Workstation (Applied Imaging, USA), which calculates the differential fluorescent ratios between the patient DNA to normal DNA hybridized to each chromosome in order to detect if there are any gains and losses in the genetic copy number of the test DNA compared to the reference DNA (Kallioniemi et al., 1992; Lomax et al., 1994).

X-chromosome inactivation assay

DNA, extracted from cord blood, amnion, chorion, chorionic villi and parental blood samples, was examined for X-chromosome inactivation (XCI) by determining the methylation status at the androgen receptor gene (AR). Following digestion with HpaII, a methylation-sensitive restriction enzyme, both the digested DNA and control undigested DNA were amplified by PCR with the appropriate primers. Methylated DNA was not cut by HpaII and therefore amplified, whereas unmethylated alleles were digested and did not amplify. Non-random XCI was revealed by a difference in quantitative intensity of the two alleles (Peñañaherrera et al., 2003).

Results

Evaluation of the G-banded chromosome preparation from the umbilical cord blood sample from the premature infant revealed a 46,XX karyotype (Figure 1a). Mosaicism was not indicated in any of the metaphases analysed.

CGH analysis (Figure 1b) also displayed a balanced, XX profile, which was in accordance with a 46,XX karyotype. FISH with an SRY probe and PCR amplification with SRY sequence primers indicated the absence of the SRY gene in all the placental tissues tested. Furthermore, 46,XX/46,XY mosaicism was not found in the trophoblast, stroma, and amnion from the placenta by CGH and FISH (Figure 2a, b). Methylation analysis at the AR locus demonstrated a skewed X inactivation pattern (81%) in the chorionic villi while random XCI was seen in amnion, chorion and cord blood from the premature infant.

Discussion

Men with a 46,XX karyotype are sterile. The frequency of 46,XX males varies from 1 in 9000 to 1 in 20 000 in newborn males (de la Chapelle, 1972; Nielsen and Sillesen, 1975). Clinical appearance of a 46,XX male may range from a classic XX male with an apparently normal male phenotype, to non-classic XX male with some degree of sexual ambiguity, to XX true hermaphrodites (Boucckiene et al., 1994).

Presence or absence of SRY, or hidden mosaicism in gonads, may be associated with the clinical manifestation of the male phenotype (Fechner et al., 1992; Kolon et al., 1998; Vernole et al., 2000). Our case has been thoroughly investigated in both cytogenetic and molecular aspects: the absence of the SRY gene was confirmed, and at least sex chromosomal mosaicism was not detected in the placental tissues (other tissues from the infant were not available for study). As far as we are aware, this is the first report of a 46,XX male from ICSI. Whether ICSI has enhanced the production of this 46,XX male is unclear. It seems that the mechanism for the production of the monosomy 21 abortus in the first ICSI cycle from this couple is different from that of this second pregnancy with the 46,XX male infant. The former was due to a maternal meiotic error as evidenced by

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the detection of a paternally derived chromosome 21 (Ma et al., 2001). The latter may be associated with a gene mutation either on an autosome or the X chromosome, which may be involved in the sex determination cascade (Vernole et al., 2000). Obviously, ICSI has assisted the fertilization of those two conceptions in this family because the sperm used for ICSI were made available only through TESE as a result of obstructive azoospermia in the male partner. The losses of the pregnancy experienced by this family may possibly be explained by the injection of the more immature sperm from testis.

There is a substantial number of XX males (~20%) in whom no Y sequences can be identified by probing for Y-specific probes, including SRY (Palmer et al., 1989; Ferguson-Smith et al., 1990). The majority of these Y-negative cases have ambiguity of external genitalia. A total of six classic XX male individuals and one fetus with an absent SRY gene have been reported to date (Vilain et al., 1992; Zenteno et al., 1997; Kolon et al., 1998; Vernole et al., 2000; Abusheikha et al., 2001). Our case provides further evidence that the SRY gene does not act alone in the determination of male phenotype and a mutation on an as-yet-unidentified autosomal or X-linked sex-determining gene may participate in the sex-determining cascade. This gene may have a broad function to inhibit male development and may normally be repressed by SRY (Kolon et al., 1998).

Although the theory was proposed that X-inactivation may play a role in the expression of a downstream gene in the aetiology of a 46,XX male (Kolon et al., 1998), no study has been reported in regard to the pattern of XCI. The finding of skewed XCI in the chorionic villi of the present XX male is difficult to explain. As indicated by Kolon et al. (1998), random XCI in premature infants occur in conjunction with true hermaphroditism 50% of the time. Our case may be an example of this; however, biopsy on the gonad was not performed in the infant.

Different types of mosaicism can play a role in fetal loss and the pathogenesis of abnormal intrauterine development. Among them are chromosome abnormalities limited to the placenta with complete dichotomy between placenta and fetus. This genetic inconsistency within the conceptus is known as confined placental mosaicism (CPM) (Kalousek and Dill, 1983). The skewed XCI in the chorionic villi in this case may have been an indicator for the presence of CPM because skewed XCI has been found more often in fetuses and newborns from pregnancies complicated with CPM (Lau et al., 1997). However, CGH and FISH analyses on placental tissues failed to detect the presence of CPM in this case.

Although all our molecular and cytogenetic studies indicated a 46,XX karyotype with negative SRY, we cannot completely exclude the other genes involved in the sex-determining cascade. With ICSI being increasingly applied as treatment for male factor infertility and follow-up studies for ICSI pregnancies, we may discover that as-yet-unrecognized genetic consequences do exist. Cytogenetic and molecular analyses should be pursued in infants from ICSI with clinical phenotypes typically seen in 46,XX males, such as hypospadias (Wennerholm et al., 2000), to correlate the genotype with the clinical phenotype and to obtain a better idea about the frequency of 46,XX male infants that arise from ICSI. The information obtained from these studies would be valuable in the counselling of couples, particularly those who have conceived a 46,XX male infant.
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References


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