Fertility in a i(Xq) Klinefelter patient: importance of XIST expression level determined by qRT-PCR in ruling out Klinefelter cryptic mosaicism as cause of oligozoospermia

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The presence of an isochromosome Xq in Klinefelter syndrome (KS) is an apparently rare condition. In all cases reported so far, patients showed the classic phenotype. We here describe a case of isochromosome Xq [47,X,i(Xq),Y] in a non-mosaic KS patient. The patient exhibited a normal androgenized phenotype, normal testes and normal cognitive abilities. Semen analysis revealed a medium oligozoospermia (5 × 10⁶ spermatozoa/ml). After the patient underwent intracytoplasmic sperm injection, he generated two cytogenetically healthy normal females. Fluorescence in situ hybridization analysis showed the presence of a dicentric Xq chromosome that did not show the presence of residual Xp arm up to the 57,820,478 bp position (Xp 1.1) of X chromosome sequence. Preferential inactivation of Xq isochromosome was demonstrated by bromodeoxyuridine replication analysis and transcriptional silencing by DNA methylation at the HUMARA locus. Furthermore, we demonstrated by quantitative RT–PCR an active XIST RNA expression in blood lymphocytes from Klinefelter patients, comparable to that observed in control females and over 30 000-fold greater than in control males. In conclusion, this qRT–PCR approach could be useful for screening of pre-puberty males and for diagnosis or exclusion of cryptic Klinefelter mosaics.

Keywords: Klinefelter syndrome; isochromosome Xq; XIST expression; fertility

Introduction

The typical features of prototypic Klinefelter syndrome (KS) case are seminiferous tubules dysgenesis, androgen deficiency and high levels of follicle-stimulating (FSH) and luteinizing (LH) hormones (Simpson et al., 2003). Affected patients present, in decreasing order of frequency: azoospermia or oligozoospermia, small, firm testes, hypergonadotropic hypogonadism, gynecomastia, tall body habitus (feminized in some cases) characterized by long legs, narrow shoulders and decreased facial and pubic hair. Psychosocial and behavioural problems with a variable degree of mental retardation may be present (Stemkens et al., 2006). Unfortunately, most of Klinefelter patients are diagnosed during adolescence because of gynecomastia or in adulthood following infertility, whereas an early clinical treatment of the hypergonadotropism during puberty could have attenuated seminiferous tubules hyalinization.

It has been reported that oligozoospermic Klinefelter patients have benefited from assisted reproductive techniques, such as intracytoplasmic sperm injection (ICSI). In the presence of azoospermia, ICSI was preceded by testicular sperm aspiration (Bourne et al., 1997; Ron-El et al., 2000; Bergere et al., 2002). However, an increased frequency of aneuploidy in spermatozoa from these patients has been described (Estop et al., 1998; Foresta et al., 1999; Morel et al., 2000; Rives et al., 2000; Blanco et al., 2001). Therefore, because of the increased risk of both sex-chromosomal and autosomal aneuploidies in KS progeny, prenatal genetic diagnosis is strongly recommended.

In mammalian females, random inactivation of one X chromosome takes place for gene-dosage compensation (Lyon, 1962). In humans, the XIST gene located within the X inactivation centre (XIC) in Xq13.2 is transcribed in a cis-acting, non-coding nuclear RNA that ‘labels’ the X chromosome and induces transcriptional silencing and DNA methylation (Hendrich et al., 1997). In KS, the inactivation to a large extent of supernumerary X chromosome(s) is commonly observed. In particular, the possibility of a skewed inactivation that could explain the wide range of phenotypic abnormalities observed has been demonstrated (Iitsuka et al., 2001).

A mosaic 47,XXX/46,XY karyotype is present in about 10% of KS cases and often results in a milder phenotype (Sharara, 1999), while additional X (or Y) chromosomes progressively shift the phenotype to a more severe clinical presentation (Simpson et al., 2003; Velissariou et al., 2006). The presence of an isochromosome Xq in KS (47,X,i(Xq),Y) is an apparently rare condition. The patients described in the literature (Zang et al., 1969; Gardiner and Brown, 1978; Kalousek et al., 1978; Trunca et al., 1979; Ponzo et al., 1980; Donlan et al., 1987; Kleczkowska et al., 1988; Richer et al., 1989; Arps et al., 1996; Stemkens et al., 2007; Höckner et al., 2008) showed all typical manifestations of the syndrome, apart from exhibiting short to normal stature. In this paper, we describe a case of isochromosome Xq in an...
oligozoospermic Klinefelter patient that did not display any of the typical phenotypic features of KS, except for relatively high FSH and low inhibin B levels. They were both different to that observed in KS patients and compatible with the oligozoospermic status and the normal structure of testes. We performed detailed cytogenetic, histopathological and molecular studies. Quantitative RT–PCR was used to analyze the expression levels of XIST, in order to rule out a cryptic mosaic (47,X,i(Xq),Y/46,XY) as a cause of a milder phenotype in our patient. XIST level is almost undetectable in normal males, while in KS patients the same level as normal females can be found. XIST levels in our patient were comparable to that of sterile homogenous KS patients. The potential usefulness of this quantitative PCR approach for rapid screening of prepuberty males, for prenatal diagnosis and for diagnosis of cryptic Klinefelter mosaic is discussed.

Materials and Methods

Patients and control subjects

A 47,X,i(Xq),Y KS patient (KS/iXq) was referred to the genetic counselling centre ‘Zigote’ with an indication of couple infertility. The patient showed a normal androgenized phenotype, normal intelligence and normal testes. DNA samples were available from both parents of the KS/iXq patient. KS patients 1 (KS/p1) and 2 (KS/p2) were used as controls. They displayed 47,XXY karyotype and normal androgenized phenotype, except for scanty beard and small testes. Both patients were azoospermic. FSH and LH levels were higher, and testosterone lower, than normal adult levels. The patients were informed and agreed to participate in the study. Male and female healthy controls were recruited from the research staff.

Cytogenetic findings

The karyotype for KS/iXq, KS/p1 and KS/p2 patients was assessed using blood lymphocytes cultured skin fibroblasts by G- and R-banding according to standard procedures.

Fluorescence in situ hybridization (FISH) analysis was performed (for the KS/iXq patient) on interphase nuclei from blood lymphocytes. Cells were fixed on slides by immersion for 2 min in serial dilution of ethanol (70, 85 and 100%). Human a-satellite fluorescent probes (Aquarius probes kit, CytoCell Technologies, Cambridge, UK) specific for X (green) and Y (red) chromosomes were used for hybridization. The hybridization procedures and stringency washing were performed according to the manufacturer’s instructions. Cellular nuclei were counterstained at room temperature by DAPI anti-fade solution supplied with the kit. Slides were observed and images recorded using an epifluorescence microscope Olympus BX60 equipped with specific filter sets.

Cytological evidences of isochromosome Xq inactivation

Bromodeoxyuridine (BrdU) replication analysis was performed as described elsewhere (Camargo and Cervenka, 1984). Briefly, BrdU (0.26% in PBS) (Sigma-Aldrich, St Louis, MO, USA) was added to lymphocyte culture 6–8 h before dye treatment. The slides were treated by Hoechst 33258 (Sigma-Aldrich) dye for 1 h, exposed to ultraviolet-C radiation for 2 h and then Giemsa stained.

DNA extraction and analysis

Peripheral blood samples (10 ml) were collected in EDTA vacutainers. DNA was extracted using the Puregene-DNA-extraction kit (Gentra system, Biocompare, Inc., South San Francisco, CA, USA), according to the manufacturer’s instructions.

Determination of parental origin of isochromosome Xq

Parental origin of iXq chromosome was determined by using highly polymorphic markers specific for the X chromosome. DXS6010, 8679, DXS5789, DXS1047, FM1, DXS55, DX515, FRAX-F and DXS377 markers were informative. Complete information for the primers used to detect the loci is available at NCBi (http://www.ncbi.nlm.nih.gov/) and at GDB (http://www.gdb.org/gdb/) web sites. The genomic DNA (200 ng) was amplified using the following protocol: 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at optimum annealing temperature for each primer pairs, 45 s at 68°C and final extension for 10 min at 72°C. The amplimers were separated by 2% agarose gel electrophoresis and revealed by ethidium bromide or, for the dinucleotide repeat markers, by 6% polyacrylamide gel electrophoresis and silver-staining.

Evaluation of HUMARA locus methylation status

Methylation status in the exon 1 of human androgen receptor locus (HUMARA-01) was evaluated as described by Allen et al. (1992). Briefly, 2 μg of DNA were digested with HpaII or MspI or TaqI restriction enzymes (Roche, Basel, Switzerland), and PCR for repetitive digestion reaction were PCR-amplified using a primer pair designed in the first exon of the gene (acc. number M35844, amplifier sequence position: bp 230–485).

Evaluation of Xp arm residual sequences in the isochromosome Xq by real-time PCR

In order to detect the presence of Xp proximal sequences in the isochromosome Xq, we modified the procedure originally described by Lorda-Sanchez et al. (1991). To reduce genomic DNA length and favour quantitative amplification, 2 μg of DNA were digested by TaqI restriction enzyme overnight at 65°C. Quantitative real-time PCR was performed by an iCycler-iQ® in a 25 μl reaction mixture containing: 1× SYBR®-Green-Supermix (Bio-Rad), 25 ng of DNA and 300 nM primers. The amplification profile was: initial denaturation of 2 min at 94°C and 35 cycles of 40 s at 94°C, annealing for 30 s at 55°C and elongation for 40 s at 68°C. Fluorescence data were collected during elongation step. A final extension of 7 min was carried out at 72°C, followed by melt-curve data analysis. Optimized primers for SYBR®-Green analysis were designed by a Beacon-Designer® software 5.1 version (BiosoftInternational, Palo Alto, CA, USA), and were synthesized (HPLC-purification grade) by MWGBiotech-MMedical (Milano, Italy). The primers utilized were selected within the loci: DXS6810 in Xp11.4-11.3 (Forward: aTGTTGTGTCGTCG TCCTC; Reverse: tGTCCTCTATCTGCTGTACTTAC, e-PCR at http://www.ncbi.nlm.nih.gov/sutils/e-pcr, loc. X: bp 42 674,863-42 675,086), DXS14 in Xp 11.1 (Forward: gAAATTAAACCATGAGGTGCTC; Reverse: gACAAACAAGGGCAAGAC, e-PCR, loc. X: 2 hits bp 57,686,527-57,686,744 and 57,820,231-57,820,478) and DXS6789 in Xq22.2 (Forward: cATACATACATACATACATACATACATAC; Reverse: cTCCC AGCTATATCTTCC, e-PCR, loc. X: bp 95,255,641-95,255,845). The mean of threshold cycles (Ct) was from five independent replicate assays. Relative quantification, normalized with respect to DXS6810 locus, was performed by GENEX software (Bio-Rad) for group-wise comparison and statistical analysis (significance probability limit was P < 0.05).

RNA extraction and quantitative RT–PCR

Total RNA was extracted, analysed by a 2100 Bioanalyzer (Agilent) (RNA Integrity Number>7.5) and retro-transcribed as previously described (Matias et al., 2006). Quantitative real-time PCR was performed as described earlier, i.e. in a 25 μl reaction mixture using 20 ng of cDNA (calculated on the basis of the input RNA on RT mixture). The amplification profile consisted of an initial denaturation of 2 min at 94°C and 40 cycles of 30 s at 94°C, annealing for 30 s at TaOpt (optimum annealing temperature, see later) and elongation for 45 s at 68°C. Fluorescence data were collected during elongation step. A final extension of 7 min was carried out at 72°C, followed by melt-curve data analysis. Optimized primers for SYBR®-Green analysis (XIST acc. NR_001564 F: tCCGTATATGCTATCTTCTTCTTAG, R: aACTACCTTCAACTCATTG; Beta-actin acc. NM_001101 F: aACTACCTTCAACTCATTG; R: tGATCTTGATCTTCATTGTG; Beta-2-microglobulin acc. NR_001564 F: tCCTAGTATCTGCTGTACTTAC, e-PCR at http://www.ncbi.nlm.nih.gov/sutils/e-pcr, loc. X: bp 42 674,863-42 675,086), DXS14 in Xp 11.1 (Forward: gAAATTAAACCATGAGGTGCTC; Reverse: gACAAACAAGGGCAAGAC, e-PCR, loc. X: 2 hits bp 57,686,527-57,686,744 and 57,820,231-57,820,478) and DXS6789 in Xq22.2 (Forward: cATACATACATACATACATACATACATAC; Reverse: cTCCC AGCTATATCTTCC, e-PCR, loc. X: bp 95,255,641-95,255,845). The mean of threshold cycles (Ct) was from five independent replicate assays. Relative quantification, normalized with respect to DXS6810 locus, was performed by GENEX software (Bio-Rad) for group-wise comparison and statistical analysis (significance probability limit was P < 0.05).
Hormonal analysis

Hormonal plasma levels of KS patients were determined by radioimmuno assay in the absence of hormonal replacement therapy. The levels of testosterone, dihydrotestosterone, 17 beta-oestradiol, prolactin and LH were determined. All hormonal dosage data were from a National Health System laboratory, licensed for hormonal diagnoses.

Results

A 25-year-old man and his wife were referred to the genetic counselling centre ‘Zigote’ with an indication of couple infertility after 3 years of marriage. The patient showed a normal androgenized phenotype (height 178 cm, weight 75 kg) with arm-span/height ratio in the normal range; he showed a scanty beard and pubic hair of a male pattern. Testicular eco-Doppler revealed slightly reduced size dydimiti without varicocele. Psychomotor development and intelligence were normal and the patient had normal social relations and an average degree of social competence. Semen analysis revealed a medium to severe oligozoospermia (5 × 10⁶ spermatozoa/ml). Hormonal levels were in a normal range, except for a relatively high FSH (about 1.7-fold the upper confidence limit in man) and for a relatively low inhibin B (94 pg/ml). Both values were compatible with the oligozoospermic status (Klingmuller and Haidl, 1997) and the normal structure of testes. After karyotype analysis, a diagnosis of KS, characterized by the presence of an isochromosome Xq, was made. The patient and his wife were informed of the risks related to the use of spermatozoa from a KS patient ejaculate. ICSI was performed successfully from a single ejaculation. Three pre-embryos were transferred in uterus 48 h from fecundation. After a bichorial-biamniotic pregnancy, two cytogenetically healthy normal females were born.

Blood lymphocyte and cultured skin fibroblast chromosomal analysis revealed a male karyotype with an extra isochromosome for the long arm of X chromosome in all metaphases observed. G and R bands of Xq isochromosome were regular for distribution and intensity (data not shown). Buccal smear analysis showed the presence of a Barr body in all cells examined. Two-colour FISH analysis on interphasic nuclei (data not shown). Buccal smear analysis showed the presence of a Barr body in all cells examined. Two-colour FISH analysis on interphasic nuclei (data not shown). Buchal smear analysis showed the presence of a Barr body in all cells examined. Two-colour FISH on interphasic nuclei (data not shown).

BrdU replication was used to assess the isochromosome Xq inactivation status. This technique highlighted a modified R-banding pattern in the late-replicating inactivated X chromosome that appeared lightly stained. The isochromosome Xq was late-replicating in all the metaphases observed (Fig. 1B), thus suggesting a preferential inactivation. Transcriptional X chromosome silencing, correlated to DNA methylation status, was evaluated by analyzing the methylation of HUMARA locus (Allen et al., 1992). The androgen receptor shows in exon 1 (HUMARA-01) a variable high polymorphic CAG repeat, and in 5’ to the repeat two restriction sites sensitive to HpaII digestion are present. In the inactive X chromosome, these sites are methylated, thus protecting DNA from HpaII digestion, but not from digestion by the isoschizomer Msp1, cutting the DNA independently of the methylation status. After Hpa II digestion, the patient’s DNA was normally amplified by a primer pair located upward of the methylation sites and downward of the trinucleotide repeat, thus indicating DNA methylation in HUMARA-01 (Fig. 2). On the other hand, in the same experimental conditions, the DNAs from the patient’s father and from two healthy controls (a male and a female) were totally (for the two males) or partially (for the female, showing X random-inactivation) Hpa II digested, as qualitatively demonstrated by the amplification bands. The DNA amplification of the KS/iXq patient was completely abolished by MspI isoschizomer digestion, thus indicating a DNA amenable to restriction digestion.

In order to detect the presence of Xp proximal sequences in the isochromosome Xq, we modified a procedure previously described by Lorda-Sanchez et al. (1991). We used a quantitative real-time PCR SYBR-Green approach. Loci DXS6789 in Xq22.2, DXS6810 in Xp11.4-11.3 and DXS14 in Xp 11.1 were selected for the analysis. Fig. 3 shows the relative increase of the amplification levels of DXS14 and DXS6789 loci, normalized in respect to the amplification level of DXS6810 locus, in the KS/iXq patient as compared to a male control. In the same figure the amplification results are also summarized in terms of mean of Cts (Fig. 3, inset). For locus DXS6789, the amplification ratio between the patient and the male control was 3:1, as predicted by cytogenetic evidences, because of the presence of three Xq arms in the patient. The ratio was about 1:1 for locus DXS14, thus indicating the absence of a residual Xp arm in the isochromosome Xq up to 57,820,478 bp of the X chromosome sequence (see Materials and Methods).
The polymorphic markers DXS6810, HUMARA-01, DXS6789, DXS1047, FMR1, DXS52, DXS15, FRAXF, and DXS8377 were informative in order to determine the parental origin of the non-disjunction event. Table I shows the cytogenetic assignments, the polymorphism types, the heterozygosity for the markers tested and the result summary for patient XS/iXq and his relatives. The patient exhibited alleles of maternal origin and homozygosity even at those markers (FMR1, DXS1047, DXS52 and DXS15) for which the mother showed heterozygosity. In order to test if the homozygosity observed in our patient could be ascribed to an imbalance of target copies during DNA amplification, we performed the experiment depicted in Fig. 4, showing the electrophoretic analysis for the PCR amplification of the marker FMR1. The marker, a CGG tri-nucleotide, is difficult to amplify, and the possibility of allele drop-out during PCR amplification has been described (Chong et al., 1994). The test clearly ruled out that the homozygosity observed could be due to an unbalance of DNA-target copies.

We used quantitative RT–PCR to evaluate the transcriptional expression of XIST gene in our patient, in comparison to Klinefelter patients with 47,XXY karyotype and healthy male and female controls (Fig. 5). In control males, where X inactivation process does not take place, XIST expression levels were about 30 000-fold lower than in control females. Our patient (KS/iXq) and one of 47,XXY patients (KS/p2) exhibited expression levels very similar to that observed in control females, whereas the other 47,XXY patient (KS/p1) displayed a 3-fold lower expression level in comparison to control females (P = 0.002).

**Discussion**

The presence of an isochromosome Xq is apparently rare in KS patients, with an estimated prevalence of 1 in about 250 cases (Kleczkowska et al., 1988). To our knowledge, 19 cases of isochromosome Xq have been described so far, and incomplete molecular characterizations have been provided for most of the reported cases (Zang et al., 1969; Gardiner and Brown, 1978; Kalousek et al., 1978; Trunca et al., 1979; Ponzio et al., 1980; Donlan et al., 1987; Bleau et al., 1987; Kleczkowska

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**Table I.** Analysis of the informative loci used for the determination of parental origin of the isochromosome Xq in the 47,Xi(Xq)Y Klinefelter patient.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Cytogenetic assign.</th>
<th>Type</th>
<th>Het.</th>
<th>Marker analysis</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>DXS6810</td>
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<td>Tetra</td>
<td>0.65</td>
<td></td>
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<tr>
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</tr>
<tr>
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<tr>
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<td>Xq27.3</td>
<td>Tri</td>
<td>nr</td>
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<td>FRAX F</td>
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<td>Tri</td>
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<td></td>
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<tr>
<td>DXS8377</td>
<td>Xq28-Xq28</td>
<td>Tri</td>
<td>0.95</td>
<td></td>
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</table>

*On the basis of the karyotype findings. F, father; M, mother; P, 47,Xi(Xq)/Y Klinefelter patient; Het, heterozygosity; Di, Tetra: nucleotide-repeat; VNTR, variable number tandem repeat; nr, not reported.
et al., 1988; Richer et al., 1989; Arps et al., 1996; Stemkens et al., 2007; Höckner et al., 2008). All 47,X,i(Xq),Y patients are reported to have exhibited, although at different degrees, the main clinical features of KS, namely, reduced androgenization, small testes, azoospermia, gynecomastia and elevated FSH and LH levels. The differences observed in comparison to the prototypic KS patient were the presence (with the exception of the case reported by Kleczkowska et al.) of a short to normal stature and the absence of cognitive impairment in all subjects examined. Theoretically, the absence of a diploid Xp arm in 47,X,i(Xq),Y Klinefelter patients should correlate to a favourable prognosis and to a mild phenotype. Genes of the Xp arm, relevant for the clinical manifestations of the syndrome, could escape supernumerary X chromosome inactivation, the mechanism for gene-dosage compensation. In a study conducted on 275 genes of chromosome X, only 3 genes on the long arm escaped inactivation, in contrast to almost 40 on the short arm (Willard, 2001). The short to normal stature observed in 47,X,i(Xq),Y patients and in the present case could be associated to the absence of over-expression of genes located in the pseudoautosomal Xp/Yp region near the telomere (Richer et al., 1989). Cytogenetic analysis revealed in our patient a supernumerary dicentric Xq chromosome that showed preferential inactivation, using both cytogenetic and molecular techniques, and did not show the presence of transcriptionally active Xp arm sequences. The patient exhibited alleles of maternal origin for both X chromosomes, and maternal heterozygosity was reduced to homozgyosity. These data suggest that isochromosome Xq originated during the first zygotic mitosis, after a non-disjunction event at meiosis II, to avoid a severe disomic status for identical X chromosomes.

The present case was peculiar when compared with 47,X,i(Xq),Y patients, so far described, because of the relatively elevated spermatogenesis, as sometimes observed in mosaic KS patients (Sharara, 1999). It is debated whether or not 47,XXY spermatocytes can pass meiotic-gate and whether or not aneuploidy rate is increased in spermatozoa from KS patient. Meiotic analysis performed by FISH on germ cells from biopsied testes suggests that only 46,XY cells can undergo meiosis, in that all pachytene figures observed (Estop et al., 1998; Blanco et al., 2001; Bergere et al., 2002) were XY. On the other hand, it has been demonstrated (Foresta et al., 1999) that 47,XXY spermatogonia are able to undergo and complete the spermatogenic process. The aneuploidy rate observed in residual spermatozoa of KS patients was 6.75%, comparable to that observed in oligozoospermic patients, and has been related to the compromised structure of testes (Morel et al., 2000; Rives et al., 2000). Conversely, the relatively elevated spermatogenesis in our patient could be mainly due to the normal size and the regular echographic structure of testes. Moreover, apart from the motility in the first hour, the morphology of spermatozoa and eosin test were in the range of normality. Although the patient showed a non-mosaic karyotype in the somatic cells, it is possible to hypothesize a case of cryptic mosaicism (47,X,i(Xq),Y/46,XY) (Lenz et al., 2005), or alternatively a mosaic asset in the germinal cell lineage, to explain these findings. For ethical reasons, it was not possible to evaluate this hypothesis directly in testicular biopsy (Bergere et al., 2002), since the biopsy procedure is invasive and, in this case, was unnecessary for ICSI purpose.

The possibility that this case was a cryptic Klinefelter mosaic was ruled out by analyzing quantitatively the transcriptional level of XIST gene. In humans, the XIST gene located within the X inactivation centre in Xq13.2 is transcribed in a cis-acting, non-coding RNA that labels the chromosome and induces transcriptional silencing (Hendrich et al., 1997). In the present paper, according to the data reported by Vawter et al. (2007), we demonstrated in Klinefelter patients an active XIST RNA expression comparable to that observed in control females and a 30 000-fold down-regulation in control males. Our patient showed XIST expression levels comparable to that of a KS prototypic patient. These data, although do not permit to rule out definitely the hypothesis of mosaicism in the germinal cells lineage, suggest a non-mosaic condition in the lymphoid cell line and, in addition, that only one of the two XIC centre of Xq chromosome was transcriptionally active.

The dramatic difference in XIST RNA level between normal males and Klinefelter patients could warrant the determination of XIST...
expression level, together with other useful expression targets (Ottesen et al., 2007), as a potent qRT–PCR based screening method in prepubertal males. Furthermore, this approach could be useful for prenatal diagnosis and for the identification of cryptic Klinefelter mosaics. Boys with KS may only be identified in a small percentage before puberty through tall stature, relatively decreased penile length, clinodactyly, hypotonia, and requirement for speech therapy (Zeger et al., 2008). Prepubertal diagnosis is essential in order to avoid the abnormal skeletal growth and to identify children who may benefit from a psychotherapeutic support.

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