CLINICAL STUDY

Severe XIST hypomethylation clearly distinguishes (SRY +) 46,XX-maleness from Klinefelter syndrome

Andreas Poplinski, Peter Wieacker1, Sabine Kliesch and Jörg Gromoll
Centre of Reproductive Medicine and Andrology, University Clinics Münster, Domagkstraße 11, 48149 Münster, Germany and 1Institute of Human Genetics, Vesaliusweg 12-14, 48149 Münster, Germany

(Correspondence should be addressed to J Gromoll; Email: joerg.gromoll@ukmuenster.de)

Abstract

Objective: 46,XX-maleness affects 1 in 20 000 live male newborns resulting in infertility and hypergonadotrophic hypogonadism. Although the phenotypes of XX-males have been well described, the molecular nature of the X chromosomes remains elusive. We assessed the X inactivation status by DNA methylation analysis of four informative loci and compared those to Klinefelter syndrome (KS) and Turner syndrome.

Design and methods: Patient cohort consisted of ten sex-determining region of the Y (SRY +) XX-males, two (SRY –) XX-males, ten 47,XXY Klinefelter men, six 45,X Turner females and ten male and female control individuals each. Methylation analysis was carried out by bisulphite sequencing of DNA from peripheral blood lymphocytes analysing X-inactive-specific transcript (XIST), phosphoglycerate kinase 1 (PGK1), ferritin, heavy peptide-like 17 (FTHL17) and short stature homeobox (SHOX).

Results: XIST methylation was 18% in (SRY +) XX-males, and thus they were severely hypomethylated compared to (SRY –) XX-males (48%; P < 0.01), Klinefelter men (44%; P < 0.01) and female controls (47%; P < 0.01). Turner females and male controls displayed a high degree of XIST methylation of 98 and 94% respectively. Methylation of PGK1, undergoing X inactivation, was not significantly reduced in (SRY +) XX-males compared to female controls in spite of severe XIST hypomethylation (51 vs 69%; P > 0.05). FTHL17, escaping X inactivation, but undergoing cell-type-specific inactivation was similarly methylated in XX-males (89%), KS patients (87%) and female controls (90%). SHOX, an X inactivation escapee located in the pseudoautosomal region, displays similarly low degrees of methylation for XX-males (7%), KS patients (7%) and female controls (9%).

Conclusions: XIST hypomethylation clearly distinguishes (SRY +) XX-males from Klinefelter men. It does not, however, impair appropriate epigenetic regulation of representative X-linked loci.

European Journal of Endocrinology 162 169–175

Introduction

Sex chromosomal aberrations are a frequent cause of male and female infertility. 46,XX-maleness is a rare sex chromosomal anomaly that affects 1 in 20 000 newborn males. Ninety percent of XX-males are designated sex-determining region of the Y (SRY +) 46,XX-males as translocation of Y chromosomal material including SRY triggers testis formation in these men (1). However, 10% of XX-males lack SRY and are thus designated (SRY –) XX-males. In these men, masculinisation is induced by upregulation of the transcription factor SOX9 that triggers testis formation independently from SRY (2). XX-males are characterised by hypergonadotrophic hypogonadism and reduced body height, increased incidence of gynecomastia and maldescended testes compared to 46,XY males. In comparison to 46,XY healthy males, these men display significantly reduced testosterone levels, and severely elevated levels of FSH and LH. Reduction of bitesticular volume to one-tenth that of normal males reflects exclusive azoospermia, and as residual spermatogenesis in the testis has not yet been reported, assisted reproduction techniques (ARTs) are no option for reproduction in these patients. Detailed molecular studies of the supernumerary X chromosomes in these patients are rare. It is known, however, that X chromosome inactivation in these patients follows a random pattern with a significant proportion of them exhibiting extreme skewing (3).

The most frequent sex chromosomal aneuploidy in men is 47,XXY Klinefelter syndrome (KS) with a prevalence of 0.1–0.2% in newborn males and 11% among azoospermic infertile men (4, 5). KS patients display hypergonadotrophic hypogonadism, gynaecomastia and reduced pubic hair; the testes are small and firm due to spermatogenic impairment (6). Oligozoospermic ejaculates and residual active foci of spermatogenesis in testicular biopsies have been observed (7, 8), and the use of ART by testicular sperm extraction has been
successful in several reported cases (9–11). Although endocrinological features of these men including reduced testosterone and LH levels have been described (3), the precise molecular role of the supernumerary X chromosome has not been elucidated in KS yet.

A sex chromosomal anomaly affecting women is the 45,X Turner syndrome. It has an incidence of 1:2000, and is caused by the complete or partial absence of the second gonosome (12). Decreased body height, ovarian dysgenesis, accelerated oocyte apoptosis and oestrogen deficiency leading to severely impaired fertility are classical symptoms in these women (13).

Of particular importance for X chromosomal gene dosage compensation between the sexes is the X chromosome inactivation. It is initiated embryonically by the expression of the X-encoded X-inactive-specific transcript (XIST) that is exclusively transcribed from the inactive X chromosome (14, 15). Expression of XIST triggers silencing of the X chromosome in cis and leads to DNA methylation of promoter regions of several inactivated X-linked loci (16, 17). This silencing process is incomplete on the inactive X chromosome as about 15% of the genes on the inactive X chromosome are expressed and designated 'escapees' (18, 19). XIST expression is subjected to a strict transcriptional control through DNA methylation. Hypomethylation of XIST leads to the overexpression of XIST and inappropriate inactivation of X-linked genes (20).

X chromosomal genes can be classified into different categories depending on the mechanism of transcriptional regulation. These are represented by the following genes in this study.

Phosphoglycerate kinase 1 (PGK1) belongs to the genes subjected to X inactivation, and its status is thus directly affected by XIST expression. It is silenced by DNA methylation on the inactivated X chromosome and is expressed from the active X chromosome, and can serve as an indicator of X chromosome inactivation (21, 22).

Short stature homeobox (SHOX) is regulated in a developmental manner and is of particular importance for the phenotypic appearance of women with 45,X Turner syndrome; its expression is essential in growth plates and is in tight correlation with body height (23). It is located in the pseudoautosomal region of the gonosomes and escapes X inactivation on the inactive X chromosome.

A third gene on the X chromosome, ferritin, heavy peptide-like 17 (FTHL17), has been shown to be expressed solely in spermatogonia and silenced in somatic cells (24, 25). Hence, it represents those X-linked genes that display cell-type-specific expression.

The aim of this study was to delineate the epigenetic status of the X chromosome in XX-males in comparison with Klinefelter patients and women with Turner syndrome to analyse the impact of the sex chromosomal constitution on X inactivation. It utilises representative X chromosomal loci to depict the impact of different sex chromosomal constitutions on differential transcriptional regulation by DNA methylation. Moreover, it aims to gain new useful information about the epigenetic nature of the X chromosomes in XX-maleness.

Subjects, materials and methods

Patients

Ten hitherto untreated (SRY+) 46,XX-male patients and ten 47,XXY Klinefelter patients were chosen for epigenetic characterisation from the cohort that was genetically, endocrinologically and clinically described in detail by Vorona et al. (2007). Patients’ ages ranged from 14 to 46 for XX-males and from 20 to 42 for Klinefelter patients.

The male control group consisted of ten men who served as semen donors in the Centre of Reproductive Medicine and Andrology. They had no chromosomal aberrations, and semen analysis proved these men to be normozoospermic.

The female control group consisted of ten women with normal ovarian function as determined by repeated hormone assessments and ultrasonographic demonstration of regular ovulation. They represented the proven healthy female partners in couples seeking advice for infertility, with husbands presenting with decreased sperm parameters.

All patients and volunteers provided written informed consent, and agreed to the assessment of genetic material as approved by the Ethics Committee of the University Clinics Münster and the State Medical Board.

Genetic and cytogenetic characterisation

Cytogenetic analysis was performed on lymphocytes as described by Vermeulen et al. (1999) (26). For (SRY+) and (SRY−) 46,XX males and 47,XXY Klinefelter patients, 30 metaphase cells were assessed by fluorescence in situ hybridisation (FISH) analysis using a SRY probe (Vysis, Hoofddorp, The Netherlands).

DNA isolation

Genomic DNA was extracted from peripheral lymphocytes from 1 ml EDTA blood using the FlexiGene Kit (Qiagen) according to the manufacturer’s instructions.

Bisulphite conversion

Two micrograms of genomic DNA in a volume of 50 μl were incubated with 5.5 μl 3 M NaOH for 15 min at 37 °C. After denaturation at 95 °C for 2 min, DNA was placed on ice. Five hundred microlitres of bisulphite solution (5 M sodium bisulphite and 100 mM hydroquinone (Sigma–Aldrich)) were added, and the reaction mixture was incubated at 55 °C for 16 h. Converted DNA was desalted using the DNA cleanup Wizard.
(Promega) following the standard instructions. To the desalted DNA, 5.5 μl of 3 M NaOH were added, and it was incubated for 15 min followed by ethanol precipitation; bisulphite-treated DNA was resuspended in 20 μl aqua dest.

**Amplification of differentially methylated regions**

Regions of interest were amplified from 4 μl bisulphite DNA. PCR mixture (25 μl) contained 1× reaction buffer (Qiagen), 1.5 mM MgCl₂, 100 μM of each dNTP (Promega), primers in the concentration given below (Eurofins MWG, Ebersberg, Germany) and 1.5 U Taq Polymerase (Qiagen, 5 U/μl). PCR conditions were 3 min at 95 °C followed by 40 cycles of 94 °C; 30 s, primer annealing (see below for temperature: 30 s), 72 °C, 30 s and a final extension at 72 °C for 10 min.

PCR products were purified using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer’s manual.

**Identification of potentially methylated regions**

The sequence of the first exons and the region 1000 bp upstream of the desired genes were retrieved from Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez) and for the identification of CpG islands, the online program MethPrimer (Li & Dahiya 2002 (27); http://www.urogenew.org/methprimer/) was used. This tool yields the location of possible CpG islands, the online program MethPrimer (Li & Dahiya 2002(27); http://www.urogenew.org/methprimer/) was used. This tool yields the location of possible CpG islands. Minimum requirements for a CpG island were a ratio of observed/expected CpGs and a percentage of CG > 0.6 and a percentage of CG > 50%. Primers were picked around CpG-rich areas. Primer sequences for XIST were adapted from Song et al. (2007) (28).

**Primer sequences (5′–3′) and PCR conditions**

- **XIST_fwd:** GTAGAGAATGATTTTGTAGTTAAGT-TAAGG
  - Location: Xq13.2 (M97168)
  - Product size: 133 bp; annealing temperature: 60 °C (−0.1 °C/cycle)
  - **FTHL17_fwd:** TTTTTGTTATTTTAGTTGTTT
  - Location: Xp22.33 and Yp11.32 (U82668)
  - Product size: 285 bp; annealing temperature: 59 °C
- **SHOX_fwd:** GGGATTTTTTGTAGTTTGGTGT
  - Location: Xq13.3 (L00159)
  - Product size: 318 bp; annealing temperature: 60 °C.

**Methylation analysis by bisulphite sequencing**

PCR products were cloned into pGEM T vector (Promega) and competent *Escherichia coli* were heat-shock transformed with the resulting plasmids. The plasmids of at least ten clones were isolated and sequenced for methylation analysis.

**Methylation analysis by dye-terminator sequencing**

The extent of methylation was determined by direct sequencing of PCR fragments by Qiagen GmbH. All sequencing reaction mixes were based on the BigDye 3.1 Terminator chemistry (Applied Biosystems, Carlsbad, CA, USA). Template amounts of 5 ng per 100 bases of fragment length and 10 pmol primer per reaction were used. Reaction mixtures were cycled in a GeneAmp PCR System 9700 (Applied Biosystems) and purified using DyeEx (Qiagen). Data collection was carried out on a 3730×1 DNA Analyser (Applied Biosystems) equipped with 50 cm capillary arrays and POP-7 polymer.

After data collection, the raw data channels from the generated result files (.ab1) were processed using custom-built software developed at Qiagen. Data processing involved the compensation of the different migration properties of the four dyes, baseline correction, peak detection and base calling. For each base, the area was calculated from the corresponding peak to measure the base’s quantity. Bases that correspond to variable positions of CpG sites were identified by comparison of the obtained sequence with a reference sequence. The ratios of methylated versus unmethylated species (C/T in forward reads and G/A in reverse reads) were reported.

**Data analysis and plotting**

For bisulphite sequencing data, the ratio of methylated sites to unmethylated sites in all sequenced clones yielded the mean degree of methylation of a given region. For dye-terminator sequencing, the mean of all analysed sites yielded the mean degree of methylation.

For visual presentation of the data and for statistical analysis, GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used.

**Results**

**Severe hypomethylation of XIST in SRY(+) XX-males**

We determined the DNA methylation status of XIST in (SRY+) 46,XX-males to assess the impact of their sex chromosomal constitution on the epigenetic state of the X chromosome. We therefore analysed a 133 bp fragment (from +871 to +1003; according to Song
et al. 2007 (28)) containing five potential methylation sites (Fig. 1a) in ten SRY(+)- XX-males compared to 47,XXY KS patients and ten female controls.

A single CpG site methylation analysis revealed that KS patients and female controls displayed highly similar degrees of XIST methylation (Fig. 2a). The mean methylation per CpG site in KS patients was between 42 and 47%, which is comparable to female controls who displayed a XIST methylation of 46–48%; these values correlate with the presence of two X chromosomes. The degree of methylation was highly consistent over the five analysed CpG sites of XIST. The distribution of the overall mean XIST methylation per individual showed the same heterogeneous pattern in KS men and female controls, ranging from 20 to 70% in both groups (Fig. 2c).

In contrast, a strong deviation from the expected XIST methylation was observed in (SRY+) XX-males. They displayed XIST methylation from 16.5 to 19.3% and are thus severely hypomethylated compared to KS patients and female controls (P<0.001). On an individual basis they showed considerable variation of XIST methylation ranging from a complete unmethylation in one patient to 45% methylation in another, similar to 46,XX female controls and 47,XXY KS men (Fig. 2c).

We aimed to assess the influence of the Y chromosomal material that SRY(+) XX-males carry as translocation to one of their X chromosomes, and to further characterise the impact of different sex chromosomal constitutions on XIST methylation. We therefore compared XIST methylation of SRY(+) XX-males to that of SRY(−) XX-males, 46,XY male controls and 45,X Turner patients. SRY(−) XX males display XIST methylation of 45–50%, depending on the CpG site; this represents a significantly elevated degree of XIST methylation compared to SRY(+) XX-males (P<0.001) and is rather comparable to female controls.

Normal males have, on average, a high degree of XIST methylation ranging from 88 to 96% depending on the CpG site (Fig. 2a) and they showed a very homogeneous high degree of XIST methylation (Fig. 2b), similar to 45,X women with highly methylated XIST levels of 97–99% (Fig. 2b).

**DNA methylation state of representative X chromosomal genes**

We next addressed the question of whether sex chromosomal anomalies, in particular (SRY+) XX-maleness associated with XIST hypomethylation, result...
in an abnormal epigenetic state of X chromosomal loci. Hence, we analysed three representative X chromosomal genes, PGK1, FTHL17 and SHOX, for the presence of CpG islands using MethPrimer (27) and designed suitable PCR primers.

PGK1 was chosen as an indicator of X chromosome inactivation harbouring a CpG island in its promoter. We analysed a 318 bp fragment from −338 to −21 relative to the transcription start site (Fig. 1b) comprising 38 CpG methylation sites in (SRY +) XX-males, KS males and 45, X women controlled by healthy men and women. (SRY +) XX males and KS men displayed a non-significant (P > 0.05) reduction of PGK1 methylation to 51 and 56% respectively (Fig. 3a) compared to female controls who had 69% PGK1 methylation. PGK1 methylation was 3.4% in male controls; this value was significantly lower compared to that in female controls (P < 0.001), but comparable to PGK1 methylation in 45,X Turner women (0.4%).

FTHL17 represents X-linked genes that do not undergo X inactivation, but are regulated in a cell-type-specific manner. We hypothesised that FTHL17 might be a suitable candidate to indicate whether this differential regulation is impaired by the abnormal sex chromosomal constitution in SRY(+) XX-males and other sex chromosomal aberration.

Analysis of the coding sequence of FTHL17 revealed a predicted CpG− island in the first exon (Fig. 1c) and a 285 bp fragment from +24 to +308 relative to the transcription start site comprising 24 CpG methylation sites was chosen for subsequent analysis. Male and female controls showed a high degree of FTHL17 methylation of 91 and 90% respectively, indicative of a high inactivation status (Fig. 3b). No difference in FTHL17 methylation was found in (SRY +) XX-males (89%), KS men (87%) or 45, X women (93%).

SHOX is located in the pseudoautosomal region of the X and escapes X inactivation; it therefore represents the so-called escapees. We analysed a 321 bp fragment from −375 to −55 relative to the transcription start site of SHOX comprising 28 CpG sites (Fig. 1d). Female and male controls showed a low degree of SHOX methylation with 9 and 7% respectively (Fig. 3c). (SRY +) XX-males displayed, on average, a similar degree of SHOX methylation as male controls and KS men (7%), while in 45, X women a comparably low degree of methylation was detected (4.8%).

Discussion

X chromosome inactivation represents an essential mechanism to balance the X chromosomal gene dosage between the sexes. Sex chromosomal aberrations, however, constitute a major threat to this finely tuned process. We present the first descriptive and comparative study of the DNA methylation status of the X chromosome in XX-maleness, KS and Turner syndrome.

The study of general molecular features of XX-maleness is always limited by the naturally small study cohort due to the extreme rarity of this syndrome. It is especially difficult to get hold of samples from (SRY −) XX-males as only about 10% of 46,XX-males lack a translocation of Y chromosomal material to one of their X chromosomes (2, 29, 30). However, our cohort of ten (SRY +) XX-males represents one of the largest cohorts ever reported (31). We could clearly demonstrate that hypomethylation of XIST is a common feature of these men with a significant reduction of XIST methylation from about 50 to 20% on average when compared to female controls and KS men.

Despite this epigenetic aberration, three relevant X-linked genes representing X inactivation (PGK1), escape from X inactivation (SHOX) and cell-type-specific inactivation (FTHL17) were normally methylated at their CpG islands when compared to those in female controls and Klinefelter patients, indicating comparably normal X inactivation.

As XIST RNA expression is coupled to its DNA methylation status (20), it would obviously be a vital investigation to compare actual XIST expression in XX-males to that in KS men and female controls. This would yield the information whether XIST methylation still dictates XIST expression in (SRY +) XX-males. However, this study has been performed retrospectively and owing to the poor availability of a sufficient number of fresh blood samples from XX-males it is not feasible at the moment to back-up our epigenetic findings with expression data.
It has been described earlier that XIST expression becomes dispensable for X inactivation once it is established (32). Silencing of the inactive X chromosome is then overtaken by histone modifications, such as extensive H3K27 methylation that is conferred by the Polycomb repressive complex 2 (33). However, if this would confer relaxed stringency to the DNA methylation status of XIST, female controls and KS men could as well display severely changed XIST methylation levels. As this is not the case, other hitherto unknown factors must account for the massive hypomethylation of XIST that remain elusive. One important finding in our study is that the two (SRY−) XX-males displayed female-like 50% XIST methylation. This finding assigns a crucial role to the Y–X translocation in (SRY+) XX-males in the aetiology of severe XIST hypomethylation.

As proposed and indicated earlier (34), KS men in this study undergo appropriate X chromosome inactivation. The striking epigenetic similarities of the X chromosomes of KS patients and women in terms of methylation status and variation suggest that insufficiency and apoptosis of Leydig cell in KS men (7) could be due to the ‘female’ expression pattern in these cells that could compromise their function. Although no functional proof of escapee transcripts can be given here, the epigenetic analysis suggests that KS men, like women, express genes from the second X that escapes X inactivation. We thus strengthen the hypothesis that these ‘escapees’, which are present in women and KS men, but not in normal men, are major contributors to the phenotypes in KS (35).

Our data support the concept of inter-individual variation of X inactivation. Earlier studies demonstrated that X-inactivated genes show substantial variation of expression between different women accompanied by variable retention of epigenetic silencing mechanisms (36). Earlier findings that females show considerable heterogeneity in levels of X-linked expression (19) have recently been backed up by the report that both X-inactivated genes and escapees show significant variation of expression (37). The wide variation of XIST methylation in all XX-carrying patients and controls in this study reflects the general heterogeneity of X-linked expression in individuals with two X chromosomes.

A number of characteristics that distinguish (SRY+) XX-males from KS men have been described before. These include anthropomorphic features such as reduced body height and an increased incidence of maldescended testis, elevated LH levels, significantly reduced creatinine levels and extremely skewed inactivation of the androgen receptor located on the X chromosome (3). Karyotypically, the difference between these syndromes is that KS men possess a complete Y chromosome, whereas (SRY+) XX-males only carry an SRY-containing fragment translocated to one of their X chromosomes (38). This partially explains why the latter are exclusively infertile due to the absence of male fertility-related regions on the Y chromosome, including AZF (39), whereas the former sometimes display residual active foci of spermatogenesis (7, 8). The severe XIST hypomethylation of (SRY+) XX-males indicates that there might be more hitherto unidentified epigenetic differences.

In summary, we provide strong evidence that XIST hypomethylation in (SRY+) XX-males is a substantial epigenetic hallmark of this chromosomal anomaly. It associates the anthropomorphic and biochemical distinction of (SRY+) XX-maleness from KS with a characteristic epigenetic pattern. Both Klinefelter patients and (SRY+) XX-males share the characteristic pattern of hypergonadotrophism indicating a severely impaired endocrine feedback regulation and an in general normal inactivation of X-linked genes. The fact that presumably only XIST methylation distinguishes both entities points to a hitherto unknown impact of the Y chromosome on X inactivation. Thus, future studies will have to elucidate the impact of the X–Y-chromosomal crosstalk on the endocrine status and phenotypic characteristics in correlation to the sex chromosomal constitution.

Declaration of interest

The authors certify that they have no affiliation with or financial involvement in any organisation or entity with a direct financial interest in the subject matter or materials discussed in the manuscript. Any research support is identified.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft DFG GR 1547/8-1.

Acknowledgements

We thank Nicole Terwort for excellent technical assistance. Michael Zitzmann, Elena Vorona and Andreas Schu¨ ring are thanked for clinical characterisation of patients.

References

1 Huang WJ & Yen PH. Genetics of spermatogenic failure. Sexual Development: Genetics, Molecular Biology, Evolution, Endocrinology, Embryology, and Pathology of Sex Determination and Differentiation 2008 2 251–259.

www.eje-online.org