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Sex chromosome Aneuploides among Men with Systemic Lupus Erythematosus

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Abstract

About 90% of patients with systemic lupus erythematosus (SLE) are female. We hypothesize that the number of X chromosomes, not sex, is a determinate of risk of SLE. Number of X chromosomes was determined by single nucleotide typing and then confirmed by karyotype or fluorescent in situ hybridization in a large group of men with SLE. Presence of an *sry* gene was assessed by rtPCR. We calculated 96% confidence intervals using the Adjusted Wald method, and used Bayes' theorem to estimate the prevalence of SLE among 47,XXY and 46,XX men. Among 316 men with SLE, 7 had 47,XXY and 1 had 46,XX. The rate of Klinefelter's syndrome (47,XXY) was statistically different from that found in control men and from the known prevalence in the population. The 46,XX man had an *sry* gene, which encodes the testes determining factor, on an X chromosome as a result of an abnormal crossover during meiosis. In the case of 46,XX, 1 of 316 was statistically different from the known population prevalence of 1 in 20,000 live male births. A previously reported 46,XX man with SLE had a different molecular mechanism in which there were no common gene copy number abnormalities with our patient. Thus, men with SLE are enriched for conditions with additional X chromosomes. Especially since 46,XX men are generally normal males, except for infertility, these data suggest the number of X chromosomes, not phenotypic sex, is responsible for the sex bias of SLE.

Keywords

Systemic lupus erythematosus; Klinefelter's syndrome; male 46; XX; female bias; X chromosome

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1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, relapsing inflammatory disease that can affect virtually any tissue or organ (1). The disease is considered autoimmune on the basis of circulating antibodies that bind self-structures, classically ribonucleoprotein particles (2), as well as lymphocytic infiltrates in affected tissue. SLE is not rare among women, occurring in about 1 in 1000 women of European descent and in up to 4 times as many women of African origin (3). In contrast, men have SLE much less commonly than women, with men constituting only about 10% of patients (3).

The pathophysiological mechanism by which women are preferentially affected by SLE is not known. While less common among men than women, SLE is generally more severe when found in men (4). However, when studied in families with at least two SLE patients, women with SLE had worse disease if they had a male relative with SLE, compared to those SLE without an affected man in their family (5). Several hypotheses have been generated as an explanation of the sex-bias found in SLE, including sex hormone differences (6), acquired X monosomy (7), maternal-fetal chimerism (8), skewed X inactivation (9) and female mosaicism (10), but no compelling data supports any of these hypotheses (4). In particular, when studied at the onset of disease (11) or when compared to patients with a non-sex-biased chronic illness (12), there are no abnormalities of sex hormones in SLE patients, be they men or women.

We have hypothesized that the number of X chromosomes, not phenotypic sex, is responsible for the sex-bias of SLE. Animal data in sex-reversed mice support this idea (13). Our previous data demonstrated that men with SLE are enriched for 47,XXY karyotype (Klinefelter's syndrome) such that 47,XXY men have the same risk of SLE as women (14). We undertook the present study to comprehensively describe the X chromosome aneuploidies among a very large cohort of SLE men.

2. Methods

2.1. Patients

All patients were enrolled in the Lupus Family Repository and Registry (LFRR). The details of LFRR recruitment, consent, evaluation, biobanking and informatics have been previously reported (15,16). Briefly, all patients met the 1997 revised American College of Rheumatology classification criteria for systemic lupus (1,17). Subjects completed an extensive questionnaire, were interviewed and had medical records reviewed in order to establish the presence or absence of these criteria as well as other clinical information. Blood was collected with storage of serum, plasma, DNA, RNA, and peripheral blood mononuclear cells. In total, 316 men with SLE were included in the present study.

2.2. SNP genotyping

All subjects were screened for chromosome X abnormalities using single nucleotide polymorphism (SNP) typing with 300,000 SNP CytoSNP, 200,000 SNP Immunochip (18) or 1,200,000 SNP OMNI-1(Quad; Illumina, San Diego, CA). Copy number variations were found by visually inspecting a B allele plot of each subject's X chromosome for abnormalities using Beadstudio software (Illumina, San Diego, CA). In the B allele plot, fluorescence intensity of the B allele of each SNP is plotted over the total fluorescence for that SNP in a given individual. Results are normalized to a percentage scale. Therefore, at a SNP with BB homozygosity the result is 100%, with AA homozygosity the result is 0% and with AB heterozygosity the result is 50%. Klinefelter's syndrome (47,XXY; Figure 1b) was

identified by the heterozygous "three-band" pattern in the B allele frequency plot, which corresponds to the 0%, 50%, and 100% SNP frequencies (i.e. AA, AB, BB at any given SNP). Normal male subjects (46,XY; Fig. 1A) were identified by the homozygous "two band" pattern in the B allele frequency plot, which corresponds to the 0% and 100% possible SNP frequencies for a single chromosome X (i.e. AA, BB). Presence of a Y chromosome was shown by examination of the B allele plot. In our hands, these SNP arrays were 100% specific and 100% sensitive for detecting chromosome X monosomies, trisomies, and mosaics when compared to FISH on 277 normal subjects and 10 abnormal subjects, who were previously characterized by karyotyping (not shown).

2.3. Fluorescence *in situ* hybridization (FISH)

After SNP typing, the abnormal SLE and control samples were validated by FISH, as derived from Klingler et al. (19) and Henegariu et al. (20), which are procedures specifically designed for minuscule samples and high throughput. Cryopreserved Epstein Barr virus transformed peripheral blood mononuclear cells were used. Interphase nuclei were swollen by the addition of 20µl of water to 5µl of cell suspension in a PCR tube and incubating at 37°C for 20 min in a thermocycler. 25µl of fixative (56% acetic acid, 44% ethanol) was added to the PCR tube and gently mixed with a pipette. All 50µl of suspension was pipetted onto a silane coated slide and allowed to air dry for 45 min at room temperature. DNA was dehydrated via ethanol series and then dried on a warm surface (~60°C) for 5 min.

Commercial FISH probes for the centromeres of chromosomes X and Y were used to confirm aneuploidies of chromosome X (alpha-satellite repeats DXZ1 & DYZ3, PID# KI-20030, Veridex, LLC, Raritan, NJ, USA). Probes were diluted in hybridization buffer (50% formamide, 2 x SSC, 10% dextran sulfate (w/v), 50 mM sodium phosphate pH 7) and 9 µl was used under an 18x18mm coverslip sealed with rubber cement. Probes and DNA were co-denatured on a hot plate at 75–80°C for 5 min. Hybridization was performed at 37°C for 2 hours. Slides were washed three times for 5 min each in 50% formamide 2 x SSC at 37°C and then three times in 0.4 x SSC at ~70–72°C. DNA was counterstained with a drop of DAPI (200ng/ml in 2 x SSC) for 5 min and then briefly rinsed with water. After drying, slides were mounted in antifade solution (2.33% DABCO, 20mM Tris pH 8, 90% glycerol (v/v)) and viewed under a Zeiss Axiovert fluorescent microscope using the appropriate filters. Fifty nuclei were scored for each abnormal subject.

2.4. Karyotype

Karyotype was performed in the Clinical Cytology Laboratory of OU Medical Center by standard techniques, as we have previously reported (14).

2.5. Sry Gene PCR

Presence of the *sry* gene was determined via real time polymerase chain reaction (RT-PCR) as described by Ottesen *et al* (21). In brief, the sample had reference genes on chromosome Y (*sry*), chromosome 12 (glyceraldehyde 3-phosphate dehydrogenase or GAPDH), and chromosome X (androgen receptor) amplified by RT-PCR. Due to the exponential nature of PCR, the number of X chromosomes and Y chromosomes in the sample could be calculated from the difference in number of PCR cycles, between each reference gene, needed to reach a threshold amount.

2.6. Statistical analysis

Chi square analysis was used to determine if there was a statistically significant difference in the prevalence of each genotype between the patients and controls. An alpha value of 0.05 was considered significant. For odds ratio calculations, zeros were replaced with a value of

1. Confidence intervals of ratios were calculated using the Adjusted Wald method. The prevalence of SLE in Klinefelter's syndrome (47,XXY) and 46,XX males was estimated using Bayes' theorem.

3. Results

3.1. We studied a total of 316 men with SLE

Three hundred-eight of these men had one X chromosome and one Y chromosome based on the B allele plots of these chromosomes (see Figure 1 for examples of B plots). Seven of the SLE men had a B allele plot consistent with 47,XXY (Figure 1B and Table 1). Each of these men was conformed to have Klinefelter's syndrome by either karyotype, FISH or both (Figure 1B). Three of them had non-mosaic 47,XXY, while three had 46,XY/47,XXY mosaicism and one was a 46,XX/47,XXY mosaic. Among 1201 unrelated, control men without SLE, we did not find a single Klinefelter's syndrome ($\chi^2=27.1$, $p<0.00001$, odds ratio=27.2 with 95% CI 3.3–221.8).

The prevalence of 7 of 316 (2.2%) is 13-fold higher than the known population prevalence of Klinefelter's syndrome of 0.0017%, or 17 per 10,000 live male births (22–24). The 95% confidence intervals of 0.0098 to 0.046 (or 98 to 900 per 10,000) for 10/302 do not cross the population rate of Klinefelter's syndrome. Using the known population rate of SLE and Klinefelter's syndrome along with rate of Klinefelter's syndrome among SLE men found in the present study, we employed Bayes' theorem to estimate the rate at which Klinefelter's syndrome men have SLE. This calculation estimates that 1 in 767 of these men will have SLE, a figure similar to the population rate of SLE among women in the USA (4).

3.2. One man among the 316 with SLE had evidence of two X chromosomes but no Y chromosome in the B allele plot

Both FISH and karyotype confirmed this man was 46,XX (Figure 1C). We confirmed that an *sry* gene, which encodes the testes determining factor and is normally located on the Y chromosome, was present by rtPCR (Figure 2). There was no 46,XX man among the 1201 controls. 46,XX men are the product of an abnormal translocation event during X-Y crossover in paternal gametes that results in transfer of the *sry* gene to an X chromosome. Examination of the X chromosome B plot of this patient showed a deleted region centromeric to pseudoautosomal region 1 (see Figure 1C), indicating 46,XX was produced from an equal crossover between X and Y during meiosis in which the breakpoint was centromeric to both the X and Y pseudoautosomal region 1 on the p arm. Such a crossover results in Y chromosomal sequence that includes the *sry* gene on the X chromosome and X chromosomal sequence on the Y chromosome. Thus, the X chromosome formed in this manner has an *sry* gene and loss of sex-linked X genes that are telomeric to the crossover (25,26).

Binomial confidence interval calculated from the 1 in 316 prevalence of 46,XX among the SLE men (0.0001–0.183, or 1 in 10,000 to 183 in 10,000) do not include the estimated prevalence of 46,XX males, which is approximately 1 in 20,000 live male births (27). When applying Bayes' theorem to the one 46,XX man among the 302 SLE men, we estimate that 1 in 158 46,XX men have SLE. Of course, because the calculations are based on a single 46,XX man, the margin of error is wide.

4. Discussion

4.1. Perhaps as many as 80 human diseases are categorized as having an autoimmune pathogenesis (28)

The principle evidence of autoimmunity is circulating antibodies to self and immune attack against involved organs and tissues. In general, these diseases have a genetic component that is complex, involving genetic contribution from many genes (29), but genetics does not account for the entire risk of disease, as evidenced by twin studies in which the rate of concordance for autoimmune is generally less than 50% (30). While environmental exposure is no doubt important, specific etiological agents have not commonly been identified for autoimmune diseases.

4.2. Almost all autoimmune diseases are more common among women than men. SLE is no exception with about 10-times more women affected than men (4)

However, men have more severe disease than women (4), including increased rate of death in population-based epidemiological studies (31). There are a number of theories as to why women are more affected with SLE, or autoimmunity in general (32). First and foremost, sex hormone differences between males and females have been proposed and studied as the factor underlying the sex-bias of SLE (reviewed in 6). However, while there are clear differences between androgen and estrogen levels when comparing SLE patients to controls, Mackworth-Young and colleagues found differences when comparing SLE patients to subjects with other chronic illnesses in which there is no sex-bias (12). Further, when studied at the onset of disease prior to therapy, no differences in sex hormone levels were found when comparing SLE men to healthy men (11). Other mechanisms for sex-bias have been studied in SLE and found to not be present. These include skewed X inactivation (8), acquired X monosomy in peripheral blood mononuclear cells (7), and maternal-fetal chimerism (9). Others have proposed that inappropriate reactivation of X chromosome genes with resultant changes in the ratios of intracellular protein concentration could lead to autoimmunity (33), but we are unaware of data to support this interesting notion. Finally, Migeon has proposed that X inactivation in females could lead to autoimmunity by a breakdown in immune tolerance education. In this scenario for example, an immature T cell encounters thymic medullary epithelial cells with the same X chromosome inactivated. Then in the periphery when this T cell encounters a cell with the other X chromosome inactivated, autoimmunity occurs (10,34). We interpret this idea as predicting autoimmunity will be directed towards antigens whose genes are on the X chromosome. This is not the case in SLE, or any other autoimmune disease.

4.3. We have proposed that the number of X chromosomes predisposes to SLE (14)

This hypothesis is compatible with a specific immune system gene on the X chromosome that is over-expressed based on the number of X chromosomes, and with the notion proposed about reactivation of X chromosome genes that are not related to the immune system (33). The data presented herein for both 47,XXY and 46,XX men continue to support the X chromosome dose effect. We previously reported that 3 of 112 men with SLE enrolled in the LFRR also had Klinefelter's syndrome (14). The present data have enlarged this cohort to 316 SLE men in which an additional 4 have Klinefelter's syndrome. Thus, the prevalence of 47,XXY among SLE men has remained similar in the 200 patients identified since our original report (14).

4.4. One SLE man in our cohort has a 46,XX karyotype

This rare abnormality was first reported by de la Chapelle in 1964 (35). Based on large cytogenetic surveys, the estimated frequency is only 1 in 20,000 to 25,000 live male births

with four 46,XX karyotypes found among 96,121 live males births when combining results from several studies (27). When an *sry* gene is present, as in our patient, these men are generally fully masculinized, except undescended testes are common, with normal sex hormones, but virtually always infertile (36,37). Thus, enrichment of 46,XX men among those with SLE implies that sex hormones are not the basis of the sex-bias in this disease.

Sex reversal in 46,XX males is generally caused by abnormal genetic exchange between X and Y chromosomes during meiosis of male gametes in which Y chromosomal material containing the *sry* gene, which encodes the testes determining factor, is transferred to an X chromosome. The *sry* gene lies in close proximity to pseudoautosomal region 1 (PAR1) on the p arm of the Y chromosome. Very rarely 46,XX males have the *sry* gene on an autosomal chromosome (38,39). Our patient had a balanced crossover event in which the breakpoint was centromeric to the pseudoautosomal region and in the same relative position on both the X and Y chromosomes. Such crossovers generally occur in regions of sequence homology between the X and Y chromosomes, especially at sites of an X chromosome gene and a Y chromosome homologous pseudogene (40). Such a crossover event results in an X chromosome with an intact PAR1, deleted X sequence (and therefore genes) just centromeric to PAR1, and non-recombining Y chromosome sequence containing the *sry* gene. We have diagrammatically demonstrated the abnormal X chromosome in our patient along with the deleted genes (Figure 3).

One other SLE patient with male 46,XX has been reported (41). This was a boy who presented at only 5 years of age with SLE. Analysis of his abnormal chromosome revealed an unbalanced, or unequal, crossover event. In this case, during meiosis the breakpoint on the X chromosome is within PAR1 as is normal, but the breakpoint on the Y chromosome is centromeric to PAR1 as well as to the *sry* gene. Thus, the X chromosome formed by such a crossover has an intact PAR1 derived from the Y chromosome involved in the exchange, non-recombining Y chromosome sequence containing the *sry* gene, and a partial PAR1 that was derived from the X chromosome involved in the exchange and was centromeric to the breakpoint. A 46,XX man with unequal crossover; therefore, has an abnormal X chromosome with a part of PAR1 triplicated and Y sequence containing the *sry* gene. A diagram of the abnormal X chromosome from this 46,XX boy with SLE is shown in Figure 3. Thus, the two 46,XX males with SLE have different mechanisms that result in distinct deletions and triplications on their respective abnormal X chromosomes. Therefore, we conclude that none of the genes with abnormal copy number in these two patients are involved in the fundamental mechanism by which an X chromosome dose effect leads to a higher risk of SLE. That is, other genes on the X chromosome must give rise to the additional SLE risk found in individuals with two X chromosomes compared to one. Another 46,XX(*sry*+) man with diffuse scleroderma has been reported, but the mechanism of the abnormal crossover is not given in this paper (42).

4.5. We have assembled the largest group of men with SLE ever collected

This large number of men allows us to determine the presence of unusual and rare X chromosome aneuploidies such as Klinefelter's syndrome (47,XXY) and de la Chapelle's syndrome (46,XX male). The latter men have normal sex hormones. The enrichment of these men with two X chromosomes among men with SLE supports the hypothesis that two X chromosomes imparts risk of SLE compared to the presence of only one X chromosome.

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Research Highlights

- Klinefelter's syndrome (male 47,XXY) is associated with SLE
- Klinefelter's syndrome men have the same rate of SLE as women
- 46,XX men (de la Chapelle's syndrome) are over-represented among men with SLE
- 46,XX men have normal levels of sex hormones
- The sex bias of SLE is associated with the number of X chromosomes, not sex

Figure 1.

B plots of the X chromosome in a 46,XY man (A), a 47,XXY man (B) and a 46,XX man (C). In these plots, the fluorescence of the B allele is plotted over the total (A+B allele) fluorescence at all SNPs along the X chromosome. Thus, at any given SNP, with homozygous BB genotype the y-axis value is 1.0, with homozygous AA genotype the y-axis value is 0.0, and with heterozygous AB genotype the y-axis value is 0.5. Thus, 47,XXY (B) and 46,XX (C) men show numerous SNPs with a 0.5 value indicating the presence of two X chromosomes. At about base position 90 mb the 46,XY shows apparent heterozygosity. In fact, these are not true SNPs, instead being homologous sequence between this point of X and the distal p arm of Y at which there are a single nucleotide differences between the two sex chromosomes. Thus, 46,XY have a heterozygous pattern on the B allele plots. Klinefelter's syndrome men have a triplicated pattern here (0.0, 0.33, 0.67, and 1.0 B allele frequencies). The 46,XX man also has the triplicated pattern at this point on the X chromosome because a portion of the distal Y chromosome p arm is present. At the far right is sex chromosome FISH of these same individuals.

Figure 2.

The androgen receptor gene (AR) on the X chromosome (labeled Chr. X), the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on chromosome 12 (labeled Chr. 12) and the *sry* gene on the Y chromosome (labeled Chr. Y) were expanded by rtPCR as previously reported (21). Results are shown for the 46,XX man with SLE. A 1:1 ratio of AR:GAPDH throughout the PCR reaction indicates the presence of an equal number of copies of these two genes, that is, two X chromosomes and two chromosomes 12. Expansion of *sry* indicates the presence of this gene, which encodes the testes determining factor. This experiment was repeated three times with similar results each time.

Figure 3.

Diagram of a normal X chromosome (A) as well as the abnormal X chromosome found in the 46,XX man with SLE reported in this report (B) and the previously reported (41)46,XX male with SLE (C).

Table 1

X chromosome aneuploidies found among men with systemic lupus erythematosus (SLE).

	SLE (n=316)	Controls (n=1201)
46,XY	308	1201
47,XXY	3	0
46,XY/47,XXY	3	0
46,XX/47,XXY	1	0
46,XX	1	0

$\chi^2=27.1$, $p<0.00001$, odds ratio=27.2 (95%CI 3.3–221.8) comparing Klinefelter's syndrome (non-mosaic 47,XXY plus mosaic 46,XY/47,XXY and 46,XX/47,XXY) in patients versus