Hum Genet (2005)
DOI 10.1007/s00439-005-0119-4

ORIGINAL INVESTIGATION

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Extreme skewing of X chromosome inactivation in mothers of homosexual men

Received: 6 September 2005 / Accepted: 1 December 2005

Abstract Human sexual preference is a sexually dimorphic trait with a substantial genetic component. Linkage of male sexual orientation to markers on the X chromosome has been reported in some families. Here, we measured X chromosome inactivation ratios in 97 mothers of homosexual men and 103 age-matched control women without gay sons. The number of women with extreme skewing of X-inactivation was significantly higher in mothers of gay men (13/97 = 13%) compared to controls (4/103 = 4%) and increased in mothers with two or more gay sons (10/44 = 23%). Our findings support a role for the X chromosome in regulating sexual orientation in a subgroup of gay men.

Introduction

Variation in human sexual preference has a substantial genetic component, and a maternal bias to the inheritance pattern in males was found in some (Green and Keverne 2000; Hamer et al. 1993; Turner 1995) but not all (Bailey et al. 1999) studies. From the earliest work on the genetic basis of sexual orientation, the X chromosome was studied as a possible location for genes influencing this trait. The X chromosome has accumulated genes that influence sex, reproduction and cognition (Graves et al. 2002). Linkage to markers on the X chromosome implied a role for X chromosome genes in sexual orientation in some families (Hamer 1999).

Because male cells contain only one X chromosome and female cells contain two, each cell in a female embryo randomly inactivates one X chromosome early in development, thus creating dosage compensation. Since the choice of which X chromosome to inactivate is made randomly in most tissues, and the inactive chromosome remains inactive in all daughter cells resulting from cell divisions, a tissue sample of a female typically contains cells that have one X chromosome inactivated and cells that have the other inactivated (Brown and Robinson 2000). A non-random pattern of inactivation could be caused by either a primary non-random inactivation in which one of the two X chromosomes is inactivated preferentially or by a random inactivation followed by a secondary selection for cells that inactivated one chromosome or the other, or by chance (Brown and Robinson 2000). Another form of non-random X chromosome selection can occur when only a small precursor cell population is present at the moment of X-inactivation (Sandovici et al. 2004).

Here, we show that the number of women with extreme skewing of X chromosome inactivation is significantly higher in mothers of homosexual men than in age-matched control women without gay sons.

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Materials and methods

Sample

The sample of 200 women consisted of 40 previously reported mothers of 2 or more gay sons, of whom 4 had 3 gay sons and one had 4 gay sons (Hamer et al. 1993; Hu et al. 1995); 57 newly recruited mothers of whom 4 had 2 gay sons and 53 had 1 gay son and 103 control

women who did not report having homosexual children (Sabol et al. 1999). No detailed sexual behavior or attitude data was collected on the children of control mothers. Participants signed an informed consent approved by the NCI IRB prior to donating blood for DNA extraction. Sexual orientation of the gay sons was assessed through the Kinsey scales of sexual attraction, fantasy, behavior and self-identification, which range from 0 for exclusively heterosexual to 6 for exclusively homosexual. The gay sons had an average score of 5.65 ± 0.48 (mean $\pm$ SD).

X-inactivation assay

DNA was prepared from white blood cells using the same protocol for all samples (Bioserve Biotechnologies, Laurel, MD, USA). The fraction of white blood cell DNA in which one or the other of the X chromosomes was inactivated was determined by polymerase chain reaction (PCR) amplification of a highly polymorphic CAG repeat in the androgen receptor (AR) locus. The DNA was digested with a control enzyme that does not cut the amplified fragment (*Rsa*I), to avoid that chromatin structures would lead to preferential amplification of the more accessible alleles. Similarly, the DNA was digested with *Rsa*I and a methylation-sensitive restriction enzyme (*Hpa*II) together (Allen et al. 1992), and both digests were PCR amplified. Fluorescently labeled products were quantified on an ABI 3100 automated sequencer (Applied Biosystems). The peak height was measured using Genotyper software (Applied Biosystems). The ratio of the peak heights of the two alleles after the control *Rsa*I digest was used as a correction factor for preferential amplification of one of the alleles. The ratio of the peak heights of the two alleles after the methylation-sensitive *Hpa*II digest was, after this correction, converted into the percentage of cells inactivating each of the alleles. The largest of the two percentage values was used. The assay was performed twice, and the mean was used in further analysis. A similar assay was performed at the fragile X (FMR1) locus (Carrel and Willard 1996), with 84 cases informative for either of the two assays and 116 women informative for both assays, in which case the values of the two assays were averaged. The results of the assays in women heterozygous for both loci were highly correlated (Pearson $r = 0.75$, $n = 143$, $P < 0.001$), and the means were not different (AR mean = 70.4, SE = 0.9; FMR mean = 69.9, SE = 1.1).

Results

To study X-inactivation ratios in women with gay sons, we determined the fraction of white blood cells inactivating one or the other X chromosome in 97 mothers of homosexual men and 103 control women, matched for ethnicity and age because of the effect of age on X-inactivation ratios (Sandovici et al. 2004). Using a methylation assay, we measured X-inactivation ratios at the androgen receptor locus (AR) and at the Fragile X mental retardation locus (FMR1). The results of both assays were averaged for further analysis.

A 90% cut-off value for X-inactivation ratios is widely used (Brown and Robinson 2000; Ozbalkan et al. 2005; Pegoraro et al. 1997) to define extreme skewing of X-inactivation. By this criterion, only 4% of the controls (4/103) displayed extreme skewing, while 13% of the mothers of gay men (13/97) did ($P = 0.021$, Fisher's exact test) (Fig. 1a, b). Those with two or more gay sons showed even more skewing (10/44 mothers = 23%), and this was significantly different when compared to mothers with no gay sons ($P = 0.00093$) (Fig. 1c, d).

The results were still valid when the control group was limited to women who have sons. The extreme skewing found in controls with sons (1/51) was significantly different when compared to women with gay sons (13/97, $P = 0.035$). None of the controls with two or more sons showed extreme skewing (0/21), and this was significantly different compared to women with two or more gay sons (10/44, $P = 0.024$). Furthermore, the AR and FMR1 assays separately showed significant differences in skewing as well ($P = 0.017$ and 0.010, respectively; Table 1). The total number of sons or the number of heterosexual sons had no effect on skewing.

There was no difference in mean skewing between controls and mothers of gay men (controls: mean 70.8; mothers of gay sons: mean 69.9), but the variance differed significantly [controls: $S^2 = 112.8$ versus mothers of two or more gay sons: $S^2 = 224.7$, $P = 0.016$ (Levene's test for difference of variance)].

No evidence was detected for factors confounding the relationship between the X-inactivation ratio and having gay sons, including age, weight, height (Spearman's correlation test) or ethnicity (Kruskal–Wallis multigroup comparison test). The grandparental origin of the active versus inactive X chromosome could not be determined because DNA was not available from the parents of the middle-aged mothers. There was no preferential transmission of the active or inactive alleles of the AR or FMR1 to the gay sons.

An increased maternal aunt/uncle ratio was found for male-to-female transsexuals (Green and Keverne 2000) and homosexual men (Turner 1995). Since this increased ratio is hypothesized to be caused by male lethality, we analyzed the sister/brother ratio in our sample of mothers with and without extreme skewing of X-inactivation. The extremely skewed women did not show such increase (total of 13 sisters/15 brothers). Women without extreme skewing of X-inactivation showed a trend towards an increased sister/brother ratio, but this did not reach significance (89 sisters/70 brothers, one-sided $P = 0.077$, test of proportions for binomial distributions).

Variations in size of the AR locus DNA repeats have been correlated with certain diseases (Biancalana et al.

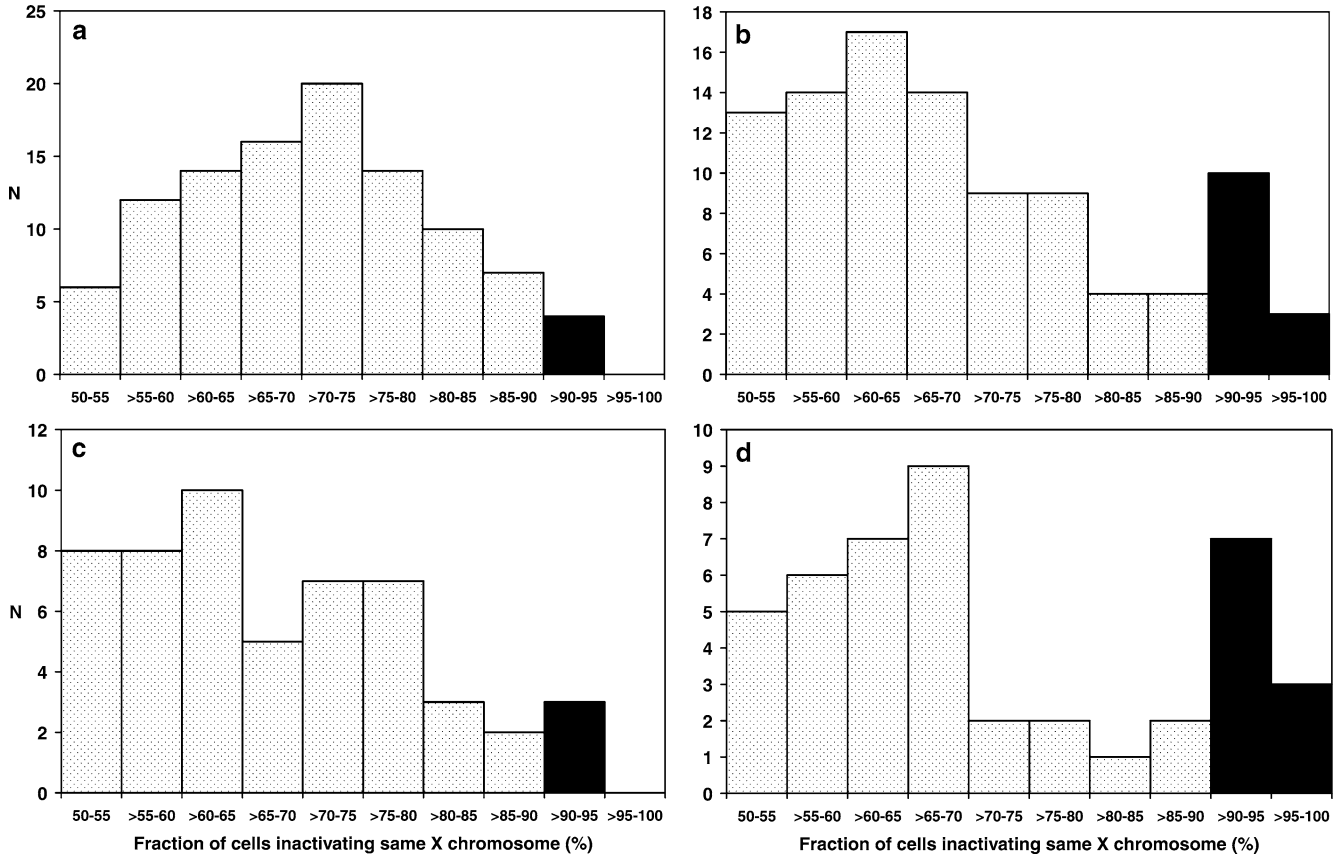

Fig. 1 Distribution of X-inactivation ratios in mothers of homosexual men and control women. The number of women for each fraction of white blood cells inactivating the same X chromosome is shown. *Black bars* represent cases of extreme skewing of X-inactivation (>90% skewing): **a** control women ($N=103$), **b** mothers of gay men ($N=97$), **c** mothers of one gay son ($N=53$), **d** mothers of two or more gay sons ($N=44$)

Table 1 X-inactivation data per assay

Number of gay sons	AR			FMR			Combined		
	Skewing of X-inactivation		%	Skewing of X-inactivation		%	Skewing of X-inactivation		%
	<90%	>90%		<90%	>90%		<90%	>90%	
0	89	4	4	74	4	5	99	4	4
1	46	1	2	32	2	8	50	3	6
2 or more	33	7	17	18	6	25	34	10	23
	$P=0.017$			$P=0.010$			$P=0.00093$		

The number of subjects in each category displaying normal (<90%) or extremely skewed (>90%) X-inactivation and the percentage of women showing extreme skewing are shown for the assay on the androgen receptor locus (AR), the fragile X mental retardation locus (FMR1) and the combination of both assays. The combined data consists of the AR and FMR1 results of those women that were informative for just one of the assays and of the average results of those women for which both assays were informative. *P* values are Fisher's exact test comparisons between 0 and 2 or more gay sons

1992). However, there was no difference in the distribution of allele sizes between mothers of gay men and controls, and a previous analysis on a large number of our samples showed that variations in the AR gene are not a common determinant of male sexual orientation (Macke et al. 1993). There was no preferential inactivation of the smaller or larger AR alleles in the extremely skewed mothers.

Discussion

We found a significant increase in extreme skewing of X chromosome inactivation in mothers of gay sons compared to women without gay sons, especially in women with two or more homosexual sons. Whether the unusual X-inactivation influenced the sexual orientation of

the sons directly through a mechanism such as the birth order effect (Blanchard 2001), or whether it is simply the consequence of a mechanism influencing sexual orientation is unclear. The extreme skewing of X-inactivation found in mothers with two or more gay sons could be the result of primary non-random inactivation, for instance by allelic differences in the X-inactivation center (Brown and Robinson 2000). Unusual imprinting of yet unidentified imprinted genes on the X chromosome has been suggested as a potential cause of extreme skewing as well (Bocklandt and Hamer 2003). Alternatively, a random inactivation might have been followed by a selection for cells that inactivated one chromosome or the other (Brown and Robinson 2000). This could happen when the sequence variation that influences sexual orientation in the children increases or decreases the growth or survival of the white blood cells or stem cells in the mother.

Recently, several identified autosomal loci suggested a multi-gene regulation of the sexual orientation pathway (Mustanski et al. 2005), as expected for a complex behavioral trait. We hypothesize that one central neuronal pathway establishes sexual attraction to either males or females, usually towards the opposite sex. However, a variety of genetic and non-genetic biological effects might intersect this pathway. Hence, there might be several subgroups of gay men and women, each with their own specific biological origin. Although these results need to be replicated, the unusual X chromosome methylation pattern in our sample of mothers of homosexual men supports a role for the X chromosome in regulating male sexual orientation and offers a path for further research on the (epi)genetic basis of a complex and biologically critical human trait.

Acknowledgements We would like to thank all the families whose participation in this study made our work possible.

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