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X Chromosome Inactivation in Women with Alcoholism

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Abstract

Background—All female mammals with two X chromosomes balance gene expression with males having only one X by inactivating one of their Xs (X chromosome inactivation, XCI). Analysis of XCI in females offers the opportunity to investigate both X-linked genetic factors and early embryonic development that may contribute to alcoholism. Increases in the prevalence of skewing of XCI in women with alcoholism could implicate biological risk factors.

Methods—The pattern of XCI was examined in DNA isolated in blood from 44 adult females meeting DSM IV criteria for an Alcohol Use Disorder, and 45 control females with no known history of alcohol abuse or dependence. XCI status was determined by analyzing digested and undigested polymerase chain reaction (PCR) products of the polymorphic androgen receptor (*AR*) gene located on the X chromosome. Subjects were categorized into 3 groups based upon the degree of XCI skewness: random (50:50–64:36), moderately skewed (65:35–80:20) and highly skewed (>80:20).

Results—XCI status from informative females with alcoholism was found to be random in 59% (n=26), moderately skewed in 27% (n=12) or highly skewed in 14% (n=6). Control subjects showed 60%, 29% and 11%, respectively. The distribution of skewed XCI observed among women with alcoholism did not differ statistically from that of control subjects ($\chi^2=0.14$, 2 df, $p=0.93$).

Conclusions—Our data did not support an increase in XCI skewness among women with alcoholism or implicate early developmental events associated with embryonic cell loss or unequal (non-random) expression of X-linked gene(s) or defects in alcoholism among females.

Keywords

Alcoholism; Women; X Chromosome Inactivation; Skewness; *AR* Gene

INTRODUCTION

Alcoholism is a chronic, relapsing illness associated with significant psychosocial, behavioral and physical dysfunction that affects the lives and relationships of millions of people. Alcoholism is believed to result from complex gene-environment interactions that involve social, biological and genetic influences. Genetic influences explain about 50% of the variance associated with the development of alcoholism (Agrawal and Lynskey, 2008; Ducci and Goldman, 2008). A combination of environmental factors and inherent physiologic differences between sexes is thought to account for alcoholism which is two to

three times more common in men (Lee et al., 2010; Wilsnack et al., 2009). Childhood onset behaviors associated with impulse dyscontrol and excessive antisocial behaviors are highly influenced by genes (Lesch et al., 2010; Loos et al., 2009; Reichborn-Kjennerud, 2010) and established as powerful risk factors for alcoholism; moreover, these “externalizing” behaviors are found more frequently among boys than girls including the more severe forms seen in specific childhood disorders such as autism, and may function as endophenotypes of alcoholism (Dawson et al., 2010; Penick et al., 2010)..

Sex chromosomes illustrate the most obvious genetic difference between males and females and thus, may contribute to sex differences seen in alcoholism (Spalt, 1979; Strauch and Baur, 2005). The fundamental chromosomal difference that genetically defines sex (XX vs XY chromosomes) provides the unique opportunity to investigate genetic influences involving the X chromosome in disorders with an unequal male to female ratio, such as alcoholism. All female mammals have two X chromosomes and achieve a balanced X-chromosome gene expression with males having only one X chromosome by inactivating one of their X chromosomes, a process known as X chromosome inactivation (XCI) (Heard et al., 1997; Lyon, 1961, 2005). This process occurs randomly during the late blastocyst stage of embryonic development (Kay et al., 1994). Once an X chromosome from the female is “selected” for inactivation within a cell, the same X chromosome remains inactivated in each subsequent daughter cell. In effect, females are a mosaic or mixture of cells with random expression of genes on a single X chromosome. Occasionally XCI can be non-random or skewed which is usually defined by at least 80% preferential inactivation of one of the two X chromosomes (Plenge et al., 1997; Talebizadeh et al., 2005).

Skewed XCI appears to play a role in the increased incidence and presentation of diseases in females with known X-linked gene involvement such as Rett syndrome (Amir et al., 2000), X-linked intellectual disability (Plenge et al., 2002) and X-linked adrenoleukodystrophy (Maier et al., 2002). Investigations are currently underway to better understand the role of skewed XCI in complex diseases such as aging, cancer, and disorders with known genetic components including behaviors that commonly precede the development of alcoholism (Orstavik, 2006).

We chose to investigate the frequency of skewed XCI in women with alcoholism because male-predominant disorders with high heritability (e.g., autism) exhibit an increased prevalence of skewed XCI in affected females when compared with control females (Muhle et al., 2004; Talebizadeh et al., 2005). In females with autism, skewed XCI reflects unequal or non-random X chromosome activity that leads to an overexpression of X-linked allele(s) conferring increased susceptibility to the development of autism. Skewed XCI may also reflect an early disruption to the developing embryo causing cell death followed by a small number of cells repopulating the embryo (Butler et al., 2007; 2009; Cassidy et al., 1992). Non-random XCI in females with alcoholism might reflect: 1) an early selection event, 2) cell death due to *in utero* insults of the developing embryo, or 3) inheritance of an X-linked gene(s) with unequal expression contributing directly or indirectly to the development of alcoholism. Theoretically, skewed XCI observed in peripheral blood samples can also result from an accelerated depletion of hematopoietic stem cell pools related to normal aging (Busque et al., 1996), exposure to nutritional deficiency, or toxins such as alcohol.

Herein, we compared the XCI status, for the first time, in a group of women with chronic and severe alcoholism to a control group of women without alcoholism. We hypothesized that XCI skewness could be observed in females with chronic alcohol dependence, possibly as a result of early pregnancy events from *in utero* risk factors including alcohol exposure, vitamin and nutrient deficiency or other environmental insults to the developing embryo or

by chance non-random X chromosome inactivation leading to increased expression of X-linked gene(s) contributing to alcoholism.

MATERIALS AND METHODS

Subjects

Adult females meeting criteria defined by the Diagnostic and Statistical Manual of Mental Disorders, version 4 (DSM IV) for an Alcohol Use Disorder and actively participating in alcohol abuse served as the experimental group and female subjects without alcoholism served as controls. All females in our study with alcoholism scored significantly higher than age-corrected normative ranges in all nine domains of the Symptom Checklist-90-Revised (PsychCorp, San Antonio, TX) indicating pathological symptoms for psychiatric disorders. They consumed alcohol at an average of four days each week for six months prior to study enrollment and consumed in excess of 4 ounces of alcohol in one setting for an average of three days a week. Eighty-three percent of females with alcoholism reported a positive family history of alcoholism although limited information was available regarding maternal or paternal drinking status. Eighty-nine percent reported past or present tobacco use and 70% reported past or present drug abuse.

DNA was obtained from blood samples taken from a total of 44 adult females with alcoholism and 45 adult females with no known history of alcoholism. The samples were obtained from 30 adult females with alcoholism who were recruited from the Greater Kansas City Metropolitan area as part of an ongoing clinical study on alcoholism (supported by the Hubert & Richard Hanlon Charitable Trust) and 35 adult females without a history of alcohol abuse were used as controls. These subjects were self-identified alcoholics meeting DSM IV diagnostic criteria for current Alcohol Dependence (American Psychiatric Association, 2000), and enrolled with oversight from the Kansas University Medical Center Human Subjects Committee in accordance with Institutional guidelines governing the use of human subjects in research programs. Additionally, 14 DNA samples were obtained from the Collaborative Studies on Genetics of Alcoholism (COGA – Newark, New Jersey) from unrelated women with a documented history of alcoholism. At the time of peripheral blood sampling, alcoholic subject ages ranged from 21–60 years with an average ($\pm$ SD) of 40.3 ± 10.2 years.

DNA samples from COGA were also obtained from 10 additional unrelated adult control females without alcoholism and combined with data from the 35 females with no history of alcoholism. The age of the control subjects ranged from 18–63 years with an average age of 35.5 ± 13.9 years.

X Chromosome Inactivation Assay

The polymorphic androgen receptor (*AR*) gene is located at Xq13. It is normally inactivated on one of the X chromosomes in females and used to determine X chromosome inactivation status. Genomic DNA isolated from blood was used as a template for polymerase chain reaction (PCR) amplification to identify the CAG polymorphic region of the *AR* gene. Prior to PCR amplification, 200 ng of genomic DNA was digested with the methyl-sensitive restriction enzyme *HpaII* as described previously (Allen et al., 1992). Approximately 50 ng of digested or undigested genomic DNA was used as a template for PCR amplification to determine the peak height of the polymorphic PCR fragment of the *AR* gene using the following primers: forward 5' TCCAGAATCTGTTCCAGAGCGTGC 3' and reverse 5' GCTGTGAAGGTTGCTGTTCTCAT 3' with the forward primer fluorescently labeled with 6-FAM. The lengths and peak heights of the resulting PCR fragments were determined with the use of capillary electrophoresis and an ABI 3100 DNA sequencer (Applied

Biosystems, Carlsbad, CA) with established protocols (Butler et al., 2007; Bittel et al., 2008).

The digestion process preferentially degrades activated (unmethylated) over inactivated (methylated) DNA. Undigested DNA is preferentially amplified producing larger peak heights. Peak height values for the digested DNA were normalized using peak height values for the undigested DNA for each subject. The percentage of X chromosome inactivation for each *AR* allele was then calculated using the following formula: $(d1/u1) / [(d1/u1) + (d2/u2)]$; d1 = peak height of digested DNA from the 1st allele; and u1 = peak height of undigested DNA from the 1st allele; d2 = peak height of digested DNA from the 2nd allele; and u2 = peak height of undigested DNA from the 2nd allele. Highly skewed XCI was defined as >80% calculated ratio for either one of the *AR* gene alleles in the digested DNA sample (see Figure 1). To ensure reproducibility of XCI results and equal amplification of both alleles the experimental approach reported by Talebizadeh (2005) was followed. Specifically, the digestion, PCR amplification and genotyping were repeated up to three times in several samples.

Statistical Analysis

XCI status for each subject was assigned to one of 3 mutually exclusive categories: 1) randomly selected inactivation of either allele from each X chromosome (XCI=50:50 to 64:36); 2) moderately skewed inactivation favoring one allele of one of the X chromosomes (XCI=65:35 to 80:20); and 3) highly skewed inactivation of a single allele representing one X chromosome (XCI>80:20). The relative frequency of random, moderate and highly skewed XCI categories was determined for both the alcoholic and control groups. The Chi Square test was used to compare the XCI categorical distributions between the two groups.

RESULTS

The X-linked androgen receptor (*AR*) gene was assayed to determine X chromosome inactivation status. A common variation (polymorphism) of the *AR* gene sequence permits discrimination of the maternally and paternally derived X chromosomes in approximately 90% of cases. Therefore, a relatively small proportion of the normal population is homozygous or “noninformative” at this *AR* locus. This variation occurs in a highly polymorphic region of exon 1 which contains varying numbers of CAG repeats. X-chromosome inactivation status was examined in 44 alcoholic female subjects and 45 unaffected female controls ranging in age from 18–63 years with allelic heterogeneity of the *AR* gene. Control subjects were within 5 years of age of the subjects with alcoholism. Figure 2 shows the relative distribution of XCI in women with and without alcoholism. XCI of females with alcoholism showed random XCI in 59% (N=26) of cases, moderately skewed XCI in 27% (N=12), and highly skewed XCI in 14% (N=6). XCI of control females showed random XCI in 60% (N=27) of cases, moderately skewed in 29% (N=13), and highly skewed XCI in 11% (N=5). The overall distribution of skewing and the prevalence of highly skewed XCI observed among women with alcoholism did not statistically differ from control subjects ($\chi^2=0.14$, df=2, p-value=0.93).

DISCUSSION

The present study found no difference in the proportion or frequency of random, moderately skewed or highly skewed XCI in blood DNA taken from women with alcoholism compared to control subjects. This conclusion is based on a clinically relevant increase in the frequency of skewed XCI observed in previous studies which found approximately 20% skewness in a predominantly pediatric population of Prader-Willi syndrome (25 females with maternal disomy 15; average age 19.1 years) compared with a 4% frequency in healthy

females (N=50; average age 18.3 years) reported by Butler et al. (2007). Using the Pearson chi-square test for two proportions, 45 subjects per group is sufficient to achieve 79% power in detecting a 20% difference in the probability of skewed XCI. There also was no evidence for a relationship between early defects during the late blastocyst stage of embryonic development leading to skewed XCI or unequal expression of X-linked genes and development of alcoholism among women. However, at least 29 genes of an estimated 1100 on the X-chromosome are known to escape the inactivation process (Mangs and Morris, 2007; Ross, 2005).

The present study design and findings cannot be extended to include these genes escaping inactivation. The present study relied upon blood samples for practical considerations. The pattern of XCI in blood appears similar to that found in brain tissue (Bittel et al., 2008). In addition, tissues derived from all three primary germ layers (endoderm, mesoderm, and ectoderm) exhibit similar patterns of XCI (Bittel et al., 2008) which buttress the claim that XCI skewness results from an early developmental event and persists in a stable fashion, more or less, throughout life. However, XCI skewness does increase at older ages possibly as a result of a dwindling hematopoietic stem cell pool (Amos-Landgraf et al., 2006; Busque et al., 1996).

Our study did not find increased XCI skewness in females with alcoholism compared with a slightly younger control population although age was not significantly different in the two subject groups. In addition to early developmental processes and X-linked genetic risk factors, XCI skewness in peripheral blood could reflect a rapid rate of hematopoietic stem cell pool depletion as a consequence of direct or indirect influences of alcohol toxicity, nutrient deficiency or other toxins. Chronic, severe alcoholism has the potential to accelerate many of these effects, but this was not observed in the present study.

In summary, our study finds no evidence that alcoholism in women is due to a disruption of early embryonic development producing a diminished cell population or due to spontaneous non-random XCI skewness with over expression of X-linked genes. Our study further suggests that alcohol abuse and alcoholism-associated nutrient deficiency leading to cell death during adulthood does not significantly reduce the size of the hematopoietic stem cell pool or contribute to XCI skewness in blood cells detectable with our methods when compared to controls.

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Figure 1.

X inactivation analysis by genotyping of the CAG repeat in the AR gene after digestion. An example of the random and highly skewed X inactivation is shown. The peak representing the active (unmethylated) X chromosome allele would be digested by the methyl sensitive enzyme and reduce in size. If skewness is present the peak height would differ between the two peaks representing each X chromosome (methylated-inactive and unmethylated-active).

Figure 2. Distribution of X chromosome inactivation categories among women with alcoholism and controls.