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The androgen receptor and DXS15-134 markers show a high rate of discordance for germline X chromosome inactivation

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EDITOR—The process of X chromosome inactivation was identified as early as 1960 when Ohno and Hauschka¹ described the presence of a pyknotic X chromosome in both benign and malignant cell lines. Mary Lyon formalised the role of X inactivation and its relationship to dosage compensation of X chromosome genetic material in a letter to *Nature* in 1961.² This phenomenon, now known as the Lyon hypothesis, states that only one X chromosome is transcriptionally active in a given female cell. While the Lyon hypothesis dictates that the X inactivation process is random, skewing of this process to the point of non-random X chromosome inactivation is a known mechanism associated with the development of X linked genetic diseases in females.³

Our laboratory has been interested in the association of non-random X chromosome inactivation (NRXI) with ovarian cancer.⁴ Not only does this process violate a basic biological principle, the Lyon hypothesis, but it also provides a mechanism to bypass one of two steps generally accepted as necessary for the development of a cancer phenotype, dictated by the Knudson two hit model.⁵ The process of X inactivation silences one of two alleles for a particular gene and hence creates a state of functional loss of heterozygosity. Thus, X linked tumour suppressor genes can require only a single mutational event (or "hit") to contribute to the process of carcinogenesis. Furthermore, with NRXI, hypothetical germline mutation of tumour suppressor genes could contribute to early onset disease. We have hypothesised that the process of NRXI may provide a mechanism for some hereditary breast or ovarian cancers independent of *BRCA1/BRCA2* associated disease because of its strong association with invasive ovarian cancers.⁴ More recently, data from our group suggest that this association extends to breast and endometrial, but not cervical or vulvar cancers.⁶

The gold standard for assessment of X chromosome inactivation status has been an evaluation of the androgen receptor (AR) gene polymorphism(s). The AR gene, localised to Xq13, is characterised by a highly polymorphic trinucleotide repeat (CAG)_n in the coding

region of its first exon. This repeat sequence is preceded by target sequences for the methylation sensitive restriction enzymes *HhaI* and *HpaII*. The X chromosome inactivation mechanism results in site specific cytosine methylation of the inactive chromosome. This renders the methylated chromosome resistant to restriction enzyme digestion. In contrast, the active (unmethylated) X chromosome is sensitive to digestion by methylation specific restriction enzymes. Polymerase chain reaction (PCR) amplification of restriction digest can therefore be used to identify the active X chromosome. The majority of papers relating to X chromosome inactivation use the AR model. While other loci have been proposed, other potentially useful loci have primarily been studied only in small cohorts and these studies have been lacking in comparative analysis between the AR and the test locus.

In order better to understand NRXI and the extent to which it may occur throughout a given X chromosome, it would be useful to evaluate the degree of methylation of multiple X chromosome markers. In 1998, Okamoto *et al*⁷ published a report of 110 Japanese females evaluated for mononuclear cell clonality, using a unique short tandem repeat (STR) site at DXS15-134.⁷ This marker has been localised distal to the AR locus at Xq28 and is characterised by a pentameric repeat pattern flanked by *HhaI* and *HpaII* restriction enzyme target sequences. These authors reported the results of AR and DXS15-134 PCR analysis of germline DNA in 91 women. Eighteen of these 91 females were heterozygous at both loci. The results of the AR and DXS15-134 PCR analysis were completely concordant. We have therefore chosen to perform a similar comparative analysis at the AR and DXS loci using germline DNA samples from probands with breast or ovarian cancer and a cohort of healthy cancer free controls.

Material and methods

Blood samples were obtained in accordance with guidelines set forth by the Committee on Human Subjects at the University of Iowa Hospitals and Clinics (UIHC). Case selection was from the Surgical (breast cancer) and

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Gynecologic (ovarian cancer) Oncology Clinics at UIHC Clinical Cancer Center. Healthy controls were consenting paid volunteers. The cancer probands were selected based on consecutive patient visits to the clinic and were not enriched for known *BRCA1/BRCA2* status or family history of breast/ovarian cancer. We used mononuclear cell DNA from 139 probands (76 healthy controls, 20 breast cancer, 43 ovarian cancer). Personnel trained in the collection and construction of cancer family pedigrees obtain all pedigrees at the UIHC.

DNA extractions followed a standard phenol/chloroform/isoamyl alcohol extraction protocol we have previously reported.^{8,9} Germline mononuclear cell DNA was subjected to restriction enzyme digestion by a combination of *HhaI* and *HpaII* (New England Biolabs, Boston, MA). Each sample was aliquotted to paired, digested, and sham digests (controls). Ten μl of germline DNA (40–80 ng of DNA) were combined with 13.7 μl of double deionised water (ddH_2O), 3 μl of 10 $\times$ concentration NEB4 buffer (New England Biolabs, Boston, MA), 0.3 μl of bovine serum albumin (New England Biolabs, Boston, MA), 1 μl (2 units) of *HhaI*, 2 μl (2 units) *HpaII* (New England Biolabs, Boston, MA) for a total volume of 30 μl . In the sham digest reaction, the combined volumes of restriction enzymes were substituted with a 50% glycerol solution (Fisher Scientific, Fairlawn, NJ). Samples were digested to completion at 37°C for 16 hours followed by heat inactivation of the restriction enzyme at 95°C for 30 minutes.

Both active and sham digested DNA (1.5 μl) were amplified at AR and DXS15-134 loci in 10 μl PCR reactions. Included in the reaction was 4.8 μl of ddH_2O , 1 μl of 10 $\times$ PCR buffer (Boehringer Mannheim, Germany), 1 μl dNTP (2 $\mu\text{mol/l}$), 0.5 pmol M_{13} forward 29/IRD 700 dye tailed primer (LI-COR®, Lincoln, NE), 1 unit of *Taq* polymerase (Boehringer-Mannheim, Germany), and 0.5 μl each of the following primers: AR-F: 5'-CACGACGTTG TAAAACGACTGCGCGAAGTGATCCAG AAC, AR-R: 5'-TACGATGGGCTTGGGGA GAA. In the DXS15-134 reaction, the M_{13} tailed DXS15-134 primers were: DXS15-134F: 5'-CACGACGTTGTAAAACGACGA ATTCTTTGCCTAGACCGG, DXS15-134R: 5'-TTGGAGCCAGGAGAATCGCTTGAAC.

Thermal cycler conditions followed a modified step down protocol. After an initial denaturing step at 95°C for five minutes, subsequent cycles included 45 second denaturation steps at this temperature. For the first five cycles, a five minute annealing step at 68°C was used to increase early amplification specificity. Next, a single 58°C step down with a two minute annealing time was followed by 25 cycles at 56°C steps with 60 second annealing time. All extensions were at 72°C for 60 seconds. The programme was completed by a final two minute extension time at 72°C. This unusual programme has been very useful in amplifying many different markers particularly in multiplex PCR reactions.

Two μl of PCR product were combined with 8 μl of LI-COR® IR 2 STOP solution (LI-COR®, Lincoln, NE). The combination was heated to 95°C for four minutes before loading onto a 25 cm, 7% Long Ranger™ (FMC Bioproducts, Rockland, ME) acrylamide gel.

The samples were electrophoresed at 50°C, 40 volts, and 40 mA for 90 minutes (AR) to 120 minutes (DXS15-134) based on fragment size. Internal size standards (LI-COR®, Lincoln, NE) were loaded in every tenth well for base pair length determination. Electrophoresis gels were evaluated using LI-COR® based ImagIR 4.0 data collection software and image manipulator software (LI-COR®, Lincoln, NE).

LI-COR® sample interpretation was based on comparison of allele banding patterns and relative intensities from the gel image files. Informative samples contained two alleles in the control lanes. Non-random X chromosome inactivation was defined as a relative loss of one allele band intensity in the digested DNA lane while the second band was unchanged relative to the control. Confirmation of the NRXI designation was via interpretation of relative band optical densities as determined by the Scanalytics GeneImagIR software (Scanalytics, Billerica, MA). An optical density differential of $\geq 3:1$ or ≤ 0.33 between bands as defined by Mutter *et al*^{10,11} was accepted as sufficient for defining NRXI. A designation of random X inactivation (RXI) was assigned when the two allele band intensities in the digest amplification maintained the same relative 1:1 ratio seen in the undigested control amplification. Occasional small differences (differential amplification) in allele intensity in the controls did not alter assignment in a sample with RXI. A reversal of the differential pattern between digest and controls necessitated a comparison of ratios between digested and control samples to determine X inactivation status in <5% of cases.

All statistical measures were performed using SPSS® 10.0 statistical software (SPSS® Inc, Chicago, IL). Comparisons of NRXI rates were performed using the chi-square test. Pearson's *r* was used to measure correlation between the two loci and Cohen's kappa (κ) was applied to assess agreement between the two loci. Comparison of mean optical density ratio for consistency analysis incorporated analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons.

In order to validate the consistency of the assay, aliquots of DNA showing SXI from a single proband was subjected to five separate restriction digests. Multiple portions of each of these digested samples were then PCR amplified at the AR locus. These reactions generated 19 separate assays on DNA from the same proband. Analysis of the band ratios confirmed the reproducibility of the assay. The mean ratio and 95% confidence intervals were 3.7479 (3.5892–3.9065). The ratios were compared among groups via ANOVA analysis. There were no significant differences detected ($0.248 \leq p \leq 1.0$) between either the varying

Figure 1 Amplification of the AR locus. Proband identification is provided along the top of the image (for example, C1, C2). Lanes are labelled as D for digested DNA and C for control or sham digested DNA. Band markers flank the gel to provide reference for base pair length determination. This image shows examples of random X inactivation for probands C1, C2, and C5 with equal numbers and intensities of bands seen in both the digest and control lanes. Probands C2 and C9 show examples of non-random X chromosome inactivation with loss of the lower allelic band for C2 and the upper band for C9 in the digest lane as a result of preferential restriction digestion of the active (unmethylated) X chromosome.

Figure 2 Amplification of the DXS15-134 locus. Proband identification (for example, BR6, BR9) and base pair ladders are provided for reference. Generally, only two alleles were amplified at DXS15-134 compared to approximately 18 allelotypes for the AR locus. Random X inactivation patterns are seen for probands BR11 and BR21 while uninformative results are shown for probands BR9 and BR22. Only BR6 shows NRXI.

Table 1 Comparison of X inactivation patterns

AR locus	DXS15-134 locus			Total
	Non-random	Random	Non-informative	
Non-random	1 (4.5%)	21 (95.5%)	23	45
Random	—	32 (100%)	45	77
Non-informative	—	9 (100%)	8	17
Totals	1 (1.6%)	62 (98.4%)	76	139

digest or amplifying identical aliquots of DNA from a single digest.

Germline DNA from a total of 139 subjects was evaluated at both the AR and DXS15-134 loci. These included samples from 76 healthy, cancer free controls, 20 breast cancer probands, and 43 ovarian cancer probands. Results for X inactivation studies were considered informative if the DNA was heterozygous at the locus, that is, there were two alleles in the control lane.

Results

Fig 1 shows a LI-COR® gel image of five DNA samples from subjects analysed at the AR locus for X inactivation. All five were informative at this locus. The upper and lower bands are of equal intensity in both the control and digest lanes reflecting random X chromosome inactivation for subjects C3 and C5. This relationship is clear to the naked eye. Software analysis of these respective samples yielded optical density ratios of 1.94 and 1.13 for these subjects. These ratios confirmed classification of these samples to the random X inactivation group. For subjects C1, C2, and C9 the ratio of optical densities between the control and digest lanes are 3.02, >100, and 3.57, respectively. Hence, since these ratios were $\geq 3:1$, the samples were designated examples of non-random X inactivation. At this locus, 122 (88%) DNA samples were informative. Seventeen (12.2%) of the DNA samples were homozygous at the AR locus and therefore were considered uninformative. Of the informative samples, 45 (37%) showed non-random X inactivation patterns, while 77 (63%) showed random X inactivation.

Fig 2 shows a LI-COR® gel image of five DNA samples from probands analysed at the DXS15-134 locus for X inactivation. Three of these five subjects (BR6, BR11, and BR21) were informative at this locus. Non-random X inactivation is seen for proband BR6 while random X inactivation is seen for probands BR11 and 21. Generally, the gel images at this locus are much clearer than for the AR locus, hence software analysis was unnecessary. Probands BR9 and 22 are homozygous at this locus and therefore were considered uninformative. Overall, 63 (45.3%) DNA samples were informative. Seventy six (54.7%) samples were homozygous at the DXS15-134 locus and therefore were uninformative at this locus for X inactivation. Of the informative samples, only one (1.6%) showed a non-random X inactivation pattern, while 62 (98.4%) showed random X inactivation.

Table 1 compares the X inactivation data between the two study loci. For informative samples, agreement between the two assays was achieved in only 33 of 54 cases for a concordance rate of 61.1%. Further, Pearson's r calculation of correlation between loci was not significant ($r=0.024$, $p=0.775$). Interassay agreement was tested using Cohen's kappa and was also found to be insignificant at $\kappa=-0.031$ ($p=0.475$). Comparing the utility of the DXS15-134 assay to the AR result, the

sensitivity of the DXS15-134 locus to detect NRXI was only 4.5% while the negative predictive value of the test was 39.6%.

Discussion

The process of X chromosome inactivation occurs as early as the blastocyst stage of embryonic life and the inactive state of the chromosome is stably maintained through subsequent cell divisions.² X inactivation results in equalisation of X chromosome gene expression between male and female cells and is therefore considered a mechanism for dosage compensation. The inactivation process has been well documented in mice¹²⁻¹⁴ and is beginning to be understood in humans.¹⁵ A *cis* acting RNA transcript known as XIST (X inactivation sequence transcript) is coded from the XIC (X inactivation centre) localised to Xq13. The XIST molecule is not translated to a protein product, but rather is distributed along the length of the X chromosome to form a complex with DNA and the histone variant MacroH2A conformational shape changes to the DNA in proximity to XIST. The final step of inactivation is site specific methylation of cytosine bases.¹⁵⁻¹⁷

The end result of the inactivation process is to abrogate gene expression from the inactive X chromosome. This mechanism does not inactivate all genes on the inactive X chromosome. A number of genes localised to a limited number of sites have been shown to escape the inactivation process; these include *ARSD*, *ARSE*, *GS1*, *STS*, *KAL*, *ANT3*, *XE7*, *MIC2*, and others.¹⁸ Initially, loci shown to escape X inactivation were localised to the pseudoautosomal region of the X chromosome, frequently had Y chromosome homologues, and appeared to cluster on the p arm of the X chromosome. Now it is known that additional genes, both with and without Y chromosome homologues, and on the q arm of the X chromosome can also escape inactivation.^{18, 19}

Studies of X chromosome inactivation were initially based upon electrophoretic analysis of the glucose-6-phosphate dehydrogenase protein. This technique pioneered by Fialkow²⁰ was unfortunately limited by its lack of informative results. Other markers and techniques were also limited by a lack of informative sites.^{21, 22} However, since the development of the AR assay by Allen *et al*,²³ this technique has gained widespread acceptance and has been used in many studies of tumour clonality as well as X chromosome inactivation. The AR gene ($(CAG)_n$ trinucleotide repeats fall within exon 1 and are polymorphic in 90% of females from all racial groups.²⁴ The utility of the AR assay is occasionally limited by difficulty in interpretation of results owing to stutter bands (as in fig 1) and software problems associated with optical scanning of gels using isotopic methods.⁴ Although trinucleotide repeats are usually much clearer on gel electrophoresis than dinucleotide repeats, the relatively high GC content of the AR repeat can potentially result in amplification difficulties. There is also a fair amount of subjectivity in the interpretation of the banding patterns that determine

inactivation status. In contrast, the pentameric character of the DXS15-134 locus contributed to the ease of amplification of two unique alleles with clear and distinct banding patterns. Theoretically, DXS15-134 X inactivation patterns should supplement an AR analysis. Unfortunately, we found that results from the two assays were discordant in 38.9% of cases.

At present, it is difficult to know which of the assays is more reliable. The overall incidence of NRXI, 37% of the informative cases, as determined by the AR assay was driven by the difference in rates between cancer probands (52.7%) and healthy controls (23.9%). Comparison of NRXI rates between several different cancers, and the influence of age on this process forms the basis of another study (Mahavni *et al*, unpublished data). Results from several cases of X linked diseases, expressed in females, can readily be explained by and are consistent with NRXI results obtained at the AR locus. Correlative studies of other X linked loci including the *MAOA* locus²⁵ and several single nucleotide polymorphisms (SNPs) are currently under way in our laboratory. One possible explanation of our results is that the DXS15-134 locus escapes X inactivation.

Three lines of evidence support the concept that the DXS15-134 locus is transcribed: (1) the study of Okamoto *et al*,⁷ (2) the single case (BR6) with NRXI, and (3) our laboratory has been able to apply our current DXS15-134 primers to a cDNA template and obtain PCR products, suggesting that the DXS15-134 locus is transcribed (data not shown). The poor sensitivity and predictive value of the locus to detect NRXI probably limit its use as a marker for X inactivation studies. Additional support for the hypothesis that the Xq28 region containing DXS15-134 escapes X inactivation was presented by Bailey *et al*¹⁷ who reported on the variable clustering of Line-1 (L1) elements in selected human chromosomes. The L1 content of genomic segments that carry genes capable of escaping X inactivation was significantly lower ($p=4.8 \times 10^{-5}$) than the X chromosome average of those genes subject to inactivation ($p=0.004$). The region of Xq28 contained a relatively low base pair fraction (0.2%) of L1 elements. This may provide support to the idea of DXS15-134 generally escaping the X inactivation process, but clearly additional studies will be required to resolve these questions.

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Evaluation of the *ELOVL4* gene in families with retinitis pigmentosa linked to the *RP25* locus

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EDITOR—Retinitis pigmentosa (RP) is the most common form of retinal dystrophy. Patients present with night blindness and progressive narrowing of the visual field, eventually leading to central vision loss. Fundus examination usually shows bone spicula pigmentation, attenuation of blood vessels in the retina, and waxy pallor of the optic disc. Typically, the electroretinogram is notably diminished or even abolished.¹

RP shows important allelic and non-allelic genetic heterogeneity (RET-GEN-NET) with different modes of inheritance, including autosomal dominant (AD), autosomal recessive (AR), X linked, and digenic.

ARRP is the most common form of RP. A locus for ARRP, *RP25*, was mapped in 1998 to the long arm of chromosome 6 between microsatellite markers D6S257 and D6S1644 (MIM 602772).² Recently, we have excluded two candidates, *GABRR1* and *GABRR2*, as the disease causing gene.³

Several loci with retinal dystrophy phenotypes have been mapped to the pericentromeric region of chromosome 6. They include autosomal dominant Stargardt-like disease (*STGD3*),⁴ autosomal dominant macular atrophy (*ADMD*),⁵ autosomal dominant cone-rod dystrophy (*CORD7*),⁶ and Leber congenital amaurosis (*LCA5*).⁷

Recently, the gene responsible for *STGD3* and *ADMD* has been identified.⁸ All affected members in four independent families with *STGD3* and one family with *ADMD* shared a common founder haplotype, indicating a single

ancestral disease specific mutation. A single 5 bp deletion of a novel gene called *ELOVL4* was identified, which segregates with all affected members of the *STGD3* and *ADMD* families. *ELOVL4* shows cone and rod photoreceptor expression in the eye, is composed of six exons, and encodes a putative transmembrane protein of 314aa with similarities to the ELO family of proteins involved in elongation of very long chain fatty acids. Based on its similarity, it has been suggested that this protein is involved in synthesis of polyunsaturated fatty acids (PUFA) in the retina, such as DHA (docosahexaenoic acid). DHA represents 50% of PUFA in the outer segment of the photoreceptor cells and plays a crucial role in photoreceptor cell functions.

It is well known that one gene can cause distinct disease phenotypes with retinal degeneration.⁹⁻¹⁹ For example, *ABCR* (also called *ABCA4*), the gene responsible for recessive Stargardt macular dystrophy (*STGD1*), can cause either ARRP or autosomal recessive cone-rod dystrophy.¹⁷⁻²⁰⁻²³

It has been known for a long time that the retina possesses unique properties in lipid metabolism. The high level of PUFA, such as DHA, in the photoreceptors is thought to form an essential lipid environment for the phototransduction function. In addition, DHA is known to be lower in the serum of patients with retinitis pigmentosa. This evidence suggests that lipid metabolism may play a role in the pathogenesis of RP.²⁴⁻²⁸

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