X Chromosome Inactivation Patterns in Normal Females

Submitted 11/04/98; revised 11/09/98
(communicated by Ernest Beutler, M.D., 11/09/98)

Omar Racchi, Rosa Mangerini, Davide Rapezzi, Michela Rolfo, Gian Franco Gaetani,
Anna Maria Ferraris

ABSTRACT: Since one of the two X chromosomes is randomly inactivated at an early stage of female embryonic development, X-linked markers have been used to study the origin and development of various neoplastic disorders in affected heterozygous women; clonality assays have provided a useful tool to the understanding of the mechanisms underlying the development of neoplasia. Recently, a technique of clonal analysis has been devised that takes advantage of a highly polymorphic short tandem repeat within the X-linked human androgen receptor (AR) gene, resulting in a heterozygosity rate approaching 90%. The rapid expansion of the number of women now suitable for X inactivation analysis has however given rise to new controversies, one of the more troublesome being the possibility of a modification of the pattern of X- chromosome inactivation pattern in blood cells of elderly women. In the present study we analyze with the AR assay a group of 166 healthy females aged between 8 and 94 years, with no history of genetic or neoplastic familial disorders. We failed to find any correlation between age and X- chromosome inactivation pattern (r = 0.17), even subdividing the subjects in different age groups according to the criteria used by other researchers, and therefore reaffirm that, when tested for with well-standardized and accurate criteria, extremely unbalanced inactivation of the X chromosome is a truly uncommon phenomenon in normal women. © 1998 Academic Press

Keywords: X inactivation, skewing, androgen receptor

INTRODUCTION

X chromosome-linked markers have been extensively used for more than three decades in order to study the origin and development of various diseases in affected heterozygous women (1-3). Inactivation of the major part of one X chromosome occurs in somatic cells of females during early embryogenesis; therefore, women heterozygous for a polymorphic X-linked gene have a mixture of cells expressing one or the other allele in their normal tissues (1,4). The first studies of mosaicism were performed in women heterozygous for variants of the enzyme glucose-6-phosphate dehydrogenase (G6PD) (5-7). Afterwards, the availability of molecular probes for X-linked polymorphic genes such as hypoxanthine phosphoribosyltransferase and phosphoglycerate kinase (8), allowed distinguishing the active and inactive copies of the alleles through differences in methylation; the use of restriction fragment length polymorphisms (RFLP) made possible evaluation of the X-chromosome inactivation pattern in nucleated cells of a greater proportion of women. More recently, a technique has been developed that takes advantage of a highly polymorphic short tandem repeat (STR) in the coding region of the

Dipartimento di Oncologia Clinica e Sperimentale, Università di Genova and Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

Reprints request to:. Anna Maria Ferraris, M.D., Ematologia Oncologica, IST, Largo Rosanna Benzi, 10, 16132 Genova, Italy, phone 390105600933, fax 39010509052, email: gaetani@mbox.ulisse.it

ISSN No. 1079-9796/98 $25.00

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Blood Cells, Molecules, and Diseases (1998) 24(21) Nov 15: 439-447
Article No. MD980213

X-linked human androgen receptor (AR) gene; the close proximity of the STR to four methylation sites makes it possible to perform a clonal analysis based on the polymerase chain reaction (PCR) (9).

The consequent availability of a large number of informative women now makes it possible to determine the true incidence of extremely unbalanced X chromosome inactivation in healthy individuals. Extreme skewing is a very rare phenomenon in normal females with no family history of genetic or neoplastic diseases (10-12). Recently however it was suggested that the aging process may exert a direct effect on the X-chromosome inactivation patterns, with an excessive number of extreme skewing in blood cells of elderly women (13-15). If correct, this theory would greatly affect the interpretation of clonal analysis in this group of subjects, by making it hazardous to distinguish a genuine clonal proliferation from the unbalanced X inactivation that results from senescence (16).

We therefore decided that a thorough investigation of a large number of normal females was warranted, in order to establish as accurately as possible the prevalence of nonrandom X chromosome inactivation in hemopoietic cells and its relationship with various parameters such as age and consanguinity.

MATERIALS AND METHODS

Subjects

X chromosome inactivation pattern was assessed in 189 healthy females without a family history of genetic or neoplastic diseases; informed consent was obtained from all subjects.

Peripheral blood samples were separated into a light-density mononuclear cell (FH) fraction and a granulocyte (PMN)-rich pellet by centrifugation through a Ficoll-Hypaque density gradient. Monocytes were then removed from the FH cells by adhesion to plastic culture dishes. Purified PMN were obtained diluting the pellet in dextran/saline at room temperature for 2 hours, followed by centrifugation of the supernatant and osmotic lysis of residual erythrocytes. The degree of contamination of each cell fraction was less than 3% by direct examination of cytocentrifuge slides stained with May-Grünwald-Giemsa. While purified PMN were obtained from all women, FH cells were isolated as well only in the subjects studied prospectively. Since several samples had been collected and stored in the past for previous clonality studies, some of the corresponding women were no longer available, and this accounts for the difference between the numbers of PMN and FH samples tested.

A complete blood count with differential and examination of erythrocyte morphology was performed by one of us in order to identify abnormalities suggestive of a myelodysplastic syndrome or other hematological disorders in all women older than 60 years, and in 22 other chosen at random.

X Chromosome Inactivation Analysis

Isolated cell populations were lysed in 10 ml 1x TNE (Tris 1M pH 8, NaCl 1M, EDTA 0.5M pH 8), 2% SDS and 100 mg/ml proteinase K at 37°C overnight. DNA was then extracted and purified following routine procedures.

Inactivation status of the X chromosome was assessed by taking advantage of the favorable characteristics of heterozygosity at the human androgen receptor (AR) locus, namely the high percentage of informative subjects, the advantage of a PCR assay, and the consistent pattern of methylation of the *Hpa*II and *Hha*I sites within a highly polymorphic CAG repeat in the coding region of the first exon of the AR gene (9). The close proximity of the repeat to the methylation-sensitive restriction enzyme sites enables the active and inactive X chromosome to be distinguished from each other. Reliability of the test has been validated by the same authors in different ways, all showing a substantial correlation between X inactivation pattern and methylation. X-chromosome inactivation pattern was determined by PCR amplification at the AR

locus according to a modification (17) of the technique of Allen et al.(9). Briefly, each DNA sample was digested overnight at 37C in a 10 µl reaction mixture containing *Rsa*I (20 units) or *Rsa*I with 40 units of *Hpa*II. After digestion, the reaction was terminated by incubating the mixture at 95°C for 10 min. Primer sequences were as follows: for the forward primer 5'-GCTGTGAAGGTTGCTGTT CCTCAT-3',and 5'-TCCAGAATCTGTTCCAGAGCGTGC-3'for the reverse primer. Five µl of the reaction mixture were amplified; PCR was performed in a total volume of 40 µl, containing 250 µM dNTP, 0.03 U Taq polymerase, 1 mM $MgCl_2$, 4 µl 10X Taq buffer, 10% DMSO and 0.38 µM of each primer. The samples were amplified for 30 cycles (each comprising 1 min at 94C, 45 s at 56C, and 1 min at 72C), with an initial denaturation at 94C for 10 min and a final extension at 72C for 10 min, on a Perkin Elmer thermocycler. The amplification product was then loaded on a denaturing 6% 19:1 acrylamide/bis-acrylamide gel (8 M Urea and 0.5x TBE) and electrophoresed at 60 W for 3 h. The gel was dried and exposed to X-ray film at -80C over-night with an intensifying screen.

Estimation of Degree of Skewing and Statistical Analysis

Visual assessment of relative hybridization intensities by inspection of the autoradiographs was confirmed by quantitation of the two AR alleles comparing radioactive intensity of the bands with densitometric scanning. All assays were performed in duplicate. Artificial mixtures made of DNA from control women homozygous for alleles with different numbers of CAG repeats in the AR gene showed that identification of the minor component was unequivocal when it constituted at least 5% of the total (17,18).

The degree of skewing (DS) was defined as the ratio of the more represented allele to the sum of both alleles, and can be identified with the percentage of cells with the same X chromosome active. Correlation between DS and age was assessed with Pearson's correlation coefficient.

In order to test the hypothesis that the skewing pattern might be influenced by hereditary transmissible factors (19,20), 26 pairs of consanguineous women (14 mother-daughter, 10 aunt-niece and 2 grandmother-granddaughter couples) were analyzed in the following way: the percentage of the high molecular weight allele of the older member of each pair was considered as an independent variable and plotted against the percentage of the high molecular weight allele of the younger woman. Correlation between the values was again assessed with Pearson's test.

Data were stored, analyzed and reported with the statistical package Statview (Abacus Concepts Inc., Berkeley, CA, USA, 1992).

RESULTS

The rate of heterozygosity at the AR locus of the 189 women tested was 88%. The X chromosome inactivation pattern was then determined in PMN's DNA from the 166 informative subjects (mean age 52 years, range 8-94). Visual assessment of the two AR alleles was confirmed by densitometric scanning of radioactive bands (Figure 1). FH samples were collected from 50% of the women (age 16-94 years).

Analysis of X-chromosome inactivation pattern showed a distribution of the degree of skewing consistent with an X inactivation process occurring on a random basis (Figure 2). A normal pattern of distribution of the high molecular weight allele was indeed found, with a mean of 45.84 ± SD 20.23. Fifty cases (30%) had a DS ≥ 75%, and only 12 (7%) had a DS ≥ 90%. There was a complete correspondence between the X- chromosome inactivation patterns of PMN and FH cells isolated from the same subject.

The age distribution of all women (range 8-94) was plotted against the DS in the attempt to verify a dependence of the latter from the former value, and no correlation was found when the data were analyzed with Pearson correlation test ($r = 0.17$, Figure 3). This result is consistent with the absence of any effect of age on skewing in normal females.

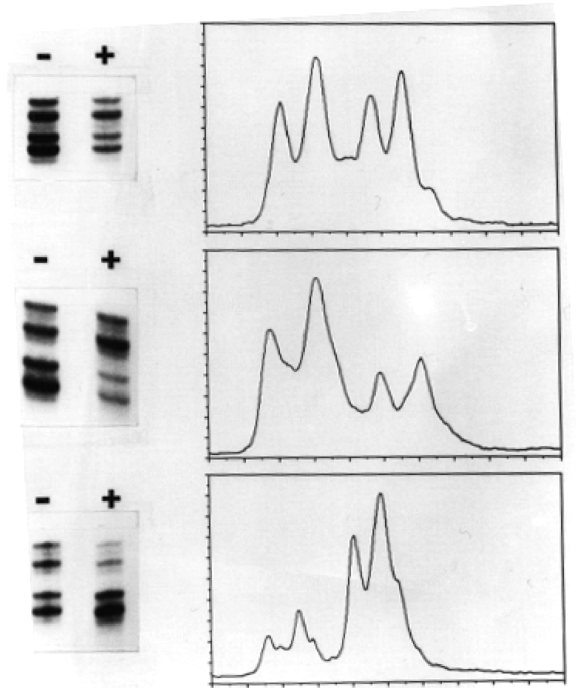

Figure 1. Representative X chromosome inactivation patterns of PMN from three normal women. On the left are shown the results of the AR PCR assay. The DNA was predigested without (lanes marked -) and with (lanes marked +) the methylation sensitive enzyme *Hpa*II; each allele is represented by two bands. On the right are reported the corresponding densitometric scannings. Upper panels: both alleles are equally represented (50:50); middle panels: 70:30; lower panels: 15:85.

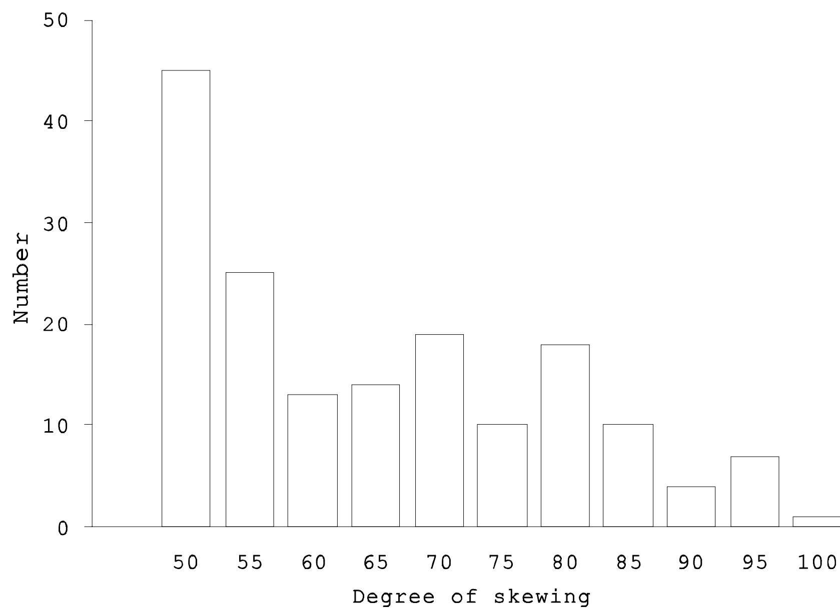

Figure 2. Frequency distribution of 166 normal females according to the X chromosome inactivation patterns. The degree of skewing (DS) is the ratio of the more represented allele to the sum of both alleles, as measured by the AR methylation assay.

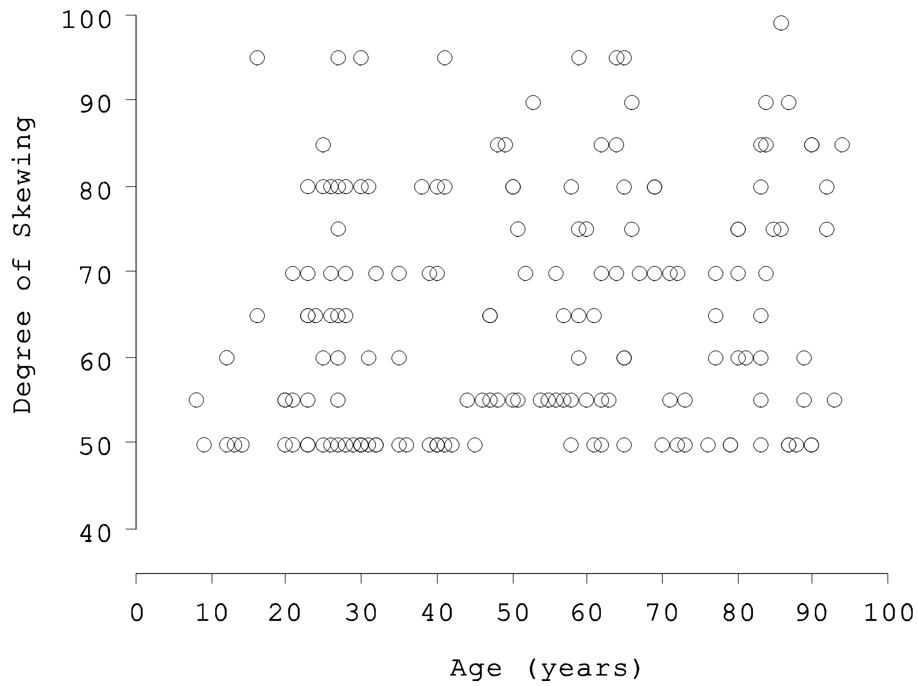

Figure 3. Correlation between age and degree of skewing of 166 normal females ($r = 0.17$).

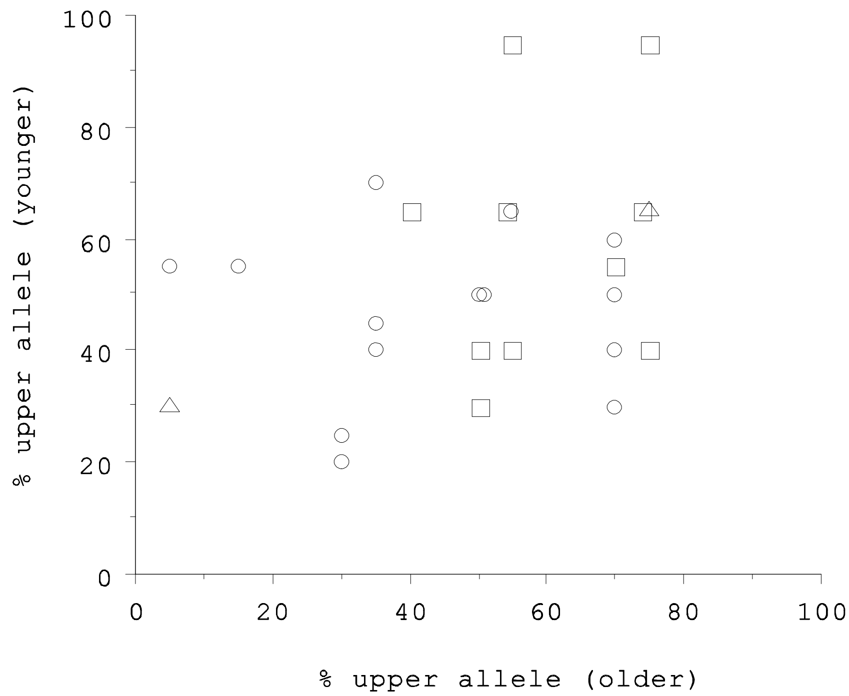

Figure 4. Correlation between the percentage of the high molecular weight allele of 26 pairs of consanguineous women ($r = 0.29$). O = mother-daughter; Δ = grandmother-granddaughter; □ = aunt-niece.

Analysis of the DS of 26 pairs of consanguineous women did not reveal any significant correlation when the percentage of the high molecular weight allele of the older member of each pair was plotted against the percentage of the high molecular weight allele of the younger (r = 0.29, Figure 4). Therefore we have been unable to demonstrate the influence of any direct hereditary factor in the determination of the X-chromosome inactivation patterns from one generation to another, at least in our rather limited sample.

DISCUSSION

Inactivation of one of the two X chromosomes in females was first described in 1962 (1,4), and has since been considered a random process, with the paternally-derived X being inactivated in some cells and the maternally-derived X in others (21-23). Given an X-linked polymorphic locus subject to inactivation, the active and inactive alleles are expected to follow a normal distribution and extreme skewing (defined by us as ≥ 90 % of cells with the same chromosome active, on the account that in our hands the AR assay allows unequivocal identification of the minor component when it constitutes at least 5% of the total) should be a rare event, accounting only for a small percentage of women (5-10%; Table 1) (6,11,12,23).

Unbalanced X inactivation has been shown to happen also as the result of complex mechanisms at action during the early phases of embryonal life (24). Mutations of genes directly involved in the X-inactivation process (XIST) (20,25), carrier status for several X-linked diseases (18, 26-29), monozygotic twinning (30), and confined placental mosaicism (31), all are rare events now known to be associated with extreme skewing (22,32,33).

The present results support the notion that inactivation of the X chromosome is in the vast majority of cases a random process and that extreme skewing is indeed a rare event. The DS clearly follows a normal distribution (Figure 2) and only 7% of all 166 subjects we examined had a DS ≥ 90%. This is in accordance with the majority of reports on the same topic published in the past twenty years with a variety of X-linked markers (6-8,12,21), and also with data presented in some studies where the AR assay was used to determine the X-chromosome inactivation pattern (Table 1).

It is conceivable that other yet unknown mechanisms are operating to induce extreme skewing, for example genes other than XIST and present on the X chromosome itself are supposed to exert a selective pressure on cell growth and survival, thereby determining unbalanced X inactivation in occasional cases. Since the phenomenon has proven so rare however, these genes should play a significant role only in a small minority of normal females. It has been reported in sporadic cases that the skewing pattern can be influenced by hereditary factors, and a few families with a high incidence of genetically transmitted unbalanced X inactivation have been recently described (19,25). This is anyway hypothesized as an uncommon phenomenon (22) and our data support this notion, since in the 26 pairs of consanguineous women analyzed we observed a random distribution of X-chromosome inactivation pattern, with no concordance between pairs of relatives (Figure 4).

In other studies age has been shown to have a direct influence on inactivation, with a much higher incidence of extremely unbalanced X-chromosome inactivation pattern in elderly women (13-16). However, it is conceivable that the use of arbitrary cutoffs of the degree of skewing at ≥ 75% and ≥ 90% by some authors may have contributed to the lack of reproducibility of their results (13-15). Furthermore, the increasing of skewing with age could likewise be due to the decision to compare distinct age groups instead of considering age as a continuous variable (13-15), and also to the questionable use of a clear-cut division between skewed and not-skewed, considered as two different categories.

Moreover, the rationale that leads to the assignment of the tag of "extremely skewed" to a 75:25 sample, instead of, say, 70:30 or 80:20 and so on, is not motivated.

Since both age and skewing are continuous variables, we decided that the most appropriate way to evaluate this relationship was with Pearson's test, and found no correlation between age and skewing (Figure 3). Our data were then analyzed with the same criteria used by the abovementioned authors, but even subdividing the subjects in limited age groups and definite skewing categories did not bring into evidence any statistically significant difference between the DS of young and elderly women (Table 2).

Table 1. Degree of skewing (DS) by different authors

Authors	Number of subjects	Age (years)	DS ≥ 75 %	DS ≥ 90 %
Lau (31)	27	35 - 50	33 %	4 %
Naumova (19)	365	—	32 %	9 %
Busque (16)	295	0 - 96	17 %	7 %
Gale (14)	174	17 - 96	38 %	17 %
Tonon (15)	68	25→ 75	23 %	—
Present study	166	8 - 94	30 %	7 %

Table 2. Comparison of Degree of skewing (DS) in different age groups.

		Age groups years				
DS	Authors	25-32	≥ 60	17-50	≥ 75	p
≥ 75 %	Busque (16)	16 %	38 %	—	—	0.006*
	Gale (14)	—	—	22 %	56 %	<0.0001**
	Tonon (15)	17 %	—	—	45 %	< 0.02†
	Present study	23 %	37 %	26 %	40 %	n.s.†
≥90 %	Busque (16)	4 %	23 %	—	—	0.002*
	Gale (14)	—	—	3 %	33 %	<0.0001**
	Present study	6 %	9 %	4 %	8 %	n.s.†

* Fisher's Exact Test; **Not indicated; † Chi-square Test; n.s.= not significant

Specifically, we applied the analysis of variance to the DS values of two discrete groups of women: those aged 25-32 and those older than 75 years, as done by Tonon et al. We found that the mean of the DS was 64.69 ± 2.49 SE for the "young" group and 67.43 ± 2.40 SE for the "old" women group. The F value was 0.619, therefore not significant for any difference between the two groups with respect to DS distribution.

Finally, since we took the effort to directly check the hematological conditions of the older women at time of testing for X-chromosome inactivation pattern, we can safely exclude the presence from this group of subjects of any hemopoietic disorder such as myelodysplasia, whose clonal development from a multipotent progenitor cell (34) could have explained at least some of other authors' findings (13-15) as they postulated but not verified.

It is conceivable that the use of arbitrary cutoffs of the degree of skewing at ≥ 75% and ≥ 90% by some authors may have contributed to the lack of reproducibility of their results.

ACKNOWLEDGMENTS

This work was partially supported by grants from Ricerca Finalizzata Ministero della Sanità 1996, RF'96.286/ICS070.1; MURST 1997-1998 and 1998-1999; and P.F. Biotecnologie (CNR Target Project on Biotechnology).

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