Age- and tissue-specific variation of X-inactivation ratios in X-linked Alport syndrome females

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Background: Alport syndrome (AS) is a progressive renal disease characterized by hematuria and progressive renal failure. X-linked dominant AS (XLAS) is the major inheritance form, accounting for almost 80% of the cases. XLAS females have variable phenotypes, from microscopic hematuria to chronic renal failure. These variable phenotypes cannot be clarified solely by mutation features of the *COL4A5* gene. X-inactivation has been suspected to be one of the reasons responsible for this phenomenon, but so far definite correlation has not been demonstrated. Moreover, it was supposed that X-inactivation ratios may vary both with age and between different tissues within an individual. This study analyzed the age- and tissue-specific variation of X-inactivation ratios in XLAS females.

Methods: Peripheral blood cells were collected from 36 XLAS females, and cultured skin fibroblasts were collected from 12 of them. The X-inactivation analysis was performed using *HpaII* predigestion of DNA followed by polymerase chain reaction (PCR) of the highly polymorphic CAG repeat of the androgen receptor (*AR*) gene.

Results: The rate of heterozygosity at the *AR* locus of the 36 female patients was 88.89%. Only 12.50% (4/32) of females detected showed skewed X-inactivation in peripheral blood cells. No individual under 30 years of age had skewed X-inactivation, and 20% (4/20) of individuals over 30 years of age had skewed X-inactivation in peripheral blood cells ($\chi^2 = 2.743$, $P = 0.098$). The X-inactivation patterns of the 12 patients showed marked variation between blood cells and skin fibroblasts. Seven of the 12 patients (58.33%) had similar X-inactivation ratios in both tissues, but the other 5 patients (41.67%) had the opposite X-inactivation ratios in both tissues. There was no correlation between the X-inactivation ratios of the mutant allele in skin fibroblasts and in peripheral blood cells ($r = 0.180$, $P = 0.575$).

Conclusion: There was no age-specific variation of X-inactivation ratios in XLAS females but there was tissue-specific variation, which maybe could explain the contradictory results between X-inactivation and the variable phenotype of XLAS females.

Keywords: Alport syndrome, female, X-inactivation

Introduction

Alport syndrome (AS) is a progressive renal disease characterized by hematuria and progressive renal failure. AS is a genetic heterogenous disorder. X-linked dominant AS (XLAS) is in the majority of families, caused by mutations in the *COL4A5* gene that encodes the type IV collagen $\alpha 5$ chain.^{1,2} XLAS males have more severe phenotypes and usually progress to end-stage renal disease (ESRD), whereas XLAS females show a wide severity of manifestations, from microscopic hematuria to chronic renal failure. The diverse phenotypes of XLAS females could not be clarified solely by mutation features of the *COL4A5* gene, especially because siblings with identical *COL4A5*

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gene mutation often exhibit different clinical phenotypes, suggesting that factor(s) other than the mutant gene may impact clinical phenotypes.^{3,4}

Other groups have revealed that the phenotypic variations in carriers of some X-linked disorders were caused by X-inactivation.^{5,6} It was also speculated that X-inactivation was responsible for the phenotype in XLAS females,^{3,7,8} but so far the present results of few reports were contradictory, and no definite correlation was demonstrated.^{9–13} It was hypothesized that X-inactivation ratios may vary both with age and between different tissues within an individual,^{14–16} which could explain the cause of contradictory results. In the present study, in order to confirm the age- and tissue-specific variation of X chromosome inactivation ratios in XLAS females, we analyzed the X-inactivation patterns in peripheral blood cells in 36 XLAS females and in skin fibroblasts in 12 of them.

Methods

Subjects

The study consisted of 36 female patients from 32 XLAS families. All patients were diagnosed through the abnormal expression and distribution of $\alpha 5$ (VI) chain in the epidermal basement membrane (EBM), in addition to fulfilling the criteria for AS proposed by Wang et al and Flinter et al.^{17,18} Mutations of the *COL4A5* gene were analyzed by PCR amplification of cDNA of skin fibroblasts firstly, then confirmed by PCR amplification of genomic DNA.¹⁷ The mutations of the *COL4A5* gene and clinical features of these female XLAS patients are shown in Tables 1 and 2, respectively. The patients were distributed to two groups. Group A included 13 patients aged under 30 years (mean age 17.85 years, standard deviation [SD] 11.68 years); group B included 23 patients aged over 30 years (mean age 38.57 years, SD 6.18 years). Samples of peripheral blood were taken after informed consent was obtained from each patient; samples of cultured skin fibroblasts were taken from 12 of them.

DNA isolation

Genomic DNA was extracted from peripheral blood lymphocytes by a simple salting out procedure.¹⁹ Genomic DNA from cultured skin fibroblasts was extracted using the wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

X-inactivation analysis

X-inactivation pattern was determined by PCR amplification of the highly polymorphic CAG repeats in the first exon

of the androgen receptor (*AR*) gene. Methylation of *HpaII* sites in close proximity to these repeats correlates with X-inactivation. These sites were methylated on the inactive X-chromosome. A PCR product was obtained only from the inactive X chromosome after digestion with *HpaII*. The *AR* (CAG)_n polymorphism method was performed according to a modified technique of Allen et al.²⁰

PCR products from both digested and undigested reactions were separated on ABI 373 automated sequencer and analyzed by GeneScan software (version 9.1). X-inactivation ratios were calculated according to the following formula: skewing = (d1/u1)/(d1+u1+d2/u2),^{16,21} where d1 and d2 represent the two peaks from the digested sample; u1 and u2 are the corresponding bands from the undigested sample. An 80% or higher percentage for one allele is considered as indication of skewed X-inactivation.^{22,23}

Statistical methods

The same procedure was performed twice for each individual; the mean value of these measurements was used for statistical analysis. Statistical analyses were performed by SPSS version 12.0. χ^2 analyses or Fisher's exact test were used to compare the proportion of females with skewed X-inactivation ratios in the various populations. The correlation between the X-inactivation ratios in skin fibroblasts and those in peripheral blood cells was tested by the Pearson correlation. *P* value of less than 0.05 was taken as statistical significance.

Results

One patient in group A and three patients in group B were uninformative for the X-inactivation assay used. The rate of heterozygosity at the *AR* locus of the 36 female patients was 88.89% (32/36). For each individual, two independent PCRs were performed, from which a mean X-inactivation ratio was calculated. In the majority of the patients, results generated from these two tests differed by only a few percentage points both in blood cells and skin fibroblasts, eg, mean difference in blood cells 3.86%, SD 3.36%; mean difference in skin fibroblasts 4.42%, SD 4.02%. This high degree of reproducibility suggests that the *AR* PCR assay was both reliable and accurate.

Variations of skewed X-inactivation with age

Based on an X-inactivation ratio of equal to or greater than 80%:20% as a criterion for skewing,^{22,23} only 12.5% (4/32) of females detected showed skewed X-inactivation in peripheral blood cells. No individual under 30 years of age had skewed

Table 1 The mutation features of the *COL4A5* gene

Number	DNA	cDNA	Amino acid change
1	g. 2550_2527 del	c. 2551_2574 del	p. P851_P858 del
2	g. 1516+1 G > A	c. 1424_1516 del	p. fs K474
3	g. 1516+1 G > A	c. 1424_1516 del	p. fs K474
4	g. 14246-1G > A	c. 1424_1516 del	p. fs K474
5	g. 1928+1 G > A	c. 1780_1948 del	p. G594 P649 del; fs P659
6	g. 1928+1 G > A	c. 1780_1948 del	p. G594 P649 del; fs P659
7	g. 3080 G > T	c. 3080 G > T	p. G1027V
8	g. 4276 C > T	c. 4276 C > T	p. P1426S
9	g. 1653_1654 del C	c. 1653 del C	p. fs L551
10	g. 5042 G > T	c. 5042 G > T	p. C1681F
11	g. 796 C > T	c. 796 C > T	p. R266X
12	g. 1490_1491 ins G	c. 1490_1491 ins G	p. fs A430
13	g. 1331 T > G	c. 1331 T > G	p. L444S
14	g. ND	c. unclear missing	
15	g. 973 G > A, 2107 A > G	c. 973 G > A, 2107 A > G	p. G325R, I703V
16	g. 2696_2705 del	c. 2696_2705 del	p. fs 899
17	g. 1949 del C	c. 1949 del C	p. fs G647
18	g. ND	c. NF	
19	g. 3319 G > A	c. 3319 G > A	p. G1107R
20	g. ND	c. NF	
21	g. 1331 T > G	c. 1331 T > G	p. L444S
22	g. ND	c. 3414_3776 del	p. fs G1137
23	g. 1208 G > T	c. 1208 G > U	p. G403V
24	g. 2605 G > A	c. 2605 G > A	p. G869R
25	g. 2267 C > A	c. 2267 C > A	p. P756H
26	g. 4271G > A	c. 4271G > A	p. G1424E
27	g. 834+5 G > T	c. 781_834 del	p. G261_P278 del
28	g. 1423+56_1423+57 ins C	c. 1387_1422 del	p. G463_K474 del
29	g. 1423+56_1423+57 ins C	c. 1387_1422 del	p. G463_K474 del
30	g. 3088 G > A	c. 3088 G > A	p. G1030S
31	g. 3088 G > A	c. 3088 G > A	p. G1030S
32	g. 4342 G > A	c. 4342 G > A	p. G1448S
33	g. 2858 G > T	c. 2858 G > T	p. G953V
34	g. 2858 G > T	c. 2858 G > T	p. G953V
35	g. 3694 G > A	c. 3694 G > A	p. G1232S
36	g. 3481 G > A	c. 3481 G > A	p. G1161R

Abbreviations: DF, not found; ND, not done.

X-inactivation, 20% (4/20) of individuals over 30 years of age had skewed X-inactivation in peripheral blood cells ($\chi^2 = 2.743$, $P = 0.098$, Figure 1). With a less stringent criterion for skewing of ratios equal to or greater than 70%:30%, 33.3% (4/12) of individuals under 30 years old had skewed X-inactivation compared with 25% (5/20) of individuals over 30 years old ($\chi^2 = 0.258$, $P = 0.612$).

Tissue-specific variations of X-inactivation

Of the 36 female patients, 4 were uninformative for the *AR* (CAG)_n polymorphism. Of the remaining 32 individuals, skin fibroblasts were obtained from 12 of them. The X-inactivation patterns of the 12 patients showed marked

variation between blood cells and skin fibroblasts. Three individuals showed skewed X-inactivation in skin fibroblasts but not in blood cells, and one individual showed skewed X-inactivation in blood cells but not in skin fibroblasts. Seven of 12 patients (58.33%) had similar X-inactivation ratios in both tissues, but the other 5 patients (41.67%) had the opposite X-inactivation ratios in both tissues (Table 2). There was no correlation between the X-inactivation ratios in skin fibroblasts and in peripheral blood cells ($r = 0.180$, $P = 0.575$, Figure 2).

Discussion

It is well known that the variable phenotype of XLAS females cannot be understood solely by analyzing mutation

Table 2 Clinical features and X-inactivation patterns of XLAS females

Patient number	Age Years	Eye abnormalities	EBM a5 (IV) staining	Renal insufficiency	Degree of proteinuria	X-inactivation rate of the mutant allele	
						Peripheral blood	Skin fibroblast
1	33	No	Mosaic	No	2+	Uninformative	ND
2	29	No	Mosaic	No	1+	73.62%	80.95%
3	27	No	Mosaic	Yes	3+	49.16%	ND
4	4	No	Mosaic	No	Negative	73.45%	74.10%
5	34	No	Mosaic	No	3+	19.96%	54.50%
6	57	No	Mosaic	No	3+	46.36%	ND
7	36	No	Mosaic	No	Negative	39.15%	ND
8	28	No	Mosaic	No	2+	Uninformative	ND
9	36	No	Mosaic	No	2+	Uninformative	ND
10	28	No	Mosaic	No	3+	22.46%	ND
11	48	No	Mosaic	No	3+	65.72%	55.87%
12	46	No	Mosaic	No	Negative	83.38%	ND
13	39	No	Mosaic	No	Negative	65.52%	ND
14	37	No	Mosaic	No	3+	16.74%	ND
15	31	No	Mosaic	No	1+	33.43%	34.34%
16	46	No	Mosaic	No	Negative	51.13%	ND
17	43	No	Mosaic	No	Negative	54.73%	ND
18	37	No	Mosaic	No	Negative	52.18%	82.56%
19	26	No	Mosaic	No	Negative	51.16%	ND
20	30	No	Mosaic	No	1+	43.33%	ND
21	36	No	Mosaic	No	Negative	55.79%	38.76%
22	32	No	Mosaic	No	Negative	Uninformative	ND
23	39	No	Mosaic	No	2+	34.70%	ND
24	41	No	Mosaic	Yes	2+	34.91%	43.41%
25	32	No	Mosaic	No	3+	21.70%	68.91%
26	28	No	Mosaic	No	1+	57.41%	ND
27	4	No	Mosaic	No	Negative	34.11%	ND
28	5	No	Mosaic	No	2+	29.72%	ND
29	35	No	Mosaic	No	1+	87.38%	ND
30	6	No	Mosaic	No	1+	37.91%	ND
31	42	No	Mosaic	Yes	2+	45.02%	ND
32	13	No	Mosaic	No	Negative	46.38%	ND
33	4	No	Mosaic	No	Negative	53.11%	9.56%
34	34	No	Mosaic	No	1+	46.01%	21.52%
35	37	No	Mosaic	No	Negative	67.29%	ND
36	36	No	Mosaic	No	2+	68.35%	39.45%

Abbreviations: EBM, epidermal basement membrane; ND, not done; XLAS, X-linked dominant Alport syndrome.

types of the *COL4A5* gene. X-inactivation was speculated to correlate this with phenotypes, and few reports provided proof.^{9,12} However, our previous study did not find any correlation between X-inactivation and the phenotype of XLAS females,¹¹ as did the study of Vetrie et al and Bell et al.^{10,13} The contradictory results may be due to the age and tissue specificity of X-inactivation, so we analyzed X-inactivation ratios in XLAS females of different ages and tissues.

Results showed that four patients were homozygous for the *AR* gene; the rate of heterozygosity for *AR* gene would be 88.89% in our study, which was quite similar to previous reports from other study groups (86.5%–90%).^{16,24} Based on

an X-inactivation ratio of equal to or greater than 80%:20% as a criterion for skewing, only 12.50% (4/32) had a skewed X-inactivation pattern in peripheral blood cells, which is consistent with data previously reported from others using the *AR* PCR assay for studying other diseases.¹⁶

No individuals under 30 years of age had skewed X-inactivation, whereas 20% of individuals over 30 years of age had skewed X-inactivation in peripheral blood cells. It seems that skewed X-inactivation can be acquired with age in XLAS females but had no statistical significance ($\chi^2 = 2.743$, $P = 0.098$). This is consistent with recent studies that show aging to have little effect on X-inactivation.^{25,26}

Figure 1 Distribution of X-inactivation ratios in blood of XLAS females aged ≤ 30 years and >30 years. Inactivation values are expressed as the percentage ratio of the predominantly inactive allele to the predominantly active allele and are arranged into 10% or 20% intervals.

That is, X inactivation was stationary because aging had little effect on it, whereas the clinical findings on proteinuria and renal insufficiency in XLAS females were dynamic, given that X activation did correlate with the phenotype of XLAS females, regardless of age, clinical marker, tissue, etc. Most of our female patients were under 50 years old and needed further follow-up studies. Furthermore, the X-inactivation pattern in blood cells was not consistent with that in skin fibroblast in our study, which was similar to the previous studies by other groups showing tissue-specific X-inactivation variation.^{15,16} Although some studies reported that there was a correlation between the X-inactivation ratios in skin and blood,^{27,28} we did not reveal any correlation between blood cells and skin fibroblasts for the X-inactivation ratios in female XLAS patients in our study ($r = 0.180$, $P = 0.575$).

In addition, except for the age and tissue specificity of X-inactivation, the different methods used in analysis of X-inactivation may also contribute to the contradictory

Figure 2 The correlation between X-inactivation ratios in peripheral blood cells and in skin fibroblasts.

results of XLAS females. The traditional method used southern hybridization to analyze the heterozygosity of the hypoxanthine phosphoribosyl transferase (*HPRT*) gene or phosphoglycerate kinase (*PGK*) gene,^{10,27} whereas, recently, most studies used methylation-sensitive restriction endonuclease digestion and PCR of the *AR* gene to analyze X-inactivation.^{20,29} It is well known that heterozygosity of the *AR* gene was much higher than that of the *HPRT* or *PGK* gene. In addition, if there is age and tissue specificity of X-inactivation, maybe only the X-inactivation pattern in renal tissues could really explain the phenotype of XLAS females. Therefore, further multicentric studies are needed, using the same method and renal tissues, in order to have an accurate result on this issue.

In conclusion, there was not age-specific variation of X-inactivation ratios in XLAS females but there was tissue-specific variation. Maybe only the X-inactivation pattern in renal tissues could really explain the phenotype of XLAS females. This could explain the contradictory results between X-inactivation and the variable phenotype of XLAS females. Nevertheless, we believe that there may be other mechanisms that impact the phenotype variations of female XLAS, such as DNA methylation, histone acetylation, and noncoding RNAs.^{30,31}

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Disclosure

No conflicts of interest were declared in relation to this paper.

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