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References


Symptoms in Carriers of Adrenoleukodystrophy Relate to Skewed X Inactivation

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Skewing of X inactivation may contribute to the manifestation of symptoms in adrenoleukodystrophy carriers. We observed highly skewed X inactivation in 32% of 22 symptomatic and asymptomatic carriers but not in 7 related and 35 unrelated controls. Skewing of X inactivation correlated with clinical neurological scores but not with the extent of very long-chain fatty acid accumulation. Transcript analysis in cultured fibroblasts revealed that skewing could occur both in favor of the mutant and the wild-type allele. Adrenoleukodystrophy carriers are more susceptible to develop skewing of X inactivation in favor of the mutant allele being associated with the manifestation of symptoms. Ann Neurol 2002;52:683–688

More than 50% of female carriers of X-linked adrenoleukodystrophy (X-ALD, MIM 300100; OMIM, http://www.ncbi.nlm.nih.gov/Omim/) show slight neurological abnormalities like hyperreflexia or a decreased vibration sense in the lower limbs. Approximately 20% develop neurological disability resembling milder forms of adrenomyeloneuropathy.1 The mean age of onset is 37.8 ± 14.6 years, and carriers frequently are misdiagnosed with multiple sclerosis.2 Approximately 85% of obligate heterozygotes show elevated concentrations of very long-chain fatty acids (VLCFAs) in plasma. Normal concentrations of VLCFAs in plasma therefore cannot exclude heterozygosity, and mutation analysis is the most reliable technique for carrier identification.2 It has been suggested that skewing of X inactivation contributes to the phenotypic variability in carriers,3

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but only a few carriers have been studied so far and data remain conflicting. To evaluate the relations between X inactivation, concentrations of plasma VLCFAs, and clinical symptoms, we analyzed patterns of X inactivation in a series of X-ALD heterozygotes compared with two control groups.

**Patients and Methods**

**Patients**

We analyzed blood samples from 22 carriers and 7 noncarriers from a total of 19 X-ALD families and 35 unrelated control individuals. Mutation analysis of the underlying *ABCD-1* gene was used to either confirm the carrier status in all carriers with the exception of C-7 or exclude carrier status in all related noncarriers. Carrier C-7 was considered to be an obligate heterozygote because her plasma VLCFAs were clearly elevated and her family history reported two affected relatives with X-ALD. Skin fibroblast cultures were available from three X-ALD carriers. The age-matched control group consisted of 40 healthy women, aged 21 to 61 years (mean, 38.6).

**X-Inactivation Analysis in Blood Leukocytes**

DNA was extracted from leukocytes by a standard salting out procedure. The patterns of X inactivation were determined by polymerase chain reaction (PCR) analysis of a polymorphic CAG repeat within the human androgen receptor gene (*HUMARA assay*) with few modifications. For each sample, aliquots of DNA (500ng) were digested with *Hpa*I and *Rsa*I, *Cfo*I and *Rsd*I, and two control enzymes *Rsd*I and *Msp*I, respectively.

DNA aliquots were incubated with 40U of each restriction enzyme (Roche Diagnostics, Mannheim, Germany) for 24 hours. The cleaved DNA (50ng) was used as a template for PCR amplification of the HUMARA repeat using AR-1 (5'-carboxy-fluorescein-GCGCGAATGGATTCGAGAAG-3') and AR-2 (5'-GCCCTCAGATGGGCTTG-3') as primers. The PCR products were analyzed on an ABI 377 sequencer (Applied Biosystems, Foster City, CA) and subsequently evaluated using GeneScan software (Applied Biosystems).

**Transcript Analysis in Cultured Fibroblast Tissues**

Because genes are transcribed only from the active X chromosome, in carriers demonstrating highly skewed X inactivation, RNA transcripts were analyzed to investigate whether the mutant or the wild-type ALD allele was predominantly active. cDNA synthesis from total RNA was performed with a first-strand cDNA synthesis kit (Pharmacia Amersham Biotech Europe, Freiburg, Germany) with the *ABCD-1*-specific primers ALD-5 (5'-TTCTGTCGCCGCTGCTGAT-3') for carriers CF-1 and CF-2 (C-11) and ALD-9 (5'-TTCTGCTGGATCCGAGCTTG-3') for carrier CF-3. The reverse transcripts were used as a template for PCR amplification of exon 1 fragment B (CF-1) and fragment C (CF-2) as reported previously. Reverse transcript PCR for CF-3 was conducted with primers ALD-20 (5'-CTCCATC-ACCCACGGGC-3') and ALD-9. For each carrier, corresponding fragments of the *ABCD-1* gene also were amplified from genomic fibroblast DNA.

**Biochemical Evaluation**

VLCFAs were quantified in plasma and/or fibroblast cultures by standard procedures.

**Clinical Evaluation**

Nineteen carriers underwent detailed neurological examination. The age of these female carriers ranged from 5 to 67 years (mean age, 42.4 years). Eight carriers reported subjectively felt neurological symptoms like paraesthesias and gait disturbances. Eleven apparently healthy carriers were examined because of their positive family history. Disability was assessed with the disability status scale (DSS) and the expanded disability status scale (EDSS). Additionally, walking ability was rated according to the ambulation index (AI). Two carriers (C-13 and C-19) refused neurological examination because they personally felt no health deficits. One carrier (C-17) could not be traced.

**Results**

**X Inactivation Analysis of X-linked Adrenoleukodystrophy Carriers, Related Noncarriers, and Controls**

Seven of 22 X-ALD carriers (32%) demonstrated highly skewed X inactivation, 8 carriers (36%) moderate skewing, and 7 carriers (32%) random X inactivation (see Fig 1A; Table). Highly skewed patterns of X inactivation were not observed in seven related noncarriers (see Fig 1B) and 35 nonrelated female controls (see Fig 1C). Both control groups showed a clear tendency to balanced patterns of X inactivation. The differences between the distribution of X inactivation patterns in X-ALD heterozygotes and female controls were significant (*p* < 0.05; Mann–Whitney *U* test). The finding of highly skewed patterns of X inactivation in seven X-ALD carriers and none of the female controls also proved to be significant (*p* < 0.05; Fisher’s exact test). Because of the small sample size, statistical evaluation was not performed in related noncarriers.

**X Inactivation and Concentrations of Very Long-Chain Fatty Acids**

Eighteen of 19 carriers available for biochemical analysis showed elevated concentrations of VLCFAs (see Table). All related noncarriers displayed normal biochemical parameters (data not shown). No significant correlation was observed between the extent of skewing of X inactivation and the carriers’ biochemical measures, namely, the plasma concentration of C26:0 (*p* = 0.532; Spearman *p* correlation), the ratio C24:0 to C22:0 (*p* = 0.936), C26:0 to C22:0 (*p* = 0.737), and the discriminant value *y* (*p* = 0.695).
Inactivation and Clinical Findings

The clinical features of the eight symptomatic carriers presenting with different degrees of spastic paraparesis, pyramidal tract abnormalities, ataxia, and somatosensory deficits were consistent with the diagnosis of mild adrenomyeloneuropathy. The median clinical scores were 3 for DSS, 3.75 for EDSS, and 2 for AI. The age of symptomatic carriers ranged from 35 to 61 years (mean, 51.6) with a mean age at onset of 43.8 years (see Table).

None of the 11 healthy carriers demonstrated abnormal findings on neurological examination. In this group, the age ranged from 5 to 61 years (mean, 33.5). The two carriers refusing clinical examination were rated AI = 0 after a telephone interview (see Table).

All symptomatic carriers showed skewing of X inactivation, whereas 6 of 12 asymptomatic carriers demonstrated random X inactivation and 4 moderate skewing \((p = 0.006; \text{Mann–Whitney} \ U \text{ test})\). The association between the extent of skewing of X inactivation and severity of clinical findings gave significant results for DSS \((p = 0.001; \text{Spearman} \ \rho \text{ correlation})\), EDSS \((p = 0.008)\), and AI \((p = 0.003; \text{see Table})\). However, two carriers did not show any neurological abnormalities despite highly skewed patterns of X inactivation (C-4 and C-5).

Transcript Analysis in Fibroblasts of Three X-linked Adrenoleukodystrophy Carriers Showing Highly Skewed X Inactivation

Transcript analysis showed solely the mutant allele (S108W) in carrier FC-1 (see Fig 2A), indicating that the mutant allele was predominantly active. In contrast, carriers FC-2 (see Fig 2B) and FC-3 (see Fig 2C) demonstrated solely wild-type allele, indicating that the wild-type allele was predominantly expressed in these two carriers. These data show that nonrandom X inactivation in X-ALD carriers can occur both in favor of the wild-type and the mutant allele.

Discussion

Inactivation of one of the two X-chromosomes in female somatic cells is considered to be a random process following a Gaussian distribution with highly skewed patterns of X inactivation being rare events in healthy female controls.12 Consistent with these postulations and the findings of other recent studies,7,13 we found random X inactivation or only moderate skewing in our two control groups. None of the control individuals showed high skewing of X inactivation. In contrast, our group of 22 X-ALD carriers showed equal proportions of random X inactivation, moderate and high skewing. This is clearly different from the rather bell-shaped normal distribution we observed in both control groups (see Fig 1) and provides proof that female carriers of X-ALD are prone to skewing of X inactivation.

We propose that the skewing of X inactivation is related to the mutant ALD allele and not due to other secondary influences. The carriers displaying highly skewed X inactivation belonged to seven unrelated kindreds. An additional genetic influence appears unlikely to have occurred, because none of the seven X-ALD
family members who were excluded as carriers showed highly skewed X inactivation.\textsuperscript{13,15} The most probable explanation for skewing of X inactivation in carriers of X-ALD is the occurrence of postinactivation selection mechanisms.\textsuperscript{12} Exceptionally, in X-ALD these selection mechanisms have been suggested to result in a growth advantage for cells expressing the mutated allele rather than the wild-type allele leading to elevated concentrations of VLCFAs and the development of neurological symptoms in carriers.\textsuperscript{3} In this study, we found no significant correlation between the extent of skewing and the concentrations of VLCFAs in plasma of 19 individual carriers. Although the accumulation of VLCFAs may contribute to the development of neurological symptoms of X-ALD patients and carriers, it cannot explain the clinical heterogeneity of the disease.\textsuperscript{2} Our findings might support the hypothesis of other yet undefined disease-causing mechanisms.\textsuperscript{16,17}

We, however, indicate a significant correlation between X inactivation and clinical scores assessing neurological symptoms. All X-ALD carriers demonstrating neurological symptoms showed skewing of X inactivation, and severely affected carriers demonstrated more pronounced skewing than mildly affected ones (see Table). These findings contrast the conclusions of a previous report on only three symptomatic carriers suggesting that skewing of X inactivation in X-ALD carriers was not related to clinical manifestations.\textsuperscript{4} A preliminary note of Naidu and colleagues recently has reported a correlation between clinical severity and the proportion of fibroblasts lacking immunoreactivity for adrenoleukodystrophy protein.\textsuperscript{18} This observation reflects X inactivation in cultured fibroblasts from X-ALD carriers and supports our findings in blood leukocytes.

### Table. Genotype, X Inactivation in Leukocytes, Clinical and Biochemical Findings of Female Carriers of X-Linked Adrenoleukodystrophy

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Age (yr)</th>
<th>Genotype</th>
<th>Mutation</th>
<th>X Inactivation\textsuperscript{a}</th>
<th>Symptoms (age of onset, yr)</th>
<th>DSS</th>
<th>EDSS</th>
<th>AI</th>
<th>C26/C24/ C22</th>
<th>C26/C22</th>
<th>Y</th>
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<tbody>
<tr>
<td>C-1</td>
<td>58</td>
<td>N148S</td>
<td>100:0</td>
<td>Yes (54)</td>
<td>3</td>
<td>4.0</td>
<td>2</td>
<td>1.004</td>
<td>0.982</td>
<td>0.032</td>
<td>0.92</td>
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<tr>
<td>C-2\textsuperscript{c}</td>
<td>56</td>
<td>Y212X</td>
<td>93:7</td>
<td>Yes (40)</td>
<td>3</td>
<td>3.5</td>
<td>2</td>
<td>0.798</td>
<td>1.280</td>
<td>0.028</td>
<td>0.96</td>
</tr>
<tr>
<td>C-3\textsuperscript{c}</td>
<td>42</td>
<td>G512S</td>
<td>92:08</td>
<td>Yes (36)</td>
<td>1</td>
<td>3.5</td>
<td>2</td>
<td>1.272</td>
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<tr>
<td>C-4</td>
<td>61</td>
<td>G512S</td>
<td>88:12</td>
<td>No</td>
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<td>0</td>
<td>0</td>
<td>0.848</td>
<td>1.048</td>
<td>0.029</td>
<td>0.88</td>
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<tr>
<td>C-5</td>
<td>56</td>
<td>c.274-311del</td>
<td>82:18</td>
<td>No</td>
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<td>0</td>
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<td>C-6</td>
<td>57</td>
<td>c.829-832insAAAT</td>
<td>82:18</td>
<td>Yes (53)</td>
<td>3</td>
<td>3.5</td>
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<td>0.508</td>
<td>1.242</td>
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<td>C-7</td>
<td>46</td>
<td>Not analyzed</td>
<td>81:19</td>
<td>Yes (39)</td>
<td>3</td>
<td>3.0</td>
<td>2</td>
<td>0.976</td>
<td>1.546</td>
<td>0.047</td>
<td>1.17</td>
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<tr>
<td>C-8</td>
<td>42</td>
<td>V208E</td>
<td>75:25</td>
<td>No</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>C-9</td>
<td>35</td>
<td>P543L</td>
<td>74:26</td>
<td>Yes (24)</td>
<td>3</td>
<td>3.0</td>
<td>3</td>
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<td>1.479</td>
<td>0.053</td>
<td>0.95</td>
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<tr>
<td>C-10\textsuperscript{a}</td>
<td>58</td>
<td>T105P</td>
<td>73:27</td>
<td>Yes (50)</td>
<td>2</td>
<td>2.0</td>
<td>2</td>
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<td>1.474</td>
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<tr>
<td>C-11</td>
<td>40</td>
<td>Q466X</td>
<td>71:29</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.017</td>
<td>1.085</td>
<td>0.035</td>
<td>0.98</td>
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<tr>
<td>C-12</td>
<td>61</td>
<td>R401Q</td>
<td>71:29</td>
<td>Yes (54)</td>
<td>2</td>
<td>2.5</td>
<td>1</td>
<td>0.643</td>
<td>1.249</td>
<td>0.039</td>
<td>0.87</td>
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<td>C-13</td>
<td>39</td>
<td>S213C</td>
<td>70:30</td>
<td>No</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.358</td>
<td>0.811</td>
<td>0.016</td>
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<tr>
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<td>14</td>
<td>P560L</td>
<td>68:32</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.759</td>
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<tr>
<td>C-15</td>
<td>26</td>
<td>G512S</td>
<td>66:34</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1.131</td>
<td>1.264</td>
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<tr>
<td>C-16</td>
<td>13</td>
<td>c.1746delC</td>
<td>64:36</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.233</td>
<td>1.346</td>
<td>0.048</td>
<td>1.20</td>
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<tr>
<td>C-17</td>
<td>54</td>
<td>L160P</td>
<td>61:39</td>
<td>n.a.</td>
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<tr>
<td>C-18</td>
<td>5</td>
<td>P560L</td>
<td>60:40</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.017</td>
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<td>C-19</td>
<td>44</td>
<td>c.1415-1416delAG</td>
<td>59:41</td>
<td>No</td>
<td>n.a.</td>
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<td>0.575</td>
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<td>32</td>
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<td>59:41</td>
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<td>14</td>
<td>V208E</td>
<td>58:42</td>
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<tr>
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<td>50</td>
<td>IVS7 + 2T→G</td>
<td>57:43</td>
<td>No</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.019</td>
<td>1.148</td>
<td>0.029</td>
<td>1.00</td>
</tr>
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</table>

\textsuperscript{a}Random X inactivation: 50:50–64:36; moderately skewed X inactivation: 65:35–80:20; highly skewed X inactivation: >80:20.\textsuperscript{19}

\textsuperscript{b}VLCFA normal ranges: C26: 0.15–0.51 g/ml; C24/C22: 0.76–0.92; C26/C22: 0.004–0.022; discriminant analysis: individuals with y values >0.725 are considered to be female carriers of X-ALD.\textsuperscript{2}

\textsuperscript{c}The clinical findings of three of the carriers in this study recently have been reported in detail.\textsuperscript{20}

\textsuperscript{d}Mann–Whitney U test.

\textsuperscript{e}Nonparametric Spearman correlation.

X-ALD = X-linked adrenoleukodystrophy; DSS = disability status scale; EDSS: extended disability status scale; AI: ambulation index; n.a. = data not available.
Although X-ALD carriers were prone to skewing of X inactivation, there was a significant share of clinically asymptomatic carriers showing random patterns of X inactivation. These individuals were younger than the symptomatic carriers (mean age, 29 vs. 51.6 years). One might suggest that selection mechanisms in X-ALD might act slowly in time because of a mild postulated growth advantage exerted by the mutant allele. This would lead to a gradual loss of the wild-type cell population with young heterozygotes having random X inactivation and older ones showing skewing.12 Our hypothesis is consistent with the finding that heterozygotes develop symptoms only at later ages.1 It cannot be answered to date whether the X-inactivation patterns in the younger individuals of our group will be stable during their lives or whether neurological symptoms will occur.

Interestingly, two carriers (C-4 and C-5) demonstrated no neurological abnormalities despite highly skewed X inactivation. This observation does not go together with the mutant allele being predominantly active. Performing transcript analysis in three cultured fibroblast cell lines, we found that skewing can be associated both with a predominance of the mutant and the wild-type allele (see Fig 2). Because selection mechanisms are supposed to occur unidirectionally,12 this is an exceptional but nevertheless conclusive finding. It implies that the demonstration of skewed X inactivation in an individual carrier does not necessarily result in a clinically detrimental influence of the mutant allele. However, the overall association of skewing with neurological symptoms is consistent with the previous hypothesis that the mutant allele is favored in most cases.3 The finding of skewed X inactivation in a carrier therefore would suggest a higher risk of becoming symptomatic. The underlying mechanism triggering this phenomenon must remain speculative to date. One could propose an influence of modifying factors affecting the postinactivation selection mechanisms as suggested for carriers of Barth syndrome13 or modifying effects postulated to explain the marked variance in the phenotypic expression of X-ALD.16,17

In this study, we provide conclusive evidence for a high frequency of skewed X inactivation in leukocytes.
of women heterozygous for X-ALD. Skewing of X inactivation was related to neurological manifestation but not to concentrations of VLCFAs in plasma.

We thank all the clinicians who provided clinical data and blood samples from X-ALD patients and their families. We are grateful to M. Dugas for assistance with the statistical analyses, T. Meitinger and K. Teuchert for helpful discussions, and A. Vielhauer and H. Hinz for excellent technical assistance.

References