J Neurol (2001) 248 : 856 – 860

ORIGINAL COMMUNICATION

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Clinical features and skewed X-chromosome inactivation in female carriers of X-linked recessive spinal and bulbar muscular atrophy

Received: 14 November 2000
Received in revised form: 5 March 2001
Accepted: 25 March 2001

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Abstract In X-linked recessive disorders, a few female gene carriers become symptomatic. Recent evidence implicates skewed X-chromosome inactivation in such female carriers. We studied the clinical features of eight female gene carriers of X-linked recessive spinal and bulbar muscular atrophy (SBMA), and evaluated the relationship between phenotype and genotype from the viewpoint of X-chromosome inactivation. Seven of eight cases were symptomatic, showing mild muscle weakness, frequent muscle cramps, slight elevation of the serum creatinine kinase level, or neurogenic changes on the electromyogram. Only one carrier was asymptomatic clinically. For the estimation of X-chromosome inactivation, the methylation status of the androgen receptor (AR) gene was determined by polymerase chain reaction-based assay. Highly skewed inactivation of the affected AR gene was found in the asymptomatic carrier, while symptomatic carriers had a random or lower inactivation pattern of the affected AR gene. These findings suggest that most female carriers of SBMA show some clinical abnormalities, and highly skewed inactivation of the affected X-chromosome seems to closely relate with escape of the manifestation in female carriers of SBMA.

Key words X-linked spinal and bulbar muscular atrophy · Skewed X-chromosome inactivation · Androgen receptor gene · Female gene carrier

Introduction

X-linked recessive spinal and bulbar muscular atrophy (SBMA) is an adult-onset, slowly progressive motor neuron disease characterized by proximal muscle weakness, bulbar palsy, contraction fasciculation, postural tremor, and gynecomastia [1]. La Spada et al. detected the elongated CAG repeat sequence in the first exon of the androgen receptor (AR) locus on the X-chromosome in SBMA patients [2]. Since then, it has been possible to find the asymptomatic patients and the female gene carriers of SBMA.

In X-linked recessive inherited disorders, only males manifest the disease. Female relatives are usually asymptomatic carriers. However, a few female carriers showing mild clinical symptoms have been reported as symptomatic carriers in some X-linked recessive disorders [3–7]. In such disorders, skewed X-chromosome inactivation has been considered to correlate with the manifestation of the symptoms in female carriers [4,5]. Subclinical symptomatic female carriers of SBMA have been described in some reports, but no relationship with X-inactivation has been demonstrated [8–10]. In this study, we examined the relationship between clinical features and the skewedness of X-chromosome inactivation in female carriers of SBMA.

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Subjects and methods

Subjects

We studied eight female SBMA gene carriers from three unrelated families. These patients and carriers all gave informed consent regarding this study, and were diagnosed by detecting the elongated CAG repeat sequence in the AR gene by the method reported previously [2].

Clinical evaluation

We collected the data about subjects' symptoms, the onset age of symptoms, neurological findings, electromyogram, and serum creatinine kinase (CK) levels prospectively according to protocol for the purpose of this study. The skilled neurologist without knowledge of the results of X-inactivation assay interviewed the subjects about their symptoms, and carried out neurological examinations and electromyography. The electromyogram was recorded from the right biceps muscle.

Digestion by Hpa II enzyme

The degree of inactivation of an X-chromosome is recognised from the methylation status of DNA [11,12]. The target of methylation is the deoxycytosine residue in CpG dinucleotides. The restriction enzyme Hpa II recognizes this sequence and digests DNA if methylation has not occurred. Allen et al. demonstrated that the methylation of the two Hpa II sites in the first exon of the human AR locus correlates with X-inactivation [13]. To determine the X-chromosome inactivation status in each female carrier, we modified a previously described method for quantifying the methylation of the AR gene [4,5,14].

Genomic DNA was extracted from peripheral lymphocytes using a DNA extraction kit (Sanko, Tokyo, Japan). For each DNA sample, two preparations were made. In one preparation, 1 μg DNA sample was incubated with the buffer containing 30 units Hpa II at 37°C for 4 hours. In the other, 1 μg of the same DNA was incubated with the buffer alone in parallel as a control. Both reactions were boiled for 5 min, spun briefly in a centrifuge, and 10 μl of the supernatant was removed for the PCR reaction.

PCR amplification of the AR gene

The DNA fragments of the AR gene including both the CAG repeat sequence and two methylation sites were amplified by PCR technique [2]. PCR was done with a thermal cycler (Koken-Rika, Tokyo, Japan) in a final volume of 30 μl containing 10 μl supernatant of the previous reactions, 14 μl H_2O, 60 pmol of each oligonucleotide primer (5'-TCCCAGAATCTGTTCCAGAGCG–3') and (5'-TGAAGGTTGCTGTTCCTCATCC–3'), 1.5 units AmpliTaq DNA polymerase (Boehringer Mannheim, Germany), 10 mM each deoxyribonucleotide triphosphate, and 3 μl 10 × PCR buffer supplied with the enzyme. The PCR conditions included an initial denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 1.5 min, and a final extension at 72°C for 8 min. Amplification occurred only if the CpG dinucleotides were methylated, since the unmethylated AR gene had already been digested by the Hpa II enzyme.

Electrophoresis and X-inactivation assay

5 μl of the product from each PCR sample was coelectrophoresed in 3 % agarose gel and stained with ethidium bromide. The signal intensity of the bands on the gel was measured by a charge-coupled device imaging system, the Densitograph AE–6900-F (Atto, Tokyo, Japan) [15]. For each carrier, the peak intensity ratio between two alleles using undigested PCR products was calculated and used as a correction factor. This correction factor was applied to compensate for unequal amplification of the alleles using digested PCR products. The degree of X-chromosome inactivation in the digested DNA was calculated by normalizing the sum of both alleles to 100 % by the following formula.

$$\% \text{ of active X} = \{1 - A/(A+B)\} \times 100\ (\%)$$

$$A: a \times d/c,\ B: b \times c/d,$$

a: small allele in digested DNA, b: large allele in digested DNA,
c: small allele in undigested DNA, d: large allele in undigested DNA.

Result

Clinical evaluation

Seven of eight carriers showed some clinically abnormal findings (Table 1). Five carriers (I–1, I–2, I–5, II–1, III–2) complained of frequent muscle cramps in the calves at night. Two carriers (I–4, II–1) had facial or limb muscle weakness. In four of five symptomatic carriers (I–2, I–3, I–5, II–1), the electromyogram showed chronic neurogenic changes with high voltage (5–7 mV) and long duration motor unit potentials. Three carriers refused electromyographic study. Mild elevation of the serum CK level was observed in one carrier (I–3). Only one carrier (III–1) had had no signs or symptoms by the age of 80 years.

Table 1 Clinical findings of female carriers of SBMA

Carrier No	Age (years)	Symptom (onset age) (years)	Muscle weakness			Serum CK (IU/l)	EMG findings
			Bulbar	Facial	Limbs		
I–1	68	frequent muscle cramp (63)	–	–	–	116	refused
I–2	57	frequent muscle cramp (50)	–	–	–	150	neurogenic change
I–3	48	none	–	–	–	285	neurogenic change
I–4	33	difficulty closing lids in sleep (29)	–	+	–	122	normal
I–5	29	frequent muscle cramp (24)	–	–	–	132	neurogenic change
II–1	72	limb weakness, muscle cramp (60)	+	+	+	70	neurogenic change
III–1	85	none	–	–	–	160	refused
III–2	73	frequent muscle cramp (60)	–	–	–	114	refused

CK: creatinine kinase (normal range 35–169 IU/l)

Table 2 Analysis of X chromosome inactivation assay

Carrier No	Clinical Abnormality	Number of CAG repeats		% of Active X	
		affected AR	wild type	affected AR	wild type
I–1	+	46	18	32	68
I–2	+	46	18	83*	17
I–3	+	46	18	78	22
I–4	+	46	17	53	47
I–5	+	46	18	37	63
II–1	+	49	17	34	66
III–1	–	49	19	8	92*
III–2	+	49	19	31	69

*: Skewed pattern of X-inactivation (cutoff point > 80 %)

CAG repeat size, age at onset, and clinical abnormality

The size of the CAG repeat is shown in Table 2. There was no apparent relationship between the size of the CAG repeat and clinical abnormalities in the female carriers. There was also no relationship between the age at onset and clinical abnormalities.

X inactivation ratio of the AR gene

The result of electrophoresis of X-inactivation assay is shown in the Figure. The allele of a male SBMA patient was digested completely by Hpa II, indicating that the male X-chromosome is not methylated at all. In the female carriers of SBMA, two alleles were obtained. The allele of the affected AR with the increased CAG repeat sequence could easily be distinguished from the one of the wild type by size. In a female with a random X-inactivation pattern, both alleles were amplified equally. On the other hand, in a female with a skewed X-inactivation pattern, the amplification was highly deviated. The ratio of 20:80 was the most often used cutoff point between random and skewed patterns of X-inactivation [14].

Two carriers (I–2, III–1) showed non-random patterns of X-inactivation (Table 2). One (III–1) was an asymptomatic carrier, and her X-inactivation was a skewed pattern showing a high inactivation rate of the affected AR gene of 92 %. The other (I–2) was a symptomatic carrier, and her X-inactivation was an inverted skewed pattern showing a low inactivation rate of the affected AR gene of only 17 %. The others were all symptomatic carriers and showed random patterns of X-inactivation with values of 22–69 %. This finding shows that the only highly skewed inactivation pattern of the affected AR gene correlates with the absence of clinical abnormality in the asymptomatic carrier.

Discussion

The incidence of both male and female patients in autosomal dominant disorders is almost equal. On the other hand, in X-linked recessive disorders, only males manifest the disorder and female gene carriers are generally asymptomatic. However, a few female carriers showing mild symptoms have been reported in some X-linked recessive disorders [3–7]. SBMA is one of the X-linked recessive disorders, but there are only few reports concerning the female carrier so far [8–10].

In our study, we found a high incidence (88 %) of symptomatic female carriers of SBMA. Although most carriers complained of only minor symptoms, a few had muscle weakness, as male SBMA patients do. On the electromyogram neurogenic changes were shown in several female carriers (75 %), as high an incidence as reported by Sobue et al. (63 %) [8]. These clinical examinations in our study suggest that the involvement of the motor neurons will also exist to some degree in female carriers of SBMA. Careful examination seems to in-

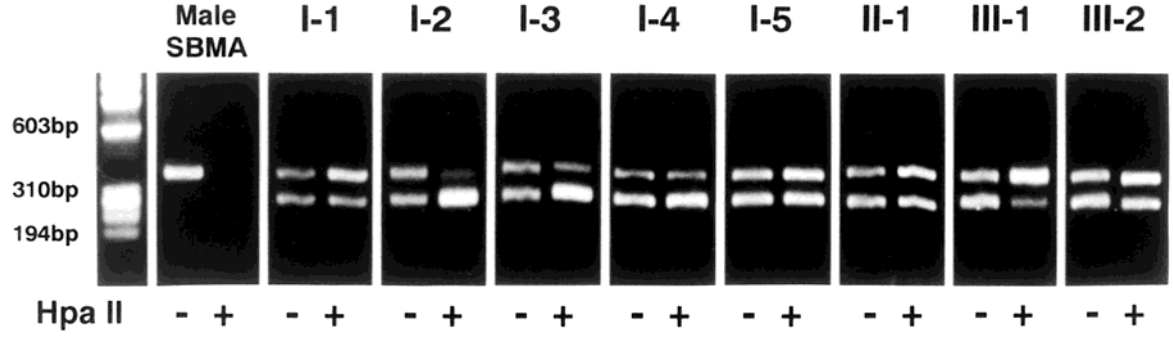

Fig. 1 X-inactivation PCR assay in female carriers of SBMA. In each lane, two alleles of the AR gene were obtained. The upper allele was from the affected X-chromosome, and the lower from the normal X-chromosome. The PCR products predigested by Hpa II are shown in the right lane, and the undigested ones in the left lane as controls. The carrier III–1 showed a highly skewed inactivation pattern of the affected AR gene. The carrier I–2 showed an inverted skewed inactivation pattern. Others showed random inactivation patterns. In a male SBMA patient, no amplification was obtained by predigestion of Hpa II, because the male X-chromosome is not methylated.

crease the recognition of symptomatic carriers in SBMA.

One of the two X-chromosomes in a female becomes inactive in an early stage during embryogenesis [16,17]. In each cell, there is equal probability of inactivation in either the paternal or the maternal X-chromosome, and random inactivation occurs in most females. The inactivation occurs when the number of the embryonic cells is very small, and females of 5–20 % have constitutional deviation of X-inactivation as the result of a chance event [14]. Recently it has been demonstrated that skewed inactivation of X-chromosomes correlates with symptomatic female carriers in some X-linked recessive disorders, such as Duchenne muscular dystrophy [4,5]. According to these reports, female carriers were symptomatic when lower inactivation had occurred in the affected X-chromosome. On the other hand, carriers were asymptomatic when random or highly skewed inactivation had occurred in the affected X-chromosome. In the female carriers of SBMA in our study, carriers were symptomatic when not only lower inactivation but also random inactivation had occurred in the affected X-chromosome. In the only asymptomatic carrier, highly skewed inactivation had occurred in the affected X-chromosome. As a result, the symptomatic carriers of SBMA showed a higher incidence than other X-linked recessive disorders do. Only highly skewed inactivation appeared to result in keeping patients asymptomatic.

Many X-linked recessive disorders develop owing to the direct loss of function by a mutant gene on the X-chromosome. Complete androgen receptor insensitivity syndrome, the so called testicular feminization syndrome, is inherited as an X-linked recessive disorder and is characterized by feminization of the male without neuromuscular disabilities. The direct loss of AR function by a point mutation or one base insertion in the AR gene causes the disease [18]. In X-linked recessive disorders caused by the direct loss of function, a gene of wild type restores the cell function to some degree even if a mutant gene has not been highly inactivated. On the other hand, in CAG repeat disorders including SBMA which is also one of the androgen receptor disorders, the cytotoxicity of expanded polyglutamine encoding by the CAG repeat sequence damages the neuron cells [19–22]. In SBMA caused by the cytotoxicity of polyglutamine to the spinal and brain stem neuron cells, the mutant gene reduces the function if the mutant gene has not been highly inactivated. The high frequency of symptomatic female carriers might be due to toxic or gain of function in SBMA that is X-linked recessive disorder.

In male patients with SBMA, a larger size of CAG repeats correlates with earlier onset and more serious symptoms. Otherwise, in female carriers of SBMA in our study, the size of CAG repeats correlates with neither the age at onset nor severity of symptoms. Our results of X-inactivation assay suggest that highly skewed X-inactivation of affected AR gene correlates with the absence of manifestation in the asymptomatic carrier. Among symptomatic carriers, however, the percentage of X-inactivation showed wide range, and did not correlate with the severity of clinical symptoms. The reason of the differences of clinical symptoms is unclear. Other environmental factors or complex factors may influence the manifestation in symptomatic carriers.

In our study, we used peripheral blood to evaluate X-inactivation status. Tissue heterogeneity of X-inactivation might influence the findings in our study. We, however, could not investigate the spinal motor neurons directly. In X-inactivation assay, most studies have been carried out on cells or DNA from the peripheral blood. Pegoraro et al. also demonstrated skewed X-inactivation using peripheral lymphocytes in symptomatic carriers of Duchenne muscular dystrophy [4]. Some reports demonstrated a similar pattern of X-inactivation when other tissues have been sampled [23]. However, there are currently no data concerning a systemic comparison of X-inactivation patterns in various tissues. Further studies will be necessary to confirm systemic X-inactivation in female carriers of SBMA.

In conclusion, most female carriers of SBMA have some clinical abnormalities, and only a few carriers have no symptoms or signs. Our clinical examinations indicated that motor neurons are also involved in the female carriers of SBMA, even though the degree of the damage is less than that in male SBMA patients. The fact that the only carrier with highly skewed inactivation of affected X-chromosome was asymptomatic suggested a relationship between skewed X-inactivation and escape of manifestation in female carriers of SBMA.

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