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ORIGINAL COMMUNICATION

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Polymorphic CAG repeat length in the androgen receptor gene and association with neurodegeneration in a heterozygous female carrier of Kennedy's disease

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Abstract Kennedy's disease (spinobulbar muscular atrophy) is an X-linked form of motor neuron disease affecting adult males carrying a CAG trinucleotide repeat expansion within the androgen receptor gene. While expression of Kennedy's disease is thought to be confined to males carrying the causative mutation, subclinical manifestations have been reported in a few female carriers of the disease. The reasons that females are protected from the disease are not clear, especially given that all other diseases caused by CAG expansions display dominant expression. In the current study, we report the identification of a heterozygote female carrying the Kennedy's disease mutation who was clinically diagnosed with motor neuron disease. We describe analysis of CAG repeat number in this individual as well as 33 relatives within the pedigree, including two male carriers of the Kennedy's mutation. The female heterozygote carried one expanded allele of the androgen receptor gene with CAG repeats numbering in the Kennedy's disease range (44 CAGs), with the normal allele numbering in the upper-normal range (28 CAGs). The subject has two sons, one of whom carries the mutant allele of the gene and has been clinically diagnosed with Kennedy's disease, whilst the other son carries the second allele of the gene with CAGs numbering in the upper normal range and displays a normal phenotype. This coexistence of motor neuron disease and the presence of one expanded allele and one allele at the upper limit of the normal range may be a coincidence. However, we hypothesize that the expression of the Kennedy's disease mutation combined with a second allele with a large but normal CAG repeat sequence may have contributed to the motor neuron degeneration displayed in the heterozygote female and discuss the possible reasons for phenotypic expression in particular individuals.

Key words Kennedy's disease · Spinobulbar muscular atrophy (SBMA) · Motor neuron disease · Androgen receptor · Polyglutamine repeat expansion

Introduction

Kennedy's disease, also known as X-linked spinobulbar muscular atrophy (SBMA), is an inherited form of motor neuron disease caused by an expansion of a (AR) CAG trinucleotide repeat in the androgen receptor gene (Fig. 1). First described in 1968 by Kennedy and colleagues [20], the genetic basis of this disease was only identified in 1991 [21]. Clinically, Kennedy's disease is

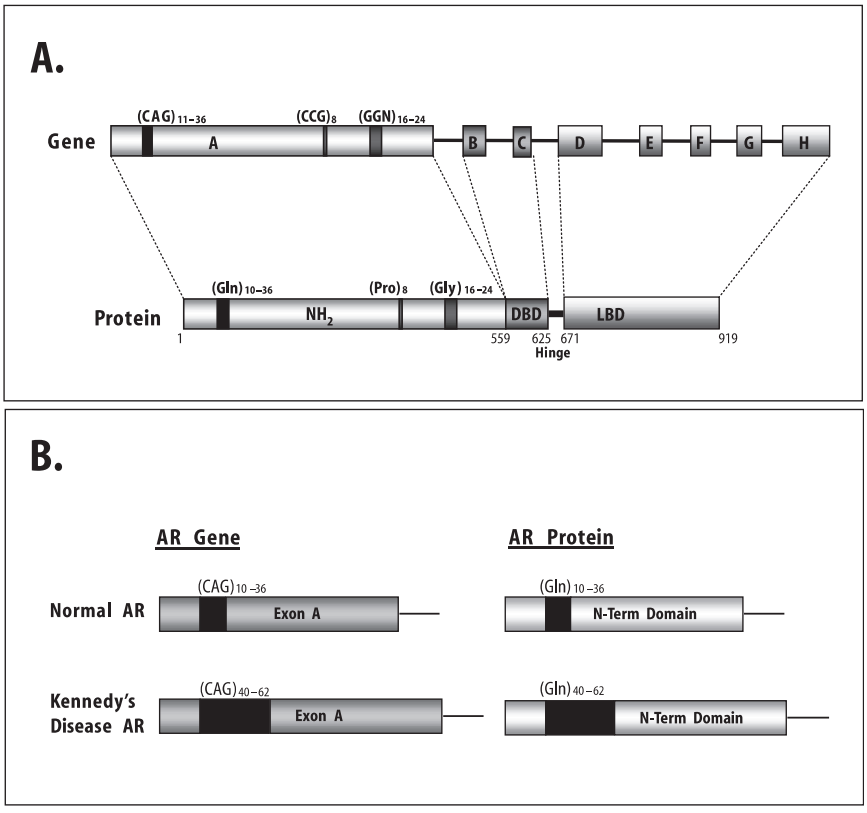

Fig. 1 Schematic diagram of androgen receptor. (**A**) Exon structure of the androgen receptor gene and the translated protein are shown. Exons A to H encode the different functional domains in the receptor protein: the N-terminal domain, the DNA binding domain (DBD), the hinge region, and the ligand binding domain (LBD). The N-terminal domain contains three trinucleotide repeat regions, a polyglutamine (CAG), a polyproline (GGN) and polyglycine (CCG) region. Amino acid numbers are indicated following the numbering used by Lubahn et al. [24]. (**B**) The CAG repeat region within exon A, encoding the polyglutamine tract within the N-terminal or transactivation domain of the receptor protein, in both the normal and pathogenic (Kennedy's disease) range

slowly progressive, presenting as proximal limb and bulbar muscular weakness, atrophy and fasciculation [3, 43]. Disease onset is normally in the fourth to fifth decades, with early symptoms such as muscle cramps commonly preceding the development of other signs by many years. Patients often have a postural tremor particularly affecting the hands, and areflexia in the lower limbs. Bulbar involvement results in dysarthria (speech impairment) and dysphagia (swallowing difficulty) and aspiration pneumonia may occur in the terminal stages of the disease. Expression of mild androgen insensitivity is also evident, with gynaecomastia, testicular atrophy, and reduced fertility occurring in approximately 50 % of patients.

Kennedy's disease was the first of eight inherited neurodegenerative diseases, including Huntington's disease, found to be caused by the expansion of a CAG repeat sequence within the coding region of affected genes [32]. The CAG repeat region in the androgen receptor is polymorphic in size within the normal population, ranging from 10 to 36 in number, whilst in Kennedy's disease the expanded repeat ranges from 40 to 62 [21]. The pathogenic mechanism involved is thought to be a 'toxic gain-of-function' conferred by the polyglutamine expansion, given that loss-of-function mutations within the androgen receptor result in a disorder of sexual differentiation, androgen insensitivity syndrome (AIS), with no evidence of neuropathy. The pathogenesis of this disease has been discussed in recent reviews [11, 23, 29].

Unlike the other polyglutamine diseases, which are autosomal dominant conditions, Kennedy's disease follows an X-linked pattern of inheritance, as the androgen receptor gene is located on the X-chromosome. Given that females do not develop Kennedy's disease, it was initially thought to be a recessive condition. However, the precise reason that heterozygote females do not present with Kennedy's disease is yet to be established. In the present study, we report a heterozygote female carrier of the Kennedy's disease mutation with features of motor neuron disease.

Subjects and methods

Subjects

Heterozygote female carrier of the Kennedy's disease mutation

The patient was a 75 year old woman with a history of progressive gait disorder who was the mother of a patient with Kennedy's disease. Weakness of the right leg had been progressing slowly for seven years. There was no clear history of swallowing difficulty or respiratory problems. Cognitive function had been declining for two years. On examination her cognition was significantly impaired. There was mild emotional lability. Significant dysarthria was present. The jaw

jerk was present and brisk. There was bilateral increased tone in the upper and lower limbs and deep tendon reflexes were present. Plantar responses were up going bilaterally. There was a tremor but cerebellar testing was normal with no nystagmus. The lower limbs were not wasted and there was no fasciculation in the upper or lower limbs or the face or tongue. Generalised spasticity was present in the upper and lower limbs bilaterally. CT revealed no evidence of cerebral infarct, tumour or other abnormality other than mild age associated cerebral atrophy. A clinical diagnosis of primary lateral sclerosis was considered. This pattern of neurological features is not typical of males with Kennedy's disease, as upper motor neuron features are not seen. The motor neuron features described do not meet fully with the El Escorial definition of amyotrophic lateral sclerosis (ALS), and fit more closely with the diagnosis of progressive lateral sclerosis [5]. The patient became progressively worse with increasing weakness and died of bronchopneumonia. Biochemical investigations reflected her general illness and the CK level was normal. Tissue retrieved from previous surgery on a basal cell carcinoma was obtained from which we were able to extract genomic DNA for analysis.

Other subjects

Informed consent was obtained from all participating subjects in this study and genetic counseling provided as appropriate. Ethical guidelines of the Royal Children's Hospital Research Committee (EHRC Ref. No. 22105 A; Melbourne, Australia) were adhered to, and were therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Clinical details were obtained for each participant in the study, including evidence of muscle weakness, cramps, fasciculation, or problems with swallowing or speech. None of the heterozygote female carriers of the Kennedy's disease mutation, apart from the mother of the index case, were found to display any symptomatology associated with motor neuron degeneration. Participants from this study are represented in generations III and IV in the pedigree in Fig. 2.

Note that individuals in generation V were, at the time of this study, below the age of consent for testing of asymptomatic individuals. Like Huntington's disease, Kennedy's disease is a late onset disease for which there is no currently established treatment and therefore no inherent advantage to knowledge of carrying the mutant allele. Given the characteristic late onset of Kennedy's disease, it is deemed appropriate that individuals who may carry the mutant allele of the AR gene make an informed decision to be genetically tested when they are adults. Such decisions may be important for family planning and vocational choice.

Individuals from generation I and II were deceased and therefore not able to be genetically tested, with the exception of the mother of the index case. A medical history of the remaining individuals was obtained from relatives and a number described symptoms consistent with Kennedy's disease.

The male index case from generation III (index case) was aged 57 years. Fasciculations in the legs were first noted in his mid 30s and cramps in the hands occurred in his 40s. At the time of this study he had weakness of the arms and legs and difficulty in walking. He had had a right mastectomy in 1980 for a breast lump which was found to be benign. Examination revealed wasting, weakness and fasciculation of the face and upper and lower limbs both proximally and distally. Bulbar weakness was present. All limb reflexes were absent. The right mastectomy was noted and the left breast was moderately enlarged. The patient was clinically depressed. MRI of the brain was structurally normal.

In generation IV, one male carrier of the Kennedy's disease allele was identified. The subject was a 31 year old male with a history of cramps affecting the shoulders, arms and thighs. He complained that his fingers locked when flexed. He also complained of his muscles "shaking". There had been a tremor of the hands for three years. There was no dysarthria and no respiratory symptoms. There were no other significant symptoms. On examination the patient was normal except for the neurological examination. Fasciculations were present in the upper limbs particularly the shoulder girdle bilaterally. There was no wasting of the upper limbs, lower limbs, face or tongue. There was mild proximal weakness of the shoulder and pelvic girdles and a

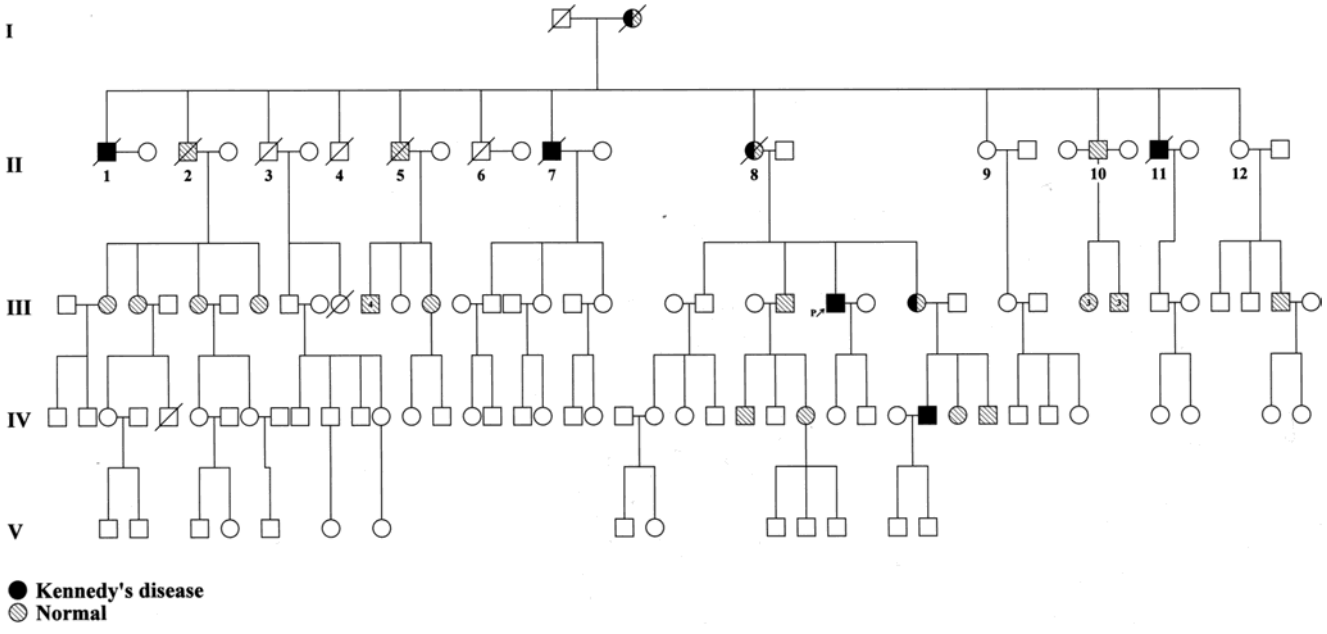

Fig. 2 Simplified pedigree of a kindred carrying the Kennedy's disease mutation. Circles and squares represent females and males, respectively, and roman numerals indicate generation. Index case in this family is indicated by an arrow. The mother of the index case is indicated by the number 8 in generation II. Individuals tested from generations III and IV have the symbol filled and genotype represented as indicated in legend. With the exception of individual number 8, genotypes of individuals from generation II were determined from detailed medical and family records kept by family members

slight decrease in the biceps reflex bilaterally. Other reflexes were normal including the lower limbs and jaw. Gag reflex was normal. There was no difficulty in swallowing or coughing. Signs of androgen insensitivity were present, including mild bilateral gynaecomastia and testicular atrophy.

Androgen receptor CAG repeat length analysis

DNA sample collection and extraction

Genomic DNA from the heterozygote mother of the index case was isolated from tissue sections from a biopsy specimen (basal cell carcinoma) embedded in paraffin using a modified version of a previously described technique [42]. Briefly, tissue samples were extracted by a 30 min incubation in histolene (Histolabs, Riverstone, NSW, Australia), followed by centrifugation (5 min, 20800 g). This step was repeated three times until paraffin was completely removed, and was subsequently followed by three washes in 100 % ethanol. Pelleted tissue was lyophilised, and resuspended in digestion buffer [50 mM Tris.Cl pH 7.5, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5 % Tween 20, 400 µg/ml Proteinase K (Sigma)]. Tissue was incubated at 55°C for 1 hour, following which a further 400 µg/ml Proteinase K aliquot was added and the sample incubated overnight at 55°C. DNA was extracted from the sample by two times phenol/chloroform extraction followed by an ethanol precipitation, following standard methodology. The resultant DNA pellet was resuspended in 10 mM Tris.Cl, pH 8.0, and stored at –20°C until analysis.

For all other subjects, 5 ml blood samples were obtained in heparinised tubes for extraction of genomic DNA. Isolation of DNA was by the sodium dodecyl sulphate (SDS)-proteinase K treatment method followed by standard phenol-chloroform extraction and sodium acetate/ethanol precipitation [37]. For each individual, duplicate blood samples were obtained and every sample found to be positive for the Kennedy's disease allele was re-tested to verify the original result, and to ensure that sample mix-ups had not occurred. All samples were resuspended in 10 mM Tris.Cl, pH 8.0 and stored at –20°C until analysis.

Polymerase Chain Reaction

For the purpose of determining CAG repeat number, polymerase chain reaction (PCR) amplification was conducted on each genomic DNA sample. Fluorescently-labelled oligonucleotide primers flanking the CAG repeat region in the first exon of the AR gene were used, with the sense and antisense primers, 5'-AGGCACCCAGAGGCCGCGAG-3' and 5'-TAGCCTGTGGGGCCTCTACGAT-3', respectively (Geneworks Pty Ltd., SA, Australia). An additional seven-base pair sequence (GTTTCTT) was added to the 5' end of the antisense primer to ensure nearly 100 % adenylation of the 3' end of the sense strand and minimize the occurrence of 1-base pair stuttering [2, 6]. PCR was performed using 1 µM primers, 1 Unit Amplitaq DNA polymerase (Perkin Elmer; Applied Biosystems), 2 mM $MgCl_2$ and 5 % dimethyl sulfoxide (DMSO; Sigma) and 25 mM dNTPs (Roche) in a final volume of 50 µl. The PCR amplification was conducted on a Perkin Elmer GeneAmp PCR System 9600 thermocycler and consisted of an initial denaturation step of 7 min at 95°C, followed by 38 cycles of 95°C denaturation for 1 min, annealing at 68°C for 45 s., and extension of products at 72°C for 45 s. The final extension step was for 7 min at 72°C. Fluorescent PCR products were electrophoresed on a 4 % denaturing polyacrylamide gel and detected via use of an ABI Prism 377 automated DNA sequencer (Perkin-Elmer). The data were subsequently analysed using the ABI Genescan® program (version 2.1, Perkin Elmer) with Genescan 350 size markers. An internal control sample of known CAG repeat number was included in each set of reactions. Pedigree analysis was conducted using the Genosketch program, available on the internet (http://ecademy.agnesscott.edu/Anthropology/SP00AntSyllabi/ANT307Households/GENOSKETCH/).

Results

Androgen receptor CAG repeat length analysis

The number of CAG repeat sequences in each of the individuals was assessed from sizing fluorescently-labelled PCR amplifications of the trinucleotide region of the androgen receptor gene from genomic DNA. The androgen receptor CAG repeat number was assessed in the heterozygote mother of the index case as well 33 relatives within the pedigree, as represented in Fig. 2. Analysis of genomic DNA from the mother revealed that she carried 44 and 28 CAG repeat nucleotides on the respective androgen receptor alleles; one in the Kennedy's disease range and the other at the upper end of the normal range. Clinical evaluation was consistent with motor neuron degeneration. Thirty-three individuals were tested from the pedigree, including 15 males and 18 females, across 3 generations. Of these, 2 males were found to carry the Kennedy's disease mutation and 13 carried normal alleles of the androgen receptor gene. Of the females tested, 14 carried normal alleles of the androgen receptor, and 4 females were found to be heterozygote carriers. The mutant allele in each of the heterozygote carriers was 44 CAGs, while the normal allele contained 24 CAG repeat sequences. None of the heterozygote female carriers of the Kennedy's disease mutation demonstrated any clinical manifestations of Kennedy's disease or other evidence of motor neuron degeneration.

Intergenerational stability of the CAG repeat in the androgen receptor gene

Genetic analysis of CAG repeat alleles across 3 generations of this pedigree was consistent with a high degree of stability. The mutant allele contained 44 CAGs in all three generations. There was no increase in size of the expansion. The pattern of inheritance through the pedigree was consistent with X-chromosome linkage.

Discussion

In the current study, we have analysed the trinucleotide repeat number within the androgen receptor alleles in the mother of a Kennedy's disease individual as well as from 33 individuals from within the pedigree. The heterozygote mother of the index case was found to have one androgen receptor allele carrying the Kennedy's disease mutation (44 CAGs) with the other allele carrying 28 CAGs, which is in the upper-normal range. A clinical diagnosis of motor neuron disease was made with a pattern not typical of a standard case of Kennedy's disease in that the patient had upper motor neuron signs. Coexistence of motor neuron disease in a carrier of

Kennedy's disease with an allele at the upper end of the normal range may be a coincidence, or the presence of 44 and 28 CAG repeats in the AR may have played a role.

Kennedy's disease has been demonstrated to be an under-diagnosed condition, with ALS being the most common misdiagnosis [31, 39]. This is likely to be due to the overlap in clinical symptoms, the lack of a clear family history in some instances, and the variability of presentation in Kennedy's disease patients. ALS is characterised by the degeneration of both upper motor neurons in the motor cortex, and lower motor neurons in the brainstem and spinal cord, resulting in spastic paralysis, flaccid muscle weakness, wasting and fasciculations [7].

The present case raises the possibility of Kennedy's disease in certain subpopulations of heterozygote females. This notion is not without precedent, as some female carriers displaying subclinical phenotypic expression of Kennedy's disease have been reported [4, 25, 28, 35]. Kennedy's disease is the only one of the eight known polyglutamine expansion diseases that is X-linked recessive, as opposed to the autosomal dominant expression of the other diseases. However, since the hypothesized mechanism underlying all of the polyglutamine expansion diseases is a 'toxic gain-of-function', it would be expected that Kennedy's would also behave in this manner. According to this theory, there would be a percentage of females that would manifest full-blown symptoms of Kennedy's disease, however, none to date have been reported.

There are a number of possible explanations for the relative absence of Kennedy's disease in female carriers. Firstly, females have low circulating levels of androgens [41], which would result in a decrease in the amount of androgen receptor protein translocated to the nucleus, a mechanism thought to be important in the pathogenesis of some of the polyglutamine expansion diseases [26]. This is supported by the presence of only minor features in females homozygous for the mutation [34], and by recent studies demonstrating exacerbation of neurodegenerative processes both in vitro [9], and in animal models of Kennedy's disease [18, 36], by androgen treatment. Secondly, it may relate to the process of X-chromosome inactivation, also known as lyonisation, which is required for dosage compensation of X-linked genes [15]. The ratio X-inactivation of the wild type and mutant alleles in a group of 8 heterozygote carriers of Kennedy's disease was addressed in a recent study where the results suggested that highly skewed X-chromosome inactivation may be closely related with escape from the manifestations of Kennedy's disease in females [17]. However, significant levels of skewing (> 80 %) were only demonstrated in a single non-symptomatic individual and all eight individuals examined had CAGs ranging from 17–19 [17]. Studies addressing this issue in a larger number of individuals are currently needed. Furthermore, inactivation levels were only determined in peripheral blood lymphocytes. Whether there is tissue heterogeneity of inactivation of the X-chromosome carrying the mutant allele of the androgen receptor needs to be determined (ie. in the central nervous system).

In the heterozygous female discussed in this current study, levels of X-chromosome inactivation could not be determined because blood samples were not available. However, the fact that this individual suffered motor neuron disease raises interesting questions about the nature of manifestations of the disease phenotype in female carriers. The possibility exists that CAG number on the non-mutant allele may influence skewing of X-chromosome inactivation. However, this notion remains to be investigated. The number of trinucleotide repeats on the non-mutant allele in the heterozygous female carrier discussed in the current study was in the upper normal range. In a study on genetic variation of tandem repeats where allele frequency was determined, 28 or more CAG repeats in the androgen receptor gene was found to be a rare allele, representing only 3.3 % of the normal Caucasian population [10]. The most frequent allele was 21 repeats [10]. Consistent with this, the incidence of CAGs numbering greater or equal to 28 in Australian males was 3.6 % in control subjects in a prostate cancer case control study [2].

The androgen receptor CAG repeat polymorphism within the normal, non-pathogenic range has been investigated and associations found with a number of conditions including prostate cancer [1], defective spermatogenesis and male infertility [38], osteoporosis [8], obesity [14], breast cancer [12], ovarian cancer [33], polycystic ovarian syndrome [16], and Alzheimer's disease [22], although the evidence for some of these is weak or remains disputed [2, 13, 27, 40]. These associations would indicate CAG length affecting androgen receptor transcriptional activity within the normal range. Indeed, several reports support decreased androgen receptor-mediated transactivation associated with increased CAG repeat length both in the normal range in vitro [3, 38], and the pathological range [19, 30]. These studies support the premise that varied CAG length within the androgen receptor gene has an effect on a variety of cellular functions and lends credence to our hypothesis that the CAG repeat number in the normal androgen receptor allele may influence expression or activity of the mutant allele.

In studies previously reporting phenotypic expression in heterozygous females carrying the Kennedy's disease mutation, the number of CAGs was either not reported or was in the lower to mid-normal range [4, 25, 28, 35]. It would be of interest to conduct a large scale study of heterozygote female carriers of the Kennedy's disease mutation and to examine repeat number in both the normal and mutant allele in association with phe-

notypic expression and X-chromosome inactivation. Increased number of repeat sequences in the upper normal range, in the presence of an allele repeat number in the disease range, may remove the protective effect normally seen in female carriers of the mutation. This indicates that the absence of a disease phenotype in female carriers may be due to a protective effect of the normal androgen receptor.

In conclusion, in the current study we report a heterozygous female carrier of the Kennedy's disease mutation who suffered from motor neuron disease. The number of repeats on the androgen receptor alleles were 28 and 44, raising the possibility that expression of the mutant allele in heterozygous females influences phenotypic expression of Kennedy's disease. Based on this, we postulate that the Kennedy's mutation may have contributed to this individual's motor neuron degeneration.

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