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Molecular features of the CAG repeats of spinocerebellar ataxia 6 (SCA6)

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Spinocerebellar ataxia 6 (SCA6) is an autosomal dominant spinocerebellar degeneration caused by the expansion of the polymorphic CAG repeat in the human α1A voltage-dependent calcium channel subunit gene (CACNL1A4 gene). We have analyzed 60 SCA6 individuals from 39 independent SCA6 Japanese families and found that the CAG repeat length is inversely correlated with the age of onset (n = 58, r = –0.51, P < 0.0001). SCA6 chromosomes contained 21–30 repeat units, whereas normal chromosomes displayed 6–17 repeats. There was no overlap between the normal and affected CAG repeat number. The anticipation of the disease was observed clinically in all eight parent–child pairs that we examined; the mean age of onset was significantly lower (P = 0.0042) in children than in parents. However, a parent–child analysis showed the increase in the expansion of CAG repeats only in one pair and no diminution in any affected cases. This result suggests that factors other than CAG repeats may produce the clinical anticipation. A homozygotic case could not demonstrate an unequivocal gene dosage effect on the age of onset.

INTRODUCTION

Spinocerebellar ataxia 6 (SCA6) is an autosomal dominant spinocerebellar degeneration, originally described in 1997 (1). SCA6 was initially identified by the expansion of polymorphic CAG repeats in the human α1A voltage-dependent calcium channel subunit gene (CACNL1A4 gene). Six isoforms of CACNL1A4 were identified and only three isoforms with the GGCAG insertion had the extended open reading frame with the polyglutamine tract (1).

It has been reported that there are two mutations disrupting the reading frame of the CACNL1A4 gene in two episodic ataxia (EA-2) families in which some patients show cerebellar atrophy (2,3). Four missense mutations were reported in the same gene in patients with familial hemiplegic migraine (FHM), which is associated with degenerative cerebellar atrophy, ataxia, nystagmus and other vestibulocerebellar ocular abnormalities, similar to those seen in EA-2 (2,4). Therefore, FHM, EA-2 and SCA6 are presumably allelic disorders.

Neurological diseases with the CAG repeat expansion include spinobulbar muscular atrophy (SBMA) (5,6), Huntington's disease (HD) (7–10), spinocerebellar ataxia 1 (SCA1) (11,12), spinocerebellar ataxia 2 (SCA2) (13–15), Machado–Joseph disease (MJD) (16,17) and dentatorubral-pallidoluysian atrophy (DRPLA) (18,19). These diseases often show unstable CAG repeats between generations and variable clinical phenotypes which correlate with the sizes of the expanded CAG repeats.

The expansion of CAG repeats between generations, commonly observed in CAG repeat diseases, has not been demonstrated for SCA6, because the numbers of CAG repeats of normal and affected SCA6 individuals are smaller and more stable between generations than that of other CAG repeat diseases (1). However, the number of SCA6 parent–child pairs examined seems too small to draw the conclusion on the change in the expansion of CAG repeats between generations (1). Initially, SCA6 had been identified by the expansion of CAG repeats in the CACNL1A4 gene, not by clinical phenotype or symptoms, and the molecular features of SCA6 have not been fully elucidated.

Here we examined the size of the polymorphic CAG repeats in the CACNL1A4 gene in affected individuals from a large number of families as well as from normal individuals. This study demonstrated characteristics of the transmission and expansion of CAG repeats, and the effect of CAG repeats on the age of onset.

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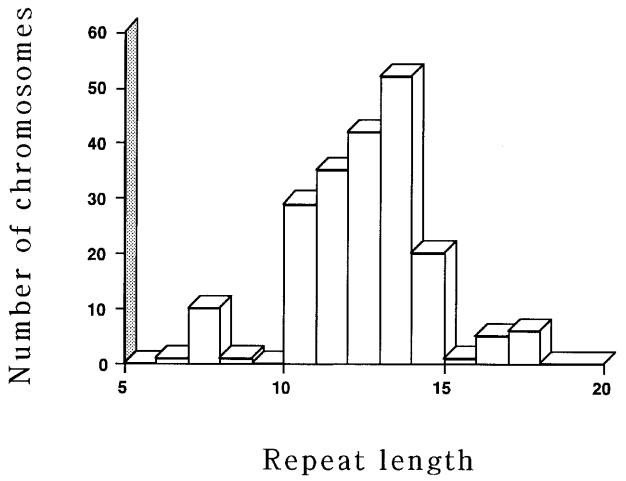

Figure 1. Distribution of the CAG repeat length in 202 normal alleles from 101 healthy Japanese. Range is from 6 to 17.

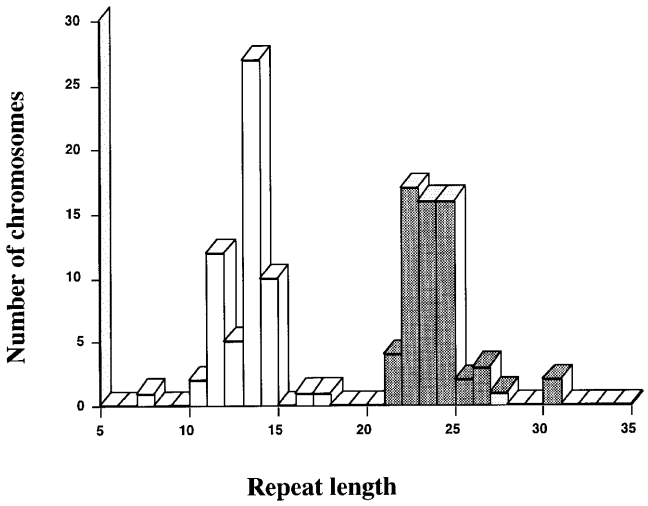

Figure 2. Distribution of the CAG repeat length in 61 expanded alleles (black bars) and 59 normal alleles from 60 SCA6 patients (including one homozygotic case). Normal alleles range from 7 to 17 and expanded alleles range from 21 to 30. SCA6 chromosomes were completely distinct from normal chromosomes.

RESULTS

Distribution of CAG repeats in the CACNL1A4 gene in the normal population

Two hundred and two normal chromosomes in normal Japanese individuals (101) displayed a range from 6 to 17 repeat units (Fig. 1). The mean was 12.0 repeats and the median was 11.5. Heterozygosity was as high as 72%.

Repeat length on SCA6 chromosomes

We screened 135 Japanese DNA samples of hereditary spinocerebellar degeneration from which SCA1, SCA2, MJD and DRPLA were excluded by PCR analysis, and 60 samples from 39 independent families had more than 21 CAG repeats on SCA6 chromosomes. The affected SCA6 chromosomes contained 21–30 repeat units (Fig. 2). The median was 25.5 repeats and the mean was 23.5 repeats. SCA6 chromosomes were completely discrete from normal chromosomes.

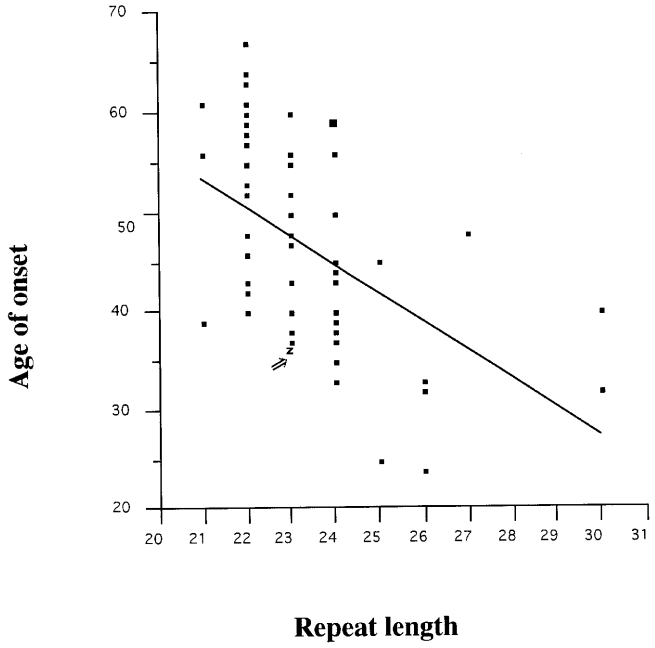

Figure 3. Relationship between age of onset and CAG repeat length in expanded alleles in affected individuals (n = 58, r = –0.51, P < 0.0001). Regression line is Y = –2.88X + 113.8 (where Y is age of onset and X is repeat length). The homozygotic case is indicated by Z and an arrow (repeat number: 23/23, just one allele plotted).

Age of onset and CAG repeat length

Figure 3 shows the relationship between the age of onset and the CAG repeat length in the expanded alleles in affected individuals. A significant inverse correlation (n = 58, r = –0.51, P < 0.0001) was noted between the length of CAG repeats and the age of onset of the disease.

Clinical anticipation

We examined a possible clinical anticipation in the age of onset in eight affected parent and affected child pairs. All parent–child pairs examined showed the clinical anticipation. The mean age of onset of the parent was 52.5 ± 3.1 years old, and that of the child, 37.8 ± 3.1 years old (mean ± standard error). The difference in the age of onset between affected parent and child was 14.7 years, and is statistically significant (P = 0.0042).

Stability of the CAG repeat length through generations

The length of CAG repeats in affected parent and child pairs was assessed to evaluate the instability of the CAG repeat in SCA6 chromosomes through generations. Although there was a clinically obvious anticipation in eight SCA6 parent–child pairs (three paternal and five maternal transmissions) examined, only one paternal transmission showed an increase in CAG repeats (Fig. 4). A decrease in the length of CAG repeats was not observed in any transmission.

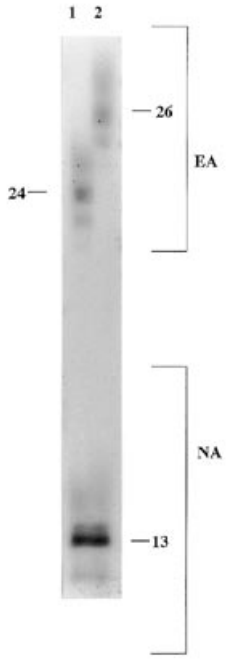

Figure 4. Analysis of the PCR-amplified products containing the CAG repeat tract. Lane 1 is the affected father (age of onset, 35 years old); lane 2, his affected son (age of onset, 24 years old). The range for the normal (NA) and expanded alleles (EA) are indicated.

Gene dosage effect (homozygosity)

We examined one homozygotic case (repeat number: 23/23), but could not demonstrate an unequivocal decrease in the age of onset, contrary to our expectation. The case was within the bivariate normal ellipse ($P = 0.60$), although the age of onset was the lowest among SCA6 patients with a repeat length of 23 (Fig. 3).

DISCUSSION

In this study we confirmed the expansion of CAG repeats in SCA6 and the report that the lowest number of the expanded CAG repeat is 21 (1). The distribution of repeat length among 60 SCA6 chromosomes was completely distinct from that observed in control chromosomes. The difference in the size of the repeat length between SCA6 and normal chromosomes was more than four repeats. These results indicate that the expanded CAG repeats would provide an accurate diagnostic tool for SCA6.

In the Chugoku area (a part of Western Japan), SCA1, SCA2, DRPLA and MJD are rather rare diseases and each of these accounts for less than 5% of inherited spinocerebellar degeneration, respectively. In contrast, SCA6 comprised more than 30% in our present examination. In addition, the frequency of normal individuals with CAG repeats larger than 15 in this area (12/202 = 5.9%) was greater than that previously reported (7/950 = 0.74%) (1), which might explain a high incidence of SCA6 in this area. No intermediate allele was observed between 17 and 21 CAG repeats.

The parent–child analysis showed the clinical anticipation of the age of onset in all eight parent–child pairs examined with an average decrease of 14.7 years per generation, whereas only one pair showed an increase in the expansion of CAG repeats in the CACNL1A4 gene. The cerebellar ataxia of SCA6 was mild and progressed slowly, so that the onset of SCA6 could not be settled definitely in some cases; however, even with this consideration, the scattering of ages of onset defined by a repeat number is too large to allow the explanation that only the CAG repeat number defines the age of onset of SCA6 (Fig 3). The line between the age of onset and the repeat length accounts for only ~26% of all the cases ($r = -0.51$). So, factors other than the CAG repeat might explain the clinical anticipation and influence the onset age of SCA6.

Clinical features of SCA6 patients are similar and consist predominantly of mild, but slowly progressive cerebellar ataxia of the limbs and gait, dysarthria, nystagmus, and mild vibratory and proprioceptive sensory loss (1). Although SCA6 does not exhibit such a phenotypic variability as MJD (17), genetically proven patients with SCA6 sometimes had double vision, increase or decrease in the deep tendon reflex, and divergence paralysis. In fact, two autopsied SCA6 cases have demonstrated that the disease involves not only the cerebellum but also the brain stem (20). Before our analysis of expansion of CAG repeats in the CACNL1A4 gene, SCA6 was clinically diagnosed as Holmes type SCD, MJD and autosomal dominant cortical cerebellar atrophy.

As mentioned above, FHM, EA-2 and SCA6 are allelic disorders in different fashions; CAG repeats are expanded in SCA6, the protein is truncated in EA-2 and missense mutations are seen in FHM. The difference in the mutational mode may produce a persistent and progressive cerebellar and brain stem dysfunction in SCA6 and mild, episodic cerebellar dysfunction in EA-2 and FHM (1,2). But SCA6 and some cases of EA-2 and FHM show cerebellar atrophy and there may be a common mechanism for them. For example, the prominent expression of P/Q Ca^{2+} channels is observed in Purkinje cells and cerebellar granule cells (21) and their mutations might lead to abnormal levels of intracellular Ca^{2+} and subsequent cell death which would cause the cerebellar atrophy and the vestibulocerebellar dysfunction.

The ataxia in a homozygotic case of SCA6 did not start from a remarkably young age in contrast to MJD (22,23). The homozygotic patient first noticed a visual disturbance and ataxia at the age of 36, but the ataxia progressed much more rapidly than the other SCA6 patients. The onset age of the homozygotic case was within the bivariate normal ellipse ($P = 0.60$), but was lower than other SCA6 patients with 23 CAG repeats (Fig. 3). In addition, because CAG repeat accounts for only ~26% of the age of onset, the effect of homozygosity may be equivocal. Thus, a possible gene dosage effect of SCA6 cannot be ruled out.

In summary, we confirmed the expansion of CAG repeats in the CACNL1A4 gene in SCA6 patients and showed an inverse correlation of the repeat length with age of onset, a clinical anticipation, and a change in expanded repeats between generations.

MATERIALS AND METHODS

Amplification of the CAG repeats

All patients and normal control DNA were obtained from leukocytes. The CAG repeat region in the human CACNL1A4 gene was amplified by PCR with the primers F6 (5′-CACGTGT-CCTATTCCCCTGTGATCC-3′) and R6 (5′-TGGGTACCTCC-GAGGGCCGCTGGTG-3′). Amplification was performed for

30 cycles (denaturation for 1 min at 95°C, annealing for 1 min at 67°C, and elongation for 1 min at 72°C), and the 5′ terminus of primer R6 was labeled with the use of bacteriophage T_4 polynucleotide kinase (Megalabel, Takara, Japan) and [r-^{32}P]ATP at 37°C. The PCR mixture (10 µl) contained 100 ng genomic DNA, 50 mM Tris–HCl (pH 9.2), 14 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 350 µM each of dCTP, dATP and dTTP, 87.5 µM dGTP, 262.5 µM 7-deaza-dGTP, 100 ng F6 primer, 90 ng unlabeled R6 primer, 10 ng ^{32}P-labeled R6 primer and 1.3 U of *Taq* and *Pwo* DNA polymerases (Boehringer Mannheim, Mannheim, Germany). PCR products (4 µl) were mixed with 1 µl of formamide loading buffer [98% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol FF, and 0.025% bromophenol blue], denatured at 99°C for 10 min, and then placed on ice. Electrophoresis was performed on 8% HydroLink Long Ranger (AT Biochem, PA, USA) polyacrylamide gels containing 7 M urea and 42% formamide (24). CAG repeat length was determined by comparison with a standard M13 sequencing ladder. For parent–child analysis, family members were analyzed side by side on the same gel and reproduced at least twice.

Clinical and statistical analysis

The diagnosis of spinocerebellar degeneration (SCD) were performed by skilful neurologists with the help of MRI, CT and electrophysiological studies. The clinical features of SCA6 patients consisted predominantly of mild, but slowly progressive cerebellar ataxia of the trunk and limbs, gait disturbance, dysarthria, and nystagmus.

The age of onset is defined as the age when the patients first noticed any symptoms. Neurologists questioned patients or family members closely about the onset of symptoms to gain as accurate an age of onset as possible. The statistical analyses were performed with JMP software (SAS Institute, Inc.).

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REFERENCES

1. Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D.W., Amos, C., Dobyns, W.B., Subramony, S.H., Zoghbi, H.Y. and Lee, C.L. (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the α1A voltage-dependent calcium channel. *Nature Genet*., **15**, 62–69.
2. Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., Eijk, R., Oefner, P.J., Hoffman, S.M.G., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Haan, J., Lindhout, D., Ommen, G.J.B., Hofker, M.H., Ferrari, M.D. and Frants, R.R. (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca^{2+} channel gene CACNL1A4. *Cell*, **87**, 543–552.
3. Vahedi, K., Joutel, A., Van Bogaert, P., Ducros, A., Maciazeck, J., Bach, J.F., Bousser, M.G. and Tournier-Lasserve, E. (1995) A gene for hereditary paroxysmal cerebellar ataxia maps to chromosome 19p. *Ann. Neurol*., **37**, 289–293.
4. Elliott, M., Peroutka, S.J., Welch, S. and May, E.F. (1996) Familial hemiplegic migraine, nystagmus, and cerebellar atrophy. *Ann. Neurol*., **39**, 100–106.
5. La Spada A.R., Roling, D.B., Harding, A.E., Warner, C.L., Spiegel, R., Hausmanowa, P.I., Yee, W.C. and Fischbeck, K.H. (1992) Meiotic stability and genotype–phenotype correlation of the trinucleotide repeat in X-linked spinal and bulbar muscular atrophy. *Nature Genet*., **2**,301–304.
6. Doyu, M., Sobue, G., Mukai, E., Kachi, T., Yasuda,T., Mitsuma, T. and Takahashi, A. (1992) Severity of X-linked recessive bulbospinal neuronopathy correlates with size of the tandem CAG repeat in androgen receptor gene. *Ann. Neurol*., **32**, 707–710.
7. The Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, **72**, 971–983.
8. Andrew, S.E., Goldberg, Y.p., Kremer, B., Telenius, H., Theilmann, J., Adam, S., Starr, E., Squitieri, F., Lin, B., Kalchman, M.A., Graham, R.K. and Hayden, M.R. (1993) The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. *Nature Genet*., **4**, 398–403.
9. Duyao, M., Ambrose, C., Myers, R., Novelletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., Abbott, M., Gray, J., Conneally, P., Young, A., Penny, J., Hollingsworth, Z., Shoulson, I., Lazzarini, A., Falek, A., Koroshetz, W., Sax, D., Bird, E., Vonsattle, J., Bonilla, E., Alvir, J., Bickham Conde, J., Cha, J.- H., Dure, L., Gomez, F., Ramos, M., Scanchez-Ramos, J., Snodgrass, S., de Young, M., Wexler, N., Moscowitz, G., Penchaszadeh, G., MacFarlane, H., Anderson, M., Jenkins, B., Srinidhi, J., Barnes, G., Gusella, J. and Macdonald, M. (1993) Trinucleotide repeat length instability and age of onset in Huntington's disease. *Nature Genet*., **4**,387–392.
10. Snell, R.G., MacMillian, J.C., Cheadle, J.P., Fenton, I., Lazarou, L.P., Davies, P., MacDonald, M.E., Gusella, J.F., Harper, P.S. and Shaw, D.,J. (1993) Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington's disease. *Nature Genet*., **4**, 393–397.
11. Orr, H.T., Chung, M.Y., Bnfi, S., Kwiatkowski, T.J., Servadio, A. and Zoghbi, H.Y. (1993) Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nature Genet*., **4**, 221–226.
12. Chung, M.Y., Ranum, L.P., Duvick, L.A., Servadio, A., Zoghbi, H.Y. and Orr, H.T. (1993) Evidence for a mechanism predisposing to intergenerational CAG repeat instability in spinocerebellar ataxia type 1. *Nature Genet*., **5**, 254–258.
13. Pulst, S.M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X.N., Cendes, I.L., Pearlman, S., Starkman, S., Diaz, G.O., Lunkes, A., DeJong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C. and Sahba, S. (1996) Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. *Nature Genet*., **14**, 269–276.
14. Sanpei, K., Takano, H., Igarashi, S., Sato, T., Oyake, M., Sasaki, H., Wakisaka, A., Tashiro, K., Ishida, Y., Ikeuchi, T., Koide, R., Saito, M., Sato, A., Tanaka, T., Hanyu, S., Takiyama, Y., Nishizawa, M., Shimizu, N., Nomura, Y., Segawa, M., Iwabuchi, K., Eguchi, I., Tanaka, H., Takahashi, H. and Tsuji, S. (1996) Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. *Nature Genet*., **14**, 277–284.
15. Imbert, G., Saudou, F., Yvert, G., Devys, D., Trottier, Y., Garnier, J.M., Weber, C., Mandel, J.L., Cancel, G., Abbas, N., DYrr, A., Didierjean, O., Stevanin, G., Agid, Y. and Brice, A. (1996) Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. *Nature Genet*., **14**, 285–291.
16. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I., Kimura, J., Narumiya, S. and Kakizuka, A. (1994) CAG expansions in a novel gene for Machado–Joseph disease at chromosome 14q32.1. *Nature Genet*., **8**, 221–228.
17. Maruyama, H., Nakamura, S., Matsuyama, Z., Sakai, T., Doyu, M., Sobue, G., Seto, M., Tsujihata, M., Ohi, T., Nishio, T., Sunohara, N., Takahashi, R., Hayashi, M., Nishino, I., Ohtake, T., Oda, T., Nishimura, M., Saida, T., Matsumoto, H., Baba, M., Kawaguchi, Y., Kakizuka, A. and Kawakami, H. (1995) Molecular features of the CAG repeats and clinical manifestation of Machado–Joseph disease. *Hum. Mol. Genet*., **4**, 807–812.

18. Koide, R., Ikeuchi, T., Onodera, O., Tanaka, H., Igarashi, S., Endo, K., Takahashi, H., Kondo, R., Ishikawa, A., Hayashi, T., Saito, M., Tomoda, A., Miike, T., Naito, H., Ikuta, F. and Tsuji, S. (1994) Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). *Nature Genet*., **6**, 9–13.
19. Nagafuchi, S., Yanagisawa, H., Sato, K., Shirayama, T., Ohsaki, E., Bundo, M., Takeda, T., Tadokoro, K., Murayama, N., Tanaka, Y., Kikushima, H., Umini, K., Kurosawa, H., Furukawa, T., Nihei, K., Inoue, T., Sano, A., Komure, O., Takahashi, M., Yoshizawa, T., Kanazawa, I. and Yamada, M. (1994) Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p. *Nature Genet*., **6**, 14–18.
20. Subramony, S.H., Fratkin, J.D., Manyam, B.V. and Currier, R.D. (1996) Dominantly inherited cerebello-olivary atrophy is not due to a mutation at the spinocerebellar ataxia-1, Machado–Joseph disease, or Dentato–Rubro–Pallido–Luysian Atrophy locus. *Movement Disord*., **11**, 174–180.
21. Stea, A., Tomlinson, W.J., Soong, T.W., Bourinet, E., Dubel, S.J., Vincent, S.R. and Snutch, T.P. (1994) Localization and functional properties of a rat brain alpha 1A calcium channel reflect similarities to neuronal Q- and P-type channels. *Proc. Natl. Acad. Sci. USA*., **91**, 10576–10580.
22. Kawakami, H., Maruyama, H., Nakamura, S., Kawaguchi, Y., Kakizuka, A., Doyu, M.and Sobue, G. (1995) Unique features of the CAG repeats in Machado–Joseph disease. *Nature Genet*., **9**, 334–335.
23. Sobue, G., Doyu, M., Nakao, N., Shimada, N., Mitsuma, T., Maruyama, H., Kawakami, H. and Nakamura, S. (1996) Homozygosity for Machado–Joseph disease gene enhances phenotypic severity. *J. Neurol. Neurosurg. Psychiatr*., **60**, 354–356.
24. Maruyama, H., Kawakami, H. and Nakamura, S. (1996) Reevaluation of the exact CAG repeat length in hereditary cerebellar ataxias using highly denaturing conditions and long PCR. *Hum. Genet*., **97**, 591–595.