Prenatal Diagnosis of Machado-Joseph Disease/Spinocerebellar Ataxia Type 3 in Taiwan: Early Detection of Expanded Ataxin-3

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Machado-Joseph disease (MJD)/Spinocerebellar Ataxia Type 3 (SCA3) is a rare autosomal dominative disorder in which one of the neurodegenerative disorders is caused by a translated CAG repeat expansion. Here, we present the first prenatal diagnosis of MJD in Taiwan in a woman whose husband was known to carry an unstable CAG repeat expansion in the MJD gene. After evaluating the couples' motivation and psychological tolerance, amniocentesis was performed at gestation of 13 weeks. The diagnosis was made using a simple nonradioactive polymerase chain reaction (PCR) for rapid detection of the presence of an expanded MJD allele. Meanwhile, using radioactive PCR, we

identified the presence of an unusual shortness of CAG expansion in the MJD gene with 74 repeats in the fetus compared with 78 repeats in the father. After termination of the pregnancy, Western blot analysis further confirmed the presence of normal and mutant ataxin-3 in the fetal tissue. In summary, we have performed the first prenatal diagnosis of MJD in Taiwan, and described our experience with an at-risk male requesting counseling, carrier testing, and prenatal diagnosis for Machado-Joseph disease. Early detection of both normal and expanded ataxin-3 in fetal tissues was first demonstrated in the present study. *J. Clin. Lab. Anal.* 17: 195–200, 2003. © 2003 Wiley-Liss, Inc.

Key words: prenatal diagnosis; Machado-Joseph disease; Spinocerebellar Ataxia Type 3; CAG trinucleotide repeats

INTRODUCTION

Machado-Joseph disease (MJD) belongs to the autosomal dominant cerebellar ataxias (ADCAs). It is an autosomal dominant neurodegenerative disorder characterized by a wide range of clinical manifestations, including cerebellar ataxia, pyramidal and extrapyramidal signs, progressive external ophthalmoplegia, dystonia with rigidity, and distal muscular atrophies (1,2). Mutations at different loci have been identified in ADCAs and some of the responsible genes have been cloned including: spinocerebellar ataxia type 1 (SCA1) on chromosome 6p (3), spinocerebellar ataxia type 2 (SCA2) on chromosome 12q (4–6), spinocerebellar

ataxia type 3 (SCA3)/Machado-Joseph disease (MJD) on chromosome 14q (7), spinocerebellar ataxia type 6 (SCA6) on chromosome 19p (8), and spinocerebellar

Grant sponsor: National Science Council of the Republic of China; Grant numbers: NSC 90-2316-B-040-002, NSC 90-2320-B-040-033, NSC90-2745-P-040-002; Grant sponsor: Chung Shan Medical University Research Fund; Grant number: CSMC 89-OM-A-074.

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Received 12 December 2002; Accepted 4 April 2003

DOI 10.1002/jcla.10092

Published online in Wiley InterScience (www.interscience.wiley.com).

ataxia type 7 (SCA7) on chromosome 3p (9). All these spinocerebellar ataxias belong to a special class of inherited neurodegenerative diseases caused by CAG trinucleotide repeat expansion in the coding region of the respective genes (10). This includes Huntington's disease (11,12), dentatro-rubral-pallidoluyian atrophy/Haw-River-Smith's disease (DRPLA) (13,14), and spinobulbar muscular atrophy (15).

SCA3/MJD is associated with an unstable expansion of CAG trinucleotide repeat contained in the MJD1 gene (7). In normal individuals, the number of repeats ranges between 12 and 44, whereas in MJD/SCA3 patients, the number of repeats of the affected allele varies between 53 and 86 (16,17). Clinical examination of the variability in MJD patients has led to the classification of four subtypes (18). Type I patients tend to have the longest repeats, the earliest onset (15–30 yr), and the most rapid progression with marked pyramidal and extrapyramidal signs in addition to the common features of cerebellar ataxia and ophthalmoplegia. The age at onset of most of the type II patients is usually between 20 and 45 yr, and the symptoms are limited to cerebellar and pyramidal signs. Type III patients show later onset (40–60 yr) and cerebellar signs, distal amyotrophic signs, marked peripheral signs, and peripheral neuropathy. The rare type IV patients are characterized by the latest onset (> 50 yr) with Parkinsonism and polyneuropathy. However, typical onset of symptoms usually occurs between the ages of 30 and 40 yr, and symptoms are slowly progressive. Anticipation, which means increasing disease severity in successive generations, is observed in these neurodegenerative disorders (10).

The long CAG repeat leads to the expression of a protein containing multiple glutamines. The pathology is thought to arise as a consequence of a gain of function by MJD1 protein or ataxin-3 (19). The polyglutamine repeat lies close to the C-terminus of ataxin-3. Ataxin-3, estimated at 42 kDa, is the smallest of the polyglutamine disease proteins. Polyglutamine diseases are predominantly inherited, typically late-onset, fatal neurodegenerative disorders. The protein is widely expressed in neurons (20) and outside the central nervous system (CNS), but the mutation ultimately leads to selective neuronal loss in restricted brain regions. The nature of the toxic insult of a poly(Q) mutation and its biological consequences in each disease are unclear. However, it has been shown that ataxin-3 accumulates in ubiquitinated intranuclear inclusions selectively in neurons of affected brain regions (21). Neuronal intranuclear inclusions have become the neuropathological sign of the CAG repeat diseases, but their cytotoxicity still remains controversial (22).

Even though the physiological role of normal or mutant ataxin-3 is still under investigation, the identi-

fication of the CAG repeat mutation has enabled the prenatal diagnosis of this disorder. In this paper, we described our first experience with couples requesting counseling, carrier testing, and prenatal diagnosis of Machado-Joseph disease in Taiwan. We also demonstrated that both normal and expanded ataxin-3 were expressed normally even in fetal tissues.

MATERIALS AND METHODS

Subject

A 30-yr-old female, whose husband had a family history of MJD, was diagnosed in the early stages of pregnancy. This couple received genetic counseling at our medical center. Amniocentesis was carried out at gestation of 13 weeks. Before amniocentesis was carried out, the couples' motivation and psychological tolerance were evaluated at Chunghau Christian Hospital, Taiwan.

DNA Isolation

Genomic DNA was isolated from peripheral lymphocytes and amniotic fluid after primary culture for two to three generations by standard phenol-chloroform methods (23).

DNA Analysis With Nonradioactive PCR

The CAG containing fragment of the MJD gene was amplified by PCR using primers MJD52 (7) and MJD25a (a slight modification of MJD25 with sequence ATCCATGTGCAAAGGCCAGCC) (7). PCR was performed in a final volume of 20 μ L, containing 100–200 ng of genomic DNA; 10 mM Tris-HCl (pH8.8); 1.5 mM MgCl₂; 50 mM KCl; 0.1% Triton X-100; 10% dimethylsulfoxide; 200 μ M dNTPs; 80 ng of each primer; and 3 units of Taq DNA polymerase. The DNA was denatured at 95°C for 5 min; then 30 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min were performed, followed by a final extension at 72°C for 10 min. To determine the size range of CAG repeat expansion, the PCR products were analyzed on 4% agarose gels in parallel with the 100 bp marker and visualized by ethidium bromide (EB). Allele size was determined by comparison with the 100 bp marker, assuming that the variation in the size of the product occurs within the repetitive CAG stretch.

Identification of the CAG Trinucleotide Repeat Expansion

For determination of CAG trinucleotide repeat at the MJD/SCA3 locus, we ran the PCR product on polyacrylamide gel electrophoresis containing fragment

of the MJD gene. This fragment was amplified by PCR using primers MJD52 and MJD25a (a slight modification of MJD25 with sequence ATCCATGTG-CAAAGGCCAGCC) (7). PCR was performed in a final volume of 20 μ L, containing 50 ng of genomic DNA; 10 mM Tris-HCl (pH8.8); 1.5 mM MgCl₂; 50 mM KCL; 0.1% Triton X-100; 10% dimethylsulfoxide; 250 μ M each dCTP, dGTP, and dTTP; 25 μ M dATP; 1.5 μ Ci³⁵S- α -dATP; 125 ng each primer; and 3 units of Taq DNA polymerase. The DNA was denatured at 95°C for 8 min; then 30 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min were performed, followed by a final extension at 72°C for 10 min. To determine the exact number of CAG repeats, the PCR products were analyzed on denaturing 6% polyacrylamide gels in parallel with a pGEM sequencing ladder (–40 primer) and were visualized by autoradiography. Allele sizes were determined by comparison to the sequence ladder and were converted to CAG unit number, assuming that the variation in size of the PCR product occurs within the repetitive CAG stretch.

Fetal Fibroblasts Culture Conditions and Treatments

Fetal fibroblasts were grown in Modified Eagle's Medium (MEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 2 mM L-Glutamine, 1% PS (100,000 U/L Penicillin G sodium, 100 mg/L Streptomycin sulfate), and 1% nonessential amino acid. The medium was

changed every 2 days and cells subcultivated every week at a ratio of 1:2. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Preparation of Cell Lysates for Analysis by SDS-PAGE

The cells were harvested with trypsin-EDTA after reaching 80% confluence as judged by microscopic examination. Cells were washed twice with phosphate buffered saline (PBS) pH 7.2, then resuspended in 500 μ L of lysis buffer (5% glycerol; 1 mM sodium EDTA; 1 mM sodium EGTA; 1 mM dithiothreitol; 40 μ g/mL leupeptin; 40 μ g/mL aprotinin; 20 μ g/mL pepstatin; 1 mM PMSF; and 0.5% Triton X-100, 1 $\times$ PBS) and incubated on ice for 15 min. The cell lysate was centrifuged at 16,000 rpm for 20 min at 4°C. Supernatant was collected, and Bio-Rad protein assay reagent (Hercules, CA) was used to determine the protein concentrations.

Gel Electrophoresis and Immunoblot Analysis

In brief, cell lysates containing 20 μ g of protein were loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Resolved proteins were electrophoretically transferred onto 0.2 μ m nitrocellulose membranes. After blocking the membrane with 5% nonfat milk in NaCl/Pi/0.1% Tween 20 for 1 hr at room temperature (RT), the antibody-binding reactions were performed in the same buffer supplement with 1% nonfat milk at 4°C

Fig. 1. Detection of the CAG expansion in the MJD gene on 4% agarose gel. PCR analysis of DNAs. Two DNA fragments with lower molecular weight below 300 bp were observed in the mother of the fetus (lane 3) and a nonrelated human wild-type control (lane 5). The expanded mutant alleles were found from the samples of the paternal grandmother, father, and fetus (lanes 1, 2 and 4) with moderated mosaic DNA fragments and a range around 400 bp. Lane 6, no template control; EX, expanded allele; NL, normal allele.

overnight for monoclonal anti-ataxin-3 (1:2000) (24) and at RT for 1 hr for secondary antibodies coupled to horseradish peroxidases (HRP)-conjugated goat anti-mouse IgG. Results were visualized by chemiluminescence.

RESULTS

The couple has a family history of MJD and requested molecular genetic testing after prenatal counseling was given at our medical center under the ethics and evaluations of psychological tolerance. The husband, who had a family history of MJD, was previously identified as carrying a 78 CAG repeat

expansion in the MJD gene (25). However, he had not developed clinical symptoms yet. The paternal grandmother, diagnosed as an MJD patient with a 78 CAG repeats in the MJD gene, is still alive. She clinically presented progressive limb and truncal ataxia, ophthalmoplegia, distal muscle atrophy, and choreoathetosis. Prominent atrophy over the cerebellar hemisphere and vermis were disclosed by a Brain MRI study. Axononeuropathy was found in a nerve conduction study.

Nonradioactive detection on agarose gel was applied to the MJD gene analysis. This method is faster, simpler, and more cost-effective than the radioactive polymerase chain reaction analysis. The PCR products resolve clear signals on 4% agarose gels (Fig. 1). On agarose gels, two DNA fragments with lower molecular weight below 300 bp were observed in the mother of the fetus, indicated as a normal subject (lane 3). The mutant alleles were found (lanes 1, 2, and 4) from the DNA samples of the paternal grandmother, father, and fetus with the mosaic DNA fragments and they range around 400 bp.

Meanwhile, the fetal DNA was studied with radioactive genomic PCR for the exact number of CAG repeats. The results of radioactive genomic PCR analysis of the mother and the fetus are shown in Fig. 2. The fetal sample showed two signals of 74 and 16 CAG repeats (lane 1), demonstrating one expanded fragment and one normal fragment, compared to the mother with normal alleles of 16 and 29 CAG repeats (lane 2). It is worth noting that the CAG repeat numbers

Fig. 2. PCR analysis of the MJD gene. PCR-amplified CAG repeats in the amniocentesis sample from the fetus (lane 1), in blood DNA sample from the mother (lane 2), and a nonrelated human wild-type control (lane 3). The four tracks on the left contain pGEM sequence, which were used to determine the size of alleles. The corresponding sizes of the alleles are indicated on the left. EX, expanded allele; NL, normal allele.

Fig. 3. Detection of mutant MJD protein from fetal fibroblasts. Fetal fibroblasts were cultured and the cultured cell lysate was subjected to Western blot analysis with anti-MJD monoclonal antibody.

of the father were previously identified to be 78 and 15 through radioactive PCR (25), as indicated in the bottom of Fig. 1. After genetic counseling regarding the results of the prenatal diagnosis, the couple requested termination of the pregnancy. The couple received counseling and psychological and social support. Subsequently, fetal fibroblasts were obtained for further analysis by Western blot. The expression of both normal and mutant ataxin-3 protein was demonstrated by the use of monoclonal antibody against ataxin-3 (see Fig. 3). The results showed that the expanded ataxin-3, with a molecular weight of about 62 kDa, migrated at a higher position than the normal ataxin-3, with a molecular weight of about 47.5 kDa.

DISCUSSION

We have successfully identified an affected MJD gene from amniocentesis sampling at gestation of 13 week. Our prenatal process was performed with a similar protocol that was reported in other countries (26). The prenatal protocol included two counseling sessions, psycho-social evaluation of the couple, and obstetrical assessment. In particular, motivations for an eventual termination and decision-making processes were evaluated. After the genetic testing, in the case of pregnancy termination, the couple received counseling and psychological and social support. In this study, we applied nonradioactive PCR and radioactive PCR analysis for prenatal diagnosis of a fetus at risk of MJD. The fetal DNA showed two PCR products with 74 and 16 CAG repeats, while the father was a presymptomatic individual with 78 and 15 CAG repeats on MJD alleles. Our data indicated that the size of the expanded MJD gene could decrease in repeat number of fetus during transmission to further generations. This was in contrast to the unidirectional expansion of the CAG repeat of the MJD gene reported by Maruyama et al. (27), but it was similar to our previous results (25). In addition, application of nonradioactive genomic PCR for detecting the CAG repeat range of the MJD gene made the molecular screening of MJD mutation easier in our laboratory. However, radioactive PCR analysis was required to deduce the exact number of CAG repeats.

Our results from Western blot analysis showed that the normal and mutant ataxin-3 migrated slower than what the molecular weight would indicate, indicating possible posttranslational modification(s) of ataxin-3. However, further experiments are required to verify this point. In addition, it has been demonstrated that the expanded polyglutamine in the Machado-Joseph disease protein induces cell death in vitro and in vivo (28). Animal models have shown that truncated expanded MJD fragments can induce the formation of intra-

nuclear inclusions that causes damage at the cellular level, whereas the normal ataxin-3 protein does not (29). All these observations suggested that it is important to understand the expression and cellular functions of ataxin-3. It was previously reported that the normal and mutant ataxin-3 are constitutively expressed in all human tissues (19). Our results further demonstrated for the first time that the expression of both normal and expanded ataxin-3 were present in the fetus. However, the cellular functions of ataxin-3 and the reasons for the late onset of the disease is still unclear.

In conclusion, we report here the first case of prenatal diagnosis of MJD in Taiwan. The diagnosis was made using a simple nonradioactive PCR for rapid detection of the presence of an expanded MJD allele. However, the accurate number of CAG expansion in the MJD gene should be resolved by the use of radioactive PCR, which is a standard assay for diagnosis of SCA3. In the case of homoallelic individuals, Southern-blot method may be used to completely exclude cases of nonamplification of expanded alleles in the homoallelic individuals (30). It is noted that a reduction in CAG triplet repeats of the MJD gene was found in this case of paternal transmission. In addition, the expression of both normal and mutant ataxin-3 was detected early in the 13-week-old fetus. In about 40% of ADCA families, the genetic background of the diseases can be identified. In these families genetic testing also enables prenatal testing. However, because the cause of ADCA still remains unidentified in most families, there is a need for training genetic counselors to understand the phenotype and genotype differences in ADCA. This will certainly benefit the patients and families with autosomal dominant cerebellar ataxias (ADCAs).

ACKNOWLEDGMENTS

We thank the family used in this study for their cooperation. We also thank Dr. Nobuyuki Nukina for the valuable MJD monoclonal antibody.

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