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Rapid detection of large expansions in progressive myoclonus epilepsy type 1, myotonic dystrophy type 2 and spinocerebellar ataxia type 8

Szybkie wykrywanie dużych ekspansji w postępującej padaczce miokloniczej typu 1, dystrofii miotonicznej typu 2 i ataksji rdzeniowo-móżdżkowej typu 8

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Abstract

Background and purpose: Human genetic disorders associated with multiple unstable repeats resulting in long DNA expansions are difficult to identify by conventional polymerase chain reaction (PCR) in routine molecular testing, and therefore require time-consuming hybridisation. To improve and expedite the diagnostic methods for progressive myoclonus epilepsy (EPM1), myotonic dystrophy 2 (DM2) and spinocerebellar ataxia 8 (SCA8) caused by dynamic mutations, we adapted a repeat primed PCR (RP-PCR) assay which was previously developed for testing of other triplet repeat disorders.
Material and methods: The new algorithm for molecular analysis was to run a standard PCR to yield alleles in an amplifiable range and then run a RP-PCR to detect larger expansions. Electrophoresis and visualisation of PCR products on an automatic sequencer were applied to determine normal and pathogenic alleles comprising $(C_4GC_4GCG)_n$ in EPM1 in 44 subjects, $(CCTG)_n$ in DM2 in 76 individuals and $(CTG)_n$ in SCA8 in 378 patients.
Results: The protocol combining conventional PCR and RP-PCR proved to be a rapid and reliable test to diagnose the above named disorders. Among 44 individuals tested for EPM1, two expanded alleles were identified in 7 patients. Out of 76 apparently homozygous subjects, RP-PCR allowed us to detect 56 expansions specific to DM2, and out of 378 ata-

Streszczenie

Wstęp i cel pracy: Oparta na konwencjonalnej reakcji łańcuchowej polimerazy (PCR) diagnostyka molekularna chorób związanych z niestabilnymi sekwencjami powtórzonymi bywa niewystarczająca do wykrycia ogromnych ekspansji, wówczas konieczne jest stosowanie hybrydyzacji. W celu usprawnienia analizy molekularnej trzech chorób wywoływanych mutacjami dynamicznymi: postępującej padaczki miokloniczej (EPM1), dystrofii miotonicznej typu 2 (DM2) i ataksji rdzeniowo-móżdżkowej typu 8 (SCA8) zaadaptowano technikę RP-PCR (*repeat primed PCR*), którą uprzednio opracowano dla chorób powodowanych ekspansjami sekwencji trójnukleotydowych.
Materiał i metody: Oprócz standardowej reakcji PCR, w której uzyskiwano allele w zakresie możliwym do amplifikacji, celem detekcji większych ekspansji stosowano RP-PCR. Elektroforetyczny rozdział produktów PCR na automatycznym sekwenatorze umożliwiał analizę alleli z zakresu prawidłowego oraz patogennego, które zawierały dwunastonukleotyd $(C_4GC_4GCG)_n$ w EPM1 u 44 osób badanych, czteronukleotyd $(CCTG)_n$ w DM2 u 76 osób i trójnukleotyd $(CTG)_n$ w SCA8 u 378 pacjentów.
Wyniki: Zastosowany protokół diagnostyczny oparty na konwencjonalnej reakcji PCR i RP-PCR pozwolił na szybkie otrzymanie wiarygodnych wyników testu genetycznego w kie-

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xia patients, a large allele of the *ATXN8OS* gene (SCA8) was found in 25 subjects.

Conclusions: Here, for the first time, we report detection of large expansions in EPM1 and SCA8 patients. This RP-PCR assay is high throughput, reproducible and sensitive enough to be successfully used for diagnostic purposes.

Key words: dynamic mutations, neurodegenerative disorders, repeat primed PCR (RP-PCR).

runku wyżej wymienionych chorób. Spośród 44 osób poddanych analizie w kierunku EPM1 wyodrębniono 7 przypadków patogennej ekspansji. Wśród 76 osób z dystrofią miotoniczną, których wyniki po standardowej reakcji PCR wskazywały na genotyp prawidłowy (homozygoty) zastosowanie reakcji RP-PCR umożliwiło identyfikację 56 osób z ekspansją powodującą DM2. Analogicznie w grupie 378 pacjentów z ataksją, w 25 przypadkach wykryto patogenne allele genu *ATXN8OS* (SCA8).

Wnioski: W pracy przedstawiono po raz pierwszy zastosowanie RP-PCR w diagnostyce molekularnej do wykrywania dużych ekspansji u pacjentów z podejrzeniem EPM1 i SCA8. Ze względu na wysoką wydajność, powtarzalność i czułość techniki RP-PCR może być stosowana do celów diagnostycznych.

Słowa kluczowe: mutacje dynamiczne, choroby neurodegeneracyjne, reakcja RP-PCR.

Introduction

Over 40 neurodegenerative disorders are known to result from unstable DNA repeat expansion in both coding and non-coding regions of implicated genes. This genetic instability, called dynamic mutation, manifests as a change in copy number (expansion and sometimes contraction) with a rate that depends to some extent on the tract length of initial repeats, tissue specificity or generational age [1]. Disorders associated with repeat multiplication include the tri-nucleotide expansion disorders, such as Huntington disease (HD), eight spinocerebellar ataxias (SCAs), myotonic dystrophy type 1 (DM1) and others. Disorders such as myotonic dystrophy type 2 (DM2), caused by tetranucleotide expansions; spinocerebellar ataxia type 10 (SCA10), caused by pentanucleotide multiplication; and progressive myoclonus epilepsy 1 (EPM1), caused by minisatellite (dodecamer) expansions, belong to the same class of human genetic diseases.

Dynamic mutations resulting in long expansions (for example, there are over 2,000 CTG repeats in the congenital form of DM1) are difficult or sometimes impossible to detect by the genotyping of PCR products. For example, testing for DM2 by routine PCR only allows detection of repeat fragments up to 300 base pairs (bp) in the *ZNF9* locus. Thus, Southern blot analysis has been used to detect large expanded alleles even though the size of the expansion can be only approximated using this method. Moreover, this technique often involves radioactive labelling and laborious hybridisation steps, and requires large amounts of DNA. Another method used to detect long expansions is called RP-PCR (repeat primed PCR). In a previous study, RP-PCR was developed as TP-PCR (triplet primed PCR) for the molecular analysis of DM1 [2] and was later successfully applied in other repeat-associated diseases such as Friedreich ataxia (FRDA), SCA2, SCA7 and SCA10 [3,4]. RP-PCR was recently used to detect tetranucleotide expansions in DM2 patients [5,6].

Progressive myoclonus epilepsy type 1 (EPM1, Unverricht-Lundborg disease, OMIM 254800) is an autosomal recessive neurodegenerative disorder caused by expansions of the $(C_4GC_4GCG)_n$ minisatellite sequence in the promoter of the *CSTB* gene that causes a defect in cystatin B, a cysteine protease inhibitor [7]. Normal alleles contain two or three copies of the dodecamer repeat, whereas a majority of pathogenic expanded alleles contain roughly 45-70 repeats. About 10% of EPM1 cases are caused by point mutations. Conventional PCR is able to detect products of about 950 bp, but amplification of these CG-rich sequences is known to be problematic and requires several modifications. The onset of clinical symptoms in EPM1 ranges from 6 to 15 years of age. Affected individuals present with stimulus-sensitive myoclonus and tonic-clonic epileptic seizures with variable progression; mental retardation or dementia as well as ataxia are also observed [8].

Myotonic dystrophy type 2 (DM2, OMIM 602668) is a progressive multisystem disorder affecting skeletal and smooth muscles and is inherited as an auto-

somal dominant trait. The DM2 corresponding gene *ZNF9* (3q13.3-q24) codes for cellular nucleic acid-binding protein (zinc finger protein 9) and contains the complex repeat motif $(TG)_n(TCTG)_n(CCTG)_n$ in the first intron. The size in normal alleles ranges from 104 to 176 bp and is usually reported in base pair length due to highly polymorphic TG and TCTG repeat tracts [9]. Moreover, the CCTG tract in normal alleles contains one or more tetranucleotide interruptions (TCTG or GCTG) [10]. A characteristic feature in DM2 patients is the loss of interruptions and a $(CCTG)_n$ repeat expansion that ranges from 75 up to 11,000 repeats; amplification of this expansion is impossible in a routine PCR. Additionally, the sensitivity of Southern blotting was estimated to be about 70% and can miss expansions containing over 5,000 CCTG repeats [11].

Spinocerebellar ataxia type 8 (SCA8, OMIM 608768) is a slowly progressive ataxia in which patients present with dysarthria, gait instability, nystagmus and other neurological signs. Patients with SCA8 were reported to have an abnormal number of CTA/CTG repeats in the *ATXN8OS* gene. The non-pathogenic range of this microsatellite region is 15–50 repeats; the size usually associated with ataxia ranges from 80 to 250 repeats, but alleles with up to 800 CTA/CTG repeats have also been observed. The mode of inheritance is autosomal dominant with a reduced penetrance, as alleles of more than 80 repeats are also observed in healthy individuals [12].

Due to high demand for molecular diagnosis in the above named neurological diseases, a reliable and rapid detection methodology must be developed. As the application of RP-PCR in testing for EPM1 and SCA8 has not previously been reported, the purpose of this study was to evaluate RP-PCR as a screening and diagnostic technique in affected individuals suspected of having these disorders.

Material and methods

DNA samples were extracted as described elsewhere [13] or by automated isolation on a Roche MagNA Pure Compact (Japan) device. Informed consent was obtained from all patients participating in the study. The following numbers of tested patients' DNA samples were analysed: 44 individuals suspected of having EPM1 and 17 family members (parents), 76 patients with clinical diagnosis of myotonic dystrophy in whom DM1 was previously excluded and a homozygous DM2 genotype in a routine PCR was determined, and 378 ataxia patients in whom expansions specific for SCA1, SCA2, SCA3, SCA17 were not found. All ataxic subjects had only one *ATXN8OS* normal allele detected, suggesting either the status of normal homozygote or the presence of large allele unamplifiable in a routine PCR. Moreover, apart from 76 affected subjects, for 4 individuals at 50% risk of DM2 molecular analysis was performed.

The patients included in the study were referred for genetic testing mainly by neurologists to confirm or to exclude clinical diagnosis of DM, SCA or EPM1. After the proband's molecular evaluation, unaffected individuals at risk were given genetic counselling and had testing done at their request.

The study was approved by the Bioethical Commission of the Institute of Psychiatry and Neurology, Warsaw.

The general principle in the RP-PCR protocol is to use three primers: a fluorescently labelled primer adjacent to the polymorphic sequence specific to the gene; a second primer consisting of several repeats, for example trinucleotide (DM1), tetranucleotide (DM2) or one dodecamer repeat (EPM1); and a 5' tail sequence complementary to the third universal primer P3R (artificially produced random DNA).

In the early amplification rounds, the PCR assay uses a locus-specific forward primer together with the reverse 5' tailed primer consisting of several microsatellite repeats to amplify a mixture of products. The mixture is due to priming within different sites of the repeated sequence. Then, after exhausting the first reverse primer with the 5' tail sequence (due to a 10 : 1 molar ratio of P3R to 5' tailed reverse primer), the third primer, P3R, which comprises the sequence complementary to the tail, preferentially binds to the end of amplicons from previous amplification cycles. In visualisation of RP-PCR products, a characteristic ladder can be observed which results from the mixture of amplicons of different length.

The molecular diagnosis of EPM1, DM2 and SCA8 was performed in two steps: (1) following the standard PCR reaction, the procedure was finished when two normal size alleles were observed; (2) the RP-PCR reaction was performed for DNA samples that showed only one visible signal in the electropherogram (suggesting possible homozygosity or presence of an abnormal allele) or in samples with no signal in the normal range (in cases of recessive EPM1).

Primer sequences used in PCR and RP-PCR are shown in Table 1; PCR thermal conditions and reac-

tions mixes are shown in Table 2 and Table 3, respectively.

The GeneAmp 9700 (Applied Biosystems, Foster City, CA USA) thermal cycler was used to perform the PCR reactions. Analysis of C_4GC_4GCG (EPM1) repeats was done by conventional PCR with primers as previously described [14]. The problematic amplification of the CG-rich region of the *CSTB* gene required the following modifications to thermal cycling conditions: 0.9°C/s RAMP and use of 150 ng/μL of genomic DNA.

The standard PCR and RP-PCR amplicons were separated on ABI PRISM 377 (Applied Biosystems, Foster City, CA USA) with the internal size marker FraX or TAMRA2500 (Applied Biosystems, UK) in a 4% denaturing gel (EPM1) and in a 5% gel (DM2) or a 4% gel (SCA8) with the internal size marker TAMRA500 (Applied Biosystems, UK). Sizing analyses were performed with Gene Scan v. 3.1.2 software. Alternatively, analysis of fluorescent RP-PCR products was done using an ABI3130 automatic sequencer (Applied Biosystems, Foster City, CA USA) on a 4 capillary array with the universal POP7 polymer and a ROX-GS500 size standard marker (Applied Biosystems, UK). Gene Mapper v. 4.0 software was used for amplicon sizing.

Results

Progressive myoclonus epilepsy type 1

The diagnostic protocol established by combining standard PCR and RP-PCR assays correctly identified the allelic sizes of the *CSTB* gene in all 44 symptomatic individuals tested: 7 subjects with two expanded alleles and 37 individuals with normal-sized alleles. Moreover,

Table 1. Routine polymerase chain reaction (PCR) and repeat primed PCR (RP-PCR) primer sequences

Disease	Primers flanking the polymorphic sequence, specific to the gene – routine PCR
EPM1	F 5'-AGC CTG CGG CGA GTG GTG-3' R 5'-**FAM**-GGC CGG GGA GGA GGC ACT-3'
DM2	F 5'-GCC TAG GGG ACA AAG TGA GA-3' R 5'-**FAM** GGC CTT ATA ACC ATG CAA ATG-3'
SCA8	F 5'-GTA AGA GAT AAG CAG TAT GAG GAA GTA TG-3' R 5'-**FAM** GGT CCT TCA TGT TAG AAA ACC TGG CT-3'
	Primers specific to the repeats – RP-PCR
EPM1	F 5'-**TAC GCA TCC CAG TTT GAG ACG** CCC CGC CCC GCG CCC CGC CCC GCG-3'
DM2	F DM2A 5'-**TAC GCA TCC CAG TTT GAG ACG** CCT GCC TGC CTG-3' R DM2B 5'-**FAM** TGA GCC GGA ATC ATA CCA GT-3'
SCA8	P4CAG 5'-**TAC GCA TCC CAG TTT GAG ACG** CAG CAG CAG CAG CAG CA-3'
	Universal primer
P3R	P3R 5'-**TAC GCA TCC CAG TTT GAG ACG**-3'

EPM1 – progressive myoclonus epilepsy type 1, DM2 – myotonic dystrophy type 2, SCA8 – spinocerebellar ataxia type 8, F – forward primer (starter forward), R – reverse primer (starter reverse)

Table 2. Polymerase chain reaction (PCR) conditions for EPM1, DM2, SCA8 and RP-PCR

	EPM1/EPM1 RP-PCR	DM2	DM2 RP-PCR	SCA8/SCA8 RP-PCR
Initial denaturation	98°C/5 min	95°C/5 min	95°C/5 min	95°C/5 min
Denaturation	98°C/1 min	95°C/45 s	95°C/1 min	95°C/1 min
Annealing	65°C/1 min	57°C/45 s	57°C/1 min	56°C/1 min
Elongation	78°C/3 min	72°C/1 min	72°C/2 min	72°C/2 min
Final elongation	78°C/10 min	72°C/10 min	72°C/10 min	72°C/10 min
Number of replication rounds	32	28	32	32

RP-PCR – repeat primed PCR, EPM1 – progressive myoclonus epilepsy type 1, DM2 – myotonic dystrophy type 2, SCA8 – spinocerebellar ataxia type 8

Table 3. Polymerase chain reaction (PCR) reaction mixes

	EPM1	DM2		SCA8
	PCR/RP-PCR	PCR	RP-PCR	PCR/RP-PCR
Polymerase	1.25 U Pfu TURBO Hot Start + buffer (Stratagene)	Ampli Tag Gold + II buffer (Applied Biosystems)		Ampli Tag Gold + II buffer
		1.25 U	5 U	5 U
dNTPs	dNTPs mix + 7-deaze (2 mM) (mix of dATP, dTTP, dCTP, without dGTP)	dNTPs 2.5 mM each		dNTPs 2.5 mM each
Others	DMSO 10%	Triton X 10% (BDH England) Gelatine 0.1% (Sigma)	DMSO 10%	DMSO 10%
Primers forward/reverse	10 μM	10 μM		10 μM
5' tailed reverse primer	1 μM	1 μM		1 μM
Total volume	20 μL	20 μL		20 μL

RP-PCR – repeat primed PCR, EPM1 – progressive myoclonus epilepsy type 1, DM2 – myotonic dystrophy type 2, SCA8 – spinocerebellar ataxia type 8

additional testing of 17 DNA samples of the affected children's parents revealed 8 carriers (heterozygotes) with one expanded allele. A range of 60-79 detectable dodecamer repeats in DNA samples from symptomatic individuals and carriers was established. In RP-PCR a signal consisting of a ladder with 12 bp periodicity corresponding to the dodecamer $(C_4GC_4GCG)_n$ was obtained (Fig. 1). This ladder confirmed the presence of the expansion. In cases in which only one allele of normal range was detected in the electropherogram, suggesting homozygosity or carrier status, re-evaluation by RP-PCR was performed to detect or to exclude the expansion. Within the group of 7 patients mentioned above (with two expanded alleles), prior testing in 2 children by a conventional PCR yielded no products and failed to detect expansions even though testing in their parents revealed

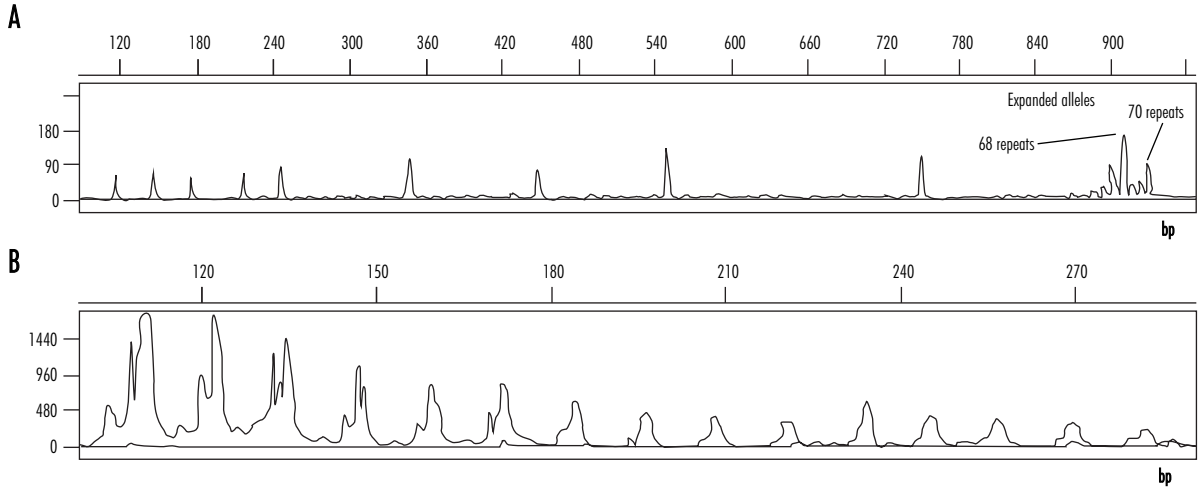

Fig. 1. Electropherograms of progressive myoclonus epilepsy type 1 (EPM1) conventional polymerase chain reaction (PCR) and repeat primed PCR (RP-PCR) products. A) Two expanded alleles (peaks) indicated by the arrows: 910 bp (68 repeats) and 934 bp (70 repeats) of *CSTB* gene in EPM1 patient. The other peaks visible in the electropherogram correspond to the internal size standard FraX from Applied Biosystems. B) Full mutation of *CSTB* gene confirmed by RP-PCR – the ladder of peaks, with 12 base pairs (bp) periodicity is shown. Peaks visible in the electropherograms represent the PCR reaction products, the x axis shows product size in bp, and the y axis shows the peak height measured by signal intensity of fluorescently labelled PCR products. Each electropherogram is presented with its own scale according to different PCR products' size and reaction efficiency

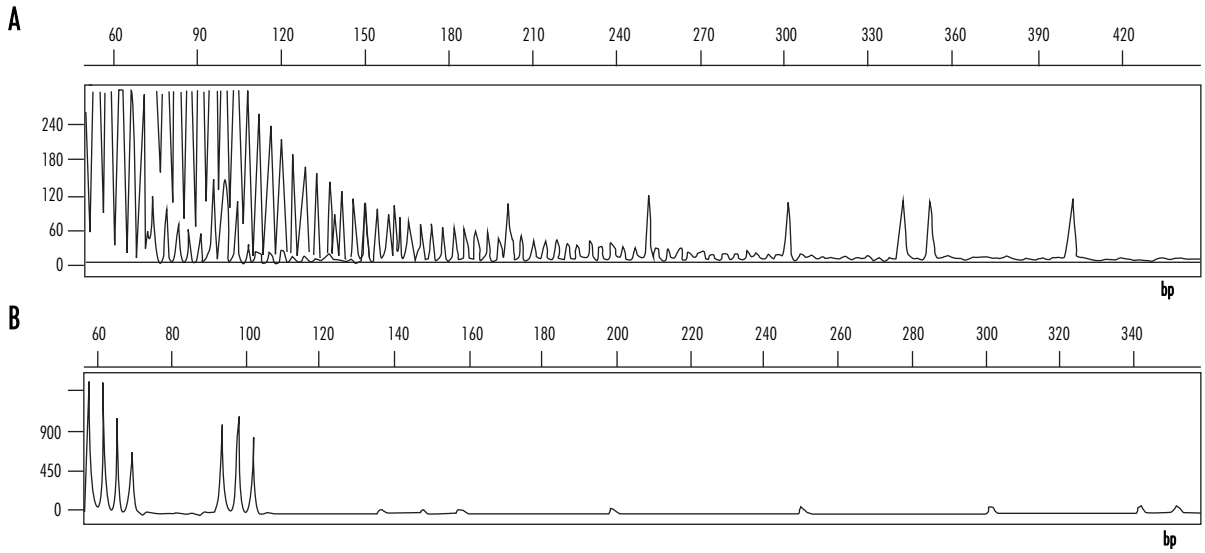

Fig. 2. Electropherograms of spinocerebellar ataxia type 8 (SCA8) repeat primed polymerase chain reaction (RP-PCR) products. A) Full range mutation in ATXN8OS gene in SCA8 patient – characteristic ladder. B) Normal SCA8 RP-PCR result excluding the diagnosis of SCA8 – no ladder of peaks. The other small peaks visible on the right in the electropherogram correspond to the internal size marker TAMRA 350 from Applied Biosystems. Peaks visible in the electropherograms represent the PCR reactions products, the x axis shows product size in bp, and the y axis shows the peak height measured by signal intensity of fluorescently labelled PCR products. Each electropherogram is presented with its own scale according to different PCR products' size and reaction efficiency

alleles of abnormal amplifiable range. The lack of products in standard PCR suggested meiotic instability resulting in larger expansions and therefore the PCR failed to amplify the two very long alleles. However, the fluorescent RP-PCR profile with a periodic 12 bp ladder was obtained for these EPM1 symptomatic siblings and confirmed the causative mutation in the *CSTB* gene.

Myotonic dystrophy type 2

In 76 individuals with previously excluded DM1, the results of conventional PCR for DM2 suggested a homozygotic status; however, the use of RP-PCR allowed us to detect the expansions specific to DM2 in 56 cases and confirmed the clinical diagnosis of myotonic dystrophy. The remaining 20 subjects were revealed to be normal homozygotes which excluded the diagnosis of DM. Following the genetic counselling, 4 individuals at 50% risk of DM2 had genetic testing done, which detected expansion specific to DM2. Altogether the RP-PCR method enabled us to identify 60 carriers of the pathogenic expansion in the *ZNF9* gene.

Spinocerebellar ataxia type 8

Out of 378 ataxia patients with homozygous alleles of the *ATXN8OS* gene, testing by RP-PCR allowed us to detect a large allele in 25 subjects, suggesting the clinical presentation of SCA8, and confirmed normal homozygotic status in the remaining 353 individuals. The characteristic ladder was observed in SCA8 patients. A representative electropherogram of RP-PCR products from a normal homozygote and a pathogenic CTG repeat expansion is shown in Fig. 2.

Discussion

The goal of this study was to introduce and evaluate a rapid PCR-based test enabling detection of large causative expansions in three neurological disorders: EPM1, DM2 and SCA8. The suggested two-step diagnosis protocol established by combining conventional PCR and RP-PCR assays correctly identified the mutations in the above named diseases.

In RP-PCR based testing for recessive EPM1, we recommend inclusion of the parents' DNA samples. It expedites the molecular evaluation of a homozygous patient's status and is helpful in the case of any amplicon's absence in standard PCR. Because the full penetrance alleles contain more than 30 dodecamer repeats (454 bp) and the largest allele comprises approximately 125 repeats (1595 bp), as detected by Southern blotting [14], we found the RP-PCR assay to be a very convenient diagnostic method.

RP-PCR also proved to be a valuable tool for DM2 and SCA8 expansion detection in homozygotic indi-

viduals (one normal allele amplified in a routine PCR), including both normal and expanded alleles. Although conventional Southern blotting is an accurate technique for molecular diagnosis in DM1, it is unsatisfactory for detection of mutations in DM2. Pathogenic alleles of the *ZNF9* gene are known to be extremely large (a mean size of approximately $(CCTG)_{5000}$). Additionally, somatic instability of the dynamic mutation has been reported. Problematic implementation of hybridisation and a sensitivity of only 70-80% in Southern blotting can result in up to 30% false-negative results. This makes Southern blotting an unreliable tool for genetic testing of DM2 [11]. Another diagnostic method called CISH (chromogenic in situ hybridisation), which involves analysis of muscle sections, has proven to be useful in CCTG expansion detection but it does not allow for accurate sizing of DM2 pathogenic alleles and requires muscle biopsy [15]. Because DM2 causative expansions within the *ZNF9* gene are too large for amplification in conventional PCR, all expansion-positive individuals will appear homozygous and thus indistinguishable from the 15% of unaffected controls who are true homozygotes [11]. Among our 76 homozygous DM2 subjects, RP-PCR distinguished 20 (26%) normal homozygotes and 56 (74%) expansion carriers.

RP-PCR based analysis in individuals with homozygous *ATXN8OS* gene alleles (according to standard PCR) revealed relatively frequent large expansions in the SCA8 locus (25 out of 378 tests – 6.6%). Therefore it may suggest that the percentage of mutation carriers could be underestimated, as the SCA8 prevalence range is thought to account for 2-5% of autosomal dominant ataxias [16] and because approximated frequencies of *ATXN8OS/ATXN8* expansions (more than 50 repeats) range from 1 : 100 to 1 : 1200 chromosomes in genotyped control groups [17-21].

Additionally, the RP-PCR assay proved to be a rapid and informative technique in prenatal diagnosis in families affected with DM1, and we have been able to document the presence of the expanded allele in the fetus (data not shown). In contrast to the large amounts of DNA required for Southern blotting, the analysis was performed with only a small amount of fetal DNA extracted from chorionic villi. The RP-PCR assay was high throughput and highly reproducible.

Conclusions

1. The RP-PCR assay allowed us to broaden the molecular diagnostic testing offered and improve detection of expanded alleles or to demonstrate their absence in three hereditary neurological disorders: EPM1, SCA8 and DM2.
2. As routine PCR analysis is unreliable in the detection of large minisatellite and microsatellite repeat expansions, and because other techniques such as Southern blotting are laborious and unsatisfactory, the sensitive and convenient RP-PCR assay may be considered a diagnostic method of choice.

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Disclosure

Authors report no conflict of interest.

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