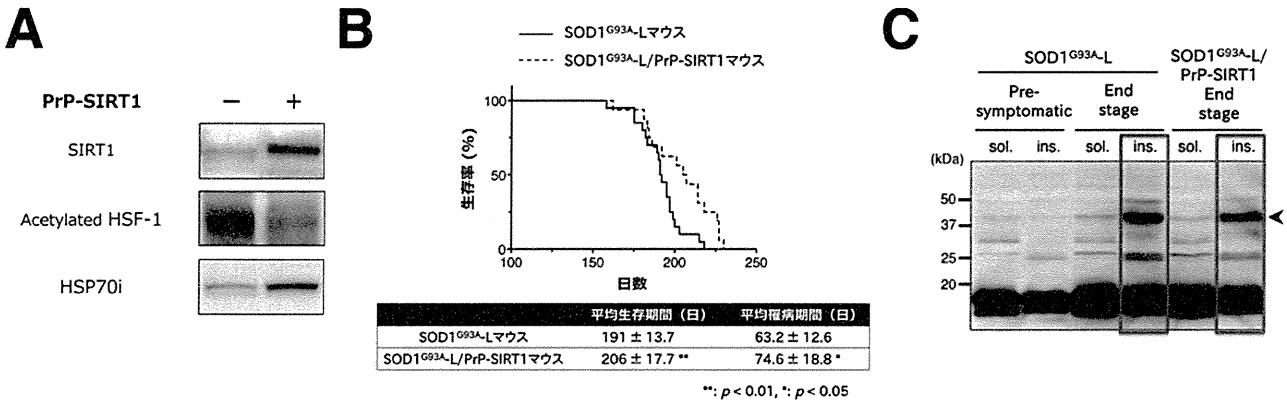
細胞に速やかに取り込まれてリソソームに局在したが、変異 SOD1 の発現下では酸化ストレス依存的に細胞質へ漏出して凝集体を形成することが判明した（図 2C）。このことから、リソソームプロテアーゼの異常化が想定され、実際、CysC のカテプシン B に対する阻害作用もオートファジーと同様に細胞保護に必須であることが明らかとなった。また、変異 CysC およびカテプシン阻害剤を用いた検討から、カテプシン B の阻害作用もオートファジーと同様に単独では細胞保護に十分でないと判明した。従って、CysC は AMPK を介したオートファジーの賦活化とカテプシン B の阻害を協調的に行うことによって、神経細胞保護的に機能していることが明らかとなった（図 2D）。

D. 考察

本研究により中枢神経系における SIRT1 の活性化が、HSF-1/HSP70i 経路の活性化による異常タンパク質の分解を介して、変異 SOD1 由来の毒性に対して神経細胞保護的に機能することを初めて明らかにした。SOD1G93A-H では生存期間の有意な延長は見られなかったが、これは HSP70i の量が SOD1G93A-H では飽和していた可能性が考えられた。従って、分子シャペロンは異常タンパク質由来の毒性から神経細胞を保護する重要な経路であるが、十分な効果を発揮するにあたっては、オートファジーやプロテアソームなど、他の異常タンパク質分解経路と併せて協調的に活性化することが必要であると考えられる。また、PrP-SIRT1 マウスは寿命や神経学的、行動学的な特徴が野生型と変わらず、今後、神経疾患などにおける SIRT1 の役割を明らかにするうえで有用なツールになると期待される。

さらに、本研究により CysC も変異 SOD1 由来の毒性に対して神経細胞保護的に機能することが判明した。興味深いことに、オートファジーの賦活化とプロテアーゼの阻害は共に細胞保護に必須であったが、単体では十分でなく、協調的に活性化される必要があった。このことは、SIRT1 の場合とも同じく、単一の細胞保護経路の活性化のみでは毒性を緩和するのに十分な効果を発揮できず、限定的な効果に留まることを示唆している。従って、今後、ALS の治療戦略を考えるうえで、複数の細胞保護経路の活性化を組み合わせていくことが必要になると考えられる。また、CysC は酸化ストレス依存的にリソソームから細胞質中へ漏出して凝集したことから、変異 SOD1 が酸化ストレスを介してリソソーム膜を傷害する可能性、およびブニナ小体は CysC の保護効果に伴う副産物である可能性が示唆された。



	平均生存期間（日）	平均罹病期間（日）
SOD1^{G93A}-Lマウス	191 ± 13.7	63.2 ± 12.6
SOD1^{G93A}-L/PrP-SIRT1マウス	206 ± 17.7 **	74.6 ± 18.8 *

**: $p < 0.01$, *: $p < 0.05$

図 1 SIRT1 は HSF-1/HSP70i 経路を活性化して異常な SOD1 を減少させ、神経保護効果を発揮する **(A)** PrP-SIRT1 マウス腰髄を用いた SIRT1, アセチル化 HSF-1, HSP70i のイムノブロット像. **(B)** SOD1^{G93A}-L マウスおよび SOD1^{G93A}-L/PrP-SIRT1 マウスの生存曲線. **(C)** Triton X-100 に可溶性（sol）および不溶性（ins）の各画分における SOD1 のイムノブロット像. 矢頭は SOD1 凝集体.

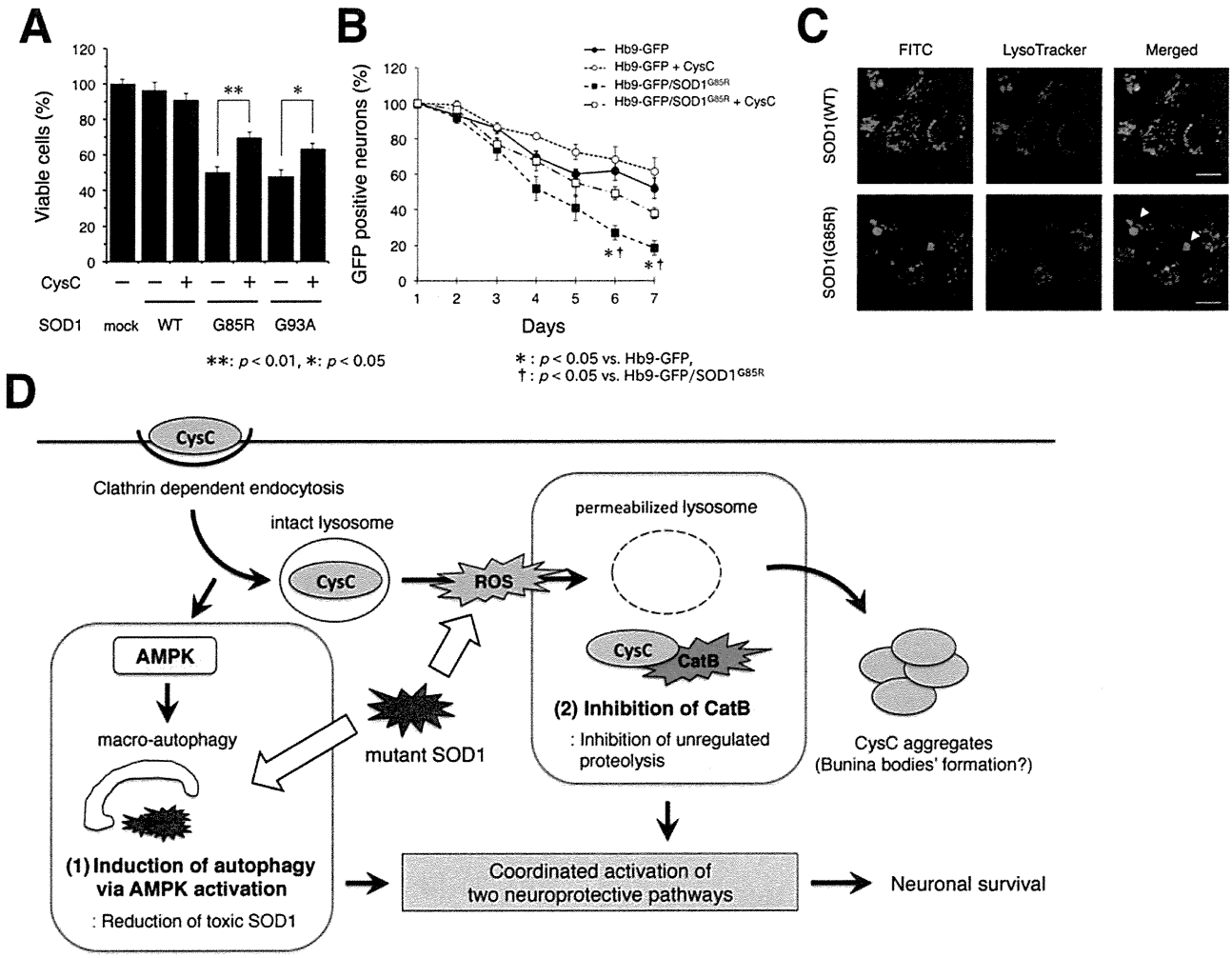


図 2 CysC はオートファジー賦活化とカテプシン阻害の協調的活性化により神経細胞を保護する (A, B) 培地に添加した CysC による変異 SOD1 を発現する Neuro2a 細胞（A）および初代培養運動神経細胞（Hb9-GFP 陽性細胞数）（B）に対する細胞保護効果. **(C)** FITC 標識した CysC の細胞内局在. 赤色はリソソームを示す. 矢頭は細胞質にある CysC 凝集体. **(D)** 本研究より明らかとなった CysC による神経細胞保護機構の模式図.

E. 結論

本研究によって SIRT1 および CysC は ALS における新規の内在性神経保護因子であることが明らかとなった。今後、これらの因子を活性化することによって効率的に運動神経細胞を保護する、新規の治療戦略が可能になると期待される。

F. 健康危険情報

該当なし

G. 研究発表

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H. 知的所有権の取得状況 (予定を含む)

1. 特許取得

該当なし

2. 実用新案登録

該当なし

厚生労働科学研究委託費　難治性疾患等克服研究事業（難治性疾患等実用化研究事業（難治性疾患実用化研究事業））
「筋萎縮性側索硬化症（ALS）新規治療法開発をめざした病態解明」

分担研究報告書

TDP-43 断片の exosome への選択的取り込みについて

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研究要旨
筋萎縮性側索硬化症(ALS)の原因蛋白質であるTDP-43を主要構成成分とする細胞質封入体は運動神経細胞死に関連し、病的性質を持ったTDP-43がcell-to-cellに拡がると考えられている。本研究では細胞外に放出される微小胞であるexosome内にTDP-43断片が選択的に存在することを明らかにし、それがin vivoでマウス脳の神経細胞内に取り込まれることを証明した。ALSの病態進展にexosomeが深く関与している事を示唆している。

A. 研究目的

筋萎縮性側索硬化症(Amyotrophic Lateral Sclerosis: ALS)における電気生理学的及び病理学的検索から、疾患の進展の機序の一つとして運動神経細胞死が発症部位から連続的に拡がっていることが示唆されるが、同時に非連続的伝播している事を疑わせる所見も報告されている．運動神経細胞死に関与するTDP-43を主要構成成分とする細胞質封入体は隣接する培養細胞間で伝播することが示されているが、遠隔的な伝播についての機序は解明されていない。

我々は、内部にmRNAやmiRNA、蛋白質などを含み細胞間コミュニケーションを担っている微小胞であるexosomeがTDP-43病理の遠隔的な伝播に関与していると考え、exosomeへのTDP-43及びその断片の取り込みについて実験を行った。

B. 研究方法

マウス神経芽細胞腫Neuro2aに野生型humanTDP-43 (hTDP-43 WT)及びC末端側にGFPやFlagなどのタグを挿入したhTDP-43を過剰発現させ、Western blot法で培養細胞の上清中に分泌されたexosome中のhTDP-43及びその断片の取り込みを調べた。同時に核、細胞質分画の抽出を行い、exosome内との分布の相違を確認した。

さらにC末端側にFlagを挿入したTDP-43-flagを過剰発現させたNeuro2aの上清から抽出したexosomeをマウス脳へ接種し、接種したTDP-43-Flagが神経細胞内へ取り込まれるかをFlag染色にて免疫組織学的に検索した。

C. 研究結果

hTDP-43 WTを過剰発現させた場合、exosome内には全長TDP-43よりも約32kDaのTDP-43C末端断片が多く含まれ、 核及び細胞質での全長TDP-43優位の分布とは異なるものだった。またexosome内の約32kDaのTDP-43断片は1% Sarcosyl不溶性分画に存在し、約25kDaの断片も少量認められた。C末端側にGFPをつけたhTDP-43を過剰発現させると、細胞内には全長のhTDP-43-GFPに加えhTDP-43を過剰発現させた時に存在した32kDa及び25kDaに相当するC末端断片が出現し、exosome内には32kDaに相当する断片が選択的に取り込まれていた。

hTDP-43-Flagを過剰発現させたNeuro2aから回収したexosomeをマウス脳の前頭葉の運動野に接種すると、接種部位の近傍にFlag染色で細胞質全体が淡く染まる細胞を確認した。さらに反対側の運動野に相当する部位にもneuropilと思われるFlag陽性の構造物を認めた。

D. 考察

exosomeは細胞質で形成される微小胞であり、細胞質では全長TDP-43が優位であるのに対しexosome内ではその断片が多いことは、exosomeへのなんらかの選択的取り込みもしくはexosome内で断片形成される機序があることを示唆している。exosome内のC末端断片は不溶化しており、これがexosomeを介して他の細胞内に取り込まれた場合、内因性のTDP-43を巻き込んで凝集するseedとして機能する可能性があると考えられる。

マウス脳の神経細胞でflag陽性の 神経細胞が確認出来た事は、in vivoでexosomeを介してTDP-43もしくはそのC末端断片がcell-to-cellに伝播した可能性があることを示している。さらにそれが反対側の運動野に運ばれていることは神経経路に沿った進展(regional spread)も起きていると考えられる。

E. 結論

Exosomeの形成過程で不溶性のTDP-43断片が存在することが示された。また、Exosome内にTDP-43断片が含まれ、TDP-43病理がexosomeを介して細胞間を遠隔的に伝播する可能性があることが示された。

F. 健康危険情報

該当なし

G. 研究発表

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H. 知的所有権の取得状況（予定を含む）

1. 特許取得

該当なし

2. 実用新案登録

該当なし

Ⅲ. 研究成果刊行一覧

研究成果の刊行に関する一覧表

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ORIGINAL ARTICLE

A blinded international study on the reliability of genetic testing for GGGGCC-repeat expansions in *C9orf72* reveals marked differences in results among 14 laboratories

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CA and AEV wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors. PMA and CK wish it to be known that, in their opinion, the last two authors should be regarded as joint last authors.

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ABSTRACT

Background The GGGGCC-repeat expansion in *C9orf72* is the most frequent mutation found in patients with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Most of the studies on *C9orf72* have relied on repeat-primed PCR (RP-PCR) methods for detection of the expansions. To investigate the inherent limitations of this technique, we compared methods and results of 14 laboratories.

Methods The 14 laboratories genotyped DNA from 78 individuals (diagnosed with ALS or FTD) in a blinded fashion. Eleven laboratories used a combination of amplicon-length analysis and RP-PCR, whereas three laboratories used RP-PCR alone; Southern blotting techniques were used as a reference.

Results Using PCR-based techniques, 5 of the 14 laboratories got results in full accordance with the Southern blotting results. Only 50 of the 78 DNA samples got the same genotype result in all 14 laboratories. There was a high degree of false positive and false negative results, and at least one sample could not be genotyped at all in 9 of the 14 laboratories. The mean sensitivity of a combination of amplicon-length analysis and RP-PCR was 95.0% (73.9–100%), and the mean specificity was 98.0% (87.5–100%). Overall, a sensitivity and specificity of more than 95% was observed in only seven laboratories.

Conclusions Because of the wide range seen in genotyping results, we recommend using a combination of amplicon-length analysis and RP-PCR as a minimum in a research setting. We propose that Southern blotting techniques should be the gold standard, and be made obligatory in a clinical diagnostic setting.

INTRODUCTION

In 2011, an expansion of a GGGGCC-repeat in the gene 'Chromosome 9 open reading frame 72' (*C9orf72*) was identified as a cause of amyotrophic lateral sclerosis (ALS, OMIM614260) and frontotemporal dementia (FTD, OMIM105550).[1 2] The following 3-years series of publications reported that a large proportion of ALS (1–30%) and FTD (6–30%) Caucasian patients carry a *C9orf72* repeat expansion,[3–5] making this mutation the most common known genetic cause of ALS and FTD, and one of the most frequent genetic alterations causing neurodegenerative diseases overall. In one of the initial reports, a combination of amplicon-length analysis, repeat-primed PCR (RP-PCR) assays, and Southern blot (SB) was used for detection and calculation of the repeat numbers.[1] SB is regarded as the gold standard for detecting large polynucleotide repeat expansions,[6] but it is relatively expensive, cumbersome and time consuming, and up to 10 μg of high-quality DNA is needed for a single analysis. It is not surprising, therefore, that in nearly all studies published during 2011–2013, the much simpler, cheaper and faster-to-perform PCR-based screening methods were used.[2] By using amplification primers flanking the repeat motif, the amplicon-length analysis allows determination of the exact repeat numbers of alleles with up to 30 repeats, and thus, is able to exclude a pathological repeat expansion if two different alleles in the wild-type range are detected. In RP-PCR, at least two primers are used: one primer that hybridises outside the repeat motif, and one primer that binds to the repeat motif itself. In most protocols a third

primer is applied that hybridises to an oligonucleotide tail of the repeat motif binding primer, also leading to the term triplet-primed PCR.[7] In this RP-PCR, a large GGGGCC-repeat expansion typically gives rise to a 'saw-tooth' or 'stutter' pattern, which has been taken as evidence for the presence of a disease-associated expansion.[1 2] Since the first reports of this mutation, a large number of studies have reported on the epidemiology, clinical, psychological and imaging features, and postmortem neuropathology of *C9orf72* repeat expansion carriers with ALS, FTD, Alzheimer disease, Parkinson's disease, and other neurodegenerative diseases, as well as in healthy individuals.[8] In many of these studies, the *C9orf72*-genotype was assessed using RP-PCR alone without a confirmatory SB analysis. Only recently, large-scale studies using SB analysis have been published.[9 10] PCR-based *C9orf72* screening is also used in clinical diagnostic testing of affected individuals, as well as in predictive testing of healthy individuals at-risk of ALS and FTD. The aim of this study is to determine the sensitivity and specificity of different *C9orf72* genotyping methods, and to establish recommendations for molecular testing of the GGGGCC-repeat expansion in *C9orf72*. Fourteen experienced genetic laboratories participated in this study, and all laboratories were requested to perform RP-PCR with or without amplicon-length analyses on the same 78 samples.

MATERIALS AND METHODS

Study design

At the initiative of the ALS research laboratory at Umeå University, Sweden, 20 laboratories with extensive experience in performing DNA analysis for neurodegenerative diseases, including ALS and FTD, were invited to participate in this study. Eleven research laboratories and two diagnostic laboratories agreed to participate, and including Umeå University a total of 14 laboratories participated. Umeå University sent 400 ng DNA from 78 familial ALS or FTD cases to 13 laboratories: eight in Europe, four in North America, and one in Asia. All participating research laboratories had already established the methodologies for identifying the GGGGCC-repeat expansion in *C9orf72* and published at least one manuscript regarding *C9orf72*. Each laboratory was asked to analyse the 78 DNA samples according to their own procedures and classify the results. Umeå University collected all results independently and analysed them in a blinded fashion. Eleven laboratories used RP-PCR and amplicon-length analysis, and three laboratories used RP-PCR alone. Independently to RP-PCR and amplicon-length analysis, SB was performed in three laboratories.

Patients and DNA extraction

Blood samples of 78 familial ALS or FTD patients from 32 families living in the Nordic countries, Switzerland and Portugal were collected. The sex ratio was 1.75 males per female. The ALS patients were diagnosed according to the EFNS consensus diagnostic criteria,[11] and the FTD patients according to the Neary criteria.[12] Autopsies were performed in 13 patients confirming the diagnosis of ALS or FTD neuropathologically. Whole venous blood was drawn into EDTA-containing vacuum tubes and following centrifugation, the buffy coat was isolated. The samples were collected during the time period 1993–2012 and stored as buffy coat in −80°C freezers until DNA extraction. The DNA was extracted according to the manufacturer's protocol with the DNA extraction kit NUCLEON BACC2 (GE Healthcare, Piscataway, New Jersey, USA) and DNA from the same extraction batch was sent to all laboratories. Ethical review boards in Sweden (The Regional Medical Review Board for Northern Sweden), Switzerland (Ethikkomission des Kantons St Gallen), and Portugal (Hospital de Santa Maria Ethics Committee, Lisbon) approved this study, and all participants gave informed written consent.

RP-PCR, amplicon-length analyses, and SB

The RP-PCR and amplicon-length analyses were done according to each laboratory's own method, and these are listed in online supplementary table S3. The SB protocols are listed in online supplementary table S5.

RESULTS

Genotyping results: comparison of the results in 14 laboratories

The overall results of 14 laboratories (laboratory A–N) are presented in table 1. When combining amplicon-length analysis and RP-PCR, sensitivity and specificity above 95% were found in seven laboratories (A–E, G and L; 50%). The mean sensitivity of the combined results was 95.0% (73.9–100%), and the mean specificity was 98.0% (87.5–100%). Using RP-PCR analysis alone, a sensitivity and specificity of more than 95% were found in six laboratories (A–D, G and L; 42.9%). The mean sensitivity of RP-PCR alone was 94.3% (71.7–100%) and the mean specificity was 97.3% (87.5–100%). The classification determined by RP-PCR alone, therefore, changed eight genotype calls in four laboratories (E, F, J and K). Or put differently, 6 of 14 laboratories (42.8%) failed to correctly classify some samples when they performed RP-PCR analysis only. By combining RP-PCR with fragment-length analysis, four laboratories still reported false negative or false positive samples. A significant number of samples (1–10) were unclassifiable/difficult to classify in eight of the laboratories even when RP-PCR and fragment-length analysis were combined.

Genotyping results: comparison of genotyped samples

The genotyping results of all 78 samples are summarised in online supplementary tables S1 and S2 available online. For 50 samples, the results among all laboratories were consistent. In the RP-PCR-only analysis, there were three individuals (6.5, 26.3 and 32.3) who were either misclassified or failed to classify in RP-PCR; these three samples had between 23 and 32 repeats on the wild-type allele, in addition to a large expansion on the other allele. In the amplicon-length analysis, two samples (samples 8.1 and 8.2) from the same family (number 8) were hard to classify: six laboratories identified two amplicons, four laboratories identified one amplicon, and one laboratory classified the samples as 'undecided'. We sequenced these samples and revealed that one allele was a wild-type allele with two repeats, and the other allele had six repeats with a complex of 15 bp deletion/17 bp insertion mutation just after the repeat motif (online supplementary figure S1A,B). We found one more sample (sample 32.1) that carried the same nucleotide variant on one allele, and this sample also carried a large repeat expansion on the other allele (online supplementary figure S1C,D). This mutation is located in the low-complexity sequence region of *C9orf72*,[13] and consequently, may interfere with the PCR-based genotyping method.

As a reference, SB was performed on all samples in a blinded fashion by three laboratories. There was complete concordance among the three laboratories: 46 samples carried a repeat expansion and 32 samples lacked the expansion (online supplementary figure S2).

Table 1 The results of 14 laboratories on 78 samples

	Laboratories													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
RP-PCR results														
GGGGCC-repeat expansion														
Yes	46	46	46	46	42	48	45	43	43	45	34	44	41	43
No	32	32	32	32	32	28	33	32	32	30	33	32	33	28
Q	0	0	0	0	4	2	0	3	3	3	11	2	4	7
False positive	0	0	0	0	0	2	0	0	0	2	1	0	0	0
False negative	0	0	0	0	1	0	1	0	0	0	2	0	1	0
Sensitivity (%)	100	100	100	100	91.3	100	97.8	93.5	93.5	93.5	71.7	95.7	89.1	93.5
Specificity (%)	100	100	100	100	96.8	87.5	100	100	100	93.8	96.9	100	100	87.5
Unclassified (%)	0	0	0	0	5.1	2.6	0	3.8	3.8	3.8	14.1	2.6	5.1	9.0
Amplicon-length analysis results														
Numbers of amplicon														
0	0	0	1	0	0	0	0	0	0	0	0	N.D.	N.D.	N.D.
1	54	54	55	56	56	54	54	51	56	54	51	N.D.	N.D.	N.D.
2	24	24	22	22	22	24	24	24	22	24	24	N.D.	N.D.	N.D.
Q	0	0	0	0	0	0	0	3	0	0	3	N.D.	N.D.	N.D.
RP-PCR+amplicon-length analysis results														
GGGGCC-repeat expansion														
Yes	46	46	46	46	46	48	45	43	43	43	34	44	41	43
No	32	32	32	32	32	29	33	32	32	30	34	32	33	28
Q	0	0	0	0	0	1	0	3	3	5	10	2	4	7
False positive	0	0	0	0	0	2	0	0	0	0	0	0	0	0
False negative	0	0	0	0	0	0	1	0	0	0	2	0	1	0
Sensitivity (%)	100	100	100	100	100	100	97.8	93.5	93.5	93.5	73.9	95.7	89.1	93.5
Specificity (%)	100	100	100	100	100	90.6	100	100	100	93.8	100	100	100	87.5
Unclassified (%)	0	0	0	0	0	1.3	0	3.8	3.8	6.4	12.8	2.6	5.1	9.0

No, sample numbers without large GGGGCC-repeat expansion; N.D., not determined; Q, sample numbers of undecided or unclassified samples Yes: sample numbers with large GGGGCC-repeat expansion.

DISCUSSION

Our blinded multicenter study demonstrates limitations of PCR-based techniques used to assess *C9orf72* GGGGCC-repeats, emphasising the need of detailed technical consensus guidelines for diagnostic and research settings.

Comparison of the methods of RP-PCR and amplicon-length analysis

Based on RP-PCR results alone, 50 samples (64.1%) showed congruent results among the participating laboratories. There are many variable RP-PCR protocols (see online supplementary table S3) and a comprehensive comparison is difficult. In summary, the RP-PCR protocols of the four laboratories (A–D) with 100% sensitivity and specificity, we found that laboratories A and C used almost identical methods. Laboratory B used primers that were a modification of the ones previously published,[1] and they deleted the unspecific linker region between the fluorescence tag and *C9orf72*-specific sequence. In laboratory D, the RP-PCR was performed with only two primers. The primers sets P1, 2, 3 and P4, 5, 6 were the most commonly used with five laboratories using each set, and two of the laboratories that obtained 100% sensitivity and specificity used primers set P1, 2, 3. Among the laboratories that failed to classify samples in concordance with the SB results (having false positive and/or false negative), we found that one used a very short PCR elongation time, the PCR products were diluted extensively before capillary electrophoresis, and the concentration of deaza-dGTP was very low, or deaza-dGTP was not used at all.

In the amplicon-length analysis alone, 72 samples (92.3%) had concordant results, highlighting the reliability of this technique, regardless of the differences in PCR reactions and PCR protocols. The set of primers were the same in all laboratories except one, but the PCR reactions and PCR protocols were all different between the laboratories (see online supplementary table S3). Thus, it is not possible to identify specific parameters that could explain the incorrect results for the six samples that were not concordant between the laboratories.

Analyses of the results

All five laboratories that obtained full concordance of the PCR-based and SB results used RP-PCR in combination with amplicon-length analysis. None of the laboratories that performed RP-PCR alone reported the correct genotype in all samples. The sensitivity and specificity increased, and the percentage of unclassified samples decreased in three laboratories (E, F and K) when they performed RP-PCR and amplicon-length analysis. Accordingly, a combination of amplicon-length analysis and RP-PCR methodology is recommended to obtain the highest level of sensitivity and specificity, but it should be emphasised that a high risk of misclassification as either false positive or false negative (6 samples in four laboratories) still exists.

The RP-PCR results alone seemed difficult to interpret if one allele with a relatively large number (20–32) of

GGGGCC-repeats was present in combination with a large repeat expansion on the other allele. This was demonstrated by three individuals who could not be genotyped using RP-PCR alone in five laboratories, possibly because the saw-tooth pattern curve of the large expanded alleles were hidden behind the peaks of the allele with 20–32 repeats (figure 1). Similarly, this intermediate 20–32 repeat allele could easily mimic an expanded allele and become a cause of false positive results.

Based on our data, there is no common definition of what a 'GGGGCC-repeat expansion' in *C9orf72* is, and laboratories classify it in different ways. For example, some laboratories used clear cut-offs, for example, of more than 24 or 30 repeats, while other laboratories used the definition that a saw-tooth pattern in RP-PCR corresponds to a GGGGCC-repeat expansion (see online supplementary table S4). In this study, there was no false positive result based on different classifications of what is an 'expansion'. This study was designed to compare the genotyping results of *C9orf72* among laboratories using the same DNA. An identical amount of DNA was send to all laboratories, but the concentration and quality of DNA may have changed

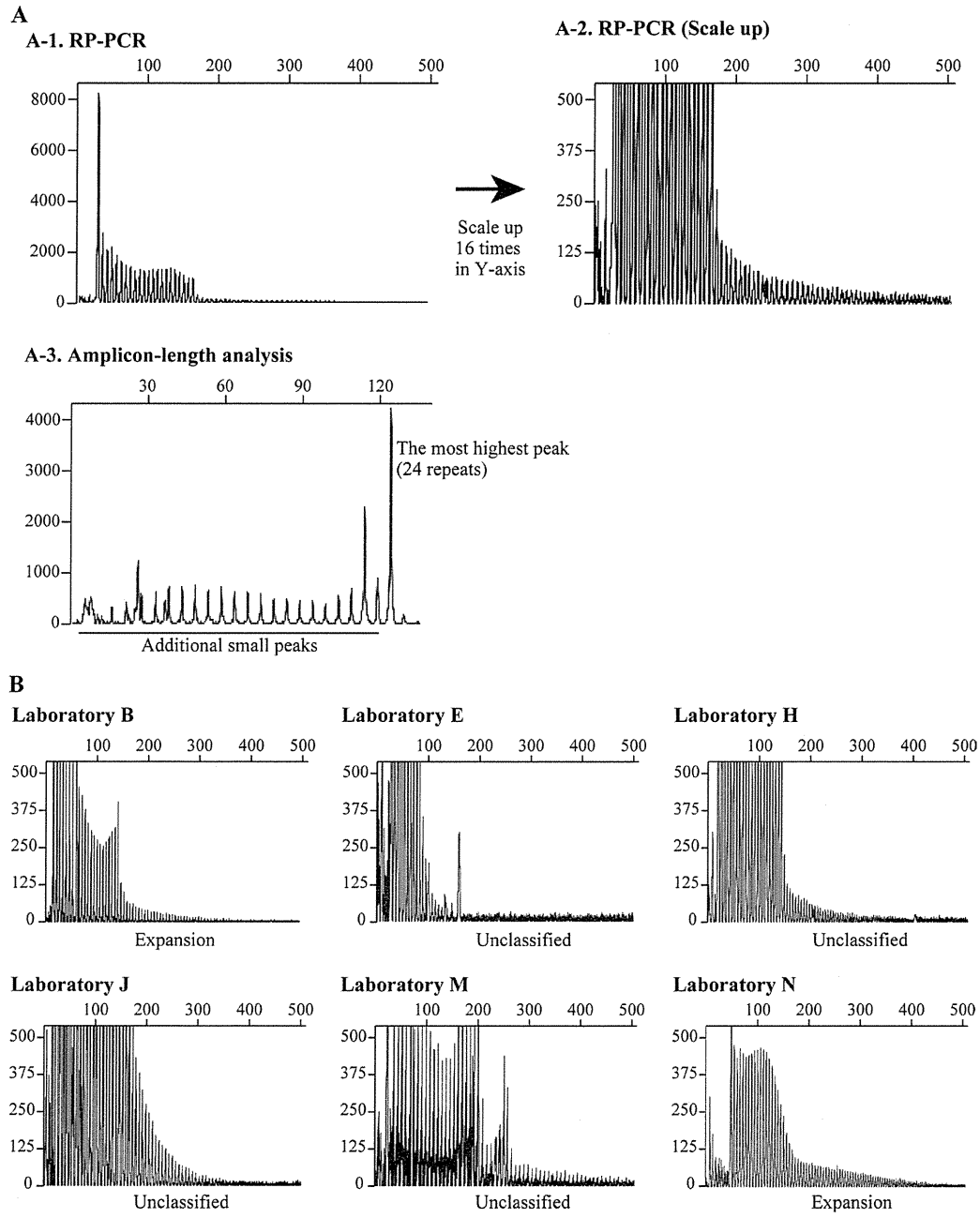

Figure 1 Results from RP-PCR and amplicon-length analysis of sample 6.5. (A) A-1 is the full shape of RP-PCR and A-2 is a 16-times scale-up (Y-axis) shape of A-1. It is possible to see the clear saw-tooth pattern after scale-up. A-3 is the result of amplicon-length analysis and there is a number of small peaks before the highest last peak. It may be hard to identify the amplicon numbers because of these small peaks. (B) The RP-PCR figures and classifications of the same sample in different laboratories. The scale of the Y-axis is the same as in A-2.

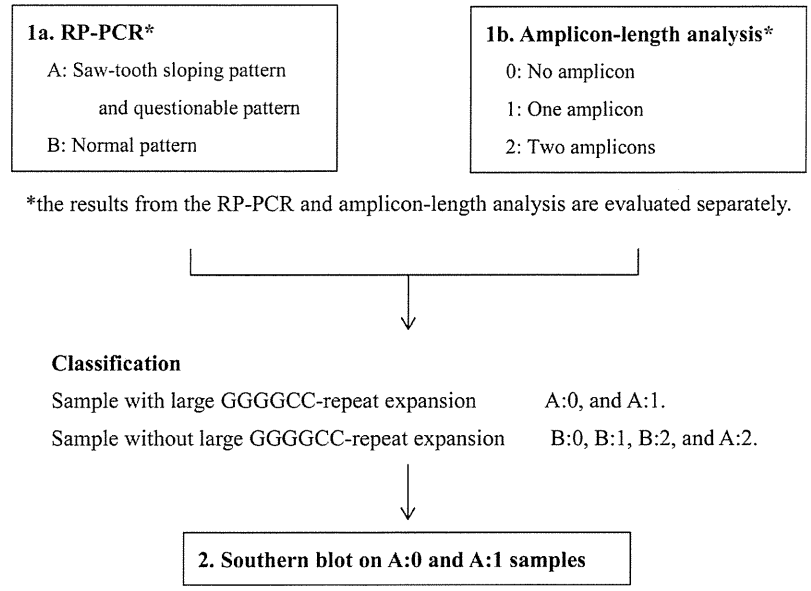

Figure 2 Flow chart for *C9orf72* genotyping in a scientific setting.

during shipping and handling. Another possible limitation of the present study is that most reactions were only performed once in each laboratory, and the accuracy may be improved if reactions were repeated in case of doubt, for example, with more DNA (however, only two laboratories requested more DNA).

Additionally, all laboratories that participated in this study used their own protocols, and hence, this study was not designed to thoroughly assess every single step in the protocol under similar circumstances within laboratories.

Proposed methods for GGGGCC-repeat expansion genotyping in *C9orf72*

There is an urgent need for broad consensus on analysing GGGGCC-repeat expansions in *C9orf72*, which is particularly important in a clinical setting (for diagnosing ALS or FTD, or when performing predictive testing of at-risk individuals), but also for research purposes. A possible algorithm for *C9orf72* genotyping is presented in figure 2. In conclusion, we recommend, that as an absolute minimum, a combination of amplicon-length analysis and RP-PCR should be performed. We recommend using good quality and quantity of DNA and primers, an appropriate concentration of deaza-dGTP, and a minimum elongation time of 3 min. When results are questionable, we suggest (1) expand the analysed scale in the analysis software, (2) use a higher concentration of PCR products in the capillary electrophoresis and (3) repeat RP-PCR with a higher amount of DNA. Though five of the 14 laboratories got full concordance with SB using PCR-based techniques only, the high risk for misgenotyping using only PCR-based techniques as performed here in nine laboratories, and the devastating consequences misgenotyping may have in clinical practise, make us conclude that SB should always be employed in a diagnostic setting, and should be the preferred method in a research setting of smaller number of samples (eg, analysis of the expansion in autopsy tissue specimens). RP-PCR plus amplicon-length analysis should be used in a research setting and when many samples are to be analysed, for example, in an epidemiological study. Optimally, also is such studies should samples with a saw-tooth pattern be confirmed to have an expansion by SB (figure 2).

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Contributors CA, AV, PMA and CK designed the study, and with MvB and RR wrote the first drafts of the manuscript. MvB, CA and AV also performed lab analysis and interpreted the results, as did MvdB, CL, SL, WC, BN, OO, WvR, BS, MP, KT, PK, AC, AR, JvdZ, HA, AB, DC, AN, DT, WJ, HD, SA, MD-H, TK, AL-J, KM, JL, JV, VS, AG, CS, GR, LvdB, CvB. SP, MdC, MW and PMA accrued the patient cohort. PMA and CK were the PIs on the project.