tivator p35 by Phos-tag SDS-PAGE. *Mol. Cell Proteomics* **9,** 1133–1143
32. Brinkkoetter, P. T., Olivier, P., Wu, J. S., Henderson, S., Krofft, R. D., Pippin, J. W., Hockenbery, D., Roberts, J. M., and Shankland, S. J. (2009) Cyclin I activates Cdk5 and regulates expression of Bcl-2 and Bcl-XL in postmitotic mouse cells. *J. Clin. Invest.* **119,** 3089–3101
33. Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K., and Koike, T. (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell Proteomics* **5,** 749–757
34. Hisanaga, S., and Endo, R. (2010) Regulation and role of cyclin-dependent kinase activity in neuronal survival and death. *J. Neurochem.* **115,** 1309–1321
35. Ohshima, T., Ward, J. M., Huh, C. G., Longenecker, G., Veeranna, Pant, H. C., Brady, R. O., Martin, L. J., Kulkarni, A. B. (1996) Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11173–11178
36. Lee, K.Y., Rosales, J. L., Tang, D., Wang, J. H. (1996) Interaction of cyclin-dependent kinase 5 (Cdk5) and neuronal Cdk5 activator in bovine brain. *J. Biol. Chem.* **271,** 1538–1543
37. Zhu, Y. S., Saito, T., Asada, A., Maekawa, S., Hisanaga, S. (2005) Activation of latent cyclin-dependent kinase 5 (Cdk5)-p35 complexes by membrane dissociation. *J. Neurochem.* **94,** 1535–1545
38. Patrick, G. N., Zhou, P., Kwon, Y. T., Howley, P. M., and Tsai, L. H. (1998) p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-proteasome pathway. *J. Biol. Chem.* **273,** 24057–24064
39. Nikolic, M., Chou, M. M., Lu, W., Mayer, B. J., and Tsai, L. H. (1998) The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* **395,** 194–198
40. Chen, G., Sima, J., Jin, M., Wang, K. Y., Xue, X. J., Zheng, W., Ding, Y. Q., and Yuan, X. B. (2008) Semaphorin-3A guides radial migration of cortical neurons during development. *Nat. Neurosci.* **11,** 36–44

JMG Online First, published on April 4, 2014 as 10.1136/jmedgenet-2014-102360

OPEN ACCESS

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

Chizuru Akimoto,[1] Alexander E Volk,[2] Marka van Blitterswijk,[3] Marleen Van den Broeck,[4,5] Claire S Leblond,[6] Serge Lumbroso,[7] William Camu,[8] Birgit Neitzel,[9] Osamu Onodera,[10] Wouter van Rheenen,[11] Susana Pinto,[12] Markus Weber,[13] Bradley Smith,[14] Melanie Proven,[15] Kevin Talbot,[16] Pamela Keagle,[17] Alessandra Chesi,[18] Antonia Ratti,[19,20] Julie van der Zee,[4,5] Helena Alstermark,[1] Anna Birve,[1] Daniela Calini,[19,20] Angelica Nordin,[1] Daniela C Tradowsky,[2] Walter Just,[2] Hussein Daoud,[6] Sabrina Angerbauer,[9] Mariely DeJesus-Hernandez,[3] Takuya Konno,[10] Anjali Lloyd-Jani,[15] Mamede de Carvalho,[12] Kevin Mouzat,[7] John E Landers,[17] Jan H Veldink,[11] Vincenzo Silani,[19,20] Aaron D Gitler,[18] Christopher E Shaw,[14] Guy A Rouleau,[6] Leonard H van den Berg,[11] Christine Van Broeckhoven,[4,5] Rosa Rademakers, Peter M Andersen,[1,21] Christian Kubisch[2]

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

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jmedgenet-2014-102360).

For numbered affiliations see end of article.

Correspondence to
Dr Peter M Andersen, Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå SE-90185, Sweden; peter.andersen@neuro.umu.se

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.

Received 13 February 2014
Accepted 10 March 2014

To cite: Akimoto C, Volk AE, van Blitterswijk M, *et al*. *J Med Genet* Published Online First: [*please include* Day Month Year] doi:10.1136/jmedgenet-2014-102360

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

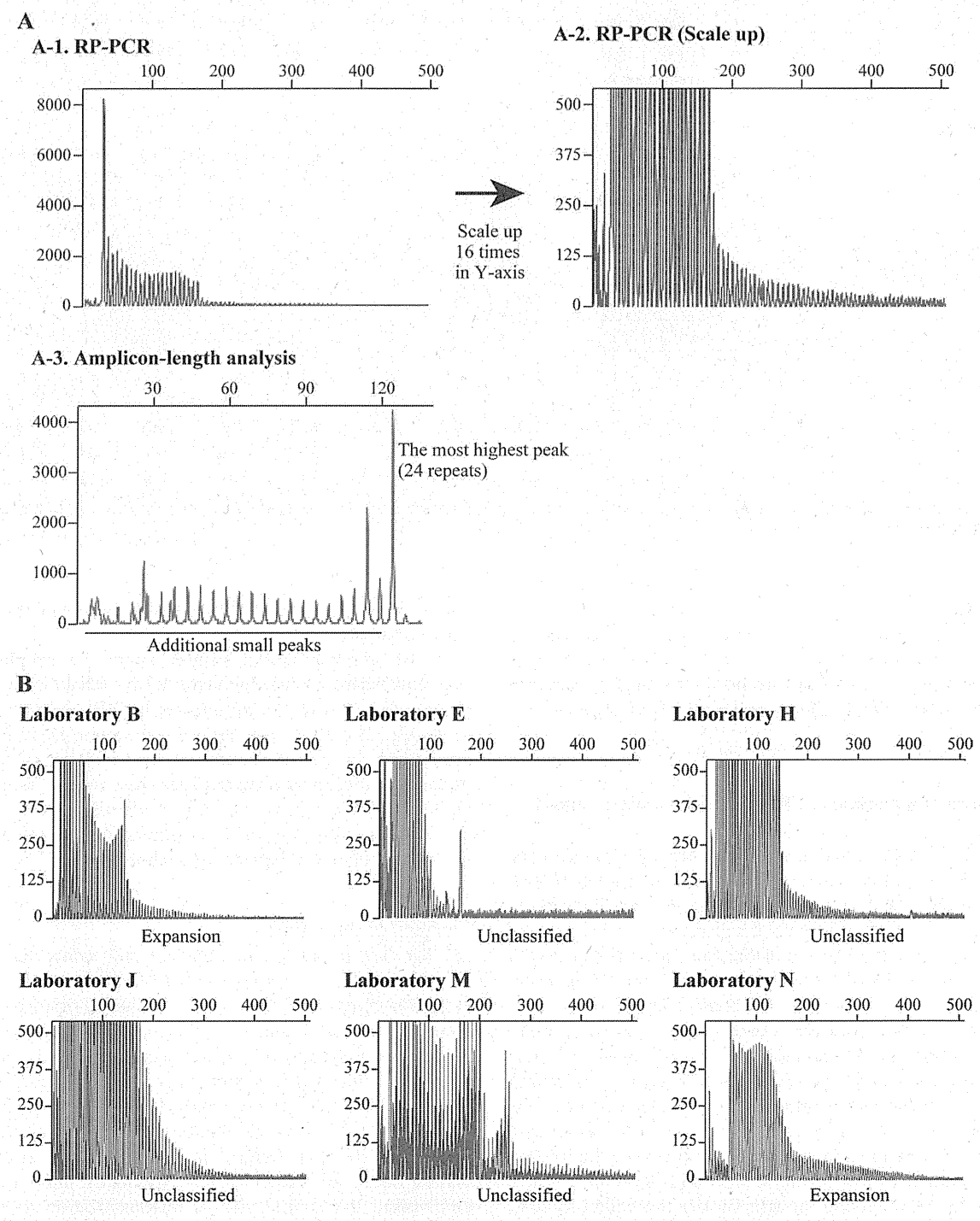

Figure 1 Results from RP-PCR and amplicon-length analysis of sample 6.5. (A) A-1 is the full shape of RP-PCR and A-2 is a 16-times scale-up (Y-axis) shape of A-1. It is possible to see the clear saw-tooth pattern after scale-up. A-3 is the result of amplicon-length analysis and there is a number of small 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.

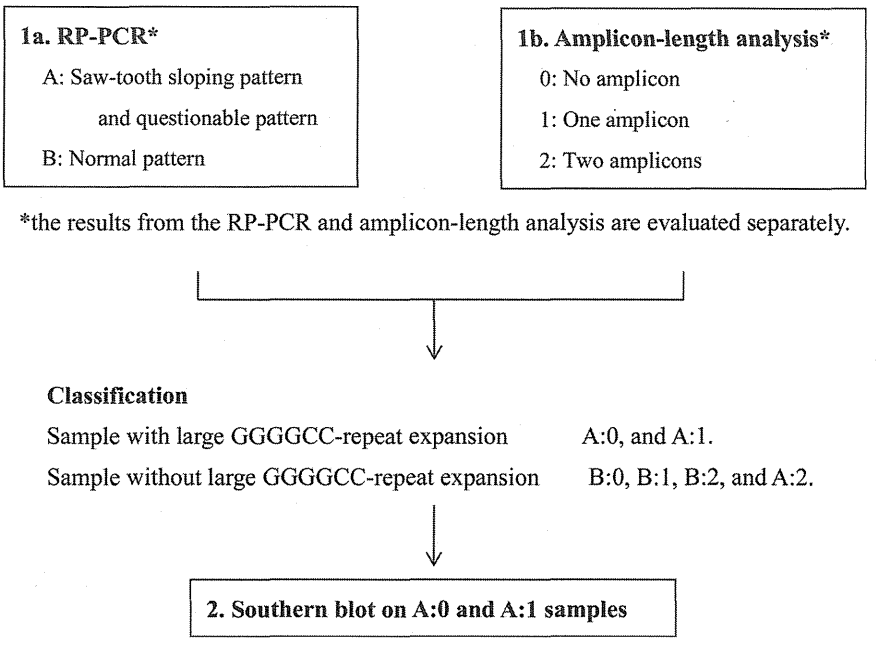

Figure 2 Flow chart for *C9orf72* genotyping in a scientific setting.

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).

Author affiliations
[1]Department of Pharmacology and Clinical Neuroscience, Umeå University, Umeå, Sweden
[2]Institute of Human Genetics, Ulm University, Ulm, Germany
[3]Department of Neuroscience, Mayo Clinic, Jacksonville, Florida, USA
[4]Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB, University of Antwerp—CDE, Antwerp, Belgium
[5]Diagnostic Service Facility, Laboratory of neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerp, Belgium
[6]Department of Neurology and Neurosurgery, Montreal Neurological Institute and Hospital, McGill University, Montreal, Quebec, Canada
[7]Department of Biochemistry, Nimes University Hospital, Nimes Cedex 9, France
[8]Center SLA, Montpellier University Hospital, Hôpital Gui-de-Chauliac, Montpellier Cedex 5, France
[9]Medizinisch Genetisches Zentrum, München, Germany
[10]Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan
[11]Department of Neurology, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, The Netherlands
[12]Faculty of Medicine-University of Lisbon, Instituto de Medicina Molecular, Hospital de Santa Maria, University of Lisbon, Alameda Universidade, Lisbon, Portugal
[13]Department of neurology, Kantonsspital St. Gallen and University Hospital, St. Gallen, Switzerland
[14]Institute of Psychiatry, King's College London and King's Health Partners, London, UK
[15]Oxford Medical Genetics Laboratories, Churchill Hospital, Oxford, England
[16]Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe hospital, Oxford, UK
[17]Department of Neurology, University of Massachusetts Medical School, Worcester, Massachusetts, USA
[18]Department of Genetics, Stanford University School of Medicine, Stanford, California, USA
[19]Department of Pathophysiology and Transplantation, "Dino Ferrari" Center, Universtà degli Studi di Milano, Milan, Italy
[20]Department of Neurology and Laboratory of Neuroscience, IRCCS Istituto Auxologico Italiano, , Milan, Italy
[21]Department of Neurology, University of Ulm, Ulm, Germany

Acknowledgements We thank the patients and their relatives for participating in this study. We also wish to thank Ann-Charloth Nilsson (Umeå University), Birgit Schmoll (Ulm University), Peter Sapp (University of Massachusetts Medical School), Jovana Kantar and Gonzague Sacaze (Nimes university hospital) for excellent technical assistance.

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.

Funding This project was funded by the Swedish Science Council, the Brain Research Foundation, Mr B Hållsten's Brain Research Foundation, The Ulla-Carin Lindquist's Fundation for ALS Research, the Knut and Alice Wallenberg Foundation, Swedish Brain Power, the European Community's Health Seventh Framework Programme (FP7/2007–2013) (grant agreement no. 259867), The Belgian Science Policy Office Interuniversity Attraction Poles (IAP) programme, the Flemish Government supported Europe Initiative on Centers of Excellence in Neurodegeneration (CoEN), the Flemish Government initiated Methusalem excellence research programme, Alzheimer Research Foundation, the Medical Foundation Queen Elisabeth, the Research Foundation Flanders (FWO) and the FWO provided a postdoctoral scientist fellowship to JvdZ, University of Antwerp Research Fund, the Swiss ALS Foundation, the Italian Ministry of Health (RF-2009-1473856), Grant-in-Aid for the Research Committee of CNS Degenerative Diseases and Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare in Japan and Dr Van Blitterswijk is supported by the Milton Safenowitz Post-Doctoral Fellowship for ALS research from the ALS Association.

Competing interests None.

Ethics approval The Medical Ethical Review Boards in Sweden, Switzerland and Portugal.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouru N, Wojtas A, Sengdy P, Hsiung GY, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR, Rademakers R. Expanded GGGGCC hexanucleotide repeat in noncoding region of *C9ORF72* causes chromosome 9p-linked FTD and ALS. *Neuron* 2011;72:245–56.

2 Renton AE, Majounie E, Waite A, Simón-Sánchez J, Rollinson S, Gibbs JR, Schymick JC, Laaksovirta H, van Swieten JC, Myllykangas L, Kalimo H, Paetau A, Abramzon Y, Remes AM, Kaganovich A, Scholz SW, Duckworth J, Ding J, Harmer DW, Hernandez DG, Johnson JO, Mok K, Ryten M, Trabuni D, Guerreiri RJ, Orrell RW, Neal J, Murray A, Pearson J, Jansen IE, Sondervan D, Seelar H, Blake D, Young K, Halliwell N, Callister JB, Toulson G, Richardson A, Gerhard A, Snowden J, Mann D, Neary D, Nalls MA, Peuralinna T, Jansson L, Isoviita VM, Kaivorinne AL, Hölttä-Vuori M, Ikonen E, Sulkava R, Benatar M, Wuu J, Chiò A, Restagno G, Borghera G, Sabatelli M, ITALSGEN ConsortiumHeckerman D, Rogaeva E, Zinman L, Rothstein JD, Sendtner M, Drepper C, Eichler EE, Alkan C, Abdullaev Z, Pack SD, Dutra A, Pak E, Hardy J, Singleton A, Williams NM, Heutink P, Pickering-Brown S, Morris HR, Tienari PJ, Traynor BJ. A hexanucleotide repeat expansion in *C9ORF72* is the cause of chromosome 9p21-linked ALS-FTD. *Neuron* 2011;72:257–68.

3 Gijselinck I, Van Langenhove T, van der Zee J, Sleegers K, Philtjens S, Kleinberger G, Janssens J, Bettens K, van Cauwenberghe C, Pereson S, Engelborghs S, Sieben A, De Jonghe P, Vandenberghe R, Santens P, De Bleecker J, Maes G, Bäumer V, Dillen L, Joris G, Couijt I, Corsmit E, Elinck E, Van Dongen J, Vermeulen S, Van den Broeck M, Vaerenberg C, Mattheijssens M, Peeters K, Robberecht W, Cras P, Martin JJ, De Deyn PP, Cruts M, Van Broeckhoven C. A *C9orf72* promoter repeat expansion in a Flanders-Belgian cohort with disorders of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum: a gene identification study. *Lancet Neurol* 2012;11:54–65.

4 Smith BN, Newhouse S, Shatunov A, Vance C, Topp S, Johnson L, Miller J, Lee Y, Troakes C, Scott KM, Jones A, Gray I, Wright J, Hortobágyi T, Al-Sarraj S, Rogeli B, Powell J, Lupton M, Lovestone S, Sapp PC, Weber M, Nestor PJ, Schelhaas HJ, Asbroek AA, Silani V, Gellera C, Taroni F, Ticonni N, Van Den Berg L, Veldink J, Van Damme P, Robberecht W, Shaw PJ, Kirby J, Pall H, Morrison KE, Morris A, de Belleroche J, Vianney de Jong JM, Baas F, Andersen PM, Landers J, Brown RH Jr, Weale ME, Al-Chalabi A, Shaw CE. The *C9ORF72* expansion mutation is a common cause of ALS+/-FTD in Europe and has a single founder. *Eur J Hum Genet* 2013;21:102–8.

5 Majounie E, Renton AE, Mok K, Dopper EG, Waite A, Rollinson S, Chio A, Restagno G, Nicolaou N, Simon-Sanchez J, van Swieten JC, Abramzon Y, Johnson JO, Sendtner M, Pamphlett R, Orrell RW, Mead S, Sidle KC, Houlden H, Rohrer JD, Morrison KE, Pall H, Talbot K, Ansorge O, Chromosome 9-ALS/FTD Consortium; French research network on FTLD/FTLD/ALS; ITALSGEN ConsortiumHernandez DG, Arepalli S, Sabatelli M, Mora G, Corbo M, Giannini F, Calvo A, Englund E, Borghero G, Floris GL, Remes AM, Laaksovirta H, McCluskey L, Trojanowski JQ, Van Deerlin VM, Schellenberg GD, Nalls MA, Drory VE, Lu CS, Yeh TH, Ishiura H, Takahashi Y, Tsuji S, Le Ber I, Brice A, Drepper C, Williams N, Kirby J, Shaw P, Hardy J, Tienari PJ, Heutink P, Morris HR, Pickering-Brown S, Traynor BJ. Frequency of the *C9orf72* hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. *Lancet Neurol* 2012;11:323–30.

6 Curtis-Cioffi KM, Rodrigueiro DA, Rodrigues VC, Cicarelli RM, Scarel-Caminaga RM. Comparison between the polymerase chain reaction-based screening and the Southern blot methods for identification of fragile X syndrome. *Genet Test Mol Biomarkers* 2012;16:1303–8.

7 Hantash FM, Goos DG, Tsao D, Quan F, Buller-Burckle A, Peng M, Jarvis M, Sun W, Strom CM. Qualitative assessment of FMR1 (CGG)n triplet repeat status in normal, intermediate, premutation, full mutation, and mosaic carriers in both sexes: implications for fragile X syndrome carrier and newborn screening. *Genet Med* 2010;12:162–73.

8 Beck J, Poulter M, Hensman D, Rohrer JD, Mahoney CJ, Adamson G, Campbell T, Uphill J, Borg A, Fratta P, Orrell RW, Malaspina A, Rowe J, Brown J, Hodges J, Sidle K, Polke JM, Houlden H, Schott JM, Fox NC, Rossor MN, Tabrizi SJ, Isaacs AM, Hardy J, Warren JD, Collinge J, Mead S. Large *C9orf72* hexanucleotide repeat expansions are seen in multiple neurodegenerative syndromes and are more frequent than expected in the UK population. *Am J Hum Genet* 2013;92:345–53.

9 Dobson-Stone C, Hallupp M, Loy CT, Thompson EM, Haan E, Sue CM, Panegyres PK, Razquin C, Seijo-Martínez M, Rene R, Gascon J, Campdelacreu J, Schmoll B, Volk AE, Brooks WS, Schofield PR, Pastor P, Kwok JB. *C9ORF72* repeat expansion in Australian and Spanish frontotemporal dementia patients. *PLoS ONE* 2013;8:e56899.

10 van Blitterswijk M, DeJesus-Hernandez M, Niemantsverdriet E, Murray ME, Heckman MG, Diehl NN, Brown PH, Baker MC, Finch NA, Bauer PO, Serrano G, Beach TG, Josephs KA, Knopman DS, Petersen RC, Boeve BF, Graff-Radford NR, Boylan KB, Petrucelli L, Dickson DW, Rademakers R. Association between repeat sizes and clinical and pathological characteristics in carriers of *C9ORF72* repeat expansions (Xpansize-72): a cross-sectional cohort study. *Lancet Neurol* 2013;12:978–88.

11 Andersen PM, Abrahams S, Borasio GD, de Carvalho M, Chio A, Van Damme P, Hardiman O, Kollewe K, Morrison KE, Petri S, Pradat PF, Silani V, Tomik B, Wasner M, Weber M. EFNS Task Force on Management of Amyotrophic Lateral Sclerosis. EFNS guidelines on the Clinical Management of Amyotrophic Lateral Sclerosis (MALS)—revised report of an EFNS task force. *Eur J Neurol* 2012;19:360–75.

12 Neary D, Snowden J, Mann D. Frontotemporal dementia. *Lancet Neurol* 2005;4:771–80.

13 van der Zee J, Gijselinck I, Dillen L, Van Langenhove T, Theuns J, Engelborghs S, Philtjens S, Vandenbulcke M, Sleegers K, Sieben A, Bäumer V, Maes G, Corsmit E, Borroni B, Padovani A, Archetti S, Perneczky R, Diehl-Schmid J, de Mendonça A, Miltenberger-Miltenyi G, Pereira S, Pimentel J, Nacmias B, Bagnoli S, Sorbi S, Graff C, Chiang HH, Westerlund M, Sanchez-Valle R, Llado A, Gelpi E, Santana I, Almeida MR, Santiago B, Frisoni G, Zanetti O, Bonvicini C, Synofzik M, Maetzler W, Vom Hagen JM, Schöls L, Heneka MT, Jessen F, Matej R, Parobkova E, Kovacs GG, Ströbel T, Sarafov S, Tournev I, Jordanova A, Danek A, Arzberger T, Fabrizi GM, Testi S, Salmon E, Santens P, Martin JJ, Cras P, Vandenberghe R, De Deyn PP, Cruts M, Van Broeckhoven C, van der Zee J, Gijselinck I, Dillen L, Van Langenhove T, Theuns J, Philtjens S, Sleegers K, Bäumer V, Maes G, Corsmit E, Engelborghs S, De Deyn PP, Cras P, Engelborghs S, De Deyn PP, Vandenbulcke M, Borroni B, Padovani A, Archetti S, Perneczky R, Diehl-Schmid J, Synofzik M, Maetzler W, Müller Vom Hagen J, Schöls L, Synofzik M, Maetzler W, Müller Vom Hagen J, Schöls L, Heneka MT, Jessen F, Ramirez A, Kurzwelly D, Sachtleben C, Mairer W, de Mendonça A, Miltenberger-Miltenyi G, Pereira S, Firmo C, Pimentel J, Sanchez-Valle R, Llado A, Antonell A, Molinuevo J, Gelpi E, Graff C, Chiang HH, Westerlund M, Graff C, Kinhult Ståhlbom A, Thonberg H, Nennesmo I, Börjesson-Hanson A, Nacmias B, Bagnoli S, Sorbi S, Bessi V, Piaceri I, Santana I, Santiago B, Santana I, Helena Ribeiro M, Rosário Almeida M, Oliveira C, Massano J, Garret C, Pires P, Frisoni G, Zanetti O, Bonvicini C, Sarafov S, Tournev I, Jordanova A, Tournev I, Kovacs GG, Ströbel T, Heneka MT, Jessen F, Ramirez A, Kurzwelly D, Sachtleben C, Mairer W, Jessen F, Matej R, Parobkova E, Danel A, Arzberger T, Maria Fabrizi G, Testi S, Ferrari S, Cavallaro T, Salmon E, Santens P, Cras P; European Early-Onset Dementia Consortium. A pan-European study of the *C9orf72* repeat associated with FTLD: geographic prevalence, genomic instability, and intermediate repeats. *Hum Mutat* 2013;34:363–73.

NEUROPATHOLOGY

Neuropathology 2014; ••, ••–••

doi:10.1111/neup.12105

Case Report

Bunina bodies in motor and non-motor neurons revisited: A pathological study of an ALS patient after long-term survival on a respirator

Tadashi Kimura,[1] Haishan Jiang,[1] Takuya Konno,[2] Makiko Seto,[3] Keisuke Iwanaga,[3] Mitsuhiro Tsujihata,[3] Akira Satoh,[3] Osamu Onodera,[2] Akiyoshi Kakita[1] and Hitoshi Takahashi[1]

Departments of [1]Pathology and [2]Molecular Neuroscience, Brain Research Institute, University of Niigata, Niigata and [3]Section of Neurology, Nagasaki Kita Hospital, Nagasaki, Japan

Bunina bodies (BBs) are small eosinophilic neuronal cytoplasmic inclusions (NCIs) found in the remaining lower motor neurons (LMNs) of patients with sporadic amyotrophic lateral sclerosis (SALS), being a specific feature of the cellular pathology. We examined a case of SALS, unassociated with *TDP-43* or *C9ORF72* mutation, of 12 years duration in a 75-year-old man, who had received artificial respiratory support for 9 years, and showed widespread multisystem degeneration with TDP-43 pathology. Interestingly, in this patient, many NCIs reminiscent of BBs were observed in the oculomotor nucleus, medullary reticular formation and cerebellar dentate nucleus. As BBs in the cerebellar dentate nucleus have not been previously described, we performed ultrastructural and immunohistochemical studies of these NCIs to gain further insight into the nature of BBs. In each region, the ultrastructural features of these NCIs were shown to be identical to those of BBs previously described in LMNs. These three regions and the relatively well preserved sacral anterior horns (S1 and S2) and facial motor nucleus were immunostained with antibodies against cystatin C (CC) and TDP-43. Importantly, it was revealed that BBs exhibiting immunoreactivity for CC were a feature of LMNs, but not of non-motor neurons, and that in the cerebellar dentate nucleus, the ratio of neurons with BBs and TDP-43 inclusions/neurons with BBs was significantly lower than in other regions. These findings suggest that the occurrence of BBs with CC immunoreactivity is intrinsically associated with the particular cellular properties of LMNs, and that the mechanism responsible for the formation of BBs is distinct from that for TDP-43 inclusions.

Key words: amyotrophic lateral sclerosis, Bunina body, cystatin C, non-motor neuron, TDP-43.

INTRODUCTION

Bunina bodies (BBs), which are small eosinophilic neuronal cytoplasmic inclusions (NCIs), are considered to be a specific feature of the cellular pathology in sporadic amyotrophic lateral sclerosis (SALS). BBs are found in lower motor neurons (LMNs) in the spinal cord and brainstem;[1] Piao *et al.* reported that they were observed in 88 (86.3%) of 102 cases of SALS.[2] However, BBs are very rare in the brainstem and in sacral LMNs innervating the striated muscles of the eye and the rectum and urethral sphincter.[1,3,4] Electron microscopy and immunohistochemical studies are important for identifying BBs in patients with SALS: they consist of electron-dense amorphous material often with inner clear areas containing cell organelles, such as filaments (neurofilaments) and vesicles,[1,2] and are immunoreactive for cystatin C (CC), a protein inhibitor of lysosomal cysteine proteases.[1,5]

In SALS, NCIs indistinguishable from BBs may also occur in non-motor neurons,[1] including those in the medullary reticular formation.[6] The ultrastructural features of such NCIs in non-motor neurons have been shown to be identical to those of BBs seen in LMNs.[1,6] However, no reported studies have yet investigated the immunoreactivity of BBs for CC or their relationship to trans-activation response DNA protein 43 (TDP-43) inclusions.

Recently, we encountered a patient with SALS who had survived for a long period on respirator support. In this patient, many small eosinophilic NCIs reminiscent of BBs,

Correspondence: Hitoshi Takahashi, MD, Department of Pathology, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan. Email: hitoshi@bri.niigata-u.ac.jp

Received 16 July 2013; revised and accepted 28 December 2013.

which were confirmed in the affected LMNs (described below), were observed in the oculomotor nucleus, medullary reticular formation and cerebellar dentate nucleus. Therefore we performed ultrastructural and immunohistochemical studies of these NCIs to gain further insight into the nature of BBs. Here we describe the clinicopathological features of this patient with new observations on Bunina bodies.

CASE REPORT

The present study was conducted with approval from the Institutional Review Board of the University of Niigata. Written informed consent was obtained from the patient's family prior to these genetic studies of the *TDP-43* and *C9ORF72* genes.

Clinical summary and pathological findings

A 63-year-old man became aware of muscle weakness in the right hand, and over the next 2 years, the muscle weakness extended to all of his extremities. On examination, fasciculation was evident in the tongue and deep tendon reflexes were increased; on this basis he was diagnosed as having ALS. About 3 years after onset, at the age of 66 years, he became bedridden with dysphagia and dyspnea, necessitating tube feeding and artificial respiratory support. Thereafter, ocular movement became limited in all directions, making communication impossible. The patient died of bronchopneumonia at the age of 75 years, about 12 years after disease onset. A general autopsy was performed 3 h after death, at which time the brain weighed 830 g, showing marked frontotemporal atrophy (frontal > temporal) (Fig. 1A).

The brain and spinal cord were fixed in 20% buffered formalin and multiple tissue blocks were embedded in paraffin. Histological examination was performed on 4-μm-thick sections using several stains, including HE, KB and Holzer. Selected sections were also immunostained with antibodies against phosphorylated TDP-43 (pTDP-43) (monoclonal, clone S409/410; Cosmo Bio, Tokyo, Japan; 1:3000, heat/autoclaving) and cystatin C (polyclonal, Dako, Glostrup, Denmark; 1:3000).

The entire spinal cord was markedly atrophic (Fig. 1B) and there was severe wasting in the anterior nerve roots. Histopathological examination revealed that except for the absence of Lewy body-like hyaline inclusions, the entire pathological picture was very similar to that shown in a case of SALS in a 71-year-old woman after long-term survival on a respirator, which we had previously reported.[7] With regard to the motor neuron system, almost complete loss of LMNs was observed in the spinal anterior horns at the levels of the cervical, thoracic and lumbar segments. The sacral anterior horns (S1 and S2), including Onuf's nucleus, contained a number of LMNs (Fig. 1C). In the brainstem, almost complete loss of LMNs was evident in the hypoglossal nucleus. The facial motor nucleus and oculomotor nucleus were relatively well preserved. BBs were found in the remaining LMNs in the sacral anterior horns, including Onuf's nucleus and the facial motor nucleus (Fig. 1D); immunostaining revealed that these BBs were

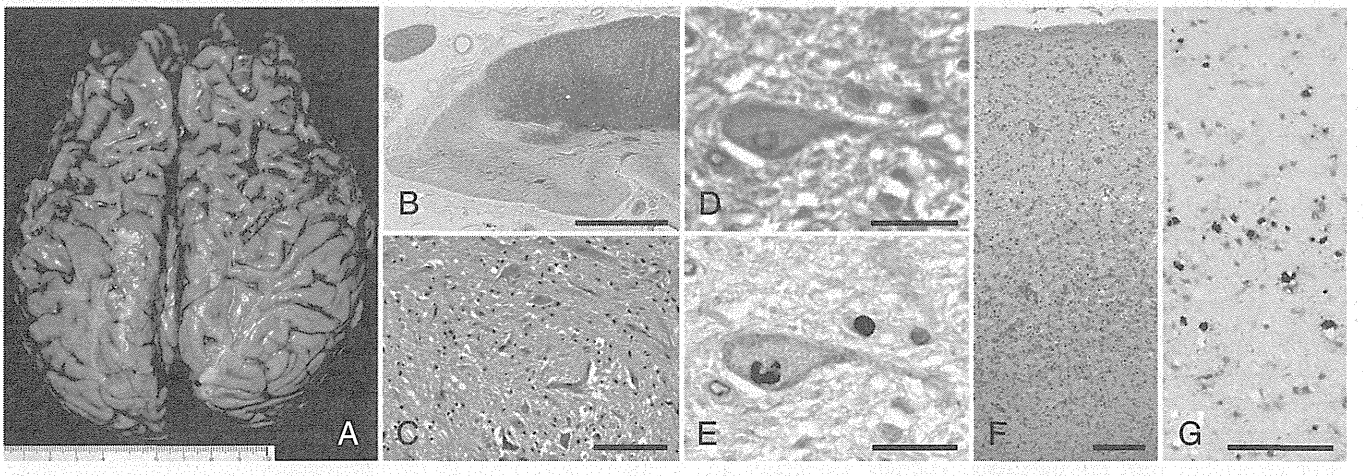

Fig. 1 Neuropathological findings in the brain and spinal cord. Sections stained by the KB method (B), HE (C,D,F) and immunostained with antibodies against cystatin C (CC) (E) and phosphorylated trans-activation response DNA protein 43 (pTDP43) (G). (A) Marked atrophy is evident in the frontal lobe, including the precentral gyrus. (B) The thoracic segment (T2), showing myelin pallor in the white matter except for the posterior columns. (C) Loss of lower motor neurons (LMNs) with gliosis is evident in the sacral (S1) anterior horn. Note that Onuf's nucleus contains a number of LMNs (lower). (D,E) Sequential staining of the same section, showing two facial motor neurons with Bunina bodies (BBs) (D) positive for CC (E). (F) Severe neuronal loss with gliosis is evident in the motor cortex. (G) Here, pTDP-43-positive neuronal cytoplasmic inclusions (NCIs) in layers II-III are shown. Scale bars = 1 mm for (B), 100 μm for (C,G), 20 μm for (D,E) and 200 μm for (F).

positive for CC (Fig. 1E). In the motor cortex, severe neuronal loss was also evident and no Betz cells were found (Fig. 1F); immunostaining revealed pTDP-43-positive NCIs mainly in layers II-III and V-VI (Fig. 1G). The histological findings are summarized in Table 1. Diffuse loss of cerebellar Purkinje cells appeared to be attributable to brain ischemia (Table 1).

Table 1 Pathological findings in the present case

Regions	Loss of neuron	pTDP-43-positive NCIs
Cerebral cortex		
Frontal	+++	+++
Motor	+++	+++
Parietal	++	+++
Cingulate	+++	+++
Insular	+++	+++
Entorhinal	++	+++
Hippocampus (DG/Sub)	+/+++	+++/++
Subcortical area		
Amygdala	++	+++
Basal nucleus of Mynert	+	+
Caudate nuclei	+++/+++	+++/+++
Globus pallidus	+	+++
Thalamus (medial/lateral)	++/+++	++/++
Subthalamic nucleus	nd	nd
Midbrain		
Midbrain tectum	+++	+++
Reticular formation	+++	+++
Oculomotor nucleus	+	+
Red nucleus	+	+
Substance nigra	+++	+
Pons		
Locus celreus	++	+
Reticular formation	++	+++
Facial nucleus (motor)	+	++
Vestibular nucleus	+	+
Pontine nucleus	+	++
Superior olivary nucleus	–	–
Medulla oblongata		
Hypoglossal nucleus	+++	–
Dorsal vagal nucleus	+	++
Reticular formation	++	+++
Nucleus ambiguus	nd	nd
Inferior olivary nucleus	+	+
Cerebellum		
Purkinje cell	+++	–
Granule cell	–	–
Dentate nucleus	+	++
Spinal cord		
Anterior horn	+++	+
Intermediate lateral nucleus	++	++
Clarke's nucleus	+++	–
Posterior horn	++	++
Anterior olfactory nucleus	++	++
Dorsal root ganglia	+	+

Loss of neurons: +, mild; ++, moderate; +++, severe. The numbers of pTDP-43-positive neuronal cytoplasmic inclusions (NCIs) were assessed using a semi-quantitative rating scale: –, absent or nearly absent; +, sparse; ++, moderate; +++, numerous. Hippocampus: DG, dentate gyrus (granule cells); Sub, subiculum. nd, not determined.

TDP-43 mutation and *C9ORF72* repeat expansion analyses

Genomic DNA was prepared from a frozen sample of cerebral cortex from the patient, and then examinations for *TDP-43* mutation and *C9ORF72* repeat expansion were carried out as previously described;[8,9] however, neither of these features was found to be present.

Bunina bodies in motor and non-motor neurons

In addition, the occurrence of many eosinophilic NCIs indistinguishable from BBs in the oculomotor nucleus, medullary reticular formation and cerebellar dentate nucleus was a feature of the present patient. Some representative inclusions in the oculomotor nucleus and medullary reticular formation were recycled for electron microscopy, and small tissue blocks from the formalin-fixed cerebellar dentate nucleus were also processed for ordinary electron microscopy. All of the studied NCIs, 2–3 in each region (Fig. 2A–C), were identified as BBs from their characteristic ultrastructural features (Fig. 2D–F). In the medullary reticular formation, the BB-containing neurons were distributed more widely than previously recorded.[6]

We then investigated the presence or absence of CC immunoreactivity in the BBs, as well as the correlation between the occurrence of BBs and that of pTDP-43-positive inclusions. Four-micrometer-thick paraffin sections that contained the bilateral oculomotor nuclei and medullary reticular formation, and unilateral cerebellar dentate nucleus were prepared, and then stained with HE, observed and photographed (Fig. 3A–C,G–I). They were then destained in absolute ethanol and finally immunostained for CC (Fig. 3D–F) or pTDP-43 (Fig. 3J–L). For comparison, the bilateral sacral anterior horns (S1 and S2) and facial motor nuclei were also similarly examined. The degrees of cytoplasmic staining intensity for CC were generally decreased in the LMNs containing BBs (Fig. 1E,3D–F). pTDP-43-positive NCIs appeared as fine to coarse granular (Fig. 3J), linear wisp-like, large irregular (Fig. 3K) or small round-to-oval inclusions (Fig. 3L); the small round-to-oval inclusions were often observed in neurons in the cerebellar dentate nucleus (Fig. 3L). In each region, the ratio of neurons containing CC-positive BBs to the total cell count of neurons containing BBs was calculated in one section. Similarly, the ratio of neurons containing both BBs and pTDP-43-positive inclusions to the total cell count of neurons containing BBs was calculated in one section. The results obtained are shown in Table 2.

DISCUSSION

Based on the distribution and severity of neuron loss and TDP-43 inclusions, the present case was considered to be

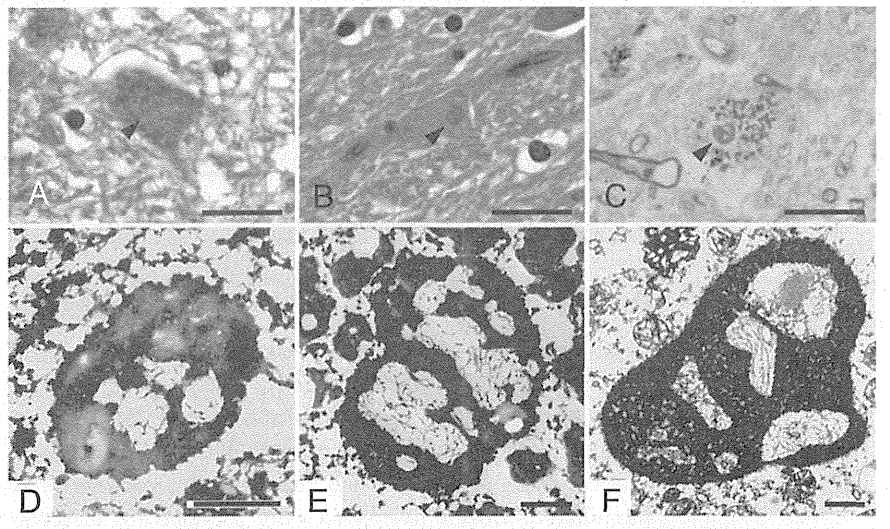

Fig. 2 Ultrastructural profiles of Bunina bodies (BBs) in neurons from the oculomotor nucleus (A), medullary reticular formation (B) and cerebellar dentate nucleus (C). Two paraffin sections stained with HE (A,B) and one Epon section stained with toluidine blue (C). Electron microscopy shows that all the BBs (A–C; arrowheads) have essentially the same ultrastructural profiles, appearing as electron-dense amorphous material with inner clear areas, in which filamentous structures are evident (D–F). In a Bunina body shown in (C), some of the filamentous structures can be identified as neurofilaments, or short fragments of the rough endoplasmic reticulum (F). Scale bars = 20 μm for A–C and 1 μm for D–F.

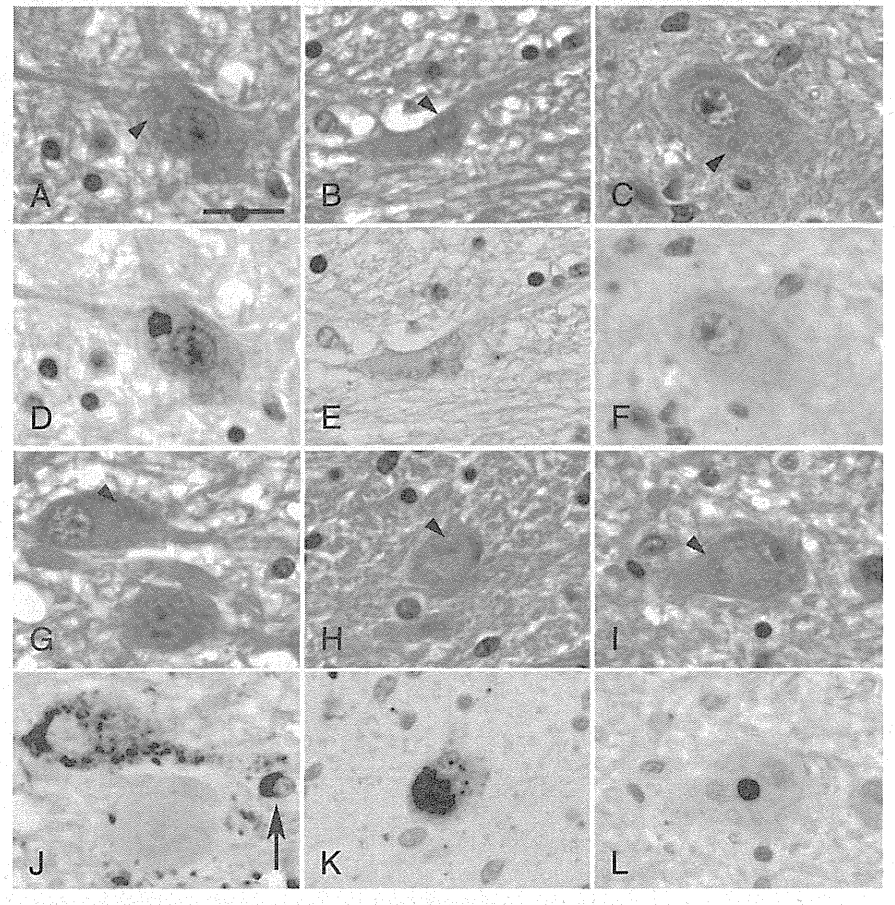

Fig. 3 Immunohistochemical profiles of Bunina bodies (BBs) in neurons from the oculomotor nucleus (A,G), medullary reticular formation (B,H) and cerebellar dentate nucleus (C,I). Sequential staining of the same sections with HE (A–C) and anti-cystatin C (CC) antibody (D–F), as well as with HE (G–I) and anti-phosphorylated trans-activation response DNA protein 43 (pTDP43) antibody (J–L). (A–F) BBs (arrowheads) seen in one lower motor neuron (A) and two non-motor neurons (B,C) are positive (D) and negative (E,F) for CC, respectively. (G–L) In all of the neurons, coexistence of BBs (arrowheads) and pTDP-43-positive neuronal cytoplasmic inclusions (NCIs) is evident; BBs themselves are negative for pTDP-43 (G,J; H,K; I,L). Arrow indicates cytoplasm of a glial cell positive for pTDP-43 (J). Scale bar = 20 μm for (A–L).

an additional example of SALS whose course had been extended by artificial respiratory support, showing widespread multisystem degeneration with TDP-43 pathology (Table 1) (Nishihira *et al.*, Type 2;[10] frontotemporal lobar degeneration – TDP pathology, Type B[11]). We reviewed seven cases in which artificial respiratory support had been used (disease duration, >10 years; Type 1 = 5, Type 2 = 2[10]) and found no NCIs indistinguishable from BBs in the oculomotor nucleus, medullary reticular formation or cerebellar dentate nucleus. In the case (disease duration = $8^2/_3$ years) reported by Nishihira *et al.*,[7] only one BB, which was confirmed by electron microscopy of recycled material, was found in the medullary reticular formation (data not shown). Therefore, the present case, which lacked *TDP-43*

Table 2 Summary of pathological findings for Bunina bodies (BBs)

Region	Ratio (cystatin C)	Ratio (pTDP-43)
Sacral anterior horn	0.88 (7/8)	1.00 (5/5)
Facial motor nucleus	1.00 (8/8)	0.90 (9/10)
Oculomotor nucleus	1.00 (10/10)	1.00 (13/13)
Medullary reticular formation	0.17 (2*/12)†	0.77 (10/13)
Cerebellar dentate nucleus	0.00 (0/36)†	0.33 (12/ /36)††

Ratio (cystatin C): neurons with cystatin C-positive BBs/neurons with BBs; Ratio (pTDP-43): neurons with BBs and pTDP-43-positive inclusions/neurons with BBs. *Regarded as weakly positive. †$P < 0.01$ versus. sacral anterior horn, facial motor nucleus or oculomotor nucleus. ††$P < 0.05$ versus sacral anterior horn, and $P < 0.01$ versus facial motor nucleus, versus oculomotor nucleus or versus medullary reticular formation. Statistical analyses were performed by Ryan's multiple comparison tests using R software (http://www.r-project.org/).

or *C9OLF72* mutation, appeared to be very unusual in terms of the occurrence of BBs even among cases of SALS whose course had been extended by artificial respiratory support.

At present, TDP-43 is widely recognized to be the pathological protein in SALS.[10,12] BBs have been reported to be negative for TDP-43,[12] which was also confirmed in the present study using a monoclonal antibody against pTDP-43. However, the presence of both BBs and TDP-43-positive NCIs has also been shown to be a characteristic feature of ALS with *TDP-43* mutations,[8,12] emphasizing anew the significance of BBs as a specific feature of the cellular pathology of ALS.

Importantly, the present case is the first reported example in which the presence of BBs exhibiting immunoreactivity for CC was a feature of LMNs, but not of non-motor neurons (Table 2). At the ultrastructural level, it is noteworthy that in LMNs, the electron-dense material considered to represent BBs themselves is negative for CC;[5,13] it has been reported that CC immunoreactivity is markedly decreased in the spinal LMNs in SALS, and that the formation of TDP-43 inclusions, but not BBs, may be linked to the CC content of these LMNs.[13] Based on the present findings, we consider that the occurrence of BBs showing CC immunoreactivity is a phenomenon confined almost exclusively to LMNs, and that this must be associated with the particular cellular properties that characterize the LMNs themselves.

The present case is also the first reported to have demonstrated BBs in neurons in the cerebellar dentate nucleus. It has been reported that there is a significant positive correlation between the occurrence of BBs and that of TDP-43 inclusions in spinal and brainstem LMNs.[14,15] This also appears to be the case in the medullary reticular formation (Table 2). However, the ratio (pTDP-43) was significantly lower in the cerebellar dentate nucleus than in other regions (Table 2), indicating that the mechanism responsible for the formation of BBs is distinct from that for TDP-43 inclusions.

Finally, even though the present study involved only a single case and revealed negativity for BBs, as in other similar cases of SALS mentioned above, the results obtained are of considerable interest. In conclusion, the nature and origin of BBs still remain uncertain. When considering why LMNs are generally most vulnerable in ALS, further studies on the formation of BBs in association with the cellular molecular properties of LMNs are needed to elucidate the pathomechanism underlying the disease.

ACKNOWLEDGMENTS

We thank C. Tanda, S. Nigorikawa, J. Takasaki, H. Saito, T. Fujita and S. Egawa for their technical assistance. This work was supported by a Grant-in-Aid, 23240049, for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, and a Grant-in-Aid from the Research Committee for CNS Degenerative Diseases, the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

1. Okamoto K, Mizuno Y, Fujita Y. Bunina bodies in amyotrophic lateral sclerosis. *Neuropathology* 2008; **28**: 109–115.
2. Piao YS, Wakabayashi K, Kakita A *et al.* Neuropathology with clinical correlations of sporadic amyotrophic lateral sclerosis: 102 autopsy cases examined between 1962 and 200. *Brain Pathol* 2003; **12**: 10–22.
3. Okamoto K, Hirai S, Amari M, Iizuka T, Watanabe M, Murakami N. Oculomotor nuclear pathology in amyotrophic lateral sclerosis. *Acta Neuropathol* 1993; **85**: 458–462.
4. Okamoto K, Hirai S, Ishiguro K, Kawarabayashi T, Takatama M. Light and electron microscopic and immunohistochemical observations of the Onuf's nucleus of amyotrophic lateral sclerosis. *Acta Neuropathol* 1991; **81**: 610–614.
5. Okamoto K, Hirai S, Amari M, Watanabe M, Sakurai A. Bunina bodies in amyotrophic lateral sclerosis immunostained with rabbit anti-cystatin C serum. *Neurosci Lett* 1993; **162**: 125–128.
6. Nakano I, Iwatsubo T, Hashizume Y, Mizutani T. Bunina bodies in neurons of the medullary reticular formation in amyotrophic lateral sclerosis. *Acta Neuropathol* 1993; **85**: 471–474.
7. Nishihira Y, Tan CF, Toyoshima Y *et al.* Sporadic amyotrophic lateral sclerosis: widespread multisystem degeneration with TDP-43 pathology in a patient after long-term survival on a respirator. *Neuropathology* 2009; **29**: 689–696.

8. Yokoseki A, Shiga A, Tan CF *et al.* TDP-43 mutation in familial amyotrophic lateral sclerosis. *Ann Neurol* 2008; **63**: 538–542.
9. Konno T, Shiga A, Tsujino A *et al.* Japanese amyotrophic lateral sclerosis patients with GGGGCC hexanucleotide repeat expansion in *C9ORF72*. *J Neurol Neurosurg Psychiatry* 2013; **84**: 398–401.
10. Nishihira Y, Tan CF, Onodera O *et al.* Sporadic amyotrophic lateral sclerosis: two pathological patterns shown by analysis of distribution of TDP-43-immunoreactive neuronal and glial cytoplasmic inclusions. *Acta Neuropathol* 2008; **116**: 169–182.
11. Mackenzie IR, Neumann M, Baborie A *et al.* A harmonized classification system for FTLD-TDP pathology. *Acta Neuropathol* 2011; **122**: 111–113.
12. Tan CF, Eguchi H, Tagawa A *et al.* TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutations. *Acta Neuropathol* 2007; **113**: 535–542.
13. Mori F, Tanji K, Miki Y, Wakabayashi K. Decreased cystatin C immunoreactivity in spinal motor neurons and astrocytes in amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 2009; **68**: 1200–1206.
14. Mori F, Tanji K, Miki Y, Kakita A, Takahashi H, Wakabayashi K. Relationship between Bunina bodies and TDP-43 inclusions in spinal anterior horn in amyotrophic lateral sclerosis. *Neuropathol Appl Neurobiol* 2010; **36**: 345–352.
15. Mori F, Kakita A, Takahashi H, Wakabayashi K. Co-localization of Bunina bodies and TDP-43 inclusions in lower motor neurons in amyotrophic lateral sclerosis. *Neuropathology* 2013. doi:10.1111/neup.12044

厚生労働科学研究費補助金　障害者対策総合研究事業(神経・筋疾患分野)

異常タンパク伝播仮説に基づく神経疾患の画期的治療法の開発
平成 26 年度　班会議プログラム

■日時:平成 27 年 2 月 13 日(金)11:00～17:40

■会場:公益財団法人東京都医学総合研究所 S 棟 2 階　会議室 2B・2C

10:30～	受付
10:55～11:00	開会挨拶 (研究代表者　長谷川成人)
11:00～12:00	≪都医学研セミナー≫ 於 講堂 (演題) 動物の脳老化および神経疾患の病理 演者:チェンバーズ ジェームズ(東京大学大学院農学生命科学研究科)
12:00～13:15	班員会議　(昼 食)
	≪タウ, αシヌクレイン, 他≫　　座長 野中 隆
13:15～13:35	1. タウ病変の伝播とその野生型マウスモデル 分担研究者: 長谷川成人 (東京都医学総合研究所 認知症プロジェクト) 演者:長谷川成人
13:35～13:55	2. αシヌクレイン凝集体のプリオン様性質 分担研究者: 野中 隆 (東京都医学総合研究所 認知症プロジェクト) 演者: 野中 隆
13:55～14:15	3. レーザーマイクロダイセクションと液体クロマトグラフィー質量分析器を用いた微量解析 分担研究者: 亀谷富由樹 (東京都医学総合研究所 認知症プロジェクト) 演者:亀谷富由樹
14:15～14:35	4. プログラニュリンの減少はタウのリン酸化を亢進させる 分担研究者: 細川雅人 (東京都医学総合研究所 認知症プロジェクト) 演者:細川雅人
14:35～14:55	5. APP による線維化タウの細胞内への取り込みと蓄積の促進 分担研究者: 久永眞市 (首都大学東京 大学院理工学研究科 神経分子機能研究室) 演者:高橋宗聖 (首都大学, 東京都医学総合研究所)
14:55～15:05	休　　憩
	≪タウ, 神経病理≫　　座長 村山 繁雄
15:05～15:25	6. 側坐核におけるタウ蓄積と神経回路 分担研究者: 秋山治彦(東京都医学総合研究所 認知症プロジェクト) 演者:河上 緒
15:25～15:45	7. Midbrain 4 repeat tauopathy 分担研究者: 村山繁雄(東京都健康長寿医療センター 高齢者ブレインバンク) 演者:村山繁雄

15:45～16:05	8. 臨床的にALS-Dと診断され、特異な病理組織学的所見を呈したGlobular glial tauopathyの1例 分担研究者：高橋 均（新潟大学 脳研究所 病理学分野） 演者：竹内 亮子
16:05～16:15	休　　憩
	≪TDP-43, RNA, αシヌクレイン≫　　座長 高橋 均
16:15～16:35	9. ELP3抗体を用いたALS剖検例の免疫組織学的検討 分担研究者：藤田行雄（群馬大学大学院 脳神経内科学） 演者：藤田行雄
16:35～16:55	10. TDP-43欠損変異体発現が与えるPoly(A)$^+$ RNA局在への影響 分担研究者：新井哲明（筑波大学 医学医療系 臨床医学域 精神医学） 演者：新井哲明
16:55～17:15	11. ALSではいつ細胞障害が始まるのか？TDP-43陽性封入体との関係 分担研究者：小野寺 理（新潟大学 脳研究所 分子神経疾患資源解析学分野） 演者：小野寺 理
17:15～17:35	12. 繊維化α-Synの線条体への接種によるパーキンソン病霊長類モデルの作成 分担研究者：横田隆徳（東京医科歯科大学大学院 脳神経病態学（神経内科）） 演者：横田隆徳
17:35～	懇親会

研究成果の刊行に関する一覧表

原著・症例報告

発表者名	論文タイトル名	発表誌	巻・号	ページ	出版年
Yamashita M, Nonaka T, Hirai S, Miwa A, Okado H, Arai T, Hosokawa M, Akiyama H, Hasegawa M	Distinct pathways leading to TDP-43-induced cellular dysfunctions.	***Hum Mol Genet***	23	4345-56	2014.
Hasegawa M, Watanabe S, Kondo H, Akiyama H, Mann DM, Saito Y, Murayama S.	3R and 4R tau isoforms in paired helical filaments in Alzheimer's disease.	***Acta Neuropathol***	127	303-305	2014.
Masuda-Suzukake M, Nonaka T, Hosokawa M, Kubo M, Shimozawa A, Akiyama H, Hasegawa M.	Pathological alpha-synuclein propagates through neural networks.	***Acta Neuropathol Commun.***	2	88	2014.
Kawakami I, Hasegawa M, Arai T, Ikeda K, Oshima K, Niizato K, Aoki N, Omi K, Higashi S, Hosokawa M, Hirayasu Y, Akiyama H.	Tau accumulation in the nucleus accumbens in tangle-predominant dementia.	***Acta Neuropathol Commun.***	2	40	2014
Yamashita S, Sakashita N, Yamashita T, Tawara N, Tasaki M, Kawakami K, Komohara Y, Fujiwara Y, et al	Concomitant accumulation of α-synuclein and TDP-43 in a patient with corticobasal degeneration.	***J Neurol***	261	2209-17	2014
Kawakami I, Hasegawa M, Arai T, Ikeda K, Oshima K, Niizato K, Aoki N, Omi K, Higashi S, Hosokawa M, Hirayasu Y, Akiyama H.	Tau accumulation in the nucleus accumbens in tangle-predominant dementia.	***Acta Neuropathol Commun.***	2	40	2014
Baborie A, Griffiths TD, Jaros E, Perry R, McKeith IG, Burn DJ, Masuda-Suzukake M,	Accumulation of dipeptide repeat proteins predates that of TDP-43 in Frontotemporal Lobar Degeneration associated with	***Neuropathol Appl Neurobiol.***		In press	2014

Hasegawa M, Rollinson S, Pickering-Brown S, Robinson AC, Davidson YS, Mann DM.	hexanucleotide repeat expansions in C9ORF72 gene.				
Davidson YS, Barker H, Robinson AC, Thompson JC, Harris J, Troakes C, Smith B, Al-Saraj S, Shaw C, Rollinson S, Masuda-Suzukake M, Hasegawa M, Pickering-Brown S, Snowden JS, Mann DM.	Brain distribution of dipeptide repeat proteins in frontotemporal lobar degeneration and motor neurone disease associated with expansions in C9ORF72.	***Acta Neuropathol Commun***	2	70	2014
Konno T, Tada M, Shiga A, Tsujino A, Eguchi H, Masuda-Suzukake M, Hasegawa M, Nishizawa M, Onodera O, Kakita K, Takahashi H.	C9ORF72 repeat-associated non-ATG-translated polypeptides are distributed independently of TDP-43 in a Japanese patient with c9ALS.	***Neuropathol Appl Neurobiol***	40	783 – 788	2014
Asada A, Yamazaki R, Kino Y, Saito T, Kimura T, Miyake M, Hasegawa M, Nukina N, Hisanaga S.	Cyclin-dependent kinase 5 phosphorylates and induces the degradation of ataxin-2.	***Neurosci Lett.***	563	112-7	2014
Sawashita J, Zhang B, Hasegawa K, Mori M, Naiki H, Kametani F, Higuchi K	C-terminal sequence of amyloid-resistant type F apolipoprotein A-II inhibits amyloid fibril formation of apolipoprotein A-II in mice	***PNAS***	112	E836-45	2015
Kametani F, Haga S	Accumulation of carboxy-terminal fragments of APP increases phosphodiesterase 8b.	***Neurobiol. Aging***	36	634-7	2014
Nakamura A, Okigaki M, Miura N, Suzuki C, Ohno N, Kametani F, Hamaoka K	Involvement of mannose-binding lectin in the pathogenesis of Kawasaki disease-like murine vasculitis.	***Clin Immunol***	153	64-72	2014

Hosokawa M, Arai T, Masuda-Suzukake M, Kondo H, Matsuwaki T, Nishihara M, Hasegawa M, Akiyama H.	Progranulin Reduction Is Associated With Increased Tau Phosphorylation in P301L Tau Transgenic Mice	***J Neuropathol Exp Neurol.***	74	158-65	2015
Serrano GE, Sabbagh MN, Sue LI, Hidalgo JA, Schneider JA, Bedell BJ, Van Deerlin VM, Suh E, Akiyama H, Joshi AD, Pontecorvo MJ, Mintun MA, Beach TG.	Positive florbetapir PET amyloid imaging in a subject with frequent cortical neuritic plaques and frontotemporal lobar degeneration with TDP43-positive inclusions	***J Alzheimers Dis.***	42	813-821	2014
Mochizuki Y, Kawata A, Hashimoto T, Akiyama H, Kawakami H, Komori T, Oyanagi K, Mizutani T, Matsubara S	An autopsy case of familial amyotrophic lateral sclerosis with FUS R521G mutation.	***Amyotroph Lateral Scler Frontotemporal Degener.***	15	305-308	2014
Beach TG, Carew J, Serrano G, Adler CH, Shill HA, Sue LI, Sabbagh MN, Akiyama H, Cuenca N; the Arizona Parkinson's Disease Consortium.	Phosphorylated α-synuclein-immunoreactive retinal neuronal elements in Parkinson's disease subjects.	***Neurosci Lett***	571	34-38	2014
Kawakami I, Katsuse O, Aoki N, Togo T, Suzuki K, Isojima D, Kondo D, Iseki E, Kosaka K, Akiyama H, Hirayasu Y.	Autopsy case of concurrent Huntington's disease and neurofibromatosis type 1.	***Psychogeriatrics***	14	81-86	2014
Adler CH, Dugger BN, Hinni ML, Lott DG, Driver-Dunckley E, Hidalgo J, Henry-Watson J, Serrano G, Sue LI, Nagel T, Duffy A, Shill HA, Akiyama H, Walker DG, Beach TG	Submandibular gland needle biopsy for the diagnosis of Parkinson disease.	***Neurology***	82	858-684	2014
Hosokawa M, Arai T, Yamashita M, Tsuji H, Nonaka T, Masuda-Suzukake M, Tamaoka A, Hasegawa M, Akiyama H	Differential diagnosis of amyotrophic lateral sclerosis from Guillain-Barré syndrome by quantitative determination of TDP-43 in cerebrospinal fluid	***Int J Neurosci***	124	344-349	2014

Kondo D, Hino H, Shibuya K, Fujisawa K, Kosaka K, Hirayasu Y, Yamamoto R, Kasanuki K, Minegishi M, Sato K, Hosokawa M, Arai T, Arai H, Iseki E	An autopsied case of corticobasal degeneration showing severe cerebral atrophy over a protracted disease course of 16 years	***Neuropathology***		In press	2015
Akitake Y, Katsuragi S, Hosokawa M, Mishima K, Ikeda T, Miyazato M, Hosoda H	Moderate maternal food restriction in mice impairs physical growth, behaviors, and neurodevelopment of offspring	***Nutrition Res***	35	76-87	2015
Arai T	Significance and limitation of the pathological classification of TDP-43 proteinopathy.	***Neuropathology***	34	578-588	2014
Tamura M, Nemoto K, Kawaguchi A, Kato M, Arai T, Kakuma T, Mizukami K, Matsuda H, Soya H, Asada T	Long-term mild-intensity exercise regimen preserves prefrontal cortical volume against aging.	***Int J Geriatr Psychiatry***			in press
Tagami S, Okochi M, Yanagida K, Kodama T, Arai T, Kuwano R, Ikeuchi T, Takeda M:	Relative ratio and level of amyloid-ß 42 surrogate in cerebrospinal fluid of familal Alzheimer's disease patients with presenilin 1 mutations.	***Neurodegener Dis***	13	166-170	2014
Tatsumi S, Mimuro M, Iwasaki Y, Takahashi R, Kkita A, Takahashi H, Yoshida M	Argyrophilic grains are reliable disease-specific features of corticobasal degeneration.	***J Neuropathol Exp Neurol***	73	30-38	2014
Kon T, Mori F, Tanji K, Miki Y, Toyoshima Y, Yoshida M, Sasaki H, Kakita A, Takahashi H, Wakabayashi K	ALS-associated protein FIG4 is localized in Pick and Lewy bodies, and also neuronal nuclear inclusions, in polyglutamine and intranuclear inclusion diseases.	***Neuropathology***	34	19-26	2014
Mori F, Kakita A, Takahashi H, Wakabayashi K	Co-localization of Bunina bodies and TDP-43 inclusions in lower motor neurons in amyotrophic lateral sclerosis.	***Neuropathology***	34	71-76	2014

Toyoshima Y, Takahashi H	TDP-43 pathology in polyglutamine diseases: with reference to amyotrophic lateral sclerosis.	***Neuropathology***	34	77-82	2014
Kimura T, Jiang H, Konno T, Seto M, Iwanaga K, Tsujihata M, Satoh A, Onodera O, Kakita A, Takahashi H	Bunina bodies in motor and non-motor neurons revisited: a pathological study of an ALS patient after long-term survival on a respiurator.	***Neuropathology***	34	392-397	2014
Hasegawa, H., Liu, L., Tooyama, I., Murayama, S., Nishimura, M.	The FAM3 superfamily member ILEI ameliorates Alzheimer's disease-like pathology by destabilizing the penultimate amyloid-beta precursor.	***Nat Commun***	5	3917	2014
Ishibashi, K., Ishiwata, K., Toyohara, J., Murayama, S. and Ishii, K.	Regional analysis of striatal and cortical amyloid deposition in patients with Alzheimer's disease.	***Eur J Neurosci***	40	2701-2706	2014
Ito S, Takao M, Hatsuta H, Kanemaru K, Arai T, Saito Y, Fukayama, M. and Murayama M.	Alpha-synuclein immunohistochemistry of gastrointestinal and biliary surgical specimens for diagnosis of Lewy body disease.	***Int J Clin Exp Pathol***	7·4	1714-1723	2014
Iwata A, Nagata K, Hatsuta H, Takuma H, Bundo M, Iwamoto K, Tamaoka A, Murayama S, Saido T and Tsuji S	Altered CpG methylation in sporadic Alzheimer's disease is associated with APP and MAPT dysregulation.	***Hum Mol Genet***	23·3	648-656	2014
Matsumoto H, Sengoku R, Saito Y, Kakuta Y, Murayama S and Imafuku I	Sudden death in Parkinson's disease: a retrospective autopsy study.	***J Neurol Sci***	343	149-152	2014
Miyashita A, Wen Y, Kitamura N, Matsubara E, Kawarabayashi T, Shoji M, Tomita N, Furukawa K, Arai H, Asada T, et al	Lack of genetic association between TREM2 and late-onset Alzheimer's disease in a Japanese population.	***J Alzheimers Dis***	41	1031-1038	2014