顕著に認められた．また複数例を検索し得たFTLD-TDPおよびAlzheimer病脳では，組織定量の結果，統計学的に有意にFTLD-TDP＞Alzheimer＞正常対照者の順でニューロピルのFUS陽性顆粒が認められた．培養下において，FUSは細胞にストレスが加わった場合，細胞質のストレス顆粒に移動することが報告されている．FTLD圏の疾患の病理機序において，神経突起における転写調節の異常が何らかの役割を果たしている可能性がある．

E.結論

ALS-6／FTLD-FUSの発病原因と考えられているFUSは，核や細胞体のみでなく，神経突起，特に樹状突起に，シナプスと関連して局在していた．この神経突起内のFUSは，変性疾患，特にFTLDで顕著に増加する傾向があり，それがFTLDの病理機序に関わっている可能性がある．

F.健康危険情報

なし

G.研究発表

1.論文発表

1. Foulds PG, Yokota O, Thurston A, Davidson Y, Ahmed Z, Holton J, Thompson JC, Akiyama H, Arai T, Hasegawa M, Gerhard A, Allsop D, Mann DM. Post mortem cerebrospinal fluid α-synuclein levels are raised in multiple system atrophy and distinguish this from the other α-synucleinopathies, Parkinson's disease and Dementia with Lewy bodies. Neurobiol Dis 2012, 45:188-95.
2. Tsuji H, Nonaka T, Yamashita M, Suzukake M, Kametani F, Akiyama H, Mann D.M.A., Tamaoka A, Hasegawa M Epitope mapping of antibodies against TDP-43 and detection of protease-resistant fragments of pathological TDP-43 in amyotrophic lateral sclerosis and frontotemporal lobar degeneration. Biochem Biophys Res Commun 2011, 417:116-21.
3. Kobayashi Z, Tsuchiy Kobayashi Z, Tsuchiya K, Kubodera T, Shibata N, Arai T, Miura H, Ishikawa C, Kondo H, Ishizu H, Akiyama H, Mizusawa H. FALS with Gly72Ser mutation in SOD1 gene: Report of a family including the first autopsy case. J Neurol Sci 2011, 300:9-13
4. Hasegawa M, Nonaka T, Tsuji H, Tamaoka A, Yamashita M, Kametani F, Yoshida M, Arai T, Akiyama H. Molecular Dissection of TDP-43 Proteinopathies. J Mol Neurosci 2011, 45:480-485
5. Maesako M, Uemura K, Kubota M, Hiyoshi K, Ando K, Kuzuya A, Kihara T, Asada M, Akiyama H, Kinoshita A. Effect of glycogen synthase kinase 3 β -mediated presenilin 1 phosphorylation on amyloid β production is negatively regulated by insulin receptor cleavage. Neurosci 2011, 177:298-307.
6. Habuchi C, Iritani S, Sekiguchi H, Torii Y, Ishihara R, Arai T, Hasegawa M, Tsuchiya K, Akiyama H, Shibayama H, Ozaki N (2011) Clinicopathological study of diffuse neurofibrillary tangles with calcification. With special reference to TDP-43 proteinopathy and alpha-synucleinopathy. J Neurol Sci 301, 77-85.
7. Aoki N, Tsuchiya K, Togo T, Kobayashi Z, Uchikado H, Katsuse O, Suzuki K, Fujishiro H, Arai T, Iseki E, Anno M, Kosaka K, Akiyama H, Hirayasu Y. Gray matter lesions in Nasu-Hakola disease: A report on three autopsy cases. Neuropathology 2011, 31:135-143
8. Kobayashi Z, Tsuchiya K, Komachi H, Miki K, Yokota O, Arai T, Miake H, Ishizu H, Akiyama H, Mizusawa H. Fatal encephalitis in a case of hypereosinophilic syndrome: MRI and autopsy findings. Intern Med. 2011, 50:1219-1225

2.学会発表

1. Hosokawa M, Arai T, Nonaka T, Yamashita M, Suzukake M, Matsuwaki T, Nishihara M, Hasegawa M, Akiyama H (2011) Progranulin reduction affects tau phosphorylation in P301L tau transgenic mice. Neuroscience 2011 Washington D.C. USA [2011/11/14]
2. Hosokawa M, Arai T, Yamashita M, Tsuji H, Nonaka T, Hasegawa M, Akiyama H (2011) Quantitative determination of TDP-43 in cerebrospinal fluid of patients with amyotrophic lateral sclerosis and other neurodegenerative disorders. International Conference on Alzheimer's Disease 2011, Paris France [2011/07/17]

H.知的所有権の取得状況（予定を含む）

なし．

厚生労働科学研究費補助金（難治性疾患克服研究事業）
筋萎縮性側索硬化症の分子病態解明と新規治療法創出に関する研究斑(分担)研究報告書

グラニュリンノックアウトマウス脳の組織化学的解析

新井哲明[1,2)],松脇貴志[3)], 長谷川成人[4)], 秋山治彦[2)],
野中隆[4)], 細川雅人[2)], 朝田隆[1)], 西原真杉[3)]

筑波大学医学医療系臨床医学域精神医学[1)], 東京都医学総合研究所
認知症・高次脳機能研究分野[2)], 東京大学大学院農学生命科学研究科獣医生理学教室[3)],
東京都医学総合研究所 病態細胞生物学研究室[4)]

研究要旨

筋萎縮性側索硬化症 (ALS)および前頭側頭葉変性症 (FTLD)の患者脳に出現する封入体の主要構成成分はTDP-43であり、その他p62とユビキチンが含まれる。グラニュリン(*GRN*)の遺伝子変異例では、プログラニュリン蛋白(PGRN)発現量が半減し、封入体の出現と神経変性が生じる。変異と封入体形成との関連を検討するため、*GRN*ノックアウトマウス脳を解析したところ、TDP-43の蓄積は認められなかったが、視床および脊髄の一部にp62陽性構造が多数観察された。以上から、少なくともマウス脳においては、PGRNの発現量低下により初期に誘導される主要な分子はp62であることが示唆された。PGRNの低下とp62の蓄積が、TDP-43蓄積を介した神経変性にどのように関与するのかを明らかにすることが今後の課題である。

A.研究目的

筋萎縮性側索硬化症 (ALS)および前頭側頭葉変性症 (FTLD)の患者脳においては、特徴的な細胞内封入体が出現する。その構成成分として、TAR DNA-binding protein of 43 kDa (TDP-43)、ユビキチン、p62などが知られている。このような封入体形成を伴う家族性ALSあるいはFTLDの原因遺伝子として、グラニュリン (*GRN*)、*TARDBP*（TDP-43の遺伝子名）、*valosin-contaning protein*、*chromosome 9 open reading frame 72* などが報告されている。これらの遺伝子変異から封入体形成に至る機序を明らかにすることは、ALSおよびFTLDの病態解明のために重要であるが、未だ不明な点が多い。

本研究では、*GRN*ノックアウト(KO)マウスの脳を解析し、プログラニュリン蛋白(PGRN)発現低下とTDP-43の蓄積および封入体形成との関連を検討した。

B.研究方法

野生型(WT)、ヘテロ接合体 (HZ)、KOの12、24ヶ月齢の脳を固定後凍結浮遊あるいはパラフィン包埋切片を作成し、免疫組織化学染色を行った。用いた抗体は、5種類の抗TDP-43抗体（リン酸化非依存性および依存性）、抗ユビキチン抗体（DAKO）、抗p62抗体 (Progen)である。

(倫理面への配慮)

すべての動物実験は、研究報告者が所属する機関の定める「動物実験取扱規程」に則り動物実験計画書を作成し、動物実験（倫理）委員会で承認を得て実施した。

C.研究結果

WT、HZ、KOの12、24ヶ月齢において、大脳、脳幹、脊髄に、TDP-43陽性構造は認められなかった。一方、p62陽性の顆粒状構造がHZとKOの視床において認められた。陽性構造の密度は、HZよりKOに有意に多く、また12ヶ月齢より24ヶ月齢に有意に多かった。これらのp62陽性構造の一部はユビキ

チンにも陽性であった。脊髄においても、24ヶ月齢のKOにのみ、神経細胞内にp62陽性のskein様構造が認められた。

D.考察

マウスでは、GRNの欠損のみではTDP-43の蓄積は生じないが、p62とユビキチンの蓄積が生じ、量的には明らかにp62陽性構造の方が多かった。以上の結果から、少なくともマウスでは、PGRNの発現量低下により初期に誘導される主要な分子はp62であることが示唆された。p62は、ユビキチン化蛋白の凝集体形成を促進するが、それ自身でも凝集体を形成する。また、p62はオートファジーよって分解され、オートファジー機能の減弱によりp62蓄積が生じる。以上から、PGRNの低下によりオートファジー系が異常を来す可能性も考えられ、さらに検討が必要と思われた。

E.結論

マウス脳では、GRNの発現低下により、ALSおよびFTLD患者脳に出現する細胞内封入体の構成成分であるp62の蓄積が生じる。PGRNの低下とp62の蓄積が、TDP-43蓄積を介した神経変性にどのように関与するのかを明らかにすることが今後の課題である。

F.健康危険情報

なし。

G.研究発表

1．論文発表

1. Ando K, Uemura K, Kuzuya A, Maesako M, Asada-Utsugi M, Kubota M, Aoyagi N, Yoshioka K, Okawa K, Inoue H, Kawamata J, Shimohama S, Arai T, Takahashi R, Kinoshita A: N-cadherin regulates p38MAPK signaling via association with JLP: Implications for neurodegeneration in Alzheimer's disease. J Biol Chem 286: 7619-28, 2011
2. Habuchi C, Iritani S, Sekiguchi H, Torii Y, Ishihara R, Arai T, Hasegawa M, Tsuchiya K, Akiyama H, Shibayama H, Ozaki N: Clinicopathological study of diffuse neurofibrillary tangles with calcification–With special reference to TDP-43 proteinopathy and alpha-synucleinopathy–. J Neurol Sci 301: 77-85, 2011
3. Aoki N, Tsuchiya K, Togo T, Kobayashi Z, Uchikado H, Katsuse O, Suzuki K, Fujishiro H, Arai T, Iseki E, Anno M, Kosaka K, Akiyama H, Hirayasu Y: Gray matter lesions in Nasu-Hakola disease: A report on three autopsy cases. Neuropathology 31: 135-143, 2011
4. Kobayashi Z, Tsuchiya K, Kubodera T, Shibata N, Arai T, Miura H, Ishikawa C, Kondo H, Ishizu H, Akiyama H, Mizusawa H: FALS with Gly72Ser mutation in SOD1 gene: report of a family including the first autopsy case. J Neurol Sci 300: 9-13, 2011
5. Kobayashi Z, Tsuchiya K, Komachi H, Miki K, Yokota O, Arai T, Hirotomo M, Ishizu H, Akiyama H, Mizusawa H: Fatal encephalitis in a case of hypereosinophilic syndrome: MRI and autopsy findings. Intern Med 50: 1219-1225, 2011
6. Foulds PG, Yokota O, Thurston A, Davidson Y, Ahmed Z, Holton J, Thompson JC, Akiyama H, Arai T, Hasegawa M, Gerhard A, Allsop D, Mann DMA: Post mortem cerebrospinal fluid α-synuclein levels are raised in multiple system atrophy and distinguish this from the other α-synucleinopathies, Parkinson's disease and Dementia with Lewy bodies. Neurobiol Dis 45: 188-195, 2012
7. Torii Y, Iritani S, Sekiguchi H, Habuchi C, Hagikura M, Arai T, Ikeda K, Akiyama H, Ozaki N: Effects of aging on the morphologies of Heschl's gyrus and the superior temporal gyrus in schizophrenia: A postmortem study. Schizophr Res

134: 137-142, 2012

8. 小林禅, 新井哲明, 秋山治彦, 水澤英洋: FUS/TLS 遺伝子変異による家族性 ALS. Clinical Neuroscience 29: 734-735, 2011
9. 新井哲明, 細川雅人, 長谷川成人, 秋山治彦, 朝田隆: TDP-43 分子による新たな認知症群. 精神経誌 113: 574-583, 2011
10. Hasegawa M, Nonaka T, Tsuji H, Tamaoka A, Yamashita M, Kametani F, Yoshida M, Arai T, Akiyama H: Molecular dissection of TDP-43 proteinopathies. J Mol Neurosci 45: 480-485, 2011
11. 新井哲明: TDP-43. 認知症学(上). 日本臨床増刊号 69: 84-87, 2011
12. 岸本由紀, 横田修, 武田直也, 池田智香子, 新井哲明: TDP-43 陽性神経細胞内封入体を伴う前頭側頭葉変性症と認知症を伴う筋萎縮性側索硬化症. 認知症学(下). 日本臨床増刊号 69: 384-388, 2011
13. 横田修, 根本清貴, 新井哲明: 認知症診療の実践テクニック－患者・家族にどう向き合うか. 非アルツハイマー型認知症とは. 精神科臨床エキスパートシリーズ, 朝田隆編, 医学書院, 東京, pp82-122, 2011
14. 新井哲明, 小林禅, 細川雅人, 長谷川成人, 秋山治彦: ALSD-FTLD-MND-FTLD-U. Clinical Neuroscience 29: 995-998, 2011
15. 小林禅, 新井哲明, 秋山治彦, 水澤英洋: FUS/TLS 遺伝子変異による家族性 ALS. Clinical Neuroscience 29: 734-735, 2011
16. 新井哲明, 山下万貴子, 細川雅人, 野中隆, 小林禅, 長谷川成人, 秋山治彦, 朝田隆: 前頭側頭葉変性症の分子病理. Dementia Japan 25: 120-128, 2011

2.学会発表

1. 新井哲明, 岩切雅彦, 水上勝義, 畑中公孝, 袖山紀子, 田中芳郎, 山形晃彦, 石井映美, 朝田隆: FTLD-FUS の 1 剖検例. 第 52 回日本神経病学会総会学術研究会, 京都, 2011 年 6 月 3 日
2. 青木直哉, 東晋二, 小林禅, 細川雅人, 近藤ひろみ, 羽賀千恵, 泉山洋子, 都甲崇, 勝瀬大海, 内門大丈, 鈴木京子, 新井哲明, 長谷川成人, 平安良雄, 秋山治彦: ビューロピルに認められる FUS陽性微細顆粒について. 第 52 回日本神経病学会総会学術研究会, 京都, 2011 年 6 月 4 日
3. Arai T, Matsuwaki T, Hasegawa M, Akiyama H, Nonaka T, Hosokawa M, Takahashi S, Mizukami K, Asada T, Nishihara M: Histopathological analysis of progranulin knockout mice. Alzheimer's Association International Conference 2011, Paris, France, July 18, 2011
4. 細川雅人, 新井哲明, 野中隆, 山下万貴子, 鈴掛雅美, 辻浩史, 長谷川成人,秋山治彦: メチレンブルーによるリン酸化タウ蓄積抑制効果の検討. 第 30 回日本認知症学会, 名古屋, 2011 年 11 月 11 日
5. 辻浩史, 野中隆, 新井哲明,山下万貴子, 亀谷富由樹, 細川雅人, 初田裕幸, 高尾昌樹, 村山繁雄, 齊藤祐子, 長谷川成人, 秋山治彦, Mann DMA, 詫間浩, 玉岡晃: FTLD-TDP および ALS に蓄積する TDP-43 蛋白の凝集体構造に関する解析. 第 30 回日本認知症学会, 名古屋, 2011 年 11 月 11 日

H.知的所有権の取得状況（予定を含む）

1.特許取得　なし

2.実用新案登録　なし

3.その他　なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
筋萎縮性側索硬化症の分子病態解明と新規治療法創出に関する研究斑(分担)研究報告書

TDP-43 proteinopathy 動物モデル構築への試み

細川　雅人
財団法人東京都医学総合研究所　高次脳機能研究分野　認知症プロジェクト

研究要旨（10 ポイント程度）
ALS の発症メカニズムを明らかにしていくとともに、病態モデルマウスを用いた薬物のスクリーニング及び薬物治療へつなげるために、TDP-43 proteinopathy モデルマウスの作製を行う。TDP-43 単独トランスジェニックマウスでは ALS の病理像再現が困難であるため、プログラニュリンの発現量が低下した神経変性疾患モデルマウス及びタウタンパク重複蓄積モデルマウスを作製する。各タンパク質の異常蓄積を同月齢の単独 Tg と比較して病変加速の有無を検討し、ALS の病理像が再現されることを確認する。

A.研究目的

筋萎縮性側索硬化症 (amyotrophic lateral sclerosis: ALS) の病理マーカーの一つである脊髄のユビキチン陽性封入体の主要構成タンパクと、前頭側頭葉変性症 (frontotemporal lobar degeneration: FTLD) で観察されるタウ陰性ユビキチン陽性封入体の主要構成タンパクが 2006 年、TAR DNA-binding protein of 43 kDa (TDP-43) であることが判明した。この封入体を有する FTLD の一部には ALS と同様の運動ニューロン疾患が合併することがすでに知られていたが、TDP-43 の発見により、FTLD と ALS が同一の病理基盤を有することが明らかになった。

ALS 患者の脊髄から調整した界面活性剤不溶性画分の生化学的解析の結果、蓄積した TDP-43 はリン酸化および断片化を受けていることが判明している。また、作製したリン酸化特異抗体を用いた免疫ブロットにより検出される断片のパターンが、TDP-43 陽性病理像と相関することを見出した。これらの結果は、TDP-43 のリン酸化および断片化が疾患の病理過程に深く関わる変化であることを示唆していると考えられる。

TDP-43 の異常蓄積はアルツハイマー病 (AD) やレビー小体病 (LBD) においても認められる。また、LBD において観察される異常なタウの蓄積はタウとα-シヌクレイン (α-syn) との間に相互促進があることを示唆する。Guam/紀伊半島の ALS・Parkinson 病・認知症複合ではタウと TDP-43 の高度な共蓄積が観察される。一方、2006 年に TDP-43 異常蓄積を伴う家族性 FTLD の原因遺伝子の一つとしてプログラニュリン (progranulin: PGRN) が同定された。PGRN 遺伝子の変異により PGRN の機能低下が TDP-43 異常を促進し、 FTLD が発症すると考えられている。これらの事より異常蓄積タンパク質の overlap は異常蓄積過程における相互作用によるものと考えられる。

これらの神経変性疾患関連タンパク質の異常が相互にどのような影響を与えるかを明らかにするため、PGRN の発現量が低下した神経変性疾患モデルマウス及び異常タンパク質重複蓄積モデルマウスを作製する。具体的には TDP-43 Tg マウスと PGRN ノックアウトマウス (KO) との交配マウス、及び TDP-43 Tg マウスと Tau-Tg マウスとの交配マウスを作出し、各タンパク質の異常蓄積を同月齢の単独 Tg マウスと比較して病変加速の有無を検討し、TDP-43 蓄積と神経変性疾患の関連を調べる。これらにより ALS 発症のメカニズムを明らかにしていくとともに、TDP-43 proteinopathy モデルマウスを用いた薬物治療へつなげる事を目的としている。

B.研究方法

1）TDP-43 proteinopathy モデル動物、PGRN 機能低

下モデル動物の作製

理化学研究所よりPGRNヘテロマウスを入手し、交配によりPGRN-KOマウスを作製した。このマウスと本研究所病態細胞生物学研究チームにより作製されたTDP-43 (G298S) Tg, TDP-43 (M337V)との交配を行った。また、Tau-Tgと TDP-43 Tgとの交配を行い、下記のマウスの作製を行った。

TDP-43 (G298S)/PGRN+/-, TDP-43 (M337V)/PGRN+/-, TDP-43 (G298S)/Tau, TDP-43 (M337V)/Tau

2）界面活性剤不溶性タンパク画分の分離および解析と免疫組織化学的検討

トランスジェニックマウスを十分に加齢させた後、脳を回収し、脳内のタンパク質をトリス buffer 可溶性画分、1% Triton X-100 可溶性画分、1% サルコシル可溶性画分および不溶性画分に分画し、イムノブロットを実施した。

また、脳と脊髄を4%パラフォルムアルデヒドで固定後10 μmの厚さで切片を作製し、抗リン酸化TDP-43抗体により免疫組織化学染色を行った。

(倫理面への配慮)

実験動物は実験動物の倫理基準に準じて実施した。具体的な実験計画は本報告者の所属研究所における動物実験倫理委員会において承認を受けた。

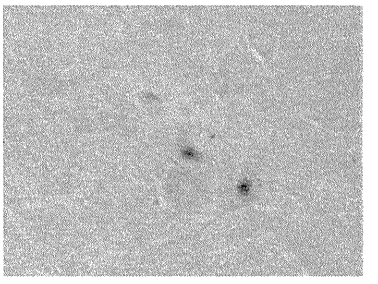
C.研究結果

TDP-43 proteinopagyモデル動物、PGRN機能低下モデル動物の作製と生化学的・免疫組織化学的検討

PGRNヘテロマウスの交配によりPGRN-KOマウスを作製した。このマウスと本研究所・病態細胞生物学チームにより作製されたTDP-43 (G298S) Tg, TDP-43 (M337V) Tg との交配を行い、現時点ですべての Tg マウスと PGRN-KO マウスとの間で子供が生まれているが (TDP-43 (G298S)/PGRN+/-、TDP-43 (M337V)/PGRN+/-)、解析可能な月齢になるまで十分に加齢させることを継続して行っている。

このうち TDP-43 (G298S)/PGRN+/-マウスにおいて TDP-43 の異常蓄積が加速しているかどうかに関して TDP-43 (G298S)Tg マウスとの比較を行った。9-10ヶ月齢の TDP-43 (G298S)/PGRN+/-, TDP-43 (G298S) Tg マウスから脳を回収し、TDP-43 の生化学的解析をイムノブロットにより行った。TDP-43 (G298S)/PGRN+/-マウスにおいて TDP-43 がサルコシル不溶性画分へ多く移行していることが期待されたが、9-10ヶ月齢の両マウスを比較したところ、トリス可溶性画分における TDP-43 タンパク量、サルコシル不溶性画分の TDP-43 タンパク量ともに変化がないと言う結果が昨年までに得られていた。本年度は23 ヶ月齢まで加齢させたマウスを用いてイムノブロットによる検討を行った。サルコシル不溶性画分中の TDP-43 量が TDP-43 (G298S)/PGRN+/-, TDP-43 (G298S) Tg マウスともに9-10ヶ月齢の時点より増えていたが、両者間での差は観察されなかった。

抗リン酸化 TDP-43 による脊髄組織の免疫組織化学染色では、TDP-43 (G298S)/PGRN+/-マウスにおいて神経細胞の細胞質内に封入体が観察され(図)、その数は TDP-43 (G298S) Tg マウスより多い傾向にあった。



(図) TDP-43 (G298S)/PGRN+/-マウス脊髄の抗リン酸化 TDP-43 抗体による染色：神経細胞の細胞質内に TDP-43 陽性封入体が観察される。

また、Guam/紀伊半島の ALS・Parkinson 病・認知症複合のマウスモデルと考えられる Tau/TDP-43 マウスを作製するために Tau Tg マウスと TDP-43 (M337V) Tgマウスの交配を行い TDP-43 (M337V)/Tau

マウスの作出を行った。昨年度は仔が生まれていなかったが、本年度は継続的に仔を産出できるようになった。現在解析可能な月齢になるまで加齢を待っている段階である。

D.考察

TDP-43 (G298S) Tgマウスでは十分なALSの病理像が得られなかったため、TDP-43異常蓄積を伴う家族性FTLDの原因遺伝子であるPGRNを欠損したTDP-43 (G298S)/PGRN+/-マウスを作製し、TDP-43蓄積に関して生化学的・免疫組織化学的検討を行った。9-10ヶ月齢及び23ヶ月齢において、生化学的解析を行ったがTDP-43 (G298S) Tgマウスと TDP-43 (G298S)/PGRN+/-マウス間でTDP-43の異常蓄積の差を検出することはできなかった。

一方、免疫組織化学的検討では TDP-43 (G298S)/PGRN+/-マウスの脊髄神経細胞において、細胞質内にリン酸化 TDP-43 抗体陽性の封入体が観察された。TDP-43 (G298S) Tg マウスではほとんどの場合、TDP-43 陽性の封入体は観察されないが、(G298S)/PGRN+/-マウスでは図のように封入体が観察され、TDP-43 proteinopathyモデル動物として期待ができる結果となった。今後は検体数を増やし、確実にALSモデルマウスとして使用できるかどうかを検討する必要がある。

また、現在加齢中である TDP-43 (M337V) Tg、TDP-43 (M337V)/PGRN+/-、TDP-43 (G298S)/Tau 、TDP-43 (M337V)/Tauに関しても解析を継続していく予定である。

E.結論

ALSの発症機序解明とその治療法・治療薬開発のため、TDP-43 proteinopathyモデルマウスを確立することが急務である。今回解析したTDP-43 (G298S)/PGRN+/-マウスはその候補となり得るが、さらなる詳細な解析が必要である。

F.健康危険情報

該当無し

G.研究発表

1. 論文発表

1. Long-term oral intake of aluminium or zinc does not accelerate Alzheimer pathology in AβPP and AβPP/tau transgenic mice.
Haruhiko Akiyama, Masato Hosokawa, Fuyuki Kametani, Hiromi Kondo, Momoko Chiba, Masako Fukushima, Takeshi Tabira
Neuropathology, 2011, Nov 30. doi: 10.1111/j.1349-7006.2011.02167.x. [Epub ahead of print]

2. 前頭側頭葉変性症の分子病理
新井哲明、山下万貴子、細川雅人、野中隆、小林禅、長谷川成人、秋山治彦、朝田隆
Dementia Japan 第25巻 第2号 120-127 (2011年)

3. ALS-D-FTLD-MND-FTLD-U
新井哲明、小林禅、細川雅人、長谷川成人、秋山治彦
Clinical Neuroscience 第29巻 第9号 995-998 (2011年)

4. TDP-43分子による新たな認知症群
新井哲明、細川雅人、長谷川成人、秋山治彦、朝田隆
精神神経学雑誌 第113巻 第6号 574-583 (2011年)

2.学会発表

1. Quantitative determination of TDP-43 in cerebrospinal fluid of patients with

amyotrophic lateral sclerosis and other neurodegenerative disorders.
Masato Hosokawa, Tetsuaki Arai, Makiko Yamashita, Hiroshi Tsuji, Takashi Nonaka, Masato Hasegawa, Haruhiko Akiyama
International Conference on Alzheimer's Disease 2011 演題番号 P1-088, Paris France [2011/07/17]

2. ALS および FTLD-TDP に蓄積するりん酸化 TDP-43 蛋白コンフォメーションに関する解析.
辻浩史, 長谷川成人, 野中隆, 亀谷富由樹, 山下万貴子, 増田雅美, 細川雅人, 秋山治彦, David Mann, 初田裕幸, 高尾昌樹, 村山繁雄, 齊藤祐子, 詫間浩, 富所康志, 中馬越清隆, 石井亜紀子, 石井一弘, 新井哲明, 玉岡晃 (2011) 第 52 回日本神経学会総会, 名古屋 [2011/05/18]

H.知的所有権の取得状況（予定を含む）

1.特許取得

なし

2.実用新案登録

なし

3.その他

J Mol Neurosci
DOI 10.1007/s12031-011-9571-x

Molecular Dissection of TDP-43 Proteinopathies

Masato Hasegawa · Takashi Nonaka · Hiroshi Tsuji · Akira Tamaoka · Makiko Yamashita · Fuyuki Kametani · Mari Yoshida · Tetsuaki Arai · Haruhiko Akiyama

Received: 30 April 2011 / Accepted: 2 June 2011

Abstract TDP-43 has been identified as a major component of ubiquitin-positive tau-negative cytoplasmic inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and in amyotrophic lateral sclerosis (ALS). We raised antibodies to phosphopeptides representing 36 out of 64 candidate phosphorylation sites of human TDP-43 and showed that the antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 labeled the inclusions, but not the nuclei. Immunoblot analyses demonstrated that the antibodies recognized TDP-43 at ~45 kDa, smearing substances and 18–26 kDa C-terminal fragments. Furthermore, the band patterns of the C-terminal fragments differed between neuropathological subtypes, but were indistinguishable between brain regions and spinal cord in each individual patient. Protease treatment of Sarkosyl-insoluble TDP-43 suggests that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between the diseases. These results suggest that molecular species of abnormal TDP-43 are different between the diseases and that they propagate from affected cells to other cells during disease progression and determine the clinicopathological phenotypes of the diseases.

Keywords Propagation · Phosphorylation · Tau · α-Synuclein · Prion · Cancer

M. Hasegawa (✉) · T. Nonaka · H. Tsuji · M. Yamashita · F. Kametani
Department of Neuropathology and Cell Biology,
Tokyo Metropolitan Institute of Medical Science,
2-1-6 Kamikitazawa, Setagaya-ku,
Tokyo 156–8506, Japan
e-mail: hasegawa-ms@igakuken.or.jp

M. Hasegawa · T. Nonaka · M. Yamashita · F. Kametani · T. Arai · H. Akiyama
Dementia Research Project,
Tokyo Metropolitan Institute of Medical Science,
2-1-6 Kamikitazawa, Setagaya-ku,
Tokyo 156–8506, Japan

H. Tsuji · A. Tamaoka
Department of Neurology, Graduate School of Comprehensive Human Sciences, University of Tsukuba,
Ibaraki 305–8577, Japan

M. Yoshida
Department of Neuropathology,
Institute for Medical Science of Aging, Aichi Medical University,
21 Karimata, Yazako, Nagakute-cho, Aichi-gun,
Aichi 480–1195, Japan

T. Arai
Department of Psychiatry, Graduate School of Comprehensive Human Sciences, University of Tsukuba,
Ibaraki 305–8577, Japan

Introduction

TAR DNA-binding protein of M_r=43 kDa (TDP-43) is a nuclear factor that functions in regulating transcription and splicing. It is structurally characterized by two RNA recognition motifs and the C-terminal tail containing a glycine-rich region, and resembles a heterogeneous ribonucleoprotein (hnRNP) (Ayala et al. 2005). It has been shown to interact with several nuclear ribonucleoproteins (RNP), including hnRNP A and B and survival motor neuron protein, inhibiting alternative splicing (Buratti et al. 2005; Bose et al. 2008). In 2006, TDP-43 was identified as a major component of ubiquitin-positive inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) (Arai et al. 2006; Neumann et al. 2006). Subsequent immunohistochemical examination demonstrated abnormal accumulation of TDP-43 in neurodegenerative disorders other than FTLD-U and ALS, including ALS/parkinsonism–

Published online: 16 June 2011

dementia complex of Guam (Geser et al. 2007; Hasegawa et al. 2007), Alzheimer's disease (AD) (Amador-Ortiz et al. 2007; Higashi et al. 2007; Arai et al. 2009), dementia with Lewy bodies (DLB) (Higashi et al. 2007; Nakashima-Yasuda et al. 2007; Arai et al. 2009), Pick's disease (Arai et al. 2006; Freeman et al. 2008; Lin and Dickson 2008), argyrophilic grain disease (Fujishiro et al. 2009) and corticobasal degeneration (Uryu et al. 2008). These diseases with TDP-43 pathologies are now referred to as TDP-43 proteinopathies. In 2008, mutations in the TDP-43 gene (*TARDBP*) were discovered in familial and sporadic cases of ALS (Yokoseki et al. 2008; Gitcho et al. 2008; Sreedharan et al. 2008; Kabashi et al. 2008; Van Deerlin et al. 2008; Barmada and Finkbeiner 2010; Pesiridis et al. 2009), FTD-MND (Benajiba et al. 2009) and FTD (Borroni et al. 2009), clearly indicating that abnormality of TDP-43 protein causes neurodegeneration.

Identification of Abnormal Phosphorylation Sites of TDP-43

Biochemical analyses of the detergent-insoluble fraction extracted from brains of patients afflicted with FTLD-TDP and ALS show that TDP-43 accumulated in these pathological structures is phosphorylated and cleaved (Arai et al. 2006; Neumann et al. 2006). By producing antibodies against synthetic phosphopeptides containing 36 different phosphorylation sites from among the 56 serine/threonine residues of TDP-43, five abnormal phosphorylation sites were identified at serine residues in the C-terminal region (Hasegawa et al. 2008). The antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD-U, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulation in neuropathological examinations (Hasegawa et al. 2008). Immunoblotting of the Sarkosyl-insoluble fraction from control, FTLD-U and ALS cases using these phospho-specific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 at ~45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD-U and ALS (Hasegawa et al. 2008).

Cellular Models of TDP-43

To establish cellular models of TDP-43 proteinopathies, several deletion mutants of human TDP-43 in SH-SY5Y cells were expressed and the accumulation of TDP-43 was analyzed by use of the phospho-TDP-43 antibodies and ubiquitin. Wild-type (WT) full-length TDP-43 was localized to nuclei and no inclusions were observed, whereas in cells transfected with C-terminal fragments as GFP fusions, round cytoplasmic inclusions with intense GFP fluorescence were formed (Nonaka et al. 2009b). In addition, a deletion mutant lacking the nuclear localizing signal (NLS) and six amino acids similar to the NLS also formed aggregates in cells without any treatment (Nonaka et al. 2009a). These inclusions are strongly positive for antibodies to phosphorylated TDP-43 and ubiquitin. Using these cellular models, the effect of pathogenic mutations of the TDP-43 gene was analyzed. Of 14 mutants examined, seven mutants showed a significantly higher number of aggregates than the WT C-terminal fragment, strongly suggesting that these mutations of TDP-43 accelerate aggregation of the C-terminal fragments (Nonaka et al. 2009b). In addition, when GFP-tagged C-terminal fragments were co-expressed with DsRed-tagged full-length TDP-43, cytoplasmic inclusions with both GFP and DsRed signals were formed, suggesting that exogenous full-length TDP-43 is trapped in cytoplasmic inclusions formed by C-terminal fragments. This may explain why normal nuclear staining of TDP-43 is lost in neuronal cells with inclusions in diseased brains (Nonaka et al. 2009b). Furthermore, we identified two cleavage sites of TDP-43 deposited in FTLD-U by mass spectrometric analysis, and confirmed that expression of these fragments as GFP fusions also afforded cytoplasmic inclusions positive for ubiquitin and phosphorylated TDP-43 (Nonaka et al. 2009b). The cleavage sites identified in the 23-kDa C-terminal fragment of FTLD were different from that of caspase-3, suggesting that caspase is not the enzyme responsible for generating the 23-kDa fragment (Nonaka et al. 2009b). These cellular models recapitulate many of the features of TDP-43 in patients, and therefore, should be useful for screening small molecules for activity to inhibit TDP-43 aggregate formation. We tested whether or not methylene blue and dimebon have the ability to suppress formation of pathological TDP-43 inclusions. Compared to controls, a 50% reduction in the number of inclusions with 0.05 μM methylene blue, a 45% reduction with 5 μM dimebon and an 80% reduction with the combination of 0.05 μM methylene blue and 5 μM dimebon were observed (Yamashita et al. 2009). The effects were statistically significant and the results were also confirmed by Western blotting. These results suggest that these two compounds may be effective in the therapy of ALS, FTLD-U and other TDP-43 proteinopathies.

TDP-43 C-Terminal Fragments

Based on neuropathological studies, TDP-43 proteinopathies have been classified into 4 subtypes (Cairns et al.

2007). Type 1 is characterized by dystrophic neurites (DNs) with few neuronal cytoplasmic inclusions (NCIs) and no neuronal intranuclear inclusions (NIIs), Type 2 has numerous NCIs with few DNs and no NIIs, Type 3 has numerous NCIs and DNs and occasional NIIs and Type 4 has numerous NIIs and DNs with few NCIs, a pattern which is specific for familial FTLD-U with mutations of VCP gene. There appears to be a strong relationship between other subtypes of TDP-43 pathology and clinical phenotype. Type 1 is associated with semantic dementia, type 2 with FTLD with motor neuron disease (MND), ALS or clinical signs of MND, and type 3 with progressive non-fluent aphasia or FTD with mutation in the progranulin gene. Recent studies of ALS have clarified the wide distribution of neuronal and glial TDP-43 pathology in multiple areas of the central nervous systems (Geser et al. 2008; Nishihira et al. 2009), suggesting that ALS does not selectively affect only the motor system, but rather is a multisystem neurodegenerative TDP-43 proteinopathy affecting both neurons and glial cells.

By immunoblot analyses of the Sarkosyl-insoluble fractions from FTLD-U and ALS patients, we found that the band patterns of the C-terminal fragments of phosphorylated TDP-43 corresponded to the neuropathological subtypes. Type 1 FTLD-U showed two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa, while type 2 ALS showed three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa. Type 3 FTD with mutation in the progranulin gene showed an intermediate pattern between those two. These results clearly indicate that TDP-43 proteinopathies subclassified by neuropathological differences can also be distinguished biochemically. This strong association between the neuropathology and the biochemistry is critical for understanding the molecular pathogenesis of TDP-43 proteinopathies.

Biochemical Analysis of TDP-43 in FTLD-U and ALS

The biochemical differences of TDP-43, as shown in the different band patterns of TDP-43 C-terminal fragments, are closely linked to the morphologies of inclusions. The properties of the abnormal TDP-43 may determine the neuropathological and clinical phenotypes of TDP-43 proteinopathies. Similar biochemical and neuropathological differences have been reported in tau between PSP and CBD. Both PSP and CBD are tauopathies with deposition of 4-repeat tau isoforms; however, distinct types of C-terminal fragments are detected, i.e., a 33-kDa band in PSP and ~3-kDa bands in CBD (Arai et al. 2004).

So, what do the different band patterns mean? It is clear that the fragments are produced by cleavage at multiple sites of TDP-43. The band patterns also suggest that the cleavage sites are slightly altered between the diseases. Based on these observations, it is likely that the event may occur after the assembly or aggregation of abnormal TDP-43, and represent relatively protease-resistant domains of TDP-43, which form beta-sheet structure. That is, the different band patterns in TDP-43 proteinopathies represent different conformations of abnormal TDP-43 in the diseases.

To test this idea, we performed protease treatment of the abnormal TDP-43 recovered in the Sarkosyl-insoluble pellets, and analyzed the protease-resistant bands. Proteins can be easily cleaved by proteases if they are denatured or unstructured, but domains that have rigid structures such as beta-sheet structure, or that are structurally buried or interacting with other molecules, are highly resistant to proteases. Figure 1 shows the result of immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment. Before treatment, hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. Upon trypsin or chymotrypsin treatment, the full-length 45-kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16–26 kDa (Fig. 1). As expected, the protease-resistant band pattern of ALS is different and clearly distinguishable from that of FTLD-U. Doublet bands at ~16 kDa and a band at 25 kDa were detected in ALS, but only a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment (Fig. 1). Similarly, multiple protease-resistant bands were detected at 16–25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U (Fig. 1). These results strongly support the idea that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between ALS and FTLD-U.

TDP-43 in Different Brain Regions

Similar protease-resistant bands and differences in the band patterns have been reported in prion diseases, CJD and BSE (Collinge et al. 1996). Protease-resistant prion from new-variant CJD cases showed a different characteristic pattern from that in sporadic CJD cases, and the band pattern is indistinguishable from that of mice infected with BSE prion. This is biochemical evidence that the BSE agent has been transmitted from bovine to human.

Applying this to TDP-43 in TDP-43 proteinopathies, it is possible to determine whether there is any difference between the abnormal TDP-43 accumulated in cortex and that in spinal cord by analyzing the band patterns of the C-

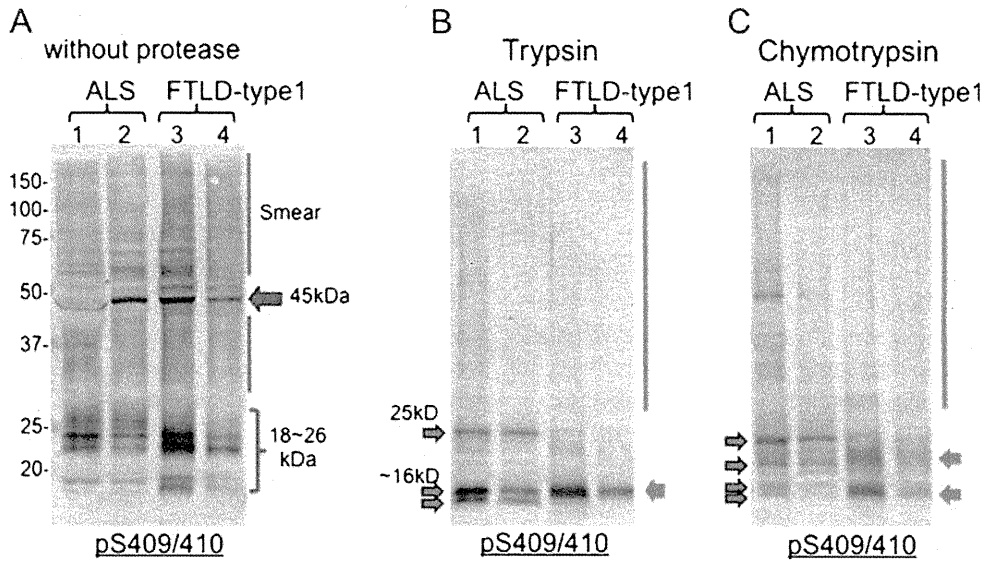


Fig. 1 Immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment with a phosphorylation dependent anti-TDP-43 rabbit polyclonal antibody (pS409/410). **a** Hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410 before treatment. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. **b** Upon trypsin treatment, the full-length 45 kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16~26 kDa. Doublet bands at ~16 kDa and a band at 25 kDa were seen in ALS, but a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment. **c** Multiple protease-resistant bands were detected at 16~25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U

terminal fragments of TDP-43. Thus, we have prepared Sarkosyl-insoluble fractions from cortex and spinal cords of three ALS cases, immunoblotted them with pS409/410 and compared the results. In all three cases, type 2 C-terminal fragments of TDP-43 were detected, and there was no significant difference between the band pattern in cortex and that in spinal cord (data not shown). This strongly suggests that the same form of abnormal TDP-43 molecule is deposited in different brain regions. Similar results were also obtained from the analysis of the C-terminal band pattern of TDP-43 in FTLD-U. It seems highly unlikely that the same conformational change would occur synchronously in different brain regions. Instead, it seems more likely that abnormal protein produced in cells is transferred to different regions and propagated. These biochemical data obtained from the brains of patients provide biochemical evidence that abnormal species of TDP-43 are transmitted from cell to cell and propagated in vivo.

Discussion

Amyloid-like protein deposition is a common neuropathological feature of many neurodegenerative diseases. Hyperphosphorylated tau in Alzheimer's disease and related tauopathies, hyperphosphorylated alpha-synuclein in Parkinson's disease and other alpha-synucleinopathies, and expanded polyglutamines in polyglutamine diseases have been identified. Importantly, the extent of the abnormal protein pathologies is closely correlated with the disease progression (Braak and Braak 1991; Braak et al. 2003; Saito et al. 2003). The proteins or protein fibrils deposited in cells in these diseases have been shown to have a common structural feature. Cross-beta structure, which is the same as in abnormal prion protein, has been demonstrated in filaments or fibrils composed of tau (Berriman et al. 2003), alpha-synuclein (Serpell et al. 2000) or expanded polyglutamines (Perutz 1999). It has not been demonstrated in TDP-43 yet, but we have shown by electron microscopy that phosphorylated TDP-43 in motor neurons of ALS patients has a fibrous structure (Hasegawa et al. 2008), suggesting that TDP-43 is also an amyloid-like protein.

For the assembly of amyloid fibrils, nucleation-dependent protein polymerization has been proposed. This comprises nucleation and elongation phases, and nucleation is the rate-limiting step. It takes a long time to form the first aggregated seed from the monomer, but once the seed is formed, the elongation step proceeds relatively quickly. More importantly, by addition of amyloid-seed, proteins are often converted to the same conformation as that of the seed. For example, WT monomeric alpha-synuclein is converted to A30P-type amyloid fibrils when it is incubated with a small amount of fibril-seeds formed with A30P mutant alpha-synuclein (Yonetani et al. 2009). Differences in the conformations of the amyloid fibrils are detected based on the differences in the protease-resistant band

patterns, as in the typing of prion proteins. There is another example of nucleation-dependent amyloid fibril formation in cultured cells. We developed a novel method for introducing amyloid seeds into cultured cells using lipofectoamine, and presented experimental evidence of seed-dependent polymerization of alpha-synuclein, leading to the formation of filamentous protein deposits and cell death (Nonaka et al. 2010). Overexpression of alpha-synuclein itself in cells does not generate abnormal inclusions, but if fibril seeds formed with alpha-synuclein are introduced into cells, abundant filamentous alpha-synuclein aggregates positive for PSer129 and ubiquitin are developed, and cells with inclusions undergo cell death. This was also clearly demonstrated in cells expressing different tau isoforms by introducing the corresponding tau fibril seeds (Nonaka et al. 2010).

The above results obtained from biochemical analyses of abnormal proteins in patients strongly suggest that intracellular amyloid-like proteins, including TDP-43, propagate from cell to cell and this propagation is the cause of disease progression, analogously to metastasis of cancer cells to multiple different tissues in cancer progression. From this point of view, we have proposed as a hypothesis that neurodegenerative diseases with amyloid-like proteins can be regarded as "protein cancers." The term prion, coined in 1982 by Stanley B. Prusiner, describes an agent transmissible among humans and a variety of mammals. On the other hand, the term "protein cancers" describes diseases that involve the spreading or propagation of abnormal proteins in tissues or individuals, even though the mechanism of propagation is basically the same as that of prions. Amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation, and the amplified amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions (Fig. 2). As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. Therefore, it is important to regulate the propagation of abnormal proteins for clinical therapy.

Conclusions

1. In ALS, FTLD-U and other TDP-43 proteinopathies, abnormally phosphorylated, ubiquitinated, and truncated TDP-43 is accumulated in a filamentous form.
2. We established cellular models which recapitulate many of the features of the abnormal TDP-43 in FTLD-U and ALS
3. ALS-related pathogenic mutations of the TDP-43 gene accelerate aggregate formation by the C-terminal fragments.
4. The band pattern of the TDP-43 C-terminal fragments is different between diseases with different clinicopathological phenotypes, and it represents different conformations of the abnormal TDP-43 between the diseases.
5. The C-terminal band patterns in several brain areas and spinal cord in each individual case of sporadic ALS are indistinguishable.
6. These and other results suggest that abnormal TDP-43, tau and alpha-synuclein are transmitted and propagated from cell to cell in different regions during disease progression. It is important to find drugs that can block the propagation of abnormal proteins for clinical therapy.

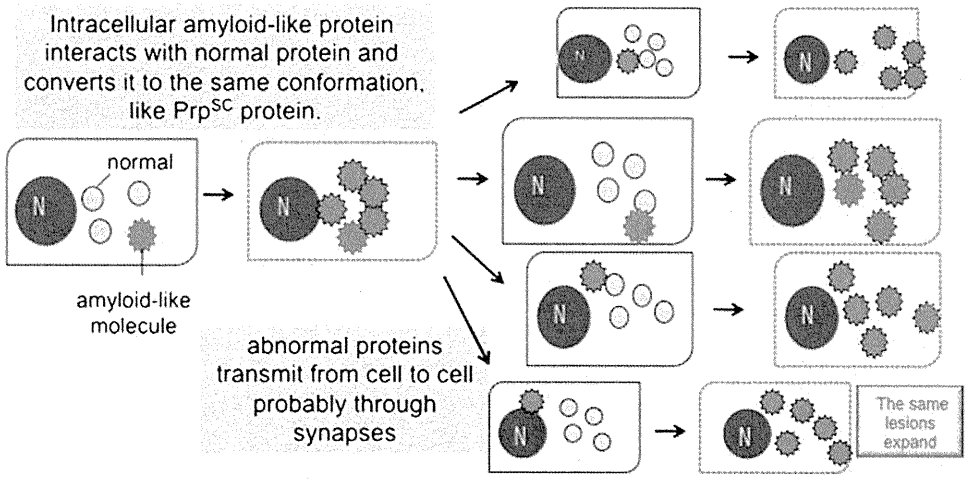


Fig. 2 Schematic representation of prion-like conversion of normal protein into amyloid-like protein and its propagation in neurodegenerative diseases. Intracellular amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation. Amplified abnormal amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions. As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. From this point of view, neurodegenerative diseases with amyloid-like proteins can be regarded as 'protein cancers"

References

Amador-Ortiz C, Lin WL, Ahmed Z et al (2007) TDP-43 immunoreactivity inhippocampal sclerosis and Alzheimer's disease. Ann Neurol 61:435–445

Arai T, Ikeda K, Akiyama H, Nonaka T, Hasegawa M, Ishiguro K et al (2004) Identification of amino-terminally cleaved tau fragments that distinguish progressive supranuclear palsy from corticobasal degeneration. Ann Neurol 55:72–79

Arai T, Hasegawa M, Akiyama H et al (2006) TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun 351:602–611

Arai T, Mackenzie IR, Hasegawa M et al (2009) Phosphorylated TDP-43 in Alzheimer's disease and dementia with Lewy bodies. Acta Neuropathol 117:125–136

Ayala YM, Pantano S, D'Ambrogio A et al (2005) Human, *Drosophila*, and *C. elegans* TDP43: nucleic acid binding properties and splicing regulatory function. J Mol Biol 348:575–588

Barmada SJ, Finkbeiner S (2010) Pathogenic TARDBP mutations in amyotrophic lateral sclerosis and frontotemporal dementia: disease-associated pathways. Rev Neurosci 21:251–272 (Review)

Benajiba L, Le Ber I, Camuzat A, Lacoste M et al (2009) TARDBP mutations in motoneuron disease with frontotemporal lobar degeneration. Ann Neurol 65:470–473

Berriman J, Serpell LC, Oberg KA et al (2003) Tau filaments from human brain and from in vitro assembly of recombinant protein show cross-beta structure. Proc Natl Acad Sci USA 100:9034–9038

Borroni B, Bonvicini C, Alberici A et al (2009) Mutation within TARDBP leads to frontotemporal dementia without motor neuron disease. Hum Mutat 30:E974–E983

Bose JK, Wang IF, Hung L, Tarn WY, Shen CK (2008) TDP-43 overexpression enhances exon 7 inclusion during the survival of motor neuron pre-mRNA splicing. J Biol Chem 283:28852–28859

Braak H, Braak E (1991) Neuropathological staging of Alzheimer-related changes. Acta Neuropathol 82:239–259

Braak H, Del Tredici K, Rub U, de Vos RA et al (2003) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197–211

Buratti E, Brindisi A, Giombi M, Tisminetzky S, Ayala YM, Baralle FE (2005) TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail. J Biol Chem 280:37572–37584

Cairns NJ, Bigio EH, Mackenzie IR et al (2007) Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration. Acta Neuropathol 114:5–22

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 383:685–690

Freeman SH, Spires-Jones T, Hyman BT, Growdon JH, Frosch MP (2008) TAR-DNA binding protein 43 in Pick disease. J Neuropathol Exp Neurol 67:62–67

Fujishiro H, Uchikado H, Arai T et al (2009) Accumulation of phosphorylated TDP-43 in brains of patients with argyrophilic grain disease. Acta Neuropathol 117:151–158

Geser F, Winton MJ, Kwong LK et al (2007) Pathological TDP-43 in parkinsonism-dementia complex and amyotrophic lateral sclerosis of Guam. Acta Neuropathol 115:133–145

Geser F, Brandmeir NJ, Kwong LK et al (2008) Evidence of multisystem disorder in whole-brain map of pathological TDP-43 in amyotrophic lateral sclerosis. Arch Neurol 65:636–641

Gitcho MA, Baloh RH, Chakraverty S et al (2008) TDP-43 A315T mutation in familial motor neuron disease. Ann Neurol 63:535–538

Hasegawa M, Arai T, Akiyama H et al (2007) TDP-43 is deposited in the Guam parkinsonism-dementia complex brains. Brain 130:1386–1394

Hasegawa M, Arai T, Nonaka T et al (2008) Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Ann Neurol 64:60–70

Higashi S, Iseki E, Yamamoto R et al (2007) Concurrence of TDP-43, tau and alpha-synuclein pathology in brains of Alzheimer's disease and dementia with Lewy bodies. Brain Res 1184:284–294

Kabashi E, Valdmanis PN, Dion P et al (2008) TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. Nat Genet 40:572–574

Lin WL, Dickson DW (2008) Ultrastructural localization of TDP-43 in filamentous neuronal inclusions in various neurodegenerative diseases. Acta Neuropathol 116:205–213

Nakashima-Yasuda H, Uryu K, Robinson J et al (2007) Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. Acta Neuropathol 114:221–229

Neumann M, Sampathu DM, Kwong LK et al (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 314:130–133

Nishihira Y, Tan CF, Hoshi Y et al (2009) Sporadic amyotrophic lateral sclerosis of long duration is associated with relatively mild TDP-43 pathology. Acta Neuropathol 117:45–53

Nonaka T, Arai T, Buratti E, Baralle FE, Akiyama H, Hasegawa M (2009a) Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells. FEBS Lett 583:394–400

Nonaka T, Kametani F, Arai T, Akiyama H, Hasegawa M (2009b) Truncation and pathogenic mutations facilitate the formation of intracellular aggregates of TDP-43. Hum Mol Genet 18:3353–3364

Nonaka T, Watanabe ST, Iwatsubo T, Hasegawa M (2010) Seeded aggregation and toxicity of alpha-synuclein and tau: cellular models of neurodegenerative diseases. J Biol Chem 285:34885–34898

Perutz MF (1999) Glutamine repeats and neurodegenerative diseases. Brain Res Bull 50:467

Pesiridis GS, Lee VM, Trojanowski JQ (2009) Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis. Hum Mol Genet 18:R156–R162

Saito Y, Kawashima A, Ruberu NN, Fujiwara H et al (2003) Accumulation of phosphorylated alpha-synuclein in aging human brain. J Neuropathol Exp Neurol 62:644–654

Serpell LC, Berriman J, Jakes R et al (2000) Fiber diffraction of synthetic alpha-synuclein filaments shows amyloid-like cross-beta conformation. Proc Natl Acad Sci USA 97:4897–4902

Sreedharan J, Blair IP, Tripathi VB et al (2008) TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science 319:1668–1672

Uryu K, Nakashima-Yasuda H, Forman MS et al (2008) Concomitant TAR-DNA-binding protein 43 pathology is present in Alzheimer disease and corticobasal degeneration but not in other tauopathies. J Neuropathol Exp Neurol 67:555–564

Van Deerlin VM, Leverenz JB, Bekris LM et al (2008) TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. Lancet Neurol 7:409–416

Yamashita, M., Nonaka, T., Arai, T et al (2009) Methylene blue and dimebon inhibit aggregation of TDP-43 in cellular models. FEBS Lett 583:2419–24

Yokoseki A, Shiga A, Tan CF et al (2008) TDP-43 Mutation in familial amyotrophic lateral sclerosis. Ann Neurol 63:538–542

Yonetani M, Nonaka T, Masuda M et al (2009) Conversion of wild-type alpha-synuclein into mutant-type fibrils and its propagation in the presence of A30P mutant. J Biol Chem 284:7940–7950

Biochemical and Biophysical Research Communications 417 (2012) 116–121

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Epitope mapping of antibodies against TDP-43 and detection of protease-resistant fragments of pathological TDP-43 in amyotrophic lateral sclerosis and frontotemporal lobar degeneration

Hiroshi Tsuji [a,b], Takashi Nonaka [a], Makiko Yamashita [a], Masami Masuda-Suzukake [a], Fuyuki Kametani [a], Haruhiko Akiyama [c], David M.A. Mann [d], Akira Tamaoka [b], Masato Hasegawa [a,*]

[a] Department of Neuropathology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan
[b] Department of Neurology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tenodai, Tsukuba-City, Ibaraki 305-8576, Japan
[c] Dementia Research Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-0057, Japan
[d] Mental Health and Neurodegeneration Research Group, Greater Manchester Neuroscience Centre, University of Manchester, Hope Hospital, Salford M6 8HD, UK

ARTICLE INFO

Article history:
Received 8 November 2011
Available online 22 November 2011

Keywords:
Aggregation
Tau
Alpha-synuclein
ALS
FTLD

ABSTRACT

TAR DNA-binding protein of 43 kDa (TDP-43) is the major component of the intracellular inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Here, we show that both monoclonal (60019-2-Ig) and polyclonal (10782-2-AP) anti-TDP-43 antibodies recognize amino acids 203–209 of human TDP-43. The monoclonal antibody labeled human TDP-43 by recognizing Glu204, Asp205 and Arg208, but failed to react with mouse TDP-43. The antibodies stained the abnormally phosphorylated C-terminal fragments of 24–26 kDa in addition to normal TDP-43 in ALS and FTLD brains. Immunoblot analysis after protease treatment demonstrated that the epitope of the antibodies (residues 203–209) constitutes part of the protease-resistant domain of TDP-43 aggregates which determine a common characteristic of the pathological TDP-43 in both ALS and FTLD-TDP. The antibodies and methods used in this study will be useful for the characterization of abnormal TDP-43 in human materials, as well as in vitro and animal models for TDP-43 proteinopathies.

1. Introduction

TDP-43 is a nuclear ribonucleoprotein implicated in exon splicing, gene transcription, regulation of mRNA stability, mRNA biosynthesis, and formation of nuclear bodies [1–5]. It has been identified as the major component of the ubiquitin-positive tau-negative intracytoplasmic inclusions in frontotemporal lobar degeneration (FTLD), amyotrophic lateral sclerosis (ALS) [6,7] and other neurodegenerative disorders [8–12]. Identification of mutations in familial and sporadic ALS and FTLD cases demonstrated a direct link between the genetic lesion and development of TDP-43 pathology [13–16]. Immunohistochemical studies using anti-TDP-43 antibodies revealed that TDP-43 translocates from its normal nuclear localization into the cytoplasm in these disorders. Furthermore, biochemical analysis detected abnormally phosphorylated TDP-43 of 45 kDa, high-molecular-weight smearing and C-terminal fragments of approximately 25 kDa, as well as normal TDP-43 of 43 kDa in the detergent-insoluble, urea-soluble fraction from affected brains. The antibodies generated by immunizing C-terminal phosphopeptides of TDP-43, such as pS409/410 and pS403/404, strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulations in neuropathological examination [17]. Immunoblotting of the Sarkosyl-insoluble fractions from FTLD and ALS cases using these phosphospecific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 of 45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD and ALS, and the band patterns of the C-terminal fragments of phosphorylated TDP-43 correspond to the neuropathological subtypes.

Anti-TDP-43 monoclonal antibody (mAb) (60019-2-Ig; Proteintech Group Inc., Chicago, IL) and polyclonal antibody (pAb) (10782-2-AP; Proteintech Group Inc., Chicago, IL) are widely used for the investigation of TDP-43 pathology [6,7,9,18–21]. According to the manufacturer's specifications, anti-TDP-43 mAb and pAb were generated against the N-terminal 260 amino acids (aa) of the protein, but the precise epitope has not yet been identified. Another mouse monoclonal antibody against TDP-43 (2E2-D3; Abnova Corporation, Taipei, Taiwan) is also commercially available; it recognizes residues 205–222 of human TDP-43, but does not recognize mouse or rat TDP-43 [22].

* Corresponding author. Fax: +81 3 6834 2349.
E-mail address: hasegawa-ms@igakuken.or.jp (M. Hasegawa).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.11.066

In this study, we mapped the epitope for anti-TDP-43 mAb and pAb (Proteintech Group Inc.). We also showed that anti-TDP-43 mAb recognizes human TDP-43, but not mouse TDP-43. Using these antibodies, we investigated the abnormal forms of TDP-43 from ALS and FTLD brains, and found that the antibodies recognized the amino-terminus of the TDP-43 C-terminal fragments of 24–26 kDa. Immunoblot analysis of Sarkosyl-insoluble fractions after treatment of proteases also demonstrated that the epitope is apparently resistant to trypsin and chymotrypsin in the abnormal TDP-43, suggesting that the epitope region is important for the formation of the pathological structure of TDP-43 in ALS and FTLD.

2. Materials and methods

2.1. Construction of plasmids

GFP-tagged TDP-43 C-terminal or N-terminal fragments were constructed as described [23] by amplifying a cDNA encoding full-length TDP-43 by means of PCR and inserting the fragment into the pEGFP-C1 vector (Clontech). To investigate the specificity of TDP-43 mAb for human TDP-43, site-directed mutagenesis of GFP-tagged full-length TDP-43 was carried out to substitute Glu204 to Ala (E204A), Asp205 to Glu (D205E), Arg208 to Gln (R208Q), Glu209 to Gln (E209Q), Ser212 to Cys (S212C), Asp216 to Glu (D216E), and Met218 to Val (M218V), using a site-directed mutagenesis kit (Strategene)(Fig. 4). All constructs were verified by DNA sequencing.

2.2. Antibodies

TDP-43 polyclonal antibody, 10782-2-AP, and TDP-43 monoclonal antibody, 60019-2-Ig, were purchased from Proteintech Group Inc. Anti-GFP monoclonal antibody was purchased from MBL (Nagoya, Japan). A polyclonal antibody specific for phosphorylated TDP-43 (pS409/410) was prepared as described [17].

2.3. Cell culture and expression of plasmids

Human neuroblastoma cell line SH-SY5Y and mouse neuroblastoma cell line Neuro 2a were maintained in appropriate medium as described previously [24,25]. Cells were then transfected with expression plasmids using FuGENE6 (Roche) according to the manufacturer's instructions.

2.4. Immunoblotting

Expressed proteins in cell lysates were separated by 10% SDS–PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 3% gelatin, membranes were incubated overnight with primary antibodies (1:1000) at room temperature. After incubation with an appropriate biotinylated secondary antibody, labeling was detected using the ABC system (Vector Lab., Burlingame, CA) coupled with a diaminobenzidine (DAB) reaction intensified with nickel chloride.

2.5. Analysis of abnormal TDP-43 in ALS and FTLD-TDP brain

Brains from two cases with Alzheimer's disease (AD), two with ALS, two with FTLD-TDP (type A), two with FTLD-TDP (type B) and two with FTLD-TDP (type C) were employed in this study. The two AD cases had no TDP-43 pathology. The age, sex, brain weight, and diagnosis are given in Table 1. Sarkosyl-insoluble, urea-soluble fractions were extracted from these brains as previously described [6,9]. The samples were loaded onto 15% polyacrylamide gel and transferred onto a membrane. The membrane was cut in the center of the loaded lane, and the same samples were reacted separately with anti-TDP-43 Abs and pS409/410 as described above.

Table 1
Description of subjects.

Case No.	Diagnosis	Age (years)	Sex	BW (g)
1	AD	65	F	1165
2	AD	70	F	1126
3	ALS	62	M	1230
4	ALS	42	F	1140
5	FTLD-TDP (type A)	71	F	863
6	FTLD-TDP (type A)	66	F	1100
7	FTLD-TDP (type B)	45	M	1260
8	FTLD-TDP (type B)	67	M	1280
9	FTLD-TDP (type C)	67	M	na
10	FTLD-TDP (type C)	59	M	na

BW, brain weight; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; FTLD-TDP, frontotemporal lobar degeneration with TDP-43 pathology; na, not available.

2.6. Protease treatment of TDP-43

Sarkosyl-insoluble fractions extracted from neocortical regions of the brains were treated with trypsin (at a final concentration of 100 μg/ml, Promega, Madison, USA) or chymotrypsin (at a concentration of 10 μg/ml, Sigma–Aldrich, St. Louis, USA) at 37 °C for 30 min. The reaction was stopped by boiling for 5 min. After centrifuging at 15,000 rpm for 1 min, the samples were analyzed by immunoblotting with anti-TDP-43 pAb and mAb as described above.

3. Results

3.1. Epitope mapping of anti-TDP-43 antibody

Our previous study showed that both TDP-43 mAb and pAb reacted with GFP-tagged TDP-43 C-terminal fragment (GFP-TDP 162–414), but failed to detect GFP-TDP 218–414 [23]. To map the epitope of these antibodies, we expressed a series of GFP-tagged human TDP-43 C-terminal fragments (Fig. 1A) in SH-SY5Y cells and immunoblotted them with the antibodies. Both anti-TDP-43 pAb and mAb detected endogenous human TDP-43 of 43 kDa and exogenous GFP-tagged full-length, 171–414, 181–414, 191–414 and 201–414 TDP-43. However, both antibodies failed to detect 211–414 (Fig. 1A). These results suggest that the epitopes of these antibodies are located within residues 201–210.

To narrow down the epitope structure further, another series of GFP-tagged C-terminal fragments of TDP-43 was expressed in SH-SY5Y cells (Fig. 1B) and tested. Both antibodies reacted with GFP-TDP 203–414, but failed to recognize GFP-TDP 204–414, 205–414 and 207–414 (Fig. 1B), demonstrating that Thr203 forms the N-terminal border of the epitope for both antibodies.

To determine the C-terminus of the epitope, a series of GFP-tagged N-terminal fragments of TDP-43 was expressed and immunoblotted with these antibodies (Fig 1C). Anti-TDP-43 pAb reacted with all of the N-terminal fragments tested, although it stained the 1-212 fragment most strongly. This suggests that one of the pAb epitopes is located at the N-terminal region of TDP-43, in addition to the central epitope. Anti-TDP-43 mAb strongly stained GFP-TDP 1-212, moderately stained GFP-TDP 1-210, and barely stained GFP-TDP 1-209, while it failed to react with GFP 1-208 and 1-207 (Fig. 1C), indicating that Glu209 forms the C-terminus of the epitope for anti-TDP-43 mAb. Thus, anti-TDP-43 mAb recognizes residues 203–209 of human TDP-43.

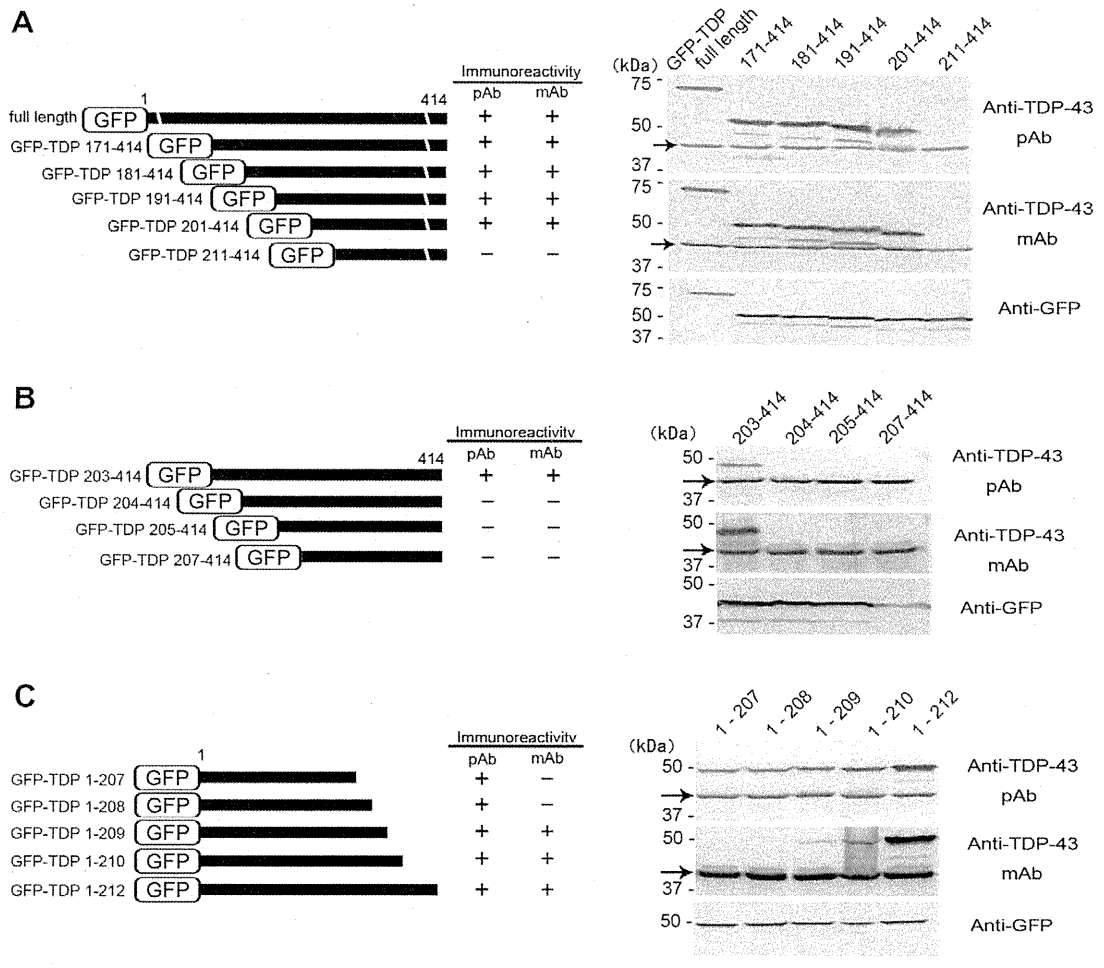


Fig. 1. Epitope mapping of anti-TDP-43 polyclonal and monoclonal antibodies. (A) Schematic diagram of GFP-tagged full-length TDP-43 (GFP-TDP) and the C-terminal fragments. Immunoblot analyses of GFP-TDP and the C-terminal fragments in SH-SY5Y cells. Both mAb and pAb reacted with GFP-TDP and the C-terminal fragments, except for 211–414. The anti-GFP antibody recognizes all the proteins expressed. (B) Further epitope mapping of anti-TDP-43 antibodies. Immunoblot analyses of the GFP tagged C-terminal fragments of TDP-43. Both mAb and pAb reacted with 203–414, but failed to recognize 204–414, 205–414, and 207–414. The anti-GFP antibody recognizes all of the fragments. (C) Epitope mapping of the C-terminus recognized by anti-TDP-43 polyclonal and monoclonal antibodies. Immunoblot analyses of GFP-TDP and N-terminal fragments in SH-SY5Y cells. Anti-TDP-43 pAb reacted with all of the N-terminal fragments, although it stained 1-212 fragment most strongly. In contrast, anti-TDP-43 mAb strongly stained GFP-TDP 1-212, moderately stained GFP-TDP 1-210, and barely stained GFP-TDP 1-209, while it failed to react with GFP 1-208 and 1-207. The anti-GFP antibody recognized all of the fragments equally. The arrows indicate endogenous TDP-43 in SH-SY5Y cells.

3.2. Amino acid sequence differences between human and mouse TDP-43

The anti-TDP-43 mAb reacted with endogenous TDP-43 of human neuroblastoma SH-SY5Y cells, but not with TDP-43 of mouse neuroblastoma Neuro2a cells (Fig. 1B, 1C, 2B). Similarly, the mAb recognized TDP-43 in human brain extract, but failed to detect TDP-43 in mouse brain extract, suggesting that the mAb does not recognize mouse TDP-43 (data not shown). The absence of reactivity with mouse TDP-43 is explained by the sequence differences around the epitope between human and mouse TDP-43 (Fig. 2A). Each different amino acid of human TDP-43 was substituted to that of mouse TDP-43. The mutated proteins were expressed in Neuro2a cells and immunoreactivity with anti-TDP-43 mAb was examined. Substitution of D216 to E and M218 to V did not affect the immunoreactivity (Fig. 2B), whereas substitutions of E204 to A, D205 to E, and R208 to Q abolished the immunoreactivity of anti-TDP-43 mAb, indicating that these residues are necessary for recognition by the mAb. Anti-TDP-43 pAb reacted with these mutants, although a marked decrease in immunoreactivity was observed in the cases of E204A, D205A, R208Q, and S212C.

3.3. Biochemical analysis of abnormal TDP-43 in ALS and FTLD brains with anti-TDP-43 mAb

On immunoblots of Sarkosyl-insoluble fractions extracted from the brain of patients with ALS and FTLD-TDP (type A), the anti-TDP-43 mAb detected phosphorylated full-length TDP-43 at 45 kDa, two bands around 25 kDa and high-molecular-weight smears, in addition to the normal TDP-43 band at 43 kDa, which can also be detected in control cases. Immunoblot analysis of the split membrane with a phosphorylation-dependent anti-TDP-43 antibody pS409/410 revealed that the two bands around 25 kDa stained with the mAb corresponded to the C-terminal fragments of 24 and 26 kDa recognized by pS409/410 (Fig. 3)[17]. These results demonstrated that these 24 and 26 kDa C-terminal fragments contain the epitope of the mAb, residues 203–209, and that the cleavage sites of these C-terminal fragments are located at the N-terminal side of Thr203.

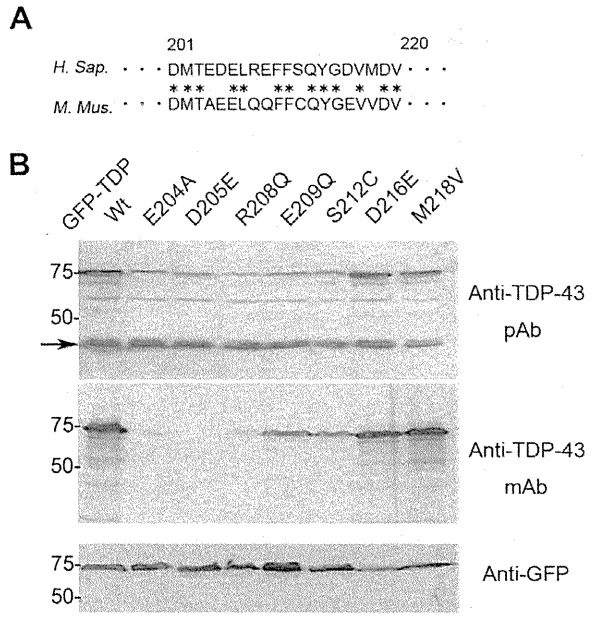


Fig. 2. Alignment of human and mouse TDP-43 (A) and immunoblot analyses of mutated TDP-43 with anti-TDP-43 antibodies. (A) The amino acid sequences of human (upper) and mouse (lower) TDP-43 around the epitope of anti-TDP-43 mAb. The asterisks show identical amino acids. (B) Immunoblot analyses of GFP-TDP wild type (Wt) and GFP-TDP mutants expressed in Neuro2a cells. Substitution of D216 to E and M218 to V did not affect the immunoreactivity, whereas substitutions of E204 to A, D205 to E, and R208 to Q, abolished the immunoreactivity of anti-TDP-43 mAb. Anti-TDP-43 pAb reacted with all these mutants, although markedly decreased immunoreactivities were observed in E204A, D205A, R208Q, and S212C. The arrows indicated endogenous TDP-43 in Neuro2A cells. Note that endogenous mouse TDP-43 in Neuro 2a cells was not recognized by anti-TDP-43 mAb.

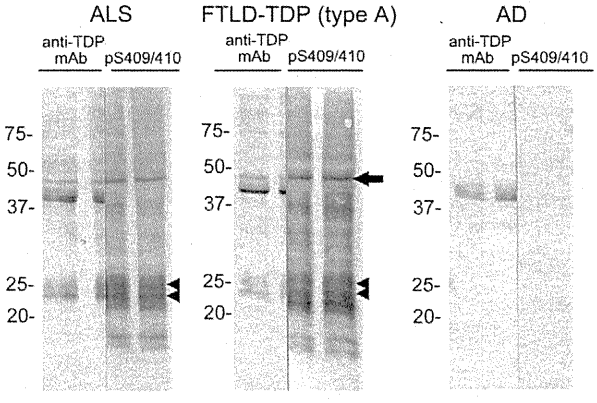


Fig. 3. Immunoblot analyses of Sarkosyl-insoluble fractions from ALS, FTLD-TDP (type A), and AD brains with anti-TDP-43 monoclonal antibody and phosphorylation-dependent anti-TDP-43 antibody, pS409/410. With pS409/410, fragments of approximately 45 kDa and 18–26 kDa, as well as smearing, were detected. The banding pattern of 18–26 kDa fragments showed three major bands at 23, 24, and 26 kDa, and 2 minor bands at 18 and 19 kDa, with the 24 kDa band being the most intense. In addition to the normal full-length TDP-43 at 43 kDa, anti-TDP-43 mAb labeled phosphorylated full-length TDP-43 at 45 kDa, high-molecular-weight smears and two bands at 26 kDa and 24 kDa (arrowheads), which were not seen in the AD case. The two bands corresponded to the major 26 and 24 kDa bands were detected with pS409/410.

3.4. The epitope of these TDP-43 antibodies constitute part of protease-resistant core domain of TDP-43 in ALS and FTLD brains

In order to characterize the epitope further, we treated the Sarkosyl-insoluble fractions extracted from brains of patients with proteases and analyzed them with these antibodies. Without protease treatment, both antibodies strongly stained normal full-length TDP-43 of 43 kDa in all cases examined including AD cases which were without TDP-43 pathology. In ALS and FTLD-TDP cases, phosphorylated full-length TDP-43 of 45 kDa (Fig 4A, arrows) and the ~25 kDa fragments (Fig 4A, arrow heads) were detected with these antibodies. After trypsin treatment, the full-length band of TDP-43 was disappeared and the protease-resistant fragments around 25 kDa (Fig 4B, white arrows) and smearing substances appeared in the ALS and FTLD-TDP cases. Similarly, after chymotrypsin treatment, protease-resistant triplet bands of 16, 20 and 25 kDa (Fig 4C, white arrow heads) and smearing substances were clearly detected in ALS and FTLD-TDP-cases with the mAb, while no such bands were seen in AD cases. On blot with the pAb, multiple bands were detected in addition to the triplet, and some of these bands were also detected in AD cases, suggesting that the pAb stained some normal fragments in addition to the abnormal TDP-43 bands. In the cases examined, apparent difference was not detected in these trypsin-resistant and chymotrypsin-resistant bands detected among the clinicopathological phenotypes of the diseases. By proteinase K treatment, immunoreactivities with these antibodies were completely abolished (data not shown), suggesting that the epitope is not entirely resistant to any proteases. However, it is obvious that the epitope of the TDP-43 deposited in the patients is fairly protease-resistant compared to the normal protein. These results indicate that the epitope of the mAb (residues 203–209 of TDP-43) constitute part of the protease-resistant domain of TDP-43 which determine a common characteristic of the abnormal TDP-43 in both ALS and FTLD-TDP.

4. Discussion

This is the first analysis of the epitopes of Proteintech's anti-TDP-43 polyclonal and monoclonal antibodies, which have often been used to research TDP-43 proteinopathies since 2006 [6,7]. We demonstrated that anti-TDP-43 mAb specifically recognizes residues 203–209 of human TDP-43, which form a part of the second RNA-recognition motif (RRM2, residues 193–257) of normal TDP-43 [26], but constitute part of the protease-resistant core domain of TDP-43 aggregates that determine the common characteristic of abnormal TDP-43 in ALS and FTLD-TDP-43.

RRM2 is a functional domain with distinct RNA/DNA binding characteristics. The anti-TDP-43 mAb recognized human TDP-43, but not mouse TDP-43. Site-directed mutagenesis and subsequent immunoblot analysis revealed that Glu204, Asp205 and Arg208 residues in human TDP-43 are important for the specific recognition by the mAb (Fig. 2). In fact, human TDP-43 shares 98.5% homology with mouse TDP-43 at the amino acid level, but the RRM2 domain has only 66% homology.

We also showed that one of the major epitopes of the pAb is located in almost the same region at that of the mAb (Fig. 1), although the pAb also recognizes the N-terminal region of TDP-43. Recently, TDP-43 transgenic mice overexpressing human TDP-43 have been produced as animal models of TDP-43 proteinopathy [27]. However, abnormal TDP-43 pathologies in these mice are very rare, so new transgenic or other animal models that develop abundant TDP-43 pathology are still required. Since the TDP-43 mAb recognizes human TDP-43, but not mouse TDP-43, it will be a useful reagent for the characterization of mouse lines transgenic for human TDP-43, together with phosphorylation-dependent antibodies.

Biochemical analyses of TDP-43 proteinopathies have demonstrated that abnormally phosphorylated full-length and C-terminal fragments of TDP-43 are the major species in the inclusions. The band patterns of the C-terminal fragments at 18–26 kDa are closely correlated with the clinicopathological subtypes of TDP-43 proteinopathies [17]. In addition, most of the pathogenic mutations

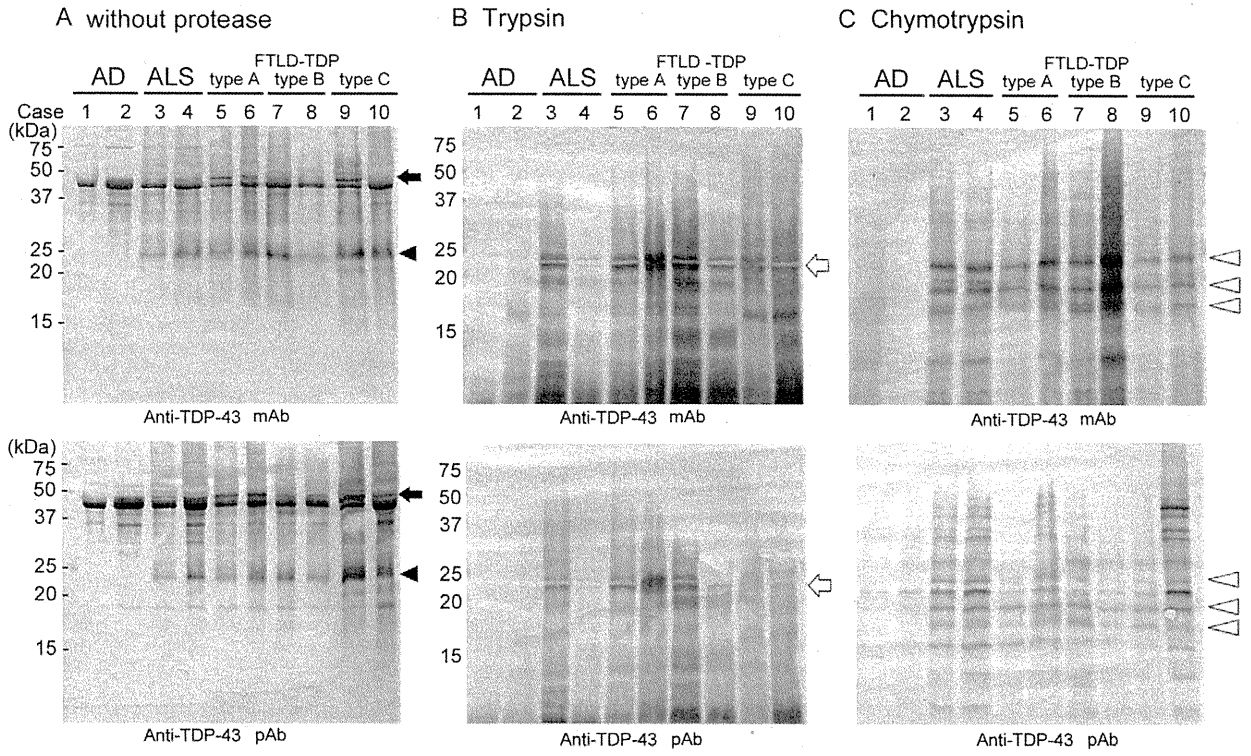


Fig. 4. Immunoblot analysis of Sarkosyl-insoluble fractions from AD and TDP-43 proteinopathies before and after protease treatment. (A) Without protease treatment, normal TDP-43 of 43 kDa was detected with these antibodies in all cases examined. In the ALS and FTLD-TDP cases, phosphorylated full-length TDP-43 of 45 kDa (arrows), high-molecular-weight smears, and the 24–26 kDa fragments (arrow heads) were detected in addition to the normal TDP-43. (B) Upon trypsin treatment, full-length TDP-43 disappeared, and the protease-resistant ~25 kDa fragments (white arrows) and smears appeared in ALS and FTLD-TDP cases, but not in AD cases. (C) After chymotrypsin treatment, triplet bands (white arrowheads) were detected in ALS and FTLD-TDP cases with the mAb and multiple bands were detected with pAb, whereas such immunoreactivities were hardly detected in AD cases.

are found in the C-terminal half of the TDP-43 [13–16]. Therefore, misfolding or structural alteration of the C-terminal half of TDP-43 seems to be the key to the pathogenesis of TDP-43 proteinopathies. By mass spectrometric analysis of the 23 kDa band in Sarkosyl-insoluble fraction from FTLD-TDP (type A), we identified the cleavage site as the N-terminus of Asp219 [23]. Another group reported cleavage at Asp208, based on N-terminal sequencing of urea extracts of FTLD-TDP brain [28]. However, the cleavage sites of the other major C-terminal fragments of 24 and 26 kDa have not been determined yet. In this study, we showed that the pathological TDP-43 C-terminal fragments of 24 and 26 kDa in ALS and FTLD-TDP type A contain the epitope of anti-TDP-43 mAb, residues 203–209, by comparing the immunoblotting results with those using pS409/410 (Fig. 3). This result suggests that the cleavage sites of pathological TDP-43 C-terminal fragments in ALS and FTLD-TDP are located at the N-terminal side of Thr203. Although the mechanisms of generation of the C-terminal fragments are still controversial, the presence of multiple cleavage sites suggests that cleavage may occur after the aggregation or assembly of TDP-43.

Structural or conformational changes in the proteins are thought to be the most important in protein aggregation in these neurodegenerative diseases. To analyze the conformational change in the epitope of TDP-43 from normal to the abnormal states further, we treated the Sarkosyl-insoluble TDP-43 with trypsin or chymotrypsin, and immunoblotted with these antibodies. The protease-resistant TDP-43 bands and smears were detected in ALS and all subtypes of FTLD-TDP with these anti-TDP-43 antibodies (Fig. 4), while no such bands were seen in AD cases. These demonstrate that the epitope is protease-resistant in the abnormal TDP-43 but not in normal TDP-43. Using an antibody pS409/410 that recognizes the C-terminal phosphorylation sites, some protease-resistant TDP-43 bands are detected, and the band patterns are slightly different between ALS and FTLD-TDP type C [29]. On immunoblots with anti-TDP-43 pAb and mAb, such difference was not observed. This is probably due to that the epitope of the mAb and pAb is located in the amino-terminus of the protease-resistant core of the TDP-43, whereas epitope of the pS409/410 located in the C-terminus. Similar protease-resistant bands have been reported in abnormal prion in prion diseases, tau in Alzheimer's disease and alpha-synuclein in Parkinson's disease and dementia with Lewy bodies. Biochemical studies in these proteinopathies suggested that the protease-resistant bands represent the core domains of the filamentous aggregates of these proteins with cross-β structures [30–32]. By analogy with these proteins we propose that these protease-resistant C-terminal fragments represent the core of the filamentous aggregates of TDP-43. Since the epitope of the mAb and pAb are determined to locate at residues 203–209, this may be important in the formation of a core region of pathological TDP-43 aggregates which is common in all TDP-43 proteinopathies. Finally, the protease treatment used in this study may be useful for detection of the abnormal TDP-43 in brains of patients, animal models, culture cells and in vitro models with these anti-TDP-43 antibodies more specifically, as used for detection of abnormal prion proteins.

Acknowledgments

Authors thank Dr Tetsuaki Arai (Tsukuba University) for helpful advice and discussions. This work was supported by a Grant-in-Aid for Scientific Research (A) (to M.H., 11000624) from Ministry of Education, Culture, Sports, Science and Technology of Japan, and grants from Ministry of Health, Labor and Welfare of Japan (to M.H.).

References

[1] Y.M. Ayala, T. Misteli, F.E. Baralle, TDP-43 regulates retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 3785–3789.

[2] Y.M. Ayala, F. Pagani, F.E. Baralle, TDP43 depletion rescues aberrant CFTR exon 9 skipping, FEBS Lett. 580 (2006) 1339–1344.

[3] E. Buratti, F.E. Baralle, Multiple roles of TDP-43 in gene expression, splicing regulation, and human disease, Front. Biosci. 13 (2008) 867–878.

[4] S.H. Ou, F. Wu, D. Harrich, et al., Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs, J. Virol. 69 (1995) 3584–3596.

[5] I.F. Wang, N.M. Reddy, C.K. Shen, Higher order arrangement of the eukaryotic nuclear bodies, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 13583–13588.

[6] T. Arai, M. Hasegawa, H. Akiyama, et al., TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Biochem. Biophys. Res. Commun. 351 (2006) 602–611.

[7] M. Neumann, D.M. Sampathu, L.K. Kwong, et al., Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Science 314 (2006) 130–133.

[8] T. Arai, I.R. Mackenzie, M. Hasegawa, et al., Phosphorylated TDP-43 in Alzheimer's disease and dementia with Lewy bodies, Acta Neuropathol. (Berl) 117 (2009) 125–136.

[9] M. Hasegawa, T. Arai, H. Akiyama, et al., TDP-43 is deposited in the Guam parkinsonism-dementia complex brains, Brain 130 (2007) 1386–1394.

[10] C.F. Tan, M. Yamada, Y. Toyoshima, et al., Selective occurrence of TDP-43-immunoreactive inclusions in the lower motor neurons in Machado-Joseph disease, Acta Neuropathol. (Berl) 118 (2009) 553–560.

[11] Y. Toyoshima, H. Tanaka, M. Shimohata, et al., Spinocerebellar ataxia type 2 (SCA2) is associated with TDP-43 pathology, Acta Neuropathol. (Berl) 122 (2011) 375–378.

[12] O. Yokota, Y. Davidson, E.H. Bigio, et al., Phosphorylated TDP-43 pathology and hippocampal sclerosis in progressive supranuclear palsy, Acta Neuropathol. (Berl) 120 (2010) 55–66.

[13] E. Kabashi, P.N. Valdmanis, P. Dion, et al., TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis, Nat. Genet. 40 (2008) 572–574.

[14] J. Sreedharan, I.P. Blair, V.B. Tripathi, et al., TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis, Science 319 (2008) 1668–1672.

[15] A. Tamaoka, M. Arai, M. Itokawa, et al., TDP-43 M337V mutation in familial amyotrophic lateral sclerosis in Japan, Intern. Med. 49 (2010) 331–334.

[16] I.R. Mackenzie, R. Rademakers, M. Neumann, TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia, Lancet Neurol. 9 (2010) 995–1007.

[17] M. Hasegawa, T. Arai, T. Nonaka, et al., Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Ann. Neurol. 64 (2008) 60–70.

[18] I.R. Mackenzie, E.H. Bigio, P.G. Ince, et al., Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations, Ann. Neurol. 61 (2007) 427–434.

[19] M. Neumann, L.K. Kwong, A.C. Truax, et al., TDP-43-positive white matter pathology in frontotemporal lobar degeneration with ubiquitin-positive inclusions, J. Neuropathol. Exp. Neurol. 66 (2007) 177–183.

[20] H. Zhang, C.F. Tan, F. Mori, et al., TDP-43-immunoreactive neuronal and glial inclusions in the neostriatum in amyotrophic lateral sclerosis with and without dementia, Acta Neuropathol. (Berl) 115 (2008) 115–122.

[21] M. Neumann, I.R. Mackenzie, N.J. Cairns, et al., TDP-43 in the ubiquitin pathology of frontotemporal dementia with VCP gene mutations, J. Neuropathol. Exp. Neurol. 66 (2007) 152–157.

[22] H.X. Zhang, K. Tanji, F. Mori, et al., Epitope mapping of 2E2-D3, a monoclonal antibody directed against human TDP-43, Neurosci. Lett. 434 (2008) 170–174.

[23] T. Nonaka, F. Kametani, T. Arai, et al., Truncation and pathogenic mutations facilitate the formation of intracellular aggregates of TDP-43, Hum. Mol. Genet. 18 (2009) 3353–3364.

[24] T. Nonaka, T. Arai, E. Buratti, et al., Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells, FEBS Lett. 583 (2009) 394–400.

[25] Y. Nishimoto, D. Ito, T. Yagi, et al., Characterization of alternative isoforms and inclusion body of the TAR DNA-binding protein-43, J. Biol. Chem. 285 (2010) 608–619.

[26] E. Buratti, F.E. Baralle, Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9, J. Biol. Chem. 276 (2001) 36337–36343.

[27] H. Wils, G. Kleinberger, J. Janssens, et al., TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 3858–3863.

[28] L.M. Igaz, L.K. Kwong, A. Chen-Plotkin, et al., Expression of TDP-43 C-terminal Fragments in Vitro Recapitulates Pathological Features of TDP-43 Proteinopathies, J. Biol. Chem. 284 (2009) 8516–8524.

[29] M. Hasegawa, T. Nonaka, H. Tsuji, et al., Molecular Dissection of TDP-43 Proteinopathies, J. Mol. Neurosci. 45 (2011) 480–485.

[30] J. Collinge, K.C. Sidle, J. Meads, et al., Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD, Nature 383 (1996) 685–690.

[31] H. Miake, H. Mizusawa, T. Iwatsubo, et al., Biochemical characterization of the core structure of alpha-synuclein filaments, J. Biol. Chem. 277 (2002) 19213–19219.

[32] M. Novak, J. Kabat, C.M. Wischik, Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament, EMBO J. 12 (1993) 365–370.