研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
佐々木裕之＆北條浩彦	「機能性小分子RNAの大規模シークエンス」	服部正平(企画)	実験医学	羊土社	東京	2009	14-19

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Furuya H, Ikezoe K, Shigeto H, Ohyagi Y, Arahata H, Araki E-I, Fujii N.	Sleep- and non sleep-related hallucinations - Relationship to ghost tales and their classifications.	*Dreaming*	**19(4)**	232-238	2009
Miyoshi K, Ohyagi Y, Sakae N, Motomura K, Ma L, Taniwaki T, Furuya H, Tabira T, Kira JI.	Enhancement of activationof caspases by presenilin 1 gene mutations and its inhibition by secretase inhibitors	*J Alzheimer Dis*	16	551-564	2009
Ma L, Ohyagi Y, Miyoshi K, Sakae N, Motomura K, Taniwaki T, Furuya H, Takeda K, Tabira T, Kira JI.	Increase in p53 protein levels by presenilin 1 gene mutations and its inhibition by secretase inhibitors.	*J Alzheimer Dis*	16	565-575	2009
Ikezoe K, Furuya H, Arahata H, Nakagawa M, Tateishi T, Fujii N, Kira J I	Amyloid-beta accumulation caused by chloroquine injections precedes ER stress and autophagosome formation in rat skeletal muscle	*Acta Neuropathol*	117	575-582	2009
Tateishi T, Hokonohara T, Yamasaki R, Miura S, Kikuchi H, Iwaki A, Tashiro H, Furuya H, Nagara Y, Ohyagi Y, Nukina N, Iwaki T, Fukumaki Y, Kira JI.	Multiple system degeneration with basophilic inclisions in Japanese ALS patients with *FUS* mutation	*Acta Neuropath*	119(3)	355-364	2010
Suzuki N, Aoki M, Warita H, Kato M, Mizuno H, Shimakura N, Akiyama T, **Furuya H**, Hokonohara T, Iwaki A, Togashi S, Konno H, Itoyama Y	FALS with FUS mutation in Japan with early onset, rapid progress and basophilic inclusion	*J Hum Genet*	*in press*		2010

古谷博和, 梅本丈二	軽症福山型筋ジストロフィー患者さんの臨床と摂食	難病と在宅ケア	*印刷中*		2010
Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, Yu PB, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, and Katagiri T.	Constitutively activated *ALK-2* and increased Smad1/5 cooperatively induce BMP signaling in fibrodysplasia ossificans progressiva.	*J Biol Chem*	284	7149-7156	2009
Kanomata K, Kokabu S, Nojima J, Fukuda T and Katagiri T.	DRAGON, a GPI-anchored membrane protein, inhibits BMP signaling in C2C12 myoblasts.	*Genes Cells*	14	695-702	2009
Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, Mundlos S, Seemann P, Kaplan FS, Mullins MC and Shore EM.	The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent and chondrogenesis and zebrafish embryo ventralization.	*J Clin Invest*	119	3462-3472	2009
Katagiri T.	Heterotopic bone formation induced by bone morphogenetic protein signaling: fibrodysplasia ossificans progressiva.	*J Oral Biosci*	52	33-41	2010
Kokabu S, Nojima J, Kanomata K, Ohte S, Yoda T, Fukuda T and Katagiri T.	Protein phosphatase magnesium-dependent 1A-mediated inhibition of BMP signaling is independent of Smad-dephosphorylation.	*J Bone Miner Res*	*in press*		2010
片桐岳信	進行性骨化性線維異形成症	*Arthritis*	7	158-163	2009
片桐岳信	進行性骨化性線維異形成症（FOP）の発症メカニズムの解明と治療法	*日本未熟児新生児学会雑誌*	22	30-32	2010

片桐岳信	進行性骨化性線維異形成症(FOP)の発症メカニズム	*Clin Nerosci*	*印刷中*		2010
Eda A., Tamura Y., Yoshida M., and Hohjoh H.	Systematic gene regulation involving miRNAs during neuronal differentiation of mouse P19 embryonic carcinoma cell.	*BBRC*	388	648-653	2009
Kitamura K., Itou Y., Yanazawa M., Ohsawa M., Suzuki-Migishima R., Umeki Y., Hohjoh H., Yanagawa Y., Shinba T., Itoh M., Nakamura K., and Goto Y.	Three human ARX mutations cause the lissencephaly-like and mental retardation with epilepsy-like pleiotropic phenotypes in mice.	*Hum Mol Genet*	18	3708-3724	2009
Hohjoh H., Akari H., Fujiwara Y., Tamura Y., Hirai H., and Wada K	Molecular cloning and characterization of the common marmoset huntingtin gene.	*Gene*	432	60-66	2009
Tamura Y., Yoshida M., Ohnishi Y., and Hohjoh H.	Variation of gene silencing involving endogenous microRNA in mammalian cells.	*Mol Biol Rep*	36	1413-1420	2009

Ⅳ. 研究成果の刊行物・別刷

機能性小分子RNAの大規模シークエンス

佐々木裕之，北條浩彦

さまざまな真核生物でmiRNA，siRNA，piRNAなどの機能性小分子RNAが遺伝子やトランスポゾンの制御に重要な役割を果たすことが明らかになってきた．小分子RNAライブラリーの大規模シークエンス解析は，新規小分子RNAの同定に有用であるばかりでなく，全体像の解明，標的の同定，発現量の推定，生合成機構の解明，修飾の同定などに大きな威力を発揮する．

キーワード● 小分子RNA，Ago，miRNA，MPSS，piRNA，RdRP，RISC，siRNA

はじめに

真核生物の機能性小分子RNAには大きく分けて3つのクラスがある（図1）．まず，miRNA（microRNA）は21～23塩基の長さの小分子RNAで，ヘアピン型の二次構造をもつ一本鎖のRNAからつくられる．miRNAは組織・発生に特異的な発現様式を示し，標的遺伝子のmRNA（メッセンジャーRNA）に結合してタンパク質への翻訳を抑制する．siRNA（small interfering RNA）も21～23塩基長の小分子RNAだが，これは主にウイルスやトランスポゾンなどに由来する長い二本鎖RNAから生成される．siRNAとmiRNAの一部は相補的な配列をもつ標的RNAに結合し，これを分解する（RNA干渉）．最後に，piRNA（piwi-interacting RNA）は24～32塩基長の小分子RNAで，主に生殖細胞において長い一本鎖RNAからつくられ，トランスポゾン由来のRNAを分解する．

近年，小分子RNAの研究が進展し，miRNA，siRNA，piRNAなどの生合成過程や機能が次々と明らかになってきた[1]．これらの研究でシークエンス情報の果たした役割は大きく，哺乳類のsiRNAやpiRNAの発見には超高速シークエンサーが大きく貢献した．ここでは，大規模シークエンス解析が生み出した最近の成果を紹介する．

1 小分子RNAライブラリーの大規模シークエンス解析

同定・未同定を問わずすべての小分子RNAを網羅的に解析するためには，小分子RNAライブラリーを作成し，大規模シークエンスによって解読する以外に方法はない．そのような研究のさきがけは，MPSS（massively parallel signature sequencing）とよばれる方法でシロイヌナズナの花序やめばえの小分子RNAの全体像を明らかにした2005年の研究である[2]．その後，パイロシークエンシングに基づく超高速シークエ

Large-scale sequence analysis of small RNAs
Hiroyuki Sasaki[1)2)]/Hirohiko Hohjoh[3)]：Division of Human Genetics, Department of Integrated Genetics, National Institute of Genetics, Research Organization of Information and Systems[1)]/Department of Genetics, School of Life Science, The Graduate University for Advanced Studies[2)]/Department of Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry[3)]（情報・システム研究機構国立遺伝学研究所総合遺伝研究系人類遺伝研究部門[1)]／総合研究大学院大学生命科学研究科遺伝学専攻[2)]／国立精神・神経センター神経研究所遺伝子工学研究部[3)]）

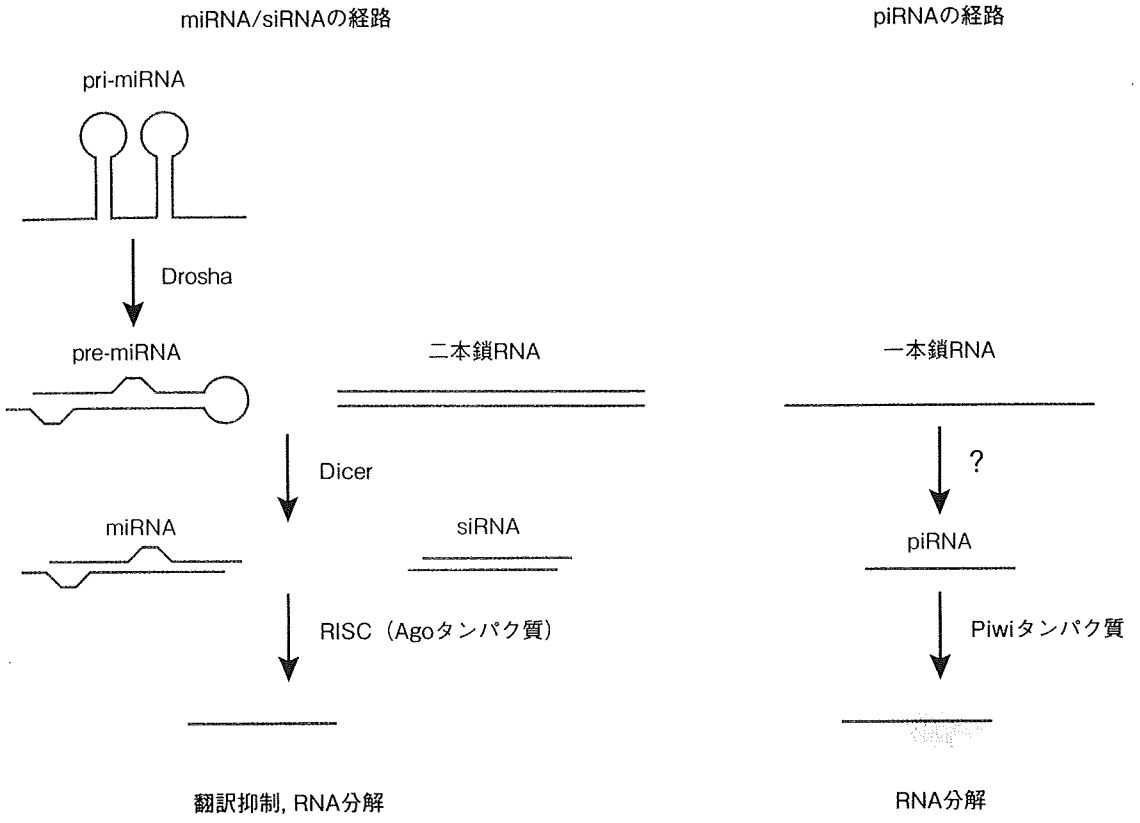

図1　3つの機能性小分子RNAの経路
miRNA，siRNA，piRNAそれぞれの生合成経路と機能を模式的に示した．miRNAはpri-miRNAとよばれる前駆体からDroshaの働きで切り出された後，Dicerによる切断を受けてつくられる．miRNAとsiRNAの経路は交差しているが，piRNAの経路は全く独立であると考えられる

ンサー等が市場に投入され，大規模解析が一気に加速された．超高速シークエンサーは，従来のシークエンサーと比べ1クローン当たりの解読塩基長が短い（数10～250塩基程度）が，高い並列性によって数千倍以上のスループットを実現している．

シークエンスに供する小分子RNAライブラリーを作成するには，まず対象となるRNA分子を集め（例えば，ポリアクリルアミドゲルで分画して切り出す，小分子RNAと結合するタンパク質に対する抗体で免疫沈降するなど），両端にRNAアダプターを結合した後，逆転写酵素を用いてcDNAを合成する．これをPCRで適度に増幅してライブラリーとし，シークエンス反応に供する[3)4)]．アダプターを含めても数10塩基程度の長さであるため，超高速シークエンサーによって十分解読可能である（最近の超高速シークエンサーはさらに解読塩基長を伸ばしつつある）．

得られたシークエンスからアダプターの配列を除き，BLAT，BLASTなどを用いて小分子RNAのアノテーションやゲノム上へのマッピングを行う．免疫沈降法を用いてサンプルを調製した場合などでは，比較的純度の高い特定の小分子RNAが得られるが，全小分子RNAを対象とする場合などは，リボソームRNAや転移RNAなど細胞内に大量に存在するRNAの分解産物を除去する必要がある．いずれにしても，出てくるデータ量が半端でないため，計算機を用いた情報処理が必須となるが，得られる生物学的情報は大量かつ貴重である．

以下，哺乳類の解析例に絞って，miRNA，siRNA，piRNAの大規模シークエンスの概要を述べる．

2 哺乳類のmiRNAとシークエンス情報

miRNAは発生・分化・組織に特異的な発現様式を示し，疾患をふくめたさまざまな生命現象とかかわる．この小分子RNAは，長い前駆体からヘアピン型の二次構造を認識するDroshaによる切断（核内），Dicerによる切断（細胞質内）を経て生成する（図1）．そして，成熟した二本鎖miRNAの一方の鎖がRISC（RNA-induced silencing complex）に取り込まれ，遺伝子抑制機能を発揮する．RISCはAgo（Argonaute）タンパク質を含むRNA干渉装置である．miRNAは進化的に保存された特定の配列をもつ．

既知のmiRNAの発現はDNAチップ，RT-PCR法，ノーザンブロットを用いて解析することができるが，実験的に新規の分子を同定するにはクローニング・シークエンスしかない．これまでに，例えば，ヒトやチンパンジーの脳[5]，ヒトやマウスのES細胞[6)7)]，ヒト由来培養細胞[8)]などのmiRNA解析に超高速シークエンサーが利用されている．2007年のLandgrafらの論文では，ヒト，マウス，ラットの26種類の器官や細胞型から250以上の小分子RNAライブラリーをシークエンスし，得られた33万の配列から33種類の新たなmiRNAを同定している[9)]（ただし，この論文では従来型シークエンサーが使われている）．2008年9月現在，ヒトで695種類，マウスで488種類のmiRNAが同定されデータベースに登録されている（Browse miRBase::Sequences by species, Sanger Institute）が，最近では1000を超えるmiRNAがあると推定されている．

大規模シークエンスにより大量のmiRNA分子の配列が得られた場合，相対的なクローニング頻度に基づいて大まかな発現プロファイルを知ることができる．しかし，実験処理などによりライブラリーの複雑度が変化する可能性も大きいので，定量的な判定ができる別法（RT-qPCR，DNAマイクロアレイ，ノーザンブロット）を用いて確認する．

miRNAはアデニンをイノシンへと置換するA-to-I RNA編集や，3′端へのアデニンやウラシルの付加といった修飾を受ける．大規模シークエンス解析では，このような修飾の位置，種類，頻度を一網打尽に同定できる（A-to-I RNA編集はアデニンからグアニンへの置換として検出される）．ただし，PCRやシークエンスのエラー率を考慮に入れ，有意に頻度の高いものを拾い上げる必要がある．RNA編集は前駆体RNAの段階で生じ，miRNAの生合成に影響を与えるほか，新たな標的遺伝子を生み出す可能性がある．Landgrafらの論文では，ヒトで143種類，マウスで109種類のmiRNAに有意な修飾があることを見つけ，RNA編集の頻度はヒトではマウスより4倍ほど高いことを報告している[9)]．また，大規模シークエンスにより，生合成過程での切断部位の違いによる5′端の付加も見つかっており[6)8)]，これも標的遺伝子を変える変化として注目される．

3 哺乳類のpiRNAとシークエンス情報
新たな機能性小分子RNA経路

マウス，ラットの精巣のpiRNAは5つのグループによりほぼ同時に発見された[10)～14)]．この小分子RNAはAgoファミリータンパク質のうちPiwiサブファミリーに属するメンバー（マウスではMili，Miwi，Miwi2の3つがある）と結合して存在し，ほかの2つの機能性小分子RNAよりやや大きいサイズをもつ（24～32塩基長）（図1）．その生合成はDicerに非依存性であり，哺乳類では生殖細胞だけで発現する．

超高速シークエンサーを用いた哺乳類の精巣piRNAの大規模解析は，われわれを含む3つのグループが報告している．1つのグループはMiwi，Miliタンパク質の抗体で免疫沈降して得られた小分子RNAを[10)15)]，別のグループはサイズ分画して得た22塩基より大きな小分子RNAを[14)]，われわれは分離した生殖細胞の全小分子RNAを[16)]シークエンスすることで，それぞれpiRNAを同定した．得られた数万～数10万の配列を解析した結果，プロ精原細胞など減数分裂期以前に存在するpiRNAの多くはLINE，SINE，LTR型などのレトロトランスポゾン※1の配列に由来していた．また，miRNAのように特定のヘアピン構造に由来することはなく，さまざまな配列をもっていた．一方，第一減数分裂パキテン期※2の精母細胞では，レトロトランスポゾン由来ではない，ユニークなまたは少コピー数の

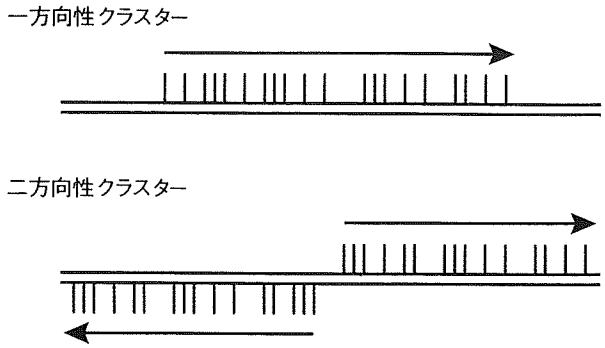

図2　マウス精巣のpiRNAクラスター
大量のpiRNA配列をゲノムにマッピングすると，一方のDNA鎖に片寄ってクラスターができる場合（上段）と，ある場所を境に逆のDNA鎖にもクラスターができる場合（下段）があることがわかった．それぞれ矢印の方向へ転写される前駆体RNAに由来すると考えられ，下段は両方向性プロモーターによるものと推測される

piRNAが多かった．

大規模シークエンスで得た情報からpiRNAの生成過程が推定された．すなわち，パキテン期の少コピー数piRNAは，マウスゲノム上の100～200カ所にクラスター状に位置していた．各クラスター（典型的には数10kbにおよぶ）のpiRNAは片鎖のみに由来することから，一本鎖RNAが前駆体であると推測される（図2）．いくつかのクラスターでは，両方向性のプロモーターを挟んで，逆鎖側にpiRNAがマップされた（図2）．これらのクラスターにはmiRNAに特徴的なヘアピンや，後出するsiRNAのような二本鎖RNAを示唆する構造もないため，全く異なる経路で生合成されるのだろう．

マウスにおいてMiliやMiwi2をノックアウトすると，精巣におけるレトロトランスポゾンの活性化がみられ，精子形成不全が起こる．しかし，少コピー数のpiRNAがいかなる機能をもつかなど，不明な点も多い．

※1　レトロトランスポゾン
RNAを鋳型とする逆転写反応によりDNAコピーをつくり，ゲノム中で増幅するタイプの分散型反復配列．レトロウイルス様のLTR（long terminal repeat）をもつ「LTR型」と，LINE（long interspersed nuclear element）やSINE（short interspersed nuclear element）などの「非LTR型」がある．

※2　パキテン期（太糸期）
第一減数分裂前期においてザイゴテン期に続く時期．二価染色体の二組の姉妹染色分体が対合し，シナプトネマ構造を形成している．また，交差が起こっている場所が組換え小節として観察される．次のディプロテン期になると対合が解離しはじめる．

4 哺乳類のsiRNAとシークエンス情報
卵子の内在性siRNAの生合成と機能

人工的なsiRNAを用いた遺伝子のノックダウンは，医学・生物学で欠くことのできない手法となっているが，最近まで，哺乳類には内在性siRNAは存在しないという説が支配的であった．その理由は，①siRNAの前駆体となる二本鎖RNAの生合成に必要なRNA依存性RNAポリメラーゼ（RdRP）の報告がないこと，②長い二本鎖のRNAはインターフェロン応答を誘導すること，③多くの組織から抽出された21塩基長の小分子RNAのほとんどがmiRNAであること，などである．

われわれは以前，マウスの未受精卵において内在性siRNAと思われる分子を報告したが[13]，シークエンスした分子の数が少なく，通説を覆すには至っていなかった．また，哺乳類の生殖細胞では小分子RNAがゲノムインプリンティングやレトロトランスポゾンの抑制にかかわるのではないかと推測していた．そこで，1万個以上のマウス成長期卵から小分子RNAライブラリーを作成し，超高速シークエンサーで解析した[17) 18)]．その結果，マウスの成長期卵には25塩基長のpiRNAのほかに，21塩基長の内在性siRNAが大量に存在することを見出した（このほかに既知の21塩基長のmiRNAも存在した）．これら内在性siRNAの多くはレトロトランスポゾンに由来していた．また，それらはDicer依存的につくられ，Ago2と結合していたほか（図1），miRNAのように特定のヘアピン構造に由来することは

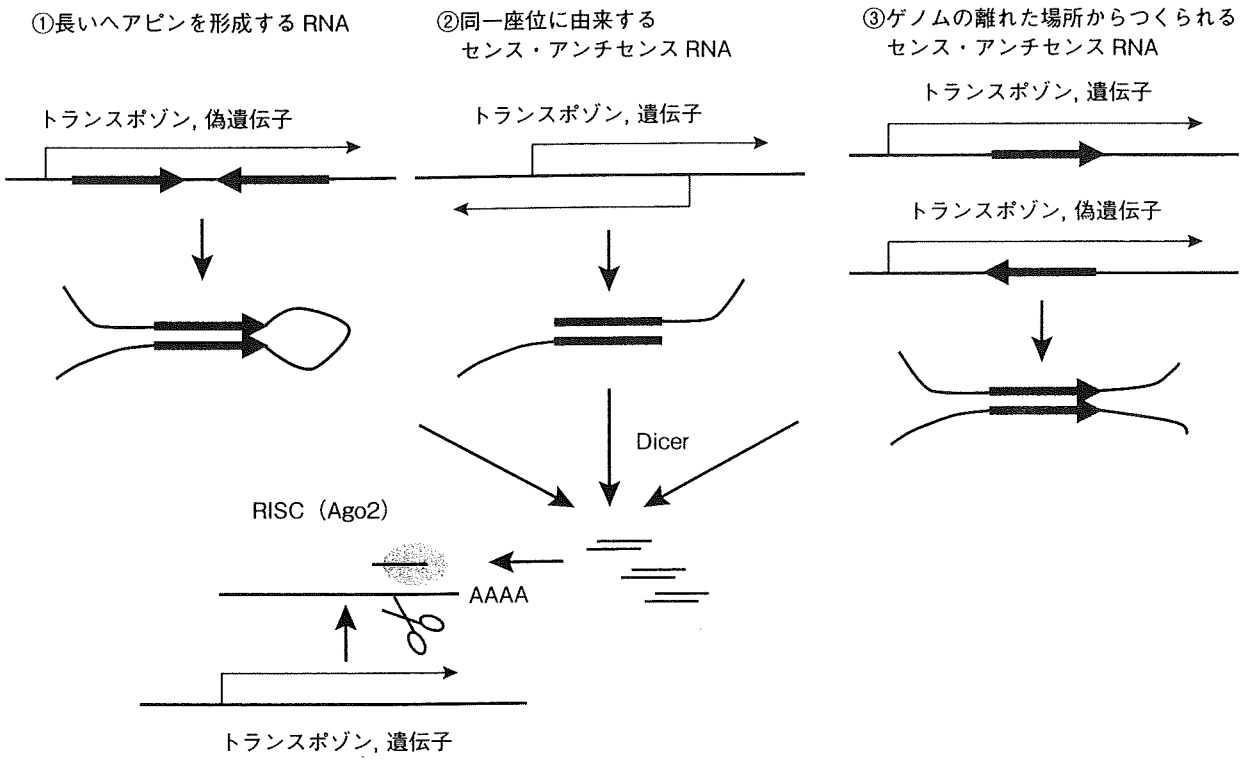

図3　マウス卵子の内在性siRNAの生成機構
内在性siRNAは自然に形成される二本鎖RNA（長いヘアピン構造，センス・アンチセンスRNAのペアなど）からDicerの作用でつくられる．また，siRNAの一部は偽遺伝子に由来する．これらの生成機構は大量の配列をゲノム上にマッピングすることではじめて明らかになった．内在性siRNAはAgo2を含むRISC複合体に取り込まれ，レトロトランスポゾンや標的遺伝子のRNAを分解する

なく，レトロトランスポゾンのさまざまな場所にマップされた．

では，RdRPをもたないマウスの卵子は，どのようにしてsiRNAの前駆体となる二本鎖RNAをつくるのだろうか？　その解答は，大量のシークエンス情報のうち，レトロトランスポゾン以外の領域に由来する少コピー数siRNAの配列をゲノム上へマッピングすることで得られた．すなわち，それらのsiRNAはゲノム上でクラスター状に分布し，①長いヘアピンを形成するRNA，②同一座位に由来するセンス・アンチセンスRNA，もしくは③ゲノムの離れた場所からつくられるセンス・アンチセンスRNA，などの転写単位に排他的にマッピングされた（図3）．つまり，自然に形成される二本鎖RNAが内在性siRNAのソースであった．おそらく，レトロトランスポゾンのsiRNAも同様な二本鎖RNAに由来すると考えられる．

興味深いことに，このような内在性siRNAの一部は偽遺伝子に由来していた．つまり，偽遺伝子由来RNA内のヘアピン構造や，タンパク質をコードするmRNAと偽遺伝子由来アンチセンスRNAとのハイブリッドからsiRNAがつくられていた[17)～19)]．最後に，卵子でDicerやAgo2をノックアウトすると，内在性siRNAが激減するとともに，レトロトランスポゾンや偽遺伝子に相同な遺伝子のmRNAが上昇していたことから，これらのsiRNAが実際に機能をもつことが証明された．体細胞の場合と異なり，卵子ではインターフェロン応答が抑制されているため[20)]，siRNAの前駆体となる二本鎖RNAが比較的安定に存在できるのであろう．

おわりに

以上，述べてきたように大規模シークエンス情報は，新規の機能性小分子RNAの同定に有用であるばかりでなく，全体像の解明，標的の同定，発現量の推定，

生合成機構の解明，RNA編集の同定などに大きな威力を発揮する．得られる大量情報の処理，データマイニング，コスト面などに課題があるが，超高速シークエンサーを有効に使うことで，さらに新たなRNA大陸が見えてくるかも知れない．

なお，本稿で紹介したわれわれの研究は，総合研究大学院大学（国立遺伝学研究所）大学院生　渡部聡朗と，理化学研究所ゲノム科学総合研究センター長　榊　佳之（現豊橋技術科学大学学長）らのグループとの共同研究によるものである．ここに深く感謝する．

文献

1）塩見美喜子：実験医学，25：794-799, 2007
2）Lu, C. et al.：Science, 309：1567-1569, 2005
3）Lu, C. et al.：Methods, 43：110-117, 2007
4）Watanabe, T. et al.：Methods Enzymol., 427：155-169, 2007
5）Berezikov, E. et al.：Nature Genet., 38：1375-1377, 2006
6）Morin, R. D. et al.：Genome Res., 18：610-621, 2007
7）Calabreses, J. M.：Proc. Natl. Acad. Sci. USA, 104：18097-18102, 2007
8）Azuma-Mukai, A.：Proc. Natl. Acad. Sci. USA, 105：7964-7969, 2008
9）Landgraf, P. et al.：Cell, 129：1401-1414, 2007
10）Girard, A. et al.：Nature, 442：199-202, 2006
11）Aravin, A. et al.：Nature, 442：203-207, 2006
12）Grivna, S. T. et al.：Genes Dev., 20：1709-1714, 2006
13）Watanabe, T. et al.：Genes Dev., 20：1732-1743, 2006
14）Lau, N. C. et al.：Science, 313：363-367, 2006
15）Aravin, A. A. et al.：Science, 316：744-747, 2007
16）Kuramochi-Miyagawa, S. et al.：Genes Dev., 22：908-917, 2008
17）Watanabe, T. et al.：Nature, 453：539-534, 2008
18）渡部聡朗，佐々木裕之：実験医学，26：2109-2111, 2008
19）Tam, O. H. et al.：Nature, 453：534-538, 2008
20）Stein, P. et al.：Dev. Biol., 286：464-471, 2005

参考図書

『躍進するRNA研究』（中村義一，塩見春彦／編），実験医学増刊，Vol.22 No.17，羊土社，2004

Profile　筆頭著者プロフィール

佐々木裕之：1987年，九州大学大学院医学系研究科修了．英ケンブリッジ大学Wellcome/CRC研究所海外リサーチフェロー，九州大学遺伝情報実験施設助教授などを経て，'98年より国立遺伝学研究所人類遺伝研究部門教授．ゲノムインプリンティングなど哺乳類のエピジェネティクス現象とその異常症，ならびに機能性小分子RNAに関する研究を行っている．著書に「エピジェネティクス入門」岩波書店（2005）など．

Sleep- and Non-Sleep-Related Hallucinations—Relationship to Ghost Tales and Their Classifications

Hirokazu Furuya and Koji Ikezoe
National Omuta Hospital and Kyushu University

Hiroshi Shigeto and Yasumasa Ohyagi
Kyushu University

Hajime Arahata, Ei-ichi Araki, and Naoki Fujii
National Omuta Hospital

To categorize four types of sleep- and non-sleep-related hallucinations experienced by normal people and classify ghost or ghost-like stories by these categories. A total of 183 reliable tales of ghosts [41 from "Tohno Monogatari" (Tohno Folktales) and 142 from "Nihon Kaidan Shu" (Ghosts Tales of Japan)] are classified into hallucinations that are sleep-related hallucinations [hypnagogic hallucination-like (HyH) and REM sleep behavior disorder or somnambulism-like (RBDS) tales] and sleep-unrelated [vivid hallucination-like (VH) and highway hypnosis-like (HHy) tales] according to the criteria. Sixty to 70% of these tales can be classified into these four types of hallucinations. Further, sleep-related hallucinations increased from 17.0% to 36.6% in about 40 years. Our criteria will be useful to classify hallucinations experienced by normal people and to elucidate the mechanisms of these kinds of hallucinations experienced in neurodegenerative or psychological disorders.

Keywords: vivid hallucination, hypnagogic hallucination, highway hypnosis, REM sleep disorder, somnambulism (sleepwalking), the vanishing hitchhiker, progressive posterior cortical atrophy, Charles-Bonnet syndrome

Supplemental materials: http://dx.doi.org/10.1037/a0017611.supp

Hirokazu Furuya and Koji Ikezoe, Department of Neurology, Neuro-Muscular Center, National Omuta Hospital, Fukuoka, Japan; and Department of Neurology Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; Hiroshi Shigeto, Department of Neurophysiology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; Yasumasa Ohyagi, Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and Hajime Arahata, Ei-ichi Araki, and Naoki Fujii, Department of Neurology, Neuro-Muscular Center, National Omuta Hospital, Fukuoka, Japan.

This study was supported in part by a Research Grant (H18-pharmaco-002) for the Research on Advanced Medical Technology, and a Research Grant (H21-Nanchi-Ippan-067) for the Incurable Disorders from the Ministry of Health, Labor and Welfare of Japan.

Supplements 1, 2, and 3 are online appendixes.

Correspondence concerning this article should be addressed to H. Furuya, Department of Neurology, Neuro-Muscular Center, National Omuta Hospital, Tachibana 1044-1, Omuta, Fukuoka 837-0911, Japan. E-mail: furuya@neuro.med.kyushu-u.ac.jp or furuya@oomuta.hosp.go.jp

Dreaming
2009, Vol. 19, No. 4, 232–238

1053-0797/09/$12.00 DOI: 10.1037/a0017611

Although there are many ghost or ghost-like stories in various cultures around the world, only two types are currently considered to arise from neurophysiologic and psychiatric mechanisms. One is the hypnagogic hallucination usually observed in narcolepsy, when experienced by normal people without other diagnostic criteria, and the other is "highway hypnosis" (Sagberg, 1999), in which fear is a component. Highway hypnosis is illustrated by tales such as "The Vanishing Hitchhiker" (Brunvand, 1981) in the United States and "The Ghost Getting into the Taxi" (Konno, 1975) in Japan. Recently, we suggested the possibility that the vivid visual hallucinations seen in progressive posterior cortical atrophy (PCA) share a similar mechanism with some of the ghost tales experienced by normal people (Furuya, Ikezoe, Ohyagi, Miyoshi, & Fujii, 2006). Furthermore, reports of rapid eye movement (REM) sleep behavior disorder (RBDS) or somnambulism (sleepwalking), sometimes resulting in violence to the bed partner, while the dreamer is dreaming of a ghost or monstrous creature (Stores, 2007) are becoming more common.

In this paper, we suggest two new classifications and outline the criteria of four types of sleep- and non-sleep-related hallucinations experienced by normal people and use them to classify the ghost or ghost-like stories.

METHOD

Sources of Ghost Tales

To exclude made-up stories or fiction, we choose collections of tales by two Japanese folklorists. One is the "*Tohno Monogatari* (Tohno Folktales)," which was originally published in 1910 (Yanagita, 2006) (Supplement 1). In this book, more than 300 short verbal records of face-to-face encounters with ghost in the early 20th century are recorded. The other is the "*Nihon Kaidan Shu* (Ghosts Tales of Japan)" (Konno, 1975), in which about 200 tales of ghosts published in newspapers or general-interest magazines by the original reporter from 1874 to 1969 are collected (Supplement 1).

Classification of Four Types of Hallucination

According to the several review papers (Furuya et al., 2006; Stores, 2007), we developed diagnostic criteria consisting of a central and core features to classify the ghost tales as far as possible into the four types described below (see Table 1).

1. Hypnagogic Hallucination-Like Ghost Tales

This type of hallucination is in principle the hypnagogic hallucination (HyH) that occurs in narcolepsy patients. The ghost image is not vivid but often rather vague, and it sometimes accompanied by a cenesthopathy such as an incubus/succubus, a sense of being touched, or feeling someone's breath. The ghost some-

Table 1. Major Criteria for Classification of Ghost Tales of Normal People. (Three Core Features Are Sufficient for a Definite Diagnosis, Two for a Probable Diagnosis, and One for a Possible Diagnosis)

1. Hypnagogic hallucination-like ghost tales (HyH)
Central features (essential for a diagnosis of HyH): HyH appears while the experiencer is sleeping or when they are awakened from sleep.
Core features:
a. The image of the ghost is clear or vague but not very vivid.
b. It sometimes accompanied by cenesthopathy such as an incubus/succubus, a feeling of being touched or a feeling of breathing.
c. The ghost sometimes speaks, makes a noise, or converses with the experiencer.
Differential diagnosis: narcolepsy, schizophrenia

2. Highway hypnosis-like ghost tales (HHy)
Central features (essential for a diagnosis of HHy): HHy is 'a tendency to become drowsy and suddenly fall asleep, sometimes into the REM stage, when driving an automobile.' In HHy, the conscious and subconscious minds appear to concentrate on different things. Thus, this hallucination appears while the experiencer does not recognize the change of consciousness level.
Core features:
a. The sleep- or trance-like state can occur with the driver (experiencer) sitting in an upright position and staring ahead.
b. The image of ghost is usually clear but sometimes vague.
c. The ghost sometimes speaks or has a conversation with the experiencer.
Differential diagnosis: complex partial seizure, temporal lobe epilepsy

3. REM sleep behavior disorder or somnambulism like ghost tales (RBDS)
Central features (essential for a diagnosis of RBDS): In REM sleep behavior disorder (RBD), the loss of motor inhibition leads to a wide spectrum of behaviors during sleep. In the case of somnambulism, it is usually defined by, or involves the person performing normal actions as if awake. Thus, RBDS is closely related to sleep.
Core features:
a. The experiencer of RBD often has a dream at the same time, which convinces him/her that the events were real.
b. The experiencer notices an abnormality in the bedroom or the experiencer himself or a bed partner after awoken, when the RBD is accompanied by somnambulism.
c. The image of ghost is not as clear as it is in HHy because it is a part of dream.
Differential diagnosis: early stage of DLB (Parkinsonism), drug abuse (including alcoholism), malingering disorder

4. Vivid hallucination-like ghost tales (VH)
Central features (essential for a diagnosis of VH): VH is similar to the hallucination occurred in the patient of diffuse Lewy body disease (DLB) or Charles-Bonnet syndrome (CBD). The ghost appears without any relation to sleep.
Core features:
a. The image of ghost is clear or vivid.
b. Hallucinations are purely visual (that is, the ghost never talks or tries to touch the experiencer, and the image of the ghost is very clear and vivid.)
c. The ghost vanishes into air when the experiencer tries to touch it or throw something at the ghost.
Differential diagnosis: early stage DLB (Parkinsonism), CBD, drug abuser (including alcoholism), schizophrenia

times speaks, makes a noise, or shakes the bed or room, and on rare occasions, converses with the sleeper.

A typical HyH-like ghost tale follows:

One night in July 1961, Mr. Ishida, a chief mechanic, had a dream of a sailor while he was sleeping in a dormitory room at Tsuiki Air Force Base, Kitsuki County in the nap room at

Tsuiki Airforce Base, Fukuoka Prefecture, which was a commando-type air force case during World War II. The sailor wore a uniform and said that he had been killed by being caught in a spinning airplane propeller. While he was dreaming, Mr. Ishida felt a heavy weight in his breast and couldn't move an inch, though he struggled to. Mr. Ishida examined the history of this air force base and learned that there had been such an accident in the past. [Newspaper story, *Nishi Nippon Shinbun* (Western Japan Newspaper), 1961; Konno, 1975].

2. Highway Hypnosis-Like Ghost Tales

Highway hypnosis (HHy; "white-line fever" or "the vanishing hitchhiker" Brunvand, 1981) has been defined as a tendency to become drowsy and suddenly fall asleep, sometimes into the REM stage, while driving an automobile (Sagberg, 1999). Theoretically, highway hypnosis is a kind of mental state that also occurs when a person concentrates on a simple mechanical task, so it may happen in a relatively frequently and in common situations; for example, workers performing simple repetitive tasks while deprived of sleep and walkers concentrating on the road in the night with the aid of faint light of lantern may experience highway hypnosis. Thus, this type of hallucination seems to be unrelated to sleep at first, and the hallucinator does not recognize the change in consciousness level. The image of the ghost is usually clear but sometimes vague.

A typical HHy-like ghost tale follows:

It is said that a ghost appeared at *Horaga Tohge* (*Horaga* Mountain Path) on the Hirakata bypass road at the border between Osaka and Kyoto Prefectures. Three years before, on a summer night, a taxi driver working in the Kyoto area was driving back from the Osaka area. He saw a lady in white clothing standing beside the road with her hand raised. When he stopped the taxi, she got in. After driving several minutes, he felt an abnormal sensation and turned back to the rear seat to find no one there. He stopped the taxi and carefully looked for her, but he couldn't find a trace of her existence. He drove the taxi with fear. Another taxi driver picked up a woman with disheveled hair, but she also disappeared while he was driving. Other drivers said that the passenger was male, and yet others said the passenger was accompanied by children. [*Shukan Yomiuri* (Weekly Journal Yomiuri), 1968; Konno, 1975].

3. REM Sleep Behavior Disorder or Somnambulism-Like Ghost Tales

In REM sleep behavior disorder (RBD), the loss of motor inhibition leads to a wide spectrum of behaviors during sleep. Somnambulism, which sometimes accompanies RBD, is usually defined by or involves the person performing normal actions as if awake (walking, opening/closing a door or window, and other acts; Stores, 2007). The sleeper thinks that someone or something like a ghost or monstrous creature has entered in the bedroom and done something. Thus, the RBD or somnambulism-like (RBDS) tale is closely related to sleep. Although it is well known that the RBD is related to REM sleep and somnambulism is related to non-REM sleep, we classify them into the same group (RBDS) in this paper because it is sometimes hard to separate them only from the report (Stores, 2007).

A typical RBDS-like ghost tale follows:

There is a small city named "*Bac Nihn,*" located 50 km southwest of the capital of Indochina (Viet Nam). In 1943, in the middle of World War II, many people were killed in the battle

between Japanese and French regiments. After the battle, the Japanese army occupied the French base camp. The Japanese lieutenant colonel lived on the third floor of this building. One August night, the lieutenant had a strange nightmare. Two or three days later, he noticed that all the windows on this floor were open in the morning, although he was certain he had closed them tightly the night before to prevent mosquitoes from entering the room. During the next night, the lieutenant was awakened by a feeling of tightness in his chest and was astonished when he unintentionally glanced at the dark edge of the room. A thin human-like figure lying on the floor suddenly stood up, showing a face without skin or muscle. It was a human skeleton. The lieutenant jumped up from the bed drawing a Japanese saber and fought with this skeleton. He swung the sword around and seemed to fight with this skeleton for one or two hours, but the fight was not settled for the skeleton did not fall down, even if his sword hit the chest or hand of the skeleton. At the minute dawn broke, the skeleton immediately disappeared, and the lieutenant colonel found himself sitting on the floor with the saber. The ghost skeleton appeared in his room for three consecutive nights while the lieutenant slept in his bed. In the fourth day, he sat up the whole night waiting for the ghost skeleton, but strange to say, it did not appear. [Newspaper story, *Shin-Yuh-Kan* Newspaper, Osaka, 1959; (Konno, 1975)].

4. Vivid Hallucination-Like Ghost Tales

Vivid hallucination (VH) is similar to the hallucinations that occur in patients with diffuse Lewy body disease (DLB), some types of dementia with Parkinson's disease (Diederich, Goetz, & Stebbins, 2005; Nagahama et al., 2007), and Charles-Bonnet syndrome (CBS) (Terao & Collinson, 2000; Furuya et al., 2006). The ghost appears without any relationship to sleep at first and is a purely visual hallucination.

A typical VH-like ghost tale follows:

This story is summarized as follows. One night, when Mr. Shinya Nishimaru was 23 years old, he saw a lady leaning on a concrete wall for the first time. Although it was a dark, moonless night, he noticed the pattern of her clothing, eyes, and the outline of her face precisely. He could even see two or three hairs falling on her face. He continued to stare at her, but she disappeared as soon as he'd crossed in front of her and she had come to the end of his visual field. Although he tried to touched her or hit her with a club, she disappeared as soon as he touched her clothes. He tried to communicate with her in some way, she only stared him without saying a word. At first, she appeared only outdoors, but she began to appear in front of the house of an acquaintance, and after one year, beside his sleeping bed. For several weeks, he ignored her, but one night, she looked directly at him. It really gave him a fright. He could hardly sleep a wink for several days, which felt like a mortal blow to his life. So he fled the town. After he had returned to Tokyo, the ghost never appeared again. [by Shinya Nishimaru, Ghost in Kamaishi City (extract), "*Michi-e-no-ashi-ire*" originally published in 1959 (Furuya et al., 2006)].

Classification and Analysis of Tales

The above tales are classified by the criteria described above in Table 1. Detailed classifications of each tale are also listed in a supplemental file (Supplement 2).

RESULTS

We analyzed 41 tales from the "*Tohno* Folktales" (Supplement 1) and 142 tales from the "Ghosts Tales of Japan" (Supplement 1). The classifications of these tales

are shown in Table 2. Although there were almost 40–50 years between these two sources, 60.9% ("*Tohno* Folktales") to 67.5% ("Ghosts Tales of Japan") of all tales were classified into four types (HyH, HHy, RBDS, and VH, Table 2). In the "*Tohno* Folktales," 17% of the tales of ghosts were sleep related (SR: HyH and RBDS), and 43.9% were non-sleep-related (NSR: HHy and VH), and the ratio of SR had increased in "Ghosts Tales of Japan" to 36.6%, while 31.0% were non-sleep-related (SR:NSR = 36.6%:31.0%).

DISCUSSION AND CONCLUSION

Based on our criteria, almost two thirds of the reliable ghost tales were classified into four types of hallucination, which means that most of them are attributable to the same mechanisms as neurophysiological and neurodegenerative or psychological disorders. These results also suggest the possibility that criteria may be useful to classify the hallucinations experienced by people with neurological disorders in order to clarify the mechanism.

Although few people in the world believe these sometimes incredible stories today, several news stories reported rumors of ghosts after the disastrous tsunami in Thailand in 2004 (Ghost stories of Koh Phi Phi Don, Thailand. (2005). *Shu-kan Shincho* (Weekly *Shincho*). Tokyo: *Shincho Sha*, 13 Jan. 2005. P. 34 (Supplement 3). Even today, rumors of ghosts become common in areas afflicted by great disasters and stir up unrest among the residents. In addition, members of cults or fanatical religious communities often have epiphanic experiences, which are induced by sleep deprivation or prolonged repetition of a simple task. These cult members sometimes complain of hallucinations at an early stage and are slowly influenced by harmful psychological effects. These criteria are so simple that every clinician who is consulted by such a patient can analyze the pattern of the halluci-

Table 2. Classification of Ghost Tales or Ghost-Like Stories in Japan

	The Tohno Folktales (41 tales; Yanagita, 2006)	**Ghosts Tales of Japan (142 tales; Konno, 1975)**
1. HyH	6/41 (14.6%)	50/142 (35.2%)
Definite	1	4
Probable	3	29
Possible	2	17
2. HHy	4/41 (9.8%)	14/142 (9.86%)
Definite	0	4
Probable	1	9
Possible	3	1
3. RBDS	1/41 (2.4%)	2/142 (1.41%)
Definite	0	1
Probable	0	1
Possible	1	0
4. VH	14/41 (34.1%)	30/142 (21.1%)
Definite	0	4
Probable	11	20
Possible	3	6
5. Unclassifiable	16/41 (39.0%)	46/142 (32.4%)

nation easily. Not only neurologists and psychologists, but general physicians should pay attention to such complaints.

In conclusion, we propose the possibility that two thirds of the ghost tales may be classified into one of four types of hallucinations experienced by normal people. Such hallucinations may share the same mechanisms as neurodegenerative or psychological disorders.

REFERENCES

Brunvand, J. H. (1981). *The Vanishing Hitchhiker: American Urban Legends and Their Meanings.* 1st ed. New York: Norton.

Diederich, N. J., Goetz, C. G., & Stebbins, G. T. (2005). Repeated visual hallucinations in Parkinson's disease as disturbed external/internal perceptions: Focused review and a new integrative model. *Movement Disorders, 20,* 130–140.

Furuya, H., Ikezoe, K., Ohyagi, Y., Miyoshi, T., & Fujii, N. (2006). A case of progressive posterior cortical atrophy (PCA) with vivid hallucination: Are some ghost tales vivid hallucinations in normal people? *Journal of Neurology, Neurosurgery, and Psychiatry, 77,* 424–425.

Konno, E. (1975). *Nihon Kaidan Shu (Ghosts Tales of Japan).* 1st ed. Tokyo: Shakaishisou-Sha. (Suppl. 1)

Nagahama, Y., Okina, T., Suzuki, N., Matsuda, M., Fukao, K., & Murai, T. (2007). Classification of psychotic symptoms in dementia with Lewy bodies. *American Journal of Geriatric Psychiatry, 15,* 961–967.

Sagberg, F. (1999). Road accidents caused by drivers falling asleep. *Accident Analysis and Prevention, 31,* 639–649.

Stores, G. (2007). Clinical diagnosis and misdiagnosis of sleep disorders. *Journal of Neurology, Neurosurgery, and Psychiatry, 78,* 1293–1297.

Terao, T., & Collinson, S. (2000). Charles-Bonnet syndrome and dementia. *Lancet, 355,* 2168.

Yanagita, K. (2006). *Tohno Monogatari* (*Tohno* Folktales). 8th ed. Tokyo: Kadokawa-Shoten. (Suppl. 1)

Journal of Alzheimer's Disease 16 (2009) 551–564
DOI 10.3233/JAD-2009-0989
IOS Press

Enhancement of Activation of Caspases by *Presenilin 1* Gene Mutations and its Inhibition by Secretase Inhibitors

Katsue Miyoshi[a], Yasumasa Ohyagi[a,*], Nobutaka Sakae[a], Kyoko Motomura[a], Linqing Ma[a], Takayuki Taniwaki[a], Hirokazu Furuya[b], Takeshi Tabira[c] and Jun-ichi Kira[a]
[a]*Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*
[b]*Clinical Research Center, National Omuta Hospital, Omuta, Fukuoka, Japan*
[c]*National Institute for Longevity Sciences, NCGG, Obu, Aichi, Japan*

Abstract. *Presenilin 1* (*PS1*) gene mutations are the major causes of early-onset familial Alzheimer's disease. Acceleration of apoptosis is one of the major pathogenic mechanisms of PS1 mutants, and PS1 mutants have also been reported to induce overproduction of amyloid-β protein 42. Here, we investigated aberrancy in activation of initiator caspases related to two *PS1* gene mutations, I143T and G384A. Acceleration of apoptosis, elevation of caspase-3/7 activity, and significant increases in caspase-4, -8 and -9 activities during apoptosis induced by several agents were found in these mutant PS1-transfected cells. Interestingly, thapsigargin treatment enhanced caspase-4 and -9 activities in I143T-mutant PS1-transfected cells, while hydrogen peroxide treatment enhanced caspase-4, -8 and -9 activities in G384A-mutant PS1-transfected cells, indicating diverse apoptosis-promoting effects of *PS1* gene mutations. In addition, treatment with a β-secretase inhibitor or γ-secretase inhibitor significantly attenuated the effects of the PS1 mutants on caspase-3/7 activation and recovered cell viability. Our present data suggest that these PS1 mutants accelerate the activation of initiator caspases and promote apoptosis, which may be associated, at least in part, with amyloid-β production.

Keywords: Alzheimer's disease, apoptosis, caspase, presenilin, β-secretase, γ-secretase

INTRODUCTION

Alzheimer's disease (AD) is the most devastating and common dementia in elderly people. To date, the amyloid-β protein (Aβ), an approximately 4-kD protein derived from the amyloid-β protein precursor (AβPP), has been suggested to play a major role in the pathogenesis of AD [1]. Aβ ending at amino acid 42 (Aβ_{42}) is a minor but pathogenic form with high aggregativity and neurotoxicity [2]. Extracellular Aβ_{42} forms oligomers that are toxic towards synapses, and this may result in cognitive dysfunction and dementia in AD [3]. Recently, a novel 56-kD Aβ_{42} oligomer, namely Aβ*56, has been reported [4].

One of the major causes of early-onset familial AD (FAD) is mutations in the *presenilin* (*PS*) *1* and *PS2* genes [5]. PS1/2 have been reported to reside in the endoplasmic reticulum (ER)-Golgi compartment [6], lysosomal membrane [7], lipid rafts [8], plasma membrane [9], and even in mitochondria [10]. PS1/2 are multifunctional proteins, and one of their major functions is γ-secretase activity by forming the γ-secretase complex with nicastrin, Aph-1 and Pen-2 [11]. γ-secretase cleaves the C-terminal fragment of AβPP to generate Aβ and AβPP intracellular domain (AICD), although many other proteins, such as Notch-1, have also been reported to be its substrates [12]. Also,

*Corresponding author: Yasumasa Ohyagi, MD, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Fax: +81 92 642 5352; E-mail: ohyagi@neuro.med.kyushu-u.ac.jp.

ISSN 1387-2877/09/$17.00

PS1/2 regulates intracellular Ca^{2+} homeostasis, protein trafficking, β-catenin stability and protein degradation, indicating that it is multi-functional [13]. Therefore, various pathogenic mechanisms of *PS1/2* gene mutations have been suggested, such as increased $A\beta_{42}$ generation [14], increased vulnerability to ER stress [15], disruption of intracellular Ca^{2+} homeostasis [16] and ultimate acceleration of apoptosis processes [17]. Neuronal apoptosis may also contribute to sporadic AD [18]. Various caspases are activated during apoptosis. Some major apoptosis pathways, the Fas-receptor-mediated pathway, mitochondrial pathway, and ER stress pathway, are well known, and caspase-8, -9 and -4/12 are specific initiators or effectors of these pathways, respectively [19]. Caspase-3 activation is the ultimate common pathway at the final stage of the apoptosis process. To date, no direct evidence for the effects has been obtained by measuring the specific activation of these initiator caspases in mutant *PS1* gene-transfected cells. Two types of *PS1* gene mutations, I143T and G384A, were reported in Belgian families with early-onset FAD [20]. These mutations were reported not to alter PS1 processing [21], but to decrease β-catenin stability [22]. I143T-mutant PS1 affects intracellular trafficking of Trk [23], while G384A-mutant PS1 causes a remarkable increase in $A\beta_{42}$ generation [24]. Here, we examined whether apoptosis and activation of initiator caspases are enhanced in cells expressing these two mutant *PS1* genes in the same or different manner.

MATERIALS AND METHODS

Cell culture, transfection and apoptosis inducers

Human neuroblastoma cell line SH-SY5Y cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Human wild-type, I143T-mutant and G384A-mutant PS1 cDNAs were subcloned into the pCEP4 vector (Invitrogen, Camarillo, CA, USA) and transfected to SH-SY5Y cells by the calcium phosphate method or lipofection [23,25]. Stably transfected cells were selected by hygromycin B-containing medium. Control cells were transfected with vector only. The following agents were used to induce apoptosis: staurosporine (STS; Biomol, Plymouth Meeting, PA, USA), a protein kinase C inhibitor; etoposide (ETP; Sigma), a topoisomerase II inhibitor; thapsigargin (TG; Biomol, Plymouth Meeting, PA, USA), an ER Ca^{2+}-ATPase inhibitor; MG132 (Calbiochem, San Diego, CA, USA), a proteasome inhibitor; and hydrogen peroxide (H_2O_2; Sigma), an oxidative stress inducer. These agents, with the exception of H_2O_2, were resolved in dimethyl sulfoxide (DMSO).

Assays for cell viability and activities of caspase-3/7, -4, -8 and -9

Cultured cells at approximately 80% confluence were harvested and distributed into 96-well plates at 1×10^4 cells/well, and were cultured on the same plates for 24 h before the assays. For cell viability assays, a CellTiter-Blue^{TM} Fluorometric Viability Assay Kit (Promega, Madison, WI, USA) was used. Since caspase-3 activity is indistinguishable from caspase-7 activity, caspase-3/7 activity was measured using the Caspase-Glo^{TM} Reagent (Promega, Madison, WI, USA) with the luminometer (MTP800 AFC Microplate-Reader; Corona Electric Japan, Tokyo, Japan).

The caspase-4, -8 and -9 activities were measured using a Fluorometric Assay Kit (BioVision, Mountain View, CA, USA). In these assays, Ac-LEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC were used as specific substrates for caspase-4, -8 and -9, respectively. Briefly, cells were distributed into 6-well dishes at 1.5×10^6 cells/well, incubated for 24 h and then treated with the above-described apoptosis-inducing agents. After lysis of the cells in lysis buffer (50 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT, 0.1 mM EDTA), the protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and adjusted to appropriate concentrations (0.8–1.2 mg/L). The caspase-4, -8 and -9 activities were measured by the fluorescence levels using a fluorescence plate reader (MTP800 AFC Microplate-Reader; Corona Electric Japan, Tokyo, Japan). The caspase activities were expressed as ΔAFU, and relative activities were expressed according to the corresponding activities in control cells treated with each agent.

Western blotting of GRP78, Bid and Bax

Western blotting was performed as reported in our other report [26]. After treatment with each apoptosis inducer for 24 h, cells were lysed in 2% sodium dodesylsulfate (SDS). Each 10 μg of total protein was electrophoresed in an SDS-15% polyacrylamide gel and electro-transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in TBST (25 mM Tris-HCl pH7.6, 150

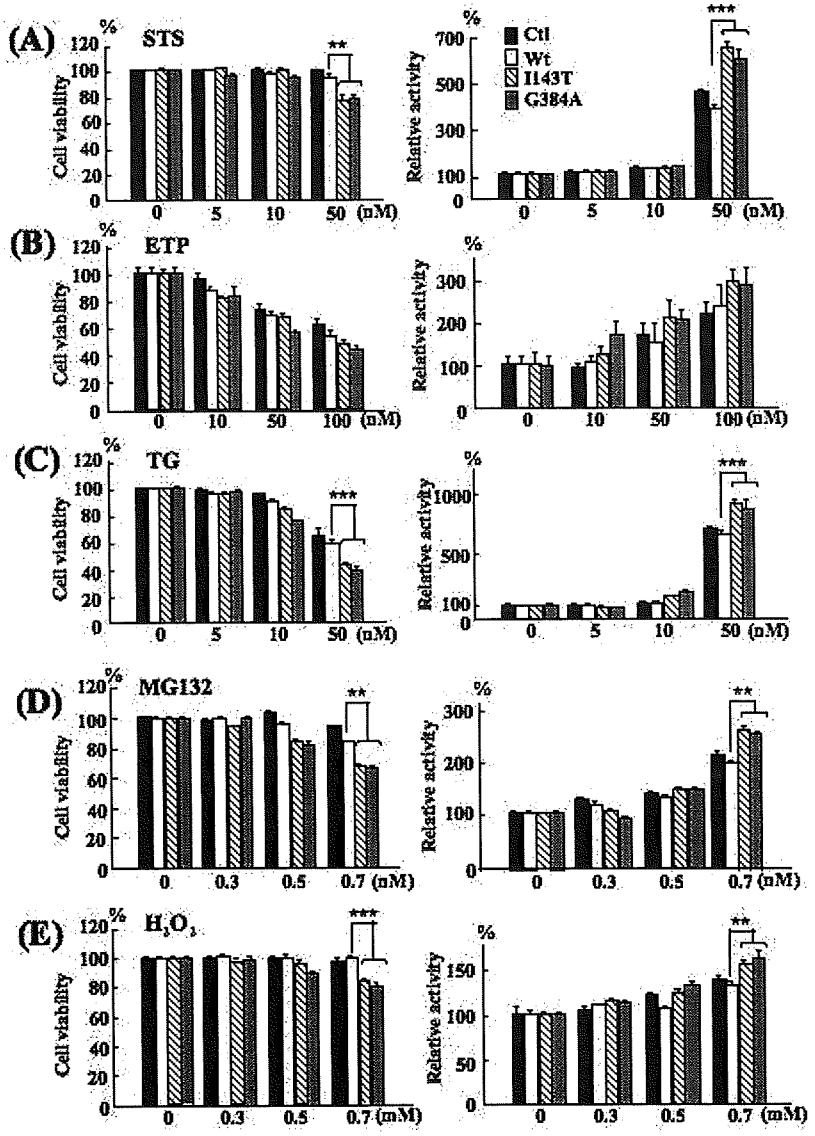

Fig. 1. Cell viability (left panels) and caspase-3/7 activity (right panels) of SH-SY5Y cells after treatments. Treatments are staurosporine (STS) for 6 h (A), etoposide (ETP) for 48 h (B), thapsigargin (TG) for 24 h (C), MG132 for 24 h (D) or hydrogen peroxide (H_2O_2) for 6 h (E). Control (Ctl), wild-type PS1-transfected (Wt) and mutant PS1-transfected (I143T or G384A) cells were examined. Cell viability was expressed relative to the viability of untreated cells. Caspase-3/7 activity was expressed relative to the corresponding baseline activity. $^{**}p < 0.001$; $^{***}p < 0.0001$.

mM NaCl, 0.1% Tween-20) for 1 h and then incubated with anti-GRP78/Bip polyclonal (1:1,000 dilution; Imgenex, San Diego, CA, USA), anti-Bid polyclonal (1:1,000 dilution; Cell Signaling Tech, Danvers, MA, USA) or anti-Bax polyclonal (1:1,000 dilution; Santa Cruz, CA, USA) primary antibodies in TBST for 2 h at room temperature. The membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution; Vector Lab, Burlingame, CA, USA) in TBST for 1 h, and antigen-antibody complexes were detected using an ECL Western Blotting Detection Reagent System (Amersham Bioscience, Piscataway, NJ, USA).

β-secretase (BACE) and γ-secretase inhibitors

The effect of Aβ on caspase activities was investigated using a β-site AβPP-cleaving enzyme (BACE) inhibitor and γ-secretase inhibitor. Appropriate concentrations were determined according to previous reports for the BACE inhibitor [27] and γ-secretase in-