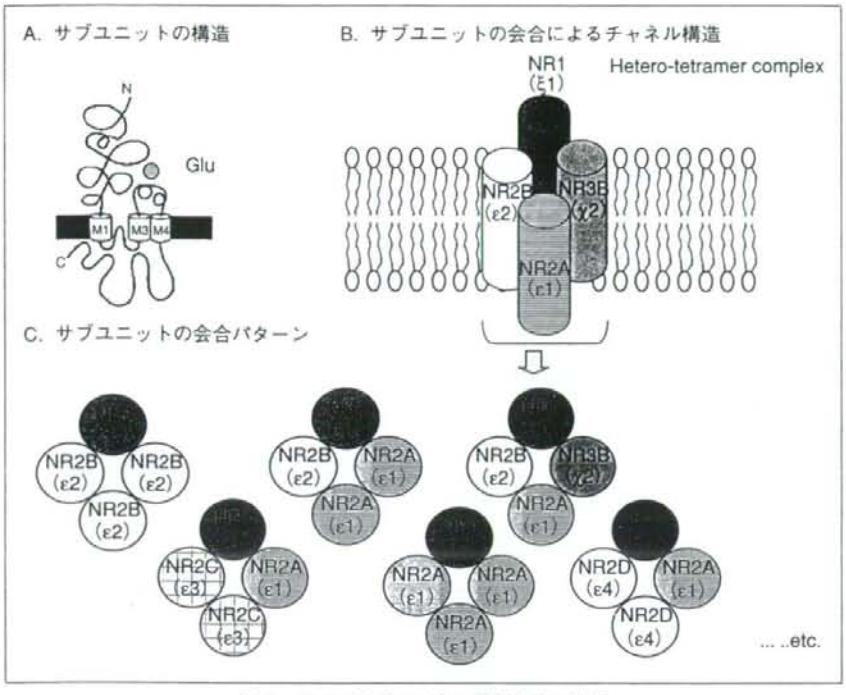

図1 NMDA型グルタミン酸受容体の構造

A：NMDA型グルタミン酸受容体(NMDAR)のサブユニット構造は，細胞外にあるN末から4つの膜貫通部位を経て細胞内側にC末が存在する共通構造をとっている．B：NMDARはGluRξ1(NMDAR1)とGluRε1-4(NMDAR2A-2D)あるいはGluRξ1(NMDAR1)とGluRχ1-2(NMDAR3A-B)といったサブユニットが4つ会合した4量体構造をとっている．C：4量体構造は，異なる種類のサブユニットの種々の会合パターンがあるとされている．

NMDA型GluR(NMDAR)は，GluRξ1(NMDAR1)とGluRε1-4(NMDAR2A-2D)あるいはGluRξ1(NMDAR1)とGluRχ1-2(NMDAR3A-B)といったサブユニット(図1-A)が4つ会合した4量体構造をとり(図1-B)，イオンチャネルとして機能しているが，種々の会合パターンがあるとされている(図1-C)．個々のサブユニットは，細胞外にあるN末から4つの膜貫通部位を経て，細胞室側にC末が存在する共通構造をとっている(図1-A)．

抗GluR抗体研究の始まり

GluRに対する自己抗体の研究は，AMPA型のGluR3に対する自己抗体がRasmussen脳炎患者血清中に存在することを1991年にRogersらが見出したのを契機に始まった[4]．抗GluR3抗体は，GluR3に結合して興奮性に作用し，神経細胞死に繋がることや[4][5]，補体非依存性[6]・補体依存性の細胞障害を起こすこと[7][8]が明らかとなっていて，Rasmussen症候群のてんかん・退行などの病態を説明し得るとされている．

抗GluRε2抗体の測定法と機能

NMDARの一つであるGluRε2(NMDAR2B)は胎生期には広く中枢神経系に発現し，生後は前脳に限局するサブユニットで，NMDAR依存的シナプス可塑性と記憶・学習にかかわる重要な分子である．抗GluRε2抗体の測定は，NIH3T3細胞中に遺伝子組み換えにより発現させたGluRε2の全長蛋白を抗原として行っている(図2)[9][10]．エピトープ解析はGluRε2のN末(細胞外ドメイン)(NT1)，C末(細胞内ドメイン)(3カ所)のペプチドをPEXシステムなどで合成し，それらの合成ペプチドを抗原として同定した．これらのGluRε2の全長蛋

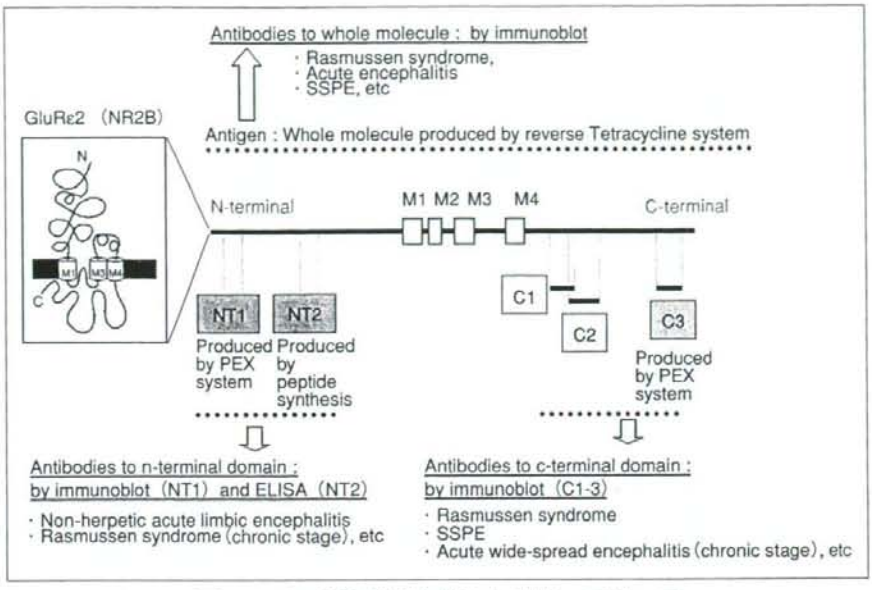

図2　GluRε2分子の構造と抗GluRε2抗体・エピトープ

GluRε2(NMDAR2B)分子全長を抗原としてイムノブロットで検出している抗GluRε2抗体は，いろいろなGluRε2分子領域をエピトープとする幅広い抗体を検出できるメリットがある．そのため，抗N末-GluRε2抗体(N末エピトープ)が主体のNHALEのみならず，抗C末-GluRε2抗体(C末エピトープ)が主体の急性期Rasmussen症候群，慢性期小児広汎性脳炎，SSPEなどでも陽性となる．抗N末-GluRε2抗体(N末エピトープ)(NT1)はGluRε2の細胞外N末ドメインを抗原としてイムノブロットで検出している．抗C末-GluRε2抗体(C末エピトープ)(C1-3)はGluRε2の細胞内ドメインの3カ所を抗原としてイムノブロットで検出している．

白・合成ペプチド抗原をポリアクリルアミド電気泳動(PAGE)後，ニトロセルロース膜に転写し，検体と反応させた後，二次抗体を用いて抗体の有無を判定した(イムノブロット法)．GluRε2のN末の合成ペプチドを抗原としたエピトープ解析(NT2)はELISA法により行った．

抗GluRε2抗体の作用機序を明らかにするために，われわれはラット海馬スライス標本を用いて，GluRε2のN末側に対するウサギ抗体・Rasmussen症候群患者のIgG(抗GluRε2抗体陽性)の興奮性シナプス後電流(EPSC)への影響を検討したが，現在までのところ抗GluR3抗体とは異なり，明らかな電気生理学的作用を見出せていない[11]．一方，SLE患者の抗ds-DNA抗体は，GluRε2などのN末細胞外ドメインにある283-287番目のアミノ酸配列(Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Gly)とds-DNAに分子相同性があるため，中枢神経系でNMDAR(NMDAR2A/2B)と交叉反応し，アポトーシスなどを起こすこと，Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Glyで免疫した動物で，その抗体が中枢神経系に至ると行動や認知機能に影響がみられることが動物実験で示されている[12)~14)]．

急性脳炎

日本の成人における急性脳炎罹患率は，19.0/100万人年(年間2,114例)と推計され[15]，小児の罹患率は，56.4/100万人年と推定されることから，成人・小児合計すると，急性脳炎・脳症は日本では年間3,100人が罹患しているものと推定され，ウィルス直接侵襲が証明できない傍感染性の病態が多いと思われる[16]．われわれは，傍感染性の病態では自己免疫的機序が働いているのではないかという仮説の下，GluRに着目して，自己免疫介在脳炎の研究を行ってきた[17)~20)]．

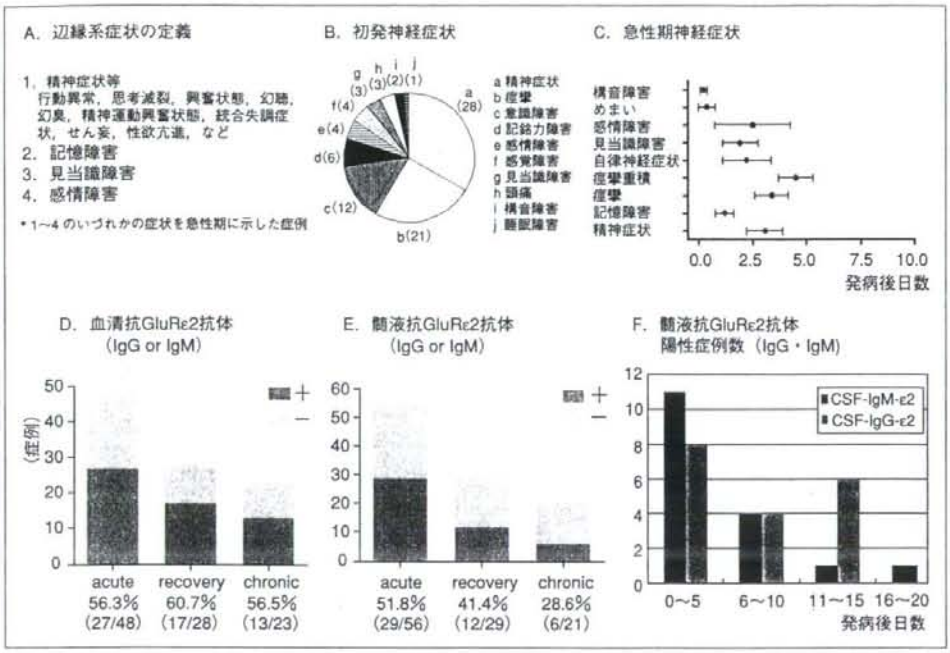

図3　成人非ヘルペス性急性辺縁系脳炎の臨床症状と抗GluRε2抗体

A：辺縁系症状の定義，1～4の症状のいずれかを意識障害の軽い急性期の段階で示した症例を辺縁系症状ありとした．B：成人非ヘルペス性急性辺縁系脳炎(NHALE)症例の初発神経症状．C：NHALE症例の急性期神経症状の出現病日を，神経症状出現日をゼロ日として，平均±SEM(standard error of the mean)で示した．D：NHALE症例の血清での抗GluRε2抗体のIgG型またはIgM型が陽性となった比率を，急性期(0～20病日)・回復期(21～60病日)・慢性期(61病日以降)に分けて示した．急性期は48例中27例(56.3%)でIgGまたはIgM抗体が陽性であった．E：NHALE症例の髄液での抗GluRε2抗体陽性率を急性期・回復期・慢性期に分けて示した．F：NHALE症例の髄液抗GluRε2抗体が陽性となった病日をIgG型，IgM型ごとに示す．

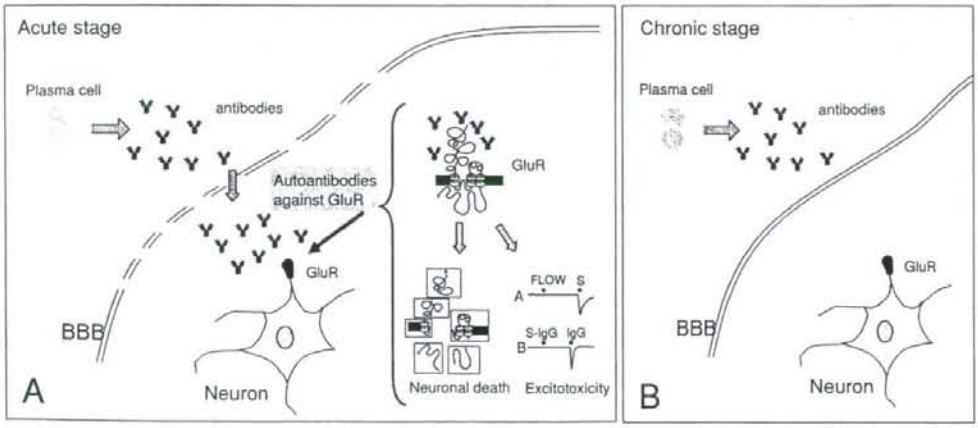

図4　抗GluRε2抗体と非ヘルペス性急性辺縁系脳炎の病態仮説

急性期(A)には，血液脳関門の破綻などによりN末に対するエピトープを含んだ抗GluRε2抗体が中枢神経系内に進入し，GluRε2(NMDAR2B)分子の細胞外ドメイン(N末など)になんらかの作用を及ぼし，脳炎症状に関与する．慢性期(B)になると血液脳関門が回復し，中枢神経系内の抗GluRε2抗体は減少する．抗N末-GluRε2抗体の作用機序，脳炎での血液脳関門の病態など，今後のさらなる研究が必要である．

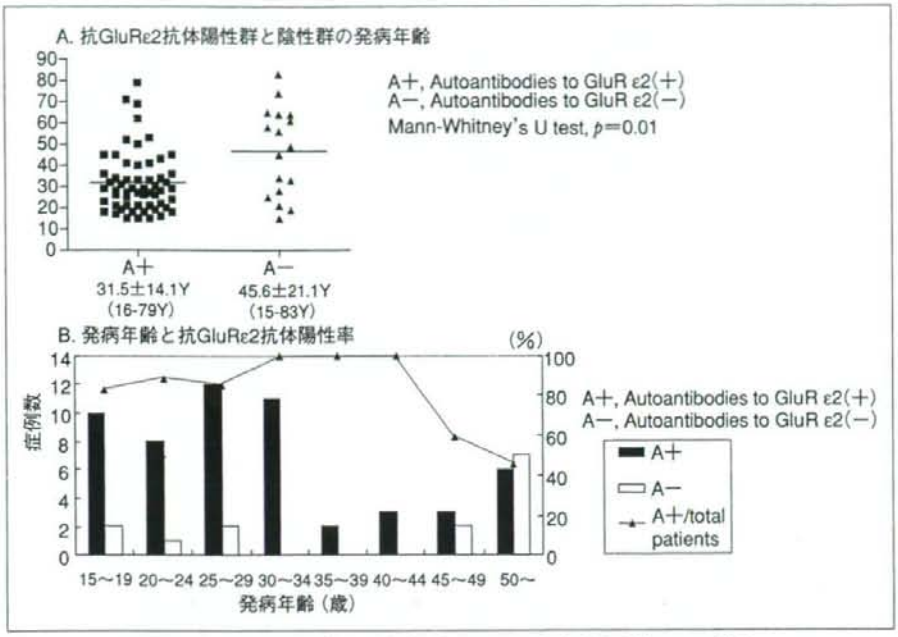

図5　非ヘルペス性急性辺縁系脳炎における発病年齢と抗GluRε2抗体
A：抗GluRε2抗体陽性群と陰性群の発病年齢，B：発病年齢と抗 GluRε2 抗体陽性率，発病年齢別にみた抗GluRε2抗体陽性者数と陰性者数を示す．A+：抗GluRε2抗体陽性症例，A−：抗GluRε2抗体陰性症例．

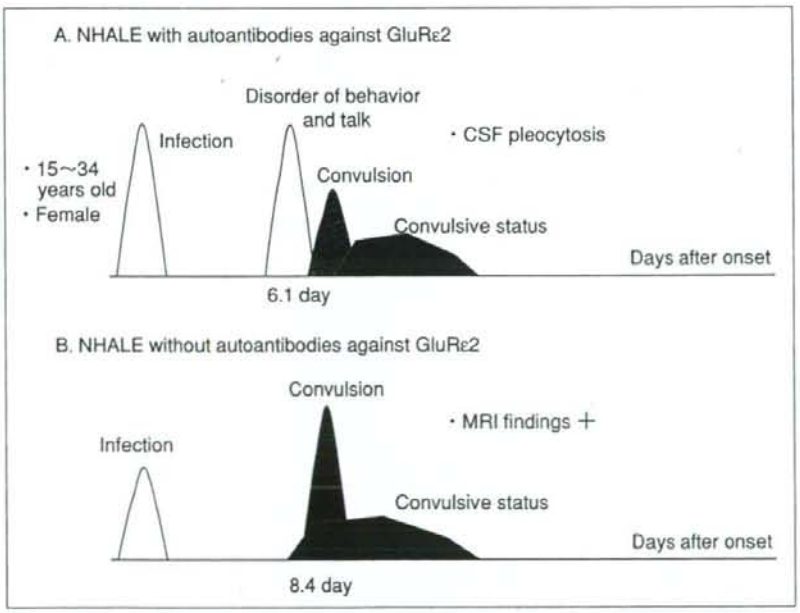

図6　非ヘルペス性急性辺縁系脳炎における抗GluRε2抗体陽性群と陰性群の臨床経過のまとめ
A：抗GluRε2抗体陽性群の経過，B：抗GluRε2抗体陰性群の経過．NHALE：nonherpetic acute limbic encephalitis(非ヘルペス性急性辺縁系脳炎)．

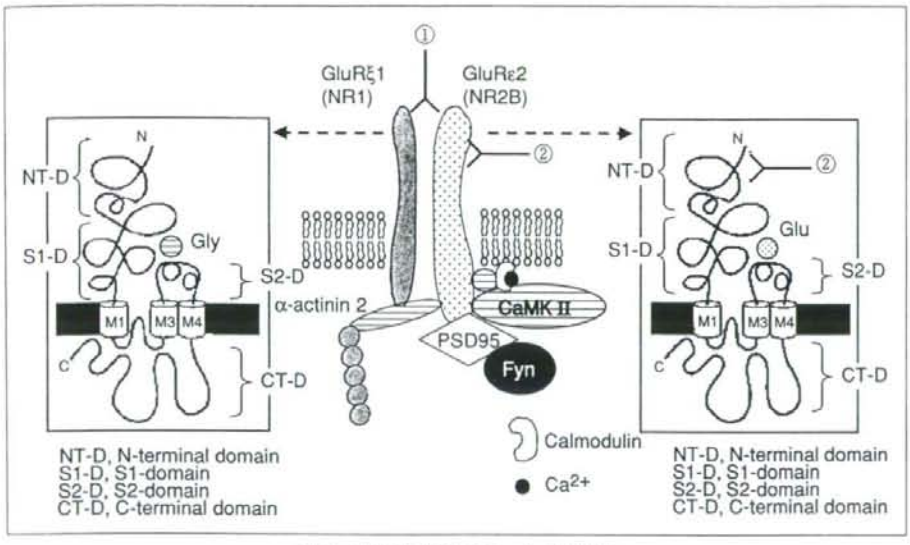

図7　NMDA型GluRチャネルの構造

NMDA型GluR(NMDAR)は異種のNMDARサブユニットが4つ会合した(ヘテロ4量体)構造の陽イオンチャネルで，GluRξ1(NMDAR1)にGluRε1-4(NMDAR2A-2D)，GluRχ1-2(NMDAR3A, 3B)が会合している．われわれの測定している抗N末-GluRε2抗体(抗NT1, 抗NT2抗体)はGluRε2(NMDAR2B)の細胞外ドメインを認識する抗体であるのに対し(図7-②で示す抗体)，Dalmauらの抗NMDAR抗体はGluRε2など単独サブユニットの細胞外ドメインを認識するのではなく，NMDAR1+NMDAR2B(一部はNMDAR1+NMDAR2A)複合体の細胞外側立体構造を認識しているとされている(図7-①で示す抗体)．〔参考文献：Inactivation of NR1 by Ca-CaM. Cell 1996；84：745-55. Neuron 1998；21：443-53. Interaction with NMDA-R locks CaMK II. Nature 2001；411：801. Ca influx by anti-calreticulin antibodies. Neurosci Res 2000；36：385-90.〕

非ヘルペス性急性辺縁系脳炎と抗GluRε2抗体

抗GluRε2抗体測定のために検体送付された急性脳炎・脳症369症例から，腫瘍合併例・再発例・慢性例・膠原病合併例・インフルエンザ脳症・単純ヘルペスウィルスPCR陽性例などを除き，辺縁系症状で神経症状が始まった15歳以上の非ヘルペス性急性辺縁系脳炎(nonherpetic acute limbic encephalitis：NHALE)91例について抗GluRε2抗体を検討した(図3)[20]．NHALEでは，血清中抗GluRε2抗体(IgGまたはIgM)は急性期から慢性期に約60%にみられ，髄液中抗GluRε2抗体は急性期に約50%，回復期に約40%，慢性期に約30%と次第に低下し，髄液中の抗GluRε2抗体は急性期でもかなり早い時期に出現した．抗GluRε2抗体のエピトープ解析は髄液中の抗GluRε2抗体陽性の4例で行い，4例とも髄液でN末エピトープ(NT1)を認めた．血液中にできた抗GluRε2抗体は，血液脳関門の破綻などにより中枢神経系に至りなんらかの急性期脳炎症状に寄与するが，回復期・慢性期になると血液脳関門の回復により髄液中から消失する病態仮説を考えている(図4)[20]．

抗GluRε2抗体陽性非ヘルペス性急性辺縁系脳炎の特徴

15歳以上のNHALEで急性期に抗GluRε2(NMDAR2B)抗体を測定できた69例(抗GluRε2抗体陽性群53例，陰性群16例)について比較検討した[21]．発病年齢は，陽性群が陰性群より若年で，陽性群の74.5%は15～34歳であった(図5)[21]．抗GluRε2抗体陽性NHALEは若年成人が75%を占め，若年成人NHALEの80%以上が抗GluRε2抗体陽性で，抗GluRε2抗体は若年成人NHALEの主たる原因となっている可能性があることがわかった．これまでに脳炎脳症の自己抗体として，辺縁系脳炎での抗VGKC抗体，橋本脳症での抗NAE抗体，傍腫瘍性辺縁系脳炎での抗Hu抗体などが報告され

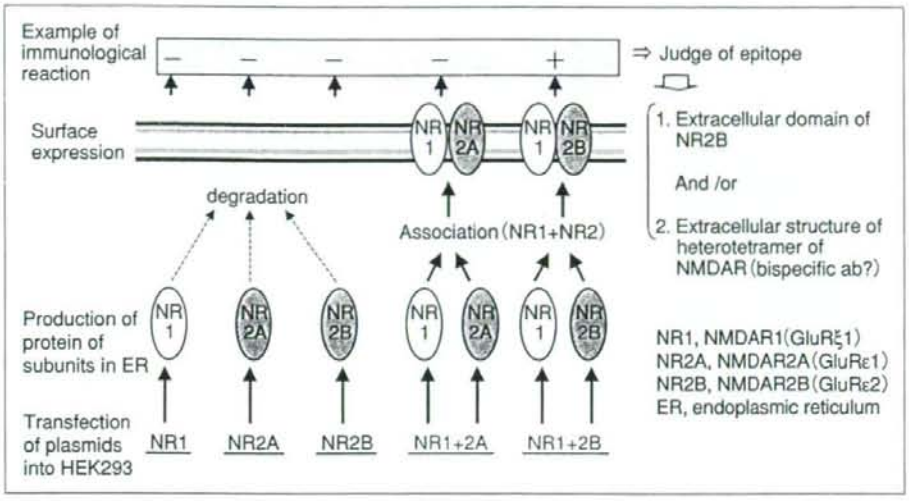

図8　ImmunocytochemistryによるNMDA型GluR複合体に対する抗体測定(Dalmau)
HEK細胞に遺伝子導入によりGluRの各サブユニットを発現させる場合，GluRξ1(NMDAR1)，GluRε1-4(NMDAR2A-2D)などの単独サブユニットの遺伝子を導入しても，細胞表面には発現できず，細胞内で分解されてしまう[2)25)]．GluRξ1とGluRε1-4の遺伝子を同時に導入するとサブユニットが会合し，NMDAR複合体として細胞表面に発現する．たとえば，髄液などが図8-上段に示すように，GluRξ1導入細胞とのimmunocytochemistryで陰性，GluRε1導入細胞で陰性，GluRε2導入細胞で陰性，GluRξ1+GluRε1導入細胞で陰性，GluRξ1+GluRε2導入細胞で陽性となった場合，Dalmauらは，個々のサブユニットに対する抗体ではなく，NMDAR複合体(GluRξ1+GluRε2)に対する抗体と判断している．しかし，個々のサブユニット単独の遺伝子導入では蛋白が細胞表面に発現できないことを考慮すると，NMDAR複合体に対する抗体である可能性に加えて，GluRε2の細胞外ドメイン(N末など)に対する抗体である可能性も否定できないことになる．〔参考文献：Annu Rev Pharmacol Toxicol 2003；43：335-58.〕

ている[20)22)]．抗VGKC抗体陽性辺縁系脳炎症例の発病年齢は34～65歳(平均50.4歳)，抗NAE抗体陽性橋本脳症は23～83歳(平均59歳)，抗Hu抗体陽性傍腫瘍性辺縁系脳炎は28～70歳(平均61.5歳)，抗Ta抗体陽性傍腫瘍性辺縁系脳炎は22～45歳(平均34歳)と，自己抗体ごとに好発年齢が異なり，自己免疫介在性NHALEに関与する自己抗体には年齢依存性の特徴があると思われる[20)22)]．

初発神経症状は，陽性群では言動の異常がもっとも多く，陰性群ではけいれんが多かった．急性期のけいれん・けいれん重積は陽性群で出現が遅かった．髄液細胞数が陽性群で高値であったが，髄液蛋白・IgGには差がなかった．予後については両群とも記憶の面での障害が強かった(図6)[21)]．

卵巣奇形腫を伴う傍腫瘍性辺縁系脳炎と抗GluRε2抗体

2007年，卵巣奇形腫を伴う傍腫瘍性辺縁系脳炎(NHALE-OT)症例12例の血清・髄液中に，NMDAR1+NMDAR2B(NMDAR2A)の複合体とは反応するが，NMDAR1あるいはNMDAR2B単独分子とは反応しない抗体が存在することがimmunocytochemistryによる検討で報告され，抗NMDAR抗体(抗NMDAR脳炎)と呼ばれている[23)24)]．当初，この抗体はNMDAR複合体を構成するGluRε2(NMDAR2B)など単独サブユニットの細胞外ドメインを認識するのではなく，NMDAR1+NMDAR2B(一部はNMDAR1+NMDAR2A)複合体の細胞外立体構造を認識しているとされていた(図7-①で示す抗体)[20)22)]．しかし，NMDAR複合体の個々のサブユニットを単独でHEK細胞に発現させることは困難であり[2)25)]，Dalmauらのimmunocytochemistryでは個々のサブユニットに対する抗体の有無は判断できない(図8)．よって，NHALE-OTの抗NMDAR抗体が，NMDAR複合体を構成する個々のサブユニットの細胞外ドメインを認識するのではなく，複合体の細胞外立体構造を認識していると判断す

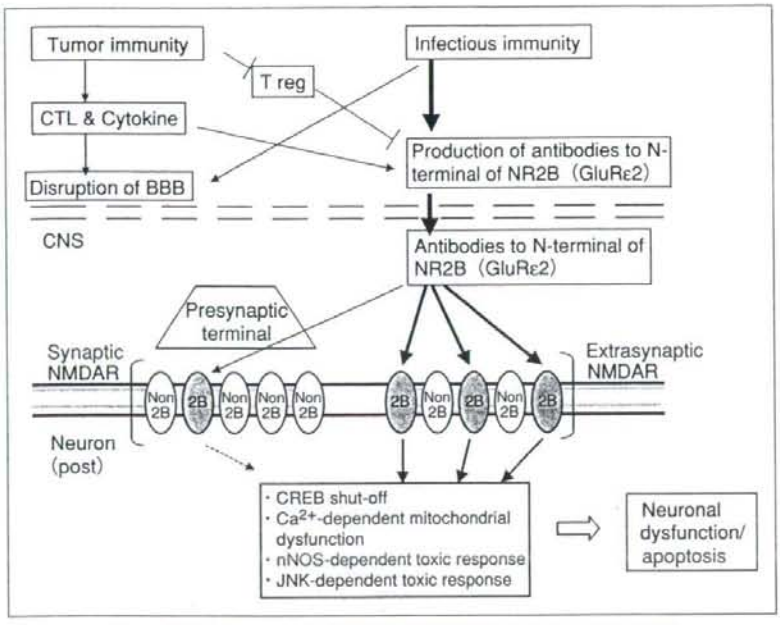

図9　卵巣奇形腫を伴う傍腫瘍性辺縁系脳炎(NHALE-OT)の病態
多くの症例で先行感染症・発熱があり，先ず感染に伴う免疫変化により抗GluRε2抗体などができる．感染・腫瘍免疫による血液脳関門(BBB)の破綻により自己抗体が中枢神経系に侵入し，主としてシナプス外のGluRε2(NMDAR2B)を含むNMDARに作用し，種々の経路に変化が起こり，ニューロンの機能障害・アポトーシスなどをきたす．CTL：cytotoxic T cell, BBB：blood brain barrier, CNS：central nervous system, 2B：NMDA receptor heteromer with MDANR2B subunit, Non 2B：NMDA receptor heteromer without NMDAR2B subunit, JNK：c-Jun N-terminal kinase, CREB：cAMP-responsive-element-binding protein.

るのは早計である．

われわれが19例のNHALE-OTの急性期髄液について抗GluRε2抗体を検討したところ，全長分子を抗原としたイムノブロット法では約40％の症例が陽性，N末のペプチドを抗原としたELISA(NT2)では約70％の症例で陽性，Dalmauらの方法のimmunocytochemistryでは約90％の症例で陽性であった[25][26]．よってNHALE-OTの抗NMDAR抗体の少なくとも半数はGluRε2(NMDAR2B)のN末を認識する抗体を含んでいるといえる．

2008年の日本神経学会(横浜)でのDalmau教授の発表では，NHALE-OTの症例においてGluRξ1(NMDAR1)の細胞外ドメイン(N末)をエピトープとする抗体の重要性が報告され，NMDAR複合体に対する抗体というより，複合体を構成するGluRξ1(NMDAR1)サブユニットに対する抗体が重要であるという仮説にシフトしてきている．

NHALE-OTの急性期髄液では，われわれの抗N末-GluRε2抗体が約70％で検出されるのに対し，Dalmauらの抗NMDAR複合体抗体の陽性率は約90％と高い．これは，Dalmauらの方法[23]がGluRε2(NMDAR2B)の細胞外ドメイン(N末)のみならず，GluRξ1(NMDAR1)の細胞外ドメイン(N末)をエピトープとする抗体も検出できるためかもしれない．NHALE-OTではGluRε2の細胞外ドメインのみならず，GluRξ1の細胞外ドメインをエピトープとする抗体など，複数の抗体が存在するのではないかと，われわれは考える．

NHALE-OTの臨床特徴は，発病年齢，先行因子から脳炎発病までの日数，初発神経症状，急性期神経症状，髄液所見などの点で，成人の非傍腫瘍性非ヘルペス性急性辺縁系脳炎・脳症ときわめてよく似ていることがわかり[26]，急性辺縁系脳炎の特徴を通常示すことがわかった．抗GluRε2抗体に

ついても，共通性がみられ，両群とも高率に，NMDARのうちのGluRε2の細胞外ドメイン(N末)をエピトープとする自己抗体を有していた．このようなことから，NHALE-OTの病態として感染免疫と腫瘍免疫が複合した病態を考えている(図9)．

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<シンポジウム 3—4>抗 NMDA 受容体抗体陽性脳症

急性辺縁系脳炎・脳症と NMDA 型グルタミン酸受容体

髙橋　幸利[1)2)]　山崎　悦子[1)]　西村　成子[1)]　角替　央野[1)]
丹羽　憲司[3)]　Josep Dalmau[4)]　今井　克美[1)]　藤原　建樹[1)]

要旨：非傍腫瘍性非ヘルペス性急性辺縁系脳炎・脳症（NPNHALE）（成人 69 例＋小児 26 例）と，卵巣奇形腫を合併する脳炎・脳症症例（NHAE-OT）（19 例）を比較検討した．NHAE-OT の臨床特徴は，発病年齢，先行因子から脳炎発病までの日数，初発神経症状，急性期神経症状，髄液所見などの点で，成人の NPNHALE ときわめてよく似ていることがわかり，卵巣奇形腫に合併する脳炎・脳症は，急性辺縁系脳炎の特徴を示すことがわかった．抗 GluRε2 抗体についても，共通性がみられ，両群とも高率に，NMDA 型 GluR のうちの GluRε2（NR2B）の細胞外ドメイン（N 末）をエピトープとする自己抗体を有していた．

（臨床神経，48：926—929, 2008）
Key words：急性辺縁系脳炎，NMDA型グルタミン酸受容体，自己抗体，卵巣奇形腫

はじめに

われわれは，グルタミン酸受容体（GluR）に対する自己抗体高感度測定系を構築し，Rasmussen 症候群，急性脳炎・脳症において検討してきた[1)〜5)]．最近，卵巣奇形腫をともなう傍腫瘍性辺縁系脳炎症例の血清・髄液中に，NMDA 型 GluR 複合体（NR1＋NR2B または NR2A）と反応する抗体が存在することが Dalmau らにより報告された[6)]．今回著者らは，卵巣奇形腫をともなう脳炎・脳症症例と非傍腫瘍性急性辺縁系脳炎・脳症症例においてそれらの臨床特徴，抗 GluRε2 抗体（抗 NR2B 抗体）を比較検討した．

対象および方法

急性脳炎・脳症の症例で当センターに抗 GluRε2 抗体の検査目的で紹介された 485 症例の中から，インフルエンザ脳症，単純ヘルペス脳炎，腫瘍合併例，再発例，慢性例，膠原病合併例などを除外した非傍腫瘍性非ヘルペス性急性辺縁系脳炎・脳症（成人 69 例＋小児 26 例）（NPNHALE と略），と，卵巣奇形腫を合併する脳炎・脳症症例（19 例）（NHAE-OT と略）を比較検討した．卵巣奇形腫は右側 10 例，左側 5 例，両側 3 例，不明 1 例である．抗 GluRε2 抗体は，全長分子を抗原としたイムノブロット法と N 末のペプチド（NT2）を抗原とした ELISA により測定した．

結　果

急性脳炎・脳症の当センター依頼症例における卵巣奇形腫合併頻度は 7.6％（19/250 例）であった．脳炎発病年齢（平均±SD）は，NPNHALE が 27.7±18.6（2〜83）歳，NHALE-OT が 27.5±6.5（14〜41）歳で，両群とも 20〜34 歳に多いことがわかった（Fig. 1）．先行因子は両群とも感冒などの上気道炎あるいは発熱が多く，NPNHALE では 63.8％ に，NHALE-OT では 89.5％ にみとめ，両群で有意差がみられた（Fisher's exact test, $p=0.025$）．先行因子から脳炎症状出現までの先行期間（平均±SD）は，成人 NPNHALE が 7.7±6.1（0〜24）日，小児 NPNHALE が 3.8±3.5（0〜15）日，NHALE-OT が 7.5±3.6（3〜14）日で，成人 NPNHALE と NHALE-OT 間に有意差はみとめなかった．脳炎の発病症状は，成人 NPNHALE では言動の異常＞発作＞意識障害が，小児 NPNHALE では言動の異常＞発作＞意識障害が，NHALE-OT では言動の異常＞発作＞見当識障害が多かった．成人 NPNHALE と NHALE-OT を比較すると NHALE-OT では辺縁系症状以外の振戦や眼振が初発症状である症例が 2 例みられた．急性期にみられる症状では，成人 NPNHALE と NHALE-OT ともに言動の異常が約 90％ の症例に，発作が約 75％ の症例に，見当識障害と記憶障害が約 20％ の症例にみられ，成人 NPNHALE と NHALE-OT では急性期症状に大きな違いはみとめなかった．

NHALE-OT の髄液細胞数（平均±SD）は 51.6±66.4/mm³，髄液蛋白は 35.4±14.7mg/dl，髄液 IgG は 6.6±4.2mg/dl で，成人 NPNHALE との間に有意差はなかった（Fig. 2）．

[1)]国立病院機構静岡てんかん・神経医療センター〔〒420-8688　静岡市葵区漆山 886〕
[2)]岐阜大学医学部小児病態学
[3)]同　腫瘍制御学講座女性生殖器学分野
[4)]Department of Neurology, University of Pennsylvania
（受付日：2008 年 5 月 16 日）

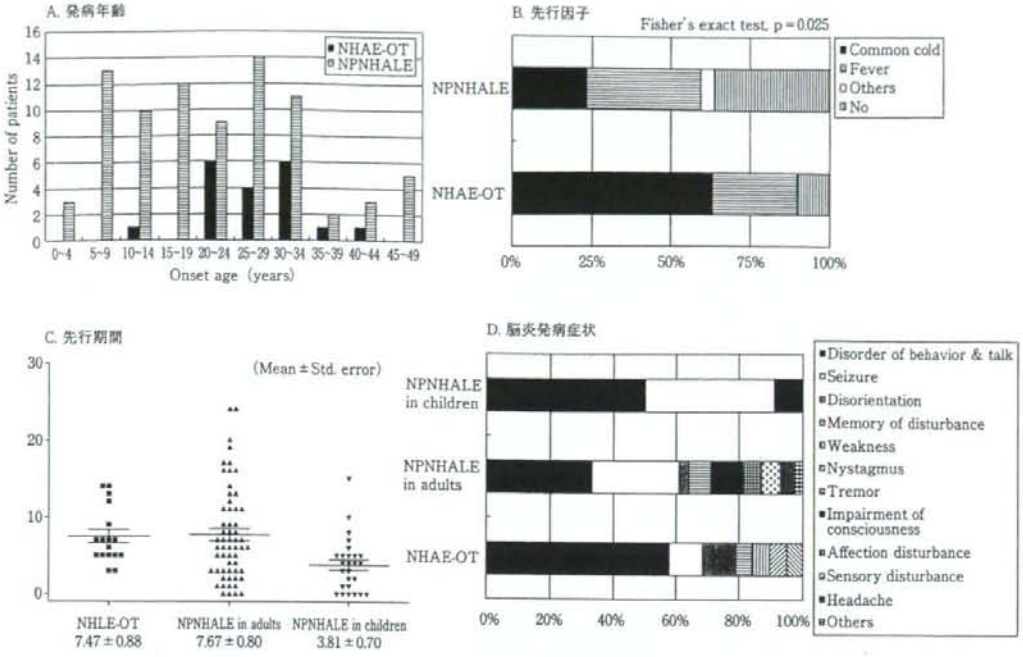

Fig. 1　非傍腫瘍性非ヘルペス性急性辺縁系脳炎・脳症（成人 69 例，小児 26 例）（NPNHALE と略）と，卵巣奇形腫を合併する脳炎・脳症症例（19 例）（NHAE-OT と略）における臨床症状の比較

入院治療日数は NHALE-OT が平均 209.0 日，成人 NPNHALE では平均 87.5 日と有意差をみとめ（Mann Whitney test，$p<0.0001$），NHALE-OT では有意に重症であることがわかった．

NHALE-OT における急性期髄液抗 GluRε2 抗体は，全長分子を抗原としたイムノブロット法では 40.0% の症例が陽性，N 末のペプチドを抗原とした ELISA（NT2）では 69.2% の症例で陽性，Dalmau らの Immunocytochemistry 法では 90.9% の症例で陽性であった．成人 NPNHALE における髄液抗 GluRε2 抗体は急性期の約 50%，回復期の約 40%，慢性期の約 30% の症例に検出された[4][5]．

考　察

卵巣奇形腫に合併する脳炎・脳症の臨床特徴は，発病年齢，先行因子から脳炎発病までの日数，初発神経症状，急性期神経症状，髄液所見などの点で，成人の非傍腫瘍性非ヘルペス性急性辺縁系脳炎・脳症ときわめてよく似ていることがわかり，卵巣奇形腫に合併する脳炎・脳症は，急性辺縁系脳炎の特徴を通常示すことがわかった．抗 GluRε2 抗体についても，共通特徴がみられ，両群とも高率に，NMDA 型 GluR のうちの GluRε2（NR2B）の細胞外ドメイン（N 末）をエピトープとする自己抗体を有していた．

Dalmau らの最初の卵巣奇形腫に合併する NMDAR 脳炎の報告では，NMDA 型 GluR を構成する個々のサブユニットではなく NMDA 型 GluR 複合体全体の細胞外構造を抗原とする抗体が関与する疾患と考えていた[6]．しかし，NMDA 型 GluR 複合体の個々のサブユニットを単独で細胞に発現させることは困難であり[7][8]，彼らの方法では個々のサブユニットに対する抗体の有無は判断できない．また，bispecific antibodies の可能性もないわけではないが，通常はひとつの分子に対する抗体と考えた方が良いと思われること，われわれの研究で NMDA 型 GluR のうちの GluRε2（NR2B）の細胞外ドメイン（N 末）をエピトープとする自己抗体を有する症例が約 50～70% 存在することなどを考慮すると，卵巣奇形腫に合併する脳炎・脳症には GluRε2（NR2B）の細胞外ドメイン（N 末）をエピトープとする自己抗体が関与する症例が少なからず存在すると推測される．第 49 回日本神経学会での Dalmau らの発表では，卵巣奇形腫に合併する脳炎症例においては GluRξ1（NR1）の細胞外ドメイン（N 末）をエピトープとする抗体が検出され，NMDA 型 GluR 複合体に対する抗体というより，複合体を構成する GluRξ1（NR1）サブユニットに対する抗体が重要であるという仮説にシフトしてきている．卵巣奇形腫に合併する脳炎・脳症の急性期髄液では，われわれの抗 N 末-GluRε2 抗体が約 70% で検出されるのに対し，Dalmau らの抗 NMDAR 複合体抗体の陽性率は 90.9% と高い．

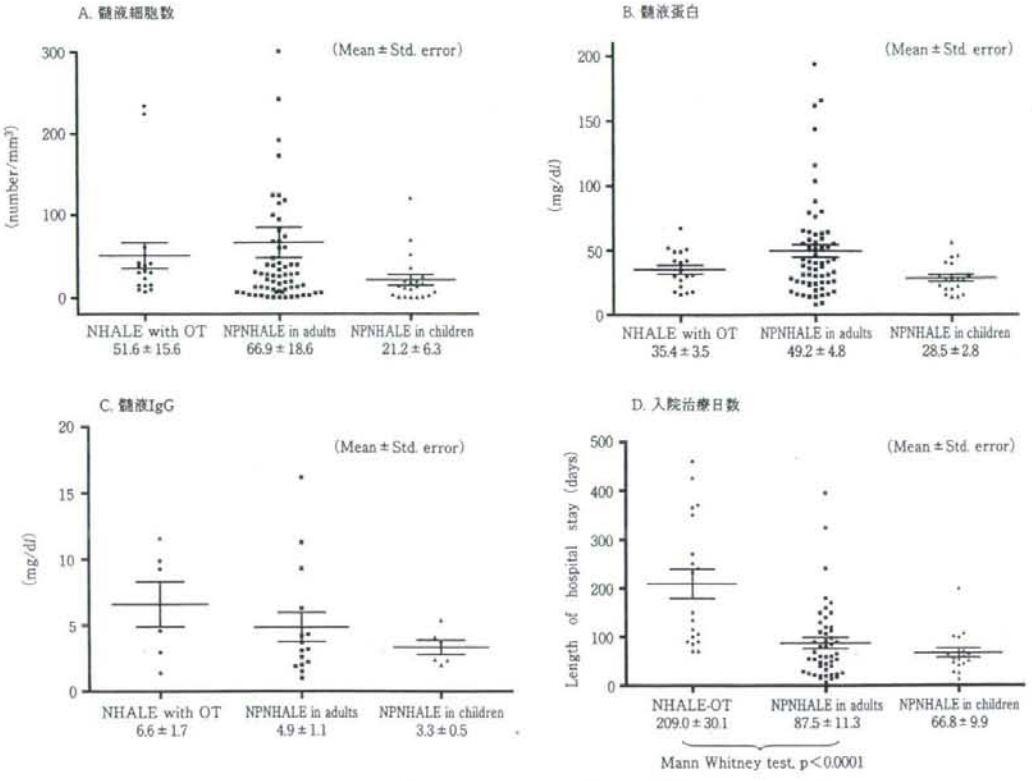

Fig. 2　非傍腫瘍性非ヘルペス性急性辺縁系脳炎・脳症（成人 69 例，小児 26 例）（NPNHALE と略）と，卵巣奇形腫を合併する脳炎・脳症症例（19 例）（NHAE-OT と略）における急性期髄液検査所見・入院日数の比較

これは，Dalmau らの方法[6]が GluRε2（NR2B）の細胞外ドメイン（N 末）のみならず，GluRξ1（NR1）の細胞外ドメイン（N 末）をエピトープとする抗体も検出できるためかもしれない．著者らは，卵巣奇形腫に合併する脳炎・脳症では GluRε2（NR2B）の細胞外ドメイン（N 末）のみならず，GluRξ1（NR1）や，GluRε1（NR2A）などの細胞外ドメイン（N 末）をエピトープとする抗体が，単独あるいは重複して存在するのではないかと考える．今後われわれは GluRξ1（NR1）の細胞外ドメイン（N 末）をエピトープとする抗体の検出方法を確立していきたい．

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Abstract

Acute limbic encephalitis and NMDA type-glutamate receptor

Yukitoshi Takahashi, M.D.[1][2], Etsuko Yamazaki, M.D.[1], Shigeko Nishimura, M.D.[1], Hisano Tsunogae, M.D.[1], Kenji Niwa, M.D.[3], Josep Dalmau, M.D.[4], Katsumi Imai, M.D.[1] and Tateki Fujiwara, M.D.[1]

[1]National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders
[2]Department of Pediatrics, Gifu University School of Medicine
[3]Department of Obstetricus, Gifu University School of Medicine
[4]Department of Neurology, University of Pennsylvania

We compared clinical characteristics and autoantibodies against GluRε2 between 95 patients with non-paraneoplastic non-herpetic acute limbic encephalitis (NPNHALE) and 19 patients with non-herpetic acute encephalitis accompanying ovarian teratoma (NHAE-OT).

Onset age (mean ± SD) was 27.7 ± 18.6 years old in NPNHALE, 27.5 ± 6.5 in NHALE-OT. Preceding factors were found in 63.8% of patients with NPNHALE and 89.5% of patients with NHALE-OT (Fisher's exact test, $p = 0.025$), and major preceding factors were upper respiratory infections or fever in both groups. Symptoms at the onset were disorder of behavior and talk > seizures > impairment of consciousness in NPNHALE, and disorder of behavior and talk > seizures > disorientation in NHALE-OT. Symptoms at the acute stage were similar between NPNHALE and NHAE-OT, but duration of hospital stay was longer in NHAE-OT (209.0 days) than NPNHALE (87.5 days) (Mann Whitney test, $p<0.0001$). At the onset, cell counts in CSF were 51.6 ± 66.4/mm^3 and protein levels were 35.4 ± 14.7 mg/dl, and IgG levels were 6.6 ± 4.2 mg/dl in NHAE-OT, and these data were not significantly different between NPNHALE and NHAE-OT.

In acute stage, autoantibodies against whole molecule of GluRε2 in CSF were detected in 51.8% (29/56) of adult NPNHALE, and 40% (6/15) of NHAE-OT patients by immunoblot. These autoantibodies in both groups included epitopes to n-terminal of GluRε2. Antibodies against NMDAR complex (Dalmau's method) in CSF were detected in 90.9% (10/11) of NHAE-OT patients.

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Key words: acute limbic encephalitis, NMDA type glutamate receptor, autoantibodies, ovarian teratoma

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Clinical Report

A Unique Case of Fibrodysplasia Ossificans Progressiva With an *ACVR1* Mutation, G356D, Other Than the Common Mutation (R206H)

Hirokazu Furuya,[1,2]* Koji Ikezoe,[1,3] Lixiang Wang,[4] Yasumasa Ohyagi,[2] Kyoko Motomura,[2] Naoki Fujii,[1] Jun-ichi Kira,[2] and Yasuyuki Fukumaki[4]

[1]Department of Neurology, Neuro-Muscular Center, National Omuta Hospital, Fukuoka, Japan
[2]Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[3]Division of Neurology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki, Okayama, Japan
[4]Division of Human Molecular Genetics, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

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Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant congenital disease characterized by progressive heterotopic endochondral osteogenesis with great-toe malformations. A 617G > A (R206H) mutation of the activin A type 1 receptor gene (*ACVR1*) has been found in all previously reported patients with FOP. Thus, this is one of the most specific of all disease-associated mutations. We report here on a 62-year-old man with slowly progressive FOP and a novel mutation in *ACVR1*. He developed difficulty in moving his shoulder since age 10 years due to contraction of the shoulder joint. The symptoms progressed slowly, and he could not walk at age 36 years and was bedridden at 55 years. He also showed rigid spine, baldness, sensorineural hearing loss, and hypodactyly accompanied by abnormal ectopic ossification. Analysis of *ACVR1* and its cDNA revealed that the patient is heterozygous for a mutation, 1067G > A (G356D). Typing of SNPs located in the ~0.5-Mb region spanning *ACVR1* and its neighbor genes suggested that 1067G > A is a de novo mutation. These results give a clue to better understanding of FOP as well as of the mild clinical symptoms in the patient. © 2008 Wiley-Liss, Inc.

Key words: fibrodysplasia ossificans progressiva (FOP); rare mutation; activin A type I receptor gene (*ACVR1*); bone morphogenetic protein (BMP); bone morphogenetic protein receptor (BMPR); single-nucleotide polymorphism (SNP)

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INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP, OMIM #135100) is a rare autosomal dominant disorder, characterized by malformed toes and extra-skeletal (heterotopic) ossification that begins in childhood [Connor, 1996; Kaplan et al., 2006; Timmerman, 2006]. Ectopic bone formation may occur after a local minor trauma but more often spontaneously. The average age at the onset of ossification is 5 years, and 95% of cases are sporadic [Cohen et al., 1993; Connor, 1996]. Recently, Shore et al. [2006] reported a mutation, 617G > A (R206H), in the activin A type I receptor gene (*ACVR1*), of all affected members from seven unrelated families and of all 32 sporadic cases in various ethnic groups [Lin et al., 2006; Shore et al., 2006; Nakajima et al., 2007]. It is of great interest that both the reported sporadic and familial cases shared the same mutation, R206H, because the findings suggest a certain genetic pressure to prevent the occurrence of another mutation, and the mutation is one of the most specific of all disease-associated mutations in the human genome [Lin et al., 2006; Shore et al., 2006; Nakajima et al., 2007].

We present a case of FOP with slowly progressive respiratory dysfunction and a novel mutation in

Grant sponsor: The Ministry of Health, Labour and Welfare of Japan.
*Correspondence to: Hirokazu Furuya, M.D., Ph.D., Department of Neurology, National Omuta Hospital, Fukuoka 837-0911, Japan.
E-mail: furuya@oomuta.hosp.go.jp; furuya@neuro.med.kyushu-u.ac.jp
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ACVR1. Significance of the mutation in FOP pathogenesis will be discussed.

MATERIALS AND METHODS

Clinical Findings

The patient (II-2, Fig. 1A) is a 62-year-old Japanese man with a history of normal birth and childhood development. Both his parents had died at advanced ages without any symptoms reminiscent of FOP, and two his siblings (II-1 and II-3, Fig. 1A) are also healthy. The patient noticed difficulty in moving his shoulder at age 10 years due to contractures of both shoulder joints. The joint contractures progressed slowly in his extremities, and he was unable to walk at age 36 years and bedridden at 55 years with rigid spine, baldness, sensory hearing-loss, accompanied by abnormal ossification but without respiratory failure. He also showed severe hypodactyly with short thumbs in both hands and a severe defect of both great toes. Neurological examination revealed no abnormality except for the joint contractures and abnormal ossification. Serum biochemical examination showed normal levels of CK, phosphorus, calcium, and parathormone. Extensive heterotopic bone formation, rigid spine, very short first metacarpal bones, toe malformations, and defect of ossa digitorum in both great toes were seen by roentgenography (Fig. 2) and three-dimensional reconstructed computed tomography. His respiratory function was preserved and blood gas analysis was normal. He was thus diagnosed to have FOP by his

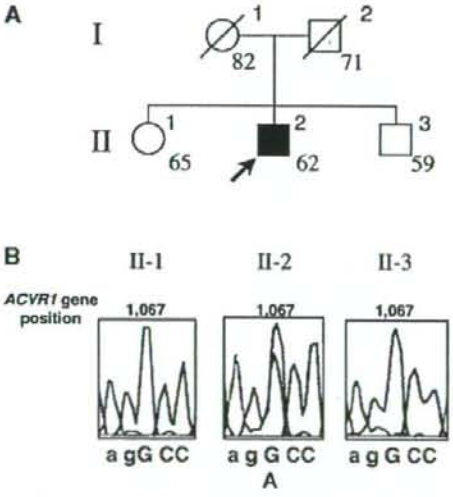

FIG. 1. Pedigree of the present family (A) and a heterozygous *ACVR1* mutation (1067G > A) in the patient (B). Arrow indicates the patient. Age of each family member is presented on the right (A).

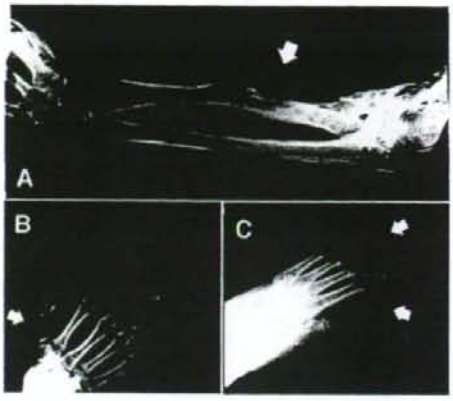

FIG. 2. Abnormal ossification of the forearm (A, arrow), short thumb and first metacarpal bone (B, arrow), and malformations and hypodactyly in the toe (C, arrow) of the patient.

clinical course and heterotopic ossification [Cohen et al., 1993].

Genetic Analysis

Genomic DNA and total RNA were obtained from peripheral blood lymphocytes of the patient and two his siblings. DNA samples were also collected from 150 normal Japanese after obtaining informed consent. All these protocols were approved by IRB. The patient's complete *ACVR1* cDNA sequence, which was subdivided into three partially overlapped fragments (Fig. 3B), was analyzed by direct sequencing after RT-PCR, and the sequences obtained were compared with normal sequences from the human genome database (BC036748, GenBank, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi). In addition, nine exons of *ACVR1* were directly sequenced after PCR using reported primer sets [Shore et al., 2006].

Single-nucleotide polymorphism (SNP) analysis was performed by direct sequencing. Tag SNPs of *ACVR1*, its intergenic regions, and 5′ and 3′ neighboring genes, *UPP2* and *ACVR1C*, were selected from the HapMap database (http://www.hapmap.org/). All primers used for SNP analysis were designed by the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the allele frequencies were referred from the SNP data base (http://www.ncbi.nlm.nih.gov/snp/; Table I, Fig. 3E).

RESULTS

PCR products and subsequent electrophoresis for the three overlapping segments of *ACVR1* cDNA of

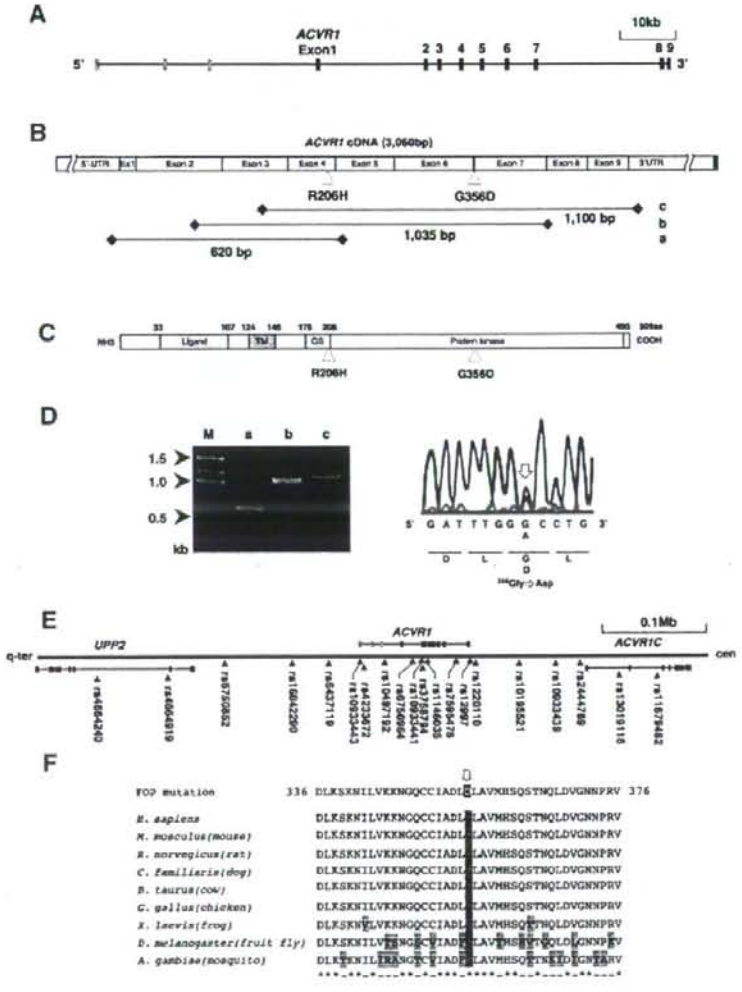

FIG. 3. Structures of the *ACVR1* gene (**A**), its cDNA (**B**), ACVR1 protein (**C**) and amplified regions after RT-PCR of *ACVR1* cDNA (**left**) and direct sequence of mutated region (**right**; **D**), localization of known SNPs (**E**), and comparison of ACVR1 protein sequences among species (**F**). *ACVR1* encodes 509 amino acid protein that contains a ligand binding region (Ligand), a transmembrane domain (TM), a glycine-serine rich domain (GS), and a protein kinase domain (C). The genomic organization of the gene and 20 SNPs were derived from the NCBI ENTREZ SNP database. White arrowhead indicates the previously reported mutation (R206H) and a novel mutation (G356D) in the present patient (B,C), and toned arrowhead the location of the SNPs with their ID numbers (E). *ACVR1* codon 356 is conserved among species, and the neighborhood of the G356D mutation site is highly conserved between vertebrates (F).

the patient showed bands with expected size without any extra-bands due to abnormal or alternative splicing (Fig. 3D). Sequencing of the segments revealed a nucleotide substitution, 1097G > A, in exon 7, leading to a missense mutation (G356D; Figs. 1B and 3D). This mutation was not observed in two his siblings or in 150 normal controls (Fig. 1B). Quantitative real-time PCR using total cDNA revealed no significant difference in the level of the *ACVR1* gene expression among the patient, his siblings and normal control (data not shown).

Genotyping of SNPs located in an about 0.5-Mb region spanning *ACVR1*, *UPP2*, and *ACVR1C* (Fig. 3E, Table I) in this family revealed four haplotypes, and the patient are his elder sister are both homozygotes for one of the haplotypes

TABLE I. SNPs in the *ACVR1* Gene and the Neighboring Region, and Genotypes in the Family Members

	Family member			Allele frequency		
Reference SNP ID	II-1	II-2	II-3	Alleles	Caucasians[a]	Japanese[a]
rs4664240	C/C	C/C	C/**G**	C/G	0.307/0.693	0.767/0.233
rs4664919	A/A	A/A	A/A	A/C	0.567/0.433	0.478/0.522
rs6750852	T/T	T/T	T/T	A/T	0.408/0.592	0.398/0.602
rs16842290	T/T	T/T	T/T	A/T	0.000/1.000	0.189/0.811
rs6437119	A/A	A/A	A/A	A/G	0.983/0.017	0.670/0.330
rs10933443	T/T	T/T	T/T	C/T	0.271/0.729	0.556/0.444
rs4233672	G/G	G/G	G/G	A/G	0.183/0.817	0.250/0.750
rs10497192	T/T	T/T	T/T	C/T	0.275/0.725	0.556/0.444
rs6756964	A/A	A/A	A/**G**	A/G	1.000/0.000	0.830/0.170
rs10933441	C/C	C/C	C/**T**	C/T	0.924/0.076	0.716/0.284
rs3768794	A/A	A/A	A/A	A/G	0.817/0.183	0.852/0.148
rs1146035	G/G	G/G	G/G	G/T	0.809/0.191	0.833/0.167
rs7595478	C/C	C/C	C/C	C/T	0.742/0.258	0.784/0.216
rs12997	T/T	T/T	T/T	C/T	0.283/0.717	0.261/0.739
rs1220110	A/A	A/A	A/A	A/T	0.258/0.742	0.250/0.750
rs10195521	T/**C**	T/**C**	T/T	C/T	0.758/0.242	0.689/0.311
rs10933439	C/**T**	C/**T**	C/C	C/T	0.202/0.798	0.144/0.856
rs2444769	A/A	A/A	A/A	A/C	0.775/0.225	0.886/0.114
rs13019116	A/A	A/A	**C/C**	A/C	0.417/0.583	0.182/0.818
rs11679482	A/**G**	A/**G**	**G/G**	A/G	0.492/0.508	0.167/0.833

[a]Cited from the database of HapMap-CEU and HapMap-JPT, respectively. Underlined are different genotypes between family members.

(Table I). Although the finding may have indicated that they would share both mutated alleles, as the sister has no mutation in either allele, the G356D observed in the patient is probably a de novo mutation. Codon 356 is located at the center of the protein kinase domain of ACVR1 protein (Fig. 3C). The amino acid encoded by codon 356 is conserved among various species of vertebrates (Fig. 3F).

DISCUSSION

We report on a patient with FOP with a slow clinical course associated with a mutation, G356D, of *ACVR1*. FOP is one of the most unusual disorders, because only one missense mutation (R206H) of *ACVR1* has been observed among various ethnic groups [Lin et al., 2006; Shore et al., 2006; Nakajima et al., 2007]. As the G356D observed in our patient is the first mutation other than R206H, it merits comments. Since his parents remained healthy even at their advanced ages, his brother and sister do not have this mutation, and the patient and his sister share the same SNP haplotype in the homozygous state, it is most likely that G356D is a de novo mutation and responsible for FOP in the patient. Clinical pictures of the patient are unique: his respiratory problem was slowly progressive, but anomalies in the great toe and thumb are unusually severe. His survival to this age is unusual for FOP. These unique manifestations as well as the rarity of the mutation may be related to its occurrence in the non-CpG region in *ACVR1*, that is, in the protein kinase domain of ACVR1. Usually, the common mutation, 617G > A (R206H), is located in the functionally important glycine-serine rich domain (GS), a CpG mutation hot-spot, leading to early onset respiratory dysfunction [Shore et al., 2006; Nakajima et al., 2007].

Ortholog comparison revealed that seven species of vertebrates and two insects showed the same codon (glycine), which is substituted by aspartic acid in our patient (Fig. 3F). The glycine residue at the relevant position is conserved in all organisms, and this region, which is located in the N-terminal of the protein kinase domain, is also conserved in all vertebrates. These findings may support that the G356D mutation observed in the patient is pathogenic.

Bone morphogenetic proteins (BMPs) act as potent osteogenic morphogens capable of inducing ectopic bone formation in animal models and interact with specific BMP receptors (BMPRs). BMPRs mediate the BMP signaling pathway, the G356D mutation may alter the expression levels of BMP, as does the R206H [Connor, 1996; Kan et al., 2004; Fiori et al., 2006; Kaplan et al., 2006].

In conclusion, we have reported on a novel *ACVR1* mutation (G356D) that may be responsible for extra-skeletal ossification and typical but severe malformation of the toe with delayed respiratory dysfunction. This is the first mutation of *ACVR1* in FOP other than the common mutation, R206H.

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A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor

Toru Fukuda [a,b], Kazuhiro Kanomata [a], Junya Nojima [a,p], Shoichiro Kokabu [a,p], Masumi Akita [b,c], Kenji Ikebuchi [b,d], Eijiro Jimi [b,e], Tetsuo Komori [b,f], Yuichi Maruki [b,g], Masaru Matsuoka [d], Kohei Miyazono [b,h], Konosuke Nakayama [b,i], Akira Nanba [b,j], Hiroshi Tomoda [b,k], Yasushi Okazaki [b,l], Akira Ohtake [b,m], Hiromi Oda [b,n], Ichiro Owan [b,o], Tetsuya Yoda [b,p], Nobuhiko Haga [q,s], Hirokazu Furuya [r], Takenobu Katagiri [a,b,s,*]

[a] Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan
[b] Project of Clinical and Basic Research for FOP at Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Saitama 250-1241, Japan
[c] Division of Morphological Science, Biomedical Research Center, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[d] Department of Laboratory Medicine, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[e] Division of Molecular Signaling and Biochemistry, Department of Biosciences, Kyushu Dental College, 2-6-1Manazuru, Kokurakita-ku, Kitakyushu-shi, Fukuoka 803-8580, Japan
[f] Department of Neurology, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[g] Department of Neurology, Saitama Neuropsychiatric Institute, 6-11-1 Honchohigashi, Chuo-ku, Saitama-shi, Saitama 338-8577, Japan
[h] Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[i] Department of Endocrinology and Diabetes, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[j] Department of Obstetrics and Gynecology, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[k] Kitasato University School of Pharmaceutical Science, 5-9-1 Shirokane, Minato-ku, Tokyo 108-0023, Japan
[l] Division of Functional Genomics & Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan
[m] Department of Pediatrics, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[n] Department of Orthopedic Surgery, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[o] Department of Orthopedic Surgery, University of Ryukyus Faculty of Medicine, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan
[p] Department of Oral and Maxillofacial Surgery, Saitama Medical University, 38 Moro Hongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[q] Department of Rehabilitation Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[r] Department of Neurology, Neuro-Muscular Center, National Omuta Hospital, Tachibana 1044-1, Omuta, Fukuoka 837-0911, Japan
[s] The Research Committee on Fibrodysplasia Ossificans Progressiva of the Ministry of Health, Labour and Welfare, Japan

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ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant congenital disorder characterized by progressive heterotopic bone formation in muscle tissues. A common mutation among FOP patients has been identified in *ALK2*, ALK2(R206H), which encodes a constitutively active bone morphogenetic protein (BMP) receptor. Recently, a unique mutation of ALK2, ALK2(G356D), was identified to be a novel mutation in a Japanese FOP patient who had unique clinical features. Over-expression of ALK2(G356D) induced phosphorylation of Smad1/5/8 and activated Id1-luc and alkaline phosphatase activity in myoblasts. However, the over-expression failed to activate phosphorylation of p38, ERK1/2, and CAGA-luc activity. These ALK2(G356D) activities were weaker than those of ALK2(R206H), and they were suppressed by a specific inhibitor of the BMP-regulated Smad pathway. These findings suggest that ALK2(G356D) induces heterotopic bone formation via activation of a BMP-regulated Smad pathway. The quantitative difference between ALK2(G356D) and ALK2(R206H) activities may have caused the phenotypic differences in these patients.

Fibrodysplasia ossificans progressiva (FOP; OMIM135100) is a rare hereditary disorder with autosomal dominant transmission. Clinical features of FOP are characterized by the presence of malformations of the great toes with hallux valgus and post-natal progressive heterotopic ossification that results in the formation of the ectopic skeleton [1–3]. Bone morphogenetic protein (BMP) signaling has been suggested to be involved in the heterotopic bone formation in FOP patients, since BMPs are capable of inducing ectopic bone formation in muscle and osteoblastic differentiation of myoblasts in vitro [4,5].

Intracellular signaling of BMPs is transduced by two types of serine/threonine kinase receptors: type I and type II [6,7]. Li-

* Corresponding author. Address: Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan. Fax: +81 42 984 4651.
E-mail address: katagiri@saitama-med.ac.jp (T. Katagiri).

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gand-bound type II receptor activates type I receptor kinase through phosphorylation of the glycine-serine (GS) domain, which is highly conserved among the type I receptors. Activated BMP type I receptor kinase, in turn, phosphorylates receptor-regulated Smads (R-Smads), which include Smad1, Smad5 and Smad8. Phosphorylated R-Smads form heteromeric complexes with Smad4, a common Smad, and translocate into the nucleus to regulate transcription of various target genes, including Id1[8]. A recurrent heterozygous mutation at 617G>A in the *ACVR1* gene encoding a BMP type I receptor ALK2, was identified in both familial and sporadic patients with FOP [9,10]. This mutation causes an amino acid substitution of Arg to His at codon 206 (R206H) within the GS domain of the ALK2 receptor. We found that ALK2(R206H) is a constitutively activated BMP receptor and cooperatively induces osteoblastic differentiation with Smad1 and Smad5, which are increased during muscle regeneration in vivo [11].

Recently, a unique mutation in the *ALK2* gene, 1067G>A, was identified in a Japanese patient who has slow progressive FOP [12]. This mutation causes an amino acid substitution of Gly to Asp at codon 356 (G356D) in the kinase domain rather than the GS domain of the ALK2 receptor. The clinical pictures of this patient seemed to be unique in comparison with other "classical" FOP patients, since his respiratory problem was slow and progressive, and he had severe hypodactyly in thumbs on both hands, as well as a severe defect in both halluces [12]. In the present study, we found that the ALK2(G356D) mutant receptor activated a Smad-dependent pathway of BMP signaling in the absence of ligands. This method of activation was similar to that for ALK2(R206H) found in the classical FOP patients. These findings suggested that ALK2(G356D) induced heterotopic bone formation by activating BMP signaling as a constitutively activated BMP type I receptor. However, the biological activities of ALK2(G356D) were lower than those of ALK2(R206H). The quantitative difference between ALK2(G356D) and ALK2(R206H) activities may have caused the differences in clinical features in these patients.

Materials and methods

Cell cultures, transfection and reporter assay. C2C12 mouse myoblasts and C3H10T1/2 clone 8 (10T1/2) fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum [13]. Cells were transfected using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The transcriptional activation induced by ALK2 receptors was measured using IdWT4F-luc or CAGA-luc reporter plasmids, as previously described [8,14]. C2C12 cells were treated with Dorsomorphin (171260, Calbiochem, Darmstadt, Germany) to examine roles of the Smad-dependent signaling on the activities of ALK2(G356D).

Immunoblotting and immunostaining. Cells were lysed in TNE buffer [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 1% Nonidet P-40] and subjected to immunoblotting, as described previously [11,15]. The following antibodies were used: anti-FLAG antibody (clone M2, Sigma, St. Louis, MO), anti-phosphorylated Smad1/5/8 antibody (Cell Signaling, Beverly, MA), anti-phospho-p38 (SC-7973, SantaCruz, Santa Cruz, CA), anti-phospho-ERK1/2 (SC-7383, SantaCruz) and anti-V5 antibody (Invitrogen). C3H10T1/2 cells were transiently transfected with MyoD to induce myogenesis, which was evaluated by immunohistochemical staining for myosin heavy chain (MHC) using anti-MHC antibody (clone MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA) [16].

Alkaline phosphatase activity. Alkaline phosphatase (ALP) activity was measured as a marker of osteoblast differentiation with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenylphosphate). Reactions were terminated by adding 3 M NaOH, and the absorbance was measured at 405 nm [13,17].

Statistical analysis. Comparisons were made by using Student's *t*-test. Results were expressed as means ± SD. $P < 0.05$ was considered statistically significant.

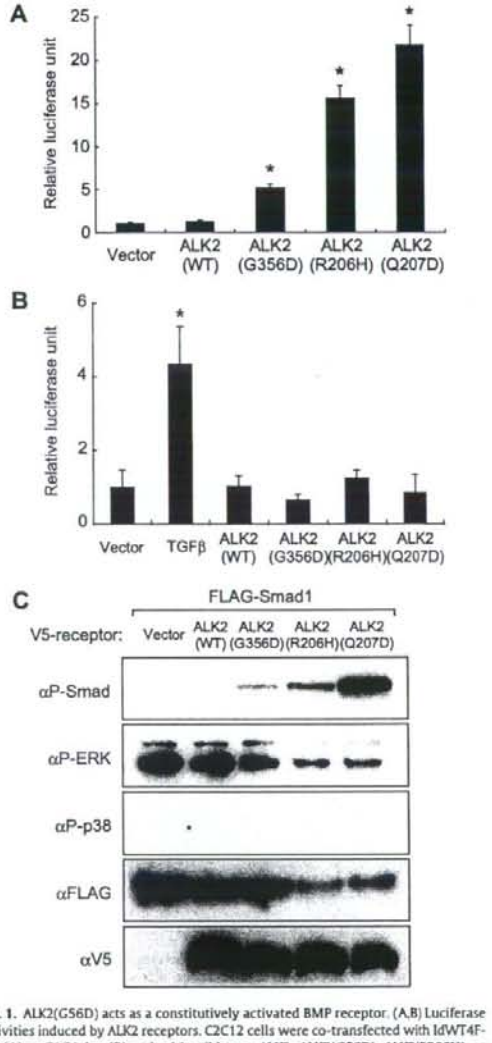

Fig. 1. ALK2(G56D) acts as a constitutively activated BMP receptor. (A,B) Luciferase activities induced by ALK2 receptors. C2C12 cells were co-transfected with IdWT4F-luc (A) or CAGA-luc (B) and with wild-type ALK2, ALK2(G56D), ALK2(R206H), or ALK2(Q207D). Constitutively active ALK2(Q207D) and 5 ng/ml of TGF-β1 were used as positive controls for IdWT4F-luc and CAGA-luc, respectively. Results are represented as means ± SD ($n = 3$). $^{*}P < 0.05$ when compared to vector transfection. (C) Intracellular signaling is activated by ALK2 receptors. C2C12 cells were co-transfected with FLAG-tagged Smad1 and a V5-tagged wild-type ALK2(WT), ALK2(G56D), ALK2(R206H), or ALK2(Q207D). Cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-phospho-p38 and anti-phospho ERK antibodies.

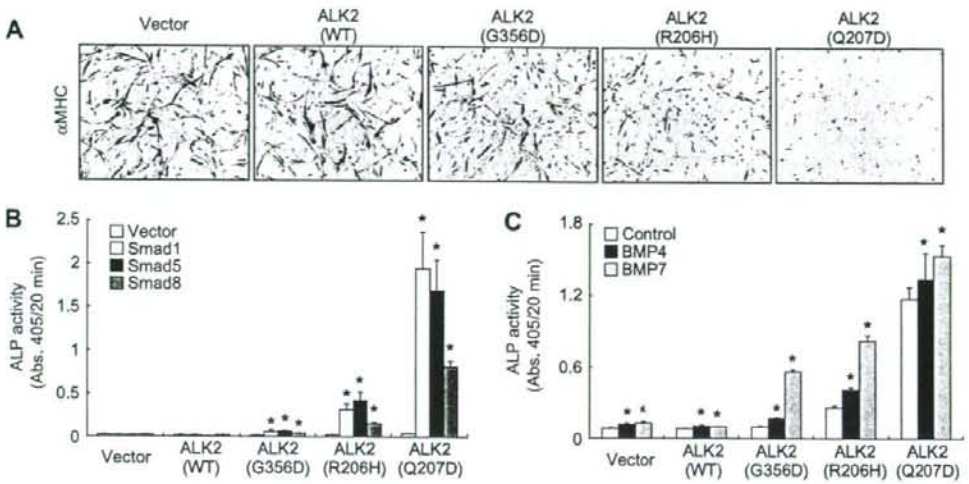

Fig. 2. ALK2(G56D) and Smad1/5 cooperatively induce osteoblastic differentiation. (A) Immunostaining of MHC in C3H10T1/2 cells co-transfected with a MyoD expression construct and an empty vector, wild-type ALK2, ALK2(G56D), ALK2(R206H), or ALK2(Q207D) construct. (B) ALP activities induced by co-operation of ALK2 receptors and Smad1/5/8. C2C12 cells co-transfected with FLAG-tagged Smad1, Smad5, or Smad8 and VS-tagged wild-type ALK2, ALK2(G356D), ALK2(R206H), or ALK2(Q207D). ALP activity was determined on day 3. Results are represented as means ± SD ($n = 3$). $^{*}P < 0.05$ when compared to vector transfection in each group. (C) ALP activities induced by co-operation of ALK2 receptors and BMPs. C2C12 cells co-transfected with Smad1 and wild-type ALK2, ALK2(G356D), ALK2(R206H), or ALK2(Q207D) were treated for 3 days with 100 ng/ml of BMP-4 or 100 ng/ml of BMP-7. ALP activities were determined on day 3. Results are represented as means ± SD ($n = 3$). $^{*}P < 0.05$ when compared with controls.

Results

ALK2(G356D) activates signaling pathway via BMP-regulated Smad in the absence of ligands

First, we asked whether ALK2(G356D) activates intracellular signaling of BMPs in the absence of ligands in a luciferase assay using IdWT4F-luc. ALK2(G356D) increased luciferase activity in C2C12 myoblasts, although the increase in activity was weaker than that induced by ALK2(R206H) and ALK2(Q207D), which had previously been shown to be constitutively active receptors (Fig. 1A). However, none of these ALK2 receptors activated CAGA-luc, a TGF-β/activin reporter (Fig. 1B). The specificity of these mutant ALK2 receptor kinases was further examined in co-transfection with FLAG-Smad1 followed by western blots. All of the mutant receptors induced FLAG-Smad1 phosphorylation in the order of ALK2(G356D) < ALK2(R206H) < ALK2(Q207D), although the receptors did not activate the phosphorylation of p38 or ERK1/2 (Fig. 1C).

ALK2(G356D) inhibits myogenesis and induces osteoblastic differentiation

Next, we examined the effect of ALK2(G356D)-induced signaling on myogenic differentiation. The number of MHC-positive muscle cells induced by transfection of MyoD in 10T1/2 cells was decreased by co-transfecting with one of the ALK2 receptors in an order of wild-type ALK2 < ALK2(G356D) < ALK2(R206H) < ALK2(Q207D) (Fig. 2A).

We recently reported that co-expression of ALK2(R206H) with Smad1, Smad5 or Smad8 cooperatively induced osteoblastic differentiation of C2C12 myoblasts [11]. Co-transfection of ALK2(G356D) with Smad1, Smad5 or Smad8 also significantly induced ALP activity, although the enzyme activities were much lower than those induced by ALK2(R206H) or ALK2(Q207D) (Fig. 2B).

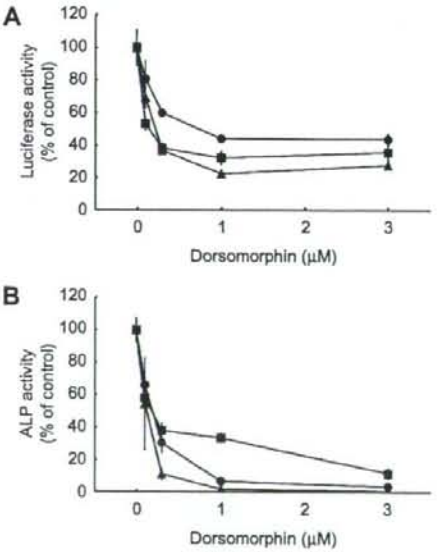

Fig. 3. Dorsomorphin inhibits ALK2(G356D) activity. The biological activities of ALK2(G356D) as determined by IdWT4F-luc (A) and ALP activity (B) were suppressed by Dorsomorphin in C2C12 cells. C2C12 cells were transfected with ALK2(G356D) (closed squares), ALK2(R206H) (closed triangles) or ALK2(Q207D) (closed circles) and treated with graded concentrations of Dorsomorphin. Luciferase activity (A) and ALP activity (B) were determined on day 3. Results are indicated as percentages of control (without Dorsomorphin).