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特 集 生体防御能の異常・免疫障害で見られる感染症の特徴とメカニズム

8. ケモカイン受容体変異とHIV感染抵抗性

Genetic pormorphisms in chemokine receptors and resistance to HIV infection

前田 洋助*

HIVの補助受容体としてのケモカイン受容体の発見は，ウイルスの侵入や細胞指向性を決定する分子として重要な知見であっただけでなく，個体間の感染感受性の違いやAIDS発症の予測など多くの情報をもたらした。特に感染初期に補助受容体として使用されるCCR5の遺伝的欠損である32塩基欠損は，実験的手法を用いてその感染抵抗性の機序が解明されただけでなく，多くのコホート研究でその感染抵抗性や病態進行遅延が実証された。

Key Words : HIV／AIDS／CCR5／CXCR4／CCR5Δ32

I HIV補助受容体としてのケモカイン受容体CCR5・CXCR4の発見

1981年，米国ロサンゼルスでホモセクシャルを中心にカリニ肺炎やカポジ肉腫が頻発し，これらの病態はまとめてAIDS（後天性免疫不全症候群）として報告された。この病気はCD4陽性Tリンパ球の減少と，それにともなう免疫不全が特徴で，ホモセクシャル以外にも薬物常習者や血友病患者などに広がりをみせていたことから原因としてウイルスが想定され，1983年，CD4陽性細胞であるTリンパ球やマクロファージに特異的に感染するヒトレトロウイルスHIV（ヒト免疫不全ウイルス）が分離された。ウイルス分離後すぐに，CD4分子に対する抗体が感染を完全に阻害することや，CD4を発現していないヒトの上皮系細胞にCD4を強制発現させると感染が成立することから，Tリンパ球やマクロファージ上に存在するCD4分子が主要なHIVの受容体であることが明らかになった。

しかしながら，マウス細胞にヒトCD4分子を強制発現させても感染が成立しないことから，何らかのCD4以外のヒト由来分子が補助受容体（コレセプター）として必要であることが当時から想定されていた。実際にHIV感染者からウイルスを分離してみると，末梢血CD4陽性Tリンパ球とマクロファージで増殖可能なウイルスと，末梢血CD4陽性Tリンパ球とCD4陽性T細胞株で増殖するウイルスの2種類が分離され，ヒトの細胞においてもCD4分子だけではその細胞指向性が説明できなかった。

前者はマクロファージで増殖可能なウイルスということからマクロファージ指向性ウイルス（M-tropic）と，後者はT細胞株で増殖可能なことからT細胞株指向性ウイルス（T-tropic）と分類された。また，末梢血Tリンパ球，マクロファージ，T細胞株のすべてで増殖可能なウイルスは両細胞指向性ウイルス（Dual-tropic）と呼ばれた。

興味深いことに，通常は感染全体を通じてマクロファージ指向性ウイルスが分離されるが，同じ

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患者でも AIDS 発症など病態の悪化した患者からのみ T 細胞株・両細胞指向性ウイルスが分離された。このような知見から，マクロファージ指向性ウイルスは持続感染成立に重要であり，一方，T 細胞株・両細胞指向性ウイルスは AIDS 病態に関与していることが示唆された。

CD4 分子が HIV の受容体であることが発見されてから 10 年以上の年月を経て，1996 年にケモカイン受容体である CXCR4 と CCR5 分子が HIV の補助受容体（コレセプター）として同定された[1]。これらの補助受容体の標的細胞における発現パターンから，CXCR4 を補助受容体として使用するウイルスが T 細胞株指向性ウイルスであり，CCR5 を使用するウイルスがマクロファージ指向性ウイルスであることが判明した（図 1）。

実際に，末梢 CD4 陽性 T リンパ球やマクロファージは CCR5 を発現しているためマクロファージ指向性ウイルスが感染可能であり，一方，T 細胞株においては CXCR4 の発現は認められるが CCR5 がまったく発現していないため CCR5 を使用するマクロファージ指向性ウイルスが感染しなかったのである。これらの補助受容体は G タンパク質共役型受容体（G protein-coupled receptors：GPCR）と呼ばれる 7 回膜貫通タンパク質であり，これらの立体構造がウイルス侵入に重要な働きをしていると考えられている。

その後，CCR2，CCR3，CCR8，CCR9，CXCR6，CX3CR1，ChemR23，APJ，Bob/GPR1，GPR1，RDC1 など多数の GPCR が HIV の感染を支持することが明らかとなったが，実際の *in vivo* の感染で使われている可能性はきわめて低いことから，これらはマイナー補助受容体と呼ばれ，CCR5 と
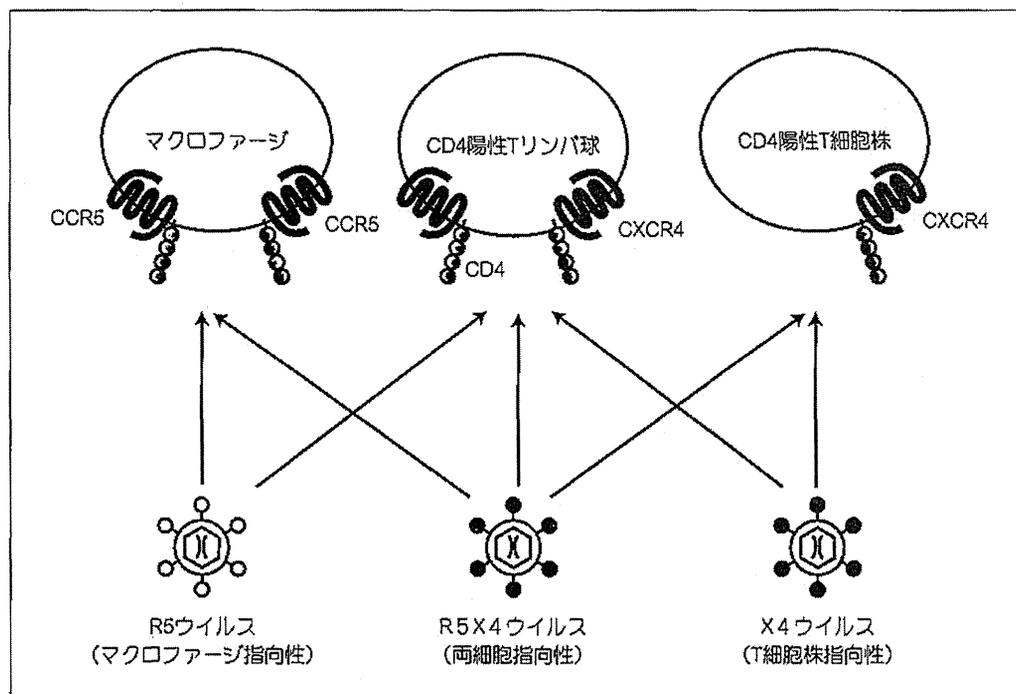

図 1 HIV の補助受容体利用性と細胞指向性
マクロファージ指向性ウイルスは T リンパ球とマクロファージの表面に存在する CCR5 を使用して感染することから R5 ウイルスと呼ばれている。T 細胞株指向性ウイルスは T リンパ球や T 細胞株に存在する CXCR4 を使用して感染することから X4 ウイルスと呼ばれている。

（筆者作成）

GPCR (G protein-coupled receptors；G タンパク質共役型受容体)

CXCR4 をメジャー補助受容体と呼んでいる。現在では，このような補助受容体利用能の差異を指標として HIV の機能的分類がなされており，CXCR4 を補助受容体として利用するものを X4 ウイルス，CCR5 を利用するものを R5 ウイルス，両者を利用する能力をもつものを R5X4 ウイルスと呼んでいる。したがって，感染初期に分離される，当初，マクロファージ指向性ウイルスと呼ばれていたウイルスが R5 ウイルスであり，感染後期，特に AIDS 期に分離される T 細胞株指向性ウイルスが X4 ウイルス，両細胞指向性ウイルスが R5X4 ウイルスということになる。

II CCR5Δ32 の発見

感染のハイリスクでありながら感染抵抗性である個体 (exposed-uninfected：EU) や，一方，感染しているにもかかわらず長期にわたって AIDS 発症から免れている長期未発症者 (long-term non-progressor：LNTP) の存在が補助受容体発見以前より報告されていたが，この原因の一部が CCR5 の遺伝的欠損で説明できることが，HIV の補助受容体としての CCR5 発見からほどなくして明らかとなった[2][3]。これらの感染抵抗性の個体においては CCR5 遺伝子中に 32 塩基の欠損がホモ接合型で存在しており (CCR5Δ32)，その結果，CCR5 のコドンにフレームシフトが起こり，結果として異なるアミノ酸が翻訳された後，途中で翻訳が止まってしまっていた (premature termination) (図2)。このような CCR5 は小胞体で停滞してしまい膜上に発現しないため (図3)，CCR5 を利用する R5 ウイルスが感染できなくなっていたのである。
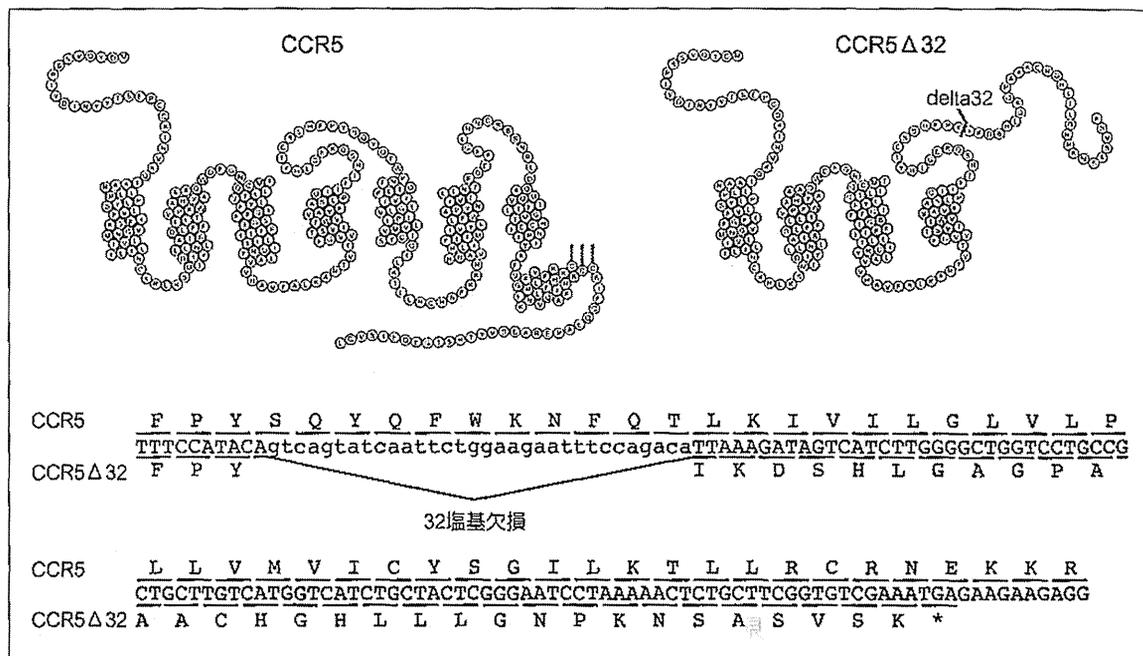

図2　CCR5 分子の構造と 32 塩基欠損

ケモカイン受容体である CCR5 は 7 回膜貫通タンパク質であり，細胞外に N 末端領域と 3 つの細胞外ループ領域を有する。HIV の感染には N 末端領域と第 2 細胞外ループが重要と考えられている。CCR5Δ32 は第 2 細胞外ループに当たる領域で CCR5 遺伝子が 32 塩基欠損しており，フレームシフトの結果，異なるポリペプチド鎖が翻訳され途中で終始してしまい，HIV の感染を支持しない分子になる。

(文献 3 より改変)

EU (exposed-uninfected)　　　　　　　　　　　　　LNTP (long-term non-progressor；長期未発症者)

	遺伝型	HIV 感受性
WT 小胞体	WT/WT	あり
Δ32	Δ32/WT	あり（AIDS 発症の遅延）
	Δ32/Δ32	高度耐性
m303	Δ32/m303	高度耐性

図3　CCR5Δ32 の細胞表面への発現抑制と HIV 感染感受性との関係
野生型 (WT) CCR5 は細胞表面へ発現するが，CCR5Δ32 は細胞表面に発現
せず，小胞体に停留している。両者のヘテロの個体では野生型 CCR5 の細胞表
面への発現はやや減弱するものの，HIV 感染感受性である。*m303* も *Δ32* と同
様に細胞表面に発現せず，*Δ32* とのヘテロの個体でのみ感染抵抗性が付与され
る。

(文献4より)

　興味深いことに，このような変異をホモでもつ個体は一般には何らかの病的素因を有していることが多いが，これらの個体では一見健康であった。

　一方，*CCR5Δ32* をヘテロでもつ個体では感染リスクの低下はないものの，AIDS 発症の遅延が明らかとなった（図3）[4]。その機序としては，CCR5Δ32 の発現が通常の CCR5 の発現を低下させている結果，ウイルスの侵入効率や増殖効率が減弱していることが考えられている。また，この多型には人種差が認められており，欧米を中心

としたコーカサス人（白人）では，約1％がホモで，9〜20％がヘテロでこの変異を保持しているが，アフリカ，アジアではこの変異の頻度はきわめて低いことが明らかとなった。このことは，HIV の発見以前の過去の何らかの脅威によりこれらの変異が地域的に選択を受けたことを意味しているが，どのような脅威であったのかは憶測の域を出ない。

　一方，このような個体ではもうひとつの主要な補助受容体である CXCR4 の発現は正常であり，R5X4・X4 ウイルスは感染可能であったにもかか

わらず HIV 感染から逃れることができているわけであり，ヒトからヒトへの感染では R5 ウイルスのみが感染を成立させるという発見につながった。

ところで，CCR5 の発現が消失している Δ32 をホモで有する個体が一見健康であるということは，CCR5 を標的とした感染予防や治療法が可能であるということを意味している。実際，種々の CCR5 に結合する薬剤が開発され，その中でマラビロクがすでに臨床で使用されている。宿主側を標的として開発される薬剤であることから耐性ウイルスの誘導が起こりにくいことが期待されたが，患者の中に CXCR4 を使用するウイルスが含まれている場合はそのようなウイルスを選択してしまうこと，さらには CCR5 阻害剤耐性ウイルスの出現も報告されている。また最近，CCR5Δ32 個体ではウエストナイルウイルス感染からの発症率が高いことが明らかにされ[5]，やはりこれらの個体も何らかの病的素因を有していることが明らかとなってきている。

III CCR5 のその他の多型

Δ32 以外に CCR5 のアミノ酸をコードしている領域において種々の多型が報告されており[6][7]，その多くは CCR5 の立体構造に影響を与えているものの，これらは比較的まれな多型であり，また，ホモの個体は観察されないため病態との関連は明らかにされていない。

アジアで比較的多いものとしては，G106R，C178R，R223Q，K26R，FS299（299 番のフレームシフト）などがあり，アフリカで多い多型としては，Y339Q，R60S などが報告されている。この中で，G106R や K26R はケモカインとの結合障害や HIV の感染抵抗性が報告されている。また，C20S や C269S の多型ではこの両者でのジスルフィド結合が障害を受けるため，細胞表面での発現低下やケモカインとの結合，HIV に対する補助受容体機能の障害が報告されている。アフリカに多い A29S は MCP-2 との結合は保持されているものの，RANTES，MIP-1α，MIP-1β との結合能を失っている。

また，C101X (m303) や FS299 は Δ32 と同様に，CCR5 の翻訳途中での終止コドンの挿入により機能的 CCR5 の発現が抑制される。FS299 はアジアに多い多型であり，野生型 CCR5 発現の低下をともなうことが報告されているものの，コホート研究では HIV 感染への影響はないとされている。C20S や C101X (m303)[8] では Δ32 のアリルを有する個体の他方のアリルに見出されるときに HIV に対して防御的に働いているものと考えられている（図3）。

IV CCR5 プロモーター領域の多型

CCR5 のプロモーター領域（図4）の変異により CCR5 の発現量に違いがあることが報告された。特にコーカサス人においては6つの主要なハプロタイプが示され，この中で CCR5P1 と呼ばれるハプロタイプをホモでもつ個体において AIDS に急速に進行することが示された[9]。しかしながら，CCR5 の発現量においてはその他のハプロタイプに比較して顕著な違いは認められなかった。

一方，CCR5 の最初のイントロンにある多型である 59029A/G（図4）の中で，59029-A/A 個体において急速に AIDS 発症に至ることが報告された[10]。59029-A/G（303A/G）多型は CCR5P1 の主要なアリルであり，この多型が CCR5P1 による病態との関連を説明しているのかもしれない。しかしながら，このようなハプロタイプと CCR5 の発現量についての関連については必ずしも一致しているわけではなく，AIDS 発症との関連は第3番染色体に存在する未知の遺伝子の単なる遺伝的マーカーとして存在しているだけなのかもしれない。現在は次の項目 V で示す CCR2-64I と，前述の CCR5Δ32 を含めたハプロタイプ（HHA-HHG）（図4）[11] により解析がなされている。

V その他のケモカイン受容体の多型

マイナー補助受容体の中で HIV 感染病態との関連が報告されているケモカイン受容体としては CCR2 と CX₃CR1 がある。CCR2 の 64 番目のアミノ酸（第1膜貫通領域に位置する）は通常バリンであるが，イソロイシンに置換しているアリル

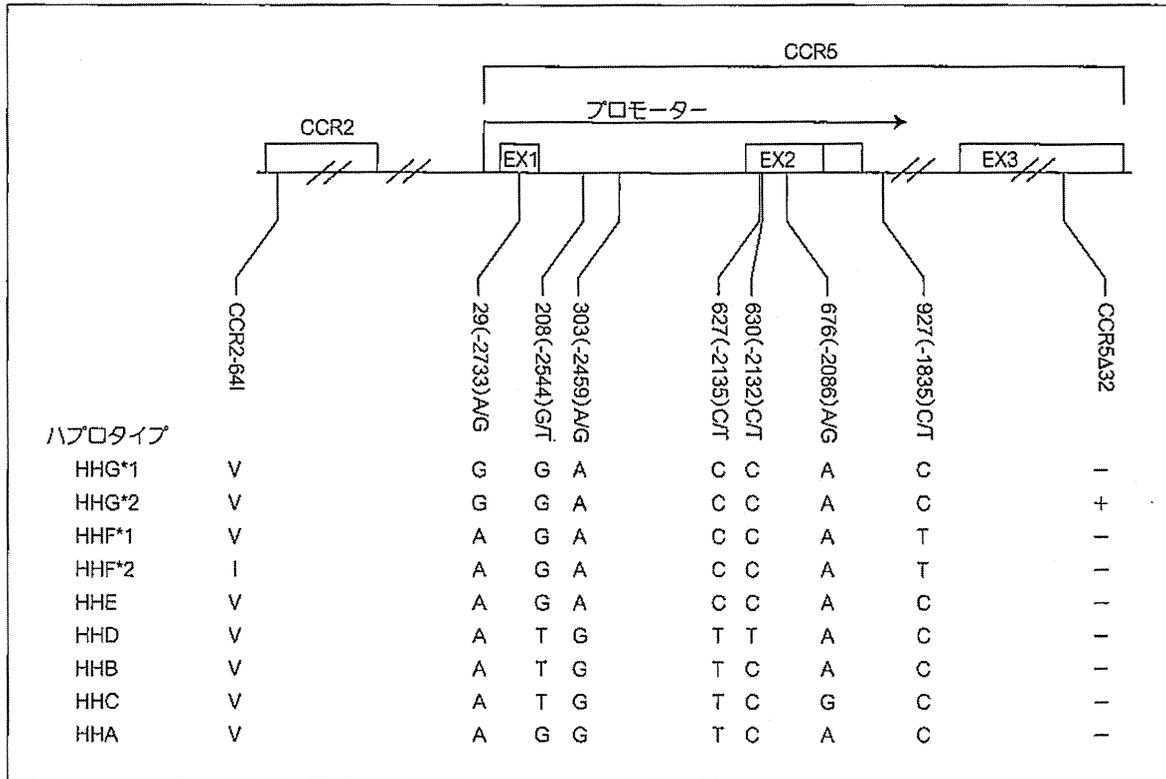| ハプロタイプ	CCR2-64I	29(-2733)A/G	208(-2544)G/T	303(-2459)A/G	627(-2135)C/T	630(-2132)C/T	676(-2086)A/G	927(-1835)C/T	CCR5Δ32
HHG*1	V	G	G	A	C	C	A	C	−
HHG*2	V	G	G	A	C	C	A	C	+
HHF*1	V	A	G	A	C	C	A	T	−
HHF*2	I	A	G	A	C	C	A	T	−
HHE	V	A	G	A	C	C	A	C	−
HHD	V	A	T	G	T	T	A	C	−
HHB	V	A	T	G	T	C	A	C	−
HHC	V	A	T	G	T	C	G	C	−
HHA	V	A	G	G	T	C	A	C	−

図4 CCR2, CCR5 遺伝子の構造とハプロタイプ

CCR2 と CCR5 は染色体 3p21.3 に存在し，さらに CCR2 は CCR5 の 17.5kb 上流に存在することから両者の連鎖が考えられている。CCR2 には病態の遅延に関連するアリルである 64I，CCR5 のアミノ酸をコードする領域には HIV 感染抵抗性と関連する Δ32 があり，その上流に存在する CCR5 のプロモーター領域を含む非翻訳領域にはいつくかの多型が報告されており，ハプロタイプを形成している。

（文献 11 より改変）

(V64I)（図4）は人種差なく 10 ～ 20%でみられる。この変異は HIV の感染感受性にはまったく影響を与えないものの，CCR2-64I をヘテロで有する個体において AIDS への進行が遅延することが報告された[12]。確かに CCR2 は一部の株において補助受容体として機能していることが報告されているものの，マイナー補助受容体であり，CCR5Δ32 のような補助受容体の機能障害によってこの病態遅延が説明できるとは考えにくい。CCR2 は CCR5 と同じく染色体 3p21.3 に存在し，さらに CCR5 の 17.5kb 上流に存在することから（図4），前述した CCR5 のプロモーター領域など CCR5 のイントロン領域の変異との連鎖が示唆されており，このような連鎖不平衡な関係から CCR5 の発現量に影響を与えている可能性がある。

一方，コーカサス人において同じくマイナー補助受容体として機能している CX3CR1 （Fractalkine）のハプロタイプ I249/M280 をホモで有する個体では，短期に AIDS 発症に至ることが報告された[13]。CCR2 と同様に HIV の感受性に影響を与えているとは考えにくく，またその後，明らかな有意差が認められておらず，さらなる検証が必要と考えられる。

また，HIV の補助受容体としては機能しないが，三日熱マラリアの赤血球内侵入に重要であると考えられているケモカイン受容体 DARC（duffy an-

DARC (duffy antigen receptor for chemokines)

tigen receptor for chemokines) の HIV の病態への関与が示された。DARC は赤血球表面に発現しており RANTES (regulated on activation, normal T cell expressed and secreted) をはじめとして種々のケモカインとの結合能を有していることから，これらのケモカインの血中レベルを制御していることが想定されている。実際に *DARC* の *T-46C* 多型はアフリカに多く，DARC の細胞表面発現が低く，病態進行の遅延がアフリカにおけるコホート研究で示されたが[14]，欧米におけるコホート研究では逆の結果が示されており，結論に至っていない。

VI *SDF-1 3' A*

もうひとつのメジャー補助受容体である CXCR4 の立体構造に変容をきたす可能性のある多型は現時点では認められない。これは，CXCR4 が発生過程で重要な分子であることと，リガンドである SDF-1 と1対1の関係であり，他のケモカイン受容体ではその機能が代替できない唯一無二の分子であることに由来していると考えられる。しかしながら，*SDF-1* の非翻訳領域である 3' 領域 (ATG 開始コドンの上流 801bp) には多型があり，*SDF-1 3' A/3' A* において AIDS 発症の遅延が報告された。この多型は *CCR5Δ32* と違って逆にアジアに多く (25 ～ 35%)，特にオセアニアで高い (50 ～ 70%)。

この変異は *SDF-1* の mRNA の安定性や翻訳効率に関与している可能性が示唆され，結果として，SDF-1 の発現増大から CXCR4 を使用する HIV の感染を阻害することが想定されたが，実験ではそのようなデータは得られていない。実際には逆に AIDS の進行を促進するという報告もあり，HIV の自然経過や AIDS 発症に対する影響はないものと現時点では結論されている。

VII MIP1α P/CCL3L1

ケモカイン受容体である CCR5 に結合する正常リガンドとしては，CCL3 (MIP-1α)，CCL4 (MIP-1β)，CCL5 (RANTES) 等があり，これらは R5 ウイルスの感染を阻害する活性をもっている。このうち CCL3 には通常の CCL3 (MIP-1α，LD78α) の他に CCL3L1 (MIP-1α P, LD78β) と呼ばれる isoform があり，遺伝子増幅 (gene duplication) の結果，*CCL3L1* 遺伝子のコピー数は2～10個と個体によって異なることが明らかとなった。CCL3L1 は特に細胞表面に存在する CD26/DPP IV (dipeptidyl peptidase IV) によってその N 末端の2アミノ酸が切断され，R5 ウイルスの感染阻害効果が通常の CCL3 に比較して30倍強いことことから，感染感受性や病態遅延に関与している可能性が考えられた。

実際に，*CCL3L1* のコピー数が低い個体で感染の危険性が高いという知見や[15]，感染感受性の違いは認められなかったものの，*CCL3L1* のコピー数が低い感染者ではウイルスコピー数が高値で CD4 陽性 T 細胞数の減少速度が速いことが報告された[16]。CCR5 の多型だけでなく，リガンド遺伝子のコピー数も CCR5 の細胞表面での発現や，HIV や AIDS の防御作用があることが示された。

VIII おわりに

HIV の感染を支持するケモカイン受容体変異により HIV 感染感受性や病態進行にかかわっている分子は，現時点では R5 ウイルスの補助受容体である *CCR5* 遺伝子の 32 塩基欠損のみである。*Δ32* に関しては *in vitro* の実験と *in vivo* における感染抵抗性や病態進行遅延等の疫学的データがほぼ一致しており，科学的に証明されたと思う。しかしながら，その他の多型に関してはデータが蓄積されるに従い有意性を失っていく傾向にある。ヒトゲノム配列決定のスピードとコストが改善されてきていることもあり，今後，より大規模なコホート研究や過去の研究のメタ解析，さらにはより精度の高い解析手法等の開発により感染・病態制御にかかわる新たな発見がなされ，その結果が HIV の治療や病態解明に資することを期待したい。

RANTES (regulated on activation, normal T cell expressed and secreted)

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Short communication
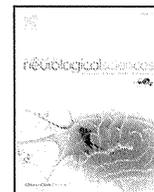
Failure of mefloquine therapy in progressive multifocal leukoencephalopathy: Report of two Japanese patients without human immunodeficiency virus infection

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ABSTRACT

Although progressive multifocal leukoencephalopathy (PML) cases showing responses to mefloquine therapy have been reported, the efficacy of mefloquine for PML remains unclear. We report on the failure of mefloquine therapy in two Japanese patients with PML unrelated to human immunodeficiency virus. One of the patients was a 47-year-old male who had been treated with chemotherapy for Waldenström macroglobulinemia, and the other was an 81-year-old male with idiopathic CD4+ lymphocytopenia. Diagnosis of PML was established based on MRI findings and increased JC virus DNA in the cerebrospinal fluid in both patients. Mefloquine was initiated about 5 months and 2 months after the onset of PML, respectively. During mefloquine therapy, clinical and radiological progression was observed, and JC virus DNA in the cerebrospinal fluid was increased in both patients. Both patients died about 4 months and 2 months after initiation of mefloquine, respectively. Further studies are necessary to clarify the differences between mefloquine responders and non-responders in PML.

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1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a brain disorder caused by JC polyomavirus, which causes death in one-half of patients within 1 year [1]. Primary infection usually occurs during childhood and is often asymptomatic. The initial site of JC virus (JCV) infection is thought to be the tonsils, and it is then carried by lymphocytes to the kidneys and bone marrow. Reactivation of JCV occurs due to severe cellular immunodeficiency, and the virus crosses the blood–brain barrier (BBB) and infects oligodendrocytes, causing widespread demyelinating lesions. A recent study revealed promyelocytic leukemia nuclear bodies as an intranuclear target of JCV [2].

A study of 9675 cases of PML between 1998 and 2005 showed that 82% of patients had human immunodeficiency virus (HIV), 8.4% hematologic malignancies, 2.83% solid organ cancers, and 0.44% rheumatologic diseases [3]. Recently, a new category of PML patients has emerged among patients treated with immunomodulatory medications including natalizumab, rituximab, and efalizumab. PML may also occur in patients with minimal or occult immunosuppression including idiopathic CD4+ lymphocytopenia [4]. In Japan, the proportion of hematological malignancies or rheumatologic diseases as underlying diseases is relatively high, whereas that of HIV infection is low [5,6].

The estimated probability of survival at 1 year is reported to be 52% in HIV related PML [1] and variable in PML unrelated to HIV among reports. Some patients with PML do survive for extended periods of time after diagnosis [7,8]. Survival in PML is influenced by the presence of JCV-specific cytotoxic T-lymphocytes, CD4+ cell counts, or JCV DNA levels [1,9]. One study reported that estimated 1-year survival was 48% in patients with HIV related PML with CD4+ cell counts<200/μl at PML diagnosis compared to 67% in those with CD4+ cell counts>200/μl [1]. Another study showed that JCV DNA levels>4365 copies/ml of cerebrospinal fluid (CSF) correlated significantly with shorter survival in patients with HIV related PML not receiving highly active antiretroviral therapy (HAART) [9].

To date, although antiviral drugs such as cytarabine and cidofovir show activity against JCV in vitro [10,11], large clinical studies have failed to establish the efficacies of these drugs in the treatment of PML [12–14]. The reason for this may be that these drugs are not able to cross the BBB and accumulate throughout the entire brain parenchyma at a dose sufficient to suppress JCV proliferation [15].

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In 2008, mefloquine, an anti-malarial drug, was reported to show activity against JCV *in vitro* [15]. Since then, there have been at least 5 reported cases of PML in which mefloquine was effective [16–20]. In contrast, a recent mefloquine trial of 24 patients with PML (21 HIV-positive and 3 HIV-negative) reported failure in reducing JCV DNA levels in the CSF [21], although it is pending publication. Because there have been no reports describing the details of PML patients demonstrating mefloquine treatment failure, we report two HIV-negative patients with PML in whom mefloquine was not effective.

2. Case reports

2.1. Case 1

A 47-year-old male presented with progressive left hemiparesis. The patient had been treated with chemotherapy including rituximab for Waldenström macroglobulinemia for six years in our hospital. The interval between the last administration of rituximab and occurrence of hemiparesis was about 1 month. Diffusion weighted images (DWI) of brain MRI about 3 months after the onset of hemiparesis demonstrated high intensity areas with internal low intensity areas in the white matter of the right frontal lobe. The apparent diffusion coefficient (ADC) values of the lesion were increased. Because serum IgM had been prominently elevated (around 5000 mg/dl) in association with Waldenström macroglobulinemia, we presumed that the hyperviscosity syndrome resulted in brain infarction.

About 4 months after the onset of hemiparesis, the patient was admitted to our hospital because a convulsion occurred in the left upper and lower limbs. At that time, the patient did not receive any immunosuppressive therapy. On admission, neurological examination revealed upper limb-dominant left hemiparesis, and Babinski's sign and Chaddock's reflex on the left. MRI on admission demonstrated lesion expansion and extension to the right parietal and insular white matter, right putamen, right internal capsule, right thalamus, corpus callosum, left frontal white matter, and midbrain. There was no edema or gadolinium-enhanced lesions. Peripheral blood tests showed white blood cell count (WBC): 3790/μl (normal range: 4500–9000), hemoglobin: 10.4 g/dl (normal range: 13–16), and platelet count: 3.7×10^4/μl (normal range $15–30 \times 10^4$), indicating pancytopenia. C-reactive protein (CRP) was below 0.1 mg/dl. Testing for HIV was negative. On the next day of admission, a nasogastric feeding tube was inserted because of dysphagia. Four days after admission, CSF examination demonstrated cell count: 1 cell per 3 μl, total protein: 97 mg/dl, and glucose: 67 mg/dl. PCR was positive for JCV DNA in the CSF and detected 1200 copies/ml of DNA. A diagnosis of PML

was established based on MRI findings and increased JCV DNA in the CSF.

After diagnosis, the patient developed right hemiparesis and apraxia of speech. Brain MRI 18 days after admission demonstrated lesion expansion and extension to the left insular white matter and left putamen (Fig. 1A). The JCV DNA copy number in the CSF was increased to 4300 copies/ml. CD4$^+$ cell count of the peripheral blood was 219/μl (normal range: 500–1300). Nineteen days after admission, about 5 months after the onset of PML, mefloquine was initiated at a dose of 275 mg/day orally for 3 days, followed by 275 mg once a week [17]. We used Mephaquin Hisamitsu tablets (Hisamitsu Pharmaceutical, Tosu, Japan), which show maximum concentration (C_{max}) of 3.1 μM, time at which C_{max} is observed (T_{max}) of 5.2 h, and terminal half-life (T1/2) of 400.1 h when 1100 mg of drug is once administered. Treatment with mefloquine was approved by the Ethics Committee in our hospital. We obtained written, informed consent from the patient's family. We also used 1 mg/day of risperidone, a 5HT2A receptor blocker at the same time. After initiation of mefloquine, we observed no symptoms suggestive of mefloquine neurotoxicity such as nausea, dizziness, sleep disturbances, anxiety, and psychosis [22]. Eight days after initiation of mefloquine, the JCV DNA copy number in the CSF was increased to 150,000 copies/ml, and the dose of mefloquine was returned to 275 mg/day for 3 days per week (Fig. 2).

However, the JCV DNA copy number in the CSF 22 days after initiation of mefloquine was increased to 850,000 copies/ml. Because of severe aspiration pneumonia, tracheotomy was performed 37 days after initiation of mefloquine. Brain MRI 38 days after initiation of mefloquine demonstrated lesion expansion and extension to the right temporal and occipital white matter and pons (Fig. 1B). The JCV DNA copy number in the CSF 50 days after initiation of mefloquine increased to 3,700,000 copies/ml. Changes in the JCV DNA load are shown in Fig. 2. Brain MRI about 3 months after initiation of mefloquine demonstrated lesion expansion and extension to the left temporal and parietal white matter, left internal capsule, left thalamus, and medulla oblongata (Fig. 1C). The patient died of respiratory failure about 4 months after initiation of mefloquine. The total clinical course of PML was about 9 months. Autopsy could not be performed.

2.2. Case 2

An 81-year-old male with a three-week history of gait disturbance presented with muscle cramp in the bilateral upper limbs and was taken to another hospital by ambulance. Past medical history included hypertension, hyperuricemia, chronic heart failure, and chronic renal failure due to renal sclerosis. A diagnosis of brain infarction of the
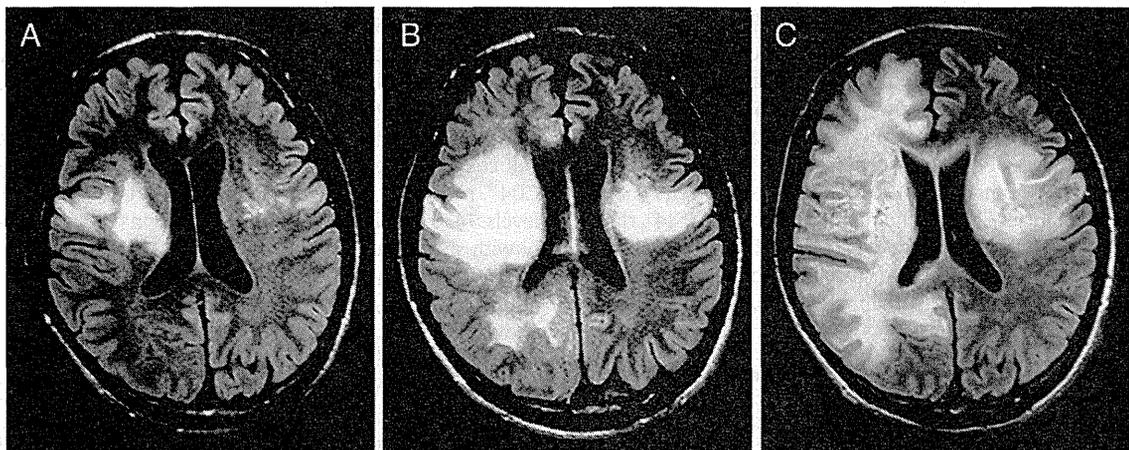
Fig. 1. A. Fluid-attenuated inversion recovery (FLAIR) sequence of brain MRI before initiation of mefloquine demonstrated high intensity areas in the white matter of the bilateral frontal lobes. B, C. FLAIR sequence of brain MRI 38 days (B) and about 3 months (C) after the initiation of mefloquine showed lesion expansion.
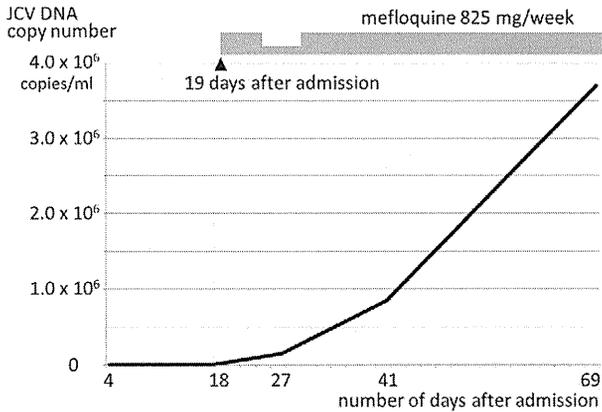
Fig. 2. Changes in the JCV DNA load of case 1 are shown. The JCV DNA copy number in the CSF was increased even after initiation of mefloquine.

subacute phase and the worsening of renal failure was made in the emergency room and the patient was transferred to our hospital.

Physical examination on admission demonstrated muscle cramp in the bilateral upper limbs and face, and right hemiparesis. Consciousness was slightly disturbed, but the orientation to time and place was preserved. Peripheral blood tests showed WBC: 5240/µl, hemoglobin: 7.8 g/dl, platelet count: 17.6×10^4/µl, albumin: 2.7 g/dl (normal range: 3.9–4.9), blood urea nitrogen (BUN): 147 mg/dl (normal range: 8–20), creatinine: 7.83 mg/dl (normal range: 0.6–1.1), creatinine kinase (CK): 445 IU/l (normal range 50–200), CRP: 0.3 mg/dl (normal range <0.1), and glucose: 103 mg/dl. Testing for HIV was negative. Hemodialysis was started on the next day of admission.

DWI of brain MRI 3 days after admission demonstrated high intensity areas in the white matter of the left frontal and parietal lobes and right parietal lobe. ADC values of the lesions were increased. Right hemiparesis progressed after admission, and 18 days after admission, the left hemiparesis emerged. Because of dysphagia, a nasogastric feeding tube was inserted 19 days after admission. CSF examination 20 days after admission demonstrated cell count: 6 cells per 3 µl, total protein: 35 mg/dl, and glucose: 60 mg/dl. PCR was positive for JCV DNA in the CSF, and detected 2223 copies/ml of DNA. A diagnosis of PML was established based on MRI findings and increased JCV DNA in the CSF.

Brain MRI 32 days after admission demonstrated lesion expansion and extension to the corpus callosum and right frontal white matter.

Thirty six days after admission, the patient manifested akinetic mutism. Thirty eight days after admission, about 2 months after the onset of PML, mefloquine was initiated at a dose of 275 mg/day orally for 3 days per week. Treatment with mefloquine was approved by the Ethics Committee in our hospital. We obtained written, informed consent from the patient's family. At that time, the JCV DNA copy number in the CSF was increased to 2,790,000 copies/ml. The CD4+ cell count of the peripheral blood was 294/µl. Because whole body CT demonstrated no mass lesions or abnormal lymph node swelling, underlying diseases causing immunodeficiency remained unclear in this patient.

After initiation of mefloquine, we observed no acute neurological deterioration suggesting mefloquine neurotoxicity. Brain MRI 15 days after initiation of mefloquine demonstrated lesion expansion and extension to the bilateral temporal and occipital white matter (Fig. 3A). Twenty nine days after initiation of mefloquine, the JCV DNA copy number in the CSF was increased to 24,075,000 copies/ml. Changes in the JCV DNA load are shown in Fig. 4. Brain MRI 31 days after initiation of mefloquine demonstrated lesion expansion (Fig. 3B). Thirty three days after initiation of mefloquine, hemodialysis was discontinued because of hypotension. The patient died 19 days later. The total clinical course of PML was about 4 months. Autopsy could not be performed.

3. Discussion

Because there is no known specific antiviral agent against JCV, we treated PML in the two HIV-negative patients with mefloquine based on case reports describing the efficacy of mefloquine for PML [16–20]. However, during mefloquine therapy, clinical and radiological progression was observed, and JCV DNA in the CSF was increased in both patients.

Our case 1 had been treated with chemotherapy including rituximab for Waldenström macroglobulinemia. The interval between the last administration of rituximab and diagnosis of PML was about 6 months. Although it is difficult to exclude the possibility that the immunodeficiency due to Waldenström macroglobulinemia itself was related to the occurrence of PML [23], rituximab is well known to cause PML [7]. Rituximab is an anti-CD20 monoclonal antibody that targets human B cells. The pathogenesis of rituximab in PML is considered to decrease B cells in the cerebral perivascular spaces, resulting in decreased antigen presentation to T cells and subsequent alterations in the cellular immune response [7]. One study reported that a median CD4+ cell count was 216/µl in 25 patients who received rituximab [24]. The interval between the last administration of rituximab and
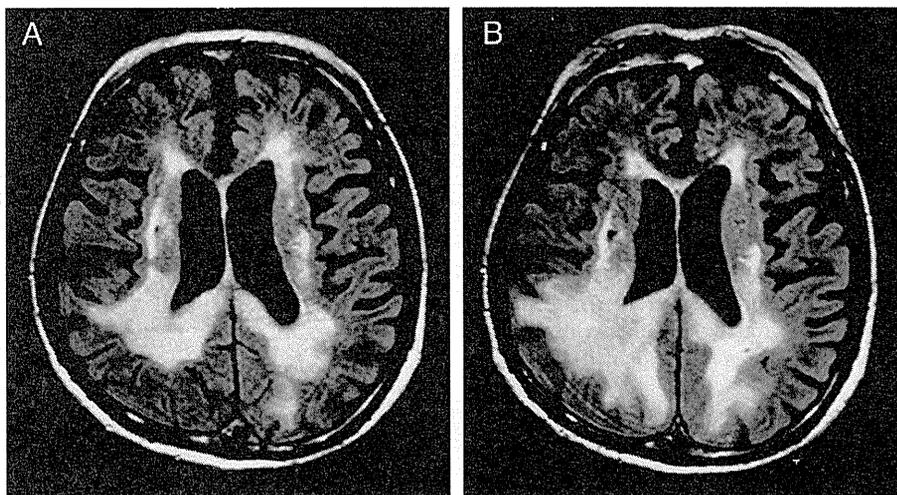

Fig. 3. A. FLAIR sequence of brain MRI 15 days after initiation of mefloquine demonstrated abnormal high intensity areas in the bilateral temporal and occipital white matter.
B. FLAIR sequence of brain MRI 31 days after the initiation of mefloquine showed lesion expansion.
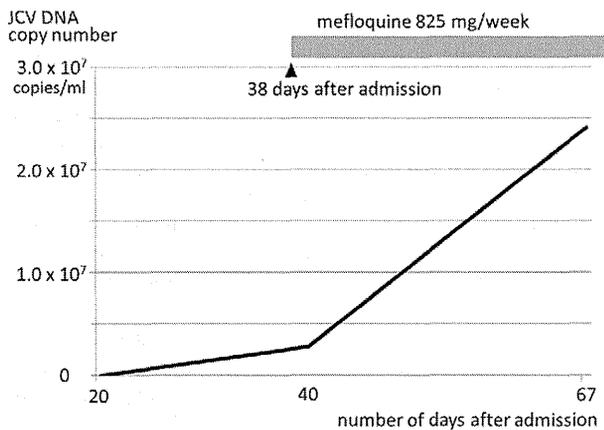
Fig. 4. Changes in the JCV DNA load of case 2 are shown. The JCV DNA copy number in the CSF was increased even after initiation of mefloquine.

diagnosis of PML has been reported to be 5.5 months [25]. Considering that 90% of patients with PML after rituximab therapy die [25], the unfavorable clinical course of our case 1 may be associated with the use of rituximab. In case 2, while CD4$^+$ lymphocytopenia was documented, there were no underlying diseases causing immunodeficiency. However, as PML may occur in patients with minimal or occult immunosuppression [4], idiopathic CD4$^+$ lymphocytopenia may be associated with the occurrence of PML in this patient.

Mefloquine is an anti-malarial drug used both for prophylaxis and treatment of chloroquine resistant *Plasmodium falciparum*. Because mefloquine is highly lipophilic and has a long terminal half-life of more than 1 week [26], a single dose of 15–25 mg/kg is used for treatment and 250 mg/week for prophylaxis. Among subjects administered 250 mg weekly, blood concentrations vary between 1 μM to 5 μM [27]. Mefloquine readily crosses the BBB, where active efflux by the P-glycoprotein membrane transporter prevents its accumulation in the brain [27].

In 2008, mefloquine was reported to show activity against JCV *in vitro* [15]. Brickelmaier et al. showed that mefloquine inhibits viral DNA replication, using quantitative PCR to quantify the number of viral copies in cultured cells. In this study, mefloquine reduced the number of infected cells by 50% or more at a concentration of 3.9 μM [15]. Brickelmaier et al. presumed that efficacious concentrations of mefloquine for PML are achieved in the brains of patients receiving approved doses of the drug [15].

Since the publication by Brickelmaier et al. [15], there have been at least 5 reported cases of PML in which mefloquine was effective [16–20]. The underlying diseases or conditions included sarcoidosis [16], umbilical cord blood transplant [17], HIV infection [18], and systemic lupus erythematosus [19]. CD4$^+$ cell counts in the peripheral blood of patients were described in 3 reports, and were 187/μl [18], 419/μl [17], and 420/μl [16], respectively. JCV DNA loads in the CSF before mefloquine therapy were available in these reports, and were 33,700 copies/ml [16], 535,500 copies/ml [18], and 911,175 copies/ml [17], respectively. The intervals between symptom onset and initiation of mefloquine therapy were about 3 months [17,19], 5 months [18], and 6 months [16,20], respectively. In 4 reports [16–19], the authors stated that PCR for JCV in the CSF became negative after mefloquine therapy. At present, the patients' background or laboratory data common among these cases showing responses to mefloquine therapy is unclear.

In contrast to these cases, a recent mefloquine trial of 24 patients with PML (21 HIV-positive and 3 HIV-negative) reported failure in reducing JCV DNA levels in the CSF [21]. Participants took 250 mg of mefloquine 4 times daily, followed by 250 mg weekly. The failure of this trial and the poor outcome of our patients raise the possibility that the improvement observed in mefloquine therapy in reported

PML patients [16–20] may actually reflect the natural favorable course of those patients.

At present, we cannot tell the difference in patient backgrounds or laboratory data between patients showing responses to mefloquine [16–20] and our patients. Regarding the presence of both mefloquine responders and non-responders in PML, Nevin stated that responses to mefloquine may correlate with polymorphisms in the *MDR1* gene coding for P-glycoprotein that affect drug efflux across the BBB [28]. In cases of unsuccessful treatment of PML, active efflux as a result of drug induced upregulation of P-glycoprotein expression in the BBB may be preventing therapeutic concentrations of mefloquine [28]. From this point of view, co-administration of P-glycoprotein inhibitors or substrates such as risperidone may be recommended in the treatment of PML [27]. On the other hand, considering the failure of the mefloquine trial and the poor outcome of our patients, re-evaluation of the anti-JCV activity of mefloquine may be required. If the anti-JCV activity of mefloquine is verified again, further studies are necessary to clarify whether the response to mefloquine in PML is influenced by the presence of HIV infection, CD4$^+$ cell counts, JCV DNA levels in the CSF, blood concentration of mefloquine, interval between disease onset and initiation of therapy, or *MDR1* polymorphism.

Conflict of interest statement

The authors have no conflicts of interest.

Acknowledgment

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blood

EZH2 overexpression in natural killer/T-cell lymphoma confers growth advantage independently of histone methyltransferase activity

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LYMPHOID NEOPLASIA

EZH2 overexpression in natural killer/T-cell lymphoma confers growth advantage independently of histone methyltransferase activity

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Key Points

- This study has uncovered an oncogenic role of EZH2 independent of its methyltransferase activity in NKTL.
- This study suggests that targeting EZH2 may have therapeutic usefulness in NKTL.

The role of enhancer of zeste homolog 2 (EZH2) in cancer is complex and may vary depending on the cellular context. We found that EZH2 is aberrantly overexpressed in the majority of natural killer/T-cell lymphoma (NKTL), an aggressive lymphoid malignancy with very poor prognosis. We show that EZH2 upregulation is mediated by MYC-induced repression of its regulatory micro RNAs and EZH2 exerts oncogenic properties in NKTL. Ectopic expression of EZH2 in both primary NK cells and NKTL cell lines leads to a significant growth advantage. Conversely, knock-down of EZH2 in NKTL cell lines results in cell growth inhibition. Intriguingly, ectopic EZH2 mutant deficient for histone methyltransferase activity is also able to confer growth advantage and rescue growth inhibition on endogenous EZH2 depletion in NKTL cells, indicating an oncogenic role of EZH2 independent of its gene-silencing activity. Mechanistically, we show that EZH2 directly promotes the transcription of cyclin D1 and this effect is independent of its enzymatic activity. Furthermore, depletion of EZH2 using a PRC2 inhibitor 3-deazaneplanocin A significantly inhibits growth of NK tumor cells. Therefore, our study uncovers an oncogenic role of EZH2 independent of its methyltransferase activity in NKTL and suggests that targeting EZH2 may have therapeutic usefulness in this lymphoma. (*Blood*. 2013;121(22):4512-4520)

Introduction

Nasal-type natural killer/T-cell lymphoma (NKTL) is an aggressive lymphoid malignancy associated with very poor survival outcomes.[1] A better understanding of the molecular abnormalities underlying this disease will provide important insights into biology of this tumor; however, studies on NKTL are often limited by the lack of adequate tissue in small nasal biopsies and the presence of necrosis in biopsy specimens. Although more effective therapy is now available, treatment is still completely reliant on radiotherapy and combinations of chemotherapy.[2,3]

We and others have recently performed whole-genome gene expression studies and identify a number of genes that are differentially expressed in NKTL as well as pathways that are activated in NKTL. Enhancer of zeste homolog 2 (EZH2), one of the genes identified in our study to be aberrantly overexpressed in NKTL,[4] is a H3K27-specific histone methyltransferase and a component of the polycomb repressive complex 2 (PRC2), which plays a key role in the epigenetic maintenance of repressive chromatin mark. EZH2 protein contains a catalytic domain (SET domain) at the

COOH-terminus that provides the methyltransferase activity. The catalytic domain must partner with other noncatalytic proteins, such as EED and SUZ12, to form the PRC2 in order to attain robust histone methyltransferase activity. Genome-wide approaches have demonstrated the importance of the PRC2 complex in the transcriptional regulation through H3K27 methylation and gene repression.[5]

Published literature reveals a number of possible mechanisms of EZH2 upregulation in different types of human cancers.[6] It has been shown that EZH2 expression can be transcriptionally activated by a fusion oncoprotein EWS-FLI1 in Ewing sarcoma.[7] EZH2 expression in the breast tumor–initiating cell population is particularly enhanced by hypoxia through HIF1a-mediated transactivation.[8] In addition to transcriptional regulation, the EZH2 transcript is known to be regulated by tumor suppressor micro RNAs (miRNAs). For example, miR-26a binds to and inhibits EZH2 transcript expression in B-cell lymphoma.[9] miR-101 is frequently lost in metastatic prostate cancer, thus releasing EZH2 from miR-101–mediated repression.[10] EZH2 can also be modulated by post-translational modifications through
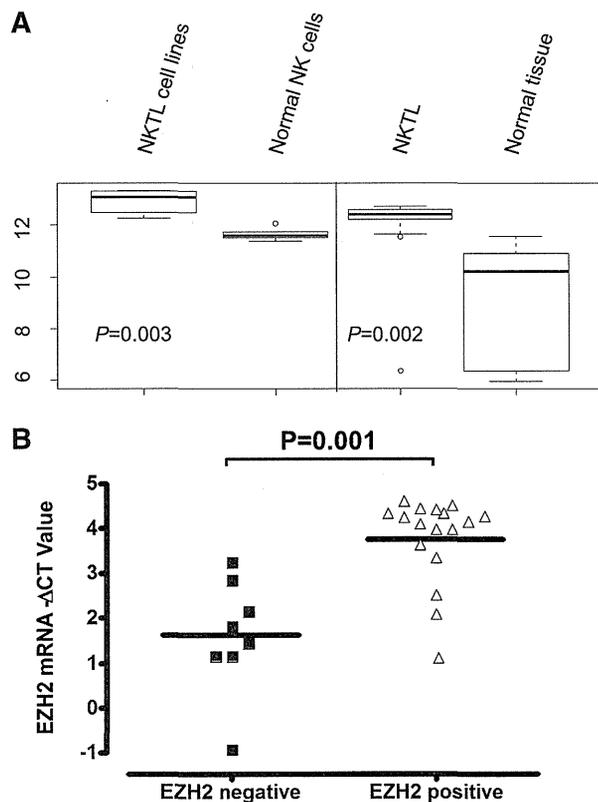
Figure 1. EZH2 mRNA levels are elevated in NKTL and cell lines. (A) Expression score for EZH2 mRNAs in NKTL GEP dataset. EZH2 gene expressions in NKTL FFPE samples were compared with that in respective normal FFPE tissue controls, as well as the NK cell lines and normal NK cells using significance analysis of microarray. (B) Correlation between EZH2 transcript levels determined by qRT-PCR and EZH2 protein levels measured by IHC for NKTL samples.

methyltransferase activity. Instead, EZH2 directly promotes cyclin D1 expression. Thus, this study demonstrates a noncanonic role of EZH2 in NKTL.

Methods

Immunohistochemistry

A total of 38 clinical cases of NKTL that fulfill the World Health Organization diagnostic criteria were used for immunohistochemistry (IHC) studies. The clinicopathologic data of the cases are included in supplemental Table 1. There were 27 cases that came from the tissue microarray used in our previous study (GEO accession no. GSE31377).[4] IHC analysis was performed for EZH2, Ki67, and H3K27me3 on 4-μm sections of NKTL using the conditions listed in supplemental Table 2. IHC study was also performed on cell blocks of normal NK cells for comparison. Appropriate positive tissue controls were used. Details of scoring and imaging are appended in the supplemental Methods.

Primary NK cell isolation and retroviral transduction

Highly purified (90%-99%) normal human NK cells were isolated and cultured as described previously.[19] Retroviruses were generated by transfection of empty plasmid vector polymorphonuclear neutrophil (pMN)-enhanced green fluorescence protein (EGFP) or vectors containing EZH2 using Fugene HD6 into Phoenix-amphotropic packaging cells. At 48 hours after transfection, the supernatants were collected and filtered. A total of 200 000 cells were mixed with 1.6 mL of retroviral supernatant in 12-well plates with 10 μg/mL of Polybrene added. The infection was repeated at 72 hours after transfection.

Luciferase reporter assay

The cyclin D1 (CCND1) promoter construct pGL4-CCND1-Luc has been described previously.[20] Cells were harvested 24 hours after transfection and were analyzed with the Dual Luciferase system (Promega). See the supplemental Methods and Materials for details.

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously.[21] See the supplemental Methods and Materials for details of the antibodies used and primer sequences.

Western blotting

Cells were lysed in radioimmunoprecipitation assay buffer and were subjected to sonication. The primary antibodies used included cell-signaling antibody EZH2 (4905), H3K27me3 (07-449), H3K27me2 (9755), Total H3 (9715) and SantaCruz antibody CCND1 (DCS-6 and A12), and cleaved poly (ADP-ribose)polymerase (PARP; F2, sc-8007).

Full methods are provided in the supplemental information.

Results

EZH2 is overexpressed in NKTL

In our previously published genome-wide gene expression profiling (GEP) of extranodal nasal-type NKTL,[4] the EZH2 transcript level was significantly higher in NKTL compared with normal NK cells (Figure 1A). In corroboration with the GEP findings, we observed a significant percentage of cases (61%) showing positive expression of EZH2 protein in the tumor cells in our 38 cases of NKTL (tissue microarrays or whole-tissue sections) by IHC studies (supplemental Figure 1; supplemental Table 3), whereas the normal NK cells only showed a minimal level of EZH2 (staining in ≤5% cells). Indeed,

phosphorylation by AKT and cyclin-dependent kinase.[11,12] To the best of our knowledge, the mechanism of EZH2 overexpression in NKTL has not yet been described.

A high level of EZH2 expression is associated with aggressiveness and poor outcome in solid tumors such as prostate, breast, and endometrial cancers. The oncogenic role of EZH2 overexpression in these tumor types has been studied extensively. In human B-cell malignancies, mutations of Y641 and A677 have been documented to be associated with profoundly increased activity for methylated H3K27, which may promote the development of lymphoma.[13-15] On the other hand, recent discoveries of recurrent somatic EZH2 mutations in myelodysplastic syndromes and myeloproliferative neoplasms indicate that inactivation of EZH2 may contribute to the pathogenesis of myeloid malignancies.[16,17] Genetic inactivation of EZH2 has also been identified in T-cell acute lymphoblastic leukemia, and the study by Ntziachristos and colleagues suggests a tumor suppressor role for EZH2 in human leukemia by a hitherto unrecognized dynamic interplay between oncogenic NOTCH1 and EZH2.[18] Taken together, the role of EZH2 and the underlying mechanisms of gene regulation by EZH2 in cancer are complex, and further studies need to be performed in a cell context–dependent manner.

In our study, we demonstrated the overexpression of EZH2 in NKTL, deciphered the molecular mechanisms underlying the overabundance of EZH2, and investigated its functional role as an oncogene in this disease. Contrary to our expectations, we found that EZH2 overexpression is not associated with H3K27 trimethylation in NKTL, and its oncogenic activity does not require its histone

Figure 2. Inhibition of EZH2 expression by miRNA-101 and miRNA-26, which are suppressed by MYC in NKTL cells. (A, left) MYC induction by tet-on. MYC overexpression was induced by treating cells with doxycycline. MYC expression was quantified by qRT-PCR analysis. (A, middle) Decreased levels of miR-101, miR-26a, and miR-26b in NKYS upon MYC induction by tet-on. (A, right) MYC induction by tet-on increases EZH2 mRNA levels. (B, left) Depletion of MYC by siRNAs results in induction of miR-101, mir-26a, and miR-26b transcription in NKYS cells. Cells were transfected with MYC siRNA or nontargeting siRNA as a control. Cells were harvested 48 hours after transfection for mRNA analysis of miR-101 and miR-26 gene levels by real-time PCR. (B, right) ChIP-qPCR for endogenous MYC binding to miR-101, miR-26a, and miR-26b genes. Fold enrichment in the ChIP experiment represents the signal obtained after MYC immunoprecipitation followed by qPCR amplified by primer pairs that spanned gene promoters. (C, left) Correlation between MYC activation index and EZH2 mRNA levels. Cross: Normal tissue; Plus sign: Normal NK; Triangle: NKTL; Circle: Cell Lines. R>0.95, $P < 2.2*10^{-16}$. (C, right) Scatterplot showing the correlation between IHC MYC staining and EZH2 expression. Spearman correlation coefficient r for MYC v EZH2 = 0.76; $P < .0001$.

EZH2-positive samples have significantly higher EZH2 messenger RNA (mRNA) levels (Figure 1B). These data confirm that EZH2 is overexpressed in NKTL at both the mRNA and protein levels.

Loss of miR-26 and miR-101 contributes to the EZH2 upregulation in NKTL

Next, we sought to identify the mechanisms leading to EZH2 upregulation in NKTL. The genomic locus containing EZH2 is not commonly amplified in NKTL.[22] In our previous miRNA expression–profiling study, we found several miRNAs that were predicted to target EZH2 by various computational algorithms (supplemental Table 4) to be downregulated in NKTL.[19] Among these, the expression of miR-26a, miR-26b, and miR-101 has a significant inverse correlation with EZH2 when our previous GEP and miRNA profiling data were analyzed. miRNAs negatively regulate protein translation by predominantly destabilizing and, hence, decreasing their target mRNA levels.[23] In prostate, muscle, and B-cell lymphoma, miR-101, miR-26a, and miR-26b can negatively regulate EZH2 expression by binding to the highly conserved predicted binding sites within the 3'UTR of EZH2.[9,10] Using lentiviral transduction to express miR-101, miR-26a, and miR-26b in NKYS (supplemental Figure 2A, left),

we observed that EZH2 expression was effectively attenuated (supplemental Figure 2A, right). These data suggest that EZH2 overexpression may be attributed to the deregulation of miR-101, miR-26a, and miR-26b in NK tumor cells.

MYC activation suppresses the expression of miR-26 and miR-101 in NKTL

As the genomic loci containing miR-101, miR-26a, and miR-26b are not recurrently deleted in the NKTL (data not shown), we looked for other mechanisms for their repression. On the basis of our previous study of gene expression in NKTL, which showed MYC activation,[4] and a recent paper showing that MYC activation can lead to repression of a many miRNAs in tumorigenesis,[24] we investigated whether MYC is involved in the suppression of miR-101 and miR-26 in malignant NK cells. When MYC expression was induced in NKYS cells using a tet-on system (Figure 2A, left), miR-101, miR-26a, and miR-26b was downregulated (Figure 2A, middle) with a corresponding (1.53-fold) increase in EZH2 mRNA (Figure 2A, right). Conversely, these 3 miRNAs were up-regulated by depletion of MYC using small interfering RNA (siRNA)–mediated knockdown (Figure 2B, left), and MYC depletion reduced EZH2 3'UTR luciferase reporter activity (supplemental

Figure 3. EZH2 overexpression in NKTL promotes cell growth independent of histone methyltransferase activity. (A) Primary NK cells transduced by EZH2 exhibit a growth advantage. Primary NK cells expressing ectopic EZH2 were monitored by a coexpressed GFP marker. Using our established viral infection protocol, we routinely obtained a transduction efficiency of 4.3% for normal NK cells. If EZH2 infection does not alter the cell growth, the EZH2-infected cell will not gain a growth advantage; thus, the percentage of GFP+ cells should remain at 4.3%. However, the percentage of GFP+ cells increased from approximately 4.3% at day 2 to ~34.4% at day 5, indicating an acquired growth advantage in these EZH2-infected cells. Antibiotic selection of positively infected cells was not done. (B) Scatterplot representation of the correlation between the percentage of Ki-67–positive cells and the percentage of EZH2-positive cells. Spearman correlation coefficient r for EZH2 v Ki67 = 0.73; $P < .0001$. (C) MTS proliferation assay showing that ectopic expression of EZH2 promotes cell growth of NKTL cell lines without requiring SET domain activity. Cells were cotransfected with pMAX-GFP and the control empty vector pcDNA4.1 or EZH2 expression plasmids. Cells transfected were subjected to proliferation assays for up to 96 hours. The cell growth (expressed as a percentage of the empty vector control) was determined by MTS assay as described in Materials and Methods. The mean values of triplicate samples are shown, and error bars indicate standard deviations. (D) Western blot analysis of EZH2, H3K27m3, and H3K27m2 in indicated samples. Expression of EZH2 WT and the SET-domain mutant was detected by the MYC-tag antibody. H3 was used as a loading control.

Figure 2B). However, this effect was still seen when the miR-101 and miR-26 binding sites in the EZH2 3'UTR were mutated, suggesting that other miRNAs or factors regulated by MYC may be involved. Furthermore, ChIP-quantitative polymerase chain reaction (qPCR) results revealed that MYC binds to the genomic locus of miR-26a and miR-26b (Figure 2B, right), consistent with previous reports.[24] Interestingly, it also revealed an association of MYC specifically with a conserved region upstream of miR-101 on chromosome 1 (Figure 2B, right). In summary, MYC activation suppresses the expression of miR-26a, miR-26b, and miR-101 in NKTL cell lines by direct binding to their genomic locus, and that MYC may stimulate EZH2 overexpression by repression of its negative regulatory miRNAs. Consistent with these in vitro findings, we observed a strong correlation between EZH2 transcript levels and MYC activation as measured by the gene expression–based MYC activation index[4] in our clinical GEP dataset (Figure 2C, left). In addition, there is also a positive correlation between the percentage of cells expressing EZH2 and nuclear MYC, which is a marker for MYC activation, detected by IHC analysis in NKTL tissue microarrays (Figure 2C, right). In addition, a significantly

greater number of cells with nuclear MYC are detected in EZH2-positive samples (supplemental Figure 3A), suggesting that the data from NKTL cell lines are applicable to clinical samples.

EZH2 overexpression in NKTL confers a growth advantage independently of histone methyltransferase activity

Next, we investigated whether EZH2 is functionally important in NKTL. When EZH2 was introduced into primary NK cells purified from normal human peripheral blood, we observed an eightfold increase in the percentage of GFP(+) cells from day 2 to day 5 compared with vector control (Figure 3A), demonstrating that the overexpression of EZH2 is able to provide a growth advantage in normal primary human NK cells. We next analyzed the effect of increasing EZH2 on cell proliferation in NKTL cell lines. Ectopic expression of EZH2 through transient cotransfection with the GFP expression construct in NKYS cells for 3 days resulted in significantly higher percentage of viable GFP(+) cells in EZH2-transfected cells than in empty vector-transfected cells (supplemental Figure 4). This result suggests that EZH2 overexpression by transfection leads to

a competitive cell growth advantage. Consistent with these observations in the cell lines, a positive correlation between the expression of EZH2 and Ki-67, a marker of cell proliferation, was also observed in NKTL tumor samples (Figure 3B). Furthermore, EZH2 positivity is associated with a greater percentage of tumor cells expressing Ki-67 (supplemental Figure 3B).

Although EZH2 commonly exerts its oncogenic properties through H3K27 trimethylation (H3K27me3) and gene repression, we did not observe an association between EZH2 expression and the abundance of H3K27me3 by IHC studies in our clinical NKTL samples (supplemental Figure 3C). Interestingly, a lack of association between EZH2 and H3K27me3 has also been described in breast tumor subtypes[25,26] and in ovarian and pancreatic cancers.[26] This finding raises the possibility that EZH2 may have functions other than its activity on H3K27me3 in cancers, including NKTL. To investigate this possibility, we compared the ability of EZH2 wild-type (WT) and an EZH2 SET domain deletion mutant (EZH2 SETΔ) to increase the cell growth of NKTL cells. Interestingly, EZH2 SETΔ, which lacked the methyltransferase activity for H3K27me3, was still able to strongly promote cell growth of NKYS cells. This effect was as potent as EZH2 WT, as indicated by a similar increase in the percentage of GFP(+) cells with time (supplemental Figure 4), and increase in cell growth as measured by the MTS assay (Figure 3C). Both western blot (Figure 3D) and quantitative reverse-transcription PCR (qRT-PCR) analysis using a pair of primers that specifically amplifies the SET domain (supplemental Figure 5) confirmed the ectopic expression of EZH2 SETΔ in transfected cells. The ability of EZH2 SETΔ to deplete the H3K27me3 was also validated (Figure 3D; supplemental Figure 6C). These results indicate that the proproliferative property of EZH2 in NKTL is not mediated by its histone methyltransferase activity.

EZH2 directly activates CCND1 transcription by binding to its promoter independent of its methyltransferase activity in NKTL

To better understand how EZH2 promotes proliferation in NK cell lines, we explored the mechanism by which EZH2 regulates cell cycle genes. The study by Bracken and colleagues showed that suppression of EZH2 by RNA interference (RNAi) significantly decreased positive regulators of cell proliferation such as G1/S-cyclins,[27] so it is tempting to speculate that EZH2 directly regulates the transcription of these genes by binding to their genomic locus. The CCND1 transcript is reported to be upregulated in NKTL tissues compared with normal NK cells,[28] and high expression of CCND1 correlates with poor prognosis and decreased survival duration in NKTL.[29] Therefore, we examined whether the transcription of CCND1 is affected by EZH2 overexpression. Consistent with our findings on cell growth, qRT-PCR indicated that CCND1 mRNA levels increased substantially after EZH2 overexpression in NKYS (Figure 4A). Higher induction of CCND1 by EZH2-SETΔ could be explained by a previous study that ectopic EZH2 SETΔ depletes endogenous EZH2 and consistently displays higher levels of protein accumulation compared with the ectopic EZH2 WT.[30]

Next, we evaluated whether EZH2 can activate the activity of the CCND1 promoter in NKYS cells. Cotransfection of the EZH2 WT expression vector with the pGL4-CCND1-Luc reporter resulted in considerable activation of CCND1 promoter activity (Figure 4B). Consistent with prior results, activation of luciferase activity was also observed in cells transfected with EZH2 SETΔ (Figure 4B). Collectively, these results indicate that EZH2 positively regulates CCND1 transcription independent of its histone methyltransferase activity.

We then addressed whether EZH2 binds to the CCND1 promoter using ChIP-qPCR assays. Six pairs of primers, located sequentially along the proximal promoter, first exon, and intron 1 of CCND1 were used to quantify the ChIP-enriched DNA by real-time PCR (Figure 4C). A peak representing EZH2 binding was observed (~33-fold above background) at a region very close to the transcriptional start site of CCND1 (Figure 4D). ChIP using a control IgG showed no significant enrichment over the entire surveyed region (Figure 4D). By performing ChIP assays using a His-Tag antibody in NKYS transfected with His-Tagged EZH2 SETΔ, we showed that ectopically expressing EZH2 SETΔ resulted in significant enrichment of EZH2-DNA complexes, which was even higher than EZH2 WT (Figure 4E). This effect is specific to the SET domain, as EZH2 SANT domain deletion mutants abolished EZH2 transcriptional activity on the CCND1 promoter (Figure 4F). Furthermore, data from tumor samples that CCND1 mRNA levels correlate well with EZH2 mRNA levels are consistent with this finding (Figure 4G). Consistently, western blot analyses showed that CCND1 protein is expressed together with EZH2 in NK cell lines but not in normal NK cells (Figure 4H). Taken together, our findings identify CCND1 as a bona fide direct target of EZH2 in NKTL. Importantly, EZH2 acts as a transcriptional activator for the CCND1 gene without requiring histone methylation catalytic activity, which provides a mechanistic explanation for the proproliferative role of EZH2 SETΔ in NKTL.

Growth inhibition on depletion of endogenous EZH2 can be rescued by exogenous EZH2 SET mutants

Given that EZH2 is of functional importance in NKTL raises the possibility that targeting EZH2 may be a feasible strategy in NKTL. We first investigated the effects of depleting EZH2 on cell growth. Using 3 different short hairpin RNAs (shRNAs) targeting the EZH2 gene (Figure 5D; supplemental Figure 7A) in NKYS, we showed that depletion of EZH2 resulted in a substantial decrease in cell numbers, as revealed by the percentage of GFP+ cells (Figure 5A), and a specific concomitant decrease of CCND1 expression but not in other gene transcripts such as PRDM1 and IGF1 (Figure 5B; supplemental Figure 8). This result strengthens the postulation that EZH2 is required for expression of the proliferative gene CCND1 and suggests that downregulation of CCND1 is responsible for the reduction in cell growth in NKTL cells after EZH2 depletion.

Next, we sought to clarify if the reduction in cell growth is dependent on the methyltransferase activity of EZH2. Because EZH2 SETΔ is immune to EZH2 shRNA-2, which targets the SET domain, EZH2 shRNA-2 only depleted endogenous WT EZH2 but not the EZH2 SETΔ after being introduced into the cells, as expected (supplemental Figure 7B). Indeed, EZH2 SETΔ was able to prevent the reduction in cell numbers mediated by EZH2 shRNA-2, whereas reintroduction of EZH2 WT could not (Figure 5C, left). These rescue experiments excluded the possibility that the cell death phenotype occurred because of an off-target effect of EZH2 shRNA and, at the same time, confirming that the prosurvival effect of endogenous EZH2 does not require its enzymatic activity. Similar results were obtained in KHYG1 (Figure 5C, right), another NKTL cell line, indicating a consistent requirement for expression of EZH2 in the growth and survival of NK tumor cells.

Inhibition of EZH2 by 3-deazaneplanocin A (DZNep) induced growth inhibition and apoptosis of malignant NK cell lines

The effects seen with EZH2 knockdown suggest that EZH2 may be a therapeutic target in NKTL. We next explored the use of a