厚生労働科学研究費補助金

エイズ対策研究事業

HIV陽性者における進行性多巣性白質脳症に対する高精度検査技術の開発および診断への応用

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研究要旨

進行性多巣性白質脳症(Progressive Multifocal Leukoencephalopathy, PML)は、JCウイルス(JCV)に起因する脱髄疾患であり、HIV陽性者を中心とした免疫不全患者等において発生する。PMLの診断では、脳脊髄液を用いたJCVゲノムDNAのリアルタイムPCR検査が主流となっている。この検査手法は極めて鋭敏であるが、病原性のない持続感染型JCVの混入、もしくは検体間の汚染によって偽陽性を生じる危険性を有している。本検査における偽陽性はPMLの診断や治療に大きな悪影響を与えるリスクが大きく、脳組織を用いたJCVの確認検査は侵襲性が高い。脳脊髄液を用いたJCVのPCR検査における偽陽性およびウイルス変異の有無を迅速にモニターするための新たな検査技術の開発が必要である。本研究は、「高解像度融解曲線分析(High-Resolution Melting analysis, HRM)法を用いて、JCVのゲノムDNAに生じるランダムな変異を迅速に識別するための検査系を確立し、PMLの高精度診断技術へと応用すること」を目的とする。前年度での本研究では、計4系統のリアルタイムPCR-HRM検出系を確立し、持続感染型および変異型のJCVのDNAクローンを用いて検出系の至適化を行った。今年度では、PML疑い患者の脳脊髄液を用いて各検出系の感度や特異性、変異識別能を解析し、それらの臨床検査技術としての実用性を評価した。4系統のリアルタイムPCR-HRM検出系のうち、1系統の検出系が極めて高い変異識別能を有していたが、感度が不十分であった。そこで、標的配列をNestedプライマーによって前増幅するステップを追加することで本検出系の感度を向上させた。臨床検体を用いたバリデーションの結果、本検出系は、JCVゲノムの変異解析に用いられるNested-PCRと同程度の感度および特異性を有することが分かった。また、本検出系を用いてPML患者の脳脊髄液DNA中に含まれるJCVの変異パターンを解析したところ、JCVゲノムに生じる不特定かつ多様な変異を患者個人レベルで識別することが可能であった。上記の結果より、確実なPMLの診断や治療に貢献しうる新たな検査技術の実用化に成功した。

研究分担者
該当なし。（若手育成型の研究課題であり、研究代表者が単独で実施する。）

A.研究目的

進行性多巣性白質脳症(Progressive Multifocal Leukoencephalopathy, PML)は、ポリオーマウイルス科のJCウイルス(JCV)に起因する脱髄疾患であり、患者の約30～50%をHIV陽性者が占める。JCVは多くの成人に持続感染しており、免疫抑制に伴って変異型ウイルスが出現し、大脳白質等を破壊する。治療がなされない場合、ほとんどの患者が発症から1年以内に死に至る。PMLの診断では脳脊髄液を用いたJCVゲノムDNAのPCR検査が有効である。近年では国内外の研究機関および民間企業等によって高感度な定量的リアルタイムPCRを用いた脳脊髄液JCV検査が数多く導入され、PMLの診断技術の主流を占めている。この手法は鋭敏な検査結果が得られるが、持続感染型JCVの混入もしくは陽性検体から陰性検体への汚染によって偽陽性を生じるリスクを有している。JCVのPCR検査において偽陽性が疑われた場合の対策としては、ウイルスゲノムの一部に生じるランダムな変異を解析する手法が古くから行われている。しかし、多くの場合、PML患者の脳脊髄液には複数のJCV変異体が混在しており、一般的なダイレクトシークエンシングによる解析が困難である。そのため、確認検査を行うためには、JCVのDNAを大腸菌プラスミドにクローニングして選別する工程が必要になる。本解析の結果が得られるまでには多数の煩雑で長時間の作業、ならびに大きなコストを要する。そのため、ルーチン検査において本解析を迅速に実施することは難しい。そこで、一般的なリアルタイムPCR検査において陽性の結果が得られた際に、post-hoc検査として偽陽性や変異の有無を容易にモニターするための新技術の開発を着想した。本研究は、「JCVのゲノムDNAに生じるランダムな変異を標的として、ウイルスの亜型を迅速に同定するためのスキャニング技術を開発し、PMLの高精度診断技術へと応用すること」を目的とする。目的を達成するために、JCVゲノムの配列の相違を解析するための検査系の開発、および参照DNAや臨床検体を用いた検査系の評価を体系的に行う。

研究を開始した平成24年度では、JCVの全長、および同領域を3分割した配列を標的とした合計4系統のリアルタイムPCR-HRM検出系を確立し、持続感染型および変異型のJCV-DNAクローンを用いて検査系の確認を行った。本年度では、PML疑い患者の脳脊髄液を用いてこれらの検出系の有用性を評価し、臨床検査技術としての実用化を目指した。

B.研究方法

一般的に、健常人に持続感染しているJCVが病原性を示すことはなく、そのゲノム配列は宿主の終生にわたって安定している。一方、PMLを引き起こすJCVのゲノムは、ウイルス遺伝子の発現を司るプロモーター領域(転写調節領域)においてランダムな変異が生じる。本検査系は、一対のDNAプライマーを用いたリアルタイムPCR法によって転写調節領域を増幅した後、高解像度融解曲線分析(High-Resolution Melting analysis, HRM)法を用いて、配列の相違に伴うDNA断片の解離温度の差を測定することを原理としている。

臨床面での評価に用いた検査系候補の選定

には、前年度において確立した4系統のリアルタイムPCR-HRM検出系を用いた。これらの検出系はJCVの転写調節領域を対象とした*in silico*解析によって、持続感染型およびPML型の双方のウイルスを検出するようにデザインされている。標準DNAとして、JCVの実験室株(持続感染型CY株、PML型Mad-1株)のゲノムDNAを用いた。リアルタイムPCR-HRMに必要な試薬およびHRM対応のリアルタイムPCR機器については、前年度と同様にRoche社の製品を用いた。

PML患者の脳脊髄液中には微量のJCVゲノムしか放出されていない症例が多いため、リアルタイムPCR-HRMにおいて標的としている調節領域の外縁に、1対のDNAプライマーを設計した。これらのプライマーセットおよびコンベンショナルPCRキット(Toyobo社製)を用いて、鋳型となるDNAの標的配列を前増幅した。このPCR産物を希釈した後、リアルタイムPCR-HRMの鋳型として用いた。持続感染型およびPML型のJCVのゲノムの希釈系列を作製し、それらを鋳型とすることで、検査系の候補となる検出系の検出限界を決定した。

次に、PML疑い患者の脳脊髄液DNA(陽性75検体、陰性453検体)を対象として本リアルタイムPCR-HRMを行い、①PML患者の脳脊髄液中に検出されたJCVのゲノム型(持続感染型、変異型)を判別しうるか否か、②PML患者において個人レベルで生じるJCVのランダムな変異を識別しうるか否か、③JCV陰性の脳脊髄液を検査に用いた場合に非特異的増幅による偽陽性が生じるか否か、を解析した。

(倫理面への配慮)

本研究は、国立感染症研究所ヒトを対象とする医学研究倫理審査委員会の承認を受けた後、適切な配慮のもとに実施された。

C.研究結果

約100種類のJCV-DNAパネルを用いた解析の結果、JCVゲノムの調節領域の全長を標的とした1系統のリアルタイムPCR-HRMを用いることで、調節領域の変異の有無や配列の相違をスキャンすることが可能であった。また、調節領域の部分的な配列を標的とした3系統のリアルタイムPCR-HRMを用いることで、変異が生じている部分の位置をより精緻に把握することが可能であった。ただし、JCVの調節領域は一般的にPCRによる増幅効率が高くないため、この検出系のみによって臨床検体中の微量のJCV-DNAを検出することは困難であった。

そのため、標的配列の外縁にプライマーセットを設計し、これらのプライマーによってサンプル中のJCVを前増幅した後の産物をリアルタイムPCR-HRMの鋳型として用いた。この手法を用いることで、検出系の感度を向上した。本検出系の所要時間は合計2時間であり、20コピー/反応の標的配列を検出することが可能であった。また、JCVゲノムDNAの希釈系列を用いた解析の結果、これらの検査系は20～10^4コピー/反応までの幅広い鋳型濃度において安定して変異を識別しうることが分かった。そこで、実際のPML疑い患者の脳脊髄液DNAサンプルを用いて、検出系の感度および精度を調べた。この評価においては、JCVの変異解析において一般的に用いられるNested PCR法を比較対象とした。

約530検体(うち75検体がJCV陽性)を用いた解析の結果、調節領域の全配列を増幅する検出系の感度および精度が、ともに100%であった。調節領域を3分割した配列を標的とした3系統の検出系のうち、変異の頻度が少ない配列を標的にした1系統の検出系では、感度および精度は100%であったが、変異の識別能が劣っていた。また、変異が生じやすい配列を標的とした2系統の検出系では、精度こそ100%であったが、

プライマー結合部位に変異が入っている場合には標的が増幅されず、感度が約80%であった。

上記の成績を鑑み、調節領域の全配列を標的としたリアルタイムPCR-HRMを本検査の基盤技術として採用することとした。JCV陽性の脳脊髄液を用いて本検査の変異識別能を調べたところ、DNAの解離温度データを差分法に基づく数理学的アルゴリズムによって演算することで、JCVゲノムに生じる変異を患者個人レベルで識別することに成功した。また、各患者の脳脊髄液DNA、および検体から単離されたJCV-DNAを用いた場合、両者が極めて類似した変異パターンを示すことを確認した。

D.考察

リアルタイムPCRはウイルス検査における汎用技術となっており、優れた迅速性、感度、特異性を有する。一方、本法の最大のリスクである偽陽性については未だ検討の余地があり、汚染の可能性を迅速に調べるためのpost-hoc検査系の開発が十分になされていない。PCR検査において最も偽陽性を引き起こす可能性が高い事象は、陽性対照DNAによるサンプル汚染である。このリスクに対しては、対照DNAの配列や断片長を改変するといった対策が講じられている。しかし、陽性検体から陰性検体へのキャリーオーバーによる汚染が生じた場合には、偽陽性の判別が困難である。複数の陽性検体を対象としてウイルスの核酸配列を比較した際に、同一のジェノタイプを示した場合には、ウイルスが個別のサンプルに含まれていたのか、もしくは一方の陽性検体から混入したのか、を迅速に判定することは難しい。検査環境のクリーンアップを行った後、当該サンプルについて、再度の核酸抽出もしくは検体採取といった非効率的な対応に迫られるケースもありうる。

PML型のJCVは、ゲノムの転写調節領域にランダムな変異を生じるというユニークな性質を有している。この点に着目し、ルーチンのPCR検査において陽性を示した場合の確認検査として、転写調節領域の配列を解析するというアプローチが古くから実施されている。しかし、PML患者の脳脊髄液には、複数のJCV変異体が含まれており、ダイレクトシークエンシングによる塩基配列の決定が困難となるケースが多い。そのため、PCR検査において偽陽性が疑われた場合には、ウイルスゲノムの転写調節領域をプラスミドにクローニングした後、多数のDNAクローンの塩基配列を決定し、ランダムに生じた変異部分の配列を解析する必要がある。これら一連の工程には、煩雑かつ長期間の作業、および大きな経済的コストを要するため、ルーチン検査での実施は困難である。

本年度における研究では、前年度において確立した4種類のリアルタイムPCR-HRM検出系および臨床検体を用いて、検査技術としてのバリデーションを実施した。

一般的なリアルタイムPCR検査と異なり、HRMによる変異の解析では、標的DNAの増幅がプラトーに達する必要がある。しかし、JCVの転写調節領域のDNA配列はPCRによる増幅効率が低いことが知られており、その増幅にはNested PCRが用いられる。本検査系においても、標的配列を短時間のPCRによって前増幅することで、その検出感度を飛躍的に向上させることが可能であった。また、臨床検体を用いて4系統の候補検出系の感度と精度、変異識別能を比較したところ、転写調節領域の全配列を標的とした検出系が最も安定した成績を示したことから、本検出系を検査において採用することとした。本検査系は、変異型JCVのゲノムを患者個人レベルで識別することが可能であり、複数の陽性検体が認められた場合においても、検査結果を迅速に確認することが可能であった。

本年度の研究は、開始当初の計画に従って着実に進行した。また、臨床面での信頼性を厳密

に確認するため、計画時に想定した以上の数の患者検体を用いて検査系のバリデーションを実施した。本検査技術は、臨床での使用に耐えうるだけの信頼性を有しており、現時点においてその実用化に成功したと判断している。本研究において開発した検出系は、患者個人レベルで陽性検体を識別するほどの高い解像度を有している。このようなアプローチは他の病原体においても珍しく、臨床検査学分野での学術的価値を有する。本研究成果は、第18回日本神経感染症学会学術集会において臨床医から高い評価を受け、同学会の学会賞を受賞した。

前年度および今年度において、本研究の柱となるPMLの新たな検査技術の開発が完了したため、次年度では、臨床分野における本検査系の応用性を重点的に調べる。具体的には、①本検査系が、PMLの治療時の脳脊髄液のフォローアップ検査において有用か否かを明らかにする。研究代表者らは、脳脊髄液中のJCVゲノムの変異を経時的にモニターすることでPMLの進行をウイルス学的に評価しうることをすでに明らかにしており、その際の検査ツールとしての可能性を調べる。また、②脳脊髄液ではなく、ヒトDNAが多量に含まれる脳組織を検体として本検査を実施し、検出時の変異識別能や非特異的なノイズの有無を調べる。

E.結論

PMLを引き起こすJCVはウイルスゲノムにランダムな変異を有する。これらの変異をリアルタイムPCR-HRMによってスキャンし、検体中に含まれているJCVの変異パターンを患者個人レベルで識別するための検査技術を開発した。また、多数の患者の脳脊髄液を用いた検査系のバリデーションを経て、その実用化に成功した。本検査系は、PMLの診断を目的とした脳脊髄液検査において、検出されたウイルスの病原性の有無、ならびに偽陽性の可能性を排除する上で有用である。

F.健康危険情報

該当なし

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H．知的財産権の出願・登録状況

該当なし。

研究成果の刊行に関する一覧表

書籍

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Detection of human herpesviruses in the cerebrospinal fluid from patients diagnosed with or suspected of having progressive multifocal leukoencephalopathy

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Abstract

Background: Progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease caused by JC virus (JCV), occurs mainly in immunocompromised patients. While JCV DNA is detected in the cerebrospinal fluid (CSF) from a certain proportion of patients suspected of having PML, JCV-negative patients may also develop brain lesions due to other infectious agents. This study assessed the prevalence of six herpesviruses in the CSF from patients diagnosed with or suspected of PML.

Methods: Two hundred and ninety-nine CSF specimens and clinical data were collected from 255 patients, including 31 confirmed PML cases. Quantitative PCR assays were carried out to detect the genomic DNA of JCV, herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpesvirus 6 (HHV-6).

Results: Herpesvirus DNAs were detected in the CSF specimens from 29 of 255 patients (11.4%). HSV-1 and CMV were detected in JCV-negative patients, whereas VZV and EBV were detected in both CSF JCV-positive and -negative individuals. The herpesvirus-positive patients had underlying disorders that caused immunosuppression, such as HIV infection, congenital immunodeficiencies, and hematologic malignancies, and presented with neurologic symptoms and MRI lesions, mainly in the cerebral white matter. The median values of CSF cell counts and protein levels in the herpesvirus-positive patients were slightly higher than those in the PML patients.

Conclusions: The results demonstrate that herpesviruses are occasionally detected in the CSF from PML patients and immunocompromised individuals suspected of having PML. Thus, this study provides a significant basis for the diagnosis and treatment of neurological disorders in immunocompromised patients.

Keywords: Cerebrospinal fluid, Human herpesvirus, JC virus, Progressive multifocal leukoencephalopathy, Quantitative PCR testing

Background

Progressive multifocal leukoencephalopathy (PML) is a rare but fatal demyelinating disease of the central nervous system (CNS) caused by JC virus (JCV), a small DNA virus belonging to the family *Polyomaviridae*, genus *Polyomavirus* [1-3]. Humans are infected with JCV asymptomatically during childhood and are persistently infected with it throughout life. From 50–90% of adults have been reported to be serologically positive for JCV [1-4]. In some severely immunocompromised patients, JCV activates and causes a lytic infection in the oligodendrocytes, leading to PML [1-4]. Although PML is mainly diagnosed in patients with HIV-infection, it is also observed in patients with immunodeficiency due to a hematological malignancy, chemotherapy, transplantation, lymphocyte depletion, or autoimmune disorders, such as systemic lupus erythematosus, and in those under treatment with immunosuppressive agents [1,3,5]. In addition, PML has recently been identified in patients receiving immunomodulatory therapies with monoclonal

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antibodies, such as natalizumab, rituximab, and efalizumab [1-3,6].

The detection of JCV DNA in the cerebrospinal fluid (CSF) by PCR is a reliable and less-invasive diagnostic marker of PML, particularly when combined with typical magnetic resonance imaging (MRI) patterns [1,7]. CSF testing for JCV DNA using a quantitative PCR technique has become the current diagnostic standard [6]. In Japan, real-time PCR testing for JCV DNA in CSF specimens has been partly supported by the Laboratory of Neurovirology, Department of Virology 1, National Institute of Infectious Diseases (NIID), Tokyo, Japan, since 2007. The CSF from approximately 11% of patients (48 of 419) was found to be positive for JCV DNA and these patients were diagnosed with PML [8]. However, no JCV could be detected in the CSF samples from the remaining approximately 89% of patients, implying that a large proportion of these subjects might have developed brain disorders due to other infectious or non-infectious causes.

Herpesviruses, in particular, herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), or human herpesvirus 6 (HHV-6), are major etiological agents of encephalitis and other CNS infections in immunocompromised persons [9-13]. This study sought to assess whether these herpesviruses contribute to the CNS involvement in patients diagnosed with or suspected of having PML.

Methods

Collection of CSF specimens and clinical data

The study was conducted under the approval from the Ethical Committee for Biomedical Science in the NIID (approval number 339). Informed consent from patients or their family members was also obtained. Patients suspected of having PML on the basis of neurological symptoms and/or MRI patterns were enrolled in this study. Upon request to the patients' physicians, CSF testing for JCVDNA was routinely performed regardless of patient age, gender, underlying disease, or medical history. Two hundred and ninety-nine CSF specimens were collected by lumbar puncture from 255 patients from April 2007 to the end of January 2010, immediately frozen, and then transferred to the NIID for PCR testing. For 44 of the patients, CSF testing was repeated during their follow-up period. Clinical data were collected from the patients' physicians through standardized questionnaires. The following data were analyzed: age, gender, underlying diseases, manifestations of neurologic symptoms, pattern of brain MRI lesions, and CSF leukocyte counts and total protein levels.

Real-time PCR testing for viral DNA in CSF specimens

Total DNAs were extracted from the CSF specimens using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and then used as PCR templates as described previously [8,14,15]. Since most of the CSF specimens had been frozen at the hospitals, they also contained cellular components. Quantitative real-time PCR assays targeting the DNAs of JCV [8,14,15], VZV [16], CMV [17], and HHV-6 [17] were carried out as described in the earlier reports. HSV-1/2 and EBV DNAs were quantified using an artus HSV-1/2 LC PCR Kit (Qiagen) and LightCycler EBV Quantification Kit (Roche, Penzberg, Germany), respectively, according to the protocols supplied by the manufacturers.

Statistical analysis

The detection rates of herpesvirus DNAs in the CSF specimens and the sex ratios of patients in each group were statistically compared by means of a two-tailed Fisher's exact test. For multiple testing, the resulting *P*-value was corrected using the Benjamini-Hochberg method. Differences in the ages and CSF cell counts and protein contents between patient groups were compared by nonparametric analyses using the Mann–Whitney *U* test. All *P*-values less than 0.05 were judged to be statistically significant.

Results

Detection of JCV in CSF from patients suspected of having PML

The study population comprised 166 males and 89 females. The mean age of the subjects was 56.3 years (median 59.0 years, range 4–89 years, SD = 18.0), excluding 1 male patient whose age was not definitely stated. The underlying diseases of 255 subjects were as follows: HIV infection ($n = 52$, 50 males, 2 females), hematologic disorders ($n = 51$, 39 males, 12 females), autoimmune disorders ($n = 33$, 10 males, 23 females), other diseases ($n = 46$, 29 males, 17 females), and unknown ($n = 73$, 38 males, 35 females). A total of 299 CSF specimens from 255 patients were subjected to the real-time PCR assay for JCV DNA, and 42 samples (14%) were found to be positive for JCV DNA. The median JCV load in these specimens was 3.2×10^4 copies/mL (range $1.5 \times 10^2 - 4.8 \times 10^8$ copies/mL, SD = 7.8×10^7). The prevalence of JCV DNA and underlying diseases in the patient population are shown in Table 1. Thirty-one of 255 patients (12%) were diagnosed with PML based on clinical findings and JCV DNA-positive CSF. These PML patients had HIV infection (10 patients, 19%), hematologic disorders (13 patients, 26%), autoimmune disorders (3 patients, 9%), or other diseases (5 patients, 11%). No JCV DNA was detected in the CSF specimens from 73 patients who had no clinically apparent underlying disorders.

Detection of herpesvirus DNA in CSF from patients diagnosed with or suspected of having PML

The next series of analyses were conducted to clarify the etiological contribution of herpesviruses to CNS disease

Table 1 Prevalence of CSF JCV DNA and underlying diseases in the patient population

	No. (%) of patients		
	Total	JCV-positive	
Underlying disease	(*n* = 255)	(*n* = 31)	
HIV infection	52	10	(19.2)
Hematologic disease	51	13	(25.5)
Autoimmune disease	33	3	(9.1)
Other disease	46	5	(10.9)
Unknown	73	0	(0)

by the detection of herpesvirus DNA in 299 CSF specimens from 255 patients diagnosed with or suspected of having PML. Among the 299 CSF samples, 31 were positive for herpesvirus DNA (Table 2). HSV-1, VZV, CMV, and EBV were detected in 1 (0.3%), 8 (2.7%), 5 (1.7%), and 19 (6.4%) specimens, respectively. Two specimens were positive for CMV and either HSV-1 or EBV. No amplification signal was observed for HSV-2 and HHV-6 in any sample. HSV-1 and CMV were detected only in JCV-negative CSF specimens. In contrast, VZV and EBV were detected in both JCV-positive and -negative samples. The viral DNA level of HSV-1 in 1 specimen was 1.3×10^3 copies/mL. The median viral loads of VZV, CMV, and EBV were 3.3×10^2, 1.1×10^3, and 1.5×10^3 copies/mL, respectively (Figure 1). Although the DNA levels of these viruses in most specimens ranged from 10^2 to 10^4 copies/mL, more than 10^4 copies/mL of VZV, CMV, and EBV DNAs were found in some samples.

Proportion of patients for whom CSF specimens were herpesvirus DNA positive

Table 3 shows the numbers and proportion of patients for whom the CSF specimens were positive for herpesvirus DNA. Among the 255 subjects, CSF herpesvirus DNA was detected in 29 (11%). HSV-1 and CMV were detected in CSF JCV-negative patients, while VZV and EBV were detected in JCV-negative and positive-patients. The detection

Table 2 Number of herpesvirus DNA-positive and -negative CSF specimens

	No. (%) of CSF specimens		
Herpesvirus	Total[a]	JCV-positive	JCV-negative
DNA	(*n* = 299)	(*n* = 42)	(*n* = 257)
HSV-1	1 (0.3)	0 (0)	1 (0.4)
HSV-2	0 (0)	0 (0)	0 (0)
VZV	8 (2.7)	1 (2.4)	7 (2.7)
CMV	5 (1.7)	0 (0)	5 (1.9)
HHV-6	0 (0)	0 (0)	0 (0)
EBV	19 (6.4)	5 (11.9)	14 (5.4)

[a]Two specimens were positive for CMV and either HSV-1 or EBV DNA.

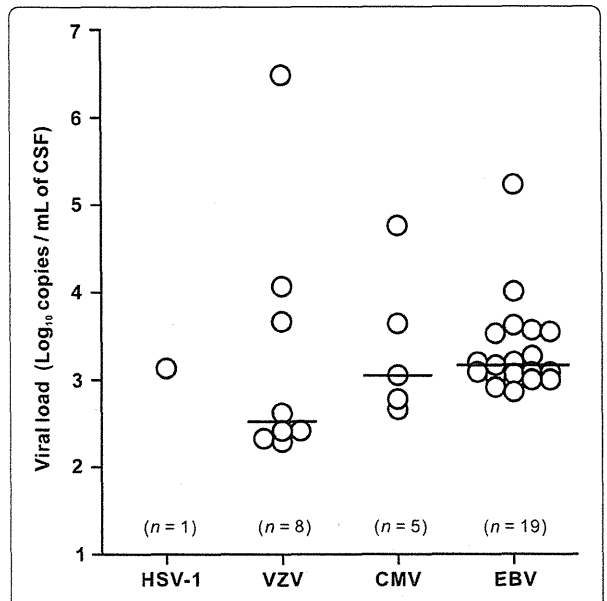

Figure 1 Viral loads of herpesvirus DNAs in the CSF specimens from patients. The CSF viral loads of HSV-1, VZV, CMV, and EBV are shown (left to right). Each open circle indicates the copy number of viral DNA in each sample, and the horizontal lines represent the medians.

rate of herpesvirus DNAs in JCV-positive patients (19%) was higher than that in JCV-negative individuals (10%), but this difference was not statistically significant. VZV was detected in 8 patients, from one of whom EBV-positive CSF specimens were obtained during the follow-up. Five patients were found to be positive for CMV, and HSV-1 and EBV were concomitantly detected in CMV-positive samples from 1 patient each. Sixteen individuals were found to be positive for EBV but not for other herpesviruses. The EBV-positive rate appeared to be higher than those of other herpesviruses. In addition, the detection rate of EBV in JCV-positive patients was statistically higher than that in JCV-negative patients ($P = 0.032$).

Table 3 Number of patients in whom herpesvirus DNA was detected in the CSF

	No. (%) of patients		
Herpesvirus	Total	CSF JCV-positive	CSF JCV-negative
DNA in CSF	(*n* = 255)	(*n* = 31)	(*n* = 224)
Total	29 (11.4)	6 (19.4)	23 (10.3)
HSV-1 and CMV	1 (0.4)	0 (0)	1 (0.4)
VZV	7 (2.7)	1 (3.2)	6 (2.7)
VZV or EBV[a]	1 (0.4)	0 (0)	1 (0.4)
CMV	3 (1.2)	0 (0)	3 (1.3)
CMV and EBV	1 (0.4)	0 (0)	1 (0.4)
EBV	16 (6.3)	5 (16.1)	11 (4.9)

[a]VZV and EBV DNA was detected in different CSF specimens collected from the same patient during the follow-up period.

Underlying diseases of herpesvirus DNA-positive patients

The clinical data of 29 herpesvirus DNA-positive patients were analyzed. The patients comprised 23 males and 6 females. The mean age of all except 1 patient was 52.5 years (median 53.5 years, range 30–84 years, SD = 14.7). There were no statistically significant differences in the age and sex ratios between the herpesvirus DNA-positive and -negative patients. The underlying diseases of the patients who provided herpesvirus DNA-positive CSF specimens are summarized in Table 4. Of the 29 patients, 27 (93%) were found to have underlying disorders that may cause immunosuppression. Sixteen patients (55%) had HIV infection, and the severe loss of peripheral blood CD4-positive T cells was observed in most cases. VZV, CMV, and/or EBV were detected in CSF from the HIV-positive patients. Eight patients (28%) suffered from hematologic diseases, such as non-Hodgkin's lymphoma and aplastic anemia, and had been treated with hematopoietic stem cell transplantation, combination chemotherapy, or other immunosuppressive drugs. Three patients (10%) had other underlying diseases, such as lupus nephritis, chronic renal failure, or primary angiitis of the CNS, and received immunosuppressive therapy. Among the subjects in each group, the proportion of the herpesvirus DNA-positive patients with HIV infection (31%) and that with hematologic diseases (16%) were statistically higher than that with other underlying diseases (4%), as compared by multiple statistical testing ($P = 0.000$ and 0.048, respectively). In addition, both JCV and herpesvirus DNAs were detected in the CSF specimens from 6 patients with either HIV infection or hematologic diseases.

Clinical features of herpesvirus DNA-positive patients

In the final set of analyses, the clinical features of the patients with herpesvirus-positive CSF were compared to those of PML patients. Table 5 shows the appearance patterns of neurologic symptoms and brain lesions. In the study population, 25 patients were positive for JCV but provided herpesvirus-negative CSF specimens. These PML patients presented with diverse neurologic symptoms, such as paralysis, dementia, dysarthria, dysphagia, and/or visual impairment (data not shown). MRI lesions were found mainly in the cerebral white matter (CWM) (84%), and a smaller proportion of patients showed lesions in other sites, such as the cerebellum (16%) and brain stem (28%). Among the 24 patients in whom herpesviruses were detected in the CSF, 20 individuals (83%) had neurologic manifestations. MRI lesions were identified in the CWM (75%), cerebellum (29%), brain stem (8%), and other sites (13%). There were no statistically significant differences in the proportion of individuals with lesions at each site between the PML and herpesvirus-positive patients. The VZV- or EBV-positive patients displayed lesions not only in the CWM, but also in other areas of the brain. In contrast, the lesions were localized in the CWM in the HSV-1- and/or CMV-positive patients. Figure 2 shows the results of CSF cell counts and total protein contents of the PML and CSF herpesvirus-positive patients. Since the CSF cell counts and/or total protein contents were not defined in the questionnaires in some cases, the numbers of patients are not identical in Figure 2A and 2B. In both patient groups, the median values of cell counts for both mono- and polynuclear cells (Figure 2A) and protein contents (Figure 2B) were at normal or near-normal levels. However, a considerable proportion of herpesvirus-positive patients exhibited higher cell numbers and protein levels when compared to those of PML patients, and these differences were statistically significant ($P = 0.049$ and 0.004, respectively).

Discussion

The present study aimed to comprehensively assess the prevalence of six human herpesviruses in the CSF specimens from patients diagnosed with or suspected of having PML. Since the aim of this study was the detection of herpesviruses DNA using PCR from the CSF samples of the patients diagnosed with or suspected PML associated with several immuno-suppressive underlying diseases, the

Table 4 Underlying diseases of patients in whom herpesvirus DNA was detected in the CSF

Herpesvirus DNA in CSF	No. of patients	Underlying disease (%)							
		HIV infection		Hematologic disease		Other disease[a]		Unknown	
Total	29	16	(55.2)	8	(27.6)	3	(10.3)	2	(6.9)
HSV-1 and CMV	1	0	(0)	1	(100)	0	(0)	0	(0)
VZV	7	2[b]	(28.6)	3	(42.9)	2	(28.6)	0	(0)
VZV or EBV	1	1	(100)	0	(0)	0	(0)	0	(0)
CMV	3	3	(100)	0	(0)	0	(0)	0	(0)
CMV and EBV	1	0	(0)	0	(0)	1	(100)	0	(0)
EBV	16	10[c]	(62.5)	4[d]	(25.0)	0	(0)	2	(12.5)

[a]The data include patients with autoimmune disorders.
[b]One patient was positive for JCV.
[c,d]The results include 2 and 3 JCV-positive patients, respectively.

Table 5 Neurologic symptoms and brain MRI patterns in the PML and herpesvirus-positive patient groups

Detected Viral DNA in CSF	No. of patients	Neurologic symptom (%)	MRI(T2/FLAIR) lesion (%)[a]				
			Cerebral white matter	Cerebellum	Brain stem	Other	Unknown
JCV[b]	25	25 (100)	21 (84.0)	4 (16.0)	7 (28.0)	2 (8.0)	2 (8.0)
Herpesviruses[c]	24	20 (83.3)	18 (75.0)	7 (29.2)	2 (8.3)	3 (12.5)	1 (4.2)
HSV-1 and CMV	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
VZV	6	6 (100)	5 (83.3)	1 (16.7)	1 (16.7)	0 (0)	1 (16.7)
VZV or EBV	1	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)
CMV	3	2 (66.7)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)
CMV and EBV	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
EBV	12	9 (75.0)	7 (58.3)	5 (41.7)	1 (8.3)	2 (16.7)	0 (0)

[a]The data include patients with multiple lesion sites.
[b]The patients were negative for herpesvirus DNA in their CSF.
[c]The patients were negative for JCV DNA in their CSF.

detection of virus DNA indicates the existence of these viruses, but the data do not always indicate the main contribution to brain damage.

One of the most important findings is that EBV was present in the CSF of approximately 16% of the confirmed PML patients. Although the detection of EBV as well as JCV in the CSF has been reported previously [18-20], the prevalence of EBV in the CSF specimens from the relatively large number of patients suspected of having PML has not been determined previously. This data suggest that it is not rare to concomitantly detect JCV and EBV in PML cases. Although the EBV-positive patients had not been diagnosed with EBV-related diseases when the specimens were collected, it would be interesting to see whether these patients later developed EBV-associated neurological disorders. It would be attractive to hypothesize that EBV infection is involved in the progression of PML progression as the detection rate of EBV in the CSF from JCV-positive patients was higher than that in JCV-negative patients. However, this result might be due to differences in the proportion of immunocompromised individuals between the JCV-negative and -positive populations. VZV was detected in the CSF from one PML patient with AIDS (CD4 cell count, 8 cells/μl). The detection rate of VZV appeared to be lower than that of EBV, and the co-detection of JCV and VZV in CSF was reported in one previous report [20]. It is possible that this patient developed both PML and VZV encephalitis.

It is of interest to note that HSV-1/2, CMV, and HHV-6 were not detected in the CSF specimens from any PML patient. Two previous reports demonstrated that JCV was concomitantly detected with CMV or HSV-1 in the CSF [20,21]. The data obtained in this study indicate that the CSF from some PML patients was HSV-1 and/or CMV DNA-positive, although the prevalence was low. Another important finding in this study is that

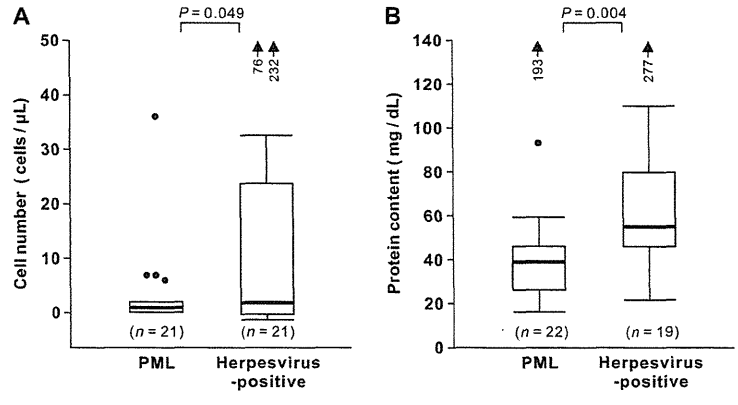

Figure 2 CSF cell counts (A) and total protein contents (B) in PML and herpesvirus-positive patients. Since the CSF cell counts and/or total protein contents were not defined in the questionnaires in some cases, the numbers of patients are not identical in panels **A** and **B**. The data of patients positive for both CSF JCV and herpesvirus DNAs are not included in each panel. In box-and-whisker plots, the thick horizontal line within each box is the median; the lower and upper boundaries are the 25th and 75th percentiles, respectively; vertical whiskers extend over the range; and dots and arrows show outliers.

HSV-1, VZV, CMV, and EBV were detected in more than 10% of the CSF specimens from patients suspected of having but not diagnosed with PML. Based on neurological symptoms, MRI lesion patterns, and underlying disease, it seems reasonable that these patients were suspected of having PML. A large proportion of the patients that were found to be positive for herpesviruses had HIV infection or hematologic disorders, suggesting that there is a significant relationship between the presence of herpesviruses in the CSF and severe immunosuppression due to AIDS, chemotherapy, or hematopoietic stem cell transplantation. As the distribution of CSF cell numbers and protein contents partially overlapped in the PML and herpesvirus-positive patients, these parameters may not directly contribute to a diagnosis of PML. It is likely that the inflammatory response was inhibited under the immunosuppressive conditions in both patient groups. However, it is worth focusing on the significant proportion of the herpesvirus-positive patients showing high CSF cell counts. In these patients, it can be postulated that the brain inflammation was induced by the lytic herpesvirus infection. In such a situation, the amount of herpesvirus DNA might be increased by the migration of the infected cells into the CSF, as the PCR assays were performed using total DNA extracted from frozen CSF.

In some herpesvirus-positive cases, a combination of two herpesviruses, such as CMV and HSV-1, CMV and EBV, and VZV and EBV, were detected in the CSF, which is consistent with previous reports describing the co-detection of herpesviruses in CSF [18,20,22,23]. It was also observed that VZV and EBV were detected in different CSF specimens from one patient during the follow-up period. This patient presented with lesions in the CWM and basal ganglia, at which time VZV was detected. At repeat CSF testing 3 months later, EBV, but not VZV, was detected in the CSF, and lesions were identified in the CWM and cerebellum. This observation indicates that although VZV propagation in the CNS was reduced, EBV infection or reactivation occurred during the follow-up period in this patient.

Currently, no specific treatment has been established for PML. Restoration of the immune system, either by combination antiretroviral therapy for patients with AIDS or by moderating the immunosuppressive therapies for non-AIDS patients, is the only treatment option for the management of PML, although several experimental treatments are being investigated [1]. In contrast, acyclovir is effective in the treatment of encephalitis caused by HSV or VZV [24]. It is also known that ganciclovir and foscarnet are beneficial for patients with CMV-related encephalitis [24]. Thus, the present data suggest that comprehensive testing for these herpesviruses as well as JCV is important for early diagnosis and proper management of patients suspected of having PML.

Conclusions

In summary, as herpesviruses can contribute to CNS disorders in a significant proportion of patients suspected of having PML, comprehensive testing for the herpesviruses as well as for JCV is required for the accurate diagnosis and treatment of CNS diseases in patients diagnosed with or suspected of having PML.

Abbreviations

PML: Progressive multifocal leukoencephalopathy; CNS: Central nervous system; JCV: JC virus; CSF: Cerebrospinal fluid; HSV: Herpes simplex virus; VZV: Varicella-zoster virus; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; HHV-6: Human herpesvirus 6; MRI: Magnetic resonance imaging; NIID: National Institute of Infectious Diseases; CWM: Cerebral white matter.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KN and MS conceived of the study. KN and NI carried out real-time PCR testing, and KN created the database of patients. KN analyzed the clinical data and drafted the manuscript. TS performed the statistical analyses. MS, NI, TS, IK, and CKL participated in the study design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Case Report

Does anti-JCV therapy improve the prognosis of AIDS-related PML?

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1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a subacute, fatal demyelinating disease of the brain that occurs in immunosuppressed patients. The causative agent is the JC virus (JCV). Approximately 5% of patients with AIDS contract PML. The only known intervention for these patients is combination antiretroviral therapy (cART): survival at 1 year is reported to be between 38.6% and 56%, and those patients who survive are usually left with severe neurological sequelae. Although several medications with in vitro activity against JCV have been employed during cART for PML patients, they have proven largely ineffective. However, the anti-malarial drug mefloquine, which is known to have anti-JCV activity in vitro, has produced successful outcomes in some PML patients [1]. We report here the use of mefloquine alongside cART in a case of AIDS-related PML in which the JCV level in cerebrospinal fluid (CSF) was relatively high compared to previously reported cases.

2. Case report

In May 2011, a 61-year-old man presented with weakness in all limbs. He had been well until 2 months earlier. He was diagnosed with HIV-1 infection at his first visit. Viral load was 99,000 copies/mL and the CD4 count was 58 cells/μL. Brain MRI showed asymmetric, patchy foci in the cerebral hemispheres, with perifocal edema. Real-time PCR testing for JCV genomic DNA in CSF revealed 315,000,000 (8.50 log) copies/mL. On this basis, we diagnosed him with AIDS-related progressive multifocal leukoencephalopathy (PML). He was immediately started on a cART regimen of abacavir, lamivudine, and efavirenz according to national protocol, but he lost consciousness 14 days after admission. Because the JCV viral load in CSF was extremely high, concomitant administration of mefloquine hydrochloride tablets was started at this time (initially 275 mg daily for 3 days, then 275 mg once weekly).

Immunological variables showed rapid improvement, with CD4 increased up to 234 μL^{-1} and HIV-1 viral load decreased to 41 copies/mL at 3 months after the initiation of cART. At this time the patient's Karnofsky Performance Scale Index score for functional impairment had progressively worsened from 70/100 on admission to 20/100. At 4 months after starting cART and with concomitant mefloquine therapy, the JCV viral load in CSF was markedly suppressed, below the detection sensitivity limit and comparable to previous reports. Follow-up MRI showed extension

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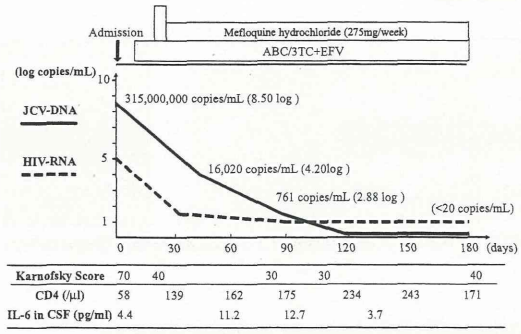

Fig. 1. Clinical course after administration of the mefloquine.

of an area of T2 hyperintensity within the white matter, but no further extension occurred after disappearance of JCV in the cerebrospinal fluid. As of December 2011, he had recovered the ability to make eye contact with medical staff and his clinical appearance had improved, with a Karnofsky Performance Scale Index score of 40/100. Unfortunately, he died in hospital from pneumonia (Fig. 1).

3. Discussion

The use of highly active cART, which is the only known intervention for AIDS-related PML, can leave many patients who survive with severe neurological sequelae. In the present case, the JC viral load was high at 8.50 log copies/mL and the eradiation rate with cART and mefloquine was fast at 7.0×10^{-2} log copies per month, compared with the highest JC viral load in CSF of 7.30 log copies/mL reported previously and a median eradiation rate with cART of 3.0×10^{-3}–1.4×10^{-2} log copies per month [2–4]. García de Viedma et al. previously reported a significant difference in survival between patients with a JCV viral load >4.68 log copies/mL in CSF and those with a JCV viral load below this [5]. Another report highlighted that early eradication or suppression of JCV in CSF was associated with the stabilization of symptoms caused by PML. It appears that our patient's neurological condition improved because of the rapid eradication of JCV.

Immune reconstitution inflammatory syndrome (IRIS) may develop secondary to restoration of immunity in HIV-positive patients with PML receiving cART and may cause paradoxical clinical deterioration despite recovery of the immune system. Risk factors for IRIS include a decrease in CD4 T cells at the initiation of cART, with a count <50 μL^{-1} posing a high risk, and a decrease in HIV-1 RNA levels in AIDS patients of >2 log copies/mL within 90 days of starting cART. Our patient was therefore at high risk for IRIS, with a CD4 count of 58 μL^{-1} at the initiation of cART and his HIV-1 RNA level falling >3 log copies/mL within 90 days of starting cART. It must be noted that the severity of PML-IRIS is varied however: some cases are mild and resolve with continued cART, while others may lead to significant morbidity and even mortality because of a severe inflammatory response characterized histopathologically by a marked influx of CD8 T cells and macrophages in areas of demyelination and inflammatory reaction. In the present case, we cannot determine whether the additional contribution of mefloquine improved the patient's clinical status because of an antiviral effect against JCV or an anti-inflammatory effect against IRIS. Accordingly, our observations here should be considered as preliminary findings only and require confirmation with a larger number of patients.

Although the clinical effectiveness of mefloquine combined with cART is controversial and remains to be proven, our case demonstrates its potential in eradicating JCV from the CSF and slowing clinical exacerbation.

4. Conclusion

The potent management of HIV infection is crucial because HIV-induced immune suppression can lead to rapid and widespread dissemination of JCV in the brain and subsequent demyelination. At that point, co-therapy with mefloquine may help with the early eradication of JCV from the CSF and contribute to neurological improvement.

Conflict of interest

The authors of this manuscript report no disclosures or conflicts of interest.

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Ethical approval

This study was approved by the National Defense Medical College Research Ethics Committee (reference Biyou-4).

Contributors

All authors were involved in discussion about diagnosis, care of the patient, and preparation of the report. Written consent to publish was obtained.

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Case Report

Progressive multifocal leukoencephalopathy developed 26 years after renal transplantation

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1. Introduction

Progressive multifocal leukoencephalopathy (PML) is a disease of the central nervous system (CNS) that is characterized by destructive infection of oligodendrocytes by the JC virus. PML has been reported in transplant recipients due to immunosuppressive treatment [1], and was recently found to develop in patients receiving treatment with monoclonal antibodies including natalizumab and efalizumab for multiple sclerosis and psoriasis respectively. Here, we report a patient with PML that developed 26 years after renal transplantation.

2. Case report

A 54-year old woman was admitted to our hospital because of dysarthria and ataxic gait. She had received a living-donor kidney transplantation at 28 years of age and was taking oral immunosuppressants. Five months before admission, the regimen was changed from a combination of mizoribine, azathioprine, and methylprednisolone to low-dose mycophenolate mofetil (MMF) and methylprednisolone, because of liver injury due to azathioprine.

Neurological examinations revealed saccadic eye movement, slurred speech, truncal ataxia, right ataxic hemiparesis, and increased deep tendon reflexes in the lower limbs. A complete blood cell count, and the results of serum chemistry tests and tumor marker screening were normal, while a lumbar puncture yielded cerebrospinal fluid (CSF) containing a normal number of mononuclear cells, as well as normal levels

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