第7章　新しいウイルス検出法，ウイルス診断法，ウイルス試験

5）病原微生物の網羅的検査系の開発と応用

はじめに

2002年に東京医科歯科大学医学部附属病院に再生医療・細胞治療の臨床研究用の無菌培養施設“細胞治療センター”が設置され，現在では厚生労働省認可の先進医療として「活性化Tリンパ球移入療法（原発性若しくは続発性の免疫不全症の難治性日和見感染症又は慢性活動性EBウイルス感染症に係るものに限る）」が，臨床研究として滑膜由来軟骨幹細胞を利用した軟骨再生医療が行われている．細胞治療センターでは，細胞製剤のウイルス安全性に関する取り組みを主要な研究課題と位置づけ，再生医療・細胞治療用細胞製剤の安全管理に関連するウイルス16種類をリストアップし，それらのウイルスを網羅的・迅速・安価に検査できる新しい検査系を開発し，細胞治療センターの安全性検査として実用化した．

一方，東京医科歯科大学医学部附属病院では，小児科と血液内科で造血幹細胞移植が実施されている．移植患者は治療過程で深い免疫不全状態となるため様々な感染症に罹患するが，移植に伴う副作用である移植片対宿主病graft versus host disease（GVHD）との鑑別が難しい場合も多く，GVHDと感染症では治療法が全く異なるため治療に難渋することが多く経験される．筆者らは，造血幹細胞移植後の疾患の原因究明に使用するため，ウイルスに加え細菌・真菌・マイコプラズマ・クラミジア・原虫などを検査項目として加えた網羅的微生物検査系を開発した．開発した検査系は東京医科歯科大学医学部附属病院に公開しており，感染症が疑われる様々な疾患の原因究明の一助として利用されている．本節では，我々が開発した網羅的微生物検査系の詳細とその応用に関して紹介する．

1．網羅的ウイルス検査系の開発の経緯と細胞製剤のウイルス安全性検査への応用

ウイルスの増殖には生きた細胞への感染が必須であるが，多くのウイルスは細胞特異性が強くウイルスを組織培養により検出することは容易ではない．さらに，未だ*in vitro*での感染増殖系がないウイルスも多く，疾患の原因ウイルスを特定するためPCR（polymerase chain reaction）によるウイルスゲノムを直接検出する手法が用いられている．一方，ほとんどのヒトには複数のヘルペスウイルス科に属するウイルス（単純ヘルペスウイルス，水痘帯状疱疹ウイルスなど8種類が知られている）が持続感染しており，B型・C型肝炎ウイルスやHTLV-1キャリアも多数存在する．したがって，これらの持続感染ウイルスの病気への関与や治療経過を調べるためにはウイルスゲノムの

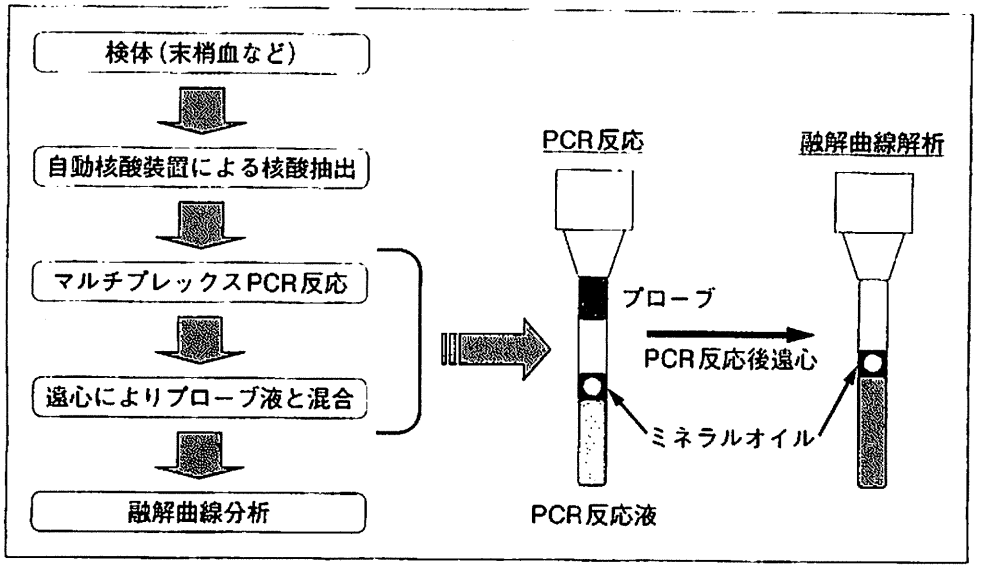

図1　網羅的ウイルス検査法の概略
マルチプレックスPCRを行う際，多くのプライマーとプローブを初めから混合しておくと感度が著しく低下するため，PCR反応を行う際にはプローブ液はキャピラリー上部にとどめ，PCR反応終了後に遠心により混合する．この方法の導入により感度が100倍程度上昇した．

定性的な検出では不十分であり，ウイルスゲノムの定量的検査が必要となる．近年，ウイルスゲノム量を短時間で直接定量することができるリアルタイムPCR法が実用化され，現在ではウイルス性疾患の診断や治療の評価に欠かせない検査法となっている．我々は，リアルタイムPCR機を使用し，マルチプレックスPCR法により多種類のウイルス同時検出系と各ウイルス定量検査系を作製し，再生医療・細胞治療のウイルス安全性検査，ウイルス性疾患の診断・経過観察およびウイルス学研究に役立てている．

我々が開発・実用化した網羅的ウイルス検査法は，短時間にPCR反応が完了するキャピラリー型リアルタイムPCR機（ライトサイクラー：ロッシュ社，50サイクルの増幅反応を20分程度で完了可能）を利用し，マルチプレックスPCR法による多種類のウイルスの定性的同時検出（偽陽性を避けるため，プローブによる検出を採用）と通常のリアルタイムPCR法による個別定量法の2段階法による検査法である．**図1**に検査法の詳細を記載した．

実際の検査では，末梢血，尿など検体から自動核酸抽出装置（BioRobot EZ1 キアゲン，検体から核酸を30分程度で抽出）により核酸を抽出し，マルチプレックスPCRを行う．その後プローブ液（各ウイルスに対応するハイブリプローブの混合液：プローブはFITCまたはLcRed640か705で標識されている）と混合して増幅産物とハイブリダイズさせ，融解曲線分析 melting curve analysisにより複数のウイルスを同時・定性的に検出・識別する．マルチプレックスPCRを行う際は，プローブを混合せずにPCR反応を行い，PCR反応終了後に遠心してプローブを混合する．マルチプレックスPCR法は感度低下が問題となることが多いが，この方法を採用することで感度が約100倍上昇し，実用的な感度が得られるようになった．

陽性ウイルスの種類は，蛍光色素の種類とTm値から判定できる（**図2**）．さらに，β-globin検出系を内部コントロールとして加えることにより，全ての被検ウイルスが陰性の場合にもβ-globinの低いピークが検出されるため，何らかの原因でPCR反応が進まないために生じる偽陰性を防止できる（**図3**）．**図2**で示した例では，1本のキャピラリーで8種類のウイルスを同時検出することが可能である．筆者は細胞治療センターの管理委員として，本検査システムを利用した再生医療・細胞治療用の細胞製剤のウイルス安全性の確保に関する研究を行っている．我が国ではiPS細

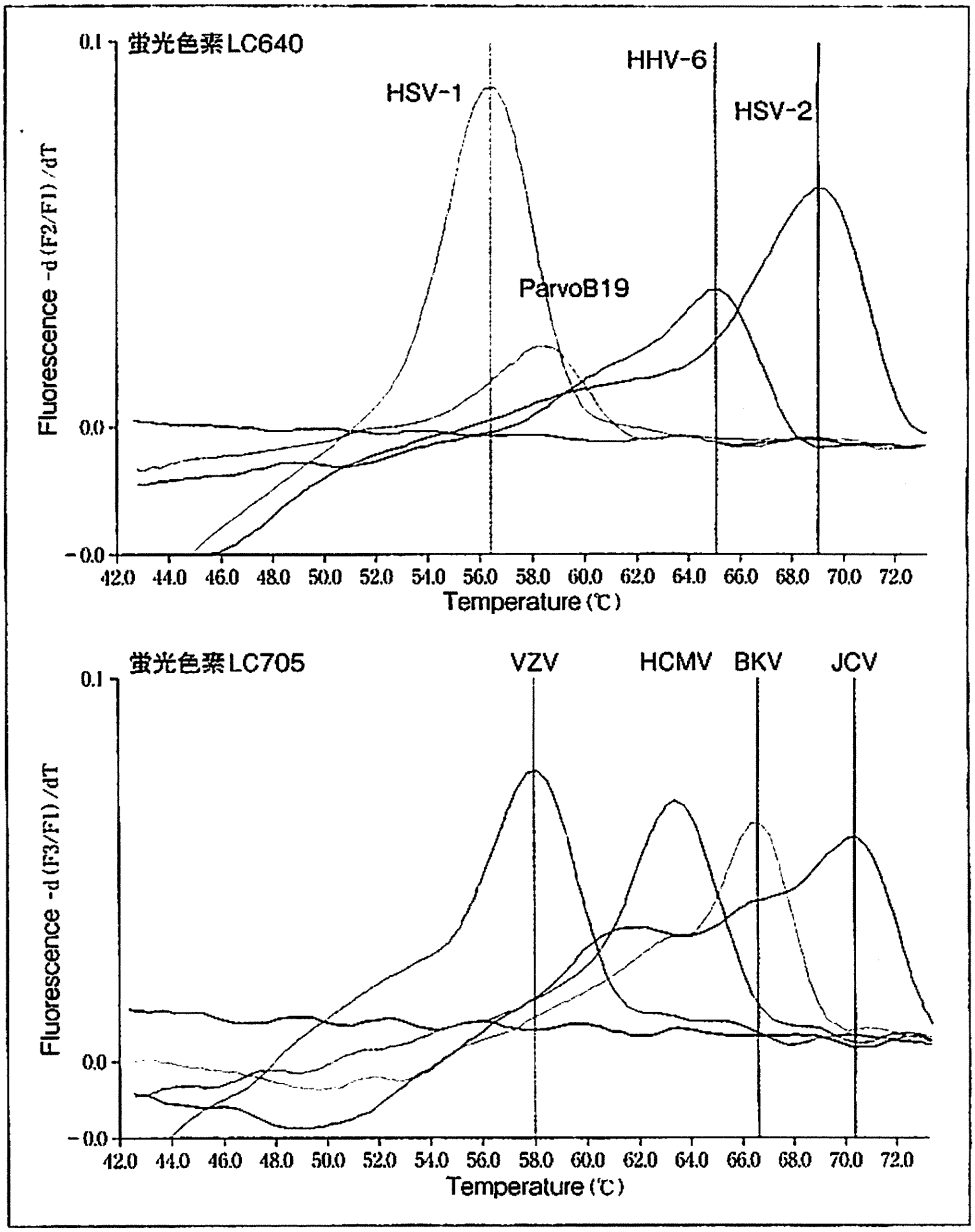

図2 融解温度解析
波長の違う2種類の蛍光色素を利用することにより，1本のキャピラリーで合計8種類のウイルスの定性的解析ができる．

胞の研究の進展により再生医療の実用化研究が国策として進められているとともに，体外で活性化培養した自己Tリンパ球を投与する癌免疫療法が広く実施されている．これらの魅力的な治療法を実用化・確立していくためには，細胞製剤の安全性確保が大前提となる(詳細は本書の「第4章 1) 細胞治療のウイルス安全性確保に関する取り組み」を参照)．

関連指針に記載のウイルス(HBV，HCV，HIV，HTLV-1など)や細胞培養中に増殖が懸念される複数の持続感染ウイルス(主にヘルペスウイルス科に属するウイルス群)を個別に検査するのは検査費用と時間的に現実的ではない．細胞治療センターでは，我々が開発した網羅的ウイルス検査系を応用した独自のウイルス検査系を作製し，培養した細胞は全てウイルス検査を行った後に出荷する取り決めとしている．

細胞治療センターで実施中の検査は，図4に示すように4本のキャピラリーを使用し，16種類のウイルス検査を実施している．なお，R1，R2 Setでは，1本のキャピラリー内で，逆転写・マ

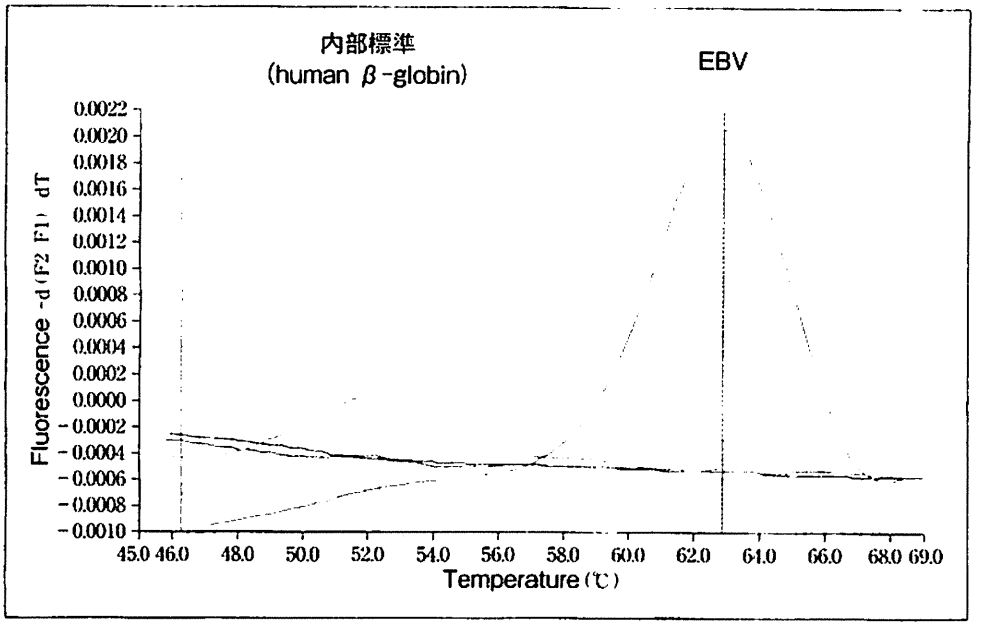

図3　内部標準による偽陰性の防止
全ての被検ウイルスが陰性の場合にはβ-globinの小さな波形が検出され，例えばEBVが陽性の場合にはEBVの陽性波形のみが検出される．

A Set	HSV-1, HSV-2, VZV, CMV, ParvoB19, BKV, JCV
B Set	EBV, HHV-7, HHV-8, HBV
R1 Set	HIV-1, HCV
R2 Set	HIV-2, HTLV-1, HTLV-2

図4　東京医科歯科大学細胞治療センターから出荷する細胞製剤に対して行っているウイルス検査項目

ルチプレックスPCR・融解曲線解析を順次行っている．RNAウイルスやレトロウイルスの検査系で1本のキャピラリーあたりの検査項目が少ないのは，これらのウイルスはゲノム配列の変異が激しいため，検査漏れを防ぐため1つのウイルスあたり複数のプライマー・プローブを使用しているためである．本ウイルス検査により，Tリンパ球を活性化培養する過程で，HHV-6やHHV-7が増殖することがあること，培養の初期過程においてEBV，BKV，JCV，ParvoB19が陽性となる例があること(特にEBV陽性例が多い)，間葉系幹細胞培養中にHHV-6やCMVが増殖する例があること，などが明らかとなり，ウイルス検査の重要性が示されている．

2. 網羅的微生物検査法への発展と臨床検査への応用

東京医科歯科大学細胞治療センターでは，上記のようにウイルス安全性検査を経た細胞治療製剤による臨床研究が進んでいる．一方，本検査系による臨床検体検査の要望が多くの臨床科より寄せられたことを受けて，本検査法を東京医科歯科大学医学部附属病院に公開し，現在年間1,000件以上のウイルス検査を受託している．その中で，造血幹細胞移植を行っている本学医学部小児科・血液内科とぶどう膜炎の治療を積極的に行っている眼科との共同研究として，検査対象を細菌・真

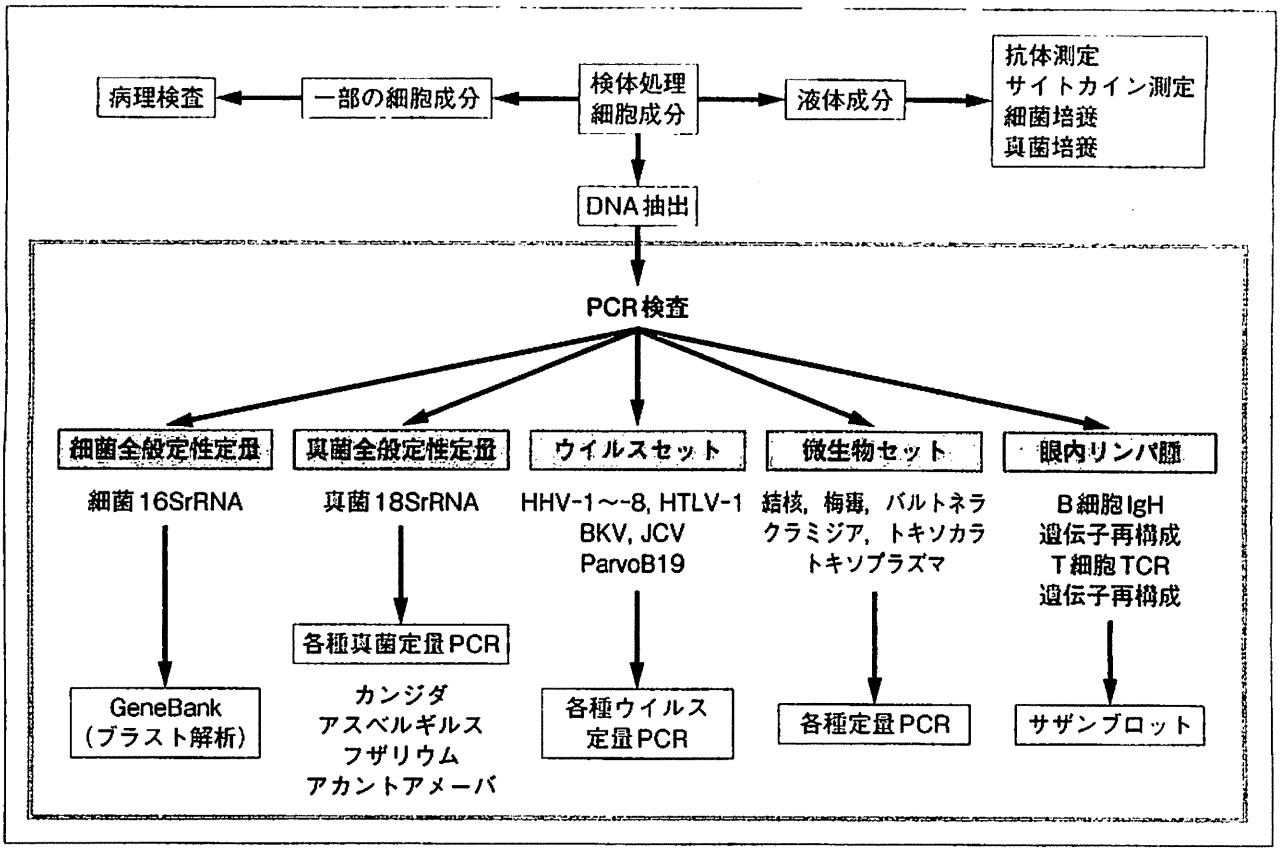

図5　網羅的微生物検査系を使用した眼疾患検査の概要
多くの細菌・真菌に共通な16SrRNAと18SrRNAの検出による網羅的検査後にシークエンス・ブラスト解析，個別PCRにより菌の種類を特定する．ウイルスセットや微生物セットでは，マルチプレックスPCRによる定性的検出に引き続き個別PCRによる定量を行う．

菌・マイコプラズマ・クラミジア・原虫などウイルス以外の微生物に拡大する取り組みを行っている．一例として感染症が疑われる眼疾患(主にぶどう膜炎)の検査の概要を**図5**に示す．造血幹細胞移植後の免疫不全状態では様々な日和見感染症に罹患するが，移植の副作用であるGVHDとの鑑別が困難な例も多いが，また感染症とGVHDでは治療法が全く違うためその鑑別診断は極めて重要であり，さらに感染症の場合その病原体を決定することは治療薬を選択するために必須である．ぶどう膜炎でも同様で，自己免疫疾患か感染症か，感染症の場合はその病原体を迅速に同定することは患者の予後とも直結するため，極めて重要である．我々が開発したマルチプレックスPCR法を用いた多項目迅速検査系はそのような疾患の診断に極めて有用であり，特に眼疾患の検体は量が限られているためマルチプレックスPCR法を使用しなくては多くの病原体の同時・迅速検査を行うことは極めて難しい．本共同研究により様々な成果が上がっているが，その一部を**表1**に示す．

3. 網羅的微生物検査法の改良

キャピラリーPCR機を用いたマルチプレックスPCRは，多くの検査項目を同時・迅速・安価に測定可能，少量の検体量でも多項目の検査が可能，などのメリットがある反面，コピー数が非常に

表1　網羅的迅速微生物検査により得られた成果

1. 急性網膜壊死（桐沢型ぶどう膜炎）検体よりHSV-1，HSV-2，VZV，EBVを検出し，前房水中のウイルス量が薬物療法のみで経過観察するか硝子体手術が必要かの目安になることが示唆された．
2. 難治性ぶどう膜炎の眼内液からHHV6-Aを検出し，本ウイルスがぶどう膜炎発症に関与している可能性を示した．
3. 造血幹細胞移植後の発熱イベントのうち43％にウイルスが関与しており，そのうちの30％が複数ウイルスの同時感染であった．
4. 造血幹細胞移植後のウイルス血症例ではCMV，HHV-6，EBV，BKV，JCVの頻度が高く，CMV，BKVはGVHDの重要度と相関した．
5. 移植後尿路感染症の70％からウイルスが検出され，BKV，JCV，ADV，CMVの順に頻度が高かった．少量のADV陽性でも血尿を生じるが，BKVは10^6 copies/mL以下では無症状の場合が多かった．
6. 造血幹細胞の意識障害患者の髄液検査では，半数からウイルスが検出され，HHV-6脳炎の頻度が最も高かった．

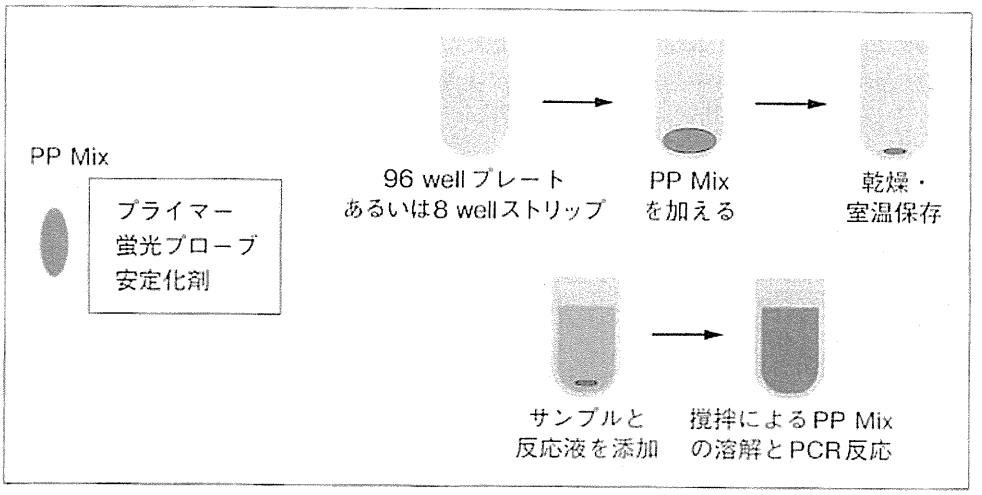

図6a　Ready-to-Use検査プレートの作製と検査実施の概要

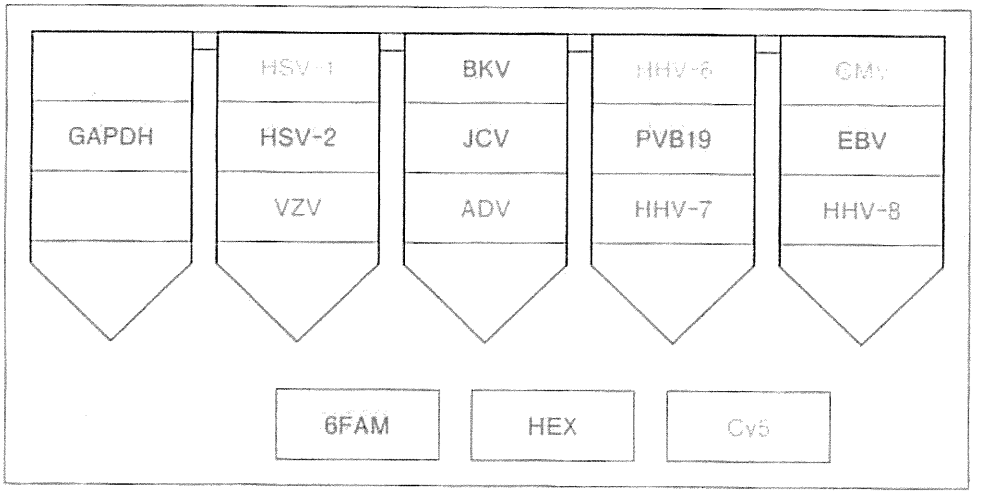

図6b　PP Mixと固相化したReady-to-Use検査ストリップの1例
1 wellで3種類，合計12種類のウイルスの測定ができる（GAPDHはポジティブコントロール，6FAM，HEX，Cy5は蛍光色素）本セットは造血幹細胞移植後の日和見ウイルス感染症の検査用に使用されている．

多い検査項目があるとdNTP等を消費してコピー数が少ない検査項目が陰性化する懸念がある，検査は定性的で定量検査を別途行う必要がある，検査のセットアップが煩雑，キャピラリーの取扱いに習熟していないと破損してコンタミネーションを引き起こすおそれがある，などの欠点がある．筆者の研究室では，これらの欠点を克服したReady-to-Useの検査セットの作製を目指し，図6aに示すような新しい検査系を作製した．

この検査系は96 well型のリアルタイムPCR装置を使用する．各検査項目のプライマーと蛍光

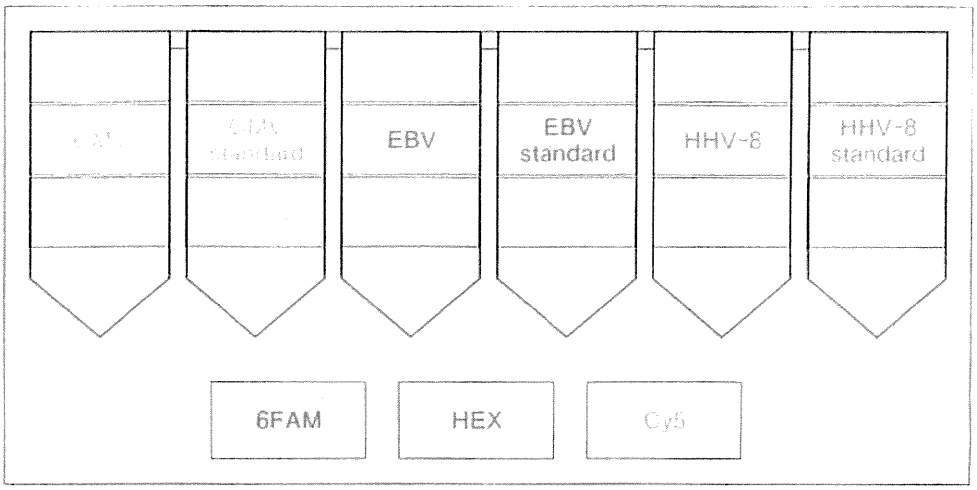

図6c　図6bで陽性が出た場合に使用する定量用ストリップ
ここでは，4番目のwellから陽性シグナルが出た場合に使用する定量用ストリップを示す．スタンダードの値からCt法で陽性項目の定量結果を得ることができる．1 wellで1ウイルスの測定を行うため，複数のウイルスが陽性な場合でも正確に定量ができる．

表2　現在行われている検査セットの開発・実用化研究

細胞治療セットⅠ	HBV，HCV，HTLV-1，-2，HIV-1，-2，HHV-1～-8，BKV，JCV，ParvoB19，AdV，Mycoplasma
細胞治療セットⅡ	HBV，HCV，HTLV-1，-2，HIV-1，-2，Mycoplasma
移植セット	HHV-1～-8，BKV，JCV，ParvoB19，AdV
肝炎ウイルスセット	HAV，HBV，HCV，HDV，HEV，GBV，TTV，EBV，CMV
呼吸器ウイルスセット	RSV，MPV，BoV，FluA，S-OIV，FluB，AdV，PIV1，2，3，RV，CoV，PeV

MPV：metapneumovirus，S-OIV：novel swine-origin influenza A (H1N1) virus，PIV：parainfluenza virus，RV：rhinovirus，CoV：coronavirus，PeV：parechovirus．

プローブを安定化剤とともに検査プレートあるいは8連ストリップのwellに固相化し，検査にあたっては検体とバッファー，dNTP，増幅酵素の混合液を加えた後に撹拌してプライマー・蛍光プローブを溶解し，PCR反応を開始する．**図6b**は造血幹細胞移植後のウイルス検査系で，マルチプレックスPCR法により1 wellで3種類のウイルスを測定し，12種類のウイルスを4つのwellで検査することができる．3種類程度のマルチプレックスPCRであれば，組み合わせにもよるが，比較的に高感度は維持されている．陽性ウイルス種は陽性シグナルが出る蛍光色素の違いで識別し，半定量的な解析が可能である．本検査系では，**図6c**に示すように既知濃度のスタンダードを加えたwellを含む定量用ストリップをあらかじめ用意し，2段階で定量検査を行っている．これは，使用する検体量を少なくし，さらに試薬代の節約と強陽性に隠れた弱陽性項目の見落としをなくすためであり，迅速性が優先される場合には初めから1 wellで1ウイルスの検査を行うシステムとすることも可能である．本検査ストリップの保存安定性を検討したところ，室温で1ヵ月保存しても感度低下が全くみられないことを確認しており，さらなる長期保存後の安定試験を行っている．

固相化プローブ・蛍光プライマーを使用する検査系は，RT-PCR法に応用することが可能であり，現在**表2**に示すように様々な検査セットの開発・実用化研究を行っている．

おわりに

本節では，網羅的病原微生物検出法開発の経緯と，現在筆者の研究室で実際に稼働している2つの検査法に関して説明した．眼科検体（前房水や硝子体液など）は100μL以下の少量しか得られないことが多く，PCR法により多数の検査項目を同時に検査するためには，キャピラリーを使用したマルチプレックスPCR法が極めて有用である．一方，プライマー・蛍光プローブを固相化した検査プレート・ストリップを用いる方法は，多くの施設に普及しているプレートタイプのリアルタイムPCR機が使用でき，試薬も長期間室温保存でき，検査のセットアップも簡便なため，手軽に多数の微生物検査を同時に行える利点があるが，比較的多くのwellを使用するため，増幅酵素代が嵩むのが欠点である．このような点を踏まえ，筆者らの研究室では検査目的，検体の種類，検査対象項目の種類，などにより検査系を使い分けているとともに，新しい検査セットの開発を継続的に進めている．本節では詳しく触れられなかったが，眼科の感染症検査のようにウイルス検査に加えて他の微生物の検査も同時に行える検査系の開発も順次進めている．今後，誰でも簡便に使用できるように検査試薬をキット化し，本学以外の施設・研究者との共同研究も積極的に進めていく予定である．本検査系の技術移転や技術指導を希望する場合は，下記のアドレスにご連絡いただきたい（清水則夫：nshivir@tmd.ac.jp）．

（清水則夫，渡邊　健）

文　献

1）北條浩彦（編）：実験医学別冊　原理からよくわかるリアルタイムPCR実験ガイド，羊土社，2007
2）Miyanaga M, Sugita S, Shimizu N et al：A significant association of viral loads with corneal endothelial cell damage in cytomegalovirus anterior uveitis. Br J Ophthalmol 94：336-340, 2010
3）Takahashi H, Sugita S, Shimizu N et al：A high viral load of Epstein-Barr virus (EBV) DNA in ocular fluids in a HLA-B27 negative acute anterior uveitis patient with psoriasis. Jpn J Ophthalmol 52：136-138, 2008
4）Sugita S, Shimizu N, Watanabe K et al：Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. Br J Ophthalmol 92：928-932, 2008
5）Kido S, Sugita S, Horie S et al：Association of varicella zoster virus load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. Br J Ophthalmol 92：505-508, 2008
6）Kawaguchi T, Sugita S, Shimizu N, et al：Kinetics of aqueous flare, intraocular pressure and virus-DNA copies in a patient with cytomegalovirus iridocyclitis without retinitis. Int Ophthalmol 27：383-386, 2007
7）Sugita S, Shimizu N, Kawaguchi T et al：Identification of human herpesvirus 6 variant A in a patient with unilateral panuveitis. Arch Ophthalmol 125：1426-1427, 2007

Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR

Sunao Sugita,[1] Norio Shimizu,[2] Ken Watanabe,[2] Miki Katayama,[2] Shintaro Horie,[1] Manabu Ogawa,[1] Hiroshi Takase,[1] Yoshiharu Sugamoto,[1] Manabu Mochizuki[1]

[1]Department of Ophthalmology & Visual Science, Medical Research Institute, Tokyo Medical and Dental University Graduate School of Medicine and Dental Sciences, Tokyo, Japan
[2]Department of Virology, Medical Research Institute, Tokyo Medical and Dental University Graduate School of Medicine and Dental Sciences, Tokyo, Japan

Correspondence to
Dr Manabu Mochizuki, Department of Ophthalmology & Visual Science, Tokyo Medical and Dental University Graduate School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; m.manabu.oph@tmd.ac.jp

Accepted 4 April 2010
Published Online First 31 July 2010

ABSTRACT

Aim To measure the bacterial genome in ocular fluids and to analyse the clinical relevance of infectious endophthalmitis.

Methods Nineteen ocular fluid samples (eight aqueous humour and 11 vitreous fluid samples) were collected from 19 patients with suspected bacterial endophthalmitis. Fifty ocular samples from uveitis patients were also collected along with 40 samples from patients without ocular inflammation and used as controls. Bacterial ribosomal DNA (16S rDNA) was measured by a quantitative PCR assay.

Results Bacterial 16S rDNA was detected in patients with clinically suspected bacterial endophthalmitis (18/19, 95%). With the exception of one case, high copy numbers of bacterial DNA were detected (1.7×10^3–1.7×10^9 copies/ml) in these patients. There were 10 samples (53%) with positive bacterial cultures while there were nine samples (47%) with positive Gram-staining. Real-time PCR detected bacterial 16S rDNA in three (6%) of the 50 samples from the control uveitis patients. In addition, none of the samples from the control patients without intraocular inflammation were positive.

Conclusions Quantitative broad-range PCR of bacterial 16S rDNA is a useful tool for diagnosing bacterial endophthalmitis.

INTRODUCTION

Bacterial infectious endophthalmitis occurs due to exogenous infections, such as those arising from trauma and intraocular surgery, or from endogenous infections, such as systemic infectious disorders. Previous studies have used PCR to demonstrate the presence of bacterial DNA in the ocular fluids in patients with infectious endophthalmitis.[1–10] PCR has often been used to provide evidence of bacterial involvement in the eyes with suspected intraocular infections.[8] These suspected infections include idiopathic endophthalmitis and uveitis. Recent advances in molecular biology along with the use of real-time PCR have made it possible to determine quantitative measurements of the viral load associated with viral diseases in the eye.[11–13] Several studies have recently reported finding the bacterial ribosomal RNA gene (16S rDNA) in the ocular fluids of patients with infectious endophthalmitis.[4 8 10] With primers of the bacterial 16S rRNA gene, broad-range PCR can be used to detect the presence of bacteria within the samples. In endophthalmitis patients with previous intravitreal administration of antibiotics, PCR methodology has been shown to be more effective than bacterial cultures in detecting bacterial DNA in the ocular fluids.[10] However, even broad-range PCR has not been able to determine quantitative information for the bacterial genome in the ocular sample.

In the present study, after collecting ocular samples from patients with suspected intraocular infections, which included bacterial infectious endophthalmitis, we attempted to detect and then measure the bacterial genome using real-time quantitative PCR with primers for 16S rDNA amplifications.

MATERIAL AND METHODS

Subjects

Based upon medical history and clinical observations, 69 patients with endophthalmitis and uveitis were consecutively enrolled in a prospective study that was conducted from 2008 to 2009 at the Tokyo Medical and Dental University Hospital. Samples of aqueous humour and vitreous fluids were collected from all patients. Nineteen patients (19 eyes: eight aqueous humour and 11 vitreous fluids) had bacterial infectious endophthalmitis. Of these 19 patients, six had acute postoperative endophthalmitis, four had late postoperative endophthalmitis, one had post-traumatic endophthalmitis, five had endogenous endophthalmitis, two had keratitis-associated endophthalmitis, and one had endophthalmitis after intravitreal injections of bevacizumab.

The second patient group was also a prospective study, and 50 ocular samples were collected from various patients with uveitis. The underlying pathology included idiopathic uveitis (n=21), herpetic keratouveitis (n=3), herpetic anterior iridocyclitis (n=3), acute retinal necrosis (n=5), cytomegalovirus retinitis (n=2), toxoplasmosis (n=3), toxocariasis (n=2), sarcoidosis (n=2), HTLV-1-associated uveitis (n=1), toxic lens syndrome (n=1), *Candida* endophthalmitis (n=2) and intraocular lymphoma (n=5). In this study, fungal endophthalmitis cases such as *Candida* endophthalmitis were classified as being part of this patient group. All the patients displayed active intraocular inflammation at the time of sampling.

In addition to the patient groups, we also analysed samples from a control group. These patients were enrolled in this prospective study in 2009. Forty samples (20 aqueous humour and 20 vitreous fluids) were collected from patients who did not have any type of ocular inflammation (age-related cataract, macular oedema secondary to branch retinal vein occlusion, retinal detachment, idiopathic macular hole or idiopathic epiretinal membrane).

For the ocular sampling (asepsis), the following procedures were performed in all subjects. In all of the eyes that were sampled, the ocular surfaces, including the conjunctival sacs, were rinsed once with an aqueous povidone iodine solution. Subsequently, all of these eyes were then rinsed once with a balanced-salt solution. A 0.1 ml aliquot of aqueous humour was collected aseptically in a syringe with a 30 G needle. Half of the sample was then transferred into a pre-sterilised microfuge tube and used for PCR.

In patients with endophthalmitis/uveitis who were undergoing vitreous surgery, uncontaminated non-diluted vitreous fluid samples (0.5–1.0 ml) were collected during diagnostic pars plana vitrectomy (PPV). Immediately after collection, 100 µl of the sample was transferred into a pre-sterilised microfuge tube and used for PCR. None of the asepsis samples used for analysis came from patients being given systemic antibiotics or from patients who were receiving intraocular antibiotic injections.

Conventional microbiological investigations

The Bacteria Work Station of the Tokyo Medical and Dental University Hospital processed all specimens (aqueous humour and vitreous fluids) within 1 h after the sample collection, with standard methods followed for the isolation and identification of the aerobic and anaerobic bacterial cultures. The culture methods followed conventional techniques that have been previously published.[14 15] Cultures were incubated for up to 7 days, with those lacking growth designated as culture-negative. Cytospin smears of the specimens were stained using Gram's method for detection of bacteria.

Quantitative PCR

DNA was extracted from samples using a DNA minikit (Qiagen, Valencia, California, USA) installed on a Robotic workstation for automated purification of nucleic acids (BioRobot E21, Qiagen). The real-time PCR was performed using Amplitaq Gold and the Real-Time PCR 7300 system (Applied Biosystems, Foster City, California, USA). Primers and probes of bacterial 16S rDNA and the PCR conditions are described elsewhere.[16] The sense primer (Bac349F) was 5′-AGGCAGCAGTDRGGAAT-3′ and the antisense primer (Bac806R) was 5′-GGACTACYVGGGTATCTAAT-3′. The TaqMan probe (Bac516F) was 5′-FAM-TGCCAGCAGCCGCGGTAATACRDAG-TAMRA-3′. Products were subjected to 50 cycles of PCR amplification, with cycling conditions set at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Bacterial copy number values of more than 100 copies/ml in the sample were considered to be significant.

Sensitivity of TaqMan real-time PCR

To confirm the real-time PCR assay sensitivity, the 458 bp fragments were amplified from the DNA of *Staphylococcus aureus* (NBRC 12732) with Bac349F and Bac806R. The PCR fragments were inserted into the pGEM cloning plasmid with the pGEM T-Easy Vector Cloning System I kit (Promega, Tokyo, Japan). The plasmid was digested with restriction enzyme ScaI. Linearised plasmid was controlled by gel electrophoresis and quantified by using the Smart Ladder DNA size and mass marker (Wako, Tokyo, Japan) and the OD260 measurement. Standard curves were constructed from serial 10-fold dilutions of linearised plasmid DNA with 10 ng/µl MS2 RNA (Basel, Roche, Switzerland). The detection limit and standard range of the TaqMan real-time PCR were determined by using serial 10-fold dilutions of linearised plasmid. The standard range of DNA was linearly quantified from one to nine log DNA copies, with a detection limit of 10 copies. The negative control (nuclease-free water) was not detected.

PCR FOR 16S rRNA GENE AND SEQUENCE ANALYSIS

PCR mix (50 µl volumes) was prepared from Low-DNA AmpliTaq Gold DNA polymerase LD (Applied Biosystems). The mix comprised dATP, dGTP, dCTP, dTTP, 2 mM $MgCl_2$ and 1×Gold buffer, along with each of the primers (500 nM) (forward primer fD1-AGAGTTTGATCCTGGCTCAG; reverse primer rp2-ACGGCTACCTTGTTACGACTT).[17]

Template DNA, 1.25U of AmpliTaq Gold DNA polymerase LD (Applied Biosystems), and nuclease-free water were added to the sample. The PCR assay was performed using the Takara Thermal Cycler TP-400 (Takara Bio Inc., Shiga, Japan). The cycling conditions used were: 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 42°C for 30 s, and 72°C for 4 min. Gel electrophoresis was performed using a 0.8% agarose gel (Takara Bio Inc.) in 40 mmol/l Tris, 1 mmol/l EDTA for 30 min at 100 V, followed by ethidium bromide staining. Before cycle sequencing, amplicons were purified using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's protocol. Cycle sequencing was performed by forward and reverse priming using the Big Dye v3.1 Terminator Reaction kit (Applied Biosystems). The PCR assay was performed using a Perkin Elmer 9700 with cycling conditions set at: 95°C for 30 s, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Electrophoresis was conducted in a 3130xl genetic analyser (Applied Biosystems).

We used the DNA sequence analysis to examine patients suspected of having bacterial endophthalmitis (patient samples that only had high amounts of total DNA and detected high copy numbers of bacterial 16S rDNA). Basic local alignment search tool (BLAST) analysis was used to examine the DNA sequences. The 16S rDNA sequences obtained were compared with those available in the GenBank BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Using a previously published method,[18] positive identification of the species level was defined as identification of a 16S rDNA sequence that had 99% similarity or greater with that of the GenBank BLAST strain sequence.

Prevention of bacterial contamination

To ensure that no contamination of the PCR preparation occurred, the DNA amplification and the analysis of the amplified products were done in separate laboratories. The preparation was performed on a laminar flow workbench and employed single-use aliquots of reagent and dedicated pipettes. Microfuge tubes and mineral oil aliquots were carefully sterilised prior to use.

RESULTS

Our initial PCR results indicated that bacterial 16S rDNA was positive in 18 ocular fluids of the clinically suspected bacterial endophthalmitis patients (18/19, 95%, table 1). These positive patients had high copy numbers of 16S rDNA ranging from 1.7×10^3 to 1.7×10^9 copies/ml, which indicated the presence of bacterial infection. In the one PCR-negative case (case 16 in table 1), PCR did not detect any bacterial genome in the vitreous fluid (<100 copies), although *Klebsiella pneumoniae* was detected in the biopsy sample of the liver abscess.

In the conventional bacterial cultures, 10 (53%) out of the 19 samples were positive (table 1). In addition, positive Gram staining was found in nine (47%) out of these samples. There were only two patients (cases 2 and 4 in table 1) that received

Table 1 Detection of bacterial 16S rDNA in suspected bacterial endophthalmitis and uveitis

Case	Diagnosis	Sample	Bacterial 16S rDNA	Culture	Smear	BLAST analysis	Treatment
1	Postoperative (acute)	AH	2.8×10^8 copies/ml	*Staphylococcus* spp.	Negative	nt	PPV, IAI, SA
2	Postoperative (acute)	VF	1.5×10^8 copies/ml	Negative	Negative	nt	PPV, IAI, SA
3	Postoperative (acute)	AH	1.5×10^6 copies/ml	*Staphylococcus epidermidis*	G (+)	*Staphylococcus epidermidis*	PPV, IAI, SA
4	Postoperative (acute)	VF	7.5×10^6 copies/ml	Negative	Negative	nt	PPV, IAI, SA
5	Postoperative (acute)	VF	9.0×10^7 copies/ml	Negative	G (+)	nt	PPV, IAI, SA
6	Postoperative (acute)	VF	1.9×10^7 copies/ml	*Streptococcus sanguinis*	G (+)	*Streptococcus sanguinis*	PPV, IAI, SA
7	Postoperative (late)	VF	8.1×10^7 copies/ml	Negative	Negative	*Bradyrhizobium elkanii*	PPV, IAI, SA
8	Postoperative (late)	AH	1.7×10^3 copies/ml	Negative	Negative	nt	SA
9	Postoperative (late)	AH	3.9×10^4 copies/ml	Negative	Negative	nt	SA
10	Postoperative (late)	AH	8.6×10^4 copies/ml	*Pseudomonas aeruginosa*	G (−)	nt	PPV, IAI, SA
11	Post-traumatic	VF	1.4×10^6 copies/ml	*Enterococcus faecalis*	G (+)	*Enterococcus faecalis*	PPV, SA
12	Endogenous	VF	1.3×10^7 copies/ml	*Pseudomonas* sp.	G (−)	*Pseudomonas* sp. PR	PPV, IAI, SA
13	Endogenous	VF	1.7×10^9 copies/ml	*α-Streptococcus*	G (+)	*Streptococcus mitis*	PPV, IAI, SA
14	Endogenous	AH	1.1×10^4 copies/ml	Negative	Negative	nt	IAI, SA
15	Endogenous	VF	5.5×10^6 copies/ml	*Staphylococcus aureus*	Negative	*Staphylococcus aureus*	PPV, IAI, SA
16	Endogenous	AH	<100 copies/ml	Negative	Negative	nt	PPV, IAI, SA
17	Keratitis	AH	3.1×10^6 copies/ml	*Streptococcus pneumoniae*	G (+)	*Streptococcus pneumoniae*	IAI, SA
18	Keratitis	VF	6.8×10^4 copies/ml	Negative	Negative	nt	IAI, SA
19	Intravitreal injection*	VF	1.8×10^6 copies/ml	*Streptococcus oralis*	G (+)	*Streptococcus* sp.	PPV, IAI, SA
20	Idiopathic uveitis	AH	1.4×10^3 copies/ml	Negative	nt	nt	IAI
21	Idiopathic uveitis	VF	6.1×10^4 copies/ml	Negative	Negative	nt	SA
22	CMV retinitis	AH	4.2×10^3 copies/ml	Negative	nt	nt	IAI, SA

AH, aqueous humour; BLAST, basic local alignment search tool; CMV, cytomegalovirus; IAI, intravitreal antibiotic injection; nt, not tested; PPV, pars plana vitrectomy; SA, systemic antibiotics; VF, vitreous fluids.
Using broad-range quantitative PCR, bacterial 16S rDNA could be detected in the ocular samples of the suspected bacterial endophthalmitis cases (18/19, 95%). Broad-range quantitative PCR was also used to measure the bacterial genome in the ocular samples collected from the uveitis patients (n=50) and from the three patients (6%) that were positive.
*Bacterial endophthalmitis after intravitreal injections of bevacizumab.

intravitreal injections of antibiotics prior to the PCR analysis. As shown in table 1, after examinations that included PCR, all patients received antibiotics (systemic and/or local medications).

With the exception of three out of the 50 uveitis patients, real-time PCR indicated the patients were negative for the bacterial 16S rDNA. Details for the three exceptions are shown in table 1.

The 16S rDNA was detected in two patients with idiopathic uveitis and one with cytomegalovirus (CMV) retinitis. Clinically, all of these patients were diagnosed with unilateral uveitis. Bacterial cultures were negative in all of the tested samples. In addition, bacterial 16S rDNA was not detected in any of the 40 control samples collected from the patients without ocular inflammation.

To identify the specific bacterial species, we used BLAST analysis to examine some of the bacterial infectious endophthalmitis patients. Analysis was only possible when the patient's samples had high amounts of total DNA and there was a detected high copy number of the bacterial 16S rDNA. As summarised in table 1, BLAST analysis identified *Staphylococcus epidermidis* (case 3), *Streptococcus sanguinis* (case 6), *Bradyrhizobium elkanii* (case 7), *Enterococcus faecalis* (case 11), *Pseudomonas* sp. *PR* (case 12), *Streptococcus mitis* (case 13), *Staphylococcus aureus* (case 15), *Streptococcus pneumoniae* (case 17) and *Staphylococcus* sp. (case 19). The results of the BLAST analysis were identical to the results of the bacterial culture with the exception of case 7, who was found to have a negative culture. However, even though the bacterial examinations such as bacterial cultures and smears were negative in this patient with late postoperative endophthalmitis, broad-range real-time PCR analysis of the vitreous sample yielded positive results (8.1×10^7 copies/ml). In the present study, once we were able to determine the bacterial species via the BLAST analysis and conclusively diagnose bacterial endophthalmitis, we were then able to begin treatment with antibiotics.

Case report

As seen in table 1, case 7 was a 75-year-old man who was referred to the uveitis clinic at our hospital during July 2007 due to keratic precipitates, cells and fibrin in the anterior chamber along with hypopyon and anterior vitreous opacity in his right eye (figure 1). The patient had undergone cataract surgery in his right eye 1 year prior to being seen in our clinic. Although visual acuity of his right eye at the time of his initial presentation to our clinic was 0.8, 2 months later, his visual acuity was less than 0.1. A vitreous sample was collected during the pars plana

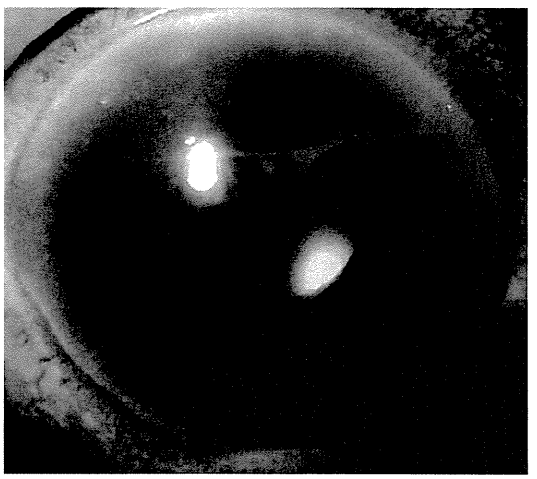

Figure 1 Case 7 (late postoperative endophthalmitis). Slit-lamp photograph in suspected bacterial endophthalmitis. In the right eye, cyclitic membrane, height of the hypopion, and severity of vitritis were seen. In this patient, broad-range quantitative PCR revealed a high copy number of the bacterial genome (8.1×10^7 copies/ml). Basic local alignment search tool (BLAST) analysis detected *Bradyrhizobium elkanii*.

vitrectomy. While bacterial culture and the Gram-staining of the vitreous sample were negative, broad-range and real-time PCR detected 8.1×10^7 copies/ml of bacterial 16S rDNA (table 1). In addition, the BLAST analysis detected *Bradyrhizobium elkanii*. After the patient was given an intravitreal antibiotic injection (vancomycin and ceftazidime) and systemic antibiotics (levofloxacin), inflammation in his right eye completely disappeared. After receiving treatment, visual acuity in his right eye recovered to 0.9 and there was no severe intraocular tissue damage noted.

DISCUSSION

In the present study, with the exception of one patient, we detected bacterial 16S rDNA in all of the cases that were clinically suspected to have bacterial endophthalmitis. In these patients, high copy numbers of the bacterial DNA were detected, which indicated the presence of a bacterial infection. In the single patient who was suspected of having infectious endophthalmitis but had no bacteria in the ocular sample, *K. pneumoniae* was detected by biopsy culture for liver infection. Thus, we were ultimately able to diagnose the patient as having endogenous endophthalmitis.

On the other hand, conventional microbiological investigations of the ocular fluid samples, such as bacterial cultures and smears, were negative in about one-half of these patients. Only three of the 50 samples collected from the patients with other clinical entities of uveitis were positive for the broad-range real-time PCR analyses of the bacterial 16S rDNA. In addition, no bacterial 16S rDNA was detected in any of the samples from the control patients without ocular inflammation.

The potential advantage of using PCR is that minute numbers of bacteria can be detected from the very small specimens that are required for the analysis. Chen *et al*[19] developed this PCR detection method for the eubacterial genome based on the conserved regions of the 16S rRNA sequence (16S rDNA) of *Escherichia coli*. As the universal primers chosen from 16S rDNA have a large amount of sequence information and highly conserved regions of the gene, primers can be synthesised for a wide variety of bacteria. In addition, the eubacterial primers used had both a high specificity and sensitivity, which was comparable to previous studies.[1 3] Hykin *et al*[1] examined 29 control vitreous samples and found four that were positive for the eubacterial genome using PCR. In a further study by Therese *et al*,[3] only a single control sample (5%) was found using the eubacterial-based PCR. In the present study, we did not detect any bacterial 16S rDNA (<100 copies/ml) in any of the samples from the control non-infectious patients when using our broad-range real-time PCR. Thus, another potential advantage of our PCR system is that it provides quantitative information for the bacterial infection. In the present study, we found false positive results (1–100 copies/ml) in only two control samples that we tested, a result that could be due to contamination caused by the conjunctival ocular flora present during the collection of the samples. Other possible causes of the contamination might be related to technical errors that occurred during the PCR preparation or perhaps due to bacterial exposure when collecting the ocular sample.

In cases of bacterial infectious endophthalmitis, it is often difficult to differentiate between inflammation caused by non-infectious and infectious agents. For example, to determine the cause of postoperative inflammation in the eye, we must consider many different possibilities, such as surgical manipulation, toxic lens syndrome, recurrent uveitis (especially if the patient has a previous history) or bacterial endophthalmitis. In the past, microbiological investigations of the ocular fluids have often failed to detect the infectious agent in bacterial endophthalmitis, resulting in a clinical dilemma regarding therapy. Deciding to use antibiotics and steroids necessitates determining whether an inflammation is infectious or sterile. Therefore, an aetiological diagnosis is essential in such cases. The use of PCR with universal eubacterial primers, which possesses broad specificities for all Gram-positive and -negative bacteria, has been recently found to be much more useful for detecting the eubacterial genome in ocular samples of postoperative endophthalmitis cases compared to the routine microbiological investigations.[2 3 5 6 8–10] In the present study, our broad-range real-time PCR for the eubacterial genome showed high correlation with the bacteriologically positive samples. This suggests that bacteriologically negative samples may include the bacterial genome. In a recent report by the French Institutional Endophthalmitis Study Groups, eubacterial PCR was found to be much more effective than bacterial cultures in detecting bacteria in vitreous samples from patients with previous intravitreal administration of antibiotics.[10] Although the previous administration of antibiotics in the PPV vitreous fluids may inhibit bacterial growth, it is assumed that PCR may still be able to detect bacterial DNA of either living or killed bacteria.

As revealed in this study, real-time PCR found only three (6%) of the 50 ocular samples from patients with unilateral uveitis to be positive. However, high copy numbers of bacterial DNA were detected in these uveitis patients, which included idiopathic uveitis (n=2) and cytomegalovirus retinitis (n=1). Endophthalmitis and uveitis positive cases with low quantification of DNA (eg, 1×10^3–1×10^4 copies/ml) cannot be differentiated according to the number of copies. Although topical or systemic steroids were administered for long periods in the idiopathic uveitis patients, the inflammation remained uncontrolled. It has also been reported that viral PCR has found cytomegalovirus DNA in the eyes of cytomegalovirus retinitis cases.[13] When these patients were given intravitreal administration of an antiviral injection (Ganciclovir), an anterior vitreous opacity was subsequently observed. There were three cases that received antibiotics (intravitreal injection and/or systemic) in our study and the intraocular inflammation, such as vitreous opacity, was well controlled by this antibiotic therapy. Although bacterial DNA amplification in such cases usually suggests contamination, antibiotic administration proved to be effective in our study. Thus, the bacterial PCR-based evidence suggests bacterial involvement in eyes that have a suspected intraocular infection. While PCR for eubacterial detection is necessary for rapid and accurate diagnosis in patients suffering from an unknown intraocular inflammatory disorder, it can also be used to accurately determine samples that are not infected. In our study we found 47 samples (94%) that had negative PCR results. Overall, our results suggest that a sensitive and rapid diagnostic test not only allows for confident verification of the diagnosis (non-infectious inflammation vs infection), but also allows for early commencement of specific and appropriate treatment. In addition, PCR analysis is able to exclude bacterial infections as the potential cause of an ocular disorder.

In conclusion, this new PCR system is an excellent diagnostic system for intraocular specimens and can be used as an alternative to further examine specimens determined to be bacteriologically negative by conventional methods. Our study also clearly demonstrated that a new diagnostic PCR system using eubacterial detection with broad-range PCR along with quantitative evaluation with real-time PCR could be extremely useful for detecting bacterial DNA within ocular samples. Recently, Goldschmidt *et al* reported that a new diagnostic test for

Propionibacteriaceae was designed using TaqMan real-time PCR.[20] Therefore, the ability to be able to collect quantitative information on bacterial infections in the eye should be useful in helping to determine clinical diagnoses and therapeutic follow-ups. Moreover, using a combination of the quantitative PCR method and the BLAST analysis to detect bacterial species is a very valuable tool for diagnosing suspected bacterial endophthalmitis. However, the DNA in 10 of 19 samples could not be sequenced using this technique and thus could not identified, which could potentially limit the clinical usefulness of this technique at the present time. In order for clinicians to be able to obtain bacterial identifications, we may need to consider additional options for the sequence analysis. In addition, in the future we will need to further verify whether this broad-range PCR can detect candidate bacterial DNA including *K. pneumoniae* in bacterial endophthalmitis.

Acknowledgements Dr Masaru Miyanaga of Miyata Hospital, and Drs Kazuichi Maruyama and Kenji Nagata of the Department of Ophthalmology, Kyoto Prefectural University of Medicine, kindly collected and sent the samples used in this study. We are very grateful for the expert technical assistance of Ms Shizu Inoue. This work was supported by Grants-in-Aid for Scientific Research (C) 20592073 and (B) 19390440 of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Ethics Committee of Tokyo Medical and Dental University. The research followed the tenets of the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Hykin PG,** Tobal K, McIntyre G, *et al*. The diagnosis of delayed post-operative endophthalmitis by polymerase chain reaction of bacterial DNA in vitreous samples. *J Med Microbiol* 1994;**40**:408–15.
2. **Lohmann CP,** Heeb M, Linde HJ, *et al*. Diagnosis of infectious endophthalmitis after cataract surgery by polymerase chain reaction. *J Cataract Refract Surg* 1998;**24**:821–6.
3. **Therese KL,** Anand AR, Madhavan HN. Polymerase chain reaction in the diagnosis of bacterial endophthalmitis. *Br J Ophthalmol* 1998;**82**:1078–82.
4. **Knox CM,** Cevallos V, Margolis TP, *et al*. Identification of bacterial pathogens in patients with endophthalmitis by 16S ribosomal DNA typing. *Am J Ophthalmol* 1999;**128**:511–12.
5. **Lohmann CP,** Linde HJ, Reischl U. Improved detection of microorganisms by polymerase chain reaction in delayed endophthalmitis after cataract surgery. *Ophthalmology* 2000;**107**:1047–51.
6. **Anand AR,** Madhavan HN, Therese KL. Use of polymerase chain reaction (PCR) and DNA probe hybridization to determine the Gram reaction of the infecting bacterium in the intraocular fluids of patients with endophthalmitis. *J Infect* 2000;**41**:221–6.
7. **Okhravi N,** Adamson P, Lightman S. Use of PCR in endophthalmitis. *Ocul Immunol Inflamm* 2000;**8**:189–200.
8. **Okhravi N,** Adamson P, Carroll N, *et al*. PCR-based evidence of bacterial involvement in eyes with suspected intraocular infection. *Invest Ophthalmol Vis Sci* 2000;**41**:3474–9.
9. **Chiquet C,** Lina G, Benito Y, *et al*. Polymerase chain reaction identification in aqueous humour of patients with postoperative endophthalmitis. *J Cataract Refract Surg* 2007;**33**:635–41.
10. **Chiquet C,** Cornut PL, Benito Y, *et al*. Eubacterial PCR for bacterial detection and identification in 100 acute postcataract surgery endophthalmitis. *Invest Ophthalmol Vis Sci* 2008;**49**:1971–8.
11. **Sugita S,** Shimizu N, Kawaguchi T, *et al*. Identification of human herpes virus 6 in a patient with severe unilateral panuveitis. *Arch Ophthalmol* 2007;**125**:1426–71.
12. **Kido S,** Sugita S, Horie S, *et al*. Association of varicella zoster virus load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. *Br J Ophthalmol* 2008;**92**:505–8.
13. **Sugita S,** Shimizu N, Watanabe K, *et al*. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. *Br J Ophthalmol* 2008;**92**:928–32.
14. **Allen SD.** Anaerobic bacteria. In: Lennete Edwin H, ed. *Manual of clinical microbiology*. 4th edn. Washington DC: American Society for Microbiology, 1985:413–72.
15. **Baron EJ,** Peterson LR, Finegold SM. *Bailey and Scott's diagnostic microbiology*. 9th edn. St Louis: Mosby, 1994:79–136.
16. **Takai K,** Horikoshi K. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl Environ Microbiol* 2000;**66**:5066–72.
17. **Weisburg WG,** Barns SM, Pelletier DA, *et al*. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;**173**:697–703.
18. **Goldenberger D,** Kunzle A. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J Clin Microbiol* 1997;**35**:2733–9.
19. **Chen K,** Neimark H, Rumore P, *et al*. Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. *FEMS Microbiol Lett* 1989;**48**:19–24.
20. **Goldschmidt P,** Ferreira CC, Degorge S, *et al*. Rapid detection and quantification of Propionibacteriaceae. *Br J Ophthalmol* 2009;**93**:258–62.

Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR

Sunao Sugita, Norio Shimizu, Ken Watanabe, et al.

Br J Ophthalmol 2011 95: 345-349 originally published online July 31, 2010
doi: 10.1136/bjo.2009.171504

Journal of Pathology
J Pathol 2011; 223: 496–510
Published online 5 January 2011 in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.2823

ORIGINAL PAPER

Activated oncogenic pathways and therapeutic targets in extranodal nasal-type NK/T cell lymphoma revealed by gene expression profiling

Siok-Bian Ng,[1]* Viknesvaran Selvarajan,[1] Gaofeng Huang,[2] Jianbiao Zhou,[3] Andrew L Feldman,[4] Mark Law,[4] Yok-Lam Kwong,[5] Norio Shimizu,[6] Yoshitoyo Kagami,[7] Katsuyuki Aozasa,[8] Manuel Salto-Tellez[1,3] and Wee-Joo Chng[2,3]*

[1] Department of Pathology, National University Health System, Singapore
[2] Department of Haematology-Oncology, National University Cancer Institute of Singapore, National University Health System, Singapore
[3] Cancer Science Institute Singapore, National University of Singapore, Singapore
[4] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, USA
[5] Division of Haematology/Oncology and Bone Marrow Transplantation, Queen Mary Hospital, Hong Kong
[6] Department of Virology, Tokyo Medical and Dental University, Japan
[7] Department of Hematology, Toyota Kosei Hospital, Japan
[8] Department of Pathology, Osaka University Graduate School of Medicine, Japan

*Correspondence to: Dr Siok-Bian Ng, MBBS, FRCPA, Department of Pathology, National University Hospital, 5 Lower Kent Ridge Road, Main Building, Level 3, Singapore 119074. e-mail: patnsb@nus.edu.sg
*Correspondence to: Dr Wee-Joo Chng, MB ChB, MRCP, FRCPath, Department of Haematology–Oncology, National University Hospital, 5 Lower Kent Ridge Road, Main Building, Level 3, Singapore 119074. e-mail: mdccwj@nus.edu.sg

Abstract

We performed comprehensive genome-wide gene expression profiling (GEP) of extranodal nasal-type natural killer/T-cell lymphoma (NKTL) using formalin-fixed, paraffin-embedded tissue ($n = 9$) and NK cell lines ($n = 5$) in comparison with normal NK cells, with the objective of understanding the oncogenic pathways involved in the pathogenesis of NKTL and to identify potential therapeutic targets. Pathway and network analysis of genes differentially expressed between NKTL and normal NK cells revealed significant enrichment for cell cycle-related genes and pathways, such as PLK1, CDK1, and Aurora-A. Furthermore, our results demonstrated a pro-proliferative and anti-apoptotic phenotype in NKTL characterized by activation of Myc and nuclear factor kappa B (NF-κB), and deregulation of p53. In corroboration with GEP findings, a significant percentage of NKTLs ($n = 33$) overexpressed c-Myc (45.4%), p53 (87.9%), and NF-κB p50 (67.7%) on immunohistochemistry using a tissue microarray containing 33 NKTL samples. Notably, overexpression of survivin was observed in 97% of cases. Based on our findings, we propose a model of NKTL pathogenesis where deregulation of p53 together with activation of Myc and NF-κB, possibly driven by EBV LMP-1, results in the cumulative up-regulation of survivin. Down-regulation of survivin with Terameprocol (EM-1421, a survivin inhibitor) results in reduced cell viability and increased apoptosis in tumour cells, suggesting that targeting survivin may be a potential novel therapeutic strategy in NKTL.

Keywords: NK/T-cell lymphoma; gene expression profiling; survivin; Myc; NF-κB; p53; paraffin-embedded tissue

Received 9 September 2010; Revised 3 November 2010; Accepted 4 November 2010

No conflicts of interest were declared.

Introduction

Extranodal nasal-type natural killer/T-cell lymphoma (NKTL) is a distinct clinicopathological entity most commonly affecting Asians and Central and South Americans, and characterized by a clonal proliferation of NK or T cells with a cytotoxic phenotype [1]. There is a strong association with Epstein–Barr virus (EBV), which manifests a type II latency pattern [2,3]. EBV is detected in the neoplastic cells in a clonal episomal form, supporting the role of the virus in tumour pathogenesis. There have been few studies investigating the oncogenic mechanisms of NKTL. These reports have identified mutations of genes regulating apoptosis, such as *FAS* and *p53*, which may contribute to the development of this tumour [4–6]. In addition, the expression of P-glycoproteins [7] and absence of granzyme B inhibitor PI9 [8] may account for the poor prognosis of patients with NKTL who were treated with chemotherapy. Like many haematolymphoid malignancies, NKTL is frequently associated with genetic alterations involving loss or gain of genetic material, the

commonest being del(6)(q21–q25) [9]. However, no specific chromosomal translocation has been identified. Iqbal *et al* [10] performed array comparative genomic hybridization on NK-cell malignancies and identified *PRDM1* as the likely target gene in del6q21. Recently, Hwang *et al* [11] performed genome-wide GEP on NKTL and identified overexpression of several genes related to vascular biology, EBV-induced genes, and platelet-derived growth factor receptor α. Deregulation of several oncogenic pathways, such as AKT, STAT3, and nuclear factor-κB pathways, was also detected. Nevertheless, comprehensive genome-wide profiling of NKTL is scarce as extensive research has been limited by the rarity of this entity, the difficulty in obtaining adequate biopsy specimens, extensive tumour necrosis, and the lack of availability of frozen tumour tissue.

Gene expression profiling (GEP) has been extensively used in cancer research in recent years. One major drawback is the requirement for frozen tissue for current methods of genome-wide expression profiling. Recently, Hoshida *et al* demonstrated the feasibility of genome-wide expression profiling of formalin-fixed, paraffin-embedded (FFPE) tissues of hepatocellular carcinoma and identified a molecular signature that correlated with survival [12]. In this study, we performed genome-wide expression profiling on a series of NKTLs using FFPE tissues in relation to normal NK cells and NK tumour cell lines, with the main objective of understanding molecular pathways deregulated in NKTL. In the process, we hope to identify potential new therapeutic targets in a disease where the outcome is poor with current treatment modalities.

Material and methods

Case selection and construction of tissue microarray

Patients with a diagnosis of NKTL were identified from the archives of the Department of Pathology, National University Hospital (NUH), from 1990 to 2009. Additional immunohistochemistry and *in situ* hybridization for EBV-encoded small RNA (EBER) were performed and the cases were classified according to the 2008 WHO lymphoma classification. Cases with no additional tissue available for immunohistochemical or genetic analysis were excluded. A total of 33 cases of NKTL were selected. According to the WHO criteria, all 33 cases expressed CD3, cytotoxic markers (granzyme B and/or TIA-1), and EBER. Immunoreactivity for CD56, CD8, and CD4 was present in 64% (21 cases), 16% (five cases), and 3% (one case), respectively. The clinical and immunophenotypic data of the cases are summarized in the Supporting information, Supplementary Table 1. Tissue microarrays (TMAs) of the 33 cases of NKTL were also constructed (see Supporting information, Supplementary methods).

Nine cases of NKTL with adequate FFPE tissue remaining and good-quality RNA were selected for GEP. In addition, two cases each of normal skin and soft tissue, intestinal, nasal, and lymph node FFPE tissue were also included for GEP analysis as control tissue. The study was approved by the Domain Specific Review Board of the National Healthcare Group, Singapore.

Immunohistochemistry (IHC)

Four-micrometre sections from the TMA blocks were cut for IHC. IHC was performed for c-Myc, p53, survivin, and five NF-κB transcription factors including p50, p52, p65, RelB, and C-Rel (see the Supporting information, Supplementary methods and Supplementary Table 2 for more details). Appropriate positive tissue controls were used.

The immunohistochemical expression for all the antibodies was scored as a percentage of the total tumour cell population per 1 mm core diameter (×400) by one of the authors (NSB). The majority of the cases (97%) had two to four cores represented on the TMA and the final score was obtained as an average of all the individual cores. For c-Myc, p53, and survivin antibodies, positive expression was defined as nuclear staining in 10% or more of the tumour population. For the five NF-κB antibodies (p50, p52, p65, RelB, and C-Rel), only nuclear immunoreactivity was regarded as constitutive NF-κB activation and positive expression was defined as nuclear staining in 10% or more of tumour cells, similar to previous published reports [13].

NK cell lines and cultures

The NK-tumour cell lines used in this study included NK-92 (American Type Culture Collection), KHYG-1 (Japanese Collection of Research Bioresources), HANK-1 (a gift from Dr Y Kagami), SNK-6, SNT-8 (a gift from Dr N Shimizu), and NK-YS (a gift from Dr YL Kwong). Culture conditions are given in the Supporting information, Supplementary methods. The phenotypic and genotypic characteristics of the NK cell lines have been well characterized in previous studies [14,15] and are summarized in the Supporting information, Supplementary Table 3.

Isolation of normal NK cells from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll Paque Plus density gradient centrifugation (Amersham Biosciences, Piscataway, NJ, USA) from whole blood samples obtained from healthy donors and buffy coat packs of whole blood samples from the Blood Donation Centre, NUH. Highly pure untouched normal human NK cells were isolated using the NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated NK cells as determined by flow cytometry was between 90% and 99%. The isolated NK cells were subsequently stimulated by culturing in the presence of human recombinant IL-2 (Miltenyi Biotec). For cell block preparation, Bouin's solution was added to the

isolated normal NK cell samples, followed by centrifugation. The packed cell sediments were fixed in formalin for tissue processing.

RNA extraction from FFPE, NK cell lines, and normal NK cells

Total RNA from NKTL FFPE tissues and FFPE normal tissue controls was isolated using a High Pure RNA Paraffin Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. All the sections were deparaffinized with xylene, subjected to proteinase K digestion, and RNA was extracted according to the manufacturer's protocol.

Total RNA was extracted from freshly isolated cells from NK cell lines and normal NK cell samples obtained from healthy donors using the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) protocol with DNaseI treatment included. The concentration and purity of the total RNA extracted were measured using a NanoDrop ND 3.0 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and an RNA 6000 LabChip Kit (Agilent Technologies). Of the samples with RNA extracted, nine met the quality requirements and were used for GEP and subsequent analysis.

Gene expression profiling and analysis

GEP was performed according to the complementary DNA-mediated annealing, selection, extension, and ligation (DASL) assay (Illumina, Inc, San Diego, CA, USA) [16,17]. Raw signal intensities of each probe were obtained from data analysis software (Beadstudio; Illumina, San Diego, CA, USA). The data were normalized using a linear calibration method. Hierarchical clustering of samples was performed using the Pearson coefficient and the Ward method as the similarity and linkage methods, respectively, using the Bioconductor packages of R. To derive the NKTL-specific gene expression profiles, we extracted genes differentially expressed between NK cell lines and NKTL FFPE samples, and normal NK cells and normal FFPE controls using significance analysis of microarray (SAM) [18] (see Supporting information, Supplementary methods).

To further understand the functional and biological relevance of differentially expressed genes, gene ontology and pathway/network analysis was performed using the web-based software MetaCore (GeneGo, St Joseph, MI, USA). The software contains an interactive, manually annotated database derived from literature publications on proteins and small molecules that allows for representation of biological functionality and integration of functional, molecular, and clinical information. Several algorithms to enable both the construction and the analysis of gene networks were integrated as previously described [19]. The output p values reflect scoring, prioritization, and statistical significance of networks according to the relevance of input data.

Quantitative PCR for validation of selected genes

A reverse transcription reaction was carried out using the High Capacity cDNA Reverse Transcription Kit system (ABI, Foster City, CA, USA). Real-time fluorescent monitoring of the PCR products was performed using the Power SYBR Green PCR Master Mix (ABI) and gene-specific primers (Supporting information, Supplementary Table 4). Real-time PCR was performed in the ABI PRISM 7300 Sequence Detection system (Applied Biosystems, foster city, CA, USA) and analysed utilizing Sequence Detection Software v1.4 (ABI). Using endogenous GAPDH as an internal control for comparison, relative quantification of gene expression levels was performed and calculated using $2(-\Delta\Delta CT)$.

FISH for c-Myc translocation

MYC breakapart (BAP) probes were designed using the University of California Santa Cruz Genome Browser (http://www.genome.UCSC.edu) to identify bacterial artificial chromosomes (BACs) flanking the genes of interest (Supporting information, Supplementary Table 5). DNA was isolated (Plasmid Maxi Kit; Qiagen, Valencia, CA, USA) from BAC clones (ResGen™; Invitrogen, Carlsbad, CA, USA) and labelled with Texas Red-dUTP (Molecular Probes, Invitrogen, Carlsbad, CA, USA) or SpectrumGreen-dUTP (Abbott Molecular, Des Plaines, IL, USA). FISH was performed on TMA sections and scored as previously described [20]. Sections were analysed qualitatively by a microscopist (ML) and a minimum of 50 cells with strong FISH signals was required for a sample to be considered informative.

Terameprocol (EM-1421) treatment of NK cell lines

NK cell lines were treated with varying concentrations of the BIRC5 inhibitor Terameprocol (EM-1421; Erimos Pharmaceuticals, Houston, TX, USA) [21, 22] and control. Following incubation for 48 h, the cells were harvested, washed in PBS, and subjected to (i) MTS assay to evaluate the degree of cell viability (see Supporting information, Supplementary methods); (ii) flow cytometric analysis to assess the degree of apoptotic cell death using Annexin-V-APC and propidium iodide (PI) staining (BD Pharmigen, CA, USA); and (iii) western blot analysis to confirm successful inhibition of BIRC5 (Supporting information, Supplementary methods). The flow cytometry analysis was performed on a BD LSR II flow cytometer (Becton Dickinson, CA, USA) using BD FACSDiva™ software.

Table 1. Pathways and cellular processes enriched in genes differentially expressed between NKTL and normal NK cells

Map	Map folders	Cell process	*p* value	Objects in gene list	Objects in map
Cell cycle_The metaphase checkpoint	Congenital, Hereditary, and Neonatal Diseases and Abnormalities Regulatory processes/Cell cycle	Cell cycle	4.01E-05	10	36
Cell cycle_Chromosome condensation in prometaphase	Regulatory processes/Cell cycle	Cell cycle	1.21E-04	7	20
Cell cycle_Transition and termination of DNA replication	Regulatory processes/Cell cycle	Cell cycle	7.48E-04	7	26
Cell cycle_Start of DNA replication in early S phase	Regulatory processes/Cell cycle	Cell cycle	2.30E-03	7	31
Cell cycle_Role of APC in cell cycle regulation	Regulatory processes/Cell cycle	Cell cycle	2.79E-03	7	32
Cell cycle_Initiation of mitosis	Congenital, Hereditary, and Neonatal Diseases and Abnormalities Regulatory processes/Cell cycle	Cell cycle	3.42E-03	6	25
Cell cycle_Spindle assembly and chromosome separation	Regulatory processes/Cell cycle	Cell cycle	1.22E-02	6	32
DNA damage_ATM/ATR regulation of G2/M checkpoint	Congenital, Hereditary, and Neonatal Diseases and Abnormalities Regulatory processes/Cell cycle Regulatory processes/DNA damage	Cell cycle	1.96E-02	5	26
Transcription_Sin3 and NuRD in transcription regulation	Regulatory processes/Transcription	Transcription	2.73E-02	6	38
Cell cycle_Cell cycle (generic schema)	Congenital, Hereditary, and Neonatal Diseases and Abnormalities Regulatory processes/Cell cycle	Cell cycle	3.72E-02	4	21
Development_MAG-dependent inhibition of neurite outgrowth	Congenital, Hereditary, and Neonatal Diseases and Abnormalities Protein function/Growth factors Regulatory processes/Development/Neurogenesis	Intracellular receptor-mediated signalling pathway, response to extracellular stimulus	4.44E-02	5	32

Results

Analysis of GEP of NKTL

We compared the gene expression of NKTL FFPE samples ($n = 9$) with that of normal NK cells, as well as the respective normal FFPE tissue controls from nasal, skin and soft tissue, intestinal tract, and lymph node (see Supporting information, Supplementary methods). Among the genes showing at least a two-fold and statistically significant difference ($p < 0.05$), 339 were found to be up-regulated and 737 were down-regulated in NKTL compared with normal NK cells (Supporting information, Supplementary Table 6). We performed quantitative PCR validation on a few interesting genes frequently involved in tumour oncogenesis, including *BIRC5* (*survivin*), *EZH2*, *STMN1*, and *WHSC1*. On the whole, the quantitative PCR results were consistent with GEP data showing overexpression of *BIRC5*, *EZH2*, and *STMN1* in NK cell lines compared with normal NK cells (see the Supporting information, Supplementary Figure 1).

These differentially expressed genes were significantly enriched for cell cycle-related pathways and processes (Table 1), suggesting that NKTLs are significantly more proliferative than normal NK cells. Amongst the key proteins involved in cell cycle and mitosis are PLK1, CDK1, and Aurora-A. These proteins may also be potential therapeutic targets because of their involvement in carcinogenesis [23–25].

Using MetaCore, we found that the differentially expressed genes between NKTL and normal NK cells were enriched for targets of a number of transcription factors including Myc, p53, and NF-κB subunits (Table 2). When we further examined the expression of the Myc transcription targets and their relationship with Myc, it was striking that most of the genes up-regulated and down-regulated in our list of differentially expressed genes have been shown by previous experiments to be concordantly expressed and repressed by Myc (Figure 1A). This result suggests that Myc is activated in NKTL compared with normal NK cells. In contrast, the transcription targets of p53 in our list of differentially expressed genes showed a predominantly opposite effect to what is expected under normal p53 control, ie genes that are normally repressed by p53 were up-regulated in NKTL compared with normal NK cells, and vice versa (Figure 1B), indicating deregulation of the p53 transcription factor in NKTL compared with normal NK cells.

We further assessed the expression of a previously published NF-κB signature [26] and a validated MYC signature (unpublished) in the gene expression dataset.

Table 2. Targets of transcription targets enriched amongst genes differentially expressed between NKTL and normal NK cells

No	Network	GO processes	Total nodes	Root nodes	*p* value	zScore	gScore
1	c-Myc	Cell cycle (33.8%; 4.867e-13), cell division (21.1%; 2.325e-12), cell cycle process (26.8%; 4.968e-11), cell cycle phase (23.9%; 5.019e-11), mitotic cell cycle (21.1%; 5.843e-10)	73	72	3.28E-149	89.37	89.37
2	SP1	Cell division (13.8%; 2.728e-06), positive regulation of cellular process (35.4%; 3.296e-06), positive regulation of catalytic activity (18.5%; 4.041e-06), regulation of catalytic activity (23.1%; 4.731e-06), regulation of molecular function (24.6%; 4.773e-06)	65	64	2.66E-132	84.18	84.18
3	p53	Regulation of cell cycle (26.7%; 3.272e-11), cell cycle (33.3%; 5.758e-11), cell division (20.0%; 7.506e-10), cell cycle process (26.7%; 1.779e-09), cell cycle checkpoint (13.3%; 2.766e-09)	62	61	5.59E-126	82.15	82.15
4	ESR1 (nuclear)	Regulation of cell cycle (25.0%; 2.165e-08), cell division (18.8%; 1.881e-07), cell cycle checkpoint (12.5%; 4.328e-07), regulation of mitotic cell cycle (14.6%; 6.202e-07), negative regulation of cellular process (39.6%; 1.318e-06)	53	52	4.48E-107	75.73	75.73
5	CREB1	Cell cycle (31.7%; 2.639e-07), M phase (22.0%; 3.244e-07), regulation of cell cycle (24.4%; 4.238e-07), cell division (19.5%; 6.635e-07), cell cycle phase (22.0%; 4.111e-06)	42	41	4.25E-84	67.07	67.07
6	RelA (p65 NF-κB subunit)	Regulation of cell proliferation (38.9%; 2.214e-08), positive regulation of biological process (52.8%; 6.173e-08), developmental process (69.4%; 8.591e-08), biological regulation (94.4%; 8.778e-08), positive regulation of cellular process (50.0%; 8.804e-08)	38	37	8.86E-76	63.63	63.63
7	GATA-1	Cell division (22.9%; 1.798e-07), regulation of cell cycle (25.7%; 9.966e-07), M phase (22.9%; 1.068e-06), cell cycle checkpoint (14.3%; 2.108e-06), cell cycle (31.4%; 2.446e-06)	35	34	1.49E-69	60.92	60.92
8	Androgen receptor	Regulation of cell cycle (33.3%; 1.553e-08), M phase (30.0%; 1.661e-08), cell cycle (40.0%; 3.930e-08), cell division (26.7%; 4.844e-08), negative regulation of cellular process (53.3%; 5.874e-08)	33	32	2.07E-65	59.04	59.04
9	c-Fos	Biological regulation (93.8%; 8.492e-07), regulation of metabolic process (62.5%; 2.756e-06), regulation of cellular process (87.5%; 4.280e-06), regulation of cell cycle (25.0%; 5.084e-06), M phase (21.9%; 7.025e-06)	32	31	2.44E-63	58.09	58.09
10	c-Rel (NF-κB subunit)	Regulation of cell cycle (31.0%; 1.674e-07), cell cycle (34.5%; 2.717e-06), M phase (24.1%; 3.459e-06), mitotic cell cycle (24.1%; 9.697e-06), cell division (20.7%; 1.212e-05)	31	30	2.85E-61	57.11	57.11
11	HNF6	Regulation of cell cycle (31.0%; 1.674e-07), M phase (27.6%; 2.200e-07), cell cycle (37.9%; 2.788e-07), cell division (24.1%; 7.233e-07), mitotic cell cycle (27.6%; 7.253e-07)	30	29	3.33E-59	56.12	56.12
12	PPAR-gamma	Regulation of cell cycle (31.0%; 1.674e-07), cell cycle (37.9%; 2.788e-07), cell division (24.1%; 7.233e-07), mitotic cell cycle (27.6%; 7.253e-07), cell cycle checkpoint (17.2%; 7.936e-07)	29	28	3.87E-57	55.11	55.11
13	STAT3	M phase (27.6%; 2.200e-07), cell cycle (37.9%; 2.788e-07), cell division (24.1%; 7.233e-07), mitotic cell cycle (27.6%; 7.253e-07), cell cycle process (31.0%; 1.649e-06)	29	28	3.87E-57	55.11	55.11
14	E2F1	Cell division (42.9%; 2.758e-14), cell cycle (57.1%; 1.936e-13), mitotic cell cycle (42.9%; 2.870e-12), cell cycle phase (42.9%; 1.646e-11), DNA metabolic process (42.9%; 3.985e-11)	28	27	4.49E-55	54.08	54.08
15	HIF1A	Regulation of cell cycle (37.0%; 4.751e-09), negative regulation of cellular process (55.6%; 7.431e-08), cell cycle (40.7%; 1.168e-07), negative regulation of biological process (55.6%; 2.183e-07), regulation of mitotic cell cycle (22.2%; 2.953e-07)	27	26	5.19E-53	53.03	53.03
16	CDP/Cux	Cell cycle (59.3%; 8.763e-14), M phase (37.0%; 2.405e-10), mitotic cell cycle (37.0%; 1.107e-09), mitosis (29.6%; 2.891e-09), M phase of mitotic cell cycle (29.6%; 3.407e-09)	27	26	5.19E-53	53.03	53.03

Table 2. (*Continued*)

No	Network	GO processes	Total nodes	Root nodes	*p* value	zScore	gScore
17	N-Myc	Cell cycle (46.2%; 5.436e-09), cell division (30.8%; 1.378e-08), regulation of cell cycle (34.6%; 5.685e-08), mitotic cell cycle (30.8%; 2.836e-07), cell cycle checkpoint (19.2%; 4.460e-07)	26	25	5.97E-51	51.96	51.96
18	GCR-alpha	Negative regulation of cellular process (57.7%; 3.722e-08), negative regulation of biological process (57.7%; 1.105e-07), cell division (26.9%; 3.196e-07), cell cycle (38.5%; 8.437e-07), regulation of cell cycle (30.8%; 8.926e-07)	26	25	5.97E-51	51.96	51.96
19	AML1 (RUNX1)	Regulation of cell cycle (38.5%; 3.078e-09), cell cycle (46.2%; 5.436e-09), cell division (30.8%; 1.378e-08), mitotic cell cycle (34.6%; 1.544e-08), cell cycle process (38.5%; 4.140e-08)	26	25	5.97E-51	51.96	51.96
20	TCF7L2 (TCF4)	Negative regulation of cellular process (60.0%; 1.775e-08), regulation of cell cycle (36.0%; 3.824e-08), cell cycle (44.0%; 4.443e-08), negative regulation of biological process (60.0%; 5.327e-08), cell cycle process (36.0%; 3.906e-07)	25	24	6.85E-49	50.87	50.87
21	VDR	Cell division (28.0%; 2.373e-07), cell cycle (40.0%; 5.473e-07), regulation of cell cycle (32.0%; 6.354e-07), M phase (28.0%; 1.154e-06), mitotic cell cycle (28.0%; 3.281e-06)	25	24	6.85E-49	50.87	50.87
22	ATF-2	Cell cycle (40.0%; 5.473e-07), regulation of cell cycle (32.0%; 6.354e-07), M phase (28.0%; 1.154e-06), mitotic cell cycle (28.0%; 3.281e-06), cell division (24.0%; 4.806e-06)	25	24	6.85E-49	50.87	50.87
23	IRF1	Regulation of cell cycle (36.0%; 3.824e-08), cell cycle (40.0%; 5.473e-07), M phase (28.0%; 1.154e-06), mitotic cell cycle (28.0%; 3.281e-06), cell division (24.0%; 4.806e-06)	25	24	6.85E-49	50.87	50.87
24	FKHR	Cell cycle (41.7%; 3.461e-07), regulation of cell cycle (33.3%; 4.443e-07), negative regulation of cellular process (54.2%; 8.335e-07), M phase (29.2%; 8.479e-07), post-translational protein modification (45.8%; 1.468e-06)	24	23	7.83E-47	49.75	49.75
25	Brca1	Regulation of cell cycle (41.7%; 1.203e-09), cell cycle (50.0%; 1.694e-09), cell cycle checkpoint (25.0%; 5.340e-09), cell cycle process (41.7%; 1.649e-08), mitotic cell cycle (33.3%; 1.401e-07)	24	23	7.83E-47	49.75	49.75
26	RARalpha	Cell division (29.2%; 1.736e-07), cell cycle (41.7%; 3.461e-07), regulation of cell cycle (33.3%; 4.443e-07), negative regulation of cellular process (54.2%; 8.335e-07), M phase (29.2%; 8.479e-07)	24	23	7.83E-47	49.75	49.75
27	MYOD	Cell cycle (41.7%; 3.461e-07), regulation of cell cycle (33.3%; 4.443e-07), M phase (29.2%; 8.479e-07), cell differentiation (54.2%; 1.202e-06), post-translational protein modification (45.8%; 1.468e-06)	24	23	7.83E-47	49.75	49.75
28	HMGI/Y	Cell cycle (43.5%; 2.128e-07), regulation of cell cycle (34.8%; 3.046e-07), M phase (30.4%; 6.128e-07), mitotic cell cycle (30.4%; 1.754e-06), cell cycle process (34.8%; 2.387e-06)	23	22	8.91E-45	48.61	48.61
29	HOXD13	Cell division (30.4%; 1.249e-07), cell cycle (43.5%; 2.128e-07), regulation of cell cycle (34.8%; 3.046e-07), M phase (30.4%; 6.128e-07), regulation of cell proliferation (43.5%; 6.871e-07)	23	22	8.91E-45	48.61	48.61
30	PPARGC1 (PGC1-alpha)	Cell cycle (43.5%; 2.128e-07), regulation of cell cycle (34.8%; 3.046e-07), M phase (30.4%; 6.128e-07), mitotic cell cycle (30.4%; 1.754e-06), cellular biopolymer metabolic process (87.0%; 1.992e-06)	23	22	8.91E-45	48.61	48.61

Consistent with the MetaCore analysis, we observed that the MYC and NF-κB signatures were highly expressed in the NK cell lines and tumour samples compared with normal NK cells. When these two signatures were summarized into indices using the median expression of genes constituting the respective signatures, both the NF-κB index and the MYC index were significantly higher in NK cell lines and NKTL than in normal NK cells (Figure 1C).

As NKTL is an aggressive lymphoma often with extranodal involvement, we wanted to see if there was any enrichment among dysregulated genes for cell