presence of Asc 2-P. These results suggested that Asc 2-P promoted differentiation of UE7T-13 cells into osteoblasts. We removed Asc 2-P from hESF10 medium for hMSCs, and designated the new formulation D-hESF10.

To confirm the characteristics of UE7T-13 cells expanded in the absence of serum, we performed flow cytometry with antibodies to markers for hMSCs and pluripotent cells (Fig. 3A). Cells grown in D-hESF10 medium were positive for CD44, CD73, CD90, CD105, and TRA-2-54 (tissue non-specific alkaline phosphatase antibody), but negative for CD45 (a marker of all hematopoietic cells) and CD56 (a neural cell adhesion molecule). We further stained the cells with antibodies to CD105 and SSEA-3 (Fig. 3B). The immunocytochemical analysis showed that SSEA-3$^+$/CD105$^+$ double positive cells were present in the UE7T-13 population grown in D-hESF10 although cells positive for either CD105 or SSEA-3 were also detected in the population. The cell growth rate in D-hESF10 was comparable to that in conventional culture conditions (Fig. 4).

We subsequently examined the properties of UE7T-13 cells serially passaged in D-hESF10 medium. The morphology of serum-free expanded UE7T-13 cell populations was comparably small, spindle-shaped cells compared with that in conventional medium (Fig. 5A). The expression of hMSC and hES cell pluripotency markers were determined by real-time PCR analysis (Fig. 5B) in UE7T-13 cells cultured for 4 passages in D-hESF10 medium. The expression of hMSC markers, *CD105*, *THY1*, and *integrinβ1* (*ITGB1*), and the hES cell pluripotency markers, *OCT3/4* (*POU5F1*) and *NANOG* were similar in the cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. *SOX2* expression was significantly higher in cells cultured in D-hESF10 compared with cells cultured in conventional culture conditions. On the other hand, the expression levels of *IBSP*, *BGP*, *SPOCK2*, and *SPP1* were significantly lower in cells cultured in D-hESF10 compared with those in the cells cultured in conventional culture conditions. These results suggest that serum-free expanded UE7T-13 cells retain an undifferentiated phenotype.

We determined the differentiation capacity of the serum-free expanded UE7T-13 cells. After the UE7T-13 cells were cultured in D-hESF10 for 7 passages, the cells were cultured in medium designed to induce differentiation into osteoblasts or adipocytes (Fig. 6). Culturing in osteoblastic differentiation medium induced the formation of nodules that stained positive with Alizarin red, suggesting that the cells had the potential to differentiate into osteoblasts. When the cells were cultured in

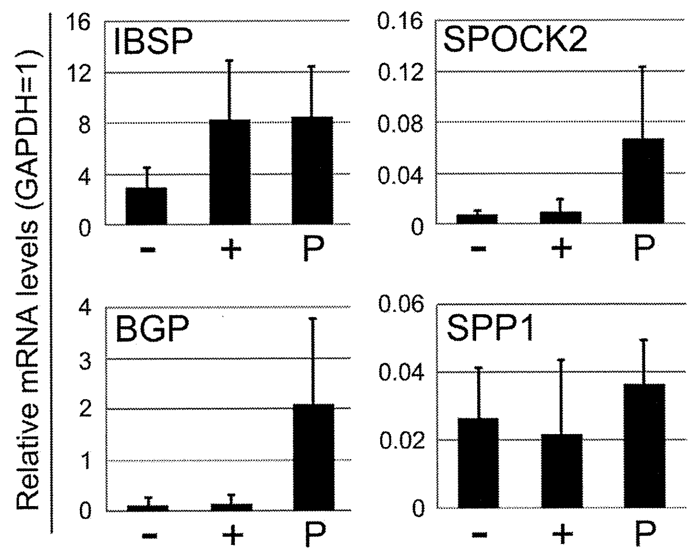

Fig. 2 (above). The effect of culture conditions on osteoblastic marker expression. *The gene expression in the cells cultured on gelatin in hESF10 without (-) or with (+) Asc 2-P for 6 days, in comparison with the cells grown in POWERDBY10 (P) was analyzed by the quantitative RT-PCR. The gene expression was normalized by the amount of* GAPDH. *The values are the mean ± SD (n=3).*

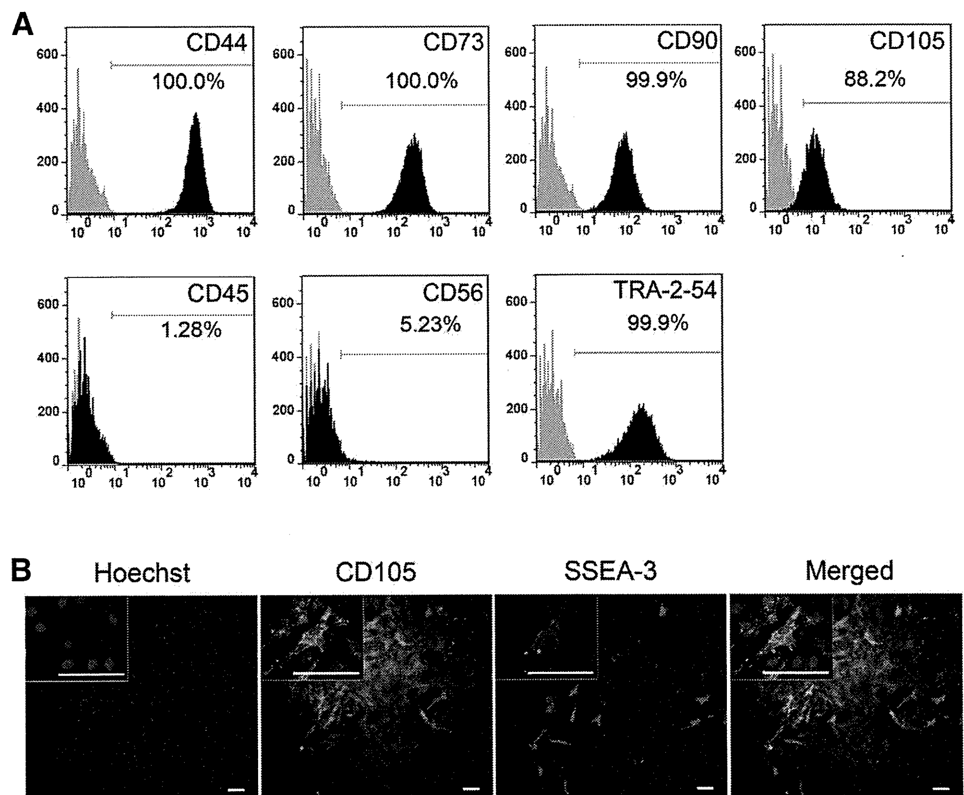

Fig. 3. Expression of hMSC markers in UE7T-13 cells. (A) *Flow cytometric profiles for CDs in UE7T-13 cells. hMSC marker expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days was analyzed by flow cytometric analysis. Antigen histogram (black); control histogram (gray); the horizontal bar indicates the gating used to score the percentage of antigen-positive cells.* **(B)** *Immunocytochemical analysis of SSEA-3 and CD105 expression in UE7T-13 cells cultured on gelatin in D-hESF10 for 4 days. Scale bars, 100 μm.*

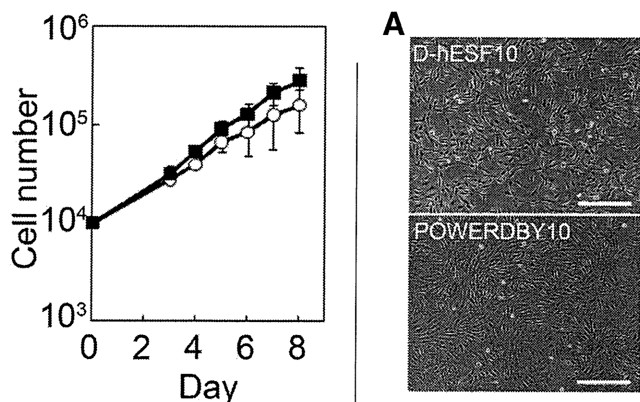

Fig. 4 (above left). A comparison of the growth of different UE7T-13 cells in the defined serum-free medium and conventional culture conditions. *The cells were seeded in a 24-well plate coated with gelatin in D-hESF10 (open circle), or in a 24-well plate in POWERDBY10 (closed square) at a cell density of 1x10^4 cells per well. Cell numbers were counted every day. The values are the mean ± SD (n=3).*

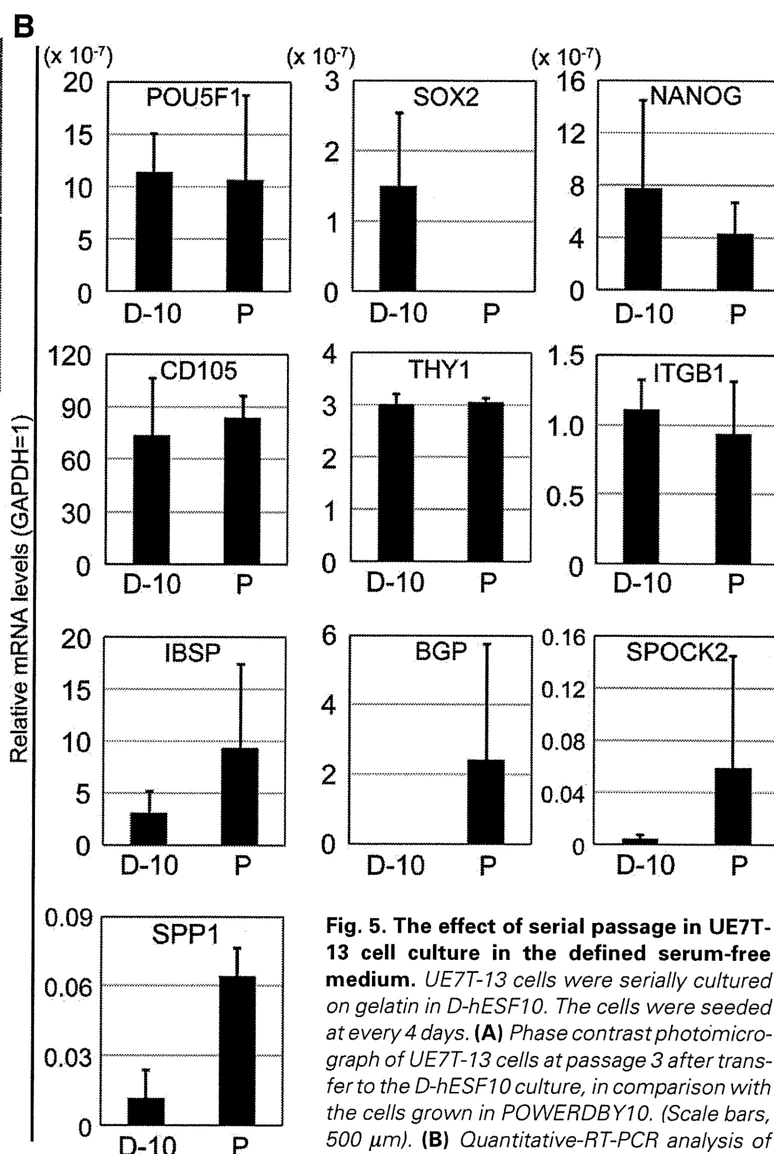

Fig. 5. The effect of serial passage in UE7T-13 cell culture in the defined serum-free medium. *UE7T-13 cells were serially cultured on gelatin in D-hESF10. The cells were seeded at every 4 days.* **(A)** *Phase contrast photomicrograph of UE7T-13 cells at passage 3 after transfer to the D-hESF10 culture, in comparison with the cells grown in POWERDBY10. (Scale bars, 500 μm).* **(B)** *Quantitative-RT-PCR analysis of gene expression in UE7T-13 cell cultured on gelatin in D-hESF10 at passage 4 (D-10), in comparison with the cells grown in POWERDBY10 (P). The name of each gene is noted in each bar graph. Gene expression was normalized with respect to* GAPDH. *The values are the mean ± SD (n=3).*

adipocytic differentiation medium, Oil red O-positive cells appeared. Taken together these results suggest that the serum-free expanded UE7T-13 cells have maintained the capacity to differentiate into osteoblasts or adipocytes.

Discussion

Developing clinical serum-free media for maintaining and expanding human stem cells is a major research topic in regenerative medicine. Our current results indicate that it is possible to culture hMSCs on gelatin in a defined medium, designated D-hESF10, in which human recombinant insulin, human transferrin, a low concentration of fatty acid-free bovine albumin conjugated with oleic acid, FGF-2, and TGF-β1 are the protein components. The basal medium ESF was developed for mouse ES cells (Furue *et al.*, 2005). For hES cell culturing, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) was removed from ESF but Asc 2-P was added (Furue *et al.*, 2008). For propagating hMSCs, Asc 2-P was removed from the hES cell culture medium because we found that Asc 2-P increased osteoblastic marker expression in hMSCs. These findings indicated that signaling by Asc 2-P in hMSCs is different from that in hES cells.

FGF-2 is a heparin-binding growth factor which stimulates the proliferation of a wide variety of cells. The biological activity of FGF-2 is efficient in the concentration range of 0.1 to 10.0 ng/ml. Addition of FGF-2 has been shown to increase the growth rate and life span of hMSCs from different species (Tsutsumi *et al.*, 2001; Benavente *et al.*, 2003), suggesting that FGF-2 play an important role in self-renewal of hMSCs. In hES cells, FGF-2 is a crucial to maintain the undifferentiated state (Amit *et al.*, 2004; Hoffman and Carpenter, 2005). We previously reported that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hES cells in serum-free without feeders (Furue *et al.*, 2008). In this study, we found that FGF-2 at 10 ng/ml together with heparin supported the cell proliferation of hMSCs in a serum-free medium. These findings suggest that they share the same signal pathway to support self-renewal. Heparin at 1 mg/ml promoted hMSC cell proliferation, and we previously reported that heparin at 1 mg/ml inhibited hES cell proliferation. Thus the sensitivity to heparin is different between hMSCs and hES cells.

The TGF-β1 pathway has been reported to be important in hMSC differentiation into the osteogenic and chondrogenic lineages (Li and Xu, 2005; Kulterer *et al.*, 2007). While we have shown that TGF-β1 alone did not promote cell proliferation of hMSCs, the combination with FGF-2 and heparin enhanced cell proliferation of hMSCs. Chase *et al.* reported the combination of TGF-β1, FGF-2, and PDGF-BB in a commercial serum-free medium for the expansion of hMSCs although the optimal concentrations of these factors were not disclosed. The cell growth rate in D-hESF10 medium was similar with that in the conven-

tional culture conditions suggesting that addition of TGF-β1 and FGF-2 is sufficient to replace serum in supporting hMSC cell growth. A culture medium consisting of the minimum components necessary to support survival and proliferation would be beneficial to understand the characteristics of naïve hMSCs. Therefore, we think that addition of PDGF-BB is not crucial for an hMSC culture medium.

Several studies reported that two distinct cell morphologies are seen in early-passage hMSC cultures: small, spindle-shaped cells that are rapidly self-renewing and large, flat cells that replicate slowly and appear more mature (Mets and Verdonk, 1981; Colter *et al.*, 2001; Sekiya *et al.*, 2002). The morphology of serum-free expanded UE7T-13 cell population contained comparably small, spindle-shaped cells. However, specific undifferentiated markers of hMSCs have not been identified yet (Pochampally *et al.*, 2004). Further, although the cells are cloned, cells within an individual colony are heterogeneous in morphology, growth rates, and efficiency with which they differentiate (Mets and Verdonk, 1981; Bruder *et al.*, 1997; Colter *et al.*, 2001). The International Society for Cellular Therapy (ISCT) has proposed three criteria to define hMSCs (Dominici *et al.*, 2006). hMSC population must be positive at least for several antigens such as CD105, CD73, and CD90, and negative for CD45. CD105 is usually used to identify an hMSC population. Many studies reported that hMSCs also expressed hES cell pluripotency markers, SSEA-3, -4, NANOG, OCT3/4, and alkaline phosphatase (Pochampally *et al.*, 2004; Roubelakis *et al.*, 2007; Battula *et al.*, 2008; Conrad *et al.*, 2008; Pang *et al.*, 2010). We also detected the expression of NANOG, OCT3/4, and SOX2. These findings suggested that hES cell pluripotency markers may be universal stem cell markers in humans. Dezawa's group recently reported that double positive CD105 and SSEA-3 cells have the ability to generate multiple cell types derived from the three embryonic germ layers (Kuroda *et al.*, 2010). We also confirmed the existence of CD105 and SSEA-3 double positive cells in the hMSC population expanded in D-hESF10. In this study, we confirmed the differentiation potential of hMSCs to generate osteoblasts or adipocytes, but in the future we will examine the ability of hMSCs to generate cells from all three germ layers.

To facilitate the transition of human stem cell biology from basic research to clinical application all the components of maintenance and differentiation media should be publicly disclosed so they can be evaluated by many researchers. A commercial xeno-free serum-free medium for hMSCs was reported recently (Chase *et al.*, 2010). However, the non-disclosure of components is problematic as the medium formulation cannot be usefully modified or improved. Because all the components of D-hESF10 medium are disclosed here, the medium can be modified to study signaling pathways involved in maintaining multipotency and to develop differentiation protocols.

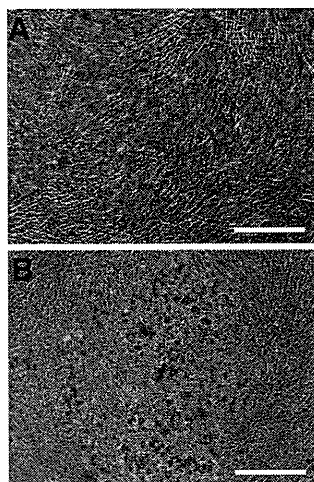

Fig. 6. The differentiation ability of UE7T-13 cell grown in the defined medium. *The UE7T-13 cells were serially cultured in D-hESF10 at passage 7, and then cultured in the differentiation medium.* **(A)** *Osteoblastic differentiation was induced in osteoblastic medium for 20 days. The nodules were stained with Alizarin Red S (red).* **(B)** *Adipocytic differentiation was induced in adipocytic medium for 24 days. The cells were stained by Oil red O staining (red). Scale bars: 500 μm.*

Materials and Methods

Cell Cultures

An immortalized hMSC line UE7T-13 (Mori *et al.*, 2005) (JCRB 1154, JCRB Cell Bank, Osaka, Japan) was used in this study. Cells were maintained on 100 mm dish (BD Falcon, Oxnard, CA) in POWERDBY10 (MED-SHIROTORI, Tokyo, Japan) that was also used in the experiments as a control medium. The cells were harvested with 0.25% trypsin in 1 mM EDTA-4Na.

Serum-free Cell Culture Media

hESF9 comprises ESF basal medium (Furue *et al.*, 2005) without HEPES supplemented with nine defined factors: Asc 2-P, 6-factors (human recombinant insulin, human transferrin, 2-mercaptoethanol, 2-ethanolamine, sodium selenite, oleic acid conjugated with fatty acid-free bovine serum albumin (FAF-BSA)), bovine heparan sulfate sodium salt, and human recombinant FGF-2 (Sigma, St. Louis, MO), as described previously (Furue *et al.*, 2008) (Supplementary Table 1). ESF basal medium without HEPES supplemented with Asc 2-P (hESF-GRO), and ESF basal medium without HEPES and Asc 2-P (hESF-DIF) were purchased by the Cell Science & Technology Institute (CSTI, Sendai, Japan). All other reagents were from Invitrogen (Carlsbad, CA) and Sigma. D-hESF10 medium consists of hESF-DIF medium supplemented with 6-factors, FGF-2, heparin, and TGF-β1 (R&D Systems, Minneapolis, MN). To harvest cells, 0.25% trypsin in 1 mM EDTA-4Na was used and the trypsin was inactivated with 0.1% soybean trypsin inhibitor (Sigma). For differentiation into osteoblasts or adipocytes, the cells were cultured according to the instruction by the suppliers (Lonza, Basel, Switzerland). The differentiated cells were stained by Alizarin Red S (Wako Pure Chemical Industries, Osaka, Japan) or Oil Red O (Wako).

Cell proliferation

Before the serum-free experiments, cells grown in POWERDBY10 were incubated by in hESF9 medium without heparin and FGF-2 (hESF9(-/-)) overnight to starve the effect of serum. Cells were replaced at the cell density of 1×10^4 cells /well on 24-well plate (BD Falcon) coated with 0.1% porcine gelatin solution (Millipore, Billerica, MA) and cultured in hESF9(-/-) medium in the presence of varying growth factors. The cell numbers were counted by Coulter Counter (Beckman Coulter, Hialeah, FL).

Gene expression

A detailed reverse transcription-polymerase chain reaction (RT-PCR) protocol was described previously (Furue, *et al.*, 2005). Total RNA was extracted from hMSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany) and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the provider's instructions. Q-RT-PCR was carried out using the TaqMan gene expression Master Mix on in ABI PRISM 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the supplier's instructions (ABI). Specific primers-probe set were listed in Supplementary Table 2. Expression levels were all normalized by the expression level of *GAPDH*. The relative level of each gene in cDNA of undifferentiated hES cells was defined as "1." The KhES-3 cell line was used as a control; the cells were obtained from the Institute for Frontier Medical Science, Kyoto University, and the Review Board of the National Institute of Biomedical Innovation approved this research.

Antigen expression

For *in situ* immunocytochemistry, the cells were immunostained with antibodies, as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, fluorescence images were acquired using by IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, England). Flow cytometry was performed with BD FACS Canto flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Draper *et al.*, 2002; Furue *et al.*, 2008). In this study, the labeled primary antibodies were used, but the biding of anti-SSEA-3, anti-CD56, and Tra-2-54 antibodies was visualized with RPE-conjugated goat anti-mouse Ig (Dako, Carpinteria, CA) or Alexa Fluor 647 goat anti-rat IgM (Invitrogen). The primary antibodies used are listed in Supplementary Table 3.

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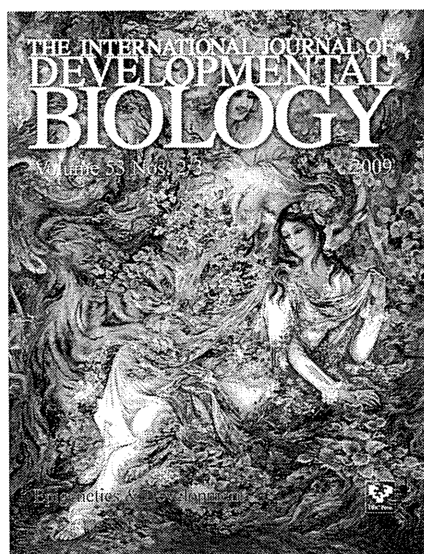

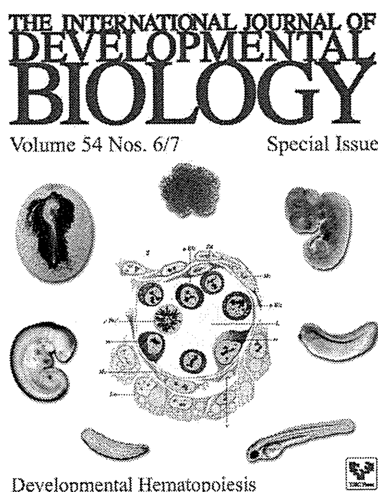

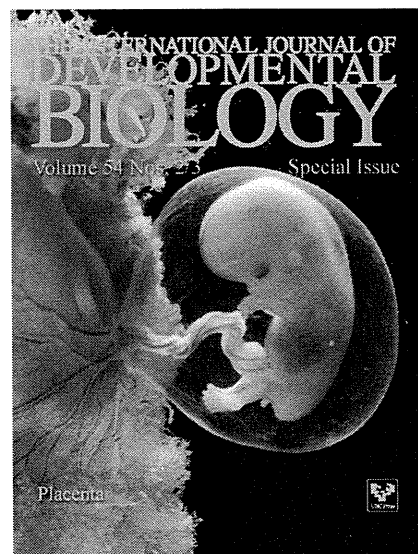

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ヒト多能性幹細胞の命名法の国際統一規格案について

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菅　三佳[1)]　高田　圭[2)]　小原 有弘[1)]　末盛 博文[3)]
青井 貴之[4)]　中村 幸夫[5)]　古江 - 楠田 美保[1) 2)]

1）独立行政法人 医薬基盤研究所 難病・疾患資源研究部 培養資源研究室
2）京都大学再生医科学研究所附属幹細胞医学研究センター 細胞プロセシング研究領域
3）京都大学再生医科学研究所附属幹細胞医学研究センター 霊長類胚性幹細胞研究領域
4）京都大学 iPS 細胞研究所 規制科学研究部門
5）独立行政法人 理化学研究所バイオリソースセンター 細胞材料開発室

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summary

In a few years, thousands of human embryonic stem (ES) / induced pluripotent stem (iPS) cell lines have been established in laboratories around the world. To date, confusions have arisen due to duplicate or redundant naming of cell lines. In addition, not all the important information such as provenance, derivation method and characterization are provided by researchers. To address these issues, a convention for naming and reporting human ES/iPS cell lines is urgently called. Recently Stem Cell Banks and researchers in the US, UK, China, Australia and the other countries proposed a new nomenclature system and a minimum set of criteria for reporting newly generated human ES/iPS cell lines. In this review, we have introduced their recommendations for developing a rule for naming and reporting of human ES/iPS cell lines.

はじめに

1998年にヒト胚性幹細胞(embryonic stem cell：ES細胞)[1)]が樹立され，2007年には，ヒト人工多能性幹細胞(induced pluripotent stem cell：iPS細胞)[2)]が開発された。これらの多能性幹細胞は，発生や疾患メカニズム解明など基礎研究のみならず，再生医療や創薬，毒性評価，ワクチン作製などへ応用の期待が高まっている。ヒトES/iPS細胞の株数は急ピッチで増加しており，すでに数千株にも及ぶ。実用化への研究を進めるために，国際幹細胞バンキングイニシアティブ(International Stem Cell Banking Initiative：ISCBI)では各国の細胞バンクや樹立機関が協力して世界中の研究者が相互に利用できる環境の整備を推進している。ところが，ヒトES/iPS細胞株の命名法について整備されておらず，混乱が生じている。このような現状から，2011年４月に，米国，英国，オーストラリア，中国などの幹細胞バンクや幹細胞研究者らから，「ヒトES/iPS細胞株の命名法および発表に関する標準化」[3)]が提案され，さらにその提案に対する意見[4)5)]が寄せられた。ISCBIや国際細胞バンク・ワーキンググループに参加する筆者らが，その内容を概説したい。

Suga, Mika[1)] / Takada, Kei[2)] / Kohara, Arihiro[1)] / Suemori, Hirofumi[3)] / Aoi, Takashi[4)] / Nakamura, Yukio[5)] / Kusuda Furue, Miho[1)2)]
1) Laboratory of Cell Cultures, Department of Disease Bioresources, National Institute of Biomedical Innovation
2) Laboratory of Cell Processing, Stem Cell Resarch Center, Institute for Frontier Medical Sciences, Kyoto University
3) Laboratory of Embryonic Stem Cell Research, Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University
4) Department of Regulatory Science, Center for iPS Cell Research and Application (CiRA), Kyoto University
5) Cell Engineering Division, RIKEN BioResource Center
E-mail : mikasuga@nibio.go.jp / mkfurue@nibio.go.jp

ヒトES/iPS細胞の国際的な相互利用に向けて

2003年に設置された日本を含む22ヵ国からなる国際幹細胞フォーラム(http://www.stem-cell-forum.net/ISCF/)からの助成を受けて，2005年から英国シェフィールド大学Andrews教授が中心となって推進しているInternational Stem Cell Initiatives (ISCI)プロジェクトでは，日本(京都大学再生医科学研究所・中辻憲夫教授)を含めた世界11ヵ国のヒトES細胞樹立研究者らが連携して，ヒトES細胞株を登録し，樹立の方法，未分化/分化マーカーの発現などの解析方法とその結果を公表し[6)7)]，ヒトES細胞研究の標準化を進めてきた(http://www.stem-cell-forum.net/ISCF/initiatives/)。ISCIワークショップには筆者らも加わり標準化についての議論を行った。2008年からは，ヒトiPS細胞も含めて検討されている。さらに，ヒトES/iPS細胞株を各国間で相互に利用する体制を構築する必要があるとの認識のもとに，2007年から英国UK Stem cell Bankをはじめとする世界各国の細胞バンクが連携し，ISCBIプロジェクトが開始され，筆者らが参加している。このプロジェクトにおいては，ヒトES細胞のドナーの情報管理，資源化，品質管理法や分譲について，国際的にコンセンサスを図ってヒトES細胞を資源化するためのガイドラインを作成している[8)9)](和訳は，京都大学再生医科学研究所・細胞プロセシング・高田らより本誌Vol.10 No.4, p79-96, 2011に掲載されているので参照されたい)。さらに，相互利用するためには不可欠な細胞登録における「細胞株の命名法」に関しても統一規定を設けることが現在の重要課題であり，国際的に活発な議論が展開されている。

これまでの現状

国内において細胞バンクが整備される1984年以前は，日本組織培養学会が細胞株を認定してJTCの番号を付与して登録する事業を実施していた。現在は，細胞バンクが整備され，JCRB (医薬基盤研究所細胞バンク，旧国立医薬品食品衛生研究所細胞バンク)，RCB (理化学研究所バイオリソースセンター細胞バンク)に，研究者が細胞株を寄託し，バンクの略称とともに登録番号で管理され，データベース上で公開されている。海外においても，米国のATCC，国立がん研究所(National Cancer Institute：NCI)，欧州細胞培養コレクション(European Collection of Animal Cell Cultures：ECACC)などの細胞バンクが各機関の略称や独自の登録番号(カタログ番号)を用いて管理し，情報を公開している。このように整備されていても，細胞株の情報や原著論文を検索するときに不都合が起こる。たとえば，“3T3 Swiss Albino”“3T3-Swiss albino”“Swiss-3T3”は同種の細胞株名であるが，データベースや論文での記載方法は他にも何通りも存在する。3T3と入力して検索すると，“3T3 (+3)”，“3T3-L1”，“3T3-SV40”など，別種の細胞株やサブクローンも検索にかかる。

まだ歴史の浅いヒトES/iPS細胞株においても，異なる研究機関で樹立された別個の細胞に全く同じ名前がつくといった問題がすでに生じている。たとえば，全く別の患者から採取した羊水(amniotic fluid：AF)に由来する2つのiPS細胞株の両方ともが“AF-iPS”と命名されたり[10)11)]，ジストロフィン遺伝子に異なる箇所に変異をもつ2人のデュシェンヌ型筋ジストロフィー (Duchenne muscular dystrophy：DMD)患者から樹立した全く別のiPS細胞であるにも関わらず，両方ともが“DMD-iPS1”と命名されたりしている[12)13)]。“iPS-1”や“iPS-WT”といった名称は汎用され，その名称のみから細胞株を特定することはできない[4)]。また，“KhES-1”“KhES-3”“HES-3”など，ヒトES細胞の名称に汎用される“HES”は，ヒト胎児皮膚(human embryonic skin：HES)由来線維芽細胞の株名“HES 5”[14)]などとも同じ表記であるため混同されやすい。細胞株を混同してしまえば，研究成果の妥当性，重要性

を正当に評価できなくなる。このように細胞命名法の国際的な統一規定がなかったことがデータベースの管理・利用を不便なものにしている。

ヒトES/iPS細胞の命名法の提案

2010年の国際幹細胞学会(International Society for Stem Cell Research：ISSCR, 2010年7月15日開催)，およびISCI(2010年9月15日開催)のワークショップで議論された内容に準拠して，米国マサチューセッツ医科大学ヒト幹細胞バンクのInternational stem cell registry(ISCR)が代表として提案する「ヒトES/iPS細胞株の命名法および細胞登録に関する統一規定の案」が米国科学誌「Cell Stem Cell」2011年4月8日号[3]に掲載された。これに対し，京都大学iPS細胞研究所(CiRA)山中伸弥所長らの意見[4]と米国細胞バンクAmerican Type Culture Collection(ATCC) Brian Pollok所長らの意見[5]が同誌の6月3日号に掲載された。両者ともISCRの提案に大筋で同意した上で，幹細胞研究の将来展望をもとに想定される問題を提起し，改善案を提示した。

ヒトES/iPS細胞の命名法についての統一規定案

ISCRによる命名法の統一規定案(図)[3]は，特に次に示す5点に配慮したものである。①独自の識別方法(樹立機関IDと細胞株シリアル番号)を採用し，細胞株間で混同しないようにすること。②細胞株に関する情報が直感的に認識できること。③既存の細胞株名の表記方法(例：KhES-1, KhES3, CT4, B124-2)と同じフォーマットを採用すること。④異なる系統の細胞株であること(例：TSRI68iとSHEF4e-ALS)や，同じ系統の細胞株であること(例：SHEF3とSHEF5)を容易に認識できること。⑤柔軟性のあるルールにすること。

その細胞の名称の表記方法は図[3]に示すような4つの構成要素からなるものであり，(a)細胞株の樹立機関のID，(b)細胞株のシリアル番号(ID)，(c)ES細胞またはiPS細胞を区別する略号，および(d)細胞の特徴を示す情報を記載する。さらに，データベース上で処理するため，規定された場所にハイフンを使用する，文字数と数字の桁数を規定する，スペースを使用しない，ハイフンを含めて14桁に統一することを提案[3]している。

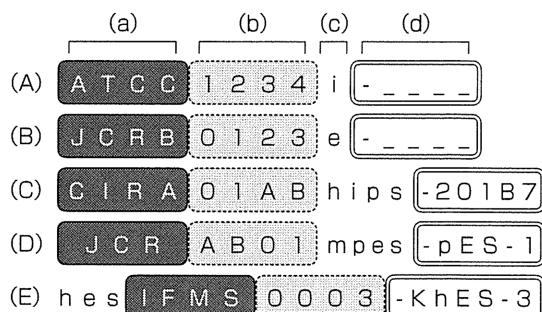

図1 ヒトES/iPS細胞株の命名法の案

(A)(B) ISCR[3]，(C)(D)山中教授ら[4]，(E)筆者らの案による細胞株名表記の例
(a)細胞株を樹立した研究機関(研究室または研究所)のID
(b)細胞株のID
(c)細胞種や由来を識別する記号。ISCRの提案[3]によると“iPS細胞”を“i”，“ES細胞”を“e”で表す。
(d)“-(ハイフン)”とその後に続くアルファベットまたは数字で細胞株の特徴やクローン番号等を表す。ISCR[3]は，(c)と(d)の部分は任意とし，(a)と(b)のみで細胞名を表すことも考慮している。それぞれの要素を表す部分に使用する文字数と数字の桁数に自由度をもたせることも可能だが，データベース管理及び検索の便宜上，①スペースを含まない，②アルファベットの大文字や小文字の表記法，数字の桁数，ハイフンの位置なども統一し，③全体で14文字に限定したものが望ましいとしている。細胞株名の表記法については，さらに議論が必要である。(文献3より引用改変)

ヒトES/iPS細胞サブクローン株の数への対応

iPS細胞は1種類のドナー細胞から100種類以上のクローンを作製することもある。山中所長らは，細胞株を識別するためのIDの表記(図(b)の部分)は増大する株数に対応し得る方式でなければならないと提言[4]

している。また，サブクローンを作製した場合，オリジナルのクローン番号とサブクローン番号の両方を含むよう命名するべきであるとも提言している。現在，世界中の研究室でiPS細胞の樹立が進められており，その株数は数年のうちに数万という値に達することが予想される。シリアル番号やIDをつけていくとしたら，このような莫大な細胞株数に対応できるものでなければならない。アラビア数字のみではなく，アルファベットなどの文字や記号とアラビア数字を組み合わせてIDを表記すれば，細胞株数が膨大になっても対応できるのではないかと山中所長ら[4]は提案している。

ヒトES/iPS細胞の既存の細胞株について

山中所長ら[4]は，すでに世界中に広く知られている細胞株(例：201B7, hFIB2-iPS2)に新規にシリアル番号等で設定し直す場合にも，オリジナルの名称およびクローンIDを継承することができるような柔軟性をもたせるべきであると提言している。オリジナルの名称から細胞株の情報や原著論文を簡単に収集できるなど，研究者にとって都合が良い点が多いとしている[4]。すでに独自の方式で命名し，細胞株を管理している研究機関は多いため，すべての研究機関の樹立細胞株に対して公平にIDを分配するには多くの困難が予想される。しかし，集積された細胞情報や研究成果を活用するためにも，国際規格のIDを公平に付与できるよう整備し，細胞株のデータベース化を推進していく必要があるのではないだろうか。

すべての多能性幹細胞へ適応

ISCRの提案は，図の(c)の部分には，“i”あるいは“e”を表記することによりその細胞株がヒトiPS細胞株とヒトES細胞株のいずれかであることを識別できるようにするというものである[3]。この点に関して，山中所長らとPollok所長ら両者ともに，マウスiPS細胞，体細胞核移植ES細胞(NT-ESC)，単為発生胚由来ES細胞(parthenogenetic-ESC)，胚性腫瘍細胞(embryonal carcinoma cell：ECC)，胚性生殖系細胞(embryonal germ cell：EGC)，エピブラスト幹細胞(epiblast stem cell：EpiSC)などの多能性細胞をすべてこの命名法規定の対象に含めるべきであり，これらを正確に識別できるよう動物種や由来細胞を表すコードを図の(c)に表記することを提案している[4][5]。しかし，筆者らは，細胞種を識別するコードを頭につけたほうが分類しやすいのではないかと考える(図)。

ヒトES/iPS細胞において特定の病名を表すのは適当ではない

名称に病名を含めることに関して，Pollok所長ら[5]は懸念を抱いている。多くのヒトiPS細胞は，新生児表皮線維芽細胞(neonatal foreskin fibroblasts)から人工的に誘導され，“正常(non-diseased)”な指標細胞としても使用されている。しかしながら，組織を採取する段階でドナーの異常を検出できることは難しく，その匿名性からドナーの病歴の追跡は不可能である。現段階では，ヒトES/iPS細胞や分化させた細胞の病気に対する感受性を明らかにすること(どのような病気になりやすいかを予測すること)も不可能である。細胞登録の際にはドナーの病歴などに関する情報もわかっている範囲で報告すべきであるが，遺伝子の変異や欠失などの確定された情報の表記を提案している。

ヒトES/iPS細胞を培養する現場での作業

山中所長ら[4]とPollok所長ら[5]は両者とも，細胞株の名称に使用する文字数はできるだけ短くするよう主張している。データベース上で管理する際の利便性も重要だが，現場での作業も考慮するべきである。培養デッシュや凍結チューブにグローブをした手で書きやすく，読み取りやすくすることが重要である。ATCCで1.5mLチューブを用いる場合，細胞株の名称が10文字以下であることが理想的であるとPollok所長ら[5]は述べている。山中所長ら[4]も，ISCRの提案した14

表1　海外の細胞登録サイト

	機関	アドレス
Stem Cell Registry	ISCI	http://www.stem-cell-forum.net/ISCF/initiatives/isci/stem-cell-registry/
ISCR	UMass	http://www.umassmed.edu/iscr/index.aspx
hESCreg	EU連携	http://www.hescreg.eu/
NIH Human Embryonic Stem Cell Registry	NIH	http://grants.nih.gov/stem_cells/registry/current.htm

ISCI：The International Stem Cell Initiative
ISCR：The International Stem Cell Registry
UMass：The University of Massachusetts Medical School, Human Stem Cell Bank and Registry
hESCreg：European Human Embryonic Stem Cell Registry

文字[3)]は不便を感じる長さであり，簡略化した名称を使用し始めるようになることを危惧する。簡略化した名称の使用は，細胞の混同のリスクにつながる。医薬基盤研JCRB細胞バンクや理化学研究所バイオリソースセンター細胞バンクでは，場合によってバーコードラベルを用いて管理している。最近は安価なバーコードリーダーもあり，研究室レベルにおいても利用が可能ではないだろうか。

これまでに命名法が規定され，広く活用されている例がある[3)]。分化抗原群は，“CD42a”“CD42b”のようにCD番号で表記され，個別の抗原が認識される。また，遺伝子や蛋白質などについてはさまざまな名称が使用されるが，データベースに登録されたアクセッション番号によって識別され，容易にその原著論文まで確認できる。利便性の高い命名法の策定とデータベースの構築を行い，現場のニーズに対応する鍵となるのが，やはり細胞登録システムの整備である。細胞株の名称やIDとともに細胞情報を登録し，管理していくことが必要であろう。

ヒトES/iPS細胞の登録

ヒトES/iPS細胞を樹立した際，具体的な報告方法に関する国際的な統一規定はなく，新規の細胞の樹立を含めた研究成果の報告項目などは研究者やジャーナルの査読者に任されている。今後，産業応用される可能性があることから，各国の倫理規定を尊重して共有できるよう倫理的妥当性および科学的合理性を将来にわたって確保することが肝要である。**表1**の記載の通り，海外のヒトES細胞については，ISCBI，EUヒトES細胞登録(European Human Embryonic Stem Cell Registry：hESCreg)が連携して，それぞれのホームページで公開をしている。また，NIHヒトES細胞登録(NIH Human Embryonic Stem Cell Registry)では，NIHの研究費を使用して研究が可能な細胞が掲載されている。細胞登録に必要な情報として，**表2**に示す5つの項目が提案されている[3)8)]。このようなヒトES/iPS細胞の情報整備は，新規細胞株の樹立に必要な基本データ作成とその情報公開の推進につながると思われる。

おわりに

幹細胞研究者の方々には，細胞樹立の際に前記の問題をご一考いただければ幸いである。一方で，幹細胞研究者らの声をさらに集約し，想定される問題を回避し，かつ利便性の高い命名法を早期に確立することが望まれる。ES/iPS細胞株を含む多能性幹細胞の命名法および発表に関するルールを設け，情報をデータベース化し，世界中で共有することは，幹細胞研究の推進につながる。本総説が日本の幹細胞研究推進の一助となれば幸いである。

表2　ES/iPS細胞の登録や研究成果の報告の際に必要な情報

細胞株の由来（source） 細胞のタイプ，由来組織，継代数など ドナーから採取された場合：ドナーの年齢，性別，人種（自己報告または解析結果）* 細胞バンクや民間企業から入手した場合：細胞株のアクセッション番号
樹立方法（derivation method） 細胞の株化までの方法，培地および添加物，培養期間，継代数など詳細な培養方法 ES細胞の場合：胚の取り扱い方法，胚盤胞を得るための透明帯除去方法，胚盤胞からの内部細胞塊の単離方法 iPS細胞の場合：リプログラミングに用いたベクターシステム，低分子，蛋白質，mRNAやmiRNAとその導入・誘導方法
細胞特性（characterization） 未分化状態の確認（免疫染色，フローサイトメトリー，遺伝子発現プロファイリングなど） 多能性の確認（*in vitro*分化，テラトーマ形成，遺伝子発現プロファイリングなど） 核型，SNP（一塩基多型）によるゲノム解析結果*
細胞同一確認（genetic identity）と無菌性（sterility） STR（short tandem repeat）やSNP解析による細胞認証試験結果* 無菌試験結果およびマイコプラズマ否定試験結果
細胞の来歴（provenance） ドナーに対する説明および同意（インフォームド・コンセント），利益相反についての確認

研究者から提供される細胞株の情報を名称・登録番号と合わせて管理していくべきだが，特に個人を特定できる情報(*)は，各国の倫理規定を尊重し，慎重に管理されなければならない。

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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

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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).

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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′-GGACTACYVGGGTATCT-AAT-3′. The TaqMan probe (Bac516F) was 5′-FAM-TGCCAGC-AGCCGCGGTAATACRDAG-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

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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

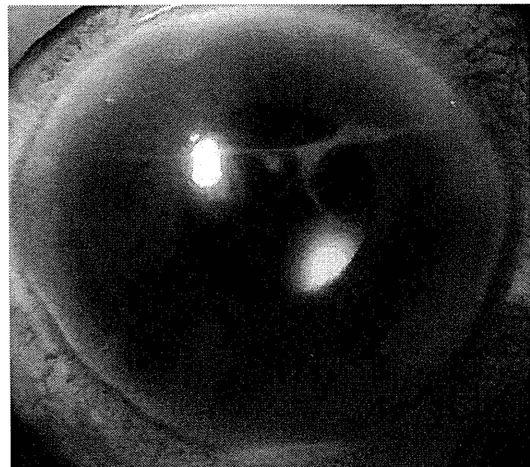

Figure 1 Case 7 (late postoperative endophthalmitis). Slit-lamp photograph in suspected bacterial endophthalmitis. In the right eye, cyclitic membrane, height of the hypopion, and severity of vitritis were seen. In this patient, broad-range quantitative PCR revealed a high copy number of the bacterial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

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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

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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.

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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.

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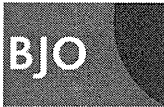

Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR

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

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

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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

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