Table 2 Septic reactions after transfusion of bacterially contaminated platelet cocentrate.

Transfusion date*	Symptoms	Clinical outcome	Bacterial species contaminated in the blood unit
Day 3	Nausea, back pain, shock	Death	*Streptococcus pneumoniae*
Day 3	Fever, chillness, leukopenia	Death	*Staphylococcus aureus*
Day 4	Fever, chillness, shivering, hypotension	Improved	*Staphylococcus aureus*
Day 4	Vessel pain, hypotension, chest discomfort	Improved	*Streptococcus dysgalactiae ssp. equisimilis*
Day 4	Chillness, shivering, fever, hypotension, dyspnea	Recovered	*Serratia marcescens*
Day 3	Chillness, shivering, hypotension, wheezing	Recovered	*Streptococcus agalactiae*
Day 4	Chillness, fever, hypotension, hypoxemia	Recovered	*Streptococcus dysgalactiae ssp. equisimilis*
Day 4	Chillness, shivering, dyspnea	Recovered	*Streptococcus pyogenes*

*Date of platelet transfusion with day 1 being the date of platelet collection.

当然細菌の種類によって病原性は大いに異なり，またエンドトキシンを産生するものとしないものとがある。一部の細菌は十分に増殖した後に自己融解を起こし，培養で完全に陰性となることがある。以上のように血液製剤の細菌汚染は極めて多くの条件に左右されるので，汚染を完全に防ぐこと，また汚染を的確に検出することは非常に困難であるといえる。例えば，欧米では血小板製剤の一部をサンプリングして培養に供し，陰性のものを出庫しているが，それでも汚染事故はなくならない[79]。主な理由は，サンプリング時の細菌濃度がまだまだ低く，サンプル中に細菌が含まれず偽陰性となってしまうためである[80]。

細菌に汚染された製剤の輸血によって患者に重篤な病態を引き起こすのは，血液製剤中の細菌の濃度が $10^5/ml$ 以上の場合と言われている[81]。細菌汚染の危険性は常温で保存される血小板製剤が最も高いが，日本の血小板製剤の有効期限は世界で最も短く（採血日を1日として4日目の夜12時まで），これによって細菌が危険な濃度まで達する前に輸血に使用されることを目指している[82]。当然この有効期限では培養法を導入することはできない。同様の考えはフランスとドイツでも取り入れている。

細菌汚染の状況は製剤の保存中にダイナミックに変化するので，輸血直前の製剤の状態の把握が非常に重要であり，血液センターの努力だけではなく臨床の現場の協力も必要である。簡便に行える検査は，製剤の外観のチェックであり，凝集・凝固物の有無，色調の変化，スワーリングの消失などのチェックは，感度は決して高くないが最悪の事態を少しでも避けるために重要である[83]。敗血症の症状は輸血による発熱反応を重篤化したものといってよく，疑った場合には直ちに輸血を中止し，血液培養用に血液をサンプリングし，抗生物質を投与すべきである。製剤の残りは，解放口からの二次汚染

を防ぐ手立てを施して，血液センターに連絡をしていただきたい。

なお日赤の調査では，現在の血小板製剤の細菌汚染頻度は 5,400 本に 1 本と推定される[84]。上記のようにそのすべてが敗血症を起こすわけではない。2000 年以降確定した細菌汚染製剤による敗血症は，血小板製剤によるもの 8 件，そのうち死亡例が 2 件 (Table 2)，赤血球製剤によるものが 3 件，そのうち 2 例は *Yersinia enterocolitica* によるものであった。また血液センター内での外観チェックによって年間に数件ずつ細菌汚染製剤が確認されている。

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Degenerate polymerase chain reaction strategy with DNA microarray for detection of multiple and various subtypes of virus during blood screening

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BACKGROUND: The risk of transferring blood-borne infections during transfusion is continually increasing because of newly emerging and reemerging viruses. Development of a rapid screening method for emerging viruses that might be transmitted by transfusion is required to eliminate such pathogens during blood donor screening. Owing to increased use of human materials in organ transplants and cell therapy, the risk of donor-transmitted viral infections is also increasing. Although nucleic acid amplification technology (NAT) is dedicated to blood screening, a small, convenient detection system is needed at the laboratory and hospital level.

STUDY DESIGN AND METHODS: We developed a new pathogen detection system that can detect multiple viruses simultaneously, using originally designed degenerate polymerase chain reaction primers to amplify a wide range of viral genotypes. Amplified samples were identified using a DNA microarray of pathogen-specific probes.

RESULTS: We detected very low copy numbers of multiple subtypes of viruses, such as human hepatitis C virus (HCV), human hepatitis B virus (HBV), human parvovirus B19 (PVB19), and West Nile virus (WNV), using a single plate. We also detected all genotypes of human immunodeficiency virus (HIV) but sensitivity was less than for the other viruses.

CONCLUSION: We developed a microarray assay using novel primers for detection of a wide range of multiple pathogens and subtypes. Our NAT system was accurate and reliable for detection of HIV, HBV, HCV, PVB19, and WNV, with respect to specificity, sensitivity, and genotype inclusivity. Our system could be customized and extended for emerging pathogens and is suitable as a future NAT system.

Quality and safety in blood products are major public health concerns. In addition to general quality control (QC) testing, introduction of good manufacturing practice and routine screening of blood material and products have assured consistency and quality in production and increased blood transfusion safety in recent decades. Newly developed serologic tests and nucleic acid technology (NAT) have markedly reduced the risk of transmitting human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) from infected blood.[1] Currently, several Food and Drug Administration (FDA)-licensed NAT assays are available to screen blood donors for HIV, HCV, HBV, and West Nile virus (WNV). However,

Pathogens	Primer sequence (5'-3')	GenBank Accession Number	Position (NT)	Gene name	Amplicons (bp)
HIV	RARAGGGGGGATTGGGGGGTA	NC_001802	4336-4356	Integrase	129
	YTGTCYCTGWAATAAACCCGA		4444-4464		
HCV	GAAAGCGYCTAGCCATGGCGT	D90208	59-327	5'-UTR	269
	TGCACGGTCTACGAGACCTCC		307-327		
HBV	AYTAYCAAGGTATGTTGCCCG	X70185	450-470	S	266
	GGAAAGCCCKRCGMACCACTG		695-715		
PVB19	AGTGGTGGTGAAAGCTCTGAA	NC_000883	2148-2168	NS1	122
	TCTCCTGAACTGGTCCCG		2252-2269		
WNV	GGHTGTTGGTATGGNATGGA	NC_009942	3451-3590	NS1	141
	CTCCTGGGTGRCCAAGAAC		3573-3591	NS2A	
IC	TCGAAGACGATCAGATACCGT	M10098	1147-1157	18S rRNA	129
	ATACTCCCCCCGGAACC		1259-1275		

TABLE 1. Pathogen-specific dPCR primers for microarray detection*

* Code base description: M, A/C; R, A/G; W, A/T; S, C/G; Y, C/T; K, G/T; V, A/C/G; H, A/C/T; D, A/G/T; B, C/G/T; N, A/T/C/G.

the continuous development of a highly sensitive screening system is a challenging task for NAT. The focus is mainly on assay sensitivity rather than the range and diversity of viral species detected; therefore, the current NAT systems only ensure detection of a restricted range of viruses and their subtypes and not newly diverged, emerging, or reemerging viruses.[2] It has been reported that the NAT sensitivity for HCV detection differs slightly in relation to virus subtype.[3] Recent advances in organ transplantation and cell therapy have also increased the risk of donor-transmitted viral infections, such as cytomegalovirus, Epstein–Barr virus, WNV, and lymphocytic choriomeningitis virus.[4] Further development of multiple virus detection systems is required to increase coverage of a range of virus strains and subtypes. We have experienced pandemics, such as WNV in the United States in 2003 and the chikungunya virus on Reunion Island in 2006; thus, there is a need to develop a rapid virus detection system that uses a more flexible blood-testing platform and meets the safety requirements for transfusion.

HBV is one of the most geographically widespread viruses and is subdivided into eight main genotypes (A–H),[5,6] causing liver cirrhosis and hepatocellular carcinoma. Although most infectious blood units are removed by screening for hepatitis B surface antigen (HBsAg), there is clear evidence that transmission by HBsAg-negative components occurs during the serologically negative window period and late stages of infection.[7] In addition to the window period of infection, HBV blood screening is required to detect all virus genotypes. Similar to HBV, several false-negative results in minipool NAT screening were reported after the introduction of WNV NAT because of the low viral load. Moreover, WNV continues to diverge rapidly from the originally isolated strain.[8,9] Multiplex NAT assays have become the modern method for detecting several viruses, and in conjunction with automated systems, they have the potential to improve processes that ensure blood safety. Candotti and colleagues[10] have reported the feasibility of a multiplex real-time quantita-

tive reverse-transcriptase polymerase chain reaction (PCR) for HBV, HCV, and HIV-1, suggesting that simultaneous amplification of multiple pathogens is an effective approach for improving pathogen detection methods. The flexibility provided by multiplex PCR is limited, however, because the PCR primers are designed in commonly preserved regions of the viral genomes. To increase the detectable range for multiple pathogens, PCR using degenerate primers has been developed. Recently, bioinformatics has strongly improved the design of degenerate primers, allowing the coverage of a wide range of virus subtypes. We have developed a new method for designing degenerate primers.[11] Here, we used the diamond-like carbon-coating microarray chip (DLC-chip) to reduce background noise and increase the detection sensitivity of the system.[12,13] We combined two newly developed technology platforms for a multiple pathogen detection system using a degenerate PCR–based NAT system (dPCR-NAT).

MATERIALS AND METHODS

Design of dPCR primers and microarray oligoprobes

We designed dPCR primers that hybridized with HIV, HCV, HBV, human parvovirus B19 (PVB19), and WNV genomic sequences (Table 1). We used the CoCoMo (Coordination of Common Motifs) algorithm (www.geneknot.info/cocomo)[14] for dPCR primer design. CoCoMo determines primer regions in commonly conserved nucleotide regions in the assembled nucleotide sequences of virus strains. In each case, all viral sequences were identified from GenBank and EMBL, and a low degeneracy primer set was selected as a candidate using the CoCoMo algorithm. We collected data on 2072 HIV-1 nucleotide sequences and selected 14 complete genome sequences that corresponded to each genotype of HIV-1. We designed dPCR primer sets for detecting 14 complete genome sequences, resulting in approximately 3897

TABLE 2. Oligonucleotide probe sequences of DNA microarray assay

Virus	Probe name	Sequence (5′-3′)	Tm (°C)*
HIV	IR1-1	ACTATTCTTTCCCCTGCACTGTACCCCCCAATCC	78
	IR1-2	TCTGTTGCTATTATGTCTACTATTCTTTCCCC	66
	IR1-3	CTTTAGTTTGTATGTCTGTTGCTATTATGTCTAC	63
	IR1-4	GTAATTTGTTTTTGTAATTCTTTAGTTTGTATGTCTG	66
	IR3-1	GGGATTGTAGGGAATTCCAAATTCCTGCTTGATT	76
	IR3-3	CTTTAATTCTTTATTCATAGATTCTACTACTCCTTGACTTTG	69
HCV	CF1-1	AACCGGTGAGTACACCGGAATTGCCAGGAC	77
	CF1-2	TTTCTTGGATCAACCCGCTCAATGCCTGGAGATTTGGGCG	88
	CF1-3	TGCCCCCGCAAGACTGCTAGCCGAGTAGTGTTGGG	85
	CF2-1	AGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGA	86
	CF2-2	CTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTG	81
	CF2-3	GCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCT	82
	CR2-1	TCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCT	86
HBV	BF4-1	CTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGG	75
	BF4-2	CTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCCTC	78
	BF4-3	TGTCCTCTAATTCCAGGATCAACAACAACCAGTAC	73
	BF4-4	ATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACC	84
	BF4-5	CCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTC	84
	BF4-6	GTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAG	80
	BR4-1	CCAGAAGAACCAACAAGAAGATGAGGCATAGCAG	75
PVB19	PVB19F-1	GGCGCCTGGAACACTGAGACCCCGCGCTCTAGTAC	85
	PVB19F-2	GGCGCCTGGAACACTGAAACCCCGCGCTCTAGTAC	84
	PVB19F-3	GAACTCAGTGAAAGCAGCTTTTTCAACCTCATCACTCC	78
	PVB19R-1	GTACTAGAGCGCGGGGTCTCAGTGTTCCAGGCGCC	85
	PVB19R-2	GTACTAGAGCGCGGGGTTTCAGTGTTCCAGGCGCC	84
	PVB19R-3	GGAGTGATGAGGTTGAAAAAGCTGCTTTCACTGAGTTC	78
WNV	WNVF-1	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-2	ATGATTGACCCTTTTCAGTTGGGCCTTCTGGTCG	80
	WNVF-3	ATGATTGATCCTTTTCAGCTGGGCCTTCTGGT	77
	WNVF-4	ACGCCGACATGATTGATCCTTTTCAGTTGGGCCT	81
	WNVR-1	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-2	CGACCAGAAGGCCCAACTGAAAAGGGTCAATCAT	80
	WNVR-3	ACCAGAAGGCCCAGCTGAAAAGGATCAATCAT	77
	WNVR-4	AGGCCCAACTGAAAAGGATCAATCATGTCGGCGT	81
IC	IC-1	GTCGTAGTTCCGACCATAAACGATGCCGACCGG	81
	IC-2	GGCGATGCGGCGGCGTTATTCCCATGACCC	86
	IC-3	CCGCCGGGCAGCTTCCGGGAAACCAAAGTCTTTG	87
	IC-4	TCGAAGACGATCAGATACCGTCGTAGTTCCGACC	78
QC	QC	TTGGCAGAAGCTATGAAACGATATGGG	69

* The melting temperature (Tm) was calculated using NetPrimer (PREMIER Biosoft International, http://www.premierbiosoft.com/).

primer sets. For HCV, we collected 978 sequences and selected 167 complete genome sequences to design dPCR primer sets, generating 31 primer sets. For HBV, 1461 sequences were collected and 1344 complete genome sequences were selected to generate the dPCR primer sets, generating approximately 29 primer sets. For WNV, we collected 17,172 sequences and used 111 complete genome sequences to design the dPCR primer sets, generating 1649 primer sets. For PVB19, we collected 1145 sequences and selected seven complete genome sequences to design the dPCR primer sets, generating 2517 primer sets. Selected primers are listed in Table 1. The sequences of the oligonucleotide detection probes on the DLC-chip are indicated in Table 2. The probes were manually designed from regions amplified by the degenerate primers. Thirty to 42 oligomers that had a GC content between 50 and 60% were selected. The resultant melting temperature values were 62 to 88°C. The hybridization stability of the PCR fragments was biased according to strand; therefore, we designed probes on each strand of the PCR products (sense strand—same sense as forward primer).

Synthesis of genotype panel oligomers for screening primers

Genotype panel oligomers of HIV-1, HBV, PVB19, and WNV were prepared by overlap-extension PCR (OE-PCR;[15] Fig. 1A). Regions for OE-PCR were selected according to the nucleotide sequences amplified with our primers for each virus genotype. The nucleotide sequences of HBV subtypes B and C panel oligomers had the same sequence. The joining oligonucleotides listed in Supplemental Table S1 (available as supporting information in the online version of this paper) were designed using DNA works (http://helixweb.nih.gov/dnaworks/).[16] The nucleotide sequences of each genotype panel oligomers are listed in Supplemental Table S2 (available as supporting information in the online version of this paper). OE-PCR

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A

Preparation of genotype panel by OE-PCR

Prepare specific 4-6 oligomers for each genotype
by DNA works ver 2 (Supplement Table S1)

~300 bp

Check with electrophoresis (Figure 1B)
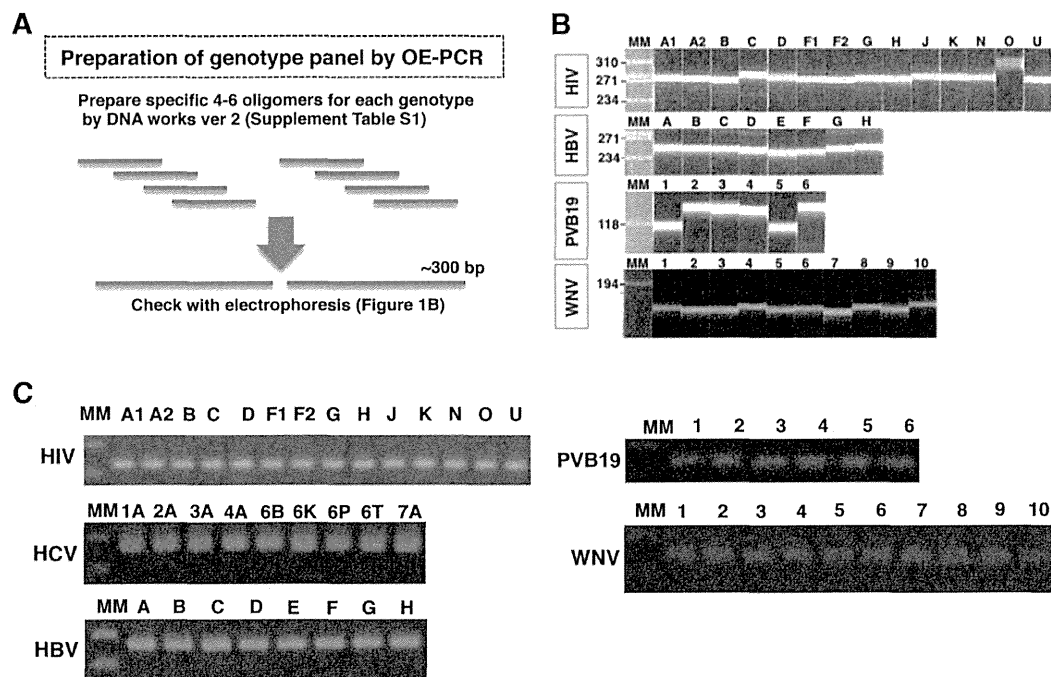
B

C

Fig. 1. Synthesis of genotype panel oligomers for HIV, HCV, HBV, PVB19, and WNV. (A) Preparation of genotype panel oligomers by PCR-based gene synthesis, OE-PCR. dPCR primers were validated with genotype panel oligomers. (B) Genotype panel oligomers were synthesized against 14 subtypes of HIV-1, eight genotypes of HBV, nine genotypes of HCV, six genotypes of PVB19, and 10 genotypes of WNV. (C) Each of the HIV-1 subtype oligomers was amplified by dPCR primers for HIV-1. The top panel shows the agarose gel electrophoresis analysis of PCR products to identify the 14 HIV-1 subtypes. Similar results were obtained from HCV, HBV, PVB19, and WNV genotype panel oligomers with specific dPCR.

was carried out according to a two-step reaction method by using a PCR kit (Prime Star, Takara-bio, Otsu, Japan; Fig. 1). The first reaction was carried out using a mixture of OE-PCR oligomers in 30 cycles of 98°C for 15 seconds, 55°C for 10 seconds, and 72°C for 15 seconds. One microliter of the first PCR products was transferred to the second PCR solution, which included 5 pmol/μL each of the 5'- and 3'-end primers. The second reaction consisted of 30 cycles at the temperature conditions used in the first reaction. The molecular weights of the OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu, Kyoto, Japan; Fig. 1B). HCV genotype panel oligomers were synthesized and obtained from Invitrogen (Carlsbad, CA; custom DNA oligonucleotide synthesis service).

Viral samples for dPCR-based NAT

For more accurate analysis, we purchased PVB19 NAT-based assays genotype panel (First International

Standard; Category Number, 09/110 National Institute for Biological Standards and Control [NIBSC], UK); HIV-1 RNA genotype panel (Category Number 08/358 NIBSC); HCV RNA genotype panel (Category Number 08/264 NIBSC); HCV for NAT (Fourth WHO International Standard; Category Number 06/102 NIBSC); HBV for NAT (Third WHO International Standard; Category Number 10/264 NIBSC); and PVB19 DNA NAT assays (Second International Standard; Category Number 99/802 NIBSC). To evaluate the specificity of our dPCR-NAT system, we diluted each genotype panel with defibrinated plasma (Basematrix 53, SeraCare BBI Diagnostics, Milford, MA) to give a final concentration of 151 to 9722 copies/mL (HIV), 500 to 1500 IU/mL (HCV), 5754 to 123,027 IU/mL (HBV), and 870,964 to 954,933 IU/mL (PVB19), respectively. To evaluate the sensitivity of our dPCR-NAT system, we diluted each international standard with defibrinated plasma to give a final concentration of 1 to 10,000 IU/mL. Samples containing the New York strain of WNV RNA (NY 2001-6263; NATtrol, Category Number NATWNV-0005,

1 mL, 50,000 copies/mL) were purchased from Zepto-Metrix (Buffalo, NY) and were diluted with defibrinated plasma to give a final concentration of 1 to 1000 copies/mL. Blood specimens from healthy volunteer donors who were confirmed as negative for HCV, HBV, and HIV were provided from Japan Red Cross and used as a negative control.

DNA and RNA extraction, reverse transcription, and PCR amplification

Each viral DNA and RNA was extracted from 200 μL of diluted sample with a viral nucleic acid kit and a viral RNA kit (High Pure, Roche, Basel, Switzerland). Total RNA of HCV, HIV-1, and WNV were all reverse transcribed with a cDNA synthesis kit (Superscript III RT, Invitrogen) according to the manufacturer's protocol. Twenty-microliter cDNA samples were prepared for PCR amplification of each virus. The PCR was carried out with PCR mixture (GoTaq, Promega, Madison, WI). In the PCR mixture, diluted nucleic acid and 50 μmol/L of each degenerate primer were included. The reaction consisted of 50 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. We used 5 ng of human cDNA fragment as an internal control (IC).

Microchip fabrication

We purchased 3-mm² silicon DLC-chip from Toyo Kohan (Tokyo, Japan). Each probe was spotted by Spotarray 72 (Perkin-Elmer, Waltham, MA) with a 250-μm spot distance and 100-μm-diameter spots. Spotted probes were baked for 60 minutes at 80°C. We made DNA chips to evaluate probe sensitivity and for detection of viral samples, including WHO International Standards and WHO geno-type panels. Each sequence for detection probes on the chip is listed in Table 2.

Synthesis and hybridization of fluorescently labeled DNA samples

PCR amplification of extracted DNA or cDNAs was performed for fluorescent labeling using polymerase (GoTaq, Promega) with Cy-5 dCTP. The PCR mixture included template DNA or cDNA, 50 μmol/L primers, 0.5 μL of Cy-5 dCTP (Perkin-Elmer), 1 μL of dNTP mixture (2.5 mmol/L each, 0.25 mmol/L dCTP), 5 μL of 5× PCR buffer (GoTaq, Promega), and 0.25 μL of polymerase (GoTaq, Promega). The 50-cycle PCR profile was 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Amplification was carried out in a PCR system (GeneAmp 9700, Applied Bio-systems, Foster City, CA). Two microliters of the PCR-amplified reaction mixture was hybridized with the chip for 30 minutes at 50°C. The hybridized chip was washed with saline-sodium citrate buffer and scanned with a fluo-rescent scanner (FLA-8000, Fujifilm, Tokyo, Japan). Geo-graphic origin was estimated from the obtained fluorescence patterns, thereby indicating specific genotypes.

Transcription-mediated amplification assay

To validate our assay sample preparation including viral DNA or RNA, we performed transcription-mediated amplification (TMA) assays for HBV, HCV, and HIV-1 by using an assay kit (Ultrio, Novartis Pharma, Tokyo, Japan) according to the manufacturer's protocol.

RESULTS

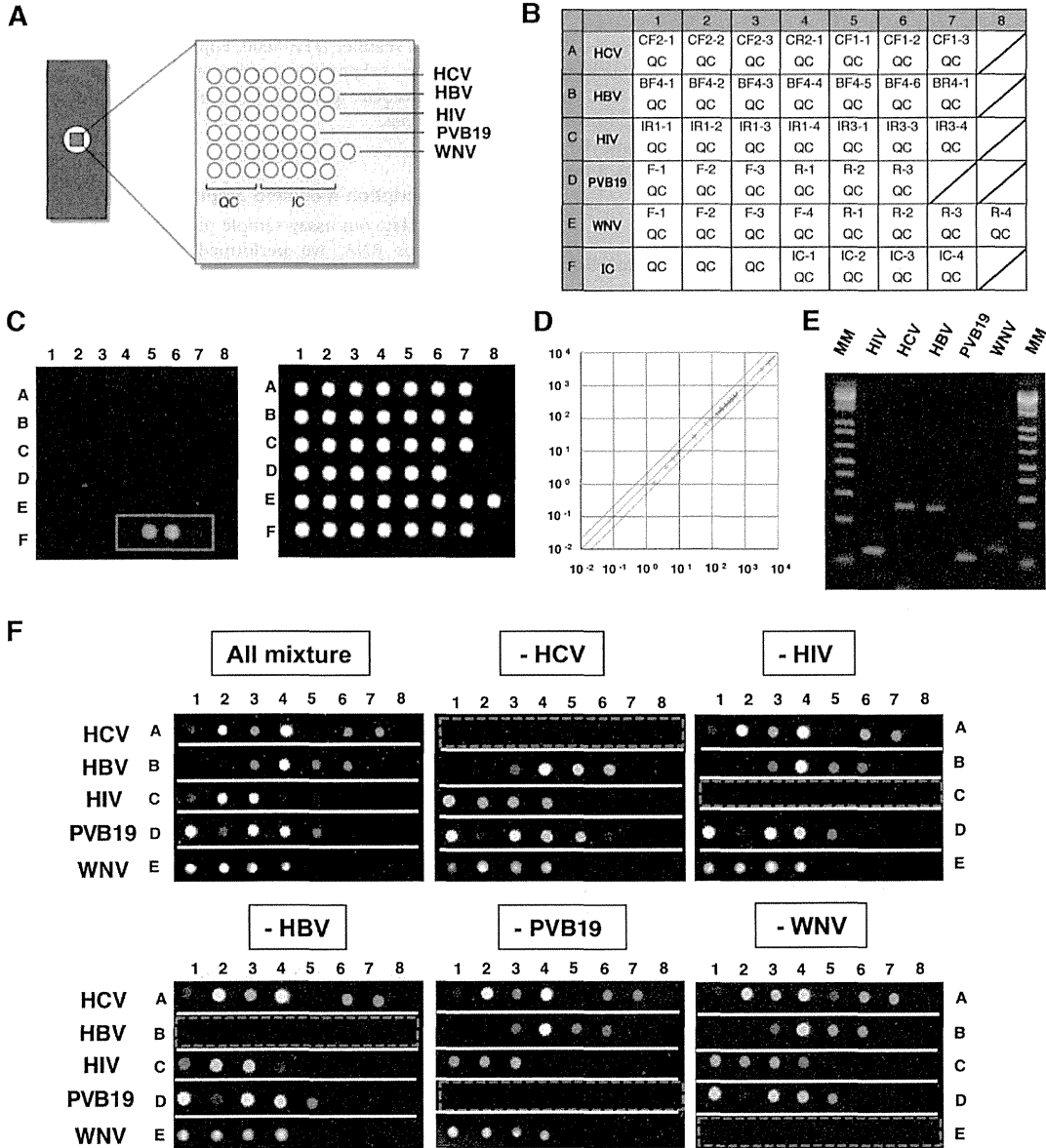### Synthesis of genotype panel oligomers by OE-PCR

To verify our designed dPCR primers, we prepared geno-type panel oligomers (100-300 bp) as viral genotype reference materials for HIV (A1, A2, B, C, D, F1, F2, G, H, J, K, N, O, and U), HBV (A–H), WNV (1-10), and PVB19 (1-6) made by OE-PCR (Fig. 1A). The target regions for OE-PCR were selected according to the nucleotide sequences of the standard strain for dPCR and the DLC-chip detection system (Supplemental Table S2). The joining oligo-nucleotides were designed using an online computer program (DNAWorks, Version 2, http://helixweb.nih.gov/dnaworks/). The nucleotide sequences of each genotype panel oligomer are listed in Supplemental Table S1. OE-PCR was carried out according to a two-step reaction method. The OE-PCR products were checked on a chip electrophoresis system (Multina 202, Shimazu). As expected, we could detect our OE-PCR products at appro-priate molecular size (Fig. 1B). For HCV (1A, 2A, 3A, 4A, 6B, 6K, 6P, 6T, and 7A), genotype panel oligomers were designed and made by custom service (Invitrogen).

Validation of dPCR primer for detection of virus genotype panel oligomers

We confirmed whether our designed primers could spe-cifically amplify each virus subtype by PCR. The PCR prod-ucts for all HIV, HCV, HBV, PVB19, and WNV subtypes were detected at the expected size by gel electrophoresis (Fig. 1C).

QC of our pathogen detection DNA microarray system

To evaluate specificity of each probe in our DLC-chip, the detection ability of our probes was analyzed. We selected 36 specific probe sets that had high detection abilities (>50% of genotypes) from the 53 originally designed evaluated probes (data not shown) and then spotted them on DLP-Chip (Fig. 2A, B). DNA microarray images were

A

HCV
HBV
HIV
PVB19
WNV

QC   IC

B

		1	2	3	4	5	6	7	8
A	HCV	CF2-1 QC	CF2-2 QC	CF2-3 QC	CR2-1 QC	CF1-1 QC	CF1-2 QC	CF1-3 QC	
B	HBV	BF4-1 QC	BF4-2 QC	BF4-3 QC	BF4-4 QC	BF4-5 QC	BF4-6 QC	BR4-1 QC	
C	HIV	IR1-1 QC	IR1-2 QC	IR1-3 QC	IR1-4 QC	IR3-1 QC	IR3-3 QC	IR3-4 QC	
D	PVB19	F-1 QC	F-2 QC	F-3 QC	R-1 QC	R-2 QC	R-3 QC		
E	WNV	F-1 QC	F-2 QC	F-3 QC	F-4 QC	R-1 QC	R-2 QC	R-3 QC	R-4 QC
F	IC	QC	QC	QC	IC-1 QC	IC-2 QC	IC-3 QC	IC-4 QC	

C

1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8
A B C D E F

D

10^4
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}

10^{-2} 10^{-1} 10^0 10^1 10^2 10^3 10^4

E

MM HIV HCV HBV PVB19 WNV MM

F

| All mixture | - HCV | - HIV |

1 2 3 4 5 6 7 8

HCV A
HBV B
HIV C
PVB19 D
WNV E

| - HBV | - PVB19 | - WNV |

1 2 3 4 5 6 7 8

HCV A
HBV B
HIV C
PVB19 D
WNV E

captured by an image analyzer (FLA-8000, Fuji Photo Film, Tokyo, Japan). Hybridized slides were inserted in the FLA-800, and the scan conditions were set as 10 μm resolution, standard scan mode, and photomultiplier tube high-value 100% laser. Saved DNA microarray images were analyzed, and fluorescent intensity of each spot was measured using a computer program (ArrayGauge Software, Fuji Photo Film). After removing the background signal, we defined the positive signal standard as radio intensity more than five times higher than background. The signal intensity was calculated as the total pixel value minus the global background. The signal intensities were then normalized to the mean for all the spots in the array.

To determine the specificity of the new system, we applied two different QCs: an IC probe (oligonucleotide complementary to the Cy5-labeled amplicon of the 18S

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Fig. 2. (A) Schematic design of DLC-chip, including HIV, HCV, HBV, WNV, and PVB19 probes (B) and their relative position in the slide layout. Spotted probes of seven subtypes of HIV-1, seven genotypes of HCV, seven genotypes of HBV, six genotypes of PVB19, and eight genotypes of WNV were selected. All of the probes were spotted together on the chip, along with the QC probes. (C) Evaluation of the detection system using the IC (left panel) and QC (right panel). Anti-IC probes specifically hybridized to and amplified 18S rRNA PCR product from human DNA. (D) QC by using same amplified sample. Correlation coefficients using scatter plot indicated that each DLC-chip was highly reproducible, with a correlation of 0.99905. (E) Multiple detection system for five virus genomes. Agarose gel electrophoresis analysis of PCR products to detect HIV-1, HCV, HBV, PVB19, and WNV. These PCR products were positive on the microarrays. (F) Multiple detection system for five viruses by DLC-chip. The PCR products of five viruses were mixed at the following concentrations: HCV, 10 IU/mL; HBV, 10 IU/mL; HIV-1, 10,000 IU/mL; PVB19, 10 IU/mL; and WNV, 10 copies/mL. Each panel used a mixture of the five viruses that lacked HIV-1, HCV, HBV, PVB19, or WNV. The red line indicates the row where excluded virus was not detected. Cross-hybridization between the five viruses was not confirmed.

rRNA gene; Fig. 2C, left) and a QC probe (oligonucleotide complementary to the Cy3-labeled QC probe; Fig. 2C right). This allowed us not only to monitor the spot uniformity, but also to detect potential irregularities during the hybridization process. To evaluate the reproducibility of our DLC-chip, we hybridized the same PCR-amplified samples to different DLC-chips and measured each signal intensity. Correlation coefficients using scatter plots indicated that each DLC-chip was highly reproducible, showing a correlation of 0.99905 (Fig. 2D).

Multiple detection of five viruses in one test

We determined whether our designed probes could detect the PCR products of all the HIV genotypes. On the DLC-chip, seven different HIV-1–specific probes were aligned. After being labeled with Cy5, PCR products were detected by hybridization to HIV-specific probes on the DLC-chip. We considered that a sample was positive if at least two different probes showed a positive signal. These HIV-1–specific degenerative primer and probe sets detected all of the following genotypes: A1, A2, B, C, D, F1, F2, G, H, J, K, N, O, and U (data not shown). Similar to the HIV detection system, different probe sets detected all the HCV, HBV, PVB19, and WNV genotypes (data not shown). To determine the ability to detect multiple viruses on one DNA chip, we separately performed virus-specific genome amplification (Fig. 2E) and mixed each PCR product in one tube and hybridized the PCR products onto the DLC-chip. The mixed viral PCR product (HIV, HCV, HBV, PVB19, and WNV) was readily detected as a hybridized spot on the DCL-chip (Fig. 2F). We prepared a mixed sample minus one virus amplicon as a negative control for cross-hybridization and nonspecific binding. We confirmed that no cross-hybridization occurred with HIV-1, HCV, HBV, PVB19, and WNV.

Specificity and sensitivity of our dPCR-NAT system by using WHO genotype panels and international standards

To determine the specificity of our DNA microarray system to detect each virus genotype, we prepared the WHO genotype panel samples for each virus. For WNV, we used the genotype panel oligomer described in Figure 1 as an NAT genotype panel because there was no commercially available panel. We extracted DNA or RNA from each genotype panel for HIV, HCV, HBV, PVB19, and WNV. Each template DNA or cDNA was amplified with each dPCR primer listed in Table 1. The amplified PCR products for all genotypes of each virus were confirmed on 3% agarose gels and DNA chips. We detected all the HIV, HCV, HBV, PVB19, and WNV genotypes (Fig. 3B). Our DNA microarray data are summarized in Supplemental Table S4 (available as supporting information in the online version of this paper). To validate the sensitivity of our NAT system, we used WHO International Standards as reference materials. To check our reference samples, we performed FDA-licensed NAT assays using a TMA assay (Supplemental Table S3, available as supporting information in the online version of this paper) before analysis. For sensitivity assay, we prepared HCV RNA, HBV, and PVB19 from NIBSC. These materials were used as international standards for NAT quality assurance. We prepared each sample to give a final concentration of 1 to 10,000 IU/mL and isolated DNA or RNA from 200-μL samples. Thus, each sample was assumed to contain 0.2 to 2000 IU virus if extraction efficiency was 100%. DNA and RNA were extracted. RNA samples were all reverse transcribed with a cDNA synthesis kit (Superscript III RT, Invitrogen), and all cDNA samples were used for PCR. We detected 1 IU/mL HCV, 1 IU/mL HBV, 1 IU/mL PVB19, and 1 copy/mL WNV (Fig. 3C). Similar results were obtained from at least three independent experiments. For HIV, we estimated the detection limit in at least five independent tests. We detected 10,000 IU/mL for 100%, 1000 IU/mL for 77%, 100 IU/mL for 7%, 10 IU/mL for 0%, and 1 IU/mL sample for 0%. Thus we conclude that our system could detect 1000 IU/mL equivalent to 200 IU/PCR sample for HIV. Detection limits for each virus are listed in Table 3.

DISCUSSION

We investigated the performance of the new NAT system using dPCR primers and a DLC-chip. We showed that our NAT system was specific for HIV-1, HCV, HBV, PVB19, and
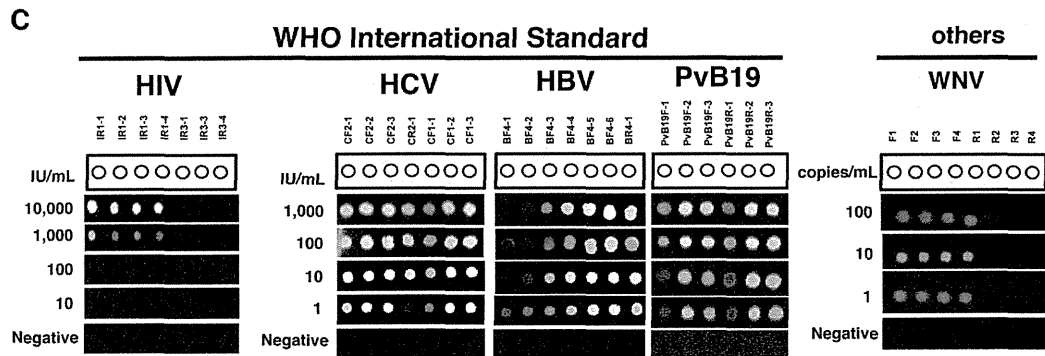
A

| Preparation samples | Extraction of DNA/RNA | PCR Amplification |

200uL sample

DNA RNA Extraction

PCR reaction

1-10,000 IU/mL sample

- WHO genotype panel
- WHO International Standard
- WNV: NATrol and OE-PCR
- Prepare 1 to 10,000 IU/mL samples

1 IU/mL = 0.2 IU/sample
10 IU/mL = 2 IU/sample
100 IU/mL = 20 IU/sample
1,000 IU/mL = 200 IU/sample
10,000 IU/mL = 2,000 IU/sample

HIV/HCV/WNV RNA→RT
HBV/PvB19 DNA

Detection with DNA Chip

Check with DNA microarray

B

WHO Genotype Panel

HIV   HBV   HCV   PvB19

OE-PCR

WNV

C

WHO International Standard

HIV   HCV   HBV   PvB19
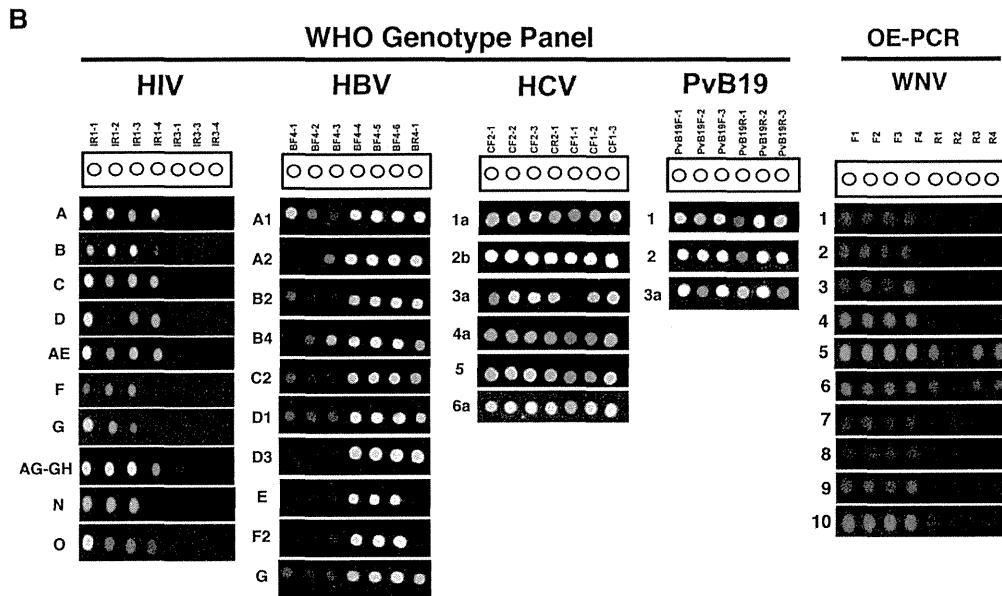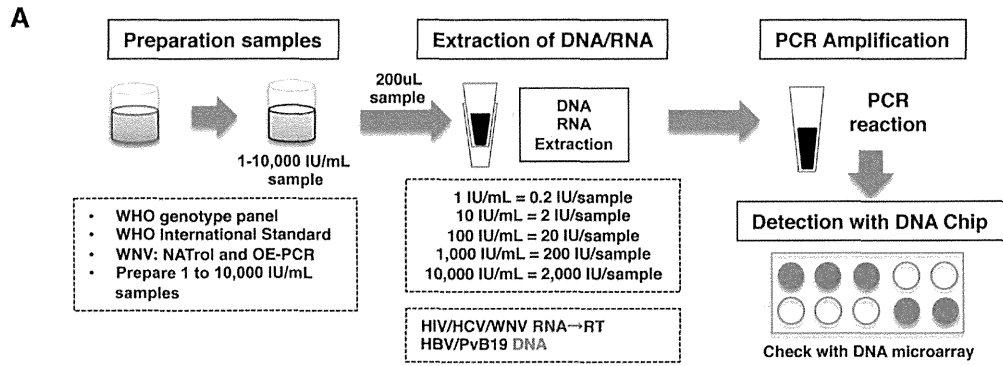
others

WNV

114

Fig. 3. (A) Schematic illustration of sensitivity analysis of our detection system, including extraction process. Detection of the isolated virus genomes from human plasma sample. Samples containing HIV-1, HCV, and HBV were diluted with defibrinated plasma (Basematrix 53; SeraCare) at 1 to 10,000 IU/mL. (B) For more accurate analysis, we prepared a WHO genotype panel for HIV-1, HBV, HCV, and PVB19. We detected all genotypes by using our designed degenerated primer. (C) For sensitivity analysis of our NAT system, we prepared WHO International Standards for HIV-1, HCV, HBV, and PVB19. We diluted these materials with negative sera at 1 to 10,000 IU/mL and extracted DNA and RNA from 200-μL samples. All RNA samples were transcribed with Superscript III. We detected HIV at 1000 IU/mL, HCV at 1 IU/mL, HBV at 1 IU/mL, and PVB19 at 1 IU/mL.

TABLE 3. Detection limit of our dPCR-NAT system*

IU/mL	HIV (%)	HCV (%)	HBV (%)	PVB19 (%)
10,000	100	100	100	100
1,000	77	100	100	100
100	7	100	100	100
10	0	100	100	100
1	0	50	25	75

* % positive: Reactive/Tested (Percent Reactive). The measurement obtained in each specimen was tested with two sample lots in two independent test assays. For HIV, we tested five sample lots in five independent test assays.

WNV at low viral loads. In addition, we showed that our system detected various virus genotypes. Degenerate primers are useful not only for detecting unknown genes, but also for the simultaneous amplification of mutated genes.[17] In the case of viruses, many mutated strains appear in a particular geographical area and at a specific time.[18] Recently, we used CoCoMo primers, a fully automatic design pipeline for PCR primers, according to the CodeHop primer design strategy, by which others can analyze the oligonucleotide motif incidence.[14] The CoCoMo program is available online (www.geneknot.info/cocomo). We utilized CoCoMo primers to design the primer sequences in this study. The algorithm-designed primers were confirmed to fit most subtypes or genotypes of the target viruses (Table 1) and enabled efficient detection of a wide range of viruses. In general, PCR procedures with the degenerate primers had lower sensitivity than that of the specific primers. To overcome this disadvantage, we used fluorescence detection on the DLC-chip, which provided higher sensitivity.[12] Additionally, the degenerate primer set was designed to detect the polymorphic region of the viral genome; therefore, subtypes or genotypes could be discriminated on the DLC-chip. The combination of the primers and DLC-chips was therefore validated. These results suggest that the combination of dPCR and DLC-chips is beneficial for blood-borne virus detection. To increase the safe use of the system, automation of our detection system will be required in the future.

A low level of HBV may proliferate in transfused recipients who are immunocompromised or immunosuppressed. In addition, the window period of HBV is relatively long, but the presence of HBV DNA without detectable HBsAg outside the window period, known as occult HBV infection, has been reported.[7] This suggests that the development of a highly sensitive detection system for HBV is particularly important. Although the current TMA sensitivity corresponds to 1 to 5 IU/mL at the reproductive level, using the same viral samples in this study we detected 1 to 10 IU/mL HBV. These data suggest that the sensitivity of our analysis system is equivalent to that of the TMA assay (Fig. 3C). Thus far, HBV Genotype C is the most prevalent genotype in Japan (85%), while the prevalence of Genotypes A and D is 1.7 and 0.4%, respectively.[19-21] Currently, the level of Genotype A is increasing in the younger generation because of horizontal infection.[11,22,23]

For HIV-1 detection and quantification, various methods have been developed, but most real-time techniques involve their sensitivity to point mutations within primer and probe target sequences. Our dPCR-NAT system could detect a wide range of HIV genotypes by using dPCR primers. Despite the wide range of genotype detection, sensitivity was not high. We could detect 200 IU HIV/PCR procedure. Improvement is needed for HIV dPCR sensitivity. Similarly for HBV and HCV, the next-generation virus detection system must be able to cope with this situation, namely, by possessing a wide detectable viral genotype range and a low detection level. With our detection system, all of the virus genotypes were detected at 1 to 1000 IU/mL sample. Previous studies by Hsia and coworkers[24] combined multiplex PCR and DNA chips and detected three different viruses in a single sample. Our mixed PCR product data (Fig. 2F) indicated that our system could simultaneously detect five different viruses in one DNA chip. These data suggest that our system is suitable for multiple pathogen testing.

In conclusion, the dPCR-NAT system is an accurate and reliable test for HIV, HBV, HCV, PVB19, and WNV detection with respect to specificity, sensitivity, and genotype inclusivity and a reproducible assay for the detection of multiple blood-contaminating pathogens.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Sequence of each oligomer set for preparing genotype panel oligomers.

Table S2. Sequence of each genotype panel oligomers.

Table S3. Validation of our sample using current NAT system.

Table S4. Summary of DLC-chip analysis of genotype panels and international standards.

MEETING REPORT

Meeting report on the possible proposal of an extranodal primary cutaneous variant in the lymphoma type of adult T-cell leukemia-lymphoma

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ABSTRACT

Based on the advances in research on the clinicopathophysiology of adult T-cell leukemia-lymphoma (ATL), Japanese researchers collected and evaluated cases of smoldering ATL exhibiting primary cutaneous manifestation but showing poor prognosis. Macroscopic findings of skin eruptions were categorized into the patch, plaque, multipapular, nodulotumoral, erythrodermic and purpuric types, as previously reported. Pathological findings were divided into low or high grade based on epidermotropism, tumor cell size and perivascular infiltration. Eight eligible cases were evaluated among 14 collected cases. Macroscopic findings were nodulotumoral in six cases, a subcutaneous tumor in one case and plaque in one case, and the number and size were heterogeneous in each case. Pathological findings of all eight cases were T-cell lymphoma, high-grade type (pleomorphic, medium or large size), with prominent perivascular infiltration and scant epidermotropism. To diagnose such cases as the "lymphoma type of ATL, extranodal primary cutaneous variant", it is essential to examine each case carefully, including cutaneous lesions at onset, lymph nodes and other organ involvement using computed tomography (CT) and/or positron emission tomography/CT, as well as the percentage of abnormal lymphocytes in peripheral blood. Based on the results of an ongoing nationwide survey on ATL, ATL with cutaneous lesions will be analyzed to investigate the incidence and prognosis of the so-called "lymphoma type of ATL, extranodal primary cutaneous variant".

Key words: adult T-cell leukemia/lymphoma, extranodal primary cutaneous variant, lymphoma type adult T-cell leukemia/lymphoma, smoldering adult T-cell leukemia/lymphoma.

PURPOSE OF THE MEETING

On the basis of the modes of initial presentation and natural history of patients with adult T-cell leukemia/lymphoma (ATL), the four clinical subtypes of acute, lymphoma, chronic and smoldering have been recognized. Diagnostic criteria for the clinical subtypes were proposed[1] and significant prognostic factors were determined in 1991.[2] Since then, patients with ATL were stratified into two groups, aggressive ones consisting of acute, lymphoma and unfavorable chronic types, and indolent ones consisting of favorable chronic and smoldering types, in which the chronic type was further divided into favorable

and unfavorable according to significant prognostic factors. This stratification was useful for the selection of treatment, in which most patients with aggressive forms were treated with systemic chemotherapy, while those with indolent forms underwent watchful waiting or local therapy only.

In the clinical subtype classification, however, the lymphoma type did not include extranodal variants because of the rarity of such cases at that time. Since then, variants of extranodal lymphoma type such as primary cutaneous ATL and primary gastrointestinal ATL have been reported. The extranodal primary cutaneous variant included in smoldering type made it particularly difficult for physicians to choose the initial treatment.[3-6] Furthermore, the extranodal primary gastrointestinal variant included in the acute type was reported to respond to treatment and be associated with long-term survival. On the contrary, the localized lymphoma type, which was rare in the initial survey in Japan, was reported to consist of approximately 10% of acute and lymphoma types of ATL, and was associated with relatively favorable prognosis after chemotherapy in a recent nationwide survey in Japan.[7]

Based on the advances in research on the clinicopathophysiology of ATL as described above, Japanese researchers, focusing on ATL, joined by the support of a grant (H23-gan rinsho-ippan-022), collected and evaluated cases such as of the localized lymphoma type and extranodal variants originating from several organs to reconsider the subclassification for the appropriate selection of treatment.

This research group, consisting of Japanese hematologists, dermatologists, pathologists, epidemiologists and oncovirologists, aimed at collecting cases as follows: smoldering type with primary cutaneous manifestation resulting in poor prognosis, acute type with the manifestation of an extranodal variant of primary gastrointestinal or nasopharyngeal type, and localized lymphoma type, reviewing clinicopathological findings and proposing the consensus report.

This report summarizes the discussion of the first meeting on this project, focusing on the extranodal primary cutaneous variant.

ELIGIBILITY CRITERIA OF PATIENTS FOR THE EVALUATION

Eligibility criteria included smoldering ATL with only cutaneous lesions confirmed by histopathology, and with survival after diagnosis of less than 1 year as a rule but less than 3 years being allowed. Each dermatologist/hematologist picked up the cases, and filled out the case report forms with macro-photographs and histological specimens of cutaneous lesions. We categorized the macroscopic findings of skin eruptions into the patch, plaque, multipapular, nodulotumoral, erythrodermic and purpuric types, as previously reported.[6] When multiple types of skin eruption exist in a patient, the most severe type should be described if a consensus on the hierarchy of severity in the types exists: patch and plaque were considered the lowest and second lowest severity, respectively, and nodulotumoral was most severe. There was no consensus on multipapular, erythrodermic and purpuric types, but multipapular

and purpuric types were considered intermediate between nodulotumoral and plaque, and should be described separately. Erythrodermic type should still be carefully evaluated. Subcutaneous tumors were specified but included as the nodulotumoral type.

Pathological findings were divided into low or high grade based on epidermotropism, the cell size and perivascular infiltration.[3]

RESULTS

Fourteen cases were evaluated, but six of them were deemed ineligible because of the period from the onset of cutaneous lesions to the diagnosis of ATL being more than 4 months in five cases and concurrent lymph node lesions at onset not indicating the smoldering but acute type in one case. Case reports were provided by Dr Y. Sawada (University of Occupational and Environmental Health, Fukuoka), Dr Y. Uchida (Kagoshima University, Kagoshima), Dr T. Johno (Kumamoto University, Kumamoto), Dr M. Takenaka (Nagasaki University, Nagasaki), Dr K. Uchimaru (Tokyo University, Tokyo) and Dr K. Tobinai (National Cancer Center Hospital, Tokyo).

All of the eight eligible cases were diagnosed as smoldering ATL. Macroscopic findings were nodulotumoral in six cases, a subcutaneous tumor in one case and plaque in one case, and the number and size were heterogeneous in each case. Pathological findings of all eight cases were consistent with T-cell lymphoma, high-grade type (pleomorphic, medium or large size), with prominent perivascular infiltration and scant epidermotropism. Median times from the diagnosis to acute crisis, and onset of the cutaneous lesion to acute crisis, were 6 and 7 months, respectively (data not shown).

DISCUSSION (PROBLEMS AND TO-DO LIST)

Accurate evaluation is essential at onset: cutaneous lesion at onset, lymph nodes and other organ involvement using computed tomography (CT) and/or positron emission tomography (PET)/CT, as well as the percentage of abnormal lymphocytes in peripheral blood (PB).

The clinical course of each lesion including cutaneous lesions should be evaluated with respect to the timing of diagnosis. As for the "extranodal primary cutaneous variant", further case evaluation is essential, including those with a relatively favorable prognosis.

As for the pathological diagnosis of cutaneous lesions, the biopsy site including macroscopic findings should be described. It is possible that specimens were biopsied at sites with a poor prognostic hierarchy in this case series.

In general, pathological findings of cutaneous lesion of ATL appear to be epidermotropic and non-epidermotropic. All of the cases in this meeting were high-grade peripheral T-cell lymphoma (PTCL)-like, and no case was low-grade cutaneous T-cell lymphoma-like because cases with a poor prognosis were collected.

Seven out of eight eligible cases were the "extranodal primary cutaneous variant", consisting of six cases of nodulotu-

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moral and one of plaque macroscopically, and all seven were high-grade PTCL microscopically. The remaining one was described as a "primary subcutaneous tumor". There was a comment against the "primary subcutaneous tumor" type included in the "primary cutaneous variant".

To diagnose such cases as the "lymphoma type of ATL, extranodal primary cutaneous variant", it is essential to examine each case carefully, including cutaneous lesions at onset, lymph nodes and other organ involvement using CT and/or PET/CT, as well as the percentage of abnormal lymphocytes in PB (Appendix 1).

The application of clinical staging based on the extension of cutaneous lesions requires further investigation.

Some ATL patients with multipapular type cutaneous lesions were reported to show a rapidly progressive clinical course.[6] Such cases should also be collected and analyzed.

FUTURE PLAN

More cases should be collected and investigated.

Based on the results of an ongoing nationwide survey on ATL, ATL with cutaneous lesions will be analyzed to investigate the incidence and prognosis of the so-called the "lymphoma type of ATL, extranodal primary cutaneous variant".

CONFLICT OF INTEREST: None.

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APPENDIX I

For the diagnostic criteria of the lymphoma type of the extranodal primary cutaneous variant, no definite appearance of abnormal cells in PB ($\leq$1%) is essential.

Evaluation of abnormal lymphocytes by flow cytometry as well as based on the morphology is warranted to calculate the cells as a real number. However, such criteria are quite different from the original criteria for the definition of ATL and clinical subtype classification of ATL. Therefore, such a proposal requires careful analyses and evaluation. Discussion on this issue is currently limited in this meeting.

For ATL, quantitative evaluation of cutaneous lesions such as using the modified Severity Weighted Assessment Tool should be investigated; however, it is not easily applicable.

Macroscopic findings of cutaneous lesions are a significant prognostic factor in ATL. However, the combination of other parameters, for example, tumor markers such as lactate dehydrogenase and soluble IL-2 receptor, needs to be investigated.

Adult T-cell leukemia cells are characterized by abnormalities of *Helios* expression that promote T cell growth

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Molecular abnormalities involved in the multistep leukemogenesis of adult T-cell leukemia (ATL) remain to be clarified. Based on our integrated database, we focused on the expression patterns and levels of Ikaros family genes, *Ikaros, Helios*, and *Aiolos*, in ATL patients and HTLV-1 carriers. The results revealed profound deregulation of Helios expression, a pivotal regulator in the control of T-cell differentiation and activation. The majority of ATL samples (32/37 cases) showed abnormal splicing of Helios expression, and four cases did not express Helios. In addition, novel genomic loss in *Helios* locus was observed in 17/168 cases. We identified four ATL-specific short Helios isoforms and revealed their dominant-negative function. Ectopic expression of ATL-type Helios isoform as well as knockdown of normal Helios or Ikaros promoted T-cell growth. Global mRNA profiling and pathway analysis showed activation of several signaling pathways important for lymphocyte proliferation and survival. These data provide new insights into the molecular involvement of Helios function in the leukemogenesis and phenotype of ATL cells, indicating that Helios deregulation is one of the novel molecular hallmarks of ATL. (*Cancer Sci* 2013; 104: 1097–1106)

A dult T-cell leukemia (ATL) is a highly aggressive malignancy of mature CD4+ T cells and is caused by HTLV-1. After HTLV-1 infection, ATL is thought to develop following a multitude of events, including both genetic and epigenetic changes in the cells. Although many aspects of HTLV-1 biology have been elucidated, the detailed molecular mechanism of ATL leukemogenesis remains largely unknown.[1,2] Therefore, to precisely define the comprehensive abnormalities associated with ATL leukemogenesis, we previously carried out global mRNA and miRNA profiling of ATL cells derived from a large number of patients.[3,4] In this study, we focused on Ikaros family genes, especially Helios, on the basis of our integrated profiling of expression and gene copy number in ATL cells, which revealed the deregulated expression of this family of genes and genomic loss of *Helios* locus.

Ikaros family genes are specifically expressed in the hematopoietic system and play a vital role in regulation of lymphoid development and differentiation.[5–11] In addition, they are known to function as tumor suppressors during leukemogenesis according to several genetic studies carried out in mouse models.[12–15] Recently, many studies reported the deregulated splicing of Ikaros and the deletion of *Ikaros* locus in several human leukemias.[16–23] These abnormalities are associated with poor prognoses.[24–27] Helios is mainly expressed in the T-cell lineage.[10,11] Genomic changes and abnormal expression of Helios are also observed in some

patients with T-cell malignancies.[18,28–31] However, in contrast to Ikaros, the substantial impact of aberrant Helios expression remains to be elucidated because of the absence of functional information, including the target genes of Helios.

In this study, we carried out a detailed expression analysis of Ikaros family genes in a large panel of clinical samples from ATL patients and HTLV-1 carriers and consequently identified a novel molecular characteristic, that is, abnormal splicing of Helios and loss of expression, which seems to be a significant key factor in leukemogenesis affecting the regulation of T-cell proliferation.

Materials and Methods

Cell lines and clinical samples. HeLa and 293T cells were cultivated in DMEM supplemented with 10% FCS. Human leukemic T cells, Jurkat, Molt-4, and CEM, ATL-derived, MT-1 and TL-Om1, and HTLV-1-infected MT-2 and Hut-102 cell lines were all maintained in RPMI-1640 with 10% FCS. The PBMCs from ATL patients of four clinical subtypes[32] and healthy volunteers were a part of those collected with informed consent as a collaborative project of the Joint Study on Prognostic Factors of ATL Development. The project was approved by the Institute of Medical Sciences, University of Tokyo Human Genome Research Ethics Committee (Tokyo, Japan). Clinical information of ATL individuals is provided in Table S1.

RNA isolation and RT-PCR analysis. The preparation of total RNA and synthesis of the first strand of cDNA were described previously.[3] The mRNAs of Ikaros family genes were examined by PCR with Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). The PCR products were sequenced by automated DNA sequencer. Nested PCR amplification was carried out with diluted full-length PCR products by Accuprime Taq DNA polymerase High Fidelity (Invitrogen). Quantitative PCR was carried out as previously described.[3] The specific primer sets for each PCR are described in Table S2.

Immunoblot analysis. Cells were collected, washed with PBS, and lysed with RIPA buffer. For immunoprecipitation, cells were lysed with TNE buffer and incubated with specific antibody. Proteins samples were then analyzed by immunoblots with specific antibodies: anti-tubulin, anti-Ikaros, and anti-Helios antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-FLAG antibody (M2) was from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-HA

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antibody was from MBL (Nagoya, Japan). Anti-mouse, rabbit, and goat secondary antibodies were from Promega (Fitchburg, WI, USA).

Immunostaining. HeLa cells were cultured on coverslip slides and transfected with the indicated expression vectors by Lipofectamine LTX (Invitrogen). At 24 h post transfection, cells were washed three times with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Then, cells were stained with primary antibodies (diluted 1:500 to 1:2000). Alexa-488 or 546-conjugated secondary antibodies (Molecular Probes, Life Technologies, Carlsbad, CA, USA) were used for detection of specific targets, and DAPI was used for nuclear staining. Images were acquired by using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

Electrophoretic mobility-shift assay. Experimental conditions and detail methods were previously reported.[3] For evaluation of DNA binding activity, 3–5 µg nuclear extracts from each transfectant were used per each lane of electrophoresis. The oligonucleotide sequences used as a probe are provided in Table S2.

Luciferase assay. The pGL4.10-firefly vector (Promega) containing *Hes1* promoter was used as a reporter vector and RSV-renilla vector was used as a control vector. HeLa cells were transiently transfected with these reporters and each Ikaros or/and Helios expression vector by Lipofectamine 2000 reagent (Invitrogen). The luciferase activities were quantified by the Dual-Luciferase Reporter Assay System (Promega) at 24 h post-transfection.

Retroviral construction and transduction. The FLAG-Hel-5 cDNA sequence was subcloned into retrovirus vector pRx-puro. Stable cell populations expressing Hel-5 were selected by puromycin. The shRNA-expressing retroviral vectors and virus production procedures have been established.[3] The shRNA sequences are listed in Table S2. Stable cell populations were obtained by puromycin or G418 selection.

Proliferation assays. Cells (0.5 or 1.0 × 10⁴) were plated in 96-well plates with media supplemented with 10% or 0.2% FCS. The cell numbers were evaluated for 4 days by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The averages of at least three independent experiments are shown.

Gene expression microarray analyses. Gene expression microarray used the 4 × 44K Whole Human Genome Oligo Microarray (Agilent Technologies, Santa Clara, CA, USA); detailed methods were previously reported.[3] Coordinates have been deposited in the Gene Expression Omnibus database with accession numbers GSE33615 (gene expression microarray), GSE33602 (copy number analyses), and GSE41796 (Jurkat models).

Results

Abnormal expression of short Helios transcripts in primary ATL cells. To characterize the gene expression signature in primary ATL cells, we previously carried out mRNA microarray analyses on a large number of samples. The comprehensive survey unveiled deregulated expression of Ikaros family genes; transcription levels of Ikaros and Aiolos were downregulated in ATL samples, whereas Helios was upregulated (Fig. S1). Thus,

we examined the detailed expression patterns and levels of Ikaros family members in PBMCs derived from a panel of ATL patients and HTLV-1 carriers (Fig. 1a). Compared with control PBMCs from normal volunteers (Fig. 1b), the expression levels of Ikaros and Aiolos seemed to be downregulated in ATL samples, consistent with our microarray results. However, there were obvious abnormalities in the expression patterns of Helios. The main isoform of Helios was changed from full-length Hel-1 to Hel-2, which lacks exon 3 that contains the first N-terminal zinc finger in the DNA-binding domain. In addition, four ATL-specific Helios short transcripts were identified (Fig. 1c). Among them, Hel-5 and Hel-6 have been reported to be expressed in ATL.[29] We also identified two novel variants, Hel-v1 that lacks exons 3 and 4 and Hel-v2 that lacks exons 2, 3, and 6. These abnormal Helios variants were also expressed in the samples of high-risk HTLV-1 carriers, who subsequently developed ATL in the next few years. Furthermore, nested PCR revealed that Hel-5 or Hel-6 were expressed in a majority of ATL samples (17/22 acute cases, 10/10 chronic cases, and 5/5 smoldering cases; total, 32/37 cases) (Fig. 1d, upper panels), whereas Hel-v1 was expressed only in limited cases of ATL (Fig. 1d, lower panels). In four cases, Helios was not expressed. Collectively, our mRNA analysis showed that Helios expression was generally deregulated in ATL cells.

Genomic abnormalities at the *Helios* locus in primary ATL cells. To investigate the *Helios* locus in ATL, we retrieved data from our gene copy number analysis[3] and found that specific genomic deletion was accumulated at the *Helios* locus in ATL samples (17/168 cases, Fig. 2). All 17 cases were aggressive-type ATL (12/17 lymphoma types and 5/17 acute types). Furthermore, we found that two acute ATL cases in Figure 1(a) (#9 and #14), which showed severely deregulated or lost Helios expression, had a genomic deletion of the *Helios* locus.

Dimerization ability of ATL-type Helios isoforms with wild-type Helios or Ikaros. Consistent with a previously published report,[33] co-immunoprecipitation analyses confirmed that wild-type Hel-1 formed homodimers with themselves and heterodimers with wild-type Ikaros (Ik-1) protein (Fig. 3a, top panel, lane 1 and lane 4). In contrast, the dimerization activity of another artificial Helios mutant (Hel-ΔC), which lacks the dimerization domain at the C-terminal region, was dramatically declined (Fig. 3b, top panel, lane 1 and lane 4). We confirmed that all ATL-type Helios proteins could interact with Hel-1 and Ik-1, despite the fact that all of them lack various sets of the N-terminal exons (Fig. 3c–f).

Cytoplasmic localization of ATL-type Helios isoforms lacking exon 6. Ectopically expressed Hel-1 and Ik-1 were localized in the nucleus (Fig. 4a, top two panels). Regarding the ATL-type Helios isoforms, we found that Hel-5 and Hel-v1 were localized in the nucleus, whereas Hel-6 and Hel-v2, both of which lack exon 6, were substantially localized in the cytoplasm (Fig. 4a, middle four panels). We also confirmed the cytoplasmic localization of Hel-Δexon 6, which is an artificial Helios mutant lacking only exon 6 (Fig. 4a, bottom panel). Thus, exon 6 appears to be critical for nuclear localization of Helios proteins. Furthermore, defect of exon 6 led to disruption of the

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Fig. 1. (On the next page) Abnormal expression of Helios mRNA in primary adult T-cell leukemia (ATL) cells. (a) Expression analysis of Ikaros family genes in PBMCs by full-length RT-PCR (Acute, *n* = 22; Chronic, *n* = 10; Smoldering, *n* = 5; HTLV-1 carriers, *n* = 5; High-risk carriers, *n* = 4). To detect and distinguish alternative splicing variants, PCR analyses were carried out with the sense and antisense primer sets designed in the first and final exons of each full-length transcript of Ikaros family genes. Obtained cDNAs were cloned and their sequences were analyzed. The samples acute #4, 4', and 4'' were derived from the same patient, but were studied independently. (b) Expression of Ikaros family genes in PBMCs from normal volunteers (*n* = 10). (c) Schematic representation of Hel-1, Hel-2, and ATL-type Helios isoforms identified in this study. Hel-variant 1 (Hel-v1) and Hel-variant 2 (Hel-v2) are novel isoforms in ATL. Arrows indicate primer locations of full-length PCR for Helios. Ex, exon; F1–F6, functional zinc-finger domains. (d) Nested PCR with specific primer sets, which were designed at exon junction of exon 1–5 or exon 2–5 for detection of Hel-5 and Hel-6 (upper panel), or detection of Hel-v1 (lower panel), respectively. Arrows indicate primer locations.