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Novel Application of Loop-mediated Isothermal Amplification for Rapid Detection of Gene Translocation

Ibu Matsuzaki[1], Hideto Iguchi[1], Yurina Mikasa[1], Hiromu Morishita[1], Katsuya Okuda[1], Keita Nakaguchi[1], Yuki Mori[1], Yoshifumi Iwahashi[1], Kenji Warigaya[1], Masakazu Fujimoto[1], Fumiyoshi Kojima[1] and Shin-ichi Murata[1]

[1]*Department of Human Pathology, Wakayama Medical University, 811–1 Kimiidera, Wakayama 641–8509, Japan*

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Identification of fusion genes in cancer is essential for pathological diagnosis and clinical therapy. Although methods for detection of fusion genes, such as fluorescence *in situ* hybridization (FISH) and real-time polymerase chain reaction (PCR), have been developed in last two decades, these methods are not ideal for detection of these genetic alterations owing to their high cost and time-consuming procedures. In this study, we developed novel application for detection of gene translocations using loop-mediated isothermal amplification (LAMP). We verified the amplified DNA products of echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (*EML4-ALK*), synaptotagmin and synovial sarcoma, X breakpoint (*SYT-SSX*), and immunoglobulin heavy chain gene and B cell leukemia/lymphoma 2 (*IgH/BCL2*) by real-time PCR, agarose-gel electrophoresis, and the naked eye after the LAMP procedure. Fusion genes were detected in samples diluted 10^3 times within 60 min. Because of the advantages of rapid amplification, simple operation, and easy detection without requiring sophisticated equipment or technical skill, LAMP may have potential applications as an on-site analytical approach in hospitals for pathological diagnosis and decision making regarding appropriate therapeutic approachs.

Key words: loop-mediated isothermal amplification, gene translocation, echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase; synaptotagmin and synovial sarcoma, X breakpoint, immunoglobulin heavy chain gene and B cell leukemia/lymphoma 2

I. Introduction

Identification of specific genomic aberrations in cancers, including point mutations, chromosomal translocations, amplifications, and deletions, is important for pathological diagnosis and establishment of therapeutic approaches. The first report of a specific translocation in human neoplasia was the t(9;22)(q34;q11) rearrangement involving the *ABL1* and *BCR* genes; this fusion yields the Philadelphia chromosome [21]. Fusion genes have been reported in not only hematologic malignancies but also solid tumors [17]. Methods to detect fusion genes are based on fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR), and sequencing. However, these methods are time consuming, expensive, and technically difficult.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification assay that can be used as an alternative to PCR. This method involves autocycling strand displacement DNA synthesis performed by Bst DNA polymerase. LAMP assays typically use four primers, including two inner primers and two outer primers, which recognize six different regions within the target DNA (Fig. 1). This method has been widely applied in various fields.

Correspondence to: Shin-ichi Murata, Department of Human Pathology, Wakayama Medical University, 811–1 Kimiidera, Wakayama 641–8509, Japan. E-mail: smurata@wakayama-med.ac.jp

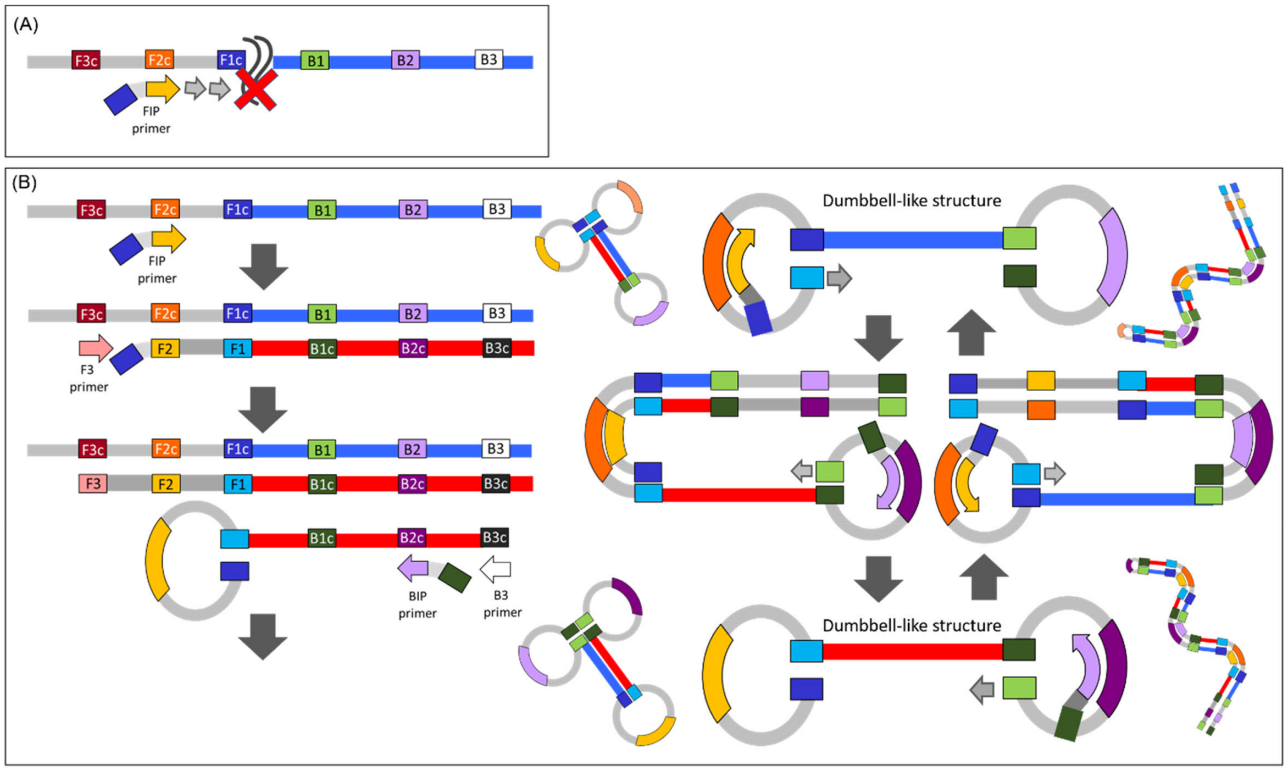

Fig. 1. Schematic representation of detection of fusion genes by LAMP assays. **A**: cDNA from cells lacking a fusion gene cannot react in LAMP assays. **B**: cDNA from cells having a fusion gene can be analyzed by LAMP assays. The inner primers FIP (BIP) were composed of F1c (B1c) and F2 (B2). The outer primers were F3 and B3. Because dumbbell-like structures cause cyclic reactions, the LAMP reaction synthesizes various products of different sizes. The schematic shows a case of detection of the *IgH/BCL2* fusion gene.

Commercial kits using LAMP have been developed, some of which have been adopted for routine identification and surveillance of pathogens, including viruses [14], bacteria [27], fungi [19], and protozoa [3]. Additionally, LAMP is a low-cost, high-specificity, efficient, simple, rapid methodology [11].

We previously reported novel methodology for rapid detection of *KRAS* mutations using artificial DNAs: peptide nucleic acid (PNA)- and locked nucleic acid (LNA)-mediated LAMP [9]. In this study, we developed a novel application of LAMP for detection of fusion genes. We assessed the *EML4-ALK* fusion gene, originating from the chromosomal inversion inv(2)(p21;p23), which is associated with non-small cell lung cancer (NSCLC); the *SYT-SSX* fusion gene, originating from the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma; and the *IgH/BCL2* fusion gene, originating from the t(14;18)(q32;q21) translocation, a hallmark of follicular lymphoma found in 20–30% of *de novo* diffuse large B-cell lymphoma.

II. Materials and Methods

Cell lines

We used 10 human cancer cell lines: H2228, HS-SY-II (HS), TK, HuH-7 (HuH), KTC-1 (KTC), BxPC-3-Luc#2 (Bx), QGP-1 (QGP), HT1197 (HT), RT4, and MCF10A. Details of the cell lines are shown in Table 1. H2228, HS, and TK cells contained the fusion genes *EML4-ALK* variant 3a, *SYT-SSX1*, and *IgH/BCL2*, respectively. We used other cell lines as controls. All cell lines were cultured with appropriate media, according to the manufacturer's instructions, in a standard humidified incubator at 37°C in an atmosphere containing 5% CO_2.

FISH analysis

FISH was performed using an *EML4-ALK* fusion FISH probe (Kreatech, Leica Biosystems, Nussloch, Germany), *SYT-SSX* break-apart FISH probe (Abbott Molecular, Des Plaines, IL, USA), or *IgH/BCL2* fusion FISH probe (Kreatech, Leica Biosystems) following the manufacturers' instructions. Results were analyzed using a fluorescent microscope (Olympus, Tokyo, Japan) with Metamorph imaging system software (Molecular Devices, Eugene, OR, USA).

Real-time PCR

Total RNA was isolated from cell lines using TRIzol Reagent (Thermo Fisher Scientific). cDNA was synthesized from 2 μg total RNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). Primers used for *EML4-ALK* and *SYT-SSX* fusion genes

Table 1. *List of cell lines*

Cell line	Origin	Fusion gene	Provider
H2228	Pulmonary adenocarcinoma	*EML4-ALK* v3a	ATCC
HS-SY-II (HS)	Synovial sarcoma	*SYT-SSX1*	NBRP
TK	B-cell lymphoma	*IgH/BCL2*	JCRB Cell Bank
HuH-7 (HuH)	Hepatocellular carcinoma	control	NBRP
KTC-1 (KTC)	Papillary thyroid carcinoma	control	Dr. Kurebayashi [13]
BxPC-3-Luc#2 (Bx)	Pancreatic adenocarcinoma	control	JCRB Cell Bank
QGP-1 (QGP)	Pancreatic adenocarcinoma	control	JCRB Cell Bank
HT1197 (HT)	Urothelial carcinoma	control	DS Pharma Biomedical
RT4	Urothelial papilloma	control	DS Pharma Biomedical
MCF10A (MCF)	Breast epithelial cell	control	ATCC

ATCC: American Type Culture Collection (USA). NBRP: National Bio-Resource Project of the MEXT (Japan). JCRB Cell Bank: Japanese Collection of Research Bioresources Cell Bank (Japan). Dr. Kurebayashi at Kawasaki Medical School (Japan).

Table 2. *Sets of PCR and LAMP primers*

Primers	Sequence (5'-3')
PCR primers	
EML4-ALK (F)	GCATAAAGATGTCATCATCAACCAAG
EML4-ALK (R)	CGGAGCTTGCTCAGCTTGTA
SYT-SSX1 (F)	AGACCAACACAGCCTGGACCAC
SYT-SSX1 (R)	ACACTCCCTTCGAATCATTTTCG
IgH/BCL2 (F)	CAGATGGCAAATGACCAGCA
IgH/BCL2 (R)	CGGGAGGGTCTGTGTTGAAA
GAPDH (F)	GAGTCAACGGATTTGGTCGT
GAPDH (R)	AATGAAGGGGTCATTGATGG
LAMP primers	
EML4-ALK (FIP)	TCCATCTGCATGGCTTGCAG-AAAGATGTCATCATCAACCAA
EML4-ALK (BIP)	GCAGAGCCCTGAGTACAAGC-TTGGGGTTGTAGTCGGTC
EML4-ALK (F3)	TTACCAAAACTGCAGACAAG
EML4-ALK (B3)	TTGCCAGCAAAGCAGTAG
SYT-SSX1 (FIP)	TGGTGGTCCAGGCTGTGTTG-AACTACCCACAGGGACAAGG
SYT-SSX1 (BIP)	ACCAGATCATGCCCAAGAAGCC-CCAGATGCTTCTGACACTCC
SYT-SSX1 (F3)	GGTCCAGGTCCTCAGTATCC
SYT-SSX1 (B3)	TGCAGTTGTTTCCCATCGTT
IgH/BCL2 (FIP)	TGAATCTCATGGGTTTAACCA-CTGGGA ATTCCCGATT TAATTC
IgH/BCL2 (BIP)	AGATGGCAAATGACCAGCA-GGCCACGTAAAGCAACTCTCT
IgH/BCL2 (F3)	CTTGTTTCTTGA AGGTTTCCTCG
IgH/BCL2 (B3)	CGGGAGGGTCTGTGTTGAAA

were synthesized according to previous reports [1, 30]. Primers for *IgH/BCL2* fusion genes were designed using the NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Real-time PCR amplification was performed with a CFX96 C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following components: 400 nM of each primer (Table 2), 200 μM dNTPs, 0.0325 U/μL Go Taq DNA Polymerase (Promega), 1× SYBR Green I (Sigma, St. Louis, MO, USA), and 1.6 μL synthesized cDNA diluted 10^0–10^3-fold.

Real-time PCR cycling conditions were as follows: 95°C for 3 min; followed by 45 cycles of 95°C for 10 sec, annealing of primers for 20 sec at annealing temperatures of 60°C for *EML4-ALK* and *SYT-SSX* or 56.3°C for *IgH/BCL2*, and 72°C for 30 sec; followed by a melting curve from 60 to 95°C. Each measurement was performed with three replicates. Data analysis was carried out with CFX Manager software (Bio-Rad Laboratories).

LAMP assay

For LAMP primer design, we used PrimerExplorer V5 software (Eiken Chemical, Japan) and added slight modifications for LAMP reaction optimization. LAMP assays were carried out using 0.2 μM for each outer primer (F3 and B3), 1.6 μM for each inner primer (FIP and BIP; Table 2), 1.4 mM dNTP mixture (Nippon Gene, Tokyo, Japan),

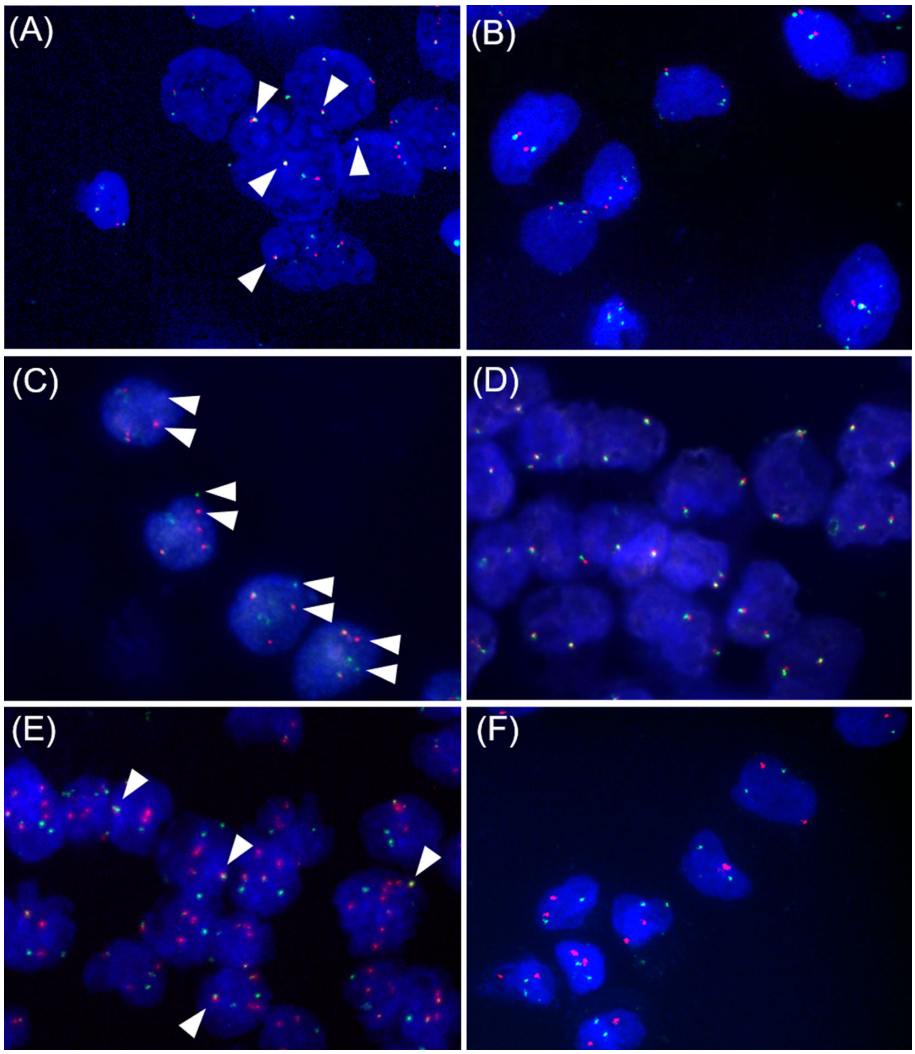

Fig. 2. Detection of gene rearrangements with FISH. **A**: The *EML4-ALK* fusion gene in H2228 cells. The arrows indicate the merged red signal (telomeric side of *ALK*) and green signal (telomeric side of *EML4*) as a yellow signal. **C**: *SYT-SSX* FISH using break-apart probes was positive in HS cells. Arrows indicating split orange and green signals showed the rearrangement of *SYT-SSX*. **E**: *IgH/BCL2* FISH-positive results in TK cells. Arrows indicate the merged red signal (*BCL2*) and green signal (*IgH*) as a yellow signal. **B, D**, and **F**: *EML4-ALK* FISH-negative (**B**), *SYT-SSX* FISH-negative (**D**) and *IgH/BCL2* FISH-negative (**F**) results in HuH cells. Original magnification: 600×.

8 U Bst DNA polymerase (Nippon Gene), and 1.6 µL synthesized cDNA diluted 10^0–10^3-fold. The total volume of each LAMP reaction was 20 µL. LAMP products were detected using a real-time PCR instrument (CFX96; Bio-Rad Laboratories), by agarose gel electrophoresis, or by the naked eye. When LAMP products were detected using real-time PCR equipment, 6.25 nM YO-PRO-1 (Thermo Fisher Scientific) was added to the mixture before the LAMP reaction. To detect LAMP products by agarose gel electrophoresis, LAMP products were separated by electrophoresis on 1.5% agarose gels and visualized using a Blue/Green LED illuminator (NIPPON Genetics, Tokyo, Japan) with Red Green Direct (NIPPON Genetics). For detection by the naked eye, we added a fluorescent detection reagent (Eiken Chemical) to the samples, and the LAMP products were then observed by the naked eye under UV light after reaction for 50 min. The reaction temperatures were 64.7°C for *SYT-SSX* and 63.2°C for *EML4-ALK* and *IgH/BCL2*, followed by incubation at 95°C for 5 min for deactivation of Bst DNA polymerase.

III. Results

Detection of fusion genes by FISH and real-time PCR

We confirmed the presence of the *EML4-ALK*, *SYT-SSX*, and *IgH/BCL2* fusion genes in various cell lines by FISH and real-time PCR (Figs. 2 and 3). The *EML4-ALK* rearrangement was detected in H2228 cells (Figs. 2A and 3A), the *SYT-SSX* fusion gene was found in HS cells (Figs. 2C and 3B), and the *IgH/BCL2* fusion gene was detected in TK cells by FISH and real-time PCR (Figs. 2E and 3C). No other cell lines contained these fusion genes. Each real-time

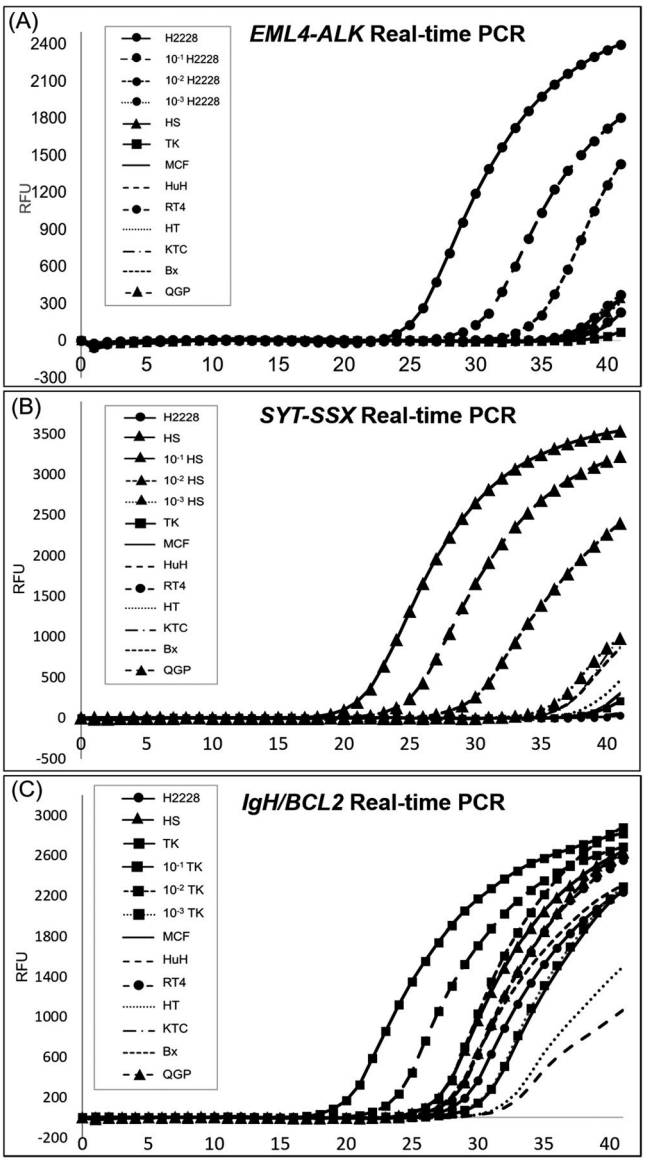

Fig. 3. Detection of fusion genes using real-time PCR. Amplification curves obtained from real-time PCR assays for *EML4-ALK* (**A**), *SYT-SSX* (**B**), and *IgH/BCL2* (**C**). H2228 cells (**A**), HS cells (**B**), and TK cells (**C**) were diluted from 10^0- to 10^3-fold.

PCR product was checked by melting curve analysis (data not shown).

Detection of fusion genes by LAMP

LAMP products were detected using real-time PCR equipment, agarose gel electrophoresis, and visualization by the naked eye (Fig. 4). LAMP products were produced in samples obtained from cell lines in which rearrangements were detected by real-time PCR and FISH; H2228 cells harbored *EML4-ALK*, HS cells harbored *SYT-SSX*, and TK cells harbored *IgH/BCL2*. Because LAMP assay yielded products of various sizes, the LAMP products appeared as a ladder-like pattern by gel electrophoresis. When a fluorescent detection reagent containing calcein and magnesium ions reacted with LAMP products containing a pyrophosphate ion, the product could be detected by the naked eye. LAMP products were not determined in other cell lines. For each LAMP assay, reaction temperatures ranging from 61°C to 65°C yielded similar results (data not shown). We selected a faster and more sensitive reaction temperature for detection of each fusion gene. These results from the LAMP assay were similar to the results from FISH and PCR assays.

Comparison of the sensitivity of fusion gene detection by real-time PCR and LAMP

Sensitivity assays for detecting the *EML4-ALK* rearrangement in cDNA from H2228 cells were performed using real-time PCR and LAMP. The detection limit by real-time PCR was 10^2-fold diluted cDNA (Fig. 3A), whereas that of LAMP detection was 10^3-fold diluted cDNA (Fig. 5A). Analysis of the *SYT-SSX* fusion gene showed that the detection sensitivity was similar to that of *EML4-ALK* (Figs. 3B and 5B). Notably, the *IgH/BCL2* fusion gene could be identified only in 10-fold diluted cDNA from TK cells by real-time PCR (Fig. 3C), but was detectable in 10^3-fold diluted cDNA by LAMP (Fig. 5C).

IV. Discussion

Genomic aberrations occur in many cancers [6, 26] and lead to changes in protein expression. Genomic aberrations and abnormal protein expression are useful for pathological diagnosis. Moreover, molecular targeted therapies that target abnormal proteins resulting from gene mutations in cancer cells have become popular in recent clinical strategies for the management of cancers. For example, among patients with colorectal tumors having mutated epidermal growth factor receptor (*EGFR*), some with mutated *KRAS* did not benefit from cetuximab, whereas others with wild-type *KRAS* did benefit from cetuximab [10]. Thus, detection of genomic aberrations may be useful for clinical treatment as well as pathological diagnosis.

LAMP is a method for amplifying DNA under isothermal conditions [20]. LAMP can also be applied to target RNA by adding reverse transcriptase in the reaction (RT-LAMP). For example, using AMV reverse transcriptase, LAMP can be carried out under the same conditions as for DNA amplification and has therefore been developed for use in the detection of various pathogens, including RNA viruses [18]. One of the clinical applications of RT-LAMP is the one-step nucleic acid amplification (OSNA) method for the detection of sentinel lymph node metastasis [28]. Based on the advantage of high sensitivity of OSNA assays, these assays have already been used in the clinical setting for the diagnosis of lymph node metastases in breast cancer, colorectal cancer, and gastric cancer in Japan [7, 12, 31].

Previously, we developed a novel methodology for rapid detection of *KRAS* mutations using artificial DNAs

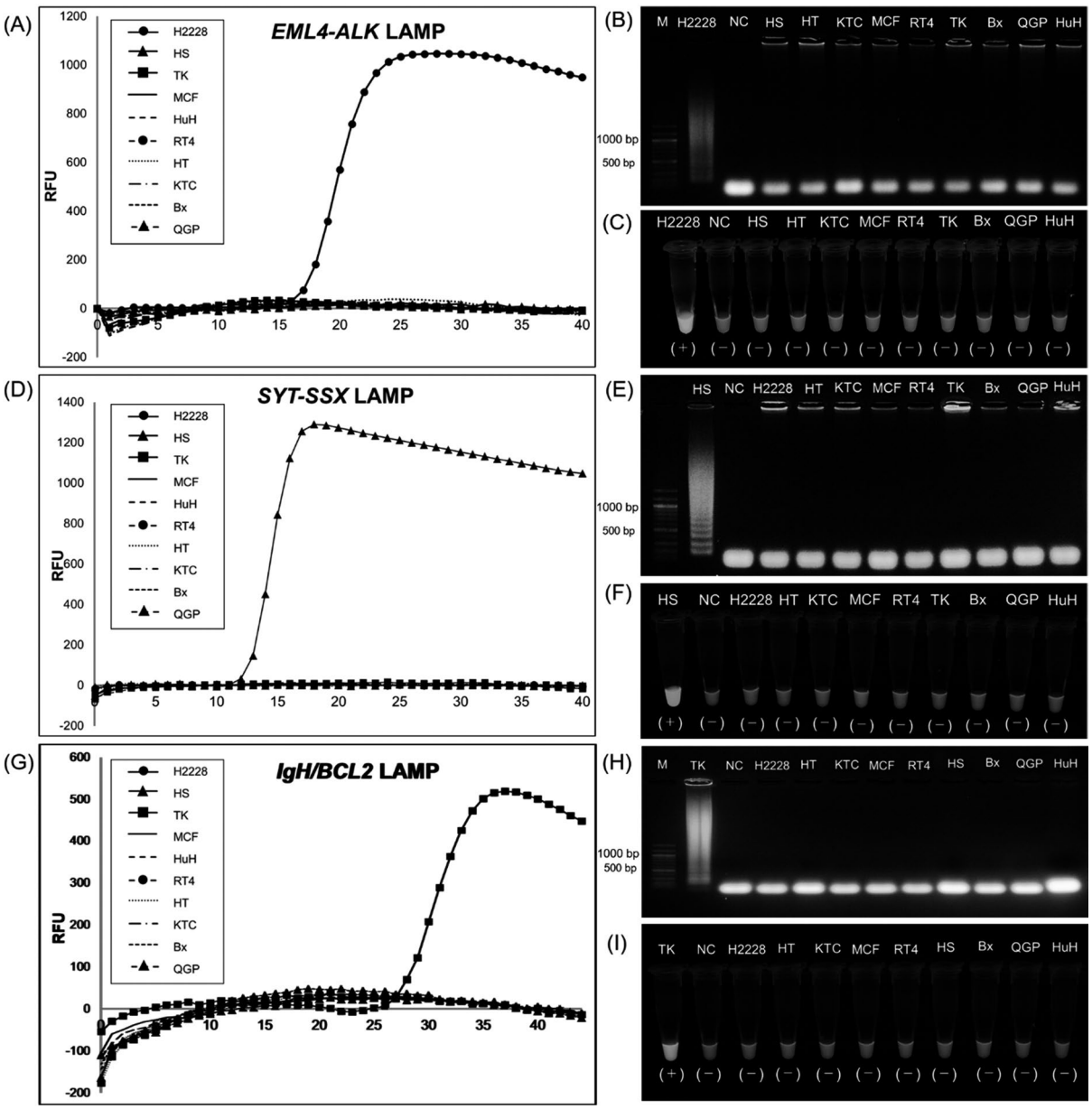

Fig. 4. Specificity of the LAMP assay. LAMP assays for *EML4-ALK* (**A–C**), *SYT-SSX* (**D–F**), and *IgH/BCL2* (**G–I**). LAMP products were detected using real-time PCR equipment (**A, D, G**), agarose gel electrophoresis (**B, E, H**), and the naked eye (**C, F, I**). M: size marker, NC: negative control without cDNA.

and the LAMP method [9]. In this novel method, we applied two types of artificial DNAs: PNAs and LNAs. The PNA-LNA-mediated LAMP method was able to detect *KRAS* mutations within a shorter time and with more accuracy than the other conventional methods, including direct sequencing assays and PNA-clamp PCR. Furthermore, the LAMP-based method did not require specialized equipment [9].

There are many genetic fusions in various human malignancies. Approximately 3–7% of patients with lung adenocarcinoma have the *EML4-ALK* fusion oncogene [8, 22, 23]. More than 95% of patients with synovial sarcoma have the translocation t(X;18)(p11;q11), which involves the *SYT* gene on chromosome 18q11 and almost always the *SSX1*, *SSX2*, or *SSX4* gene on chromosome Xp11. The translocation results in a chimeric transcriptional activator protein [15, 16, 25]. Eighty-five percent of follicular lymphomas and 20–30% of diffuse B-cell lymphomas contain the t(14;18) translocation. This rearrangement involves a translocation of the *BCL2* locus at chromosome segment 18q21 and the immunoglobulin heavy chain locus at 14q32, resulting in deregulated expression of Bcl-2 [2, 4, 5, 29]. These fusion genes are generally detected by PCR or FISH. However, the instruments required to perform these assays

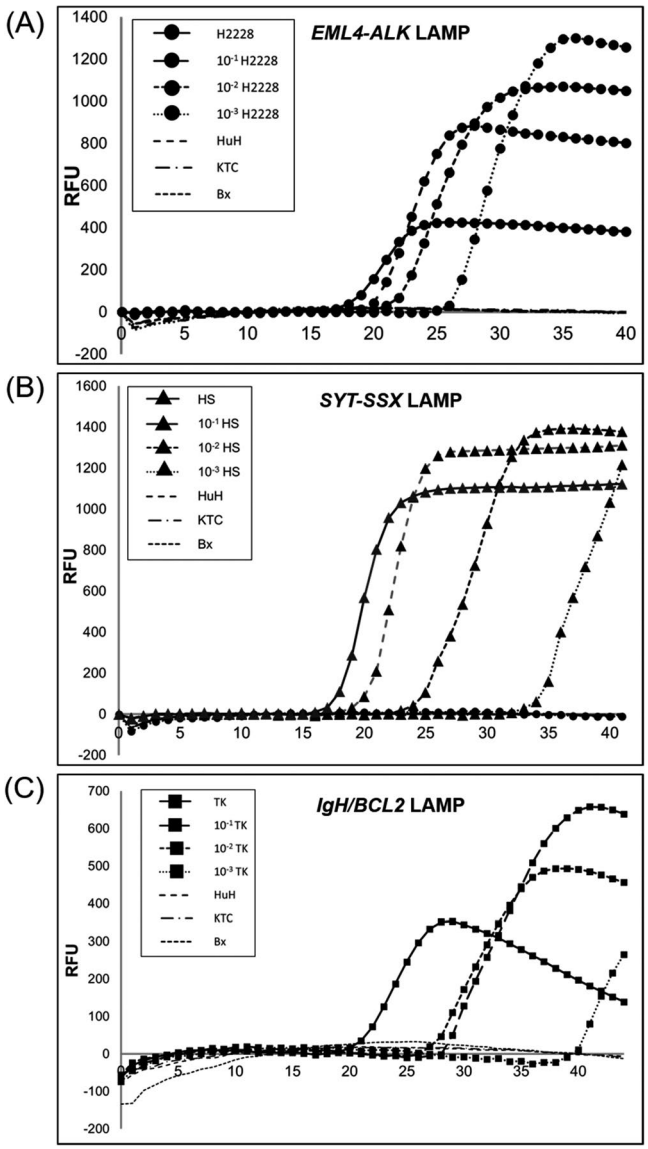

Fig. 5. Sensitivity of the LAMP assay. LAMP assays for *EML4-ALK* (**A**), *SYT-SSX* (**B**), and *IgH/BCL2* (**C**). H2228 cells (**A**), HS cells (**B**), and TK cells (**C**) were diluted from 10^0 to 10^3-fold.

are expensive. In contrast, LAMP assays can be carried out under isothermal conditions, and LAMP products can be observed with the naked eye under UV light. Therefore, LAMP assays do not require special instruments. In this study, we demonstrated a novel application of the LAMP method, i.e., highly sensitive detection of fusion genes, without the need for any additional materials or equipment, including artificial DNA. Therefore, this method is more cost-effective than both conventional PCR methods and FISH assays. Additionally, this method is highly sensitive, with a detection limit of almost 10^{-3}, and is quick, yielding results within 1 hr. Thus, this method is comparable to PNA/LNA-mediated LAMP.

Based on our previous report and current results, LAMP can detect not only gene expression and point mutations but also fusion genes in cancer. We expect that this highly specific, rapid, and cost-effective method will be applicable to clinical samples, such as biopsy, surgical tissue, and cytology samples, to be assessed by on-site analysis in hospitals.

Similar to our study, Spinelli *et al.* found that LAMP was a useful method for detecting gene translocations in acute promyelocytic leukemia [24]. Our study and the study by Spinelli *et al.* demonstrated that the LAMP method can be widely applied in clinical cytogenetic analysis of various tumors as well as hematopoietic disease. Furthermore, we will be able to detect fusion variants as *SYT-SSX1*, *SYT-SSX2*, and *SYT-SSX4* at the same time in different tubes using the same cDNA. We believe that we can further improve the diagnostic accuracy for pathological diagnosis and facilitate appropriate decision making for therapeutic purposes in the clinical setting.

In summary, we established a new application of LAMP assays for the detection of fusion genes. This method detected cDNA diluted to 10^3-fold and was more specific, rapid, and practical than previous methods, including real-time PCR. In our future studies, we will detect fusion genes from clinical samples using this method; subsequently, LAMP assays may be shown to be applicable for pathological diagnosis and on-site decision making regarding clinical therapies at hospitals.

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