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 approaches.

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.
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% CO₂.

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
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), and 1.2 μL of each cell line DNA diluted 10^{-3}-fold. LAMP assays were performed in a real-time PCR instrument (RapidLens, Eiken Chemical).
8 U Bst DNA polymerase (Nippon Gene), and 1.6 μL synthesized cDNA diluted 10⁰–10⁻⁴-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
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 10²-fold diluted cDNA (Fig. 3A), whereas that of LAMP detection was 10³-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 10³-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.
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

![Graph](image)

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.
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 cytotgenetic 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.

V. References

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