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Detection of *EGFR* Gene Mutation by Mutation-oriented LAMP Method

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Abstract. *Background/Aim: Epidermal growth factor receptor (EGFR) is a target of molecular therapeutics for non-small cell lung cancer. EGFR gene mutations at codons 746-753 promote constitutive EGFR activation and result in worst prognosis. However, these mutations augment the therapeutic effect of EGFR-tyrosine kinase inhibitor. Therefore, the detection of EGFR gene mutations is important for determining treatment planning. The aim of the study was to establish a method to detect EGFR gene mutations at codons 746-753. Materials and Methods: EGFR gene mutation at codons 746-753 in six cancer cell lines were investigated. A loop-mediated isothermal amplification (LAMP)-based procedure was developed, that employed peptide nucleic acid to suppress amplification of the wild-type allele. Results: This mutation-oriented LAMP can amplify the DNA fragment of the EGFR gene with codons 746-753 mutations within 30 min. Moreover, boiled cells can work as template resources. Conclusion: Mutation oriented-LAMP assay for EGFR gene mutation is sensitive on extracted DNA. This procedure would be capable of detecting EGFR gene mutation in sputum, pleural effusion, broncho-alveolar lavage fluid or trans-bronchial lung biopsy by chair side.*

Epidermal growth factor receptor (EGFR) belongs to a family of receptor tyrosine kinases. The binding of its ligand induces cell proliferation (1). Approximately 35% of patients with non-small cell lung carcinoma (NSCLC) in the east Asia have tumor-associated *EGFR* mutations (2). These mutations preferentially occur within EGFR exons 18-21, which encode a part of the EGFR kinase domain. Approximately 90% of these mutations are exon 19 deletions that remove residues 746-750 (p.E746-A750del) or exon 21 p.L858R point mutations (3). The p.E746-A750del, in the region encoding the kinase domain, is the most frequent mutation (4). These mutations increase the kinase activity of EGFR, leading to hyper-activation of downstream pro-survival signaling pathways (5). In contrast, the clinical correlation between the presence of mutations in exon 19 and therapeutic response to EGFR tyrosine kinase inhibitor (TKI) has been reported (2). Thus, detection of *EGFR* gene mutations in exon 19 has an important role for determining treatment plan.

Previously, we developed a rapid mutation detection system named the mutation-oriented (MO) loop-mediated isothermal amplification (LAMP) (6). The MO-LAMP can detect various types of mutations in the *KRAS* gene at codons 12 and 13, in a single tube, within 30 minutes. This would enable screening of genes for amino acid substitutions at specific codons in a way that saves significantly on cost and time and perhaps provides enough information to make more effective clinical decisions. To test this principle, we focused on *EGFR* mutations. The aim of this study was to establish a method to detect *EGFR* gene mutations at codons 746-753.

Materials and Methods

Sample preparation. In this study, we used purified genomic DNA from several cancer cell lines and subjected them to the experiment as template samples. Moreover, boiled cultured cell lines were examined to explore availability for template sample. These template samples were prepared from a lung adenocarcinoma cell line (HCC-827: c.2335-2249del15, p.E746-A750del), a pancreatic cancer cell line (NCI-H1650: c.2335-2249del15, p.E746-A750del), a malignant melanoma cell line (SK-MEL-28: c.2257C>T, p.P753S), a tongue squamous cell carcinoma cell line (HSC-3: wild type allele), a breast adenocarcinoma cell line (MDA-MB-231: wild type allele), and a colorectal adenocarcinoma cell line (HT-29: wild allele) (Table I). Information of *EGFR* gene mutations was obtained from COSMIC Cell Lines Projects (Sanger Institute Genome Research Limited, Hinxton, Cambridge, UK) (7). All cell lines

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Key Words: EGFR mutation, LAMP, molecular targeted therapy, stratification biomarker.

Table I. *Characteristics of investigated cell lines.*

Cell line	Histological typing	Organ	*EGFR* mutation
HCC-827	Adenocarcinoma	Lung	Heterogeneous, c.2235-2249del15, p.E746-A750del
NCI-H1650	Adenocarcinoma	Pancreas	Heterogeneous, c.2235-2249del15, p.E746-A750del
SK-MEL-28	Melanoma	Skin	Homogeneous, c.2257C>T, p.P753S
HSC-3	Squamous cell carcinoma	Tongue	None
MDA-MB-231	Adenocarcinoma	Breast	None
HT-29	Adenocarcinoma	Colon	None

Table II. *Primer sequences used in this study.*

Primer and probe	Sequence
F3	GCATCGCTGGTAACATCCAC (DNA)
B3	CCCCACACAGCAAAGCA (DNA)
FIP	ATGACAGAGAGAGAAGGAAGACGTTAACAGATCACTGGGCAGCATGT (DNA)
BIP	GATCCCAGAAGGTGAGAAAGTTAAAATTCCCGAGGATTTCCTTGTTGGCTT (DNA)
LF	CTGGCAATTGTGAGATGGTGC (DNA)
LB	CGCTATCAAGGAATTAAGAGAAGCA (DNA)
PNA-probe	GAATTAAGAGAAGCAACATCTC (PNA)

F3, B3: Forward and backward outer primers; FIP, BIP: forward and backward inner primers, LF, LB: forward- and backward-loop primer.

except for MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). These cell lines were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), and maintained under standard cell culture conditions at 37˚C and 5% CO_2 in a humid environment.

Genomic DNA was purified with DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Both the quality and quantity of DNA were assessed by NanoDrop-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cultured cells were washed and suspended in phosphate buffered saline (PBS, pH 6.0) and boiled for 1 min. The boiled cells were examined as crude sample.

Primer sets. A primer set for amplification of *EGFR* gene was designed for LAMP and MO-LAMP methods: forward and backward outer primers (F3, B3), forward and backward inner primers (FIP, BIP), forward and back-loop primer (LF, LB). These six primers recognize a region within *EGFR* encompassing codons 746-753. A PNA polymer with exact homology to the wild type allele was used as the PNA probe (Table II).

LAMP and MO-LAMP reactions. The MO-LAMP reaction was carried out using an Isothermal Master Mix (Nippon Gene Material Co., Ltd., Tokyo, Japan) in a 25 μl volume. The reaction mixture contained template sample, 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3 primers, 20 pmol each of LF and LB primers, and 15 pmol of PNA probe. The PNA probe was omitted from the MO-LAMP reaction. The reaction mixture was incubated at 65˚C. The time to fluorescence positive values (Tp) was monitored using a thermally controlled fluorimeter (Genie II; Optigene Limited, West Sussex, UK) and the Tp values of amplification were automatically recorded. Differences in Tp value between the MO-LAMP and LAMP reaction was presented as ΔTp.

Specificity and sensitivity of MO-LAMP reaction. The sensitivity of MO-LAMP to detect an EGFR mutation was assessed by serial dilutions of DNA or boiled cells. Furthermore, specificity of MO-LAMP reaction was assessed by mixing DNA from a mutant and a wild type allele of the *EGFR* gene. These data were confirmed by using cloned EGFR gene fragment in a plasmid, as template. The primer pair F3/B3 was used to amplify the DNA fragment including target sequences of EGFR, exon 19, codons 746–753. The polymerase chain reaction (PCR) products were amplified from the genomic DNA of HCC-827 and HSC-3 cell lines. PCR was carried out using KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan) for 40 cycles (95˚C, 10 sec; 62˚C, 30 sec; 68˚C, 20 sec). Each amplified fragment was treated with A-attachment mix (Toyobo CO., Ltd.), and then cloned into pGEM-T easy vector (Promega Co., Madison, WI, USA), followed by sequence confirmation by the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA), and the 3130 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, USA). The sequences of the PCR products were identical to the announced *EGFR* mutations by ATCC.

Statistical analysis. To examine the difference of ΔTp values, the Kruskal–Wallis H-test was performed. All experiments were performed five times. Differences were considered statistically significant at *p*-values of less than 0.05.

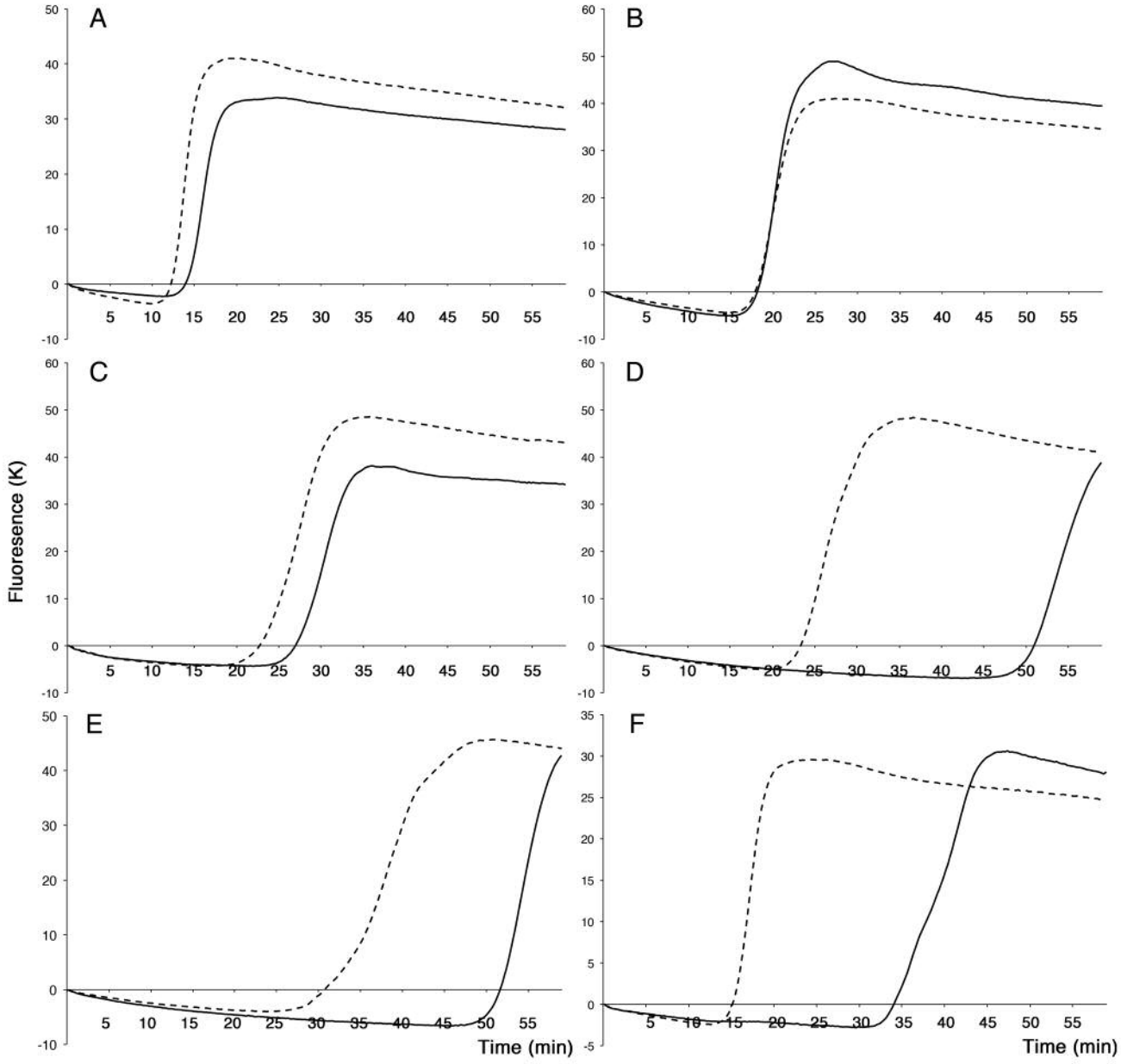

Figure 1. *Loop-mediated isothermal amplification (LAMP) and the mutation-oriented LAMP reaction in cancer cell lines. The mutation-oriented-LAMP reaction (solid line) and LAMP reaction (dashed line) were conducted to investigate EGFR gene mutation status in six cancer cell lines. (A) HCC-827 (p.E746-A750del), (B) NCI-H 1650 (p.E746-A750del), (C) SK-MEL-28 (p.P753S), (D) HSC-3 (wild type allele), (E) MDA-MB-231 (wild type allele), (F) HT-29 (wild type allele). MO-LAMP reaction inhibited amplification of EGFR gene fragment in in the group of wild type allele and resulted delay of Tp value.*

Results

LAMP and the MO-LAMP reaction. We compared conventional LAMP with the MO-LAMP reaction for amplification of the wild and mutant allele of *EGFR* gene. In the LAMP reaction, all examined samples exhibited a positive reaction for *EGFR* gene. Amplification of the gene was observed within 30 min (range=14 min 53 sec–26 min 33 sec). The ΔTp value reflects the suppression level of MO-LAMP reaction. There was no difference in the ΔTp value among the mutated alleles. In contrast, the wild type allele exhibited delayed gene amplification in MO-LAMP and the

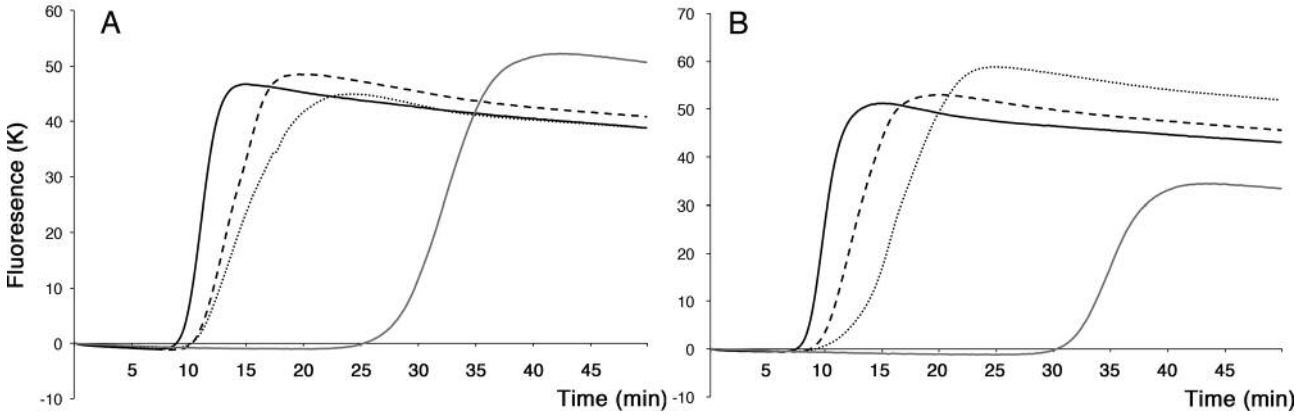

Figure 2. *Detection limit for EGF gene mutation in the LAMP and MO-LAMP reactions. The limit of detection of EGFR gene mutation in the two assays was examined. (A, B) Genomic DNA template from HCC-827 cells that are EGFR-mutant. (A) LAMP analysis showed amplification in 0.01 to 10 ng of genomic DNA from HCC 827 cells. (B) Although MO-LAMP analysis showed amplification in 0.01 to 10 ng of genomic DNA, the reaction in 0.01 ng of genomic DNA sample was inhibited (10 ng, solid line; 1 ng, dashed line; 0.1 ng, dotted line; 0.01 ng, grey line).*

Table III. *Differences between LAMP and MO-LAMP reaction.*

	Mutant allele			Wild type allele		
	HCC-827	NCI-H 1650	SK-MEL-28	HSC-3	MBA-MB-231	HT-29
LAMP	14'53"	21'03"	26'33"	27'03"	39'33"	18'23"
MO-LAMP	17'08"	21'18"	29'33"	54'03"	55'03"	37'23"
ΔTp	2'15"	0'15"	3'00"	27'00"	15'30"	19'00"
p-value	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01

ΔTp: Time to positive value (Tp) of mutation-oriented LAMP minus the Tp value of LAMP.

ΔTp value was significantly increased (Figure 1). The ΔTp was significantly lower in the mutated allele of *EGFR* gene (HCC-827: 15 sec, NCI-H1650: 2 min 15 sec, SK-MEL-28: 3 min) compared with wild allele (HSC-3: 27 min, MDA-MB-231: 15 min 30 sec, HT-29: 19 min) (Table III).

Sensitivity of MO-LAMP reaction. Both LAMP and MO-LAMP analysis showed amplification of a gene mutation when more than 0.01 ng of template gDNA of HCC-827 cell was used; however, the ΔTp value was significantly increased at the gDNA concentrations of 0.01 ng (Figure 2A and B). We therefore infer that a concentration of more than 0.1 ng of template gDNA, which is compatible with approximately 10.5 cells of HCC 827 cells (average ploidy=2.84) (7).

Specificity of MO-LAMP reaction. The specificity of MO-LAMP for an *EGFR* gene mutation was assessed by mixing DNA from mutant allele (HCC-827 cell) and wild-type allele (HSC-3 cell). A total of 10 ng DNA mixture was subjected to LAMP and MO-LAMP assays. The ΔTp value of the 100 and 10% concentration of mutant DNA samples were 0 sec and 13sec, respectively (Figure 3A, Table IV). Meanwhile, ΔTp value of the 1% concentration of mutant DNA sample

Table IV. *Specificity of MO-LAMP reaction.*

	% mutant DNA			
	100%	10%	1%	0%
LAMP	9'15"	10'38"	10'27"	13'31"
MO-LAMP	9'15"	10'51"	17'53"	42'15"
ΔTp	0'00"	0'13"	7'26"	28'44"
p-value	>0.05	>0.05	<0.01	<0.01

ΔTp: Time to positive value (Tp) of mutation-oriented LAMP minus the Tp value of LAMP.

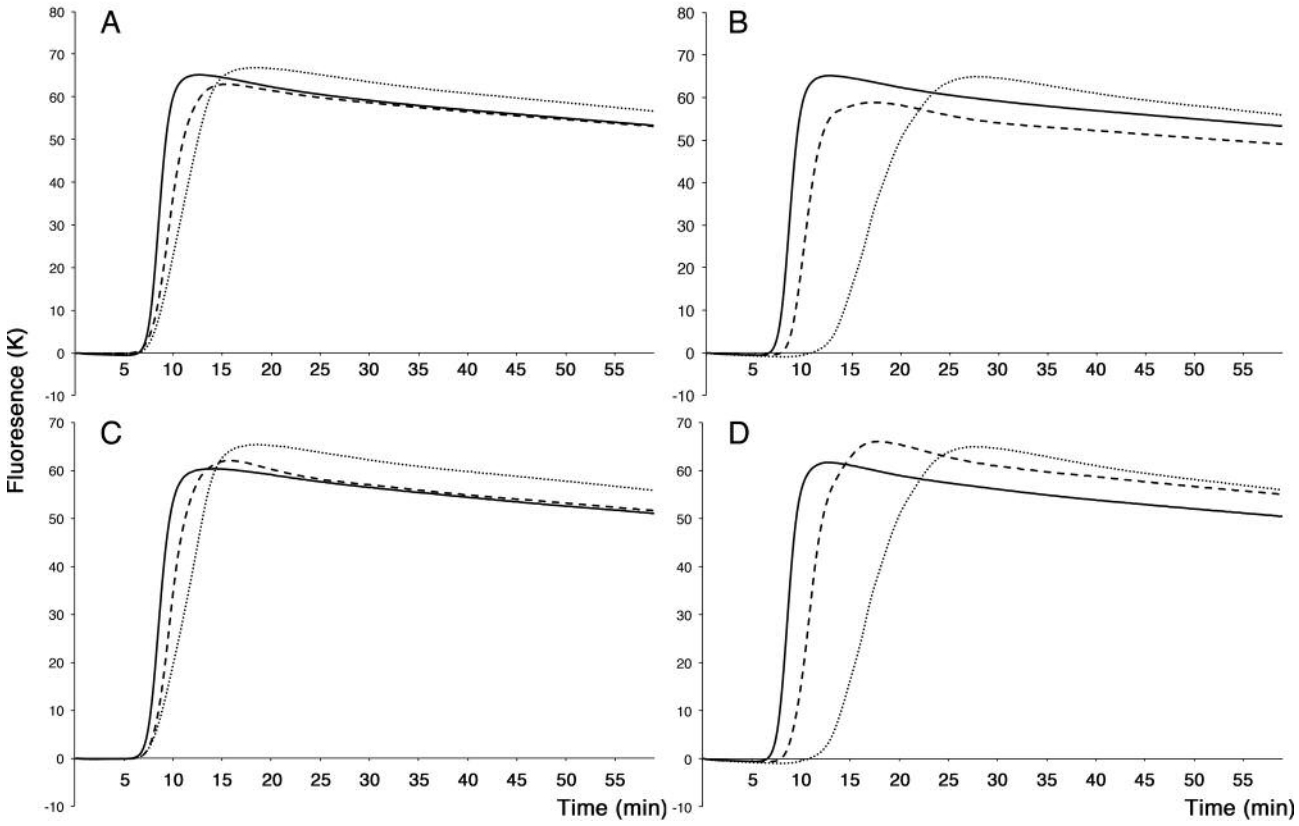

Figure 3. *Specificity of MO-LAMP reaction. Specificity of MO-LAMP reactions were investigated using template DNA with mixing mutant and wild type alleles. (A) A total of 10 ng genomic DNA mixture was subjected to LAMP and (B) MO-LAMP assays. There were statistically not different between 10-100% of mutant DNA samples. Similarly, a total of 10 ng plasmid DNA, containing EGFR gene fragment was subjected to (C) LAMP and (D) MO-LAMP assays. There were statistically no differences between 10 to 100% of mutant DNA samples, but not in 1% of mutant DNA samples (100%, solid line; 10%, dashed line; 1%, dotted line).*

exhibited 7 min 26 sec, which was significantly increased. These data indicate the specificity of MO-LAMP for the *EGFR* gene suspected to be less than 10% of mutant DNA concentration. These data were confirmed by recombinant plasmid DNA carrying an *EGFR* gene fragment. Ten ng of plasmid DNA, which contain positive allele of *EGFR* gene was mixed with mock plasmid DNA. The results indicated that the ΔTp value of the 100 and 10% concentration of mutant DNA samples were 0.5 sec and 27sec, respectively (Figure 3B).

Detection of gene mutation using crude samples. Moreover, we tried to detect *EGFR* mutations by investigating boiled cells as a crude sample. The ΔTp values of a mutant allele (HCC-827) was significantly lower than that of a wild-type allele (MDA-MB-231). We examined the detection limit of a number of cells, and amplification was observed in 100 and more cells (Figure 4).

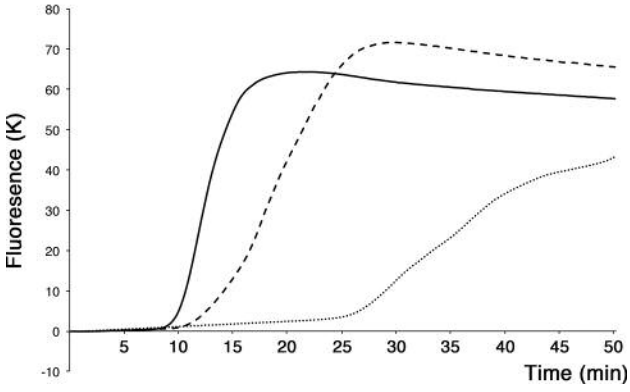

Figure 4. *Detection of gene mutation using boiled cells. Boiled cells were examined by MO-LAMP. Mutant EGFR gene was detected by MO-LAMP analysis when 100 or more boiled HCC-827 cells were used in the reaction mixture (500 cells, solid line; 100 cells, dashed line; 10 cells, dotted line).*

Discussion

Predictive markers are parameters for predicting treatment outcome, and may refer to response or survival (8). To date, the clinicopathological staging system has been the standard for determining prognosis, although its value for the individual patient is questionable (9). Recently, EGFR TKIs has become standard treatment for the patients with NSCLC. EGFR mutation at exon 19, codons 746-750, is a predictive marker of EGFR TKIs (2, 8). Thus, *EGFR* mutation assessment has become the standard approach in NSCLC management to establish treatment plan. There are many techniques available but there is no strong consensus on optimum approach. The aim of our study was to develop a rapid and specific detection method for *EGFR* gene mutations at exon19, codons 746-753, on the basis of MO-LAMP method. Previously, our group developed the MO-LAMP method, which amplifies DNA fragments of mutant alleles of the *KRAS* gene (6). This procedure suppressed amplification of wild type allele by PNA probe, one artificial nucleic acid, which binds to the full sequence of DNA and prevents amplification of a gene fragment. In this study, the MO-LAMP can amplify the DNA fragment of the *EGFR* gene within codons 746-753 within 30 min. Moreover, crude samples from cultured cells can work as template resources. The limit of detection was over 0.1 ng of DNA or 100 boiled cells. The sensitivity of the method was 10%.

The p.E746-A750del is the most frequent *EGFR* mutation and constitutes approximately 48% of all mutations (4). EGFR TKIs were developed as an inhibitor of EGFR tyrosine kinase that is often over-expressed in many cancers. The drug was used in combination with other traditional chemotherapies in patients with NSCLC. Subsequently, an *EGFR* mutation in exon 19 was seen in a subset of NSCLC with a good response to EGFR TKIs (10). Thus, the MO-LAMP assay for EGFR mutations may avail to decide on a course of NSCLC treatment.

Different alleles of the *EGFR* gene mutations at codons 460-453 have been reported, including deletion, insertion, and compound mutations (11, 12). Over the past decade, various methods have been proposed for the detection of *EGFR* gene mutations (13). However, most other methods use mutation specific primers. Because of the need for mutation specific primers, these methods would overlook unknown types of mutations. Thus, comprehensive detection of mutations in *EGFR* exon 19 is not possible. These results are a disadvantage for cancer treatments. In this study, the MO-LAMP assays for EGFR exon 19 mutations in codons 746-753, overcome this drawback, which uses mutation-specific primers to detect small variable insertions or deletions. MO-LAMP approach can be more widely applied for detection of cancer mutations where codon changes result in amino acid substitutions, insertion, and deletion.

The benefit of MO-LAMP assay is the use of crude sample prepared simply by boiled cells in PBS. Mutant *EGFR* alleles were detected by MO-LAMP analysis when 100 or more boiled cells were used in the reaction mixture. In contrast, most other methods require purified DNA. DNA isolation and purification are archived by several steps, including overnight digestion in proteinase K. Thus, simple preparation of MO-LAMP samples would enable to reduce sample contamination and mix-up. In addition, MO-LAMP assay is more sensitive than direct sequencing, with the ability to detect mutations in samples containing <10% mutant alleles (14). These results prompt that the MO-LAMP assay is capable of detecting the *EGFR* gene mutations in sputum, pleural effusion, broncho-alveolar lavage fluid or trans-bronchial lung biopsy by chair side. The test uses a small aliquot of the samples isolated to establish the diagnosis of cancer, and therefore, patients do not need to undergo additional or excess sampling. These may minimize patient discomfort.

Regarding the sensitivity of this assay, there are some differences between DNA samples and boiled cells. These differences may be due to an unknown inhibitor in boiled cell samples.

A limitation of the current study is that although the MO-LAMP method successfully identified several types of mutations at codons 746-753, the method has not been able to differentiate the DNA sequence. In addition, the detectable region was limited at the specific region. Although, the authors did not explore the all reported mutations at codons 746-753, on the basis of principle, the MO-LAMP would be able to detect any type of mutations at the region. To apply the clinical examination, the threshold of ΔTp in MO-LAMP should be investigated. MO-LAMP suppressed amplification of wild type allele samples, and ΔTp value was increased. However, we could not completely suppress the amplification in wild type allele samples. The reason is thought to be that the *EGFR* mutations are usually heterozygous (15). Indeed, the cultured cells used in this study have heterozygosity in the *EGFR* gene (Table I).

A large-scale screening requires a rapid and sensitive technique. In the present study, we established a MO-LAMP assay for the detection of mutations occurring in the *EGFR* codons 746-753. The MO-LAMP makes it possible to screen for all possible mutations in one tube, without post-amplicon processing. The MO-LAMP assay was thought to be clinically useful as a diagnostic tool.

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