Melting Curve Analysis for Mutations of *EGFR* and *KRAS*

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Abstract. *Epidermal growth factor receptor (EGFR) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations are common in non-small cell lung cancer (NSCLC) and colorectal cancer (CRC). The aim of the present study was to develop a simple and versatile tool to determine EGFR and KRAS mutations for pre-clinical research in the laboratory. We developed a melting curve analysis to detect exon 19 deletion, L858R mutation, and T790M mutation of EGFR, and codon 12/13 and codon 61 mutations of KRAS using LightCycler480 with mutation-specific sensor and anchor probes. The analytical method was applicable to determine the approximate rate of heterogeneity of mutation in the genomic DNA of cancer cell lines. In conclusion, our melting curve analysis is a rapid and semi-quantitative method to screen for exon 19 deletion, L858R or T790M mutations of EGFR and codon 12/13/61 mutations of KRAS in cancer cell lines.*

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 80% of lung cancers. About 50% of patients with NSCLC in Japan harbor somatic mutations of the epidermal growth factor receptor (*EGFR*) gene (1) which is a member of the erythroblastic leukemia viral oncogene homolog (ERBB) receptor tyrosine kinase family. Activation of EGFR results in cell proliferation and inhibition of apoptosis by promoting the downstream signaling pathways that involve rat sarcoma viral oncogene homolog (RAS)/extracellular signal-regulated kinase (ERK)1/2 and phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (AKT) (2). The most common *EGFR* mutations, observed in about 90% of cases and termed 'activating mutations' (3), are in-frame E746-A750 deletion in exon 19 and single-point substitutional L858R mutation in exon 21, which consist of eight and two different types of mutations in Japanese patients with NSCLC, respectively (Table I) (4). EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, block phosphorylation of EGFR, with subsequent inhibition of the downstream signaling pathways. Remarkable response rates to EGFR-TKIs are associated with the occurrence of *EGFR*-activating mutations (3), however, almost all patients will eventually develop acquired resistance to EGFR-TKIs within several years (5), and a second mutation of T790M in exon 20 of *EGFR* is observed in about 50% of these resistant cases (6).

v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations are found in 33% of NSCLC and are mainly restricted to the following specific sites: common codons 12/13 in exon 2 and rare codon 61 in exon 3 (7, 8). They consist of five and two different types of mutations in patients with NSCLC in Japan and Hong Kong, respectively (Table I) (9, 10). *KRAS* is a member of the RAS gene family that encodes small G proteins with intrinsic GTPase activity and plays a key role in RAS/ERK signal transduction (7). *KRAS* mutations lead to impaired GTPase activity, which results in the constitutive activation of RAS/ERK signaling pathway, and tend to be associated with lack of responsiveness to EGFR-TKIs (11, 12).

For known mutations in a target gene, real-time detection by polymerase chain reaction (PCR) followed by melting curve analysis is a convenient method in the laboratory because it is easy, less costly, highly sensitive and rapid in detecting mutations (13, 14). The melting curve method utilizes fluorescent sensor and anchor probes complementary to the target amplicon that can distinguish genetic alterations of target by measuring the differences in the melting temperature (Tm) needed to dissociate a probe from the target. When a mutation is present in the target, the probe-target duplex is separated at a lower temperature than a completely matching wild-type sequence. The differences in Tm are detected by a loss of fluorescence as a function of increasing temperature. Although the melting curve

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methodology for deletion in exon 19, single-point substitution in exon 21 of *EGFR* and codon 12/13 mutation in exon 2 of *KRAS* has been previously described, each method uses different PCR conditions (15, 16). In addition, no melting curve method for T790M in exon 20 of *EGFR* and codon 61 of *KRAS* has been developed until now. In the present study, we developed and validated optimum melting curve methods to detect these types of *EGFR* and *KRAS* mutations using LightCycler480 under the same PCR conditions, allowing us to investigate concurrently any type of mutation status of *EGFR* and *KRAS* in one run in the laboratory.

Materials and Methods

Samples. As listed in Table I, DNA fragments containing wild-type or mutated *EGFR* and *KRAS* were synthesized in pMA plasmid vector (Life Technologies Co., Ltd., Carlsbad, CA, USA). The sequences were referenced from the University of California Santa Cruz (UCSC) Genome Browser database. Human cancer cell lines HCC827, NCI-H292, NCI-H1975, NCI-H1355, NCI-H2122, NCI-H460, AsPC-1, CFPAC-1, RPMI8226, Calu-6, HCT116 and SW948 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); PC-9 was purchased from Immuno-Biological Laboratories (IBL, Gunma, Japan). Human genomic DNA (Promega, Madison, WI, USA) and NCI-H292 cell line contain no mutations of *EGFR* and *KRAS*. The *EGFR* and *KRAS* mutation status of cell lines other than NCI-H292 were referenced from the COSMIC database (17) (Table I). The genomic DNA was extracted from cells using DNeasy Blood&Tissue kit (Qiagen, Valencia, CA, USA).

PCR. The forward and reverse primers used to amplify each amplicon of *EGFR* and *KRAS* mutations are shown in Table II. PCR reactions were performed in a final volume of 20 μl that contained 0.5 μM of each forward and reverse primer, LightCycler 480 Genotyping Master (Roche Diagnostics, Ltd., Basel, Switzerland), 10 ng of genomic DNA or 0.5 pg of plasmid DNA, and were thermally cycled in a LightCycler 480 (Roche Diagnostics) as follows: 95˚C for 10 s, followed by 45 cycles of 95˚C for 10 s, 60˚C for 10 s, and 72˚C for 10 s.

Melting curve analysis. Fluorescent sensor and anchor probes are shown in Table II. Melting curve analyses were performed in a final volume of 24 μl containing 20 μl of PCR products and 0.33 μM of sensor and anchor probes. Reactions were performed in a LightCycler 480 with one cycle of 95˚C for 1 min, 40˚C for 2 min, and ramped up to 95˚C at 0.11˚C/second. The melting curve was analyzed by LightCycler 480 software. Representative melting curve plots of three independent experiments are shown.

Direct sequencing analysis. PCR primers and sequencing primers are listed in Table II. PCR products were sequenced directly using the BigDye terminator v3.1 cycle sequencing kit (Life Technologies Co., Ltd.) with ABI PRISM 3100 genetic analyzer (Life Technologies Co., Ltd.) according to the manufacturer's instructions.

Mouse xenograft models. Male 5-week-old BALB *nu/nu* mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). Animals were housed in a pathogen-free environment under controlled conditions (temperature: 20-26˚C, humidity: 40-70%, light-dark cycle: 12-12 h). Chlorinated water and irradiated food were provided *ad libitum*. The animals were allowed to acclimatize and recover from shipping-related stress for more than seven days prior to the study. The health of mice was monitored daily. All animal protocols were reviewed by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd. and all animal experiments were performed in accordance with the 'Guidelines for the Accommodation and Care of Laboratory Animals' of Chugai Pharmaceutical Co., Ltd. A cell suspension of HCC827 cells was subcutaneously inoculated into the right flank of each mouse. When the tumor grew to about 500 mm^3, genomic DNA was extracted from tumor samples using DNeasy Blood&Tissue kit (Qiagen).

Results

Detection of exon 19 deletion of EGFR by melting curve analysis. Detection of E746-A750 deletion type I in exon 19 of *EGFR* was tested by templates that mixed E746-A750 deletion type-I pMA plasmid with wild-type pMA plasmid at ratios of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% and 5%. The position of the melting peaks was different between deletion and wild-type, and the mutation peak of 5% deletion in the mixture with wild-type was clearly detected (Figure 1A). The melting peaks of the other types of deletion plasmids (E746-A750 type II, L747-T751, L747-P753 S ins, E746-T751 A ins, E746-S752 V ins, L747-A750 P ins, L747-K754 ST ins, L747-P753) were also detected (Figure 1B). By direct sequence analysis, PC-9 and HCC827 cells were seen to have E746-A750 deletion type-I and type-II of *EGFR*, respectively (Figure 1C). By melting curve analysis of genomic DNA of PC-9 and HCC827 cells, the melting peaks of deletions were detected and found to be different from those of NCI-H292 cells and human genomic DNA (Figure 1D).

Detection of L858R and T790M mutations of EGFR by melting curve analysis. Detection of L858R and T790M mutations were also investigated in the way described above. The position of the melting peaks was different between L858R/wild-type and T790M/wild-type, and each mutation peak of 5% deletion in the mixture with wild-type was clearly detected (Figure 2A). By direct sequence analysis, NCI-H1975 cells were found to have double mutations of L858R and T790M (Figure 2B). By melting curve analysis of genomic DNA of NCI-H1975 cells, the melting peaks of mutations were detected and were found different from those of NCI-H292 cells and human genomic DNA (Figure 2C).

Detection of codon 12/13 and codon 61 mutations of KRAS by melting curve analysis. Detection of codon 12/13 (G12C) and codon 61 (Q61H) mutations were also investigated in the same way as described above. The position of the melting peaks was different between G12C/wild-type and Q61H/wild-type, and each mutation peak of 10% deletion in the mixture with wild-type was clearly detected (Figure 3A).

Table I. *List of mutations of epidermal growth factor receptor (EGFR) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) in non-small cell lung cancer (NSCLC). Frequency of mutations in exon 19 deletion and L858R/L861Q mutations of EGFR, exon 2 and 3 of KRAS in NSCLC was determined by Yokoyama et al. (4), Tam et al. (9), Araki et al. (10). Amino acid change, nucleotide change, synthesized pMA plasmid DNA of each subtype of mutation, and cell lines containing each mutation are shown. del, Deletion; ins, insertion.*

Gene	Exon	Frequency (%)	Amino acid change	Nucleotide change	Cell line with mutation
EGFR	19	41	E746-A750 del type II	del(2236_2250)	HCC827
		34	E746-A750 del type I	del(2235_2249)	PC-9
		7	L747-T751 del	del(2239_2253)	-
		7	L747-P753 del S ins	del(2240_2257)	-
		5	E746-T751 del A ins	del(2237_2251)	-
		2	L747-A750 del P ins	del(2239_2247), G2248C	-
		2	E746-S752 del V ins	del(2237_2254), C2255T	-
		2	L747-K754 del ST ins	del(2240_2257), A2261C	-
		0	L747-P753 del	del(2239_2259)	-
	21	98	L858R	T2573G	NCI-H1975
		2	L861Q	T2582A	-
	20	No data	T790M	C2369T	NCI-H1975
KRAS	2	23	G12C	G34T	NCI-H2122
		45	G12D	G35A	AsPC-1
		19	G12V	G35T	CFPAC-1
		10	G12A	G35C	RPMI8226
		3	G12F	GG34_35TT	-
		0	G13C	G37T	NCI-H1355
		0	G13D	G38A	HCT116
	3	50	Q61H	C183T	NCI-H460
		50	Q61L	A182T	SW948
		0	Q61K	C181A	Calu-6

Table II. *List of polymerase chain reaction (PCR) primers and fluorescent probes in melting curve analysis. Anchor probes were 3' labeled with fluorescein, and sensor probes were 5' labeled with LC-Red 640 and 3' phosphorylated. *Sequencing primers.*

Gene	Exon	PCR primer sequence	Probe sequence
EGFR	19	Forward*: 5'-catctcacaattgccagttaacgtcttcc-3'	Sensor: 5'-caacaaggaaatcctcgatgtgagtttctg-3'
		Reverse: 5'-gatgtggagatgagcagggtctagag-3'	Anchor: 5'-tcccgtcgctatcaagacatctccgaaag-3'
	21	Forward: 5'-aaacaccgcagcatgtcaa-3'	Sensor: 5'-gggtgcggaagagaaagaatacc-3'
		Reverse*: 5'-ggctgacctaaagccacctc-3'	Anchor: 5'-ggctggccaaactgc-3'
	20	Forward*: 5'-tccaggaagcctacgtgatg-3'	Sensor: 5'-ccttcggctgcctcctggac-3'
		Reverse: 5'-gtctttgtgttcccggacat-3'	Anchor: 5'-cagctcatcacgcagctcatg-3'
KRAS	2	Forward: 5'-aggcctgctgaaaatgactg-3'	Sensor: 5'-cctacgccaccagctcca-3'
		Reverse*: 5'-cctctattgttggatcatattc-3'	Anchor: 5'-ttctgaattagctgtatcgtcaaggcactctt-3'
	3	Forward*: 5'-ccttctcaggattcctacagga-3'	Sensor: 5'-gtcgagaatatccaagagacaggtttctc-3'
		Reverse: 5'-gcatggcattagcaaagactc-3'	Anchor: 5'-tactcctcttgacctgctg-3'

The melting peaks of the other types of mutation plasmids (G12D, G12V, G12A, G12F, G13C, G13D and Q61L, Q61K) were also detected (Figure 3B). By direct sequence analysis, NCI-H2122, AsPC-1, CFPAC-1, RPMI8226, NCI-H1355, HCT116, NCI-H460, SW948 and Calu-6 were found to have G12C, G12D, G12V, G12A, G13C, G13D, Q61H, Q61L and Q61K mutations, respectively (Figure 4). By melting curve analysis of genomic DNA of CFPAC-1, RPMI8226, NCI-H1355, HCT116, SW948 and Calu-6 cells, each melting peak for the different mutations was detected and was found different from those of NCI-H292 cells and human genomic DNA (Figure 3C).

Detection of heterogeneity of EGFR or KRAS mutation by melting curve analysis. The applicable amount of template genomic DNA in melting curve analysis was determined as 0.01 ng to 10 ng of human genome DNA. The melting curves for exon 19 deletion of *EGFR* and codon 12/13 mutation of *KRAS*

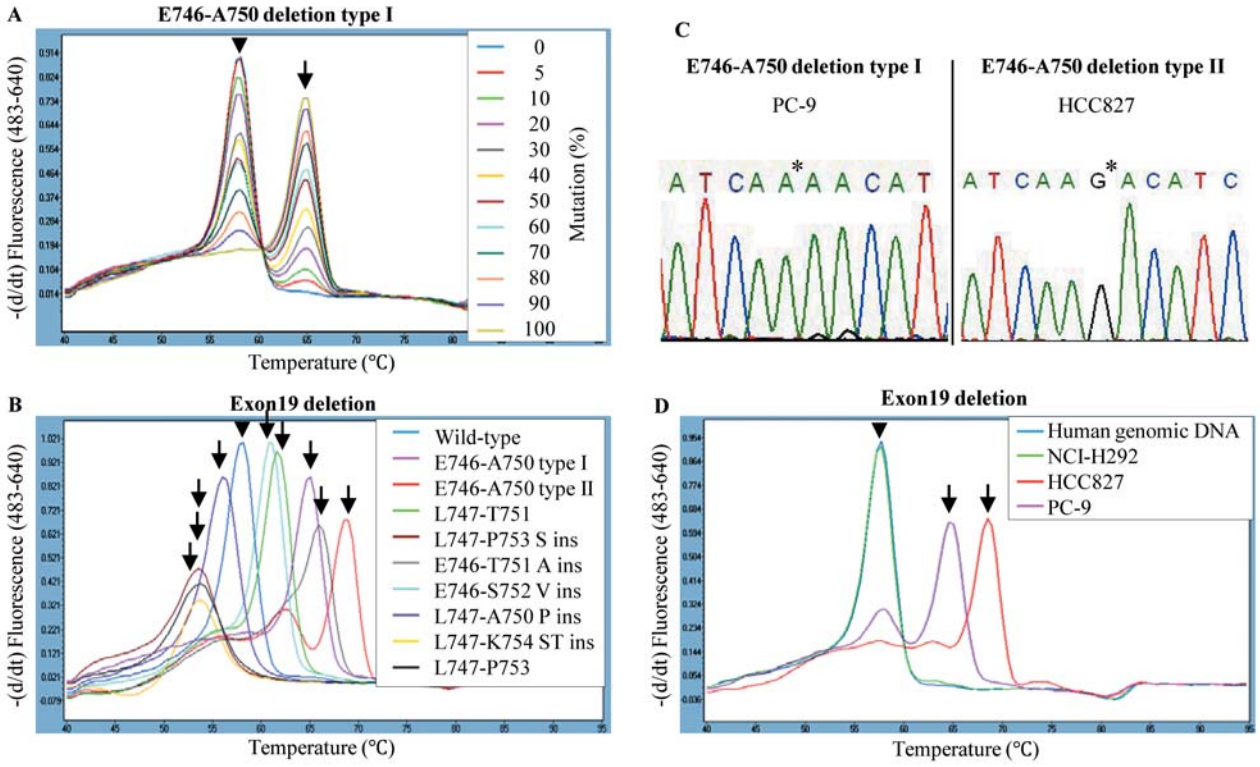

Figure 1. *Melting curve analysis for exon 19 deletions of epidermal growth factor receptor (EGFR). A: Mixtures of E746-A750 deletion type I pMA plasmid with wild-type pMA plasmid at different ratios were used as polymerase chain reaction (PCR) templates. B: pMA plasmids containing different types of exon 19 deletion were compared with that of wild-type. C: Direct sequencing analysis of PC-9 and HCC827 cells. *Position of deletion. D: Genomic DNAs of PC-9 and HCC827 cells were used as PCR templates. Arrowheads and arrows indicate the peaks of wild-type and deletions, respectively.*

from 1 ng and 10 ng of human genome DNA almost matched, but some non-specific peaks were found in the curves of 0.1 ng to 0.01 ng of the template DNA (Figure 5A). In addition, some non-specific peaks were also found in the curves with 0.1 ng of the template DNA in the melting curve of L858R/T790M mutation of *EGFR* and codon 61 mutation of *KRAS* (data not shown). By direct sequence analysis, NCI-H1975 and CFPAC-1 cells have heterozygous mutation of L858R and G12V because there were two peaks of mutation and wild-type, respectively (Figure 2B and 3C). We investigated the ratio of heterogeneity of mutation in these cells by melting curve anaysis using pMA plasmids containig a known ratio. NCI-H1975 and CFPAC-1 cells showed similar melting curves to 70% of L858R pMA and 60% of G12V pMA plasmids, respectively (Figure 5B). In addition, all melting curves of five xenografted tumor samples of HCC827 had almost the same melting peaks as those of *in vitro* cultured HCC827 cells (Figure 6).

Discussion

Currently, various methods can be used to assess *EGFR* and *KRAS* mutation status in clinical tumor samples, including direct sequencing, PCR-Invader, PNA-LNA-PCR clamp, Cycleave PCR, mutant-enriched PCR and Scorpion-ARMS analysis (18-20). In particular, direct sequencing analysis has been used as the standard method because it can essentially detect all base substitutions, insertions and deletions with or without known mutations. However, on the downside, it is more time-consuming and has limited sensitivity for detectable mutations (20%) (21). Methods other than direct sequencing are less time-consuming and have much higher sensitivity (1%), but also have disadvantages of high cost, inability to detect unknown mutations, and require special apparatuses (20).

In contrast, although a methodology of melting curve analysis has been established for *EGFR* and *KRAS* mutation in clinical NSCLC, it has not been used clinically because of its only moderate sensitivity (10%). The tumor content was only about 5% in some lung tissue samples and the formalin-fixed, paraffin-embedded specimens available for analysis are often small in size in many NSCLC cases (15, 16, 19). In contrast, the sensitivity of melting analysis (10%) may be sufficient to detect mutations for pre-clinical research because commercially available cancer cell lines consist of a single population. Our melting curve method in this study detected over 5% E746-A750 del type I/L858R of *EGFR* or 10% T790M of *EGFR* and G12C/Q61H of *KRAS* and was validated with genomic DNA

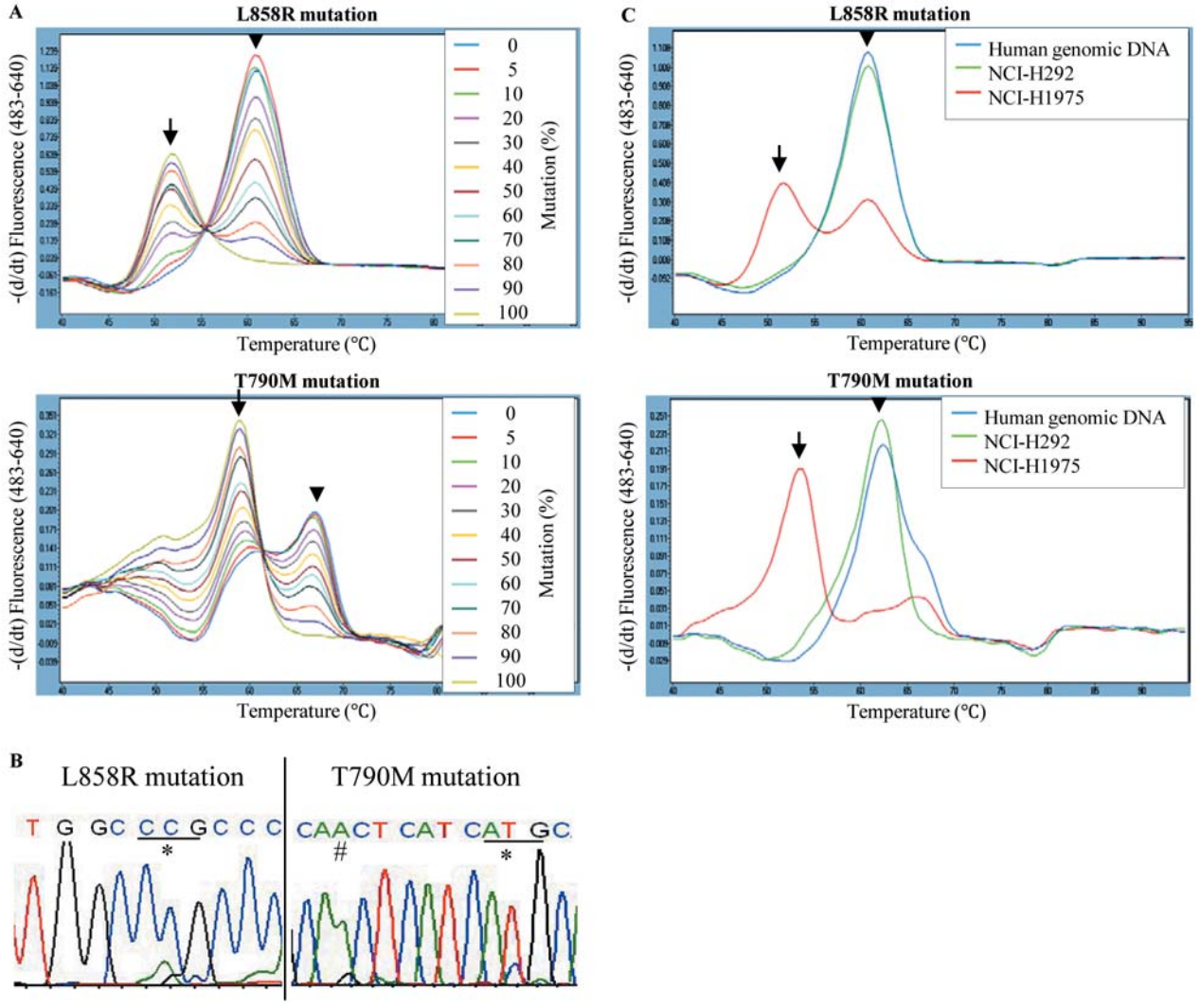

Figure 2. *Melting curve analysis for L858R and T790M mutations of epidermal growth factor receptor (EGFR). A: Mixtures of L858R or T790M pMA plasmids with wild-type pMA plasmid at different ratios were used as polymerase chain reaction (PCR) templates. B: Direct sequencing analysis of NCI-H1975 cells. *Position of heterozygous mutation; codon involving substitution is underlined (L858R, CAG>CCG; T790M, ACG>ATG); #position of synonymous substitution (Q787Q, CAA>CAG). C: Genomic DNA of NCI-H1975 cells was used as a PCR template. Arrowheads and arrows indicate the peaks of wild-type and mutations, respectively.*

not only from *in vitro* cells but also from xenografted tumors. These findings indicate that our melting curve analysis would be useful not only for *in vitro* but also for *in vivo* pre-clinical samples, if not applicable to clinical samples. In order to use our method for detecting mutations in clinical samples for diagnosis, breakthrough in the sensitivity of detection is needed, by designing sensor/anchor probes containing most suitable sequence/length and high-fluorescence intensity to discriminate fractional genetic alterations, or by developing a more sensitive machine to detect smaller changes of fluorescence intensity in the melting reaction.

All PCR products amplified from plasmid DNA and genomic DNA had a single band in agarose gel electrophoresis, indicating that only target regions containing the mutation were specifically amplified (data not shown). Moreover, an advantage of melting curve analysis is that the PCR amplification and analysis are performed in one run without the need for any post-PCR sample manipulation, and the results are available for analysis at the end of the run. The running time for assay and analysis of 96 samples with our method is about 1.5 h. Thus, the faster PCR method and elimination of additional steps in analyzing PCR products

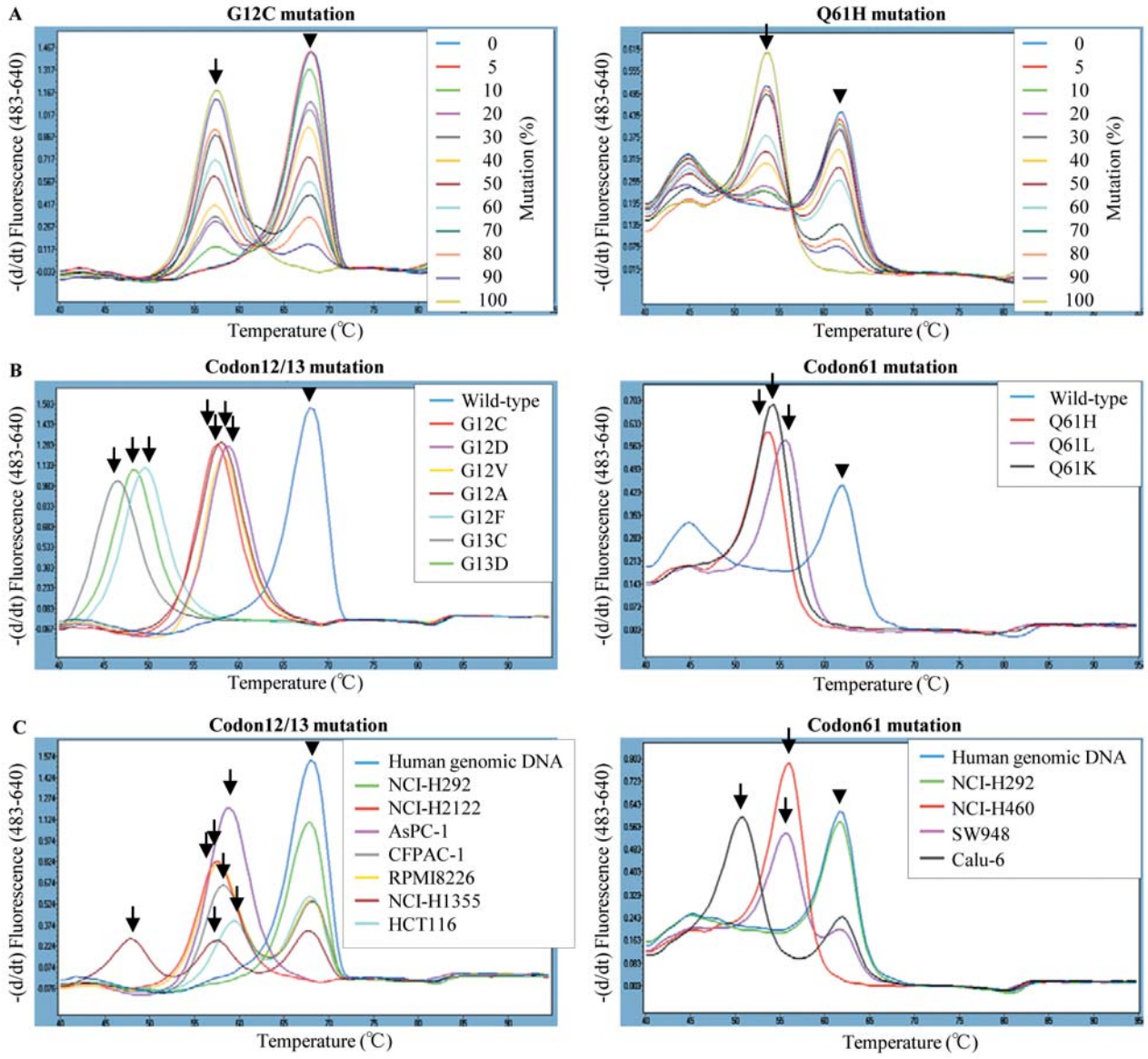

Figure 3. *Melting curve analysis for codon 12/13 and codon 61 mutations of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). A: Mixtures of G12C or Q61H pMA plasmid with wild-type pMA plasmids at different ratios were used as polymerase chain reaction (PCR) templates. B: pMA plasmids containing various types of mutations were used as PCR templates. C: Genomic DNA from cancer cells were used as PCR templates. Arrowheads and arrows indicate the peaks of wild-type and mutations, respectively.*

save time and money, and minimize the risks of DNA contamination. Handling is facilitated because toxic reagents, such as ethidium bromide, are avoided.

Compared to other solid tumors, NSCLC is well-known for cell population heterogeneity in individual lesions (22), and intra-tumoral heterogeneous distribution of *EGFR* mutation has also been reported (23-25). Chmielecki *et al.* demonstrated that sensitivity to EGFR-TKI, erlotinib, is reduced when T790M-containing cells made up >25% of the population (26). *KRAS* mutations are found in up to 40% of colorectal cancer, and 68% of these mutations are localized at codon 12 and the remaining 32% are localized at codon 13 (27, 28). Treatment with antibody to EGFR, such as cetuximab and panitumumab, has been established in patients with wild-type *KRAS* (29, 30). Intra-tumoral heterogeneity of *KRAS* mutation is detected in 35% of patients with colorectal cancer with *KRAS* mutation (31). It is reported that the presence of very low numbers of *KRAS*-mutated cells provides an explanation, at least in part, for the therapeutic failure of therapies utilizing EGFR antibody,

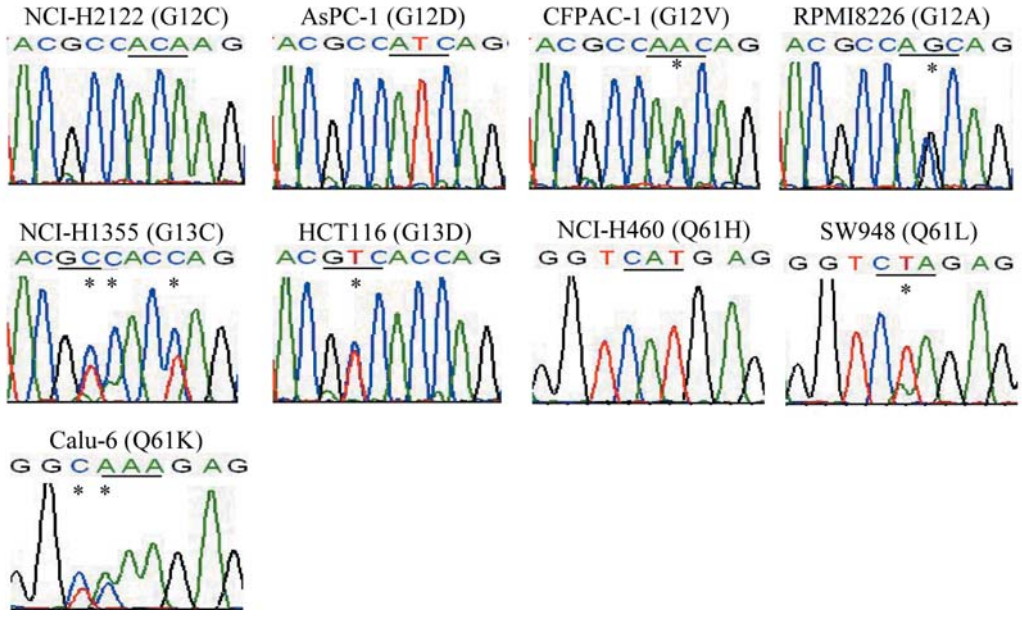

Figure 4. *Direct sequencing analysis for codon 12/13 and codon 61 mutations of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). Direct sequencing analysis of cancer cell lines. * Position of heterozygous mutation; codon involving substitution is underlined (G12C, D, V, A and F; ACC>ACA, ATC, AAC and AGC. G13C and D; GCC>GCA and GTC).*

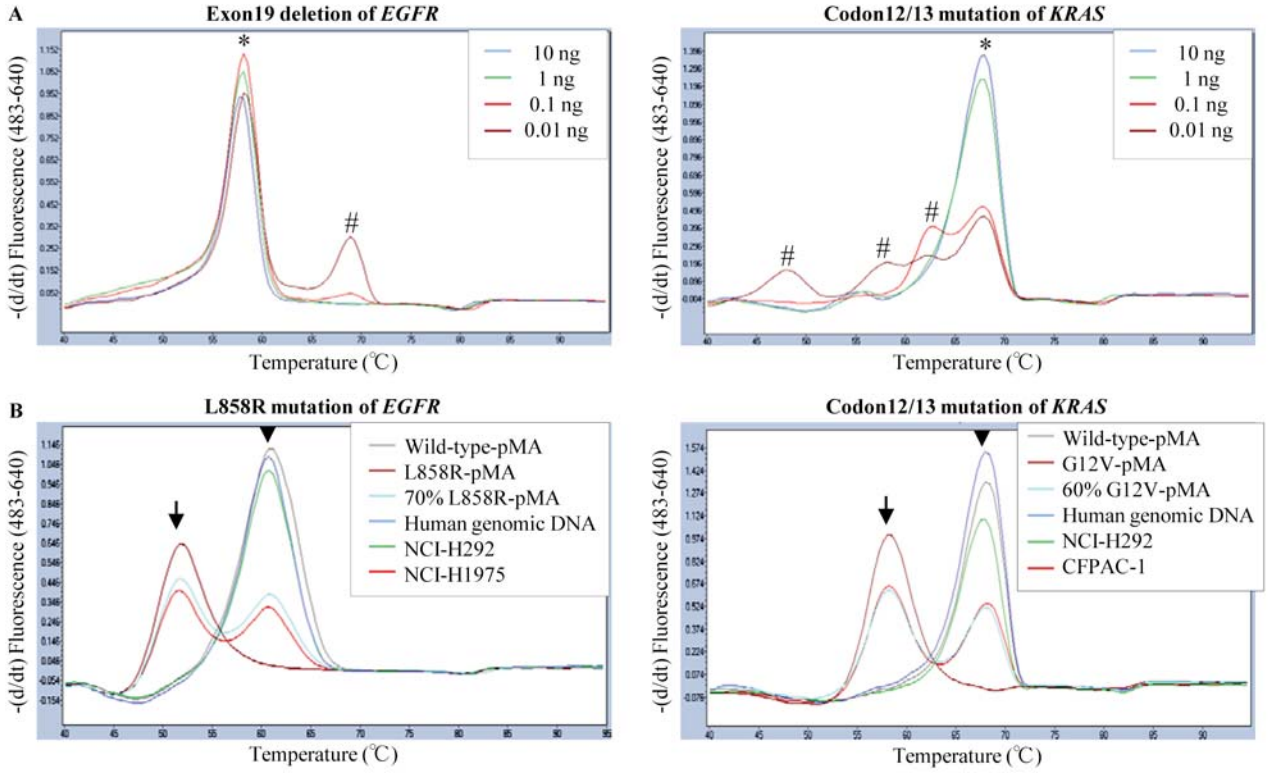

Figure 5. *Melting curve analysis for heterogeneity of epidermal growth factor receptor (EGFR) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation. A: Amounts of human genomic DNA ranging from 0.01 to 10 ng were used as PCR templates. *Normal peak and #non-specific peak positions. B: Melting peaks of NCI-H1975 and CFPAC-1 genomic DNAs were compared to those of plasmid DNA containing mixtures of L858R or G12V pMA plasmids with wild-type pMA plasmid at a ratio of 70% and 60%, respectively. Arrowheads and arrows indicate the peaks of wild-type and mutations, respectively.*

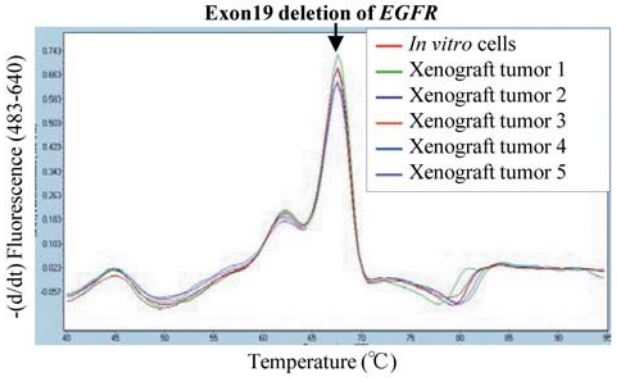

Figure 6. *Melting curve analysis for exon 19 deletions of epidermal growth factor receptor (EGFR) using HCC827 xenograft tumors. Melting peaks of HCC827 genomic DNAs of in vitro cells were compared to those of five in vivo xenograft tumors. Arrow indicates the peaks of mutation.*

which has been found to occur in 40%-60% of cases classified as *KRAS* wild-type by direct sequencing analysis. These findings indicate that such genetic heterogeneity of *EGFR* or *KRAS* mutation could contribute to acquired resistance to EGFR-targeted treatment. Our melting curve analysis could be a useful method not only to identify the presence of *EGFR* or *KRAS* mutation but also to determine the approximate ratio of heterogeneity of mutation in unknown preclinical samples.

In conclusion, our melting curve analysis is an easy, useful and semi-quantitative tool for pre-clinical screening for exon 19 deletion, L858R or T790M mutations of *EGFR*, for codon 12/13/61 mutations of *KRAS*, and for heterogeneity of mutation, and is applicable to cancer cell lines. In the future, the measurement of the heterogeneity of *EGFR* or *KRAS* mutation by melting curve analysis may be a useful method in predicting response to EGFR-targeted therapy.

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