Detection of *EGFR* Mutations in Archived Cytologic Specimens of Non–Small Cell Lung Cancer Using High-Resolution Melting Analysis

Kiyoaki Nomoto, CT,[1] Koji Tsuta, MD,[1] Toshimi Takano, MD,[2] Tomoya Fukui, MD,[2] Karin Yokozawa,[3] Hiromi Sakamoto, MD,[4] Teruhiko Yoshida, MD,[4] Akiko Miyagi Maeshima, MD,[5] Tatsuhiro Shibata, MD,[5] Koh Furuta, MD,[3] Yuichiro Ohe, MD,[2] and Yoshihiro Matsuno, MD[1]

Key Words: Epidermal growth factor receptor; DNA mutation analysis; High-resolution melting analysis; Non–small cell lung cancer

DOI: 10.1309/N5PQNGW2QKMX09X7

Abstract

*Mutations of the epidermal growth factor receptor (*EGFR*), particularly deletional mutations (DEL) in exon 19 and L858R in exon 21, are reportedly correlated with clinical outcome in patients with non–small cell lung cancer (NSCLC) receiving the EGFR tyrosine kinase inhibitors gefitinib and erlotinib, suggesting that detection of* EGFR *mutations would have an important role in clinical decision making. We established and validated an easy, inexpensive, and rapid method for detecting DEL and L858R from cytologic material by high-resolution melting analysis (HRMA). Dilution for sensitivity studies revealed that DEL and L858R were detectable in the presence of at least 10% and 0.1%* EGFR*-mutant cells, respectively. We analyzed 37 archived cytological slides of specimens from 29 patients with advanced NSCLC and compared the results with direct sequencing data obtained previously. Of 37 samples, 34 (92%) yielded consistent results with direct sequencing, 2 were false negative, and 1 was indeterminate. The sensitivity of this analysis was 90% (19/21) and specificity, 100% (15/15). These results suggest that HRMA of archived cytologic specimens of advanced NSCLC is useful for detecting* EGFR *mutations in clinical practice.*

Increased expression of epidermal growth factor receptor (EGFR) has been reported in carcinomas of various organs, including of the lung, and has been shown to have a crucial role in tumor progression.[1,2] Gefitinib (Iressa, AstraZeneca, Osaka, Japan) is an orally active, selective EGFR tyrosine kinase inhibitor that binds to the adenosine triphosphate binding pocket of the kinase domain and blocks downstream signaling pathways. Clinical phase 2 studies have demonstrated gefitinib antitumor activity in patients with advanced non–small cell lung cancer (NSCLC).[3,4] Although some of these studies have shown that the rate of response to gefitinib is higher in women, patients with adenocarcinoma, patients who have never smoked, and Japanese and East Asians,[3-5] no predictive molecular marker had been discovered until April 2004, when somatic mutations in the kinase domain of *EGFR* were suggested to be correlated with gefitinib sensitivity.[6,7] Thereafter, several studies revealed a strong association between *EGFR* mutations and clinical outcome in parameters such as response rate, time to progression, and overall survival in consecutive NSCLC patients treated with gefitinib.[8-10]

Many types of *EGFR* mutation have been identified.[6-16] They are concentrated in exons 18 to 21 of *EGFR*, close to the region encoding the adenosine triphosphate binding pocket, and about 90% of patients with *EGFR* mutations have mutations in 2 hotspots: in-frame deletions including amino acids at codons 747 to 749 (DEL) in exon 19 and a missense mutation at codon 858 (L858R) in exon 21.

The mutational status of *EGFR*, especially DEL and L858R, is a strong predictor of gefitinib sensitivity, and detection of such mutations would provide patients and physicians with important information for optimal choice of therapy. Therefore, analysis of a sufficient number of tumor samples in

good condition and direct sequencing after laser capture microdissection (LCM) is considered the "gold standard" for detecting *EGFR* mutations. However, this approach is not necessarily practical for clinical use for a number of reasons. First, tumor samples with a large volume and in good condition are difficult to obtain in most cases of advanced NSCLC. Second, LCM and direct sequencing require special instruments and are time-consuming and costly. Therefore, it is necessary to establish practical and precise methods for detecting *EGFR* mutations from easily obtainable diagnostic samples, which usually contain a small number of tumor cells and a large number of normal cells.

The real-time reverse transcription–polymerase chain reaction (PCR) assay has been reported for detection of *EGFR* mutations.[17] In this method, many samples can be genotyped within a few hours without the need for post-PCR sample manipulation, although expensive fluorescence-labeled probes and restriction enzymes are needed. A new inexpensive dye, SYBR Green I, has been developed,[18] but this limits the melting resolution because of dye redistribution during melting.

Recently, studies have validated the usefulness of high-resolution melting analysis (HRMA) using LCGreen I dye for mutational analysis,[19-22] and another study has validated analysis using cytologic samples for c-kit.[23] The advantages of this approach are that labeling of either primer with dye is not needed and PCR amplification and melting analysis can be performed in the same capillary tube, minimizing sample handling and reducing the possibility of error and sample contamination. HRMA is easy, rapid, and inexpensive to perform and has considerable potential for mutation detection in clinical practice.

We report a new method for detecting DEL and L858R from archival Papanicolaou-stained cytologic slides by HRMA. We validated the method by comparing the results with direct sequencing data from specimens surgically resected from the same patients. We also performed a titration assay to evaluate the lower limit of the proportion of tumor cells for detection of *EGFR* mutations by using a mixture of wild-type (WT) and *EGFR*-mutant lung cancer cell lines.

Materials and Methods

Cell Lines and Titration Assay

We performed dilution for sensitivity studies using 3 lung adenocarcinoma cell lines, NCI-H1650, NCI-H1975, and NCI-A549, obtained from the American Tissue Cell Collection (Manassas, VA). The H1650 cell line contains a DEL mutation (delE746-A750), the H1975 cell line contains the L858R mutation,[24] and the A549 cell line contains WT *EGFR*.[25] *EGFR* copy numbers in the H1650, H1975, and A549 cells are reported to be 2, 3, and 2.48 per cell, respectively.[25,26] Dilutions of the *EGFR*-mutant cells (H1650 or H1975) with A549 cells were prepared using proportions of *EGFR*-mutant cells of 100% (no A549 cells), 10%, 1%, 0.1%, and 0% (no mutant cells). DNA extracted from each dilution was subjected to subsequent PCR assay.

DNA Extraction From Archived Cytologic Slides

With approval of the National Cancer Center Institutional Review Board, Tokyo, Japan, we performed *EGFR* gene analysis. Among the 66 cases analyzed in a previous study, diagnostic Papanicolaou-stained cytologic samples were available for 29. Of the patients, 5 had multiple (2 to 4) metachronous samples, and the total number of available cytologic samples was 37. Two clinical cytologists (K.N. and K.T.), who were unaware of the patients' characteristics and mutational status, examined these 37 samples. Cytologic parameters described for each slide included sampling procedure, approximate number of nucleated cells on each slide (<100, 100-499, 500-999, ≥1,000), and proportion of tumor cells among total nucleated cells (<10%, 10%-49%, 50%-89%, ≥90%). After this assessment, DNA was extracted from the cells on the slides using a QIAamp DNA Micro Kit (catalog No. 56304, QIAGEN, Valencia, CA) as follows: Coverslips were removed by immersion in xylene for 72 hours, and the slides were rinsed in 95% ethanol 3 times. Cells on the slides were removed by using sterilized disposable knives and suspended in ATL buffer containing Proteinase K in 1.5-mL tubes. Further procedures were performed according to the manufacturer's protocol.

In 2 samples with a small proportion of tumor cells, tumor cell–rich parts on the slides were marked with a diamond pen and selectively retrieved manually with a knife to enrich the proportion of tumor cells.

Polymerase Chain Reaction

Primer A was designed to amplify a region containing nucleotides 2235 to 2277 (amino acids E746 to I759) of *EGFR*, in which almost all reported deletional mutations in exon 19 occur.[6-16] The sequences of primer A were AAAATTCCCGTCGCTATC (forward) and AAGCAGAAACTCACATCG (reverse). Primer B was designed to amplify a region containing nucleotides 2573 and 2582, at which point mutations L858R and L861Q in exon 21 occur, respectively. L858R and L861Q account for about 96% and 2%, respectively, of all reported point mutations in exon 21.[6-16] The sequences of primer B were AGATCACAGATTTTGGGC (forward) and ATTCTTTCTCTTCCGCAC (reverse).

PCR was performed using these primers, Fast Start *Taq* Polymerase (Roche Diagnostics, Indianapolis, IN), and LCGreen I Gene Scanning Reagents (Idaho Technology, Salt Lake City, UT) on a LightCycler (Roche Diagnostics). The samples were denatured at 95°C for 10 minutes and then subjected

DOI: 10.1309/N5PQNGW2QKMX09X7

to 37 cycles of denaturing for 10 seconds at 95°C, annealing for 10 seconds at 60°C, and extension for 5 seconds at 72°C with primer A and 45 cycles of denaturing for 10 seconds at 95°C, annealing for 5 seconds at 56°C, and extension for 5 seconds at 72°C with primer B.

High-Resolution Melting Analysis

The PCR products were denatured at 95°C for 5 minutes and cooled to 40°C in the LightCycler to form heteroduplexes. The LightCycler capillary was transferred to an HR-1 (Idaho Technology), an HRMA instrument, and heated at a transition rate of 0.3°C per second. Data were acquired and analyzed using the accompanying software (Idaho Technology). After normalization and temperature-adjustment steps, melting curve shapes from 78.5°C to 85.5°C were compared between samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as a control sample with wild-type *EGFR*.

Direct Sequencing

In a previous study, we performed direct sequencing of *EGFR* in 66 cases of NSCLC that relapsed after primary surgery. Methanol-fixed, paraffin-embedded surgical specimens of primary NSCLC were obtained, and DNA was extracted from laser capture microdissected tumor tissue. Nested PCR was performed to amplify exons 18 to 24 of *EGFR* using primers described previously.[8] Direct sequencing of the PCR products was performed using the ABI PRISM 3700 and 3100 DNA sequencers (Applied Biosystems, Foster City, CA).

Results

In the melting analysis using primer A (exon 19), 100% H1650 cells (EGFR DEL) gave a skewed curve from 100% A549 cells (EGFR WT). Mixtures of both cells gave gradual curves, and DEL could be detected in the presence of 10% but not 1% H1650 cells **Figure 1A**. In the analysis using primer B (exon 21), 100% H1975 cells (EGFR L858R) gave a left-shifted curve from 100% A549 cells. Mixtures of both cells gave gradual curves, and L858R could be detected in the presence of 0.1% H1975 cells **Figure 1B**.

We analyzed 37 archival cytologic samples from 29 patients by HRMA, and the results are summarized in **Table 1**, in comparison with the results obtained by direct sequencing from surgically resected specimens of each patient. Eleven samples were obtained by bronchial brushing or washing, 4 by transbronchial fine-needle aspiration (FNA), 4 by percutaneous FNA of lung tumors, 2 by FNA of superficial lymph nodes, 14 from pleural effusion, and 2 from pericardial effusion. The median time between sampling and analysis was 3 years (range, 1-8 years).

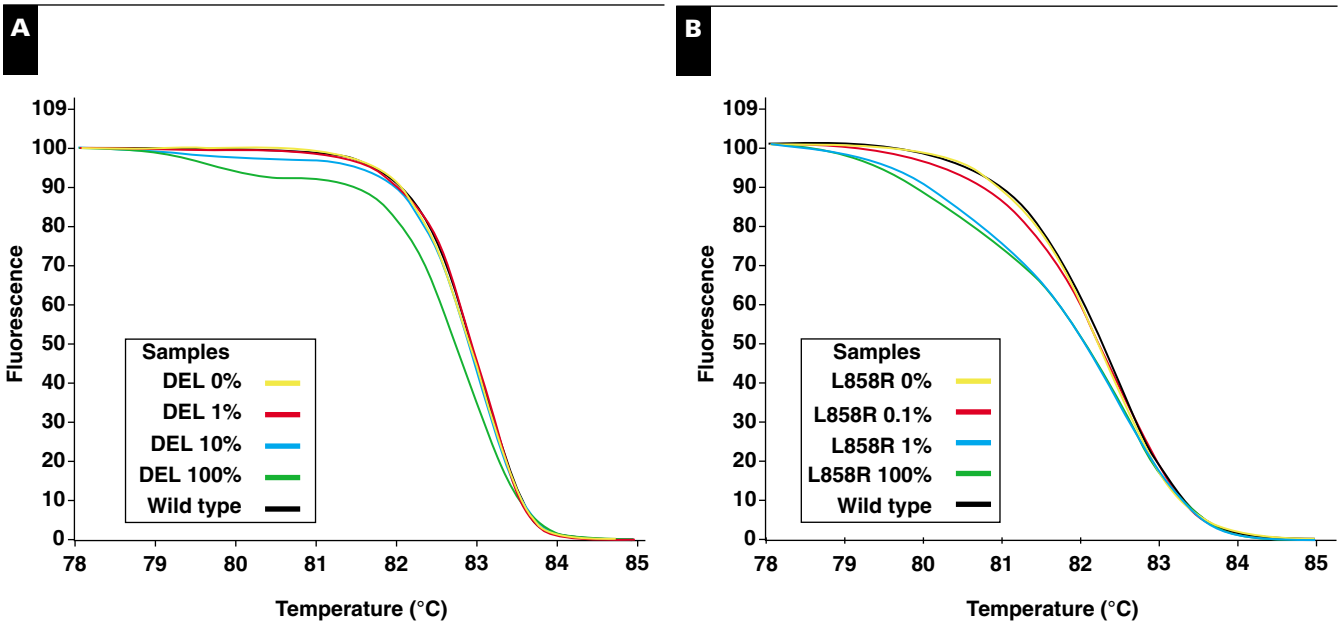

Figure 1 Adjusted melting curves obtained by high-resolution melting analysis of lung adenocarcinoma cells with primers designed to detect mutations in epidermal growth factor receptor (*EGFR*) exon 19 (**A**) or exon 21 (**B**). **A**, Mixtures of H1650 cells ($EGFR^{DEL}$) and A549 cells ($EGFR^{WT}$) revealed gradual curves; 100% and 10% H1650 cells were identified as containing a DEL mutation, and 1% H1650 cells were identified as wild type. **B**, Mixtures of H1975 cells ($EGFR^{L858R}$) and A549 cells ($EGFR^{WT}$) revealed gradual curves, and 100% to 0.1% H1975 cells were identified as containing the L858R mutation. DEL, deletional.

DOI: 10.1309/N5PQNGW2QKMX09X7

In the analysis of exon 19, thorough melting curves were obtained in 35 samples, whereas the other 2 samples (5 and 21) could not be analyzed because PCR was not complete in these cases. Among the 35 samples, 12 gave curves that were different from a WT obtained for cell line A549, as shown in **Figure 2A**, and 23 samples revealed almost the same curves with a WT **Figure 2C** (Figure 2A). Because the skewed curves for the 12 samples were analogous to the curve for H1650 cells, we judged that they had DEL. In the analysis of exon 21, 7 and 2 samples gave left- and right-shifted curves from a WT, respectively, and 28 samples gave almost identical curves with a WT **Figure 2B** and **Figure 2D**. Because the left-shifted curves of the 7 samples were analogous to the curve for H1975, we judged that they had L858R.

As mentioned previously, 2 samples (21 and 25) showing right-shifted curves were considered inadequate for evaluation because of incomplete PCR (Figures 2C and 2D). Taken together, DEL was detected in 12 samples (8 patients) and L858R was detected in 7 samples (6 patients) among 37 samples (29 patients). Samples 5 and 25 were insufficient for judging genotypes of 1 hotspot but judged as containing mutations in the other hotspot (L858R and DEL, respectively). Therefore, the genotype was indeterminate in only 1 sample (case 21) and determined as WT in 17 samples (14 patients). Analysis of the 5 cases with multiple (2 to 4) metachronous samples revealed no differences of genotype in each case.

The results of HRMA were consistent with those of direct sequencing in all samples except samples 19 and 20 (Table 1), which revealed WT curves by HRMA, although

Table 1
HRMA Results for 37 Archival Cytologic Samples From 29 Patients

Sample No.	Histologic Type	Sampling Method	No. of Nucleated Cells	Proportion of Cancer Cells/Nucleated Cells (%)	Mutational Analysis by HRMA		Mutational Analysis by Direct Sequencing
					DEL	L858R	
1	Ad	TBAC	≥1,000	≥90	WT	L858R	L858R
2	Ad	PAC	500-999	≥90	WT	L858R	L858R
3	Ad	BC	500-999	≥90	WT	L858R	L858R
4	Ad	BC	500-999	10-49	DEL	WT	delL747-E749
5	Ad	BC	500-999	50-89	NE	L858R	L858R
6	Ad	BC	≥1,000	<10	WT	WT	WT
7	Ad	BC	100-499	≥90	WT	WT	WT
8	Ad	LN	≥1,000	50-89	WT	WT	WT
9a	Ad	BC	500-999	50-89	WT	WT	WT
9b		BC	500-999	≥90	WT	WT	WT
9c		BC	100-499	<10	WT	WT	WT
10	Ad	PL	≥1,000	10-49	DEL	WT	delE746-A750
11a	Ad	PL	≥1,000	≥90	WT	L858R	L858R
11b		PL	500-999	50-89	WT	L858R	L858R
12	Ad	PL	≥1,000	<10	WT	WT	WT
13	SCC	PAC	500-999	50-89	WT	WT	WT
14a	Ad	PC	≥1,000	50-89	DEL	WT	delE746-A750
14b		PL	500-999	50-89	DEL	WT	delE746-A750
14c		PL	≥1,000	50-89	DEL	WT	delE746-A750
14d		PL	500-999	10-49	DEL	WT	delE746-A750
15a	Pleo	BC	100-499	50-89	DEL	WT	delE746-A750
15b		BC	500-999	50-89	DEL	WT	delE746-A750
16	Ad	PAC	100-499	10-49	WT	WT	WT
17	Ad	PL	500-999	50-89	WT	L858R	L858R/E709K
18	Ad	TBAC	500-999	≥90	WT	WT	WT
19	Ad	PL	≥1,000	10-49	WT	WT*	L858R/S768I
20	Ad	PL	≥1,000	<10	WT	WT†	L858R
21	Ad	LN	100-499	≥90	NE	NE	delE746-A750
22	Ad	PL	≥1,000	<10	WT	WT	WT
23	Ad	TBAC	500-999	≥90	DEL	WT	delE746-A750
24	Ad	PL	≥1,000	50-89	WT	WT	WT
25	Ad	PL	100-499	<10	DEL	NE	delE746-A750
26a	Ad	TBAC	500-999	50-89	WT	WT	WT
26b		PL	≥1,000	<10	WT	WT	WT
27	Ad	PAC	≥1,000	50-89	WT	WT	WT
28	Ad	PC	500-999	50-89	DEL	WT	delE746-A750
29	Ad	BC	100-499	50-89	DEL	WT	delE746-A750

Ad, adenocarcinoma; BC, bronchial brushing or washing cytology; DEL, deletional mutation; HRMA, high-resolution melting analysis; LN, fine-needle aspiration cytology of superficial lymph nodes; NE, not evaluable; PAC, percutaneous fine-needle aspiration cytology; PC, pericardial effusion; PL, pleural effusion; Pleo, pleomorphic carcinoma; SCC, squamous cell carcinoma; TBAC, transbronchial fine-needle aspiration cytology; WT, wild type.
* WT after tumor cell–enrichment procedure.
† L858R after tumor cell–enrichment procedure.

DOI: 10.1309/N5PQNGW2QKMX09X7

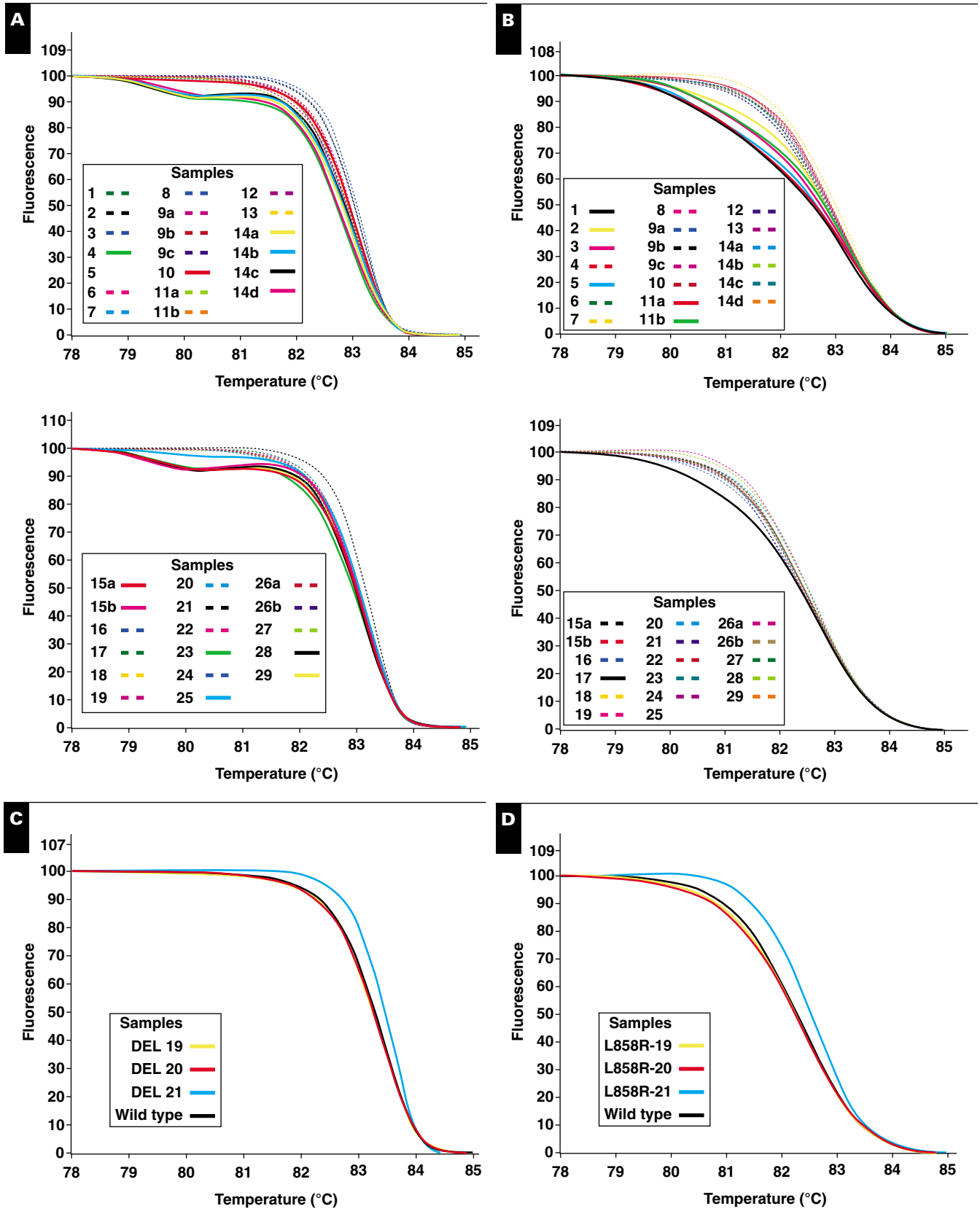

Figure 2 Adjusted melting curves of DNA extracted from archived cytologic slides in the analysis of epidermal growth factor receptor (*EGFR*) exon 19 (**A**) and exon 21 (**B**). Samples 4, 10, 14a-d, 15a-b, 23, 25, 28, and 29 were identified as containing deletional (DEL) mutations, and samples 1, 2, 3, 5, 11a-b, and 17 were identified as containing the L858R mutations. **C** and **D**, The curves of 3 samples (19-21) are shown in **C** (DEL) and **D** (L858R), but the curves were not obtained in 2 samples (5, DEL; 25, L858R) because of incomplete polymerase chain reaction.

DOI: 10.1309/N5PQNGW2QKMX09X7

surgical specimens from the same patients showed the L858R mutation by direct sequencing. Thus, the results for these samples were considered false-negative. The cytologic appearances of these samples are shown in **Image 1**. Sample 20 contained only a small proportion (<10%) of cancer cells in a background of numerous benign nucleated cells, possibly explaining the false-negative result. In fact, we were able to detect the L858R mutation after tumor cell enrichment by manual dissection in sample 20. However, this was not the case for sample 19, which contained a moderately small proportion (10%-50%) of cancer cells, and the result remained negative even after tumor cell enrichment.

In summary, we identified DEL or L858R in 19 samples (14 patients) and WT *EGFR* in 15 samples (12 patients) accurately by HRMA, but 2 samples (2 patients) gave false-negative results and 1 sample (1 patient) was indeterminate. Accuracy was 92% (34/37) based on the number of samples and 90% (26/29) based on the number of patients. Among the 36 samples in which the genotype was determined, sensitivity was 90% (19/21) and specificity was 100% (15/15), or 88% (14/16) and 100% (12/12), respectively, based on the number of patients. These data indicate that this new method is useful for clinical decision making, especially when a patient is given a positive result.

Discussion

In the present study, we established and validated a new method for detecting 2 major *EGFR* mutations (DEL and L858R) using HRMA for cytologic samples. In a study using a cell line, the sensitivity of HRMA indicated that if at least 10% of cells in a sample were cancer cells, then both DEL and L858R were detectable. L858R was detectable even in 0.1% of L858R cells, whereas DEL could not be detected in 1% of DEL cells. Although the reason for this difference is unclear, the sensitivity is still sufficiently high for application to clinical practice.

We performed the HRMA using archival cytologic samples from 29 patients with NSCLC, and the results were quite consistent with the data obtained for the corresponding 26 cases by LCM plus direct sequencing, which were performed in a previous study.[8] The HRMA was completed in 8 of 9 tumor samples with known DEL mutations, and all 8 samples were identified as having DEL. DEL was detected accurately even in sample 25, in which tumor cells accounted for fewer than 10% of the cells on the slides. In the analysis of archived cytologic samples, no marked difference in detection accuracy was observed between DEL and L858R.

Two samples that contained a relatively small proportion of tumor cells gave false-negative results for L858R; one of these (sample 20) gave a positive result after tumor cell enrichment, but the other (sample 19) did not. The sensitivity of this analysis was 88% (14/16) based on the number of patients, and it increased to 94% (15/16) if tumor cells were enriched in the samples with a small proportion of tumor cells. These results indicate that cytologists will be required not only to diagnose whether samples are benign or malignant but also to evaluate whether they are suitable for molecular analysis.

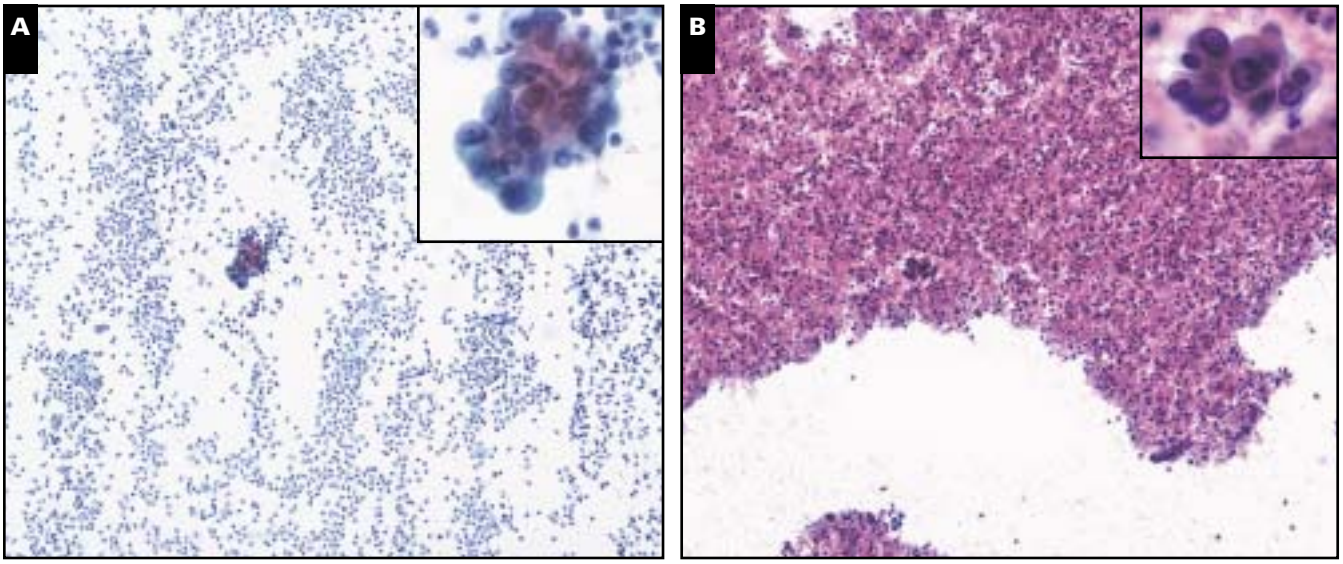

Image 1 Samples 19 (**A**) and 20 (**B**) show a few cancer cells in a background of many normal nucleated cells. L858R was not detected in these samples by high-resolution melting analysis (HRMA), although direct sequencing showed that DNA extracted from surgical specimens from the same patients had L858R. After retrieving tumor-rich parts selectively, L858R was detected by HRMA from a cytologic slide obtained simultaneously with sample 20, but this was not the case with sample 19 (×10). Insets, The tumor showed 3-dimensional clusters with nuclear atypia compatible with adenocarcinoma (×40).

DOI: 10.1309/N5PQNGW2QKMX09X7

The mutational status of *EGFR* is a strong predictor of gefitinib sensitivity, and detection of such mutations would provide patients and physicians with important information for optimal choice of therapy. However, mutation detection has not become a common procedure in clinical practice because it often is difficult and impractical. Direct sequencing, which is a standard method for detecting mutations, requires high-quality DNA extracted from an adequate amount of pure tumor cells to obtain precise data and is costly and time-consuming.

Many researchers have tried to establish new methods for detecting *EGFR* mutations using small tumor samples contaminated with normal cells. To date, a number of nonsequencing methods for detecting mutations have been suggested, such as single-strand conformation polymorphism,[15,27] restriction fragment length polymorphism,[28,29] PCR amplification of specific alleles (also known as amplification refractory mutation system and allele specific amplification),[30-32] peptide nucleic acid–mediated PCR clamping,[33,34] peptide nucleic acid–locked nucleic acid PCR clamping,[35] denaturing gradient gel electrophoresis,[36] temperature gradient capillary electrophoresis,[37,38] denaturing high-performance liquid chromatography,[39,40] and high-density oligonucleotide arrays.[41] Some of these methods have been reported to give good results for detection of *EGFR* mutations[15,29,35,40]; however, they often require intensive labor or sophisticated instruments and, therefore, have not been adopted in clinical practice.

HRMA is one of these new methods and has the advantage of being able to distinguish specific mutations from the WT sequence with less labor, time, and cost; PCR and the melting analysis can be performed in the same capillary tube within a few hours, and the running cost is only about $1 (US) per sample.

Detection of DEL and L858R using HRMA is accurate even when archived cytologic samples are used. Because HRMA involves little labor, time, and cost, it is expected to become one of the most practical and useful methods for detecting major *EGFR* mutations in cytologic materials from patients with NSCLC.

From the [1]Clinical Laboratory Division, [2]Division of Internal Medicine, and [3]Clinical Support Laboratory, National Cancer Center Hospital; and the [4]Genetics Division and [5]Pathology Division, National Cancer Center Research Institute, Tokyo, Japan.

Supported in part by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology; a program for the promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency; and a Health and Labour Science research grant from the Ministry of Health, Labour and Welfare, Japan.

Address reprint requests to Dr Matsuno: Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.

References

1. Ozanne B, Richards CS, Hendler F, et al. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. *J Pathol*. 1986;149:9-14.
2. Haeder M, Rotsch M, Bepler G, et al. Epidermal growth factor receptor expression in human lung cancer cell lines. *Cancer Res*. 1988;48:1132-1136.
3. Fukuoka M, Yano S, Giaccone G, et al. A multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non–small cell lung cancer (the IDEAL 1 Trial). *J Clin Oncol*. 2003;21:2237–2246.
4. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non–small cell lung cancer: a randomized trial. *JAMA*. 2003;290:2149-2158.
5. Takano T, Ohe Y, Kusumoto M, et al. Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non–small cell lung cancer treated with gefitinib. *Lung Cancer*. 2004;45:93-104.
6. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
7. Paez JG, Janne PA, Lee JC, et al. *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
8. Takano T, Ohe Y, Sakamoto H, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non–small-cell lung cancer. *J Clin Oncol*. 2005;23:6829-6837.
9. Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non–small cell lung cancer with postoperative recurrence. *J Clin Oncol*. 2005;23:2513-2520.
10. Han S-W, Kim T-Y, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non–small-cell lung cancer patients treated with gefitinib. *J Clin Oncol*. 2005;23:2493-2501.
11. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004;101:13306-13311.
12. Kosaka T, Yatabe Y, Endoh H, et al. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res*. 2004;64:8919-8923.
13. Huang SF, Liu HP, Li LH, et al. High frequency of epidermal growth factor receptor mutations with complex patterns in non–small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clin Cancer Res*. 2004;64:8195-8203.
14. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst*. 2005;97:339-346.
15. Marchetti A, Martella C, Felicioni L, et al. *EGFR* mutations in non–small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol*. 2005;23:857-865.
16. Tokumo M, Toyooka S, Kiura K, et al. The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non–small cell lung cancers. *Clin Cancer Res*. 2005;11:1167-1173.

DOI: 10.1309/N5PQNGW2QKMX09X7

17. Sasaki H, Endo K, Konisi A, et al. *EGFR* mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler. *Clin Cancer Res*. 2005;11:2924-2929.
18. Giglio S, Monis PT, Saint CP. Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. *Nucleic Acids Res*. 2003;31:e136-e140.
19. Dobrowolski SF, McKinney JT, Filippo CAS, et al. Validation of dye-binding/high-resolution thermal denaturation for the identification of mutations in the *SLC22A5* gene. *Hum Mutat*. 2005;25:306-313.
20. Zhou L, Vandersteen J, Wang L, et al. High-resolution DNA melting curve analysis to establish HLA genotypic identity. *Tissue Antigens*. 2004;64:156-164.
21. Wittwer CT, Reed GH, Gundry CN, et al. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem*. 2003;49(6 pt 1):853-860.
22. Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem*. 2004;50:1748-1754.
23. Carlynn WP, Lester JL, Joseph AH, et al. c-*KIT* mutation analysis for diagnosis of gastrointestinal stromal tumors in fine needle aspiration specimens. *Cancer*. 2005;105:165-170.
24. Sordella R, Bell DW, Haber DA, et al. Gefitinib-sensitizing *EGFR* mutations in lung cancer activate anti-apoptotic pathways. *Science*. 2004;305:1163-1167.
25. Tracy S, Mukohara T, Hansen M, et al. Gefitinib induces apoptosis in the EGFRL858R non–small-cell lung cancer cell line H3255. *Cancer Res*. 2004;64:7241-7244.
26. Zhao X, Weir BA, LaFramboise T, et al. Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. *Cancer Res*. 2005;65:5561-5570.
27. Orita M, Iwahana H, Kanazawa H, et al. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A*. 1986;86:2766-2770.
28. Felley-Bosco E, Pourzand C, Zijlstra J, et al. A genotypic mutation system measuring mutations in restriction recognition sequences. *Nucleic Acids Res*. 1991;19:2913-2919.
29. Pan Q, Pao W, Ladanyi M. Rapid polymerase chain reaction–based detection of epidermal growth factor receptor gene mutations in lung adenocarcinomas. *J Mol Diagn*. 2005;7:396-403.
30. Okayama H, Curiel DT, Brantly ML, et al. Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J Lab Clin Med*. 1989;114:105-113.
31. Newton CR, Graham A, Heptinstall LE, et al. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). *Nucleic Acids Res*. 1989;17:2503-2516.
32. Sarkar G, Cassady J, Bottema CD, et al. Characterization of polymerase chain reaction amplification of specific alleles. *Anal Biochem*. 1990;186:64-68.
33. Ørum H, Nielsen PE, Egholm M, et al. Single base pair mutation analysis by PNA directed PCR clamping. *Nucleic Acids Res*. 1993;21:5332-5336.
34. Thiede C, Bayerdorffer E, Blasczyk R, et al. Simple and sensitive detection of mutations in the *ras* proto-oncogenes using PNA-mediated PCR clamping. *Nucleic Acids Res*. 1996;24:983-984.
35. Nagai Y, Miyazawa H, Huqun, et al. Genetic heterogeneity of the epidermal growth factor receptor in non–small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid–locked nucleic acid PCR clamp. *Cancer Res*. 2005;65:7276-7282.
36. Fischer SG, Lerman LS. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc Natl Acad Sci U S A*. 1983;80:1579-1583.
37. Gao Q, Yeung ES. High-throughput detection of unknown mutations by using multiplexed capillary electrophoresis with poly (vinylpyrrolidone) solution. *Anal Chem*. 2000;72:2499-2506.
38. Li Q, Liu Z, Monroe H, et al. Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis*. 2002;23:1499-1511.
39. Liu W, Smith DI, Rechtzigel KJ, et al. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res*. 1998;26:1396-1400.
40. Jänne PA, Borras AM, Kuang Y, et al. A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer Res*. 2006;12:751-758.
41. Hacia JG, Brody LC, Chee MS, et al. Detection of heterozygous mutations in *BRCA1* using high density oligonucleotide arrays and two-colour fluorescence analysis. *Nat Genet*. 1996;14:441-447.

DOI: 10.1309/N5PQNGW2QKMX09X7