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

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Abstract

Lung cancer is one of the leading causes of the cancer death worldwide. Gefitinib is an inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and has been introduced in the treatment of advanced lung cancers. The responsiveness to gefitinib has been linked to the presence of EGFR mutations. Clinical samples contain many normal cells in addition to cancer cells. A method capable of detecting EGFR mutations in a large background of wild-type EGFR genes could provide a superior clinical test. We developed a rapid and sensitive detection system for EGFR mutations named the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp that can detect EGFR mutations in the presence of 100-to 1,000-fold background of wild-type EGFR. We used this method to screen 30 non–small cell lung cancer cell lines established from Japanese patients. In addition to 11 cell lines that have mutations, we found 12 cell lines in which specific mutations are observed only in the subpopulation(s) of the cells. Genetic heterogeneity of EGFR suggests that the *EGFR* gene is unstable in established cancers and the heterogeneity may explain variable clinical responses of lung cancers to gefitinib. (Cancer Res 2005; 65(16): 7276-82)

Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide (1). Although advanced lung cancers are treated by the combination of chemotherapy and radiotherapy (2), the outcome is still not satisfactory (3). Gefitinib is an inhibitor of tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and has recently been introduced in the treatment of advanced lung cancers (4). Gefitinib is dramatically effective in some patients, whereas it is completely ineffective in others. In addition, Japanese show a high response rate (5). Factors that govern the responsiveness to gefitinib are under intense investigation. EGFR mutations in cancer cells are the first such factors reported (6–8). EGFR mutations are found in a portion of lung cancers, mostly those with adenocarcinoma histology. These mutations are activating mutations that enhance the tyrosine kinase activity of EGFR, which is effectively suppressed by gefitinib. The mutations are much more frequent in Japanese than in U.S. patients, consistent with the difference in the response rate (6, 9). The association of the presence of EGFR mutations and the responsiveness to gefitinib is so tight that the former may be used as a predictor of the latter.

Clinical specimens used to diagnose lung cancers (i.e., sputum, pleural effusion, bronchial washing, and surgically resected tissue) contain many normal cells. A method capable of detecting EGFR mutations in a large background of wild-type EGFR genes could provide a superior clinical test. To serve this purpose, we have developed a method able to detect all 11 different EGFR mutations in the initially reported (6, 7) in the presence of 100- to 1,000-fold wild-type EGFR background. These 11 mutations account for >95% of EGFR mutations found in Japan (9). As an application of the method and to investigate its performance, we screened non–small cell lung cancer (NSCLC) cell lines established from Japanese patients and found several cell lines that have EGFR mutations. Unexpectedly, we found that many cell lines have subpopulations that harbor specific EGFR mutations.

Materials and Methods

Plasmid. Wild-type fragments that contain exons 18, 19, or 21 of the *EGFR* gene (UniGene Cluster Hs.77432) were amplified by PCR from normal human genomic DNA. Primers used were F18, 5′-GGTAGCTGTTCAGTTAAAGAACACC-3′ and B18, 5′-CCTTTGGTCTGTGAATTGGTC-3′ for exon 18; F19, 5′-CTGGATGAAATGATCCACACG-3′ and B19, 5′-TGGGTAGATGCCAGTAATTGC-3′ for exon 19; and F21, 5′-CTGGATGGAGAAAAGTTAATGGTC-3′ and B21, 5′-CAGCAAGTACCGTTCCCAAAG-3′ for exon 21. The amplified fragments were cloned into pCR-Script vector (Stratagene, La Jolla CA). DNA fragments harboring individual EGFR mutations were made from the cloned fragments by site-directed mutagenesis using the Megaprimer method (10), and the fragments were then inserted into pCR-Script. Linear wild-type and mutant DNA fragments about 500 bp long were amplified from individual plasmids by PCR and used.

Cell lines and genomic DNA isolation. Human NSCLC cell lines which were established from Japanese patients with well-documented histologies were collected. 11-18, 1-87, LCSC#1, LCSC#2, and LK87 (adenocarcinomas); 86-2 (large cell carcinoma); Lu99 (giant cell carcinoma); EBc-1, LK2, LK79, Sq-1, Sq5, and Sq19 (squamous cell carcinomas); and A431 (squamous cell carcinoma of the skin) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Japan. ABC-1, PC-3, RERF-LC-Ad1, RERF-LC-Ad2, and RERF-LC-MS (adenocarcinomas); RERF-LC Sq-1 (squamous cell carcinoma); and Lu65 (giant cell carcinoma) were from the Japanese Collection of Research Bioresources (Tokyo, Japan). LC2/ad, PC-14, RERF-LC-KJ, and RERF-LC-OK (adenocarcinomas) and RERF-LC-A1 (squamous cell carcinoma) were from the Riken Bioresource Center

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(Tsukuba, Japan). PC-7, PC-9, and PC-13 (adenocarcinomas) were from IBL (Takasaki, Japan). KTA-7 (adenocarcinoma) and KTSq-1 were kindly provided by Drs. Toru Kameya and Shi-Xu Jiang (Kitasato University, Japan). Genomic DNA was prepared by QIAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands) from the cells in the first passage of expansion after being obtained from the establisher (KTA-7 and KTSq-1) or from the cell banks (all other cells). Normal human genomic DNA was purified in the same way from the peripheral blood of normal volunteers.

PCR primers and solutions. All PCR reaction solutions (25 μL) were based on the Basic Mixture containing 25 mmol/L TAPS (pH 9.3), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L 2-mercaptoethanol, 200 μmol/L each of deoxynucleotide triphosphates, and 1.25 units of Takara Ex Taq HS (Takara Bio, Shiga, Japan). For conventional PCR, PCR primers (200 nmol/L each) were added to the Basic Mixture. For peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, PCR primers (200 nmol/L each), fluorogenic probes (100 nmol/L each), and a PNA clamp primer (5 μmol/L) were added to the Basic Mixture. PCR primers were designed manually or by using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) so that the T_m values were between 55°C and 60°C. Fluorogenic probes containing LNA were manually designed and confirmed by the LNA T_m prediction tool (http://lna-tm.com/) to have T_m values between 54°C and 56°C. PNA clamp primers, 14- to 18-mer in length, were designed according to the guidelines (11). LNA-containing oligos were synthesized by IDT (Coralville, IA) and PNA oligos were by Greiner Japan (Tokyo, Japan).

PCR reactions. PCR and the real-time amplification monitoring for both conventional and the PNA-LNA PCR clamp were done using Smart Cycler II (Cepheid, Sunnyvale, CA). PCR cycling was a 30-second hold at 95°C followed by 45 cycles of 95°C for 3 seconds and 62°C (exons 18 and 19) or 56°C (exon 21) for 30 seconds. Nested PCR for the PNA-LNA PCR clamp was done using the same reaction conditions except that the inner primers and 1 μL of a $1:10^6$ dilution of the first PCR reaction were used.

Nucleotide sequencing. PCR products from both conventional PCR and the PNA-LNA PCR clamp were purified by Wizard PCR Prep DNA purification kit (Promega, Madison, WI) and directly sequenced by an automated DNA sequencer.

Results

Establishment of peptide nucleic acid-locked nucleic acid PCR clamp. The PNA-LNA PCR clamp is a method that can quickly detect specific mutation or deletions occur at known positions. In the PNA-LNA PCR clamp, PNA (12) and LNA (11) are used to construct PCR clamp reactions (13). Positive signals are detected by the 5′ nuclease assay (14). The PNA-LNA PCR clamp system is schematically presented in Fig. 1. Here, PNA clamp primers suppress amplification of the wild-type sequences, thereby enhance preferential amplification of the mutant sequences. LNA probes were employed to specifically detect mutant sequences in the presence of wild-type sequences. Because PNA clamp primers that have wild-type sequences and LNA probes that have mutant sequences are located in the position, PNA clamp primers competitively inhibit mutant LNA probes to bind to the wild type, further increasing the specificity of detection. Thus, individual EGFR mutations can be detected in the presence of a 100- to 1,000-fold wild-type EGFR background molecules (Fig. 2*A* and *B*). We multiplexed the reactions by using multiple probes labeled with different dyes to detect 11 mutations by five reactions (Table 1). Even after being multiplexed, each mutation was detected in the presence of 100- to 1,000-fold background (data not shown). The mutant/wild type ratio of a given sample can be semiquantified by plotting the second derivatives of the amplification curves (Fig. 2*C*). Samples with signals close to the baseline are further resolved by optional nested PCR reactions (Fig. 2*D*).

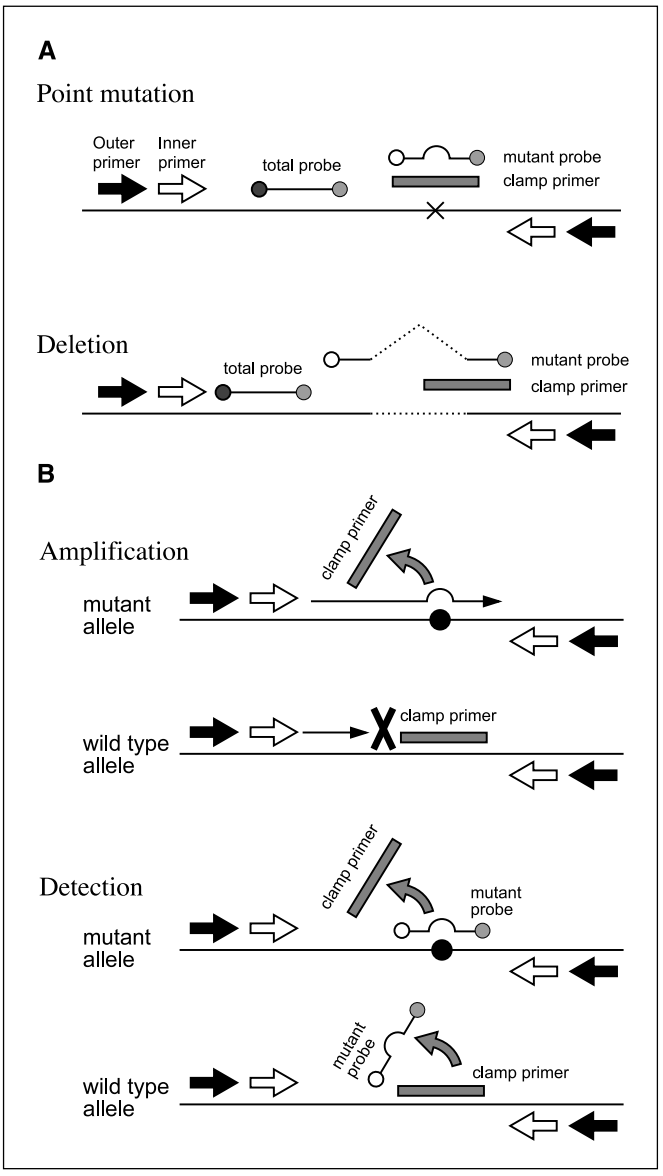

Figure 1. PNA-LNA PCR clamp system. *A,* primer and probe positions. Point mutation: both the clamp primer and the mutant probe are located on the mutation site. The "Total probe" to detect both mutant and wild-type fragments are on the adjoining sequence. *X,* mutation. Deletion: the clamp primer partly covers the deletion. Sequences located on both sides of the deletion are joined to make the sequence of the mutation probe. *Dashed line,* deletion. Outer (*black arrow*) and inner (*white arrow*) PCR primers. *B,* schematic presentation of the reaction to detect point mutations. In the amplification process, the clamp primers fail to bind to the mutation sequences but binds to the wild type, resulting in the preferential amplification of mutant sequences. In the detection process, the mutant probes bind to the mutant sequences but fail to bind to the wild type partly because of mismatches and partly because of the competitive displacement by the clamp primers. The signals are detected by the 5′ nuclease assay. *Black circle,* mutation.

Screening of the non–small cell lung cancer cell lines established from Japanese patients. As an application of the method, we screened NSCLC cell lines established from Japanese patients. It has been reported that the rate of EGFR mutations shows apparent ethnic differences and mutations are frequently found in lung cancer specimens from Japanese patients (6, 9).

Therefore, we considered that mutations would also be frequent in the cell lines. We found six different mutations in 11 of the 30 cell lines examined (Table 2, "mutation present in most of the cells"). In addition, we unexpectedly found many cell lines presented amplification curves that indicated a subpopulation(s) of the cells harbored EGFR mutation(s).

We first confirmed the presence of the subpopulations by nucleotide sequencing. Direct sequencing of the conventional PCR product revealed a wild-type sequence, whereas that of the PNA-LNA PCR clamp product revealed a mutant sequence (Fig. 3*A*). The mutant sequence is considered to be derived from the sample DNA, because the PNA-LNA PCR does not have any reaction steps that artificially produce specific mutation fragments. Consistently, nucleotide sequencing of both the conventional and the PNA-LNA PCR products from 17 normal DNA samples presented only the wild-type sequences (data not shown).

We next semiquantified the size of the subpopulations by two methods. First, the PCR cycle number that gives the peak of the second derivative of the amplification curve was compared with that of standards (Fig. 3*B, left*). Second, a shift in the amplification curve was observed after incrementally adding mutant DNA fragments as an internal positive control. Here, adding mutant DNA fragments at a copy number less than or close to that of the mutant *EGFR* gene do not affect the curve while adding more shifts the curve to the left and upwards (Fig. 3*B, right*). For all cell lines, these two methods gave consistent results that are summarized in Table 2 [mutation present in a subpopulation(s) of the cells]. Results suggesting a subpopulation size of ≤0.1% were removed from the table because 0.1% is close to the detection limit of the first round of the PNA-LNA PCR clamp; therefore, the result may be erroneous. Taken together, 19 of the 30 cell lines had an EGFR mutation(s) either in their entire population (11 cell lines) and/or in their subpopulation(s) (12 cell lines; Table 2).

The *EGFR* gene is amplified in some types of cancers such as glioblastomas (15). Our hypothesis that subpopulations of EGFR mutants exist may be disputed by the possibility that the *EGFR* gene is amplified in the cell lines and only a single copy within the amplified *EGFR* gene is mutated, resulting in what seems subpopulations. To disprove this argument, we semiquantified the copy number of the *EGFR* gene in a cell using two different methods. First, individual exons are amplified by the conventional

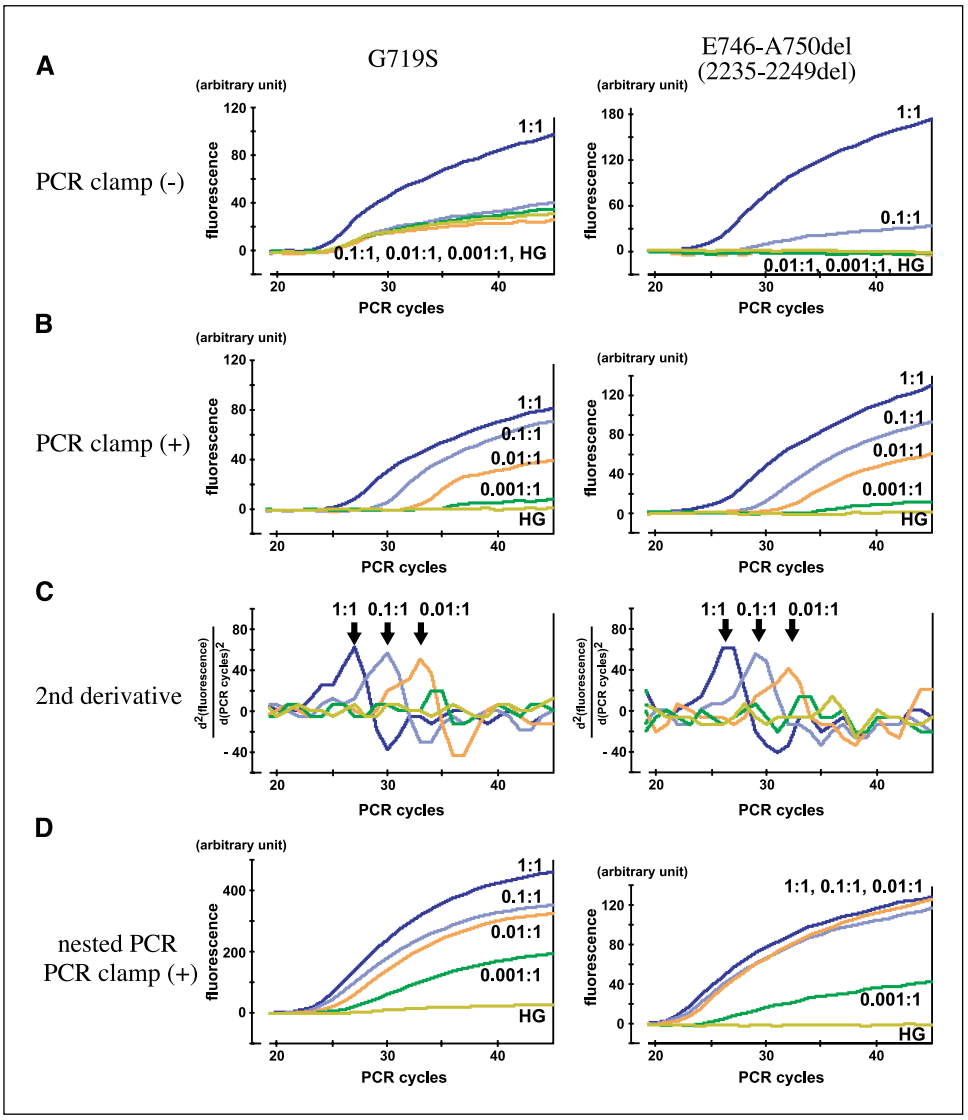

Figure 2. PNA-LNA PCR clamp reactions. Detection of a point mutation (G719S) and a deletion (E746-A750del). To 25 ng of normal human genomic DNA mutant, DNA fragments that contained either a point mutation or a deletion were added at a ratio of 1, 0.1, 0.01, 0.001, or 0 molecules of mutant DNA per normal human haploid genome (1:1, 0.1:1, 0.01:1, 0.001:1, or HG). *A,* reactions without a PNA clamp primer. *B,* reactions with a PNA clamp primer. *C,* second derivative of the curves for the reaction in (*B*). Positions of the peaks depend on the copy numbers of the mutant DNA. *D,* a 10-millionth of the PCR clamp (+) reaction solution was used to do the nested PCR reaction. Signals are stronger and clearly differentiate the mutants from the wild types.

Table 1. Primers and probes used

	Oligo name	Oligo sequence 5′ to >3′	Final concentration (nmol/L)
Reaction 1 (G719C, G719S)			
PCR primer	ex18o-F	TCCAAATGAGCTGGCAAGTG	200
	ex18o-B	TCCCAAACACTCAGTGAAACAAA	200
Mutation probe	G719Cp	6-FAM/accggagcAcagcactt/BHQ1	100
	G719Sp	TET/accggagcTcagcactt/BHQ1	100
Total probe	Ex18t	Cy5/ccaagctctcttgaggatcttg/BHQ2	100
PNA clamp primer	G179cl	NH2-GAGCCCAGCACTTT-CONH2	5,000
Reaction 2 (E746-A750del type 1, E746-A750del type 2, L747-A750del T751S)			
PCR primer	ex19o-F	GTGCATCGCTGGTAACATCC	200
	ex19o-B	TGAGGTTCAGAGCCATGGAC	200
Mutation probe	E746-A750del-1p	6-FAM/ctatcaaAaCatctccgaaagc/BHQ1	100
	E746-A750del-2p	TET/cgctatcaaGacatctccg/BHQ1	100
	L747-A750del T751Sp	Texas red/ctatCaagGaaTCatctcc/BHQ2	100
Total probe	Ex19t	Cy5/ttaaCtTTCtCaCct/BHQ2	100
PNA clamp primer	Delcl	NH2-AGATGTTGCTTCTCTTAA-CONH2	5,000
Reaction 3 (L747-S752del P753S, L747-E749del A750P, L747-S752del E746V, S752-I759del)			
PCR primers, total probe and PNA clamp primer is the same as in Reaction 2			
Mutation probe	L747-S752del P753Sp	6-FAM/ctatcaaggaatCgaaagcca/BHQ1	100
	L747-E749del A750Pp	TET/atcaaggaaCcaacatctcc/BHQ1	100
	L747-S752del E746Vp	Texas red/tatcaaggttCcgaaagcca/BHQ2	100
	S752-I759delp	6-FAM/agagaagCaaCactcgat/BHQ1	100
Reaction 4 (L858R)			
PCR primer	ex21o-F	GCATGAACTACTTGGAGGAC	200
	ex21o-B	ACCTAAAGCCACCTCCTTAC	200
Mutation Probe	L858Rp	6-FAM/tttggccCgcccaa/BHQ1	100
Total probe	Ex21t	Cy5/ccaGgaaCgtaCtg/BHQ2	100
PNA clamp primer	L858cl	NH2-CAGTTTGGCCAGCCCA-CONH2	5,000
Reaction 5 (L861Q)			
PCR primers and total probe are the same as in Reaction 4			
Mutation probe	L861Qp	TET/acccagcTgtttGg/BHQ1	100
Clamp primer	L861cl	NH_2-ACCCAGCAGTTTGGC-CONH2	5,000
Inner primers for nested PCR			
Reaction 1 (exon 18)	ex18i-F	CTTACACCCAGTGGAGAAGC	200
	ex18i-B	GGACCTTACCTTATACACCG	200
Reactions 2 and 3 (exon 19)	ex19i-F	TGTCATAGGGACTCTGGATCC	200
	ex19i-B	AGCAGAAACTCACATCGAG	200
Reaction 4 and 5 (exon 21)	ex21i-F	CTTGGAGGACCGTCGCTTG	200
	ex21i-B	CCACCTCCTTACTTTGCCTC	200

NOTE: A total of 11 mutations and deletions of the *EGFR* gene were detected by five separate reactions. PCR primers to amplify the target sequences, fluorogenic probes to detect mutations and deletions, fluorogenic probes to detect total amplification, and PNA clamp primers are listed. For fluorogenic probes, usual deoxynucleotide residues are written in lowercase letters and LNA residues are in uppercase. The inner PCR primers are used when nested reactions are set up to increase sensitivity. Final concentration of each oligo in the reaction solution is shown. Correspondences of the deletion names in this Table: in ref. (7): in ref. (6) are E746-A750del type 1: patient 1: Del-1a; E746-A750del type 2: —:Del-1b; L747-A750del T751S: patient 2: —; L747-S752del P753S: patient 3: Del-4; L747-E749del A750P: —:Del-3; L747-S752del E746V: —: Del-5; S752-I759del: —: Del-2, where — indicates not reported.

Abbreviation: BHQ; Black hole quencher.

PCR (i.e., a quantitative PCR) and the PCR cycle number that gave the peak of the second derivative of the amplification curve was compared with that of standards (Fig. 3*C, left*). Second, a shift in the amplification curve was observed after incrementally adding wild-type DNA fragments as an internal control (Fig. 3*C, right*). Both methods gave consistent results for all cell lines and showed that the copy number of the *EGFR* gene in all the cell lines with EGFR mutations in the subpopulation(s) is close to 2, indicating that the *EGFR* gene is not amplified.

Discussion

In this study, we have established a rapid and sensitive method, the PNA-LNA PCR clamp, for detecting EGFR mutations. The PNA-DNA heteroduplex with a completely matched sequence has a higher melting temperature (T_m), whereas that with a single base mismatch has a lower T_m than the corresponding DNA-DNA duplex (16). In addition, PNA is resistant to the 5′ nuclease activity of Taq DNA polymerase. These characteristics make a PNA oligo a superior clamp primer for inhibiting PCR

amplification of wild-type sequences. The LNA-DNA heteroduplex with a completely matched sequence has a higher T_m, whereas that with a single base mismatch has a lower T_m than the corresponding DNA-DNA duplex (17). LNA can be mixed with other nucleotides to synthesize fluorogenic probes for detecting mutant sequences. The amount of DNA used for one reaction is 25 ng (i.e., the amount of DNA from 8,000 haploid genome). Therefore, the resolution of one copy of mutant in 100 to 1,000 copies of wild type is considered sufficient for many of clinical and experimental applications. This method is applicable to other mutations where known point mutations or small deletions at fixed positions need to be detected rapidly and at a high sensitivity. An example is the detection of K-ras mutations, in which we successfully detected in the presence of 100- to 1,000-fold background of the wild-type gene. We found many PCR primers and fluorogenic probes that have T_m described in Materials and Methods worked fine. For designing PNA primers A + G contents and positions are important so that the primer is soluble in water (16).

The finding that many cell lines are mixtures of the cells without EGFR mutations and of cells with specific EGFR mutations (i.e., genetic heterogeneity of EGFR) surprised us. Cell lines are usually isolated from advanced cancers and have expanded many passages in culture medium. Therefore, they are considered to better represent the later stages of cancers and to possess genetic changes that better provide a growth advantage (18, 19). Thus, they are often considered a clone, although they may actually contain a variety of subclones (20, 21). Coexistence of the wild-type cells and the mutant cells in the same culture suggests that mutation of the *EGFR* gene does not confer cells with a major growth advantage. It also suggests that the *EGFR* gene is unstable and that mutation(s) may occur in established cancers. Wild-type EGFR transmits growth signals to extracellular signal-regulated kinase (Erk) to promote cell division and to Akt and to signal transducer and activator of transcription to promote cell survival (22). L858R (T2573G) and L747-E749del A750P (2239-2247del, G2248C), two major mutations that together account for two thirds of the mutations in lung cancers (6, 7), produce EGFR protein that only

Table 2. Mutations of *EGFR* gene in NSCLC cell lines established from Japanese patients

Cell line name	Cytology	Mutation present in nearly 100% of the cells	Mutation present in a subpopulation(s) of the cells	
			10%	1%
11-18	Ad	L858R (T2573G)	–	–
1-87	Ad	–	L858R (T2573G)	–
ABC-1	Ad	–	L858R (T2573G)	–
KTA-7	Ad	L861Q (T2582A)	–	–
LC2/Ad	Ad	L858R (T2573G)	–	–
LCSC#1*	Ad	L858R (T2573G)	–	–
LCSC#2	Ad	–	–	–
LK87	Ad	–	–	–
PC-3	Ad	L747-E749del, A750P (2239-2247del, G2248C)	–	L858R (T2573G)
PC-7	Ad	G719S (G2155A)	–	–
PC-9	Ad	E746–A750del type 1 (2235-2249del)	–	L858R (T2573G)
PC-13	Ad	–	L858R (T2573G)	–
PC-14	Ad	†E746-A750del type 1 (2235-2249del)	–	–
RERF-LC-Ad1	Ad	–	–	–
RERF-LC-Ad2	Ad	L747-E749del, A750P (2239-2247del, G2248C)	–	–
RERF-LC-KJ	Ad	–	–	–
RERF-LC-MS	Ad	–	–	–
RERF-LC-OK	Ad	–	–	L858R (T2573G)
86-2	Lg	–	–	–
Lu65	Gt	–	L858R (T2573G)	–
Lu99	Gt	–	–	–
EBc-1	Sq	–	L858R (T2573G)	G719S (G2155A)
KTSq-1	Sq	E746-A750del type 2 (2236-2250del)	–	L858R (T2573G)
LK2	Sq	–	–	L858R (T2573G)
LK79	Sq	–	–	–
RERF-LC Sq-1	Sq	–	–	–
RERF-LC-A1	Sq	L858R (T2573G)	–	G719S (G2155A)
Sq-1	Sq	–	L858R (T2573G)	–
Sq5	Sq	–	–	–
Sq19	Sq	–	–	–

NOTE: EGFR mutations in cell lines. Mutations found are listed by the approximate proportions of the mutant cells (nearly 100%, 10%, or 1%) in each cell line. Mutations are written in the form of amino acid changes (nucleotide changes). –, mutations were not detected.
Abbreviations: Ad, adenocarcinoma; Lg, large cell carcinoma; Gt, giant cell carcinoma; Sq, squamous cell carcinoma.
*The *EGFR* gene is amplified.
†Homozygous mutation.

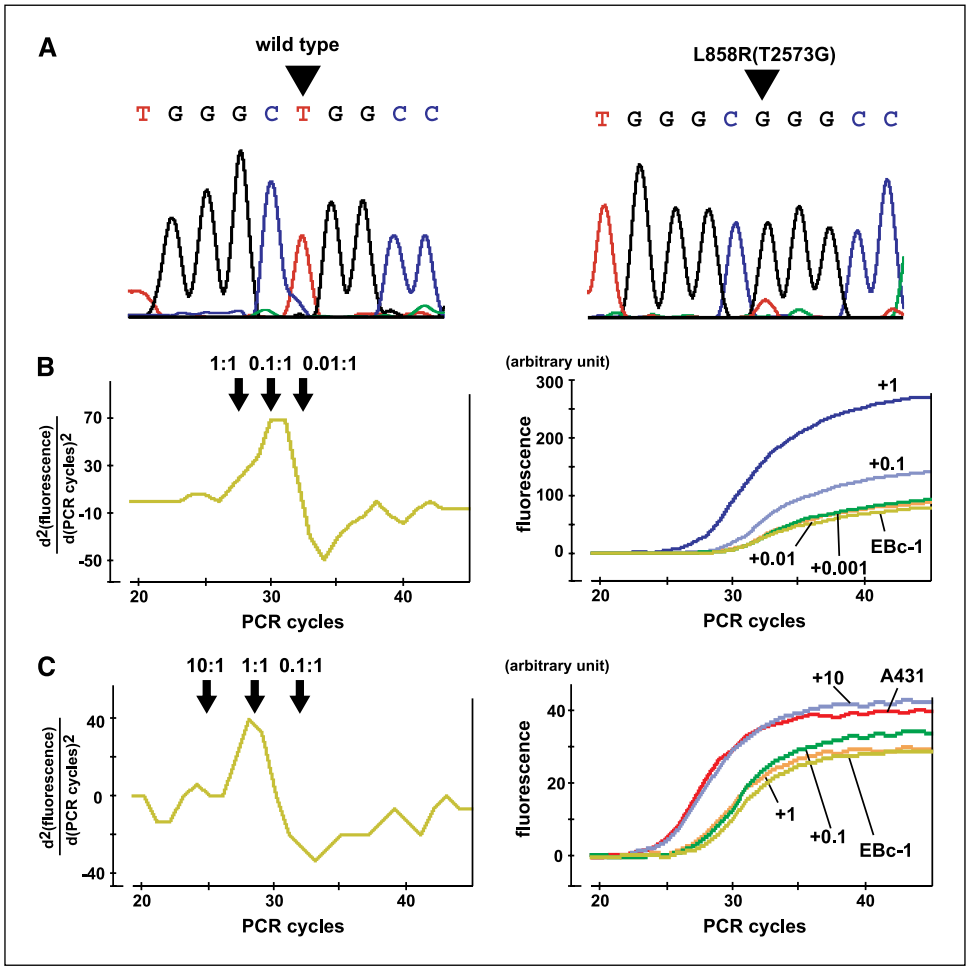

Figure 3. Detection of the subpopulation with an EGFR mutation. Representative results obtained from EBc-1 cells that have L858R (T2573G) mutation in about 10% of the cells (Table 2) are shown. *A,* direct nucleotide sequencing of exon 21 of the *EGFR* gene amplified by the conventional PCR (*left*) and by the PNA-LNA PCR clamp Reaction 4 lacking L858Rp probe (*right*, Table 1). *B,* semiquantification of the copy number of the mutant *EGFR* gene using the PNA-LNA PCR clamp Reaction 4 that detects L858R (T2573G) mutation. *Left,* second derivative of the amplification curve. *Right,* incremental addition of mutant DNA fragments at ratios of 0.001, 0.01, 0.1, or 1 molecule per haploid genome (+0.001, +0.01, +0.1, and +1). *EBc-1,* samples to which no mutant DNA fragment was added. *C,* semiquantification of the copy number of the *EGFR* gene. Exon 21 was amplified by the conventional PCR and the amplification signal was detected by Ex21t probe (Table 1) that detects exon 21 sequence located outside of the mutation sites. *Left,* second derivative of the amplification curve. *Right,* incremental addition of wild-type DNA fragments at ratios of 0.1, 1, or 10 molecules per haploid genome (+0.1, +1, and +10). *EBc-1,* samples to which no mutant DNA fragment was added. A431 that is known to have 30-fold increase in the copy number of the *EGFR* gene (26) was used as a control.

stimulates the survival signaling to Akt (23) in cooperation of Erb-B3 (24) but not the growth signaling to Erk (23). Therefore, these EGFR mutations may not dramatically change the rate of cell growth, which in turn may allow the coexistence of both wild-type and mutant cells. In several cell lines, almost 100% of the cells have mutations. In such cells, the mutation may provide a strong survival advantage.

Genetic heterogeneity of EGFR may contribute to the variable clinical response to gefitinib. For example, we have seen patients in whom primary tumors responded poorly to gefitinib but metastatic legions responded well, and patients in whom tumors initially responded to gefitinib but became refractory during therapy and the tumor relapsed. Genetic heterogeneity of EGFR in the tumor may explain the disease in these patients. Genetic heterogeneity may also explain tumors with two different EGFR mutations (25), a situation we also observed in PC-3, PC-9, EBc-1, KTSq-1, and RERF-LC-A1 cells (Table 2). Serial examination of tumor samples for changes in EGFR mutations during the course of gefitinib therapy, and examination of different parts in a single tumor using microdissection are warranted.

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References

1. Shibuya K, Mathers CD, Boschi-Pinto C, Lopez AD, Murray CJ. Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. BMC Cancer 2002;2:37.
2. Pfister DG, Johnson DH, Azzoli CG, et al. American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: update 2003. J Clin Oncol 2004;22:330–53.
3. Ramalingam S, Belani CP. State-of-the-art chemotherapy for advanced non-small cell lung cancer. Semin Oncol 2004;31:68–74.
4. Raben D, Helfrich BA, Chan D, Johnson G, Bunn PA Jr. ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. Semin Oncol 2002;29:37–46.
5. Nakagawa K, Tamura T, Negoro S, et al. Phase I pharmacokinetic trial of the selective oral epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa,' ZD1839) in Japanese patients with solid malignant tumors. Ann Oncol 2003;14:922–30.
6. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
7. 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;350:2129–39.
8. 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; 101:13306–11.
9. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004;64:8919–23.
10. Sarkar G, Sommer SS. The "megaprimer" method of site-directed mutagenesis. Biotechniques 1990;8:404–7.
11. Ugozzoli LA, Latorra D, Pucket R, Arar K, Hamby K. Real-time genotyping with oligonucleotide probes containing locked nucleic acids. Anal Biochem 2004;324: 143–52.
12. Wittung P, Nielsen PE, Buchardt O, Egholm M, Norden B. DNA-like double helix formed by peptide nucleic acid. Nature 1994;368:561–3.
13. Aebersold DM, Landt O, Berthou S, et al. Prevalence and clinical impact of Met Y1253D-activating point mutation in radiotherapy-treated squamous cell cancer of the oropharynx. Oncogene 2003;22:8519–23.
14. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996;6: 995–1001.
15. Hill C, Hunter SB, Brat DJ. Genetic markers in glioblastoma: prognostic significance and future therapeutic implications. Adv Anat Pathol 2003;10: 212–7.
16. Nielsen PE, editor. Peptide nucleic acids: protocols and applications. 2nd ed. Norwich (U.K.): Horizon Bioscience; 2004.
17. McTigue PM, Peterson RJ, Kahn JD. Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. Biochemistry 2004;43:5388–405.
18. Le Rhun Y, Duthu A, Ehrhart JC, Michiels F, May E, May P. Directional selection associated with clonal expansion of p53 mutant cells during neoplastic development of carcinogen-treated rat embryo lung epithelial cells. Oncogene 1994;9:263–71.
19. Chow M, Rubin H. Clonal selection versus genetic instability as the driving force in neoplastic transformation. Cancer Res 2000;60:6510–8.
20. Chu YW, Yang PC, Yang SC, et al. Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line. Am J Respir Cell Mol Biol 1997;17:353–60.
21. Mueller MM, Peter W, Mappes M, et al. Tumor progression of skin carcinoma cells *in vivo* promoted by clonal selection, mutagenesis, and autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. Am J Pathol 2001;159:1567–79.
22. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003;284:31–53.
23. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
24. Engelman JA, Janne PA, Mermel C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci U S A 2005;102:3788–93.
25. 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;10:8195–203.
26. Merlino G, Xu Y, Ishii S, et al. Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. Science 1984;224:417–9.