TABLE 2. Distribution of Germline T790M Mutation Carriers Stratified by Smoking Status, Gender, and Lung Cancer

Smoking Status	Sex	Case[a,b]	Control[b]
Ever smoker	Male	1	5
	Female	1	0
Never smoker	Male	2	1
	Female	9	3

[a]Out of 19 lung cancer cases, there are two cases with unknown sex and four cases with unknown smoking status; overall, there are 13 cases with both sex and smoking status known (Table 1). Although there are 10 mutation carriers, we do not know whether the proband inherited the mutation from I:1 or I:2 (Figure 2), so we can confirm the smoking status and gender in only nine controls.

[b]Case = Lung cancer arising in germline T790M mutation carrier; control = germline mutation carrier that has not developed lung cancer.

(ESs), which was significantly smaller (p = 6.2E-07) than the expectation—a conservative estimate 0.81—in the general lung cancer cases. The p-value remained significant (p = 7.4E-06) even if the two cases of smokers with uncertain gender were taken into account, that is, four out of 15 cases were ESs.

Prevalence of T790M Germline Mutations in Lung Cancer Patients and the General Population

We estimated the overall penetrance of the germline mutation and its penetrance in both ESs and NSs without regard to age by modeling the conditional probability of family phenotypes and genotypes given the proband's phenotype. We estimate the penetrance to be 0.15 and 0.31 in ESs and NSs, respectively, and the crude penetrance to be 0.23. We examined 627 resected Japanese NSCLC lung cancers for the presence of *EGFR* mutations before the onset of TKI or other therapies. Of these cases, 553 were from frozen tissues (552 tumors, one malignant pleural effusion) and 74 from formalin-fixed, paraffin-embedded materials of resected tumors. *EGFR* mutations were detected in 209 of 627 samples (33.3%). One patient harbored both L858R and an acquired T790M mutation. We compared the reported prevalence of germline T790M mutations in lung cancer patients in the United States (5 of 503)[18] with the prevalence in our Japanese cases (0 of 627) (Fisher's exact test p-value = 0.017).

As germline T790M mutations predispose to the development of lung cancer, the prevalence of germline mutations in lung cancer cases may exceed the prevalence in the general population. The T790M mutation was absent in the 1000 Genomes Project database[25] and in the genomes of 6503 individuals from the NHLBI GO Exome Sequencing Project (http://evs.gs.washington.edu/EVS/). Thus, without adjusting for ethnicity, we estimate that the prevalence of germline T790M mutations in the general population is less than 1 in 7500 subjects. Only one T790M germline mutation has been identified during non-*EGFR* gene targeted sequencing—in a patient with an unrelated malignancy.[26]

DISCUSSION

As the rare germline T790M mutations predispose to lung cancer, they represent a rare but interesting lung cancer familial predisposition gene. Based on the data learned from our family, germline T790M is a major cancer predisposition gene, with an estimated 31% risk for lung cancer in NS carriers. We report the investigation of several cases from a single family, with the 29-year-old proband inheriting the mutation maternally. As we obtained relevant family history from several family members for five generations, and tested for the germline mutation in members from three generations, our family represents the most extensively studied pedigree reported. We combined the family data with the scant published literature data to summarize our current knowledge of this familial form of lung cancer. Our review of the literature was complicated by double reporting of some cases,[13,17] deliberate alteration of some patient data for confidentiality reasons,[13] and incorrect or difficult to interpret data from another report (Supplemental data, Supplemental Digital Content, http://links.lww.com/JTO/A545).[20]

A comparison of lung cancers arising in patients with sporadic activating mutations in the *EGFR* gene versus cancers arising in T790M carriers demonstrates similarities and differences (Table 3). Germline T790M mutations are rare, and, with the exception of family member testing, are almost always detected during analysis of lung cancers for *EGFR* mutations. As germline inheritance predisposes to lung cancer, the prevalence in lung cancer cases is likely to be higher than in controls without cancer. Vikis et al.[9] sequenced the probands of 237 families with predisposition to lung cancer, but did not identify any germline T790M mutations, confirming that these mutations are very rare, even in families with a genetic predisposition for lung cancer. Although approximately 1% of NSCLC cases have heterozygous germline mutations, an analysis of public databases indicated that the prevalence in the general population was probably less than one in 7500. Although sporadic *EGFR* mutations are more frequent in lung cancers arising in East Asian ethnicities, our data from 627 Japanese cases indicated that the prevalence of germline mutations in lung cancer cases in Japan was lower than that reported for U.S. cases. To date, not a single case of germline T790M mutation has been reported in East Asians.

Almost all of the lung cancers having acquired *EGFR* mutations and those arising in T790M carriers have adenocarcinoma histology. While some sporadic adenocarcinomas of the lung arise as multiple apparently independent tumors and may be associated with multiple preinvasive lesions,[25–27] these findings are characteristic of many inherited cancer syndromes.[28–32] We obtained computed tomography (CT) scans of six of the germline mutation carriers, including the proband and five unaffected carriers. The proband had multiple bilateral ground glass and solid nodular lesions, histologically proven to represent the entire spectrum of preneoplastic, preinvasive, and microinvasive lesions associated with peripheral adenocarcinomas.[21] One or more subcentimeter solid or ground glass nodules of uncertain etiology were identified in all the unaffected carriers (Supplemental Table 2, Supplemental Digital Content, http://links.lww.com/JTO/A545).

Females were overrepresented in the germline cases. Female predominance is present in both lung cancers with sporadic *EGFR* mutations and for germline T790M cases. Of

the 22 lung tumors arising in 11 patients whose tumors had *EGFR* gene sequencing, 16 (73%) had a second activating mutation. Thus, the important gain-of-function properties of these double mutants may explain their frequent presence in lung cancers arising in T790M carriers. The median age of the cancer cases was about 63 years, with our proband at 29 years being the youngest case identified. The median age for the T790M cases is considerably higher than for breast cancer development in *BRCA1* carriers (median age, 40 yr), although the age ranges are similar.[35] Female predominance is present in both lung cancers with sporadic *EGFR* mutations and for germline T790M cases.

Perhaps the most interesting and unexpected finding of our study was the effect of smoking status on the appearance of lung cancer in germline carriers. The distribution between smoking status in the germline carriers did not deviate from the expected in the U.S. population. However, the distribution of smoking status among lung cancer cases indicated a considerable, highly significant overrepresentation of NSs (p = 3.2E-07), based on the expectation that 86% of lung cancers in the United States arise in ESs.[24] The calculations are based on our proband being classified as an ES, even though her lifetime tobacco exposure was 0.1 pack-years and was highly unlikely to have contributed to the development of her lung cancer. We estimated the penetrance to be 0.15 and 0.31 in ESs and NSs, respectively, and the crude penetrance to be 0.23. However, as we were unable to adjust for age and given the small sample size, our estimates must be interpreted with caution.

These findings are puzzling, as smoking is universally accepted as the major risk factor for lung cancer. Our hypothetical explanation is illustrated in Figure 3. While the T790M mutation is a weak oncogene by itself, when combined with a common activating *EGFR* mutation such as L858R, the oncogenic potential of both mutations is greatly enhanced, and 73% of lung cancers arising in germline carriers contained a second activating mutation. Multiple studies have demonstrated that sporadic activating EGFR mutations favor never smoking status and female gender.[3] *KRAS* mutations, a major driver mutation in adenocarcinomas arising in ESs, are mutually exclusive with *EGFR* mutations.[3,33] Because somatic mutations of *EGFR* and *KRAS* are mutually exclusive, germline T790M mutations and *KRAS* mutations in ES carriers may also be mutually exclusive. Thus, second activating *EGFR* mutations in germline carriers may arise more frequently in NSs and women and predispose to cancer development. Of interest, both lung cancers with sporadic *EGFR* mutations and the germline T790M cases favor female gender, adenocarcinoma histology, and never smoking status. However, one notable difference is the absence of reported T790M germline mutations in those of East Asian ethnicity.

The management and prevention of lung cancers in germline carriers present unusual and possibly controversial options. The presence of the T790M mutation with or without being accompanied by a more typical activating mutation at presentation predicts for resistance to the standard TKIs. The only germline cases reported to have received TKI therapy were the two sisters reported by Tibaldi et al.,[20]

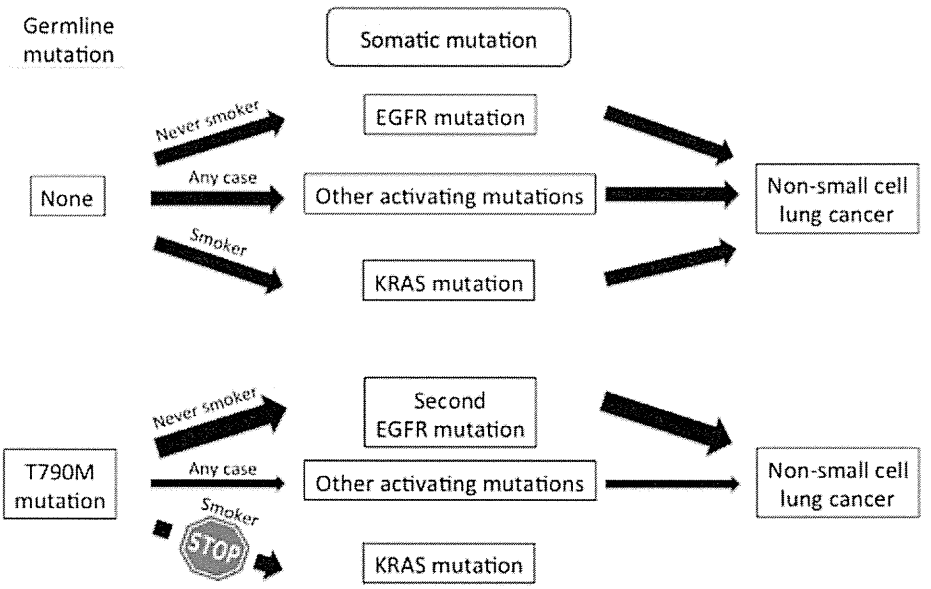

FIGURE 3. Hypothetical mutation-driven pathways to NSCLC development in patients with and without germline T790M mutations. In subjects without the germline mutation, the major pathways are via *EGFR* mutations (in NS) or via *KRAS* mutations (in ES), although other driver mutations may occur in either group. Subjects with germline T790M mutations, a weak driver mutation, usually require one or more other driver mutations for NSCLC development. In NS, the second mutation is usually another *EGFR* mutation although other pathways may occasionally be activated. In ES, the presence of the germline *EGFR* gene mutation and the smoking status decrease or preclude the possibility of developing a *KRAS* mutation, although alternative driver mutations may occasionally occur. ES, ever smoker; NS, never smoker; NSCLC, non–small-cell lung cancer.

TABLE 3. Comparison of Lung Cancers Associated with Sporadic and Germline Mutations of *EGFR* Gene

Feature	Sporadic	Germline
Frequency	Relatively common	Exceedingly rare
Lung cancer type	Mostly adenocarcinoma	Mostly adenocarcinoma
Ethnic preference	East Asian	White[a]
Gender preference	Female	Female
Smoking status	More frequent in never smokers	More frequent in never smokers
Field cancerization effects	Occasional	Occasional[a]
Presence of T790M	Rare at initial diagnosis	Present at initial diagnosis (by definition)
Presence of second activating *EGFR* mutation	Rare at initial diagnosis	Frequent at initial diagnosis
Inheritance	Not applicable	Dominant
Penetrance	Not applicable	May vary with smoke exposure[a]
Response to TKIs	Frequent initial response	Predicted to be resistant[a]

[a]Given the exceedingly rare prevalence of germline T790M mutations, several of the observations are tentative and need confirmation by study of further cases.
TKI, tyrosine kinase inhibitor.

who had partial responses. Thus, in the absence of another known or suspected molecular target, conventional chemotherapy seems to be the preferred first-line therapy option. Several newer third- and even fourth-generation TKIs have entered clinical trial, and some were designed to overcome T790M-mediated resistance[34,35] and offer the possibility of effective targeted therapy in the future. Regular CT scans are one method for the early detection of lung cancers developing in carriers, and the carriers in the family of our proband are being offered this option. Clearly, optimal management and prevention strategies will require more information obtained from observation, prevention, and therapy of many more carriers and cases. For these reasons, The Dana-Farber Cancer Institute has initiated the "INHERIT EGFR study: Investigating the hereditary risk from T790M" (http://clinicaltrials.gov/ct2/show/NCT01754025).

CONCLUSIONS

Germline T790M mutations result in a very rare and unique lung cancer hereditary syndrome that targets female NSs. The risk of lung cancer development in never smoking carriers is greater than the risk of heavy smokers with or without the mutation. Unaffected carriers with this mutation are at increased risk for developing lung cancer irrespective of their smoking status and should be followed by increased surveillance including low-dose CT scans. The resultant cancers share several features with lung cancers containing sporadic *EGFR* mutations and known or suspected differences.

ACKNOWLEDGMENTS

Funding was provided by the Texas Specialized Program of Research Excellence in Lung Cancer (P50CA70907), with additional support from the Harold C. Simmons Comprehensive Cancer Center, UT Southwestern Medical Center, Dallas, TX. The sponsors of the study had no role in study design, data collection, analysis or interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

REFERENCES

1. Linardou H, Dahabreh IJ, Bafaloukos D, et al. Somatic EGFR mutations and efficacy of tyrosine kinase inhibitors in NSCLC. *Nat Rev Clin Oncol* 2009;6:352–366.
2. Sharma SV, Bell DW, Settleman J, et al. Epidermal growth factor receptor mutations in lung cancer. *Nature Rev Cancer* 2007;7:169–181.
3. Shigematsu S, 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.
4. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS medicine* 2005;2:e73.
5. Yu HA, Arcila ME, Rekhtman N, et al. Analysis of Tumor Specimens at the Time of Acquired Resistance to EGFR-TKI Therapy in 155 Patients with EGFR-Mutant Lung Cancers. *Clin Cancer Res* 2013;19:2240–2247.
6. Godin-Heymann N, Bryant I, Rivera MN, et al. Oncogenic activity of epidermal growth factor receptor kinase mutant alleles is enhanced by the T790M drug resistance mutation. *Cancer Res* 2007;67:7319–7326.
7. Suda K, Onozato R, Yatabe Y, et al. EGFR T790M mutation: a double role in lung cancer cell survival? *J Thorac Oncol* 2009;4:1–4.
8. Regales L, Balak MN, Gong Y, et al. Development of new mouse lung tumor models expressing EGFR T790M mutants associated with clinical resistance to kinase inhibitors. *PLoS One* 2007;2:e810.
9. Vikis H, Sato M, James M, et al. EGFR-T790M is a rare lung cancer susceptibility allele with enhanced kinase activity. *Cancer Res* 2007;67:4665–4670.
10. Gandhi J, Zhang J, Xie Y, et al. Alterations in genes of the EGFR signaling pathway and their relationship to EGFR tyrosine kinase inhibitor sensitivity in lung cancer cell lines. *PLoS One* 2009;4:e4576.
11. Rosell R, Moran T, Carcereny E, et al. Non-small-cell lung cancer harbouring mutations in the EGFR kinase domain. *Clin Transl Oncol* 2010;12:75–80.
12. Soh J, Okumura N, Lockwood WW, et al. Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells. *PLoS One* 2009;4:e7464.
13. Oxnard GR, Miller VA, Robson ME, et al. Screening for germline EGFR T790M mutations through lung cancer genotyping. *J Thorac Oncol* 2012;7:1049–1052.
14. Asano H, Toyooka S, Tokumo M, et al. Detection of EGFR gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay. *Clin Cancer Res* 2006;12:43–48.
15. Borras E, Jurado I, Hernan I, et al. Clinical pharmacogenomic testing of KRAS, BRAF and EGFR mutations by high resolution melting analysis and ultra-deep pyrosequencing. *BMC cancer* 2011;11:406.
16. 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.

17. Bell DW, Gore I, Okimoto RA, et al. Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nature genetics 2005;37:1315–1316.
18. Girard N, Lou E, Azzoli CG, et al. Analysis of genetic variants in never-smokers with lung cancer facilitated by an Internet-based blood collection protocol: a preliminary report. Clin Cancer Res 2010;16:755–763.
19. Prudkin L, Tang X, Wistuba, II. Germ-line and somatic presentations of the EGFR T790M mutation in lung cancer. *J Thorac Oncol* 2009;4:139–141.
20. Tibaldi C, Giovannetti E, Vasile E, et al. Inherited germline T790M mutation and somatic epidermal growth factor receptor mutations in non-small cell lung cancer patients. *J Thorac Oncol* 2011;6:395–396.
21. Travis W, Brambilla E, Noguchi M, et al. The new IASLC/ATS/ERS international multidisciplinary lung adenocarcinoma classification. *Journal of Thoracic Oncology* 2009;4:244–285.
22. Stellman SD, Takezaki T, Wang L, et al. Smoking and lung cancer risk in American and Japanese men: an international case-control study. *Cancer Epidemiol Biomarkers Prev* 2001;10:1193–1199.
23. Wakelee HA, Chang ET, Gomez SL, et al. Lung cancer incidence in never smokers. *J Clin Oncol* 2007;25:472–478.
24. Yi JH, Huh J, Kim HJ, et al. Genome-wide single-nucleotide polymorphism array-based karyotyping in myelodysplastic syndrome and chronic myelomonocytic leukemia and its impact on treatment outcomes following decitabine treatment. *Annals of hematology* 2013;92:459–469.
25. Gazdar AF, Minna JD. Multifocal lung cancers--clonality vs field cancerization and does it matter? *Journal of the National Cancer Institute* 2009;101:541–543.
26. Flieder DB, Vazquez M, Carter D, et al. Pathologic findings of lung tumors diagnosed on baseline CT screening. *Am J Surg Pathol* 2006;30:606–613.
27. Chung JH, Choe G, Jheon S, et al. Epidermal growth factor receptor mutation and pathologic-radiologic correlation between multiple lung nodules with ground-glass opacity differentiates multicentric origin from intrapulmonary spread. *J Thorac Oncol* 2009;4:1490–1495.
28. Ashworth M. The pathology of preclinical medullary thyroid carcinoma. *Endocr Pathol* 2004;15:227–231.
29. Lohmann D. Retinoblastoma. *Advances in experimental medicine and biology* 2010;685:220–227.
30. Marsh DJ, Gimm O. Multiple endocrine neoplasia: types 1 and 2. *Advances in oto-rhino-laryngology* 2011;70:84–90.
31. Frank TS. Hereditary cancer syndromes. *Arch Pathol Lab Med* 2001;125:85–90.
32. Gatalica Z, Torlakovic E. Pathology of the hereditary colorectal carcinoma. *Familial cancer* 2008;7:15–26.
33. Tsao A, Tang X, Sabloff B, et al. Clinical-pathological characteristics of the EGFR gene mutation in non-small cell lung cancer. *J Thorac Oncol* 2006;1:231–239.
34. Hirsch FR, Janne PA, Eberhardt WE, et al. Epidermal Growth Factor Receptor Inhibition in Lung Cancer: Status 2012. *J Thorac Oncol* 2013;8:373–384.
35. Ohashi K, Maruvka YE, Michor F, et al. Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease. *J Clin Oncol* 2013;31:1070–1080.

Presence of the minor *EGFR* T790M mutation is associated with drug-sensitive *EGFR* mutations in lung adenocarcinoma patients

SHINSUKE HASHIDA[1], JUNICHI SOH[1], SHINICHI TOYOOKA[1,2], TOMOAKI TANAKA[3], MASASHI FURUKAWA[1], KAZUHIKO SHIEN[1,2], HIROMASA YAMAMOTO[1], HIROAKI ASANO[1], KAZUNORI TSUKUDA[1], KOICHI HAGIWARA[3] and SHINICHIRO MIYOSHI[1]

Departments of [1]Thoracic Surgery and [2]Clinical Genomic Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Okayama 700-8558; [3]Department of Respiratory Medicine, Saitama Medical University, Moroyama, Saitama 350-0495, Japan

Received March 4, 2014; Accepted April 22, 2014

DOI: 10.3892/or.2014.3197

Abstract. The T790M mutation in the epidermal growth factor receptor (*EGFR*) gene is known to be associated with the acquired resistance of lung adenocarcinoma patients to EGFR-tyrosine kinase inhibitors (EGFR-TKIs). The minor T790M mutant allele is occasionally detected in EGFR-TKI-naive tumor samples, yet findings concerning the clinical impact of the minor T790M mutation vary among previous studies. In the present study, we assessed the clinical impact of the minor T790M mutation using a novel, highly sensitive assay combining high-resolution melting (HRM), mutant-enriched PCR and co-amplification at a lower denaturation temperature (COLD)-PCR. We determined the T790M mutational status in 146 surgically resected lung adenocarcinomas without a history of EGFR-TKI treatment using mutant-enriched COLD-HRM (MEC-HRM) and standard HRM assays. The sensitivities of the MEC-HRM and standard HRM assays for the detection of T790M-mutant alleles among wild-type alleles were 0.01 and 10%, respectively. Although the T790M mutation was not detected using a standard HRM assay, we identified 19 (13%) T790M mutations using the MEC-HRM assay and defined these 19 mutations as minor T790M mutations. The proportion of T790M alleles was <0.1% in 17 (84%) of the 19 samples. Multivariate analyses revealed that a minor T790M mutation was significantly associated with the presence of *EGFR* exon 19 deletions or the L858R mutation (both of which are drug-sensitive *EGFR* mutations) (P=0.04). In conclusion, the minor *EGFR* T790M mutations were present in 13% of EGFR-TKI-naive surgically resected lung adenocarcinomas and were associated with drug-sensitive *EGFR* mutations.

Correspondence to: Professor Shinichi Toyooka, Department of Thoracic Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
E-mail: toyooka@md.okayama-u.ac.jp

Abbreviations: HRM, high resolution melting; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; COLD-PCR, CO-amplification at lower denaturation temperature PCR; MEC-HRM, mutant-enriched COLD-HRM

Key words: T790M mutation, high resolution melting analysis, mutant-enriched PCR, COLD-PCR, drug resistance

Introduction

Activating mutations of the epidermal growth factor receptor (*EGFR*) gene are characteristic genetic alterations in non-small lung cancer (NSCLC) patients, particularly in those with lung adenocarcinomas (1-3). *EGFR* exon 19 deletions or the exon 21 L858R mutation account for more than 90% of all *EGFR* mutations (4,5). Of particular importance, these mutations are known as predictors of a favorable clinical outcome in response to treatment with EGFR-tyrosine kinase inhibitors (EGFR-TKIs) (1,2). Although ~80% of NSCLC patients with drug-sensitive *EGFR* mutations such as *EGFR* exon 19 deletions or the exon 21 L858R mutation initially show satisfactory responsiveness to EGFR-TKI treatment (1,2), acquired resistance to EGFR-TKIs occurs in most cases (6). Half of all resistance to EGFR-TKIs is caused by an acquired T790M mutation in exon 20 of the *EGFR* gene (7-9). The T790M mutation has been reported to cause EGFR-TKI resistance by sterically hindering the binding site of gefitinib and erlotinib, two first-generation EGFR-TKIs (7), thereby causing a relative decrease in binding with EGFR-TKIs (10).

The T790M mutation has been detected in some patients who have not been treated with EGFR-TKIs (11-16). Since the incidence of a T790M mutation has been reported to range from 0.02 to 0.05% in all surgically resected NSCLC patients based on studies using direct sequencing (2,17), it is difficult to clarify the association due to the T790M mutation and clinicopathological factors because of the limitation in the number of cases. Using highly sensitive assays, the clinical impact of the minor T790M mutation in NSCLC patients with EGFR-TKI treatment has been previously investigated (11,14,15). However, the impact on EGFR-TKI-naive NSCLC patients has not been adequately investigated (12,13,16). Recently, various therapies to overcome the T790M mutation are being developed. These include next generation EGFR-TKIs (18) and combination

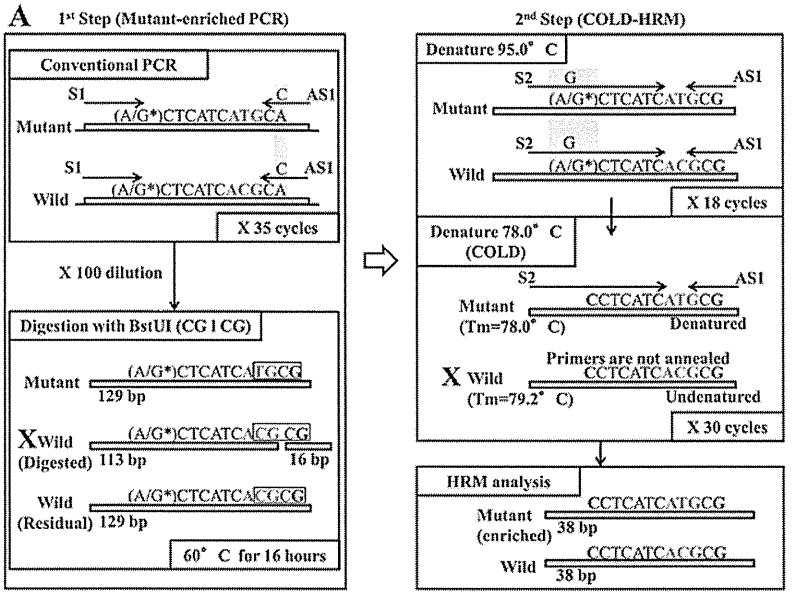

Figure 1. (A) Principle of the mutant-enriched COLD-HRM (MEC-HRM) assay. In the first step (left box), the upper box indicates the conventional PCR step for the amplification of *EGFR* exon 20, including codon 790. The mismatched site of the AS1 primer to introduce the CGCG sequence (framed) is emphasized with light gray shading. The exchanged bases are emphasized with bold print. An asterisk indicates the SNP site. The lower box indicates the step for the selective digestion of the wild-type amplicon. The crosses are applied on reduced amplicons. After digestion, the product that includes the mutant amplicon and residual undigested wild-type amplicon is used for the second step. The upper box indicates the first step of the real-time PCR for the amplification of the template product. The mismatched site of the S2 primer to diminish the influence of SNP is emphasized in light gray shading. The lower box indicates the second step, which is a COLD-PCR step for the selective amplification of the mutant. The Td (78.0°C) was lower than Tm of the wild-type amplicon; thus, they were not denatured and the primers were not annealed. The final products were analyzed using an HRM analysis.

therapies (19). Minor T790M mutated clones are enriched by EGFR-TKI treatment (12,16); thus, early detection of a minor T790M mutation using a highly sensitive assay may be useful for predicting the cause of resistance to EGFR-TKIs and for selecting optimum therapeutic strategies.

In the present study, we determined the T790M mutational status using high-resolution melting (HRM) analysis combined with mutant-enriched (12,20) and co-amplification at lower denaturation temperature (COLD)-polymerase chain reactions (PCRs) (21-24) to investigate the relationship between the presence of minor T790M mutations and clinicopathological factors in EGFR-TKI-naive lung adenocarcinoma patients with pulmonary resection.

Materials and methods

Clinical samples, cell lines and DNA extraction. We studied 146 patients with lung adenocarcinomas who underwent surgical resection without a preoperative history of EGFR-TKI treatment at our institute between 2006 and 2009. Approval of the Institutional Review Board and the informed consent of each patient were obtained. After pulmonary resection, fresh tissue was immediately frozen and stored at -80°C. DNA was extracted from the frozen tissue using proteinase K treatment followed by phenol-chloroform extraction (25). We also used a human bronchial epithelial cell line (HBEC-5KT) harboring the wild-type *EGFR* gene and the NCI-H1975 cell line (H1975) harboring the *EGFR* mutations L858R and T790M as negative and positive controls, respectively. These cell lines were kindly provided by Dr Adi F. Gazdar (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). DNA of the cell lines was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

Plasmids containing exon 20 of the EGFR gene. We used a plasmid containing *EGFR* exon 20 with the T790M mutation, which is one of the standardized plasmids containing each of the *EGFR* mutations occurring in the exons, and was used in a study by Goto *et al* (26). We also constructed a plasmid containing wild-type *EGFR* exon 20 using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) as previously reported by us (27). The sequences of each plasmid were confirmed by direct sequencing.

Detection of EGFR exon 19 deletions and the L858R mutation. *EGFR* exon 19 deletions and the exon 21 L858R mutation were examined using a restriction fragment length polymorphism (RFLP) assay without the enrichment of mutant alleles, as previously reported by us (20). We defined these *EGFR* exon 19 deletions and the exon 21 L858R mutation as drug-sensitive *EGFR* mutations.

Detection of the EGFR T790M mutation. Mutant-enriched COLD-HRM (MEC-HRM) is a two-step PCR-based assay combining a standard HRM assay with a mutant-enriched PCR (12,20) that enriches the mutant allele by the intermittent restriction digestion of the wild-type allele and a COLD-PCR (21) that enriches the mutant allele by means of the difference in melting temperatures between the mutant and wild-type alleles. The principle of the MEC-HRM assay is shown in Fig. 1A.

As the first step, conventional PCR was performed to amplify the target region (129 base pairs), including codon 790

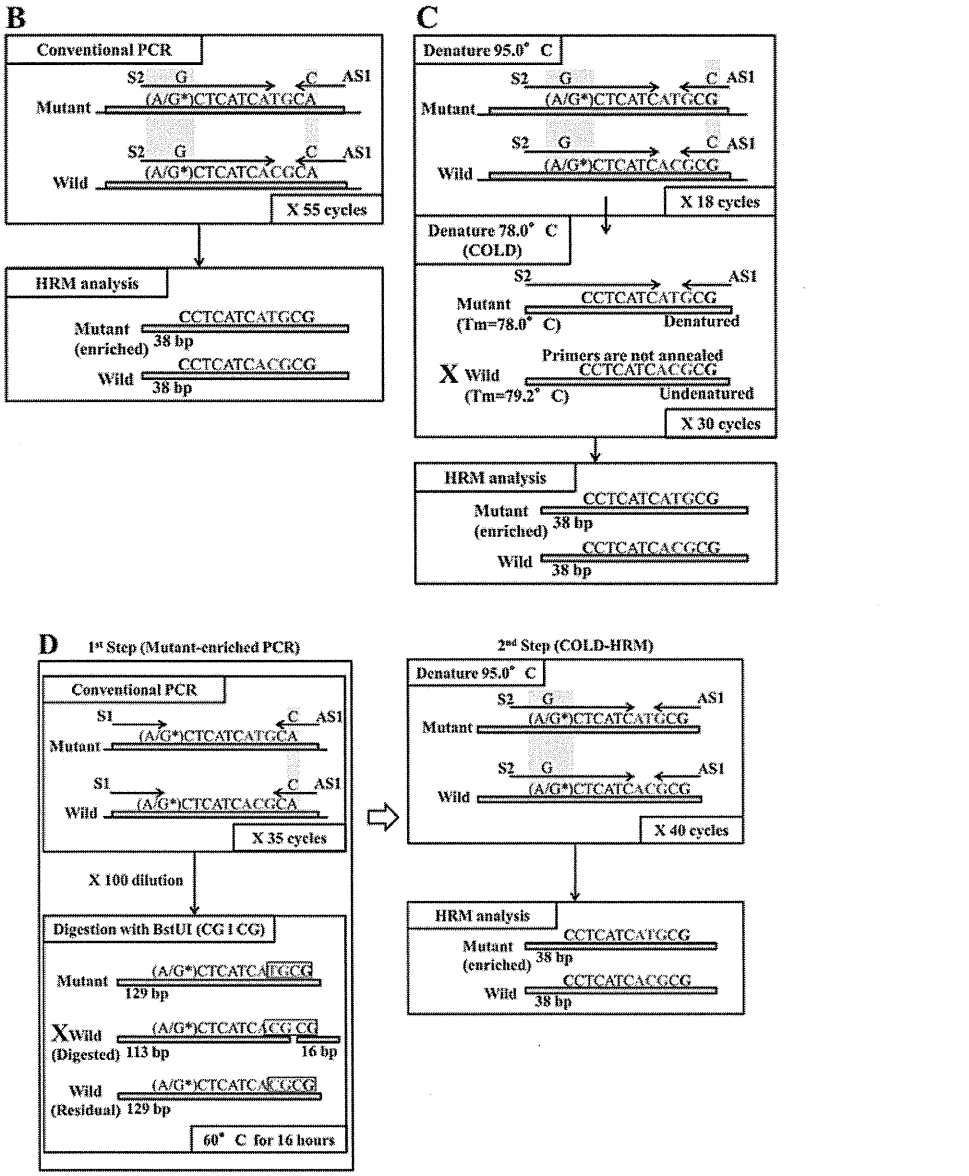

Figure 1. Continued. (B) Principle of standard HRM assay. The box indicates the real-time PCR step using the S2 primer (forward) and the AS1 primer (reverse). The real-time PCR conditions are as follows: an initial denaturation step at 95°C for 10 min, and an amplification step for 55 cycles (95°C for 15 sec, 60°C for 60 sec). The final products are analyzed using HRM analysis. (C) Principle of COLD-HRM assay. The upper box indicates the first step of 18 cycles of real-time PCR for the amplification of the template product, and the lower box indicates the second step, which is a COLD-PCR step, for the selective amplification of the mutant allele. In the second step, the denaturing temperature (Td) is decreased to 78°C and amplification is performed for 30 cycles. The final products are analyzed using HRM analysis. (D) Principle of mutant-enriched HRM assay. In the first step (left box), conventional PCR and digestion are performed, similar to that shown in the left box of (A). The second step (right box) is performed in the same manner as standard HRM (B) using the products from the first step. Forty cycles are used for the mutant-enriched HRM assay, instead of 55 cycles for the standard HRM assay.

of the *EGFR* gene, using the GeneAmp® 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The forward primer sequence was 5'-ACTGACGTGCCTCTCCCTCC-3' (S1). The reverse primer sequence was 5'-CGAAGGGCATG AGCC*GC-3' (AS1), harboring one mismatched site (* T to C) to introduce the CGCG sequence into the amplicon of the wild-type. The final volume of the PCR mixture was 10 μl contained 100 ng of sample DNA, 150 nmol of deoxynucleotide triphosphate, 2 pmol of each primer, and 1 unit of HotStarTaq DNA Polymerase Plus (Qiagen). The PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 20 sec. Diluted PCR products (100-fold dilution with distilled water) were treated with the restriction enzyme *Bst*UI (New England BioLabs, Ipswich, MA, USA), which digests the wild-type allele but not the mutant allele, for 16 h at 60°C, resulting in the enrichment of the mutant alleles, as previously reported by us (12).

After the first step, the second step (COLD-PCR, including melting curve analysis) was performed using the StepOnePlus™ real-time PCR system (Applied Biosystems). The forward primer sequence was 5'-CCTCCACCGTGCAC*CTCATC-3' (S2). Since one SNP site (rs1050171 A/G) exists in the S2 primer sequence, we designed a mismatched-base (* C) at the SNP position to diminish the influence of the SNP (28). We used the AS1 primer as a reverse primer. The final volume of the PCR mixture was 20 μl, containing 10 μl of MeltDoctor™ Master Mix (Applied Biosystems), 12 pmol of each primer and

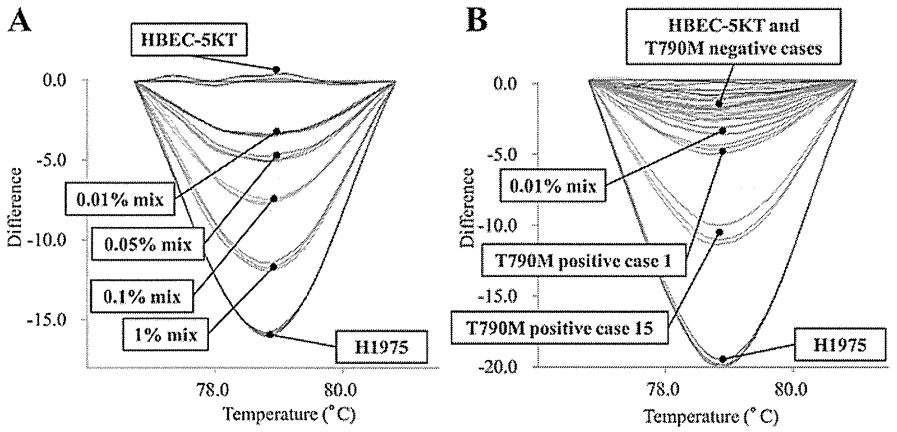

Figure 2. Sensitivity of MEC-HRM assay and representative clinical samples. (A) Difference plot showing the sensitivity of the MEC-HRM assay. We selected wild-type DNA (HBEC-5KT) for the standard of difference plots (horizontal line). Each percentage indicates the proportion of T790M-mutant DNA out of wild-type DNA. Consequently, the MEC-HRM assay was able to detect a T790M-mutant DNA (H1975) level of 0.01% among wild-type DNA (HBEC-5KT). (B) The red curves indicate a DNA mixture containing 0.01% H1975 DNA among HBEC-5KT DNA. The green curves indicate the clinical samples determined as T790M-negative, and the blue curves indicate the samples determined as T790M-positive. Case 1 contains a 0.01-0.1% proportion of T790M clones, and case 15 contains a 0.1-1% proportion of T790M clones.

1 μl of the first-step product. The real-time PCR conditions were as follows: an initial denaturation step at 95°C for 10 min, 18 cycles for the first round of standard amplification (95°C for 15 sec, 60°C for 60 sec), and 30 cycles for the second round of amplification to enrich the mutant-amplicons (78°C for 15 sec and 60°C for 60 sec). The denaturing temperature (Td) of the COLD-PCR step (78°C) was determined using a melting curve analysis of the standard HRM [the melting temperatures (Tm) of the mutant-type and wild-type amplicons were 78°C and 79.2°C, respectively] and an evaluation of the sensitivity of COLD-PCR from Td 79.2 to 78°C every 0.2°C. After the amplification step, a melting curve was generated and analyzed using HRM software ver. 3.0.1 (Applied Biosystems).

We also performed standard-HRM, COLD-HRM and mutant-enriched HRM assays. The principles of these three assays are provided in Fig. 1B-D. All the samples, including the standard DNA mixtures, were analyzed in triplicate in all the assays. The sensitivities of all four assays were determined using multiple DNA mixtures of T790M mutant and wild-type alleles (0.01, 0.05, 0.1, 1, 10, 20 or 50% of T790M mutant allele).

Statistical analysis. Chi-square tests or Fisher's exact tests were used to examine the differences in categorical factors across groups, as appropriate. The multivariate logistic regression model was used to identify clinicopathological factors that might independently predict the presence of T790M mutations.

After pulmonary resection, imaging studies were repeated every 3 months for at least 2 years and every 6 months thereafter for 3 years. After 5 years, medical examinations were repeated every year. The progression-free survival (PFS) was calculated from the date of surgery until confirmed disease recurrence or death. The overall survival (OS) was calculated from the date of surgery until the date of death or the last follow-up. The survival curve was calculated using the Kaplan-Meier method, and the difference between groups was compared using the log-rank test. Multivariate analyses were performed using the Cox proportional hazard model. All the data were analyzed using JMP, version 9.0.0 (SAS Institute Inc., Cary, NC, USA). All the statistical tests were two-sided, and probability values (P) <0.05 were considered statistically significant.

Results

Sensitivity of each assay for the detection of the T790M mutation. The MEC-HRM assay was able to detect the T790M mutant allele in the sample with a mutant allele content of 0.01%. In other words, it detected the mutant allele in the presence of a 10,000-fold background of wild-type allele (Figs. 2 and 3). Meanwhile, the detection limits of standard HRM, COLD-HRM and mutant-enriched HRM assays were a mutant allele content of 10, 1 and 0.1%, respectively (Fig. 4). Based on these data, we investigated the T790M mutation in 146 clinical samples using the standard HRM using a DNA mixture with T790M content of 10% as a positive control, and the MEC-HRM assays using a DNA mixture with T790M content of 0.01% as a positive control. Clinical samples and positive controls were investigated in the same plate in triplicate. When all three signals from a clinical sample exceeded the maximal signal from the control, the sample was identified to be positive for the T790M mutation (Fig. 2).

EGFR mutations in the clinical samples. Drug-sensitive *EGFR* mutations were found in 54 (37%) of the 146 lung adenocarcinomas (26 exon 19 deletions and 28 L858R mutations). Drug-sensitive *EGFR* mutations were significantly associated with females (P<0.01) and never-smokers (P<0.01).

Although standard HRM did not detect any *EGFR* T790M mutations, *EGFR* T790M mutations were detected in 19 (13%) of the 146 lung adenocarcinomas using the MEC-HRM assay. Thus, these 19 patients were defined as harboring minor T790M mutations. We confirmed that these 19 tumor samples had a tumor cell composition of at least 20% by examining the proportion of tumor cells in the tissue using hematoxylin and eosin staining. Next, we determined the dosage of the T790M mutant allele in 19 mutant samples by reanalyzing these samples and comparing them with 10, 1, 0.1 and 0.01% standard T790M mutant DNA mixtures. We subcategorized the

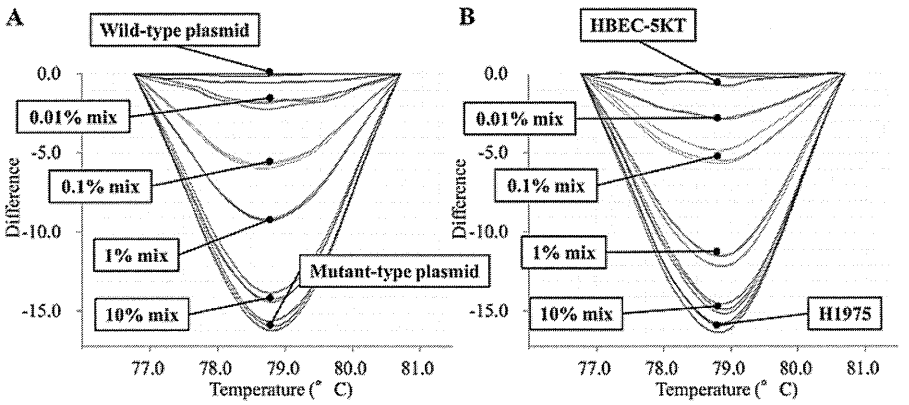

Figure 3. Comparison of sensitivities between plasmid and genomic DNA mixes. (A) The MEC-HRM assay was able to detect the T790M mutant allele at a level of 0.01% among wild-type alleles using the plasmid mixture. (B) The MEC-HRM assay showed similar sensitivities for the genomic DNA mixtures (H1975 and HBEC-5KT) and the plasmid mixtures.

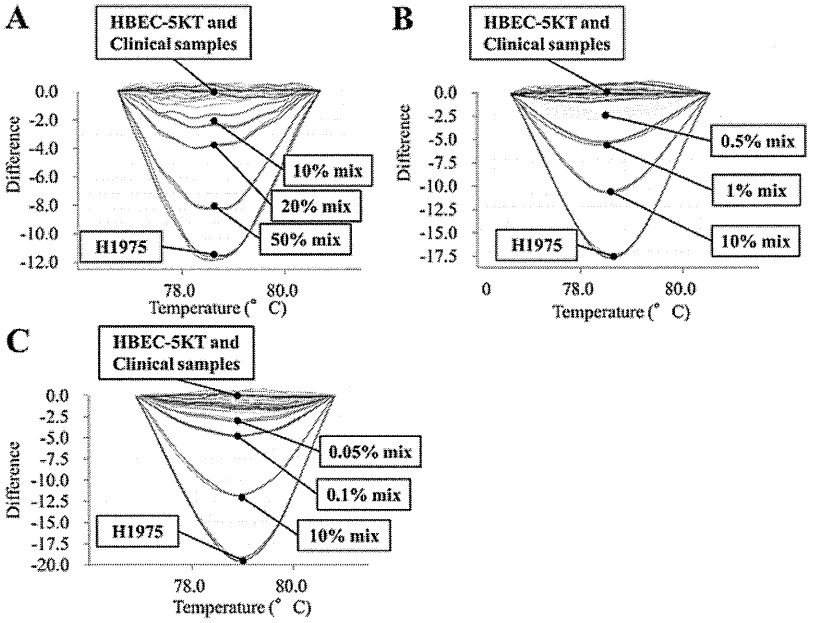

Figure 4. Sensitivities of standard HRM, COLD-HRM and mutant-enriched HRM assays. The difference plots for all three assays are shown. Wild-type DNA was used as a negative standard, and the percentages in each figure indicate the proportion of T790M-mutant DNA out of wild-type DNA. Consequently, the (A) standard HRM, (B) COLD-HRM and (C) mutant-enriched HRM assays were able to detect T790M-mutant DNA at levels of 10, 1 and 0.1% among wild-type DNA, respectively.

19 minor T790M mutations into groups with T790M mutant DNA levels corresponding to 0.01-0.1%, 0.1-1% and 1-10%. As shown in Table I, 17 of the 19 patients (84%) had a T790M mutant allele proportion of <0.1%.

The details of the 19 patients harboring minor T790M mutations and the relationships between a minor T790M mutational status and clinicopathological factors are shown in Tables I and II, respectively. Minor T790M mutation was not significantly associated with age, gender, pathological stage, smoking history or drug-sensitive *EGFR* mutations in univariate analyses. However, drug-sensitive *EGFR* mutations are considered to be associated with age, gender and smoking status (27,29,30); thus, a multivariate analysis was performed to identify independent factors associated with a minor T790M mutation. Consequently, the minor T790M mutation was found to be independently associated with drug-sensitive *EGFR* mutations (OR, 3.0; 95% CI, 1.0-9.0; P=0.04) (Table III).

Regarding the prognostic impact of the minor T790M mutation, it was not associated with either the OS or the PFS in our cohort (Table IV).

Discussion

In the present study, we determined the T790M mutational status using our newly developed, highly sensitive assay, the MEC-HRM assay. We previously developed a mutant-enriched PCR assay (sensitivity, 0.1%) and found that the T790M mutation was detected in 3.8% of NSCLC patients without a history of treatment with EGFR-TKIs (12). Su *et al* (16) found that the T790M mutation was present in 25.2% of EGFR-TKI-naive NSCLC patients using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (sensitivity, 1.5%), and Oh *et al* (13) found that the T790M mutation was present in 8.2% of EGFR-TKI-naive NSCLC

Table I. Details of the 19 T790M-positive patients.

Case	Age (years)	Gender	Smoking history	Pathological stage	Drug-sensitive *EGFR* mutation	Proportion of T790M clones
1	59	Male	Never-smoker	I	ex19 del.	0.01-0.1%
2	75	Male	Never-smoker	I	ex19 del.	0.01-0.1%
3	75	Female	Never-smoker	I	L858R	0.01-0.1%
4	75	Female	Never-smoker	I	L858R	0.01-0.1%
5	79	Female	Never-smoker	I	L858R	0.01-0.1%
6	60	Female	Never-smoker	I	WT	0.01-0.1%
7	60	Male	Never-smoker	III	WT	0.01-0.1%
8	66	Male	Smoker	I	ex19 del.	0.01-0.1%
9	57	Female	Smoker	I	ex19 del.	0.1-1%
10	76	Male	Smoker	I	ex19 del.	0.01-0.1%
11	82	Male	Smoker	I	L858R	0.01-0.1%
12	58	Male	Smoker	I	L858R	0.01-0.1%
13	56	Male	Smoker	I	WT	0.01-0.1%
14	32	Female	Smoker	I	WT	0.1-1%
15	59	Female	Smoker	I	WT	0.01-0.1%
16	78	Female	Smoker	I	WT	0.01-0.1%
17	68	Male	Smoker	I	WT	0.01-0.1%
18	74	Male	Smoker	I	WT	0.01-0.1%
19	84	Female	Smoker	II	L858R	1-10%

ex19 del., exon 19 deletion; WT, wild-type. Drug-sensitive *EGFR* mutations include detection of exon19 deletions and the L858R mutation.

Table II. Relationship between minor T790M mutations and clinicopathological factors.

Subsets (n=146)	T790M-positive (n=19) n, (%)	T790M-negative (n=127) n, (%)	P-value
Age (median; range) (68; 32-87 years)			
<68 (n=72)	9 (47.4)	63 (49.6)	0.9
≥68 (n=74)	10 (52.6)	64 (50.4)	
Gender (male vs. female)			
Male (n=71)	10 (52.6)	61 (48.0)	0.7
Female (n=75)	9 (47.4)	66 (52.0)	
Smoking history			
Smoker (n=74)	12 (63.2)	62 (48.8)	0.2
Never-smoker (n=72)	7 (36.8)	65 (51.2)	
Pathological stage			
I (n=103)	17 (89.5)	86 (67.7)	0.06
II (n=12)	1 (5.3)	11 (8.7)	(I vs. others)
III (n=21)	1 (5.3)	20 (15.7)	
IV[a] (n=10)	0 (0.0)	10 (7.9)	
Drug-sensitive *EGFR* mutation			
Mutant (n=54)	10 (52.6)	44 (34.6)	0.1
Wild (n=92)	9 (47.4)	83 (65.4)	

[a]Stage IV includes 4 recurrent patients. Drug-sensitive *EGFR* mutations include detection of exon19 deletions and the L858R mutation.

Table III. Multivariate analysis of the minor T790M mutation-related factors.

Variables	OR (95% CI)	P-value
Age (years) (<68 vs. ≥68)	1.0 (0.4-2.6)	1.0
Gender (Male vs. female)	0.6 (0.2-2.5)	0.5
Smoking history (Smoker vs. never-smoker)	3.8 (0.9-18)	0.08
Drug-sensitive *EGFR* mutation (Mutant vs. wild)	3.0 (1.0-9.0)	0.04

OR, odds ratio; CI, confidence interval. Drug-sensitive *EGFR* mutation include detection of exon19 deletions and the L858R mutation.

patients using peptide nucleic acid (PNA)-clamping PCR with a melting curve analysis (sensitivity, 0.01%). The present study revealed that the MEC-HRM assay could detect the T790M mutation in 13% of EGFR-TKI-naive adenocarcinomas. Of note, our standard HRM assay (10% sensitivity) did not detect any T790M-positive specimens, and we considered all the T790M mutations that were detected using the MEC-HRM assay to be minor mutations (2,17).

As novel findings, the minor T790M mutation was significantly associated with drug-sensitive *EGFR* mutations in a multivariate analysis. Previous reports have described that drug-sensitive *EGFR* mutations and the T790M mutation generally occur *in cis* (31-33), although the statistical relationship between minor T790M mutation and drug-sensitive *EGFR* mutations has never been revealed in clinical samples. Our present study is, to the best of our knowledge, the first report to confirm that the minor T790M mutation was significantly associated with drug-sensitive EGFR mutations in lung adenocarcinomas. To understand this situation, two issues should be discussed: i) the relationship between drug-sensitive *EGFR* mutations and the T790M mutation, and ii) the presence of the T790M mutation as a minor population. Regarding the first issue, while the reason for this association is unknown, lung cancers with germ-line *EGFR* mutations such as T790M (31), V843I (34), and R776H (35) are accompanied by additional *EGFR* mutations in the development of lung cancer, suggesting that *EGFR* mutations themselves cause genetic instability, thereby predisposing cells to additional mutations within the gene (34). The second issue can be explained by the following hypothesis. During the carcinogenic process of *EGFR*-mutant-related lung cancer, drug-sensitive *EGFR* mutations occur first in the progenitor cells of lung cancer, followed by the T790M mutation. At this stage, the tumor consists of a heterogeneous population of drug-sensitive *EGFR* mutant cells with or without the T790M mutation. Previous studies have reported that drug-sensitive *EGFR* mutant cells with an additional T790M mutation exhibit an indolent progression, indicating that the possession of the T790M mutation is a disadvantage for cell proliferation (36). This fact suggests that cancer cells with only drug-sensitive *EGFR* mutations may be dominant in tumors, in addition to those with T790M and drug-sensitive *EGFR* mutations as a minor population.

Our study also suggested that the T790M mutation tended to be associated with a smoking history in this study. Drug-sensitive *EGFR* mutations are known to be frequent in never-smokers with NSCLCs. However, drug-sensitive *EGFR* mutations themselves have not been reported to be associated with a never-smoking habit (30). Matsuo *et al* (30) indicated that *EGFR* mutations presumably occur in both smokers and never-smokers with a similar incidence, but other smoking-related mutations such as TP53 or KRAS mutations preferentially occur in smokers resulting in the enrichment of the prevalence of *EGFR* mutations in never-smokers. In fact, the average mutation frequency has been reported to be >10-fold higher in smokers than in never-smokers (37). Considering these observations, the *EGFR* mutation at codon

Table IV. Multivariate analysis for recurrence and mortality.

	PFS		OS	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (<68 vs. ≥68 years)	0.3 (0.1-0.6)	<0.01	0.3 (0.1-0.9)	0.02
Gender (Male vs. female)	0.7 (0.2-1.9)	0.4	1.0 (0.3-3.6)	0.9
Smoking history (Smoker vs. never-smoker)	2.3 (0.8-7.3)	0.1	2.1 (0.6-8.5)	0.3
Pathological stage (I vs. II-IV)	0.2 (0.09-0.4)	<0.01	0.2 (0.05-0.4)	<0.01
Drug-sensitive *EGFR* mutation (Mutant vs. wild)	1.0 (0.4-2.2)	1.0	0.5 (0.1-1.4)	0.2
T790M mutation (Mutant vs. wild)	0.5 (0.08-1.8)	0.3	1.7 (0.4-5.9)	0.4

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval. Drug-sensitive EGFR mutations include detection of exon19 deletions and the L858R mutation.

790, unlike drug-sensitive *EGFR* mutations, might be susceptible to tobacco-related carcinogens.

In conclusion, we revealed an association between the minor T790M mutation and clinicopathological factors in surgically resected lung adenocarcinoma patients without a history of EGFR-TKI treatment using our novel, highly sensitive assay. Minor T790M mutations are independently associated with drug-sensitive *EGFR* mutations.

Acknowledgements

The authors thank Ms. Fumiko Isobe (Department of Thoracic, Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan) for her technical support. The present study was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (S.T.).

References

1. Mitsudomi T, Morita S, Yatabe Y, *et al*: Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 11: 121-128, 2010.
2. Sequist LV, Martins RG, Spigel D, *et al*: First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic *EGFR* mutations. J Clin Oncol 26: 2442-2449, 2008.
3. 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 350: 2129-2139, 2004.
4. Yamamoto H, Toyooka S and Mitsudomi T: Impact of *EGFR* mutation analysis in non-small cell lung cancer. Lung Cancer 63: 315-321, 2009.
5. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T and Mitsudomi T: Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 64: 8919-8923, 2004.
6. Araki T, Yashima H, Shimizu K, *et al*: Review of the treatment of non-small cell lung cancer with gefitinib. Clin Med Insights Oncol 6: 407-421, 2012.
7. Kobayashi S, Boggon TJ, Dayaram T, *et al*: *EGFR* mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 352: 786-792, 2005.
8. Sequist LV, Waltman BA, Dias-Santagata D, *et al*: Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. Sci Transl Med 3: 75ra26, 2011.
9. Oxnard GR, Arcila ME, Sima CS, *et al*: Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. Clin Cancer Res 17: 1616-1622, 2011.
10. Eck MJ and Yun CH: Structural and mechanistic underpinnings of the differential drug sensitivity of EGFR mutations in non-small cell lung cancer. Biochim Biophys Acta 1804: 559-566, 2010.
11. Maheswaran S, Sequist LV, Nagrath S, *et al*: Detection of mutations in *EGFR* in circulating lung-cancer cells. N Engl J Med 359: 366-377, 2008.
12. Inukai M, Toyooka S, Ito S, *et al*: Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. Cancer Res 66: 7854-7858, 2006.
13. Oh JE, An CH, Yoo NJ and Lee SH: Detection of low-level *EGFR* T790M mutation in lung cancer tissues. APMIS 119: 403-411, 2011.
14. Fujita Y, Suda K, Kimura H, *et al*: Highly sensitive detection of EGFR T790M mutation using colony hybridization predicts favorable prognosis of patients with lung cancer harboring activating EGFR mutation. J Thorac Oncol 7: 1640-1644, 2012.
15. Rosell R, Molina MA, Costa C, *et al*: Pretreatment EGFR T790M mutation and BRCA1 mRNA expression in erlotinib-treated advanced non-small-cell lung cancer patients with EGFR mutations. Clin Cancer Res 17: 1160-1168, 2011.
16. Su KY, Chen HY, Li KC, *et al*: Pretreatment epidermal growth factor receptor (*EGFR*) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. J Clin Oncol 30: 433-440, 2012.
17. Toyooka S, Kiura K and Mitsudomi T: *EGFR* mutation and response of lung cancer to gefitinib. N Engl J Med 352: 2136, 2005.
18. Sakuma Y, Yamazaki Y, Nakamura Y, *et al*: WZ4002, a third-generation *EGFR* inhibitor, can overcome anoikis resistance in *EGFR*-mutant lung adenocarcinomas more efficiently than Src inhibitors. Lab Invest 92: 371-383, 2012.
19. Regales L, Gong Y, Shen R, *et al*: Dual targeting of EGFR can overcome a major drug resistance mutation in mouse models of *EGFR* mutant lung cancer. J Clin Invest 119: 3000-3010, 2009.
20. Asano H, Toyooka S, Tokumo M, *et al*: Detection of *EGFR* gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay. Clin Cancer Res 12: 43-48, 2006.
21. Li J, Wang L, Mamon H, Kulke MH, Berbeco R and Makrigiorgos GM: Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med 14: 579-584, 2008.
22. Luthra R and Zuo Z: COLD-PCR finds hot application in mutation analysis. Clin Chem 55: 2077-2078, 2009.
23. Milbury CA, Li J and Makrigiorgos GM: COLD-PCR-enhanced high-resolution melting enables rapid and selective identification of low-level unknown mutations. Clin Chem 55: 2130-2143, 2009.
24. Song C, Milbury CA, Li J, Liu P, Zhao M and Makrigiorgos GM: Rapid and sensitive detection of KRAS mutation after fast-COLD-PCR enrichment and high-resolution melting analysis. Diagn. Mol Pathol 20: 81-89, 2011.
25. Fan H and Gulley ML: DNA extraction from fresh or frozen tissues. Methods Mol Med 49: 5-10, 2001.
26. Goto K, Satouchi M, Ishii G, *et al*: An evaluation study of *EGFR* mutation tests utilized for non-small-cell lung cancer in the diagnostic setting. Ann Oncol 23: 2914-2919, 2012.
27. 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 11: 1167-1173, 2005.
28. Do H, Krypuy M, Mitchell PL, Fox SB and Dobrovic A: High resolution melting analysis for rapid and sensitive *EGFR* and *KRAS* mutation detection in formalin fixed paraffin embedded biopsies. BMC Cancer 8: 142, 2008.
29. Ueno T, Toyooka S, Suda K, *et al*: Impact of age on epidermal growth factor receptor mutation in lung cancer. Lung Cancer 78: 207-211, 2012.
30. Matsuo K, Ito H, Yatabe Y, *et al*: Risk factors differ for non-small-cell lung cancers with and without *EGFR* mutation: assessment of smoking and sex by a case-control study in Japanese. Cancer Sci 98: 96-101, 2007.
31. Bell DW, Gore I, Okimoto RA, *et al*: Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nat Genet 37: 1315-1316, 2005.
32. Soh J, Okumura N, Lockwood WW, *et al*: Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells. PLoS One 4: e7464, 2009.
33. Pao W, Miller VA, Politi KA, *et al*: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2: e73, 2005.
34. Ohtsuka K, Ohnishi H, Kurai D, *et al*: Familial lung adenocarcinoma caused by the *EGFR* V843I germ-line mutation. J Clin Oncol 29: e191-e192, 2011.
35. van Noesel J, van der Ven WH, van Os TA, *et al*: Activating germline R776H mutation in the epidermal growth factor receptor associated with lung cancer with squamous differentiation. J Clin Oncol 31: e161-e164, 2013.
36. Chmielecki J, Foo J, Oxnard GR, *et al*: Optimization of dosing for EGFR-mutant non-small cell lung cancer with evolutionary cancer modeling. Sci Transl Med 3: 90ra59, 2011.
37. Govindan R, Ding L, Griffith M, *et al*: Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell 150: 1121-1134, 2012.

Cardiovasc Intervent Radiol
DOI 10.1007/s00270-014-0926-x

CIRSE

CLINICAL INVESTIGATION

Percutaneous Radiofrequency Ablation of Lung Cancer Presenting as Ground-Glass Opacity

Toshihiro Iguchi · Takao Hiraki · Hideo Gobara · Hiroyasu Fujiwara · Yusuke Matsui · Junichi Soh · Shinichi Toyooka · Katsuyuki Kiura · Susumu Kanazawa

Received: 9 January 2014 / Accepted: 21 April 2014

Abstract
Purpose We retrospectively evaluated the outcomes of lung cancer patients presenting with ground-glass opacity (GGO) who received radiofrequency ablation (RFA).
Methods Sixteen patients (5 men and 11 women; mean age, 72.6 years) with 17 lung cancer lesions showing GGO (mean long axis diameter, 1.6 cm) underwent a total of 20 percutaneous computed tomography (CT) fluoroscopy-guided RFA sessions, including three repeated sessions for local progression. Lung cancer with GGO was defined as a histologically confirmed malignant pulmonary lesion with a GGO component accounting for >50 % of the lesion on high-resolution CT. Procedure outcomes were evaluated.
Results There were no major complications. Pneumothorax occurred in 15 of 20 treatment sessions: 14 were asymptomatic, and 1 required chest tube placement but resolved satisfactorily within 48 h. Minor pulmonary hemorrhage occurred in two and mild pneumonitis in one. The median tumor follow-up period was 61.5 (range 6.1–96.6) months. The effectiveness rates of the primary and secondary techniques were 100 and 100 % at 1 year, 93.3 and 100 % at 2 years, and 78.3 and 92.3 % at 3 years, respectively. The median patient follow-up period was 65.6 (range 6.1–96.6) months. One patient died owing to recurrent other cancer 11.7 months after RFA, whereas the other 15 remained alive. Overall survival and disease-

T. Iguchi (✉) · T. Hiraki · H. Gobara · H. Fujiwara · Y. Matsui · S. Kanazawa
Department of Radiology, Okayama University Medical School, 2-5-1 Shikata-cho kita-ku, Okayama 700-8558, Japan
e-mail: iguchi@ba2.so-net.ne.jp

T. Hiraki
e-mail: takaoh@tc4.so-net.ne.jp

H. Gobara
e-mail: gobara@cc.okayama-u.ac.jp

H. Fujiwara
e-mail: hirofujiwar@gmail.com

Y. Matsui
e-mail: wckyh140@yahoo.co.jp

S. Kanazawa
e-mail: susumu@cc.okayama-u.ac.jp

J. Soh · S. Toyooka
Department of General Thoracic Surgery, Okayama University Medical School, 2-5-1 Shikata-cho kita-ku, Okayama 700-8558, Japan
e-mail: soh-j@cc.okayama-u.ac.jp

S. Toyooka
e-mail: shintoyooka@gmail.com

S. Toyooka
Department of Clinical Genomic Medicine, Okayama University Medical School, 2-5-1 Shikata-cho kita-ku, Okayama 700-8558, Japan

K. Kiura
Department of Respiratory Medicine, Okayama University Medical School, 2-5-1 Shikata-cho kita-ku, Okayama 700-8558, Japan
e-mail: kkiura@md.okayama-u.ac.jp

Published online: 18 June 2014

specific survival rates were 93.3 and 100 % at 1 year and 93.3 and 100 % at 5 years, respectively.
Conclusions RFA for lung cancer with GGO was safe and effective, and resulted in promising survival rates.

Keywords Radiofrequency ablation · Lung · Cancer

Introduction

With recent advances in diagnostic imaging technologies, such as computed tomography (CT), pulmonary lesions with ground-glass opacity (GGO) often are detected incidentally by chest CT. Lesions with GGO cannot be ignored, even if they are small, because some of them may have malignant potential. Usually, small lesions with GGO are followed up with periodic CT. Indications of malignancy, such as size progression and/or the appearance of solid components, are occasionally found on follow-up CT scans [1]. In such lesions, pathological confirmation is required to determine whether they are malignant or benign. Surgical resection is performed for lesions with GGO that are histologically diagnosed as malignant, but the preferred type of surgery (lobectomy vs. limited resection) is controversial.

Percutaneous radiofrequency ablation (RFA) has been used to locally treat both primary and secondary lung cancers, mainly for patients who are not candidates or who are considered high-risk patients for surgery. Several studies have shown mid- or long-term results. Hiraki et al. [2] and Okuma et al. [3] reported that the overall control rates for tumors <2 cm were 89 and 77 % at 1 year and 66 and 73 % at 2 years, respectively. A study by de Baère et al. [4] found that the overall control rate at 18 months was 95 % for tumors ≤2 cm and 87 % for tumors >2 cm. In a multicenter, prospective, clinical trial, overall survival was 70 % at 1 year and 48 % at 2 years in patients with non-small cell lung cancer [5]. RFA appears to be an appropriate local therapy for lung cancer with GGO without lymph node metastasis. In this study, we retrospectively evaluated outcomes of RFA in these patients.

Materials and Methods

Our institutional review board approved this retrospective study and waived the informed consent requirement for the use of medical data from these patients.

Patients and Tumors

GGO was defined as homogeneous visualization on high-resolution CT (HRCT) with hazy increased attenuation of the lung and preservation of bronchial and vascular margins [6, 7]. The percentage of the GGO component was calculated as follows: (DGGO − *D*)/DGGO × 100, where DGGO is the greatest diameter of the lesion (including GGO area), and *D* is the greatest diameter of the lesion without GGO [6, 7].

Patients who met the following criteria were included in the study: (1) a pulmonary lesion with a GGO component >50 % on HRCT images before RFA (definition of HRCT is 1 mm or sub mm slice thickness), (2) histological diagnosis of malignancy, and (3) unsuitability for or patient refusal of surgery. Exclusion criteria included the presence of regional lymph node metastasis or distant metastasis.

Between July 2001 and October 2013, 556 patients with 1,489 lung tumors underwent RFA under CT fluoroscopic guidance at our institution. From this group, 16 patients (5 men and 11 women; mean age, 72.6 years ± 8.4; range 58–88 years) with 17 tumors were included in this study. The mean lesion size was 1.6 cm ± 0.6 (range 0.7–3.3 cm). Seventeen tumors showed no contrast enhancement and/or could not be detected on CT scans in the mediastinal window setting. Tumor location was described as central (tumor center located in the inner half of the lungs on the axial CT images obtained before RFA; $n = 5$) or peripheral (tumor center located in the outer half of the lungs on the axial CT images obtained before RFA; $n = 12$). CT-guided lung biopsies confirmed that all 17 tumors were histologically adenocarcinoma. A total of 20 ablation sessions were performed, including three repeated sessions for local tumor progression. Twelve tumors had a GGO component >90 % (lung cancer with pure GGO). Tumors were incidentally detected on chest CT scans ($n = 4$) or were detected by follow-up CT after surgical resection of previous lung cancers ($n = 7$) or treatment for other types of cancers ($n = 6$).

Positron emission tomography (PET)/CT scanning was performed in 11 patients 24.5 days (mean) before RFA, and there was no abnormal accumulation of 18F-fluorodeoxy glucose. Eleven lung cancers with pure GGO in 10 patients and 5 tumors with non-pure GGO in 5 patients were included in our previous publication that reported the results of 50 patients with stage I non-small cell lung cancer between July 2002 and September 2009 [8].

Radiofrequency Ablation Technique

The detailed technical aspects of our institution's RFA procedure have been described previously [9]. Briefly, RFA was performed percutaneously using CT fluoroscopy (Asteion or Aquilion; Toshiba, Tokyo, Japan) in all sessions.

After the administration of local anesthesia with lidocaine, an electrode connected to a generator was introduced

into the tumor, and then ablation started. The electrodes used for ablation included a multi-tined expandable electrode (LeVeen; Boston Scientific, Natick, MA) with an array diameter of 2 cm ($n = 7$), 3 cm ($n = 10$), or 3.5 cm ($n = 1$), and a single internally cooled electrode (Cool-tip; Covidien, Mansfield, MA, USA) with a 3 cm ($n = 2$) noninsulated tip. The procedure aimed at ablation of tumor plus at least 5-mm margin. The mean total ablation time per tumor was 26.8 min ± 11.0 (range 10.0–54.5 min).

Immediately after RFA, chest CT with 5-mm thickness was performed to evaluate the ablation zone and procedural complications. Chest radiographs were obtained 3 h after completion of RFA and the following morning to check for complications.

Follow-Up Examinations

Patients were followed up whenever possible at 1, 3, 6, 9, and 12 months after RFA with contrast-enhanced chest CT with 5-mm thickness to assess local efficacy. Thereafter, follow-up visits were conducted every 6 months.

The tumor is considered to be completely treated when the entire ablation zone is not contrast-enhanced (i.e., enhancement ≤15 HU) or when the ablation zone exhibits contrast enhancement that is peripheral, concentric, symmetric, and uniform, which was considered to correspond to the ablated marginal parenchyma [10, 11]. Thereafter, local efficacy was evaluated by comparing the size and geometry of the ablation zone in the previous CT images. In contrast, local progression of the tumor was indicated by the appearance of an irregular, scattered, nodular, or eccentric enhancement focus in the ablation zone or when the ablation zone was circumferentially enlarged [11, 12]. Although PET/CT was not included in routine follow-up, eight patients underwent PET/CT examinations to evaluate the outcomes of RFA.

Statistical Analysis

Technique effectiveness was defined as the complete ablation of the macroscopic tumor at imaging follow-up [13]. The primary and secondary technique effectiveness rates were calculated by Kaplan–Meier analysis. The primary effectiveness rate was defined as the percentage of the tumor that was successfully eradicated after the initial procedure [13]. The secondary effectiveness rate was defined as the percentage of the tumor that underwent successful repeated ablation after identification of local tumor progression [13]. Patient survival rates were estimated using Kaplan–Meier analysis.

Seventeen tumors and 17 initial sessions were divided into 2 groups according to the occurrence of local progression after initial RFA and complications, respectively. Age, sex, tumor size, tumor location (central or peripheral), GGO type (pure or non-pure), total ablation time, and electrode type (multi-tined expandable or internally cooled) were compared between the two groups by using the Mann–Whitney U test for numerical values and by using Fisher's exact test for categorical values.

P value <0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences software (version 11.0; SPSS Inc, Chicago, IL, USA).

Results

Local Tumor Control

The mean and median follow-up periods were 55.1 and 61.5 months (range 6.1–96.6 months), respectively. Follow-up CT scan was performed 3–19 times (median, 12 times). Periodic follow-up CT showed that completely treated tumors increased in size from baseline and then after a gradual decrease in size, remained stable. Although, in 1 tumor, the ablation zone exhibited peripheral contrast enhancement 1 month after RFA, 14 completely treated tumors showed no contrast enhancement (Fig. 1). In the 3 cases of lung cancer with pure GGO (17.6 % of 17 cases), local progression was observed at 18.3, 28.2, and 31.2 months after RFA (Fig. 2). CT images of these tumors showed that GGO with ($n = 1$) or without nodular enhancement focus ($n = 2$) appeared adjacent to the ablated tumor and continued to increase in size. Two of these three patients underwent PET/CT scanning, and 18F-fluorodeoxy glucose did not accumulate in two tumors diagnosed with local progression by CT. The primary technique effectiveness rate was 100 % at 1 year, 93.3 % at 2 years, and 78.3 % at 3 years. Two of the three tumors with local progression were retreated with RFA. One showed a complete response while the other showed local progression again 25.4 months after the second RFA. This tumor underwent a third RFA, but local progression occurred again 12.6 months after the third RFA. The remaining patient was treated with subsequent partial resection, not repeated RFA. The secondary technique effectiveness rates were 100.0 % at 1 year, 100.0 % at 2 years, and 92.3 % at 3 years.

There were no significant risk factors for local progression (Table 1).

Procedural Complications

The definition of major complication was an event that lead to substantial morbidity and disability, increasing the level of care, or results in hospital admission or substantially

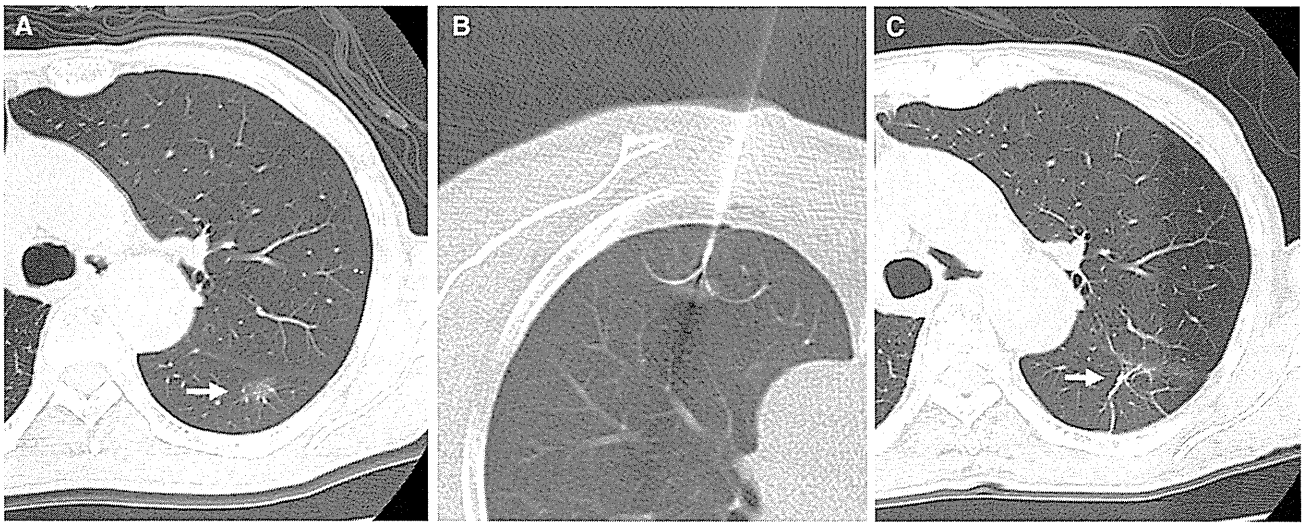

Fig. 1 Lung cancer with GGO was incidentally detected in a 79-year-old man on follow-up CT after treatment of hepatocellular carcinoma and histologically proven adenocarcinoma. **A** CT image before RFA in the lung field window setting shows a tumor, 1.3 cm in diameter, located in the left lower lung (*arrow*). **B** CT fluoroscopic image during RFA shows that an electrode is inserted in the tumor. **C** CT image 53.1 months after RFA in the lung window setting shows involution of the ablated tumor (*arrow*), indicating complete ablation

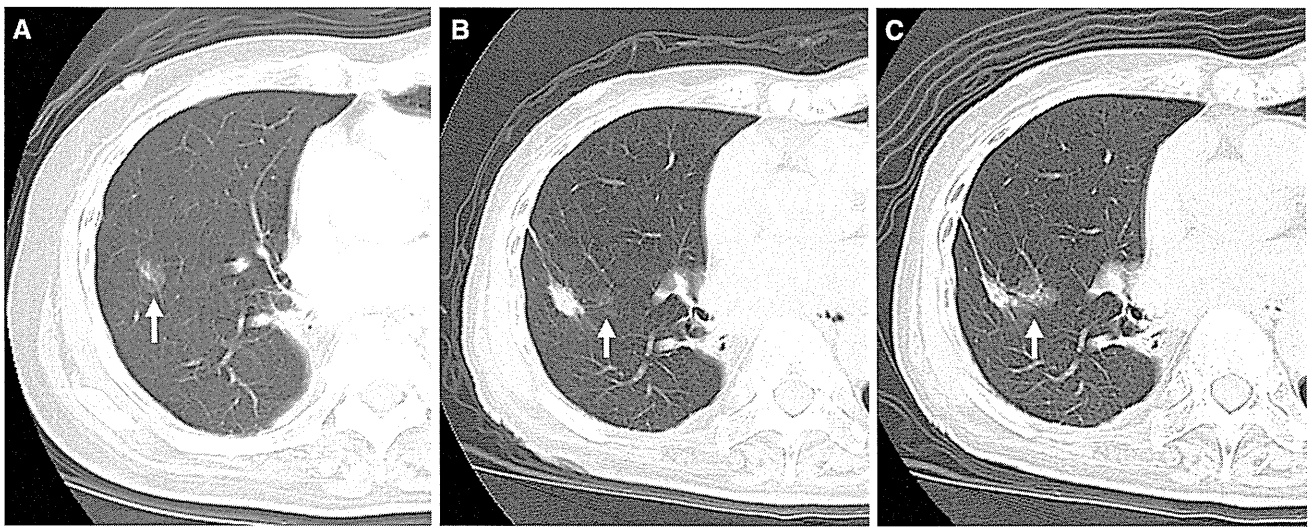

Fig. 2 Lung cancer with GGO was detected on follow-up CT in an 82-year-old woman after right lung lower lobectomy for lung cancer, histologically confirmed as adenocarcinoma. **A** CT image before RFA in the lung window setting shows a tumor, 1.5 cm in diameter, located in the right upper lung (*arrow*). CT images 28.2 months (**B**) and 39.7 months (**C**) after RFA in the lung window setting shows that the GGO inside the ablated tumor is increasing in size (*arrow*), indicating local progression

lengthened hospital stay; all other complications were considered minor [13].

There were no major complications. Pneumothorax occurred in 15 of 20 treatment sessions: 14 were asymptomatic, and 1 required chest tube placement but resolved satisfactorily within 48 h. Minor pulmonary hemorrhage occurred in two and mild pneumonitis in one. None of the patients died from the procedure. For the 17 initial sessions, male sex was a significant risk factor for complications (Table 1).

Patient Survival and Outcome

By the end of the study, 1 patient died due to recurrence of other cancer 11.7 months after RFA; the remaining 15 patients were alive when this report was written. The mean and median follow-up periods were 58.4 and 65.6 months (range 6.1–96.6 months), respectively. The overall survival and disease-specific survival rates were 93.3 and 100 % at 1 year and 93.3 and 100 % at 5 years (mean survival time 90.9 months), respectively. Of the 15 patients who

Table 1 Results of analyses of multiple variables to determine factors for local progression and complication

Variable	Local progression			Complication		
	No ($n = 14$)	Yes ($n = 3$)	P value	No ($n = 6$)	Yes ($n = 11$)	P value
Age (year)						
Mean ± SD	73.5 ± 8.0	73.7 ± 11.2	0.768	75.0 ± 11.0	71.8 ± 6.7	0.462
Sex						
Male/female	5/9	1/2	1.000	0/6	6/5	0.043
Tumor size (mm)						
Mean ± SD	17.1 ± 6.1	12.0 ± 4.4	0.300	17.8 ± 3.3	15.3 ± 7.1	0.078
Tumor location						
Central/peripheral	4/10	1/2	1.000	1/5	4/7	0.6
GGO type						
Pure/nonpure	10/4	2/1	1.000	4/2	8/3	1.000
Total ablation time (min)						
Mean ± SD	29.0 ± 11.9	16.8 ± 5.9	0.197	25.8 ± 12.0	27.4 ± 12.5	0.884
Electrode type						
Multitined expandable/internally cooled	12/2	3/0	1.000	5/1	10/1	1.000

SD standard deviation

survived, 11 are cancer-free. Two patients who had had a contralateral lung cancer treated prior to ablation of the GGO, developed recurrence in the contralateral hemithorax—a hilar node and a chest wall mass. The distribution of disease was much more in keeping with recurrence from the contralateral cancer rather than the GGO. These patients are now being treated with chemotherapy and/or other therapy. New lung cancer with GGO occurred in 1 patient, and a treatment strategy is currently undecided. One patient who had failure of the third RFA received stereotactic radiation therapy (SRT) 4 months before the end of the study.

Discussion

In 1995, Noguchi et al. [14] reported the histological classification of 236 surgically resected peripheral-type adenocarcinomas (≤2 cm in diameter) into 6 groups (Noguchi types A–F) according to tumor growth pattern. Noguchi type A and B tumors without foci of active fibroblastic proliferation are less invasive, do not display lymph node metastasis, and are associated with an excellent prognosis (100 % 5-year survival rate). Thus, these types of tumors represent in situ adenocarcinoma, and limited surgical resection is adequate. In contrast, Noguchi type C tumors with foci of active fibroblastic proliferation resemble small adenocarcinoma with regard to both the rate of lymph node metastasis (24.5 %) and prognosis (74.8 % 5-year survival rate).

Recently, pulmonary lesions with GGO have become a great concern for thoracic surgeons and physicians because of their increased incidental detection by chest CT. As a result, many studies have assessed the radiological-pathological correlation in pulmonary lesions with GGO. Small, well-circumscribed pure GGO on HRCT often is considered to represent either atypical adenomatous hyperplasia or Noguchi type A or B tumors. Mixed-typed GGO on HRCT often is considered indicative of Noguchi type C tumors in which a presumptive preoperative diagnosis of in situ adenocarcinoma can be made [15].

Although patients with lung cancer with GGO usually undergo surgical resection, the mode of surgical resection (limited resection vs. lobectomy) is still controversial. Current ongoing prospective studies are evaluating outcomes of limited resection. Asamura et al. [16] demonstrated that 28 patients with 28 lung cancer lesions showing GGO ≤2 cm in diameter had no recurrence during a median follow-up period of 1,436 days after surgery (12 wedge resections, 3 segmentectomies, and 13 lobectomies). The 5-year disease-free survival rate of these patients was 100 %. Nakao et al. [17] studied the long-term outcomes of prospective limited resection for 50 pulmonary lesions with GGO <2 cm in diameter, including 2 Noguchi type A, 23 Noguchi type B, 15 Noguchi type C, and 10 benign tumors. None of the patients had recurrence within 5 years after surgery.

SRT also is sometimes used as a local treatment for non-small cell lung cancer, mostly for patients unfit for surgery. Hamamoto et al. [18] reported that the 2-year local failure-

free rate after SRT was 96 % in 28 patients mainly with GGO lung cancer. Their short-term result was similar to ours.

The reported overall and long-term survival rates after RFA are promising as follows: 78 and 94 % at 1 year, 36 and 40 % at 3 years, and 27 and 25 % at 5 years for 75 and 57 patients with stage I non-small cell lung cancer [19, 20]. Our results (93.3 % 5-year overall survival rate) are better than those of previous reports. This likely reflects our exclusive focus on small GGO type malignancy which carries a better prognosis.

Follow-up CT appearances of completely ablated lung cancer with GGO, including both the morphological and enhancement pattern, were similar to those of solid lung tumors [11, 21]. In all three locally progressed tumors, GGO was observed adjacent to the ablated tumor and continued to increase in size. These appearances were judged as local progression although the histology was not confirmed. This might be a characteristic recurrent pattern of lung cancer with GGO after RFA.

In 17 initial sessions, male sex was a significant risk factor for complications. This may be because of smoking, although we did not evaluate the smoking history in all patients. Pneumothorax occurred in 10 of 11 sessions with complications. We speculated that the man had more smoking related lung damage.

There are some differences between RFA for lung cancer with GGO and RFA for solid lung tumors. First, the contour of the target lesion becomes unclear on CT immediately after ablation because of the emergence of ground-glass attenuation around the ablated tumor. Anderson et al. [22] suggested that lack of a circumferential ground-glass halo at the time of treatment may be an early predictor of treatment failure. Difficulty evaluating the ablation zone surrounding the lesion may result in treatment failure. Coregistration of CT images before and after RFA would be effective in overcoming this disadvantage and reducing the recurrence rate because of the possibility of a more accurate evaluation of the ablation region (both GGO lung cancer and the margin) [23, 24]. Second, clear detection of recurrence on CT takes more time because these tumors grow slowly. The mean volume doubling times for tumors with pure GGO and partial GGO with a solid central component are 813 and 457 days, respectively [25]. In our three tumors, local progression was observed 18 months or later after the initial RFA. Third, PET is not usually useful for evaluating therapeutic efficacy of RFA for lung cancer with GGO, unlike solid lung cancer [26].

RFA has some potential advantages over other therapies. RFA can be performed under local anesthesia, and the associated mortality and morbidity are low [27], although RFA often is performed in patients who are unfit for surgery. Repeated RFA is possible after detection of local progression, unlike conventional external beam radiation therapy or SRT [28]. Repeated RFA significantly improves overall local control outcomes [28]. In our study, the local tumor control rate at 3 years also was improved from 78.8 to 92.3 % by repeated RFA.

This study showed that the local progression rate after RFA was 17.6 %. Thus, we suggest that the use of RFA for lung cancer with GGO should be limited to inoperable patients or patients who refuse to undergo surgery, because RFA has lower local efficacy than surgical resection. However, we anticipate that we can reduce the likelihood of local tumor progression by scrupulous attention to the GGO margin. If local tumor progression rates can be reduced then RFA will become the preferred therapeutic modality, as preservation of lung parenchyma is so important in these patients who are likely to develop new primary cancers.

This retrospective study was limited to a single-institution study and included a small sample size. Furthermore, our study was not designed to compare the outcomes of RFA and other treatments such as surgery or SRT. However, the results of this study, including the lack of major procedure-related complications or deaths and the high survival rates, suggest that RFA is a therapeutic option for nonsurgical candidates or patients with a history of prior treatment with radiation therapy.

In conclusion, RFA for lung cancer with GGO was safe and effective, and resulted in promising survival rates. Therefore, RFA may be a treatment option for nonsurgical candidates.

Conflict of interest Toshihiro Iguchi, Takao Hiraki, Hideo Gobara, Hiroyasu Fujiwara, Yusuke Matsui, Junichi Soh, Shinichi Toyooka, Katsuyuki Kiura, and Susumu Kanazawa have no conflicts of interest to declare.

References

1. Godoy MC, Naidich DP (2009) Subsolid pulmonary nodules and the spectrum of peripheral adenocarcinomas of the lung: recommended interim guidelines for assessment and management. Radiology 253(3):606–622 PMID: 19952025
2. Hiraki T, Sakurai J, Tsuda T et al (2006) Risk factors for local progression after percutaneous radiofrequency ablation of lung tumors: evaluation based on a preliminary review of 342 tumors. Cancer 107(12):2873–2880 PMID: 17096433
3. Okuma T, Matsuoka T, Yamamoto A et al (2010) Determinants of local progression after computed tomography-guided percutaneous radiofrequency ablation for unresectable lung tumors: 9-year experience in a single institution. Cardiovasc Intervent Radiol 33(4):787–793 PMID: 19967367
4. de Baère T, Palussière J, Aupérin A et al (2006) Midterm local efficacy and survival after radiofrequency ablation of lung tumors with minimum follow-up of 1 year: prospective evaluation. Radiology 240(2):587–596 PMID: 16864679

5. Lencioni R, Crocetti L, Cioni R et al (2008) Response to radiofrequency ablation of pulmonary tumours: a prospective, intention-to-treat, multicentre clinical trial (the RAPTURE study). Lancet Oncol 9(7):621–628 PMID: 18565793
6. Kim TJ, Lee JH, Lee CT et al (2008) Diagnostic accuracy of CT-guided core biopsy of ground-glass opacity pulmonary lesions. AJR Am J Roentgenol 190(1):234–239 PMID: 18094317
7. Hur J, Lee HJ, Nam JE et al (2009) Diagnostic accuracy of CT fluoroscopy-guided needle aspiration biopsy of ground-glass opacity pulmonary lesions. AJR Am J Roentgenol 192(3): 629–634 PMID: 19234257
8. Hiraki T, Gobara H, Mimura H et al (2011) Percutaneous radiofrequency ablation of clinical stage I non-small cell lung cancer. J Thorac Cardiovasc Surg 142(1):24–30 PMID: 21529847
9. Hiraki T, Gobara H, Mimura H et al (2011) Radiofrequency ablation of lung cancer at Okayama University Hospital: a review of 10 years of experience. Acta Med Okayama 65(5):287–297 PMID: 22037265
10. Yamamoto A, Nakamura K, Matsuoka T et al (2005) Radiofrequency ablation in a porcine lung model: correlation between CT and histopathologic findings. AJR Am J Roentgenol 185(5):1299–1306 PMID: 16247153
11. Abtin FG, Eradat J, Gutierrez AJ, Lee C, Fishbein MC, Suh RD (2012) Radiofrequency ablation of lung tumors: imaging features of the postablation zone. Radiographics 32(4):947–969 PMID: 22786987
12. Hiraki T, Gobara H, Mimura H et al (2010) Does tumor type affect local control by radiofrequency ablation in the lungs? Eur J Radiol 74(1):136–141 PMID: 19231125
13. Goldberg SN, Grassi CJ, Cardella JF et al (2009) Image-guided tumor ablation: standardization of terminology and reporting criteria. J Vasc Interv Radiol 20(7 Suppl):S377–S390 PMID: 19560026
14. Noguchi M, Morikawa A, Kawasaki M et al (1995) Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer 75(12):2844–2852 PMID: 7773933
15. Kondo T, Yamada K, Noda K, Nakayama H, Kameda Y (2002) Radiologic-prognostic correlation in patients with small pulmonary adenocarcinomas. Lung Cancer 36(1):49–57 PMID: 11891033
16. Asamura H, Suzuki K, Watanabe S, Matsuno Y, Maeshima A, Tsuchiya R (2003) A clinicopathological study of resected subcentimeter lung cancers: a favorable prognosis for ground glass opacity lesions. Ann Thorac Surg 76(4):1016–1022 PMID: 14529977
17. Nakao M, Yoshida J, Goto K et al (2012) Long-term outcomes of 50 cases of limited-resection trial for pulmonary ground-glass opacity nodules. J Thorac Oncol 7(10):1563–1566 PMID: 22878750
18. Hamamoto Y, Kataoka M, Yamashita M et al (2012) Factors affecting the local control of stereotactic body radiotherapy for lung tumors including primary lung cancer and metastatic lung tumors. Jpn J Radiol 30(5):430–434 PMID: 22450903
19. Simon CJ, Dupuy DE, DiPetrillo TA et al (2007) Pulmonary radiofrequency ablation: long-term safety and efficacy in 153 patients. Radiology 243(1):268–275 PMID: 17392258
20. Ambrogi MC, Fanucchi O, Cioni R et al (2011) Long-term results of radiofrequency ablation treatment of stage I non-small cell lung cancer: a prospective intention-to-treat study. J Thorac Oncol 6(12):2044–2051 PMID: 22052222
21. Yasui K, Kanazawa S, Sano Y et al (2004) Thoracic tumors treated with CT-guided radiofrequency ablation: initial experience. Radiology 231(3):850–857 PMID: 15105453
22. Anderson EM, Lees WR, Gillams AR (2009) Early indicators of treatment success after percutaneous radiofrequency of pulmonary tumors. Cardiovasc Intervent Radiol 32(3):478–483 PMID: 19127381
23. Kim YS, Lee WJ, Rhim H, Lim HK, Choi D, Lee JY (2010) The minimal ablative margin of radiofrequency ablation of hepatocellular carcinoma (>2 and <5 cm) needed to prevent local tumor progression: 3D quantitative assessment using CT image fusion. AJR Am J Roentgenol 195(3):758–765 PMID: 20729457
24. Kim KW, Lee JM, Klotz E et al (2011) Safety margin assessment after radiofrequency ablation of the liver using registration of preprocedure and postprocedure CT images. AJR Am J Roentgenol 196(5):W565–W572 PMID: 21512046
25. Hasegawa M, Sone S, Takashima S et al (2000) Growth rate of small lung cancers detected on mass CT screening. Br J Radiol 73(876):1252–1259 PMID: 11205667
26. Kim TJ, Park CM, Goo JM, Lee KW (2012) Is there a role for FDG PET in the management of lung cancer manifesting predominantly as ground-glass opacity? AJR Am J Roentgenol 198(1):83–88 PMID: 22194482
27. Kashima M, Yamakado K, Takaki H et al (2011) Complications after 1000 lung radiofrequency ablation sessions in 420 patients: a single center's experiences. AJR Am J Roentgenol 197(4):W576–W580 PMID: 21940529
28. Hiraki T, Mimura H, Gobara H et al (2008) Repeat radiofrequency ablation for local progression of lung tumors: does it have a role in local tumor control? J Vasc Interv Radiol 19(5):706–711 PMID: 18440459

Acta Med. Okayama, 2014
Vol. 68, No. 4, pp. 191-200

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Review

Drug Resistance to EGFR Tyrosine Kinase Inhibitors for Non-small Cell Lung Cancer

Kazuhiko Shien[a,b], Hiromasa Yamamoto[b], Junichi Soh[b], Shinichiro Miyoshi[b], and Shinichi Toyooka[a,b]*

Departments of [a]Clinical Genomic Medicine, and [b]General Thoracic Surgery and Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

Non-small cell lung cancer (NSCLC) harboring an activating mutation within the epidermal growth factor receptor (*EGFR*) was defined as a clinically distinct molecular group. These lesions show oncogene addiction to EGFR and dramatic responses to the EGFR tyrosine kinase inhibitors (TKIs). Several large Phase III trials have shown that EGFR-TKIs improved the progression-free survival of patients with *EGFR* mutant NSCLC compared to conventional chemotherapy. However, the long-term effectiveness of EGFR-TKIs is usually limited because of acquired drug resistance. To overcome this resistance to EGFR-TKIs, it will be essential to identify the specific mechanisms underlying the resistance. Many investigators have attempted to identify the mechanisms using preclinical models and drug-resistant clinical samples. As a result, several mechanisms have been showed to be responsible for the resistance, but not all of the relevant mechanisms have been uncovered. In this review, we provide an overview of mechanisms underlying drug-resistance to EGFR-TKIs, focusing on results obtained with preclinical models, and we present some possible strategies to overcome the EGFR-TKI resistance.

Key words: non-small cell lung cancer, *EGFR* mutation, tyrosine-kinase inhibitor, drug resistance, cancer stem cell

Lung cancer continues to be the leading cause of death among patients with malignant tumors worldwide [1]. Many patients are diagnosed after the cancer has already spread to distant sites or directly beyond the primary site, resulting in an inoperable stage. In 2004, mutations in the epidermal growth factor receptor (*EGFR*) that cause oncogene addiction to EGFR were discovered in non-small cell lung cancer (NSCLC) [2, 3]. Because these mutations are strongly associated with sensitivity to EGFR-tyrosine kinase inhibitors (TKIs), a great deal of knowledge has been uncovered in regard to both *EGFR* and other genes in the EGFR family and their downstream genes.

EGFR-TKIs have exhibited significant antiproliferative effects against NSCLC with *EGFR*-activating mutations in preclinical studies [2, 3] and their use in the treatment of NSCLC patients has also resulted in prolonged progression-free survival (PFS) in randomized Phase III studies [4-7]. However, patients with *EGFR* mutations who initially respond to EGFR-TKIs eventually acquire resistance, which is a critical problem in the treatment of patients with advanced

Received February 25, 2014; accepted June 12, 2014.
*Corresponding author. Phone:+81-86-235-7436; Fax:+81-86-235-7437
E-mail:toyooka@md.okayama-u.ac.jp (S. Toyooka)

Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.