expression loss in our resistant sublines. The expression of PTEN has also been reported to be regulated by the EGR1 transcription factor, and three EGR1-binding sites have been identified in the *PTEN* gene promoter (29, 30, 43). In this study, we showed a close correlation between PTEN expression and EGR1 expression in drug-resistant PC-9/GEF cells, suggesting that the nuclear translocation of EGR1 directly regulates the expression of PTEN: EGR1 activates transcription of the *PTEN* gene through binding to its consensus motif in the promoter (44). Our present study showed downregulation of some EGR1 target genes, cyclin D1, FGF2, and ICAM-1, but not other EGR1 target gene, PTP1B (Fig. 2E). Together, whether expression of PTEN or other EGR1 target genes could be upregulated or downregulated might in part depend on how activation of EGR1 is controlled in response to environmental stimuli. Exposure of PC-9 cells to low doses of gefitinib up to 5 days resulted in unchanged expression of PTEN (Supplementary Fig. S1). Because gefitinib-resistant cell lines were selected after stepwise exposure to increasing doses of the drug for 6 to 8 months, continuous exposure for longer periods might be required to induce loss of both PTEN expression and nuclear EGR1 translocation. However, it remains unclear how nuclear translocation of EGR1 is specifically blocked by acquirement of drug resistance to gefitinib. Further elucidation of relevant underlying mechanisms at molecular basis should be required.

In both immunocytochemical and Western blot analyses using EGFR mutation-specific antibodies (26, 28), the EGFR delE746-A750 mutation was found to be conserved in PC-9/GEFs (Fig. 1C and D), suggesting that there was no change in the original EGFR deletion mutation that sensitizes the effect of gefitinib under selection by drug resistance. Onitsuka and colleagues (27) have recently characterized 10 NSCLC patients showing acquired resistance to monotherapy by gefitinib. In the primary tumor, T790M was observed in 7, and HGF overexpression was observed in 5, of 10 refractory tumors. In one refractory patient, immunohistochemical analysis showed that PTEN was expressed in cancer cells harboring the delE746-A750 mutation, both in the primary tumor before treatment and also in the secondary refractory

tumors after treatment with gefitinib (Fig. 4). Immunohistochemical analysis also showed that Met and EGFR were expressed in both the primary tumor, before gefitinib treatment, and in the refractory tumors derived from three metastatic foci from the lung of this patient. By contrast, PTEN expression was much lower in the refractory tumors than the primary tumor, and Akt phosphorylation was much higher in two of the refractory tumors (Fig. 4). About the frequency of PTEN loss, Sos and colleagues (24) have reported co-occurrence of homozygous gene deletion of PTEN and EGFR mutation in 1 of 24 clinical NSCLC samples with EGFR mutations. It seems likely that PTEN loss is less frequent compared with T790M, HGF expression, and Met amplification. To evaluate the frequency of PTEN loss in gefitinib- or erlotinib-resistant tumors, quantitative analysis will be required with large number of refractory tumors.

In conclusion, we selected gefitinib-resistant cell lines from PC-9 cells harboring an activating EGFR mutation and observed PTEN loss in these resistant cell lines. PTEN loss was also observed in one NSCLC patient who had become refractory to gefitinib treatment. Together with recent results published by another group (24, 38, 39), loss of PTEN expression together with Akt activation could act as a predictive marker for a therapeutic response to EGFR-targeted drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

References

1. Parkin DM. Global cancer statics in the year 2000. Lancet Oncol 2001;2:533–43.
2. Spiro SG, Silvestri GA. One hundred years of lung cancer. AM J Respir Crit Care Med 2005;172:523–9.
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 2004;350:2129–39.
4. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304:1497–500.
5. 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.
6. Ono M, Hirata A, Kometani T, et al. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular

signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther 2004;3:465–72.
7. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
8. 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.
9. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–46.
10. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. New Engl J Med 2009; 361:947–57.
11. Kwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. Clin Cancer Res 2006;12:7242–51.

12. Reinmuth N, Meister M, Muley T, et al. Molecular determinants of response to RTK-targeting agents in nonsmall cell lung cancer. Int J Cancer 2006;119:727–34.
13. Anido J, Matar P, Albanell J, et al. ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells. Clin Cancer Res 2003;9:1274–83.
14. Hirata A, Hosoi F, Miyagawa M, et al. HER2 overexpression increases sensitivity to gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, through inhibition of HER2/HER3 heterodimer formation in lung cancer cells. Cancer Res 2005;65:4253–60.
15. Cappuzzo F, Ligorio C, Toschi L, et al. EGFR and HER2 gene copy number and response to first-line chemotherapy in patients with advanced non-small cell lung cancer (NSCLC). J Thorac Oncol 2007;2:423–9.
16. Thomas RK, Greulich H, Yuza Y, et al. Detection of oncogenic mutations in the EGFR gene in lung adenocarcinoma with differential sensitivity to EGFR tyrosine kinase inhibitors. Cold Spring Harb Symp Quant Biol 2005;70:73–81.
17. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
18. 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 2005;2:e73.
19. Massarelli E, Varella-Garcia M, Tang X, et al. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res 2007;13:2890–6.
20. Riely GJ, Marks J, Pao W. KRAS mutations in non-small cell lung cancer. Proc Am Thorac Soc 2009;6:201–5.
21. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
22. Engelman JA, Jänne PA. Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. Clin Cancer Res 2008;14:2895–9.
23. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with activating epidermal growth factor receptor-activating mutations. Cancer Res 2008;68:9479–87.
24. Sos ML, Koker M, Weir BA, et al. PTEN loss contributes to erlotinib resistance in EGFR-mutant lung cancer by activation of Akt and EGFR. Cancer Res 2009;69:3256–61.
25. 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;65:7276–82.
26. Kawahara A, Yamamoto C, Nakashima K, et al. Molecular diagnosis of activating EGFR mutations in non-small cell lung cancer using mutation-specific antibodies for immunohistochemical analysis. Clin Cancer Res 2010;16:3163–70.
27. Onitsuka T, Uramoto H, Nose N, et al. Acquired resistance to gefitinib: the contribution of mechanisms other than the T790M, MET, HGF status. Lung Cancer 2010;68:198–203.
28. Yu J, Kane S, Wu J, et al. Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. Clin Cancer Res 2009;15:3023–8.
29. Okamura H, Yoshida K, Morimoto H, Haneji T. PTEN expression elicited by EGR-1 transcription factor in calyculin A-induced apoptotic cells. J Cell Biochem 2005;94:117–25.
30. Fantini D, Vascotto C, Deganuto M, et al. APE1/Ref-1 regulates PTEN expression mediated by Egr-1. Free Radic Res 2008;42:20–9.
31. Adamson ED, Mercola D. Egr1 transcription factor: multiple roles in prostate tumor cell growth and survival. Tumour Biol 2002;23:93–102.
32. Yan YX, Nakagawa H, Lee MH, Rustgi AK. Transforming growth factor-α enhances cyclin D1 transcription through the binding of early growth response protein to a cis-regulatory element in the cyclin D1 promoter. J Biol Chem 1997;272:33181–90.
33. Maltzman JS, Carmen JA, Monroe JG. Transcriptional regulation of the Icam-1 gene in antigen receptor- and phorbol ester-stimulated B lymphocytes: role for transcription factor EGR1. J Exp Med 1996;183:1747–59.
34. Biesiada E, Razandi M, Levin ER. Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. J Biol Chem 1996;271:18576–81.
35. Wang D, Mayo MW, Baldwin AS, Jr. Basic fibroblast growth factor transcriptional autoregulation requires EGR-1. Oncogene 1997;14:2291–9.
36. Fukada T, Tonks NK. The reciprocal role of Egr-1 and Sp family proteins in regulation of the PTP1B promoter in response to the p210 Bcr-Abl oncoprotein-tyrosine kinase. J Biol Chem 2001;276:25512–9.
37. Li L, Ross AH. Why is PTEN an important tumor suppressor? J Cell Biochem 2007;102:1368–74.
38. Endoh H, Yatabe Y, Kosaka T, Kuwano H, Mitsudomi T. PTEN and PIK3CA expression is associated with prolonged survival after gefitinib treatment in EGFR-mutated lung cancer patients. J Thorac Oncol 2006;1:629–34.
39. She QB, Solit D, Basso A, Moasser MM. Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. Clin Cancer Res 2003;9:4340–6.
40. Soria JC, Lee HY, Lee JI, et al. Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clin Cancer Res 2002;8:1178–84.
41. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004;22:2954–63.
42. Noro R, Gemma A, Miyanaga A, et al. PTEN inactivation in lung cancer cells and the effect of its recovery on treatment with epidermal growth factor receptor tyrosine kinase inhibitors. Int J Oncol 2007;31:1157–63.
43. Virolle T, Adamson ED, Baron V, et al. The Egr-1 transcription factor directly activates PTEN during irradiation-induced signaling. Nat Cell Biol 2001;3:1124–8.
44. Sukhatme VP, Cao XM, Chang LC, et al. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 1988;53:37–43.

Molecular Diagnosis of Activating EGFR Mutations in Non–Small Cell Lung Cancer Using Mutation-Specific Antibodies for Immunohistochemical Analysis

Akihiko Kawahara[1], Chizuko Yamamoto[4], Kazutaka Nakashima[1], Koichi Azuma[2], Satoshi Hattori[3], Masaki Kashihara[4], Hisamichi Aizawa[2], Yuji Basaki[4], Michihiko Kuwano[5], Masayoshi Kage[1], Tetsuya Mitsudomi[6], and Mayumi Ono[4]

Abstract

Purpose: Therapeutic responses of non–small cell lung carcinoma (NSCLC) to epidermal growth factor receptor (EGFR)–targeted drugs, such as gefitinib and erlotinib, are closely associated with activating EGFR mutations. The most common mutations are delE746-A750 in exon 19 and L858R in exon 21, accounting for ~90% of all EGFR mutations. Recently, EGFR mutation-specific antibodies were developed and did well in immunohistochemical analysis, giving a sensitivity of ~90%. We have investigated whether this method detects activating EGFR mutations with sensitivity comparable with direct DNA sequencing, which is used to detect these mutations in NSCLC.

Experimental Design: We used antibodies specific for the E746-A750 deletion mutation in exon 19 and the L858R point mutation in exon 21 in Western blot analysis and immunohistochemistry to determine the presence of these mutations in NSCLC cell lines. We also examined these EGFR mutations in NSCLC tumor samples from 60 patients by immunohistochemically and direct DNA sequencing.

Results: We were able to identify EGFR mutations in NSCLC tumor samples immunohistochemically with a sensitivity of 79% using the anti–delE746-A750 antibody and 83% using the anti-L858R antibody. Additional DNA sequencing markedly improved the sensitivity obtained by immunohistochemistry.

Conclusions: This simple and rapid assay for detecting EGFR mutations, even in the small bronchial biopsies obtained in stage IV NSCLC patients, will be useful for diagnosing responsiveness to EGFR-targeted drugs in patients with NSCLC. Combining this with DNA sequencing is recommended for the development of improved personalized EGFR-targeted therapeutics. *Clin Cancer Res; 16(12); 3163–70. ©2010 AACR.*

Lung cancer is the most common cause of death from cancer worldwide. Non–small cell lung carcinoma (NSCLC) is the major type of lung cancer and is classified into three histologic types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (1, 2). Since the introduction of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib and its approval

Authors' Affiliations: [1]Department of Diagnostic Pathology, Kurume University Hospital; [2]Division of Respirology, Neurology, and Rheumatology, Department of Internal Medicine, Kurume University School of Medicine; [3]Biostatistics Center, Kurume University, Kurume, Japan; [4]Department of Pharmaceutical Oncology; [5]Laboratory of Molecular Cancer Biology, Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan; and [6]Department of Thoracic Surgery, Aichi Cancer Center Research Institute, Nagoya, Japan

Note: A. Kawahara, C. Yamamoto, and K. Nakashima contributed equally to this work.

Corresponding Author: Mayumi Ono, Department of Pharmaceutical Oncology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan. Phone: 81-92-642-6296; Fax: 81-92-642-6296; E-mail: mono@phar.kyushu-u.ac.jp.

for clinical use in the treatment of advanced NSCLC (3), a critical question has been how to optimize its therapeutic efficacy in NSCLC patients. Subsequent studies have shown a significant association between the presence of EGFR-activating mutations in lung tumors and their sensitivity to gefitinib and another EGFR tyrosine kinase inhibitor, erlotinib. Most of these mutations occur in exons 18 to 21 in the tyrosine kinase domain, the most common being deletions in exon 19, such as delE746-A750, and the L858R point mutation in exon 21 (4–6). These mutations are found more frequently in female patients, individuals who have never smoked, and patients of East Asian ethnicity (7–11).

Of the various molecular mechanisms that bring about EGFR activation and that affect responses to gefitinib, erlotinib, and other EGFR-targeted drugs (12), activating EGFR mutations, especially delE746-A750 and the L858R point mutation, are closely associated, with favorable clinical outcomes in ~80% of patients with NSCLC. The delE746-A750 mutation in exon 19 and the L858R mutation in exon 21 are the most common mutations found in NSCLC, accounting for ~90% of all EGFR mutations. The presence

Translational Relevance

Activating mutations in the kinase domain of the epidermal growth factor receptor (*EGFR*) gene are critical for determining the therapeutic efficacy of EGFR-targeted drugs for patients with non–small cell lung carcinoma. DNA sequencing of the EGFR tyrosine kinase domain has been used to determine treatment strategies for these patients. Recently, mutation-specific anti-EGFR antibodies recognizing delE746-A750 in exon 19 and L858R in exon 21 have been developed and used immunohistochemically to identify EGFR mutations in cancer cells. The identification of EGFR mutations immunohistochemically and in Western blots is further investigated in this article. Our results suggest that a simple immunohistochemical diagnosis using these antibodies can provide important quantitative and tissue-specific expression data to complement DNA sequence results. We show that the sensitivities of the immunohistochemical and DNA tests are comparable and that the two methods show good correlation in determining the EGFR mutations present in non–small cell lung carcinoma in a Japanese population.

of these activating EGFR mutations is often determined by direct PCR-based sequencing of seven exons of the EGFR tyrosine kinase domain, exons 18 to 24. Yu and colleagues (15) have developed specific antibodies recognizing the delE746-A750 and L858R mutations, which can be used to identify the EGFR status of tumor samples and provide a simple immunohistochemical method for diagnosing EGFR mutations in human tissue. In this study, we have further investigated the use of these mutation-specific antibodies in immunohistochemistry and their application to the diagnostic screening of lung cancer patients and their responsiveness to EGFR-targeted drugs.

Materials and Methods

Cell lines and tissue culture

PC9 and QG56 cells were kindly provided by Dr. Yukito Ichinose (Kyushu Cancer Center, Fukuoka, Japan) and 11-18 cells were kindly provided by Dr. Kazuhiko Nakagawa (Kinki University, Osaka, Japan). LK2 cells were purchased from the Japanese Collection of Research Bioresources, and H1975 and HeLa cells were purchased from the American Type Culture Collection. PC9, QG56, LK2, H1975, and 11-18 cells were cultured in RPMI supplemented with 10% fetal bovine serum. HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum as described previously (16). The cells were maintained under standard cell culture conditions at 37°C in a humid environment in 5% CO_2.

Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in 50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, and 10% glycerol containing 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate (Triton X-100 buffer). Cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore Corp.). After transfer, the membrane was incubated with blocking solution, probed with primary antibodies, and then washed. The primary antibodies were mutation-specific anti-EGFR antibodies recognizing the wild-type (WT) EGFR (D38B1; 16), the delE746-A750 mutation in exon 19 (6B6), and the L858R mutation (43B2) in exon 21 (15), all kindly provided by Cell Signaling Technology. The protein content was visualized using horseradish peroxidase–conjugated secondary antibodies, followed by enhanced chemiluminescence (Amersham).

Tumor samples

We retrospectively examined 45 primary NSCLC adenocarcinomas showing moderate to strong expression of total EGFR that had been completely removed surgically from patients at the Department of Surgery, Kurume University Hospital, between 1995 and 2005 (Kurume tumor samples). We also examined 15 primary NSCLC tumors surgically removed from patients at the Aichi Cancer Center (Nagoya tumor samples).

DNA extraction and direct DNA sequencing

Exon 19 (delE746-A750) and exon 21 (L858R) mutations in the *EGFR* gene were identified by direct DNA sequencing. In brief, genomic DNA was purified from paraffin-embedded tissues using a QIAamp DNA Micro kit (QIAGEN). The exon sequences of the EGFR kinase domain were amplified by nested PCR using specific primers, and exons 19 and 21 were done.

Immunohistochemistry for activating EGFR mutations in cultured lung cancer cells

Cells cultured on slides were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes. After fixation, the slides were washed briefly in water and boiled in a microwave for 30 minutes in 1 mmol/L EDTA (pH 9.0) target retrieval solution (DakoCytomation) to recover antigens. Intrinsic peroxidase activity was blocked by treatment with peroxidase-blocking reagent (DakoCytomation) for 5 minutes. After washing in TBS (DakoCytomation) for 5 minutes, primary antibodies, as used for Western blotting, were diluted 1:100 and applied to the cells. The slides were incubated at room temperature for 30 minutes, washed in TBS for 5 minutes, and incubated with labeled polymer–horseradish peroxidase secondary antibody (ChemMate ENVISION Kit, DakoCytomation) for 30 minutes at room temperature. After washing in TBS for 10 minutes, the slides were visualized using 3,3′-diaminobenzidine.
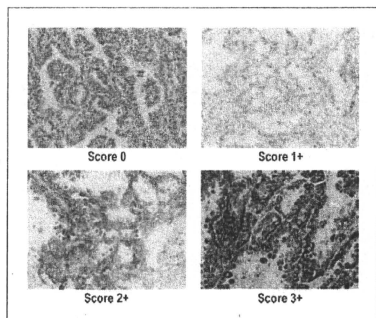
Fig. 1. Immunohistochemical staining and scores for NSCLC adenocarcinoma tumor samples labeled with anti–delE746-A750 EGFR antibody. Staining intensity was scored as 0, 1+, 2+, and 3+ (Materials and Methods).

Immunohistochemistry for activating EGFR mutations in clinical samples from NSCLC patients

Paraffin-embedded tissue samples of human lung cancer tissues were used to cut 4-μm sections, which were mounted on coated glass slides and incubated with the same mutation-specific anti-EGFR antibodies used for Western blotting 4°C overnight. We used DAKO autostainer (DakoCytomation). In evaluating the expression of EGFR mutations as biomarkers, we assumed that staining intensity of the cancer cell membranes or cyto-

plasm with the mutation-specific antibodies represented the level of EGFR expression in the cancer specimens. The intensity of staining was scored using the following scale: no staining, 0; weak staining, 1+; moderate staining, 2+; and strong staining, 3+ in >10% of cancer cells (Fig. 1). We classified scores of 0 and +1 as negative and scores of 2+ and 3+ as positive.

Results

Immunocytochemical analysis of activating EGFR mutations in human lung cancer cell line

We first determined whether the mutation-specific antibodies can specifically recognize EGFR mutations in Western blots (Fig. 2) using five human lung cancer cell lines (QG56, LK2, PC9, 11-18, and H1975) and HeLa cells, a cervical cancer cell line. DNA sequence analysis showed that PC9 carried the delE746-A750 in exon 19 of the EGFR, and 11-18 and H1975 carried the L858R mutation in exon 21, whereas the QG56, LK2, and HeLa cell lines carried no EGFR mutations in either of these two exons. In Western blots, five of these cell lines showed comparable levels of EGFR expression when labeled with the control anti-EGFR antibody, but LK2 showed a lower level of expression, consistent with a previous study (16). The deletion-specific antibody recognized the mutant EGFR with a E746-A750 deletion in PC9 cells, whereas the antibody specific for the L858R mutation recognized EGFR in the 11-18 and H1975 cell lines (Fig. 2A). However, the deletion-specific antibody did not recognize EGFR in 11-18 and H1975 cells carrying the L858R mutation, and the antibody specific for the L858R mutation did not recognize EGFR in PC9 cells with the E746-A750 deletion,
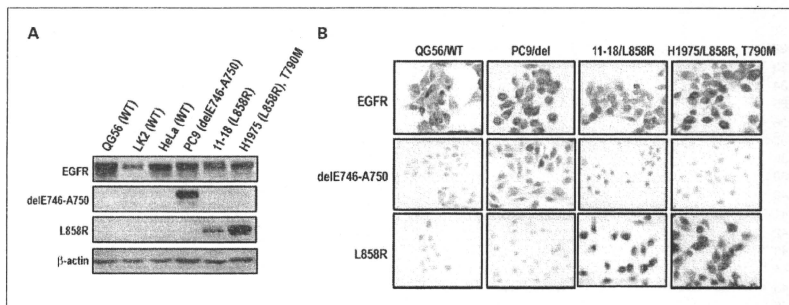

Fig. 2. Identification of the EGFR mutations delE746-A750 and L858R in NSCLC lines. The cervical cancer cell line HeLa was used as a control. A, Western blots showing the expression of EGFR, the delE746-A750 mutation, and L858R mutation in five NSCLC lines (QG56, LK2, PC9, 11-18, and H1975) under normal cell culture conditions. EGFR expression was identified using a control anti-WT EGFR antibody. Anti–delE746-A750 antibody labeled only PC9 carrying an exon 19 deletion. Anti-L858R antibody labeled 11-18 and H1975 cells carrying point mutations in exon 21. Expression of EGFR protein was determined in immunoblots using 100 μg protein of each cell lysate per lane. The loading control used was β-actin. B, immunohistochemical analysis of four NSCLC lines (QG56, PC9, 11-18, and H1975). Anti-WT EGFR antibody stained all cell lines, anti–delE746-A750 antibody stained only PC9 cells, and anti-L858R antibody stained 11-18 and H1975 cells.

Table 1. Immunohistochemistry and DNA sequence analysis of 60 NSCLC tumor samples

Patient no.	DNA sequencing of EGFR mutations	Immunohistochemistry		
		delEGFR	L858R	WT EGFR
1	E746-A750	2+	0	3+
2	E746-A750	3+	0	3+
3	E746-A750	3+	0	3+
4	E746-A750	1+	0	2+
5	E746-T751>A*	3+	1+	3+
6	E746-A750	2+	0	3+
7	E746-A750	2+	0	3+
8	E746-A750	2+	0	2+
9	E746-A750	2+	0	3+
10	E746-A750	1+	0	3+
11	E746-A750	1+	0	3+
12	E746-A750	3+	0	3+
13	S752-I759*	1+	2+	3+
14	L747-T751>P*	1+	1+	3+
15	L858R	0	3+	3+
16	L858R	1+	3+	3+
17	L858R	0	2+	3+
18	L858R	0	3+	3+
19	L858R	0	2+	3+
20	L858R	0	3+	3+
21	L858R	0	1+	2+
22	L858R	0	3+	3+
23	L858R	0	3+	3+
24	L858R	1+	3+	3+
25	L858R	1+	1+	3+
26	L858R	0	3+	3+
27	L858R	0	1+	3+
28	L858R	0	3+	3+
29	L858R	0	2+	3+
30	L858R	0	2+	2+
31	L858R	0	1+	3+
32	L858R	0	2+	3+
33	L858R	0	3+	3+
34	No mutation	0	0	3+
35	No mutation	0	0	3+
36	No mutation	0	0	3+
37	No mutation	0	0	3+
38	No mutation	0	0	3+
39	No mutation	0	0	3+
40	No mutation	0	0	3+
41	No mutation	0	0	3+
42	No mutation	0	0	3+
43	No mutation	0	0	3+
44	No mutation	0	0	3+
45	No mutation	0	0	3+
46	T751-I759>NKA*	2+	0	3+
47	L747-P753>S*	1+	0	2+
48	L747-T751>Q*	0	0	3+
49	L747-A750>P*	1+	0	3+
50	E746-A750	3+	0	3+

(Continued on the following page)

Table 1. Immunohistochemistry and DNA sequence analysis of 60 NSCLC tumor samples (Cont'd)

Patient no.	DNA sequencing of EGFR mutations	Immunohistochemistry		
		delEGFR	L858R	WT EGFR
51	E746-A750	3+	0	3+
52	E746-A750	3+	0	2+
53	L858R	0	2+	3+
54	L858R	0	3+	2+
55	L858R	0	3+	3+
56	L858R	0	3+	3+
57	No mutation	0	0	2+
58	No mutation	0	0	3+
59	No mutation	0	1+	3+
60	No mutation	0	0	2+

*Rare exon 19 deletion mutations.

confirming that these two antibodies were specific for the two mutations and would function in Western blots.

We next asked whether these mutation-specific antibodies were able to recognize mutant EGFRs in cultured lung cancer cells in immunohistochemistry (Fig. 2B). Apparent expression of EGFR was seen in all four lung cancer cell lines, QG56, PC9, 11-18, and H1975, when labeled with the control anti-WT EGFR antibody. The deletion-specific antibody labeled only PC9 cells carrying the delE746-A750 EGFR mutation, whereas the antibody specific for the EGFR point mutation labeled only 11-18 and H1975 cells carrying the L858R mutation. Therefore, Western blotting and immunohistochemical analysis consistently showed that each mutation-specific antibody was able to identify the appropriate EGFR-activating mutation present in lung cancer cell lines.

Immunohistochemical analysis of activating EGFR mutations in NSCLC patients
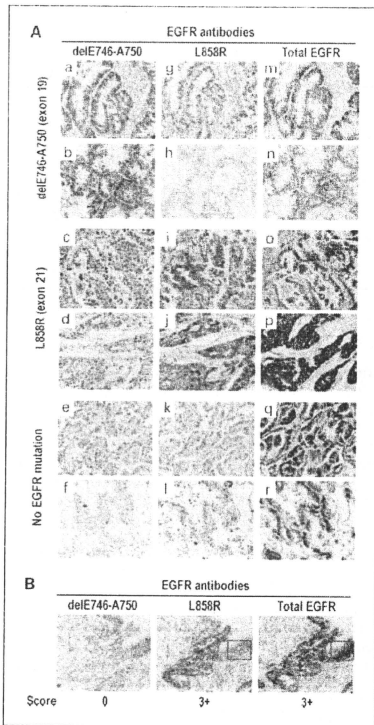
We investigated EGFR mutation status in NSCLC adenocarcinomas showing moderate to strong total EGFR expression from 45 patients who were treated at the Kurume University Hospital by direct DNA sequence analysis (Table 1, patients 1-45). Deletion mutations in exon 19, including delE746-A750, delE746-T751>A, delS752-I759, and delL747-T751>P, were present in 14 patients, the L858R point mutation in exon 21 was present in 19 patients, and WT EGFR was present in 12 patients (Table 1). We then labeled paraffin-embedded samples of the NSCLC adenocarcinomas immunohistochemically for the EGFR mutations. Figure 3A shows representative images of two examples in each case of cancers carrying the delE746-A750 mutation, the L858R mutation, and WT EGFR. The two cases carrying the delE746-A750 mutation were stained strongly by the anti–delE746-A750 and anti–WT EGFR antibodies but not with the anti-L858R antibody; those carrying the L858R mutation were stained strongly by the anti-L858R and anti-WT EGFR antibodies but not

with the anti–delE746-A750 antibody; those carrying only WT EGFR were only stained by the control anti-EGFR antibody (Fig. 3A).

Among the 45 cases of primary NSCLC, 12 samples had been shown to carry delE746-A750 deletion mutation, including a delE746-T751>A mutation (patient 5), and 75% (9 of 12) of these were stained by the deletion-specific antibody with a score of 2+ or 3+. Of the three samples that were identified by DNA sequencing as carrying rare exon 19 deletion mutations (patients 5, 13, and 14), the tumor sample carrying a delS752-I759 mutation (patient 13) and a delL747-T751>P mutation (patient 14) was not positively stained by the delE746-A750–specific antibody (Table 1).

All of the tumor samples from the 19 patients shown to carry the exon 21 L858R point mutation by direct DNA sequencing analysis (patients 15-33) were also stained by the anti-L858R antibody. Fifteen of 19 cases were positively stained with a score of 2+ or 3+ (Table 1). Twelve patients (patients 34-45), whose tumors carried WT EGFR according to DNA sequencing, were not stained by either of the mutation-specific antibodies. As shown in Fig. 3B, in one tumor (patient 23), the cancer cells and bronchial epithelial cells in the sample were strongly stained by the control EGFR antibody, but only the cancer cells were strongly stained by the anti-L858R antibody.

We further investigated whether these two mutation-specific antibodies can be useful for diagnosing rare exon 19 deletion mutations in 15 NSCLC patients who had been treated at the Aichi Cancer Research Hospital (patients 46-60). Paraffin-embedded tissue samples, which included four rare exon 19 deletions (patients 46-49), three delE746-A750 mutations (patients 50-52), four L858R mutations (patients 53-56), and four WT EGFR (patients 57-60), were examined immunohistochemically (Table 1). Of rare exon 19 deletion mutation, L747-P753>S (patient 47), L747-T751>Q (patient 48), and L747-A750>P (patient 49) were negatively stained by the anti–delE746-A750 antibody, and only T751-I759>NKA

A

EGFR antibodies

delE746-A750 | L858R | Total EGFR

(rows labeled: delE746-A750 (exon 19); L858R (exon 21); No EGFR mutation; panels a–r)

B

EGFR antibodies

delE746-A750 | L858R | Total EGFR

Score   0   3+   3+

Fig. 3. A, immunohistochemical analysis of human NSCLC tumor samples. Control EGFR antibody stained all six tumor samples shown, the EGFR deletion-specific antibody stained cancer cells only in the two samples with delE746-A750 mutations (a and b), and the L858R-specific antibody stained only the cancer cells in the two samples with L858R mutations (i and j). B, differential diagnosis by immunohistochemical analysis of WT and mutant EGFRs in a NSCLC patient. In one tumor sample (Table 1, patient 23), labeling for total EGFR showed strong EGFR expression in both bronchial epithelial cells (red) and cancer cells. However, labeling with the anti-L858R antibody only stained the cancer cells and did not stain the bronchus.

(patient 46) was moderately stained (+2). None of these four samples carrying deletions were stained by the anti-L858R antibody, but tumor samples carrying L858R mutations (patients 53-56) by DNA sequencing were positively stained by the anti-L858R antibody.

The diagnostic data in Table 1 for EGFR mutations identified by immunohistochemistry are summarized in Table 2. We observed a high correlation between the results from

DNA sequencing and immunohistochemistry. When staining +2 and +3 were determined as positive, EGFR mutation-specific antibodies detected delE746-A750 mutations in 79% (11 of 14) of cases identified by DNA sequencing, including patients from Kurume University Hospital and Aichi Cancer Research Hospital, and detected L858R mutations in 83% (19 of 23) of cases, indicating that this type of immunohistochemical analysis would be capable of diagnosing activating EGFR mutations. Thus far, rare exon 19 deletion mutations were examined using the anti-delE746-A750 antibody (Table 2); shorter (patients 14, 48, and 49) or longer (patients 13 and 47) deletion mutations than 15 bp were not positively stained. One (T751-I759>NKA, patient 46) harboring six–amino acid deletion, which was moderately (2+) stained and 1 (E746-T751>A, patient 5) was positively stained. Furthermore, of the samples without these EGFR mutations, immunohistochemistry with these two specific antibodies identified 100% (16 of 16) as negative for the deletion and point mutations in EGFR.

We next investigated whether these two mutation-specific antibodies can be useful in the small bronchial biopsies from stage IV NSCLC patients. Each stage IV patient harboring delE746-A750 or L858R showed strongly positive staining with the anti–delE746-A750 and anti-L858R, respectively (Fig. 4). In contrast, a stage IV patient without EGFR mutations showed strongly positive staining with anti-WT EGFR antibodies but not with both the anti–delE746-A750 and anti-L858R antibody.

Discussion

The ability to selectively administer EGFR-targeted drugs, such as gefitinib and erlotinib, to NSCLC patients carrying activating EGFR mutations is essential to the establishment of personalized anticancer therapy. A recent study has shown favorable clinical outcomes for patients with NSCLC adenocarcinoma carrying EGFR mutations after administering gefitinib compared with cisplatin-paclitaxel (17). The diagnosis of the activating EGFR mutations that are closely associated with the therapeutic efficacy of EGFR-targeted drugs is clearly essential to this strategy. The development of rapid and precise diagnostic techniques for activating EGFR mutations is particularly important for personalizing therapeutics in East Asian patients because these activating EGFR mutations (delE746-A750 and L858R) are significantly more frequent in this ethnic group. These EGFR mutations have been observed in 27.0% NSCLC patients in Japan, 36.8% in China, 19.2% in Korea, and 38.6% in Taiwan (8, 18–20).

The identification of EGFR mutations using mutation-specific antibodies would be a very useful diagnostic method for use in conjunction with DNA sequencing. Yu et al. (15) have generated antibodies specific for delE746-A750 and L858R mutations in EGFR and reported that the sensitivity of immunohistochemical assays using these antibodies was 92% in tests on 340 paraffin-embedded NSCLC tumor samples compared with a sensitivity of

Table 2. Summary of immunohistochemistry and DNA sequence analysis of NSCLC tumor samples

(A) Exon 19 deletions

Immunohistochemistry	DNA sequencing								
	delE746-A750	delE746-T751>A	delS752-I759	delL747-T751>P	delT751-I759>NKA	delL747-P753>S	delL747-T751>Q	delL747-A750>P	WT*
delE746-A750 (+)	11	1	0	0	1	0	0	0	0
delE746-A750 (−)	3	0	1	1	0	1	1	1	16

(B) L858R

Immunohistochemistry	DNA sequencing	
	L858R	WT*
L858R (+)	19	0
L858R (−)	4	16

*The same tumor samples were tested immuhistochemically in (A) and (B).

99% for DNA sequencing. This suggested that this simple immunohistochemical approach can be useful for establishing a rapid, sensitive, and cost-effective method to identify NSCLC patients responsive to EGFR-targeted therapeutics (15). In one tumor sample (patient 23) from the series used in this study, tumor cells, but not normal bronchial epithelial cells, were specifically immunostained with anti-L858R antibody (Fig. 3B), indicating that differential diagnosis between the WT and mutant EGFR in a single pathologic section was possible using this immunohistochemical approach. This result also suggested that a somatic EGFR mutation was present in cancer cells but not in normal cells.

In this study we have confirmed the usefulness of EGFR mutation-specific antibodies for the identification of activating EGFR mutations. The sensitivity of the delE746-A750– and L858R-specific antibodies was found to be 79% to 83% when all samples from Kurume University Hospital and Aichi Cancer Research Hospital were scored. Several other rare deletion mutations are also known to occur close to the E746-A750 deletion in exon 19 (21). In this study, to further investigate the presence of other EGFR mutations in detail, we also carried out direct DNA sequencing of exons 19 and 20 and confirmed that several deletion mutations occurred close to delE746-A750. We identified rare deletions in exon 19 of the EGFR

EGFR antibodies

Fig. 4. Immunohistochemical analysis of bronchial biopsy samples of stage IV NSCLC patients. A sample with delE746-A750 mutation was stained with anti delE746-A750–specific antibody, and a sample with L858R mutation was stained with L858R-specific antibody. These samples were stained with WT antibody. No sample without EGFR mutations were stained with these two mutation-specific antibodies. Samples were stained with H&E.
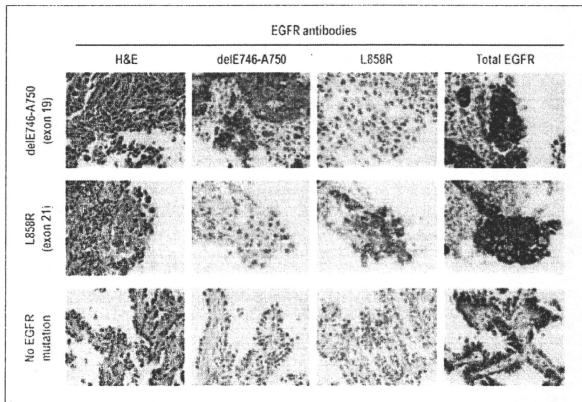
gene in seven tumor samples: one 8–amino acid deletion (S752-I759, patient 13), two 6–amino acid deletion (T751-I759.NKA, patient 46; L747-P753>S, patient 47), one 5–amino acid deletion (E746-T751>A, patient 5), two 4–amino acid deletion (L747-T751>P, patient 14; L747-T751>Q, patient 48), and one 3–amino acid deletion (L747-A750>P, patient 49). Of these seven rare exon 19 deletion mutations, five samples (patients 13, 14, and 47-49) were not positively stained, one sample (patient 46) was moderately stained, and one sample (patient 5) that harbor 5–amino acid deletion with T751A was positively stained by the anti–delE746-A750 antibody (Tables 1 and 2), suggesting that the del E746-A750 antibody may not be useful for identification of these rare exon 19 deletion mutations. Yu et al. (15) also reported rare deletion mutations in exon 19, one of which (E746-T751) was stained by the anti–delE746-A750 antibody, whereas the other (L747-A750) was not. Further refinement of these mutation-specific antibodies will be required to encompass these rare exon 19 deletion mutations and to improve the sensitivity of molecular diagnosis using immunohistochemistry.

Our immunohistochemical data described here for EGFR mutation-specific antibodies suggest that this approach will be very useful for identifying the EGFR mutations that are known to be closely associated with the therapeutic efficacy of EGFR-targeted drugs. One particular merit of immunohistochemical diagnosis is that it provides a measure of the expression levels of mutant EGFRs in the cancer cells in tumors from individual patients. Combining DNA sequencing and immunohistochemistry will be very useful for further refining personalized diagnoses for patients with NSCLC.

References

1. Devesa SS, Bray FI, Vizcaino AP, Parkin DM. International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. Int J Cancer 2005;117:294–9.
2. Parkin DM, Bray FI, Devasa SS. Cancer burden in the year 2000: the global picture. Eur J Cancer 2001;37:4–66.
3. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell-lung cancer. J Clin Oncol 2003;21:2237–46.
4. 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.
5. 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.
6. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "neversmokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306–11.
7. 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.
8. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–46.
9. Taron M, Ichinose Y, Rosell R, et al. Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. Clin Cancer Res 2005;11:5878–85.
10. Tokuooka S, Matsuo K, Shigematsu H, et al. The impact of sex and smoking status on the mutational spectrum of epidermal growth factor receptor gene in non-small cell lung cancer. Clin Cancer Res 2007;13:5763–8.
11. Bell DW, Brannigan BW, Matsuo K, et al. Increased prevalence of EGFR-mutant lung cancer in women and in east Asian populations: analysis of estrogen-related polymorphisms. Clin Cancer Res 2008; 14:4079–84.
12. Ono M, Kuwano M. Molecular mechanisms of epidermal growth factor receptor activation and response to gefitinib and other EGFR-targeting drugs. Clin Cancer Res 2006;12:7242–51.
13. Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small cell lung cancer with postoperative recurrence. J Clin Oncol 2005;23:2513–20.
14. Takano T, Ohe Y, Sakamoto H, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 2005;23:6829–37.
15. Yu J, Kane S, Wu J, et al. Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. Clin Cancer Res 2009;15:3023–8.
16. Ono M, Hirata A, Kometani T, et al. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther 2004;3:465–72.
17. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361: 947–57.
18. Wu XL, Li LY, Zhang XT, et al. Gefitinib-sensitive mutations of the epidermal growth factor receptor tyrosine kinase domain in Chinese patients with non-small cell lung cancer. Clin Cancer Res 2005;11: 4289–94.
19. Han SW, Kim TY, Lee KH, et al. Clinical predictors versus epidermal growth factor receptor mutation in gefitinib-treated non-small cell lung cancer patients. Lung Cancer 2006;54:201–7.
20. 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.
21. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer 2007;7: 169–81.

ELSEVIER

Original contribution

The close correlation between 8-hydroxy-2'-deoxyguanosine and epidermal growth factor receptor activating mutation in non–small cell lung cancer[☆]

Akihiko Kawahara PhD[a,b], Koichi Azuma MD, PhD[b,c], Satoshi Hattori PhD[d],
Kazutaka Nakashima BS[a,b], Yuji Basaki PhD[e], Jun Akiba MD, PhD[f],
Sinzo Takamori MD, PhD[g], Hisamichi Aizawa MD, PhD[c], Takashi Yanagawa PhD[d],
Hiroto Izumi MD, PhD[h], Kimitoshi Kohno MD, PhD[h], Suminori Kono MD, PhD[i],
Masayoshi Kage MD, PhD[a,b], Michihiko Kuwano MD, PhD[b,j], Mayumi Ono PhD[b,e,i,*]

[a]Department of Diagnostic Pathology, Kurume University Hospital, Kurume 830-0011, Japan
[b]Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume 830-0011, Japan
[c]Division of Respirology, Neurology, and Rheumatology, Department of Internal Medicine,
Kurume University School of Medicine, Kurume 830-0011, Japan
[d]Biostatistics Center, Kurume University, Kurume 830-0011, Japan
[e]Department of Pharmaceutical Oncology, Graduate School of Pharmaceutical Sciences, Kyushu University,
Fukuoka 812-8582, Japan
[f]Department of Pathology, Kurume University School of Medicine, Kurume 830-0011, Japan
[g]Department of Surgery, Kurume University School of Medicine, Kurume 830-0011, Japan
[h]Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health,
Kitakyushu 807-8555, Japan
[i]Department of Preventive Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[j]Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka 812-8582, Japan

Keywords:
8-OHdG;
EGFR activating
mutations;
Gefitinib;
YB-1;
NSCLC

Summary Patients with non–small cell lung cancer harboring mutations in the epidermal growth factor receptor gene, including delE746-A750 and L858R, are highly sensitive to therapy with epidermal growth factor receptor–targeting drugs, such as gefitinib and erlotinib, in comparison with those harboring wild-type epidermal growth factor receptor. It remains unclear how such epidermal growth factor receptor mutations are induced. In this study, we examined whether 8-hydroxy-2'-deoxyguanosine, a representative oxygen nucleotide of DNA, could play a role in activating mutations of the epidermal growth factor receptor gene and also whether Y-box binding protein–1 and 8-

oxoguanine DNA glycosylase that are involved in repair of oxidative stimuli-induced DNA damages could play any role in epidermal growth factor receptor activating mutations. Immunohistochemistry was used to evaluate the expression of 8-hydroxy-2'-deoxyguanosine, Y-box binding protein-1, and 8-oxoguanine DNA glycosylase in patients with non–small cell lung cancer (N = 170). We analyzed mutations of delE746-A750 and L858R in the epidermal growth factor receptor gene using peptide nucleic acid–locked nucleic acid polymerase chain reaction clamping. In non–small cell lung cancer patients, nuclear 8-hydroxy-2'-deoxyguanosine expression was strongly associated with these epidermal growth factor receptor mutations. Furthermore, nuclear expression of Y-box binding protein-1 was inversely associated with epidermal growth factor receptor mutations; but nuclear expression of 8-oxoguanine DNA glycosylase was not. Among 51 patients who were treated with gefitinib, progression-free survival was substantially better when 8-hydroxy-2'-deoxyguanosine expression was positive, when epidermal growth factor receptor mutations were present, and when nuclear Y-box binding protein-1 expression was negative. Thus, activating mutations of the epidermal growth factor receptor gene in non–small cell lung cancer were closely associated with a decrease in the damage repair process for 8-hydroxy-2'-deoxyguanosine in oxidized DNA.

1. Introduction

Lung cancer continues to be a leading cause of cancer death worldwide [1], and non–small cell lung cancer (NSCLC) is the most common type of the disease. Despite many clinical trials of platinum-based chemotherapy in combination with various drugs, the prognosis of patients with NSCLC remains poor [2]. The development of molecular-targeting drugs, including gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR), has improved the efficacy of therapy for NSCLC. The absence or presence of activating mutations within the kinase domain of the EGFR gene in adenocarcinoma of NSCLC has a key role in determining the therapeutic efficacy of EGFR-targeting drugs [3-5]. The presence of activating EGFR mutations in lung cancer cells confers an EGF/TGFα-independent growth capacity together with susceptibility to the cytotoxic effect of gefitinib [6]. About 80% of tumors possessing EGFR-activating mutations respond to EGFR-tyrosine kinase inhibitors. In NSCLC patients, more than 90% of EGFR mutations are located in exon 19 (delE746-A750) or 21 (L858R point mutation). Several factors have been reported to be associated with the frequency of EGFR mutations, including an adenocarcinoma phenotype, female sex, never smoker, and East Asian ethnicity [7]; but how mutations that increase sensitivity to the therapeutic effect of gefitinib or erlotinib are induced remains unclear. A recent study by Mok et al (2009) [8] reported that gefitinib is superior to carboplatin-paclitaxel as an initial treatment of NSCLC adenocarcinoma among nonsmokers in East Asia.

Oxidative DNA damage and repair contribute to the development of various human pathologies, including cancer. In both nuclear and mitochondrial DNA, the oxygenated nucleotide 8-hydroxy-2'-deoxyguanosine (8-OHdG) has been implicated in the type of somatic mutations found in human cancers [9]. The major pathway for oxidative DNA damage repair is base excision repair, which in humans involves the MutM human homolog 8-oxoguanine DNA glycosylase (OGG1), the MutY homolog MUTYH, and the Mth1 homolog MTH1 [10]. Among these base excision repair-related genes, OGG1 appears to be very important. Oka et al [11] have demonstrated 2 distinct pathways of cell death by oxidative damage to nuclei and mitochondria, and OGG1 plays key role in the repair of oxidative DNA damage in both pathways. On the other hand, OGG1-null mice develop adenomas and carcinomas of the lungs with a marked increase of 8-OHdG [12]; and base excision repair-defective mice (myh$^{-/-}$, OGG1$^{-/-}$) show significantly increased accumulation of 8-OHdG in the liver, small intestine, and lung DNA in comparison with wild-type mice [13]. Frequent loss of heterozygosity has been observed in the region of the OGG1 gene in lung and kidney cancer [14], and some genetic polymorphisms of the OGG1 gene are associated with an increased risk for various cancers [15]. Elevated levels of urinary 8-OHdG have also been detected in patients with various cancers, including those of the breast, bladder, and prostate [16]. Furthermore, 8-OHdG and base excision repair modulation are expected to be risk factors for human cancers [17]. 8-OHdG has also been highlighted as a marker of oxidative stress and damage related to occupational and environmental exposure [18,19]. These basic and clinical findings strongly suggest that 8-OHdG plays a key role in somatic mutations and human carcinogenesis.

On the other hand, the Y-box binding protein-1 (YB-1) has been implicated in numerous functions, such as drug resistance, cell growth/proliferation, malignant transformation, and DNA repair through its regulation of transcription and translation, and its suppression of oxidative stress [20,21]. Increased expression of the YB-1 gene has been shown to induce both the development of breast cancers of many histologic types and genome instability in an experimental animal model, suggesting that YB-1 has oncogenic activity [22]. A recent study by de Souza-Pinto et al (2009) [23] showed that YB-1 depletion in human cancer cells increases mitochondrial DNA mutagenesis,

suggesting YB-1 as a key candidate for mitochondrial mismatch-binding protein. YB-1 binds specifically to DNA/RNA that has been damaged or modified by DNA-damaging agents, including hyperoxide [24,25]. YB-1 may thus play a protective role against various types of genotoxic damage, including oxidative DNA damage. Furthermore, it has been shown that the nuclear expression of YB-1 is associated with the favorable outcome of patients with NSCLC [26,27].

In the present study, we investigated whether 8-OHdG is associated with EGFR activating mutations and also whether 8-OHdG is associated with the nuclear expression of YB-1 or OGG1. We also examined the outcome after gefitinib treatment of patients with NSCLC harboring EGFR mutations in relation to 8-OHdG expression and EGFR mutations. On the basis of our clinicopathologic findings, we discuss the possible role of base excision repair of oxidized DNA in EGFR mutations in NSCLC.

2. Materials and methods

2.1. Patients, tumor samples, and treatment

We retrospectively examined 170 patients with primary NSCLC whose tumors had been completely removed surgically at the Department of Surgery, Kurume University Hospital, between 1995 and 2005. Among these patients, 102 were diagnosed histologically with adenocarcinoma; and the other 68, with squamous cell carcinoma (Table 1). The age ranged from 41 to 82 years, with a median of 68 years. Two patients had received adjuvant chemotherapy, and the other patients had not received neoadjuvant or adjuvant chemo-
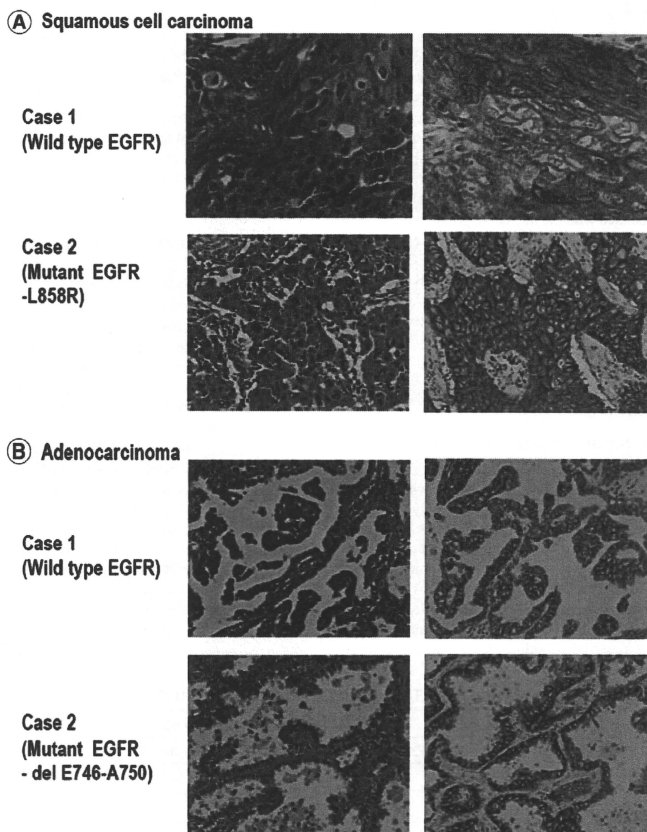
therapy. Among 170 patients, 51 had received gefitinib therapy for the recurrence after surgical resection between July 2002 and February 2009. Thirteen patients had received gefitinib as the initial therapy; and the others, as second- or third-line therapy (33 patients, platinum doublets as first line; 5 patients, monotherapy, nonplatinum doublets, and platinum doublets as second line). Tumor response was evaluated after chemotherapy according to the Response Evaluation Criteria for Solid Tumors. This study was approved by the Institutional Review Board of Kurume University.

2.2. Antibodies and immunohistochemistry

Paraffin-embedded tissue samples were cut at a thickness of 4 μm, examined on coated glass slides, and labeled with the following antibodies using the BenchMark XT (Ventana Automated Systems, Inc, Tucson, AZ). Anti-8-OHdG antibody was obtained from the Japan Institute for the Control of Aging (Shizuoka, Japan). Anti-YB-1 polyclonal antibody was generated against a 15–amino acid synthetic peptide in the COOH-terminal domain, and this antibody was used at a working dilution of 1:2000. OGG1 used the ChemMate ENVISION method (DakoCytomation, Glostrup, Denmark). Endogenous peroxidase activity was inhibited by incubating the slides in 3% H_2O_2 for 5 minutes. Each slide was heat-treated using Target Retrieval Solution, pH 9.0 (DAKO, Glostrup, Denmark) for 30 minutes and incubated with the antibody at 4°C overnight. Immunohistochemistry (IHC) analysis was performed as described previously. Positive and negative controls were used for each section. Fig. 1 shows representative images of hematoxylin and eosin and IHC with anti-EGFR antibody for 2 samples of each

Table 1 Characteristics of 170 patients with NSCLC according to EGFR status and 8-OHdG expression

Characteristic	EGFR status		P value	8-OHdG		P value
	Wild type (n = 122)	Mutation (n = 48)		Positive (n = 97)	Negative (n = 73)	
Age						
≤65	44	30	.002	49	25	.042
>65	78	18		48	48	
Sex						
Female	31	30	<.001	44	17	.004
Male	91	18		53	56	
Smoking status						
Never	29	33	<.001	44	18	.006
Ever	93	15		53	55	
Histologic type						
Squamous cell carcinoma	62	6	<.001	28	40	<.001
Adenocarcinoma	60	42		69	33	
Bronchioloalveolar carcinoma (BAC)	13	11	.640	17	7	.806
Non-BAC	47	31		52	26	
Pathologic stage						
I	57	16	.257	49	24	.010
II	29	13		16	26	
III	36	19		32	23	

(A) Squamous cell carcinoma

Case 1
(Wild type EGFR)

Case 2
(Mutant EGFR
-L858R)

(B) Adenocarcinoma

Case 1
(Wild type EGFR)

Case 2
(Mutant EGFR
- del E746-A750)

Fig. 1   Typical histologic findings of squamous cell carcinoma and adenocarcinoma. IHC with anti-EGFR antibody for 2 samples of each squamous cell carcinoma and adenocarcinoma (A and B). Two samples with or without activating EGFR mutation are presented.

squamous cell carcinoma and adenocarcinoma. Two samples with or without activating EGFR mutation are presented.

The intensity of nuclear-positive cancer cells of 8-OHdG was expressed as follows: none, weak, or strong (Fig. 2A). We classified 8-OHdG nuclear expression of none and weak as negative (Fig. 2A, a and b) and strong as positive (Fig. 2A,

c and d). YB-1 was expressed in the cytoplasm alone, in both nuclei and cytoplasm, or in cytoplasm in NSCLC (Fig. 2B). The extent of staining of nuclear YB-1 and OGG1 was classified based on cells with strongly stained nuclei; 5% or more tumor cells had nuclear-positive YB-1 or OGG1 (Fig. 2B, c and dl and 2C, a), and less than 5% had nuclear

Fig. 2   Examples of IHC showing negative and positive expressions of 8-OHdG (A), YB-1 (B) ,and OGG1 (C) in NSCLC. A, Expression of 8-OHdG in 4 clinical specimens: 8-OHdG negative (no or weak immunostaining) (a, b) and 8-OHdG positive (strong immunostaining) (c, d). B, Nuclear expression of YB-1 in 2 clinical samples: negative samples show no or weak immunostaining in the nucleus; and nuclear YB-1− positive samples show strong immunostaining in the nucleus, as indicated by arrowheads. C, OGG1 in 2 clinical specimens: defined as positive (a) and negative (b).

(A) 8-OHdG

Negative

Positive

(B) Nuclear YB-1

Negative

Positive

(C) OGG1

Positive   Negative
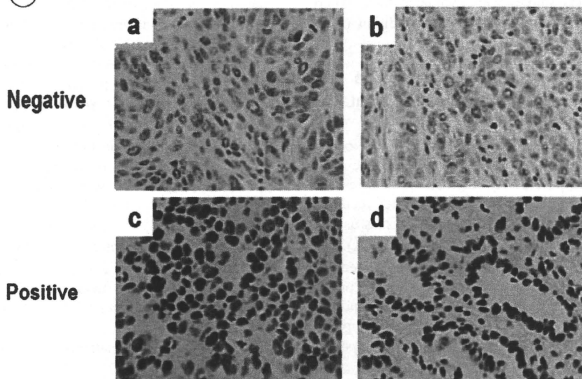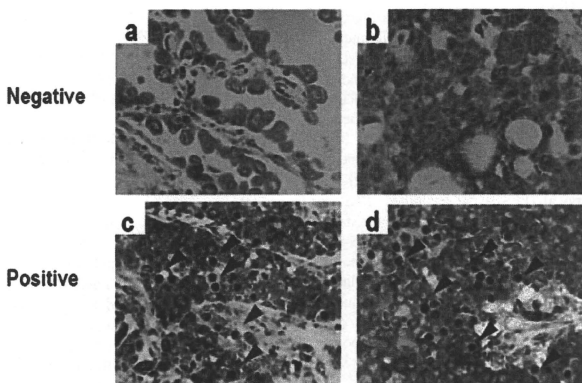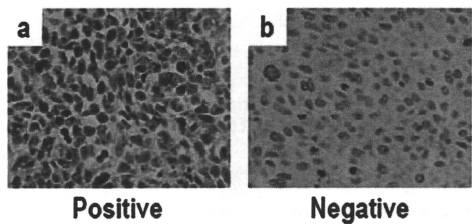
negative (Fig. 2B, a and b; and 2C, b) [25]. All immunohistochemical studies were evaluated by 2 experienced observers who were blind to the condition of the patients (A. K. and M. Ka.).

2.3. Peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp for EGFR mutation

Mutations of the EGFR gene were examined in exons 19 (delE746-A750) and 21 (L858R) by peptide nucleic acid–locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp as described previously. In brief, genomic DNA was purified from paraffin-embedded tissues using a QIAamp DNA Micro kit (QIAGEN, Valencia, CA). PCR primers used were synthesized by Invitrogen Inc (Carlsbad, CA). PNA clamp primers and LNA mutant probes were purchased from FASMEC (Kanagawa, Japan) and IDT (Coralville, IA), respectively. PNA-LNA PCR clamp was performed using a SDS-7500 System (Applied Biosystems, Foster City, CA).

2.4. Statistical analysis

Histologic type and clinicopathologic factors (age, sex, smoking status, and pathologic stage) by EGFR mutation and 8-OHdG expression were tested by Fisher exact test. The association between EGFR mutations and 8-OHdG and also between 8-OHdG and YB-1 was tested by Fisher exact test. It was also applied to examine whether they are associated with the nuclear expression of YB-1 and OGG1. In 51 patients who were treated with gefitinib after recurrence, the effects of 8-OHdG, EGFR mutation, and nuclear YB-1 on progression-free survival (PFS) were examined. *PFS* was defined as the time until disease progression from the start of gefitinib treatment. Kaplan-Meier estimators for PFS were calculated according to 8-OHdG, EGFR mutation, and nuclear YB-1, respectively. Log-rank tests were applied to examine the effects of 8-OHdG, EGFR mutation, and nuclear YB-1; and hazard ratios and 95% confidence intervals (CIs) were estimated with the Cox proportional hazards models. Statistical significance was declared if the 2-sided *P* value was less than .05. Statistical analysis was performed with SAS version 9.1 (SAS Institute Inc, Cary, NC), R version 2.8.1, and StatXact 7 (Cytel Inc, Cambridge, MA).

3. Results

3.1. Nuclear expression of 8-OHdG was associated with activating mutations of the EGFR in NSCLC

Fig. 2 shows representative examples of IHC staining for 8-OHdG (Fig. 2A), YB-1 (Fig. 2B), and OGG1 (Fig. 2C) in NSCLC. IHC staining showed very clear differences between positive and negative expressions of 8-OHdG,

YB-1, and OGG1 in the nucleus. The clinical and pathologic characteristics at the time of diagnosis according to EGFR mutations are summarized in Table 1. Among the 170 patients, 48 (28.2%) harbored activating mutations of the EGFR; delE746-A750 and L858R mutations were observed in 26 and 22 patients, respectively. None showed simultaneous mutations at the 2 loci. There were higher proportions of younger patients, women, and nonsmokers among patients with the EGFR mutation. Adenocarcinoma was also more frequent in patients with the EGFR mutation. All of these differences were statistically significant. In 102 cases of adenocarcinoma, 24 cases of pure bronchioalveolar carcinoma and 78 cases of invasive adenocarcinomas were observed; and there was no minimally invasive bronchioalveolar carcinoma. EGFR mutations of pure bronchioalveolar carcinomas and invasive adenocarcinomas were observed in 11 (45.8%) of 24 and in 31 (39.7%) of 78, respectively.

Of 170 patients, 97 patients were found to be positive for 8-OHdG expression (57.1% of the total). The predominance of younger patients, men, smokers, and adenocarcinomas was evident in patients positive for 8-OHdG expression (Table 1). On the other hand, more patients showing positivity for 8-OHdG were at the earliest stage. On the basis of 8-OHdG expression and EGFR mutation in the 170 patients, EGFR mutation showed significantly (*P* < .001) higher frequency in patients positive for 8-OHdG expression than in those who were negative (Table 2).

3.2. Nuclear YB-1 expression was associated with EGFR mutations, but not with 8-OHdG

Because YB-1 or OGG1 is expected to be involved in repair process for oxidized DNA, we next examined whether the nuclear expression of YB-1 or OGG1 was associated with the 8-OHdG expression or EGFR mutations. We observed that those with delE746-A750 or L858R mutation in the EGFR gene had a significantly (*P* < .001) lower prevalence of the nuclear positivity for YB-1 expression than those who were negative (Table 3). Concerning the possible correlation between 8-OHdG and nuclear YB-1 expression, nuclear YB-1 expression was found to be positive in 32 (33.0%) and negative in 65 (67.0%) of 8-OHdG–positive

Table 2 Correlation between EGFR mutation status and 8-OHdG expression

| EGFR status	8-OHdG		*P* value
	Positive (n = 97)	Negative (n = 73)	
Wild type (n = 122)	58 (59.8%)	64 (87.7%)	<.001
Mutation (n = 48)	39 (40.2%)	9 (12.3%)	
delE746-A750	22	4	
L858R	17	5	

Table 3 Correlation of 8OHdG expression and EGFR mutation with nuclear expression of YB-1 and OGG1

	8-OHdG		P value	EGFR status		P value
	Positive (n = 97)	Negative (n = 73)		Wild type (n = 122)	Mutation (n = 48)	
Nuclear YB-1						
Positive (n = 66)	32 (33.0%)	34 (46.6%)	.082	58 (47.5%)	8 (16.7%)	<.001
Negative (n = 104)	65 (67.0%)	39 (53.4%)		64 (52.5%)	40 (83.3%)	
OGG1						
Positive (n = 43)	28 (28.9%)	15 (20.5%)	.285	30 (24.6%)	13 (27.1%)	.845
Negative (n = 127)	69 (71.1%)	58 (79.5%)		92 (75.4%)	35 (72.9%)	

patients (n = 97), whereas there was similar number of YB-1 positive in 34 (46.6%) and YB-1 negative in 39 (53.4%) of 8-OHdG–negative patients (n = 73). However, the correlation between 8-OHdG expression and nuclear YB-1 expression was just short of statistical significance ($P = .082$). In contrast, OGG1 expression was not statistically significantly
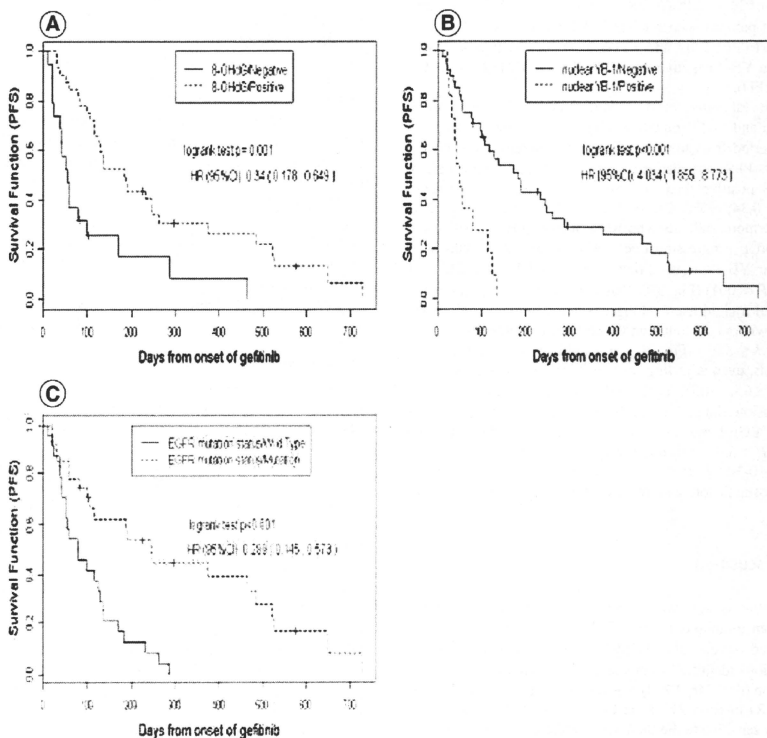
Fig. 3 Kaplan-Meier estimate of PFS from the start of gefitinib therapy in relation to 8-OHdG expression (A), nuclear YB-1 expression (B), and EGFR mutation (C) in 51 patients who received the drug for NSCLC recurrence after surgical resection. Abbreviation: HR, hazard ratio.

associated with 8-OHdG expression or EGFR mutation (Table 3).

3.3. Correlation of 8-OHdG and EGFR mutations with PFS in patients who received gefitinib

Among 51 patients who received gefitinib for recurrence after surgical resection, tumors in 46 were histologically diagnosed as adenocarcinoma; and the other 5, as squamous cell carcinoma. Fifteen patients were men and 36 were women. Fifteen were smokers and 36 were nonsmokers. Six patients were classified as stage III and the other 45 as stage IV at the start of gefitinib therapy. The response rate in patients with mutant EGFR was 10 of 27 = 37.0% (10 partial response [PR], 12 stable disease [SD], and 5 progressive disease [PD]), whereas that in patients with wild-type EGFR was 6 of 24 = 25.0% (6 PR, 11 SD, and 7 PD). The response rate in patients with nuclear YB-1 positive was 4 of 11 = 36.4% (4 PR, 2 SD, and 5 PD), whereas that in patients with nuclear YB-1 negative was 12 of 40 = 30.0% (12 PR, 21 SD, and 7 PD).

The estimated product-limit survival functions of 8-OHdG and EGFR mutation with respect to the progression-free period from the start of gefitinib therapy are shown in Fig. 3. PFS was distinctly better in patients who were 8-OHdG positive than in those who were negative (hazard ratio, 0.34; 95% CI, 0.18-0.65; $P = .001$) (Fig. 3A). Furthermore, patients who were nuclear YB-1 positive had a shorter progression-free period than those who were nuclear YB-1 negative (hazard ratio, 4.03; 95% CI, 1.86-8.77; $P < .001$) (Fig. 3B). Patients with EGFR mutation also showed a significantly longer progression-free period than those without the mutation (hazard ratio, 0.29; 95% CI, 0.15-0.57; $P < .001$) (Fig. 3C). By multivariate Cox regression analysis, even adjusting for possible confounding factors of age (≤65, >65), sex, and smoking status, 8-OHdG expression (hazard ratio, 0.34; 95% CI, 0.17-0.68; $P = .002$), EGFR mutation (hazard ratio, 0.28; 95% CI, 0.14-0.57; $P < .001$) and nuclear YB-1 (hazard ratio, 4.80; 95% CI, 2.10-10.99; $P < .001$) were found to be independent prognostic factors with regard to the progression-free period.

4. Discussion

In this study, we demonstrated a strong association between mutations in the EGFR gene and the presence of elevated levels of 8-OHdG in patients with NSCLC. Mutations identified in this study included a small in-frame deletion (delE746-A750) in exon 19 and a missense mutation (L858R) in exon 21 of the EGFR gene, both of which are highly sensitive to the therapeutic effects of EGFR-targeting drugs, such as gefitinib and erlotinib [3-6]. These mutations in NSCLC are well known to be significantly associated with female sex and never having smoked [28]. Toyooka et al

(2008) [29] examined the impact of sex and smoking status on the mutational spectrum of the EGFR gene in NSCLC (n = 1467) and found that, in women, mutations in exons 19 and 21 were significantly less frequent in ever smokers than in never smokers, whereas in men, mutations in exons 19, 21, and 18 were significantly less frequent in ever smokers than in never smokers.

Concerning the induction of 8-OHdG, smoking has been identified as an important factor. Although some studies concluded that 8-OHdG is a biomarker of oxidative stress associated with chemical exposure, including smoking, benzene, and asbestos, various occupational studies did not reveal higher levels of 8-OHdG in smokers [18]. It remains to be further studied how 8-OHdG is induced in response to oxidative stress in lung cancer and also how 8-OHdG could affect mutations in the EGFR gene in lung cancer. One possible mechanism whereby 8-OHdG affects EGFR mutations in NSCLC could be failure of the base excision repair process for eliminating oxidized DNA, thus resulting in augmentation of EGFR mutations and promotion of lung carcinogenesis. Two large independent case-control studies of lung cancer demonstrated that the rate of base excision repair of 8-OHdG was decreased in blood leukocytes of cancer patients in comparison with controls [30,31]. Furthermore, Speina et al (2003) [32] reported that repair capacity was significantly lower in blood leukocytes of lung cancer patients than in those of controls. Our findings suggest that decreased efficacy of base excision repair to eliminate 8-OHdG in oxidized DNA lesions may enhance not only the development of lung cancers, but also mutations in EGFR genes.

Our present study demonstrated that YB-1 expression was inversely associated with EGFR-activating mutations in NSCLC. A protective effect of YB-1 against genotoxic damage may explain the inverse relationship between EGFR mutation and nuclear YB-1 expression. YB-1 shows much higher affinity for DNA/RNA that has been damaged by oxidation or genotoxic drugs than for undamaged DNA/RNA [21,33]; and this molecule interacts with the repair-related proteins PCNA, p53, and HMGB1 to promote the repair of genotoxic damage. In particular, YB-1 harboring endonuclease III is considered to mediate base excision repair and strand separation of damaged DNA [34,35]. On the other hand, DNA base excision repair by OGG1 and relevant molecules is known to be a major pathway for repair of oxidative DNA damage. Some genetic changes of OGG1 are associated with increased risks of various human malignancies [14,15]. However, in our present study, OGG1 expression was not significantly correlated with 8-OHdG expression or EGFR mutation status. The reparative property of YB-1 might play a rather more important role in activating EGFR mutations than that of OGG1.

The present study further demonstrated a substantially better prognosis after gefitinib treatment among NSCLC patients with 8-OHdG expression and among those with EGFR mutation. No previous study has evaluated the effect

of gefitinib in relation to oxidative DNA damage, and we present for the first time evidence that EGFR mutations are positively correlated with 8-OHdG expression in NSCLC. Our study also demonstrated that 8-OHdG expression and EGFR mutation were associated with nonsmoking status and female sex. Thus, it is considered that 8-OHdG is a biomarker for mutagenesis in the EGFR gene and could be used to optimize anticancer therapeutics using EGFR-targeting drugs.

Acknowledgments

References

[1] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533-43.

[2] Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non–small-cell lung cancer: Four-Arm Cooperative Study in Japan. Ann Oncol 2007;18:317-23.

[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 2004;350:2129-39.

[4] 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.

[5] Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancer from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004;101:13306-11.

[6] Ono M, Kuwano M. Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. Clin Cancer Res 2006;12:7242-51.

[7] Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339-46.

[8] Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361: 947-57.

[9] Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: induction, repair and significance. Mutat Res 2004;567:1-61.

[10] Tsuzuki T, Nakatsu Y, Nakabeppu Y. Significance of error-avoiding mechanisms for oxidative DNA damage in carcinogenesis. Cancer Sci 2007;98:465-70.

[11] Oka S, Ohno M, Tsuchimoto D, Sakumi K, Furuichi M, Nakabeppu Y. Two distinct pathways of cell death triggered by oxidative damage to nuclear and mitochondrial DNAs. EMBO J 2008;27:421-32.

[12] Sakumi K, Tominaga Y, Furuichi M, et al. Ogg1 knockout-associated lung tumorigenesis and its suppression by Mth1 gene disruption. Cancer Res 2003;63:902-5.

[13] Russo MT, De Luca G, Degan P, et al. Accumulation of the oxidative base lesion 8-hydroxyguanine in DNA of tumor-prone mice defective in both the Myh and Ogg1 DNA glycosylases. Cancer Res 2004;64:4411-4.

[14] Chevillard S, Radicella JP, Levalois C, et al. Mutations in OGG1, a gene involved in the repair of oxidative DNA damage, are found in human lung and kidney tumours. Oncogene 1998;16:3083-6.

[15] Hung RJ, Hall J, Brennan P, Boffetta P. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. Am J Epidemiol 2005;162:925-42.

[16] Chiou CC, Chang PY, Chan EC, Wu TL, Tsao KC, Wu JT. Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers. Clin Chim Acta 2003;334:87-94.

[17] Tudek B. Base excision repair modulation as a risk factor for human cancers. Mol Aspects Med 2007;28:258-75.

[18] Pilger A, Rüdiger HW. 8-Hydroxy-2'-deoxyguanosine as a marker of oxidative DNA damage related to occupational and environmental exposures. Int Arch Occup Environ Health 2006;80:1-15.

[19] De Vizcaya-Ruiz A, Barbier O, Ruiz-Ramos R, Cebrian ME. Biomarkers of oxidative stress and damage in human populations exposed to arsenic. Mutat Res 2009;674:85-92.

[20] Matsumoto K, Wolffe AP. Gene regulation by Y-box proteins: coupling control of transcription and translation. Trends Cell Biol 1998;8:318-23.

[21] Kohno K, Izumi H, Uchiumi T, et al. The pleiotropic functions of the Y-box-binding protein, YB-1. Bioessays 2003;25:691-8.

[22] Bergmann S, Royer-Pokara B, Fietze E, et al. YB-1 provokes breast cancer through the induction of chromosomal instability that emerges from mitotic failure and centrosome amplification. Cancer Res 2005; 65:4078-87.

[23] de Souza-Pinto N, Mason P, Hashiguchi K, et al. Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. DNA Repair 2009;8:704-19.

[24] Ise T, Nagatani G, Imamura T, et al. Transcription factor Y-box binding protein 1 binds preferentially to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen. Cancer Res 1999;59: 342-6.

[25] Hayakawa H, Uchiumi T, Fukuda T, et al. Binding capacity of human YB-1 protein for RNA containing 8-oxoguanine. Biochemistry 2002; 41:12739-44.

[26] Shibahara K, Sugio K, Osaki T, et al. Nuclear expression of the Y-box binding protein as a novel marker of disease progression in non–small cell lung cancer. Clin Cancer Res 2001;7:3151-5.

[27] Kashihara M, Azuma K, Kawahara A, et al. Nuclear Y-box binding protein-1 (YB-1), a predictive marker of prognosis, is correlated with expression of HER2/ErbB2 and HER3/ErbB3 in non–small cell lung cancer. J Thorac Oncol 2009;4:1066-74.

[28] Subramanian J, Govindan R. Lung cancer in never smokers: a review. J Clin Oncol 2007;25:561-70.

[29] Toyooka S, Takano T, Kosaka T, et al. Epidermal growth factor receptor mutation, but not sex and smoking, is independently associated with favorable prognosis of gefitinib-treated patients with lung adenocarcinoma. Cancer Sci 2008;99:303-8.

[30] Gackowski D, Speina E, Zielinska M, et al. Products of oxidative DNA damage and repair as possible biomarkers of susceptibility to lung cancer. Cancer Res 2003;63:4899-902.

[31] Paz-Elizur T, Krupsky M, Blumenstein S, Elinger D, Schechtman E, Livneh Z. DNA repair activity for oxidative damage and risk of lung cancer. J Natl Cancer Inst 2003;95:1312-9.

[32] Speina E, Zielińska M, Barbin A, et al. Decreased repair activities of 1, N6-ethenoadenine and 3, N4-ethenocytosine in lung adenocarcinoma patients. Cancer Res 2003;63:4351-7.

[33] Kuwano M, Oda Y, Izumi H, et al. The role of nuclear Y-box binding protein 1 as a global marker in drug resistance. Mol Cancer Ther 2004; 3:1485-92.

[34] Gaudreault I, Guay D, Lebel M. YB-1 promotes separation in vitro of duplex DNA containing either mispaired bases or cisplatin modifications, exhibits endonucleolytic activities and binds several DNA repair proteins. Nucleic Acids Res 2004;32:316-27.

[35] Das S, Chattopadhyay R, Bhakat KK, et al. Stimulation of NEIL2-mediated oxidized base excision repair via YB-1 interaction during oxidative stress. J Biol Chem 2007;282:28474-84.

Y-box binding protein-1 (YB-1) promotes cell cycle progression through CDC6-dependent pathway in human cancer cells

Yuji Basaki [a], Ken-ichi Taguchi [b], Hiroto Izumi [c], Yuichi Murakami [a], Takuya Kubo [a], Fumihito Hosoi [a], Kosuke Watari [a], Kenji Nakano [d], Hidetoshi Kawaguchi [e], Shinji Ohno [e], Kimitoshi Kohno [b], Mayumi Ono [a], Michihiko Kuwano [d,*]

[a] Department of Pharmaceutical Oncology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan
[b] Department of Pathology and Clinical Laboratory, National Kyushu Cancer Center, Fukuoka, Japan
[c] Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan
[d] Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka, Japan
[e] Department of Breast Oncology, National Kyushu Cancer Center, Fukuoka, Japan

ARTICLE INFO

ABSTRACT

Y-box binding protein-1 (YB-1) plays pivotal roles in acquisition of global drug resistance and cell growth promotion through transcriptional activation of genes for both drug resistance and growth factor receptors. In this study, we investigated whether YB-1 is involved in regulation of the cell cycle and cell proliferation of human cancer cells. Treatment with YB-1 siRNA caused a marked suppression of cell proliferation and expression of a cell cycle related gene, CDC6 by cancer cells. Of cell cycle of cancer cells, S phase content was specifically reduced by knockdown of YB-1. The overexpression of CDC6 abrogated this inhibition of both cell proliferation and S phase entry. ChIP assay demonstrated that YB-1 binds to a Y-box located in the promoter region of the CDC6 gene. Expression of cyclin D1, CDK1 and CDK2 was also reduced with increased expression of $p21^{Cip1}$ and $p16^{INK4A}$ when treated with YB-1 siRNA. Furthermore, the nuclear YB-1 expression was significantly associated with the level of CDC6 nuclear expression in patients with breast cancer. In conclusion, YB-1 plays an important role in cell cycle progression at G1/S of human cancer cells. YB-1 thus could be a potent biomarker for tumour growth and cell cycle in its close association with CDC6.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

The Y-box binding protein-1 (YB-1) whose cold shock domain is highly conserved plays essential roles in transcriptional and translation regulation and DNA repair. It has been involved in cell growth, apoptosis, drug resistance, embryogenesis and carcinogenesis.[1,2] Specifically, YB-1 activation enhances expression of the ABC transporter gene encoding ABCB1 (P-glycoprotein) in cultured human cancer cells in response to genotoxic stimuli.[3,4] Nuclear expression of YB-1 has been significantly correlated not only with the expression of the ABCB1 gene in various human malignancies[5–11] but also with expression of non-P-glycoprotein-mediated drug resistance-related genes,[2] suggesting that YB-1 could be a biomarker of global drug resistance in human cancer.[12]