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様式第19

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委託業務題目
「BIM多型陽性癌におけるHDAC阻害薬の耐性克服効果を最適化する薬力学的効果の指標を探索する研究(H26-革新的がん-一般-113)」
機関名　金沢大学

1．学会等における口頭・ポスター発表

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Therapeutic and preventive antiemetic effect of aprepitant in Japanese patients with thoracic malignancies who truly need it	Ito S, Tsukiyama I, Ando M, Katakami M, Hamanaka R, Kosaka K, Matsubara A, Nishimura M, Tanaka H, Asai N, Yokoe N, Takahashi A, Baba K, Matsuura K, Yamaguchi E, and Kubo A	Support Care Cancer	2014	国外
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A predictive power of prothrombin time and serum total bilirubin for postoperative mortality after major hepatectomy with extrahepatic bile duct resection	Yokoyama Y, Ebata T, Igami T, Sugawara G, Ando M, and Nagino M	Surgery	2014	国外
A phase III study comparing amrubicin and cisplatin with irinotecan and cisplatin for the treatment of extensive-disease small cell lung cancer (ED-SCLC): JCOG0509	Satouchi M, Kotani Y, Shibata T, Ando M, Nakagawa K, Yamamoto N, Ichinose Y, Ohe Y, Nishio M, Hida T, Takeda K, Kimura T, Minato K, Akira Y, Atagi S, Fukuda H, Tamura T, and Saijo N	J Clin Oncol	2014	国外

（注1）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。
（注2）本様式はexcel形式にて作成し、甲が求める場合は別途電子データを納入すること。

Priority Report

Cancer Research

EGFR-TKI Resistance Due to *BIM* Polymorphism Can Be Circumvented in Combination with HDAC Inhibition

Takayuki Nakagawa[1,4], Shinji Takeuchi[1], Tadaaki Yamada[1], Hiromichi Ebi[1], Takako Sano[1], Shigeki Nanjo[1], Daisuke Ishikawa[1], Mitsuo Sato[2], Yoshinori Hasegawa[2], Yoshitaka Sekido[3], and Seiji Yano[1]

Abstract

BIM (BCL2L11) is a BH3-only proapoptotic member of the Bcl-2 protein family. BIM upregulation is required for apoptosis induction by EGF receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKI) in *EGFR*-mutant forms of non–small cell lung cancer (NSCLC). Notably, a *BIM* deletion polymorphism occurs naturally in 12.9% of East Asian individuals, impairing the generation of the proapoptotic isoform required for the EGFR-TKIs gefitinib and erlotinib and therefore conferring an inherent drug-resistant phenotype. Indeed, patients with NSCLC, who harbored this host *BIM* polymorphism, exhibited significantly inferior responses to EGFR-TKI treatment than individuals lacking this polymorphism. In an attempt to correct this response defect in the resistant group, we investigated whether the histone deacetylase (HDAC) inhibitor vorinostat could circumvent EGFR-TKI resistance in *EGFR*-mutant NSCLC cell lines that also harbored the *BIM* polymorphism. Consistent with our clinical observations, we found that such cells were much less sensitive to gefitinib-induced apoptosis than *EGFR*-mutant cells, which did not harbor the polymorphism. Notably, vorinostat increased expression in a dose-dependent manner of the proapoptotic BH3 domain-containing isoform of BIM, which was sufficient to restore gefitinib death sensitivity in the *EGFR* mutant, EGFR-TKI–resistant cells. In xenograft models, while gefitinib induced marked regression via apoptosis of tumors without the *BIM* polymorphism, its combination with vorinostat was needed to induce marked regression of tumors with the *BIM* polymorphism in the same manner. Together, our results show how HDAC inhibition can epigenetically restore BIM function and death sensitivity of EGFR-TKI in cases of *EGFR*-mutant NSCLC where resistance to EGFR-TKI is associated with a common *BIM* polymorphism. *Cancer Res; 73(8); 2428–34.* ©*2013 AACR.*

Introduction

The EGF receptor (EGFR) tyrosine kinase inhibitors (TKI), gefitinib and erlotinib, have shown marked therapeutic effects against non–small cell lung cancer (NSCLC) with *EGFR*-activating mutations, such as exon 19 deletions and L858R point mutations (1). About 20% to 30% of patients, however, show intrinsic resistance to EGFR-TKIs despite having tumors harboring these *EGFR* mutations. In addition, patients who respond initially later develop acquired resistance to EGFR-TKIs after varying periods of time (2). Among the molecular mechanisms associated with acquired resistance to EGFR-TKIs are (i) gatekeeper mutations in *EGFR* (i.e., a T790M second mutation), (ii) activation of bypass signaling caused by *Met* amplification or hepatocyte growth factor overexpression, (iii) transformation to small-cell lung cancer, and (iv) epithelial-to-mesenchymal transition (3, 4). Several therapeutic strategies, including new generation EGFR-TKIs and the combination of an EGFR-TKI and a Met-TKI, have been evaluated clinically in patients with *EGFR*-mutant NSCLC who acquired resistance to EGFR-TKIs (2). The mechanisms of intrinsic resistance, however, remain poorly understood.

Recently, a *BIM* deletion polymorphism was reported to be a novel mechanism of intrinsic resistance to EGFR-TKIs (5). BIM, also called BCL2L11, is a proapoptotic protein and a member of the Bcl-2 family. Gene products (such as BIM_{EL}, BIM_L, and BIM_S) with a BH3 domain, which is essential for apoptosis induction, antagonize antiapoptotic proteins (such as Bcl-2, Bcl-X_L, and Mcl-1) and activate proapoptotic proteins (such as BAX and BAK), thereby inducing apoptosis (6, 7). Activation of BAX and BAK induce cytochrome *c* release into the cytoplasm and result in activation of the caspase cascade (8). BIM is pivotal in apoptosis induced by EGFR-TKIs in *EGFR*-mutant NSCLC cells (9). The expression and degradation of BIM is regulated mainly by the MEK-ERK pathway (10). The *BIM* deletion polymorphism is relatively common in East Asian populations (12.9%), with 0.5% of individuals being

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-12-3479

homozygous for this deletion. During the transcription of *BIM*, either exon 3 or exon 4, the latter of which encodes the BH3 domain, is spliced out due to the presence of a stop codon and a polyadenylation signal within exon 3 (11). The *BIM* deletion polymorphism involves the deletion of a 2903 bp fragment in intron 2 and results in the preferential splicing of exon 3 over exon 4, generating a *BIM* isoform that lacks the BH3 domain (5). A retrospective analysis in patients with *EGFR*-mutant NSCLC showed that progression-free survival (PFS) following EGFR-TKI treatment was significantly shorter in patients with the *BIM* polymorphism (6.6 months) than with wild-type *BIM* (11.9 months; ref.5). Another study in patients with *EGFR*-mutant NSCLC treated with EGFR-TKIs also reported that PFS was significantly shorter in patients with BIM-low (4.3 months) than BIM-high (11.3 months) expressing tumors (12), suggesting that reduced expression of BIM with a BH3 domain is associated with an unfavorable response to EGFR-TKIs. To date, however, no therapeutic strategy has yet been developed for patients with *EGFR*-mutant NSCLC with low BIM expression.

Histone deacetylase (HDAC) is an enzyme that regulates chromatin remodeling and is crucial in the epigenetic regulation of various genes (13). Many compounds targeting HDAC have been developed, including vorinostat, an HDAC inhibitor approved by the United States Food and Drug Administration (FDA) for the treatment of patients with cutaneous T-cell lymphoma (14). In mantle cell lymphoma (MCL) cell lines and in cells from patients with MCL, vorinostat induced histone hyperacetylation on promoter regions and consequent transcriptional activation of proapoptotic *BH3*-only genes, including BIM (15). Using *in vitro* and *in vivo* models, we assessed whether the combination of vorinostat and gefitinib restored the expression of BIM protein with a BH3 domain in *EGFR*-mutant NSCLC cells with the *BIM* polymorphism and overcame EGFR-TKI resistance associated with this polymorphism.

Materials and Methods

Cell lines and reagents

The NSCLC cell lines, PC-9, HCC827, and HCC2279, all of which have *EGFR* mutations, were obtained from Immuno-Biological Laboratories Co., ltd., the American Type Culture Collection (ATCC), and Dr. John Minna (University of Texas Southwestern Medical Center, Dallas, TX), respectively. PC-3 cells, established from a Japanese female patient with NSCLC and with an exon 19 deletion in *EGFR*, and differing from the prostate cancer cell line PC-3 (ATCC CRL1435), were purchased from Human Science Research Resource Bank (JCRB0077: http://cellbank.nibio.go.jp/~cellbank/cgi-bin/search_res_det.cgi?DB_NUM=1&ID=252 = 1&ID = 252). PC-3 and the other 3 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium, respectively, each supplemented with 10% FBS and antibiotics. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza). The cell lines were authenticated at the laboratory of the National Institute of Biomedical Innovation (Osaka, Japan) by short tandem repeat analysis. Vorinostat and gefitinib were obtained from Selleck Chemicals and AstraZeneca, respectively.

Genotype and expression analysis of *BIM*

Genomic DNA was extracted from cells using DNeasy Blood and Tissue Kits (Qiagen), according to the manufacturer's protocol. Total RNA was extracted from cells using RNeasy PLUS Mini kits (Qiagen). PCR methods were used to detect the *BIM* deletion polymorphism in the samples and the level of expression of *BIM* isoforms (5).

Cell apoptosis

Cells (3×10^3) were seeded into each well of 96-well, white-walled plates, incubated overnight, and treated with the indicated compounds or vehicle [dimethyl sulfoxide (DMSO)] for 48 hours. Cellular apoptosis was analyzed with Caspase-Glo 3/7 assay kits (Promega), which measure caspase-3/7 activity, and PE-Annexin V Apoptosis Detection Kits (BD Biosciences, in accordance with the manufacturers' directions.

Apoptotic cells in tumor xenografts were detected by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) staining, using the DeadEnd Fluorometric TUNEL system (Promega), according to the manufacturer's protocol.

RNA interference

Duplexed Stealth RNAi (Invitrogen) against *BIM* and Stealth RNAi-negative control low GC Duplex #3 (Invitrogen) were used for RNA interference (RNAi) assays as described (4). The siRNA target sequences were 5′-CAUGAGUUGUGACAAAUCAACACAA-3′ and 5′-UUGUGUUGAUUUGUCACAACUCAUG-3′ for BIM #1, and 5′-UGAGUGUGACCGAGAAGGUAGACAA-3′ and 5′-UUGUCUACCUUCUCGGUCACACUCA-3′ for BIM #2.

Western blot analysis

Western blotting was conducted with antibodies against phospho-EGFR (Tyr1068), Akt, phospho-Akt (Ser473), cleaved PARP, cleaved caspase-3, histone H3, acetylated histone H3 (Lys27), BIM, and β-actin (Cell Signaling Technology); and against phospho-Erk1/2 (Thr202/Tyr204), Erk1/2, and EGFR (R&D Systems). Blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies specific to mouse or rabbit immunoglobulin G, with signals detected by enhanced chemiluminescence (Pierce Biotechnology).

Subcutaneous xenograft models

Male BALB/cAJcl-nu/nu mice, ages 5 to 6 weeks, were obtained from CLEA Japan Inc and injected subcutaneously into their flanks with cultured tumor cells (5×10^6 cells/0.1 mL/mouse). When tumor volumes reached 100 to 200 mm^3, the mice were randomized and treated once daily with gefitinib and/or vorinostat. Each tumor was measured in 2 dimensions, and the volume was calculated using the formula: tumor volume (mm^3) = 1/2 × length (mm) × width $(mm)^2$. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval No. AP-081088).

Statistical analysis

Between group differences were analyzed by one-way ANOVA. All statistical analyses were conducted using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc.), with $P < 0.05$ considered statistically significant.

Results

EGFR-mutant NSCLC cell lines harboring the *BIM* deletion polymorphism have low susceptibility to gefitinib-induced apoptosis

We first examined the *BIM* deletion polymorphism in *EGFR*-mutant NSCLC cell lines by PCR. PC-9 and HCC827 had wild-type alleles, with a PCR product 4.2 kb in size. Consistent with a previous report (5), HCC2279 cells were heterozygous for the *BIM* deletion polymorphism, with PCR products 4.2 kb (wild-type) and 1.3 kb (2.9 kb deletion polymorphism) in size. Among the 7 additional cell lines with *EGFR* mutations (Supplementary Table S1), PC-3 was heterozygous for the *BIM* deletion polymorphism (Fig. 1A). Western blot analyses reveal that the expression of the proapoptotic BIM protein was markedly lower in PC-3 and HCC2279 than in PC-9 and HCC827 cells. Analysis of *BIM* isoform transcripts showed that cells with the *BIM* polymorphism expressed more exon 3- than exon 4-containing transcripts (Supplementary Fig. S1A and S1B). Treatment with gefitinib enhanced BIM expression, caspase-3/7 activities, and apoptosis in PC-9 and HCC827 cells much more than in PC-3 and HCC2279 cells (Fig. 1B; Supplementary Fig. S1C, S1D, and S2). Moreover, gefitinib did not increase caspase-3/7 activity in PC-9 and HCC827 cells treated with *BIM* siRNA (Fig. 1C), indicating the crucial role of BIM in apoptosis induction in *EGFR*-mutant NSCLC cells treated with EGFR-TKI. These observations clearly showed that *EGFR*-mutant NSCLC cells with the *BIM* deletion polymorphism are much less sensitive to gefitinib, as shown by induction of apoptosis, than cells with wild-type *BIM*.

Vorinostat upregulates BIM and efficiently induces apoptosis when combined with gefitinib

Because HDAC inhibition modulates the expression of various genes, including proapoptotic molecules (13), we hypothesized that the HDAC inhibitor, vorinostat, may sensitize *EGFR*-mutant NSCLC cells with the *BIM* polymorphism to gefitinib. In *EGFR*-mutated NSCLC cell lines, including PC-3 and HCC2279 cells, vorinostat dose dependently increased the expression of acetylated histone H3 and BIM with the BH3 domain (Fig. 2A, Supplementary Fig. S3A). We further explored whether the addition of vorinostat to gefitinib induced apoptosis in *EGFR*-mutant NSCLC cells with the *BIM* polymorphism (Fig. 2B and D). In HCC827 and PC-9 cells, which contain only wild-type *BIM*, gefitinib inhibited downstream signaling,

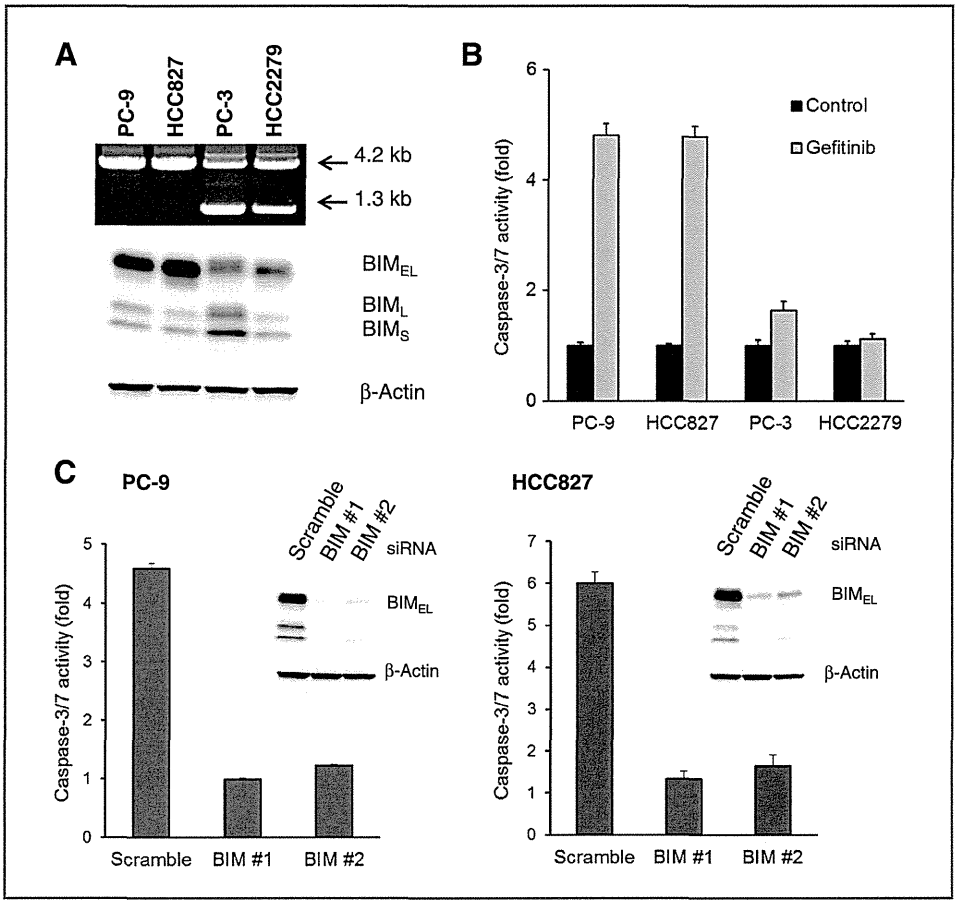

Figure 1. *EGFR*-mutated NSCLC cell lines harboring the *BIM* deletion polymorphism show low susceptibility to gefitinib-induced apoptosis. A, top, PCR products from the 4 *EGFR*-mutated NSCLC cell lines generated by primers flanking the deletion. PCR products 4.2 kb and 1.3 kb in size correspond to the alleles without and with the deletion, respectively, with the presence of both products indicating heterozygosity for the deletion polymorphism. Bottom, the levels of expression of the proteins BIM$_{EL}$, BIM$_L$, and BIM$_S$ in each cell line. B, cell lines were treated with gefitinib (1 μmol/L) or DMSO control for 48 hours, and the activity of caspase-3/7 was measured using Caspase-Glo3/7 assay kits. Each bar represents the mean ± SD. C, PC-9 (left) and HCC827 (right) cells were transfected with *BIM* or control siRNA for 24 hours before gefitinib (1 μmol/L) treatment for 48 hours, and the activity of caspase-3/7 was measured as in B. Each bar indicates the mean ± SD. Lysates were collected and proteins were analyzed by Western blotting.

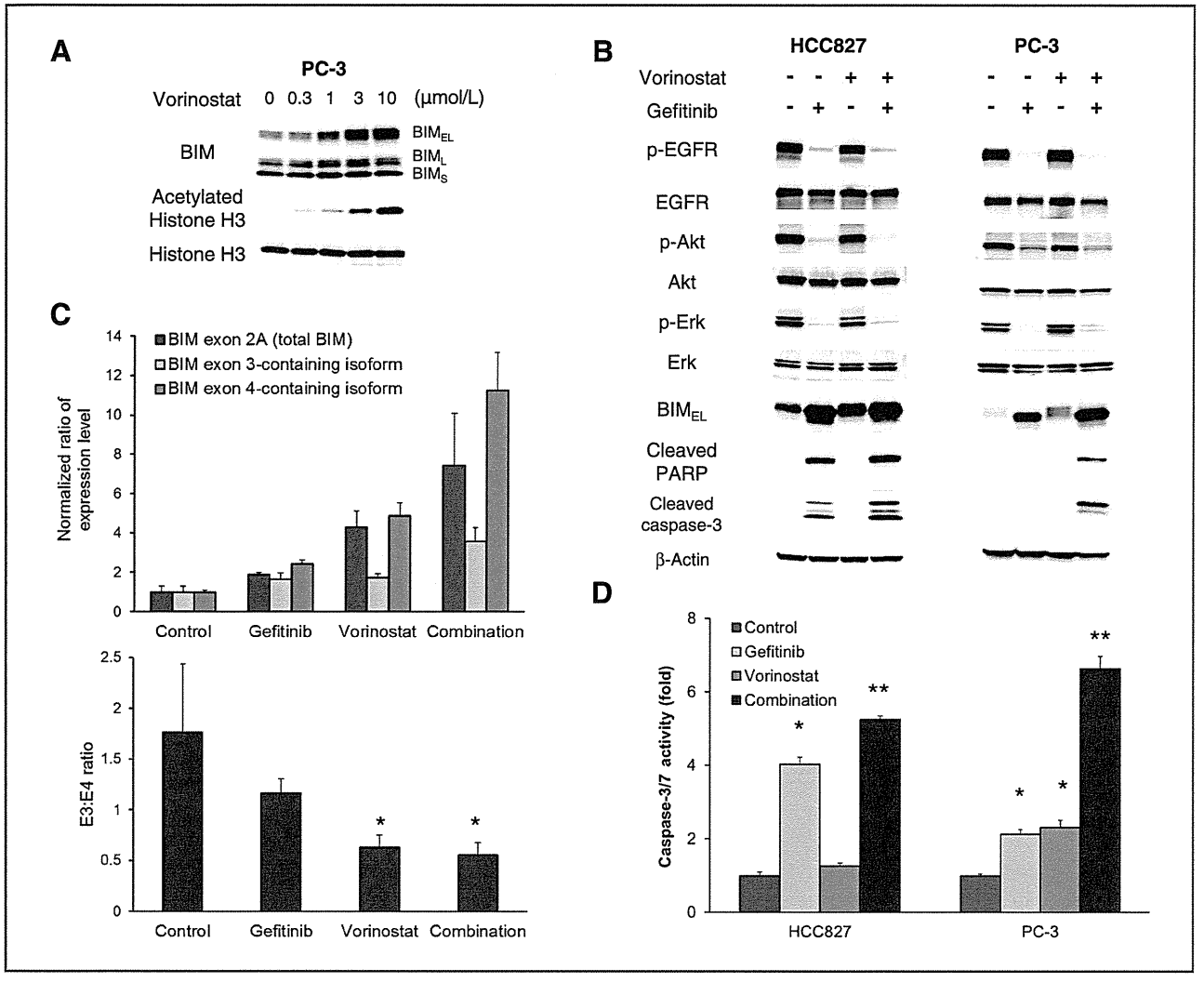

Figure 2. Upregulation of BIM by vorinostat enhances induction of apoptosis in *EGFR*-mutated NSCLC cell line with the *BIM* polymorphism. A, PC-3 cells were incubated with serial dilutions of vorinostat for 24 hours. The cell lysates were harvested and the indicated proteins were analyzed by Western blotting. B, HCC827 cells (left) and PC-3 cells (right) were incubated with gefitinib (1 μmol/L) and/or vorinostat (3 μmol/L) for 48 hours. The cell lysates were harvested and the indicated proteins were determined by Western blotting. C, PC-3 cells were treated with gefitinib (1 μmol/L) and/or vorinostat (3 μmol/L) for 12 hours. The amounts of the various transcripts containing exon 2A, 3, or 4 are expressed as normalized ratios relative to actin (top). Ratio of exon 3-containing transcripts to exon 4-containing transcripts in PC-3 cells after treatment with each compound. *, $P < 0.05$ versus control. Bar indicates the mean ± SD. D, apoptosis was analyzed by measurement of caspase-3/7 activity. *, $P < 0.05$ gefitinib or vorinostat versus control; **, $P < 0.05$ combination versus control and single agents. Bars represent the mean ± SD.

including the phosphorylation of EGFR, Erk, and Akt, resulting in apoptosis, as shown by the expression of cleaved PARP and cleaved caspase-3. The further addition of vorinostat augmented BIM expression and caspase-3/7 activity. In PC-3 and HCC2279 cells, which contain the *BIM* polymorphism, however, treatment with gefitinib alone induced minimal apoptosis, although the phosphorylation of EGFR, Erk, and Akt was inhibited, whereas the combination of vorinostat and gefitinib markedly increased the expression of BIM, as well as of cleaved PARP and cleaved caspase-3 (Fig. 2B and Supplementary Fig. S3B). This combination also augmented caspase-3/7 activity compared with that of gefitinib or vorinostat alone (Fig. 2D and Supplementary Fig. S3C), but this activation of caspase-3/7 was inhibited by knockdown of *BIM* (Supplementary Fig. S4A and S4B). Conversely, overexpression of BIM_{EL} itself stimulated caspase-3/7 activities in cells with the *BIM* polymorphism, with these activities further enhanced by gefitinib treatment (Supplementary Fig. S4C and S4D). These results indicate that BIM mediates the activation of caspase-3/7 induced by gefitinib and vorinostat. Analysis of *BIM* transcripts revealed that vorinostat alone induced *BIM* mRNA, which was enhanced by the inclusion of gefitinib. Moreover, vorinostat treatment preferentially induced transcripts containing exon 4 over those containing exon 3 (Fig. 2C). These results indicate that the combination of vorinostat and gefitinib inhibits HDAC and increases the expression of BIM protein with the BH3 domain, thereby sensitizing *EGFR*-mutant NSCLC cells with the *BIM* polymorphism to apoptosis *in vitro*.

Combined treatment with vorinostat with gefitinib shrinks tumors produced by *EGFR*-mutant NSCLC cells with the *BIM* polymorphism

We next determined the *in vivo* efficacy of vorinostat and gefitinib. Gefitinib alone almost completely shrunk xenograft tumors induced by HCC827 cells (Fig. 3A). Although gefitinib monotherapy prevented the enlargement of tumors produced by PC-3 cells, which harbor the *BIM* polymorphism, it did not induce their complete regression, indicating that PC-3 cells remained less susceptible to gefitinib *in vivo*. Under these experimental conditions, vorinostat monotherapy inhibited tumor growth slightly, whereas the combination of vorinostat with gefitinib resulted in marked tumor shrinkage (Fig. 3B). None of the mice treated with these agents showed any macroscopic adverse effects, including loss of body weight (data not shown).

To clarify the mechanisms by which vorinostat and gefitinib act *in vivo*, we assessed tumor-cell apoptosis by TUNEL staining. Gefitinib treatment increased the number of apoptotic cells in HCC827 tumors but had little effect on PC-3 tumors (Fig. 4A and B), indicating that *EGFR*-mutant NSCLC cells with the *BIM* polymorphism are refractory to gefitinib-induced apoptosis *in vivo* as well as *in vitro*. Importantly, although vorinostat alone had little effect on apoptosis, the combination of vorinostat and gefitinib induced marked apoptosis in PC-3 tumors (Fig. 4A and B). Western blot analyses showed that gefitinib induced cleavage of caspase-3 in HCC827, but not in PC-3, tumors. In PC-3 tumors, treatment with gefitinib or vorinostat had little effect on caspase-3 cleavage, whereas their combination increased BIM expression and the cleavage of caspase-3 (Fig. 4C and D). These findings indicate that the combination of vorinostat and gefitinib increases BIM protein expression and induces tumor-cell apoptosis, thereby shrinking tumors produced by *EGFR*-mutant NSCLC cells with the *BIM* polymorphism.

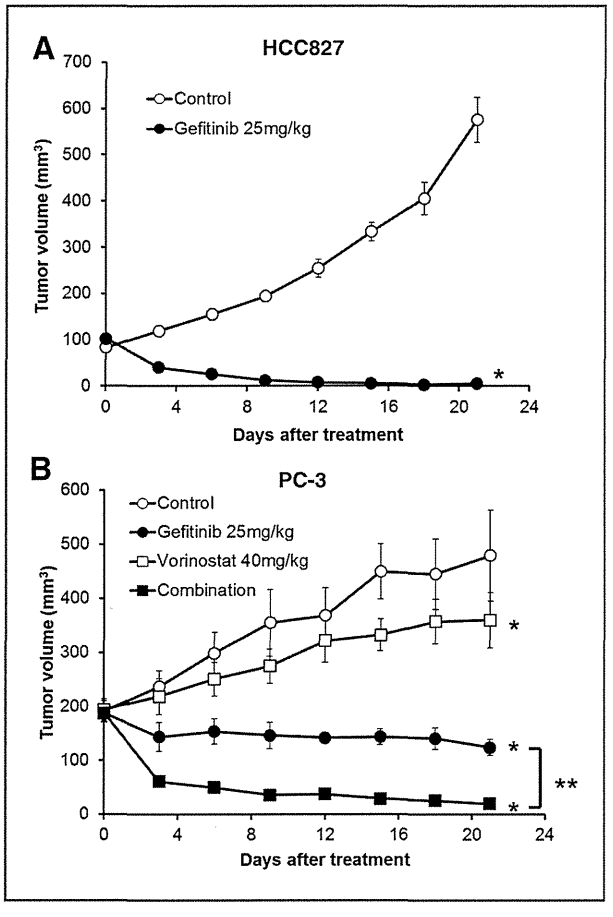

Figure 3. Antitumor activity of gefitinib and/or vorinostat in mouse xenograft models of HCC827 and PC-3 tumors. Nude mice bearing established tumors with HCC827 (A) or PC-3 (B) cells were treated with 25 mg/kg gefitinib and/or 40mg/kg vorinostat once daily for 21 days. Tumor volume was measured using calipers on the indicated days. Mean ± SE tumor volumes are shown for groups of 4 to 5 mice. *, $P < 0.05$ versus control, **, $P < 0.05$ versus gefitinib by one-way ANOVA.

Discussion

EGFR-mutant NSCLC cells with the *BIM* deletion polymorphism show impaired generation of BIM with the proapoptotic BH3 domain, as well as resistance to EGFR-TKI–induced apoptosis (5). We have shown here that treatment of cells with the combination of vorinostat, a HDAC inhibitor, and gefitinib, an EGFR-TKI, restored the expression of BIM protein with a BH3 domain (predominantly BIM_{EL}), induced apoptosis, and overcame gefitinib resistance *in vitro* and *in vivo*.

Although vorinostat preferentially induced expression of BIM containing the BH3 domain, its exact mechanisms of action remain unclear. The wild-type allele may be more susceptible to the effects of HDAC inhibition than the deletion allele due to differences in the acetylation status of these alleles. Alternatively, vorinostat may affect the splicing process, resulting in the production of exon 4- rather than exon 3-containing transcripts from the deletion polymorphism allele as HDAC has been found to affect the splicing of RNA (16).

Vorinostat has been shown to induce the expression of several genes other than *BIM* (13). However, we found that BIM was pivotal not only for gefitinib-induced apoptosis but also when combined with vorinostat. Moreover, the combination of vorinostat and gefitinib increased BIM expression and markedly induced apoptosis in PC-3 and HCC2279 cells. Collectively, these findings strongly suggest that vorinostat promotes gefitinib-induced apoptosis in *EGFR*-mutant NSCLC cells with the *BIM* polymorphism, primarily by increasing BIM expression. Several other mechanisms, including inhibition of epigenetic modifications leading to a drug-tolerant state (17) and transition of cancer cells from a resistant mesenchymal state to an E-cadherin–expressing epithelial state (18) may be also involved.

Both the *BIM* polymorphism and *EGFR* mutations are more prevalent in East Asian than in Caucasian populations. Few East Asian patients with *EGFR*-mutant NSCLC show a complete response to EGFR-TKIs (1). This incomplete response, including intrinsic resistance, may be due, in part, to low BIM expression associated with the *BIM* polymorphism (6). Our preclinical data indicate that vorinostat increases BIM even in *BIM*-wild type *EGFR*--mutant NSCLC cells. However, a clinical trial with erlotinib and entinostat, an HDAC inhibitor, in

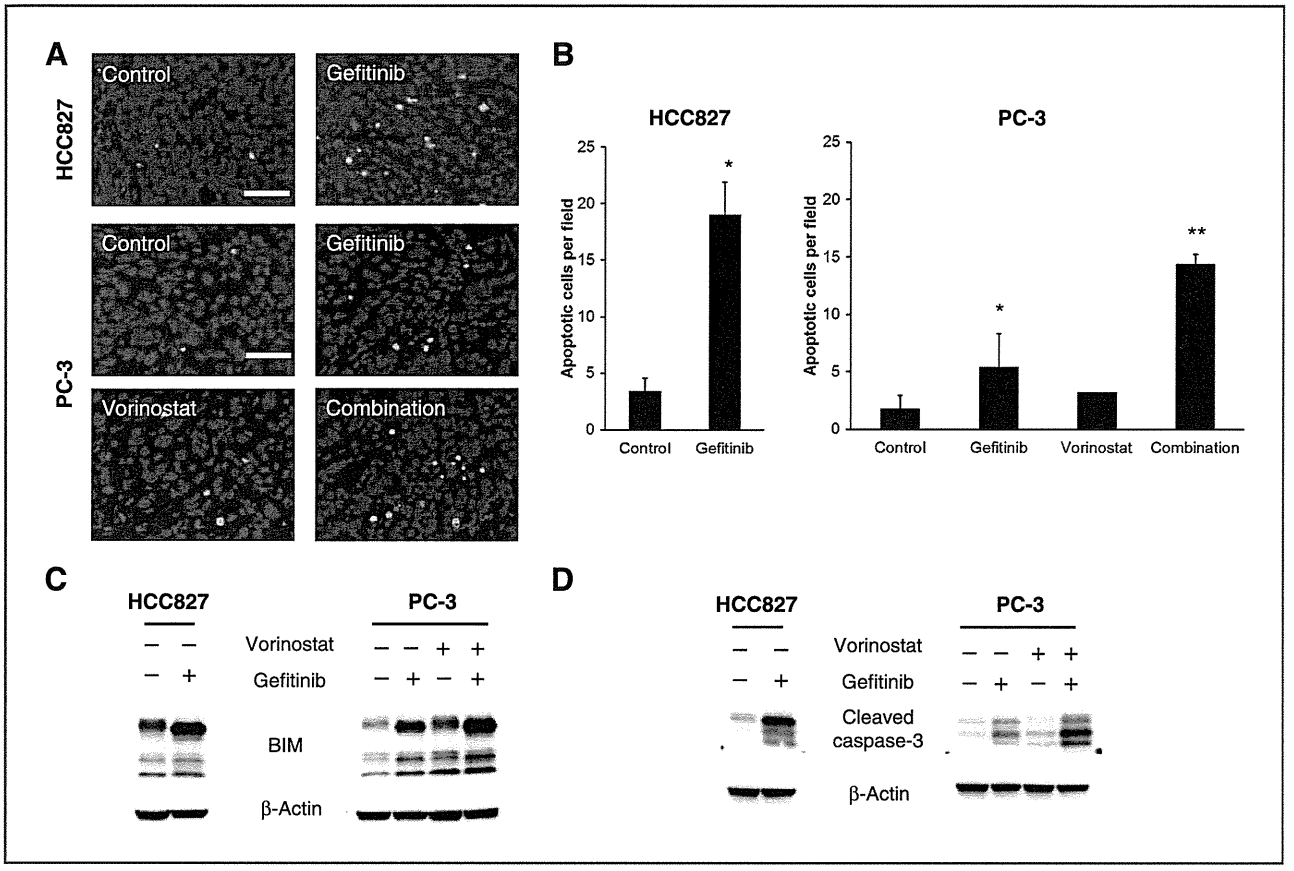

Figure 4. Vorinostat combined with gefitinib increases apoptosis in xenograft tumors with the *BIM* polymorphism. HCC827 and PC-3 xenograft tumors were resected from mice treated with 25 mg/kg gefitinib and/or 40mg/kg vorinostat for 4 days. A, analysis of apoptosis by TUNEL staining. Representative fluorescent images are shown. Green fluorescence indicates apoptotic cells. Bar indicates 50 μm. B, quantitation of number of apoptotic cells. *, $P < 0.05$ gefitinib or vorinostat versus control; **, $P < 0.05$ combination versus control and single agents. Bars represent mean ± SD. C, tumors were harvested 8 hours after 2 consecutive treatments with each compound, and the levels of protein in tumor lysates were determined by Western blotting. D, tumors were harvested 24 hours after 4 consecutive treatments with each compound. Protein expression levels in the tumor lysates were determined by Western blotting.

unselected patients with NSCLC, more than 65% of whom were Caucasian, failed to show therapeutic benefits (19). These findings suggest that the combination of vorinostat and an EGFR-TKI should be tested in selected patients with NSCLC with *EGFR* mutations and the *BIM* polymorphism.

Resistance to EGFR-TKIs associated with the *BIM* deletion polymorphism may be overcome by treatment with BH3 mimetics, such as ABT-737 (5). Although ABT-737 antagonized antiapoptotic proteins, such as Bcl-2 and Bcl-X_L, it did not antagonize the antiapoptotic protein Mcl-1, which is overexpressed in NSCLC (20), suggesting that the effects of BH3 mimetics may be limited to overcoming EGFR-TKI resistance caused by the *BIM* polymorphism in NSCLC. BH3 mimetics are being evaluated in early-phase clinical trials but are not ready for use in clinical practice. In contrast, vorinostat has been approved by the FDA for the treatment of patients with advanced primary cutaneous T-cell lymphoma (15). Therefore, the combination of gefitinib and vorinostat could easily be tested clinically.

The *BIM* polymorphism can be detected in formalin-fixed paraffin-embedded tumor tissues and peripheral blood (5). Moreover, a convenient and easy access PCR screening method can detect this polymorphism in circulating DNA from serum (Supplementary Fig. S5A and S5B). As the *BIM* polymorphism is a germline alteration, it can be assayed in serum obtained at any time point. Collectively, our findings illustrate the importance of clinical trials testing the ability of combinations of vorinostat and EGFR-TKIs to overcome EGFR-TKI resistance associated with the *BIM* polymorphism in patients with *EGFR*--mutant NSCLC.

Disclosure of Potential Conflicts of Interest

T. Nakagawa is an employee of Eisai Co., Ltd. for oncology research. Y. Hasegawa received research funding from Chugai Pharmaceutical Co., Ltd., Merck Sharp & Dohme Corp., AstraZeneca, and TAIHO Pharmaceutical Co., Ltd. S. Yano received honoraria from Chugai Pharmaceutical Co., Ltd. and AstraZeneca and received research funding from Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., and Eisai Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Nakagawa, S. Takeuchi, S. Nanjo, S. Yano
Development of methodology: T. Nakagawa, S. Takeuchi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Nakagawa, D. Ishikawa, Y. Hasegawa

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Nakagawa, S. Yano
Writing, review, and/or revision of the manuscript: T. Nakagawa, S. Takeuchi, H. Ebi, M. Sato, Y. Hasegawa, Y. Sekido, S. Yano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Yamada, T. Sano, M. Sato, Y. Sekido
Study supervision: S. Takeuchi, Y. Sekido, S. Yano

Acknowledgments

The authors thank Dr. John Minna (University of Texas Southwestern Medical Center) for the HCC2279 cells.

Grant Support

This study was supported by Grants-in-Aid for Cancer Research (21390256 to S. Yano; 11019957 to S. Takeuchi), Scientific Research on Innovative Areas "Integrative Research on Cancer Microenvironment Network" (22112010A01 to S. Yano), and Grant-in-Aid for Project for Development of Innovative Research on Cancer Therapeutics (P-Direct) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

Received September 3, 2012; revised December 20, 2012; accepted January 19, 2013; published OnlineFirst February 4, 2013.

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Lack of Association between the *BIM* Deletion Polymorphism and the Risk of Lung Cancer with and without *EGFR* Mutations

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Introduction: The *BIM* deletion polymorphism in intron 2 was found in a significant percent of the Asian population. Patients with epidermal growth factor receptor (*EGFR*) mutant lung cancers harboring this *BIM* polymorphism have shorter progression free survival and overall response rates to EGFR tyrosine kinase inhibitors. However, the association between the *BIM* deletion polymorphism and lung cancer risk is unknown.

Methods: The *BIM* deletion polymorphism was screened by polymerase chain reaction in 765 lung cancer cases and 942 healthy individuals.

Results: Carriers possessing one allele of the *BIM* polymorphism were observed in 13.0% of control cases and 12.8% of lung cancer cases, similar to incidence rates reported earlier in healthy individuals. Homozygote for the *BIM* polymorphism was observed in four of 942 healthy controls and three of 765 lung cancer cases. The frequency of the *BIM* deletion polymorphism in lung cancer patients was not related to age, sex, smoking history, or family history of lung cancer. The *BIM* deletion polymorphism was found in 30 of 212 patients with *EGFR* wild type lung cancers and 16 of 120 patients with *EGFR* mutant lung cancers. The frequency of the *BIM* polymorphism is similar between cancers with wild type *EGFR* and mutated *EGFR* ($p = 0.78$).

Conclusion: The *BIM* deletion polymorphism was not associated with lung cancer susceptibility. Furthermore, the *BIM* polymorphism is not associated with *EGFR* mutant lung cancer.

Key Words: BIM polymorphism, Lung cancer, Susceptibility, *EGFR* mutation.

(*J Thorac Oncol.* 2015;10: 59–66)

Lung cancer is a leading cause of cancer death in developed countries. Loss of apoptosis is critical for both tumorigenesis and resistance to drug therapies. The BCL-2 family member proteins play important roles in regulating apoptosis in response to a wide variety of cellular signals, including DNA damage and growth factor withdrawal.[1,2] The BCL-2 family consists of three subfamilies[1,2]: pro-survival members (e.g., BCL-2 and MCL1), pro-apoptotic members (i.e., BCL-2 homology domain 3 [BH3]-only proteins including BIM and PUMA, and the pro-apoptotic BAX and BCL-2 antagonist/killer [BAK]). BIM is a member of the BH3-only proteins that binds and neutralizes the anti-apoptotic BCL2 family members, as well as directly activating BAX and BAK to induce apoptosis. In a number of different cancer types, both in vitro and in vivo studies have evidenced that BIM is essential for apoptosis following targeted therapy administration.[2–10]

Activating mutations in the epidermal growth factor receptor (*EGFR*) renders EGFR the primary driver oncogene in lung cancer. *EGFR* tyrosine kinase inhibitors (EGFR-TKIs) have provided significant survival benefit in patients harboring *EGFR* mutations. However, these studies have indicated that 20 to 40% of patients are primarily resistant to EGFR-TKIs.[11–13] In oncogene addicted cancers like *EGFR* mutant lung cancers, survival signals derived from the oncogene regulate the expression and the interaction of BCL-2 family members. In particular, BIM is a key mediator of apoptosis in response to EGFR-TKIs.[3–5,10,14–16] EGFR-TKIs downregulate MAPK signaling that leads to upregulation of BIM expression in these cancers. Importantly, several studies have shown that low levels of pretreatment, functional BIM in tumor cell lines and patients' tumors is related to a mitigated apoptotic

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Disclosure: The authors declare no conflict of interest.

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DOI: 10.1097/JTO.0000000000000371

ISSN: 1556-0864/15/1001-0059

response and lack of efficacy following EGFR-TKI treatment.[4,5,10,15–21] Although degradation of BIM is mainly regulated by MAPK signaling, a number of other mechanisms, such as alternative splicing, transcriptional and posttranscriptional regulation, posttranslational modification, and epigenetic silencing also effects BIM expression.[18] Recently, paired-end DNA sequencing identified a deletion polymorphism in *BIM*.[14] This polymorphism is located in intron 2 of the *BIM* gene that results in the expression of BIM isoforms lacking BH3 domain. This polymorphism is commonly found in the East Asian population yet absent in the Caucasian population. Intriguingly, lung cancer patients harboring this *BIM* germ line polymorphism have shorter progression free survival (PFS) to EGFR-TKIs[14,19–22] thus contributing substantially to primary resistance. Tests to identify the *BIM* polymorphism in the clinic are being developed. Given the clinical significance of the *BIM* polymorphism, we sought to investigate the relationship between this *BIM* polymorphism and the risk to develop lung cancer in general and lung cancer specifically with *EGFR* activating mutations.

PATIENTS AND METHODS

Subjects

All subjects were first-visit outpatients at the Aichi Cancer Center Hospital (ACCH) aged 18 to 79 who gave written informed consent for enrollment in the Hospital-based Epidemiological Research Program at Aichi Cancer Center (HERPACC) during 2001–2005. Information on lifestyle factors was collected using a self-administered questionnaire, checked by a trained interviewer. The outpatients were also asked to provide blood samples. Approximately 95% of eligible subjects completed the questionnaire and 60% provided blood samples. Details of this program have been described elsewhere.[23,24] The lung cancer cases consisted of 765 patients who were newly and histologically diagnosed as having lung cancer. Controls ($n = 942$) were randomly selected from outpatients who completed the questionnaire, provided blood samples, and were confirmed cancer free.[25] The study protocol was approved by the ethics committee of Aichi Cancer Center and complied with the declaration of Helsinki.

Genotyping of the BIM Deletion Polymorphism and Other Polymorphisms

DNA of each subject was extracted from the buffy coat fraction using a DNA Blood mini kit (Qiagen, Tokyo, Japan) for the use of genotyping. Primers detecting wild type *BIM* and *BIM* deletion polymorphism were developed previously.[26] The primer sequences are F: 5′-CCACCAATGGAAAAGGTTCA-3′, R: 5′-CTGTCATTT CTCCCCACCAC-3′ for detecting wild-type *BIM* and F: 5′-CTGTCATTTCTCCCCACCAC-3′, R: 5′- GGCACAGCC TCTATGGAGAA-3′ for identifying the *BIM* deletion polymorphism. The primer pairs yield a 362 bp and 284 bp of PCR products, respectively. Screening was performed by primer sets identifying the *BIM* deletion polymorphism. DNA from PC-3 cells, known to harbor the *BIM* deletion polymorphism, was used as a positive control. Positive samples were then determined to be homozygote or heterozygote by performing PCR with both primer sets. In addition to the *BIM* deletion polymorphism, genotyping data on 14 other polymorphisms (rs2289321, rs1439287, rs2015454, rs1837369, rs17041869, rs13396983, rs1877330, rs724710, rs3789068, rs17041887, rs616130, rs13405741, rs726430, rs9308742) that locate ±30,000-bp to the *BIM* polymorphism was adopted from previously genotyped data by an Illumina Human 610-Quad BeadChip (Illumina, San Diego, CA). Briefly, 576,736 SNP markers were examined at the Center for Genomic Medicine of Kyoto University Graduate School of Medicine. After removing SNPs that failed the quality control criteria (Hardy–Weinberg equilibrium $p < 1 \times 10^{-6}$ [excluded SNPs: $n = 277$]; SNP call rate > 0.95 [$n = 2921$]; and minor allele frequency [MAF] < 0.01 [$n = 82,414$]), 491,738 markers were selected as a source for this analysis (some SNPs were excluded based on two or more criteria).

Assessment of Smoking and Fruits and Green–Yellow Vegetable Intake

All exposures were assessed from the self-administered questionnaire, as completed at the first visit to ACCH before the diagnostic procedure was conducted. Subjects were questioned specifically about their lifestyle before the onset of the symptoms that prompted their visit to ACCH. Smoking status was divided into three categories: never, former, and current. Former smokers were defined as those who quit smoking at least 1 year before the time of the survey. The intake of fruits and green–yellow vegetables was determined using a food frequency questionnaire (FFQ), described in detail elsewhere.[25] Briefly, FFQ enables estimating quantity of intake by the information of frequency of the intake in eight categories: never or seldom, 1 to 3 times/month, 1 to 2 times/week, 3 to 4 times/week, 5 to 6 times/week, once/day, twice/day, and three or more times/day. The intake was adjusted for total energy intake, and was classified into tertiles.

Clinicopathological Information

Clinicopathological information was obtained by linking clinical cohort data[27] with HERPACC database. Pathological staging was based on UICC version 7. Mutation status of *EGFR* (exon 18 to 21) and *KRAS* (exon 1 and 2) were examined by sequencing of PCR products as previously described.[28] EML4-ALK fusion was screened with RT-PCR and immunohistochemistry as described elsewhere.[27]

Statistical Analysis

Differences in categorized demographic variables between cases and controls were tested by a chi-squared test or Fisher's exact test as appropriate. To verify that the allele distribution for each SNP was in the Hardy–Weinberg equilibrium (HWE), we used a chi-squared test with one degree of freedom.

We applied odds ratios as measures of association and they and their 95% confidence intervals were estimated using unconditional logistic regression models adjusted for potential confounders. Potential confounders considered in this analysis were age, sex, smoking evaluated as pack-years (PY), and the energy-adjusted intake of fruit and green–yellow vegetables.