PTEN inactivation in lung cancer cells and the effect of its recovery on treatment with epidermal growth factor receptor tyrosine kinase inhibitors

RINTARO NORO, AKIHIKO GEMMA, AKIHIKO MIYANAGA, SEIJI KOSAIHIRA, YUII MINEGISHI, MICHII YARA, YUTAKA KOKUBO, MASAHIRO SEIKE, KIYOKO KATAOKA, KUNIKO MATSUDA, TETSUYA OKANO, AKINOBU YOSHIMURA and SHOJI KUDOH

Department of Pulmonary Medicine/Infection and Oncology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

Received June 14, 2007; Accepted August 2, 2007

Abstract. To understand the mechanisms of PTEN inactivation, which is reported to be involved in tumor progression and drug resistance in lung cancer, we analyzed the expression levels of PTEN at mRNA and protein levels, along with the genetic and epigenetic status of the PTEN gene, in a panel of lung cancer cell lines. Western blot analysis showed that six out of 25 (24%) cell lines displayed low expression of PTEN protein. The level of PTEN mRNA correlated well with corresponding protein expression in each of these six cell lines. In two of the six cell lines genomic analysis revealed homozygous deletions of the PTEN gene. Another two of the six cell lines displayed hypermethylation of the PTEN gene promoter assessed by methylation-specific PCR. The levels of PTEN mRNA and protein expression in PC9/f9 and PC9/f14 cells, which are gefitinib-resistant derivatives of the gefitinib-sensitive cell line, PC9, were reduced compared to the parental line. After treatment with the demethylating agent 5-aza-2'deoxycytidine (5-AZA) and the histone deacetyltransferase (HDAC) inhibitor Trichostatin A (TSA), the expression levels of PTEN mRNA and protein in these four cell lines (PC9/f9, PC9/f14, PC10 and PC14) were actually restored. In summary, reduction in PTEN protein expression was regulated by histone deacetylation and hypermethylation of the gene promoter, as well as homozygous deletion. In addition, we demonstrated that the combination treatment of gefitinib and TSA induced significant growth inhibition in gefitinib-resistant PC9/f9 and PC9/f14 cells. These findings suggest that the combination of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib with the demethylating agent 5-AZA and the HDAC inhibitor TSA may be a useful strategy for the treatment of some lung cancers.

Introduction

Lung cancer is one of the leading causes of cancer mortality in both men and women and its incidence is increasing worldwide (1). The 5-year survival rate of lung cancer patients is only 15% and this situation has not remarkably improved over the last 30 years. Improving survival rate of patients with this disease requires a better understanding of tumor biology and the subsequent development of novel therapeutic strategies.

PTEN is a tumor-suppressor gene located on chromosomal position 10q23.3 (2). PTEN is a lipid phosphatase that acts by dephosphorylating the 3-position of phosphatidylinositol 3,4,5-triphosphate, a second messenger of PI3K (3,4). PTEN antagonizes PI3K activity and negatively regulates its downstream target, the serine/threonine kinase Akt, which is a cytosolic signal transduction protein implicated in carcinogenesis and chemoresistance (5,6). According to recent reports, loss of PTEN is associated with poor prognosis for lung cancer patients (7-9), as well as resistance towards many anti-cancer agents, including gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (10-12).

Alterations of the PTEN gene have been demonstrated in 8-16% of examined non-small cell lung cancer (NSCLC) cell lines suggesting that PTEN is infrequently targeted in NSCLC tumorigenesis. Studies of PTEN in NSCLC have focused exclusively on searching for mutations or deletions of the gene, with little emphasis on examining alterations at the protein level (13-16). DNA hypermethylation as a mechanism for PTEN inactivation has been reported in the context of lung carcinomas (17). Soria et al found that 24% of the lung cancers tested showed a lack of staining for PTEN protein expression, although PTEN methylation was detected in only 35% of the PTEN-negative NSCLC samples using methylation-specific PCR (17). Other mechanisms, that have yet to be developed, are required to explain the basis of reduced PTEN protein expression in lung cancer. It also remains unknown whether the expression of PTEN mRNA and protein expression in these cells can be restored after treatment with the demethylating agent, 5-aza-2'deoxycytidine.
(S-AZA). In an effort to clarify these issues, we took a panel of lung cancer cell lines and examined the levels of PTEN protein expression using Western blot analysis and determined the genetic and epigenetic status of the PTEN gene. We also attempted to recover PTEN protein expression in a subset of lung cancer cell lines by exposure to S-AZA and the histone deacetyltransferase (HDAC) inhibitor, Trichostatin A (TSA). We also investigated the recovery of the antitumor activity of gefitinib by the recruitment of PTEN expression via TSA in gefitinib-resistant sublines of PC9 cells.

Materials and methods

Cell lines. Twelve adenocarcinoma cell lines (ABC-1, A549, PC3, PC7, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCK, PC14, PC9, PC9/f9 and PC9/f14), eight squamous-cell carcinoma cell lines (NCI-H69, SBC3, NCI-N231, Lu135 and MS-1), as well as an immortalized tracheal cell line (BET2A), were subjected to PTEN gene mutational analysis and mRNA and protein expression analysis. The Lu135 cell line was provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan). The LCD and LCK cell lines were provided by S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). The NCI-N231, A549, NCI-H69 and BET2A cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19). The PC1, PC3, PC7, PC9, PC10, PC14 and QG-56 cell lines were obtained from IBL (Gunma, Japan). The RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). The ABC-1, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19).

Western blot analysis. Western blot analysis was performed as previously described (12). The membranes were first incubated overnight at 4°C with primary antibodies specific for either PTEN (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (from Cell Signaling Technology, Beverly, MA). The membranes were then incubated with peroxidase-conjugated secondary antibodies, with antibody-antigen complexes detected via an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) (21,23).

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and sequencing of genomic DNA. From each genomic DNA sample, all exons of the PTEN gene were amplified separately using previously described PCR primers (16) via the GeneAmp XL PCR kit (Perkin-Elmer/Roche, Branchburg, NJ, USA). PCR amplification conditions for analysis of genomic DNA were as follows: 40 cycles at 94°C for 40 sec, 60°C for 30 sec and 68°C for 90 sec, followed by 68°C for 8 min. Each reaction mixture contained 1X XL buffer, 200 μM deoxynucleotide triphosphate, 1100 μM Mg(OAc)2, 0.5 U rTth DNA polymerase XL, 0.3 mM of each primer (one of each pair) (16) and 25 ng of genomic DNA. PCR products were loaded onto 1.2% agarose gels and analyzed following electrophoretic separation. PCR-SSCP and DNA sequence analyses were performed as previously described (22).

Real-time RT-PCR analysis. Real-time quantitative RT-PCR assessment of PTEN gene expression was performed using the ABI PRISM 7700 Sequence Detector system (Perkin-Elmer/Applied Biosystems, Fosters City, CA). Both the PCR primers and associated TaqMan fluorogenic probe were designed using the Primer Express software program (Perkin-Elmer/Applied Biosystems). The relevant oligonucleotide sequences were as follows: PTEN: sense, TGGCTAAGTTGAAGTACAATGG; antisense, TGCACATATCATTACCAGCTTCGT; probe, TTCACGCAATCTGTAAGC TGAAAAG. GAPDH: sense, GAAGGTGAAGGTCGGAACTAC; antisense, GAAGATGTGTAGTGATGGTGTAGATTTC; probe, CAAGGCTCGTCCTCAGCC.

Total RNA was extracted from cultured cells and reverse transcribed using the RevaTrA Ace Kit, with a random hexamer being used as primer (Toyobo Co., Osaka, Japan). A portion of the resulting cDNA was used for quantitative PCR in a 50-μl final volume, together with the desired primers, TaqMan probe, and Master Mix, the latter being composed of PCR buffer, MgCl2, dATP, dCTP, dGTP, dUTP, AmpEraseUNG, and AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems). The initial thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, as recommended by the manufacturer. The PC1, PC3, PC7, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). The ABC-1, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the National Cancer Center Research Institute, Tokyo, Japan. The PC1, PC3, PC7, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). The NCI-H69, SBC3, NCI-N231, Lu135 and MS-1, as well as an immortalized tracheal cell line (BET2A), were subjected to PTEN gene mutational analysis and mRNA and protein expression analysis. The Lu135 cell line was provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan). The LCD and LCK cell lines were provided by S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). The NCI-N231, A549, NCI-H69 and BET2A cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19). The PC1, PC3, PC7, PC9, PC10, PC14 and QG-56 cell lines were obtained from IBL (Gunma, Japan). The RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). The ABC-1, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19).

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and sequencing of genomic DNA. From each genomic DNA sample, all exons of the PTEN gene were amplified separately using previously described PCR primers (16) via the GeneAmp XL PCR kit (Perkin-Elmer/Roche, Branchburg, NJ, USA). PCR amplification conditions for analysis of genomic DNA were as follows: 40 cycles at 94°C for 40 sec, 60°C for 30 sec and 68°C for 90 sec, followed by 68°C for 8 min. Each reaction mixture contained 1X XL buffer, 200 μM deoxynucleotide triphosphate, 1100 μM Mg(OAc)2, 0.5 U rTth DNA polymerase XL, 0.3 mM of each primer (one of each pair) (16) and 25 ng of genomic DNA. PCR products were loaded onto 1.2% agarose gels and analyzed following electrophoretic separation. PCR-SSCP and DNA sequence analyses were performed as previously described (22).

Real-time RT-PCR analysis. Real-time quantitative RT-PCR assessment of PTEN gene expression was performed using the ABI PRISM 7700 Sequence Detector system (Perkin-Elmer/Applied Biosystems, Fosters City, CA). Both the PCR primers and associated TaqMan fluorogenic probe were designed using the Primer Express software program (Perkin-Elmer/Applied Biosystems). The relevant oligonucleotide sequences were as follows: PTEN: sense, TGGCTAAGTTGAAGTACAATGG; antisense, TGCACATATCATTACCAGCTTCGT; probe, TTCACGCAATCTGTAAGC TGAAAAG. GAPDH: sense, GAAGGTGAAGGTCGGAACTAC; antisense, GAAGATGTGTAGTGATGGTGTAGATTTC; probe, CAAGGCTCGTCCTCAGCC.

Total RNA was extracted from cultured cells and reverse transcribed using the RevaTrA Ace Kit, with a random hexamer being used as primer (Toyobo Co., Osaka, Japan). A portion of the resulting cDNA was used for quantitative PCR in a 50-μl final volume, together with the desired primers, TaqMan probe, and Master Mix, the latter being composed of PCR buffer, MgCl2, dATP, dCTP, dGTP, dUTP, AmpErase UNG, and AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems). The initial thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, as recommended by the manufacturer. The PC1, PC3, PC7, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the National Cancer Center Research Institute, Tokyo, Japan. The ABC-1, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19). The PC1, PC3, PC7, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the National Cancer Center Research Institute, Tokyo, Japan. The NCI-H69, SBC3, NCI-N231, Lu135 and MS-1, as well as an immortalized tracheal cell line (BET2A), were subjected to PTEN gene mutational analysis and mRNA and protein expression analysis. The Lu135 cell line was provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan). The LCD and LCK cell lines were provided by S. Hirohashi (National Cancer Center Research Institute, Tokyo, Japan). The NCI-N231, A549, NCI-H69 and BET2A cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19). The PC1, PC3, PC7, PC9, PC10, PC14 and QG-56 cell lines were obtained from IBL (Gunma, Japan). The RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). The ABC-1, RERF-LCMS, RERF-LCKJ, VMRC-LCD, RERF-LCAI, and MS-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD) (18,19).
Universal PCR buffer, 250 μM deoxynucleotide triphosphate, 0.2 μM of each primer, 1.25 U of AmpliTaq Gold, and 2 ng of template DNA in a final volume of 25 μl. PCR amplification was performed as follows: denaturation at 95˚C for 12 min, followed by denaturation at 95˚C for 45 sec, annealing at 60˚C for 45 sec, and extension at 72˚C for 60 sec for 35 cycles. Each allele-specific PCR sample (10 μl) was mixed with 2 μl of loading dye and loaded on a 2% agarose gel, followed by staining with ethidium bromide.

5-AZA and TSA treatment. Lung cancer cells (5x10^5/dish) were seeded into 100-mm dishes. One day later, 10 μM 5-AZA and 1 μM TSA (both from Sigma-Aldrich Japan K.K., Tokyo) were added in RPMI-1640 containing 10% FCS. After 3 days of treatment, the total RNA and the protein were extracted from these cells. The effect of these drugs was analyzed by Western blot analysis and real-time RT-PCR using the above-mentioned methods.

Drugs and growth-inhibition assay. Gefitinib was provided by AstraZeneca and dissolved in dimethyl sulfoxide (DMSO) for in vitro studies. We used the colorimetric MTT assay to examine the activity of TSA and gefitinib against PC9, PC9/f9 and PC9/f14 cells as previously reported (12,26). Cell suspensions (200 μl, 10^5 cells/ml) were seeded into 96-well microtiter plates and 10 μl of drug solution added, at various concentrations (TSA, 0.5-50 μM; gefitinib, 0.5-50 μM).

After incubation for 72 h at 37˚C, 20 μl of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubation then continued for a further 4 h at 37˚C. In addition, the effect of combination treatment with TSA and gefitinib at the concentrations indicated (TSA, 0.5 μM; gefitinib 1.0 μM) was analysed in the above-mentioned assay.

Each experiment was repeated at least three times. Statistical analysis was performed using the student’s t-test to compare the cells treated with the combinations to the cells treated with either drug alone. A p<0.05 was considered statistically significant.

Results

PTEN mRNA and protein expression in lung cancer cell lines. We analyzed the expression levels of PTEN protein in a panel of 25 lung cancer cell lines, as well as BET2A cells (Fig. 1). Six of the 25 cell lines (24%) displayed low PTEN expression. BET2A cells showed moderate PTEN expression. Ad, adenocarcinoma cell lines; Sq, squamous cell carcinoma cell lines; Sm, small cell carcinoma cell lines.

Figure 1. Western blot analysis of PTEN protein expression. The expression levels of PTEN and β-actin protein in all 25 lung cancer cell lines, as well as BET2A cells, was analyzed. Six out of the 25 cell lines (24%) showed low PTEN expression. BET2A cells showed moderate PTEN expression. Ad, adenocarcinoma cell lines; Sq, squamous cell carcinoma cell lines; Sm, small cell carcinoma cell lines.
PTEN mutational analysis via PCR-SSCP and DNA sequencing. Homozygous deletion of the PTEN gene is reported to frequently occur in lung cancer (13,27,28). Accordingly, we performed PTEN mutational analysis, via PCR-SSCP and DNA sequencing, on genomic DNA extracted from the entire panel of lung cancer cell lines, as well as the BET2A cells. Homozygous deletions of the PTEN gene were detected in two of the six cell lines that displayed low PTEN protein expression. Both of these cell lines, QG56 and N231, had homozygous deletions in the exon 1 region of the PTEN gene. No other mutations in the PTEN gene were found in the remainder of samples (Fig. 3).

Sodium bisulfite modification and methylation-specific PCR (MSP). To determine the methylation status of the PTEN gene in our series of lung cancer cell lines, MSP analysis was performed on the six lung cancer cell lines with low PTEN protein expression, as well as BET2A cells and the PC9 cell line (both of which display moderate PTEN expression). The PC10 and PC14 cell lines showed evidence of hyper-methylation in the promoter region of the PTEN gene (Fig. 4). Crucially, expression of both PTEN mRNA and protein was increased in PC10 and PC14 cells, following treatment with 5-AZA (Fig. 5).

Histone deacetylation analysis by TSA treatment. To further understand the contribution of epigenetic mechanisms to regulation of PTEN expression, we treated the subset of six lung cancer cell lines displaying low PTEN protein expression, as well as PC9 cells, with 1 μM TSA for 3 days. Following

Figure 2. Real-time RT-PCR analysis of PTEN mRNA expression. The level of PTEN mRNA, as determined by real-time RT-PCR analysis, was expressed as the ratio of PTEN to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA. Each sample was measured three times. The bar chart was prepared with the StatView program (SAS Institute Inc., Cary, NC), with the error bars referring to variation in expression levels between independent measurements.

Figure 3. Mutational analysis of the PTEN gene. The genetic status of the PTEN gene across the 25 lung cancer cell lines, as well as BET2A cells, was assessed via PCR-SSCP analysis and DNA sequencing. The status of exons 1, 5 and 9 regions of the PTEN gene is shown. QG56 and N231 had homozygous deletions in the exon 1 region of the PTEN gene.
TSA treatment, the expression of PTEN mRNA and protein in PC9/f9 and PC9/f14 cells had recovered to the same level as native PC9 cells. However, no increase in expression of PTEN was seen in the other four cell lines (PC10, PC14, QG56 and N231) following TSA treatment (Fig. 5).

**Drugs and growth-inhibition assay.** We have previously reported that two subpopulations of the PC9 cell line, PC9/f9 and PC9/f14, show natural resistance to gefitinib (12). Combination treatment with gefitinib (1 μM) and TSA (0.5 μM) induced significant growth inhibition in PC9/f9 and PC9/f14.
DNA sequence analysis of the PTEN mutations in lung cancer cell lines (14,16). Two other studies have demonstrated an absence of no other mutations in the remaining lung cancer cell lines determined by immunohistochemistry, was higher than the activation (24%) at the protein level in 125 NSCLC tissues, as determined by Kohno homozygous deletions of the gene have also been reported Absence of PTEN expression in lung cancer cell lines with homozygous deletion in the exon 1 region of the gene. In this study, 4 of 6 cell lines with low PTEN expression showed evidence of epigenetic alterations. Two of these cell lines (PC10 and PC14) displayed hypermethylation in the promoter region of the PTEN gene, as determined by MSP analysis. Khan et al reported that 60% of invasive breast cancer samples with PTEN inactivation showed hypermethylation in the promoter region of the PTEN gene (30).

In our study, the expression levels of PTEN mRNA and protein actually recovered in PC10 and PC14 cells, following treatment with the demethylating agent 5-AZA. Another key epigenetic event is histone deacetylation. When the histones are deacetylated, the chromatin is more compact and transcriptionally inactive (31). In our study, expression of PTEN mRNA and protein in the gefitinib-resistant PC9/f9 and PC9/f14 cell lines recovered following treatment with the HDAC inhibitor TSA to the level observed in the parental gefitinib-sensitive PC9 cell. Thus, diminished levels of PTEN, a regulator of EGFR pathways, might decrease gefitinib sensitivity, as previously reported (12). HDAC inhibitors, such as TSA, have been demonstrated to inhibit the expression of anti-apoptotic genes (i.e. c-Myc, cyclin D1 and BCL-XL) and increase the transcription of pro-apoptotic genes (p21/waf1/cip1) (32-34). Currently, several hydroxamate-based inhibitors, including TSA, have been shown to selectively inhibit tumor growth in animals at apparently nontoxic doses (35). Recently, SAHA, a hydroxamic acid, has been used clinically for the treatment of cutaneous T-cell lymphoma (35).

Using the growth-inhibition assay, we demonstrated that the combination treatment of gefitinib and TSA at low concentrations induced significant growth inhibition in gefitinib-resistant PC9/f9 and PC9/f14 cells. The important role of the EGFR signaling pathway in some lung cancers and the findings in our recovery study suggest that the combination of the EGFR TKI gefitinib with the demethylating agent 5-AZA and the HDAC inhibitor TSA may be a useful strategy for the treatment of some lung cancers.

Discussion

Loss of PTEN function increases the cellular concentration of PIP-3, which in turn leads to Akt hyperactivation, suggesting that the tumor suppressor function of PTEN is exerted through the negative regulation of the PI3K/Akt cell pathways (4). According to recent reports, loss of PTEN is associated with poor prognosis for patients with lung cancer (7-9), as well as resistance to many anti-cancer agents, including gefitinib (10-12). To understand the mechanisms leading to loss of PTEN protein expression in lung cancer, we performed mutational and epigenetic analyses of the PTEN gene. In this study, 6 out of 25 (24%) lung cancer cell lines displayed low PTEN expression. We detected homozygous deletions of the PTEN gene in two of these six cell lines that had low PTEN expression. Both of these cell lines, QG56 and N231, displayed homozygous deletion in the exon 1 region of the PTEN gene. Absence of PTEN expression in lung cancer cell lines with homozygous deletions of the gene have also been reported by Kohno et al (5% of cells) and Forgacs et al (4% of cells) (13,15). DNA sequence analysis of the PTEN gene showed no other mutations in the remaining lung cancer cell lines (15). Two other studies have demonstrated an absence of PTEN mutations in lung cancer cell lines (14,16).

Soria et al reported that the frequency of PTEN inactivation (24%) at the protein level in 125 NSCLC tissues, as determined by immunohistochemistry, was higher than the rate (23%) of PTEN genetic alteration (17). In our study, the expression levels of PTEN mRNA in the subset of six cell lines were reduced compared to the PC9 cell line and BET2A cells, this was consistent with the results obtained at the protein level. Another possible mechanism of PTEN inactivation may be epigenetic inactivation of the gene through hypermethylation (17,29) and/or histone deacetylation. In our study four of the six cell lines with minimal PTEN expression showed evidence of epigenetic alterations. Two of these cell lines (PC10 and PC14) displayed hypermethylation in the promoter region of the PTEN gene, as determined by MSP analysis.

Acknowledgements

IRESSA is a trademark of the AstraZeneca group of companies. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Japan Society for the Promotion of Science.

References


