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Original Article

Effects of (−)-epigallocatechin-3-gallate on EGFR- or Fusion Gene-driven Lung Cancer Cells

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(−)-Epigallocatechin-3-gallate (EGCG) has been shown to bind to several receptors including epidermal growth factor receptor (EGFR). EGFR tyrosine kinase inhibitors and anaplastic lymphoma kinase (ALK) inhibitors are effective for non-small cell lung cancers harboring activating EGFR mutations and ALK or c-ros oncogene 1 (ROS1) fusion genes, respectively. We investigated the effects of EGCG on EGFR- or fusion gene-driven lung cancer cells such as PC-9, RPC-9, H1975, H2228 and HCC78. The five cell lines had similar sensitivity to EGCG. Phosphorylated (p)EGFR, pAkt and pErk in PC-9, RPC-9 and H1975 cells were suppressed by EGCG (50 or 100 μM). EGCG also inhibited pALK in H2228, pROS1 in HCC78, and pErk and pAkt in both cell lines. All the xenograft tumors established using the 5 cell lines in EGCG-treated groups were significantly smaller than the tumors in the vehicle-treated groups. The numbers of tumor blood vessels of xenograft tissues in EGCG-treated mice were significantly lower than those in vehicle-treated mice. In conclusion, EGCG may be effective for EGFR-driven lung tumors irrespective of the presence of T790M, and for ALK or ROS1 fusion gene-driven lung tumors.

Key words: epigallocatechin-3-gallate, lung cancer, EGFR, ALK, ROS1

Tea is the most widely consumed beverage in the world. Green tea contains a variety of polyphenols known as catechins. Major components of tea polyphenols are epicatechin, epigallocatechin, epicatechingallate and (−)-epigallocatechin-3-gallate (EGCG) (Fig. 1) [1, 2]. EGCG is the most abundant and biologically active compound in green tea [3]. It has been shown to inhibit enzyme activities and some signal transduction pathways, resulting in the suppression of cell proliferation and enhancement of apoptosis, as well as the inhibition of cell invasion, angiogenesis and metastases [4, 5].

Lung cancer is the most common cause of cancer death in many countries. Oncogene drivers—including epidermal growth factor receptor (EGFR) mutation and fusion gene rearrangement between echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinases (ALK)—play important roles in the carcinogenesis and tumor progression of non-small cell lung cancer (NSCLC). These EGFR mutations and fusion gene rearrangements can be suppressed by EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib [6] and ALK TKIs such as crizotinib [7], respectively. Recently, c-ros oncogene 1 (ROS1) fusion genes whose partners were SLC34A2, CD74 or FIG

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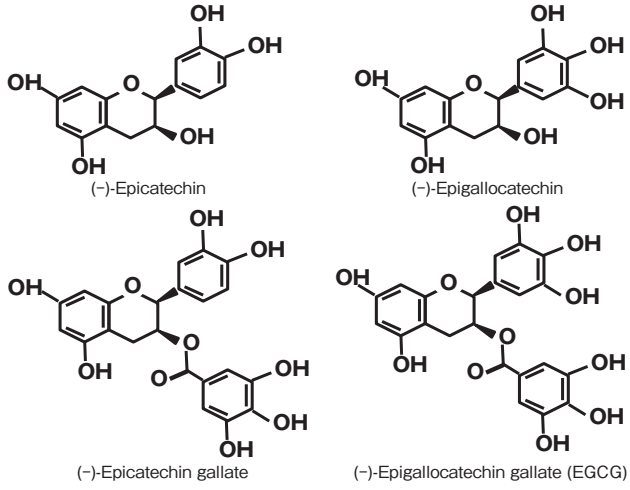

Fig. 1 Structure of the green tea polyphenols.

were discovered to be oncogene drivers, and crizotinib found to be effective for these lung cancers as well [8, 9]. In this study, we attempted to clarify whether EGCG has an inhibitory effect against EGFR-, ALK- or ROS1-driven lung tumors.

Materials and Methods

Reagents and antibodies. EGCG (>95% purity; Sigma-Aldrich, Tokyo, Japan) was prepared as a 10 mM stock solution, and diluted into culture media immediately prior to the experiments. Rabbit antisera against EGFR, phospho-specific (p) EGFR (pY1068), Erk, pErk, Akt, pAkt (pSer473), ALK, pALK, ROS1, pROS1 and GAPDH were purchased from Cell Signaling Technology (Tokyo, Japan). Antibody against hypoxia-induced factor 1α (HIF)-1α was purchased from BD Biosciences (Tokyo, Japan). Antibody against CD31 (platelet endothelial cell adhesion molecule) used in the immunohistochemical analyses was also purchased from Cell Signaling Technology.

Cell lines and culture. The NSCLC cell lines PC-9 and H1975 were purchased from Immuno-Biological Laboratories (Takasaki, Japan). H2228 and HCC78 were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS; complete medium). All cells were maintained under a 5% CO_2-humidified atmosphere at 37°C. Cells were subcultured once and reached 80-90% confluency. RPC-9 cells with an EGFR T790M mutation were established from parental PC-9 cells harboring an EGFR exon 19 deletion mutation in our laboratory [10]. RPC-9 cells showed a 400-fold resistance to gefitinib compared with parental PC-9 cells. H1975 cells carried an EGFR exon 21 missense mutation (L858R) and exon 20 T790M mutation [6]. H2228 cells and HCC78 cells harbored the EML4-ALK fusion gene and SLC34A2-ROS1 fusion gene, respectively [7, 8].

Cell growth assay. Cells were plated in 96-well plates at 3,000 cells per well in complete medium and then were incubated with different concentrations of EGCG for 96 h. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [11]. The percentage of surviving cells was calculated using the following formula: [(mean fluorescence in 6 test wells - fluorescence in background wells) / (mean fluorescence in control wells - fluorescence in background wells)] × 100. The drug concentration required to inhibit the growth of tumor cells by 50% (IC_{50}) for 96 h exposure was determined by plotting the logarithm of drug concentration versus the percentage of surviving cells. Determinations were carried out in quadruplicate in each experiment and results were confirmed in 3 or more separate experiments.

Western blotting. After treating with EGCG (50 or 100 μM) for 20 h, cells were immediately harvested and disrupted. Cells were lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerol phosphate, 10 mmol/L NaF, and 1 mmol/L sodium orthovanadate-containing protease inhibitor tablets (Roche Applied Sciences GmbH, Mannheim, Germany)]. Proteins were separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies followed by detection with Enhanced Chemiluminescence Plus (GE Healthcare Biosciences, Tokyo, Japan). Protein expression was assessed by densitometry using ImageJ software (developed at the National Institutes of Health).

Xenograft model. Female BALB/C nu/nu mice (6-week-old; Japan Charles River Co., Kanagawa, Japan) were injected with 2×10^6 PC-9, RPC9, H1975, H2228 or HCC78 cells in 100 μl phosphate buffered saline and 100 μl matrigel subcutaneously into the upper flank region. The mice were orally treated with 0.5% (w/v) EGCG or water in a water bottle for 6 weeks (PC-9, RPC-9 and H1975) or 8 weeks (H2228 and

HCC78) after injection of cancer cells. Tumors became measurable by day 10 post-implantation and were measured with digital calipers twice a week. Tumor volumes were calculated by the equation: volume= length × width^2 × 0.5. The mice were sacrificed at of day 42 (PC-9, RPC-9 and H1975) or day 56 (H2228 and HCC78) post-implantation and the tumors were surgically removed.

Immunohistochemistry. Deparaffinized sections were unmasked in antigen-unmasking solution (DAKO, Copenhagen, Denmark) in a microwave oven for antigen retrieval. Endogenous peroxidase was quenched using 3% H_2O_2, and then the sections were blocked for 1 h at room temperature and incubated with CD31 antibody (1:20) overnight at 4°C. Biotin-conjugated secondary antibody (1:200) and avidin–biotin peroxidase complex (1:100) were then applied. Finally, the sections were counterstained with hematoxylin. CD31-positive blood vessels were counted in ten random fields (×200).

Statistical analysis. All data were presented as the mean ± standard deviation (SD). Student's *t*-test was used to test differences between 2 groups. Values of $p < 0.05$ were considered to indicate statistical significance in the two-tailed comparison. All data were analyzed using Microsoft Office Excel 2010 (Microsoft Japan Corporation, Tokyo, Japan).

Results

Effect of EGCG in vitro. EGCG was found to have effects on all 5 cell lines (PC-9, RPC-9, H1975, H2228 and HCC78) in a dose-dependent manner. The IC_{50} values ranged between 22 μM and 57 μM (Table 1). Next, we examined the effects of EGCG on the phosphorylation of EGFR, ALK and ROS1 in the cells (Fig. 2A). pEGFR was inhibited by a 20-h pretreatment with 50 or 100 μM EGCG in PC-9, RPC-9 and H1975 cells. EGCG also suppressed pAkt and pErk, downstream signals of EGFR, in the same 3 cell lines. In H2228 cells, pALK, pAkt and pErk were also inhibited. In the HCC78 cells, pEGFR, pAkt, pErk and pROS1 were suppressed. The ratios of pEGFR to total (t) EGFR in PC-9, RPC-9 and H1975 cells, of pALK to tALK in H2228 cells, and of pROS1 to tROS1 in HCC78 cells were significantly reduced by EGCG treatment (Fig. 2B).

Effect of EGCG in vivo. EGCG or vehicle treatments of the tumor xenografts from each of the five cell lines are shown in Fig. 3. On day 42 after xenograft, the sizes (mm^3; mean ± SD) of the tumors derived from PC-9 cells were 1,114 ± 478 (n = 10) in the EGCG group and 2,803 ± 973 (n = 9) in the control group ($p < 0.05$). Similarly, the xenograft tumors derived from RPC-9 cells in the EGCG group on day 42 were significantly smaller (371 ± 262, n = 13) than those (760 ± 352, n = 11) in the control group ($p < 0.05$). H1975 xenografts in the EGCG group on day 42 were significantly smaller (1,579 ± 1,104, n = 9) than those (3,435 ± 1,719, n = 7) in the control group ($p < 0.05$). On day 56 after xenograft, the tumors derived from H2228 cells in the EGCG group were significantly smaller (170 ± 128, n = 9) than those (260 ± 240, n = 9) in the control group ($p < 0.05$). The HCC78 tumors on day 56 after xenograft were also significantly smaller in the EGCG group (51 ± 10, n = 12) than in the control group (116 ± 29, n = 6) ($p < 0.05$). There were no significant differences in body weight between the EGCG group and control group (Fig. 4).

Table 1 Sensitivity to EGCG

Cell line	IC_{50} (μM)
PC-9	43.33 ± 7.03
RPC-9	37.48 ± 3.64
H1975	32.47 ± 0.61
H2228	57.46 ± 7.64
HCC78	22.42 ± 0.92

EGCG, (−)-epigallocatechin-3-gallate; IC_{50}, inhibitory concentration of cell growth by 50%.

In order to evaluate the antiangiogenic properties of EGCG, we utilized immunostaining. CD31-positive blood vessels in the tumors treated by EGCG or vehicle are shown in Fig. 5A. In all xenograft models, the tumors treated with EGCG exhibited significantly fewer tumor vessels (all $p < 0.05$; Fig. 5B). To examine the cause of the suppressive effect of EGCG on neovascularization, we measured the HIF-1α levels in the cells. The results showed that the levels of HIF-1α in PC-9, RPC-9, H1975, H2228 and HCC78 cells were all inhibited by 100 μM of EGCG (Fig. 6).

Discussion

EGCG was previously shown to inhibit both EGFR mutant and wild type lung cancer cells [12, 13]. In the present study, we revealed that EGCG inhibited cell growth irrespective of EGFR-, ALK- or ROS1-

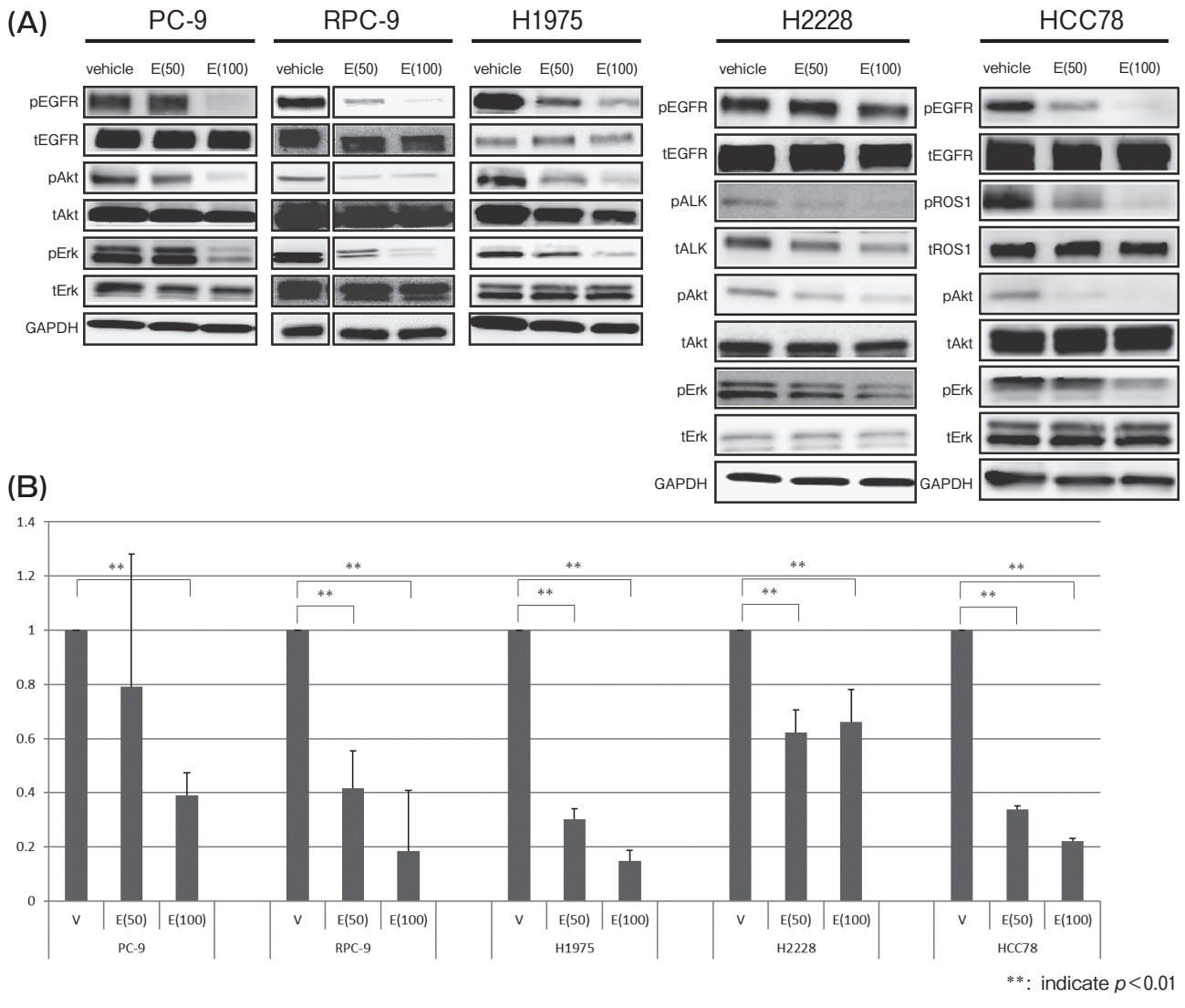

Fig. 2 (A) Effects of EGCG on EGFR, ALK, ROS1 and its downstream signaling in PC-9, RPC-9, H1975, H2228 and HCC78 cells. Cells were incubated with EGCG 50 μM or 100 μM for 20 h and cell lysates were subjected to immunoblot analysis with the antibodies. E(50): EGCG 50 μM; E(100): EGCG 100 μM. (B) Protein expression was assessed by densitometry using ImageJ software (developed at the National Institutes of Health). The ratios of pEGFR to total (t)EGFR in PC-9, RPC-9 and H1975 cells, of pALK to tALK in H2228 cells, and of pROS1 to tROS1 in HCC78 cells were significantly reduced by EGCG treatment. Student's *t*-test. $^{**}p < 0.01$.

dependency. In addition, we showed that EGFR TKI-resistant cells harboring a T790M mutation were suppressed by EGCG. Finally, we revealed that lung cancer cells driven by EML4-ALK or SLC34A2-ROS1 fusion genes were also inhibited by EGCG.

EGFR-activating mutations in NSCLC represent an example of oncogene addiction [4], and tumors harboring the mutations are quite sensitive to gefitinib and erlotinib. But even in cancers with the activating mutations, resistance to EGFR TKIs frequently develops after several months of the treatment. Approximately half of the resistant tumors would have a secondary T790M mutation.

Although osimertinib was recently approved and shown to have great efficacy in patients with T790M-positive advanced NSCLC [14], no other drugs have been proven effective and made clinically available for these resistant tumors. Therefore, T790M-positive

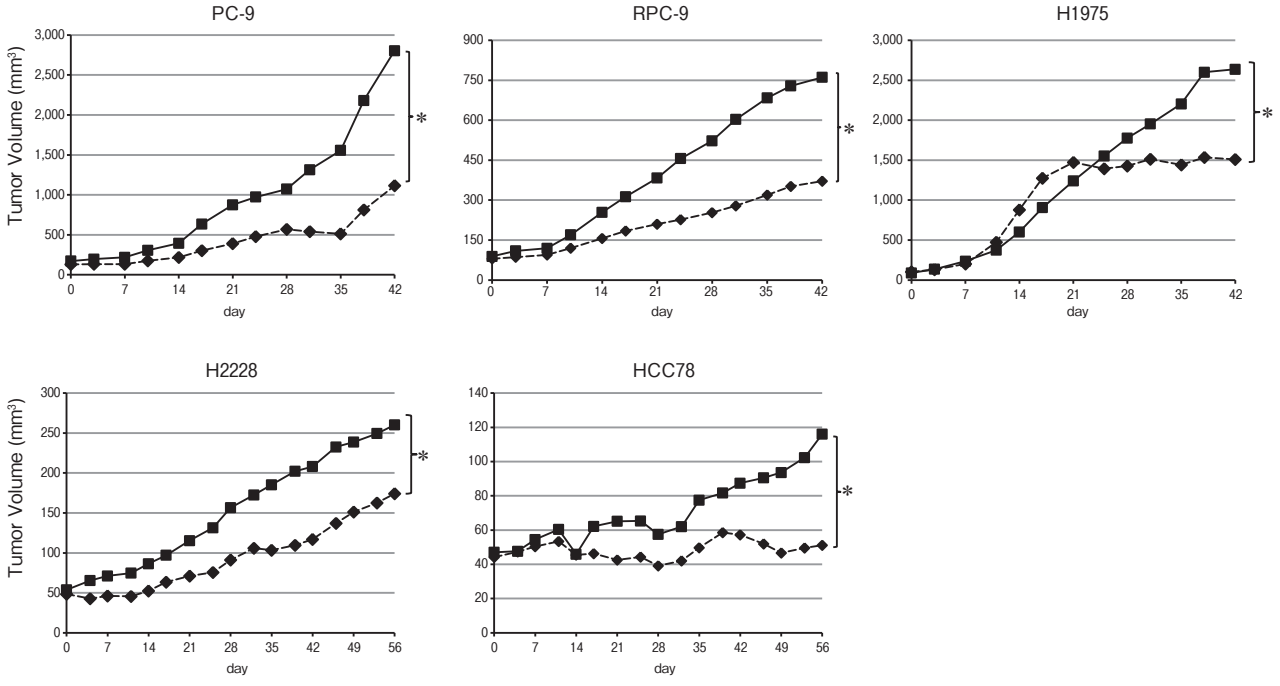

Fig. 3 Growth curves of PC-9, RPC-9, H1975, H2228 and HCC78 xenograft tumors in mice receiving 0.5% (w/v) EGCG (broken line) or water (solid line). Mean tumor volumes of 6–13 tumors per group are shown. Differences in the tumor volume at day 42 (PC-9, RPC-9 and H1975) or day 56 (H2228 and HCC78) were compared using Student's *t*-test. $^{*}p < 0.05$.

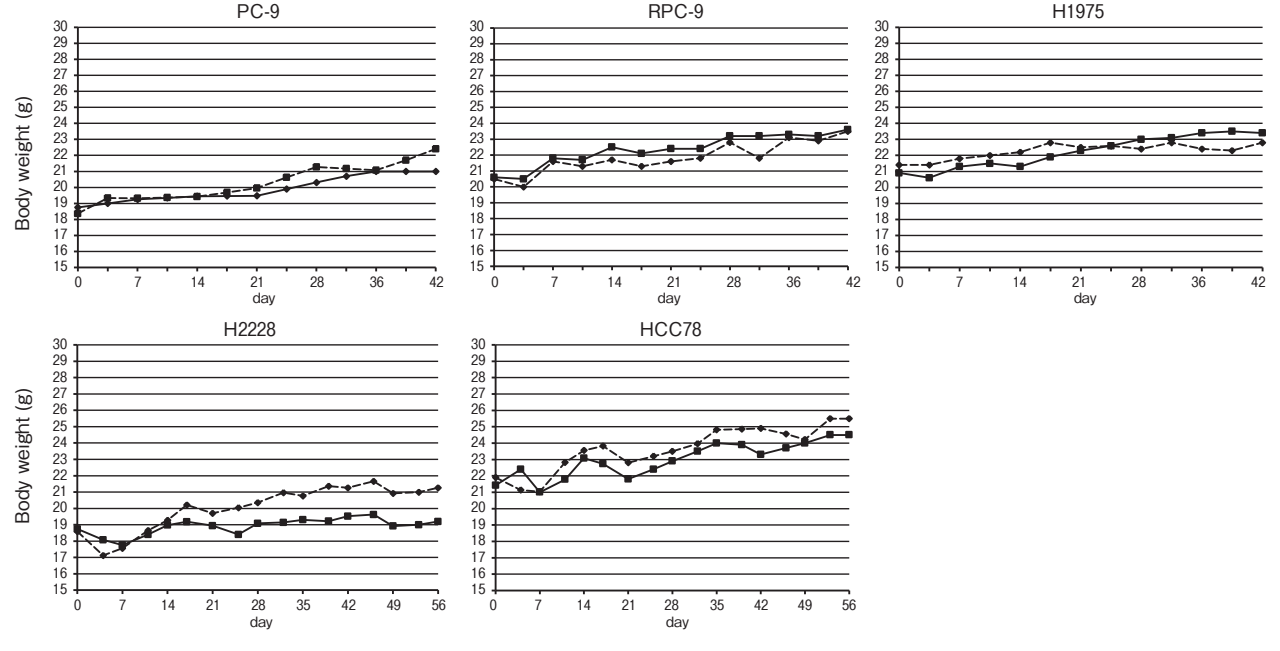

Fig. 4 Changes in the body weights of PC-9, RPC-9, H1975, H2228 and HCC78 xenograft mice. Body weight was measured twice a week. There were no significant differences of body weights between the EGCG group (broken line) and control group (solid line).

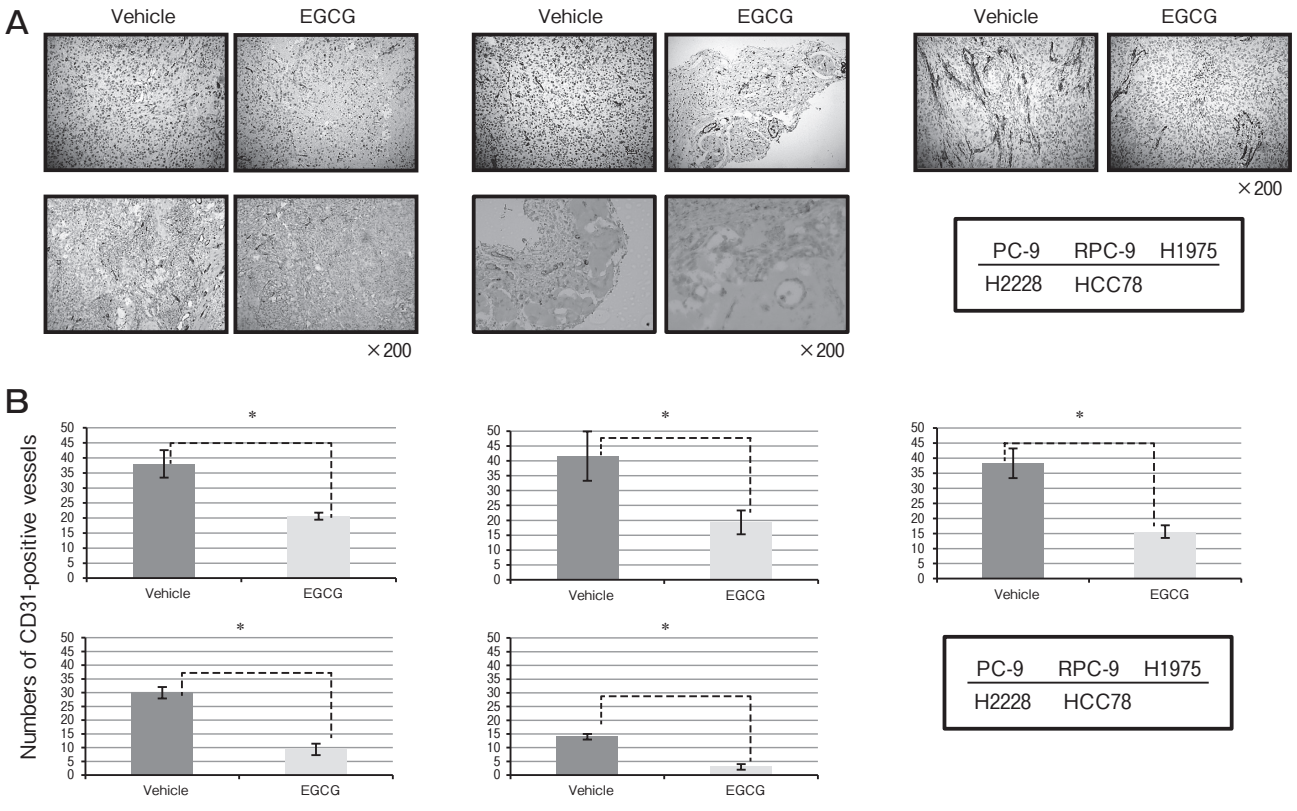

Fig. 5 (A) Tumor angiogenesis assessed by CD31 immunohistochemical staining. The mice were treated with EGCG or water. The xenograft tumor specimens grown from PC-9, RPC-9, H1975, H2228 and HCC78 cells are shown. (B) Quantification of angiogenesis. The tumors treated with EGCG exhibited a significant decrease in tumor vessels in all cell lines. Student's *t*-test. $^{*}p < 0.05$.

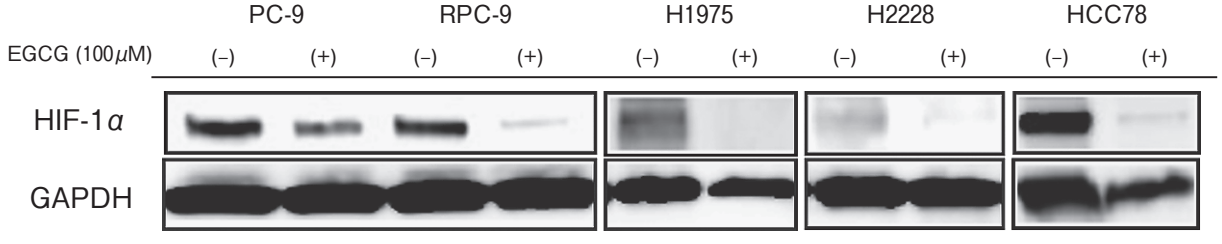

Fig. 6 Effects of EGCG on HIF-1α. Western blotting analysis showed that HIF-1α was suppressed in PC-9, RPC-9, H1975, H2228 and HCC78 cells with EGCG treatment.

advanced NSCLC patients who cannot use osimertinib may be good candidates for EGCG therapy. The NSCLC tumors harboring ALK or ROS1 fusion genes would also be resistant to crizotinib after approximately 1 to 1.5 years of treatment [7, 15, 16]. The response rate of ceritinib, another recently approved ALK inhibitor, was previously reported as 58% among 80 ALK-positive patients who had been treated with crizotinib [17]. Despite these promising results with ceritinib, however, EGCG may be effective in cases of fusion gene-driven lung tumors refractory to crizotinib. Unfortunately, since we did not have crizotinib-resistant H2228 or HCC78 cells, we could not examine this issue in the present work.

Angiogenesis, the process of blood-vessel growth, is a critical event during development, as well as during tumor invasion and metastasis [18]. We showed that all the xenograft tumors derived from the 5 cell lines used

here were significantly inhibited by EGCG treatment. The overexpression or accumulation of HIF-1α protein was associated with oncogenic events by up-regulating vascular endothelial growth factor (VEGF) expression and promoting the tumor-associated angiogenesis, which is a mechanism for cancer cells to survive hypoxia in the tumor microenvironment [19-21]. EGCG has been reported to suppress the activity of HIF-1α [22,23]. We also confirmed that HIF-1α was suppressed in all the cell lines used here. Although several mechanisms are already thought to contribute to the inhibition of angiogenesis by EGCG, including those involving VEGFR, MET, and fibroblast growth factor [4], suppression of HIF-1α may also have played a role in the anti-angiogenesis in our study. In addition, a previous report using xenograft models showed that EGCG had antiproliferative effects on A549 lung cancer cells harboring KRAS mutation [24]. In that study, the antiangiogenic potential of EGCG was partially responsible for the growth suppression of tumors, similarly to our experiments. EGCG seems to be effective for KRAS mutant lung cancers in addition to EGFR, ALK, and ROS1 mutant lung cancers.

EGCG treatment has been shown to impose a blockade of G1-to-S transition, leading to G0/G1 arrest, in an epidermoid carcinoma cell line (A431) [25]. In biliary tract cancer cell lines treated with EGCG, a decrease in G2/M and an increase in G0/G1 were observed [26]. In the future, it will be important to further analyze the cell cycle in lung cancer cells treated with EGCG, since such analysis could provide a clue to the possibility of combination therapy with chemotherapeutic agents including cisplatin [26].

The mechanisms by which EGCG inhibits EGFR have been reviewed previously [4,5]. EGCG can inhibit EGFR activation by interfering with the binding of EGF to EGFR; alter lipid organization in the plasma membrane and inhibit EGF binding to EGFR; and induce internalization of EGFR into endosomes [4]. In addition, the phosphorylation of Akt and Erk were also suppressed because their upstream molecules, such as EGFR, MET, and IGFR, were inhibited by EGCG [4]. However, the fact that the principal mechanisms of action of EGCG remain unknown constitutes a limitation of this study.

Although Akt and Erk, which are downstream signals from EGFR, ALK and ROS1, were suppressed by 50 or 100 μM of EGCG, these EGCG concentrations were quite high. Dietary EGCG treatment increased the serum EGCG level in the xenograft in a dose-dependent manner [27]. However, the serum concentration of EGCG in mice was reported to be 2 orders of magnitude lower than the IC_{50} of 20-100 μM observed in *in vitro* experiments [4]. It remains unresolved whether the therapeutic efficacy of EGCG observed *in vivo* reflected the mechanisms obtained in *in vitro*, although there have been several studies evaluating the effect of the differences in the effective concentrations between the *in vitro* and *in vivo* experiments [4,28-30]. Moreover, we have no data concerning the IC_{50} values of EGCG for non-malignant cells. Because human cholangiocarcinoma cells were 25 times more sensitive to EGCG than human embryonic kidney 293 T cells [31], we expect that EGCG might have a tumor-selective antiproliferative effect.

In summary, we demonstrated that EGCG possessed inhibitory effects on the growth of all the cell lines we tested. PC-9 cells with EGFR mutation, RPC-9 and H1975 cells with resistant T790M mutation, and H2228 and HCC78 cells with fusion mutation were similarly sensitive to EGCG *in vitro* and *in vivo*. EGCG suppressed angiogenesis, and this effect may have been attributable to the inhibition of HIF-1α. Treatment with EGCG may be warranted for this intractable cancer.

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