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Dual inhibition of GTP-bound KRAS and mTOR in lung adenocarcinoma and squamous cell carcinoma harboring KRAS G12C

Masaoki Ito[1,2*], Yoshihiro Miyata[1*], Shoko Hirano[3], Nagisa Morihara[3], Misako Takemoto[3], Fumiko Irisuna[3], Kei Kushitani[4], Kenichi Suda[2], Junichi Soh[2,5], Yukio Takeshima[4], Yasuhiro Tsutani[2] and Morihito Okada[1]

Abstract

Background *Kirsten rat sarcoma* (*KRAS*) mutations are somatic variants in lung adenocarcinoma. One of the most prevalent mutations, G12C, has led to the clinical approval of targeted inhibitors for advanced stages in lung cancer. Research has increasingly focused on the efficacy of combination therapies that target multiple tumorigenic pathways. Cases harboring *KRAS* G12C mutation are heterogenous. We explored alternative changes in genetic pathways and evaluated the effectiveness of combination therapy using several types of cell lines and *KRAS* inhibitors.

Methods We comprehensively investigated genetic changes induced by *KRAS* G12C inhibition using RNA sequences and the candidate to inhibit in combination therapy was explored. Three lung cancer cell lines (two adenocarcinoma and one squamous cell carcinoma) and three *KRAS* G12C inhibitors (AMG 510, MRTX849, and ARS-1620) were used. *KRAS* G12C and candidate gene were simultaneously inhibited in cell lines and the efficiency of combination therapy was evaluated using clonogenic assays and MTS assay. Pathway activation was assessed via western blotting. A combination index (CI) < 0.8 was considered statistically synergistic.

Results RNA sequences revealed treatment with two of the three *KRAS* G12C inhibitors led to a significant increase in *mTOR* expression across all three cell lines. *mTOR* was targeted in combination therapy; each *KRAS* G12C inhibitor and *mTOR* inhibitor (RAD001) combination exhibited synergism (CI < 0.8) in MTS and clonogenic assays. Single inhibition of *mTOR* induced activation of guanosine triphosphate (GTP)-RAS, thereby activating the *RAS-MEK-ERK* and *PI3K-AKT-mTOR* pathways in WB, suggesting *mTOR* activation is crucial for *KRAS*-driving lung cancer. A combination strategy targeting *KRAS* G12C and *mTOR* abrogated GTP-RAS, pmTOR (Ser2448), and pERK (Thr202/Tyr204) more efficiently.

Conclusions *KRAS* G12C inhibitor plus RAD001 consistently revealed synergism. Targeting *KRAS* G12C and *mTOR* abrogates the *RAS-MEK-ERK* and *PI3K-AKT-mTOR* pathways. Our data suggests that a combined strategy targeting GTP-bound *KRAS* G12C and *mTOR* shows promise for primary lung cancers with *KRAS* G12C mutations. This approach may also be effective even for lung cancers harboring *KRAS* G12C mutation but having different profiles.

*Correspondence:
Masaoki Ito
itooo_m@yahoo.co.jp
Yoshihiro Miyata
ymiyata@hiroshima-u.ac.jp

Full list of author information is available at the end of the article

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Background

Kirsten rat sarcoma (*KRAS*) is a major onco-driving gene in lung cancer. Somatic *KRAS* mutations are likely harbored in lung adenocarcinoma patients with a history of smoking [1] and can also be harbored in squamous cell carcinoma patients [2]. Targeting inhibitors for *KRAS* G12C have been clinically approved for advanced cases [3].

In gene target therapies, acquired resistance is inevitable and its mechanisms have been revealed in treatment targeting *epidermal growth factor receptor* (*EGFR*) mutations and *anaplastic lymphoma kinase* (*ALK*) rearrangements [4–7]. Likewise, resistant mechanisms in other target therapies, secondary nucleotide changes have been reported in *KRAS* G12C inhibition [8, 9]. Activations of alternative or downstream pathways have been also revealed as a resistant mechanism in *KRAS*-driving tumors. Clinical data and in vitro studies have unveiled that secondary mutations in *KRAS* or amplification in *MET* can emerge as resistant mechanisms for *KRAS* G12C inhibitors [8, 9]. Other studies showed that *KRAS* signaling can be maintained after *KRAS* G12C inhibition and then drug-resistance is acquired by *EGFR* or *AURKA* signaling [10], or epithelial-mesenchymal transition (EMT) via *PI3K* pathway activation [11]. *KRAS*-mutant cases are heterogeneous. EMT is also one of the key mechanisms for acquired resistance [12, 13]. Different tumorigenesis is suggested between epithelial and mesenchymal types [14]. *KRAS* inhibition alone can be thwarted by compensatory or parallel pathway activation [15, 16] and monotherapy may be an insufficient approach.

To overcome the resistance or achieve greater efficiency of target therapy, the usefulness of combination therapies targeting several signaling pathways has been suggested [17–20]. Co-inhibition by *KRAS* G12C plus *SHP2* [21] or *KRAS* G12C plus *mTORC1* kinase has been suggested to be promising [22]. Solanki et al. evaluated combination therapy by dividing *KRAS*-mutant cases into epithelial and mesenchymal types; the former is activated by *IGFRs* and *ERBB2/3* and responsive to co-inhibition with *SHP2* or *SOS1*, and the latter is activated by *IRAS1* or *AXL* signaling [14]. Combination treatment for tumors harboring *KRAS* G12C is a promising approach and is not limited to lung cancer [23, 24].

Combination strategies have been attempted to conquer the *KRAS*-driving tumors [15, 25, 26]. Phase II-III clinical trials of the combination of the *KRAS* G12C inhibitor with cytotoxic chemotherapy or immune checkpoint inhibitors (ICIs) for non-small cell lung cancer (NSCLC) are ongoing (NCT05920356, NCT04613596, NCT05472623, NCT05609578, NCT05375994, NCT05840510, NCT05578092, NCT06024174, NCT05118854, NCT05054725, NCT05074810) [27].

We aimed to reveal the alternative pathway changes as a potential mechanism of resistance using several types of *KRAS*-mutant cell lines (adenocarcinoma or squamous cell carcinoma, epithelial or mesenchymal type, or oncogenic mutations in *STK11* and/or *TP53*) and evaluate the efficiency of combination therapy targeting the alternative pathway change.

Methods

Overview of study design

We aimed to evaluate alternative changes in the tumorigenesis pathway induced by *KRAS* target treatment. Three lung cancer cell lines (including squamous cell carcinoma) harboring *KRAS* G12C were cultured with three types of *KRAS* G12C inhibitors, and the frequency of *KRAS* G12C was explored in clinical adenocarcinoma and squamous cell carcinoma patients. After exposure to each inhibitor, RNA-Seq was performed and changes in the mRNA expression of tumor-related genes were evaluated. The candidates detected by RNA-Seq were further examined to determine whether their inhibition was effective in *KRAS*-targeted therapy. The efficiency of combination therapy for *KRAS* G12C was also determined. All the procedures involving human participants were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent for collecting samples for use in the study and publication of results was obtained from all patients before surgery. This study was approved by the Institutional Review Board of Hiroshima University (E2020-2081-01).

Detection of *KRAS* G12C in clinical primary lung cancer cases

The frequency of *KRAS* G12C was evaluated in consecutive patients with pN0M0 adenocarcinoma and squamous cell carcinoma resected at Hiroshima University Hospital between 2007 and 2019 or 2014 and 2018. DNA was extracted from the resected frozen or formalin-fixed paraffin-embedded tissues using the QIAamp DNA Micro Kit or the QIAamp DNA FFPE Tissue Kit (56304, 56404; Qiagen GmbH, Hilden, Germany). *KRAS* G12C was detected using commercially available primers and probes (1863506; Bio-Rad, Hercules, CA, USA), and QX100 Droplet Digital PCR System (1863111, Bio-Rad,

Hercules, CA, USA). The detailed ddPCR conditions have been described previously [28].

Cell lines and inhibitors

Three lung cancer cell lines harboring *KRAS* G12C were used: H23 (adenocarcinoma), HOP-62 (adenocarcinoma), and Calu-1 (squamous cell carcinoma). As a control, H520 cells (*KRAS* wild type, lung squamous cell carcinoma) were used in the clonogenic assay. RPMI 1640 Media (11875093, Thermo Fisher Scientific, MA, USA) and McCoy's 5 A (Modified) Medium (16600082, Thermo Fisher Scientific, MA, USA) medium supplemented with 10% fetal bovine serum and 50 μg/mL penicillin-streptomycin were used for H23/HOP-62/H520 and Calu-1, respectively. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. AMG 510, MRTX849, and ARS-1620 were used as *KRAS* G12C inhibitors, while RAD001 was used as a *mammalian target of rapamycin* (*mTOR*) inhibitor. The profiles of each cell line are shown in Additional file 1: Supplementary Table 1. Information on the inhibitors is provided in Additional file 2: Supplementary Table 2.

RNA sequencing (RNA-Seq) with next-generation sequencing (NGS)

To evaluate changes in mRNA expression levels, we performed RNA-Seq using cell lines treated with *KRAS* G12C inhibitors. Cells (50,000) were disseminated in a 60 mm dish (83.3901; SARSTEDT, Nümbrecht, Germany). The cells were cultured for 24 h to attach, and the medium was replaced with a fresh medium containing a *KRAS* G12C inhibitor or dimethyl sulfoxide (DMSO). The number of seeded cells and the concentration of inhibitors were the same regardless of the cell line. The medium containing the inhibitor or DMSO was changed every 3 d for approximately 14 d before reaching cell confluency. RNA was extracted using the Tri Reagent (TR118, COSMO BIO. Tokyo, Japan).

For cDNA synthesis, we used PrimeScript™ Double Strand cDNA Synthesis Kit (6111 A, TAKARA BIO INC., Kusatsu, Siga, Japan) to make cDNA from the mRNA purified with Oligotex™-dT30 < Super > mRNA Purification Kit (9086, TAKARA BIO INC., Kusatsu, Siga, Japan). For library preparation, 14 ng of covaris sheared cDNA was used as a direct input for the xGen Prism DNA Library Prep Kit (10006202, Integrated DNA Technologies Inc., Coralville, IA, USA) and indexed by xGen UDI Primer Pairs, Index 1–96 (Integrated DNA Technologies Inc., Coralville, IA, USA). Libraries were pooled at 500 ng each and subsequently hybridized with the xGen Hybridization and Wash Kit (1080577, Integrated DNA Technologies Inc., Coralville, IA, USA) and xGen Customs Hyb Panels (Integrated DNA Technologies Inc., Coralville, IA, USA) according to the manufacturer's instructions. The final captured libraries were quantified using a Qubit dsDNA HS Assay Kit (Q32854, Thermo Fisher Scientific, MA, USA) and a Bioanalyzer High Sensitivity DNA Analysis Kit (5067 – 4626, Agilent Technologies, Santa Clara, CA USA). The expression levels of 48 oncogenes and beta-actin were assessed. The genes evaluated are listed in Additional file 3: Supplementary Table 3. We pooled six capture pools together at 4 nM and sequenced them on a HiSeq 2500 (Illumina, PE Rapid v2 workflow) with 2 × 67 bp paired-end reads.

Real-time PCR

mRNA expression levels were examined using real-time PCR and performed independently of RNA sequencing. Predesigned primers and probes (TaqMan Gene Expression Assays, Thermo Fisher Scientific, MA, USA) were used for *mTOR* (Hs00234508_m1) and β-actin was used as an internal control (Hs01060665_g1). RNA was extracted as described above and purified using the SuperScript IV VIL Master Mix with ezDNase (11766050, Thermo Fisher Scientific, MA, USA). Real-time PCR was performed using StepOnePlus (Thermo Fisher Scientific, MA, USA). Delta-delta cycle threshold (Ct) was employed to quantify expression level using the following formula; $2^{-(\mathrm{DCt\ sample})}$, in which delta Ct (DCt) values were determined by subtracting the Ct value of the house-keeping gene (β-actin) from the Ct value of the target gene.

Clonogenic assay

Clonogenic assays were performed as previously described [15]. Briefly, 300 cells were seeded in 6-well plates (83.3920, SARSTEDT, Nümbrecht, Germany). The cells were cultured for 24 h and the medium was replaced with inhibitor(s) or DMSO. The number of seeded cells and the concentration of inhibitors were the same regardless of the cell line. The medium containing inhibitor(s) or DMSO was changed every 3 d for 11–14 d before reaching cell confluency. The medium was removed, and the cells were washed with phosphate-buffered saline (PBS). The colonies were fixed with methanol for 25 min on ice and stained with 0.5% crystal violet (031-04852, Wako, Japan) for 15 min at room temperature. The colonies were then washed and photographed. For semiquantitative measurements, the crystal violet was reabsorbed using 2% SDS, and the absorbance was measured at 570 nm using a TriStar LB941 (BERTHOLD, Tokyo, Japan).

MTS assay

The antitumor effect of the drug combination was assessed by cell viability using the Thiazolyl Blue Tetrazolium Bromide assay and the drug concentration required for 50% growth inhibition (IC50) was determined using

GraphPad Prism Version 7.05 (GraphPad Software, San Diego, CA, USA). Tumor cells were seeded at a density of 2000 (H23) or 3000 (Calu-1) per well in 96-well plates (83.3924; SARSTEDT, Nümbrecht, Germany) and allowed to attach for 24 h. Subsequently, the cells were treated with a medium containing several inhibitor(s) for 3 d. After treatment, the cells were incubated with a medium containing reagent for MTS assay (G3581, Promega) for 2 h at 37°C. Cell viability was determined by measuring the absorbance at 450 nm using Multiskan GO (N10588, Thermo Fisher Scientific, MA, USA). To evaluate the combination index (CI), the concentration of each inhibitor ranged from 2^{-6} and 2^{2} IC50. The average of the CIs from each concentration (from 2^{-6} to 2^{1} IC50) was used as the final CI.

Western blotting

Cells (35×10^4) were seeded in 35 mm dishes (83.3900, SARSTEDT, Nümbrecht, Germany) with medium and allowed to attach for 24 h. The medium was replaced with fresh medium with or without the inhibitor(s). After 24 h of incubation, the cells were washed with PBS and lysed in RIPA buffer (9806, Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitor (04080-24, Nacalai Tesque, Kyoto, Japan) and PMSF (8553, Cell Signaling Technology, Danvers, MA, USA). Following centrifugation at 14,000 × g for 10 min at 4°C, the supernatant was collected as the total cell lysate and then diluted using the Laemmli Sample Buffer (1610737, BIO-RAD, Hercules, CA, USA) and 2-mercaptoethanol (161–0710, BIO-RAD, Hercules, CA, USA). After heating for 5 min at 95°C, the lysates containing proteins were electrophoresed on 5–20% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (1704156, BIO-RAD, Hercules, CA, USA). All target proteins were immunoblotted with the appropriate primary and secondary antibodies using the Can Get Signal/PVDF Blocking Reagent (NKB101/NYPBR; TOYOBO LIFE SCIENCE, Tokyo, Japan). β-actin was used as an internal control. Chemiluminescent bands were detected, and expression levels were measured using an Amersham Imager 680 (General Electric, CT, USA). The protein expression levels were quantified using ImageQuant TL (Cytiva, Tokyo, Japan). The antibodies and their concentrations are listed in Additional file 2: Supplementary Table 2. The GTP-binding RAS was extracted and detected using an Active RAS Pull-Down and Detection Kit (16117, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. To detect another protein in the same membrane, the attached antibody was removed using the Stripping Solution (193-16375, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Generation and transfection of *mTOR*-targeting SiRNA

siRNA targeting *mTOR* was generated using Silencer Select Validated siRNA (4427038, Thermo Fisher Scientific, MA, USA). siRNAs designed to block *mTOR* expression (4390824, siRNA ID s603/s604, Thermo Fisher Scientific, MA, USA) or Silencer Select Negative Control (4390843, Thermo Fisher Scientific, MA, USA) was transfected with Lipofectamine RNAiMAX Reagent (3778-030, Thermo Fisher Scientific, MA, USA). After incubation of cell lines with siRNA for 72 h, the cells were seeded in 96-well plates (83.3924; SARSTEDT, Nümbrecht, Germany). The MTS assay was performed as described above, and the effect of silencing *mTOR* in *KRAS* G12C cell lines was evaluated.

Data analysis

The reads resulting from the capture of 49 genes were subjected to RNA-seq analysis using the StrandNGS software, version 2.7 (Tomy Digital Biology). The reads were aligned against a human genome reference (hg19) using RefSeq transcript annotations. Low-quality read removal and QC were then performed, and quantification was performed using the DEseq algorithm. The impact of combination therapy was evaluated using the CI calculated using the Chou–Talalay method [29]. Data for CI calculation was referred to in quantified cell viability in the MTS assay or concentration of crystal violet in the Clonogenic assay. A combination index of less than 0.8 was regarded as statistically synergistic. The significance of the frequency was evaluated using the chi-squared or Yates chi-squared test. Protein and mRNA expression levels were compared using the multiple T test or Mann–Whitney U test. Statistical significance was set at $p < 0.05$. Statistical analyses and figure creation were performed using SPSS Version 29.0.1.0 (IBM Corp., Armonk, NY, USA), StatMate V (ATMS Co., Ltd., Tokyo, Japan), and GraphPad Prism Version 7.05 (GraphPad Software, San Diego, CA, USA).

Results

Distribution of KRAS G12C in lung adenocarcinoma and squamous cell carcinoma

We tested *KRAS* mutations using ddPCR in 877 resected adenocarcinoma and 113 squamous cell carcinoma samples. *KRAS* G12C was detected in 2.9% (25/877) and 1.8% (2/113) of adenocarcinoma and squamous cell carcinoma, respectively. Among *KRAS* G12C cases, 85.2% (23/27), including two squamous cell carcinoma cases, were current or ex-smokers.

mRNA expression change induced by KRAS G12C Inhibition

RNA-Seq NGS revealed increasing or decreasing changes in mRNA expression. The results are presented

as volcano plots in Fig. 1A. The expression of four, 17, and 11 genes was significantly increased by AMG 510, MRTX849, and ARS-1620 treatment, respectively. The expression level of *mTOR* was significantly increased in all three cell lines by two of the three inhibitors (H23 treated with MRTX849 and ARS-1620, HOP-62 treated with AMG 510 and MRTX849, and Calu-1 treated with AMG 510 and ARS-1620) (Fig. 1B). Although the expression of *mTOR* in H23 cells after AMG 510 treatment did not significantly increase by NGS, a significant increase in *mTOR* RNA expression after AMG 510 treatment was confirmed by real-time PCR (Fig. 1C). RNA-Seq revealed that the increased expression of *mTOR* was induced by several *KRAS* G12C inhibitors.

Synergism of KRAS G12C inhibitors and mTOR inhibitor

A clonogenic assay revealed synergism between each *KRAS* G12C and *mTOR* inhibitor (RAD001). The colony formation was suppressed in a dose-dependent manner. CIs were less than 0.8 in all three cell lines and three *KRAS* G12C inhibitors (Fig. 2). Synergism or dose-dependent suppression by monotherapy has not yet been confirmed in *KRAS* wild type squamous cell carcinoma cell lines (H520). The MTS assay also showed synergism between *KRAS* G12C and *mTOR* inhibition in adenocarcinoma (H23) and squamous cell carcinoma (Calu-1) cell lines (Fig. 3).

Impact of mTOR on tumor growth in lung adenocarcinoma and squamous cell carcinoma harboring KRAS G12C

Next, we evaluated the effect of *mTOR* suppression on tumor growth. Expression of *mTOR* was suppressed by siRNA and cell growth was compared with that of intact H23 and Calu-1 cell lines. The MTS assay showed suppressed cell growth following *mTOR* inhibition in both cell lines (Fig. 4A). These results suggest that the activation of *mTOR* is involved in the tumor growth of *KRAS* G12C-driving lung cancer.

Alternative pathway activation by mono- or combination treatment targeting KRAS G12C and/or mTOR

Western blotting revealed that KRAS G12C inhibition suppressed ERK and PI3K- protein kinase B (AKT)-mTOR pathways. Expression of activated GTP-bound RAS was induced by a single mTOR inhibitor. The expression of phosphorylated ERK (Thr202/Tyr204) and AKT (Ser473) was also elevated, suggesting the activation of the *RAS-MEK-ERK* and *PI3K-AKT-mTOR* pathways. The

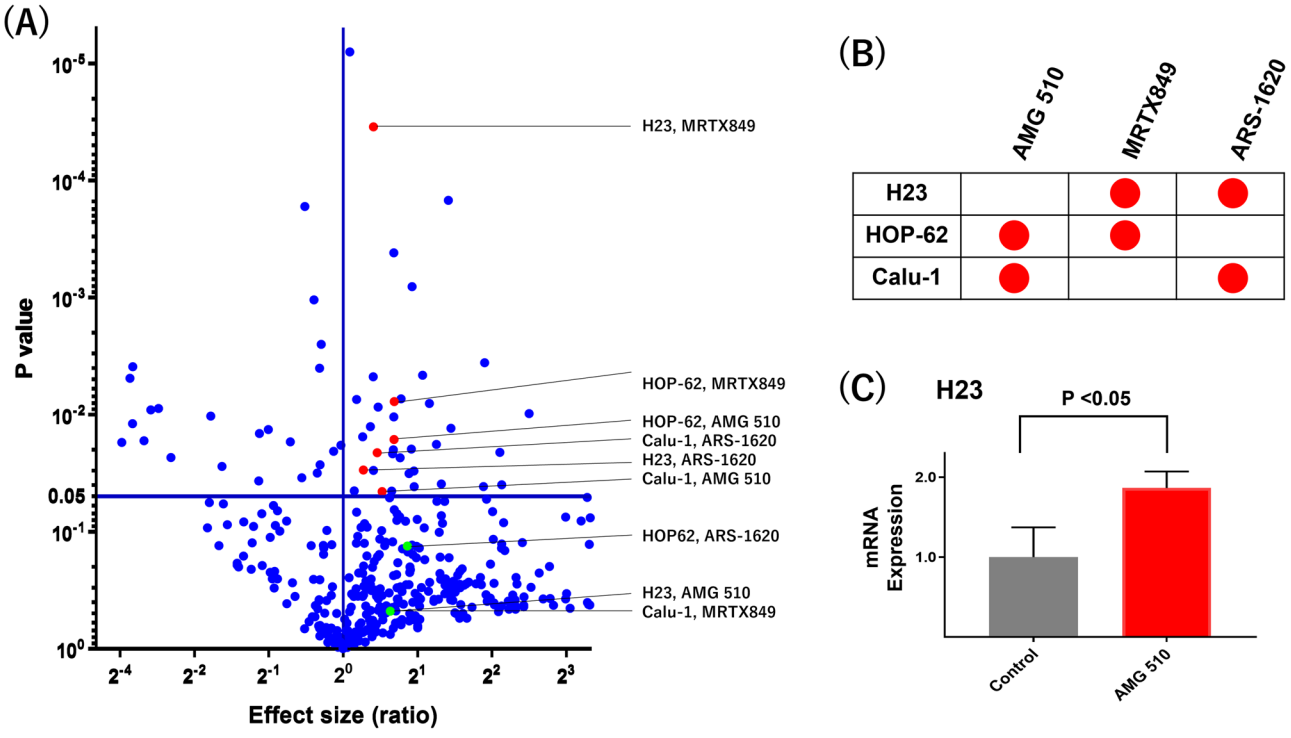

Fig. 1 Expression of mRNA after *KRAS* G12C inhibition in three cell lines. (**A**) Comprehensive RNA-Seq results are shown as volcanic plots. Each dot represents mRNA expression and its statistical significance compared to intact cell lines. Red and green dots indicate *mTOR* expression levels with and without statistical significance, respectively. (**B**) Summary of *mTOR* expression as determined by RNA-Seq. The expression level of *mTOR* is significantly elevated in all three cell lines by two different *KRAS* G12C inhibitors. The red circles denote significantly increased *mTOR* expression levels. (**C**) Comparison of mRNA expression levels in intact and H23 cell lines treated with AMG 510. Real-time PCR analysis confirmed a significantly greater increase in *mTOR* mRNA levels in the H23 cell line compared to the intact cell lines

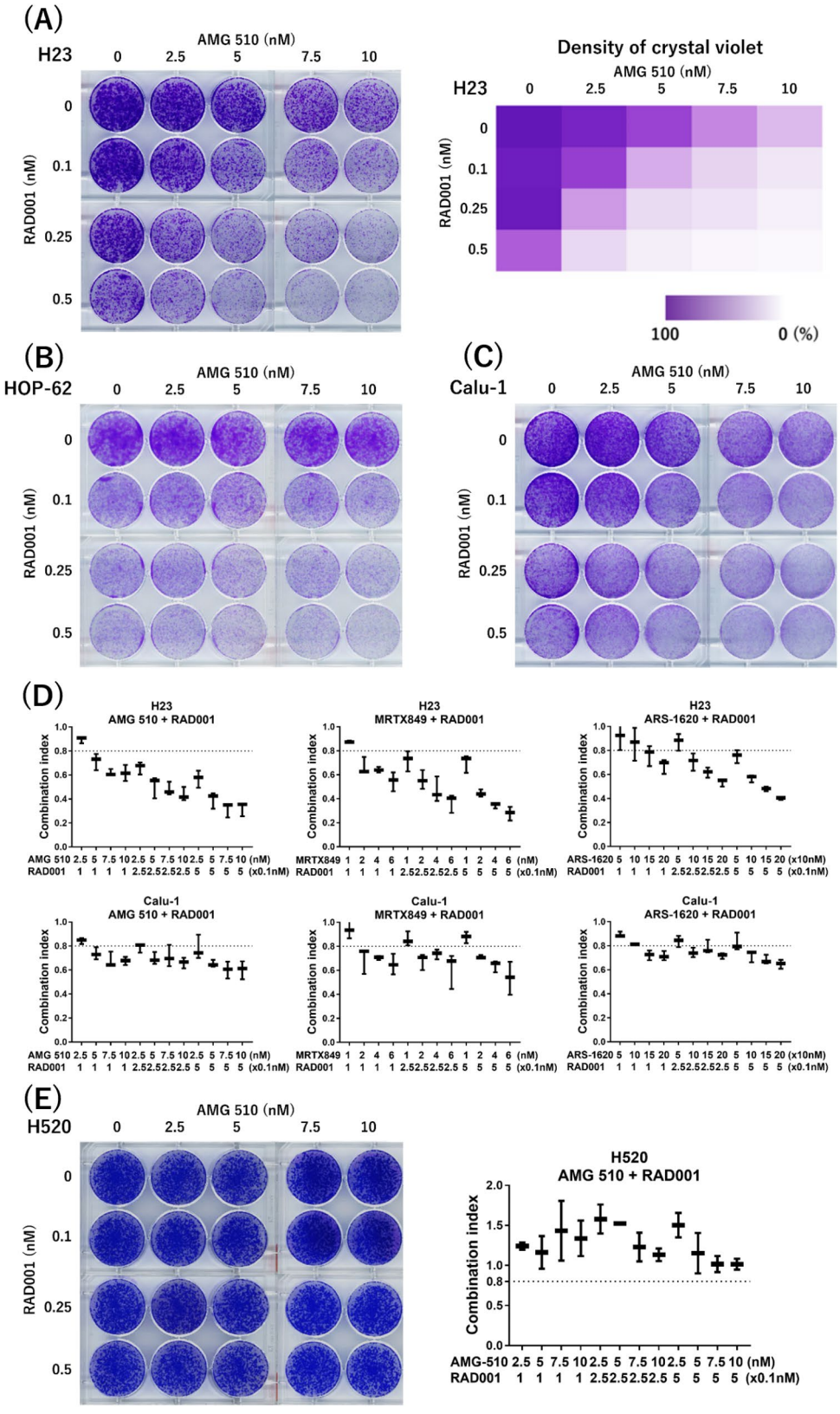

Fig. 2 (See legend on next page.)

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Fig. 2 Clonogenic assay in three cell lines treated using three *KRAS* G12C inhibitors and *mTOR* inhibitor. (**A**) Representative image of the clonogenic assay in H23 cell lines treated with AMG 510 and RAD001. The colony growth is suppressed in a dose-dependent manner by monotherapy and stronger inhibited by combination therapy. The heatmap represents the amount of colony growth by the density of crystal violet. (**B**, **C**) Two representative images of the clonogenic assay in HOP-62 (**B**) and Calu-1 (**C**) cell lines after treatment with AMG 510 and RAD001. In both cell lines, the colony growth is suppressed in a dose-dependent manner and stronger in combination therapy. (**D**) Summary of the clonogenic assay in cell lines treated with three different *KRAS* G12C inhibitors and RAD001. Cell growth is suppressed in a dose-dependent manner. Combination index is below 0.8 (dashed line) in all three cell lines in three different agents. (**E**) Results of the clonogenic assay in H520, which is lung squamous cell carcinoma cell lines without *KRAS* mutation. Neither colony growth inhibition in a dose-dependent manner nor higher suppression by combination treatment is observed. Combination index is above 0.8 (dashed line) in all concentrations, being non-synergistic in H520 cell lines

combination treatment inhibited these alterations and significantly suppressed the expression of GTP-bound RAS and phosphorylated mTOR (Ser2448) (Fig. 4B).

The elevation of phosphorylated ERK (Thr202/Tyr204), AKT (Ser473), and GTP-bound RAS induced by mTOR single inhibition suggested that mTOR activation is crucial for maintaining tumor growth in KRAS G12C-driven lung cancer, and suppression of mTOR was restored by the alternative activation of GTP-bound KRAS, resulting in ERK elevation as a downstream change of the *RAS-MEK-ERK* pathway. Targeting KRAS G12C and mTOR in combination abrogates GTP-RAS, pmTOR, and pERK expression Although pAKT was elevated even with combined inhibition, this may be due to the steady suppression of mTOR in the *PI3K-AKT-mTOR* pathway (see Fig. 5).

Discussion

KRAS is one of the most frequently identified oncodriving genes in lung cancer, particularly in Western countries [30]. In oncogenic *KRAS*-mutant cases, RAS proteins are binary molecular switches that convert active GTP-bound to inactive guanosine diphosphate (GDP)-bound forms. *KRAS* mutations maintain their active form by compromising GTPase and GTPase-activating protein (GAPs) activity, which hydrolyzes GTP to GDP [16, 31, 32].

Several treatment approaches have been suggested for RAS-mutant tumors: inhibition of RAS processing, inhibition in the upstream or downstream of the RAS pathway, or synthetic lethality strategies [3, 33, 34]. As combination therapy, preclinical studies and clinical trials have been conducted [35].

We used three *KRAS* G12C inhibitors, including two clinically approved agents with RAD001 (everolimus), which is clinically available for treating breast cancer, kidney cancer, and neuroendocrine tumors.

Sotorasib (AMG 510) and adagrasib (MRTX849) monotherapies have shown therapeutic efficacy in NSCLC in clinical phase I-II trials [36–38]. ARS-1620 inhibited RAS-GDP and atropisomerically inhibited *KRAS* G12C activity [39].

Combination therapy suppressing GTP-bound *KRAS* and *mTOR* is classified as "co-targeting a critical downstream node" [35]. A previous study suggested *mTOR*, IGF1R, and *KRAS* G12 inhibition by ARS-1620 markedly increased tumor regression in three different *KRAS*-driven lung cancer mouse models compared to *mTOR*, *IGF1R*, and *MEK* inhibition [40]. The combination of the PI3K inhibitor and ARS-1620 showed efficacy in the *KRAS* G12C NSCLC model [28]. Phase I-II trials combining sotorasib and several inhibitors, including *mTOR* inhibitors, are ongoing (NCT05840510 and NCT04185883) [27].

Here, we showed that the activation of *mTOR* was induced by *KRAS* G12C inhibition in three different types of cell lines, and a single inhibition of *mTOR* induced a feedback enhancement in GTP-bound RAS, leading to *RAS-MEK-ERK* pathway activation. Dual inhibition of *KRAS* G12C and *mTOR* abrogated the *RAS-MEK-ERK* and phosphatidylinositol-3-kinase (PI3K)-AKT-mTOR cascades and suppressed tumor growth more effectively. The *PI3K-AKT-mTOR* pathway is activated by RAS in *KRAS*-mutant lung cancers [41, 42] and has been suggested as a promising candidate target for combination therapy.

Genetically targeted therapies have been developed almost exclusively for adenocarcinomas. We confirmed similar alternate changes by *KRAS* G12C or *mTOR* inhibition in different phenotypes of lung cancer cell lines. *KRAS* G12C can also occur in non-adenocarcinoma lung cancers. The incidence of *KRAS* mutations in lung squamous cell carcinoma ranged 0–14% (median: 4.5% from 13 studies) [2]. Targeted therapy has been suggested to be effective in non-adenocarcinoma cases harboring targetable genetic variants [43, 44]. We have previously shown that targeting specific gene expression could be effective in both adenocarcinoma and squamous cell carcinoma, even if the target signal does not stem from oncogenic mutations [45]. Skoulidis et al. divided *KRAS*-mutant lung adenocarcinoma by co-occurring genomic alterations into three subtypes and found that *mTORC1* signaling was inactivated in a subtype characterized by the absence of *TP53* or *STK11*/*LKB1* mutations [46]. H23 included cancer gene mutations in *STK11* and *TP53*, and HOP-62 harbored mutations in TP53 (Additional file 1: Supplementary Table 1). De novo *mTORC1* suppression might not occur in H23 and HOP-62, but no data regarding Calu-1 cell lines exist. We tried to reveal the genetic features, including *TP53*/*STK11*/*LKB1* mutations

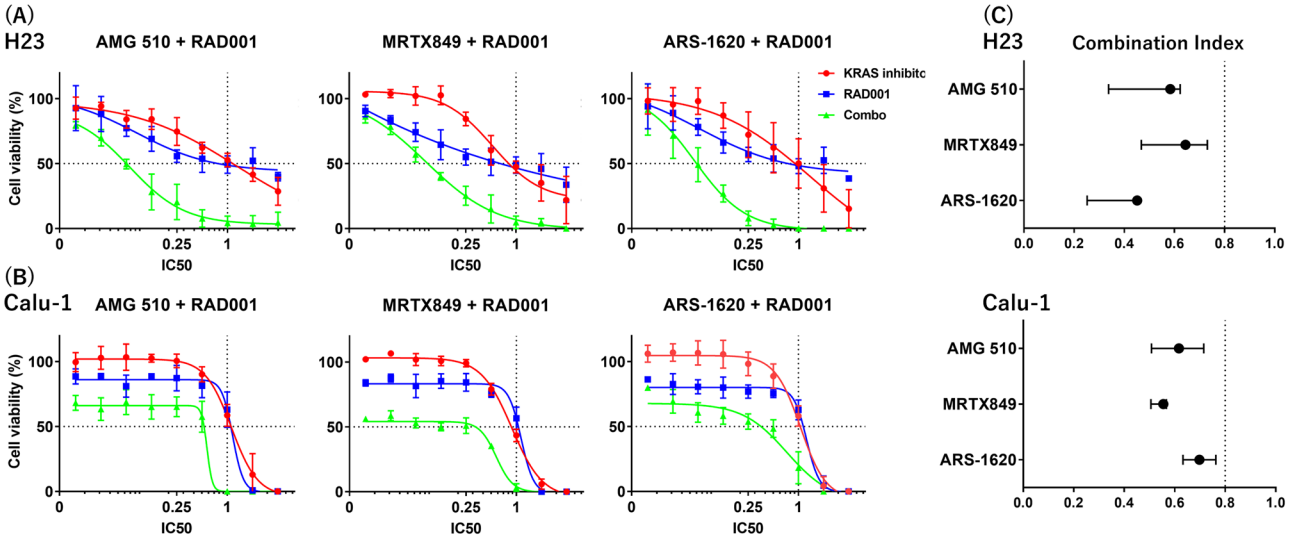

Fig. 3 Results of MTS assay using *KRAS* G12C and *mTOR* inhibitor. (**A**-**B**) Results of MTS assay in H23 and Calu-1 cell lines using three different *KRAS* G12C inhibitors with RAD001. Tumor growth is assessed in a range of 2^{-6} to 2^{2} times of concentration of IC50. Cell viability is lower in combination therapy in both cell lines. The dashed lines indicate the 50% cell viability and drug concentration for IC50. (**C**) Summary of the MTS assay representing the combination index. Combination indexes are lower than 0.8 (dashed line) in both cell lines with three different agents

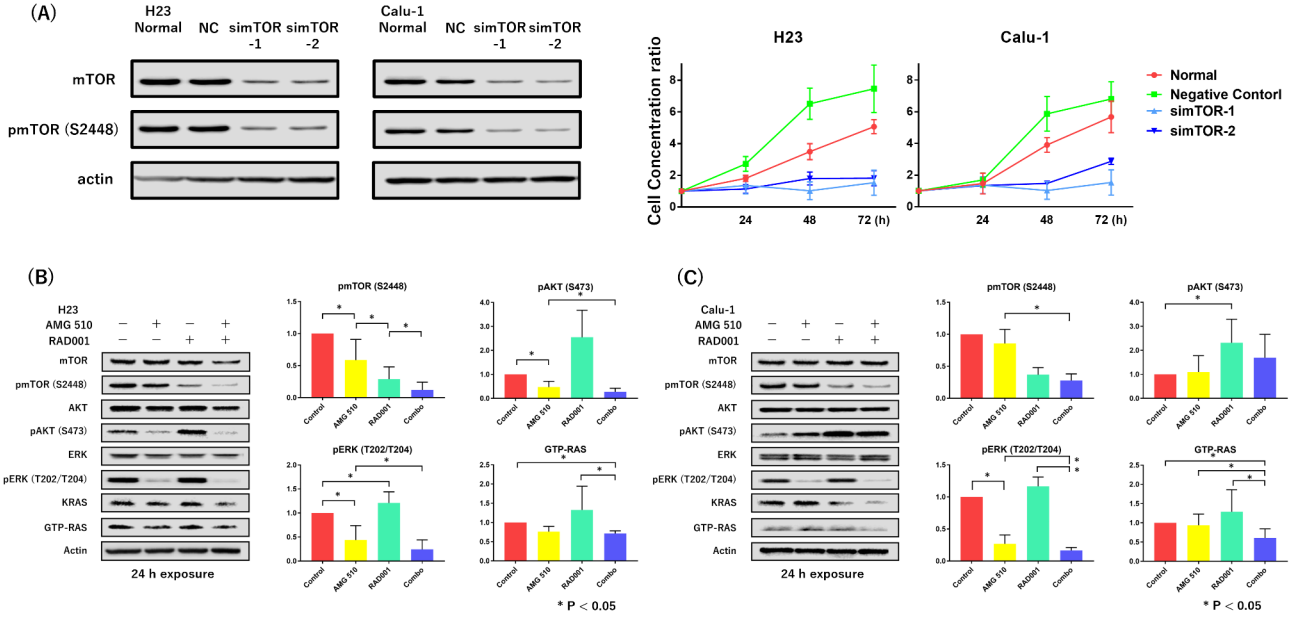

Fig. 4 Impact of *mTOR* expression on cell growth and western blotting results in H23 and Calu-1 cell lines. (**A**) Growth curves for intact or *mTOR*-inhibited H23 or Calu-1 cell lines. Cell growth was assessed using the MTS assay at 24, 48, and 72 h after allowing cells to adhere overnight. Significant suppression of cell growth was observed with *mTOR* inhibition. (**B**) Western blot analysis shows elevated levels of pAKT (Ser473), pERK (Thr202/Tyr204), and GTP-RAS following RAD001 monotherapy. In contrast, combination therapy with AMG 510 and RAD001 leads to reduced levels of pmTOR (Ser2448), pAKT (Ser473), pERK (Thr202/Tyr204), and GTP-RAS

and *mTOR* expression, in non-adenocarcinoma *KRAS*-mutant cases, and compare the genetic features between *KRAS*-mutant adenocarcinoma and squamous cell carcinoma cases using clinical samples. Although the incidence of *KRAS* G12C in squamous cell carcinoma was not significantly different compared with adenocarcinoma (1.8% (2/113) vs. 2.9% (25/877), p-value = 0.721), the number of squamous cell carcinoma cases was too small. Further exploration of a combination strategy targeting *mTOR* is warranted in the *mTORC1*-suppressed subtype and more non-adenocarcinoma cases with *KRAS* G12C mutation. We evaluated the pathway change as a potential mechanism in drug resistance. However, we did not directly confirm cell lines with *mTOR* activation

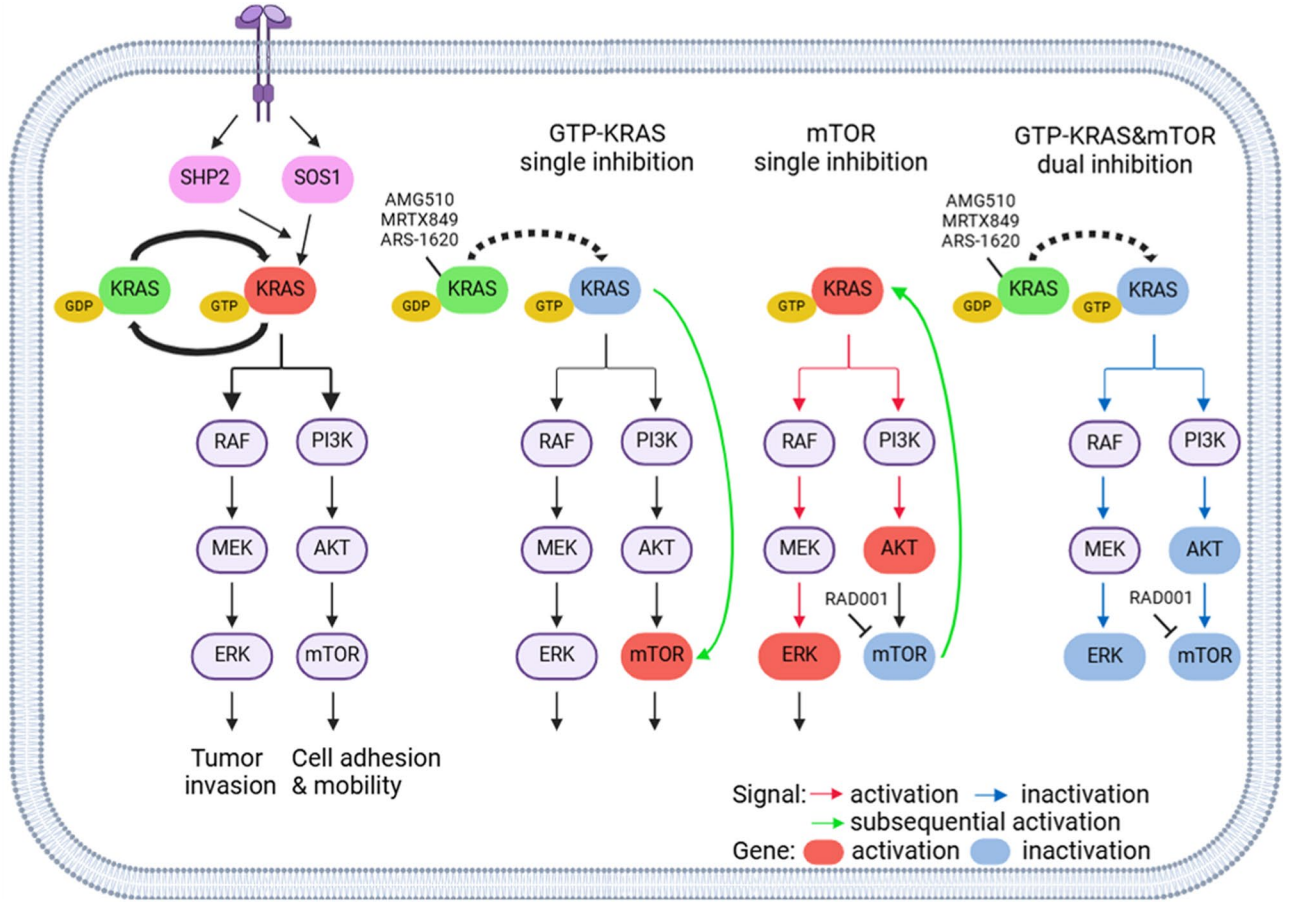

Fig. 5 Illustration summarizing pathway alterations in lung cancer with *KRAS* G12C under monotherapy and combination treatment. Continuous *KRAS* G12C inhibition induces *mTOR* activation, while single-agent *mTOR* inhibition causes feedback activation of GTP-bound *KRAS*, resulting in activation of both the *RAS-MEK-ERK* and *PI3K-AKT-mTOR* pathways. Simultaneous dual inhibition of *KRAS* G12C and *mTOR* abrogates both cascades, leading to tumor suppression

showed the drug resistance. Further research which validates *mTOR* activation induced by *KRAS* G12C inhibition can be a resistant mechanism in several lung cancer phenotypes is warranted.

Conclusions

Activation of *mTOR* has been suggested as an alternative change induced by *KRAS* G12C inhibition, and *mTOR* solo-inhibition induces GTP-bound *KRAS* activation. A combination strategy inhibiting GTP-bound *KRAS* G12C and *mTOR* is promising for primary lung cancers harboring a *KRAS* G12C mutation. This combination strategy might be effective for lung cancers harboring *KRAS* G12C mutation regardless of difference in histology, treatment agent, coexisting oncological mutations, or EMT phenotype.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9: Supplementary Table 1. Mutational status of ***KRAS***, ***TP53***, ***STK11***, ***KEAP***, and ***mTOR*** in cell lines

Supplementary Material 10: Supplementary Table 2. List of treatment agents used, and primary and secondary antibodies

Supplementary Material 11: Supplementary Table 3. List of genes evaluated in RNA-Seq

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Author contributions
Conceptualization; MI, YM. Data collection and curation; MI, KK, YT, Interpretation; MI, YM, SH, NM, KS, JS, Formal analysis; MI, SH, NM, MT, FI. Investigation; MI, SH. Methodology; MI, YM, SH. Project administration; MI, YM. Supervision; YT, MO, Visualization; MI, Roles/Writing– original draft; MI, Writing–review & editing and approving the final manuscript; all authors.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All the procedures involving human participants were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent for collecting samples for use in study and publication of results was obtained from all patients before surgery. This study was approved by the Institutional Review Board of Hiroshima University (E2020-2081-01).

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
[2]Division of Thoracic Surgery, Department of Surgery, Kindai University Faculty of Medicine, Osaka-Sayama, Japan
[3]Analysis Center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan
[4]Department of Pathology, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
[5]Present address: Department of Thoracic Surgery, Osaka Metropolitan University Graduate School of Medicine, Osaka, Japan

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