

ARL4C is associated with initiation and progression of lung adenocarcinoma and represents a therapeutic target

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Abstract

Lung adenocarcinoma is the most common histological type of lung cancer and is classified into adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA) and invasive adenocarcinoma (IA). Atypical adenomatous hyperplasia (AAH) lesions are possible precursors to adenocarcinoma. However, the mechanism underlying the stepwise continuum of lung adenocarcinoma is unclear. In this study, the involvement of ADP-ribosylation factor (ARF)-like (ARL) 4C (ARL4C), a member of the small GTP-binding protein family, in the progression of lung adenocarcinoma and the possibility of ARL4C as a molecular target for lung cancer therapy were explored. ARL4C was frequently expressed in AAH and ARL4C expression in immortalized human small airway epithelial cells promoted cell proliferation and suppressed cell death. In addition, **ARL4C was expressed with increased frequency in AIS, MIA and IA in a stage-dependent manner, and the expression was correlated with histologic grade, fluorine-18 fluorodeoxyglucose uptake and poor prognosis.** An anti-sense oligonucleotide (ASO) against ARL4C (ARL4C ASO-1316) inhibited RAS-related C3 botulinum toxin substrate activity and nuclear import of Yes-associated protein and transcriptional coactivator with PDZ-binding motif, and suppressed in vitro proliferation and migration of lung cancer cells with KRAS or epidermal growth factor receptor (EGFR) mutations. In addition, transbronchial administration of ARL4C ASO-1316 suppressed orthotopic tumor formation induced by these cancer cells. Thus, ARL4C is involved in the initiation of the premalignant stage and is associated with the stepwise continuum of lung adenocarcinoma. ARL4C ASO-1316 would be useful for lung adenocarcinoma patients expressing ARL4C regardless of the KRAS or EGFR mutation.

KEYWORDS

anti-sense oligonucleotides, ARL4C, atypical adenomatous hyperplasia, lung adenocarcinoma, nucleic acid medicine

1 | INTRODUCTION

Lung cancer ranks among the most lethal malignancies worldwide.¹ Approximately 85% of lung cancers are non-small cell lung cancers, which included adeno, squamous and large-cell undifferentiated carcinomas.² Among them, adenocarcinoma is the most common histologic subtype and progress in molecular biology and oncology has led to the discovery of epidermal growth factor receptor (EGFR) mutations and prediction of their response to EGFR tyrosine kinase inhibitors (TKI). Based on multidisciplinary approaches for diagnosing lung adenocarcinoma, a new classification was proposed.³ Namely, adenocarcinoma is classified into preinvasive lesions, such as atypical adenomatous hyperplasia (AAH) and adenocarcinoma in situ (AIS), and invasive lesions, such as minimally invasive adenocarcinoma (MIA) and invasive adenocarcinoma (IA). AAH are the possible precursor lesions that develop to adenocarcinoma.⁴ Indeed, some of the well-differentiated types are believed to develop stepwise from AAH to IA through AIS and MIA. However, some adenocarcinomas may be developed through the de novo pathway independently of AAH.^{5,6} Thus, the mechanism underlying the stepwise continuum of lung adenocarcinoma is unclear at the molecular level and new molecular events must exist to explain the progression from preinvasive to invasive states.

ADP-ribosylation factor (ARF)-like (ARL) 4C (ARL4C) is a member of the ARF small GTP-binding protein subfamily.⁷⁻⁹ Reportedly, ARL4C stimulates cholesterol efflux,¹⁰ is involved in the transport of transferrin from early endosomes to recycling endosomes by interacting with α -tubulin,¹¹ is required for osteogenesis of adipose-derived stem cells,¹² and functions with filamin-A to promote filopodia formation and cell migration.¹³ ARL4C expression is induced by the activation of Wnt/ β -catenin or RAS/MAPK signaling^{14,15} and DNA hypomethylation of the ARL4C gene¹⁶ in a cell-context-dependent manner. ARL4C activates RAS-related C3 botulinum toxin substrate (RAC) and inhibits RAS homolog family member (RHO), followed by the intracellular nuclear translocation of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ), resulting in the stimulation of cell proliferation and migration.¹⁴

Consistent with ARL4C functions, ARL4C expression is associated with progression of tumorigenesis, including colorectal,^{15,17} tongue,¹⁶ liver,¹⁷ gastric,¹⁸ renal cell¹⁹ and ovarian²⁰ cancers as well as glioblastoma.²¹ Therefore, ARL4C may represent a molecular target for the treatment of these cancers. The direct injection of ARL4C siRNA into xenograft tumors induced by HCT116 colorectal cancer cells inhibited tumor growth in immunodeficient mice.¹⁵ In addition, subcutaneous injection of an anti-sense oligonucleotide (ASO) against ARL4C (ARL4C ASO-1316) suppressed liver tumor formation induced by HLE hepatocellular carcinoma cells.¹⁷

In lung cancer, ARL4C is also frequently overexpressed in the tumor lesions of both adenocarcinoma and squamous cell carcinoma but not in non-tumor regions.^{15,16} Clinicopathological analysis has shown that ARL4C expression in adenocarcinoma is not associated with the T and N grade, indicating that ARL4C is involved in the initiation of lung cancer. However, the relationship between ARL4C expression and lung tumor progression and the in vivo

pharmaceutical effects of ARL4C ASO on lung cancer have not been studied. Therefore, in the present study, the role of ARL4C in pre-malignant lesions using human small airway epithelial cells (SAEC) and the effects of administration by inhalation of an ARL4C ASO-1316 on lung tumor formation were investigated.

2 | MATERIALS AND METHODS

2.1 | Patients and cancer tissues

ARL4C expression was immunohistochemically examined in 161 patients who underwent surgical resection at Osaka University Hospital between July 2011 and March 2018. The specimens were diagnosed as 27 AAH, 30 AIS, 22 MIA and 83 IA, according to standard lung adenocarcinoma guidelines.³ In our previous study, immunostaining results showed that lung adenocarcinoma patients were positive for ARL4C¹⁵ and 33 of those patients were incorporated in the present study. The AAH cases included patients with lung adenocarcinoma.

Tumors were staged according to the Union for International Cancer Control TNM staging system. Histological specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Paraffin-embedded samples were stored in a dark room at room temperature. The tissues were sectioned into 4- μ m-thick slices. The protocol for this study was approved by the ethical review board of the Graduate School of Medicine, Osaka University, Japan (No. 13 455, No. 18518) according to the Declaration of Helsinki and the study was performed in accordance with the Committee guidelines and regulations.

2.2 | Materials

Small airway epithelial cells were purchased from Lonza. Six human lung adenocarcinoma cell lines, A549, H358, H441, HCC827, H1650 and H1975 cells, were purchased from the American Type Culture Collection (ATCC). A549 (G12S), H358 (G12C) and H441 (G12V) harbor the KRAS mutation.²² HCC827 (E746-A750 deletion), H1650 (E746-A750 deletion) and H1975 (L858R and T790M) harbor the EGFR mutation.²³ All human cell lines were authenticated prior to obtaining them from ATCC or Lonza. Initial cell lines were frozen in liquid nitrogen and early passages of cells (<1 month in culture) were used in all experiments. All cultured cells were negative for the mycoplasma testing.

Small airway epithelial cells stably expressing a dominant-negative p53, CDK4 and hTERT (kindly provided by Dr RA Weinberg; SAEC-Triple) were generated using retroviral vectors as previously described.²⁴ SAEC-Triple stably expressing ARL4C-WT, ARL4C^{G2A}, KRAS^{G12V}, or ARL4C-WT and KRAS^{G12V} were generated using a lentivirus as previously described.¹⁷ A549 and H1975 cells stably expressing GFP or ARL4C-GFP and stably expressing luciferase (Luc) were generated using a lentivirus as previously described.¹⁷ Anti-ARL4C antibody was purchased from Proteintech. Anti-HSP-90 and anti-RAC antibodies were purchased from BD Biosciences. Anti-YAP/TAZ

antibody was from Cell Signaling Technology. Anti-Ki-67 antibody was obtained from BioLegend. VivoGlo luciferin was from Promega.

2.3 | Transpleural orthotopic model of human lung cancer and in vivo anti-sense oligonucleotide treatment

Eight-week-old male BALB/cAJcl-nu/nu mice (CLEA Japan) were anesthetized with an intraperitoneal injection of medetomidine (0.75 mg/kg), midazolam (4 mg/kg) and butorphanol (5 mg/kg). The orthotopic transplantation model was generated by implanting human lung cancer cells into the left lung of mice as previously described.²⁵⁻²⁷ Mice were placed in the right lateral decubitus position. A small skin incision to the left chest wall was made approximately 5 mm to the tail side of the scapula. Approximately 2×10^6 A549/Luc cells or 5×10^4 H1975/Luc cells suspended in 10 μ L of PBS and 10 μ L of Matrigel (BD Biosciences) were directly injected through the intercostal space into the left lung to a depth of 2 mm using a 29 G needle attached to a 0.5 mL insulin syringe (BD Biosciences). The skin incision was closed using a 5-0 polypropylene suture. Mice were then allowed to rest on a heating carpet until fully recovered.

One week after the tumor was implanted, the colonization of the tumor was confirmed using the IVIS imaging system (Xenogen). For the in vivo imaging, 100 μ L of VivoGlo luciferin (30 mg/mL) was intravenously administered via tail vein and then bioluminescence imaging was recorded 5 min after administration of luciferin. Next, the mice were divided into two groups, control ASO inhalation and ARL4C ASO-1316 inhalation groups, to ensure the same level of IVIS signal intensity. ASO (100 μ g/body in A549, 200 μ g/body in H1975) in 25 μ L of saline was administered via intratracheal routes on days 7, 11 and 15 in A549 (or days 7, 9 and 11 in H1975) after the tumor was implanted. Transbronchial administration using a 22 G intravenous catheter was performed as previously described.²⁸ On day 21 (or day 14 in H1975) after tumor implantation, tumor sizes were measured using the IVIS imaging system. Next, the mice were killed and histological analysis was performed. All protocols used for animal experiments in this study were approved by the Animal Research Committee of Osaka University, Japan (No. 26-032-048).

Detailed methods for immunohistochemical studies, clinical data analyses using open sources, cell culture, knockdown of protein expression by ASO and RAC activation assay are available in the supplementary material (Doc S1).

3 | RESULTS

3.1 | ARL4C is expressed in premalignant lesions and is associated with poor prognosis of lung adenocarcinoma

A total of 161 patients who underwent pulmonary resection for lung adenocarcinoma at Osaka University Hospital between 2010 and 2018

were histologically diagnosed as AAH, AIS, MIA and IA (Figure 1A) according to the IASLC/ATS/ERS classification updated in 2011.³ Twenty-six patients had AAH (16.1%), which was consistent with the rate previously reported²⁹ (Table S1). Twenty-seven AAH lesions from those patients were used to evaluate ARL4C expression. Specifically, 6 patients had AAH adjacent to lung adenocarcinoma and 20 patients had either a single AAH lesion or AAH not adjacent to lung adenocarcinoma. Consistent with our previous report,¹⁵ cytoplasmic staining for ARL4C expression was observed in tumor specimens. Results were considered high ARL4C expression when the total area of the tumor stained with anti-ARL4C antibody was >20%. ARL4C was highly expressed in 18 of 27 (66.7%) AAH cases. Patient characteristics and AAH status were not statistically significantly different between the high ARL4C expression and low ARL4C expression groups. Furthermore, in the high ARL4C expression group, 14 of 18 (77.8%) lesions were not adjacent to lung adenocarcinomas, indicating that ARL4C expressed in the premalignant stage is independent of the continuity of adenocarcinoma lesions.

Among 135 lung adenocarcinoma patients without AAH lesions, 30, 22 and 83 cases were diagnosed as AIS, MIA and IA, respectively, and ARL4C was highly expressed in 7 (23.3%), 8 (36.3%) and 42 (50.6%) cases, respectively, based on pathological classification (Table 1). Notably, higher ARL4C expression was detected more frequently in AAH than in adenocarcinoma patients (Figure 1B). The combined TCGA and GTEx datasets retrieved from the UCSC Xena browser (<http://xena.ucsc.edu>), showed that the ARL4C mRNA levels in lung adenocarcinoma are significantly higher than in normal lungs (Figure 1C).

In lung adenocarcinoma patients, significant difference was not observed between low and high ARL4C expression based on age, gender, smoking history, presence of chronic obstructive pulmonary disease (COPD), interstitial pneumonia (IP), tumor markers, tumor location, tumor size, presence of micropapillary pattern, presence of EGFR mutation and pathological stage (Table 1). Higher ARL4C expression was significantly associated with histologic grade and the maximum standardized uptake value (SUVmax). This value is the main parameter used in clinical practice to estimate fluorine-18 fluorodeoxyglucose uptake, which can be used to evaluate metabolic activity and malignancy of tumors.³⁰ Relapse-free survival was significantly decreased in patients with tumors showing high ARL4C expression compared with tumors showing low ARL4C expression ($P = 0.0128$; Figure 1D). Consistently, the Kaplan-Meier plotter (KM plotter; www.kmplot.com)-generated data from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) database showed that both progression-free survival ($P = 0.0004$) and overall survival ($P = 0.0001$) were decreased in patients with a higher ARL4C mRNA expression level compared with patients with a lower ARL4C mRNA expression level (Figure 1E).

Univariate analysis showed that ARL4C expression, carcinoembryonic antigen (CEA), SUVmax and tumor size were associated with poorer relapse-free survival (Table 2). Furthermore, multivariate analysis showed that higher ARL4C expression and tumor size >2 cm were independent prognostic factors ($P = 0.0341$ and 0.0012 , respectively; Table 2). Taken together, these results indicate that the expression level of ARL4C in adenocarcinomas correlates with the aggressiveness of lung adenocarcinoma.

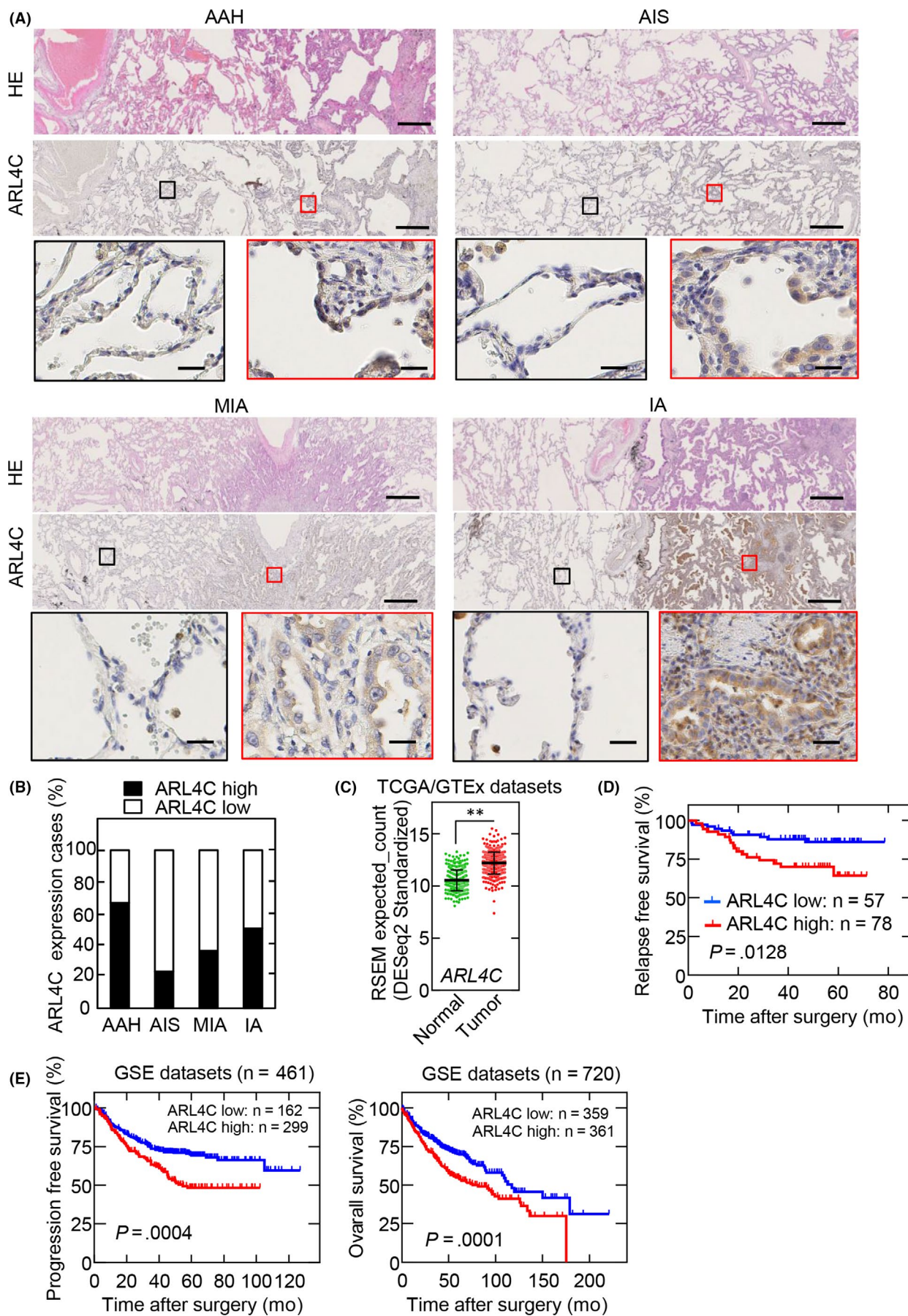


FIGURE 1 ARL4C is expressed in AAH and lung adenocarcinoma. A, Lung adenocarcinoma specimens were stained with H&E, or ARL4C antibody and hematoxylin. Upper panels show representative images of H&E staining and middle panels show representative images of ARL4C staining in consecutive specimens of AAH (n = 27), AIS (n = 30), MIA (n = 22) and IA (n = 83). Black squares show enlarged images of the normal lung tissue, and red squares show enlarged images of the lesion. B, Percentages of ARL4C-positive cases in the AAH, AIS, MIA and IA lesions are shown. High ARL4C expression and low ARL4C expression groups were classified based on the area of positive staining for ARL4C as follows: low expression, <20%; high expression, ≥ 20%. C, The ARL4C mRNA levels in adenocarcinoma and normal tissues of the lung were compared using the UCSC Xena platform (<http://xena.ucsc.edu>) to search the TCGA and GTEx datasets. D, The relationship between relapse-free survival and ARL4C expression in lung adenocarcinoma patients was analyzed. The log-rank test was used for statistical analysis. E, Clinical outcome data for lung adenocarcinoma were retrieved from the Kaplan-Meier plotter (www.kmplot.com). High ARL4C expression and low ARL4C expression groups were classified based on the median value of ARL4C expression in lung adenocarcinoma patients in the dataset. The data were analyzed using Kaplan-Meier survival curves and the log-rank test was used for statistical analysis. Scale bars, 0.5 mm in upper and middle panels (A); 50 μm in lower panels (A). **P < 0.01. AAH, atypical adenomatous hyperplasia; AIS, adenocarcinoma in situ; IA, invasive adenocarcinoma; MIA, minimally invasive adenocarcinoma

TABLE 1 Clinicopathological features of low and high ARL4C expression in lung adenocarcinoma

Factors	ARL4C-low n = 78 (57.8%)	ARL4C-high n = 57 (42.2%)	P-value
Age (mean, range)	65.4 (32-84)	66.9 (40-85)	0.48
Gender (M/F)	34/44	32/25	0.17
Smoking history			
BI	456.1	409.5	0.75
COPD (-/+)	65/13	47/10	1.00
IP (-/+)	75/3	52/5	0.28
Tumor marker			
CEA (≤3.1/>3.1)	58/20	34/23	0.09
CYFRA (≤3.5/>3.5)	72/4	52/4	0.72
Tumor location			
Right/Left	47/31	35/22	1.00
UL/ML/LL	34/43/1	24/28/5	0.11
PET			
SUVmax (mean, range) (n = 108)	2.1 (0-10.8)	3.4 (0-11.2)	0.01
Tumor size (cm) (<2.0/≥2.0)	37/41	20/37	0.16
Pathologic stage (I/II,III,IV)	69/9	43/14	0.06
Histology (AIS/MIA/IA)	23/14/41	7/8/42	0.02
Micropapillary (-/+)	72/6	53/4	1.00
EGFR mutation (-/+)	7/8	18/5	0.08

Abbreviations: AIS, adenocarcinoma in situ; BI, Brinkman Index; CEA, carcinoembryonic antigen; COPD, chronic obstructive pulmonary disease; EGFR, epidermal growth factor receptor; F, female; IA, invasive adenocarcinoma; IP, interstitial pneumonia; LL, lower lobe; M, male; MIA, minimally invasive adenocarcinoma; ML, middle lobe; UL, upper lobe.

3.2 | ARL4C expression in small airway epithelial cells promotes cell proliferation and survival

To examine the role of ARL4C expression in premalignant lesions of the lung, immobilized human SAEC were used. SAEC expressing hTERT, CDK4 and a dominant-negative form of

TABLE 2 Univariate analysis and multivariate analysis of relapse-free survival using Cox's proportional hazard model

Parameters	Hazard ratio	95% CI	P-value
Univariate analysis			
ARL4C high vs low expression	2.60	1.21-5.91	0.014
Age (≥70 vs <70)	0.70	0.31-1.51	0.37
BI (<1000 vs ≥1000)	1.12	0.38-2.75	0.81
CEA (≥3.0 vs <3.1)	3.13	1.47-6.84	0.0033
SUVmax (≥2.5 vs <2.5)	11.0	4.09-38.2	0.0001
Tumor size (≥2.0 vs <2.0)(cm)	4.93	1.89-16.8	0.0005
Multivariate analysis			
ARL4C high vs low expression	2.30	1.06-5.22	0.0341
Tumor size (≥2.0 vs <2.0) (cm)	4.52	1.73-15.5	0.0012

Abbreviations: BI, Brinkman index; CEA, carcinoembryonic antigen; CI, confidence interval.

p53 (SAEC-Triple) were immortalized but not transformed.²⁴ When SAEC-Triple were grown in 3D Matrigel, expression of wild-type (WT) ARL4C (ARL4C-WT) enlarged the sphere areas (Figure 2A). ARL4C harboring a mutation of Gly² to Ala (ARL4C^{G2A}) was an inactive form^{10,31} and the mutant did not affect sphere formation (Figure 2A). Because the function of ARL4C in the precancerous AAH lesion was considered to be involved in the pathological condition of its monoclonal growth, a single colony formation assay was conducted as an in vitro experiment. Similarly, the number of colonies formed by cells plated at low density was higher in ARL4C-WT-expressing SAEC-Triple than in control SAEC-Triple (Figure 2B). Consistent with our previous results using IEC6 cells,¹⁴ ARL4C-WT, but not ARL4C^{G2A}, induced nuclear import of YAP/TAZ and activated RAC (Figure 2B,C).

KRAS^{G12V} expression enhanced the colony formation ability of SAEC-Triple and was further stimulated with the simultaneous expression of ARL4C-WT (Figure 2D). Depletion of growth factors induced cell death based on PI staining in SAEC-Triple, and ARL4C-WT or KRAS^{G12V} prevented cell death (Figure 2D). The simultaneous expression of ARL4C-WT and KRAS^{G12V} further suppressed cell death (Figure 2E). However, SAEC-Triple expressing both KRAS^{G12V} and ARL4C-WT or KRAS^{G12V} or ARL4C-WT did not form xenograft

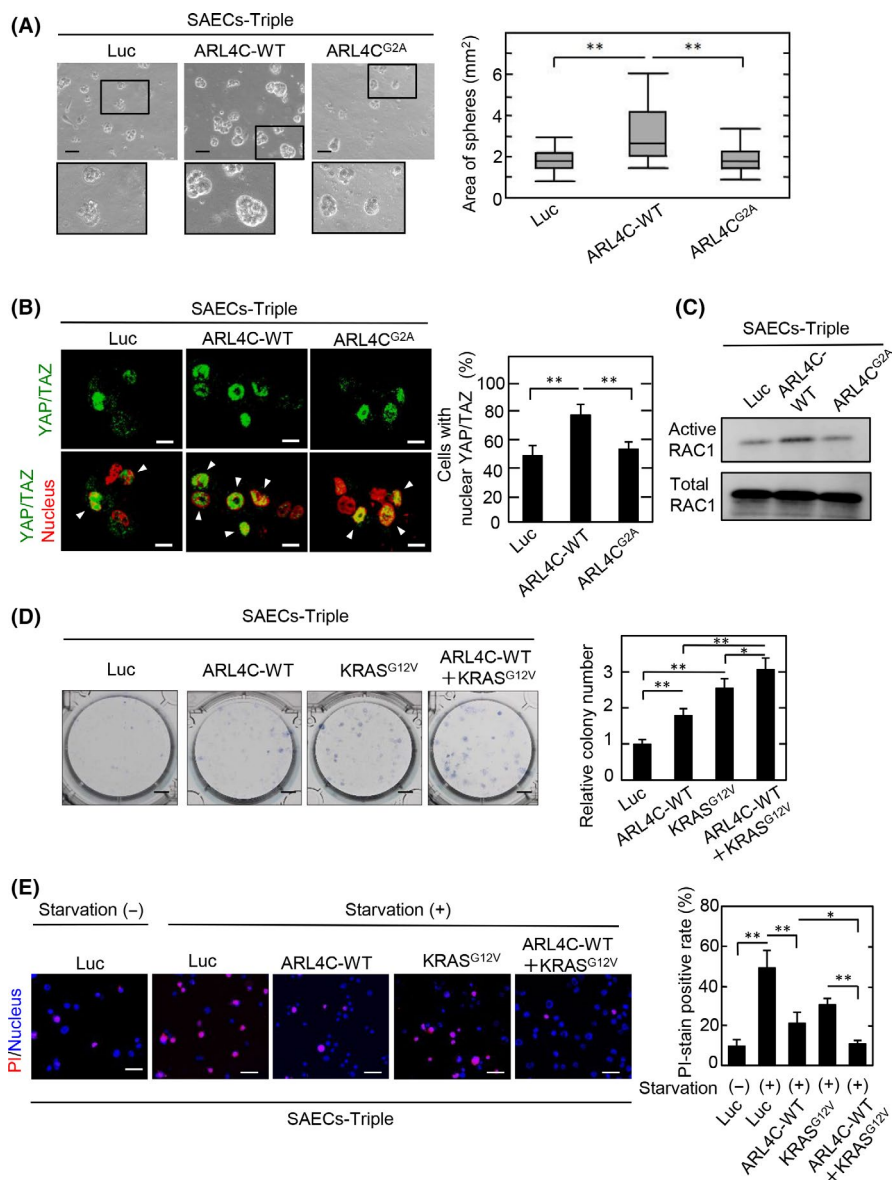


FIGURE 2 ARL4C expression promotes cell proliferation and survival in immortalized human SAEC. A, SAEC-Triple stably expressing Luc, ARL4C-WT or ARL4C^{G2A} were cultured for 6 days in 3D Matrigel. The cells were photographed using phase-contrast microscopy. The area of spheres was calculated ($n = 40$). B, SAEC-Triple stably expressing Luc, ARL4C-WT or ARL4C^{G2A} were cultured for 24 h in 3D Matrigel, and cells were stained with anti-YAP/TAZ antibody and Hoechst 33342. White arrowheads indicate YAP/TAZ in the nucleus. Cells with nuclear YAP/TAZ were expressed as the percentage of nuclear YAP/TAZ-positive cells of Hoechst 33342-stained cells per field ($n = 40$). C, After growth factor was deprived for 24 h, SAEC-Triple stably expressing Luc, ARL4C-WT and ARL4C^{G2A} were conducted to the RAC assay. The total lysate (total RAC1) and precipitates (active RAC1) were probed with anti-RAC1 antibody. D, SAEC-Triple stably expressing Luc, ARL4C-WT, KRAS^{G12V}, or ARL4C-WT and KRAS^{G12V} were cultured in six-well plates for the single colony formation assay. Relative colony numbers are expressed as fold changes compared with SAEC-Triple expressing Luc. E, PI staining of SAEC-Triple stably expressing Luc, ARL4C-WT, KRAS^{G12V}, or ARL4C-WT and KRAS^{G12V} were cultured for 72 h in medium without all growth factors included in the supplement kit. Then, the cells were incubated with PI and Hoechst 33342. PI-positive cells are expressed as the percentage of positively stained cells compared with total Hoechst 33342 stained cells per field ($n = 40$ –60). Results are shown as means $\pm$ SD. Scale bars, 100 μ m in (A); 5 mm in (B); 50 μ m in (C). * $P < 0.05$; ** $P < 0.01$. Luc, luciferase; PI, propidium iodide; SAEC, small airway epithelial cells; TAZ, transcriptional coactivator with PDZ-binding motif; WT, wild type; YAP, Yes-associated protein

tumors in vivo (data not shown). Taken together, these results indicate that ARL4C expression is sufficient for the stimulation of proliferation and prevention of cell death of normal bronchial epithelial cells but insufficient for the cells to be fully transformed even in the presence of the KRAS mutation.

3.3 | ARL4C ASO-1316 inhibits proliferation and migration of lung cancer cells in vitro

ARL4C represents a molecular target for the treatment of lung, colon and liver cancers, and ARL4C ASO-1316 is effective in primary and

metastatic liver tumors expressing ARL4C.^{15,17} Whether ARL4C ASO-1316 affects proliferation and migration of lung cancer cells harboring KRAS or EGFR mutations was examined. Among various lung adenocarcinoma cell lines, A549 cells harboring KRAS^{G12S}, HCC827 cells with EGFR^{ΔE746-A750} and H1975 cells with EGFR^{L858R+T790M} showed higher ARL4C expression than H358, H441 and H1650 cells (Figure 3A).

ARL4C ASO-1316 reduced endogenous ARL4C expression in A549 and H1975 cells expressing GFP (A549/GFP and H1975/GFP cells) but did not affect exogenously expressed ARL4C-GFP, which is designed to be ARL4C ASO-1316-resistant (Figure 3B). Consistent with the knockdown effects by ARL4C siRNA,¹⁵ ARL4C ASO-1316 inhibited the nuclear import of YAP/TAZ and decreased RAC activity in A549 and H1975 cells (Figure S1).

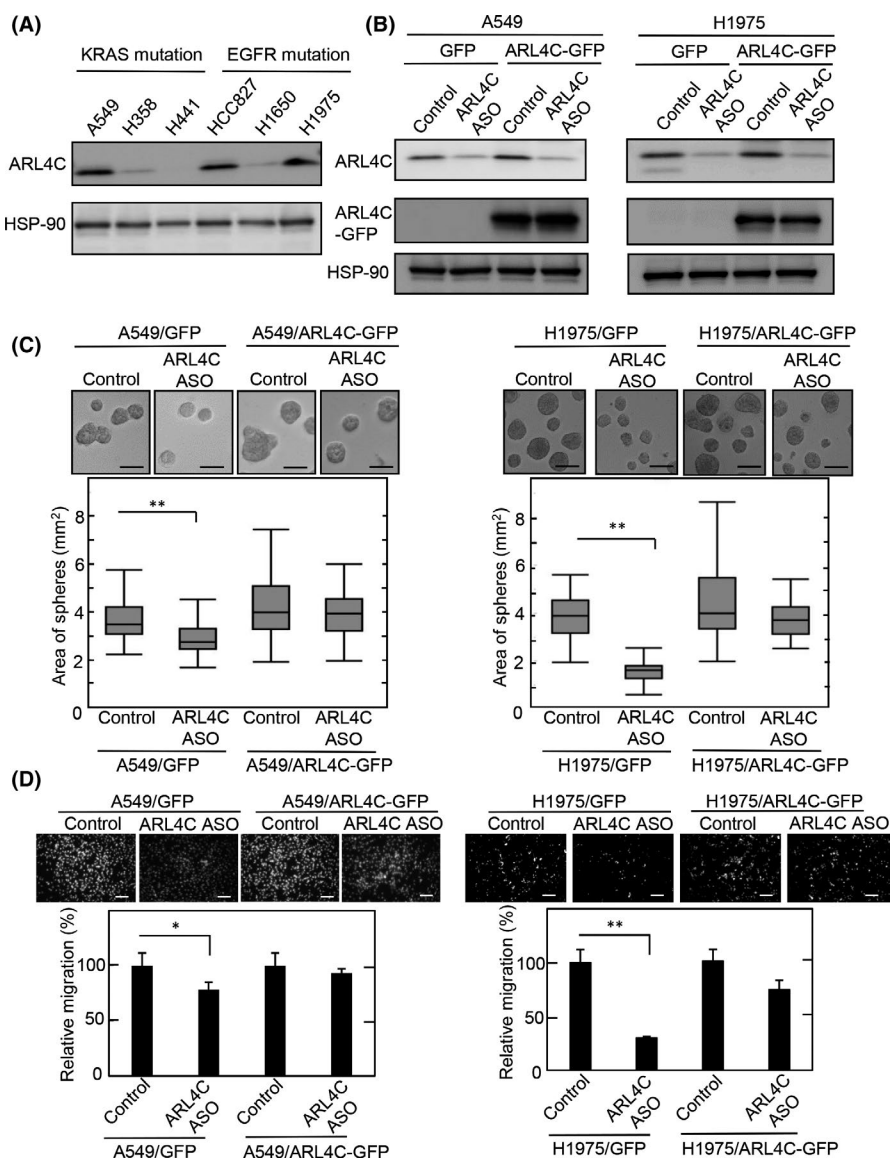
ARL4C ASO-1316 reduced sphere areas of A549/GFP and H1975/GFP cells but not of A549/ARL4C-GFP and H1975/ARL4C-GFP cells (Figure 3C). ARL4C ASO-1316 also decreased the ARL4C expression and sphere formation in HCC827 cells (Figure S2) but did

not affect the sphere formation of H441 cells, which little expressed ARL4C (Figure S3). Similarly, ARL4C ASO-1316 decreased the migration ability of A549/GFP and H1975/GFP cells, but not of A549/ARL4C-GFP and H1975/ARL4C-GFP cells (Figure 3D). Thus, ARL4C ASO-1316 was confirmed to inhibit the nuclear import of YAP/TAZ and RAC activity and to suppress the proliferation and migration of both A549 and H1975 cells in vitro by decreasing endogenous ARL4C expression.

3.4 | ARL4C ASO-1316 inhibits tumor formation induced by lung cancer cells

To overcome inefficient delivery of nucleic acids to target cells, ARL4C ASO-1316 was intrabronchially administered to mice in which A549/Luc cells were orthotopically transplanted into the lung beginning on day 7. The effects of treatment on tumor formation were analyzed on day 21 using bioluminescence measurements (Figure 4A).

FIGURE 3 ARL4C ASO-1316 reduces ARL4C expression and inhibits lung cancer cell proliferation and migration in vitro. A, The lysates of lung adenocarcinoma cell lines with KRAS mutation (A549 [G12S], H358 [G12C] and H441 [G12V]) or EGFR mutation (HCC827 [E746-A750 deletion], H1650 [E746-A750 deletion] and H1975 [L858R and T790M]) were probed with anti-ARL4C and anti-HSP90 antibodies. B, A549 or H1975 cells stably expressing GFP or ARL4C-GFP were transfected with control anti-sense oligonucleotide (ASO) or ARL4C ASO-1316 and the lysates were probed with anti-GFP, anti-ARL4C and anti-HSP90 antibodies. C, A549 or H1975 cells stably expressing GFP or ARL4C-GFP were transfected with control ASO or ARL4C ASO-1316. Then, the cells were cultured in 3D Matrigel for 5 days in A549 or 4 days in H1975. The area of spheres was calculated (n = 40-100). D, A549 and H1975 cells stably expressing GFP or ARL4C-GFP were transfected with control ASO or ARL4C ASO-1316 and then subjected to the migration assay. Migration activity is expressed as the percentage of GFP-expressing control cells. Scale bars, 100 μ m in (C); 200 μ m in (D). Results are shown as means $\pm$ SD. *P < 0.05; **P < 0.01. Control, control ASO; ARL4C ASO, ARL4C ASO-1316



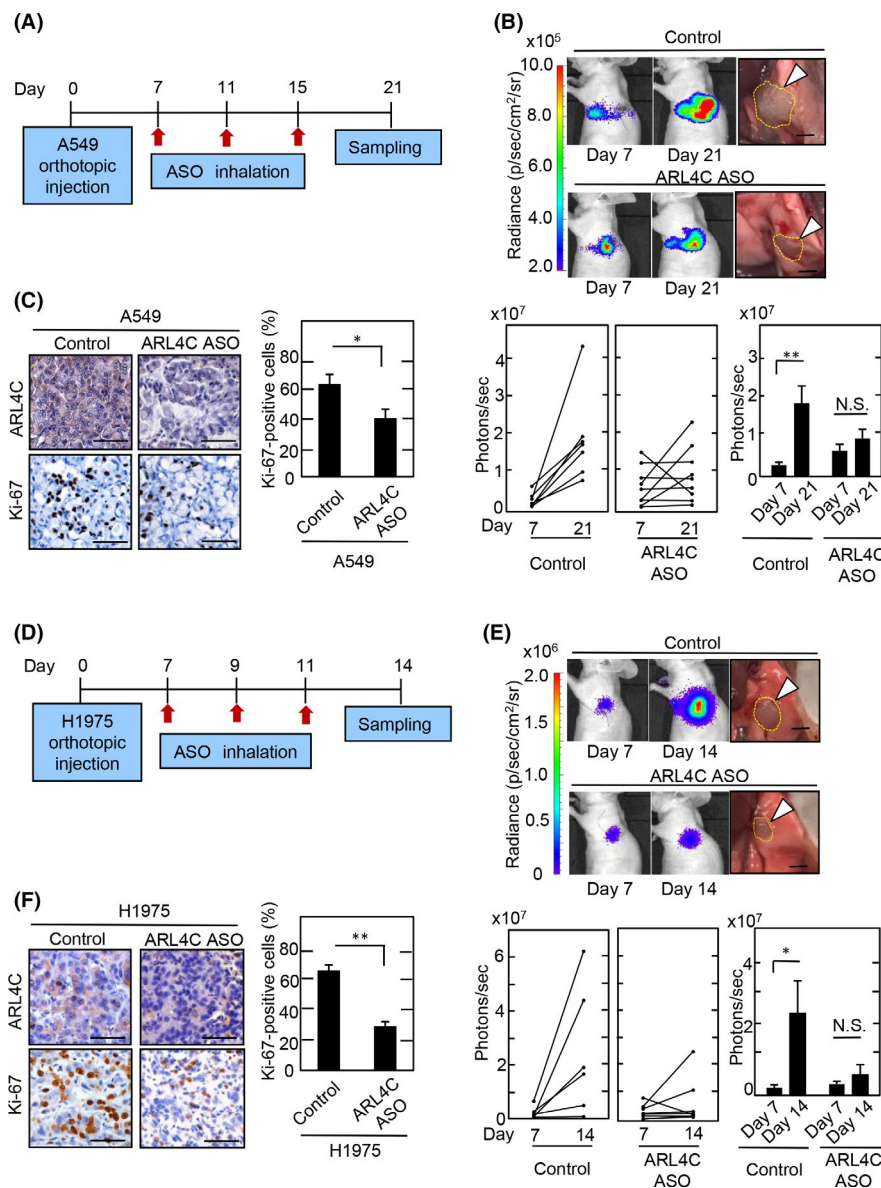


FIGURE 4 ARL4C ASO-1316 inhibits tumor growth in the orthotopic lung cancer model. A, The experimental protocols using A549 cells are shown. B, Representative bioluminescence images at day 7 and 21 after transplantation of A549 cells are shown in the left two panels. Representative images of the lung tumors at day 21 after transplantation are shown in the right panels. White arrows indicate the position of the tumors and yellow dotted lines indicate tumor outlines. The individual data of bioluminescence intensity in A549 cells with control ASO ($n = 7$) or ARL4C ASO-1316 ($n = 9$) are shown in lower left panels. In the lower right panel, results of the bioluminescence intensity in A549 are shown as means $\pm$ SE. C, Sections were prepared from the lung tumors of A549 cells and stained with anti-ARL4C or anti-Ki-67 antibody, and the Ki-67-positive cells were counted. Results are shown as means $\pm$ SD and expressed as the percentage of positively stained cells compared with the total number of cells. D, The experimental protocols using H1975 cells are shown. E, Representative bioluminescence images at day 7 and 21 after transplantation of H1975 cells are shown in the left two panels. Representative images of the lung tumors at day 21 after transplantation are shown in the right panels. White arrows indicate the position of the tumors and yellow dotted lines indicate tumor outlines. The individual data of bioluminescence intensity in H1975 cells with control ASO ($n = 6$) or ARL4C ASO-1316 ($n = 7$) are shown in the lower left panels. In the lower right panel, Results of the bioluminescence intensity in H1975 cells are shown as means $\pm$ SE. F, Sections were prepared from the lung tumors of H1975 cells and stained with anti-ARL4C or anti-Ki-67 antibody, and the Ki-67-positive cells were counted. Results are shown as means $\pm$ SD expressed as the percentage of positively stained cells compared with the total number of cells. Scale bars, 50 μ m (G, H); 2 mm (B, E). * $P < 0.05$; ** $P < 0.01$. N.S., non-significant. Control, control ASO; ARL4C ASO, ARL4C ASO-1316

Bioluminescence, which indicated the growth of A549/Luc cells, increased 6.0-fold from day 7 to day 21 in the ASO-control-treated mice, whereas luminescence in the ARL4C ASO-1316-treated mice increased only 1.4-fold (Figure 4B). In addition, ARL4C expression and the number of Ki-67-positive cells were reduced in the lung

tumors of the ARL4C ASO-1316-treated mice (Figure 4C), indicating that ARL4C ASO-1316 inhibited tumor formation induced by A549/Luc cells. The effects of ARL4C ASO-1316 were also confirmed in H1975/Luc cells (Figure 4D-F). Therefore, ARL4C ASO-1316 effectively inhibited lung tumor formation in vivo.

4 | DISCUSSION

One putative model for the development of lung adenocarcinoma shows that IA develops sequentially from AAH through AIS and MIA. However, because this model is simple, other possibilities have been proposed.⁶ Our immunohistochemical analysis revealed that ARL4C expression was frequently detected in AAH, decreased in AIS and MIA once, and was frequently observed in IA again. Because it was reported that KRAS and BRAF mutations are more frequently observed in AAH than in AIS and MIA,^{5,6} the ARL4C expression patterns are similar to those of RAS and RAF mutations. Because ARL4C expression is upregulated by RAS/MAPK signaling,¹⁴ ARL4C signaling may be activated in the early stage of hyperplasia of lung epithelial cells with EGFR or KRAS mutations. ARL4C is a unique small G protein in a constitutively active form irrespective of wild type.^{7,32} Therefore, ARL4C could be active without any active mutations, and its activity could be controlled by quantitative changes through transcriptional upregulation. However, ARL4C was not expressed in some lung cancer cells with KRAS and EGFR mutations. Thus, ARL4C signaling is not always correlated with EGF-RAS signaling; alternatively, other mechanisms may be required for inducing ARL4C expression in these types of lung cancers.

To elucidate the role of ARL4C in precancerous epithelial cells, a carcinogenic model was used to introduce oncogenes into immortalized normal SAEC.²⁴ ARL4C expression enhanced sphere formation in 3D Matrigel and colony formation of SAEC, and decreased cell death, resulting in promotion of hyperplasia, which is characteristic of AAH. Because the combined KRAS^{G12V} and ARL4C expressions promoted SAEC proliferation in vitro rather than either expression on its own, ARL4C may promote cell proliferation by cooperating with the EGF-RAS pathway. Reportedly, the combined expression of KRAS^{G12V} and c-Myc, PI3KCA^{H1047R}, cyclin D1 or LKB1^{D194A} is sufficient for immortalized SAEC to induce xenograft tumor formation in nude mice, although to variable degrees.²⁴ Therefore, taken together with the observation that SAEC expressing ARL4C did not grow in vivo, ARL4C signaling is not sufficient for lung epithelial cells to be fully transformed compared with well-known oncogene products. ARL4C might enable the epithelial cells to abnormally proliferate and resist cell death thereby separating from the basement membrane and develop AAH.

When the relationship between IHC staining of ARL4C and prognosis in surgically resected lung cancer patients was evaluated, ARL4C expression in lung adenocarcinoma was inversely correlated with relapse-free survival. These results are consistent with the analyzed data from GSE datasets of lung cancer and our previous reports on liver cancers and liver tumors that metastasize from colon cancer.¹⁷ Furthermore, multivariate analysis using clinical data in this study identified high ARL4C expression as an independent prognostic factor in relapse-free survival. A significant difference in overall survival was not found between the high ARL4C expression and the low ARL4C expression groups in the present study, although analysis using GSE datasets of lung cancer showed a poorer prognosis in the high ARL4C expression group than in the low ARL4C expression

group. The present study included numerous p-Stage I patients with relatively good overall survival, which might have caused a discrepancy in the study results. Thus, ARL4C expression can be used as a molecular marker for the prognosis of lung cancer in clinical applications.

The ARL4C ASO-1316 used in this experiment was flanked by AmNA with a phosphorothioate backbone. This type of chemically-modified ASO was shown to be stable in the blood in vivo. Nucleic acid drugs, such as ASO and siRNA, accumulate more in the lung with intratracheal administration than with intravenous administration.^{33,34} An orthotopic lung tumor model is considered to provide better understanding of the biology of lung cancer and more clinically relevant systems for characterizing novel therapeutic drugs compared with a subcutaneous xenograft model.^{26,27} Intratracheally administered ARL4C ASO-1316 reduced ARL4C expression and suppressed the growth of orthotopic lung tumors consisting of both the KRAS mutant cell line A549 and the EGFR mutant cell line H1975. Notably, H1975 cells were positive for the T790M EGFR mutation, which confers resistance to the first-generation EGFR TKI.³⁵ The development of TKI resistance results in the recurrence of tumors, and >50% of all acquired secondary resistance to EGFR TKI is attributed to the development of the T790M secondary mutation.³⁵⁻³⁸ Third generation of TKI, such as osimertinib, was developed to treat patients with EGFR^{T790M}.³⁹ However, it is anticipated that other EGFR mutations that are resistant to new TKI will appear in lung cancer cells in the future. If ARL4C is highly expressed in these types of lung cancers, ARL4C ASO-1316 may have therapeutic potential for these cases, because its mechanism of inhibitory action is different from that of TKI.

Intratracheal administration of ASO can be selectively administered to lung cancer patients using a bronchoscope and may enable treatment of patients with multiple lung tumors that are difficult to treat surgically. Ground glass opacity nodules (GGN) are a CT finding indicating a probability of lung cancer.⁴⁰ GGN frequently appear as multiple nodules. Previous genetic research on multiple GGN indicates that these tumors are mostly multifocal.⁴¹ When GGN lesions are scattered in different lobes of the lung in patients with multifocal GGN, resection of all lesions is difficult and a longer follow-up period is required to confirm subsequent GGN growth. Due to the common clinical settings of treatment for patients with synchronously multiple GGN that are not easily treated with surgical resection, an inhaled drug delivery system of ARL4C ASO-like therapeutic agents for asthma and COPD could be an ideal treatment instead of surgery. However, the administration period and dose of ASO differ between A549 and H1975. Therefore, these factors should be further analyzed before consideration for clinical application. In conclusion, high ARL4C expression is a poor prognostic factor in lung adenocarcinoma and intratracheal administration of ARL4C ASO could be a novel treatment for lung cancer.

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DISCLOSURE

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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