





Alpha-actinin-4 (ACTN4) gene amplification is a predictive biomarker for adjuvant chemotherapy with tegafur/uracil in stage I lung adenocarcinomas

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Funding information

Japan Agency for Medical Research and
Development (AMED), Grant/Award
Number: 17ck0106295h0001

Abstract

Although adjuvant tegafur/uracil (UFT) is recommended for patients with completely resected stage I non-small-cell lung cancer (NSCLC) in Japan, only one-third of cases has received adjuvant chemotherapy (ADJ) according to real-world data. Therefore, robust predictive biomarkers for selecting ADJ or observation (OBS) without ADJ are needed. Patients who underwent complete resection of stage I lung adenocarcinoma with or without adjuvant UFT were enrolled. The status of *ACTN4* gene amplification was analyzed by FISH. Statistical analyses to determine whether the status of *ACTN4* gene amplification affected recurrence-free survival (RFS) were carried out. Formalin-fixed, paraffin-embedded samples from 1136 lung adenocarcinomas were submitted for analysis of *ACTN4* gene amplification. Ninety-nine (8.9%) of 1114 cases were positive for *ACTN4* gene amplification. In the subgroup analysis of patients aged

Abbreviations: ACTN4, actinin-4; ADJ, adjuvant chemotherapy; CI, confidence interval; FPPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ hybridization; HR, hazard ratio; NSCLC, non-small-cell lung cancer; OBS, observation; OS, overall survival; RFS, recurrence-free survival; UFT, tegafur/uracil.

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65 years or older, the ADJ group had better RFS than the OBS group in the ACTN4-positive cohort (hazard ratio [HR], 0.084, 95% confidence interval [CI], 0.009-0.806; $P = .032$). The difference in RFS between the ADJ group and the OBS group was not significant in ACTN4-negative cases (all ages: HR, 1.214; 95% CI, 0.848-1.738; $P = .289$). Analyses of ACTN4 gene amplification contributed to the decision regarding postoperative ADJ for stage I lung adenocarcinomas, preventing recurrence, improving the quality of medical care, preventing the unnecessary side-effects of ADJ, and saving medical costs.

KEYWORDS

ACTN4 gene amplification, adjuvant chemotherapy, lung adenocarcinoma, recurrence-free survival, tegafur-uracil

1 | INTRODUCTION

Surgery is a standard treatment option in patients with early-stage NSCLC, but even such patients who undergo complete surgical resection have a risk of recurrence and death from lung cancer.¹ The indication for ADJ for early-stage NSCLC has remained controversial. In the Adjuvant Navelbine International Trialist Association (ANITA) trial,² survival benefit was reported for patients with stage II NSCLC, but not for those with stage IB disease. In the CALGB 9633 trial, only stage IB NSCLC patients with tumors larger than 4 cm benefited from ADJ with paclitaxel and carboplatin.³ Based on these reports, the Cancer Care Ontario and American Society of Clinical Oncology Joint Panel decided not to recommend routine adjuvant therapy for stage I NSCLC.⁴

The Japanese nationwide lung cancer registry report analyzed a total of 11 663 patients who underwent surgery in 2004. This study showed that the 5-year overall survival was 85.9% for pathological p-stage IA and 69.3% for p-stage IB.⁵ As the common form of relapse after local curative surgery is distant metastasis, the use of ADJ has been considered to improve the outcome of these early-stage lung cancers. In 2004, a large Japanese phase III trial showed that postoperative oral UFT (a prodrug of 5-fluorouracil developed in Japan) monotherapy significantly improved overall survival compared with surgery alone for resected p-stage I adenocarcinoma, especially for p-stage IB (T2 disease in TNM 6th edition) adenocarcinoma (HR, 0.48; 95% CI, 0.29-0.81).⁶ A meta-analysis of 2003 patients enrolled in six clinical trials in Japan showed the efficacy of UFT (HR for adenocarcinoma, 0.69; 95% CI, 0.56-0.85; HR for squamous cell carcinoma, 0.82; 95% CI, 0.57-1.19).⁷ A subsequent exploratory analysis indicated a survival benefit of UFT for p-stage IA disease with T1 larger than 2 cm (HR, 0.62; 95% CI, 0.42-0.90).⁸ Based on these results, the current Japanese treatment guideline recommends postoperative UFT therapy for patients with completely resected p-stage I (T1 >2 cm and T2 in TNM 6th edition) NSCLC (recommendation 1A).

Although UFT was recommended in the lung cancer clinical practice guideline, it was not administered to many patients in

clinical practice. Yoh et al⁹ examined how much UFT was prescribed in actual clinical practice, and they reported that only 33% of patients received postoperative ADJ, mainly UFT. Only 25% of elderly patients aged 70 years or more received ADJ. Approximately 70% of patients did not receive the additional 4.3-percentage point 5-year survival benefit shown in the meta-analysis.⁷ It has been calculated that approximately 1000 people a year in Japan do not achieve 5-year survival because they are not given UFT, which is a nonnegligible number.

From these data, decision biomarkers that can help determine whether to start UFT as postoperative ADJ are critically needed.

Actinin-4 (ACTN4) is an actin-binding protein and a nonmuscle alpha-actinin that we identified in 1998.¹⁰ We have reported that overexpression of ACTN4 leads to an aggressively malignant phenotype of cancer cells with metastatic potential.¹¹⁻²⁴ Numerous clinical studies have shown that strong expression of actinin-4 protein was correlated with aggressiveness, invasion, and metastasis in certain tumors. In particular, gene amplification of ACTN4, which is located on 19q13, is strongly involved in the metastatic ability of adenocarcinoma of the lung. Experimental manipulations with ACTN4 expression and amplification further confirmed its involvement in cell proliferation, motility, and epithelial-mesenchymal transition.¹⁶⁻²⁶

We recently reported that patients with gene amplification of ACTN4 in stage I lung adenocarcinoma who never underwent ADJ with any drug definitely had a worse prognosis than patients without gene amplification of ACTN4, as well as the potential clinical applicability of ACTN4 as a prognostic biomarker of stage I adenocarcinoma.¹⁴ These samples were resected before the recommendation of adjuvant UFT chemotherapy was made. Metastatic potential is strongly associated with actinin-4 expression. In addition, when protein expression of actinin-4 was reduced by ACTN4 shRNA in A549 cells, an adenocarcinoma of the lung cell line with gene amplification of ACTN4, metastatic potential was significantly decreased in *in vitro* assays and in an animal transplantation model. ACTN4 gene expression predicts the efficacy of ADJ including cisplatin + vinorelbine for NSCLC with stage IB-II using a published

microarray of JBR10. The clinical and preclinical data suggested that ACTN4 is a potential predictive biomarker for the efficacy of ADJ in stage IB/II patients with NSCLC by reflecting the metastatic potential of tumor cells.¹⁵

Because the efficacy of adjuvant UFT for resected stage I adenocarcinoma of the lung has been proven, a prospective trial of UFT based on ACTN4 would be ethically problematic. Thus, a retrospective study of all eligible patients treated at high-volume centers was undertaken to evaluate the metastatic activity of NSCLC and predict the effect of ADJ.

2 | MATERIALS AND METHODS

2.1 | Patients and methods

This was a retrospective, multicenter, observational study carried out according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) (Figure 1).²⁷ Archival tissue samples from a total of 1136 patients who underwent surgical resection of histologically proven primary lung adenocarcinoma and diagnosed as pathological stage I (TNM 7th edition)²⁸ from January 1, 2007 to December 31, 2014 in three institutions were used. The number of tissue samples from Nippon Medical School Hospital, National Cancer Center Hospital, and Tokyo Medical University Hospital was 158, 518, and 460, respectively.

The protocol of this study was reviewed and approved by each institutional ethics board (Nippon Medical School Hospital, 29-12-869; National Cancer Center Hospital, 2018-063; and Tokyo Medical University Hospital, SH 4101).

The enrollment criteria were as follows: (a) pathological stage I (TNM 7th edition); (b) naive for neoadjuvant chemotherapy

and radiotherapy; (c) maximal tumor diameter from 2 to 5 cm including visceral pleural invasion; (d) complete (R0) resection was confirmed pathologically; (e) older than 20 years of age; (f) resections with lobectomy or segmentectomy; and (g) standard hilar/mediastinal lymph node dissection was performed. The exclusion criteria included: (a) patients with multiple cancers (with a disease-free period of 5 years or less); (b) patients with pathologically lepidic growth only; (c) patients with interstitial pneumonia and pulmonary fibrosis; (d) patients with serious postoperative complications; (e) patients with high-grade neuroendocrine tumor; (f) patients with major organ dysfunction; and (g) ECOG performance status of 3 or 4.

Formalin-fixed, paraffin-embedded tissue slides were collected from the three institutes. Fluorescence in situ hybridization staining techniques allowed for visualization of nucleic acid sequences within a cell under a fluorescence microscope through the precise annealing of a single-stranded, fluorescently labeled DNA probe to a complementary target sequence. The target area was confirmed, and the hybridization areas were marked using an H&E-stained slide. After the FFPE tissue slides completed the four main steps (deparaffinization, pretreatment of slides with 1× paraffin pretreatment solution at 95°C for 30 minutes, protease treatment with protease solution, and dehydration), hybridization was examined using an ACTN4/CEN19p (orange/green) FISH probe. Following hybridization, the unbound probe was removed by a series of washes, and the nuclei were counterstained with DAPI, a DNA-specific stain that fluoresces blue. The ACTN4/CEN19p (Orange/Green) FISH probe was observed under a fluorescence microscope with appropriate filters, orange (ACTN4) and green (CEN19p) signals were counted, and the ACTN4/CEN19p ratio was calculated by Ariol SL200 (Leica Microsystems). ACTN4 gene amplification-positive was defined

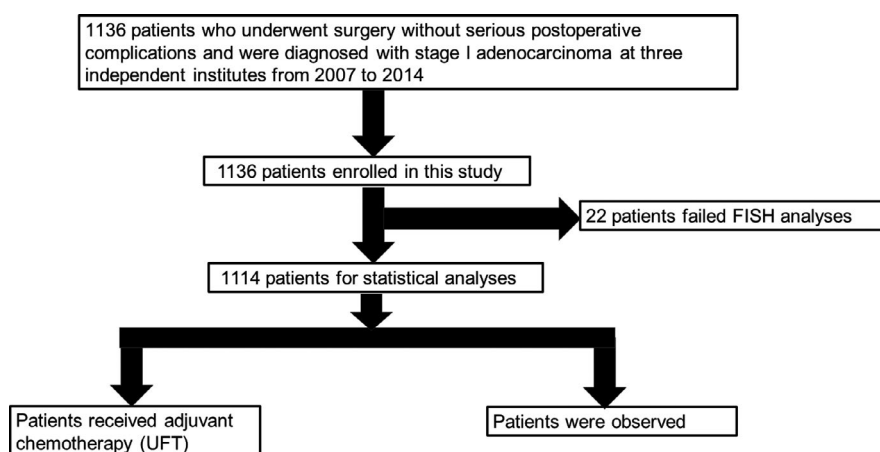


FIGURE 1 Eligibility of patients with stage I adenocarcinoma of the lung. A total of 1136 patients who underwent complete surgery for adenocarcinoma without serious postoperative complications between 2007 and 2014 and were diagnosed as having pathological stage I were initially eligible, and 1114 of the 1136 patients who completed successful FISH analysis according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) were ultimately analyzed. Serious postoperative complications were classified as continued subsequent complications that needed home oxygen therapy and all deaths during the first 30 days after operation due to cardiac and respiratory disorders including pneumonia, pneumonitis, pulmonary embolism, bronchial fistula, empyema, and heart failure, in the retrospective study. UFT, tegafur/uracil

as $ACTN4/CEP19p \geq 2$, and $ACTN4$ gene amplification-negative was defined as $ACTN4/CEP19p < 2$ (Figure S1).¹⁴

2.2 | Statistical methods

Recurrence-free survival was compared between the ADJ and OBS groups with $ACTN4$ amplification-positive patients, between the $ACTN4$ amplification-positive and -negative patients in the OBS group, and between the ADJ and OBS groups in the $ACTN4$ amplification-negative patients.

For the baseline variables, summary statistics were calculated using frequencies and proportions for categorical data and medians and ranges for ordinal variables. Patients' characteristics were compared using Fisher's exact test for categorical variables and the Wilcoxon rank-sum test for ordinal variables.

To evaluate the superiority of ADJ in the $ACTN4$ amplification-positive patients, the HR between the OBS and ADJ groups and its 95% CI were estimated using a Cox regression model adjusted for age (≤ 65 or > 65 years), smoking status (no smoking or smoking), and sex (male or female). The other analyses for RFS were done in the same manner. Landmark analyses were undertaken with 180 days from the start of follow-up as the landmark time point; 180 days was chosen as the landmark because it was considered a sufficiently long treatment period in the ADJ group for postsurgical ADJ.⁶ In addition, subgroup analyses of older patients (age ≥ 65 years) were carried out. Compliance with UFT was calculated based on the number of patients who took UFT and the number of patients in the ADJ group at 60, 120, 180, and 365 days.

All analyses were planned prior to database lock except for the subgroup analysis, and all P values and 95% CIs were two-sided. A P value less than .05 was considered significant. Regarding the RFS analysis between the ADJ and OBS groups in the $ACTN4$ amplification-negative patients, no difference was defined as a point estimation of the HR between the ADJ and OBS groups of 1.3 or less. All statistical analyses were undertaken using SAS version 9.4 (SAS Institute).

3 | RESULTS

3.1 | Fluorescence in situ hybridization of $ACTN4$ and patients' characteristics

As shown in Figure 1, 1114 of the 1136 patients after lobectomy completed FISH analysis successfully. A total of 364 patients (32.7%) received ADJ, and 750 patients (67.3%) did not receive ADJ. In addition, 572 patients (53.5%) had a smoking history, 374 patients (33.6%) were younger than 65 years of age, 594 patients (53.3%) were female, and 614 patients (55.1%) and 500 patients (44.9%) were defined as having pathological IA (T1b) and pathological IB (T2a) disease, respectively. There were no correlations between $ACTN4$ amplification and clinical factors such as the status of ADJ, smoking status, age, sex, or pathological staging.

3.2 | Clinical outcome of ADJ for patients with stage I adenocarcinoma by $ACTN4$

The difference in RFS between the ADJ group and the OBS group for all patients was not significant (HR, 1.162; 95% CI, 0.835-1.618; $P = .373$; $n = 1086$). The total number of patients in this study was 1136. The number of patients with missing covariate data was 46 ($1136 - 46 = 1090$) and the number missing event time data was 4 ($1090 - 4 = 1086$) (Figure 2). The point estimate of RFS in the ADJ group tended to be better than that in the OBS group in $ACTN4$ -positive cases (HR, 0.686; 95% CI, 0.234-2.012; $P = .492$; $n = 97$). The number of patients with missing covariate (smoking status) data was 2 ($99 - 2 = 97$) (Figure 3A). The RFS of $ACTN4$ -negative cases tended to be better than that of $ACTN4$ -positive cases in the OBS group (HR, 0.581; 95% CI, 0.315-1.072; $P = .082$; $n = 710$). The number of patients with missing event time data was 3 ($750 - 3 = 747$). The number of patients with missing covariate data was 37 ($747 - 37 = 710$) (Figure 3B). The difference in RFS between the ADJ group and the OBS group was not significant in $ACTN4$ -negative cases (HR, 1.214; 95% CI, 0.848-1.738; $P = .289$; $n = 968$). The number of patients with missing event time data was 4 ($1015 - 4 = 1011$). The number of patients with missing covariate data was 43 ($1011 - 43 = 968$) (Figure 3C).

3.3 | Duration of UFT treatment with conditional landmark analysis and sensitivity analyses

The median duration (range) of UFT treatment in all cases, $ACTN4$ -negative cases, and $ACTN4$ -positive cases was 674 (1-1444), 670 (1-1444), and 700 (28-887) days, respectively. As a sensitivity analysis, for the purpose of evaluating the therapeutic effect of UFT treatment for a certain period of time, a landmark analysis was undertaken excluding UFT treatment for less than 180 days and the patients whose RFS was less than 180 days (Table S1).

The RFS tended to be better in the ADJ group than in the OBS group in $ACTN4$ -positive cases (HR, 0.695; 95% CI, 0.217-2.225; $P = .540$; $n = 89$) (Figure S2A). The RFS tended to be better in $ACTN4$ -negative cases than in $ACTN4$ -positive cases in the OBS group (HR, 0.614; 95% CI, 0.325-1.160; $P = .133$; $n = 697$) (Figure S2B). Recurrence-free survival was similar in the ADJ group and the OBS group in $ACTN4$ -negative cases (HR, 1.131; 95% CI, 0.756-1.693; $P = .550$; $n = 885$) (Figure S2C); the point estimate of the HR for RFS was greater than 1.

3.4 | Subgroup analyses

In the subgroup of patients aged 65 years or mover, RFS was better in the ADJ group than in the OBS group in $ACTN4$ -positive cases (HR, 0.084; 95% CI, 0.009-0.806; $P = .032$; $n = 64$). The number of patients with missing covariate data was 1 ($65 - 1 = 64$) (Figure 4A). Recurrence-free survival was better in $ACTN4$ -negative cases

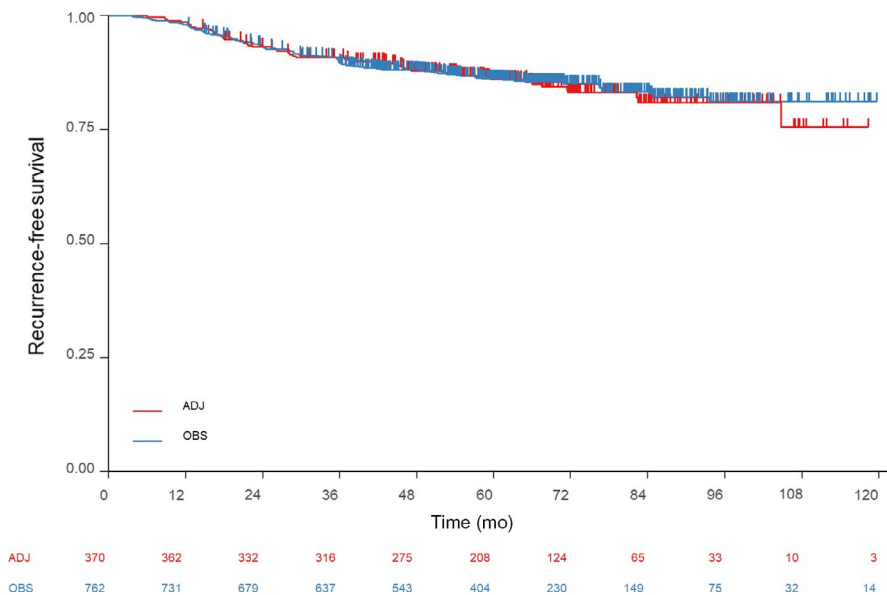


FIGURE 2 Clinical outcome of adjuvant chemotherapy with tegafur/uracil (ADJ) for patients with stage I adenocarcinoma (ADJ and observation [OBS] group in all patients). The difference in recurrence-free survival between the ADJ group and OBS group in all patients is not significant (hazard ratio, 1.162; 95% confidence interval, 0.835-1.618; $P = .373$; $n = 1086$)

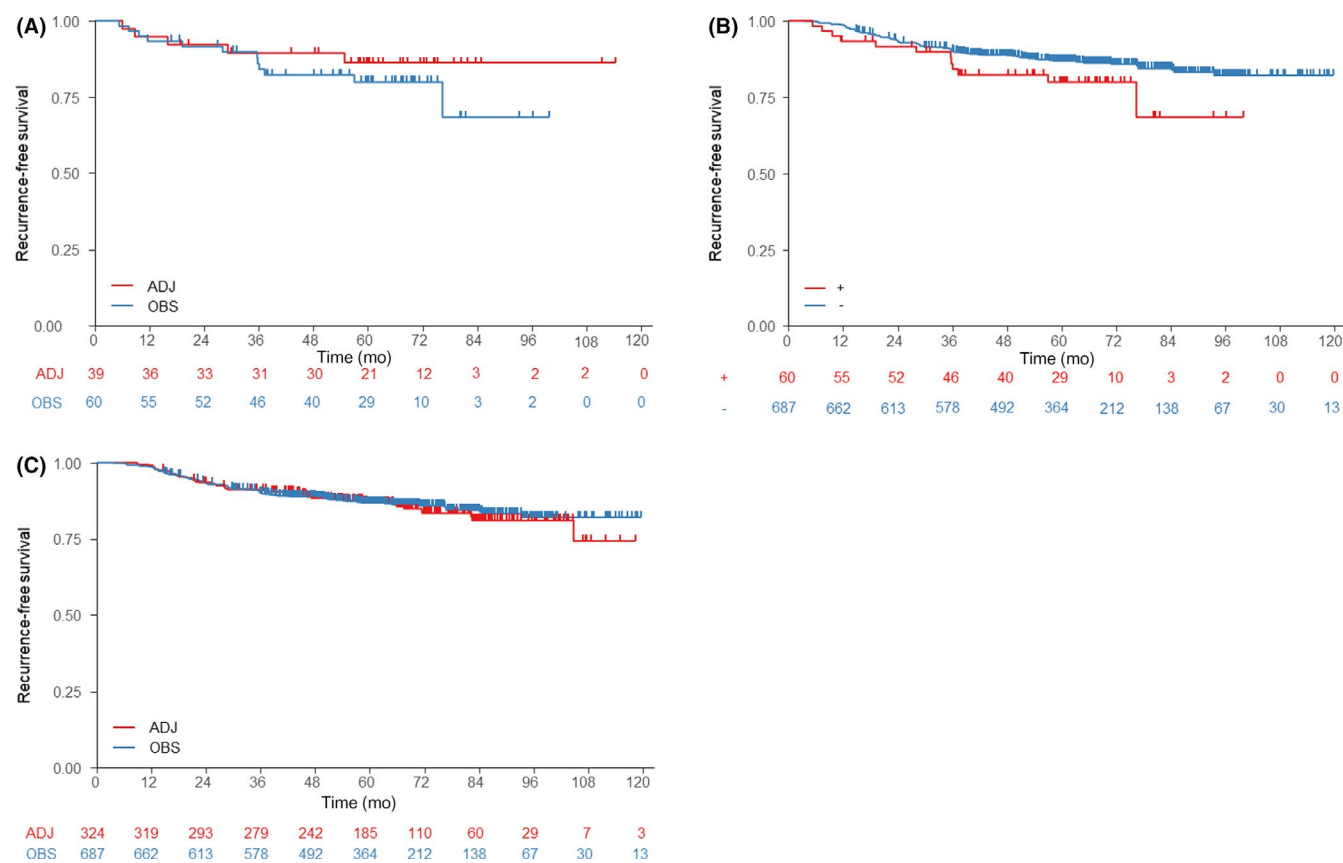


FIGURE 3 Clinical outcomes of adjuvant chemotherapy with tegafur/uracil (ADJ) for patients with stage I adenocarcinoma (in all patients). A, Estimated recurrence-free survival (RFS) in the ADJ group and the observation (OBS) group in actinin-4 (ACTN4)-positive cases. B, Estimated RFS in ACTN4-negative cases and ACTN4-positive cases in the OBS group. C, Difference in RFS between the ADJ group and the OBS group

than in ACTN4-positive cases in the OBS group (HR, 0.475; 95% CI, 0.239-0.946; $P = .034$; $n = 497$). The number of patients with missing event time data was 2 (518 - 2 = 516). The number of patients with missing covariate data was 19 (516 - 19 = 497) (Figure 4B). There was no difference in RFS between the ADJ

group and the OBS group in ACTN4-negative cases (HR, 0.923; 95% CI, 0.566-1.506; $P = .748$, $n = 649$). The number of patients with missing event time data was 3 (675 - 3 = 672). The number of patients with missing covariate data was 23 (672 - 23 = 649) (Figure 4C). The median age in the present study was 69 years.

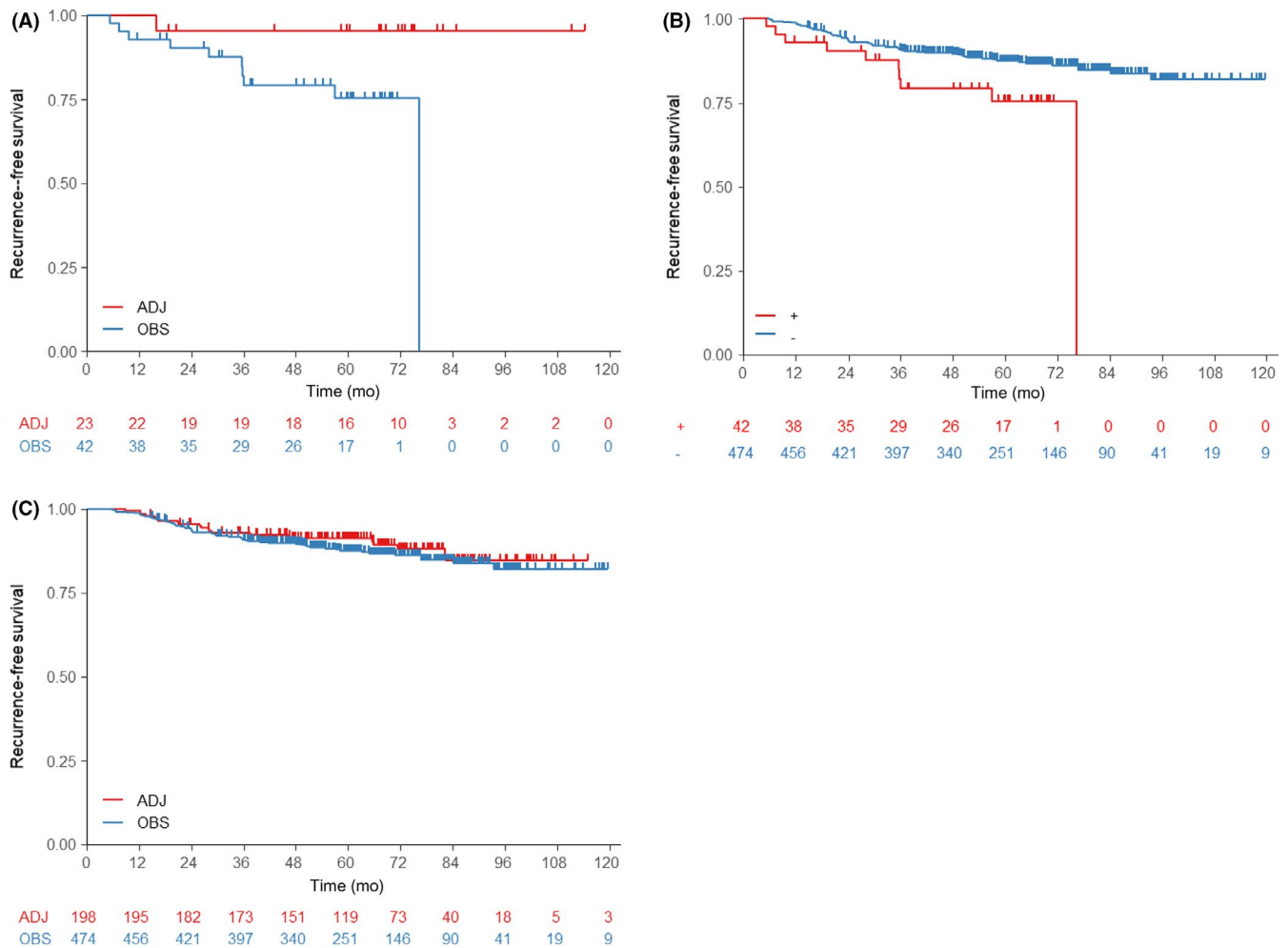


FIGURE 4 Subgroup analyses of patients with stage I adenocarcinoma who received adjuvant chemotherapy with tegafur/uracil (ADJ) or underwent observation. In the subgroup of patients aged ≥ 65 years, (A) the estimated RFS in the ADJ group and the OBS group in actinin-4 (ACTN4)-positive cases, (B) the estimated RFS in ACTN4-negative cases and ACTN4-positive cases in the OBS group, and (C) the difference in RFS between the ADJ group and the OBS group

In the subgroup of patients aged 69 years or more, RFS was better in the ADJ group than in the OBS group in ACTN4-positive cases (HR, 0.041; 95% CI, 0.000-0.500; $P = .007$; $n = 54$). One patient was excluded due to missing covariate data (Figure 3A). Recurrence-free survival was better in ACTN4-negative cases than in ACTN4-positive cases in the OBS group (HR, 0.481; 95% CI, 0.232-0.996; $P = .049$; $n = 395$). Seventeen patients were excluded due to missing event time or covariate data (Figure 3B). There was no difference in RFS between the ADJ group and the OBS group in ACTN4-negative cases (HR, 0.772; 95% CI, 0.430-1.386; $P = .386$; $n = 493$). Twenty-one patients were excluded due to missing event time or covariate data (Figure 3C).

4 | DISCUSSION

The present study showed that UFT did not add significant survival benefit with respect to RFS, suggesting that thoracic oncologists have the potential of selecting high-risk patients in real clinical

settings. However, there are no robust biomarkers to efficiently predict the efficacy of ADJ with UFT. Especially in elderly patients who might have other diseases and the possibility of developing severe side-effects of ADJ, the risk and benefit of ADJ should be strictly evaluated by easy-to-understand metrics. The metastatic potential that individual cancers have is one of the biomarkers for predicting the benefit of ADJ.

In the analysis of the public database JBR.10, a randomized trial of adjuvant cisplatin + vinorelbine in patients with stage IB/II NSCLC showed that OS was higher in the ADJ group than in the OBS group in ACTN4-positive patients (HR, 0.273; 95% CI, 0.079-0.952; $P = .032$; $n = 25$), and there was no difference in OS between the ADJ and OBS groups in ACTN4-negative patients (HR, 1.008; 95% CI, 0.574-1.767; $P = .979$; $n = 108$).¹⁵

In the present study, the analysis comparing RFS of the OBS and ADJ groups in the ACTN4-positive population favored the ADJ group (adjusted HR, 0.686; 95% CI, 0.234-2.012; $P = .492$), but failed to show the statistical superiority of the ADJ group. In fact, the difference shown in the analysis (ie, adjusted HR, 0.686 and 5-year

RFS, 79.9% [OBS] vs 86.1% [ADJ]) is considered clinically meaningful. Furthermore, the subset analyses of patients aged 65 years and over showed that the RFS of the ADJ group was significantly better than that of the OBS group in ACTN4-positive patients (adjusted HR, 0.084; 95% CI, 0.009-0.806; $P = .032$). The median age of patients enrolled in the Kato et al⁶ study was 62 years, but lung cancer patients in that study have been aging and are now over 70 years old, indicating the clinical significance of ACTN4.

More importantly, there was no difference in RFS between the ADJ and OBS groups by the predetermined criteria in the ACTN4-negative population. Although one should be careful to apply the present data to clinical practice, because there was no difference in RFS between patients with and without adjuvant UFT in the total population, the results would also be useful in clinical decision-making in ACTN4-negative patients. In fact, cautious use of UFT was described in the Japan Lung Cancer Society's guideline, because 74% of patients who underwent curative surgery had no recurrence.

There are limitations to the present study. First, the study was retrospective, and several biases could not be excluded. However, all eligible patients treated in the high-volume centers participated in the study, and all analyses were undertaken blindly by the statistician (K.N.), and statistical significance was predefined. Second, it was difficult to compare OS between groups in this study due to the low mortality rate. Several new agents, including immune checkpoint inhibitors and molecular-targeted therapies, have become available for patients with relapsed NSCLC, and RFS would be an appropriate endpoint in this setting. Finally, the percentage (8.9%) of ACTN4-positive cases was lower than anticipated (10%). Our previous report showed that positive rates with p-stage I, stage IA (size <3 cm), and stage IB (size ≥3 cm) were 7.9%, 6.0%, and 11.3%, respectively, in the patients examined.¹⁴ The enrollment criterion for T factor (size, 2-5 cm) was different from that (size, not limited) of our previous report.¹⁴

The present findings showed that ACTN4 is useful for deciding whether to give postoperative ADJ to patients with pathological stage I lung adenocarcinoma who have undergone complete resection. The use of ACTN4 in general medical care contributes to improving the long-term survival of patients with stage I lung adenocarcinoma and improving the quality of medical care, and it also contributes to lowering costs. Further clinical trials of ACTN4 are warranted in the setting of ADJ for NSCLC.

ACKNOWLEDGMENTS

The authors would like to thank Ming Hui of Abnova Corp for preparation of the FFPE FISH Accessory Kit (Abnova Catalog#: DS0002), ACTN4 normal control slides (Abnova Catalog#:S169W0), and ACTN4 positive control slides (Abnova Catalog#:S170W0), provision of ACTN4/CEN19p FISH Probe (Orange/Green), and technical support of the FISH procedure, Kota Zama of Geneticlab Corp for the FISH procedure and signal counting, and Hiroshi Kutsumi, Hayato Yoshinaka, and Daisuke Oabata for advice on clinical performance testing. This work was supported by the Japan Agency for Medical Research and Development (AMED): 17ck0106295h0001.

CONFLICT OF INTEREST

Dr Noro received honoraria from AstraZeneca and Chugai Pharmaceutical. Dr Honda received research expenses not related to this research from Toray. Dr Noro, Dr Honda, and Dr Kubota are inventors of and hold a patent on the ACTN4 biomarker. Dr Motoi received research grants from Ono Pharmaceutical and NEC. Dr Takeuchi received honoraria for lectures, presentations, and speakers bureaus from Taiho Pharmaceutical. Dr Usuda received a research grant from Taiho Pharmaceutical Co., Ltd. Dr Ikeda received research grants from Astra Zeneca, Chugai Pharma, Pfizer, Taiho Pharma, MSD, Boehringer Ingelheim, Eli Lilly, Ono Pharma, Teijin, Nihon, and Medipysics, and honoraria from Astra Zeneca, Chugai Pharma, Pfizer, Taiho Pharma, MSD, Boehringer Ingelheim, Eli Lilly, Ono Pharma, Teijin, Nihon Medipysics, Bristol-Meyers, Olympus, Medtronics, and Johnson & Johnson. Dr Seike received payment or honoraria for lectures, presentations, and speakers bureaus from Boehringer Ingelheim Pharmaceuticals, Taiho Pharmaceutical, and Eli Lilly Japan. Dr Gemma received payment or honoraria for lectures, presentations, and speakers bureaus from Boehringer Ingelheim Pharmaceuticals. Dr Kubota received payment or honoraria for lectures, presentations, and speakers bureaus from Chugai Pharmaceutical, Taiho Pharmaceutical, MSD, Nippon Boehringer Ingelheim, Bristol-Myers Squibb, Kyowa-Hakko Kirin, AstraZeneca, and Ono Pharmaceutical. The other authors have no conflict of interest.

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SUPPORTING INFORMATION

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How to cite this article: Noro R, Honda K, Nagashima K, et al. *Alpha-actinin-4 (ACTN4) gene amplification is a predictive biomarker for adjuvant chemotherapy with tegafur/uracil in stage I lung adenocarcinomas.* *Cancer Sci*. 2022;113:1002-1009. doi:[10.1111/cas.15228](https://doi.org/10.1111/cas.15228)