Upregulation of long non-coding RNA LINC00460 in EGFR-mutant lung cancer indicates a poor prognosis in patients treated with osimertinib

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Abstract. The long non-coding RNA (lncRNA) LINC00460 is involved in tumor growth, metastasis and drug resistance. The present study investigated the clinical significance of LINC00460 expression in patients with epidermal growth factor receptor (*EGFR*) mutation-positive lung cancer treated with osimertinib. Osimertinib-resistant cells we derived from *EGFR*-mutant non-small-cell lung cancer (NSCLC) cell lines, after which, small interfering RNA (siRNA)-mediated silencing and *in vitro*-transcribed (IVT), synthetic LINC00460 RNA transfection were used to investigate the effects of LINC00460 expression on acquired resistance to osimertinib. Reverse transcription-quantitative polymerase chain reaction was performed to evaluate LINC00460 expression in 54 samples (RNA extracted from the tumor tissues of 30 cases and cell-free RNA from 24 cases) obtained from patients with *EGFR* mutation-positive lung cancer who had received osimertinib as the initial treatment. The acquisition of osimertinib resistance increased the expression of LINC00460 in the *EGFR*-mutant NSCLC cell lines. By contrast, knockdown of LINC00460 in osimertinib-resistant cell lines increased their sensitivity to osimertinib, whereas treatment of NSCLC cells with IVT LINC00460 RNA decreased their sensitivity to osimertinib. The present study examined LINC00460 expression at the primary tumor site and demonstrated that compared with in the low-expression group (n=24), the high-expression group (n=6) had a significantly lower best overall response rate to osimertinib (16.6% vs. 60.0%; P=0.044), significantly shorter median progression-free survival (PFS; 224 days vs. 669 days; P=0.001) and significantly shorter median overall survival (724 days vs. not reached; P=0.011). Moreover, following osimertinib therapy, PFS was significantly shorter for patients with high LINC00460 expression in plasma cell-free RNA (n=12) than for those with low LINC00460 expression (n=12) (median PFS: 655 days vs. 210 days; P=0.020). In conclusion, the upregulation of LINC00460, the expression of which is implicated in osimertinib resistance, in the primary site and plasma of patients with *EGFR* mutation-positive lung cancer may be associated with a poor prognosis in those treated with osimertinib.

Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) have shown efficacy in patients with lung adenocarcinoma with *EGFR*-activating mutations, leading to improved progression-free survival (PFS) and overall survival (OS) (1,2). Most lung adenocarcinoma patients with *EGFR*-activating mutations including exon 19 deletions and L858R have shown dramatically positive responses to EGFR-TKIs including gefitinib, erlotinib, afatinib, and osimertinib (3-6). Osimertinib, a third-generation EGFR-TKI, was shown to significantly prolong the PFS and OS compared with first-generation EGFR-TKIs including gefitinib and erlotinib, and to cause mild adverse events (7). Therefore, osimertinib is the first-line treatment for lung adenocarcinoma with *EGFR*-activating mutations (8,9). However, despite this favorable initial response to osimertinib, most patients eventually develop resistance to the drug. The acquisition of *EGFR* C797S, amplification of wild-type *EGFR*, *MET* and *HER2*, activation of a bypass pathway, and small-cell lung cancer transformation may contribute to the acquired resistance to osimertinib (10,11). However, the mechanism of resistance is unknown in approximately 60% of all cases (12). Therefore, the development of effective therapeutic strategies

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Abbreviations: NSCLC, non-small-cell lung cancer; *EGFR*, epidermal growth factor receptor; EGFR-TKI, EGFR tyrosine kinase inhibitor; lncRNA, long non-coding RNA; IVT, *in vitro*-transcribed; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PFS, progression-free survival; OS, overall survival

Key words: lncRNA, LINC00460, NSCLC, osimertinib

to overcome osimertinib resistance requires the identification of novel predictive biomarkers and a better understanding of the molecular mechanisms leading to malignancy and drug resistance.

Long noncoding RNAs (lncRNAs) are noncoding RNAs that do not code for proteins and are greater than 200 bases in length (13,14). Although lncRNAs were initially thought to be transcriptional noise, recent studies have shown that lncRNAs play important roles in cancer pathogenesis, tumorigenesis, angiogenesis, and drug resistance (14). The aberrant expression of lncRNAs has been detected in various carcinomas and may be correlated with carcinogenesis through effects on the expression of associated genes (14). Furthermore, in preclinical experiments, lncRNA-LINC00460 was implicated in promoting tumor proliferation, resistance to apoptosis, and epithelial-mesenchymal transition (EMT) in lung cancer (13,15-18). Wang *et al* (15) showed that lncRNA-LINC00460 competitively binds to miR-539 to suppress its inhibitory effect on NSCLC cell proliferation. Zhao *et al* (16) reported that nicotine promoted the development of NSCLC by activating the LINC00460 and PI3K/AKT signaling pathways. Furthermore, Ma *et al* (19) demonstrated that LINC00460 was involved in gefitinib resistance in NSCLC cells by sponging miR-769-5p. In addition, we reported the role of LINC00460 in lung adenocarcinoma with an *EGFR* mutation (20). Our previous study suggested that activating mutations of *EGFR* significantly increased the expression levels of LINC00460, which functioned as a competitive decoy for miR-149-5p, promoting interleukin (IL)-6 expression, and inducing EMT-like phenotypes, followed by the development of resistance to EGFR-TKIs (20). Therefore, LINC00460 might be a novel predictive marker and potential therapeutic target for *EGFR* mutation-positive lung cancer.

In vitro studies using growth inhibition and RNA interference tests are often performed using cancer cells to validate predictive markers. In the present study, we conducted an *in vitro* experiment to examine the relationship between LINC00460 expression and osimertinib drug resistance. We established osimertinib-resistant cells derived from *EGFR*-mutant NSCLC cell lines and used siRNA-mediated silencing and treatment with *in vitro*-transcribed (IVT) synthetic LINC00460 molecules to investigate the effect of LINC00460 expression on acquired resistance to osimertinib.

Because it is not easy to prospectively identify valid predictive markers, they are often found via retrospective clinical analyses. To clarify the clinical significance of LINC00460 expression in *EGFR* mutation-positive lung cancer patients treated with osimertinib, we evaluated the relative expression levels of LINC00460 in primary tumors from patients with *EGFR*-mutant NSCLC following osimertinib therapy. We then correlated the LINC00460 expression with the PFS and OS of patients with *EGFR*-mutated lung cancer following osimertinib therapy.

Recently, biomarkers released into the blood such as circulating DNA/RNA (cell-free DNA/RNA), circulating tumor cells, and exosomes, have gained increasing attention for use in the diagnosis, prognosis prediction, and monitoring of drug resistance. Liquid biopsy is an ideal test that allows rapid, painless, and safe serial testing, which is not possible by conventional cancer diagnosis that requires invasive tests. The advantage of liquid biopsy is its potential to detect disease progression and drug resistance in real time before clinical symptoms are found or identified on diagnostic imaging. In addition, a liquid biopsy can be conducted using plasma collected from patients with tumors, when it is difficult to harvest a sufficient amount of tumor tissue for biomarker testing. Therefore, to further explore the usefulness of LINC00460 as a predictive marker, we also focused on the relationship between the expression of LINC00460 in plasma and the PFS and OS of patients harboring *EGFR*-mutant NSCLC treated with osimertinib.

Materials and methods

Cell culture and reagents. NSCLC cell lines, PC9 (EGFR W746-A750 del) and H1975 (EGFR L858R + T790M), were used in the present study. PC9 cells were kindly provided by Dr Yuichiro Kanno (Faculty of Pharmaceutical Sciences at Toho University, Chiba, Japan). H1975 cells were obtained from the American Type Culture Collection (ATCC, USA). These cell lines were verified to be mycoplasma free. The cell lines were cultured in DMEM medium (Fujifilm Wako Pure Chemical) supplemented with 10% fetal bovine serum and 100 μg/ml streptomycin. These cells were incubated in a humidified 5% CO_2 atmosphere at 37˚C in an incubator. Osimertinib (AZD9291) was purchased from MedChemExpress LLC. Osimertinib was dissolved in DMSO and stored at -80˚C. Cell lines were cultured in osimertinib-free medium for 5 days before all experiments.

Cell viability assay. The cells were seeded in 96-well plates at a density of $1x10^3$ cells/well and cultured in a 5% CO_2 incubator overnight. The following day, the cells were treated with osimertinib (0.001-10 μM). Cell viability was determined using Cell Counting Kit-8 containing WST-8 (Dojindo Laboratories) according to the manufacturer's protocol. Drug sensitivity was determined by the IC_{50} value (half-maximal inhibitory concentration).

Small interfering RNA (siRNA)-mediated silencing. siRNAs targeting LINC00460 (Silencer Select, Catalog #4390771, The assay ID: n360772) and non-specific siRNA (Silencer Select Negative Control No. 1 siRNA, Catalog #4390843) were custom synthesized by Thermo Fisher Scientific. The cells were transfected with a specific siRNA and a non-specific siRNA (negative control) using LipoTrust EX Oligo (Hokkaido System Science). The cells were detached and diluted in growth medium without antibiotics and then plated in single wells ($1x10^4$ cells/well). Next, siRNA (100 nM) and LipoTrust EX Oligo were mixed in Opti-MEM (Gibco) and incubated for 15 min at room temperature. The siRNA/LipoTrust EX Oligo complexes were added to the wells containing the cells. The cells were then incubated for 24 h at 37˚C and subsequently treated with osimertinib.

Preparation of LINC00460 RNA using IVT. LINC00460 RNA molecules were produced through IVT using a Takara IVTpro mRNA Synthesis Kit (Takara Bio) according to the manufacturer's instructions. DNA template preparation for the IVT LINC00460 RNAs was designed to include a T7 promoter

sequence (5'-TAATACGACTCACTATAAGG-3'). The linearized DNA fragment was synthesized by a gene synthesis service provided by Eurofins Genomics. The designed DNA sequence for LINC00460 was as follows: 5'-TAATACGAC TCACTATAAGGCTTCCTGCAGAAATCCTCCAGCCCT GTTAGAAATGCCTCAGCCAGGGGGACTCATCTCCTC AAACCTGGGGGACCGAGACCTATGAGAGGTCACAGC ATGAGCCAGGACATCGGAGGTACCCAGACATTGTTA TGAAACTCCGCATGTGCCCCTGGTGGACGCTGCTGG ACCCAGCATGCACACTTCTCGGCTAAGAGTCACCCT GGATGAACCACCATTGCCAGCGGGGAGCATGTTGCA GCTTTCCCACGCAGTGGATGAGAACGAAGGTTACGA CCATTGTGTGGGAGGCGTCTGTGTAGCAATTGCTGG AATCACTTGTGGCATTGTAGAAAGACTGAGCGTGGG AAAGAAGACGCATTCTGAAGTCACCCCGATTTATGT TAAATTATCACCTTGACTACTGCTATAGAACGAATG TTTATGTCCCCCACCCAAATTCGTATGCTAAGACCT AATAGCCAATAAGATAGTATTAATAGATGGGGCCTT TGGGAGGTGAGTGGCTCATGAGGGCAGAGTCCTCAA AACCAGATAAGTGCCCGAATAAAAGGGGCCCCAGAG AGATCCCTTGCACCGTCTACCATGTGAAGTGTAGAA CACAGCGAGAAGGCCACCTATGAGCCAGAACGTGGG CCCTCACCAGAACCCAGTTGTGCTGGCACCCTGATC CTGGACTTCCAGCCTCCAAAATGACGACAAAGAAGT TTGTTTTTCCTGAGCCATCCACTTCAAAGTATTCTG TCATAGCTCCCCAAATAGACTAAGACATCTACTTAA CCTTGGTCAAACGTTTAACCTTGGAGTCCACGCCTC TGAAATGGTGACAATAACACTGTGTATTTCCTACTT TATGATCAGGATTAATAAATGTAAT-3'

Synthesized LINC00460 RNAs were used for transfection using LipoTrust EX GENE (Hokkaido System Science) according to the manufacturer's instructions. The RNA molecules and LipoTrust EX GENE were mixed in Opti-MEM (Gibco) and incubated for 20 min at room temperature. The LINC00460 RNA/LipoTrust EX GENE complexes were added to wells containing cells (0.2 μg/100 μl). Then, the cells were incubated for 24 h at 37˚C and subsequently treated with osimertinib.

Clinical samples. This study included a cohort of 54 patients with recurrent post-operative *EGFR*-mutant lung adenocarcinoma who received osimertinib between August 2018 and May 2021. L858R and 19del mutations in tissues were detected using the Cobas® EGFR Mutation Test v2.0 kit. The relative expression levels of LINC00460 were analyzed in RNA extracted from the tumor tissues of 30 cases and plasma cell-free RNA from 24 cases. Both of these samples were collected directly from the patients themselves.

Of the 21 specimens obtained for stage IV lung cancer, 10 had bronchoscopic lung biopsies and 11 had CT-guided biopsies. Of the nine postoperative recurrence specimens collected, four were surgical specimens and five were bronchoscopic lung biopsies. All tissues and peripheral blood were collected within 1 month of osimertinib administration. Patient cohorts in which LINC00460 was tested in tissues and plasma cell-free RNAs were completely separate, and no case had tests for both. Written informed consent was obtained from all patients prior to their participation in this study. RNA was extracted from intratumoral lung tissues, which were defined as a surgical specimen excluding normal lung cells and/or mesenchymal cells, including inflammatory cells. The specimens were preserved as intact blocks, devoid of any cut surfaces. The tissue thickness was established at a range between 10 and 20 μm, accompanied by a surface area of approximately 30 to 50 mm^2.

Peripheral blood was collected in a direct-draw, whole-blood collection tube, Cell-Free RNA BCT (STRECK) for the stabilization of cell-free RNA and extracellular vesicles. In this study, a 5 ml sample of whole blood was collected from each patient. To separate the plasma, whole blood was centrifuged at 1,800 x g for 15 min at room temperature, followed by centrifugation of the plasma at 2,800 x g for 15 min at room temperature.

This single-center study was conducted at the Department of Respiratory Medicine, Toho University School of Medicine as a single-center study and received approval from Toho University Human Genome/Gene Analysis Research Ethics Committee (authorization no. A20101_A17117).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In accordance with the manufacturer's protocol, total RNA was extracted and purified using an RNeasy Mini kit or RNeasy FFPE kit (Qiagen). Plasma cell-free RNA was extracted and purified using a QIAamp Circulating Nucleic Acid kit (Qiagen). To synthesize cDNA, the purified RNAs were reacted with PrimeScript RT Master Mix (Takara Bio, Japan).

RT-qPCR was performed using specific primers and TB Green Premix Ex Taq II (Takara Bio). The PCR amplification program consisted of an initial denaturation step at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. The relative expression levels of the RNAs were determined using the comparative $2^{-\Delta\Delta Cq}$ method (21). The specificities of the primers were validated by melting curve analysis. The relative amount of target RNA in NSCLC cell lines was normalized to that of *GAPDH* mRNA as an internal control. The following primers were used for the analyses: LINC00460 forward, 5'-GTGGATGAGAACGAAGGTTAC G-3' and reverse, 5'-CTTTCCCACGCTCAGTCTTTC-3'; human *GAPDH* forward, 5'-GCACCGTCAAGGCTGAGA AC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

Clinical outcomes. We estimated the PFS and OS of patients whose expressions of LINC00460 had been measured. Our primary experimental data were derived from the RT-qPCR analysis, which was performed using patient samples. This study evaluated the PFS of patients who received osimertinib treatment. The PFS was determined from the start of osimertinib therapy to the earliest signs of disease progression, as determined by computed tomography or magnetic resonance imaging, and assessed according to the Response Evaluation Criteria In Solid Tumors (RECIST) criteria. The OS was defined as the time between the date of diagnosis and death from any cause. Complete response (CR), partial response (PR), stable disease (SD), progressive disease, or not evaluable were determined according to RECIST criteria. Best response refers to the best objective assessment of tumor shrinkage documented from the initiation to the termination of osimertinib therapy. Determination of the best response CR and PR requires confirmation of a sustained response for 4 weeks

or longer. In addition, the determination of SD must be SD at 6 weeks from enrollment. Also, the best overall response rate (ORR) was the proportion of CR and PR with the best response. The disease control rate (DCR) was defined as the proportion of cases with CR, PR, and SD at best response.

We classified patients with EGFR-mutated lung adenocarcinoma by LINC00460 expression in the primary site into two groups and used the values from RT-qPCR to calculate the Youden Index: a low expression group using the Youden Index from receiver operating characteristic (ROC) curve analysis according to the relative expression level of LINC00460, and a high expression group. The tumor lung histology and ORR (i.e., complete and partial responses) were examined. A similar approach was used to establish the cutoff value for plasma.

Statistical analysis. Statistical analyses were conducted using SPSS software version 12.0 for Windows (SPSS Inc., Tokyo, Japan) and Python 3.11.3. The Kaplan-Meier method was used to draw survival curves, and the log-rank test was used for statistical analysis. Differences in the clinical characteristics, ORR and DCR between high LINC00460 expression patients and low LINC00460 expression patients were compared using Fisher's exact test and Welch's t-test. Prior to t-test analysis, Levene's Test was performed to assess the equality of variances. Welch's t-test was utilized to compare data between two groups. One-way ANOVA with Tukey's post-hoc test was used for comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment of osimertinib-resistant cells. To investigate the relationship between LINC00460 expression and acquired resistance to the third-generation EGFR-TKI, osimertinib, we established osimertinib-resistant *EGFR*-mutant NSCLC cell lines by their long-term exposure to osimertinib.

PC9 cell lines, expressing *EGFR* exon 19 deletion mutations without T790M and H1975 cells, and harboring *EGFR* L858R/T790M mutations, were gradually exposed to increasing concentrations of osimertinib over six months. This resulted in the acquisition of osimertinib-resistant cells, which were named PC9-OR and H1975-OR, respectively. PC9 cells were sensitive to osimertinib, whereas PC9-OR cells showed high resistance to the drug and had higher IC50 values than the parent PC9 cells. Similarly, H1975-OR cells exhibited resistance to osimertinib, and parent H1975 cells were sensitive to osimertinib (Fig. 1A and B). Notably, the expression of LINC00460 was significantly higher in PC9-OR and H1975-OR cells than in their respective parent cells (Fig. 1C). These results indicate that LINC00460 overexpression was associated with the resistance of these cells to osimertinib.

Effect of LINC00460 expression on responses to osimertinib in NSCLC cell lines. To examine whether LINC00460 inhibition could overcome the acquired resistance to osimertinib in PC9 cells and H1975 cells, LINC00460 expression in PC9-OR and H1975-OR cells was silenced by specific siRNAs (si-LINC00460). The expression of LINC00460 was significantly suppressed by si-LINC00460, which was confirmed by RT-qPCR analysis (Fig. 2A). The sensitivity of the cells to osimertinib was significantly restored in PC9-OR cells by silencing LINC00460 expression (Fig. 2B), as evidenced by a significant decrease in the IC_{50} value for osimertinib in PC9-OR cells after LINC00460 silencing (Fig. 2C). Similar results were obtained with H1975-OR cells transfected with si-LINC00460 (Fig. 2B and C). In addition, we investigated the effect of LINC00460 expression on osimertinib sensitivity. Because previous studies have shown that the upregulation of LINC00460, which localizes primarily to the cytoplasm and functions as a decoy for some miRNAs, is involved in lung cancer progression (13,14), we directly introduced IVT-synthetic LINC00460 RNA molecules into the cytoplasm of PC9 and H1975 cells via transfection (Fig. 3A and B). Treatment of the PC9 cell lines with IVT LINC00460 induced resistance to osimertinib compared with the parental cells (Fig. 3C and D). Similar results were observed for H1975 cells (Fig. 3C and D). Taken together, these findings suggest that the expression of LINC00460 was associated with osimertinib resistance in NSCLC cell lines.

Clinical characteristics of high and low LINC00460 expression groups. First, to investigate the relationship between the expression of LINC00460 in primary tumors of patients treated with osimertinib and their response and prognosis, we investigated the LINC00460 status of 30 patients with *EGFR*-mutant lung adenocarcinoma who received osimertinib treatment. The established cutoff value of 0.417 for the relative expression of LINC00460 in the primary site was used to classify patients into high and low expression groups. The area under the ROC curve (AUC) was 0.732. The 95% confidence interval (CI) was 0.549 to 0.915. The similarly calculated cut-off value for plasma was 6.5 (ROC AUC=0.722, 95% CI=0.513-0.931). There were no significant disparities in the clinical features, including age, sex, performance status, smoking history, exon 19 deletion or L858R mutations, EGFR-TKI treatment, and metastatic site, between the two cohorts. There were no other significant differences in patient characteristics between the two groups (Table I). The ORR was significantly higher in the low-LINC00460 expression group than in the high-LINC00460 expression group (60.0% vs. 16.6%, P=0.044) (Table II).

LINC00460 expression predicts shorter PFS and OS in patients with EGFR-mutant NSCLC following osimertinib therapy. The PFS was significantly longer in the low-LINC00460 expression group than in the high-LINC00460 expression group (median 669 days vs. 224 days, P=0.001) and the ORR was significantly higher in the low-LINC00460 expression group than in the high-LINC00460 expression group (60.0% vs. 16.6%, P=0.044). Similarly, the OS was significantly longer in the low-LINC00460 expression group than in the high-LINC00460 expression group (median not reached vs. 724 days, P=0.011) (Fig. 4A and B). To explore the potential of LINC00460 as a non-invasive biomarker for osimertinib responses, we examined the LINC00460 expression in plasma cell-free RNA derived from patients with *EGFR*-mutated lung cancer treated with osimertinib. Interestingly, patients with high LINC00460 expression in plasma cell-free RNA (n=12) who received osimertinib therapy had a significantly shorter PFS than those with low

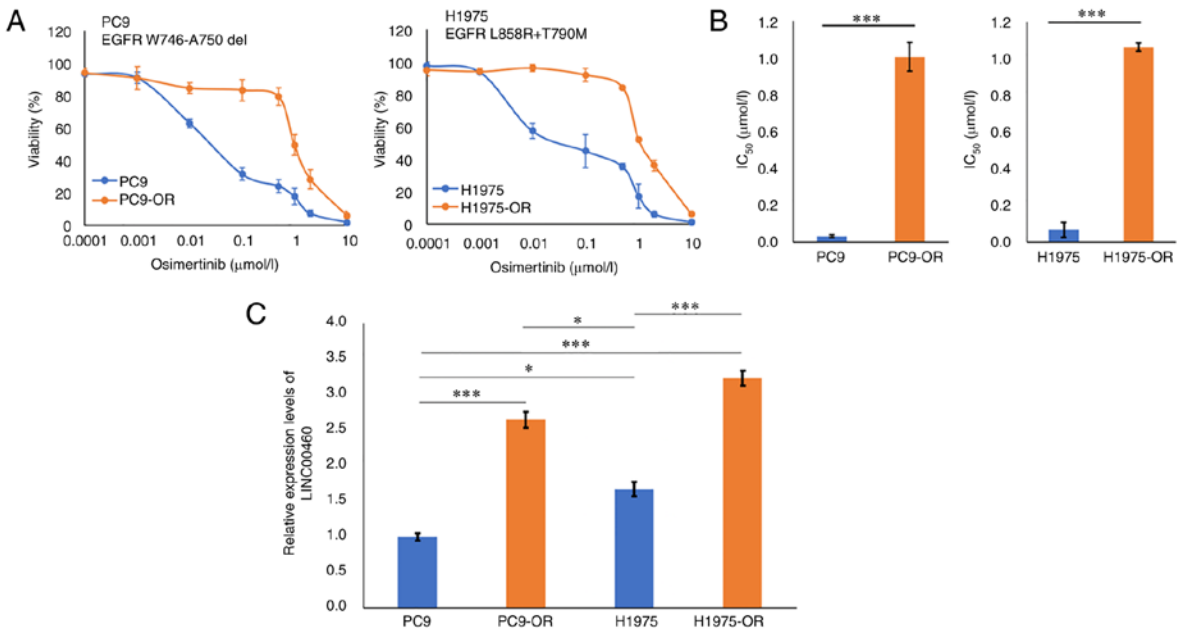

Figure 1. Establishment of osimertinib-resistant NSCLC cells. (A) Parental NSCLC cell lines (PC9 and H1975) and osimertinib-resistant NSCLC cell lines (PC9-OR and H1975-OR) were treated with osimertinib at the indicated concentrations or were left untreated (control). Each point is expressed as a percentage of viable cells relative to the untreated controls (n=3, error bar represents SEM). (B) The bar graph shows the IC_{50} values of osimertinib for the parent and osimertinib-resistant NSCLC cell lines (n=3, error bar represents SEM). ***P<0.001 using the Welch's t-test. (C) The expression of LINC00460 was analyzed in NSCLC cell lines (PC9 and H1975) and osimertinib-resistant NSCLC cell lines (PC9-OR and H1975-OR) by RT-qPCR (n=3, error bar represents SEM). *GAPDH* mRNA was used as an internal control. ***P<0.001 and *P<0.05 using one-way ANOVA with Tukey's post-hoc test. NSCLC, non-small cell lung cancer; OR, osimertinib-resistant; *EGFR*, Epidermal growth factor receptor; SEM, standard error of the mean; del, deletion.

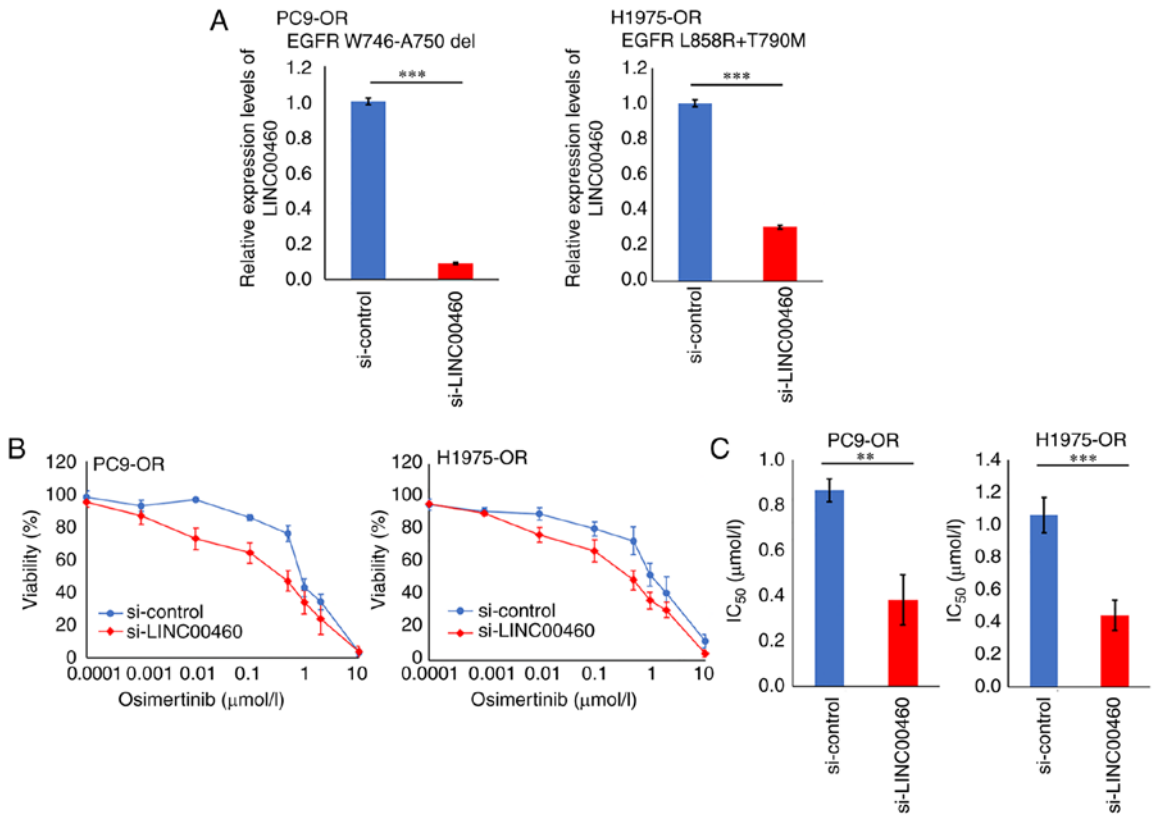

Figure 2. Effects of LINC00460 knockdown on osimertinib sensitivity. (A) Relative expression levels of LINC00460 in osimertinib-resistant NSCLC cells following transfection with siRNAs (si-control and si-LINC00460). The results of RT-qPCR are presented as the means ± SEM (n=3). *GAPDH* mRNA was used as an internal control. (B) Sensitivity to osimertinib in osimertinib-resistant NSCLC cells following transfection with si-LINC00460. Each point is expressed as a percentage of the viable cells relative to the untreated controls (n=3, error bar represents SEM). (C) The bar graph shows the IC_{50} values of osimertinib for osimertinib-resistant NSCLC cell lines following LINC00460 silencing (n=3, error bar represents SEM). ***P<0.001 and **P<0.01 using the Welch's t-test. NSCLC, non-small cell lung cancer; OR, osimertinib-resistant; *EGFR*, epidermal growth factor receptor; siRNA, small interfering RNA; del, deletion; si-control; control siRNA; si-LINC00460, siRNA-LINC00460; SEM, standard error of the mean.

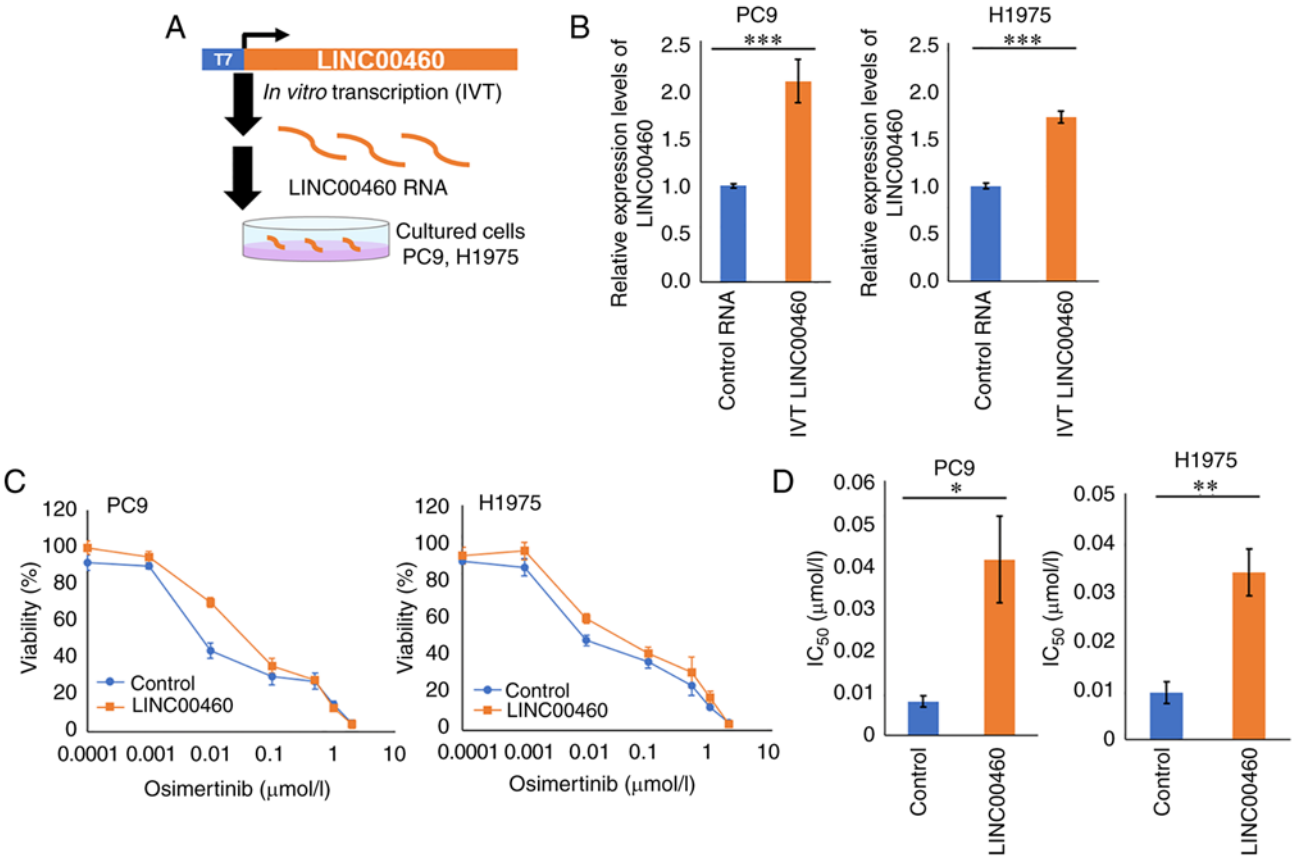

Figure 3. Effects of treatment with LINC00460 on osimertinib resistance in NSCLC cell lines. (A) Schematic of transfection with *in vitro*-transcribed (IVT) synthetic LINC00460 RNA molecules. (B) Relative expression levels of LINC00460 in NSCLC cells following treatment with synthetic LINC00460. The results of RT-qPCR are presented as the means ± SEM (n=3). *GAPDH* mRNA was used as an internal control. (C) Sensitivity to osimertinib in parental NSCLC cells following transfection with LINC00460 molecules. Each point is expressed as a percentage of the viable cells relative to the untreated controls (n=3, error bar represents SEM). (D) The bar graph shows the IC_{50} values of osimertinib for parental NSCLC cells following treatment with LINC00460 (n=3, error bar represents SEM). ***P<0.001, **P<0.01 and *P<0.05 using the Welch's t-test. NSCLC, non-small cell lung cancer; IVT, *in vitro* transcription; T7, T7 promoter; SEM, standard error of the mean.

LINC00460 expression (n=12) (median PFS: 655 days vs. 210 days, P=0.020, respectively) (Fig. 5).

In addition, we examined the expression of LINC00460 across different stages of LC in our cohort of 30 tissue cases and 24 plasma cell-free RNA cases. Our analysis revealed no statistically significant differences in the expression levels of LINC00460 between the subgroups within each group. Specifically, for the 30 tissue cases, we found no significant differences in LINC00460 expression levels when comparing stage III, IV, and surgical recurrence (Fig. 6A, P=0.57). Similarly, for the 24 plasma cell-free RNA cases, LINC00460 expression levels showed no significant variation between the stage IV and surgical recurrence subgroups (Fig. 6B, P=0.25). This result suggests that LINC00460 expression was not significantly correlated with clinical stage in our sample of EGFR-mutant NSCLC patients treated with osimertinib.

Discussion

The molecular mechanisms of cancer growth and drug resistance are still poorly understood. Therefore, it is challenging to identify effective predictive markers other than drug target molecules and their expression levels. However, effective predictive markers are often found in clinical retrospective analyses.

Researchers have recently shown interest in non-protein-coding RNAs. In our previous study, based on the analysis of publicly available databases of transcriptome data, we identified an lncRNA, LINC00460. In addition, we obtained the clinical stage information of lung cancer patients and their corresponding LINC00460 expression levels (FPKM) from RNA-seq datasets. We then compared the expression levels of LINC00460 across different clinical stages. The results demonstrated a tendency for LINC00460 expression levels to increase as the clinical stage advanced. Notably, the expression levels of LINC00460 were significantly higher in stage III patients compared with stage I patients whose LINC00460 expression was significantly upregulated in lung tumor tissues with *EGFR* mutations compared with adjacent normal tissues.

We also found that the expression of LINC00460 was upregulated in lung cancer cell lines with *EGFR*-activating mutations. Furthermore, *EGFR* activation by EGF treatment also caused an increase in LINC00460 expression, whereas *EGFR* inactivation by gefitinib significantly attenuated LINC00460 expression levels. Thus, the overexpression of LINC00460 was associated with abnormal *EGFR* activation.

Table I. Patient characteristics (n=30).

Characteristic	High LINC00460 expression (n=6)	Low LINC00460 expression (n=24)	P-value
Mean ± SEM age, years	63.5±12.5	69.5±12.3	0.47
Sex, male/female	3/3	8/16	0.44
PS, 0/1/2	2/2/2	16/5/3	0.29
Clinical stage, IV/Rec	6/0	15/9	0.67
Smoking history, current/former/never	0/2/4	1/5/18	0.79
EGFR mutation, 19Del/L858R	3/3	15/9	0.73

PS, performance status; *EGFR*, epidermal growth factor receptor; 19Del, exon 19 deletion; L858R, exon 21 L858R; Rec, recurrence after surgical resection.

Table II. Comparison of clinical responses after osimertinib therapy.

Clinical response	High LINC00460 expression (n=6)	Low LINC00460 expression (n=24)	P-value
ORR, %	16.6	60	0.044
DCR, %	83.3	91.6	0.54

ORR, best overall response rate; DCR, disease-control rate.

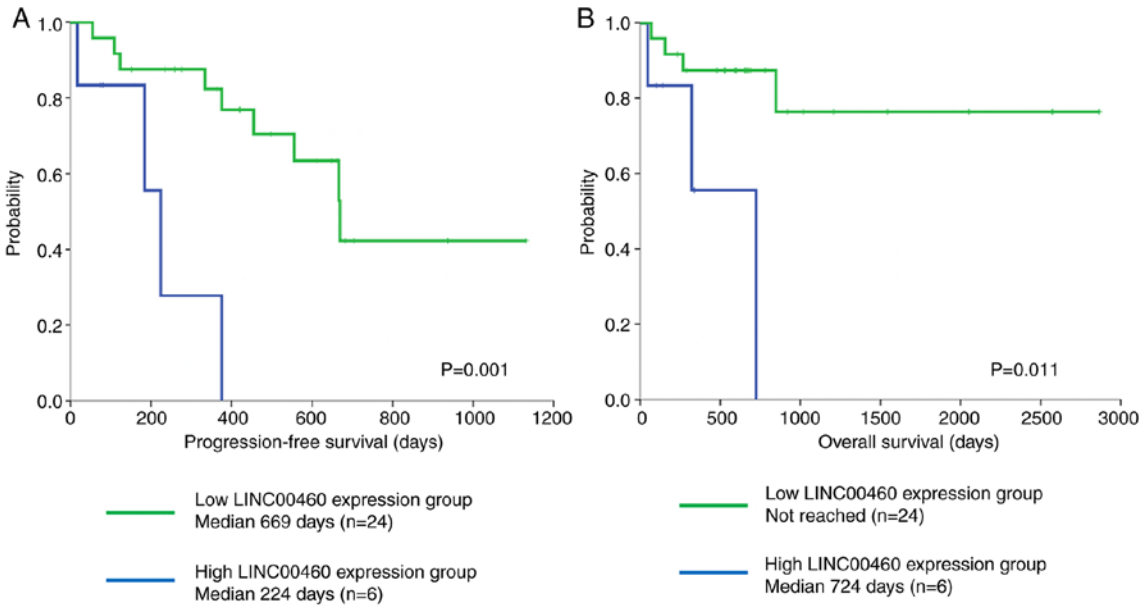

Figure 4. LINC00460 expression in primary site predicts shorter PFS and OS in patients with *EGFR*-mutant NSCLC treated with osimertinib. The expression level of LINC00460 in patients with *EGFR*-mutant lung adenocarcinoma was categorized as low or high, based on a cut-off value determined by the Youden Index from ROC curve analysis according to LINC00460 expression of ORR. (A) PFS after osimertinib therapy was significantly shorter for patients with high LINC00460 expression in tumors than for those with low tumor expression (median PFS: 224 days vs. 669 days, respectively; P=0.001). (B) OS was significantly shorter for patients with high LINC00460 expression in tumors than for those with low tumor expression (median OS: 724 days vs. not reached, respectively; P=0.011). PFS, progression-free survival; OS, overall survival; NSCLC, non-small cell lung cancer; *EGFR*, epidermal growth factor receptor; ROC curve; receiver operating characteristic curve; ORR, objective response rate.

Moreover, we found a correlation between LINC00460 and lung cancer progression, as well as mechanisms of poor efficacy and resistance to EGFR-TKIs, by regulating the miR-149-5p/IL-6 signaling pathway axis, thereby promoting an EMT-like phenotype (20). It is known that EMT regulates the metastatic potential of cancer and EGFR-TKI resistance (20). The main

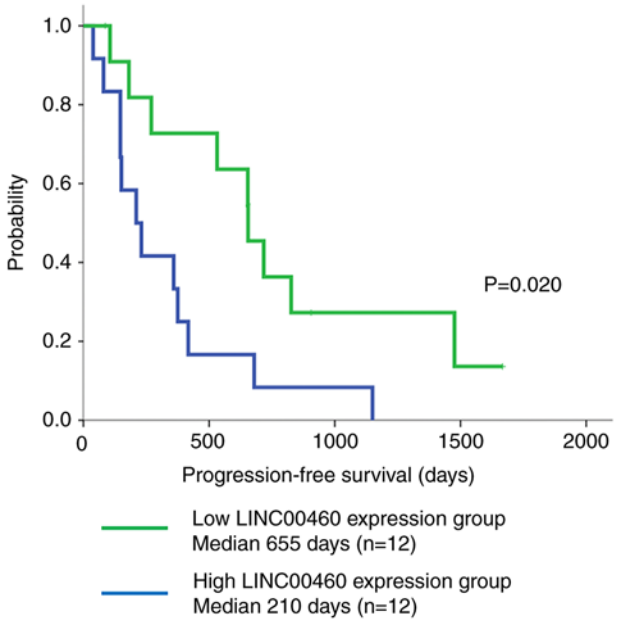

Figure 5. LINC00460 expression in plasma cell-free RNA is associated with shorter PFS in patients with *EGFR*-mutant NSCLC treated with osimertinib therapy. PFS after osimertinib therapy was significantly shorter for patients with high LINC00460 expression in tumors than for those with low tumor expression (median PFS: 655 days vs. 210 days, respectively; P=0.020). LINC00460 expression in patients with *EGFR*-mutant lung adenocarcinoma was classified as low or high based on the median value of LINC00460 expression as the cutoff. PFS, progression-free survival; *EGFR*, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

objective of this study was to clarify the clinical significance of LINC00460 expression in *EGFR* mutation-positive lung cancer patients treated with osimertinib, a third-generation EGFR-TKI.

In the present study, *in vitro*-transcribed-LINC00460-treated NSCLC cell lines harboring mutated *EGFR* tended to be less sensitive to osimertinib than the untreated cell lines. Furthermore, the sensitivity of NSCLC cell lines to osimertinib was significantly restored by silencing LINC00460, as observed in the cell viability assay.

Furthermore, we showed that the overexpression of LINC00460 in the primary tumors of *EGFR* mutation-positive lung cancer lowered the efficacy of osimertinib. Moreover, the low-LINC00460 expression group had a significantly higher ORR to osimertinib than the high-LINC00460 expression group (60.0% vs. 16.6%, P=0.044), in addition to significantly prolonged PFS and OS (median 669 days vs. 224 days, P=0.001; median not reached vs. 724 days, P=0.011). These findings suggest a close relationship between the overexpression of LINC00460 and the therapeutic effects of resistance to osimertinib. A more comprehensive understanding of LINC00460 is, therefore, of great importance in the development of prognostic and diagnostic indicators for EGFR-TKIs in patients with *EGFR* mutation-positive lung cancer and to clarify mechanisms involved in tumor pathogenesis and osimertinib resistance.

To establish an optimal treatment strategy, it is desirable to monitor the condition of the patient's cancer tissues over time. However, the development of non-invasive biomarkers is highly desirable because it is difficult to perform repeated biopsies from patients. One potential method is to diagnose the state of cancer tissues from liquid biopsies such as tumor cells, DNA, and RNA that leak into the blood circulation. In this study, the PFS was significantly shorter in patients with a high expression of LINC00460 in plasma cell-free RNA (n=12) after osimertinib treatment than in those with a low expression of LINC00460 (median PFS: 655 days vs. 210 days, P=0.020, respectively). This suggests that the isolation of plasma cell-free RNA from blood, followed by the detection of LINC00460, might predict treatment responses and prognosis in patients receiving osimertinib therapy. However, the clinical significance of LINC00460 in plasma requires further investigation.

Interestingly, LINC00460 was detectable in blood and tissue samples, although RNA was generally more prone to degradation than DNA. Recent analyses of gene expression in exosomes suggested that LINC00460 was internalized in exosomes secreted by some tumor cells (22). Thus, one reason for the stability of LINC00460 in the blood of lung cancer patients may be that LINC00460 is enriched in exosomes.

Hellyer *et al* (23) reported that the L858R mutation was associated with a shorter duration of response to EGFR-TKI therapy compared with the 19del mutation (23). However, the results of our previous study demonstrated that the forced expression of the *EGFR* 19del mutant gene or L858R gene in H1299 cells, which have wild-type *EGFR* genes, increased the expression of LINC00460. Nevertheless, there was no significant difference in LINC00460 expression levels between the two mutations. Furthermore, knocking down LINC00460, whose expression was increased by the forced expression of the L858R gene, resulted in heightened sensitivity to gefitinib and osimertinib (20). In the cohort of this study, the difference in *EGFR* genotype was not associated with LINC00460 expression levels. Therefore, this suggests that the expression level of LINC00460 is not directly related to the type of active *EGFR* mutation, such as 19del or L858R, but rather to the signaling pathway activated downstream of EGFR signaling upon abnormal EGFR activation, or to the type and presence of co-mutations. Understanding the association of LINC00460 expression with the presence of bypass signaling pathways or co-mutations associated with EGFR-TKI resistance is a future challenge.

This study had several limitations. First, this was a retrospective, single-center study with a small sample size. Although we identified differences in the clinical efficacy and survival of osimertinib-treated patients with different LINC00460 expression levels, the number of enrolled patients was too small to allow any in-depth discussion of the association between LINC00460 expression and the PFS and OS. Therefore, a large multicenter study is needed to confirm the validity of our results. Second, in the current study, tissue and plasma samples were investigated from different populations, so it is unclear whether patients with high LINC00460 expression had a high expression in the plasma. Further studies should be performed to clarify the differential expression of LINC00460 in tissues and plasma. We acknowledge that our current study design had some limitations, and it would be valuable to examine the relationship between tissue and plasma LINC00460 expression in the same patient population in future studies. By doing

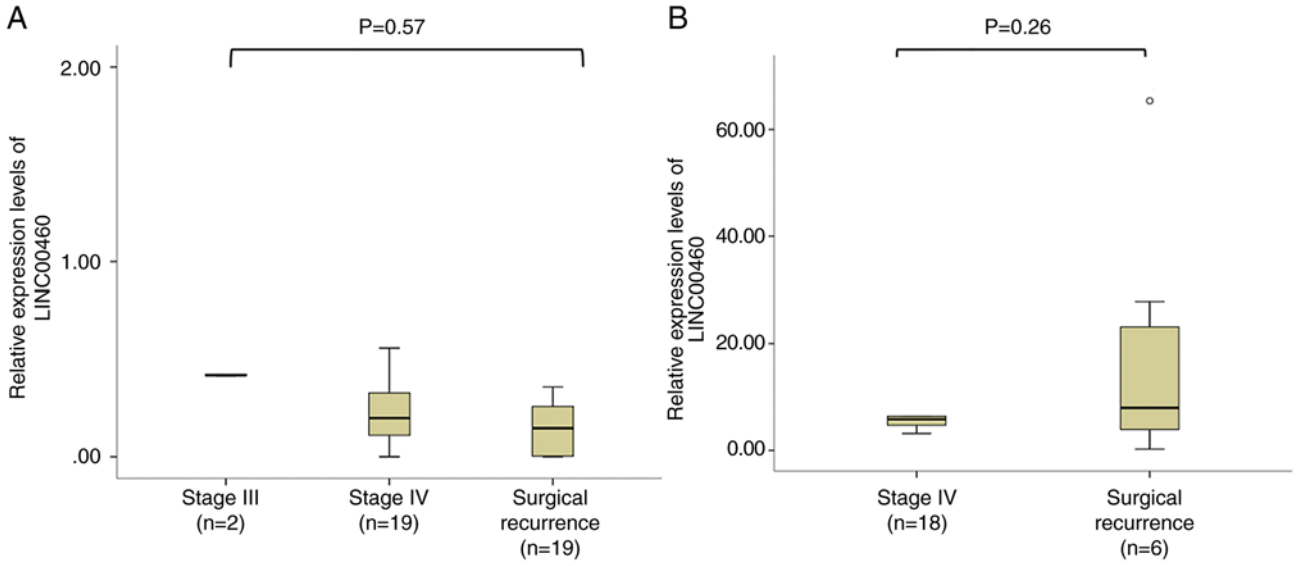

Figure 6. LINC00460 expression across the different clinical stage in patients with *EGFR*-mutant NSCLC treated with osimertinib. (A) The expression levels of LINC00460 between the sub-groups (stage III vs. IV vs. surgical recurrence) in 30 tissue cases were not significantly different (P=0.57). (B) The expression levels of LINC00460 between the sub-groups (IV vs. surgical recurrence) in 24 plasma cell-free RNA cases were not significantly different (P=0.26). *EGFR*, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

so, we could provide a more conclusive understanding of the association between LINC00460 expression and osimertinib resistance, as well as the clinical significance of monitoring plasma LINC00460 levels.

In conclusion, our findings suggest that LINC00460 expression is associated with a poor response to osimertinib, and its overexpression in the primary site of *EGFR* mutation-positive lung cancer might be an indicator of poor prognosis in patients treated with osimertinib. The high expression of LINC00460 based on liquid biopsy might be a predictive marker of poor osimertinib responses in patients with *EGFR*-mutated lung cancer. Thus, understanding the significance of LINC00460 may have important implications when considering it as a molecular target for pharmaceuticals, and as a diagnostic and prognostic indicator for EGFR-TKIs. Recent advancements in nucleic acid biomarker detection technologies, preclinical and clinical development of RNA-targeted drugs, and drug delivery systems have significantly enhanced the drug and diagnostic development process (24-26). Thus, it seems natural to conclude that this discovery may lead to the development of precision therapy for *EGFR*-mutant lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YN, TY, NU and KI contributed to the conception and design of the *in vitro* experiments, analysis and interpretation of data, wrote the manuscript and selected the literature. KI, TY, NU, SH and KK were involved in the design, case enrollment, informed consent, tissue and blood sample collection, data interpretation and manuscript writing. YN and KI confirm the authenticity of all the raw data. All authors read and approved the final manuscript, and accept responsibility for the integrity and accuracy of the research, including the resolution of any issues related to the work.

Ethics approval and consent to participate

This study was received approval from the Toho University Human Genome/Gene Analysis Research Ethics Committee (authorization no. A20101_A17117). Written informed consent was obtained from all patients before their participation in the study.

Patient consent for publication

Not applicable.

Competing interests

YN was a research assistant at Toho University during the experimentation phase; however, YN was an employee of

Daiichi Sankyo RD Novare Co., Ltd. during manuscript preparation and submission. The authors other than YN declare that they have no competing interests.

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