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Safety and efficacy of SNK01 (autologous natural killer cells) in combination with cytotoxic chemotherapy and/or cetuximab after failure of prior tyrosine kinase inhibitor in non-small cell lung cancer: non-clinical mouse model and phase I/IIa clinical study

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ABSTRACT

Background Choosing treatments for epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) patients with osimertinib resistance is challenging. We evaluated the safety and efficacy of SNK01 (autologous natural killer (NK) cells) in combination with cytotoxic chemotherapy and/or cetuximab (an anti-EGFR monoclonal antibody) in treating EGFR-mutated NSCLC in this non-clinical and phase I/IIa clinical trial.

Methods We developed a cell line-derived xenograft-humanized mouse model with an osimertinib-resistant lung cancer cell line. The mice were divided into four groups based on treatment (no treatment, cetuximab, SNK01, and combination groups) and treated weekly for 5 weeks. In the clinical study, 12 patients with EGFR-mutated NSCLC who failed prior tyrosine kinase inhibitor (TKI) received SNK01 weekly in combination with gemcitabine/carboplatin (n=6) or cetuximab/gemcitabine/carboplatin (n=6) and dose escalation of SNK01 following the "3+3" design.

Results In the non-clinical study, an increase in NK cells in the blood and enhanced NK cell tumor infiltration were observed in the SNK01 group. The volume of tumor extracted after treatment was the smallest in the combination group. In the clinical study, 12 patients (median age, 60.9 years; all adenocarcinoma cases) received SNK01 weekly for 7–8 weeks (4×10^9 cells/dose (n=6); 6×10^9 cells/dose (n=6)). The maximum feasible dose of SNK01 was 6×10^9 cells/dose without dose-limiting toxicity. Efficacy outcomes showed an objective response rate of 25%, disease control rate of 100%, and median progression-free survival of 143 days.

Conclusion SNK01 in combination with cytotoxic chemotherapy, including cetuximab, for EGFR-mutated NSCLC with TKI resistance was safe and exerted a potential antitumor effect.

Trial registration number NCT04872634.

INTRODUCTION

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are effective for the treatment of EGFR-mutated non-small cell lung cancer (NSCLC)[1–3]; however, they eventually develop resistance, and EGFR T790M mutation is known as the primary mechanism of resistance to these TKI therapies.[4 5] Osimertinib, a third-generation EGFR TKI, provides an effective approach by targeting this resistance mechanism and is currently a first-line therapy for EGFR-mutated NSCLC.[6 7] Yet, osimertinib resistance is also inevitable.[8] While a definitive mechanism for osimertinib resistance has not been fully established, several known mechanisms include EGFR-dependent ones, such as C797S mutation, and EGFR-independent ones, such as mesenchymal epithelial transition amplification.[9] Several studies have been conducted to target these mechanisms and overcome osimertinib resistance,[10 11] but no definitive proven strategy has been established to date. Additionally, it is known that patients with EGFR-mutated NSCLC have a limited response to immune checkpoint inhibitors,[12] making it challenging to select subsequent treatment options following osimertinib failure.

So far, several studies have suggested that cetuximab, an anti-EGFR monoclonal antibody, in combination with TKIs, including afatinib, is beneficial in overcoming osimertinib resistance in NSCLC.[13–15] It has been hypothesized that the benefit of cetuximab on

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ It is challenging to choose subsequent treatment options in patients with osimertinib resistance in epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC). Meanwhile, antitumor effect of the antibody-dependent cellular cytotoxicity of natural killer (NK) cells mediated by cetuximab—anti-EGFR monoclonal antibody—has been reported. Therefore, we aimed to evaluate the safety and efficacy of SNK01 (non-genetically modified autologous NK cell) in combination with cytotoxic chemotherapy and/or cetuximab in NSCLC after failure to prior tyrosine kinase inhibitor (TKI) in a non-clinical cell line-derived xenograft (CDX)-humanized mouse model study and phase I/IIa clinical study.

WHAT THIS STUDY ADDS

⇒ In the non-clinical study using CDX-humanized mouse model, an increase in NK cells in the blood and enhanced NK cell tumor infiltration were observed in the SNK01 group. In the clinical study, no dose-limiting toxicities were observed during study period. Efficacy outcomes showed an objective response rate of 25%, disease control rate of 100%, and median progression-free survival of 143 days.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Autologous NK cell therapy in combination with cytotoxic chemotherapy and/or cetuximab in EGFR-mutated NSCLC with resistance to TKI was found to be safe and exerted a potential antitumor effect in this non-clinical study and early phase I/IIa clinical study. In addition, we have suggested the alternative treatment option to overcome osimertinib resistance. This study could be a basis and evidence for further investigations.

osimertinib-resistant cells involves the blockade of bypass pathways other than the EGFR pathway.[14] Additionally, cetuximab can enhance the activity of natural killer (NK) cells, which play a crucial role in innate tumor immunosurveillance,[16 17] by simultaneously binding to tumor and NK cells and using antibody-dependent cellular cytotoxicity (ADCC) process. In vitro studies have demonstrated the antitumor effects of ADCC through cetuximab in ovarian and colorectal cancers.[18–21] In a phase I clinical trial, the combination of NK cells and cetuximab showed a safe profile in patients with nasopharyngeal carcinoma.[22] Furthermore, in another phase I/II clinical trial, NK cells and cetuximab exerted both safety and favorable effects in patients with advanced NSCLC.[23] However, these studies had the following limitations: they did not provide information on the presence of EGFR mutations, and the control groups consisted of cetuximab monotherapy, which lacks sufficient evidence of treatment efficacy in NSCLC. Thus, these studies did not adequately reflect real-world clinical practice.

Therefore, we aimed to evaluate the safety and efficacy of SNK01 (non-genetically modified autologous NK cells) in combination with cytotoxic chemotherapy and/or cetuximab as a potential subsequent treatment option in NSCLC after prior TKI therapy failure in a non-clinical cell line-derived xenograft (CDX)-humanized mouse model study and phase I/IIa clinical study.

METHODS

Cell line

Cell culture and establishment of osimertinib-resistant cell lines

PC9 and HCC827 NSCLC cell lines were provided by Dr Kazuto Nishio (National Cancer Center Hospital, Tokyo, Japan) and purchased from the American Type Culture Collection (www.atcc.org). Osimertinib-resistant NSCLC cell lines (referred to as PC-9/OR, HCC827/OR) were developed by exposing NSCLC cells to progressively higher concentrations of osimertinib, as described in previous studies.[24 25] The resistant cell lines were authenticated via STR analysis and confirmed to be mycoplasma-free using standard methods. The NSCLC and osimertinib-resistant cell lines were cultured in RPMI 1640 medium (WELGENE Inc., Gyeongsan, Korea) containing 10% fetal bovine serum (Invitrogen, Carlsbad, California, USA) and 1% penicillin-streptomycin (Invitrogen) at 37°C and 5% CO_2. Then, the cell lines were subcultured at ratios of 1:3–1:6 two to three times per week. Briefly, the cell culture medium was removed and washed twice with 15 mL phosphate-buffered saline (WELGENE). The cells were detached from the flask using 2 mL Trypsin-EDTA (Invitrogen) and subsequently neutralized with 8 mL of the medium. Subsequently, the cells were moved to a 15 mL conical tube and pelleted via centrifugation at 1500 rpm for 5 min. The cells were suspended in new medium and cultured in a flask. To eliminate the effects of the drug, the resistant cells were cultured in a drug-free medium for more than 1 week before the experiment.

Cellular viability assays against cetuximab and osimertinib

Cells (1×10^4) were seeded in 96-well sterile plastic plates overnight and then treated with the relevant agents. After 72 hours, 15 µL MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 hours. Crystalline formazan was solubilized with 100 µL of 10% (w/v) SDS solution for 24 hours, and then absorbance at 595 nm was read spectrophotometrically using a microplate reader. The results are representative of at least three independent experiments, and the error bars indicate SD. The IC50 values were determined using GraphPad Prism (GraphPad, La Jolla, California, USA).

Autologous NK cell isolation and expansion

The manufacturing and testing procedures adopted to produce ex vivo expanded NK cells for non-clinical and clinical uses were performed under good manufacturing practice conditions (NKMAX, Seongnam, Korea). Peripheral blood mononuclear cells (PBMCs) were collected from the peripheral blood of the enrolled patients and then used for NK cell expansions as previously described with some modification.[26 27] The detailed methods on NK cell isolation and expansion are described in online supplemental methods.

Establishment of CDX-humanized mouse model and non-clinical study design

NOD.Cg-Prkdc^{scid}IL2rg^{tmlWjl}/SzJ (NSG) mice were developed at the Jackson Laboratory by backcrossing a complete null mutation at the Il2rg locus onto the NOD.

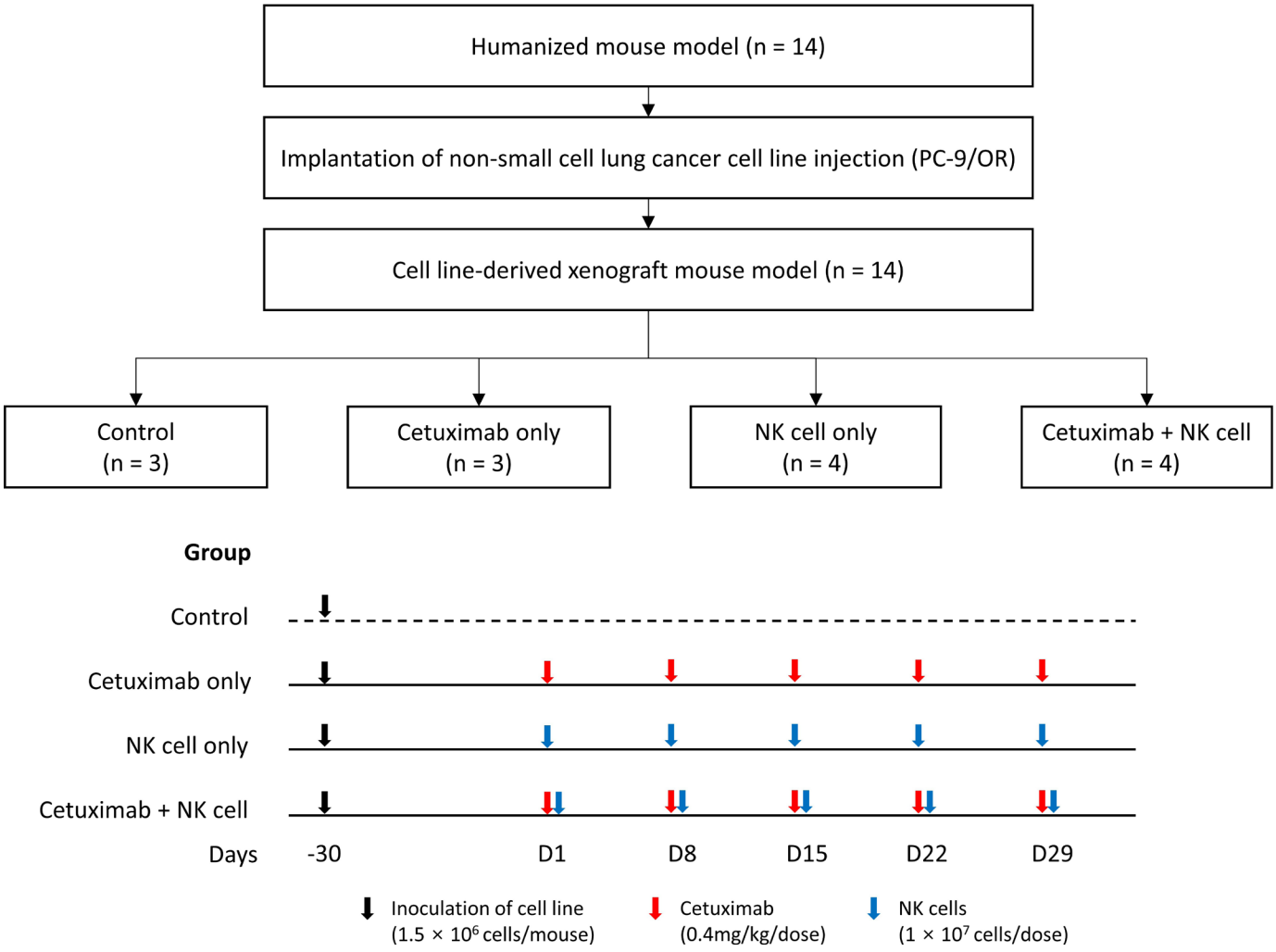

Figure 1 Study design and schematic overview of the treatment in the cell line-derived xenograft-humanized mouse model. CD34-positive hematopoietic stem cells derived from human umbilical cord blood were transplanted into mice, and osimertinib-resistant lung cancer cells (PC-9/OR) were implanted in the right intercostal of 12 weeks posthumanized NSG mice. The developed CDX-humanized mice were divided into four groups based on treatment (no treatment (n=2); cetuximab only (n=3); NK cells only (n=4); cetuximab with NK cells (n=4)) and treated weekly for 5 weeks (NK cells, 1×10^7 cells/dose; cetuximab, 0.4 mg/kg/dose). CDX, cell line-derived xenograft; OR, osimertinib resistance; NK cell, natural killer cell.

Cg-Prkdc^{scid} (NOD/SCID) strain.[28 29] CD34-positive hematopoietic stem cells derived from human umbilical cord blood were transplanted into the mouse, and osimertinib-resistant lung cancer cells (PC-9/OR) were implanted in the right intercostal of 12 weeks post-humanized NSG mice. Cetuximab (0.4 mg/kg/dose) and NK cells (2×10^7/cells) were intravenously injected (figure 1). Tumor volumes were measured using digital calipers every 3–4 days and then calculated (in mm^3) using the following formula: volume=(length×width)×0.5. In addition, tumor growth was measured by quantifying bioluminescence intensity with a small-animal in vivo imaging system (IVIS 200; Caliper Life Sciences). The mice were sacrificed 1 day after the fifth treatment injection or on day 30 in the control group. Immunohistochemistry analysis was then performed on the tumors extracted from the mice to confirm NK cell infiltration into the tumor tissue. The detailed methods of the development of CDX-humanized mouse model are described in online supplemental methods.

Flow cytometry for analyzing changes in human-specific immune cell markers of CDX-humanized mice during treatment

Flow cytometry analysis was performed using fresh PBMCs from each individual blood sample obtained a day after the injection of the treatment agents into the CDX-humanized mice. The cells were washed with 1× DPBS containing 0.5% bovine serum albumin (BSA, Fisher Scientific; Pittsburgh, Pennsylvania, USA) and then fixed in 2% PFA for 10 min at room temperature. After fixation, the cells were incubated with mouse-specific Fc blocker for 20 min at RT to prevent non-specific binding of antibodies to the cells (100 nM anti-CD16/32, BioLegend, San Diego, California, USA). All of the indicated antibodies were then mixed with the cells and incubated for

1 hour at RT protected from the light (since the antibodies were conjugated with fluorescent molecules). In total, approximately 1,000,000 events per sample were acquired by flow cytometry (BD FACS CANTO IICA). All data were analyzed by using FlowJo V.10.9.0 (BD Biosciences, San Jose, California, USA) and statistical analysis was conducted using GraphPad Prism software (V.5.01)

To identify T and B cells, the following antibodies were used: hCD3-allophycocyanin violet (APC-Vio), hCD4-Pacific blue (Bio-Blue), hCD8-phycoerythrin-cyanine 7 (PE-Cy7), and hCD19-PE. To identify Pan-NK and NKT, the following antibodies were used: hCD3-allophycocyanin violet (APC-Vio) and hCD56 (fluorescein isothiocyanate). All antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Clinical study design and populations

The present clinical study is a single-center, open-label, phase I/IIa clinical trial for patients with recurrent or metastatic NSCLC who have failed prior TKI therapy. From November 2021 to August 2022, patients with NSCLC aged 20 years or above with an Eastern Cooperative Oncology Group performance status of 0–1 were recruited from Asan Medical Center (Seoul, Korea). The inclusion criteria were patients with recurrent or metastatic NSCLC with an EGFR mutation and patients with confirmed disease progression after receiving TKI therapy for EGFR mutation at least once. Three patients were allocated to each cohort, and dose escalation followed the "3+3" design. The primary endpoint of the clinical study was the evaluation of the dose-limiting toxicity (DLT) and the safety of SNK01 administered in combination with cytotoxic chemotherapy or cytotoxic chemotherapy/cetuximab as well as the determination of the maximum feasible dose (MFD). The secondary endpoint was the efficacy, including progression-free survival (PFS), overall response rate (ORR), and disease control rate (DCR).

Treatment in the clinical study

After enrolling three patients in cohort 1, three patients were sequentially enrolled in cohort 2 (figure 2). The patients received an initial dose of SNK01 at 4×10^9 cells/dose, given every week for a total of eight doses, starting from day 1 of the second cycle of the cytotoxic chemotherapy. Furthermore, they received cytotoxic chemotherapy (gemcitabine (days 1 and 8) and carboplatin (day 1)) administered every 3 weeks for a total of four cycles, and concomitant weekly administration of cetuximab (cohort 2). If no DLTs were observed during the DLT observation period in these patients, we planned to enroll three patients each in cohorts 3 and 4. These patients received eight doses of SNK01 at a dose of 6×10^9 cells/dose, along with four cycles of gemcitabine/carboplatin every 3 weeks and weekly cetuximab (cohort 4). DLT is an adverse drug reaction related to SNK01 that is categorized as Common Terminology Criteria for Adverse Events (CTCAE) grade 3 or higher. MFD is the highest dose at which one or fewer out of six patients, or none out of three patients, experience DLT.

Follow-up and adverse events

Adverse reactions were monitored through vital signs, physical examinations, and laboratory data and assessed according to the CTCAE V.5.0. Efficacy was evaluated through CT scan, with disease evaluation performed 4 weeks after the treatment initiation, followed by disease evaluations every 6 weeks thereafter. The tumor evaluation followed the Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1. The patients were administered the therapeutic agents until disease progression was observed, and after treatment discontinuation, survival follow-up was conducted through clinical visits every 2–3 months.

NK cell activity in the patients was measured using blood samples collected at baseline and at 4, 10, and 16 weeks post-treatment. We assessed the release of IFN-γ using an ELISA with an NK Vue Kit (ATgen, Sungnam, Korea) following the manufacturer's instructions. This method detected NK-secreted IFN-γ levels by using a quantitative sandwich ELISA on the supernatant of NK cells exposed to a specific recombinant cytokine, Promoca (ATgen), as described by previous studies.[30]

Statistical analysis

The two-way analysis of variance was conducted to compare the composition of the immune cells according to treatment group in the CDX mouse model. In the present clinical trial, a paired t-test was employed to compare NK cell activity according to the treatment course. Descriptive analysis was used to present the tumor size in the CDX model and the patients' baseline characteristics in the clinical study. The error bars in the graph of tumor sizes in mice indicate the SD. The data were expressed as number (percentage), median (IQR), or mean±SD. In a box-whisker plot, the middle line represents the median, the box represents the 25th–75th percentiles, and the whiskers extend from the minimum to the maximum values. The overall survival (OS) and PFS analyses in the clinical study were conducted using Kaplan-Meier curves and log-rank test. The ORR was defined as the proportion of patients who achieved complete response (CR) or partial response (PR) compared with baseline. DCR was defined as the proportion of patients who achieved the best response of CR, PR, or stable disease (SD) according to RECIST V.1.1 evaluation from the first day of treatment. A $p<0.05$ was considered significant. Statistical analyses were conducted using GraphPad Prism V.7 and IBM SPSS V.25.0 (IBM).

RESULTS

Evaluation of the antitumor effects of NK cells in combination with cetuximab in CDX-humanized mouse model

The developed CDX-humanized mouse model with an osimertinib-resistant cell line was allocated with

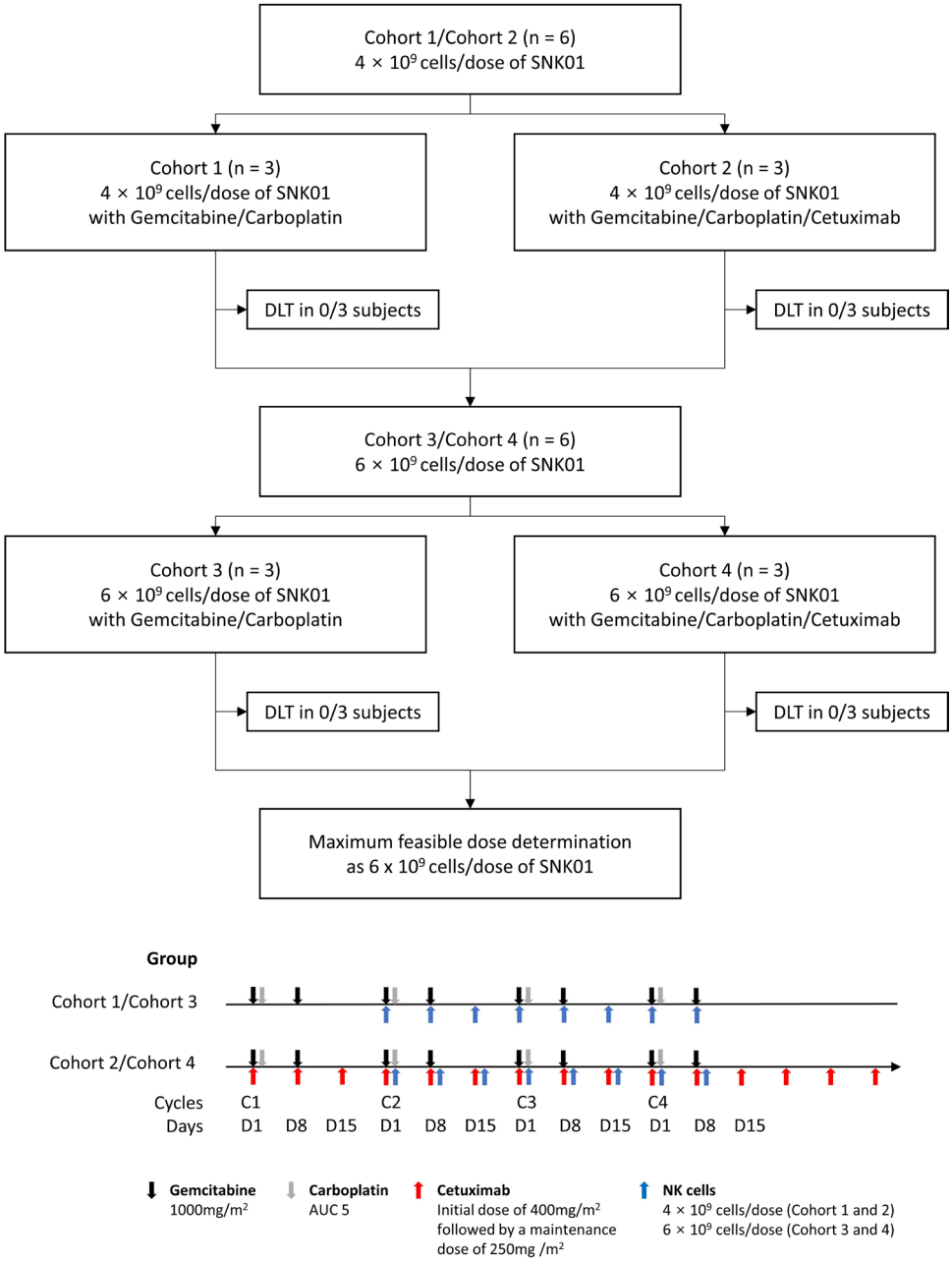

Figure 2 Flow chart of the clinical study in patients with NSCLC receiving SNK01 (NK cells) in combination with either gemcitabine/carboplatin or gemcitabine/carboplatin/cetuximab. Three patients were allocated to each cohort, and dose escalation followed the "3+3" design. A total of 12 patients who failed prior TKI therapy were finally enrolled. During the dose-limiting toxicity (DLT) evaluation period, no DLTs were observed. As the maximum planned dose of this study was found to be tolerable, the maximum tolerated dose was not determined, and a 6×10^9 cells/dose was set as the maximum feasible dose. AUC, area under the concentration; NK cell, natural killer cell; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor.

three or four mice per treatment group (figure 1 and figure 3A,B). In the tumor control group, one mouse died before the treatment completion, resulting in the analysis of 13 mice. Figure 3C presents the tracking of tumor metabolism according to the treatment using an IVIS. Tumor growth was lower in the group treated with NK cells than in the control or cetuximab group, and the volume of tumor extracted after treatment completion was the smallest in the SNK01 plus cetuximab group (figure 3D,E).

Changes in human-specific immune cell markers of CDX-humanized mouse model

We also analyzed changes in human-specific immune cell markers of the CDX-humanized mice according to the treatment course. In the groups treated with SNK01 (NK alone and combination group), an increase in pan-NK cells (CD45+/CD56+) and NK cells (CD45+/CD56+/CD3−) was observed as the treatment progressed, whereas there were no changes in NKT cells (figure 3F and online supplemental figure 1). The cetuximab monotherapy group showed a

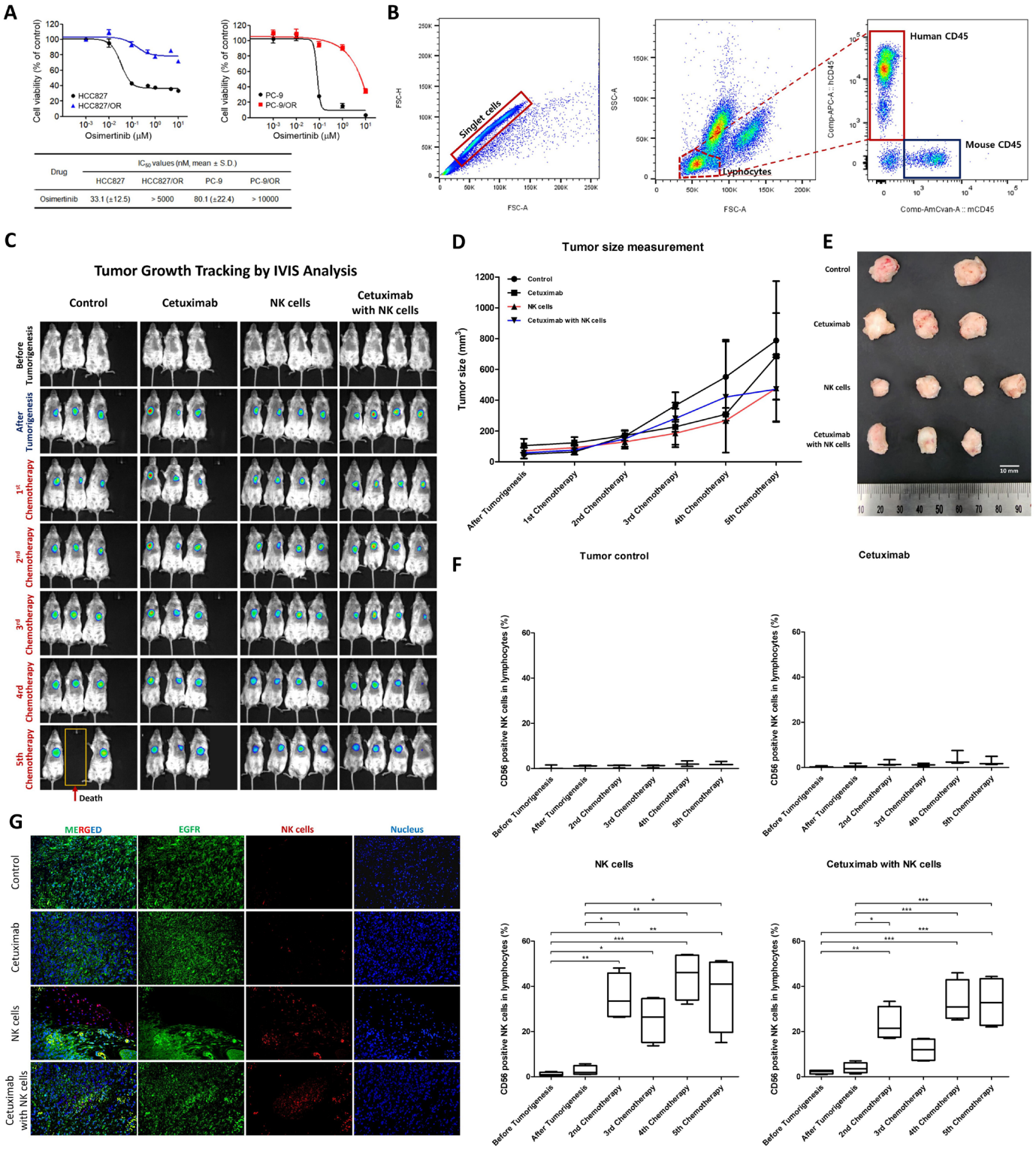

Figure 3 Changes in tumor size and immune cells during tumorigenesis and treatment in cell line-derived xenograft (CDX)-humanized mouse model. (A) Cellular viability against osimertinib using MTT assay in HCC827/OR and PC-9/OR cell lines. (B) Quantitative measurement of human-specific immune cells in preclinical reenactment humanized mouse model in this study. (C) Tumor growth tracking using an in vivo imaging system. (D) Changes in tumor size according to treatment. (E) Extracted tumor after treatment. (F) Changes in pan-NK marker (CD45+/CD56+) in immune cells of CDX-humanized mice during tumorigenesis and treatment. (G) Immunohistochemistry analysis of tumor extracted from CDX-humanized mouse model according to treatment *p<0.05, **p<0.01, ***p<0.001. MTT; 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide.

significant increase in pan-T-cells and CD8+T cells as the treatment progressed. However, in the group treated with NK cells, no significant differences were observed. In all groups, the B-cell subunit exhibited a decreasing trend over time (online supplemental figure 2).

Immunohistochemistry analysis of tumor extracted from CDX-humanized mouse model

Immunohistochemistry analysis revealed NK cell infiltration in the tumor tissue extracted from the mice (figure 3G). Only a trace amount of NK cell infiltration into the tumor tissue was detected in the control and cetuximab monotherapy group. However, in both the NK and combination groups, NK cell infiltration into the tumor tissue was observed. Furthermore, in the combination group, the NK cells visually appeared to have infiltrated more into the central part of the tumor than the NK monotherapy group, especially in areas showing high EGFR expression.

Study population in the clinical study

From November 2021 to August 2022, a total of 14 patients were screened. After the exclusion of 2 patients who died before receiving SNK01, 12 patients who failed prior TKI therapy (osimertinib, n=7; gefitinib, n=4; and afatinib, n=1) were finally enrolled (figure 2). The mean age of the patients was 60.9 years, and 33.3% of them were men (table 1). All patients had adenocarcinoma, and 58.3% of them demonstrated osimertinib resistance.

Characteristics of expanded NK cells from patients

In the freshly isolated $CD56^+$ cells from the peripheral blood of the enrolled patients, the proportion of NK cells ($CD56^+CD3^-$) varied among donors (77.82%±12.60%). However, the final products were mainly composed of NK cells (99.81%±0.22%) with minimal contamination of $CD3^+$ T cells (0.15%±0.20%), $CD14^+$ monocytes (0.32%±0.10%), and $CD20^+$ B cells (0.01%±0.01%) (online supplemental figure 3A). In the expansion culture, the NK cells were highly expanded (4,462,189±2,063,228-fold) with high viability (98.00%±1.00%) (online supplemental figure 3B,C), which was sufficient for multiple injections in all donors. The detailed characteristics of the NK cells are described in Supplemental results and depicted in online supplemental figure 3.

We also investigated the cytotoxic activity and the cetuximab-mediated ADCC activity of the expanded NK cells in vitro. The NK cell products showed higher cytotoxicity against osimertinib-resistant PC-9/OR and HCC827/OR than their parent cells PC-9 and HCC827, respectively (online supplemental figure 4). Such results were observed across all NK cell-to-cancer cell ratios (E:T ratio) regardless of donors. Moreover, the cytotoxicity of the NK cell products was significantly increased against both cell by ADCC when they were pretreated with cetuximab. A similar increase in the cytotoxicity of the NK cell products in response to cetuximab was also observed in HCC827 and HCC827/OR cells (online supplemental figure 5). Detailed results of the in vitro experiments are presented in online supplemental results.

Table 1 Baseline characteristics of patients with NSCLC who received SNK01 (NK cells) in combination with either gemcitabine/carboplatin or gemcitabine/carboplatin/cetuximab

Variables	Total number of patients (n=12)
Age	60.9±5.5
Sex, male	4 (33.3)
Pathology, adenocarcinoma	12 (100)
ECOG PS, 1	12 (100)
Previous treatment line	
1	3 (25.0)
2	7 (58.3)
≥3	2 (16.7)
Osimertinib resistance	7 (58.3)
EGFR	
Wild type	0 (0)
Mutation	12 (100)
ALK	
Wild type	11 (91.7)
Mutation	0 (0)
Unknown	1 (8.3)
PD-L1 status	
<1%	2 (16.7)
1%–49%	2 (16.7)
≥50%	1 (8.3)
Unknown	7 (58.3)
Baseline NK cell activity (pg/mL)	667.0 (145.0–902.5)

Data are presented as number (percentage), mean±SD, or median (IQR).
ALK, anaplastic lymphoma kinase; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; NK, natural killer; NSCLC, non-small cell lung cancer.

Change in NK cell activity in patients according to treatment

The median baseline NK cell activity before the treatment initiation was 667.0 pg/mL (online supplemental figure 6). The NK cell activity measured 4 weeks following cytotoxic chemotherapy (n=12) and immediately before SNK01 infusion (n=12) significantly decreased compared with that at baseline (p=0.045). The NK cell activity showed a tendency to increase again at the 10th week of treatment (n=10, p=0.090) and remained increased until the 16th week of treatment (n=10, p=0.018) compared with the level just before NK cell infusion.

MFD determination, safety, and efficacy

Of the 12 patients, 10, excluding the 2 who received seven doses (disease progression (n=1) and personal circumstances of the participant (n=1)), completed a total of 8

Table 2 Adverse events reported in clinical study (n=12)

Event	SNK01 with GC (n=6)		SNK01 with C/GC (n=6)	
	All grade AEs	≥3 grade AEs	All grade AEs	≥3 grade AEs
All AEs	6 (100)	3 (50.0)	6 (100)	2 (33.3)
AEs occurring in ≥2 patients				
Neutropenia	5 (83.3)	3 (50.0)	4 (66.7)	2 (33.3)
Anemia	3 (50.0)	1 (16.7)	2 (33.3)	0 (0.0)
Fever	3 (50.0)	0 (0.0)	1 (16.7)	0 (0.0)
Skin rash	2 (33.3)	0 (0.0)	6 (100)	0 (0.0)
Diarrhea	2 (33.3)	0 (0.0)	1 (16.7)	0 (0.0)
Thrombocytopenia	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Alopecia	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
Constipation	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)
Anorexia	1 (16.7)	0 (0.0)	1 (16.7)	0 (0.0)
Paronychia	0 (0.0)	0 (0.0)	5 (83.3)	0 (0.0)
Hypomagnesemia	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)
SNK01-related AEs*	2 (33.3)	0 (0.0)	1 (16.7)	0 (0.0)

Data are presented as number (percentage).
*All the SNK01-related AEs were fever.
AE, adverse event; C/GC, cetuximab/GC; GC, gemcitabine/carboplatin.

doses of weekly SNK01 (4×10^9 cells/dose, n=6; 6×10^9 cells/dose, n=6). During the DLT evaluation period, no DLTs were observed. As the maximum planned dose of this study was found to be tolerable, the maximum tolerated dose was not determined, and 6×10^9 cells/dose was set as the MFD. All 12 patients were included in the safety analysis and tolerated the treatment well. Grade 3 or higher adverse events (AEs) occurred in five patients (neutropenia, n=5; anemia, n=1), but all of them were related to cytotoxic chemotherapy and no grade 3 or higher SNK01-related AEs were reported (table 2).

All 12 patients were included in the efficacy analysis, with an ORR of 25% and a DCR of 100% (PR, n=3; SD, n=9) (table 3). Particularly in the SNK01 with cetuximab/gemcitabine/carboplatin group, the ORR was 50%. The median PFS for all patients was 143 days (SNK01 with cetuximab/gemcitabine/carboplatin group, 145 (114–N/E) days; SNK01 with gemcitabine/carboplatin group, 143 (107–246) days; p=0.678), and the OS did not reach the median survival (figure 4A,B). Figure 4 presents the treatment timeline and response for each patient. A PR was observed in three patients, and two representative cases are presented in online supplemental figure 7.

DISCUSSION

The safety and potential antitumor effect of SNK01 in combination with cytotoxic chemotherapy and/or cetuximab could be demonstrated in this non-clinical and phase I/IIa clinical study. By creating a CDX-humanized mouse model, a mouse model that closely mimics human immune environment was successfully made. The NK cell infusion resulted in increased blood NK cell concentration in the mouse and NK cell infiltration into the

Table 3 Efficacy outcome in the clinical study

Clinical outcome	Total (n=12)	SNK01 with GC (n=6)	SNK01 with C/GC (n=6)
Objective response rate	3 (25.0)	0 (0.0)	3 (50.0)
Disease control rate	12 (100)	6 (100)	6 (100)
Partial response	3 (25.0)	0 (0.0)	3 (50.0)
Stable disease	9 (75.0)	6 (100)	3 (50.0)
Progressive disease	0 (0.0)	0 (0.0)	0 (0.0)
Median progression-free survival (days)	143 (107–246)	143 (107–246)	145 (114–N/E)

Data are presented as number (percentage) or median (IQR).
.C/GC, cetuximab/GC; GC, gemcitabine/carboplatin.

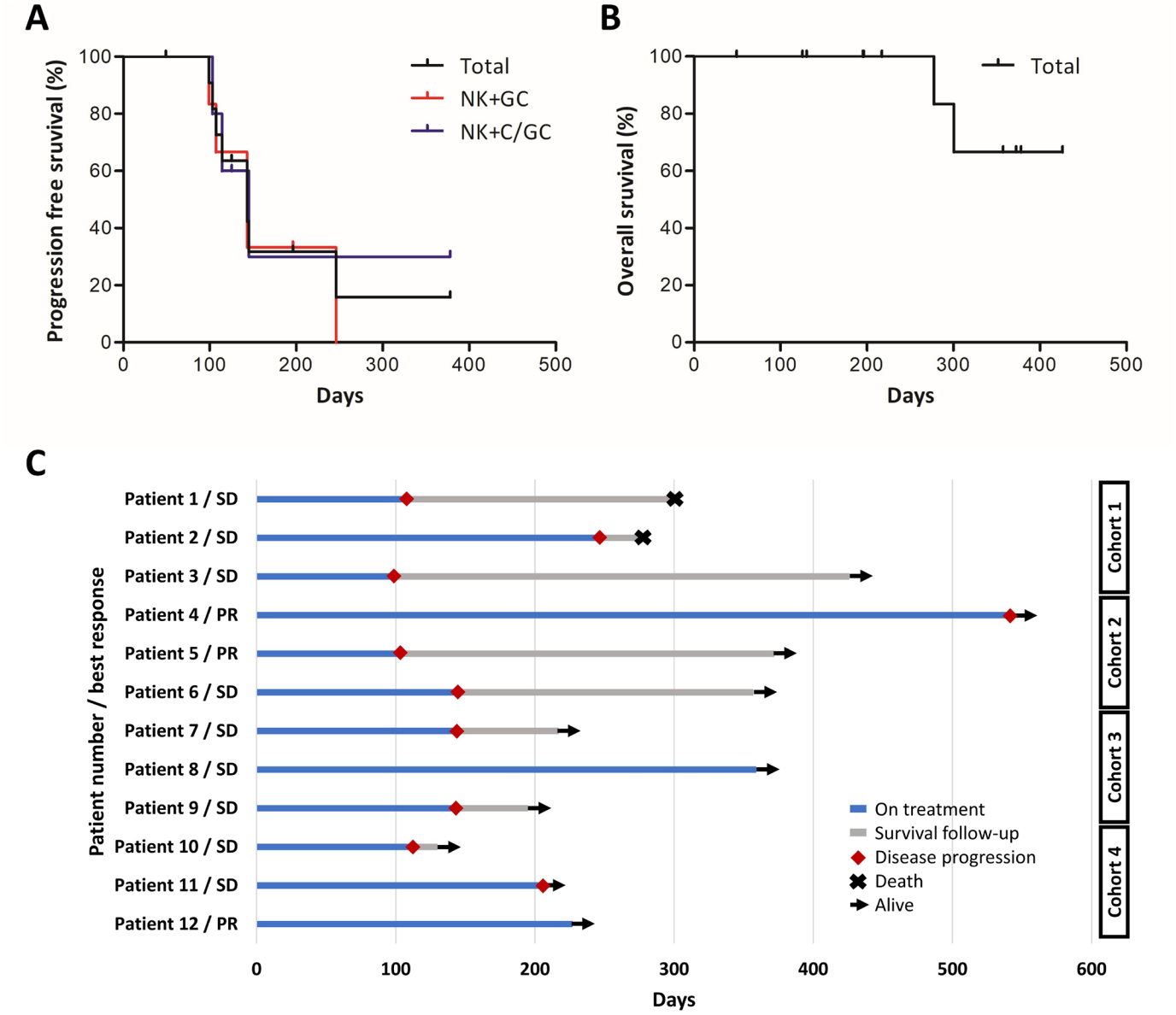

Figure 4 Kaplan-Meier curves for progression-free survival and overall survival and the timeline according to treatments in the clinical study. (A) Progression-free survival. (B) Overall survival. (C) Treatment timeline in each patient. C/GC, cetuximab/gemcitabine/carboplatin; cohort 1, SNK01 (4×10^9 cells/dose)+gemcitabine/carboplatin; cohort 2, SNK01 (4×10^9 cells/dose)+cetuximab/gemcitabine/carboplatin; cohort 3, SNK01 (6×10^9 cells/dose)+gemcitabine/carboplatin; cohort 4, SNK01 (6×10^9 cells/dose)+cetuximab/gemcitabine/carboplatin; GC, gemcitabine/carboplatin; PR, partial response; SD, stable disease;

extracted mouse tumors. In the clinical trial, NK cell activity increased after SNK01 infusion. A safe profile of SNK01 in combination with cytotoxic chemotherapy was confirmed, and an MFD of 6×10^9 cells/dose for SNK01 was determined. The DCR was 100%, and particularly in the SNK01 plus cetuximab/gemcitabine/carboplatin group, a satisfactory efficacy with an ORR of 50% was observed.

To the best of our knowledge, this study is the first to investigate the effects of NK cells with cetuximab in NSCLC, which was conducted simultaneously with non-clinical CDX mouse model study and phase I/IIa clinical trial. To date, several in vitro studies on the combination therapy of NK cells and cetuximab for various cancer types have been conducted. Veluchamy *et al* reported the antitumor effects of peripheral blood NK cells with cetuximab in a colorectal cell line in an in vitro study.[19] They specifically showed that cetuximab enhances the cytotoxic activity of NK cells against EGFR-expressing tumor cells. Mallmann-Gottschalk *et al*, in an in vitro study, reported that coincubation of cetuximab and NK cells increased the cytotoxicity of NK cells and enhanced NK-specific tumor cell lysis in an ovarian cancer cell line that had been pretreated with anti-EGFR TKIs, such as erlotinib and gefitinib.[18]

Two clinical studies on the combination of NK cells and cetuximab also have been conducted. Lim *et al* conducted a phase I trial on recurrent/metastatic nasopharyngeal carcinoma (n=7) with an EGFR positivity of more than 80%.[22] This trial involved the administration of two doses

of NK cells (1×10^6 NK cells/kg or 1×10^7 NK cells/kg on cycles 1 and 4) in combination with cetuximab. No intolerable side effects were reported, and four patients were found to have SD and three experienced PD. Phase I/II clinical trials on NK cell in combination with cetuximab have also been conducted in patients with NSCLC. Liang *et al*, in 54 patients with advanced EGFR-expressing NSCLC, compared safety and efficacy between allogenic NK cell infusion plus cetuximab (n=27) and cetuximab monotherapy (n=27).[23] All AEs were manageable, and the combination group exhibited an ORR of 14.8%, which was higher than the ORR of 7.4% in the cetuximab monotherapy group.

Regrading safety, similar to previous clinical studies,[22 23] we demonstrated the safety of autologous NK cell therapy in this clinical study. Although 5 out of 12 patients experienced ≥grade 3 AEs (neutropenia in 5/12 patients (41.7%); anemia in 1/12 patients (8.3%)), these were all AEs associated with cytotoxic chemotherapy and were similar to or lower than the rates of ≥grade 3 AEs reported in previous clinical studies of cytotoxic chemotherapy (neutropenia, 30.3%–50%; anemia, 18%–46%).[31 32] On the other hand, regarding study design, the previous study by Liang *et al*[23] of NK cells with cetuximab in NSCLC had some differences from our clinical study. First, because this study did not compare combination therapy with NK cell monotherapy, it could not be determined whether the observed effect was due to the efficacy of combination therapy or the standalone effect of NK cell therapy. In addition, there is a limitation in reflecting the current clinical setting, as there is no evidence for the effect of cetuximab monotherapy in NSCLC, and it is not currently selected as a recommended treatment option in EGFR-mutated NSCLC without prior TKIs.[33]

Thus, to reflect a real clinical setting, we aimed to conduct a study on cell lines or patients with EGFR-mutated NSCLC who have already developed resistance to osimertinib or other EGFR TKIs. A previous study by Janjigian *et al*, in a setting similar to the present study, reported that the combination of cetuximab and afatinib showed favorable efficacy in patients with EGFR-mutated NSCLC who have failed prior TKI therapy.[13] This study hypothesized, regarding the mechanism of efficacy that cetuximab, an EGFR antibody, induces ligand binding and receptor degradation, whereas afatinib, a phospho-EGFR activity inhibitor, works in synergy to achieve antitumor effects.[13] Moreover, this study suggested that cetuximab enhances ADCC. The findings of this study have been incorporated into the NCCN (National Comprehensive Cancer Network) guidelines as a therapeutic option for patients with failed prior TKI therapy.[33] By demonstrating the potential efficacy of NK cell in combination with cytotoxic chemotherapy and/or cetuximab in a similar clinical setting, the present study has contributed valuable insights for future research in patients who have failed previous treatment with TKI, including osimertinib, with limited treatment options.

It has been hypothesized that ADCC is the primary mechanism of NK cell plus cetuximab therapy. Several preclinical studies have reported ADCC in various cancer types.[18 19 21 34] The mechanism involves cetuximab binding to EGFR on tumor cells, inhibiting EGFR signaling, and simultaneous interaction with the FcγRIIIa/CD16 receptor on NK cells. This interaction triggers the release of granzyme B and perforin from NK cells, resulting in tumor cell death.[35–38] The present study also supported previous studies by identifying the effect of ADCC on NSCLC cell lines in vitro using NK cells isolated from patients in combination with cetuximab. Furthermore, this study shows that cetuximab not only induces ADCC but also maximizes its efficacy by concurrently administering additional NK cells, thereby increasing the number or activity of NK cells and NK cell infiltration into the tumor. In this study, a noticeable elevation in circulating NK cell levels was found in the group receiving NK cells in the CDX-humanized mouse model. Furthermore, one clinical study reported that NK cell activity, which initially declined after cytotoxic chemotherapy and/or cetuximab treatment, believed to be a result of lymphopenia caused by cytotoxic chemotherapy,[32 39 40] rebounded following NK cell infusion. It could be hypothesized that the increase in NK cell count and NK activity, NK cell infiltration into the tumor, and enhanced ADCC would all exert synergistic effects.

Another interesting finding of the present clinical study was that even in the SNK01 plus gemcitabine/carboplatin group, where cetuximab was excluded, a DCR of 100% was observed. Conventional chemotherapy is known to induce the release of stress molecules called damage-associated molecular patterns from dying or stressed tumor cells, which in turn alert the immune system, including NK cells.[41] Moreover, cytotoxic chemotherapy can stimulate the expression of death receptors on tumor cells to mediate NK cell recognition.[42] Li *et al* conducted a study on a combination of cytotoxic chemotherapy, including oxaliplatin and 5-FU, and NK cell therapy in patients with locally advanced colon carcinoma. In their in vitro study, they showed that cytotoxic chemotherapy sensitizes colon carcinoma cells and colon carcinoma stem cells to NK cell cytotoxicity. In a clinical study, they also reported a favorable outcome in a group receiving a combination of NK cell therapy and cytotoxic chemotherapy.[43] It is noteworthy that in our in vitro experiments, osimertinib-resistant cell lines exhibited a better response to NK cell therapy compared with their parental EGFR mutant cell lines. Similarly, another in vitro study reported that TKI-resistant cell lines demonstrated a superior response to NK cell therapy than their parental cell lines.[18] The underlying mechanism, although not definitively established, is hypothesized to be due to structural changes in surface proteins on tumor cells, such as MHC 1, MICA/B, and stress-induced NKG2D ligands including ULBP, induced by EGFR-TKI treatment, which may enhance NK cytotoxicity.[18] We believe that the effect of NK cell sensitization caused by cytotoxic chemotherapy

and increased NK cell cytotoxicity against TKI-resistant NSCLC cells should not be ignored.

The present study has several limitations. First is the small sample size in both the non-clinical and clinical studies, resulting in limited statistical power. Nonetheless, it can be expected that these results will serve as a basis for future large-scale studies. Second is that this study did not clearly elucidate the mechanism of the effect of the NK cell plus cytotoxic chemotherapy and/or cetuximab in in vivo. Given that no definitive hypothesis has been established in previous studies, various possibilities need to be considered, including ADCC, as confirmed in our in vitro study. In addition, it can be suggested that the increase in NK cell count and activity in the blood and tissues following NK cell infusion is associated with the efficacy of treatment. Furthermore, due to the short-term period of the clinical trial, it failed to show survival benefits, and we did not compare this combination treatment with other known subsequent treatments, such as immune checkpoint inhibitors or cytotoxic chemotherapy. However, as a phase I/IIa and non-clinical study, we believe that highlighting the potential of a new treatment option for patients with limited alternatives, particularly those resistant to osimertinib, is significant. Further long-term clinical studies are warranted to confirm its effect compared with immune checkpoint inhibitors or cytotoxic chemotherapy. Lastly, in the clinical trial, the treatment agents other than SNK01 used in the non-clinical and clinical trial groups were heterogeneous. This is because cetuximab does not play a significant role in NSCLC treatment, and at present, regarding the subsequent treatment following TKI failure, it is not recommended as a standard therapy by the NCCN guidelines.[33] Given the real clinical practical considerations, we had no option but to incorporate standard treatments with SNK01 and cetuximab.

In conclusion, these non-clinical and early phase I/IIa clinical studies have demonstrated that autologous NK cell therapy, in combination with cytotoxic chemotherapy and/or cetuximab, offers a safe and potentially effective treatment for patients with EGFR-mutated NSCLC who have developed resistance to TKIs, including osimertinib. In addition, we have suggested an alternative treatment option to overcome osimertinib resistance. This study provides reference information and supports further investigations into this alternative treatment.

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Contributors C-MC designed the non-clinical study and developed the protocol. JCL designed the clinical study and developed the protocol. MGC, GWS, MYC, JSJ, JKR, BGY, J-MJ, YMK, D-HK and C-MC conducted experiments, acquired nonclinical data, and interpreted nonclinical data. MGC, JSJ, WJ, JCL and C-MC acquired clinical data and interpreted clinical data. MGC, GWS, JSJ, JKR, JCL and C-MC wrote the manuscript. All authors edited the manuscript. C-MC and JCL are the guarantors of the study. The order of the co-first authors was determined based on contribution to data analysis, interpretation of results, and writing manuscript.

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Ethics approval This prospective clinical trial was approved by the institutional review board of Asan Medical Center (IRB No. 2021-0354) and registered at clinical research information service (NCT04872634). All participants provided informed consent before enrolment. The trial was designed and conducted in accordance with the principles of the Declaration of Helsinki and the ethical guidelines for clinical studies. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Asan Medical Center (IACUC No. 2021-14-287).

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