이학석사 학위논문

Cytotoxicity of NK92-CD16 cells on NSCLC cell line resistant to TKI

티로신 키나아제 억제제에 대한 획득내성이 생긴 비소세포폐암에서 NK92-CD16 세포의 항암 효과 연구

2017년 8월

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협동과정 종양생물학 전공

박 하 람

A thesis of the Degree of Master of Science

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이 논문을 이학석사 학위논문으로 제출함

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Cytotoxicity of NK92-CD16 cells on NSCLC cell line resistant to TKI

by

Ha-Ram Park

(Directed by Professor Dae Seog Heo, M.D., PhD)

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Professor _____ **Chairman**

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ABSTRACT

Cytotoxicity of NK92-CD16 cells on NSCLC cell line resistant to TKI

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Purpose: The use of tyrosine kinase inhibitor (TKIs) improved outcome of non-small cell lung cancer (NSCLC) patients harboring targetable driver mutation. However, the most patients eventually showed disease progression due to the acquired resistance to TKIs by various mechanisms including gatekeeper mutation and alternative pathway activation. As immunotherapy can be considered for these patients to override drug resistance, I investigated the efficacy of NK92-CD16 cells (CD16-transduced NK-92 cell line) to TKI-resistant NSCLC cells.

Methods: TKI-resistant NSCLC cells (H3122CR1, H3122LR1, H3122CR1LR1, EBC-CR1, EBC-CR2, PC-9GR and PC-9ER) were established from NCI-H3122 (*EML4-ALK* fusion), EBC-1 (*MET* amplification), and PC-9 (*EGFR* exon 19 deletion) after continuous exposure to crizotinib, ceritinib, capmatinib, gefitinib, and erlotinib. NK cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) using anti-EGFR monoclonal antibody (mAb) cetuximab were measured using 'off-the-shelf' NK92-CD16 as effectors and detected by the ^{51}Chromium-

release assay. Expression of the ligands for NK cell receptors and total EGFR were analyzed by flow cytometry.

Results: Most of TKI-resistant NSCLC cell lines were more susceptible to NK92-CD16 cell compared with their parental cell lines. The expression of ICAM-1, which is a ligand for LFA-1 in NK cells, is higher in TKI-resistant NSCLC cells than in parental cells and is expected to be correlated with NK92-CD16 cytotoxicity. When ICAM1-CD11a interaction was blocked during a cytotoxic assay, the cytotoxicity was decreased. Cetuximab-mediated ADCC was higher in resistant cells due to the increased expression level of total EGFR in resistant cells.

Conclusions: TKI-resistant NSCLC cells are more sensitive to NK92-CD16 cell-mediated cytotoxicity that is partially dependent on up-regulation of ICAM-1 via an immunological synapse. In addition, cetuximab, an EGFR-targeting mAb, significantly increases NK cell cytotoxicity in TKI-resistant NSCLC cells. Taken together, NK-cell based immunotherapy with cetuximab might be feasible to treat NSCLC patients with acquired resistance to TKIs.

Keywords: NK-92, Non-small cell lung cancer, acquired resistance

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INTRODUCTION

Lung cancer is one of the most causes of cancer-related death [1]. Only 15.9% of patients diagnosed with lung cancer survive for 5 years [2]. Non-small cell lung cancer (NSCLC) accounts for more than 85% of lung cancers and nearly half of all NSCLC patients have been found to have oncogenes that can be targeted and inhibited with tyrosine kinase inhibitors (TKIs) [3]. Most NSCLC patients with these oncogenic drivers showed dramatic responses to TKIs, but the cancer cells eventually acquired resistance to the TKIs within a year through various mechanisms, including gate keeper mutation, bypass tract activation, and histological transformation [4-7]. Specifically, patients with *EGFR*-mutant NSCLC who did not have *EGFR* T790M mutation showed only one-year survival after progression of EGFR inhibitor [8]. Although next-generation TKIs have been developed to overcome resistance, additional resistance mechanisms arose in the cancer cells [9]. Recently, programmed cell death-1 (PD-1) and programmed death ligand 1 (PD-L1) blockades have been developed and approved for the treatment of NSCLC [10-13]. Although oncogenic epidermal growth factor receptor (*EGFR*) mutations [14] and anaplastic lymphoma kinase (*ALK*) translocation [15] were found to upregulate PD-L1 expression, treatment with checkpoint inhibitors did not prolong survival in patients with *EGFR*-mutant NSCLC when compared with cytotoxic chemotherapy [16]. Therefore, other therapeutic strategies are needed urgently for NSCLC patients with oncogenic drivers who have acquired resistance to TKIs.

Natural killer (NK) cells can recognize and kill virus-infected or transformed cells without tumor antigen specificity, and allogeneic NK cells do not cause graft-versus-host disease, thus they can be transferred safely to recipients [17]. Studies have shown that many drug-resistant cancer cells express stem cell-like phenotypes

[18] and that NK cells preferentially target cancer stem cells [19, 20], thus NK cell-based therapy seems to be able to eradicate TKI-resistant cancer cells.

In this study, TKI-resistant NSCLC cell lines were established *in vitro* and NK cell cytotoxicity towards these resistant cells and their parental non-resistant cells was measured. Here, NK-92 cell line was used instead of primary NK cells to avoid donor variation due to a major histocompatibility complex-killer cell Ig-like receptor (MHC-KIR) match/mismatch. An 'off-the-shelf' NK-92 cell line was established from NK cell lymphoma and further developed for clinical uses [21, 22]. This NK-92 cell line has shown strong cytotoxicity against a variety of cancer cells when compared to primary NK cells because they nearly do not express inhibitory KIRs [23]. But NK-92 cells generally do not express CD16a (Fc□RIIIa). Thus, here a specific NK-92 cells were used, which was transduced retrovirally with high-affinity CD16 (V/V at amino acid 158), for the antibody-dependent cell-mediated cytotoxicity (ADCC) effect; these cells were named NK92-CD16 [24].

In this study, I found that several TKI-resistant NSCLC cell lines were efficiently killed by NK92-CD16 cells when compared to their parent cell lines. Interestingly, NK92-CD16 cells showed superior ADCC against TKI-resistant NSCLC cell lines when combined with cetuximab.

MATERIALS AND METHODS

Cell lines and drugs

Three NSCLC cell lines (NCI-H3122, PC-9 and EBC-1) were used in this study. The NCI-H3122 and PC-9 cells were kindly provided by Pasi A. Janne (Dana-Farber Cancer Institute, Boston, MA) and Dr. Mayumi Ono (Kyushu University, Fukuoka, Japan), respectively. EBC-1 cells were purchased from the JCRB Cell Bank (Osaka, Japan). These cell lines were cultured in RPMI1640 medium with 10% FBS. The CD16-transduced NK-92 cell line (ATCC PTA-8836, NK92-CD16) was obtained from ATCC and was cultured with rhIL-2-containing MEM-alpha media. Crizotinib, a first-generation ALK inhibitor, was provided by Pfizer (Milwaukee, WI), and ceritinib, a second-generation ALK inhibitor, was obtained from Active Biochem (Maplewood, NJ). A c-MET inhibitor, capmatinib, and EGFR inhibitors, gefitinib and erlotinib, were purchased from Selleck Chemicals (Houston, TX).

In vitro establishment of drug-resistant cell lines

TKI-resistant NSCLC cell lines were established *in vitro* by treating cells with stepwise dose escalations of TKIs. Crizotinib-resistant (H3122CR1) and ceritinib-resistant (H3122LR1) NSCLC sublines were established by exposing parental H3122 cells to 100nM-1μM of crizotinib or ceritinib. The H3122CR1LR1 cell line was established by treating cells with 100nM-1μM ceritinib after crizotinib resistance was established [25]. Gefitinib and erlotinib-resistant NSCLC sublines (PC-9GR and PC-9ER, respectively) were established by exposing the parental PC-9 cell line to 100nM-1μM of gefitinib or erlotinib. The capmatinib-resistant

NSCLC sublines (EBC-CR1 and EBC-CR2) were established by stepwise exposure to 1.5 and 2.2μM capmatinib, respectively.

Cell viability assay

NSCLC cells were seeded into 96-well plate at 5×10^3cells/well and incubated for overnight. After that, the cells were cultured in the presence each TKIs or vehicle for 72 hours. The cell viability was analyzed using the Ez-cytox (Dogen, Korea), and the absorbance was measured at wavelength 450nm in an Eon^{TM} Microplate Spectrophotometer (Biotech, Winooski, VT). IC_{50} values were determined by SigmaPlot12 (Hulinks, Japan).

^{51}Chromium-release assay

1×10^6 target cells (NSCLC cells) were labeled with 50μCi of ^{51}Cr for 1h at 37℃ and then were washed three times with complete media. For the ADCC and blocking experiments, target cells were cultured with anti-CD54 and/or cetuximab for 30min and then washed. In triplicate, effector cells (NK92-CD16 cells) and ^{51}Cr-labeled target cells (5×10^3/well) were co-incubated at various effector: target (E:T) ratios in a 96-well U-bottomed plate for 4h at 37℃. Maximum and spontaneous lysis data were obtained by incubating target cells with 100μl of 0.5% Triton X-100 and complete media, respectively. After incubation, 75μl of supernatant was harvested from each well and radioactivity in each sample was measured with a gamma counter (Packard Cobra II). The percent specific cytolysis was calculated using the formula: percent specific lysis = [experimental counts per minute (cpm) – spontaneous cpm/maximum cpm – spontaneous cpm] × 100.

Antibodies and flow cytometry

The following FACS mAbs were used; Mouse monoclonal antibodies against human EGFR (clone EGFR.1), ICAM-1 (clone HA58), CD58 (clone 1C3), CD48 (clone TU145), MIC A/B (clone 6D4), MHC I (clone G46-2.6) and CD95 (clone DX2) were purchased from BD Biosciences (San Jose, CA, USA). And ULBP1, ULBP2,5,6, UBBP3, DR5 were obtained from R&D systems (Minneapolis, MN, USA) and CD112 (clone TX31) and CD155 (clone SKII.4) were from BioLegend (San Diego, CA, USA). An HLA-E (clone 3D12) was purchased from Miltenyi Biotec (Bergich Gladbach, Germany). A CD54 blocking antibody (clone HDC54) was from BioLegend (San Diego, CA, USA). Flow cytometry analysis was performed using FACSCalibur (BD Biosciences) to observe the expression level. Data were analyzed using FlowJo software version 7.6 for Windows (FlowJo, LLC, Ashland, OR, USA).

Western blot

The harvested cells were lysed with lysis buffer. Equal amounts of protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred PVDF membrane. The membrane was blocked with PBS containing 5% skim milk (BD bioscience) and 0.05% Tween 20 (MP biomedicals, Illkirch, France). The proteins of interest were detected with rabbit monoclonal antibody and HRP-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, SC-2004). For visualization, the ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, UK) was used as an HRP substrate. Total EGFR (D38B1), CD133 (D4W4N), GAPDH (D16H11) antibodies were purchased from Cell Signaling Technology (Danvers, MA). The blots were subjected to imaging analysis with ImageQuant™ LAS 4000 mini (GE

Healthcare). All antibodies and reagents were used according to the manufacturer's instructions.

RT-PCR

Total RNA was extracted from each cell lines using the RNeasy® Plus Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed using Superscript® First-strand synthesis system (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed with oligo$(dT)_{20}$ Primer according to recommended protocol.

Quantitative real-time PCR

cDNA was prepared as described above. The StepOnePlus™ Real-time PCR System (Applied Biosystems, CA, USA) was used for cDNA amplification and analysis. The *Power* SYBR® Green PCR Master Mix (Applied Biosystems, CA, USA) was used according to the manufacturer's instruction. Relative quantification of the gene was calculated using $\Delta\Delta C_t$ (comparative Ct) method with β-actin as the internal reference gene. RT-qPCR cycling conditions were 95℃ for 10min, followed by 45 cycles at 95℃ for 15sec, 55℃ for 1min and 72℃ for 30sec. The Primers used for amplification of β-actin, ALDH1, CD133 are listed in Table 1.

Statistical analysis

Data comparisons and statistical significance were determined using the paired *t*-test and data were represented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism software version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA)

Table 1. Primers used in quantitative real-time PCR.

Gene	Primer	Sequence
β-actin	β-actin (F)	5’-CAA TGA GCT GCG TGT GGC T-3’
	β-actin (R)	5’-TAG CAC AGC CTG GAT AGC AA-3’
ALDH1	ALDH1 (F)	5’-CCA AAG ACA TTG ATA AAG CCA TAA-3’
	ALDH1 (R)	5’-CAC GCC ATA GCA ATT CAC C-3’
CD133	CD133 (F)	5’-CAC TAC CAA GGA CAA GGC GT-3’
	CD133 (R)	5’-TCC TTG ATC GCT GTT GCC AT-3’

RESULTS

Establishment of NSCLC cell lines with acquired resistance to various TKI

Here TKI-resistant NSCLC cell lines were generated from three parent cell lines: NCI-H3122, EBC-1, and PC-9. First, the NSCLC cell lines resistant to crizotinib, ceritinib and crizotinib + ceritinib (H3122CR1, H3122LR1, and H3122CR1LR1, respectively) from the parental cell line NCI-H3122 harboring an *echinoderm microtubule associated protein like 4* (*EML4*)-*ALK* gene rearrangement. The *EML4-ALK* gene rearrangement which is found in 4-6% of NSCLC patients, especially in never smokers [26, 27]. Even though ALK kinase inhibitor crizotinib show a great response rate in these patients, acquired resistance is eventually developed in most of the patients [28]. The second-generation ALK inhibitor ceritinib (LDK378) was developed to overcome this, but the resistance was also developed [29].

Two of EGFR TKIs (gefitinib and erlotinib) resistant NSCLC cell lines were also generated from PC-9 cells harboring a *EGFR* exon 19 deletion mutation, which is the most common mutation among *EGFR* mutations [30]. In addition, capmatinib-resistant NSCLC cell lines, EBC-CR1 and EBC-CR2, were established from an EBC-1 cell line harboring the amplification of the *mesenchymal-epithelial transition factor* (*MET*) gene, which encodes receptor tyrosine kinase c-MET. All of the TKI-resistant cells were successfully established *in vitro* from their parental cell lines by the dose escalation method [25]. Acquired cellular drug resistance was confirmed by performing cell viability assays and measuring IC_{50} values (Table 2 and Figure 1).

Table 2. TKI-resistant cell lines and inhibitory effects of TKIs.

Histology	Parent cell lines	Resistant cell line	Used TKIs	IC50(μM)	
Lung adenocarcinoma	NCI-H3122 (*EML4-ALK* fusion)			Crizotinib 0.15±0.01	Ceritinib 0.05±0.004
		NCI-H3122CR1	Crizotinib	0.96±0.02	0.35±0.03
		NCI-H3122LR1	Ceritinib	5.48±0.4	1.21±0.07
		NCI-H3122CR1LR1	Crizotinib & Ceritinib	4.45±0.26	1.16±0.09
	PC-9 (*EGFR* exon 19 deletion)			Gefitinib 0.015	Erlotinib 0.006
		PC-9GR	Gefitinib	>10	>10
		PC-9ER	Erlotinib	11.05	3.48
Lung squamous cell carcinoma	EBC-1 (*MET* amplification)			Capmatinib 0.0037±0.0001	
		EBC-CR1	Capmatinib	>10	
		EBC-CR2			

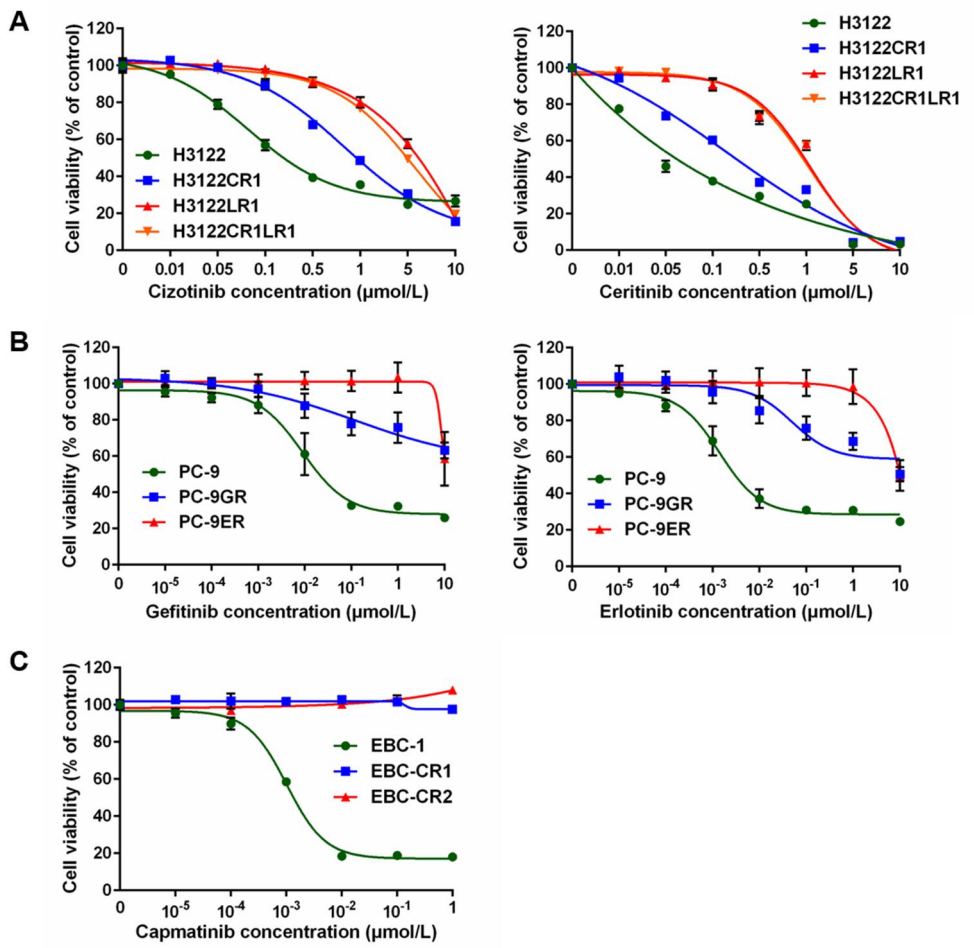

Figure 1. Establishment of TKI-resistant cells from NSCLC cell lines.

Establishment of TKI-resistant cells was confirmed using cell viability assays. Cell viability assays were performed Simultaneously with parental and TKI-resistant cells. The cells were treated with each TKI or vehicle for 72 h. (**A**) H3122, H3122CR1, H3122LR1, and H3122CR1LR1 cells were treated with 0-10μM crizotinib (left) or ceritinib (right). (**B**) PC-9, PC-9GR, and PC-9ER cells were treated with 0-10μM gefitinib (left) or erlotinib (right). (**C**) EBC-1, EBC-CR1, and EBC-CR2 cells were treated with 0-1μM capmatinib. All experiments were independently performed at three times.

NK92-CD16 cells preferentially target TKI-resistant NSCLC cells than parental cells

To evaluate the efficacy of NK cell-based immunotherapy for TKI-resistant NSCLC cells, the NK cytotoxicity was measured by ^{51}Chromium-release assay using NK92-CD16 effector cells. NK cytotoxicities were significantly higher for the TKI-resistant cells than for their parental cell liens (Figure 2). Because the NK-92 cell line does not express KIRs, which recognize cognate ligands, such as MHC class I molecules, on target cells [21], thus there was no variation due to MHC-KIR binding, which would occur in primary NK cells.

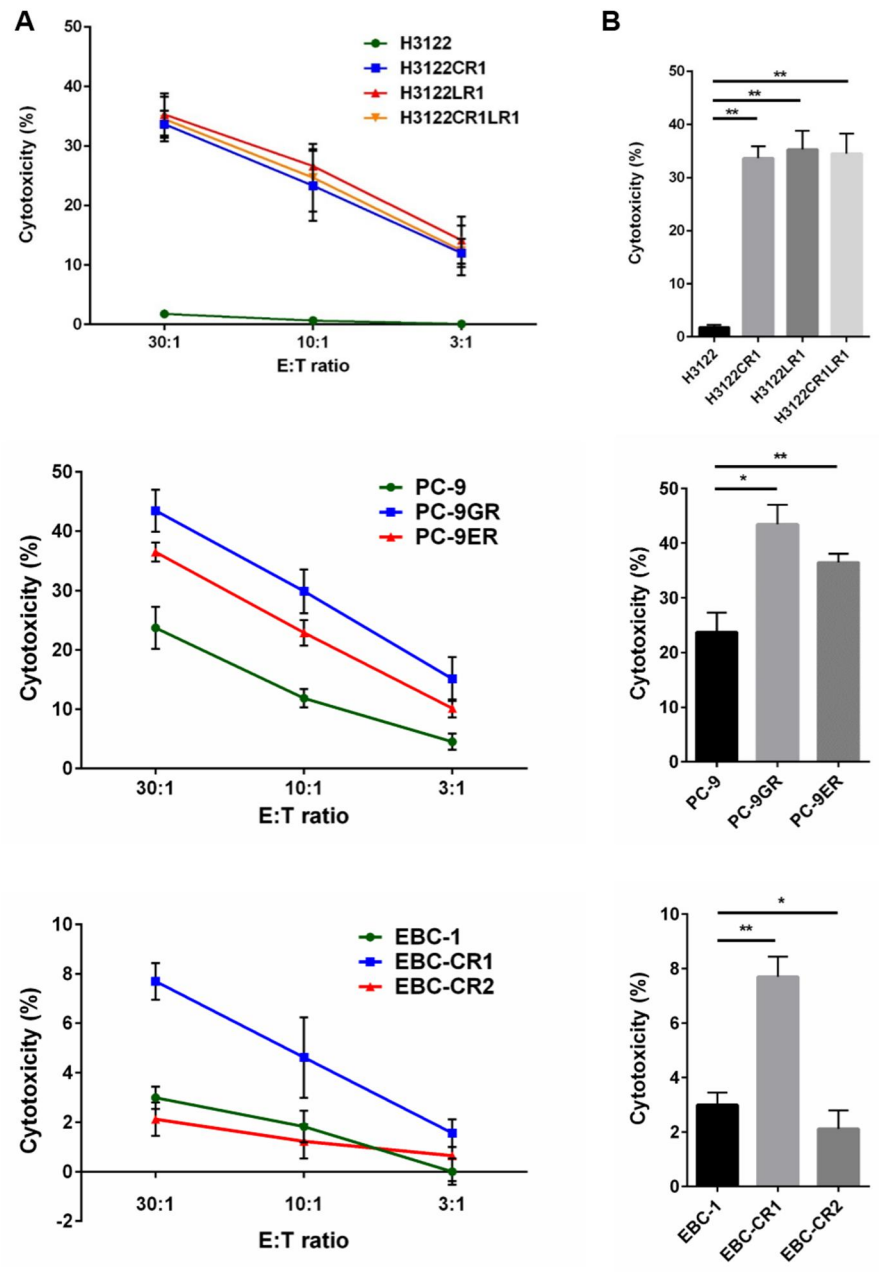

Figure 2. Sensitivities of NSCLC cell lines to NK92-CD16 cells.

(**A**) The NK92-CD16 cytotoxicity towards NSCLC cells was evaluated using the ^{51}Chromium-release assay with various E:T ratios. (**B**) The bar graphs represent the NK92-CD16 cytotoxicity at ratio E:T of 30:1. All of the experiments were performed three independent times and in triplicate. *p<0.05, **p<0.01.

ICAM-1 expression level of TKI-resistant NSCLC cells correlates with NK92-CD16 cytotoxicity

To determine whether the stem cell-like characteristics of cancer cells are related to the cytotoxicity of NK cells, the expression level of CD133 and ALDH1, which are known as cancer stem cell marker of lung cancer [31], was examined at RNA and protein levels. However, in all NSCLC cell lines, the changes of CD133 and ALDH1 were not observed in RNA level (Fig. 3A). In addition, CD133 showed no significant difference even at protein level and was extremely low expressed in all NSCLC cells (Fig. 3B)

To elucidate the mechanism of TKI-resistant NSCLC cell line susceptibility to NK92-CD16 cells, next, the changes in ligands of NK cell receptors were measured in TKI-resistant and parental cells. Several ligands, including NKG2D ligands (MIC A/B and ULBPs), 2B4 ligand (CD48), and DNAM-1 ligands (CD112 and CD155) were evaluated by flow cytometry, but no significant differences in the ligands were found between the parental and resistant cells (Figure 4). However, some changes were found that ICAM-1 (Intracellular adhesion molecule 1, also known as CD54) was upregulated in almost all of the TKI-resistant cancer cells when compared to the parental cells (Fig. 5A). I then blocked ICAM-1 in the cytotoxicity assay with a monoclonal antibody to ICAM-1 to confirm the association between ICAM-1 and NK92-CD16 cell cytotoxicity. I found that NK92-CD16 cell cytotoxicity was reduced for all of the cell lines except EBC-CR2 when ICAM-1 was blocked (Fig. 5B), and this may be due to low levels of ICAM-1 expression in EBC-1 and EBC-CR2 cells. Thus, I hypothesized that enhanced NK cell cytotoxicity is partially mediated by upregulation of ICAM-1 in TKI-resistant cells.

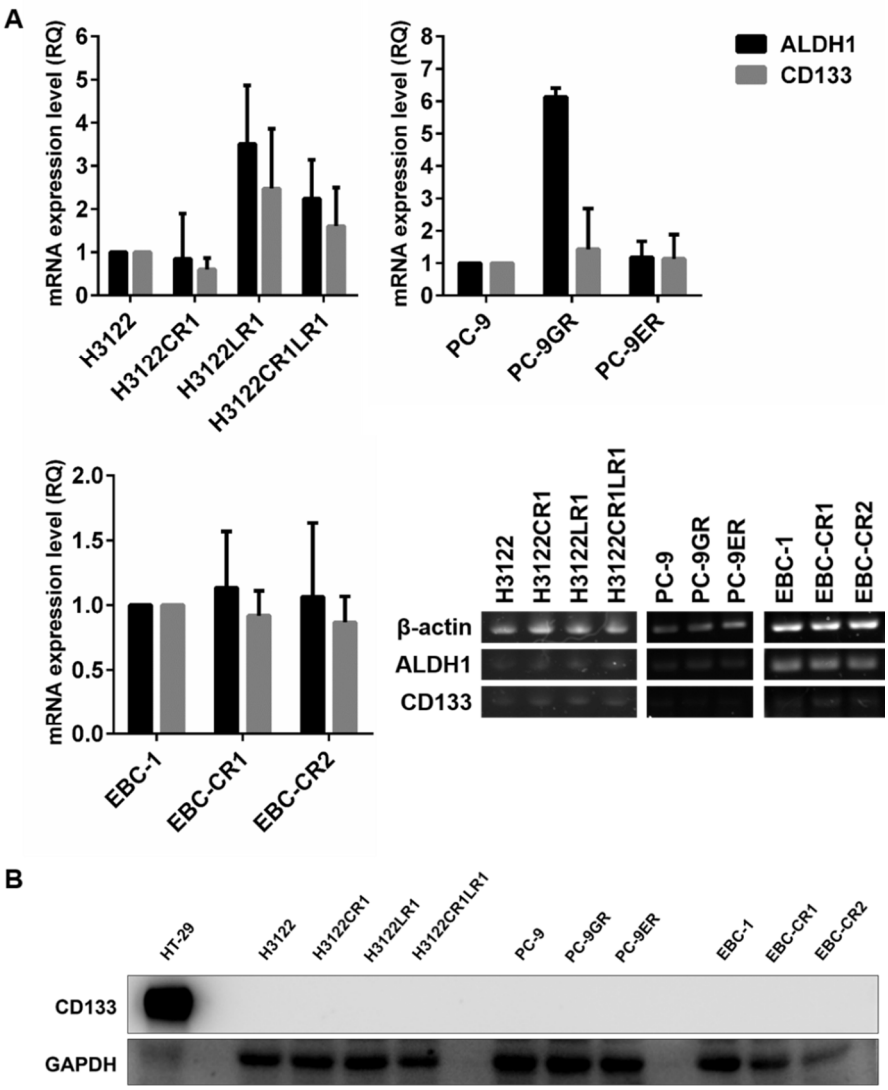

Figure 3. CD133 and ALDH1 expression level in the NSCLC cells.

(**A**) Relative mRNA expression level of CD133 and ALDH1 in NSCLC cells were determined by quantitative real-time PCR (RT-qPCR). The amplified products were confirmed by 1% agarose gel electrophoresis. (**B**) The protein expression levels of CD133 and ALDH1 were determined by western blot analysis. They were rarely expressed in all of NSCLC cells. The HT-29 human colorectal adenocarcinoma cell line (ATCC® HTB38™) was used as a positive control. All of the experiments were performed three independent times and RT-qPCR was performed in triplicate.

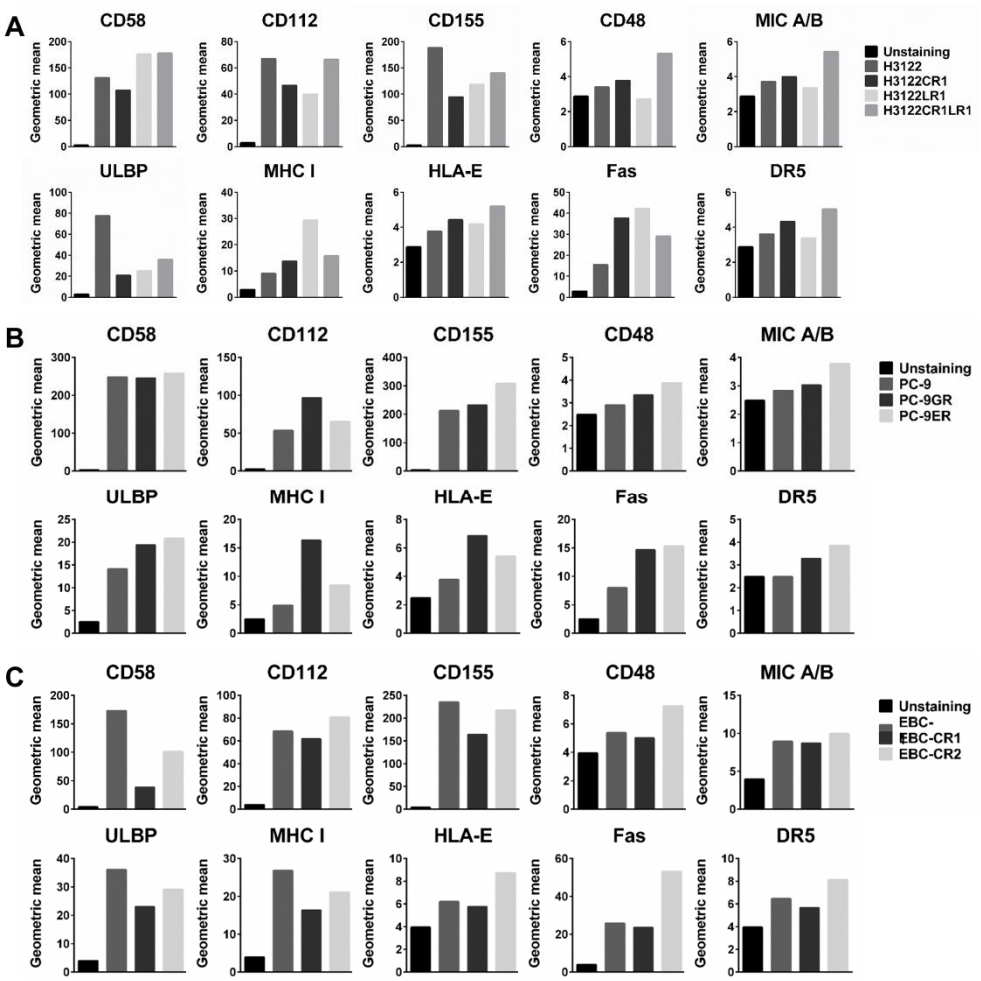

Figure 4. The expression level of ligands for NK cell receptors in NSCLC cells.

Expression of NK cell receptor ligands was measured by flow cytometry. There were no significant differences in ligand expression between parental cells and resistant cells. The bar graphs represent the expression of NK cell receptor ligands (**A**) in H3122, H3122CR1, H3122LR1, H3122CR1LR1 cells. (**B**) in PC-9, PC-9GR, and PC-9ER cells. (**C**) in EBC-1, EBC-CR1, and EBC-CR2 cells. The FMO controls are represented by black bars.

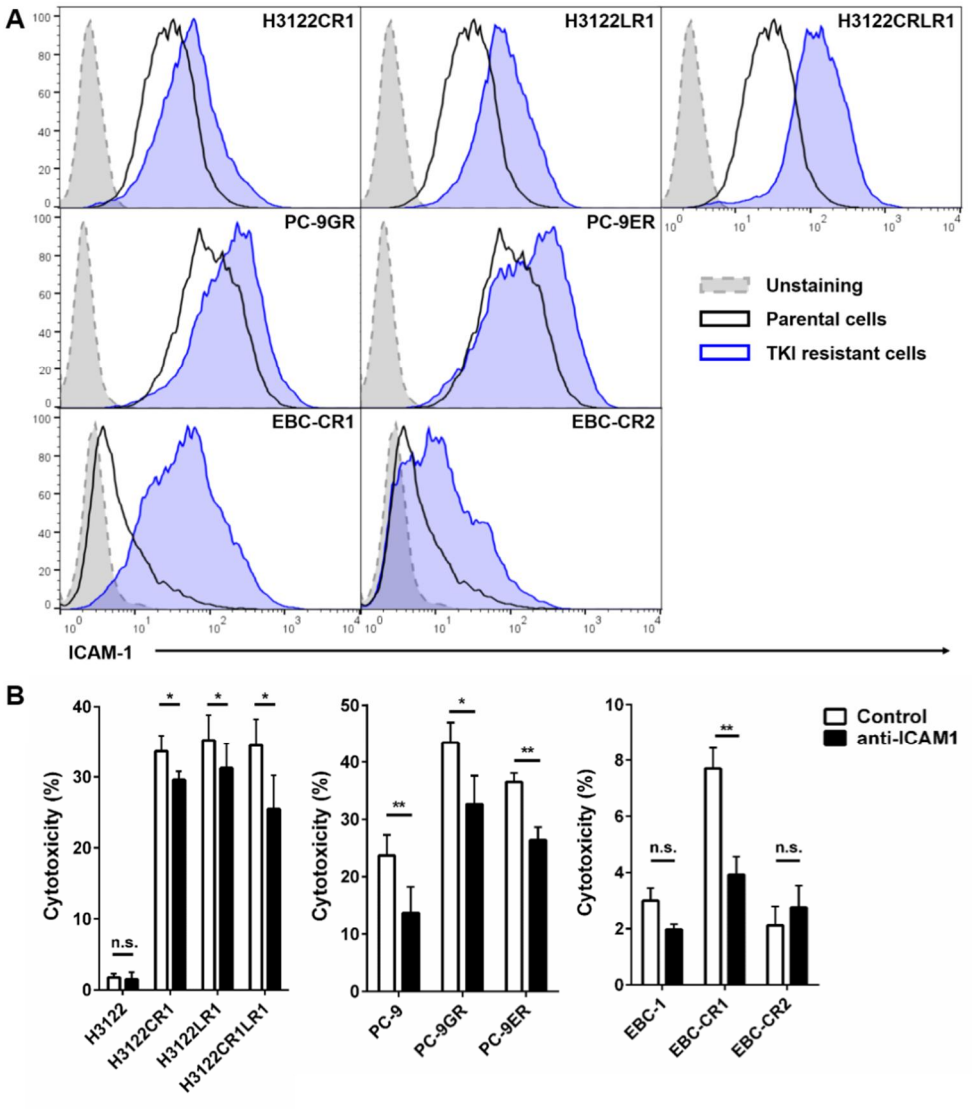

Figure 5. ICAM-1 expression levels and NK92-CD16 cell cytotoxicities to NSCLC cells.

(**A**) Flow cytometry showed that ICAM-1 expression was higher in TKI-resistant NSCLC cells than in the parent cells (**B**) The NK92-CD16 cytotoxicities in the presence or absence of an antibody that blocks ICAM-1 was evaluated by the ^{51}Chromium release assay. All of the experiments were performed three independent times in triplicates. *$p<0.05$.

Synergistic effect of NK92-CD16 and cetuximab against TKI-resistant NSCLC cells

Although ICAM-1 expression has partial effects on the NK92-CD16 cytotoxicities, activating/co-activating signals are required for efficient degranulation and killing of target cells [32-34]. To address this, I measured ADCC towards NSCLC cells in the presence of cetuximab, an EGFR-targeting monoclonal antibody. In the presence of cetuximab, NK92-CD16 cell cytotoxicity towards all of the TKI-resistant NSCLC cell lines increased significantly (Fig. 6A). Moreover, relatively NK cell-insensitive EBC-1 and EBC-1-derived TKI-resistant cells were efficiently killed upon the addition of cetuximab. EGFR expression was upregulated in PC-9-derived and EBC-1-derived TKI resistant cells when compared to their parent cells (Fig 6B and 6C). Furthermore, ADCC effect rises superior to ICAM-1 blocking (Fig 6A, black bars).

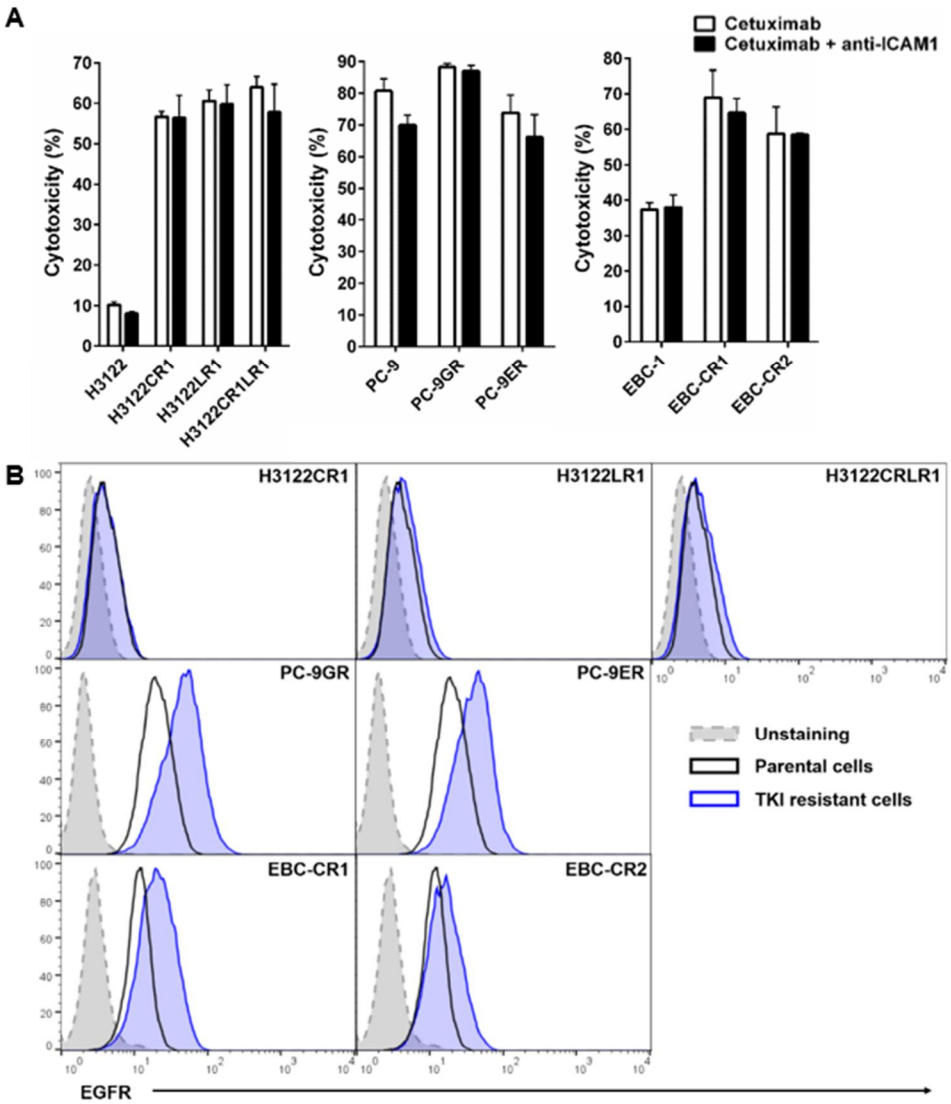

Figure 6. NK92-CD16 showed higher cytotoxicity against NSCLC cells with cetuximab.

(**A**) ADCC assays were performed with an E:T ratio of 30:1 in the presence of an antibody that blocks ICAM-1 (black bars) or in the absence of an antibody that blocks ICAM-1 (white bars). All of the experiments were performed three independent times and in triplicate. (**B**) EGFR expression level analyzed using Flow cytometry. All of NSCLC cells were expressed EGFR. And EGFR expression is upregulated in TKI-resistant NSCLC cells except H3122 cell lines.

DISCUSSION

Although TKIs are sensitive to NSCLC with driver mutations or rearrangements, their benefits were usually short due to acquired resistance to TKIs by various mechanisms. Many researchers have studied and reported various resistant mechanisms and the changes of drug-resistant cells and tried to overcome TKI-resistant cancer [4, 6, 29, 35, 36]. Although next-generation TKIs or strategies targeting bypass alterations were used in TKI-resistant NSCLC patients, duration of response was not durable. In addition, benefits of checkpoint blockades have not been well elucidated in TKI-resistant settings. Therefore, a novel and resistance overcoming strategy are urgent in patients with TKI-resistant NSCLC. Hence, I evaluated the effectiveness of NK92-CD16 cell therapy in TKI-resistant NSCLC cells in this study.

The first reason why NK92-CD16 cell line was used in this study is that NK92-CD16 expresses inhibitory KIR at low frequencies, so it shows superior cytotoxicity to primary NK cells. In addition, the safety of NK92-CD16 was proved by a few clinical trials [21]. Considering these various aspects, I thought NK92-CD16 could be easily applied cancer patients compared other cell therapies.

First of all, the TKI-resistant cells were established from various NSCLC cell lines (NCI-H3122, PC-9, EBC-1). And the resistant mechanisms (e.g. secondary mutations or any alteration) were checked in TKI-resistant cells. In the TKI-resistant cells derived form NCI-H3122, there are no secondary ALK and bypass tract mutations, but, PD-L1 expression was upregulated (unpublished data). In the TKI-resistant cell lines derived from PC-9, PC-9GR and PC-9ER, there was no *EGFR* T790M mutation and *MET* amplification (data not shown). And EBC-CR1 and EBC-CR2 showed ligand-dependent EGFR activation and MET/EGFR

heterodimers, respectively, as a result of capmatinib resistant mechanism (unpublished data).

To identify the efficacy of NK92-CD16 cells, I performed the ^{51}Chromium-release assay and observed the cytotoxicity of the NK92-CD16 cells. As a result, I found almost TKI-resistant NSCLC cells were preferentially killed by NK92-CD16 cells compared to parent cells. And also I identified the ICAM-1 expression level in cancer cells is correlated with the NK92-CD16 cytotoxicity by screening ligands interacting with NK cell receptors. ICAM-1 is a ligand of adhesion molecule LFA-1 (CD11a/CD18) expressed in NK cells (both in NK92-CD16 and primary NK cell) and involved in the formation of immunological lytic synapse [37]. Although NK92-CD16 cytotoxicity did not show a complete decrease when ICAM-1 was blocked, but significant enough. Based on these data, I hypothesized the stabilization of immunologic synapse due to up-regulated ICAM-1 in the resistant cells was one of the mechanisms, but more comprehensive study is needed.

And when NK92-CD16 cytotoxicity was measured in the presence of cetuximab, it was greatly increased through the ADCC effect. Especially, EBC-1 cell lines (EBC-1, EBC-CR1, and EBC-CR2) showed the marginal cytotoxic effect to NK92-CD16 cells alone, but their susceptibilities to the NK92-CD16 cells were greatly increased when NK92-CD16 with cetuximab were treated together. As previously mentioned, the capmatinib-resistant mechanisms of EBC-CR1 and EBC-CR2 are related to EGFR signaling activation (ligand-dependent EGFR activation and MET/EGFR heterodimer formation). Taken together, this combination therapy of NK92-CD16 and cetuximab has the synergetic effects of direct killing of tumor cells via NK92-CD16 and EGFR signal inhibition through cetuximab. In this respect, this combination therapy may be more simple and effective compared to chimeric antigen receptor (CAR)-engineered T cell or NK cell. Additionally, it also becomes an effective treatment for EGFR-upregulated NSCLC patients.

Because *EGFR* overexpression and EGFR signaling activation frequently occur in NSCLC patients and had a significantly worse outcome, it is necessary to seek a suitable treatment [38]. In this respect, combination therapy of NK92-CD16 may be helpful NSCLC patients which overexpressed or highly activated EGFR. Cetuximab is a chimeric immunoglobulin G1 monoclonal antibody and binds to EGFR with high specificity [39]. Thus it works to interrupt the down-stream signal of EGFR (cell growth, progression, and angiogenesis) through competition to EGFR ligand, internalization, and degradation of EGFR [40].

And there is one more point need to focus. As mentioned before, the PD-L1 expression level was up-regulated in TKI-resistant NSCLC cells derived from the NCI-H3122 parent cell. PD-L1 (also known as B7-H1, or CD274) interacts with PD-1 (also known as CD279) and assists immune evasion via immune cell inhibition. PD-L1 is also expressed in numerous tumors and also associated with a poor clinical outcome as a result of inhibition of immune response [41]. In this experiments, but, NK92-CD16 cells effectively killed the TKI-resistant NSCLC cells ignored the immune checkpoint mediator (i.e. PD-L1). Based on this data, a favorable prognosis could be expected on PD-L1 positive cancer patients.

In conclusion, it was confirmed that NK92-CD16 cells kill efficiently the TKI-resistance in NSCLC cells. In addition, combination NK92-CD16 with cetuximab effectively inhibited the TKI-resistant NSCLC cells which up-regulated EGFR expression. Moreover, the effect of combination therapy was not affected by other factors (e.g. ICAM-1 and PD-L1). In conclusion, this combination therapy (NK92-CD16 combined with cetuximab) might become an effective treatment strategy for EGFR-overexpressed and acquired TKI-resistant NSCLC patients.

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국문초록

연구 목적: 티로신 키나아제 억제제 사용은 비소세포폐암 환자들에서 효과적인 치료이다. 그러나 대부분의 환자에서 여러 변이를 통해 티로신 키나아제에 내성을 획득하여 암이 진행되는 것이 관찰되었다. 본 연구에서는 티로신 키나아제 억제제에 내성이 생긴 비소세포폐암 세포주에 대한 NK92-CD16 세포의 세포독성을 확인함으로써, 약물 내성을 극복할 수 있는 치료 전략으로 면역 치료의 적용 가능성을 확인하고자 하였다.

연구 방법: 먼저 세가지 비소세포폐암 세포주 (NCI-H3122, EBC-1, PC-9)에 Crizotinib, Ceritinib, Capmatinib, Gefitinib 그리고 Erlotinib 을 지속적으로 노출시킴으로서 티로신 키나아제 억제제 내성 획득 세포 (H3122CR1, H3122LR1, H3122CR1LR1, EBC-CR1, EBC-CR2, PC-9GR, PC-9ER)을 확립하여 실험에 사용하였다. 암세포에 대한 자연살해세포 독성 및 항체 의존성 세포 독성 (ADCC; Antibody-dependent cellular cytotoxicity)은 CD16 이 형질 도입된 NK92-CD16 세포주를 사용하여 ^{51}Chromium-release assay 로 확인하였으며, 자연살해세포의 수용체에 대한 리간드 및 비소세포폐암에서의 표피 성장인자 수용체 (EGFR)의 발현량은 유세포분석을 통해 분석하였다.

연구 결과: 원래의 비소세포폐암 세포주보다 대부분의 티로신 키나아제 억제제 내성 획득 비소세포폐암 세포주에서 NK92-CD16 에 대한 민감도가 더 높은 것이 확인 되었다. 기존 비소세포폐암 세포주와 비교했을 때, 내성 세포주에서 NK 세포에 있는 LFA-1 에 대한

리간드인 ICAM-1 의 발현량이 증가되는 양상이 관찰되었으며, 이는 NK92-CD16 세포독성과 관련이 있을 것으로 생각되었다. 이를 확인하고자 ICAM-1 항체를 이용해 LFA-1/ICAM-1 의 상호작용을 막았을 때, NK92-CD16 세포의 세포독성이 감소되었다. 그리고 내성 세포주에서의 EGFR 발현량 증가로 인해 EGFR 단일클론항체인 cetuximab 을 통해 매개된 항체 의존성 세포독성이 증가하는 것을 확인하였다.

결론: 티로신 키나아제 억제제에 내성을 획득한 비소세포폐암에서 NK92-CD16 의 세포독성이 더 높게 나타났으며, 이는 면역 시냅스를 형성하는 ICAM-1 의 증가에 부분적으로 의존적인 것이 확인되었다. 그리고 EGFR 단일클론항체인 cetuximab 의 사용은 NK92-CD16 의 세포독성을 상당히 증가시켰다. 이를 종합해보았을 때, NK92-CD16 세포와 cetuximab 을 함께 사용한 면역치료가 티로신 키나아제 억제제에 내성이 생긴 비소세포폐암 환자들에게 효과적인 치료 전략으로 활용될 수 있을 것으로 생각된다.

주요어 : NK-92, 비소세포폐암 , 내성 획득

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