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Development of Antigen-specific Chimeric Antigen Receptor KHYG-1 Cells for Glioblastoma

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Abstract. *Background/Aim: Glioblastoma is the most common cancer among primary brain tumors, however, its prognosis and treatment advances are very poor. Here, we investigated whether c-Met, FOLR1, and AXL proteins are promising targets for chimeric antigen receptor (CAR) T-cell therapy, for they are known to be over-expressed in a variety of solid tumors. Materials and Methods: CAR constructs were prepared and CAR KHYG-1 cells targeting c-Met, FOLR1, or AXL were made by lentiviral transduction. The activity of CAR KHYG-1 cells against cancer cells was measured by cytokine secretion and cell lysis assays. Results: c-Met and AXL were over-expressed in most glioblastoma cell lines (11/13), but not in neuroblastoma cell lines (0/8). FOLR1 was over-expressed only in one among 16 glioblastoma cell lines. Our antigen-specific CAR KHYG-1 cells eradicated target positive glioblastoma cells selectively. Conclusion: Anti-c-Met and anti-AXL CAR NK or T cells could be effective in glioblastoma cells.*

Research on chimeric antigen receptor (CAR) T-cell therapy has increased since CD19 targeting CAR T-cells has shown an impressive response rate in patients with B-cell acute lymphoblastic leukemia (B-ALL) (1). CAR T-cell therapy is an adoptive cell therapy during which engineered T or natural killer (NK) cells are reinjected into a patient. The CAR construct is composed of the single-chain variable fragment (scFv) domain for antigen recognition, a co-stimulatory domain, and a CD3ζ signaling domain for T cell activation. T cells or NK cells that stably express the CAR can specifically recognize the antigen and effectively kill the tumor cells (2). NK cells are cytotoxic immune cells that play an important role in the innate immunity and respond to infection through various receptors that mediate activation and inhibition. NK cells directly attack virus-infected cells or tumor cells, and increase activity of macrophages through cytokine secretion. Several NK cell lines have been used for research purposes (3, 4). Currently, CAR NK cell studies use a variety of NK cell lines such as NK-92, KHYG-1, and YT (5). These NK cell lines have similar characteristics to normal NK cells regarding phenotype and function. However, they present the advantage of being cultured in large scale *in vitro*. Of these NK cell lines, NK-92 is the most widely used in the CAR NK cell research. However, NK-92 cells grow slowly and require expensive culture medium. For studying CAR NK cell against cancer, we chose another NK cell line, KHYG-1, to overcome this disadvantage of NK-92 (6).

Neural cancers can be divided into gliomas and neuroblastomas. Glioma, including oligodendroglioma, medulloblastoma, astrocytoma, and glioblastoma, arise from glial cells, which are non-neuronal cells and provide protection and support for neurons, whereas neuroblastomas arise from primitive nerve cells in the fetus or embryo (7, 8). Glioblastoma is the most common brain tumor, and the 5-year survival rate is merely 4-5% (9). Neuroblastoma is a common cancer in childhood, accounting for about 10% of all pediatric cancer deaths (10). In recent years, antibody-based immunotherapy has emerged as a novel therapeutic modality for these tumors (11, 12). However, the clinical efficacy in glioblastoma is quite limited (13).

Here, we applied CAR NK cell technology to the treatment of neural cancers. We selected three receptors, c-Met, the folate receptor 1 (FOLR1), and AXL, which are known to be over-expressed in solid tumors, to target by NK cells. c-Met, a hepatocyte growth factor receptor (HGFR), is required for normal cell development, and has been found to be over-expressed in a variety of solid tumors (14, 15). FOLR1, also known as folate receptor alpha (FRA), is a member of the folate receptor family (16, 17). It mediates delivery of 5-methyltetrahydrofolate into the cell. FOLR1 has been shown to be highly expressed in several solid tumors such as ovarian cancer, renal cancer, lung adenocarcinoma, and breast cancer

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Key Words: Chimeric antigen receptor, natural killer cell, KHYG-1, c-Met, AXL, FOLR1, glioblastoma.

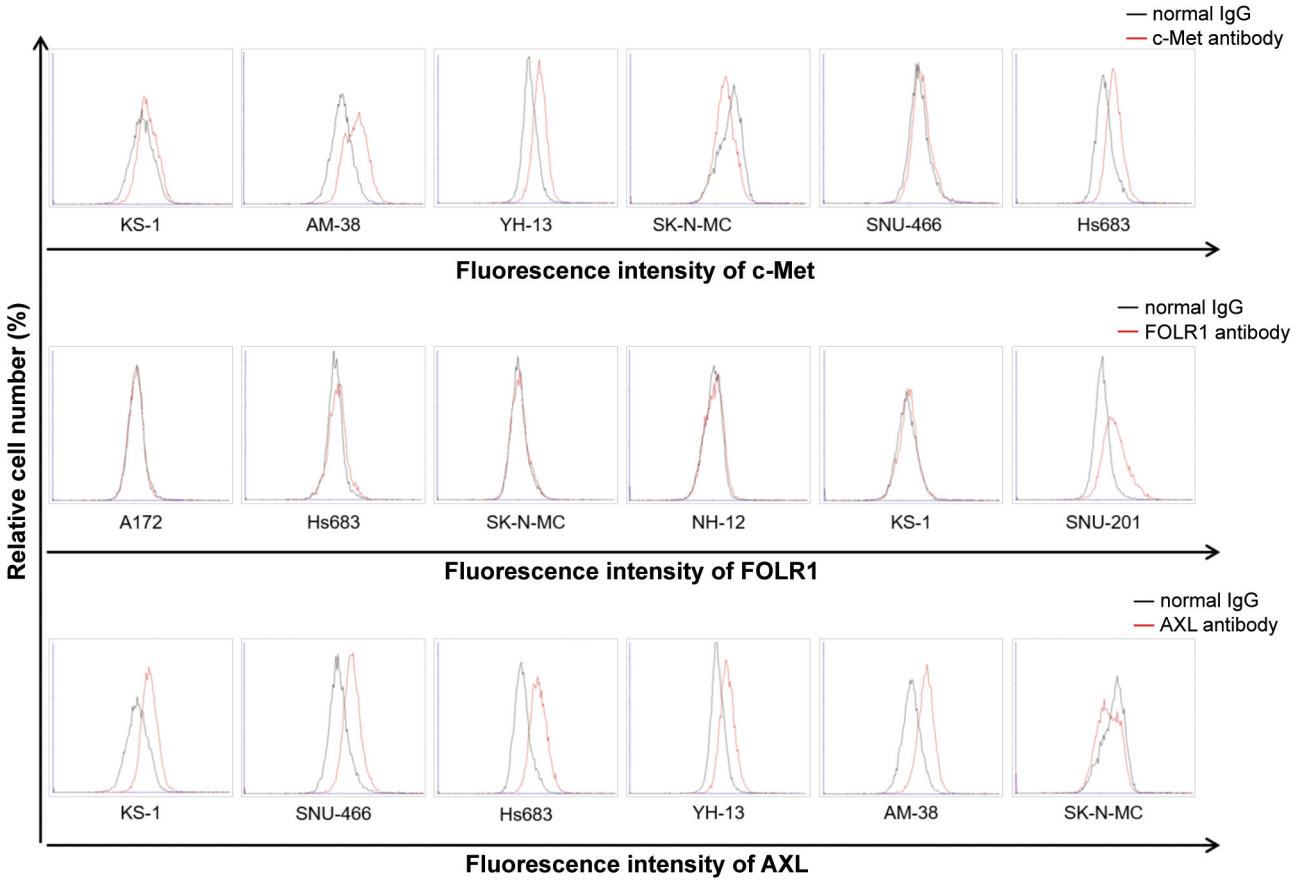

Figure 1. *Expression levels of each target protein in neural cancer cells. Various neural cancer cells were stained with anti-human HGF R/c-Met (Top, red line), anti-folate-binding protein (Middle, red line), anti-human AXL (Bottom, red line), or matched isotype control antibodies (all black lines).*

(18-20). AXL is a receptor tyrosine kinase (RTK), which is dimerized and auto-phosphorylated *via* ligand binding (21). AXL has been found to be over-expressed in renal cell carcinoma (RCC) (22, 23), acute myeloid leukemia (AML) (24), and pancreatic cancer (25). We evaluated membrane expression of c-Met, FOLR1, and AXL in 30 neural cancer cell lines. Then, anti-c-Met-CAR KHYG-1, anti-FOLR1-CAR KHYG-1, and anti-AXL-CAR KHYG-1 were prepared to measure antigen-specific tumor cell killing ability. As a result, these target-specific CAR KHYG-1 cells effectively killed only the target positive glioblastoma cells.

In this study, we developed CAR KHYG-1, specific for c-Met, FOLR1, and AXL, for cancer treatment, and proved they were effective against glioblastoma cell lines.

Materials and Methods

Cell culture. All the neural cancer cell lines and Jurkat cells were maintained in RPMI 1640 medium (HyClone, Marlborough, MA, USA; #SH30027) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA; #16000-044). KHYG-1 cells were cultured in RPMI 1640 supplemented with 10 % FBS and 200 IU/ml IL-2. All the cells were incubated in a humidified incubator at 37˚C with 5% CO_2.

CAR construction and lentivirus production. Each CAR construct was synthesized from Macrogen (Daejeon, Republic of Korea) and then inserted into pLVX-puro vector. To generate CAR KHYG-1 stable cell lines, pLVX-CAR vectors were co-transfected with lentiviral packaging plasmids, pRSV-Rev (Addgene, Watertown, MA, USA; #12253), pMDLg/pRRE (Addgene, #12251) and pMD2.G (Addgene, #12259) into 293T cells. The supernatants containing lentiviruses were collected and concentrated using the Lenti-X Concentrator (Clontech, Mountain View, CA, USA; #631232). For spin infection, lentiviruses, KHYG-1 cells and LentiBoost (Sirion Biotech, Martinsried, Germany; #SB-P-LV-101-02) were mixed in a 50 ml tube and centrifuged at 1000 × *g*, for 90 min. For selection, KHYG-1 cells were treated with 1 μg/ml of puromycin.

Measurement of surface expression of antigens. Approximately 1×10^6 cancer cells were washed with cold PBS and resuspended in resuspension buffer I (PBS, 10% FBS, 0.02% sodium azide). Each primary antibody was added to the cells and the cells were incubated on ice for 30 min. Anti-human HGF R/c-Met (R&D Systems, Minneapolis, MN, USA; #MAB3582), anti-folate binding

protein (R&D Systems, #MAB154) and anti-human AXL (Abcam, Cambridge, MA, USA: #ab3361) were used as primary antibodies, respectively. After washing with cold PBS, secondary antibody was added to the cells and incubated for 30 min on ice. Cells were washed with cold PBS and resuspended in resuspension buffer II (PBS, 3% BSA, 0.02% sodium azide) supplemented Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA; #H3570). After incubation at 37˚C for 15 min, the phenotype of the cells was analyzed using the NucleoCounter NC-3000 (ChemoMetec, Allerod, Denmark) and the cells were classified as positive or negative for each antigen.

ELISA assay. To measure secreted cytokine levels, 1×10^4 CAR Jurkat cells or CAR KHYG-1 cells were co-cultured with 1.5×10^5 cancer cells in 96-well plates (Effector:Target ratio of 15:1). After overnight incubation at 37˚C, only supernatants were collected and the amount of IL-2 or IFN-γ was determined using a human IL-2 or IFN-γ ELISA kit (BioLegend, San Diego, CA, USA; #431801 or #430104).

Cytotoxicity assay. The cytotoxic activity of CAR KHYG-1 was examined using the Bright-Glo Luciferase Assay System (Promega, Madison, WI, USA; #E2650). The luciferase gene was transfected into target neural cancer cells. Next, antigen-specific CAR KHYG-1 cells were co-cultured with the target cells expressing luciferase at an E:T ratio of 3:1 and 10:1. After 5 h of co-culture, the luciferase Bright-Glo Luciferase assay reagent was added to each well of effector/target cell mixture. Finally, plates were shaken for 5 min at room temperature and luminescence was measured *via* an EnVision reader (PerkinElmer, Waltham, MA, USA).

Western blotting. To confirm expression of the CAR construct, c-Met-CAR KHYG-1, FOLR1-CAR KHYG-1, and AXL-CAR KHYG-1 cells were harvested with 1X SDS-PAGE loading buffer (10% glycerol, 2% SDS, 50 mM Tris-HCl (pH 6.8), 3% β-mercaptoethanol). Lysates were boiled for 10 min, at 95˚C and were loaded into 4-15% gradient Mini-PROTEAN TGX gels (BioRad, Hercules, CA, USA; #456-1086). Endogenous CD3ζ was used as a loading control. (BD Biosciences, San Jose, CA, USA; #551034) antibody.

Table I. *c-Met, AXL, or FOLR1 expression in glioblastoma cell lines*.

Cell line	Disease	Expression level		
		c-Met	AXL	FOLR1
A172	Glioblastoma			–
Hs683	Oligodendroglioma	+	+	–
SNU-201	Glioblastoma			+
SNU-626	Glioblastoma			–
SNU-466	Glioblastoma	–	+	–
SNU-489	Glioblastoma	+	+	–
U-373 MG	Astrocytoma	+	+	–
YKG-1	Glioblastoma	+	+	–
KS-1	Glioblastoma	+	+	–
AM-38	Glioblastoma	+	+	–
YH-13	Glioblastoma	+	+	–
T98G	Glioblastoma	–	–	–
ONS-76	Medulloblastoma	+	+	–
U-251 MG	Astrocytoma	+	+	–
B2-17	Astrocytoma	+	+	–
SF126	Glioblastoma	+	+	–

Table II. *c-Met, AXL, or FOLR1 expression in neuroblastoma cell lines*.

Cell line	Disease	Expression level		
		c-Met	AXL	FOLR1
SK-N-MC	Neuroepithelioma	–	–	–
SK-N-SH	Neuroblastoma	–	–	–
IMR-32	Neuroblastoma	–	–	–
SH-SY5Y	Neuroblastoma	–	–	–
CHP-126	Neuroblastoma	–	–	–
TNB-1	Adrenal gland neuroblastoma	–	–	–
NH-6	Adrenal gland neuroblastoma	–	–	–
NH-12	Adrenal gland neuroblastoma	–	–	–

Results

Antigen-specific CAR Jurkat cells were activated by target positive glioblastoma cells. We targeted c-Met, FOLR1, and AXL to apply CAR NK cell therapy in neural cancer cells. Before measuring the antigen-specific cytotoxic effect of CAR KHYG-1 cells, we analyzed the expression levels of FOLR1, c-Met, and AXL proteins in a number of neural cancer cell lines. Figure 1 and Tables I and II show the expression levels of each antigen in neural cancer cell lines. Most of the glioblastoma cell lines (14/16) are c-Met and AXL positive, whereas no neuroblastoma cell line (0/8) expressed c-Met or AXL. In addition, only two cell lines, SNU-201 and TM-31, are folate receptor positive among the 20 glioblastoma cell lines, whereas no neuroblastoma cell line (0/10) expresses folate receptor. Based on the expression level analysis, we classified the cell lines as positive or negative for each target (Tables I and II).

We made CAR constructs in which the expression of CAR proteins was driven by a CMV promoter. Scfvs were fused with CD28 hinge and transmembrane domain, and CD28 and CD3ζ cytoplasmic domains (Figure 2A). To test the function of the CAR construct, the malignant T cell line Jurkat was transfected with the control CAR (FITC-CAR) vector, c-Met-CAR vector, FOLR1-CAR vector, and AXL-CAR vector, by electroporation. Because FITC-CAR recognizes the FITC compound, it was used as a negative control. In Figure 2B, western blot shows that CARs were expressed in Jurkat cell lines. Jurkat cells produce the cytokine IL-2 when they are activated. In a 96-well plate, c-Met-CAR Jurkat, FOLR1-CAR Jurkat, or AXL-CAR Jurkat cells were co-cultured overnight with cancer cells and the next day, the supernatant was collected to measure IL-2 levels. As expected, IL-2 levels were increased when CAR Jurkat cells

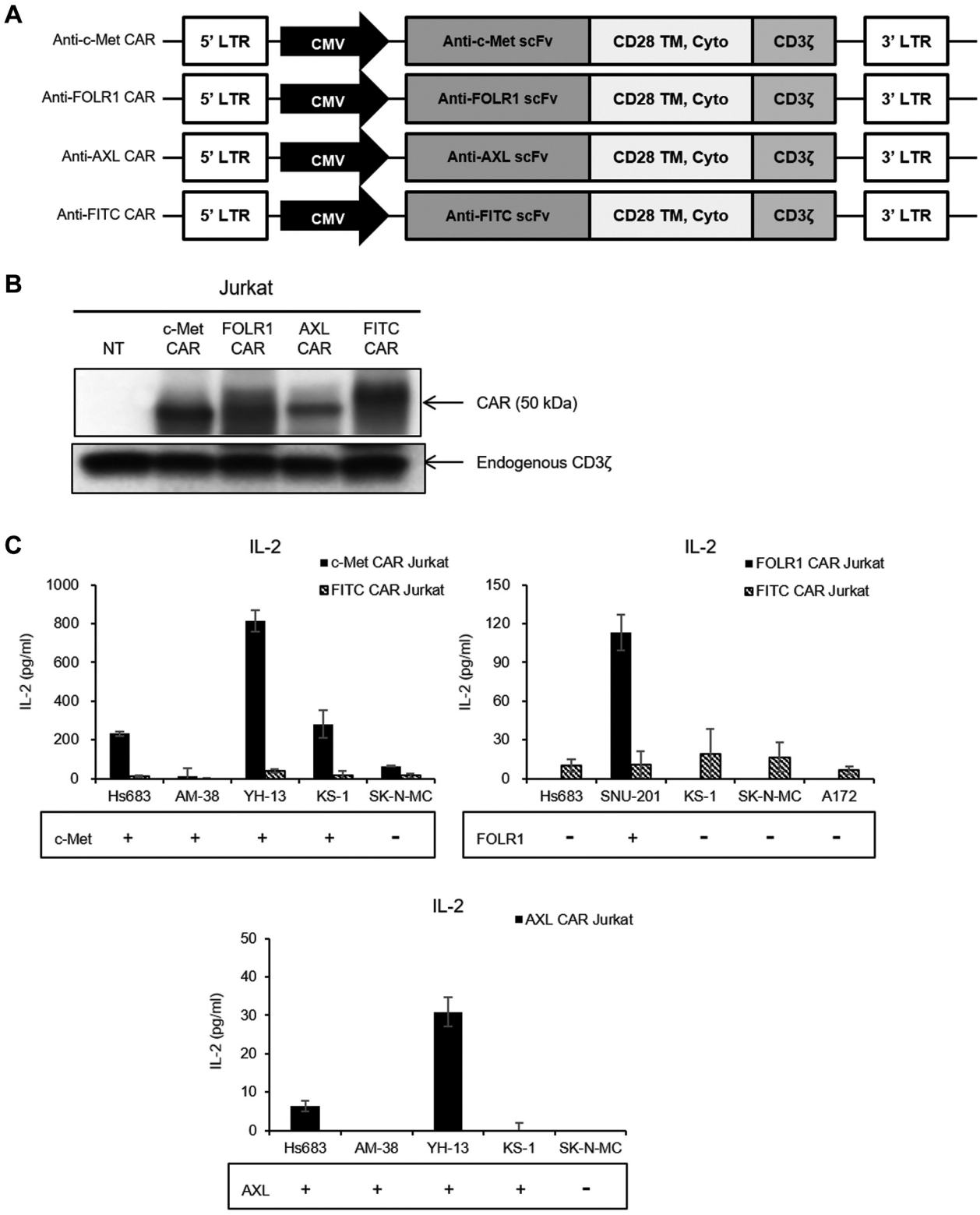

Figure 2. *Antigen-specific chimeric antigen receptor (CAR) Jurkat cells secrete IL-2 in response to antigen-positive neural cancer cells. (A) Schematic of antigen-specific CAR constructs. scFv: Single-chain variable fragment; H: hinge; TM: transmembrane. (B) After transfection of CAR plasmid into Jurkat cells, cell lysates were prepared for western blot with CD3ζ antibody. (C) After c-Met-CAR, FOLR1-CAR, and AXL-CAR Jurkat cells were co-cultured with cancer cells overnight, the levels of secreted IL-2 were measured using the IL-2 ELISA kit.*

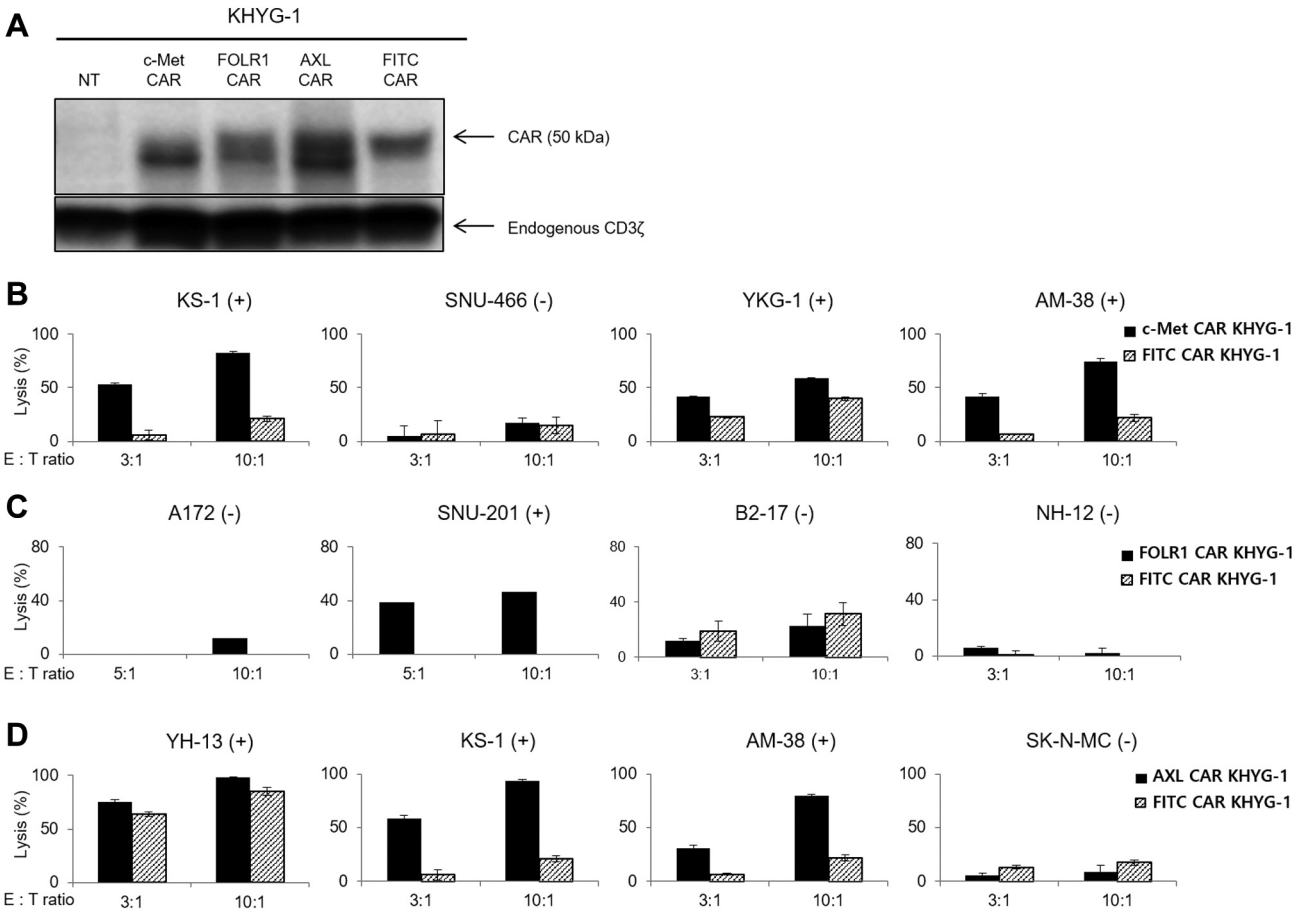

Figure 3. *Cytotoxicity assay of antigen-specific chimeric antigen receptor (CAR) KHYG-1 cells against various cancer cells. (A) After lentiviral transduction of c-Met-CAR, FOLR1-CAR, AXL-CAR, or FITC-CAR genes into KHYG-1, each CAR expression was confirmed by western blot. (B-D) c-Met-CAR KHYG-1 (B), FOLR1-CAR KHYG-1 (C), and AXL-CAR KHYG-1 (D) were co-cultured with cancer cells expressing luciferase at E:T ratio of 3:1 and 10:1 for 4 h. Target cell-killing ability of CAR KHYG-1 cells was evaluated by a Bright-Glo assay. '+', antigen positive; '–', antigen negative.*

were incubated with antigen-positive glioblastoma cells (Figure 2C). However, less IL-2 was produced when CAR Jurkat cells were incubated with the antigen-negative cell line. c-Met-CAR Jurkat cells produced high levels of IL-2 when incubated with YH-13, KS-1, and Hs683 cell lines, which are c-Met positive cell lines. However, low levels of IL-2 were produced when incubated with the SK-N-MC cell line, which is a c-Met negative cell line. The same results were obtained with FOLR1-CAR and AXL-CAR Jurkat cells. IL-2 was secreted only when FOLR1-CAR Jurkat cells were co-cultured with the FOLR1-positive cell line SNU-201 (Figure 2C). No IL-2 was secreted when FOLR1-CAR Jurkat cells were co-cultured with the FOLR1-negative cell lines KS-1, A172, and SK-N-MC. These results indicate that our CAR constructs specifically recognize their antigen, and activate T cell signaling.

Antigen-specific CAR KHYG-1 cells specifically killed target positive glioblastoma cells. We investigated the antigen-specific cytotoxic effect of c-Met-CAR KHYG-1, FOLR1-CAR KHYG-1, and AXL-CAR KHYG-1. c-Met-CAR, FOLR1-CAR, or AXL-CAR cells were transduced into KHYG-1 using lentiviral particles. After 2 weeks of puromycin selection, expression of each CAR was confirmed by western blotting (Figure 3A). c-Met-CAR KHYG-1, FOLR1-CAR KHYG-1, or AXL-CAR KHYG-1 cells were incubated with cancer cells with the indicated E/T ratios and times. CAR KHYG-1 showed higher cytotoxic effect on each target positive cancer cell line than control KHYG-1, which is FITC CAR KHYG-1 (Figure 3B-D). c-Met-CAR KHYG-1 cells lysed c-Met positive cell lines, such as KS-1, YKG-1, and AM-38, but not SNU-466, which is c-Met negative. FOLR1-CAR KHYG-1 cells eradicated only SNU-

201, which is a FOLR1 positive cell line. AXL-CAR KHYG-1 also lysed only AXL positive cell lines, including YH-13, KS-1 and AM-38, but not AXL negative cell line, SK-N-MC. These results indicate that c-Met-CAR, FOLR1-CAR, and AXL-CAR constructs effectively recognized each antigen and specifically killed antigen-positive glioblastoma cells.

Antigen-specific CAR KHYG-1 cells secret IFN-γ when incubated with antigen positive tumor cells. Because KHYG-1 is an NK cell line, it secrets IFN-γ when activated. We examined whether our CAR KHYG-1 cells secret IFN-γ when incubated with tumor cells in an antigen-dependent manner. CAR KHYG-1 cells were incubated with glioblastoma cell lines for 24 h, then supernatants were collected to measure IFN-γ levels using ELISA. As shown in Figure 4, IFN-γ was secreted when CAR KHYG-1 cells were incubated with antigen-positive cell lines. Therefore, we anticipate that CAR-KHYG-1 cells could be activated by tumor cells in an antigen-dependent manner.

Discussion

CAR T-cell therapy is attracting huge attention in cancer treatment because of its impressive effect on B-cell acute lymphoblastic leukemia (B-ALL) patients. However, limited effect has been reported on solid tumors. So far, among solid tumors, clinical studies have shown that CAR T-cells are most active in neural cancers, and have shown promising clinical results in glioblastoma and neuroblastoma patients (26, 27). In addition, CAR T-cells are able to pass the blood-brain barrier, whereas antibodies cannot enter the central nervous system (28). GD2 is the most studied target in neuroblastoma, and IL13Rα2, EGFRvIII, and Her2 have been the most studied in glioblastoma for CAR T-cell therapy (29, 30). Other targets, such as L1-CAM, Glypican 2, B7-H3, ALK, and NCAM have also been studied for neuroblastoma targeting CAR T-cell therapy (31-35), and CD70, CD133, B7-H3, EphA2, CD147, and PDL1 have been studied for glioblastoma treatment (36-39). Here, we suggest targeting the membrane proteins c-Met, FOLR1, and AXL, to apply CAR-based cell therapy to neural cancers, for they are known to be highly expressed in a variety of solid tumors (40-42). c-Met is known to be over-expressed in 30% - 80% of glioblastoma patients (43, 44), while few of primary neuroblastoma cells over-express c-Met (45). AXL is also known to be over-expressed in more than 60% of glioma patients (46). In this study, the expression of c-Met, FOLR1, and AXL at the surface of 24 neural cancer cell lines were measured. Surprisingly, c-Met and AXL were found to be over-expressed in the majority of glioblastoma cell lines (14/16). However, in neuroblastoma cell lines, c-Met and AXL were not found to be expressed (0/8). Only one cell line, SNU-201, among the 24 neural cancer cell lines, expressed surface folate receptor.

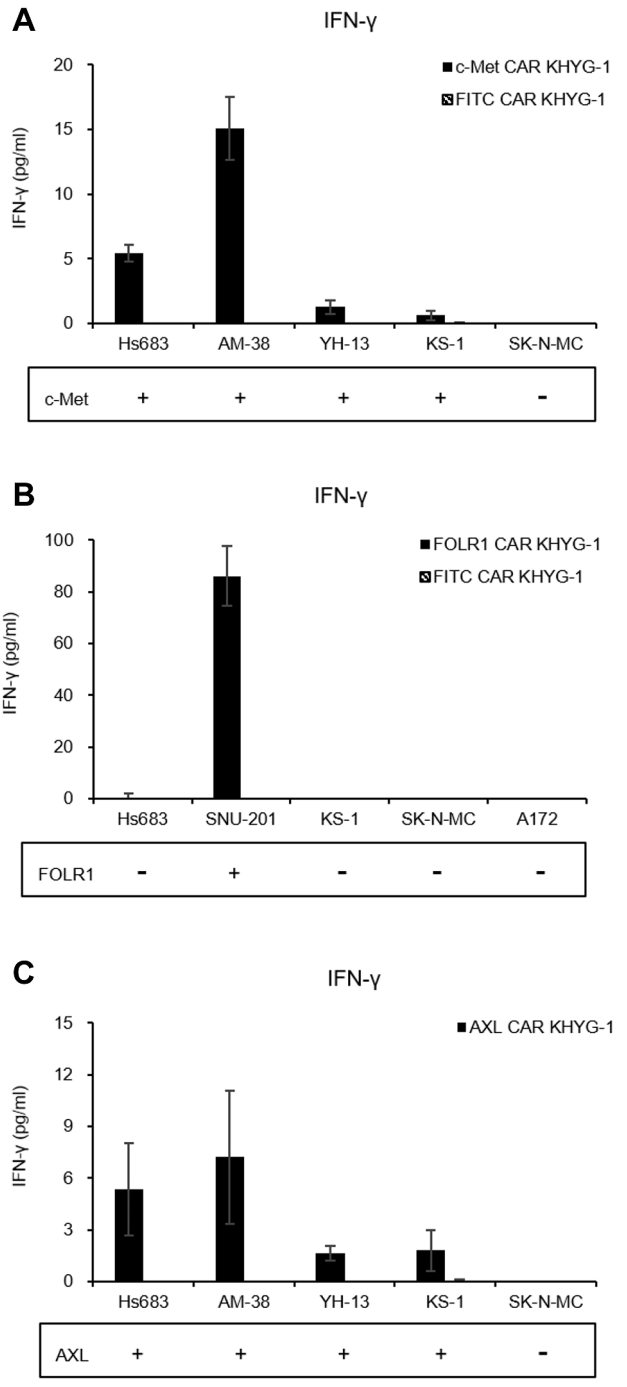

Figure 4. *IFN-γ assay of antigen-specific chimeric antigen receptor (CAR) KHYG-1. c-Met-CAR KHYG-1 (A), FOLR1-CAR KHYG-1 (B), or AXL-CAR KHYG-1 (C) cells were incubated with cancer cell lines for 24 h. Supernatant of culture medium was collected to measure the levels of IFN-γ by ELISA assay.*

The function of CAR constructs was examined by measuring the secreted IL-2 levels. IL-2 was highly secreted when c-Met-CAR, FOLR1-CAR, or AXL-CAR Jurkat cells

were co-cultured with antigen positive cancer cells. Therefore, these constructs are capable of activating T cells in response to antigen. In addition, CAR KHYG-1 cells were generated by lentiviral transduction followed by puromycin selection. CAR KHYG-1 cells capable of recognizing each antigen were prepared and their cytotoxicity against the target cells was measured. Our antigen-specific CAR KHYG-1 cells effectively recognized only antigen-positive cancer cells and showed cytotoxic effects. The CAR KHYG-1 cells did not respond to antigen-negative cancer cells. In addition, the antigen-specific KHYG-1 cells secreted IFN-γ, which is an important cytokine produced by NK cells, when co-cultured with antigen-positive cancer cells.

Our data suggest that KHYG-1 could be useful for the study of CAR NK. Especially, for c-Met and AXL that are over-expressed in most glioblastoma cell lines, anti-c-Met-CAR NK or anti-AXL-CAR NK cells would demonstrate a promising result in glioblastoma patients.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

CHK, and CHP designed the study and interpreted the results. CHK, YK, and SML performed the experiments and analyzed data. SUC provided the technical support. CHP and CHK wrote the manuscript. All Authors have approved the publication of the study.

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