Chimeric Antigen Receptor-Modified T Cells Redirected to EphA2 for the Immunotherapy of Non-Small Cell Lung Cancer

Abstract
Erythropoietin-producing hepatocellular carcinoma A2 (EphA2) is overexpressed in more than 90% of non-small cell lung cancer (NSCLC) but not significantly in normal lung tissue. It is therefore an important tumor antigen target for chimeric antigen receptors (CAR)-T-based therapy in NSCLC. Here, we developed a specific CAR targeted to EphA2, and the anti-tumor effects of this CAR were investigated. A second generation CAR with co-stimulatory receptor 4-1BB targeted to EphA2 was developed. The functionality of EphA2-specific T cells in vitro was tested with flow cytometry and real-time cell electronic sensing system assays. The effect in vivo was evaluated in xenograft SCID Beige mouse model of EphA2 positive NSCLC. These EphA2-specific T cells can cause tumor cell lysis by producing the cytokines IFN-γ when cocultured with EphA2-positive targets, and the cytotoxicity effects was specific in vitro. In vivo, the tumor signals of mice treated with EphA2-specific T cells presented the tendency of decrease, and was much lower than the mice treated with non-transduced T cells. The anti-tumor effects of this CAR-T technology in vivo and vitro had been confirmed. Thus, EphA2-specific T-cell immunotherapy may be a promising approach for the treatment of EphA2-positive NSCLC.

Introduction
Lung cancer is the leading cause of cancer-related mortality among men and the second leading cause of cancer death among women worldwide [1]. The 5-year relative survival rate of patients diagnosed with lung cancer was less than 19%, while the average rate of cancer patients at all site was 70% [2]. Non-small cell lung cancer (NSCLC) accounts for nearly 85% of all cases of lung cancer [3], in which adenocarcinoma will be the predominant histological subtype [4,5]. The current treatments including surgery, radiotherapy, chemotherapy and targeted therapy has helped improve the survival in patients with NSCLC. However, the average 5-year survival rate of lung adenocarcinoma was only 15% [6], mainly because of the poor prognosis and lack of effective treatment in late-stage, highlighting the unmet need for new therapeutic paradigms for this disease.

Immunotherapy with chimeric antigen receptor (CAR)-engineered T cells is a breakthrough treatment in hematology, such as anti-CD19 CAR-T cells in treating acute lymphoblastic leukemia (ALL) [7,8], chronic lymphocytic leukemia (CLL) [9] and B cell lymphomas [10]. In recent years, much more progress has been made in solid tumors,
including colorectal cancer [11], metastatic ovarian cancer [12],
glioblastoma [13]. Immunotherapy with CARs targeting epidermal
growth factor receptor (EGFR) in a clinical trial showed good
response with EGFR-expressing advanced relapsed/refractory
NSCLC [14]. CAR glycans α (CARα) T cells was also proved to
be a novel potential therapeutic agent for the treatment of patients
with lung squamous cell carcinoma (LSCC) [15]. However, the
studies with regards to NSCLC were still limited.

The ephrin receptors (Ephs) are the largest group within the family
of receptor tyrosine kinases (RTKs) [16]. Erythropoietin-producing
hepatocellular carcinoma A2 (EphA2) play critical roles in many
developmental processes and are implicated in a number of cancers
[17,18]. EphA2 is overexpressed in more than 90% of NSCLC but
not significantly in normal lung tissue [19], and correlates with tumor
malignancy and poor patient survival [20]. In addition, we have
found EphA2-positive cells in malignant pleural effusion of lung
adenocarcinoma patients. One study using EphA2 targeting
pegylated nanocarrier drug delivery system for treatment of lung
adenocarcinoma patients. One study using EphA2 targeting
pegylated nanocarrier drug delivery system for treatment of lung
cancer have shown improved clinical outcome [21]. Hence, EphA2 is
supposed to be an important marker with potential clinical utility in
the immunotherapy of NSCLC [22].

Here, we report the development of an EphA2-specific CAR to
redirect T cells to EphA2-positive NSCLC. These T cells are able to
recognize and kill EphA2-positive lung cancer cells. Furthermore, we
have found that IFN-γ paly role in EphA2-CAR-T therapies. The
effect of EphA2-specific CAR in vivo was also evaluated in xenograft
SCID Beige mouse model of lung cancer.

**Materials and Methods**

**Cell Lines, Pleural Effusions, and Media**

Three NSCLC cell lines (A549, PC9, H1650), the leukemia cell line
K562 and 293 T cell line were purchased from the American Type
Culture Collection (ATCC, Manassas, VA). Thirteen samples of
pleural effusions were obtained from patients diagnosed with lung
adenocarcinoma in the First Affiliated Hospital of Zhejiang University.
Peripheral blood mononuclear cells (PBMCs) derived from human donors
were collected by Ficoll–Hypaque density-gradient centrifugation
provided by the Zhejiang Blood Center. All cell lines were grown in
RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with
10% fetal bovine serum (FBS) and 100 μg/ml penicillin. Medium with
recombinant human interleukin-2 (IL-2) of 300 U/ml was used for the
expansion of T cells.

**Flow Cytometric Analysis**

Flow cytometric analysis (BD, Mountain View, CA) was used to
detect the expression of EphA2 on tumor cells and to detect the
expression of CAR on EphA2-positive T cells. EphA2 expression in
cell lines (A549, PC9, H1650, K562) was tested using a EphA2-PE
antibody (BioLegend, San Diego, CA). Cells collected from pleural
effusions were stained with both EphA2-PE antibody and
CD45-APC antibody (BD, San Jose, CA). Cells were collected and
washed once with phosphate buffered saline (PBS) containing 2%
FBS prior to the addition of antibodies, and were incubated for
20 minutes on ice in the dark, washed twice prior to analysis.

**Generation of CAR-Expressing T Cells**

The EphA2-specific single chain variable fragment was derived from
the EphA2 MAb 4H5, a humanized version of the EphA2 MAb EA2
[23,24]. A codon-optimized synthetic gene encoding 4H5 in single
chain variable fragment format was cloned into a SFG retroviral vector
upstream of an IgG1-CH2CH3 domain, a CD8α transmembrane
domain, and costimulatory domains derived from 4-1BB and CD3-ζ.
Lentiviral particles were generated by transient transfection of 293 T
cells with the EphA2-specific CAR encoding SFG retroviral vector.
PBMC were stimulated on 6-well plates in RPMI 1640 medium
containing 1×10⁶/beads/ml dynabeads human T-activator CD3/CD28
(Invitrogen, Carlsbad, CA). On day 2, vector encoding the
EphA2-specific CAR were used to transduce CD3/CD28-activated
T cells. On day 3 or 4, T cells were transferred into new wells and
subsequently expanded with 300 IU/ml IL-2. Nontransduced T cells,
used as controls, were activated with CD3/CD28 and expanded in parallel. EphA2-specific CAR expression was determined 5 to 6 days
posttransduction. In all experiments, the functionality of matched
(from the same donor) transduced and nontransduced T cells was
compared.

**Cytokine Release Assays**

The transduced and nontransduced T cells were co-cultured with
EphA2-positive cell A549 or negative cell k562 at the effector:target
ratio of 5:1, 1:1 and 1:5 for 5 h, separately. Each ratio repeated three
times. The expression of cytokines IFN-γ were measured by flow
cytometry (FCM). BD CytoTox/Cytoperm™ Fixation/Permeabilization
Kit and APC mouse anti-human IFN-γ (BD Bioscience, USA) were
used for staining.

**Cytotoxicity Assay**

The xCELLigence system, also known as the Real-Time Cell
Electronic Sensing System (RT-CES, ACEA Biosciences), was used to
test the time- and dose-dependent lung cancer cell line response
profiles to EphA2-specific T cells in vitro. Briefly, 50 μL of the
medium was added to E-plates to obtain background readings,
followed by the addition of 50 μL of the target cell (A549, 2×10⁶/ml)
suspension. The E-plates containing the indicated initial number of
cells were incubated at room temperature for 30 min before being
placed onto the reader in the incubator. The cells were allowed to
attach and grow for 24 h to reach a stable baseline before the addition
of the different number of CAR-T and T cells. The CAR-T and
T cells were co-cultured with A549 at the effector:target ratio of 1:1,
5:1 and 20:1 for 48 h, separately. Each ratio repeated three times. The
target cells were monitored every 15 min for 48 h.
Statistical Analysis

For the mouse experiments, differences in tumor radiance from baseline at each time point were calculated and compared between groups using t test. Overall survival determined from the time of tumor cell injection was analyzed by the Kaplan–Meier method and by the log-rank test. GraphPad Prism 5.0 was used for statistical calculations. \( P < .05 \) were considered significant.

Results

EphA2 is Expressed in Lung Cancer Cell Lines and Malignant Pleural Effusions

To evaluate whether EphA2 function as surface phenotypic hallmarks of lung cancer cells, we detected the distribution of this molecule on the lung cancer cell lines (A549, PC9, H1650) and leukemia cell line K562 by FCM. The results showed that EphA2 was expressed in the lung cancer cell lines but not in the leukemia cell line K562 (Figure 1A). 13 samples of pleural effusions were collected from patients diagnosed with lung adenocarcinoma. 5.4–22.1% EphA2 expression was found in 5 pleural effusions, while the other cells expressing CD45 indicated the white blood cells. Because of a shortage of pleural effusion, only one sample tested for EphA2 expression was identified with microscopic cancer cells (Figure 1B).

Generation of EphA2-Specific CAR-Modified T Cells

To redirect T cells to the EphA2 receptor, a second-generation EphA2-specific CAR was designed based on the humanized EphA2 monoclonal antibody (MAb) 4H5. A codon-optimized synthetic gene encoding 4H5 in single chain variable fragment format was cloned into a SFG retroviral vector upstream of an IgG1-CH2CH3 domain, a CD8α transmembrane domain, and costimulatory domains derived from 4-1BB and CD3ζ (Figure 2A). Lentiviral vector encoding the EphA2-specific CAR were used to transduce CD3/CD28-activated T cells from normal healthy donors. Following T-cell transduction, FCM analysis was used to determine the cell surface expression of the EphA2-specific CAR. The percentage of CAR-expressing T cells were 40.5% and 45.5% in two normal healthy donors (Figure 2B).

EphA2-Specific T Cells Recognize and Kill EphA2-Positive Lung Cancer Cells

We used FCM and RT–CES assays to test the functionality of EphA2-specific T cells in vitro. EphA2-specific T cells were cocultured with EphA2-positive and negative target cells, and the percentage of interferon-γ-expressing (IFN-γ) T cells was determined after 5 hours by FCM. EphA2-specific T cells recognized EphA2-positive lung cancer cell lines A549 as evidenced by a higher percentage of IFN-γ-expressing T cells in comparison to the EphA2-negative T cells and K562 (Figure 3A). None IFN-γ-expressing T cells was observed in non-transduced-T cells in response to any targets (Figure 3A). The cell index (CI) of A549 cocultured with different concentrations of effector cells were monitored continuously in real-time by RT-CES. In standard 48-hour assays, EphA2-specific T cells showed a significant lower CI against A549 whereas non-transduced T cells (Figure 3B), and a rapid and precipitous increase in CI occurred in the control group (only A549). And the higher effector to target (E:T) ratio showed the stronger cytotoxic activity against A549.

Figure 1. Erythropoietin-producing hepatocellular carcinoma A2 (EphA2) is expressed in lung cancer cell lines and malignant pleural effusions. (A) FCM showed that EphA2 was expressed in the lung cancer cell lines but not in the leukemia cell line K562. (B) EphA2 were found in cancerous pleural effusion of lung adenocarcinoma patients.
We used an orthotopic xenograft mouse model to evaluate the therapeutic effect of lung cancer of EphA2-specific T cells in vivo. A549 lung cancer cells were modified to express an GFP-Luciferase fusion protein (A549.GFP. Luc), allowing us to track tumor growth using serial noninvasive in vivo bioluminescence imaging (Berthold).

**EphA2-Specific T Cells Inhibit the Growth of Lung Cancer In Vivo**

We used an orthotopic xenograft mouse model to evaluate the therapeutic effect of lung cancer of EphA2-specific T cells in vivo. A549 lung cancer cells were modified to express an GFP-Luciferase fusion protein (A549.GFP. Luc), allowing us to track tumor growth using serial noninvasive in vivo bioluminescence imaging (Berthold).

**Figure 2.** Generation of erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells. (A) The EphA2-specific chimeric antigen receptors (CAR) was generated by cloning a single chain variable fragment derived from the EphA2 monoclonal antibody 4H5 upstream of an IgG1-CH2CH3 domain, a CD8α TM domain, and costimulatory domains derived from 4-1BB and CD3-ζ into an SFG retroviral vector. (B) EphA2-CAR expression was detected by staining T cells with a corresponding antibody. Fluorescence activated cell sorting analysis revealed expression of EphA2-specific CARs on the cell surface of transduced T cells as compared with controls. The percentage of CAR-expressing T cells were 40.5% and 45.5% in two normal healthy donors LTR, long terminal repeats; TM, transmembrane.

**Figure 3.** Erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells recognize and kill EphA2-positive lung cancer cells. (A) Non-transduced (NT) or EphA2-specific T cells were cocultured with target cells at 5:1, 1:1 and 1:5 ratio, and after 24 hours, percentage of IFN-γ-expressing T cells was determined by FCM. A higher percentage of IFN-γ-expressing T cells was occurred in comparison to the EphA2-negative T cells and K562. (B) EphA2-specific T cells showed a significant lower CI against A549 whereas non-transduced T cells, and a rapid and precipitous increase in CI occurred in the control group (only A549). And the stronger cytotoxic activity against A549 was observed at the higher effector to target (E: T) ratio of 20:1.
LB983, Germany). 2.5 × 10⁶ A549.GFP Luc cells were injected into the caudal vein of SCID Beige mice on day 0. Tumors engrafted and grew exponentially in the week post-tumor cell injection. On day 7, the tail vein injection was performed with 1 × 10⁷ non-transduced T cells or EphA2-specific T cells into the previous stereotactic tumor coordinates. Although the mice treated with EphA2-specific T cells and non-transduced T cells both showed continuous tumor growth, the tumor signals of mice treated with EphA2-specific T cells presented the tendency of decrease, and was significantly lower than the mice treated with non-transduced T cells at the fourth week (n = 5, \( P = .02 \); Figure 4, A and B). In addition, one mouse treated with EphA2-specific T cells had a complete response (CR). Two groups of mice die within 7–8 weeks successively, and no significant difference of mice survival was observed.

**Discussion**

In this study, the development and characterization of a novel CAR specific for the EphA2 receptor has been described. These EphA2-specific T cells can cause tumor cell lysis by producing the cytokines IFN-γ when cocultured with EphA2-positive targets, and the cytotoxicity effects was specific in vitro. The antitumor activity in vivo of these EphA2-specific T cells has also been demonstrated.

On 171 (61%)adenocarcinomas and 108 (39%)squamous cell carcinomas tissue microarrays (TMA) samples, EphA2 expression was detected in >90% of NSCLC samples [20]. Likewise, EphA2 expression was detected in all nine NSCLC cell lines assayed by Western blotting [25]. Epidermal growth factor receptor (EGFR) is expressed in majority of NSCLC. EGFR mutations are present in approximately 10 to 15% of Caucasian patients [26] and 51.4% of Asian patients [27]. The prolonged progression-free survival (PFS) and increased disease control rates were observed on treatment with EGFR tyrosine kinase inhibitor (EGFR TKI), however, inevitable drug resistance was also observed after 6–12 months’ treatment [28]. Recent studies indicated that EphA2 was more than 10-fold overexpressed in EGFR-TKI-resistant cells suggesting a potential role in developing resistance [29]. On the other hand, NSCLC patients for smokers with Kirsten rat sarcoma (K-Ras) mutations didn’t enjoy the same benefit from EGFR-TKI, and the treatment options are very limited. While the overexpression of EphA2 has been found to correlate with a poor prognosis on smokers with K-Ras mutations [20]. All the results indicate that EphA2 may be a potential molecular target for treatment of most NSCLC, including the special patients resistant to EGFR-TKI and smokers with K-Ras mutations.

Based on the above researches, we didn’t test the expression of EphA2 in tumor tissue in this study, but 5.4–22.1% EphA2 expression was found in 5 of 13 pleural effusions diagnosed with lung adenocarcinoma. As is well known, approximately 15% of lung cancer patients show a pleural effusion at the time of initial diagnosis, and 50% develop a pleural effusion later in their progress [30,31]. Hence, EphA2-specific T cells developed in this study can be injected into the thoracic cavity to treat patients with EphA2-positive malignant pleural effusions, and the subsequent researches have been started.

Actually, EphA2-specific T-cell immunotherapy has been reported in glioblastoma [13], and secretion of IFN-γ was observed when
EphA2-specific T cells cocultured with EphA2-positive target cells. In another study, patients with renal cell carcinoma (RCC), who expressing high levels of EphA2, exhibit both CD8 and CD4 T-cell responses to novel EphA2-derived epitopes [32]. The results of this study demonstrated that the EphA2-specific T-cells can be activated and expanded in the presence of EphA2-positive lung cancer cells. The in vitro cytotoxicity assay further supported that the CAR T cells could specifically eliminate EphA2-positive lung cancer cells by secretion of IFN-γ. The cytotoxic effect increased at the higher effector to target (E:T) ratio.

In vivo, the mice treated with EphA2-specific T cells and non-transduced T cells both died within 7–8 weeks. Especially, one of the five mice treated with EphA2-specific T cells, in which nearly no tumor signal was detected since the fifth week also died in the eighth week. The cause of death could not be related to tumor progress. Although no significant difference of mice survival was observed, the tumor signals of mice treated with EphA2-specific T cells was much lower than the mice treated with non-transduced T cells at the fourth week (n = 5, P = .02).

Conclusions

In this study, we provide evidence that T cells redirected to EphA2 by an EphA2-specific CAR have potent antitumor activity against NSCLC in vitro and in vivo, and might be novel therapeutic agents for patients with NSCLC.

Funding

This work was supported by the National Key R&D Program of China (No.2016YFC1303403), the National Natural Science Foundation of China (No.31501082) and the Natural Science Foundation of Zhejiang Province (No.LY15H160011).

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of The First Affiliated Hospital of Zhejiang University for the use of clinical materials for research purpose. And animal use and experiment protocol were approved by the Institutional Animal Care and Use Committee of Tongji University School of Medicine.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

Not applicable.

References

[1] Torre LA, Siegel RL, and Jemal A (2016). Lung cancer statistics. Adv Exp Med Biol 893, 1–19.
[2] National Cancer Institute (2016). SEER Cancer Statistics Review, 1975-2013; 2016 [Available online: https://seer.cancer.gov/csr/1975_2013/browse_csr.php?sectionSEL=1&cpagesEL=sect_01_table_04.html].
[3] Ramalingam SS, Owonikoko TK, and Khuri FR (2011). Lung cancer: New biological insights and recent therapeutic advances. CA Cancer J Clin 61(2), 91–112.
[4] Kaeser KM, Trajman A, and Madi K (2001). Evolving features of lung adenocarcinoma in Rio de Janeiro, Brazil. Oncol Rep 8(1), 189–192.
[5] Fry WA, Phillips JL, and Menck HR (1999). Ten-year survey of lung cancer treatment and survival in hospitals in the United States: a national cancer data base report. Cancer 86(9), 1867–1876.
[26] Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, and Boggon TJ, et al (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304(5676), 1497–1500.

[27] Shi Y, Au JS, Thongprasert S, Srinivasan S, Tsai CM, Khoa MT, Hererra K, Itoh Y, Cornelio G, and Yang PC (2014). A prospective, molecular epidemiology study of EGFR mutations in Asian patients with advanced non-small-cell lung cancer of adenocarcinoma histology (PIONEER). *J Thorac Oncol* 9(2), 154–162.

[28] Jackman D, Pao W, Riely GJ, Engelman JA, Kris MG, Janne PA, Lynch T, Johnson BE, and Miller VA (2010). Clinical definition of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. *J Clin Oncol* 28(2), 357–360.

[29] Koch H, Busto ME, Kramer K, Medard G, and Kuster B (2015). Chemical proteomics uncovers EPHA2 as a mechanism of acquired resistance to small molecule EGFR kinase inhibition. *J Proteome Res* 14(6), 2617–2625.

[30] Anderson CB, Philpott GW, and Ferguson TB (1974). The treatment of malignant pleural effusions. *Cancer* 33(4), 916–922.

[31] Memon A and Zawadski ZA (1981). Malignant effusions: diagnostic evaluation and therapeutic strategy. *Curr Probl Cancer* 5(8), 1–30.

[32] Tatsumi T, Herrem CJ, Olson WC, Finke JH, Bukowski RM, Kinch MS, Ranieri E, and Storkus WJ (2003). Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor tyrosine kinase EphA2 in patients with renal cell carcinoma. *Cancer Res* 63(15), 4481–4489.