Supplementary Data

Targeting cell surface glucose-regulated protein 94 in gastric cancer with an anti-GRP94 human monoclonal antibody

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MATERIALS AND METHODS

Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described with minor modifications (1). Briefly, a total of 27 cancer tissue samples printed on tissue slides (Supplementary Table. 1) were purchased from SuperBioChips Laboratories (Seoul, Korea). The slides were initially incubated with a rabbit anti-GRP94 polyclonal antibody (1:200; Abcam, Cambridge, UK), followed by OV HRP multimer. Immunoreactive proteins were visualized using the OptiView DAB IHC Detection Kit (Roche, Rotkreuz, Switzerland). Chromogenic reactions were initiated by incubating the slides with a freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution. All samples were counterstained with hematoxylin. GRP94 expression was observed using light microscopy on a Leica Aperio AT2 (Leica Biosystems, Wetzlar, Germany).

Interpretation of immunohistochemical staining

A semi-quantitative approach was employed to interpret the immunohistochemical staining of GRP94. The expression of GRP94 was calculated based on the intensity values of DAB and hematoxylin obtained from six randomly selected fields of each specimen. The staining intensity was rated on a scale of 1 (negative) to 4 (strong), with 2 indicating weak and 3 indicating moderate staining. The extent of positive staining in the tissue samples was assessed as follows: 0% - 9% was scored as none, 10% - 29% as 1, 30% - 39% as 2, 40% - 49% as 3, and 50% - 100% as 4. The density value of GRP94 expression was measured using Image J software version Fiji (National Institutes of Health, Bethesda, MD, USA). This approach provided a comprehensive and objective assessment of GRP94 expression in the analyzed tissue samples.

Cell culture

All cells were maintained at 37 ˚C with 5% CO_2 unless specified otherwise. Human gastric cancer (GC; MKN45, AGS, NCI-N87, and KATO III) and liver cancer (LC; Huh-7) cell lines were maintained in Roswell Park Memorial Institute 1640 medium

(Gibco, Billings, MT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). Human LC cell lines (Hep G2 and SK-HeP-1) were cultured in Dulbecco′s Modified Eagle's Medium (Gibco) with the same supplements. Expi293F cells were cultured in the Expi293 expression medium (Gibco) in a humidified shaking incubator at 37 ˚C with 8% CO_2.

Overexpression and purification of K101.1

K101.1 was produced as previously described (2). Briefly, a bicistronic mammalian expression vector encoding K101.1 was transfected into suspension-adapted Expi293™ cells using the Expi293 transfection kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Seven days after transfection, the overexpressed IgG was purified from the culture media using affinity column chromatography with Protein A Sepharose® (Repligen, Waltham, MA, USA). Following purification and subsequent dialysis in phosphate-buffered saline (PBS), the purity of the IgG antibody was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Flow cytometry

To confirm the expression of GRP94 on the cell surface, 2×10^5 human gastric (NCI-N87, AGS, MKN45, and KATO III) and liver (Hep G2, Huh-7, and SK-HeP-1) cancer cells were fixed with 4% (w/v) paraformaldehyde (PFA), blocked with PBS containing 1% (w/v) bovine serum albumin (BSA), and stained with a rabbit anti-GRP94 polyclonal antibody (1:100; Abcam) for 1 h. Additionally, the cells were incubated with Alexa Fluor 488-labeled anti-rabbit IgG (1:1000; Invitrogen, Carlsbad, CA, USA) for 1 h.

To confirm the specific binding of K101.1 to the GC cell surface GRP94, 40 μg/mL of rhGRP94 was preincubated with 4 μg/mL of K101.1 in PBS for 3 h at room temperature. Then, NCI-N87 cells fixed with 4% PFA were incubated with these mixtures for 1 h at room temperature. Following several washes with PBS containing 1% BSA, the cells were incubated with Alexa Fluor 488-labeled anti-human Fc IgG (Invitrogen, 1:200) for 1 h at room temperature in the dark. The

fluorescence intensity was measured using flow cytometry (Millipore, Burlington, MA, USA).

To evaluate the effect of K101.1 on the downregulation of cell surface GRP94, NCI-N87 cells (2×10^5 cells) were fixed with 4% (w/v) PFA. The fixed and unfixed cells were washed twice with PBS containing 1% (w/v) BSA and incubated in the presence or absence of 20 g/ml K101.1 for 4 h at 37 °C. The cells were stained with rabbit anti-GRP94 polyclonal antibody (Abcam) for 2 h at 4 °C. Subsequently, the cells were washed three times with PBS containing 1% (w/v) BSA, incubated with Alexa Fluor 488-labeled anti-rabbit IgG (1000:1; Invitrogen), and analyzed using flow cytometry (Millipore).

Public RNA-seq data analysis

Public RNA-seq data containing clinical information were obtained from the International Cancer Genome Consortium (ICGC) data portal. Specifically, we downloaded the stomach adenocarcinoma (STAD) RNA-seq data and clinical information from release 28. Our analysis focused on assessing the expression of GRP94 mRNA, using 35 samples from normal gastric tissues and 413 samples from GC tissues. To determine mRNA expression levels, we utilized the normalized read counts, which were calculated using the ICGC RNA-seq pipeline. Graphs were generated using the R program.

In Vitro measurement of GC cell growth

To examine the effect of K101.1 on GC cell growth, NCI-N87 cells were seeded into 96-well plates (3×10^4 cells/well) and then treated with 20 μg/ml control IgG and K101.1 for 60 h. Cell growth was assessed using the Cell Counting Kit-8 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The final absorbance was measured at 450 nm using a microplate reader (Synergy H1, BioTek, Winooski, VT, USA).

Measurement of antibody internalization

Antibody internalization in NCI-N87 cells was assessed using the FabFluor-pH Red

Antibody Labeling Reagent (Sartorius, Göttingen, Germany) following the manufacturer's instructions. The FabFluor reagent exhibits very low fluorescence intensity under neutral or basic pH conditions (on the cell surface or outside the cell). However, its fluorescence intensity increases in the acidic conditions of the endosomal and lysosomal compartments upon internalization of the reagent-labeled antibody. NCI-N87 cells were seeded at a density of 1×10^4 cells per well in 96-well culture plates and allowed to adhere overnight. Control IgG and K101.1 antibodies were separately labeled with the human FabFluor-pH Red Antibody Labeling Reagents at a molar ratio of 1:3 in PBS. The cells were then incubated for 15 min. Subsequently, the plates were transferred to the IncuCyte SX1 Live Cell Analysis instrument (Sartorius), and images were captured using a 10x objective over a 12-h period to measure fluorescence intensities.

Cell enzyme-linked immunosorbent assay

To assess the effect of K101.1 on GRP94 downregulation on the surface of NCI-N87 GC cells, 3×10^4 NCI-N87 cells were plated on a 96-well plate and incubated with 20 μg/ml of either control IgG or K101.1 for 0, 2, 4, 6, or 12 h at 37 ˚C. Subsequently, the cells were washed with ice-cold PBS and fixed with PBS containing 4% (w/v) PFA. Following fixation, the cells were incubated with a rabbit anti-GRP94 polyclonal antibody (1:5000, Abcam). After three washes with ice-cold PBS, the plates were further incubated with HRP-conjugated anti-rabbit Fc secondary antibody (1:5000; Invitrogen) for 1 h at 37 ˚C. Following another three washes with ice-cold PBS, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added. The optical density was measured at 450 nm using a microplate reader (Synergy H1, BioTek) after quenching with 1 M H_2SO_4 solution.

In vivo efficacy testing

The animals were maintained in a specific pathogen-free environment and acclimated to the laboratory conditions for at least one week before the experiment. The housing and care of the mice were carried out at the National Cancer Center's accredited animal facility (unit number NCC-22-849) in accordance with the AAALAC International Animal Care Policy. To assess the impact of K101.1 on NCI-

N87 tumor growth, BALB/c-nude mice were subcutaneously injected with 1×10^7 NCI-N87 cells. Once the tumor volume reached approximately 100 mm^3, the mice (n = 14) were randomly divided into groups and administered intravenous injections of PBS and 10 mg/kg of K101.1, as well as intraperitoneal injections of 40 mg/kg of 5-fluorouracil (5-FU) (Sigma-Aldrich) twice a week for three weeks. The mice were weighed, and tumor sizes were measured once a week, up to day 23.

In vivo toxicity testing

To assess *in vivo* toxicity, established procedures were followed. Briefly, BALB/c-nude mice (n = 8) received intravenous injections of 10 mg/kg K101.1 or intraperitoneal injections of 40 mg/kg 5-FU, twice weekly. The mice's body weights were recorded weekly throughout the 23-day study period. At the end of the study, the mice were euthanized, and blood samples were collected. Enzymatic activities, including glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), total bilirubin (TBIL), blood urea nitrogen (BUN), and creatinine (CRE), were analyzed using a Fuji Dri-Chem 3500 biochemistry analyzer (Fujifilm, Tokyo, Japan).

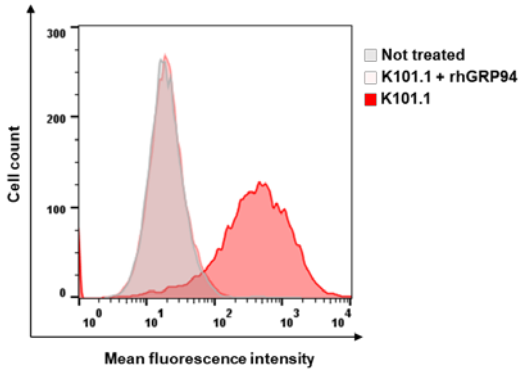
In vivo fluorescence IHC

Resected tumor specimens were embedded in an optimal cutting temperature (OCT) compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and stored at -80 °C. For IHC, frozen blocks were sectioned into 10-μm slices using a cryostat microtome (Thermo Fisher Scientific). These sections were fixed in 4% PFA in PBS for 10 min and washed three times with PBS. To assess the impact of K101.1 on microvessel density (MVD), tissue sections were blocked with 20% FBS in PBS for 30 min. Subsequently, the sections were incubated with an anti-CD31 monoclonal antibody (R&D Systems, Minneapolis, MN, USA) for 1 h. After three PBS washes, the sections were further incubated with FITC-conjugated anti-rat IgG (Invitrogen) for 1 h. To investigate the efficacy of K101.1 on apoptosis, each tissue section was subjected to individual examination of apoptosis and nuclear morphology using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Roche) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Sigma-Aldrich)

staining. Fluorescence images were captured using a confocal laser scanning microscope (Leica biosystems). Furthermore, MVD and apoptosis rates were quantified using inForm Advanced Image Analysis Software (PerkinElmer, Waltham, MA, USA) and LASX software (Leica), respectively.

Statistical analysis

The data were analyzed using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA). Two-tailed Student's *t*-tests were used for comparisons between two groups, and a two-way analysis of variance (ANOVA) with Bonferroni's correction was applied for multiple comparisons. All data are presented as means ± standard errors of the mean (SEM). Differences with *P* values below 0.05 were considered statistically significant and indicated on the graphs using the following symbols: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Supplementary Fig. 1. Specific binding of K101.1 to cell surface GRP94 on NCI-N87 cells. The specific binding of K101.1 to cell surface GRP94 on NCI-N87 cells was investigated using flow cytometry under different conditions. The analysis was performed in the absence of K101.1 (gray), in the presence of K101.1 (red), or in the presence of K101.1 preincubated with rhGRP94 (light red).

Supplementary Table. 1. Detailed information regarding cancer tissue samples printed on the tissue microarray

Organ	Diagnosis	Age	Sex
Skin	squamous cell carcinoma	65	M
Subcutis	Liposarcoma	36	M
Breast	infiltrating duct carcinoma	58	F
Lymph node	Hodgkin lymphoma	34	F
Bone	Osteosarcoma	54	F
Lung	Adenocarcinoma	61	M
Lung	squamous cell carcinoma	72	M
Liver	Cholangiocarcinoma	41	F
Liver	hepatocellular carcinoma	54	M
Liver	metastatic adenocarcinoma (from rectum)	52	M
Esophagus	squamous cell carcinoma	77	M
Gastric	Adenocarcinoma	65	F
Gastric	malignant lymphoma, diffuse large B cell	53	M
Gastric	signet ring cell carcinoma	40	F
Duodenum	gastrointestinal stromal tumor, malignant	61	M
Colorectal	Adenocarcinoma	62	M
Colorectal	Adenocarcinoma	73	M
Kidney	renal cell carcinoma	57	M
Bladder	invasive urothelial carcinoma	65	M
Prostate	Adenocarcinoma	63	M
Testis	Seminoma	35	M
Uterine	squamous cell carcinoma	65	F
Uterine	Adenocarcinoma	69	F
Ovary	metastatic adenocarcinoma (from stomach)	44	F
Ovary	mucinous cystadenocarcinoma	15	F
Ovary	serous cystadenoma of low malignant potential	44	F
Thyroid	papillary carcinoma	69	F

REFERENCES

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