Synergistic antitumor activity of a DLL4/VEGF bispecific therapeutic antibody in combination with irinotecan in gastric cancer

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SUPPLEMENTARY METERIALS AND METHODS

Western blotting

Cell lysates were prepared as described previously (1). Protein samples were loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and following electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with primary and secondary antibodies and the protein bands were visualized using ECL solution (Amersham Life science, Little Chalfont, UK) using a LAS3000 (Fujifilm, Tokyo, Japan). The FUSION SOLO S (Vilber, Eberhardzell, Germany) was used for image detection according to the manufacturer's instructions. Antibodies used in this study were anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), anti-DLL4, and anti-VEGFR2 (Cell Signaling Technology, Danvers, MA, USA).

RNA isolation and quantitative RT-PCR

Total RNA was prepared using RNA-lysis reagent (Intron) following the manufacturer's instructions. cDNA (1 μg) was synthesized using quantitative RT-PCR master mix (TOYOBO, Osaka, Japan). qRT-PCR was performed using SYBR Premix Ex Taq (Clontech Laboratories, Mountain View, CA, USA) with ABI instruments (Applied Biosystems Inc, Foster City, CA, USA). All results were normalized to β-actin.

Cell proliferation assay

Cells (3×10^3/well) were plated in 96-well culture plates. Following incubation for 24 h, the cells were treated with anti-hDLL4 and ABL001 at the indicated concentrations. Following transfection for 72 h, WST assay was performed as described previously (2). The absorbance was measured at 450 nm using an ELISA plate reader.

Transwell migration and invasion assays

Cells were isolated and added (2×10^4 cells/well) to the upper Transwell (Corning, New York, USA) chamber. The membrane (on the upper side) in the top chamber was coated with 0.5 mg/ml collagen type I (BD bioscience, Korea) for the migration assay, while the lower side of the membrane was coated with a 1/15 dilution of Matrigel (BD bioscience, Korea) for the invasion assays. RPMI 1640 supplemented with 10% FBS and 1% antibiotics was added to the lower chamber and the plates were incubated further for 24 h. Cells that migrated or invaded to the lower chamber were quantified following H&E staining by counting five randomly selected areas in each well using wide-field microscopy (3). Data are expressed as mean ± standard error of mean (SEM) from three independent experiments.

Cell cycle analysis

Cells were plated in culture plates and treated with anti-hDLL4 and ABL001. The cells were harvested, washed with cold PBS, and then resuspended in 5 ml 70% EtOH overnight at

-20°C. After fixing, the cells were resuspended in a propidium iodide staining solution (containing RNaseA 50 μg/ml, PI 50 μg/ml in PBS). Cell cycle distribution was measured using FACS analysis (4).

Apoptosis assay

Cells were plated in culture plates and treated with anti-hDLL4 and ABL001. After 24 h, the cells were washed and resuspended in FITC Annexin V and 7-AAD solution. Apoptosis was evaluated using FACS analysis following Annexin V staining.

Kaplan-Meier analysis

Kaplan-Meier analysis of survival curve was performed using cBioPortal TCGA database for patients with GC.

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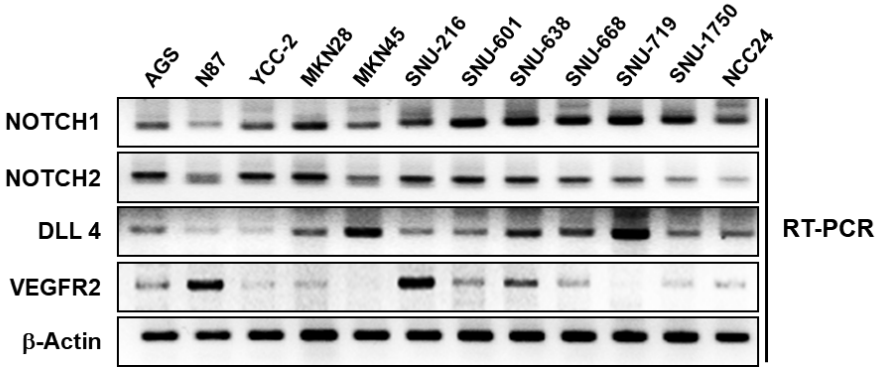
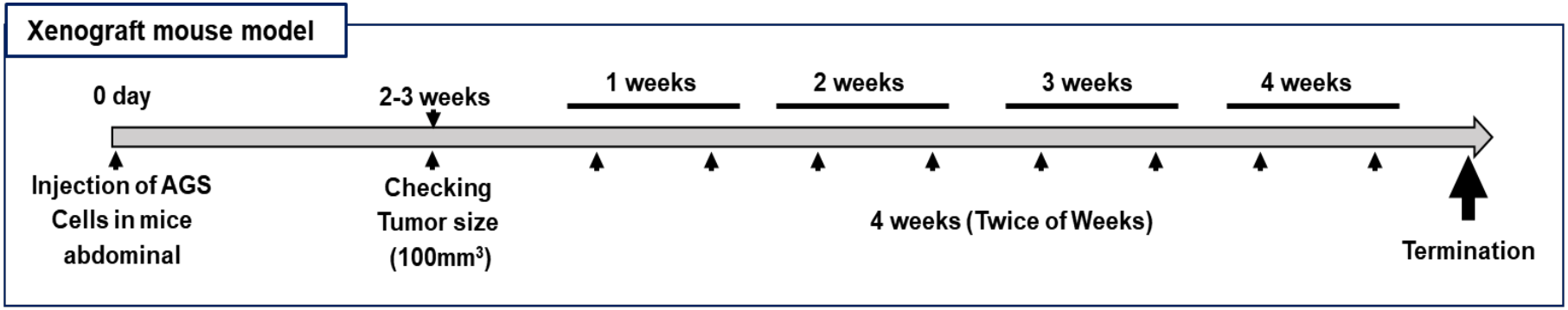
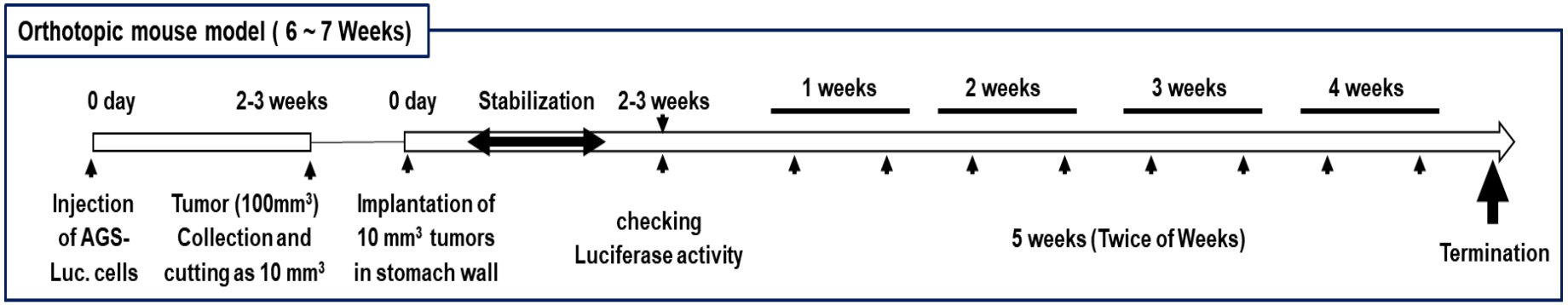
SUPPLEMENTARY FIGURES



Supplementary Figure 1. mRNA expression level of Notch related genes and VEGFR2 in the 12 GC cell lines. The basal mRNA expression level of Notch related genes, such as Notch 1, Notch 2 and DLL4, and VEGFR2 were evaluated by RT-PCR in twelve human GC cell lines. β-actin was served as the loading control.



Supplementary Figure 2. A schematic diagram of the *in vivo* xenograft mouse model illustrating the time-line of treatment with mABL001 and irinotecan



Supplementary Figure 3. A schematic diagram of the *in vivo* orthotopic mouse model illustrating the time-line of tumor implantation and treatment with mABL001 and irinotecan.