약학석사학위논문

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발암성 변이체 AIMP2-DX2에 특이적인 단일클론성항체 개발과 특성분석

2016년 8월

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이 논문을 약학석사학위논문으로 제출함

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성준식의 석사학위논문을 인준함
2016 년 8 월

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ABSTRACT

AIMP2-DX2 is known as an exon2-deleted splicing variant of AIMP2 (amonoacyl-tRNA synthetase-interacting multifunctional protein 2) showing high expression in lung cancer cells and patients' tissues. AIMP2-DX2 inhibits a pro-apoptotic role of AIMP2, which is known as an tumor suppressor, through competing for the association with common binding partners such as p53, FBP (fuse-binding protein) and TRAF2 (TNF receptor-associated factor 2) under various stimuli and stresses. According to previous studies, the cells with higher level of AIMP2-DX2 tend to show increased resistance to cell death. Also, suppression of AIMP2-DX2 in xenograft mouse model significantly retarded tumor growth.

Due to lack of monoclonal antibody specific to AIMP2-DX2, there has been a difficulty in developing diagnostic tools to classify patients with relatively higher level of AIMP2-DX2 for targeted cancer therapy. In this study, phage display technique was used to create monoclonal antibody that specifically binds to AIMP2-DX2. The newly generated monoclonal antibody was also validated and characterized by immunoblot, enzyme-linked immunosorbent assay, immunoprecipitation, surface plasmon resonance assay and immunofluorescence microscopy.

The results showed that the selected monoclonal antibody clone, H5, could specifically recognize AIMP2-DX2 but not AIMP2 and it showed a prominent binding interaction with the epitope peptide as well as purified AIMP2-DX2 protein. Based on these evidences, I concluded that the newly generated monoclonal antibody hold a great potential in both research and clinical fields and it could be employed in cancer diagnosis at in various ways.

Key Words AIMP2-DX2, Phage Display, Monoclonal Antibody, Lung Cancer, Diagnosis

Student Number 2014-24867

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ABBREVIATION LIST

AIMP2 : Aminoacyl-tRNA synthetase-interacting multifunctional Protein 2

C_H : Constant heavy chain

C_k : Constant kappa chain

C_L : Constant lambda chain

ELISA : Enzyme-linked immunosorbent assay

Fab : Fragment antigen-binding

IgG : Immunoglobulin G

IB : Immunoblot

IF : Immunofluorescence

IP : Immunoprecipitation

mAb : Monoclonal antibody

NSB : Non-specific binding

RU : Response units

SPR : Surface plasmon resonance

V_H : Variable heavy chain

V_L : Variable light chain

WCL : Whole cell lysate

INTRODUCTION

Pharmaceutical industry has entered a new era of personalized medicine for cancer diagnosis and therapy. Since the approval of Herceptin, development of targeted antibody therapy has grown rapidly. In 2014 alone, 8 out of 41 novel drugs including blinatumomab were developed for lymphoblastic leukemia (Grand View Research, 2016). Pharmaceutical industry is expecting skyrocketing increase in personalized medicine that will reach up to over 2,452 billion dollars by 2022 (Grand View Research, 2016).

Many gene mutations have been identified in various cancers and the mutant-targeting therapies have been increasingly approved by FDA. In the same context, existence of targeted therapies expands the needs of companion diagnostics development. Even though current lung cancer biomarkers such as KRAS and EGFR are taking a great portion on diagnosis, we still have over 36% of dark matter in lung cancer where there is no known diagnostic markers. Therefore we expect AIMP2-DX2 will be able to fulfill the moiety of medical unmet needs in lung cancer diagnosis (Lung Cancer Foundation America, 2016).

AIMP2-DX2 is known as a splicing variant of AIMP2 lacking exon2 produced by alternative splicing and it is highly expressed in lung cancer cells and patients tissues through competitive binding(Choi et al., 2011). AIMP2 is a potent tumor suppressor and it mediates pro-apoptotic activity of AIMP2 interaction with FBP, TRAF2, and p53 under TGF-β, TNF-α, and genotoxic stress. AIMP2-DX2 compromises pro-apoptotic activity of AIMP2 through the competitive binding to p53, TRAF2, and FBP (S. Kim et al., 2011; Choi et al., 2009; Choi et al., 2011; Choi et al., 2013). According to previous studies, the cells with higher level of AIMP2-DX2 showed higher propensity for the increase of anchorage-independent colony formation and were more resistant to chemotherapy (Choi et al., 2012). Knockdown of

AIMP2-DX2 via the chemical or small interfering RNA (siRNA) transfection suppressed tumor growth *in vivo* (Choi et al., 2012) suggesting that AIMP2-DX2 can be a promising target and diagnostic marker for anti-cancer therapeutics.

Over the past decade, many efforts have been made toward development of monoclonal antibody specific to AIMP2-DX2. A variety of methods were attempted to generate a monoclonal antibody because the only available AIMP2-DX2 antibody named under #324 detects both wild type AIMP2 and AIMP2-DX2 simultaneously, although it recognizes AIMP2 majorly with several non-specific bands in the immunoblotting. However, it was not successful to develop AIMP2-DX2 monoclonal antibody due to the protein instability and low immunogenicity. It was a big hurdle in the development of anti-cancer therapeutics targeting AIMP2-DX2, because there was a limitation for diagnosing cancer patients who have high expression of AIMP2-DX2 proteins.

In this study, I have successfully developed a new monoclonal antibody that specifically binds to AIMP2-DX2 by phage display technique. Over the past decades, phage display has evolved into well-accepted technology and it delivered high quality antibodies in short amount of time (Kretzschmar et al., 2002). Compared to other antibody producing techniques such as hybridoma, phage display led to success in generation of highly specific antibodies (Kretzschmar et al., 2002). Construction of antibody library began with immunization of two rabbits with AIMP2-DX2 antigen peptide with containing only 11 short amino acids residing in the exon 1 and exon 3 junction region. Modified pComb3XTT phagemid vector containing Fab (Fragment antigen-binding) gene and mRNA extracted from the spleen of one of the immunized rabbits was used to clone heavy and light chain of Fab fragment for building an appropriate sized antibody library. With the success of library construction, a single clone, H5, was sorted out and was tested for characterization of the selected antibody candidate. Through various experimental methods, I have reached a

conclusion that the H5 clone I discovered detects specifically an isotype of AIMP2-DX2.

MATERIALS AND METHODS

Rabbit Immunization, mRNA Extraction and cDNA Synthesis

A total of 200 μg keyhole limpet hemocyanin (KLH)-conjugated AIMP2-DX2 antigen peptide was dissolved in 500 μl phosphate-buffered saline (PBS) and mixed with 500 μg of adjuvant. Two rabbits were subcutaneously injected with 1 ml of the antigen and adjuvant mixture at 7-10 day intervals. A portion of blood was taken from each animal 1 week after each immunization and titrated by enzyme-linked immunosorbent assay (ELISA) to determine the presence of antigen-specific antibodies.

Following the final immunization, the rabbits were exsanguinated, and the spleen and blood serum were harvested. After weighing and blending, splenic tissues were placed in TRI-reagent (Molecular Research Center, Cat. TR118), and homogenized. An additional 15 ml of TRI-reagent was added and the homogenate was incubated for 5 min at room temperature. Phase separation was carried out by addition of 1.5 ml of BCP (1-bromo-3-chloropropane). The homogenate was strongly shaken by hands for 15 sec and centrifuged for 15 min at 12,000 rpm after incubation for another 15 min at room temperature. The supernatant was carefully transferred to a clean tube, avoiding contamination by the fatty layer and pellet separated on the bottom of the tube, and mixed with 7.5 ml of isopropanol for RNA precipitation. The tubes were vortex-mixed, incubated at room temperature for 10 min, and centrifuged at 12,000 rpm for 10 min at 4℃ to pellet the RNA. The supernatant was removed and the pellet was washed with 10 ml of 70% ethanol and incubated on ice for 5 min. Then, it was centrifuged at 12,000 rpm for 10 min at 4℃. Ethanol was attentively discarded and the pellets were air-dried for 3 min and resuspended in 1 ml of nuclease-free water under clean bench. To reprecipitate the pellet, 500 μl of 7.5 M lithium chloride was added to the tube. The concentration and purity were determined by measuring the absorbance at 260 nm/280 nm. The isolated RNA was stored at -80℃.

First-strand cDNA was synthesized using the reverse transcription-polymerase chain reaction (RT-PCR) kit (Thermoscientific Cat. K1651). For cDNA synthesis, 1 µl of total RNA was mixed with 1 µl of oligo$(dT)_{18}$ primer, 1 µl of 10 mM dNTP mix, 4 µl of 5x RT buffer, 1 µl of Maxima H minus enzyme mix, and water to a final volume of 20 µl. The reaction mixture was incubated for 30 min at 50℃. The reaction was terminated by heating to 85℃ for 5 min. The first-strand cDNA was immediately used for PCR amplification.

Generation of Rabbit Fab Fragment

First-strand cDNA derived from the rabbit spleen was used to amplify V_H ,variable heavy chain, and V_L ,variable light chain, genes and modified pComb3XTT phagemid vector was used for C_k, constant kappa chain and C_{H1}, constant heavy chain, cloning for construction of Fab antibody library. In order to create Fab fragment, three PCR steps were set up: a primary PCR which amplifies C_H-C_L and V_H-V_L genes, a secondary extension overlap PCR which combines V_H-C_H and V_L-V_H, and lastly third PCR which bonds chimeric light chain and Fd.

Construction of Fab Monoclonal Antibody Library

Modified pComb3XTT phagemid vector was used for the construction of chimeric Fab library. First, vector plasmid and PCR product were digested with restriction enzymes, SfiI/HindIII and BamHI/EcoRI. For digestion of DNA product, 15 µg of purified PCR product was mixed with 36 units/µg of SfiI, 20 µl of 10x buffer M, and water to a final volume of 200 µl. Digest mixtures were incubated at 50℃ for 16 hrs in waterbath followed by ethanol precipitation overnight at -20℃. Then, the digests were resuspended in 100 µl nuclease free water. Identical procedure was carried out for vector plasmid except the amount of restriction enzyme applied to the mixture (6 units SfiI/µg). Then, ligation of insert and vector was done with 140 ng of purified vector, with 3:1 molar ratio of

insert:vector, 4 µl of 5x ligase buffer, and 1 µl of T4 DNA ligase. It was incubated at room temperature overnight followed by electro-transformation with 300 µl of ER2537 competent cells and 3 ml of SOC (Super optimal broth with catabolite repression) medium. Transformed ligations were then incubated at 37℃ overnight. The total number of library was counted on 0.1 µl, 1 µl, and 10 µl on 100 mm LB plates. Roughly determined size of the antibody library was 7.2 X 10^7.

Panning of Phage Displayed Antibody Library

Phage from Fab library was panned against AIMP2-DX2-BSA antigen (3.9 mg/ml). For each round of selection, 2.6 µg of antigen in a total of 1 ml of PBS was coated on an immunotube (Nunc, Cat. 470319). The tube was coated for 1 hr at 37℃ and subsequently blocked by filling the tube with 5% skim milk in PBS for 1 hr at room temperature. The blocking solution was removed and 1ml of prepared phage from library rescue was added to the antigen coated immunotube. It was then incubated with shaking at 37℃ for 2 hrs. The unbound phages were washed away by filling the tube with 1 ml of PBST three times. The bound phages were eluted by adding 1 ml of 100 mM TEA (Triethanolamine) and incubated precisely for 10 min at 37℃ with agitation at 120 rpm. TEA detaches antigen-antibody from the wall. Then, eluted phage was mixed with 500 µl of 1M Tris-HCl (pH 7.4) for neutralization. The neutralized phage was then applied to 8.5 ml mid-log phase ER2537 and incubated for 1 hr at 37℃ with shaking at 120 rpm. Infected ER2537 was diluted to test input and output colony growth overnight at 37℃. Identical panning steps were repeated three times for the optimum affinity selection of antibody binding to target peptide.

Screening for Phage Library Clones

Following three round panning selection, pooled phages were screened by ELISA to sort antigen binding candidates. Individual clones were isolated by sterile toothpicks into 96 well round

bottom plate containing 200 µl SB (Super broth) supplemented with 100 µg/ml ampicillin and grown at 37℃ at 220 rpm overnight. Next day, these picks in 96 well round bottom plate were duplicated by use of a pin replicator (Boekel Scientific, Cat. #140500) and stored at -80℃ freezer additionally supplemented with 50% glycerol for further experiment. Cells were spun down at 3,500 *g* in a plate centrifuge for 15 min and supernatant were discarded by inverting plate. Pellets were resuspended with 1x TES (Tris(hydroxymethyl)-methyl-2-aminoethane sulfonate) and 0.2x TES in sequence to lyse the cell. Centrifuged soluble fragments were then applied to two immunoplates coated with 15.5 µl of AIMP2-DX2-BSA antigen in 6 ml PBS (final concentration 10 µl/ml) or PBS for control. Prior to incubating the antibody with immobilized antigen, blocking was done with 3% skim milk for 1 hr. Immunoplates with transferred supernatant was incubated for 1 hr at room temperature with gentle agitation and Fab specific secondary antibody (Sigma Cat. A0293) as well. After binding is completed, plates were washed three times with tap water then pre-warmed TMB (3,3', 5,5'-Tetramethylbenzidine) solution was applied evenly to each well. As blue color develops, 1 M sulphuric acid was used to stop the reaction turning yellow. Absorbance was read at 450-650 nm in a plate reader.

Sequencing of the Selected Clones

Selected fragments from the ELISA screening test were sequenced with a pelseq primer stocked at company. Beforehand, amplification was done with 1 pick of colony, pc3x-f primer, pc3x-b primer, 10x PCR buffer, 10 mM dNTP mix, taq polymerase and water to final volume of 50 µl. Sequences were analyzed at Macrogen Korea. Sequences for pc3x-f and pc3x-b primers are GCACGACAGGTTTCCCGAC and AACCATCGATAGCAGCACCG, respectively.

Cell Culture

H460 human large lung carcinoma cells and A549 human lung carcinoma cells were cultured in Rosewell Park Memorial Institute (RPMI) 1640 Medium (with 25 mM HEPES, L-Glutamine, Cat. SH30255.01) with 10% fetal bovine serum (FBS, Hyclone, Cat. SH30919.03) and 100 µg/ml of penicillin and streptomycin (10,000 units/ml penicillin/10,000 µg/ml streptomycin, Hyclone, Cat. SV30010) at 37℃ in 5% CO_2 incubator. AIMP2-DX2 isotype-expressing plasmids were transfected into A549 human lung carcinoma cells to monitor the cell proliferation.

HEK293T human embryonic kidney cells were culture in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (with 4 nM L-Glutamine, 4500 mg/l glucose, sodium pyruvate, Cat. SH30243.01) with 10% Fetal Bovine Serum (FBS, Hyclone, Cat. SH30919.03) and 100 µg/ml of penicillin and streptomycin (10,000 units/ml penicillin/10,000 µg/ml streptomycin, Hyclone, Cat. SV30010) at 37℃ in 5% CO_2 incubator.

Freestyle 293F cells for antibody purification with Protein A agarose (Pierce, Cat. 20333) were cultured in Freestyle 293 Expression Medium (with Glutamax, Gibco, Cat. 12338018) at 37℃ in an atmosphere of 5% CO_2 incubator.

Immunoblotting

Cells used in the experiments were lysed in cold RIPA lysis buffer (1 M Tris-HCl pH 7.4, 5 M NaCl, 20% NP-40, 10% sodium deoxycholate, and 20% sodium dodecyl sulfate, supplemented with protease inhibitors) for 20 min at 4℃. Then, each lysate was collected into an eppendorf tube and centrifuged at 13,000 rpm for 20 min at 4℃. The supernatant proteins were quantified by Bradford assay (BioRad, Cat. 500-0006). After boiling samples at 100℃ for 5 min, they were loaded on SDS page gels and separated by electrophoresis. After running, proteins on the gel were transferred to Amersham Protran 0.45 µm nitrocellulose blotting membranes (GE Healthcare, Cat. 10600002) at

1.3 mA, 25 mV for 1 hr. The membranes were incubated with 5% skim milk solution based 0.5% PBS-T for 1 hr to prevent non-specific bindings. After removing the skim milk solution, primary antibodies were added to the each membrane during overnight at 4℃. Next day, the membranes were washed with 0.5% PBS-T buffer for 5 min, 3 times repeatedly. Secondary antibodies were added and incubated for 1 hr. Membranes were washed equally to the previous step, and ECL solution (GE Healthcare, Cat. RPN2232) was applied to the membrane.

Immunoprecipitation (IP)

H460 cells were seeded in 100 mm plates and incubated in RPMI medium for 24 hrs. The cells were rinsed with cold PBS and lysed in 1 ml RIPA buffer for 20 min on a rotator at 4℃. Then the cell extract was centrifuged at 13,000 rpm for 30 min at 4℃. The supernatant was isolated from the pellet and incubated with Protein A agarose overnight. Next day, primary antibody was added and incubated on a rotator again at 4℃ for 2 hrs. After that, complexes were washed with lysis buffer 5 times with centrifugation at 2000 rpm for 1 min at 4℃. Thereafter, the proteins were boiled in 20 μl of 3x SDS loading buffer at 100℃ for 5 min. The collected proteins were run through 10% SDS PAGE gel for coomassie staining and immunoblotting.

Small Interfering RNA (siRNA)

Three different siRNA was designed and purchased from Oligo Center, ST Pharm Co. Ltd. Samples were named after their silencing sites: siDX2-1(exon1/3 junction), siDX2-2(exon3/4), and siDX2-3(exon4). The siRNAs targeting those three sites were designed as the sequence of CTGGCCACGTGCAGGATTA, GGAACATTGCACGTTTCTT and GCTGTCAACGCAACCCTTA, respectively.

Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance (SPR) experiments were performed using a Biacore T200 (GE Healthcare) equipped with a Series S sensor chip CM5 (GE Healthcare) at 25℃. PBS buffer (KH_2PO_4 144 mg/l, NaCl 9,000 mg/l, Na_2HPO_4 795 mg/l, without calcium or magnesium) was used as a running buffer for the immobilization procedure. Immobilization was performed by using amine coupling kit (GE Healthcare). Flow cells were activated with a 7 min pulse of a 1:1 mixture of EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) and NHS (N-Hydroxysuccinimide) according to manufacturer's recommendations. H5 (human IgG form) was diluted with 10 mM sodium acetate (pH 5.0) and immobilized to the chip giving a surface density of 3320 response units (RU). Flow cells were then blocked with a 7 min pulse of 1 M ethanolamine-HCl (pH 8.5). For the interaction analysis, epitope peptide was diluted in PBS buffer supplemented with NSB (non-specific binding) reducer (GE Healthcare) and a serial dilution series was created with a 2-fold dilution factor to give concentrations of 0.02 to 1 μM.

In another set of experiment, AIMP2-DX2 was diluted with 10 mM sodium acetate (pH 5.0) and immobilized to a Series S sensor chip CM5 to achieve immobilization levels of 590 RU. H5 antibody was diluted in PBS buffer with NSB reducer producing a 2-fold dilution with the concentration ranges from 0.04 to 1 μM. After each binding cycle, a regeneration solution (10 mM glycine-HCl, pH 1.5) was injected to remove any non-covalently bound protein. The transformed data was fitted into a 1:1 binding model in Biacore T200 Evaluation software v2.0 (GE Healthcare). For each set of experiments, sensorgrams obtained with the reference channel were subtracted from those obtained with the channel with each protein.

Immunofluorescence (IF)

Immunofluorescence microscopy was done using H5 monoclonal antibody. Approximately $1.2x10^5$/ml CHO-K1 cells were seeded with RPMI media in 12 well plates. On the second day, empty

vector (EV) and AIMP2-DX2 DNAs were transfected into the cells with Lipofectamine 3000. After the media is completely removed, 100% MeOH was added to each well for fixation and permeabilization of the cell for 7 min at room temperature. Blocking was done with 3% CAS-Blocking solution (Life Technologies, Cat. 008120) for 15 min at room temperature. Primary antibody, anti-AIMP2-DX2, was diluted and applied to at 1:100 ratio for 1 hr at RT. Next, secondary antibody, Alexa 488 (Invitrogen, Cat. A11008), was diluted and added at 1:500 ratio for 1 hr at room temperature. Fluorescent nucleic acid staining dye, hoechst (Invitrogen, Cat. H3570), was also applied to the plates at 1:1000 ratio for 10 min at RT. Stained cells were mounted on slides covered with cover glass and the pictures were taken under microscope with 20x and 60x resolution.

RESULTS

Rabbit Immunization with AIMP2-DX2 Peptide Antigen

Since AIMP2-DX2 is a splicing variant of AIMP2 full size (AIMP2-F) lacking exon2, all the sequences are the same with AIMP2 except for the exon1 and exon3 junction region (Fig. 1A), therefore we used 11 amino acids spanning the junction region as an antigen (Fig. 1B) to induce immune reaction in rabbits. To investigate whether polyclonal sera from two immunized rabbits would recognizes AIMP2-DX2, immunoblotting was carried out using H460 cell lysates which is known to show high level of AIMP2-DX2 protein (Lee et al., 2012). Both immunized and pre-immunized sera were used for the immunoblotting to compare the differences before and after immunization. The result revealed that only one serum obtained from #2 rabbit showed clear signal around the AIMP2-DX2 size whereas the other rabbit serum detected two bands whose sizes were similar to those of AIMP2-F and AIMP2-DX2, respectively (Fig. 1C). When I compared the #2 rabbit serum with #324 antibody via immunoblotting, the band size detected by the rabbit serum was smaller than the original DX2 band size (Fig. 1D). Actually the band coincided with the lower band of original AIMP2-DX2 which was detected by #324 antibody. Although the protein recognized by the rabbit serum was not matched with original AIMP2-DX2 based on the size, there is still possibility that it would be another kind of variant of AIMP2-DX2 (for convenience, I named the protein detected by the rabbit serum as AIMP2-DX2 isoform). Therefore I used the spleen from the #2 rabbit for the construction of an antibody library to generate monoclonal antibody specific to AIMP2-DX2.

Construction and Selection of Monoclonal Antibody Library

After immunization was completed, mRNA was extracted from the spleen of #2 rabbit and used for the synthesis of cDNA from which the antibody variable regions were amplified by PCR (Fig. 2A). Fab fragments were generated by three steps of overlap PCR and then transformed into ER2537 competent cells (Fig. 2B-2D) followed by ligation between Fab fragments and pComb3XTT vector. Finally, a total of 7.2 x 10^7 numbers of phage library was generated. The uniquely generated large synthetic antibody library was then used for panning against the BSA-conjugated AIMP2-DX2 peptide. Gradual increase in the number of washes over three rounds of panning selection provided selective pressure for the isolation of the high affinity monoclonal antibodies (Andris-Widhopf et al., 2000). After the third round of bio-panning, randomly picked clones were subjected to ELISA screening, and several clones showed solid binding affinity to AIMP2-DX2. Total 3840 copied library clones were tested through ELISA and the number is narrowed down to 22 candidates based on the positive signal obtained by immunoblotting. Among all the selected candidates, H5 clone was found to be the only one that specifically recognized AIMP2-DX2 isoform according to the immunoblot result, although there were several other clones which showed higher affinity to AIMP2-DX2 peptide in ELISA (Fig. 3). The heavy and light chain of H5 Fab were sequenced to guarantee the accurate selection and further characterization (data not shown).

Identification of Possible AIMP2-DX2 Isoforms

To prove whether H5 Fab truly recognizes AIMP2-DX2 isoform, exon1 and exon3 junction peptide (GAGHVQDYFALK) was incubated with *Escherichia coli* extract

containing H5 Fab during immunoblotting. The signal from H5 was considerably decreased by addition of junction peptide, suggesting that H5 specifically recognizes AIMP2-DX2 epitope (Fig. 4A). To conform whether the signal is dependent to original AIMP2-DX2 protein, I designed three different siRNA sequences specific to AIMP2-DX2 (Fig. 4B) and transfected each of them into H460 cells. The cell lysate from transfected H460 with three different siRNAs was subjected to immunoblotting to detect AIMP2-DX2 isoform. The result revealed that the signals from H5 Fab was clearly decreased by siRNA transfection with slight difference in silencing extent as those of #324 (Fig. 4C) suggesting that the protein recognized by H5 was a real AIMP2-DX2 isoform. To further validate the relationship with original AIMP2-DX2, I prepared three different AIMP2-DX2 isoforms (Δ2-DX2, Δ23-DX2, and Δ 33-DX2) and full-length AIMP2-DX2 (F-DX2) with strep tag in the N-terminal region. These isoforms are expected to be expressed endogenously in that they have different in-frame translation initiation sites or can be endogenously processed by various proteases (Fig. 4D). These isoforms were overexpressed in HEK293T cells and the cell lysate was used for immunoblotting. H5 Fab was able to detect all the strep-tagged AIMP2-DX2 isoforms prominently (Fig. 4E). The epitope peptide was incubated with the H5 Fab again and as expected, all the signals from the overexpressed AIMP2-DX2 isoforms were disappeared. All these results strongly support that the protein recognized by H5 is the real AIMP2-DX2 isoform derived from original AIMP2-DX2, although I don’t know it is a splicing variant or proteolytic fragment of AIMP2-DX2. Furthermore, overexpression of the three different AIMP2-DX2 isotypes increased the cell proliferation like original AIMP2-DX2 (Fig. 4F), suggesting that AIMP2-DX2 isoforms may have oncogenic function like original AIMP2-DX2. Taken together, these results demonstrated that H5 Fab recognizes a specific AIMP2-DX2 isoform which may enhance cell proliferation like original AIMP2-DX2.

Conversion of H5 Fab into IgG1 Form

H5 Fab fragment was converted into IgG1, a general antibody form, to deduct an accurate data along with further experiment. Heavy chain and light chain of the fragment was amplified with PCR and ligated into human pVITRO1-IgG1 vector (Fig. 5). The light chain sequence of complete H5 IgG was designed to be identical to that of H5 Fab. H5 IgG was then expressed in 293F freestyle cell, purified with protein A affinity chromatography and used for further studies.

Characterization of H5 Monoclonal Antibody by Measuring the Affinity and Specificity against AIMP2-DX2

To further evaluate the functional potency of H5 clone, immunoblotting was performed using H460 cell lysate. H5 IgG recognized endogenous AIMP2-DX2 isoform like H5 Fab as well as the original rabbit serum suggesting that the detection level of H5 IgG was clearly and accurately maintained. (Fig. 6A). To confirm the specificity of H5 IgG to AIMP2-DX2, 3 different siDX2 (Fig. 4B) were transfected into H460 cell line and the lysate was subjected to the immunoblotting with H5 IgG. As shown in Figure 6B, the signal of AIMP2-DX2 isoform detected by H5 IgG was dramatically affected by all the siRNA transfection (Fig. 6B). H5 monoclonal antibody with rabbit form of IgG was further tested for immunoprecipitation (IP) assay. The results showed us that H5 IgG can be applicable to IP also (Fig. 7). Heavy and light chain of H5 IgG was detected due to its cross-reactivity between mouse and rabbit antibody.

To investigate the binding affinity of H5 monoclonal antibody to the antigen, SPR

was performed using AIMP2-DX2 epitope as well as AIMP2-DX2 full size protein. Binding kinetics of H5 IgG and AIMP2-DX2 antigens in both peptide and protein format was analyzed. When H5 IgG antibody was immobilized on sensor chip and AIMP2-DX2 specific peptide (exon1/3 junction) was ran over the H5, the equilibrium affinity constant (Kd) was measured 22.5 nM. AIMP2-DX2 protein antigen and H5 IgG interaction was also measured as 10.7 nM (Fig. 8). As shown in the picture, association (ka) and dissociation (kd) values of H5 suggest that the association and dissociation to peptide is faster than those of AIMP2-DX2 protein. The difference can be explained from the structure of antigen and antibody. Peptide antigen is normally in linear form which leads antibody to single binding. On the other hand, protein antigen has complex folding structure that antibody cannot go on and off the binding site as fast as in the peptides. In addition the bivalency of antibody can influence the speed of association and dissociation. According to a reference (Lehninger, 2013), the typical Kd value for a good antibody is ranged from 10^{-8} to 10^{-12} and that is the case of H5 monoclonal antibody.

IF microscopy was also performed to see the possibility of H5 application. Strep-tagged AIMP2-DX2 was overexpressed in Chinese hamster ovary (CHO) cell line and IF was performed. While empty vector (EV) transfected cell showed low level of AIMP2-DX2 signal, the AIMP2-DX2 overexpressed cells revealed clear signals when H5 was applied (Fig. 9). It implies that H5 can specifically recognize AIMP2-DX2 isoform in the IF setup and be applicable to IF without non-specific background signals.

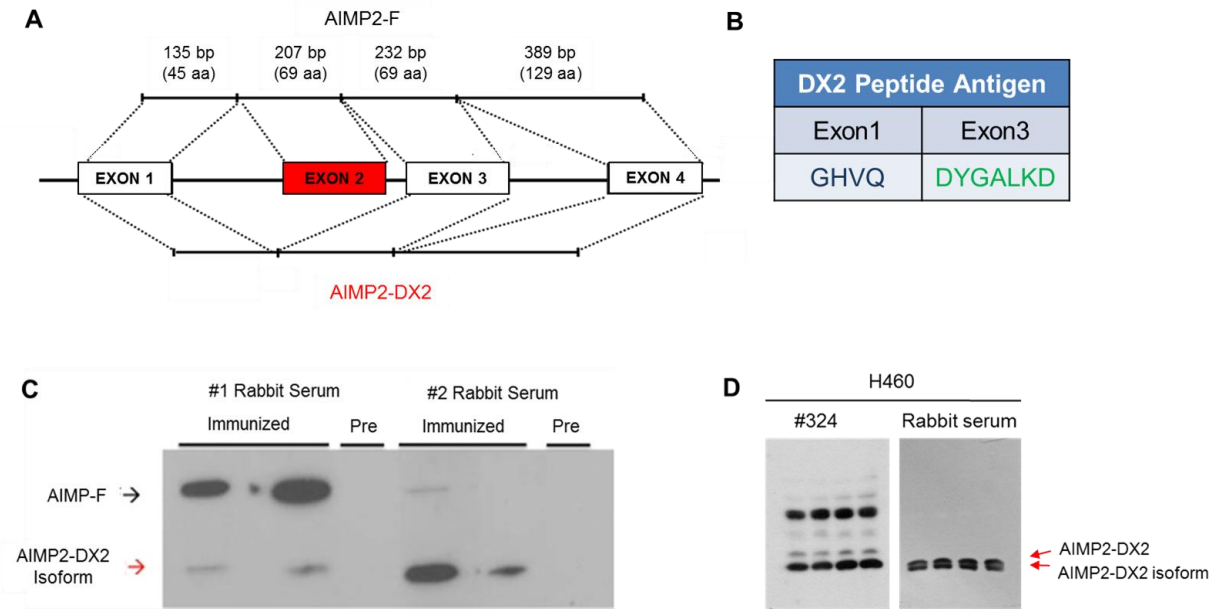

Figure 1. Rabbit Immunization with AIMP2-DX2 Peptide Antigen

(A) Schematic representation for the composition of AIMP2 full size (AIMP2-F) and exon2-deleted splicing variant AIMP2-DX2.

(B) Sequence of AIMP2-DX2 specific antigen used for rabbit immunization. Antigen containing eleven amino acids located at the junction of exon1 and exon3 were synthesized.

(C) Immunized and pre-immunized serum were subjected to immunoblotting using H460 cell lysate.

(D) The polyclonal rabbit serum from #2 rabbit was used for immunoblotting with H460 cell lysate and compared with #324 antibody which recognizes both AIMP2 and AIMP2-DX2.

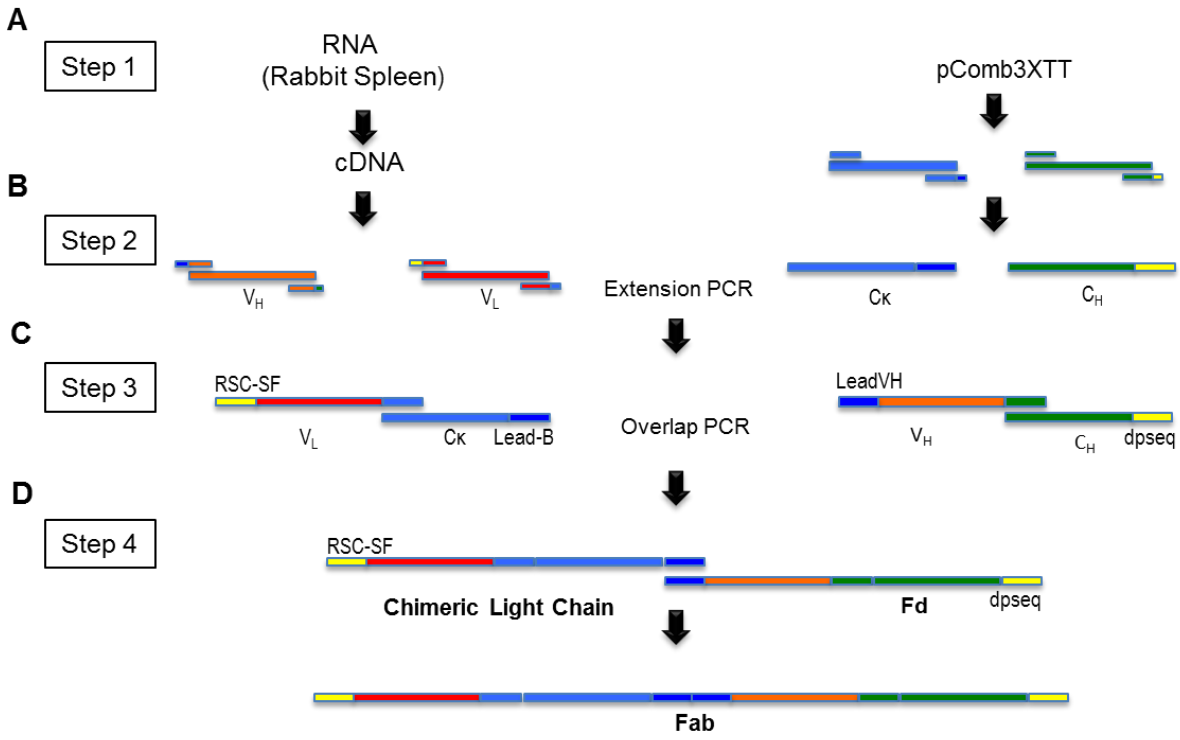

Figure 2. Cloning process of Fab fragment generation

(A) Overall schematic map of antibody fragment generation by overlap extension PCR. First step is to synthesize cDNA with mRNA extracted from spleen of immunized rabbit and clone constant light and heavy chain with pComb3XTT phage vector containing Fab gene.

(B) Second step is to amplify V_H/V_L from cDNA synthesized with extracted mRNA and C_k/C_{H1} from pComb3XTT plasmid vector.

(C) For tertiary step, light chains were fused with RSC-SF/Lead-B primers and heavy chains were overlapped with LeadVH/dpseq primers.

(D) Last step is to combine chimeric light chain and heavy chain Fd into full length Fab fragment.

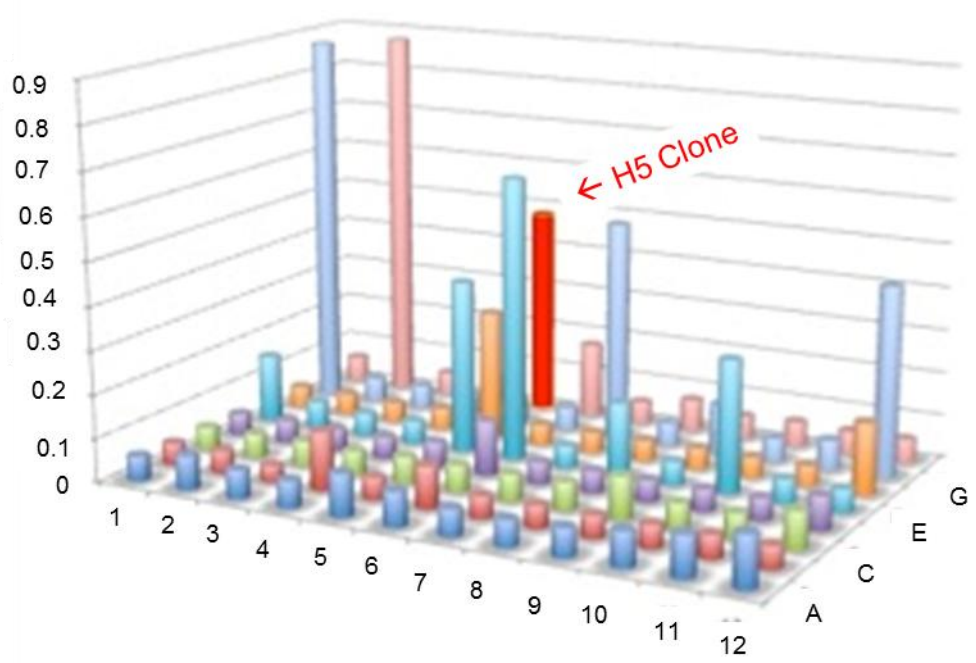

Figure 3. Phage screening of the antibody clones by ELISA

Quantitative analysis for the phage clones which showed the positive signal based on ELISA. The bar in red color indicates the selected H5 clone.

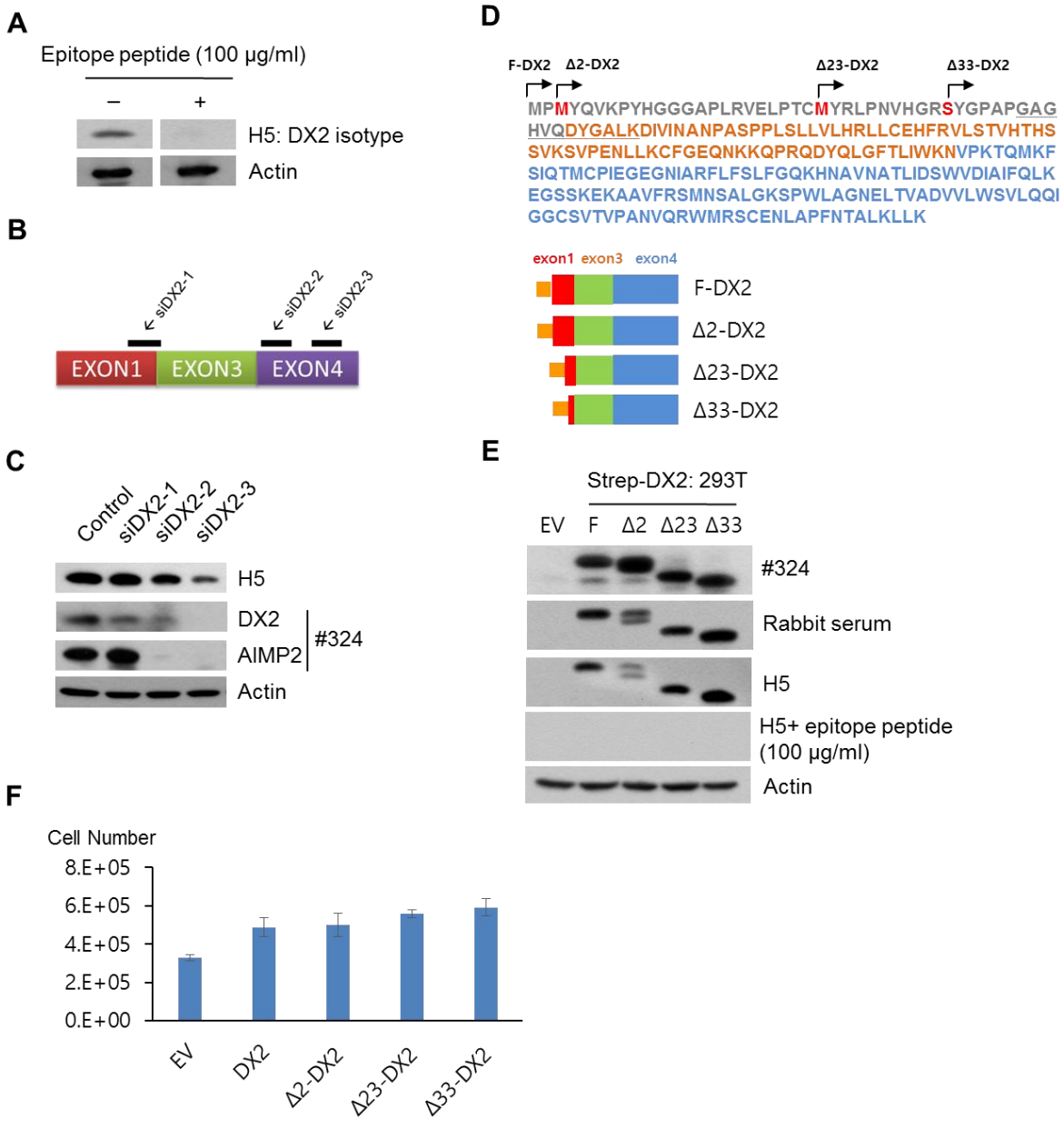

Figure 4. Identification of possible AIMP2-DX2 isoforms

(A) Masking effect of AIMP2-DX2 epitope peptide (GAGHVQDYFALK) for the band recognition by H5 Fab. AIMP2-DX2 epitope peptide was incubated with *E. coli* extract containing H5 Fab during the incubation step with PDVF membrane. The signal by H5 was clearly decreased by addition of the junction peptide.

(B) A schematic diagram for the hybridization site of three siDX2s used for transfection (Continued on following page).

(C) Levels of AIMP2-DX2 isoform detected by H5 and #324 were considerably reduced by siDX2 treatment. Three different siDX2 were used to reduce AIMP2-DX2 expression in H460 cells.

(D) Sequence of possible AIMP2-DX2 isoforms and structure of the relative size of each AIMP2-DX2 isoform used in Fig. 4E and 4F. Each exon sequence was differentiated by color and yellow colored box indicates the strep tag in the N-terminal region of each protein.

(E) H5 Fab was used for immunoblotting to see whether it can recognize overexpressed AIMP2-DX2 and its possible isoforms, and compared with #324 antibody and polyclonal rabbit serum. AIMP2-DX2 epitope peptide was also incubated with H5 Fab to see the competition effect.

(F) AIMP2-DX2 isotype-expressing plasmids were transfected into A549 cell and the cell numbers were counted to check cell proliferation.

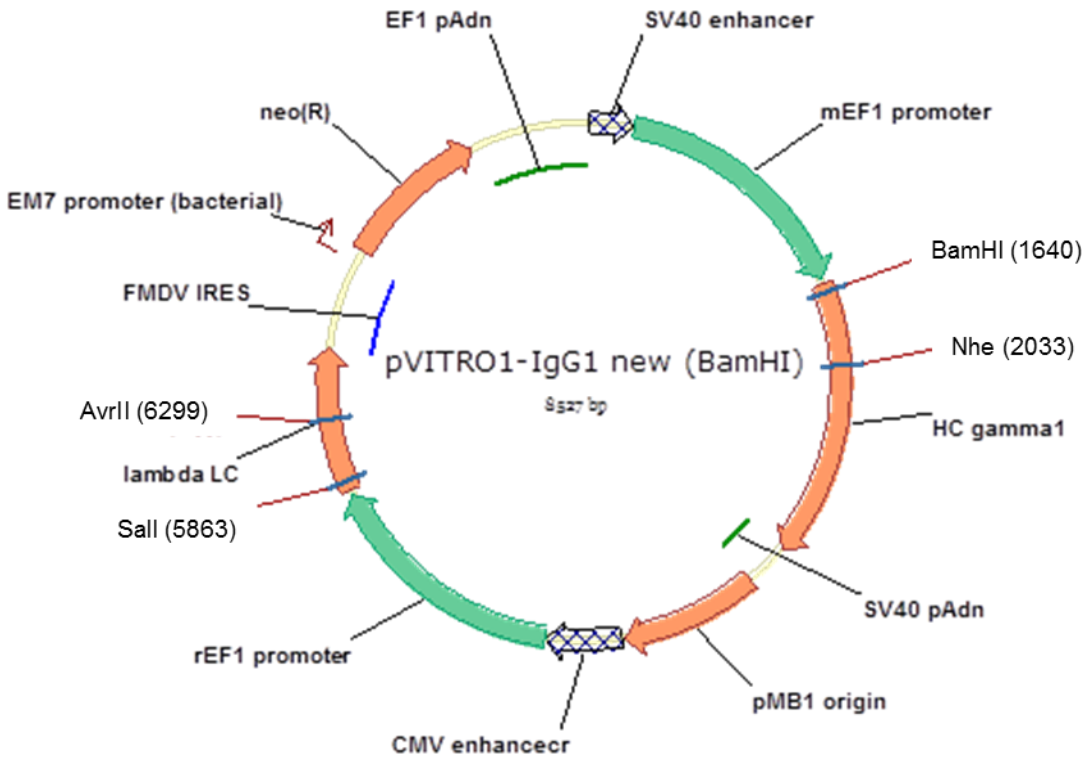

Figure 5. Map of pVITRO-IgG used to convert H5 Fab into IgG format

The pVITRO-IgG1 vector with cloning sites for both V_H and V_L genes was used for conversion. The BamHI and NheI sites are provided for cloning PCR-amplified variable heavy chain. The AvrII and SalI sites are provided for cloning PCR-amplified variable light chain.

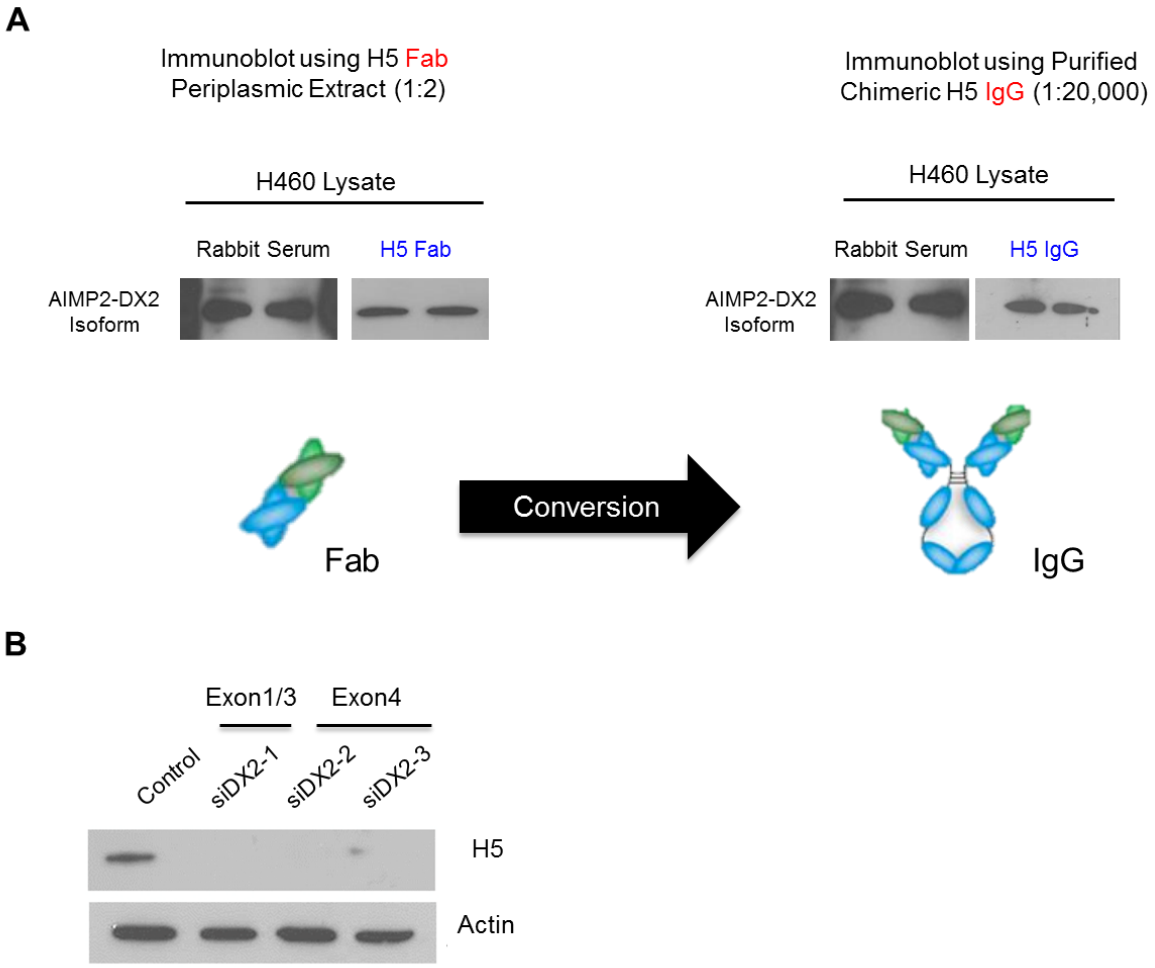

Figure 6. Characterization of H5 IgG via immunoblotting

(A) H5 Fab was converted into IgG form and then H5 Fab and H5 IgG were compared with the original rabbit polyclonal serum to see the immunoblot applicability. The H5 Fab was diluted 1:2 in 0.1% skim milk.

(B) To see the specificity of H5 IgG to AIMP2-DX2, H460 cells were transfected with three different siDX2 and used for immunoblotting with H5 IgG.

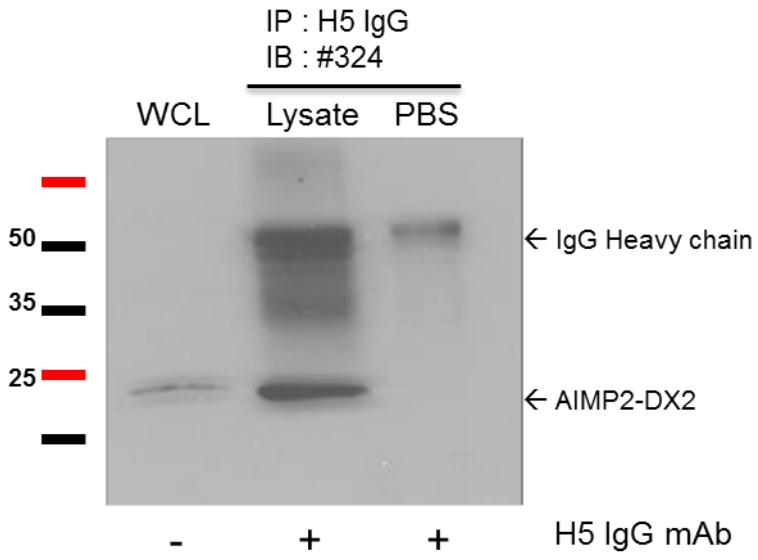

Figure 7. Immunoprecipitation analysis using H5 IgG

Purified H5 IgG form was applied to immunoprecipitation (IP) and analyzed by immunoblotting (IB). Band for AIMP2-DX2 isoform immunoprecipitated by H5 IgG was clearly visualized by immunoblotting with #324 antibody. Red and black bar with numbers are for the protein size (kDa).

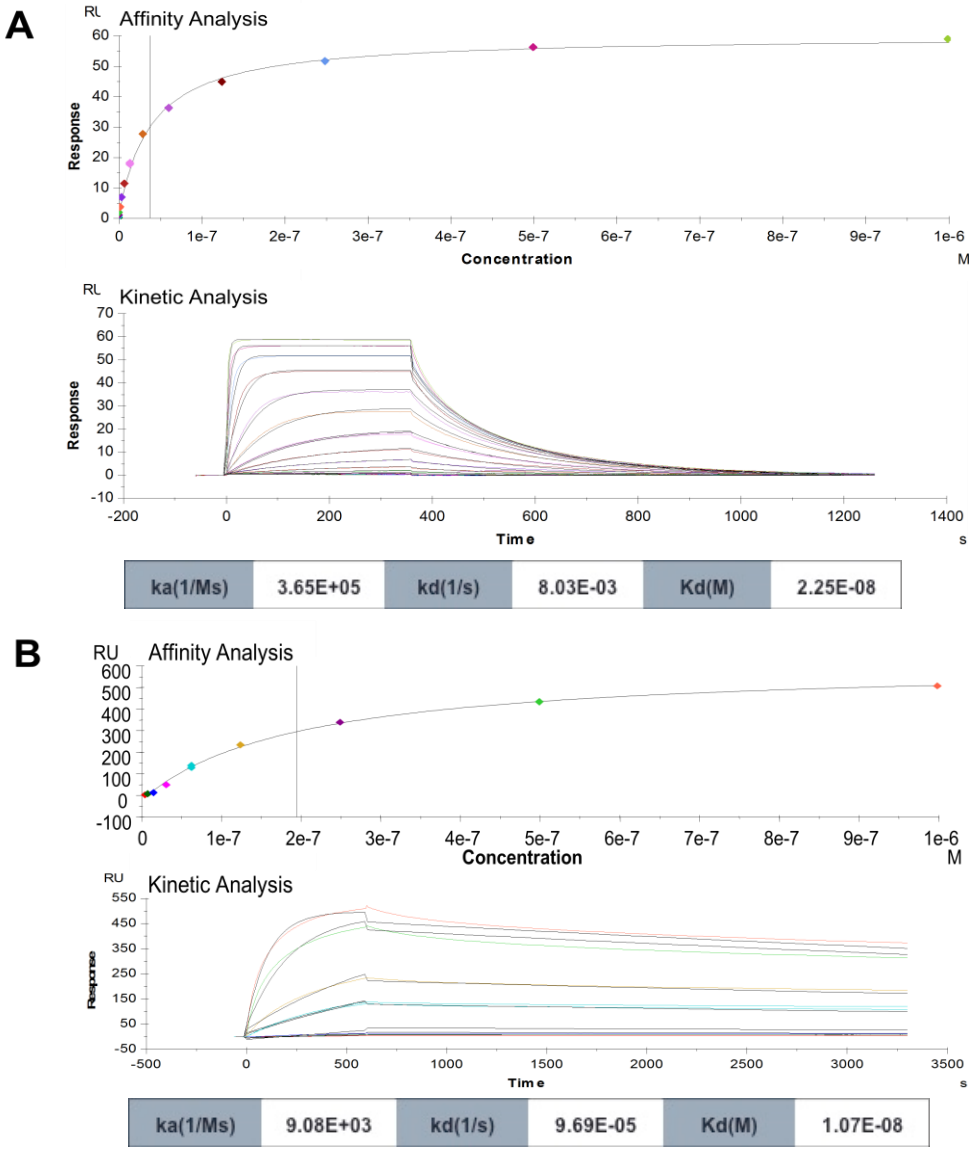

Figure 8. Surface plasmon resonance (SPR) analysis for the binding affinity between H5 IgG and AIMP2-DX2 antigens

(A) H5 IgG was immobilized on a Series S sensor chip CM5 and AIMP2-DX2 peptide (exon 1/3 junction) antigen was flowed over H5 IgG in a concentration dependent manner. Kd (M) value was measured as 22.5 nM.

(B) Identical experiment was performed with AIMP2-DX2 protein antigen instead of peptide. The protein antigen was immobilized on a Series S sensor chip CM5 and H5 IgG was ran through the chamber. Kd(M) value was measured as 10.7 nM. The interaction (A and B) was analyzed by affinity as well as kinetic methods, respectively.

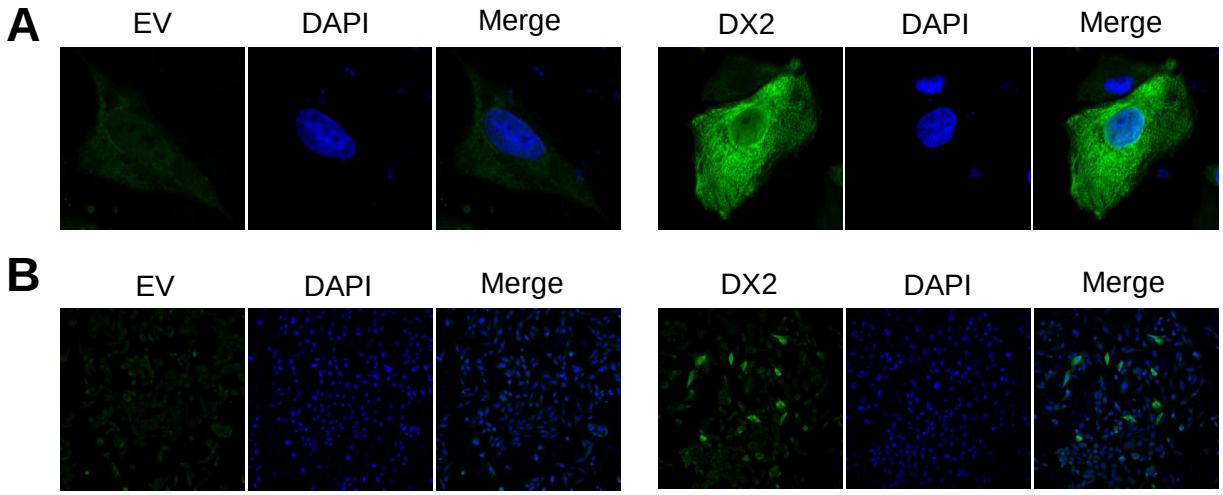

Figure 9. Immunofluorescence microscopy using H5 monoclonal antibody

H5 IgG was subjected to immunofluorescence analysis with CHO-K1 cells which were transfected with AIMP2-DX2 plasmid or empty vector (EV). H5 IgG attached to AIMP2-DX2 was visualized with green color using Alexa 488 fluorophore. Nucleus was stained with DAPI (blue). The image was presented at 60x resolution (A) and 20x resolution (B), respectively.

DISCUSSION

According to previous studies, AIMP2-DX2, exon2 deleted version of AIMP2, compromises the tumor suppressive activity of AIMP2 and induces tumor formation. It is known that high expression of AIMP2-DX2 in cancer patients is significantly correlated to poor prognosis (Choi et al., 2011). In the same context, suppression of AIMP2-DX2 suppressed tumor growth, suggesting it as a potent therapeutic target and diagnostic marker (Choi et al., 2011).

Over the past years, multiple trials were attempted to create an anti-AIMP2-DX2 antibody applicable to diagnosis but it has not been successful probably due to the bad stability and low immunogenicity of AIMP2-DX2 protein. Immunization with purified AIMP2-DX2 protein with different tags and modifications to increase the solubility and stability was tried to rabbits and mice but we could not get any reliable antibodies specific to AIMP2-DX2. We also tried various lengths of AIMP2-DX2 exon 1-3 junction sequences as epitopes but the results were not successful either. In this study rabbit immunization with the short peptide was tried and the AIMP2-DX2 specific antibody could be generated. In combination with the antibody library construction, usage of short peptide sequence was thought to be the optimal strategy for overcoming the distinct obstacles which we have faced before.

The size of the antibody library prepared in this study reached 7.2×10^7 which is appropriate for panning selection (Lehninger, 2013). Even though phage display is a powerful technique for generating monoclonal antibodies and improving their affinity, antibody cloning also has its own difficulties (Bazan, 2012). In the case of overlap extension PCR, slight and continuous adjustment of experimental condition is required to increase the

efficiency even though the whole procedure was well set up. Managing competency and stocking condition of the competent cell is also extremely important and it has to be done in a short amount of time because the process is highly sensitive to temperature and its efficiency. After constructing the novel antibody library with genetic source from the immunized rabbit spleen, individual phage clone was selected by ELISA screening for further characterization. For expression of human IgG antibodies, pVITRO-IgG vector which had been previously designed in the laboratory was employed. By cloning heavy and light chain of H5 Fab clone into the vector, expression of complete human immunoglobulin was acquired by transfection with 293F cells. The recombinant IgG antibody was properly assembled and the average concentration of purified H5 IgG was generally 0.73 mg/ml showing its stable expression and purification efficiency.

We characterized H5 IgG monoclonal antibody via immunoblotting, IP, IF and SPR and found that this antibody has high affinity to its antigens and is applicable to IF and IP (Fig. 6-9). We will further characterize the application of H5 IgG for other usage including ELISA, ICC (immunocytochemistry) and IHC (immunohistochemistry) which would be more valuable for clinical diagnosis. One discrepancy is that H5 rabbit monoclonal antibody showed better performance in IP than H5 human monoclonal antibody, although both of them are identically derived from H5 Fab sequences. There would be some factors affected by IgG backbone regardless of antigen recognition. It would be further investigated.

Interestingly, H5 antibody recognizes AIMP2-DX2 isoforms but not original endogenous AIMP2-DX2 (Fig. 1D). We proved that this band is real AIMP2-DX isoform based on the epitope competition analysis, siRNA transfection and overexpression experiments (Fig. 4). We could not identify the exact sequence of the AIMP2-DX2 isoform recognized by H5, but it is expected that there would be several types of AIMP2-DX2 in the cells delivering the same effect like original AIMP2-DX2. H5 did not recognize the

endogenous AIMP2-DX2 but it clearly detected the N-term tagged exogenous AIMP2-DX2. AIMP2-DX2 is very unstable protein and its N-term domain is very flexible blocking the crystallization of AIMP2-DX2 *in vitro* (data not shown). It is plausible that N-term strep tag may stabilize the N-term structure of exogenous AIMP2-DX2 enabling it to be recognized by H5 unlikely to endogenous AIMP2-DX2. The AIMP2-DX2 isoform recognized by H5 seems to be the same protein identified by #324. Based on our experience, this band showed a positive expressional correlation to AIMP2-DX2 when we analyzed various cell lines and cancer patients (data not shown). This AIMP2-DX2 isoform, therefore has the possibility of surrogate marker for AIMP2-DX2. Further investigation to identify the isoform and evaluate its merit as a diagnostic marker or cancer therapeutic target should be required.

Based on these results from previous studies and presented in this study, the novel H5 antibody specific to AIMP2-DX2 isoform have a prominent properties in the field of clinical development. Since personal medication comes into spotlight in cancer diagnosis and therapeutics, this antibody will give a potential for the clinical agents development against numerous solid tumors associated with AIMP2-DX2 overexpression, as in form of IHC and ELISA kit that can be easily applicable to patients diagnosis for precise and effective therapy.

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요약 (국문초록)

발암성 변이체 AIMP2-DX2에 특이적인 단일클론성항체 개발과 특성분석

성 준 식 (Junsik Sung)

AIMP2-DX2는 야생형 AIMP2의 엑손(exon)2가 결손된 돌연변이를 의미한다. AIMP2 (Aminoacyl-tRNA Synthetase-interacting multifunctional protein 2)는 아미노아실-티알엔에이 합성효소(aminoacyl-tRNA synthetase)를 포함하는 복합체의 형성에 관련된 물질이자 여러 신호전달 하에서 암 억제제로 작용하는 단백질이다. AIMP2-DX2 변이체 단백질은 여러 종류의 암 특히 폐암에서 과발현되어 있으며, AIMP2의 암억제 기능을 방해하므로 암의 발생과 진행에 밀접하게 연관되어 있다. AIMP2-DX2 과발현 환자의 선별은 추후 AIMP2-DX2 표적 항암제 적용 및 마커 개발에 필수적이나, 현존하는 AIMP2-DX2 인식 항체는 AIMP2를 동시에 인식한다는 점에서 한계를 가진다. 이에 본 연구에서는 파지 디스플레이 기법 (Phage Display)을 사용하여 AIMP2-DX2를 특이적으로 인식하는 단일클론 항체를 개발하였다. 우선 AIMP2-DX2 항원을 주입한 토끼로부터 혈청과 비장을 얻어 mRNA를 분리, 증폭시켰고, 얻어진 cDNA와 pComb3XTT 벡터를 이용해 항체조각(Fab)을 만들고 형질전환을 통해 7.2×10^7 크기의 거대 합성 항체 라이브러리를 만들었다. 이를 이용해 패닝(panning) 및 ELISA 스크리닝을 수행하여 AIMP2-DX2 항원에 특이적으로 결합하는 단

하나의 클론(H5)을 선별하였고, 선별된 항체조각(Fab)를 IgG 분자로 전환하여 재조합 항-AIMP2-DX2 항체를 개발하였다. 개발된 단일클론성 항체 H5의 기능과 특성은 면역블롯 (Immunoblot), 효소면역분석법 (Enzyme-linked Immunosorbent Assay, ELISA), 면역침강반응법 (Immunoprecipitation, IP), 표면플라스몬공명 (Surface Plasmon Resonance, SPR), 면역형광법 (Immunofluorescence) 등의 기술을 이용하여 확인하였다. 그 결과, 새로운 항-AIMP2-DX2 단일클론성 항체는 야생 AIMP2 단백질에는 반응하지 않고 엑손(exon)2가 결손된 AIMP2-DX2에만 특이적으로 결합하며 우수한 결합력을 가짐을 확인할 수 있었다. 본 연구에서 개발된 단일클론 항체는 바이오 마커 및 진단기법으로 활용될 수 있으며 추후 AIMP2-DX2 과발현 폐암환자들을 대상으로 한 맞춤 진단 치료의 가능성을 여는데 기여할 수 있을 것이다.

주요어 : AIMP2-DX2, 파지 디스플레이, 단일클론성항체, 폐암, 진단

학 번 : 2014-24867