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약학석사 학위논문

Identification of CD24 as a Therapeutic Antibody Target for Ovarian Cancer

난소암에서 항체치료제 표적으로서의 CD24 에 관한 연구

2013 년 8 월

서울대학교 대학원

약학과 병태생리학전공

박 하 연

ABSTRACT

Ovarian cancer is the second most common gynecologic cancer. It has the highest mortality rate among gynecologic cancers, and more than 50% of patients with ovarian cancer die.

CD24 is a mucin-type glycoprotein with a small protein core that is a marker of B-cell differentiation. It is highly glycosylated and is anchored on the membrane via glycosylphosphatidylinositol (GPI). Recently, the role of CD24 in tumor biology has received considerable attention. It is involved in cell adhesion, metastatic tumor spread, and p-selectin binding. It has also recently been described as a diagnostic tumor marker, a marker of neuroendocrine differentiation, and, the most intriguing of all, of patient prognosis. Because CD24 is overexpressed in a variety of carcinomas such as ovarian cancer, breast cancer, non-small cell lung cancer, prostate cancer, and pancreatic cancer, it is a promising target for cancer therapy.

Here we verified the usefulness of CD24 as a target for anticancer therapy in several breast cancer and ovarian cancer cell lines. We also studied the development of CD24 as a target protein for anticancer therapy. In a previous study in our lab, we observed p-selectin binding (an important function of CD24) by ELISA. We treated mice with mAbs targeting CD24. Compared with mice in the control group, mice treated with mAbs exhibited meaningful changes in tumor growth and apoptosis. We analyzed CD24 expression in the cell lines by RT-PCR, real-time PCR, western blotting, and FACS analysis. These results suggested that CD24 is a potential molecular target for therapeutic antibody development.

A second aim of this study was to generate a human scFv antibody against CD24 and evaluate its affinity for CD24 as a step toward developing a human monoclonal antibody against CD24.

To screen the antibodies, we identified the optimal CD24 epitope through structural and functional analyses, and then produced recombinant CD24 in a mammal cell line to reconstruct the glycosylated form of CD24 *in vivo*. Isolated, single-chain variable antibody fragments (scFv) were selected after 4 rounds of biopanning and secondary screening by ELISA and sequence analysis. The affinity of the selected scFvs was measured by flow cytometry and surface plasmin resonance (SPR) with a CD24-overexpressing cancer cell line. In conclusion, in this study, we demonstrated that CD24 expression is a marker for ovarian cancer and we identified anti-CD24 scFvs that could be developed as potential therapeutic anticancer antibodies.

Key words: Ovarian cancer, CD24, prognostic marker, target protein, single chain variable fragment, therapeutic antibody

Student number: 2011-23730

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INTRODUCTION

Ovarian cancer is a serious cancer and the leading cause of death among gynecologic cancers because it is difficult to detect in the early stage and is resistant to chemotherapy [1]. Therefore, new drugs are needed to enhance the efficiency of ovarian cancer treatment. In comparison to other cancers, studies on the response of ovarian cancer to anticancer antibodies are limited.

Over the past 30 years, target specificity in cancer treatment has improved through the development of monoclonal antibodies such as Rituxan (anti-CD20) and Herceptin (anti-HER2). Compared to traditional therapies, therapeutic mAbs have an advantage in that they are more specific for the target tumor and have fewer side effects. They induce their anticancer effects through complex mechanisms via the immune system, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [2]. The activities of ADCC and CDC play a key role in the therapeutic effects of mAbs. Recently, antibody-based therapeutics have emerged as important therapeutic components for an increasing number of human malignancies such as breast cancer, colorectal cancer, and lymphoma [3]. However, there is currently no adoptable therapeutic antibody, approved or unapproved, for ovarian cancer [4].

The putative oncogene CD24 is a GPI-anchored protein composed of 16 potential O-linked glycosylation sites (Ser and Thr) [5]. The CD24 glycosylation

patterns are highly variable and cell-type dependent [6]. Anti-CD24 monoclonal antibodies such as ML5, OKB2, and SWA11, recognize exposed cores like LAP, whereas others recognize specific glycosylation sites or site that are yet to be defined [7]. CD24 has been used as a B-cell specific marker because of its expression in B-cell development, which is limited to the early stages only [8]. Subsequent studies showed that CD24 could also be used as a marker for renal tubular epithelial cells in the developing brain [9]. Additionally, CD24, which was thought to function as an adhesion molecule, is involved in cell-cell interaction as a ligand of p-selectin (CD62P). This interaction suggests that overexpression of CD24 might be important for tumor metastasis through the thrombus [10].

In agreement with the putative functions of CD24, high rates of expression have recently been found in various solid carcinomas by immunohistochemistry, and its expression has been shown to be a prognostic marker of poor survival [11]. Furthermore, high rates of cytoplasmic CD24 expression were significantly related with aggressive tumors [12-15].

Over the past few years, a considerable number of studies have been conducted on the mechanism downstream of CD24 as an oncogene. Several experiments on CD24 down-regulation or up-regulation have shown that CD24 associates with the products of many proliferative genes, including *Lyn*, *Ras*, *MAPK*, and *BCL-2*, and cell migration related genes, including 3β1 and 4β1 integrins, which induce binding to p-selectin or collagen and actin [16-18].

Studies focused on CD24 as a therapeutic target started in the early 1990s. One mAb against CD24, SWA11, has already had been used as a toxin-carrier to treat non-small lung cancer; however, this use is not related to the natural therapeutic

mechanisms of antibodies. Furthermore, there is no good clinical data on patients with NSCLC and metastasis [19]. In 1998, an anti-human CD24 monoclonal antibody was clinically tested as a therapeutic antibody along with an anti-CD21 monoclonal antibody on B-lymphoproliferative disorder [20]. In this report, the anti-CD24 monoclonal antibody showed clear anticancer effects, and the possibility of not only diagnostic applications but also therapeutic purposes was demonstrated. Moreover, a recently published paper showed that downregulation of CD24 through RNAi and an anti-CD24 mAb was able to decrease tumorigenicity *in vitro* and *in vivo* [21].

In the present study, based on the previously shown anti-cancer effects of CD24 mAb, we explored the possibility of CD24 as a suitable anticancer target for ovarian cancer and as a potential anticancer drug. To determine whether CD24 could be a suitable target for the development of anticancer therapeutic agents, we attempted to produce CD24-specific human single chain variable fragments (scFvs) by screening an scFv phage library using CD24 as the selecting antigen. Phage display was first introduced in 1985 and it is a common technique for identifying and isolating nucleic acid sequences encoding specific binding proteins. After selecting candidate scFvs by biopanning and ELISA, they were expressed in *Escherichia coli* TOP10 cells. The binding specificity of the purified scFvs was confirmed using CD24-expressing cancer cell lines.

MATERIALS AND METHODS

RT-PCR and real-time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, CA, USA), and RNA (with an A_{260}/A_{280} ratio of 1.8–2.1) was transcribed into cDNA using the Superscript II RNase H reverse transcriptase kit (Invitrogen). Real-time RT-PCR was performed with LightCycler TaqMan Master Mix (Roche, Germany) and a LightCycler 2.0 with LightCycler 4 software (Roche). The following primers were used: CD24 forward (RT-PCR), 5′-AACGTCTTCTAAATTTCCCC-3′; CD24 reverse (RT-PCR), 5′-TGGCATTCATCATCTAGTCA-3′; CD24 forward (real-time PCR), 5′-TTTGACTAGATGATGAATGCCAAT-3′; CD24 reverse (real-time PCR), 5′-GGATGTTGCCTCTCCTTCAT-3′; PAPOLA forward (reference), 5′-AAACTTTTTGAAGCTCCAAACTTCTT-3′; PAPOLA reverse (reference), 5′-CACCAAGCCCACCCATTC-3′.

Preparation of buffy coat

Buffy coats were obtained from the whole blood of normal donors by using RBC lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2–7.4).

P-selectin binding assay

P-selectin binding was analyzed by ELISA with P-selectin-Fc coated plates. Bound CD24 in cell lysates was detected by biotin-conjugated SN3 (eBioscience, Inc., CA, USA) and HRP-conjugated streptavidin.

Animals

Female 4–5-week-old BALB/C nude mice, purchased from Central Lab Animal, Inc. (Seoul, Korea) were housed in individually ventilated cages (IVC) in a sterile facility. Cages, water, and bedding were sterilized by autoclaving. All animals were maintained in accordance with the recommendations of the National Institutes of Health (NIH).

In vivo study of cancer xenograft mouse model

A pCDNA 3.1-Luciferase construct was obtained from professor Kang Dong Min (Korean Basic Science Institute). This construct was stably transfected into SK-OV3 cells. Exponentially growing SK-OV3-Luc cells were harvested and resuspended at a final concentration of 5×10^6 cells/0.1 mL of PBS. A xenograft was obtained on the back of BALB/C nude mouse after s.c. implantation of 5×10^6 SK-OV3-Luc cells. The growth of tumors was monitored using the IVIS® Imaging System 200 (XENOGEN, CA, USA). On day 2 after tumor cell injection, mice were divided into 2 groups according to the tumor signal: a PBS-treated group

(negative control) and an SN3-antibody treated group with 4 mice in each group. The SN3 mAb was administered intravenously (i.v.) once a week for 4 weeks at the indicated doses. Tumors were measured every 3 days.

TUNEL assay

A TUNEL assay was performed using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Flowgen Bioscience) to assess apoptosis according to the manufacturer's protocol.

Antigen preparation

The optimal CD24 antigen for screening the scFvs by phage display was identified by structural and functional analyses. Our construct was designed to mimic the mature CD24 peptide. Recombinant proteins were produced through cloning. An Fc tag was introduced to enable purification of the recombinant proteins by protein A chromatography. The construct containing the cloned CD24 gene was transfected into freestyle™ 293-F cells (R790-07; Invitrogen, Carlsbad, CA, USA) with freestyle™ MAX reagent (16447-100; Invitrogen). After incubating for 7 days, the medium was harvested, and the CD24 peptide was purified by protein A chromatography.

Antibody library screening

The purified recombinant CD24 protein described above was used to screen an scFv-expressing phage library. For the first screening, 4 rounds of biopanning were performed, from which 192 colonies were selected for a secondary screening by ELISA. The 20 scFv phage clones yielding the best signals in ELISA were isolated, and the scFvs were sequenced, and then the sequences were aligned. Eight scFv clones were then selected for further study.

scFv production

The selected scFvs were transformed into *E. coli* TOP10 cells. Once the cells reached an OD_{600} of 0.7, scFv expression was induced by the addition of 1 mM IPTG and overnight incubation. After incubation, the harvested cells were lysed in a microfluidizer. The protein was purified with an AKTA purifier (GE healthcare, Waukesha, WI, USA) with a Hisprep™ FF 16/10 column (GE Healthcare).

Western blot analysis

Whole cell extracts (20 μg per lane) were resolved by 12% SDS-PAGE, and then the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked with a solution of 5% non-fat milk in Tris-buffered saline with Tween-20 (TBS-T) for 1 h. The membrane was probed overnight at 4℃ with an SN3 mouse anti-CD24 antibody (MS-1278-PABX; Thermo scientific, Hudson, NH, USA) or an ML5 mouse anti-CD24 antibody

(Santa Cruz Biotechnology, Inc., CA, USA) diluted to 1:1,000 in 1% non-fat milk in TBS-T. The membranes were then rinsed with TBS-T, incubated with an anti-mouse secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) for 1 h, and then washed with TBS-T for 1 h. As a loading control, membranes were probed in a similar manner with an anti-β-actin antibody (sc-1616; Santa Cruz Biotechnology).

Flow cytometric analysis

Cells were resuspended at a density of 2×10^5/100 μL in FACS buffer (1% fetal bovine serum [FBS] in phosphate-buffered saline [PBS]). Cells were incubated with 1 of the 8 candidate anti-CD24 scFv antibodies (1–8 in Fig. 7; 20 μg per sample), and a mouse anti-CD24 antibody-FITC (ML5) (20 μL, 555427; BD Pharmingen) or a mouse anti-CD24 human antibody (SN3) (MS-1278-PABX; Thermo scientific) for 1 h at 4℃. Cells were then washed with FACS buffer and resuspended in a 1:4 dilution of mouse anti-HA Tag Ab-FITC (A01621; GenScript) or a 1:100 rabbit anti-mouse IgG Ab-FITC (ab6724; Abclon, Inc.) in FACS buffer and incubated for 1 h at 4℃. Samples were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). At least 10,000 events were acquired for each sample.

To compare the affinities of the antibodies with cells, relative-intensity (R.I.) ratio was calculated as (mean of experiment − mean of negative control)/mean of negative control.

Surface plasmon resonance

A Reichert (part # 13206061) planar mSAM 10% COOH surface was mounted. We used PBS as the running buffer, and 40 mg of EDC, 10 mg of NHS, and 1 mL of DW were added to an empty container and mixed. The surface was activated by injecting the solution for 7 min. A solution of 200 µg/mL of poly-L-lysine in 10 mM sodium acetate buffer, pH 5.2, was prepared. Over the left channel only, the poly-L-lysine solution was injected for 7 min. Over both channels, 1 M ethanolamine HCl (pH 8.5) was injected for 8 min. The cell solution was prepared by diluting the cell stock solution 1:10 in the running buffer. The prepared solution was centrifuged for approximately 1 min. Over the left channel only, the cell solution was injected for 7 minutes at a flow rate of 25 µL/min. Then, the scFv samples were injected.

Cell culture

The ovarian carcinoma cell lines CA-OV3, OV-90, SW626, and Tov-112D were purchased from the American Type Culture Collection (Manassas, VA, USA). Other ovarian cancer cell lines, OVCAR3, SK-OV3, SNU8, and SNU119, and the breast cancer cell lines MCF7 and MDA-MB-231 were obtained from the Korean Cell Line Bank (KCLB, Korea). An immortalized ovarian surface epithelial cell line, IOSE80 (NIH), was provided by Dr. Michael Birrer (National Cancer Institute, USA) and another immortalized cell line, IOSE385 was a gift from Dr. Nelly Auersperg (The University of British Columbia, Canada). All cells were

propagated in their own specific medium according to the supplier's instructions with 10% FBS and 1% antibiotics (Thermo Scientific, Waltham, MA, USA) at 37℃ in 5% CO_2.

RESULTS

Binding of CD24 to p-selectin on cancer cell lines

To study whether CD24 expressed on cancer cell lines could bind to P-selectin, we used an ELISA method with a chimeric P-selectin-Fc. The results were compared to that of HL-60, which is known to bind to P-selectin. Attachment of SK-OV3 cells was observed (Fig. 2). The results indicated that CD24 on SK-OV3 had more glycosylation and was able to strongly interact with P-selectin more than the other cell lines.

Targeting of CD24 using a mAb decreased tumor growth rate and induced morphological changes *in vivo*

We targeted CD24 with mAbs and showed that it decreased tumor growth rate and induced morphological changes *in vivo*. To evaluate the cytotoxic potency of the SN3 mAb against CD24 *in vivo*, we established a human xenograft model by injecting 5×10^6 SK-OV3-Luc cells in the back of BALB/C nude mice. Tumor growth was measured as the luciferase activity of SK-OV3 after a Luciferin i.p. injection. The SN3-treated mice were injected with 100 μg of SN3. IV injection of the mAb SN3 through the tail vein for 30 days inhibited tumor growth by 44.1% (Fig. 3A). In the next series of experiments, tumor tissue was removed from the

mice, and the morphology of the PBS-treated control and SN3-treated groups was compared by H&E staining (Fig. 3B). The PBS-treated group had more extensive necrosis than the SN3-treated group. A TUNEL assay was performed to evaluate whether the targeting of CD24 was related to apoptosis. The histological features of TUNEL-positivity are shown in Fig. 3B and 3C. Strong TUNEL-positive apoptotic signals were detected in the SN3 treatment group.

CD24 expression profiling in several cancer cell lines

To characterize the CD24 expression in these ovarian carcinoma and breast cancer cell lines, we detected the expression level of CD24 by variable analysis (Fig. 4A-4D). Buffy coat and Mcf-7 were used as positive controls, and MDA-MB-231, which is known to express low levels, was used as a negative control. As expected, the immortalized ovarian cell lines, IOSE80 and IOSE385, were negative or expressed only low levels of CD24. Whereas in other cancer cell lines, CD24 expression was 2–8 fold higher than normal buffy coat (Fig. 4B). Interestingly, we observed that the expression of transcripts and proteins did match, but the expression detected by ML5 and SN3 clones did vary (Fig. 4C and D).

Production and purification of a recombinant CD24 protein

To determine whether CD24 could be a target for the development of anticancer therapeutics, we produced scFv antibodies by selecting CD24-binding scFvs from a

phage library. To conduct the phage library screening, we produced recombinant CD24 antigens (Fig. 5A). After cloning and transfecting 293-F cells, we purified the antigens by protein A affinity chromatography and then concentrated them for subsequent experiments. (Fig. 5B, 5C).

Selection of anti-CD24 scFv candidates by phage library screening and ELISA

To select specific CD24-binding scFv clones, a purified recombinant CD24 protein was used in a phage display assay. Four rounds of biopanning were performed (Fig. 6A, 6B), from which 192 colonies were selected for further screening by ELISA (Fig. 6C). Based on the ELISA results, 20 scFv clones were selected for sequencing. Finally, 8 clones with the correct sequences were selected (Fig. 6D).

Expression of selected scFvs and measurement of CD24 binding specificity

To produce sufficient quantities of the 8 scFv clones, the constructs were transformed into *E. coli* TOP10 cells. scFv expression was induced by IPTG (Fig. 7A) and the proteins were purified via a hexahistidine tag with a AKTA purifier and a Hisprep™ FF 16/10 column (GE Healthcare). We identified the purified scFvs on an SDS-PAGE (Fig. 7B). The CD24 binding specificity of the 8 scFv antibodies was measured by flow cytometry using the CD24-positive ovarian

cancer cell lines CAOV-3 and OVCAR-3 and the CD24-negative control cell line TOV-112D. CD24 expression in these cell lines was first confirmed by FACS analysis of whole cells and western blotting of cell extracts using a control anti-CD24 antibody (Fig. 7C). Figure 7D shows that of the 8 new anti-CD24 scFv antibodies produced, #1, #4, #5, and #6 showed more significant binding to CAOV-3 and OVCAR-3 cells, but not to TOV-112D cells, indicating that these scFv antibodies specifically recognized human CD24 on the cell surface. The affinity of the anti-CD24 scFv antibodies to wild type CD24 was demonstrated through cell-based SPR with CAOV-3 (Fig. 7E) Collectively, these results demonstrate that human CD24-specific scFv antibodies could be used to produce anti-CD24 antibodies, and have the potential for use as anticancer agents.

Figure 1. A model of CD24

The CD24 antigen is composed of a small, 27-amino acid peptide that is attached to the membrane via a GPI-anchor and has many potential glycosylation sites.

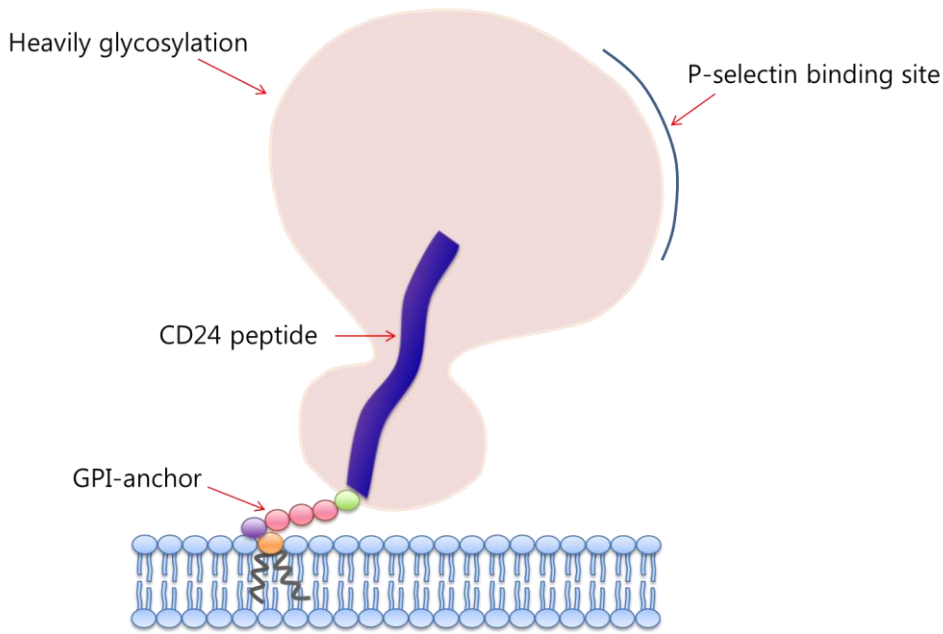


Figure 2. CD24-mediated association between SK-OV3 cells and P-selectin

Attachment analysis of various cell lines expressing CD24 to P-selectin by ELISA. Plates were coated with P-selectin-Fc to capture CD24 in the cell lysate, which was detected by biotin-conjugated SN3 and HRP-conjugated streptavidin. The binding signals for OVCAR3 and Tov-112D cells were only slightly above the basal levels, and the background signal for SNU119 was too high. Therefore, only the interaction of SK-OV3 cells with P-selectin-Fc is similar to that of HL-60 cells.

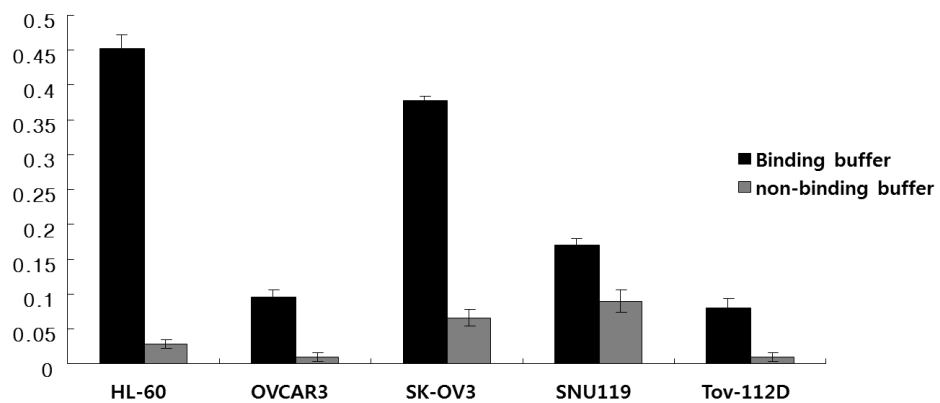


Figure 3. Treatment of mice with anti-CD24 mAb affects tumors

(A) BALB/c mice injected with SK-OV3-luc cells were treated with 100 μg of SN3 or PBS, and then tumor growth was observed every 3 days for 30 days. (B, C) H&E staining and a TUNEL assay were performed on the extracted tumors after observing tumor growth.

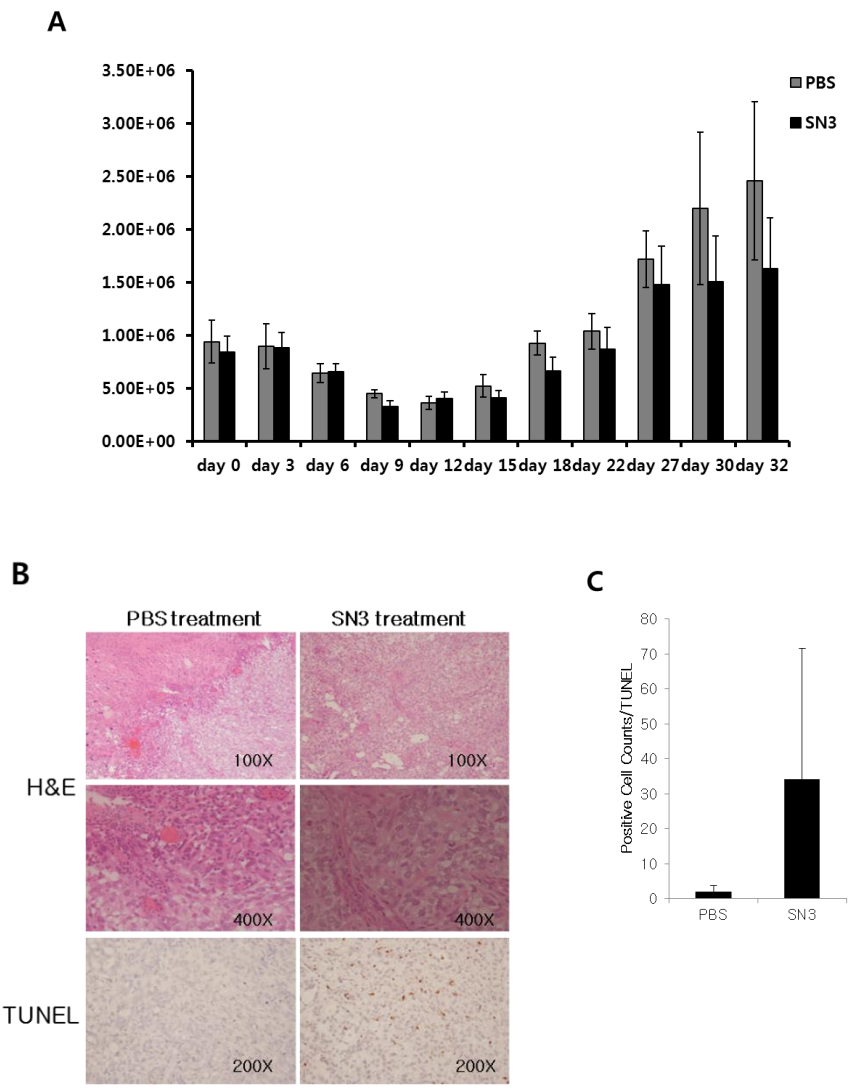
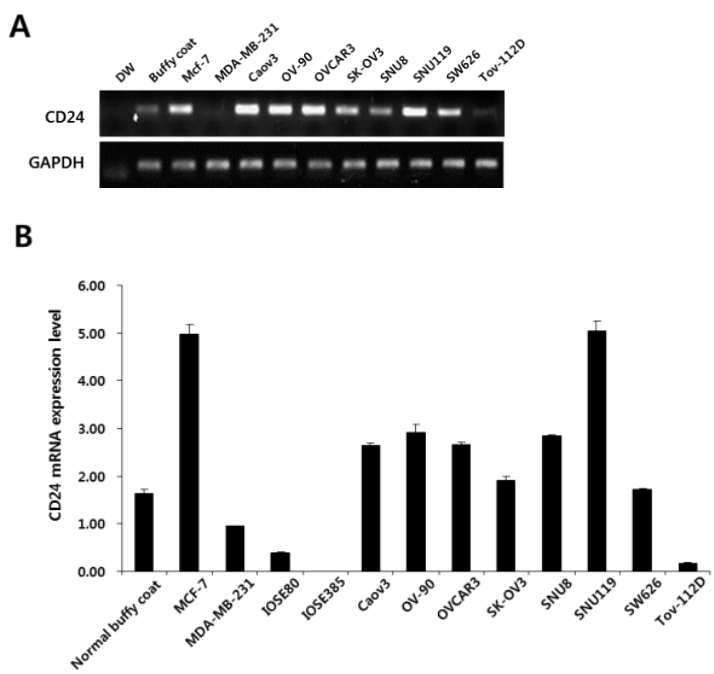


Figure 4. Expression of CD24 on several cancer cell lines

(A) CD24 transcripts were detected by RT-PCR. The reference was PAPOLA, a new validated reference gene. CA-OV3, OV-90, OVCAR3, and SNU119 cells expressed CD24 strongly, whereas CD24 expression in Tov-112D was very low. (B) Real-time PCR analysis for CD24. The same templates were used. The relative concentration ratio means the ΔCP ratio between the reference gene (PAPOLA) and CD24. The level of transcript was matched to the RT-PCR data and additional cell lines, IOSE80 and 385 had the lowest transcript levels. (C) Western blotting for CD24. CD24 was detected by an anti-CD24 monoclonal antibody, either ML5 or SN3 and an anti-β-actin antibody was used as a reference. The ML5 clone was not able to capture CD24 on CA-OV3, OV-90, OVCAR3, SK-OV3, or SNU8 cells. (D) FACS analysis for CD24. Cells were stained with ML5 and SN3; however, the tendencies for CD24 detection were not the same between the 2 clones.



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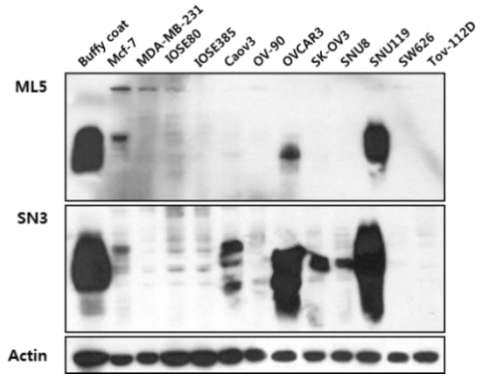
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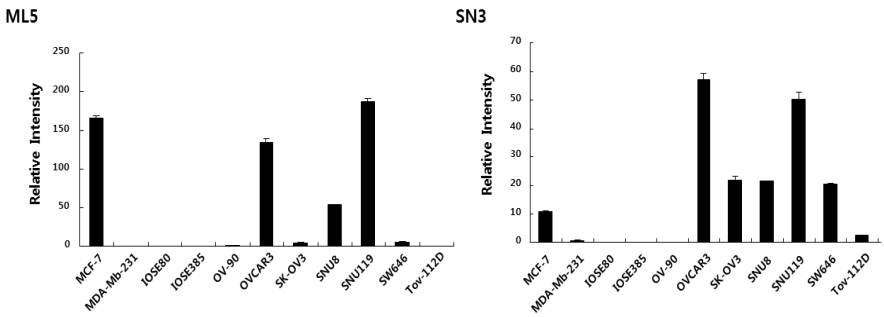
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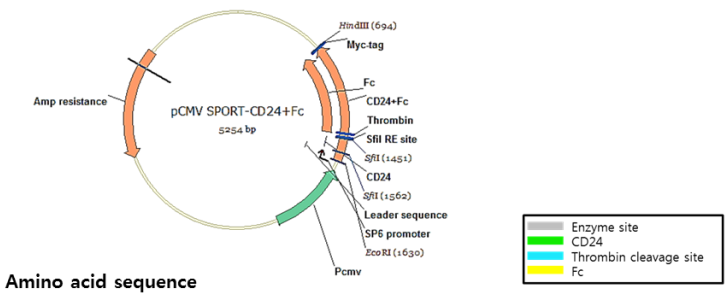
SW646
Tov-112D

Figure 5. Preparation of a recombinant CD24 protein

(A) Sequence of the recombinant CD24 antigen and the vector map. (B, C) SDS-PAGE analysis of CD24 antigen purification by protein A affinity chromatography. Antigen was detected in eluate fractions 2–7, and was concentrated.

A



Amino acid sequence

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DNA sequence

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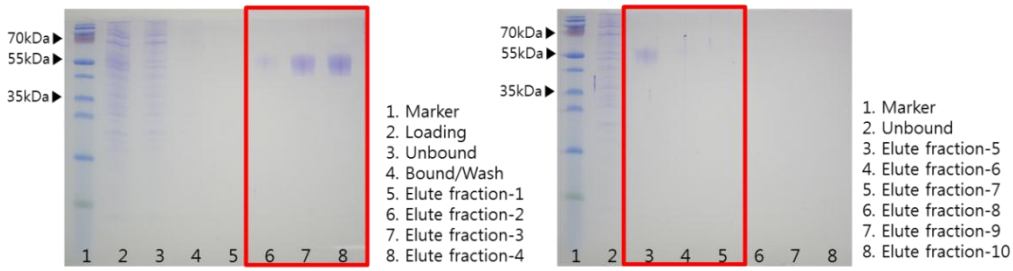
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55kDa▶
35kDa▶
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3. Unbound
4. Bound/Wash
5. Elute fraction-1
6. Elute fraction-2
7. Elute fraction-3
8. Elute fraction-4
70kDa▶
55kDa▶
35kDa▶
1 2 3 4 5 6 7 8
1. Marker
2. Unbound
3. Elute fraction-5
4. Elute fraction-6
5. Elute fraction-7
6. Elute fraction-8
7. Elute fraction-9
8. Elute fraction-10

C

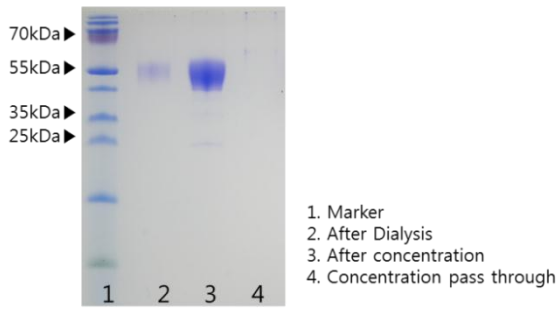
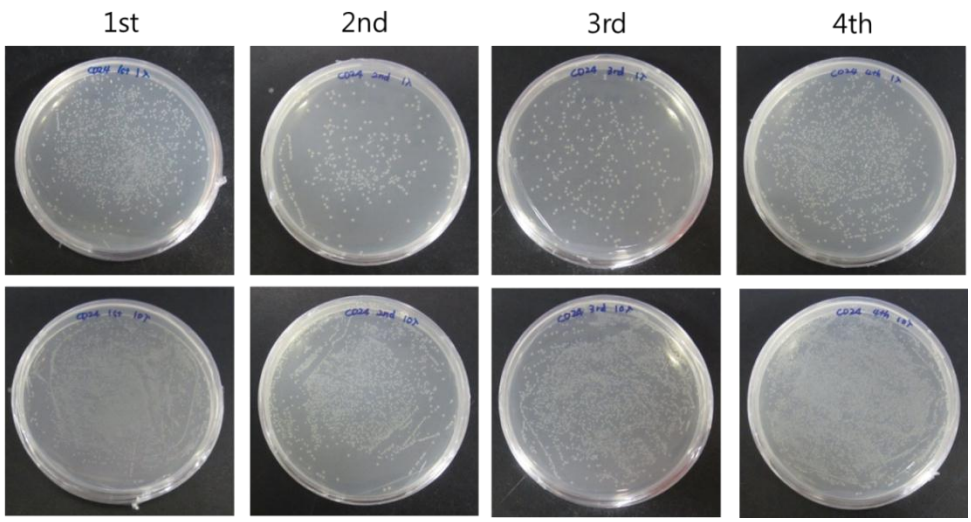

70kDa▶
55kDa▶
35kDa▶
25kDa▶
1 2 3 4
1. Marker
2. After Dialysis
3. After concentration
4. Concentration pass through

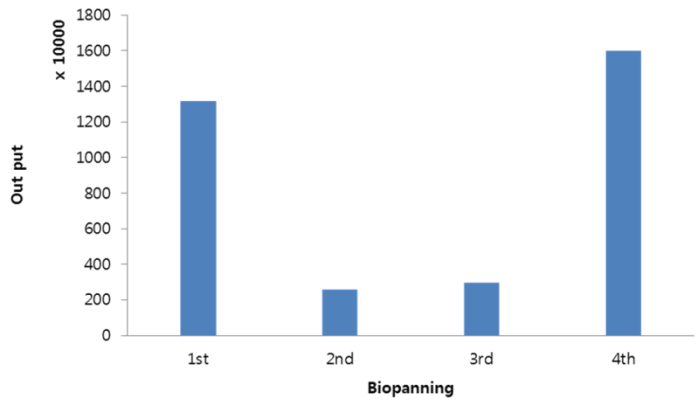
Figure 6. Selection of candidate CD24-binding scFvs by phage library screening

Candidate CD24-binding scFvs were selected by phage library screening. (A, B) Photographs of colonies selected during 4 rounds of phage biopanning on recombinant CD24 and graph of 4 rounds of phage biopanning output. (C) Absorbance values of a secondary CD24-specific ELISA screen. (D) Sequence alignment of the 8 selected scFv clones.

A



B



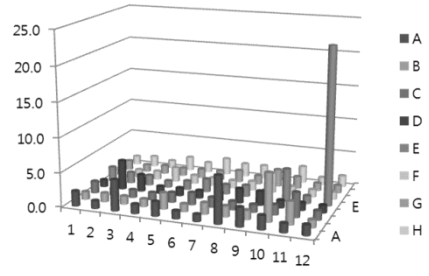
C

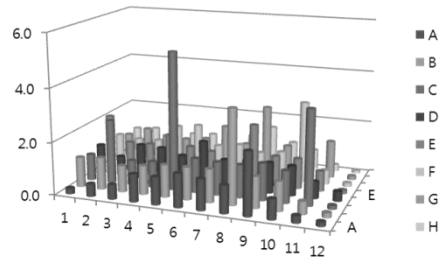
Plate 1

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	2.2	1.1	4.5	1.4	2.1	1.2	1.0	6.9	2.8	2.0	1.3	1.5
B	1.1	1.3	1.2	1.1	2.4	1.1	1.7	1.6	1.3	7.0	3.3	1.1
C	1.8	1.0	1.2	1.1	1.5	1.8	1.4	1.2	1.1	1.5	3.6	1.4
D	1.1	4.3	2.5	1.1	1.2	1.1	1.1	2.1	2.1	1.1	1.1	1.1
E	2.1	1.6	1.5	1.0	1.3	1.9	1.4	1.7	1.3	4.8	1.1	22.8
F	1.2	1.2	1.2	2.1	1.3	1.2	1.1	1.3	2.9	1.3	1.1	1.1
G	1.4	1.1	1.2	1.2	1.3	1.4	1.7	1.3	2.5	1.8	1.1	1.1
H	1.3	1.5	1.2	2.0	1.5	2.4	1.9	2.0	1.2	2.4	1.1	1.7

Plate 2

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0.2	0.5	0.6	1.1	1.0	1.3	1.2	1.0	2.4	0.8	0.3	0.2
B	1.1	1.2	1.0	1.1	1.2	1.2	1.3	3.6	1.2	1.1	0.5	0.2
C	1.0	2.4	0.9	1.0	5.2	1.3	1.3	1.3	1.4	1.2	3.6	0.1
D	1.1	0.8	1.3	1.3	1.1	1.7	1.1	1.1	1.2	1.1	0.6	0.4
E	2.1	1.1	1.1	1.6	1.2	1.1	1.5	2.3	1.2	1.2	0.8	0.1
F	1.1	1.0	1.0	1.7	1.8	1.1	1.2	1.0	1.1	3.1	0.5	0.1
G	0.9	1.2	1.0	0.9	1.0	1.6	1.0	2.6	1.1	1.1	1.4	0.1
H	0.9	1.0	1.2	0.9	1.0	1.1	1.1	1.5	1.0	0.8	0.3	0.1





D

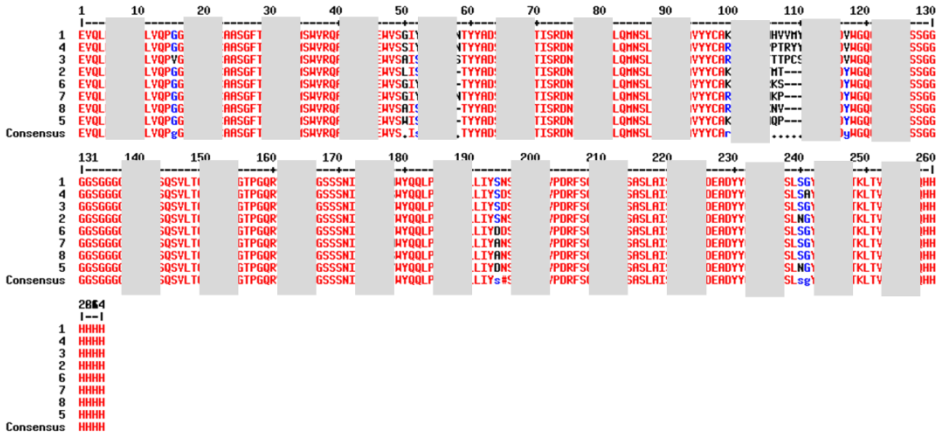
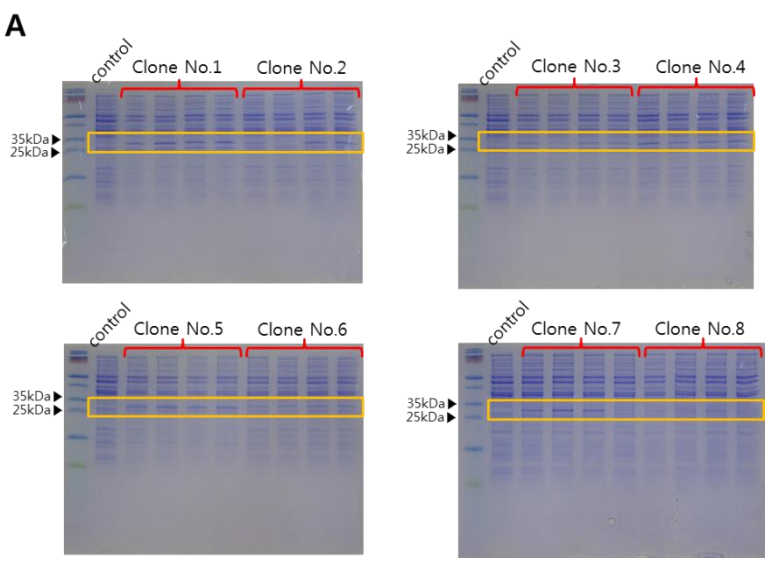
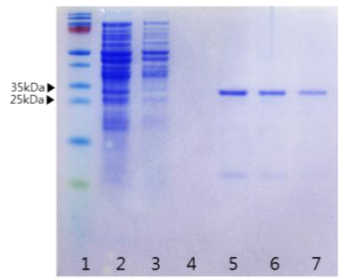


Figure 7. Expression of selected scFvs and binding to CD24-positive cells

(A) Expression of selected scFv in *E. coli* TOP10 cells was induced by the addition of 1 mM IPTG. (B) Purification of the selected scFvs with an AKTA purifier, eluted with 500 mM imidazole. (C) Verification of CD24 expression by FACS analysis and western blotting of the CD24-positive mammalian cell lines CA-OV3 and OVCAR-3. The CD24-negative cell line TOV-112D was used as a control. (D) The binding specificity of the 8 anti-CD24 scFv antibodies was confirmed by FACS analysis with CA-OV3, OVCAR-3, and TOV-112D cells. The scFv antibodies 1, 4, 5, and 6 exhibited specific binding to the CD24-positive cells. (E) The affinity of the anti-CD24 scFv antibodies to wild type CD24 was confirmed by cell-based SPR with CA-OV3 cells.

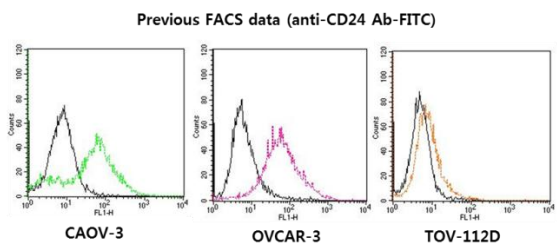


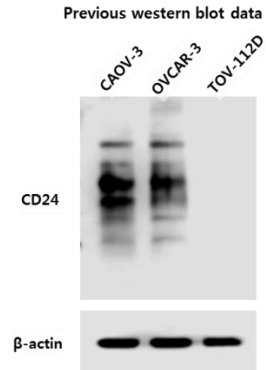
B



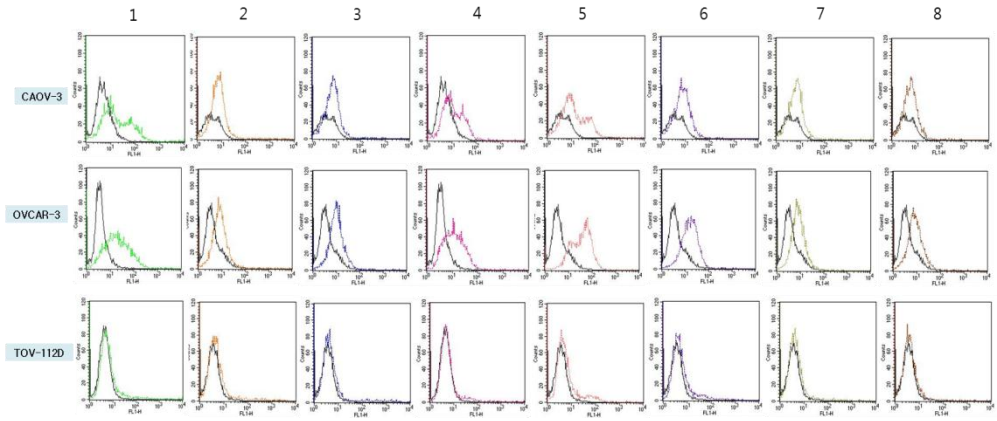
1. Marker
2. loading
3. Unbound
4. Flow though
5. Elute fraction-28
6. Elute fraction-28
7. Elute fraction-28

C

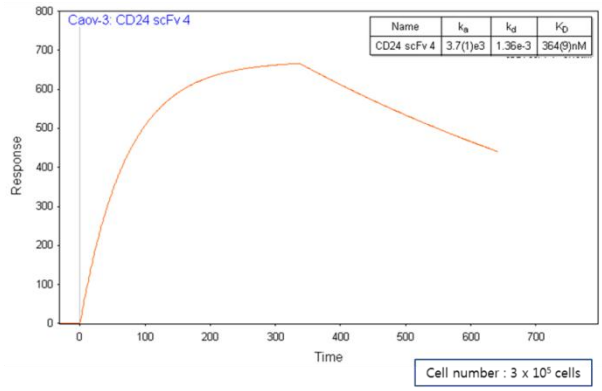




D



E



Analyte	Association	Dissociation	Buffer
CD24 scFv	5.5min	6min	1X PBS

DISCUSSION

Ovarian carcinoma is the most lethal gynecologic malignancy, with a low overall 5-year survival rate of 30%. Despite modern surgical techniques and chemotherapy, the survival rate for ovarian carcinoma has remained low due to the difficulty of early detection and its resistance to chemotherapy. Therefore, new therapies, such as monoclonal antibody treatments, that support surgery and chemotherapy are needed.

The goal of this study was to verify CD24 as a target for ovarian cancer therapy and evaluate the potential of a CD24 mAb as an anticancer agent. We confirmed that CD24 is a valuable target for anticancer therapy. To characterize cell lines, we analyzed CD24 expression level by several methods such as RT-PCR, real-time PCR, western blotting, and FACS analysis. Interestingly, we observed that the expression detected by the 2 mAb clones we used, ML5 and SN3, did not matched (Fig. 4C and D). We confirmed the specificities of the mAbs against CD24 with an siRNA test (data not shown), and these 2 clones were able to detect CD24. The ML-5 clone reportedly captures the LAP motif on CD24 protein core [5], which is less glycosylated than other portions of the protein. The MCF-7 cell line may have a protein core with low glycosylation. Therefore, a heavily glycosylation LAP motif might disturb the interaction with the ML-5 clone. In contrast, the epitope of the SN3 clone is not known, but is believed to be a part of the protein core with specific glycosylation. Judging from our data, the affinity of CD24 mAbs could be controlled by the glycosylation near the epitope. In addition, in a previous study,

overexpressed CD24 was stained in the cytoplasm by immunohistochemistry. Therefore, we believe that there is a lot of non-functional CD24 in the cytoplasm, not on the membrane. The P-selectin binding assay suggested that only CD24 on the cell scarifying all conditions, resistance of CD24 and specific glycosylation, which can contribute to P-selectin attachment, is important in the mechanism underlying metastasis. Targeting of CD24 using mAbs decreased tumor growth rate and induced morphological changes *in vivo*. This suggests that the apoptotic effect of the SN3 clone against CD24 could reduce cell tumorigenicity *in vivo*. Our *in vivo* data is similar to previous data in colorectal cancerous xenograft model in a recent paper by Sagiv, E. *et al*. [17]; however, they interpreted their result as an effect induced by the loss of CD24. Although their interpretation was reasonable, the main reaction of an animal to antibody treatment is a defensive immune mechanism. Therefore, in our opinion, the anti-tumorigenic effect of CD24 mAb is predominantly generated by ADCC and CDC.

We conclusively showed that high CD24 expression could be a strong indicator of ovarian cancer. This finding suggests that CD24 expression might have value as a molecular target for ovarian cancer therapy. With this in mind, we attempted to produce anti-CD24 scFv antibodies as a step toward producing anti-CD24 antibodies through screening and selection from a CD24 scFv-expressing phage library. The recombinant CD24 used for screening was designed to mimic the native, mature CD24 peptide. In this study, we found that 8 of the scFvs tested exhibited specific binding to CD24-expressing cells. To generate therapeutic CD24 mAbs, next, we should perform whole IgG conversion, then characterize the antibody and test its efficacy *in vitro* and *in vivo*. Once candidate therapeutic

antibodies with high affinity for the antigen have been identified, a number of experiments are necessary to confirm their suitability for use as therapeutic antibodies. These include, testing their capacity for antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, because these are the major antibody-based cytotoxicity mechanisms *in vivo*. Then, the antibodies can be tested *in vivo* for pharmacokinetics and efficacy using mouse models. Various forms of therapeutic antibodies have been tested in humans, including immunoliposomes and antibody-drug conjugates.

In conclusion, this study has shown that CD24 is a valuable marker for ovarian cancer. We also demonstrated the successful production of CD24-specific scFv antibodies as a step toward producing whole anti-CD24 human IgGs, suggesting that CD24 is a potential target for the development of therapeutic antibodies against ovarian cancer.

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국 문 초 록

난소암은 우리나라에서 자궁경부암 다음으로 발생빈도가 높은 여성생식기 암이며, 부인암 중에서도 가장 사망률이 높아 발병한 환자의 절반 이상이 사망한다. CD24는 B-cell 분화 마커로 알려져왔으며, 작은 protein core를 가지고 있다. 또한 상당한 양의 당화(glycosylation)가 되어있는 mucin 타입의 당단백질이며 GPI (glycosyl phosphatidyl-inositol)에 의해 세포막에 연결되어 있는 막단백질이다. 세포부착(cell adhesion)과 전이성 암 확산(metastatic tumor spread), P-selectin 결합과 관련이 있으며 난소암, 유방암, 비소세포성폐암, 전립선암, 췌장암 등의 다양한 암에서 과발현되기 때문에 pan-carcinoma marker로도 가능성이 있다고 알려져 있다. 여러 유방암과 난소암 세포주에서 항체치료제의 표적으로서의 CD24가치를 확인하였고, 더 나아가 CD24를 표적단백질로 한 항체치료제 개발에 관한 연구를 진행하였다. CD24의 여러 기능 중의 하나인 P-selectin과의 결합을 ELISA analysis로 확인하였다. CD24를 표적으로 하는 단클론항체의 처리가 생체 내(*in vivo*)에서 변화를 일으키는 지 확인하였다. 그 결과 대조군과 비교하여 종양성장의 억제를 보였으며, 세포사멸(apoptosis)도 유의성있는 차이를 나타내었다. 이러한 결과를 바탕으로 하여 CD24의 marker로서의 가치를 확인하며 항체치료제 개발을 위한 잠재적 표적단백질이 될 가능성을 검증할 수 있었다. 어느 세포주에서 CD24가 발현하는지 확인하기 위하여 RT-PCR, real-time PCR, western blot, FACS로 확인하였으며, 이 후 CD24의 구조적 분석을 통하여 가장 적합한 항원결정부위를 디자인하여 재조합 단백질로 제작하였고, 이것을 이용하여 단일사슬절편항체(single chain variable fragment, scFv) 스크리닝을 진행하였다. Phage display 기법을 활용하여 4회의 biopanning으로 첫 번째 스크리닝을 진행한 후 ELISA analysis를 통한 두 번째 스크리닝 후 염기서열 분석을 통하여

candidate scFv를 선별하였다. 선별된 scFv로 CD24가 과발현되는 것으로 알려진 암 세포주를 선별하여 flow cytometry, surface plasmon resonance을 수행한 결과 후보 scFv의 CD24에 대한 친화성을 확인하며 whole IgG 개발에 한 걸음 나아갈 수 있는 길을 마련하였다. 본 연구는 유방암에서 CD24의 marker로서의 가치와 표적단백질로서의 가치를 확인하였으며 이를 활용하여 항체치료제의 개발과정을 제시한다.

주요어 : CD24, 난소암, 표적단백질, single chain variable fragment, 항체치료제

학 번 : 2011-23730