약학석사 학위논문

Evaluation of CD151 as an Antibody Target for Therapy of Non-Small Cell Lung Cancers

폐암에서 항체치료제 표적으로서의 CD151 에 관한 연구

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ABSTRACT

CD151 is a member of the tetraspanin superfamily of transmembrane proteins and is overexpressed in a variety of cancers. CD151 affects the motility and metastasis of tumor cells, and its expression is closely associated with poor prognosis. Here, we investigated the potential use of CD151 expression as a prognostic marker in human non-small cell lung cancer (NSCLC). We also produced anti-CD151 antibodies as a first step in exploring the potential for CD151 to serve as a target for anticancer therapy. Tissues from 380 patients with NSCLC were collected at Samsung Hospital between 1994 and 2005. Samples were subjected to immunohistochemical staining for CD151 expression and the results were correlated with patient clinicopathological features. High expression of CD151 was significantly associated with worse disease-free survival and overall survival of males, smokers, and patients with adenocarcinoma subtype of NSCLC. CD151 expression was identified as a significant prognostic indicator of NSCLC, suggesting that it may serve as a potential molecular target for therapeutic antibody development. To investigate this possibility, we identified the optimal CD151 epitope for antibody development through structural and functional analyses, produced a recombinant CD151 protein, and isolated single chain variable antibody fragments (scFv) by screening an scFv-expressing phage library against the

recombinant protein. Candidate scFv clones were selected after 4 rounds of biopanning and a secondary screening by ELISA analysis. Selected scFvs were cloned and converted to whole IgG, and the antibodies were expressed in CHO-S cells. Four anti-CD151 antibodies were affinity purified and shown by flow cytometry to bind specifically to CD151-positive tumor cell lines. In conclusion, this study demonstrated that CD151 expression is a prognostic marker for NSCLC and identified 4 anti-CD151 antibodies that may be developed as potential therapeutic anticancer antibodies.

Key words: Non-small cell lung cancer, tetraspanin, CD151, prognostic marker, target protein, single chain variable fragment, therapeutic antibody

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INTRODUCTION

Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers[1] and is the most frequent cause of cancer-related death worldwide.[2] In the past several decades, the incidence of lung cancer has increased dramatically in Korea and is now 29.1 cases per 100,000 people.[3,4] Landmark studies have demonstrated the importance of the molecular characterization of NSCLC tumors to aid in the identification of patient-specific molecular targets for treatment.[5] However, the prognosis for patients with NSCLC remains poor, with an overall 5-year survival rate of less than 15%.[3,4] These unsatisfactory clinical outcomes highlight the need to develop new reliable predictors of survival and to identify novel therapeutic targets.[6]

Tetraspanins are a family of transmembrane proteins expressed on several cell types. The proteins are involved in a variety of biological processes such as cell-to-cell adhesion, cell motility, differentiation, activation, and proliferation, as well as metastasis and progression of many human malignancies.[7] CD151, a member of the tetraspanin superfamily, comprises 4 transmembrane domains, 2 extracellular loops, and intracellular NH_2- and COOH-terminal domains. CD151 acts as an adaptor or organizer by assembling multimolecular complexes of cell surface proteins. CD151 affects integrin-dependent cell adhesion and motility by directly

interacting with laminin-binding integrins, including α3β1 and α6β4.[7-9] CD151 is expressed on many cell types, including endothelial cells and platelets, and is frequently overexpressed on cancer cells.[10] In malignant tumors, CD151 is involved in tumor progression and metastasis and promotes tumor cell invasiveness associated with the epithelial-mesenchymal transition.[11,12]

CD151 is under investigation as a biological marker of poor prognosis in several human malignancies[13-18] and is also a potential molecular target for antibody-based cancer therapy.[10] Several anti-CD151 monoclonal antibodies have been shown to display anti-metastatic activity in vivo.[10,12,19] One study has reported that high CD151 mRNA expression is associated with a poor prognosis in NSCLC.[20] In that study, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to clarify discrepancies in the results of immunohistochemical assays. The clinicopathological significance of CD151 expression measured by immunohistochemistry, however, has not been confirmed in NSCLC.

In the present study, we sought to clarify the relationship between CD151 expression and the clinicopathological features of a large cohort of NSCLC patients and to determine whether CD151 expression could serve as a prognostic factor. We also analyzed correlations between CD151 expression and clinical outcomes according to the histological type (squamous cell carcinoma [SCC] or adenocarcinoma [ADC]) and other clinical factors such as gender and smoking history. Because immunohistochemistry is the method of choice for detection of many cell surface proteins in the clinical practice environment, we also evaluated a

semi-quantitative scoring system for CD151 immunostaining.

To determine whether CD151 could be a suitable target for the development of anticancer therapeutic agents, we attempted to produce CD151-specific human IgGs by screening of a single chain variable fragment (scFv) phage library using CD151 as the selecting antigen. Phage display was first introduced in 1985 and is a common technique for identifying and isolating nucleic acid sequences encoding specific binding proteins. After selection of candidate scFvs by biopanning and ELISA, the sequences were cloned, converted to whole IgGs, and expressed in a CHO cell line. The binding specificity of the purified antibodies was confirmed using CD151-expressing cancer cell lines.

The results of this study suggest that CD151 is a significant prognostic marker for NSCLC and that it can serve as a target for the development of therapeutic anticancer antibodies.

MATERIALS AND METHODS

Patients and histological evaluation

A total of 1897 patients with lung cancer who were diagnosed, treated, and followed at Samsung Medical Center, Seoul, Korea between 1994 and 2005 were recruited for this study. Inclusion criteria were as follows: diagnosis of primary NSCLC that was surgically resected and pathologically confirmed, no prior treatment, complete medical records, and the availability of well-preserved paraffin-embedded blocks and histopathologic slides of resected specimens. Of the final 380 NSCLC patients who were enrolled, 300 patients had undergone lobectomy, 78 had undergone pneumonectomy, and 2 had undergone wedge resection. Demographic information and other variables were retrieved from the patient medical records and tumor registry and were analyzed for clinical information, including age, gender, treatment modality, and survival or disease progression. The disease diagnosis and histological classification were based on a new multidisciplinary classification of lung cancer proposed by the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society.[21] TNM stages were based on the tumor registry database, which was classified according to the World Organization Classification

of Tumors.[22] This study was approved by the Institutional Review Board at the Samsung Medical Center (Seoul, Korea).

Fabrication of tissue microarrays

After the 380 cases were reviewed to confirm the disease diagnosis, a tissue microarray (TMA) was constructed. Representative tissue areas on the individual paraffin blocks were carefully selected based on evaluation of hematoxylin and eosin-stained parallel sections. A tissue core (2-mm diameter) was punched out from each tumor specimen using a TMA manufacturing tool. The tissue cores were arrayed on the recipient paraffin block using a trephine apparatus (ISU Abxis, Seoul, Korea), as previously described.[23]

Immunohistochemical analysis

Samples of 5-μm-thick TMA tissue sections were deparaffinized in xylene and rehydrated, and nonenzymatic antigen retrieval was achieved by heating at 100℃ in citrate buffer (pH 6.0) for 5 min. The sections were then incubated with a monoclonal mouse anti-human CD151 antibody (1:100 dilution, clone RLM30; Novocastra, Newcastle upon Tyne, UK) for 1 h at room temperature, followed by

incubation with biotinylated goat anti-mouse IgG (1:1000 dilution; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature.[13] Staining was revealed by incubation of sections with diaminobenzidine chromogen for 5–10 min, and sections were then counterstained with hematoxylin for 5 min, as previously described.[13]

CD151 expression in lepidic-, acinar-, papillary-, or micropapillary-predominant tumors was scored using the semi-quantitative method for HER2 expression in gastric cancer[24], according to the following scale: score 0 (no staining or cell membrane staining in <10% of tumor cells); score 1+ (faint perceptible membrane staining in >10% of tumor cells, with incomplete staining of the membrane); score 2+ (weak to moderate complete or basolateral membrane staining in >10% of tumor cells); and score 3+ (moderate to strong complete or basolateral membrane staining in >10% of tumor cells).

For solid-predominant ADC and SCC subtypes, CD151 expression was scored using the HER2 semi-quantitative method for breast cancers: score 0 (no staining); score 1+ (weak, incomplete membrane staining in any proportion of tumor cells, or weak, complete membrane staining in <10% of cells); score 2+ (intense complete membrane staining in ≤30% of tumor cells or complete membrane staining that is non-uniform or weak but has obvious circumferential distribution in ≥10% of cells); and score 3+ (uniform intense membrane staining of >30% of tumor cells). The scoring systems are illustrated in Fig.1 and described in Table 1.

Scores of 0 or 1+ were classified as low CD151 expression and scores of 2+ or 3+

were classified as high CD151 expression. Two pathologists (YLC and MJK), blinded to the patients' clinical data, interpreted all immunostained slides with excellent agreement (kappa value 0.90). In cases where the pathologists disagreed, the final scoring was determined by consensus.

Statistical analysis

The Chi-square test or two-tailed Fisher's exact test was used to examine associations between qualitative clinicopathological variables and CD151 expression. Disease-free survival was defined as the time from the first surgery until documented relapse, including locoregional recurrence and distant metastasis. Overall survival was defined as the time from the first surgery until death. Survival differences among groups were analyzed using the Kaplan-Meier method with a log-rank test. The Cox proportional hazards model was used for multivariate analyses of overall and disease-free survival. SPSS statistical software (version 18; IBM, Armonk, NY, USA) was used for all analyses. *P* values <0.05 were considered statistically significant.

Antigen preparation

The optimal antigen for screening of scFv by phage display was identified by structural and functional analysis of CD151. Our construct was designed to mimic the specific annular structure of the second extracellular loop of CD151 (Fig. 4). Recombinant proteins were produced through cloning. A hexahistidine tag was introduced to enable purification of the recombinant proteins by Ni-NTA agarose CL-6B (Incospharm, Daejeon, Korea) affinity chromatography. Cysteines were introduced at both thrombin cleavage sites to allow self-cyclization of the molecule following on-column oxidation during the purification. Finally, a structurally stable and pure antigen was obtained by enzymatic cleavage of the His-tag with a Thrombin Cleavage Capture Kit (Novagen-EMD Chemicals, Madison, WI, USA).

Antibody library screening

A purified recombinant CD151 protein produced as described above was used to screen an scFv-expressing phage library. Four rounds of biopanning were conducted for the first screen, from which 96 colonies were selected for a secondary screen by ELISA. The 23 scFv phage clones yielding the best signals in ELISA were isolated, the scFv were cloned and sequenced, and the sequences were aligned. Four of 5 appropriate clones were then selected for further study.

Human IgG conversion and production

The 4 scFvs selected were converted to whole human IgG molecules by cloning and ligation to human light chain and γ1 heavy chain sequences. The heavy and light chain plasmids were pOPtiVEC™-TOPO® and pcDNA™3.3-TOPO® (both Life Technologies, Carlsbad, CA, USA), respectively. The plasmids were transfected into CHO-S cells for production of whole IgGs. For this, CHO-S cells were resuspended at 5×10^5 cells/mL, placed in flasks (30 mL per flask), and shaken at 130 rpm for 24 h in an 8% CO_2 atmosphere. On the day of transfection, cell viability was checked to ensure >95% viability and the cells were resuspended at a density of 1×10^6/mL. Heavy and light chain plasmids (18.75 μg each) were added to OptiPRO SFM (Gibco-Life Technologies) in a total volume of 0.6 mL and mixed with an equal volume of OptiPRO SFM containing 37.5 μL of FreeStyle™ MAX transfection reagent (Life Technologies). The mixture was incubated at room temperature for 15 min and then added to the cells, which were cultured at 37℃ in an 8% CO_2 atmosphere to allow protein expression. After 7 days, the cell supernatant was harvested and IgG was affinity purified with rProtein A Sepharose™ Fast Flow (GE Healthcare, Uppsala, Sweden). The column was equilibrated with buffer A (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7). The supernatant was mixed with an equal volume of buffer A and loaded onto the column. The column was washed with buffer A and the protein was eluted with a linear gradient of 0 to 100% buffer B (0.1 M glycine, pH 3.5). The purity of the

eluted IgG was analyzed by SDS-PAGE; then, the solution was dialyzed against phosphate-buffered saline and finally concentrated with an Amicon® Ultra filter unit (Millipore, Billerica, MA, USA).

Western blot analysis

Whole cell extracts of A549, BT549, and MOLT-4 cells (20 μg per lane) were resolved by 12% SDS-PAGE and 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 a rabbit anti-CD151 antibody (catalog number ab125363; Abcam, Cambridge, MA, USA) diluted to 1:3000 in 1% non-fat milk in TBS-T. The membranes were then rinsed with TBS-T, incubated with an anti-rabbit secondary antibody (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, and then rinsed with TBS-T for 30 min. 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

A549, BT549, and MOLT-4 cells were resuspended at 3×10^5/100 μL in FACS buffer (1% fetal bovine serum [FBS] in phosphate-buffered saline). Cells were incubated with 1 of the 4 candidate anti-CD151 antibodies (#1–4 in Fig. 6; 4 μg per sample) or a positive control anti-CD151 antibody (0.5 μL; sc-65293, Santa Cruz Biotechnology) for 1 h at 4℃. Cells were then washed with FACS buffer and resuspended in a 1:100 dilution of goat anti-human IgG-FITC (sc-2456, Santa Cruz Biotechnology) in FACS buffer and incubated for 40 min at 4℃. Samples were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). At least 10,000 events were acquired for each sample.

Cell culture

The human lung cancer cell line A549 was obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea), the breast cancer cell line BT549 was from the American Type Culture Collection (ATCC, Manassas, VA, USA), and the T lymphoblastoid line MOLT-4 was from Seoul National University. The cells were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics (Thermo Scientific, Waltham, MA, USA).

RESULTS

Association between CD151 expression and clinicopathological features of non-small cell lung cancer

A summary of the patient characteristics and correlations with CD151 expression is given in Table 2. The mean age at diagnosis was 60.77 ± 9.22 years (range, 30–80). The number of patients with disease at the different stages was 192 (50.5%) at stage I, 88 (23.2%) at stage II, 92 (24.2%) at stage III, and 8 (2.1%) at stage IV. A total of 245 (64.5%) tumors were classified as SCC and 135 (35.5%) as ADC. The most common histologic subtype of ADC was acinar-predominant (48/135, 35.5%), followed by solid-predominant (45/135, 33.3%), lepidic-predominant (26/135, 19.3%), micropapillary-predominant (9/135, 6.7%), and papillary-predominant (7/135, 5.2%).

High CD151 expression was detected in 28.7% of NSCLC samples (109/380), of which 20.8% were SCC (51/245) and 42.9% were ADC (58/135). Of the ADC histologic subtypes, high CD151 expression was most common in solid-predominant tumors (48.3%), followed by acinar-predominant (29.3%), lepidic-predominant (10.35%), micropapillary-predominant (10.35%), and papillary-

predominant (1.7%). High CD151 expression was significantly associated with male patients ($P = 0.012$), smokers ($P = 0.005$), and ADC ($P < 0.001$). Among the 380 NSCLC, 232 (61.1%) expressed p63, 109 (28.7%) expressed TTF-1, and 263 (69.2%) expressed E-cadherin. High CD151 expression was significantly associated with p63 negativity ($P < 0.001$), TTF-1 negativity ($P = 0.001$), and E-cadherin positivity ($P = 0.002$).

Relationship between CD151 expression and overall or disease-free survival of patients with non-small cell lung cancer

We analyzed the prognostic relevance of CD151 expression and other clinicopathological parameters to overall and disease-free survival in patients with NSCLC (Table 3, Fig. 2). High CD151 expression was significantly associated with worse overall and disease-free survival; patients with high CD151 expression had shorter overall and disease-free survival periods than those with low CD151 expression ($P = 0.006$ and $P = 0.012$, respectively). High CD151 expression was confirmed as an independent worse prognostic factor affecting overall survival [$P = 0.005$, hazard ratio (HR) = 1.602, 95% confidence interval (95% CI), 1.169–2.193] using multivariate analyses (Table 4).

Other clinical variables such as gender ($P = 0.003$), smoking history ($P = 0.026$),

age (P = 0.028), AJCC stage (P < 0.001), pT stage (P < 0.001), and pN stage (P < 0.001) significantly affected overall survival, whereas AJCC stage (P < 0.001), pT stage (P < 0.001), and pN stage (P < 0.001) were associated with disease-free survival. By multivariate analysis, male patients, older age (>60 years), ADC, pT stage, and pN stage were independent prognostic factors of worse overall survival, whereas ADC, pT stage, and pN stage were independent prognostic factors associated with disease-free survival.

Correlation of CD151 expression with overall and disease-free survival according to adenocarcinoma and squamous cell carcinoma subtypes

We further analyzed the prognostic impact of high CD151 expression on overall and disease-free survival according to histologic type of NSCLC (Table 5). High CD151 expression was strongly correlated with worse overall survival of ADC (P = 0.004) but not of SCC (P = 0.251) (Fig. 3). However, CD151 expression was not significantly related to disease-free survival of ADC (P = 0.098).

Cox multivariate analyses revealed that high CD151 expression was an independent prognostic factor predicting decreased overall survival of patients with ADC [P = 0.001, HR = 2.445, 95% CI, 1.45–4.12]. Gender, age, and pN stage were also independent prognostic factors associated with overall survival [P = 0.026, HR

= 2.493, 95% CI, 1.11–5.58; *P* = 0.049, HR = 1.669, 95% CI, 1.00–2.78; *P* = 0.001, HR = 2.821, 95% CI, 1.56–5.10, respectively]. On the other hand, pN stage was the only independent prognostic factor of disease-free survival in ADC patients [*P* = 0.023, HR = 1.970, 95% CI, 1.10–3.53].

For patients with SCC, older age (>60 years), advanced AJCC stage (III-IV), and high pT and pN stages were associated with decreased overall survival (*P* = 0.024, *P* < 0.001, *P* < 0.001, and *P* < 0.001, respectively), and age, pT stage, and pN stage were independent prognostic factors of overall survival (*P* = 0.003, HR = 1.880, 95% CI, 1.23–2.87; *P* = 0.001, HR = 2.477, 95% CI, 1.48–4.15; *P* = 0.001, HR = 2.213, 95% CI, 1.41–3.48, respectively).

pT stage and pN stage were independent prognostic factors associated with decreased disease-free survival of patients with SCC (*P* = 0.004, HR = 2.371, 95% CI, 1.32–4.25; and *P* = 0.011, HR = 1.978, 95% CI, 1.17–3.35, respectively).

Relationship between CD151 expression in the predominant histologic subtypes of adenocarcinoma and overall survival

We next examined the prognostic value of CD151 expression in the predominant ADC histologic subtypes. Patients with solid-, micropapillary-, and acinar-predominant ADC had shorter overall survival than lepidic- and papillary-predominant ADC patients (*P* < 0.001). We found no significant relationship

between high CD151 expression and overall or disease-free survival in the acinar-, lepidic-, or solid-predominant subtypes (P = 0.648 and P = 0.542; P = 0.384 and P = 0.386; P = 0.191 and P = 0.398, respectively) In the solid-predominant subtype, the patients with ADC expressing high CD151 levels tended to have decreased overall and disease-free survival, although the differences were not statistically significant.

Production and purification of a cyclized recombinant CD151 protein

To determine whether CD151 could be a potential target for the development of anticancer therapeutics, we produced antibodies by selection of CD151-binding scFvs from a phage library, followed by conversion of the scFvs to whole IgG1 molecules. To conduct the phage library screening, we produced recombinant CD151 antigens. Two domains within the second extracellular loop were selected as the optimal antigens based on structural and functional analysis of CD151 (Fig. 4). After cloning and transfection of CHO-S cells, we found that only antigen CD151-2 could be expressed and purified. Antigen CD151-2 contained the tripeptide QRD (194-19), which is involved in integrin binding and plays an important role in CD151 function. Three cysteines located in the middle of the molecule were mutated to serines to prevent intramolecular disulfide bond

formation during protein folding. This allowed a cyclized recombinant protein, which was similar to the native structure, to be produced by disulfide bonding between the cysteines located at the sequence termini. Although formation of protein multimers was a concern, we found that the cyclized form of the protein was readily produced and yielded a single band by non-reducing PAGE (Fig. 4B and C).

Selection of anti-CD151 scFv candidates by phage library screening and ELISA

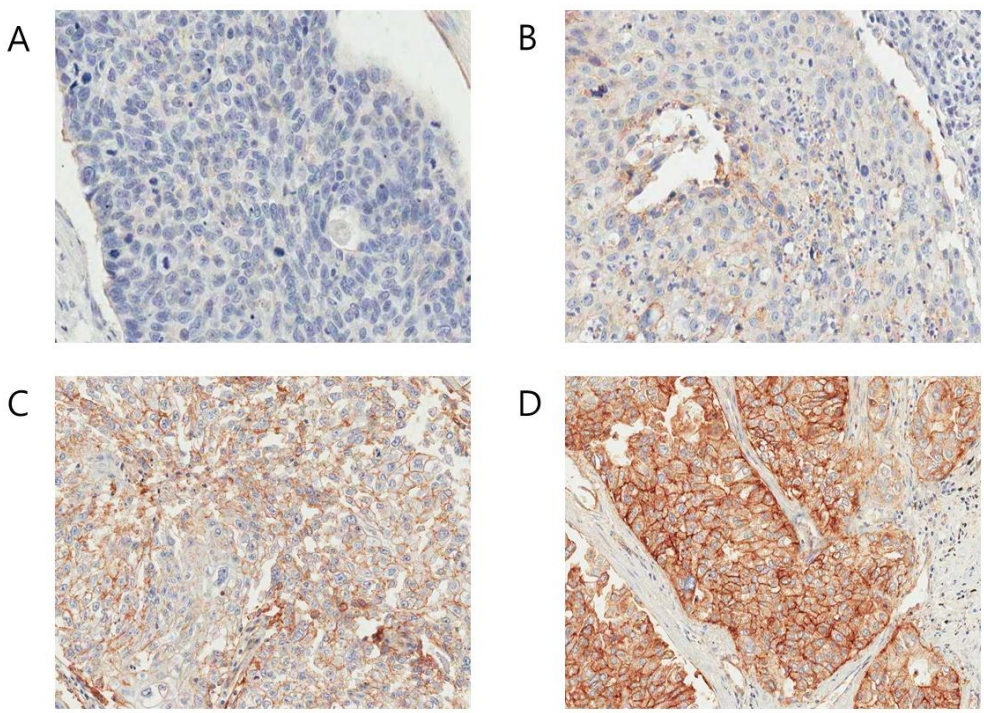
The purified recombinant CD151 protein was used in the phage display assays to select specific CD151-binding scFv clones. Four rounds of biopanning were performed (Fig. 5A), from which 96 colonies were selected for further screening by ELISA (Fig. 5B). We selected 23 scFv clones for sequencing based on the ELISA results. Finally, 4 of 5 clones with the correct sequences were selected for conversion to whole IgG (Fig. 5C).

Conversion of scFv to whole IgG and measurement of CD151 binding specificity

Four of the scFv sequences were converted to whole IgG. The candidate scFv sequences were cloned and cDNAs were ligated into plasmids encoding the human γ1 heavy and light chains (Fig. 6A). The plasmids were transfected into CHO-S cells and the secreted IgG was purified by protein A affinity chromatography. The specificity of binding of the 4 antibodies was measured by flow cytometry using the CD151-positive lung cancer cell line A549, the breast cancer cell line BT549, and the CD151-negative control cell line MOLT-4. The pattern of CD151 expression in these cell lines was first confirmed by FACS analysis of whole cells and western blotting of cell extracts using a control anti-CD151 antibody (Fig. 6B). Figure 6C shows that of the 4 new anti-CD151 antibodies produced here, #2 and #4 showed significant binding to A549 and BT549 cells, but not to MOLT-4, indicating that the antibodies specifically recognized cell surface human CD151. Collectively, these results demonstrate that human CD151-specific antibodies can be readily produced and may have utility as anticancer agents.

Figure 1.

Representative photomicrographs of CD151 immunohistochemical staining scores in non-small cell lung cancer subtypes. (A-D) solid-predominant ADC and SCC and (E-H) lepidic-, acinar-, papillary-, and micropapillary-predominant ADC. (A, E) score 0, (B, F) score 1+, (C, G) score 2+, and (D, H) score 3+.



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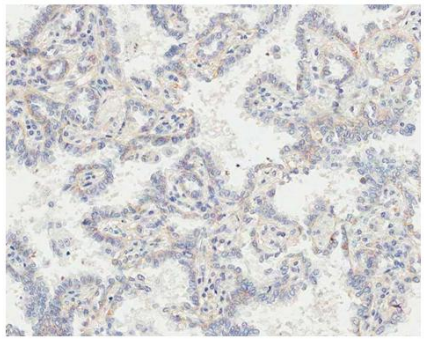

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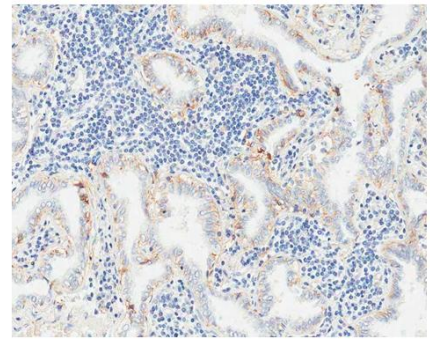

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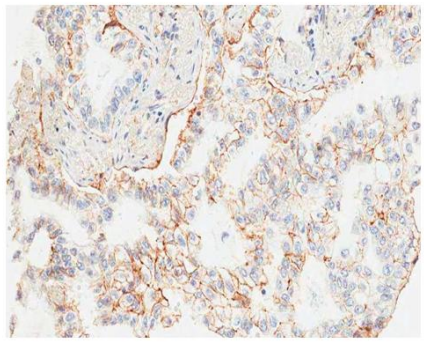

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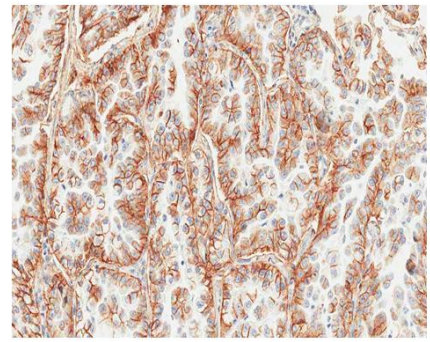


Figure 2.

Correlation of CD151 expression in non-small cell lung cancer with overall and disease-free survival.

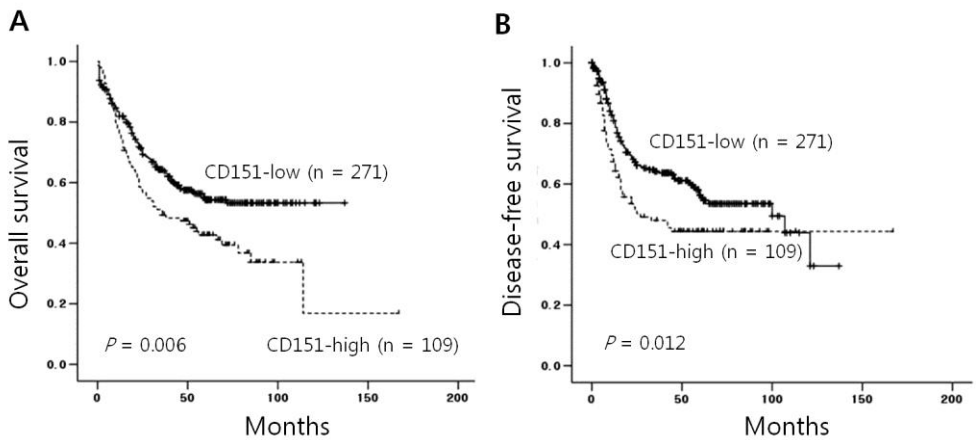


Figure 3.

Association of CD151 expression in non-small cell lung cancer with overall survival according to tumor subtype, patient gender, and patient smoking history. CD151 expression predicts unfavorable overall survival of (A) ADC patients, (C) males, and (E) smokers but was unrelated to overall survival of (B) SCC patients, (D) females, and (F) non-smokers.

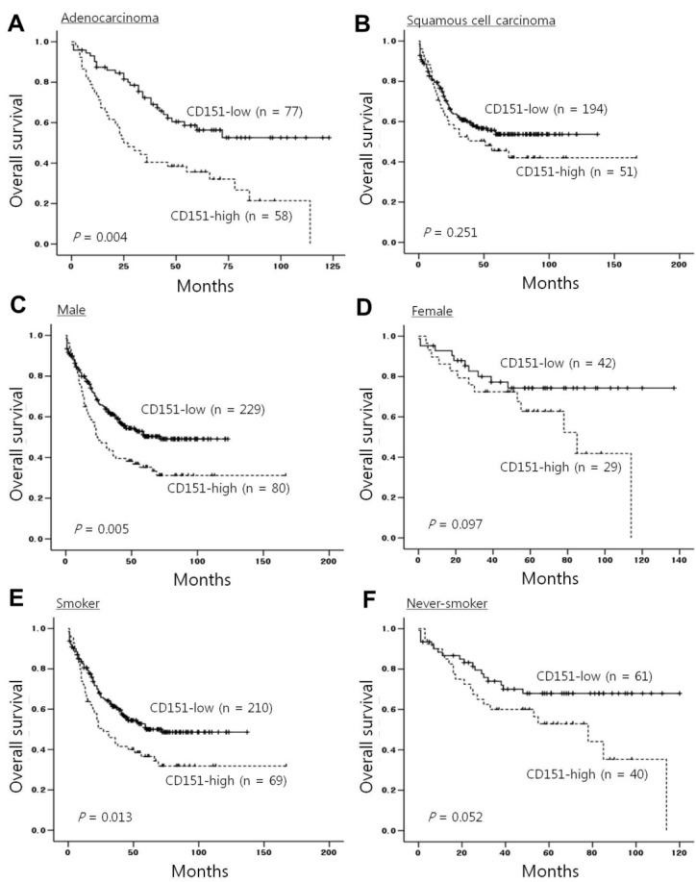


Figure 4.

Preparation of a cyclized recombinant CD151 protein. (A) Antigen domains of CD151 and sequences of constructs CD151-1 and CD151-2, which were designed to mimic the second loop of the native protein. (B) SDS-PAGE analysis of expressed CD151-2 protein; CD151-1 was not expressed. (C) Purified CD151-2 protein runs as a single band in non-reducing PAGE.

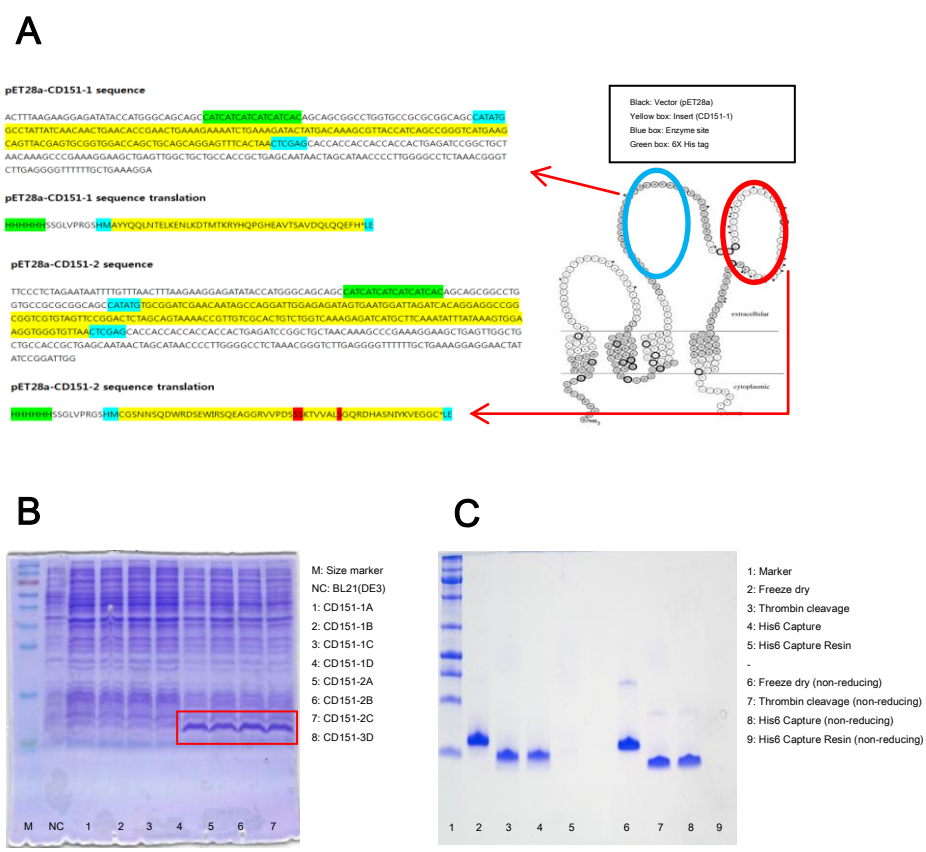
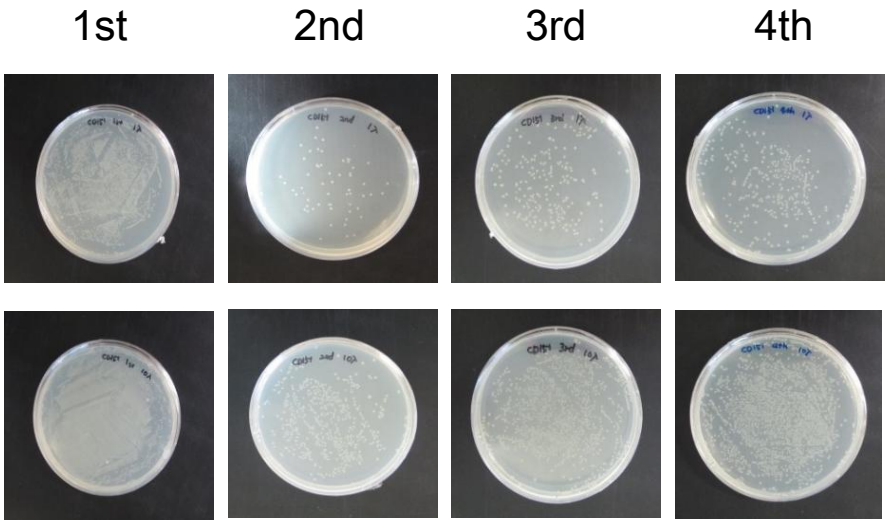


Figure 5.

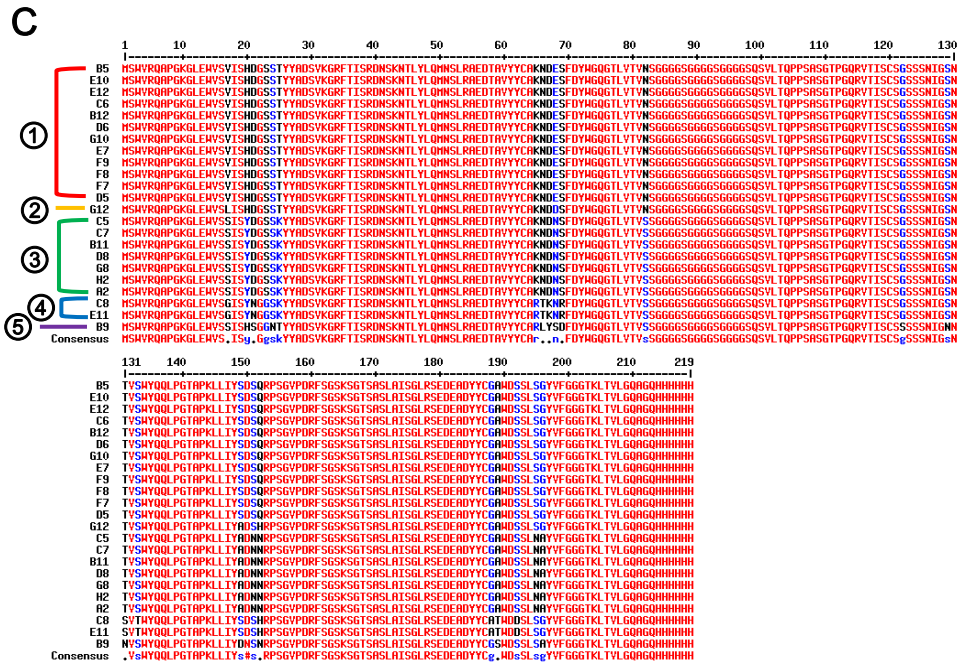
Selection of candidate CD151-binding scFvs by phage library screening. (A) Photographs of colonies selected during 4 rounds of phage biopanning on recombinant CD151. (B) Absorbance values of a secondary CD151-specific ELISA screen. (C) Sequence alignment of 5 selected scFv clones.

A



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.067	0.655	0.361	0.399	0.147	0.059	0.119	0.247	0.254	0.443	0.08	0.406
B	0.547	0.483	0.311	0.508	0.285	0.544	0.439	0.262	0.074	0.218	0.72	0.624
C	0.528	0.433	0.058	0.455	0.496	0.603	0.219	0.068	0.2	0.355	0.083	0.509
D	0.053	0.481	0.423	0.352	0.067	0.574	0.54	0.688	0.071	0.077	0.255	0.495
E	0.055	0.059	0.543	0.056	0.297	0.465	0.583	0.485	0.344	0.579	0.081	0.583
F	0.437	0.369	0.516	0.124	0.077	0.072	0.571	0.116	0.447	0.464	0.447	0.549
G	0.457	0.222	0.108	0.062	0.51	0.23	0.504	0.749	0.08	0.586	0.408	0.732
H	0.53	0.767	0.379	0.094	0.455	0.435	0.403	0.407	0.082	0.537	0.083	0.494


C
1
2
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4
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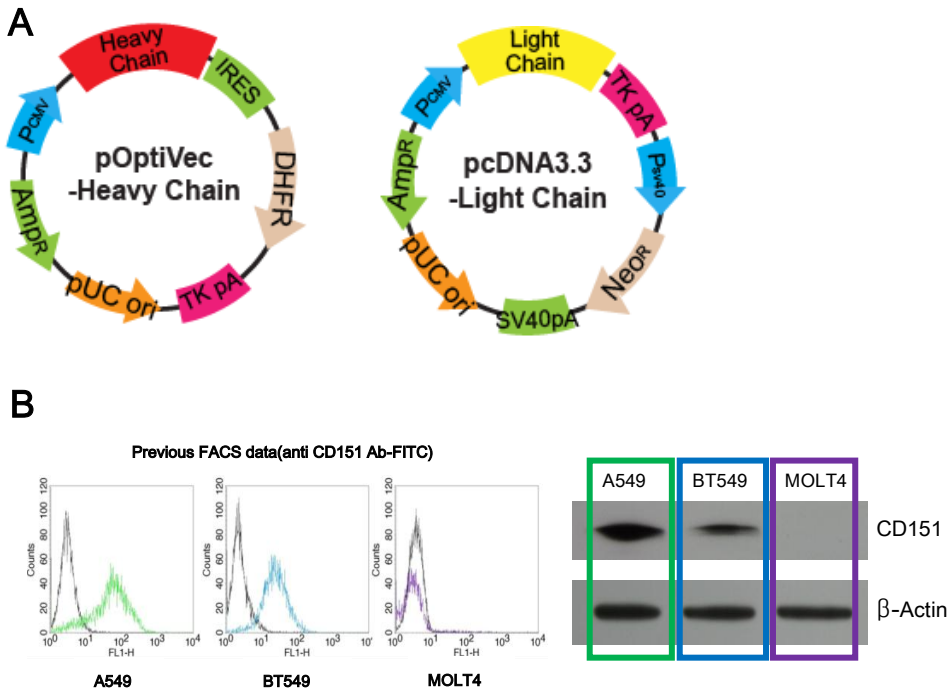
B5
E10
E12
C6
B12
D6
G10
E7
F9
F8
F7
D5
G12
C5
C7
B11
D8
G8
H2
A2
C8
E11
B9
Consensus

Figure 6.

Verification of conversion of scFvs to whole IgGs and binding to CD151-positive cells. (A) Schematic of vectors used for cloning of heavy and light chains. (B) Verification of CD151 expression by FACS analysis and western blotting of the CD151-positive mammalian cell lines A549 and BT549. The CD151-negative cell line MOLT-4 was used as a control. (C) The specificity of binding of 4 anti-CD151 antibodies was confirmed by FACS analysis with A549, BT549, and MOLT-4 cells. Antibodies 2 and 4 show specific binding to CD151-positive cells.



C

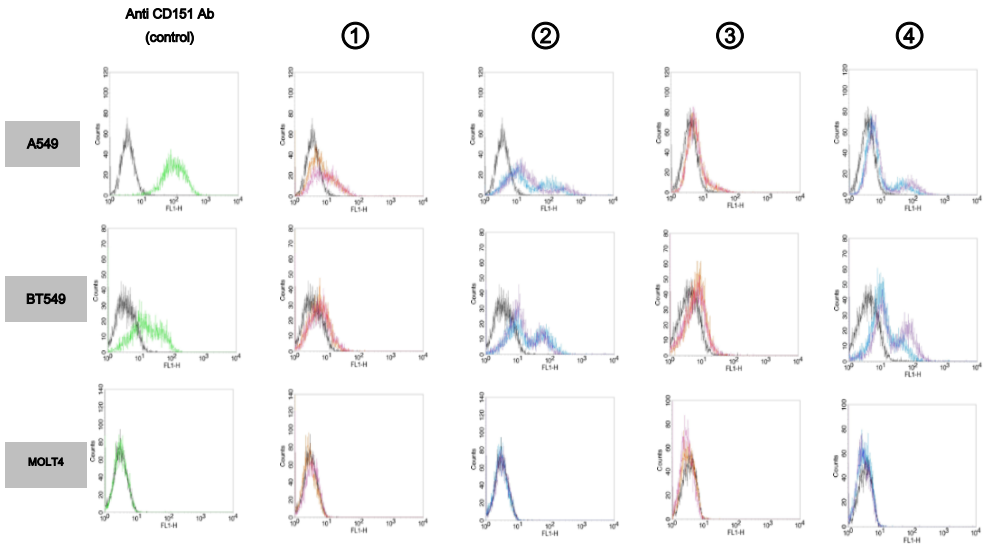


Table 1. Scoring system for immunohistochemical staining of CD151 in non-small cell lung cancer subtypes

Score	Lepidic/acinar/papillary/micropapillary-predominant ADCs	Solid-predominant ADC and SCC
0	No staining or cell membrane staining in < 10% of tumor cells	No staining in tumor cells
1+	Faint perceptible membrane staining in > 10% of tumor cells. Only part of the cell membranes are stained	Weak, incomplete membrane staining in any proportion of tumor cells, or a weak, complete membrane staining in < 10% of all cells
2+	Weak to moderate complete or basolateral membrane staining in > 10% of tumor cells	Complete membrane staining that is non-uniform or weak, but with obvious circumferential distribution ≥ 10% of cells or intense complete membrane staining in ≤ 30% of tumor cells
3+	Moderate to strong complete or basolateral membrane staining in > 10% of the tumor cells	Uniform intense membrane staining of > 30% of tumor cells

Table 2. Relationship between CD151 expression and the clinicopathological features of 380 patients with non-small cell lung cancer

Characteristic	CD151 expression		*P*
	High n = 109 (28.7%)	Low n = 271 (71.3%)	
Gender			0.012
Male	80 (73.4)	229 (84.5)	
Female	29 (26.6)	42 (15.5)	
Age (years)			
Mean ± SD	59.55±10.19	61.26±8.77	0.113
Range	30-82	34-79	
Smoking			0.005
Smoker	69 (63.3)	210 (77.5)	
Non-smoker	40 (36.7)	61 (22.5)	
Histology			<0.001
SCC	51 (46.8)	194 (71.6)	
ADC	58 (53.2)	77 (28.4)	
Lepidic predominant	6 (10.35)	20 (25.9)	
Acinar predominant	17 (29.3)	31 (40.3)	
Solid predominant	28 (48.3)	17 (22.1)	
Papillary predominant	1 (1.7)	6 (7.8)	
Micropapillary predominant	6 (10.35)	3 (3.9)	
Pathologic stage			0.210
Stage I	49 (44.9)	143 (52.8)	
Stage II	32 (29.4)	56 (20.7)	
Stage III	27 (24.8)	65 (23.9)	
Stage IV	1 (0.9)	7 (2.6)	
T stage			0.023
T1	23 (21.1)	40 (14.8)	
T2	60 (55.0)	183 (67.5)	
T3	17 (15.6)	20 (7.4)	
T4	9 (8.3)	28 (10.3)	
N stage			0.959
N0	68 (62.4)	167 (61.6)	
N1	25 (22.9)	61 (22.5)	
N2	16 (14.7)	43 (15.9)	
M stage			0.678
M0	108 (99.1)	265 (97.8)	
M1	1 (0.9)	6 (2.2)	
P63			<0.001
Negative	62 (56.9)	86 (31.7)	
Positive	47 (43.1)	185 (68.3)	
TTF-1			0.001
Negative	64 (58.7)	207 (76.4)	
Positive	45 (41.3)	64 (23.6)	
E-cadherin			0.002
Negative	21 (19.3)	96 (35.4)	
Positive	88 (80.7)	175 (64.6)	

SCC, squamous cell carcinoma; ADC, adenocarcinoma

Table 3. Univariate analyses of the overall and disease-free survival of 380 patients with non-small cell lung cancer

	Overall survival		Disease-free survival	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
CD151 expression		0.007		0.014
Low vs. High	1.520 (1.120-2.061)		1.519 (1.089-2.119)	
Gender		0.003		0.908
Female vs. Male	1.966 (1.267-3.049)		0.978 (0.670-1.427)	
Smoking		0.026		0.195
No vs. Yes	1.498 (1.049-2.138)		0.800 (0.570-1.121)	
Age (years)		0.028		0.441
≤60 vs. >60	1.403 (1.036-1.901)		0.882 (0.642-1.213)	
Histology		0.815		0.067
SCC vs. ADC	1.037 (0.766-1.403)		1.348 (0.979-1.858)	
AJCC stage		<0.001		<0.001
I, II vs. III, IV	2.619 (1.933-3.550)		1.866 (1.314-2.648)	
T stage		<0.001		<0.001
T1, 2 vs. T3, 4	2.884 (2.082-3.995)		2.357 (1.621-3.429)	
N stage		<0.001		<0.001
N0 vs. N1, 2	2.320 (1.725-3.120)		1.955 (1.419-2.695)	

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma; ADC, adenocarcinoma; AJCC, American Joint Committee on Cancer

Table 4. Multivariate analyses of the overall and disease free survival of 380 patients with non-small cell lung cancer

	Overall survival		Disease-free survival	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
CD151 expression		0.005		0.064
Low vs. High	1.602 (1.169-2.193)		1.384 (0.982-1.952)	
Gender		0.036		0.331
Female vs. Male	1.921 (1.044-3.535)		1.327 (0.750-2.347)	
Smoking		0.312		0.406
No vs. Yes	1.277 (0.795-2.052)		0.813 (0.499-1.325)	
Age (years)		0.001		0.692
≤60 vs. >60	1.752 (1.274-2.410)		1.070 (0.766-1.495)	
Histology		0.007		0.040
SCC vs. ADC	1.611 (1.141-2.275)		1.506 (1.020-2.225)	
AJCC stage		0.415		0.649
I, II vs. III, IV	1.196 (0.778-1.838)		0.891 (0.543-1.463)	
T stage		<0.001		<0.001
T1,2 vs. T3,4	2.050 (1.371-3.065)		2.246 (1.427-3.537)	
N stage		<0.001		0.001
N0 vs. N1,2	2.316 (1.618-3.313)		1.952 (1.320-2.888)	

CI, confidence interval; SCC, squamous cell carcinoma; ADC, adenocarcinoma; AJCC, American Joint Committee on Cancer

Table 5. **Univariate and multivariate analyses of the clinicopathological and biological factors affecting the overall and disease-free survival rates of patients with ADC and SCC**

	OS				DFS			
	Univariate		Multivariate		Univariate		Multivariate	
ADC	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
CD151								
Low vs. High	1.981 (1.23-3.19)	**0.005**	2.445 (1.45-4.12)	**0.001**	1.495 (0.93-2.41)	0.098	1.353 (0.82-2.24)	0.240
Gender								
F vs. M	2.807 (1.67-4.72)	**<0.001**	2.493 (1.11-5.58)	**0.026**	1.476 (0.91-2.39)	0.113	1.438 (0.71-2.93)	0.316
Smoking								
No vs. Yes	2.300 (1.42-3.73)	**0.001**	1.262 (0.63-2.55)	0.516	1.141 (0.71-1.85)	0.592	0.776 (0.39-1.52)	0.453
Age (years)								
≤60 vs. >60	1.166 (0.73-1.87)	0.524	1.669 (1.00-2.78)	**0.049**	0.783 (0.48-1.27)	0.320	0.926 (0.56-1.54)	0.767
AJCC stage								
I, II vs. III, IV	3.661 (2.20-6.09)	**<0.001**	1.543 (0.78-3.05)	0.214	2.928 (1.74-4.94)	**<0.001**	1.390 (0.67-2.87)	0.373
T stage								
T1, 2 vs. T3, 4	3.395 (1.90-6.07)	**<0.001**	1.224 (0.62-2.42)	0.562	3.237 (1.77-5.91)	**<0.001**	1.974 (0.93-4.19)	0.076
N stage								
N0 vs. N1, 2	2.814 (1.74-4.54)	**<0.001**	2.821 (1.56-5.10)	**0.001**	2.533 (1.56-4.12)	**<0.001**	1.970 (1.10-3.53)	**0.023**
SCC								
CD151 expression								
Low vs. High	1.284 (0.84-1.97)	0.256	1.345 (0.87-2.07)	0.180	1.402 (0.86-2.28)	0.174	1.405 (0.86-2.29)	0.173
Gender								
F vs. M	2.315 (0.57-9.38)	0.240	2.40 (0.56-10.40)	0.241	0.793 (0.32-1.97)	0.617	1.135 (0.40-3.26)	0.814
Smoking								
No vs. Yes	1.095 (0.59-2.04)	0.775	1.169 (0.60-2.26)	0.643	0.651 (0.35-1.20)	0.171	0.699 (0.35-1.42)	0.322
Age (years)								
≤60 vs. >60	1.599 (1.07-2.40)	**0.024**	1.880 (1.23-2.87)	**0.003**	1.000 (0.65-1.55)	1.000	1.188 (0.75-1.89)	0.466
AJCC stage								
I, II vs. III, IV	2.367 (1.61-3.48)	**<0.001**	1.028 (0.58-1.81)	0.924	1.453 (0.90-2.34)	0.124	0.638 (0.32-1.27)	0.202
T stage								
T1, 2 vs. T3, 4	2.813 (1.88-4.20)	**<0.001**	2.477 (1.48-4.15)	**0.001**	2.092 (1.29-3.39)	**0.003**	2.371 (1.32-4.25)	**0.004**
N stage								
N0 vs. N1, 2	2.174 (1.49-3.18)	**<0.001**	2.213 (1.41-3.48)	**0.001**	1.756 (1.14-2.70)	**0.010**	1.978 (1.17-3.35)	**0.011**

ADC, adenocarcinoma; SCC, squamous cell carcinoma; OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; F, female; M, male; AJCC, American Joint Committee on Cancer

DISCUSSION

The goal of this study was to determine the prognostic significance of CD151 expression in NSCLC with particular focus on disease subgroup analysis and clinicopathological variables. We confirmed that high CD151 expression was an independent prognostic factor of worse overall survival in ADC but not SCC. Based on these and published data, we hypothesized that CD151 may have additional value as a target protein for anticancer therapy, and we initiated the development of potential antibody therapeutics by producing 4 new human CD151-specific antibodies.

Previous studies have shown that CD151 overexpression has negative prognostic value for the survival of patients with breast,[13] liver,[17,18] kidney,[15] pancreas,[25] and esophageal cancers[16]. The relationship between CD151 overexpression and poor survival in NSCLC in our study is consistent with an earlier study in which high expression of CD151 mRNA was significantly associated with decreased overall survival.[20] The prognostic value of CD151 overexpression for survival of patients with NSCLC, however, has not previously been investigated by immunohistochemical analysis. The present study suggests that immunohistochemical detection of CD151 expression could serve as a prognostic biomarker for NSCLC.

CD151 overexpression is associated with poor prognosis in several cancer types, including ADC and SCC.[13,15-17,25] In this study, we found clinicopathological and prognostic relevance of CD151 expression in ADC but not SCC. Clinical variables, such as pT and pN stages, were associated with worse survival outcomes for patients with SCC. The lack of correlation between CD151 expression and SCC prognosis found here contrasts with the results of an esophageal SCC study,[16] in which CD151 overexpression was associated with tumor depth, lymph node or distant metastases, lymphatic invasion, and poor survival. Other studies have investigated CD151 expression in different types of ADC. CD151 is expressed on endothelial cells and platelets and is frequently overexpressed on cancer cells,[10] but it remains unclear whether CD151 performs cell type-specific or common biological functions in human malignancies.

High CD151 expression is associated with unfavorable clinicopathological parameters in various cancers, including high pTNM stage, high nuclear grade, large tumor size, and lymph node metastasis.[13,15-17,25] We found that high CD151 expression was prevalent in males, smokers, and patients with ADC and that it was associated with decreased overall survival of these patients. The relationship between high CD151 expression and these unfavorable factors is consistent with the known correlations between age, gender, smoking history, and the clinical prognosis of NSCLC.[26]

According to histological classification,[21] there is a widely pathologic spectrum within lung ADC. Interestingly, some histological subtypes, including lepidic-,

acinar-, papillary-, and micropapillary-predominant ADC show a basolateral staining pattern similar to that exhibited by HER2 in gastric cancer. Basolateral staining results from CD151 and integrin α3β1 colocalization on endothelial cells and keratinocytes at points of cell-to-cell adhesion. CD151 is also detected on the lateral surface of contacts in polarized epithelial cells.[27-29] Previous studies of CD151 expression in NSCLC enrolled only patients with ADC or SCC,[15-18,25] and each study used a single method to detect CD151 expression. In esophageal SCC, positive and negative CD151 expression was defined on the basis of complete membrane staining and the extent of staining, using a cut-off of 10% complete membrane staining.[16] Most other studies have used a semi-quantitative classification based on the staining intensity and the percentage of positive cells; these include studies on pancreatic ductal ADC,[25] clear cell renal carcinoma,[15] hepatocellular carcinoma,[18] and cholangiocarcinoma.[17] We used the HER2 scoring system for breast cancer to score CD151 expression in solid-predominant ADC and SCC, and the HER2 system for gastric cancer to score expression in lepidic-, acinar-, papillary-, and micropapillary-predominant ADC.

The classification of lung ADC according to subtype has revealed novel correlations between histologic subtypes and molecular and clinical features.[21] We did not observe statistically significant associations between CD151 expression and survival of patients with any of the predominant ADC subtypes. Although patients with the solid-predominant subtype and high CD151 expression tended to have decreased overall and recurrence-free survival, the differences were not statistically

significant. In contrast, a previous study showed that high CD151 expression was associated with poor overall survival of patients with specific breast cancer subtypes classified according to estrogen, progesterone, HER2, and basal marker expression.[13] This discrepancy may arise from the formation of complexes between CD151 and transmembrane proteins such as other tetraspanins, integrins, and growth factor receptors, which is required for their function.[18,30] Hence, CD151 expression might be more relevant as a prognostic marker for functional subgroups identified by immunohistochemical or molecular features than for morphologic subtypes of ADC.

We have conclusively shown that high CD151 expression can be a strong prognostic indicator for lung ADC. This finding suggests that CD151 expression may have value as a molecular target for therapy of ADC in NSCLC. With this in mind, we attempted to produce anti-CD151 antibodies by screening and selection from an scFv phage library, followed by conversion of the scFv to IgG. The recombinant CD151 used for screening was designed to mimic the native form of the second CD151 extracellular loop. In this study, we found that 2 of the 4 whole IgGs tested showed specific binding to CD151-expressing cells. Although we did not examine the affinity of the antibody-CD151 interaction here, several methods are available to measure this, such as immunoprecipitation (IP) and surface plasmon resonance (SPR) as well as western blot analysis and flow cytometry. 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 mechanisms of cytotoxicity 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 CD151 is a valuable prognostic marker for NSCLC. We also demonstrated the successful production of CD151-specific human IgGs, suggesting that CD151 may a potential target for the development of therapeutic antibodies for lung cancer.

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국 문 초 록

CD151은 tetraspanin superfamily에 속하는 막단백질 중 하나로 다양한 암종에서 과발현되어 종양 세포의 이동성, 전이 등에 기능하여 작용하며 또한 poor prognosis와 밀접한 관련이 있다고 알려져 있다. 그 중에서 prognostic significance가 명확히 밝혀지지 않은 비소세포폐암(non-small cell lung cancers, NSCLCs)에서 CD151의 발현과 prognostic marker로서의 가능성을 확인하고자 하였고 더 나아가 CD151을 표적단백질로 한 항체치료제 개발에 관한 연구를 진행하였다. CD151의 prognostic marker로서의 가치를 확인하기 위해 삼성병원에서 1994년부터 2005년까지 채취한 비소세포폐암(NSCLCs) 환자 조직을 통해 임상병리학적 요인을 분석하였고 또한 이 조직들로 immunohistochemical analysis를 수행하였다. 생존 분석결과 CD151이 과발현 되었을 경우 남성, 흡연자, 선암종(adenocarcinoma) 환자 군의 무병생존율(DFS), 전체생존율(OS)이 낮아짐을 알 수 있었으며 독립적인 prognostic marker로서의 가치를 확인하였다. 따라서 CD151은 폐암, 특히 비소세포폐암(NSCLCs)의 strong prognostic indicator로서 작용하며 항체치료제 개발을 위한 잠재적 표적단백질이 될 수 있음을 검증하였다. 이 후 CD151의 구조적, 기능적 분석을 통해 최적의 항원결정부위를 디자인하여 재조합 단백질로 제작하였고 이것을 이용한 single chain variable fragment (scFv) 스크리닝을 진행하였다. Phage display를 활용한 4차례의 bio-panning으로 1차 스크리닝을 수행한 후 ELISA analysis를 통한 2차 스크리닝을 거쳐 후보 scFv를 선별하였다. 이 후 선별된 후보 scFv는 whole IgG로의 conversion을 수행하였고 CHO-S cell을 이용한 transient expression system을 통해 IgG를 생산하였다.

CD151이 과발현 되는 것으로 알려진 암 세포주를 선별하여 flow cytometry, western blot analysis를 수행한 결과 후보 IgG의 CD151에 대한 친화성, 특이성을 확인 할 수 있었다. 본 연구는 폐암에서 CD151의 prognostic marker로서의 가능성과 표적단백질로서의 가치를 검증하고 이를 활용한 항체치료제 개발의 과정을 제시한다.

주요어 : CD151, 폐암, 비소세포폐암, prognostic marker, 표적단백질, single chain variable fragment, 항체치료제

학 번 : 2011-21750

감사의 글

이렇게 졸업을 눈 앞에 두기까지 석사과정은 2년간의 시간이었지만 그 시작은 2008년 여름쯤으로 기억합니다. 광대한 캠퍼스에 한 번 놀라고 힘겹게 찾은 29동에 위치한 실험실 모습에 또 한 번 놀랐던 순간이 엊그제 같은데 벌써 이렇게 많이 시간이 흘러 학위 과정을 마치게 되니 감회가 새롭습니다.

학부 재학 시절 가슴에 새긴 신약개발이라는 부푼 꿈과 포부를 현실로 이루어 내보고자 실험실의 구성원으로 함께하기까지 나름대로는 치열했던 시간과 노력을 거쳐 뜻 깊은 첫 발을 내딛게 되었던 것 같습니다. 돌이켜보면 여러모로 부족한 점 투성이였고 아쉬움으로 가득한 시간이었지만 인내와 관심 그리고 사랑으로 지켜봐 주신 신영기 지도교수님께 가장 먼저 감사의 말씀을 드리고 싶습니다. 학문과 연구를 대하는 과학자의 모습과 더불어 시대를 살아가는 인생의 선배이자 스승으로서 제가 받은 지도와 가르침은 앞으로의 삶에 있어 정말 큰 도움이 될 것입니다. 진심으로 존경하고 다시 한 번 감사드립니다. 또한 논문을 완성하는데 많은 힘이 되어주신 삼성병원의 최윤라 교수님께도 감사의 말씀을 전합니다. 기회가 된다면 좀 더 근사한 모습으로 노래하는 모습을 보여드리고 싶었는데 그러지 못해 아쉬운 마음입니다. 늘 좋은 말씀과 인자한 웃음으로 대해주신 이미옥 교수님, 오우택 교수님, 이호영 교수님, 정말 감사드립니다.

오랜 시간 동안 가족보다도 더 많은 시간을 함께하며 웃고 울던 실험실 선배님, 동기, 후배들에게도 진심으로 감사의 말씀을 전하고 싶습니다. 드럽게 말 안 듣는 철부지 놈 사람 만들어 보겠다고 각고의 노력과 인고의 시간을 감내하신 준영이형, 그 동안 형이 있어 실험실 생활이 즐

거웠고 또 행복했습니다. 앞으로도 오랜 시간 동안 형과 아우로서 함께 할 수 있었으면 좋겠습니다. 그리고 참치 사주세요. 박사과정 선배이며 동갑내기 친구인 태은이, 언제나 밝은 모습으로 유쾌하게 살아가는, 하지만 연구를 대하는 자세만큼은 누구보다도 진지하고 열정적인 너를 보면서 참 배울 점이 많은 사람이라고 생각했어. 너의 앞 날에 축복만이 가득하기를 멀리서나마 응원할게. 같은 팀으로는 첫 후배이자 짝지로 시작한 하연이, 강렬했던 첫 인상만큼이나 씩씩하고 매사에 열심인 지금의 모습 변치 말고 목표하는 것 이상으로 이룰 수 있기를 진심으로 바란다. 정신머리 없는 사람 하나 옆에 두고 오빠 대접 해주기가 쉽지 않았을 텐데 정말 고맙다. 너에게는 정말 미안하고 고마운 마음뿐인데 앞으로 살아가면서 그 동안 못한 선배 노릇, 오빠 노릇 다 해줄게. 또 레퍼런스바이오랩 식구이자 항체팀으로 함께한 명석이형, 혜정이, 김도형선생님, 여러모로 부족한 석사 과정 학생을 물심양면으로 끌어주고 밀어준 점, 정말 진심으로 감사하게 생각합니다. 잊지 않겠습니다.

2008년 처음 실험실을 찾아왔을 때 방장으로서 저에게 많은 이야기 해주신 헌순이형, 지금은 정박사님이라 부르지만 아직도 형이라는 호칭이 좀 더 익숙하네요. 오랜 시간 동안 관심 가져주시고 응원해주신 점, 표현은 못했지만 늘 감사하게 생각하고 있었습니다. 고맙습니다. 때로는 엄하게, 또 너그럽게 많은 것을 가르쳐주신 훈석이형, 마음으로 느껴지는 조언과 함께 든든한 힘이 되어주신 미정누나(권박사님), 은설누나(오박사님), 경누나, 성수형, Cem 박사님, 이선명 박사님, Juthika, Lina, 또 실험실 인연으로 함께한 많은 선배님들 진심으로 감사드립니다. 어디에 계시든 늘 응원하겠습니다. 지금과 비교하면 정말 협소하고 세월의 흔적 가득했던 29동 실험실 한 켠에 다같이 모여 앉아 의지를 불태우며 공부하던 해민이와 지현이 그리고 유부녀 민정이, 나중에 알게 됐지만 많은 것을 알려주고 도와준 수연이, 학부 후배이자 실험실의 실세로 자리 잡

아가고 있는 호빈이와 지금은 떠나고 없는 동기 Rony, 인도에서 온 쾌남 Nirmal에게도 감사의 마음을 전합니다. 이제 막 첫 발을 내 딛고 힘찬 걸음은 시작한 신영이, 해인이, 태순이, 혜은이, 세형이, 경수, 실험실에 생기를 불어 넣어 줄 모든 후배들에게도 격려와 함께 감사를 전합니다. 그 동안 따로 떨어져 있다는 핑계로 가까운 선배임에도 살뜰히 챙겨주지 못해 미안한 마음뿐입니다. 여러모로 많은 도움을 주시는 민정누나, 은영씨, 실험실 식구들과 더불어 오랜 시간 함께한 지희, 연진이, 시은누나와 모든 레퍼런스바이오랩 식구들에게도 심심한 감사의 말씀을 전하고 싶습니다. 특히 언제나 제게 많은 관심과 격려 아끼지 않아주신 김영덕 연구소장님께 감사를 표합니다.

이제는 모두 다른 곳에서 서로 다른 모습으로 살아가고 있지만 소중한 추억이자 큰 힘으로 언제나 함께하고 있는 경성약대 06학번 약우회 친구들, 민석이, 창윤이, 원태, 규민이, 광석이, 찬주, 대성이 모두 너무 고맙고 또 진심으로 사랑한다. 힘들었던 구직의 시간을 뒤로 하고 야구단 단장을 꿈꾸는 인규와 타지에서 외로움에 몸부림치고 있을 준식이, 가끔씩 만나도 반갑고 애틋한 창호, 너희들 때문에 안양을 떠나는 발걸음이 무거워진다. 그리고 당당하고 멋진 커리어우먼으로 거듭나고 있는 오래된 친구 소라, 여러모로 늘 고맙게 생각하고 있어. 다들 자주 만나지도, 연락하지도 못했지만 존재만으로 큰 힘이 되어준 나의 소중한 친구들 모두에게 다시 한 번 진심으로 감사의 마음을 전합니다.

끝이 있으면 새로운 시작이 있듯이 학위 과정의 끄트머리에서 만난 태원이, 수단이 아닌 목적이자 이유로 기분 좋은 생소함을 느끼게 해줘서 너무나도 감사하고 또 사랑합니다. 앞으로 맞이 할 새로운 끝과 시작의 순간에도 언제까지나 함께이기를 소원합니다. 아니 다짐합니다.

남들은 한창 철들어서 효도할 나이에 뒤늦은 바람으로 공부하는 아들 뒷바라지 해주신 아버지, 어머니, 매 순간 존경하고 사랑합니다. 가르침

과 은혜, 잊지 않고 살아가며 언제나 겸손하고 또 베풀 줄 아는 사람이 되겠습니다. 그리고 철 없는 형을 둔 까닭으로 훨씬 더 먼저 어른이 되어버린 지형이, 언제나 의젓하고 사려 깊게 행동하는 널 보면서 항상 미안하고 잘해주고 싶은 마음뿐이란다. 앞으로는 형이 너의 고민과 걱정 덜어줄 수 있는 사람이 될게. 묵묵히 내 역할까지 아니 그 이상으로 잘 해줘서 정말 고맙다. 앞으로도 서로 의지하며 힘이 되는, 우애 깊은 멋진 형제로 열심히 살아가자. 마지막으로 우리할머니, 이름만 불러도 눈물이 날 것 같은 나의 첫째, 할머니가 주시는 사랑이 복에 겨워 가끔씩은 당연한 것으로 여겨 아픔을 드릴 때면 후회스럽기 그지 없어요. 할머니는 나에겐 신앙이자 근원입니다. 이 글을 직접 읽지도 못해 직접 읽어드려야 하겠지만 옆에 앉아 들으시고 따뜻하게 계시면 그만입니다. 아무것도 바라는 게 없어요. 그저 건강하게 오래 계세요. 그 뿐입니다. 글로는 감히 다 하지 못할 정도로 사랑합니다.

그 동안 서툴고 부족했지만 나름대로의 도전과 시험을 무사히 마치고 새로운 시작을 준비하는 나 자신에게 격려와 감사의 말을 전하며 글을 줄입니다.