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Original Research

Migration and invasion of NSCLC suppressed by the downregulation of Src/focal adhesion kinase using single, double and tetra domain anti-CEACAM6 antibodies

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ABSTRACT

Carcinoembryonic antigen-related cell adhesion molecules 6 (CEACAM6) is a cell adhesion receptor. Expression of CEACAM6 in non-small cell lung cancer (NSCLC) associated with tumor progression and metastatic condition via Src/FAK signaling pathway. We established three anti-CEACAM6 antibodies with valences, which were designed to be monomeric sdAb, bivalent sdAb (2Ab), and tetravalent sdAb (4Ab). The anti-CEACAM6 antibodies can be used to target CEACAM6 overexpressing NSCLC. Anti-CEACAM6 antibodies, sdAb, 2Ab and 4Ab, were modified with different valency via protein engineering. sdAb and multivalent sdAbs (2Ab & 4Ab) were expressed and purified from *E.coli* and CHO cells, respectively. We compared the effect of anti-CEACAM6 antibodies with doxorubicin in NSCLC cell line both in vitro and in vivo. The 4Ab showed significant effect on cell viability. In addition, A549 cells treated with 2Ab and 4Ab inhibited the invasion and migration. In western blot, the 2Ab and 4Ab showed significant inhibition of phospho FAK domain Ty397 that is essential for activation of Src kinase family. Meanwhile, overall protein analysis revealed that 2Ab and 4Ab potently inhibited the phosphorylation of pSRC, pERK, pFAK, pAKT, MMP-2, MMP-9 and N-cadherin. Anti-tumor effect was observed in an A549 NSCLC xenograft model treated with 2Ab or 4Ab compared with doxorubicin. Confocal analysis showed higher targeting ability of 4Ab than that of 2Ab at 4 h incubation. Our data suggests that 2Ab and 4Ab inhibits EMT-mediated migration and invasion via suppression of Src/FAK signaling, which exhibits therapeutic efficiency for NSCLC treatment.

Introduction

Lung cancer is the most common cause of cancer related death worldwide [1]. Among them, NSCLC affects about 85% of lung cancer patients [1–6]. Standard treatment in early stages of NSCLC is surgery which can prolong the survival of patients about 50–60% in stages I and II [1]. However, owing to the metastatic diseases, which are associated with highly aggressive invasion in surgery treatment, are presented in most of NSCLC patients that is, the surgical resection in NSCLC patients are limited. Although Erlotinib, gemcitabine, and several FDA approved drugs are available to treat, NSCLC [2,3], the poor drugs response and multiple drug resistance are restricting their efficacy. Therefore, developing new approach for NSCLC treatment is critically important.

Specific antibodies or ligands conjugated liposome can recognize the receptor of cancer cells, resulting in precise delivery of drugs to the

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specific tumor site [7,8]. Thus, identifying specific receptors restricted expression on NSCLC is essential. CEACAM6 or CD66c is a member of carcinoembryonic antigen (CEA) family and normally expresses on epithelial and myeloid cell surfaces. Otherwise, CEACAM6, which is a tumor-related marker, mediates homotypic binding with other CEA family members and heterotypic binding with integrin receptors and plays crucial role in organizing tissue architecture, regulation of signal transduction pathways. Over-expressed CEACAM6 is observed and highly associated with invasion and metastasis in several human malignancies, such as pancreatic, lung, and colon cancer cells [4]. Elevated expression of CEACAM6 modulates cancer progression through apoptosis inhibition, cell proliferation enhancement, and drug resistance [9,10].

Cell adhesion plays a crucial role in tumor invasion and metastasis. Compared to the intact tissues malignant tumors are characterized by morphological damages [11]. It has been postulated that abnormal expression of cell adhesion molecules resulting in loss of cell-cell and cell-matrix interaction can promote invasion and migration [5] and closely associated with differentiation as well as metastatic potential. Integrins are belongs to cell adhesion receptor family and have been demonstrated that involved in tumor cell migration and metastasis. Moreover, integrins are associated with several tumor progressions relatively growth factors and oncogenes, such as CEACAM6. CEACAM6 overexpression promotes the migration of NSCLC by enhancing integrin expression [12–14] and remodeling of the extracellular matrix, MMP-2 and MMP-9 [15]. The activation of matrix metalloproteinases are regulated by focal adhesion kinase (FAK) signaling, which induce integrin signaling pathway [14]. FAK is upregulated in several tumors, including breast, thyroid, ovarian, colon, head and neck [16,17]. It has been reported that the binding of upstream molecule, integrin, to its ligand influences the activation of FAK. Despite integrin $\beta 1$ and $\beta 3$ transmit signal which facilitate binding with ECM, activates downstream signaling [18]. Suppression of FAK phosphorylation may halt the cell-ECM binding and inhibit the invasive ability of tumor cells. However the activated Tyr397 residue of FAK regulate complex formation with FAK/Src-homology domain 2 of PP60Src. Understanding the regulation of Fak/Src and FAK dependent signaling pathways may provide us to identify the molecular targets to inhibit the migration and metastasis in NSCLC.

Anti-CEACAM6 antibody had been used as a targeting ligand for pancreatic cancer treatment [9]. However, advanced modification with higher binding affinity and specificity of anti-CEACAM6 sdAb be worth for further clinical application. In this study, we established 2A3 sdAb-hFc (2Ab) and tetravalent 2A3 × 2A3 sdAb-hFc (4Ab) antibodies through fusion with Fc region of IgG antibody and (G4S)3 linker between two sdAbs (Fig. 1(A)). The 2Ab and 4Ab binding to CEACAM6 increases anti-tumor activity which leads to downregulation pFAK, pSRC, pAKT, pErk1/2, MMP-2, MMP-9, N-cadherin, vimentin, and increasing E-cadherin expression levels. We established therapeutically modified anti-CEACAM6 antibodies against lung adenocarcinoma and we explored the mechanism of tumor growth inhibition. We analyzed various in vitro and in vivo parameters of 2Ab and 4Ab antibodies including proliferation, migration, and invasion. Whereas, determined the targeting and therapeutic effects of 2Ab or 4Ab by growth inhibition assay, western bot and confocal microscopy. This study provides insight into mechanism of NSCLC.

Materials and methods

Preparation of recombinant anti-CEACAM6 antibodies

The codon optimization sequence of anti-CEACAM6 sdAb clone 2A3 was synthesized and cloned into pET expression vectors (GenScript). pET expression vectors harboring anti-CEACAM6 sdAb clone 2A3 sequence was used for sdAb production (Takara Bio USA). Recombinant anti-CEACAM6 sdAb was purified by a TALON his-tag purification resin (Takara Bio USA). Fc fusion protein expression vector harboring anti-CEACAM6 sdAb clone 2A3 sequence or 2A3 × 2A3 sequence was used for multivalent 2Ab and 4Ab production (*Invivo*Gen). Chinese hamster ovary (CHO) cells were transfected with sequence confirmed expression vectors via using Nucleofector Technology (Lonza) and stable clones expressing recombinant antibodies were obtained after selectable marker selection. CHO cells were cultivated with 1 L shaking flask using the CDM4PerMab serum-free medium (General Electric). Recombinant 2Ab and 4Ab antibodies were be purified from culture supernatant via MabSelect SuRe antibody purification resin (General Electric).

Cell culture

The Human lung adenocarcinoma cell line A549 (BCRC NO.60074) and human normal lung fibroblast cell line HFL-1 (BCRC NO. 60299) were obtained from the Boiresource Collection and Research Centre (BCRC, Hsinchu, Taiwan). Based on the expression level of CEACAM6, A549 lung cancer cell line (CEACAM6 ^{high}) and HFL-1 cell line (CEACAM6^{low}) were used as experimental group and normal control group respectively. A549 cells were cultured in RPMI medium containing 10% FBS at 37 ˚C, 5% CO_2. The HFL-1 human lung cells were cultured in a DMEM medium containing 10% FBS at 37 ˚C, 5% CO_2.

ELISA assay

ELISA assay was performed by coating Nunc MaxiSorp ELISA plates with 3 μg/mL recombinant CEACAM6 (SinoBiological). Different concentrations of 2Ab or 4Ab were used for binding affinity study. Bound antibodies were detected by incubation with HRP-conjugated goat anti-human IgG-Fc fragment cross-adsorbed antibody (Bethyl Laboratories). All data were calculated from average of the triplicate values and analyzed by GraphPad Prism software.

Purification and characterization of recombinant anti-CEACAM6 antibodies

First, monomeric anti-CEACAM6 sdAb was expressed and purified from *E.coli* via a Ni-NTA column. The molecular weight of monomeric sdAb is about 17 kDa (Fig. 1(B). Then we designed and generated two novel multivalent sdAb antibodies based on IgG1 Fc fusion strategy so that 2Ab harboring two sdAbs and 4Ab containing four sdAbs (Fig. 1(A)). All multivalent sdAb antibodies were expressed on CHO cells and purified through protein A chromatography and confirmed their molecular weights by SDS-PAGE shown in Figs. 1(C) and (D). The molecular weights of bivalent (2Ab) and tetravalent sdAb (4Ab) were about 85 kDa and about 115 kDa, respectively Figs.1(C) and (D). Subsequently, the binding affinities of 2Ab or 4Ab were all below 100 pM by ELISA assay Fig. 1(E), highlighting the distinguished interaction between multivalent sdAb and recombinant CEACAM6. Indeed, tetravalent sdAb (4Ab) improve binding affinity to more than 3.8 fold as compared with that of bivalent sdAb (2Ab) Fig.1 (E).

Cell viability assay

Cells were plated in 96 wells at a density of 2 × 104 cells/well overnight and were treated with CEACAM6 antibodies at different concentration. Detailed description of MTT assay was included in the supplementary methods

Western blot analysis

Cell lysate were extracted with RIPA buffer containing protease cocktail and EDTA. Protein concentration was quantified by Bradford protein assay. Detailed description of western blot analysis was included in supplementary methods.

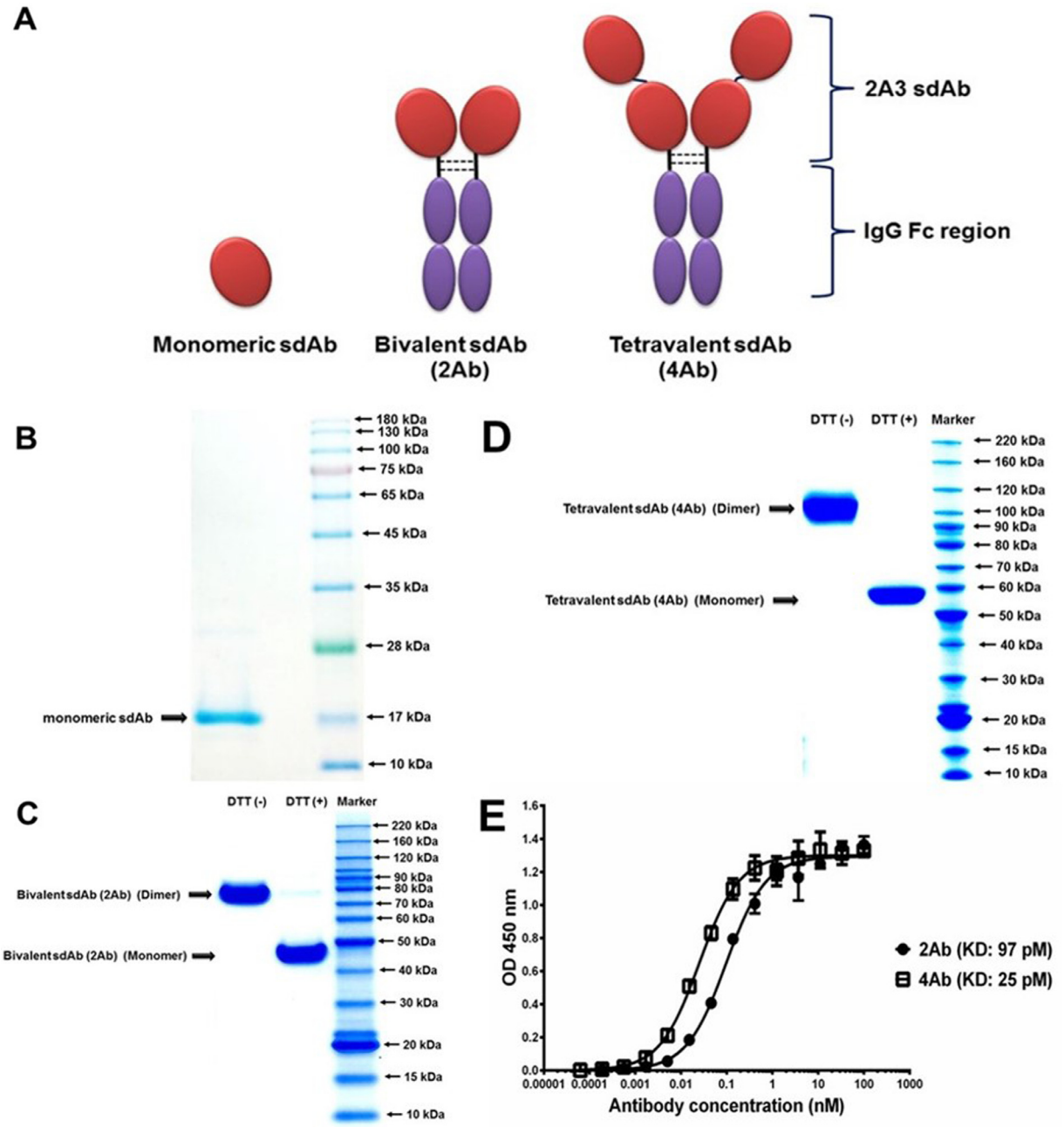

Fig. 1. Preparation and binding affinity study of recombinant anti-CEACAM6 antibodies. Monomeric, bivalent and tetravalent sdAb were electrophoresed on SDS-PAGE under nonreducing (DTT−) and reducing (DTT+) conditions. (A) A schematic diagram of monomeric sdAb, bivalent sdAb (2Ab), and tetravalent sdAb (4Ab). (B) Monomeric sdAb clone 2A3, (C) Bivalent sdAb clone 2A3 (2Ab), (D) Tetravalent sdAb clone 2A3 (4Ab). (E) ELISA assay was performed by coating wells with recombinant CEACAM6 at 3 μg/mL and the bound antibodies were detected via horseradish peroxidase (HRP)-conjugated goat anti-hFc antibody. The binding affinities of 2Ab and 4Ab were all less than 100 pM. All data were calculated from average of the triplicate values and were analyzed through GraphPad Prism software.

Cell migration & invasion assay

Cell invasion ability of A549 cells was studied using Matrigel® invasion assay. Both cell invasion and migration experiments were described in supplementary methods.

Flow cytometry

Endocytosis efficiency of antibodies was analyzed by flow cytometry. Detailed description of flow cytometry was included in supplementary file.

Confocal fluorescence microscope

Cells were seeding on a round cover slide overnight and were treated with PBS or CEACAM6 antibodies. Then the cells were fixed 15 min with 4 percent PFA, followed by PBS wash, and the cells were stained. Added 1 mL/well of 0.1% nonionic surfactant Triton X-100 in PBS for 10 min to perforate the cell membrane, washed with PBS, and added 1 mL/well of 1% FBS in PBS. After 30 min, 0.1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) was added and left it in the dark for 30 min. After dyeing washed with PBS, 2000-fold diluted primary antibody 2Ab or 4Ab (2 mg/mL) was added, cultured at 4 ˚C for 30 min, and washed

with PBS. A 2000-fold dilution of goat polyclonal secondary antibody to Llama IgG - H&L (FITC) was added and incubated at 4 ˚C for 30 min in the dark. Finally, after third wash, coverslip was fixed on the glass slide using mounting medium.

Immunohistochemical analyses

The harvested tumors and organs were fixed with 10% formalin and embedded in paraffin. Sample sections (6 μm) were stained with H&E for morphological observation. For immunohistochemical staining, tumor sections were deparaffinized and rehydrated before staining. The sections were treated with 0.3% H_2O_2 and incubated with blocking buffer (PBS containing 1% BSA and 0.25 Triton X-100) for 1 hr. Sections were incubated overnight with a rabbit anti-Ki67 antibody (1:400) (GeneTex International Corporation, Hsinchu City, Taiwan) at 4 ˚C. After PBS washing, sections were incubated with HRP-conjugated anti-rabbit IgG (1:1000) at room temperature for 1 h. Sections were washed with PBS and stained with diaminobenzidine (DAB) for 5–10 min at room temperature and counterstained with hematoxylin. Samples were dehydrated, and mounted with cover slides. The slides were observed under a microscopy at 10 × magnification.

Body weight monitoring and tumor growth rate by xenograft study

A549 and HFL-1 cell lines were subcutaneous inoculation two flank of 6-week-old female BALB/cAnN.Cg-Foxn1nu/CrlNarl mice were purchased from the national experimental animal center, Taiwan.

When the tumor volume reaching to around 200 mm^3, the conditional treatments of PBS, doxorubicin (10 mg/kg), 2Ab (10 mg/kg), and 4Ab (10 mg/kg) was administrated through tail vein injection for twice per week. Tumor volume and body weight were measured at same time. All the tumor volume representative with the mean ± standard deviation of five times replication. All animal experiments were conducted under a protocol approved by the Animal Care and Use Committee of National Chiao Tung University, Taiwan and the Affidavit of Approval of Animal Use Protocol Taipei Medical University, LAC-2018–0093.

Statistical analysis

All the values as the mean ± SD unless otherwise mentioned. A $p<0.05$ and $p<0.01$ values are considered to be significant and very significant respectively, as acquired by one-way ANOVA using SPSS software version 10.0.

Results

Purification and identification of modified CEACAM6 antibodies

The sdAb, 2Ab, and 4Ab were expressed by Escherichia coli (*E.coli*). Modified antibodies were transfected into CHO cells by NucleofectorTM technology and the stable cells were screened with Hygromycin B. Constructed antibody (2A3 × 2A3 dAb-hFc) is chimeric humanized antibody (Fig. 1).

Expression level of CEACAM6 in HFL-1 and A549 cells

Expression level of CEACAM6 was determined by flow cytometry and western blot. Moreover, A549 showed higher CEACAM6 expression than HFL-1, which is consistent with flow cytometry analysis Fig. 2(B). We observed very low CEACAM6 expression in HFL-1 cells.

Cell viability

To investigate the effect of 2Ab and 4Ab on cancer cell viability, A549 cells were treated with different concentrations for 48 and 72 h incubation. Doxorubicin showed lower cytotoxicity against A549 cells than 2Ab or 4Ab at 48 and 72 h. Nevertheless, the 2Ab had moderate effect on A549 cell with different concentration at different time points as shown in Fig. 2(C) and (D). However, 4Ab had more significantly inhibits cell proliferation at 72 hr incubation as shown in Fig. 2(D).

MMPs contributes to cell migration and invasion by interacting with CEACAM6 expression

The effect of 2Ab and 4Ab treatment (1.5 μM) on cancer cell invasion after 16 hrs was studied using Matrigel® invasion chambers. As shown in Fig. 3(A) and (C) compared to cell alone and doxorubicin, the number of invaded cells were represented by propidium iodide. The number of invaded stained cells were reduced markedly in 4Ab treated A549 cells as shown in Fig. 3(B). These results suggest that 4Ab inhibits the invasion in NSCLC. Meanwhile, scratch wound healing assay was performed to analyze the anti-migratory effect of 2Ab and 4Ab in A549 cells. Cells were treated with PBS, doxorubicin (1 μM), 2Ab, or 4Ab (1.5 μM) for 0, 6, and 24 h. Control A549 cells showed migratory potential by resulting 100% wound healing after 0, 6 and 24 h (Fig. 3(C)). In contrast, 2Ab and 4Ab treatment (1.5 μM) of A549 cells inhibited wound healing at different time points.

CEACAM6 is associated with tumor progression and metastasis. We examined the role of CEACAM6 on migration of lung cancer. Treatment with 2Ab and 4Ab significantly reduced in comparison with doxorubicin and PBS. Further, the expression of EMT markers N-cadherin and vimentin (mesenchymal marker) were significantly decreased in A549 cells treated with 2Ab and 4Ab. In contrast, EMT markers E-cadherin were upregulated in 2Ab and 4Ab as compared with PBS in Fig. 3(D) and (E)).The difference may be explained by the fact that modified anti-CEACAM6 antibody enhances the cell binding affinity and accelerates tumor targeting efficacy.

Alleviation of matrix metalloproteinase-2 (MMP-2) influence tumorigenic and metastatic activity. Several studies have been reported that crucial role of MMP in lung tumorigenesis, especially contributing to the formation of complex microenvironment to malignant state [19]. MMP-2 promotes angiogenesis via vascular endothelial growth factor expression and promotes invasive ability of tumor cells. Moreover, other family members of MMPs including MMP-9 triggered by distinct primary tumors [20]. Overexpression of MMP-2 and MMP-9, a key factor for tumorigenic and metastatic activity [21]. The result from present study demonstrate that exposure of A549 cells to 2Ab and 4Ab led to decreased migration and invasion ability. In order to explore the underlying mechanism of this phenomenon, we investigated the expression of MMP-2 and MMP-9 on protein level as shown in Fig. 3(D) and (E). The results showed that 2Ab and 4Ab exposure decreased the expression of MMP-2 and MMP-9 in A549 lung cancer cells, which described the primary role of MMP's in the inhibitory effect of anti-CEACAM6 on lung cancer metastasis.

Anti-CEACAM6 attenuated Src/Fak mediated CEACAM 6 upregulation

Downregulation of the FAK-Src signaling pathway mediates proliferation, invasion and metastasis in lung cancer cells [22]. To address these possibilities, we investigated the effect of 2Ab and 4Ab treatment on FAK/ Src phosphorylation. Besides, Integrin are the important receptors binding with ECM promotes the phosphorylation of focal adhesion kinase (FAK) [23]. Western blot analysis revealed that the expression level of N-cadherin, and vimentin, were decreased in 2Ab and 4Ab groups than PBS and doxorubicin alone. In A549 cells, 2Ab and 4Ab decreased the auto-phosphorylation (Tyr397) site of FAK domain, which is essential for the activation of FAK signaling. On other hand, which increase binding activity with Src family kinase. The activated FAK/Src kinase upregulates ERK, and AKT via PI3K pathways. However, anti-CEACAM6 antibodies promotes the downregulation of ERK, AKT, Src, FAK, vimentin, cadherin and MMPs (Fig. 4(A) and (B). Thus, these results indicated that 2Ab and 4Ab inhibits FAK-Src activation, which ex-

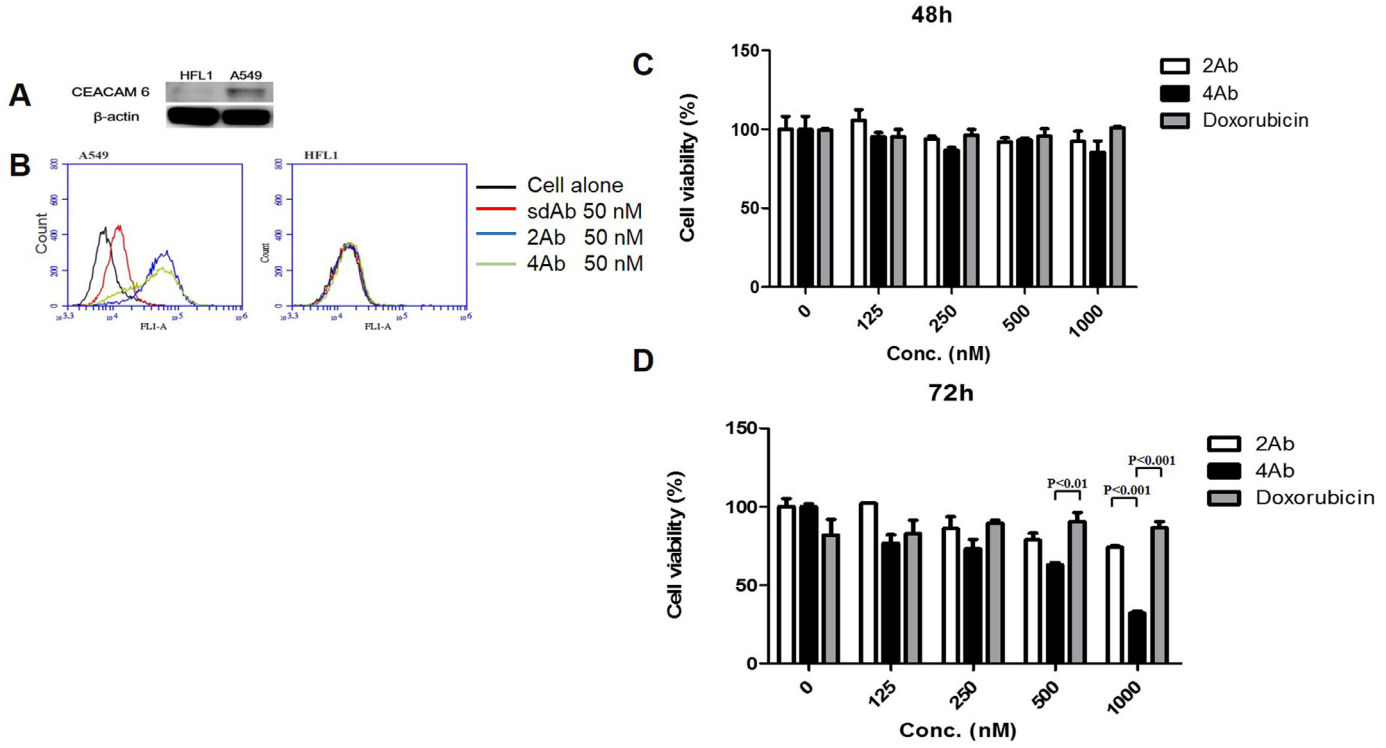

Fig. 2. Expression level of CEACAM6 in HFL-1 and A549 cell lines. (A) CEACAM6 western blot showed A549 cell line expresses higher CEACAM6 compared to HFL-1. (β-actin as an internal control.) (B) Flow cytometry analysis showed 2Ab and 4Ab have higher binding affinity than sdAb in A549 cell line. 2Ab and 4Ab have dose-dependent effect on lung cancer cells. A549 was treated with doxorubicin, 2Ab and 4Ab under the concentration of 0, 125, 250, 500, 1000 nM for (C) 48 and (D) 72 h. Cell viability was measured by MTT assay. The data represent the average ± deviation of three replicates. $P \leq 0.01$ is statistically significant when compared to 2Ab at 72 h.

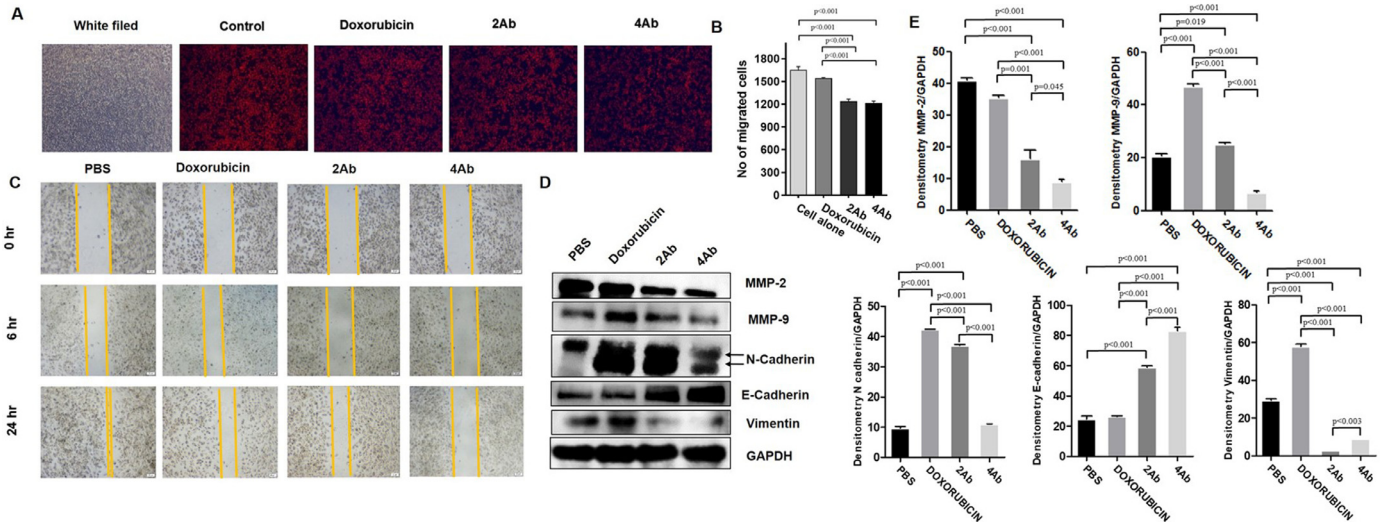

Fig. 3. The effect of 2Ab and 4Ab was studied by Matrigel Invasion assay, 2Ab and 4Ab impair invasiveness of lung cancer cell line (A) A549 cells were placed in the upper chamber of transwells after the cell was grown to 90% confluency and was pretreated with 1.5 μM of 2Ab and 4Ab for 16 h. Migrated cells to the lower surface of filter was stained with propidium iodide and the images were captured by fluorescence microscope. (B) Quantification of migrated cells per field (C) Inhibitory potential of 2Ab (1.5 μM) and 4Ab (1.5 μM) were examined by wound healing at 0, 6, and 24 h. (D) Cell lysates were harvested and EMT markers (MMP2, MMP-9, N-cadherin, E-cadherin, and vimentin) were examined by western blotting. (E) The protein bands were quantified by densitometry analysis.

plored the changes in oncogenic protein levels. A549 cells treated with the 2Ab and 4Ab exhibited a reduced migration in wound healing, transwell and western blot analysis (Fig. 3(A) and (D)). Both 2Ab and 4Ab treatment alleviated EMT markers by increasing the expression of E-cadherin in 2Ab, meanwhile reduced the expression of N-cadherin and vimentin.

Effects of anti-CEACAM6 on cellular uptake efficiency

The quantitative measurement of cellular uptake of 2Ab and 4Ab compared with doxorubicin and untreated using flow cytometry at different time points. The cellular uptake efficiency of 2Ab and 4Ab in A549 cell line showed a prominent right shift compared to the other

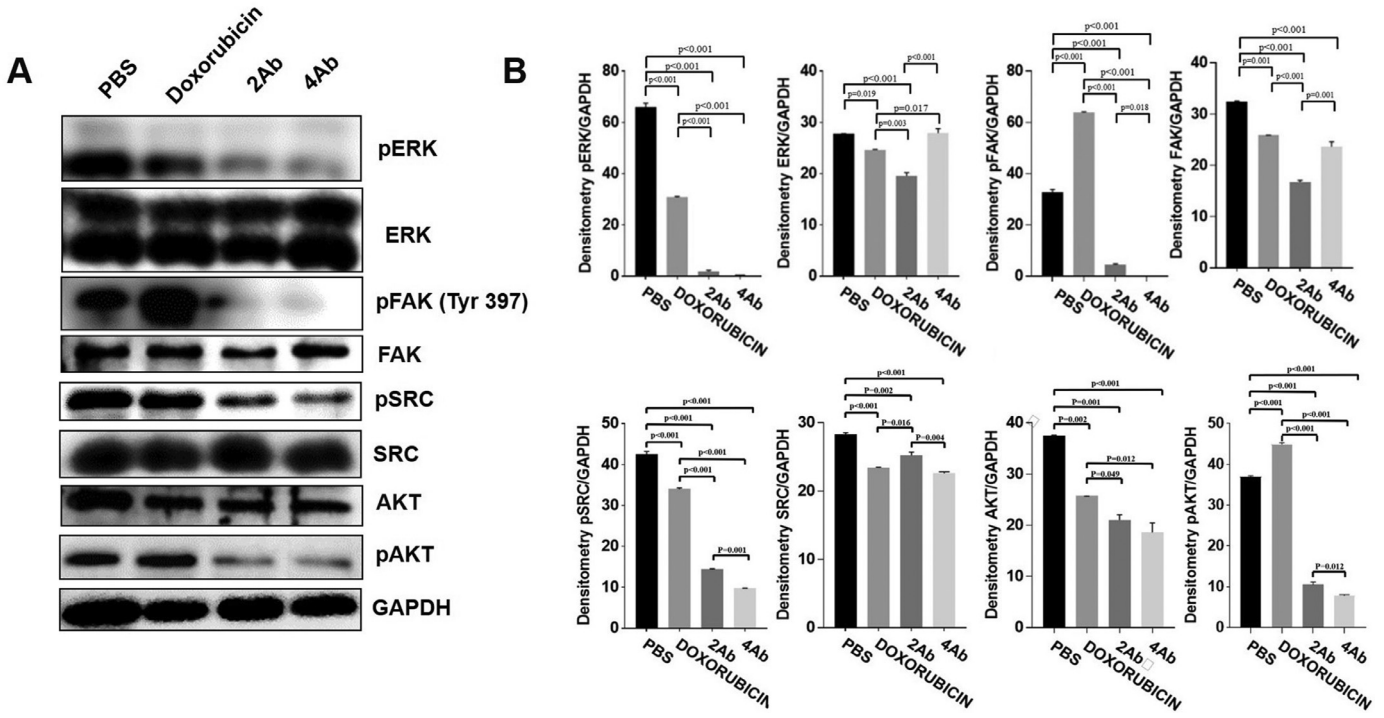

Fig. 4. Expression level of FAK/Src related proteins. (A) A549 cells were treated with PBS, Doxorubicin, 2Ab, and 4Ab. Phosphorylation of ERK, FAK, SRC, and AKT were inhibited by 2Ab and 4Ab. (B) The protein bands were quantified by densitometry analysis.

groups suggesting that the enhanced cellular uptake of 2Ab and 4Ab (Fig. 5). In addition, confocal results also showed enhanced uptake with prolonged incubation, initially there was no uptake at 0 h in 2Ab and 4Ab treated A549 cells. At 4 h of incubation, facilitate the uptake of 2Ab and 4Ab. Thus, 2Ab and 4Ab had a greater targeting efficiency to CEACAM6 expressing A549 cells.

Tumor targeting efficiency of anti-CEACAM6 antibodies

The targeting efficiency of 2Ab and 4Ab (3 μM) with different time points (0, 1, 2, and 4 h) at 37 °C was investigated using confocal microscopy. In A549 tumor cells, no uptake of 2Ab was observed at 0, h despite gradually visible at 1, 2, and 4 h. Moreover, A549 tumor cells incubated with 4Ab (3 μM) showed better targeting and uptake than 2Ab at 1, 2, and 4 h at 37 °C as shown in Fig. 6(A) and (E). Whereas, less prominent expression was appeared in A549 cells treated with 2Ab, seemingly due to higher binding affinity indicates remarkable targeting as shown in Fig. 6(B) and (E).

Therapeutic effects of anti-CEACAM6 antibodies

Mice bearing A549 tumor growth was recorded in PBS treated (control), doxorubicin treated, and 2Ab (10 mg/kg) and 4Ab (10 mg/kg) per week until 24 days. A549 tumor growth did not show significant inhibition by PBS, doxorubicin at dose of (5 mg/kg) as compared to the 2Ab and 4Ab as shown in Fig. 6(C). However, A549 tumor growth inhibition was starting and visible on day 3 after treatment. It indicates that 2Ab and 4Ab was potent enough to inhibit A549 tumor growth and showed complete inhibition of A549 tumor on day 24. However, mice body weight did not change significantly during experimental period, as shown in Fig. 6(D).

Anti-CEACAM6 attenuates tumor cell proliferation

Ki67 immunohistochemistry was performed to explore the proliferation rate of A549 cells treated with either control or 2Ab and 4Ab (1.5 μM). The cell proliferation ratio was quantified by the number of Ki67 positive cells in the tumor region, was similar in control and other groups as shown in Fig. 6(F). Proliferation ratio is higher in doxorubicin whereas we observed fewer ki67 positive cells in A549 cell treated 2Ab. A459 treated with 4Ab groups showed the significantly lower ki67 positive cells and it indicates low proliferation. Moreover, proliferation and migration are consistently lower in 4Ab. Thus, indicates proliferative and migratory effects were associated with CEACAM6 expression.

Discussion

Previous studies have suggested that CEACAM6 sdAb can be used to inhibit pancreatic cancer cell invasion, angiogenesis, and slow down cell proliferation [9]. We explored the targeting and therapeutic potential of multivalent anti-CEACAM6 antibodies in this report. Our data supports that 2Ab and 4Ab inhibiting FAK/Src pathways as shown in Fig.4 (A) and (B). We have shown that CEACAM6 has important functional roles in controlling tumor cell proliferation, migration and invasion in *in vitro* and *in vivo*. Generally, CEACAM6 express lower levels in normal epithelial, endothelial and hematopoietic cells [24]. However, in many epithelial malignancies such as pancreatic, breast, colorectal, gastric, and lung adenocarcinomas with poor clinical outcomes, CEACAM6 expression is upregulated. CEACAM6 overexpression regulates massive cell proliferation in lung adenocarcinoma cells and increased serum CEACAM6 levels are frequently observed in patients [25]. As expected, expression of CEACAM6 associated with enhanced cell growth, migration and invasion in NSCLC. Targeting and therapeutic effects have been shown to improve anti-tumor activity, accompanied by oncogenic receptors downregulation. In addition, expression of CEACAM6 elevated the expression of MMPs. In this case, modified anti-CEACAM6 antibodies could decrease the migratory and invasive activity via FAK/Src signaling pathway.

The process of tumor cell invasion and metastasis require the degradation of extra cellular matrix (ECM) or other proteins by protease. Especially, MMP-2 and MMP-9 efficiently degrade collagen IV, thereby aid metastatic tumor cells to pass through basement membrane [26,27]. Usually, MMPs expression are highly influenced by several factors.

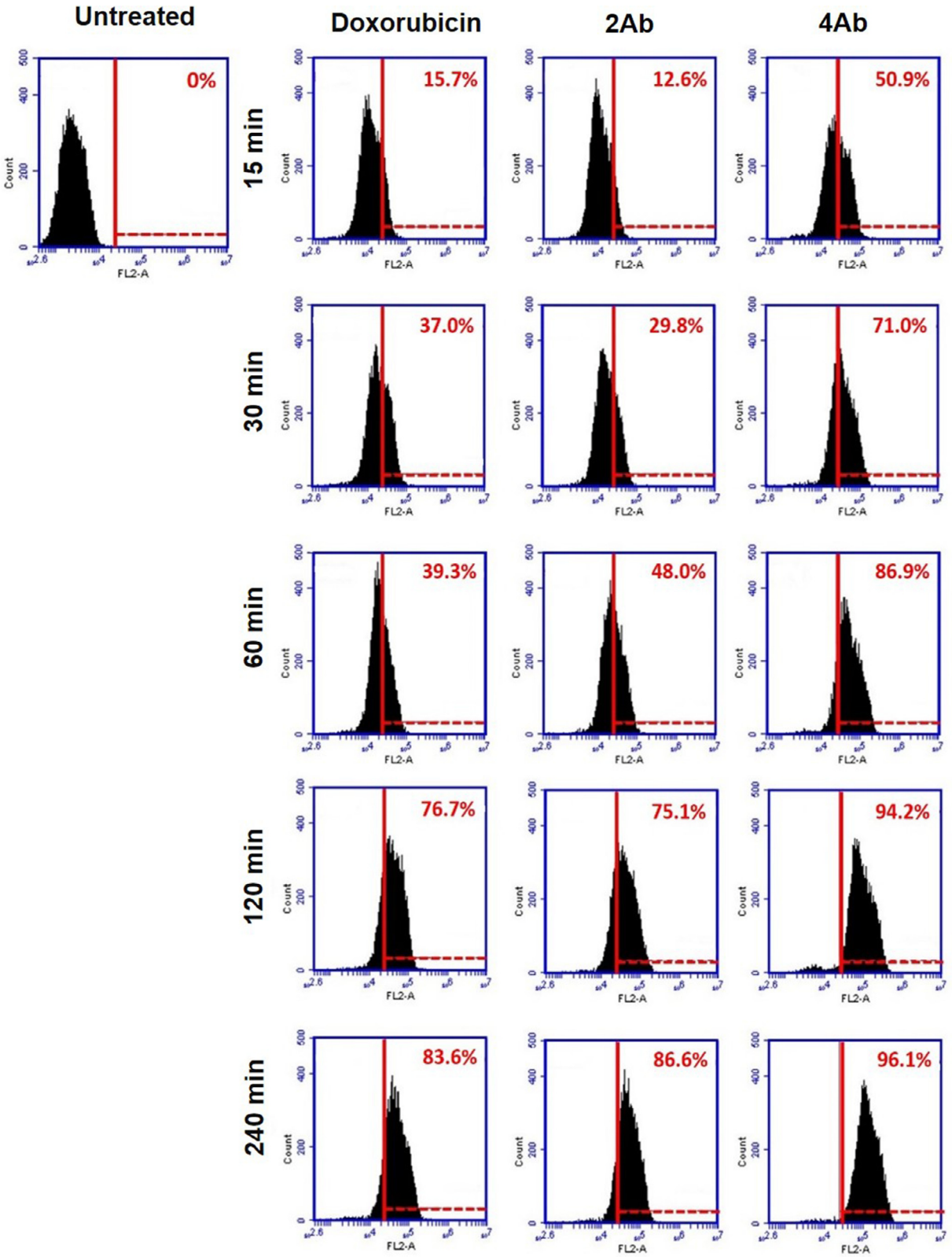

Fig. 5. Endocytosis efficiency of antibodies were analyzed by flow cytometry. A549 tumor cell was incubated with doxorubicin, 2Ab (3 μM), and 4Ab (3 μM) for 15, 30, 60, 120 and 240 mins at 37 ˚C.

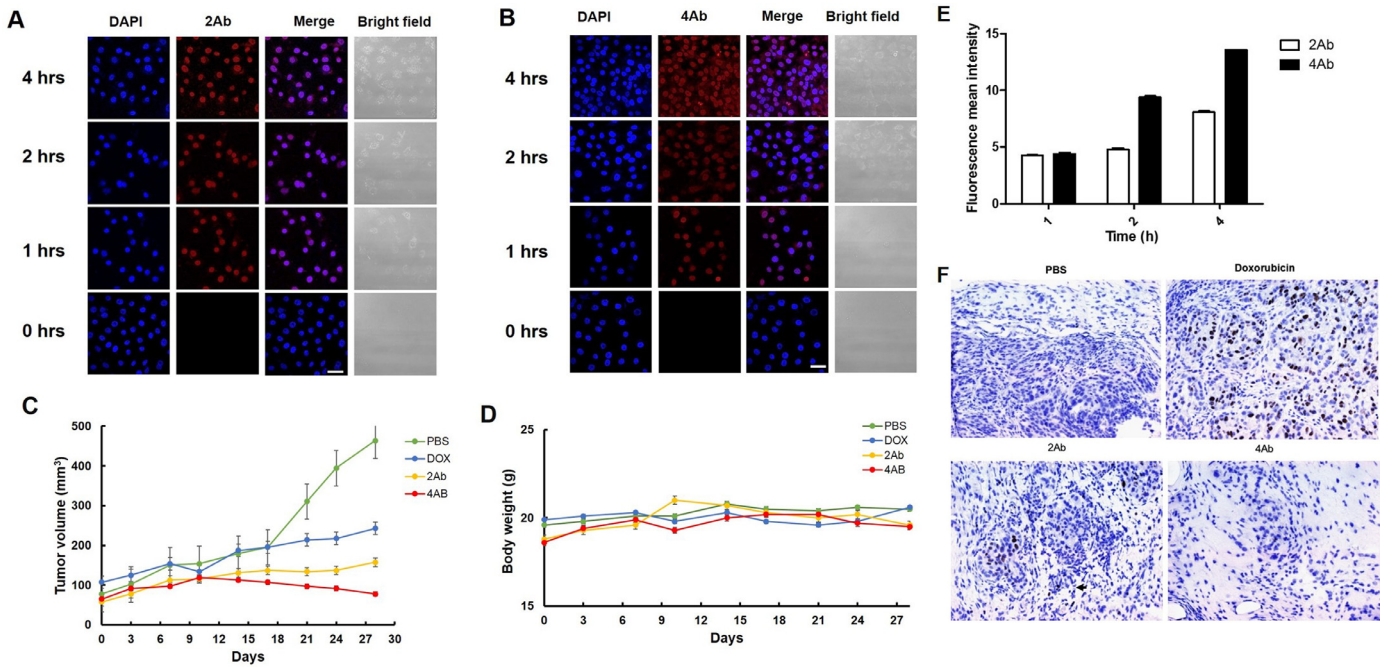

Fig. 6. Endocytosis efficiency of antibodies were analyzed by confocal microscopy. A549 tumor cells were incubated with (A) 2Ab (3 μM) and (B) 4Ab (3 μM) at 37 °C for 0, 1, 2, and 4 h. 2Ab and 4Ab were detected by fluorescence-conjugated secondary antibody (red). DAPI is counter staining (blue). The scale bar represents 45 μm. Anti-tumor effects of CEACAM6 antibody in A549 xenograft. Tumors were weekly treated with PBS, doxorubicin, 2Ab, and 4Ab for 3 weeks. (C) Tumor volume was calculated every 3 days. The statistical values are not shown because the significant results were not observed. (D) Body weight of mice were recorded 2 times a week. (E) Fluorescent intensity quantification result of 2Ab and 4Ab. Data were analyzed by linear regression. Confocal signal=2.5525 × [Ab type] +3.518167 × [Time]+(-0.9930834) $p<0.0001$ for both Ab type and Time variable. (F) Ki67 expression in A549 xenograft was treated with PBS, doxorubicin, 2Ab, and 4Ab. Dark brown represents Ki67 expression. Blue, hematoxylin, represents cell nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

FAK/Integrin signaling is an important pathway to regulate production and expression of MMPs. Through this pathway the activated MMPs can promote cancer metastasis by increasing expression of MMP-2 and MMP-9. EMT is significantly associated with cancer metastasis, induces cancer stemness, prevent apoptosis, senescence and contributes to the development of cancer progression [28]. Activation of EMT through regulation of PI3K/AKT signaling pathways [29]. CEACAM6 function as adhesion molecules, which can form bigger lipid rafts by interacting itself or other CEACAM family, thus activating downstream signaling pathways such as integrin and PI3K/AKT pathways. Therefore, we investigated the effects of 2Ab and 4Ab on AKT, ERK, FAK, and Src phosphorylation on lung cancer cells.

Here, we explored the evidences of 2Ab and 4Ab antibodies on migration and invasion in A549 cell. In addition, this study also demonstrated the anti-CEACAM6 antibodies suppress the proliferation and tumor growth activity by decreasing levels of phosphorylated FAK at Tyr397, which is indispensable for the activation of FAK, mediated signaling pathways. Anti-CEACAM6 antibodies not only showed significant downregulation of FAK/Src but also decrease the phosphorylation of FAK at Tyr397 residue, as shown in Fig. 4(A) and (B). Moreover, lung cancer with enhanced FAK/Src increased metastatic condition. Integrin family receptors play crucial role in cell division, differentiation, and movement. It promotes heterodimers of integrin receptors regulate cell-matrix and cell-cell adhesion, while their cytoplasmic tail associated with cytoskeleton. Moreover, integrin receptors can transduce information in a bidirectional manner and its subunits can able to play role in this process. Especially, Integrin $\beta 1$ and $\beta 3$ promote FAK activation. Collectively these studies provide insight into targeted binding of 2Ab and 4Ab for lung cancer treatment. Additional confirmation of targeting ability and therapeutic effects of these anti-CEACAM6 antibodies will be analyzed in future work.

Conclusion

In conclusion, we claim that the modified multivalent anti-CEACAM6 sdAb (2Ab or 4Ab) could serve as a specific moiety for CEACAM6 targeted therapeutics. Molecular heterogeneity of tumor cells has likely contributed to the lack of effective targeted therapies. We demonstrated that modified anti-CEACAM6 antibodies could suppress cell proliferation, migration and invasion of NSCLC by targeting CEACAM6 with heightened specificity. FAK/CEACAM6 axis provides new insight into the pathogenesis of NSCLC and represents a potential therapeutic target for the treatment of NSCLC.

Author contribution statement

Data curation, Original draft preparation, Formal analysis, and Investigation, were done by Shang-Jung Wu and Arivajiagane Arundhathi. Hsiang-Ching Wang helped in Resources. Editing, Visualization, Supervision, Project administration, and Funding acquisition were done by Chiao-Yun Chen, Tsai-Mu Cheng, Shyng-Shiou F. Yuan, and Yun-Ming Wang.

Declaration of Competing Interest

None declared.

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Supplementary materials

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101057.

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