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ORIGINAL RESEARCH

Elevated expression of podoplanin and its clinicopathological, prognostic, and therapeutic values in squamous non-small cell lung cancer

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Background: Squamous non-small cell lung cancer (SqNSCLC), as a leading cause of cancer-related deaths worldwide, has limited treatment options and poor prognosis. Thus, novel targeted therapies are desperately needed.

Materials and methods: SqNSCLC cases from derivation and validation cohorts were analyzed for podoplanin (PDPN) expression, and its clinicopathological correlation and prognostic prediction. The Human Proteome Map database was used to compare the expression of different lung cancer targets in normal human tissues. Two human lung cancer cell lines, H226 (a SqNSCLC line) and A549 (a non-SqNSCLC line), were examined for PDPN expression. The in vitro cytotoxicity of an anti-PDPN therapy (NZ-1-immunotoxin [NZ-1-IT]) was tested against both lines. The in vivo therapeutic effect of NZ-1-IT was examined in subcutaneous non-small cell lung cancer (NSCLC) xenograft mouse models.

Results: In the derivation cohort, 40% (28/70) were PDPN positive. There was significantly increasing pleural invasion (46.4% vs 9.5%, p=0.001), lymphovascular invasion (25.0% vs 9.5%, p=0.08), and lymph node involvement (53.6% vs 33.3%, p=0.09) in PDPN-positive vs PDPN-negative patients, along with poorer progression-free survival in PDPN-positive patients (p=0.07). The validation cohort with 224 randomly matched cases from The Cancer Genome Atlas data set also displayed significantly shorter overall survival in the group with elevated *PDPN* mRNA (p=0.05). However, PDPN showed limited expression in normal tissues. PDPN was highly and specifically expressed on the surface of H226 cells instead of A549 cells. Subsequently, PDPN-positive H226 cells were around 800 times more sensitive to anti-PDPN NZ-1-IT therapy than PDPN-negative A549 cells in vitro. Furthermore, NZ-1-IT significantly delayed tumorigenesis only in the H226 subcutaneous mouse model (p<0.05).

Conclusion: Our results demonstrate a distinctively elevated expression of PDPN in SqNSCLC, which is significantly associated with worse clinicopathological features and poorer prognosis. With promising preclinical therapeutic results, anti-PDPN targeted therapy can thus be a robust potential strategy for future SqNSCLC treatment.

Keywords: podoplanin, squamous non-small cell lung cancer, pleural invasion, prognosis, molecular targeted therapy, immunotoxins

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for 1.69 million deaths in 2015 according to the World Health Organization.[37] In China, lung cancer is also the most common cancer, with an incidence of 730,000 and deaths of 610,000.[1] Squamous non-small cell lung cancer (SqNSCLC) accounts for one-third of non-small cell lung cancer (NSCLC).[2,3] Given that there are more than 300 million

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smokers in China,[38] along with the fact that SqNSCLC is closely related to smoking in a dose-dependent manner,[2,4,5] a persistent large patient volume who suffer from voluntary and involuntary smoking-induced SqNSCLC is foreseeable. To date, around 80% of SqNSCLC patients are diagnosed as advanced stage, with a median survival of about 12 months, and a 5-year survival rate of 28.9% for regional disease and 4.5% for distant disease, despite therapeutic progress achieved in the past few decades.[2,6,7] Even though many advances have been made in understanding the molecular genetics of NSCLC, there are still limited treatment options available for SqNSCLC, and more novel and targeted therapeutics with effective and unique functions are desperately needed for SqNSCLC treatment.

For novel treatment strategies, crossover expression of some therapeutic targets has disrupted the fragile balance between cancer cell killing and normal tissue protection and has been a major impediment.[8] Throughout the last decade, advances have been made to seek more specific and reliable targets for SqNSCLC.[3,9] Such advances, focusing on identifying unique molecular abnormalities specifically associated with squamous cell lung cancer but minimally expressed or absent in normal tissue, are vital in the development of more effective SqNSCLC therapeutics.[3,9] Podoplanin (PDPN) is a 162-amino acid type I transmembrane sialomucin-like glycoprotein with a serine- and threonine-rich extracellular domain, a single transmembrane domain, and a short cytoplasmic domain containing cyclic adenosine monophosphate (cAMP) and protein kinase C phosphorylation sites. PDPN binds to C-type lectin-like receptor 2 (CLEC2) expressed on platelets and causes platelets to aggregate and promote tumor metastasis.[10] In addition, PDPN can form a complex with ezrin–radixin–moesin (ERM) protein to increase cell motility by activating RhoA signaling.[11] Therefore, PDPN is related to malignant progression involving epithelial–mesenchymal transition (EMT), metastasis, and invasion in several cancers,[11–13] which can be a potential therapeutic target for PDPN-expressing malignant tumors. Our previous study revealed a robust antitumor efficacy in a preclinical brain tumor model using an anti-PDPN NZ-1 immunotoxin (NZ-1-IT),[14] which is developed by fusing a recombinant single-chain variable-region antibody fragment (scFv) from a monoclonal antibody (mAb) with a bacterial toxin. As important derivatives of antibody–drug conjugates (ADCs), scFv-based immunotoxins provide robust tumor penetration, specific tumor binding, and unique tumor killing. These immunotoxins continue to be extensively investigated to treat many cancers, including lung cancer, malignant mesothelioma, pancreatic cancer, gastric cancer, and brain cancer.[15–18]

In the present study, we elucidated the robust clinicopathological, prognostic, and therapeutic value of PDPN in SqNSCLC. We investigated the PDPN expression and the correlation between elevated PDPN expression and pleural invasion, lymphovascular invasion (LVI), lymph node metastases, and patient survival. Subsequently, we demonstrated promising preclinical results of an anti-PDPN immunotoxin therapy in in vitro NSCLC models and in vivo xenograft mouse models.

Materials and methods

Study population

In total, 440 consecutive patients with a pathological diagnosis of single primary NSCLC who underwent curative-intent thoracic surgery in Fudan University Shanghai Cancer Center (FUSCC) from January 2012 to December 2012 were identified, including 101 patients with SqNSCLC. Among them, tissue samples from 70 SqNSCLC patients with complete pivotal clinical information were assessed, and these patients comprised the derivation cohort (Figure 1). Patients were >20 years old and newly diagnosed with the American Joint Committee on Cancer (AJCC), 7th edition, stage I–IV SqNSCLC confirmed by surgical pathology. The research was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All clinical demographic characteristics and specimens were collected from patients who had provided written informed consent for FUSCC institutional review board (IRB)-approved clinical and laboratory research protocols. Patient follow-up information was obtained from an electronic medical record (EMR) system in FUSCC.

The validation cohort consisted of 504 SqNSCLC patients with known clinical information and genomic data recorded in The Cancer Genome Atlas (TCGA) data sets. *PDPN* mRNA expression was analyzed to identify patients with high expression of *PDPN* (above the 75th percentile) and low expression of *PDPN* (below the 25th percentile). Randomized matching of the high-expression and low-expression groups was carried out with a 1:1 ratio in terms of AJCC pathological staging. Gene expression data of these two groups were analyzed with cBioPortal tools;[39] users are free to use any of the figures from the portal in their publications or presentations).[19,20] TCGA is a public data set that contains deidentified patient-related clinical and genomic information; thus, no IRB or Health Insurance Portability and Accountability Act approval was required for the validation cohort.

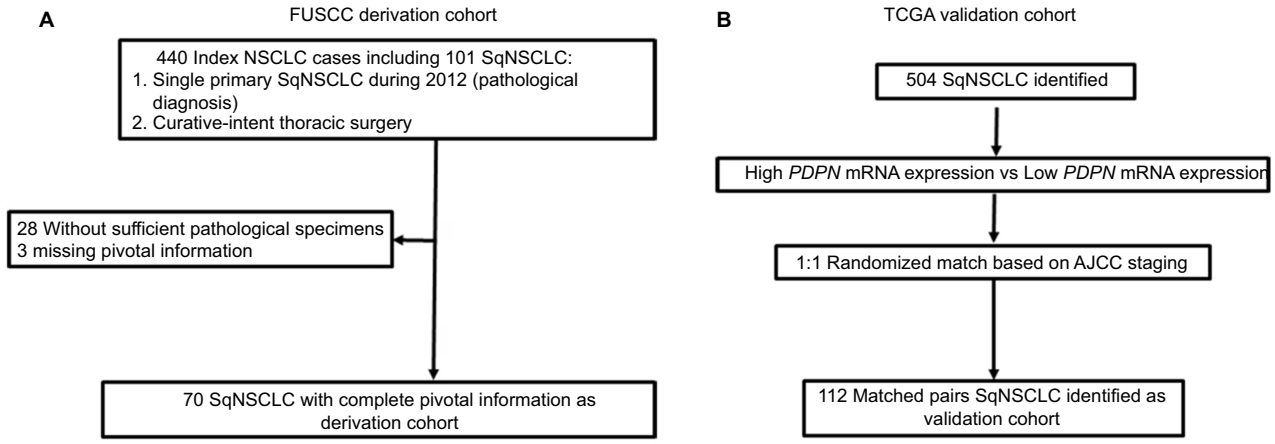

Figure 1 Study profile to show how samples from (**A**) FUSCC and (**B**) TCGA were analyzed.
Abbreviations: FUSCC, Fudan University Shanghai Cancer Center; TCGA, The Cancer Genome Atlas; NSCLC, non-small cell lung cancer; SqNSCLC, squamous non-small cell lung cancer; *PDPN*, podoplanin; AJCC, American Joint Committee on Cancer.

Normal tissue protein expression data set

Protein expression data in normal tissues were extracted from the Human Proteome Map (HPM) portal.[21,40] As part of the HPM project, 30 clinically defined normal tissues were profiled in total, of which 17 were adult human tissues and 6 were primary hematopoietic cells. The project detected proteins corresponding to over 17,000 human genes covering >84% of the annotated protein-coding regions of the human genome.

Tissue sample

Surgical tissue specimens from those 70 patients with SqNSCLC who underwent surgical treatment at FUSCC in 2012 were retrospectively collected following IRB guidelines. Representative tissue sections were obtained from the surgical pathology archive. Paraffin-embedded blocks and corresponding pathological diagnoses were confirmed by the EMR at FUSCC.

Immunohistochemistry

In brief, the formalin-fixed paraffin-embedded sections were deparaffinized and then heated in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activities were inactivated in 3% hydrogen peroxide (H_2O_2), and the sections were blocked with 3% normal goat serum in PBS (pH 7.4). Sections were incubated with an anti-PDPN antibody (Sigma-Aldrich, St. Louis, MO, USA) and then a secondary goat anti-rat antibody (Sigma-Aldrich), followed by treatment with substrate/chromogen (DAKO, Santa Clara, CA, USA). Slides were counterstained with hematoxylin. All slides with a confirmed pathological diagnosis from the EMR in FUSCC were independently re-reviewed by a senior surgical pathologist from a different institution (Q. Zhang).

Cell lines

The A549 cell line was bought from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, which was cultured in F12K medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). The H226 cell line was a generous gift from one of our collaborators (D.D. Bigner), which was cultured in RPMI-1640 medium (Thermo Fisher Scientific) modified to contain 2 mM L-glutamine, 10 mM HEPES (Thermo Fisher Scientific), 1 mM sodium pyruvate (Thermo Fisher Scientific), 4500 mg/L glucose (Sigma-Aldrich, St. Louis, MO, USA), 1500 mg/L sodium bicarbonate, and 10% FBS. Both cell lines were maintained and passed according to the American Type Culture Collection (ATCC) recommendations.[41] The use of the H226 cell line was approved by FUSCC-IRB.

Flow cytometry analysis

Flow cytometry analysis was carried out with AF488-labeled (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) NZ-1 mAb (NZ-1-AF488), while rat immunoglobulin G2a (IgG2a)-AF488 isotype control (Thermo Fisher Scientific) was used as the negative control. The fluorescence-labeled antibodies stained 10^6 cells at a concentration recommended by the manufacturer. Stained cells were analyzed on a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo V10 software (FlowJo, Ashland, OR, USA).

Western blot analysis

Protein samples were extracted from A549 and H226 cells using the Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) and protease inhibitor cocktail (Roche, Basel, Switzerland). After separation and transfer onto the membrane, protein was probed with antibodies against PDPN (1:1000; Sigma-Aldrich) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000; Santa Cruz Biotechnology, Dallas, TX, USA). Proteins of interest were detected with horseradish peroxidase-conjugated goat anti-rat IgG antibody (1:5000; Sigma-Aldrich) and visualized with the Pierce ECL Western blotting substrate (Thermo Fisher Scientific), according to the manufacturer's protocols.

In vitro cytotoxicity assay

Cells were seeded in 96-well plates at a density of 10^4 cells per well in 100 μL of complete medium overnight before the assay. NZ-1-IT was serially diluted to achieve a final concentration of 0.001–1000 ng/mL in PBS (Thermo Fisher Scientific) containing 0.2% human serum albumin (0.2% HSA-PBS; Shanghai RAAS Blood Products Co., Shanghai, China). Plates were incubated for 48 h at 37°C and 10 μL WST-1 reagent (Roche) was then added for 0.5–4 h at 37°C. The absorbance of each well was measured against a blank background control at 420–480 nm wavelength. The reference wavelength was 650 nm. The cytotoxic activity of NZ-1-IT was defined by the IC_{50}, which was the immunotoxin concentration that inhibited cell proliferation by 50% when compared to that of untreated cells. The IC_{50} was calculated using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). All the assays were performed twice in triplicate for each cell line.

In vivo subcutaneous xenograft NSCLC model

Animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University. Female athymic nude mice (7–10 weeks old) were injected subcutaneously in the right flank with 3×10^6 A549 or H226 cells suspended in 100 μL of PBS. When the implanted tumors reached around 100 mm^3 on day 35 after the implantation, five mice per arm were randomized for treatment. The test mice were treated by intratumoral injection every other day with a total of three doses (300 μg/kg per dose) of NZ-1-IT diluted in 0.2% HSA-PBS, or 0.2% HSA-PBS as the vehicle control. Tumor growth was monitored in the following 30 days, and the tumor volumes were calculated in cubic millimeters using the formula: ([Length] × [Width^2])/2. Tumor growth plots were drawn using GraphPad Prism 6.

Statistical methods

The pleural invasion, LVI, and lymph node status between PDPN-positive and PDPN-negative cases were compared with the chi-squared test and Fisher's exact test. Overall survival (OS) and progression-free survival (PFS) were defined, respectively, as the time from initial diagnosis to death from any cause, and to local, regional, or distant progression or death from any cause. The distributions of OS and PFS were estimated by the Kaplan–Meier method[22] and compared to the log-rank test.[23] HRs were estimated according to the Cox proportional hazard method.[24] The statistical analyses for clinical data were conducted using PASW Statistics version 18.0 (SPSS, Chicago, IL, USA). The nonparametric Wilcoxon each-pair multiple comparison method was used to analyze the data from the xenograft lung cancer mouse model using JMP Pro 13.0 (SAS Institute, Cary, NC, USA). Difference was considered significant at $p<0.05$.

Results

Elevated PDPN expression had a significant impact on patient clinicopathological features and prognosis in SqNSCLC

The baseline characteristics of the derivation and validation cohorts are listed in Tables 1 and 2. Among the derivation

Table 1 Clinicopathological parameters of SqNSCLC patients in the derivation cohort from FUSCC

Characteristic	PDPN(+) group (N=28)	PDPN(−) group (N=42)	*p*
Gender			0.56
Male	26 (92.9)	41 (97.6)	
Female	2 (7.1)	1 (2.4)	
Age (years)	61.6±1.4	61.8±1.3	0.91
Tumor largest dimension (cm)	4.8±0.7	4.3±0.6	0.61
Lymph node status			0.05
N0	13 (46.4)	28 (66.7)	
N1	7 (25.0)	11 (26.2)	
N2	8 (28.6)	3 (7.1)	
AJCC stage			0.30
I	8 (28.6)	18 (42.9)	
II	9 (32.1)	16 (38.1)	
III	10 (35.7)	7 (16.7)	
IV	1 (3.6)	1 (2.4)	

Note: Data are shown as n (%) or mean±SE.

Abbreviations: SqNSCLC, squamous non-small cell lung cancer; FUSCC, Fudan University Shanghai Cancer Center; PDPN, podoplanin; AJCC, American Joint Committee on Cancer.

Table 2 Clinicopathological parameters of SqNSCLC patients in the validation cohort from TCGA

Characteristic	*PDPN* mRNA high-level group	*PDPN* mRNA low-level group	*p*
	(N=112)	(N=112)	
Gender			0.55
Male	84 (75.0)	80 (71.4)	
Female	28 (25.0)	32 (28.6)	
Age (years)	66.1±8.6	68.9±9.3	0.02
Ethnicity			0.20
White	73 (65.2)	90 (80.4)	
Black or African American	9 (8.0)	5 (4.5)	
Asian	3 (2.7)	1 (0.9)	
Unknown	27 (24.1)	16 (14.3)	
AJCC stage			1.00
I	62 (55.4)	62 (55.4)	
II	33 (29.5)	33 (29.5)	
III/IV	17 (15.2)	17 (15.2)	
Lymph node status			0.37
N0	79 (70.5)	73 (65.2)	
N1	27 (24.1)	26 (23.2)	
N2	5 (4.5)	12 (10.7)	
N3	1 (0.9)	1 (0.9)	
Nx	–	–	
Smoking			0.72
Lifetime nonsmokers	3 (2.7)	5 (4.5)	
Former smokers	77 (68.8)	76 (67.9)	
Current smokers	30 (26.8)	27 (24.1)	
Unknown	2 (1.8)	4 (3.6)	

Note: Data are shown as n (%) or mean±SE.
Abbreviations: SqNSCLC, squamous non-small cell lung cancer; TCGA, The Cancer Genome Atlas; PDPN, podoplanin; AJCC, American Joint Committee on Cancer.

cohort, which consisted of consecutive patients with a diagnosis of SqNSCLC treated at FUSCC, a total of 70 patients who met the criteria were analyzed (Table 1 and Figure 1). To confirm the impact of PDPN on patient prognosis observed in the derivation group, a case-matched validation cohort was then identified with a total of 112 matched pairs of SqNSCLC (Table 2 and Figure 1).

For the derivation cohort of 70 patients, PDPN immunohistochemistry showed that 40% of the patients (28 cases) were PDPN positive (Figure 2). Cases with elevated PDPN expression had significantly increased pleural invasion (46.4% vs 9.5%, p=0.001), more LVI (25.0% vs 9.5%, p=0.08), and more lymph node involvement (53.6% vs 33.3%, p=0.09) (Figure 3). With a median follow-up of 53.4 months (95% CI 38.0–68.9 months), marginally significantly shorter PFS was observed in the PDPN-positive group (p=0.07, HR=2.15, 95% CI 0.93–6.09) (Figure 4a). The median PFS for the PDPN-positive group was 66.3 months (95% CI 39.6–92.9 months), while the median PFS for the PDPN-negative group was not reached. The estimated 5-year PFS rates were 54.7% and 72.3% for the PDPN-positive and PDPN-negative groups, respectively.

As in the case-matched validation cohort from TCGA data set, the median follow-up time was 34.6 months (95% CI 28.9–40.2 months), with a total of 90 deaths. In these patients, OS was significantly different between *PDPN* mRNA dichotomized groups, with shorter OS observed in the high *PDPN* mRNA group (p=0.05, HR=1.504, 95% CI 1.003–2.301) (Figure 4b). The median OS was 39.1 months (95% CI 12.9–65.2 months) for the high *PDPN* mRNA group and 65.2 months (95% CI 47.5–82.9 months) for the low *PDPN* mRNA group. The estimated 5-year OS rates were 45.1% and 54.3% for the high *PDPN* mRNA and low *PDPN* mRNA groups, respectively. These clinical data from FUSCC and TCGA clearly indicate that SqNSCLC patients with an elevated expression of PDPN have worse clinicopathological features and poorer prognosis.

PDPN showed only limited expression in normal human tissues

An important advance in cancer treatment is the development of targeted therapies that attack a target expressed by tumor cells instead of normal tissues to avoid an off-target effect.[25] Hence, we compared the protein expression of PDPN with other established targets[7] that have been widely investigated to treat NSCLC using the HPM portal. The results showed that PDPN had no expression in several major adult organs, including cortex, spinal cord, heart, lung, kidney, gastrointestinal tract, and major immune cells, although PDPN had moderate to high expression in fetal brain and placenta (data not shown). Only mild PDPN expression was found in the adult liver, ovaries, testis, gallbladder, and urinary bladder, especially compared to the fact that popular established tumor targets, including EGFR, FGFR1, K-Ras, MET, and PIK3CA, had higher and broader expression in normal tissues (Figure 5). These results indicated that PDPN had high specificity as a potential target for SqNSCLC.

Anti-PDPN NZ-1-IT showed specific and significant cytotoxicity in the human SqNSCLC cell line with elevated PDPN expression

According to the NCI-60 cell line study,[26] the H226 cell line (SqNSCLC cell line) showed a much higher expression (z-score 3.19) of *PDPN* mRNA than that of the negative

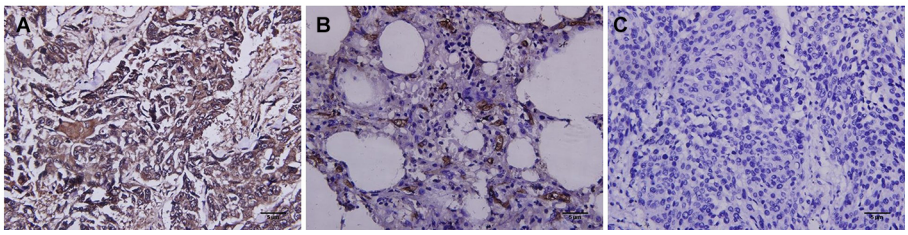

Figure 2 Immunohistochemical detection of PDPN in SqNSCLC patient samples: **(A)** PDPN-positive SqNSCLC section; **(B)** section displaying PDPN-negative staining in adjacent normal lung tissue and some positive staining in endothelial cells of lymphatic vessels; **(C)** PDPN-negative SqNSCLC section (×400, bar=5 μm).
Abbreviations: PDPN, podoplanin; SqNSCLC, squamous non-small cell lung cancer.

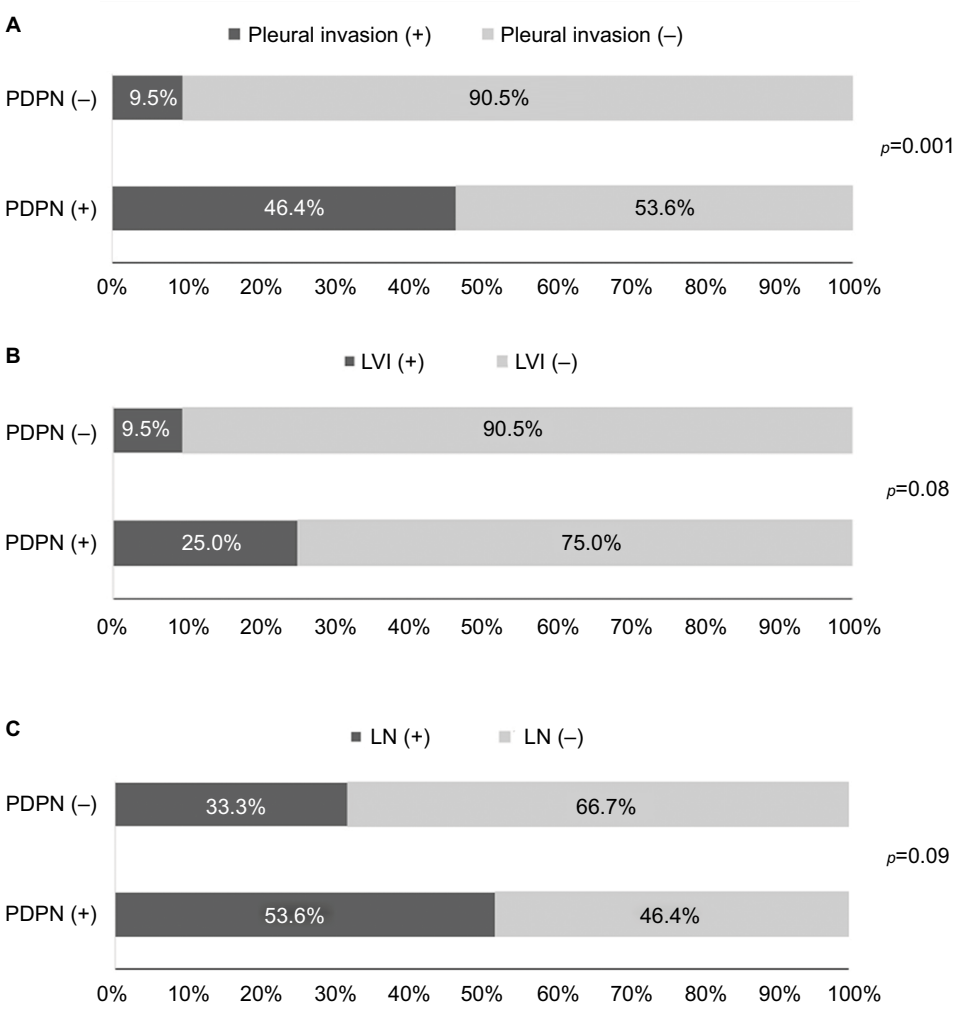

Figure 3 Pleural invasion, LVI, and LN involvement in SqNSCLC patients: **(A)** 9.5% and 46.4% of PDPN-negative and PDPN-positive SqNSCLC patients showed pleural invasion, respectively (p=0.001); **(B)** 9.5% and 25% of PDPN-negative and PDPN-positive SqNSCLC patients showed LVI, respectively (p=0.08); **(C)** 33.3% and 53.6% of PDPN-negative and PDPN-positive SqNSCLC patients showed LN involvement, respectively (p=0.09).
Abbreviations: LVI, lymphovascular invasion; LN, lymph node; SqNSCLC, squamous non-small cell lung cancer; PDPN, podoplanin.

control, A549 cell line (z-score –0.45) (Figure 6a). This was confirmed by total PDPN protein expression using Western blot analysis. PDPN had a strong enrichment in the H226 cell line, while it was not detected in A549 cell line (Figure 6b). Surface PDPN expression was also much higher in the H226 cell line than in the A549 cell line in flow cytometry analysis (Figure 6c and 6d). These experiments confirmed that the human SqNSCLC H226 cell line was a good model to investigate the efficacy of anti-PDPN therapy, whereas the A549 cell line could be used as the negative control cell line.

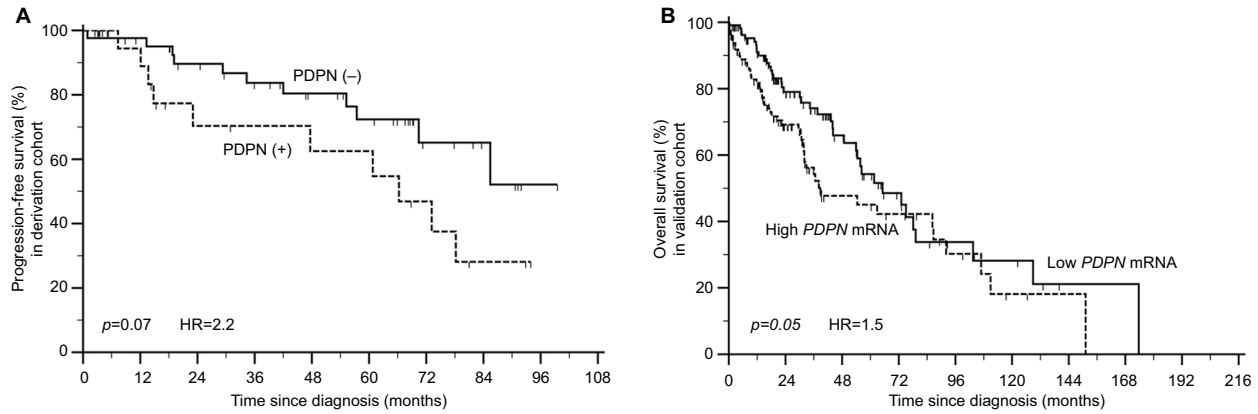

Figure 4 Association between PDPN expression and prognosis in SqNSCLC patients: **(A)** association between PDPN protein expression and progression-free survival in SqNSCLC patients in the derivation cohort (p=0.07); **(B)** association between *PDPN* mRNA expression and overall survival in case-matched SqNSCLC patients in the validation cohort (p=0.05, log-rank test, HR=1.504, 95% CI 1.003–2.301).
Abbreviations: PDPN, podoplanin; SqNSCLC, squamous non-small cell lung cancer.

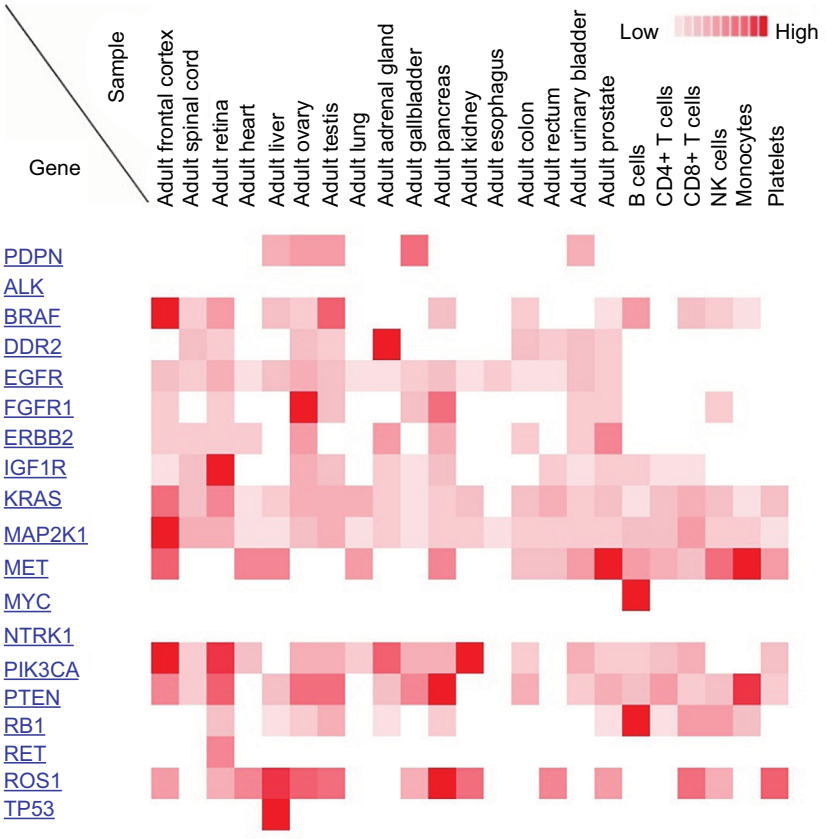

Figure 5 Expression of PDPN and other popular lung cancer targets in normal human tissues. Protein expression is depicted by a heat map (red indicates higher expression) in the Human Proteome Map portal.
Abbreviations: PDPN, podoplanin; ALK, anaplastic lymphoma kinase; BRAF, v-raf murine sarcoma viral oncogene homolog B1; DDR2, discoidin domain receptor 2; EGFR, epidermal growth factor receptor; FGFR1, fibroblast growth factor receptor 1; ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; IGF1R, insulin-like growth factor 1 receptor; K-Ras, Kirsten rat sarcoma viral oncogene homolog; MAP2K1, dual-specificity mitogen-activated protein kinase kinase 1; MET, tyrosine-protein kinase Met; MYC, myelocytomatosis oncogene cellular homolog; NTRK1, neurotrophic tyrosine kinase receptor type 1; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RB1, retinoblastoma protein; RET, rearranged during transfection proto-oncogene; ROS1, proto-oncogene tyrosine-protein kinase ROS; TP53, tumor protein p53.

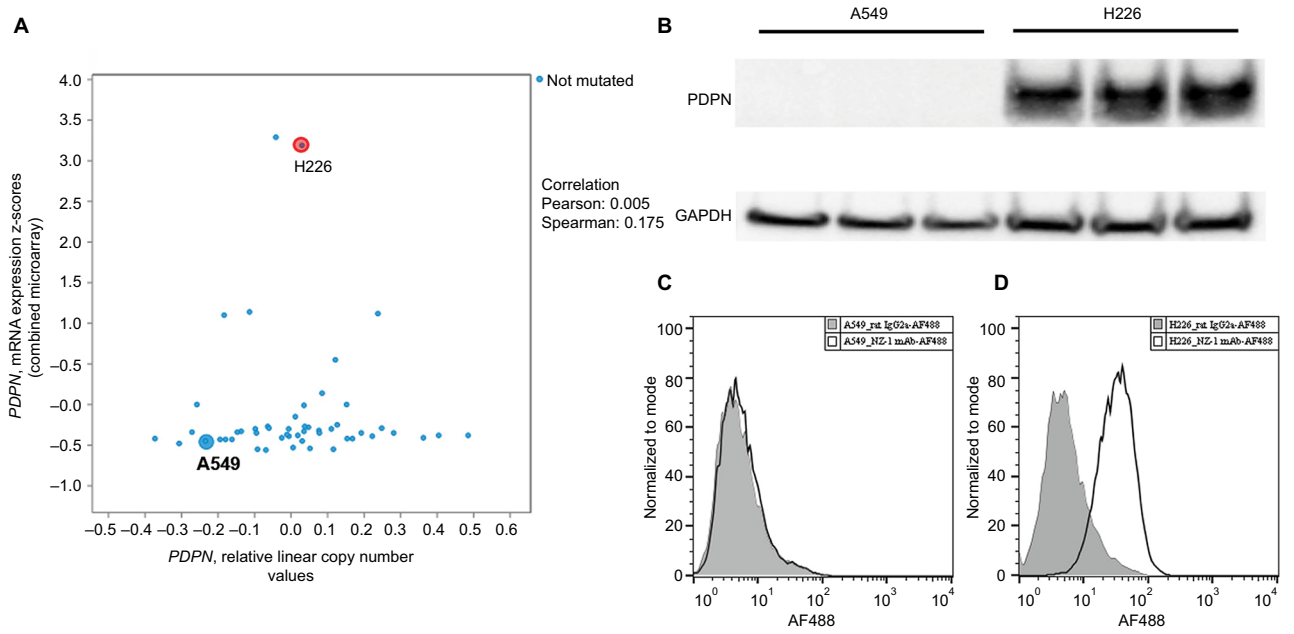

Figure 6 PDPN expression in A549 and H226 human NSCLC cell lines: **(A)** *PDPN* mRNA expression in A549 and H226 cell lines from the NCI-60 cell lines study; **(B)** Western blot analyses of PDPN expression in A549 and H226 cell lines. GAPDH was used as an internal reference. **(C, D)** Flow cytometry analyses of A549 and H226 cells to determine PDPN expression on their surface. Cell lines were stained with anti-PDPN-AF488 antibody (open black peaks) or an isotype control antibody (filled gray peaks).
Abbreviations: PDPN, podoplanin; NSCLC, non-small cell lung cancer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Based on this model, we then examined the effect of NZ-1-IT on A549 and H226 human NSCLC cell lines. The cytotoxicity of NZ-1-IT was determined by the WST-1 assay. We found that NZ-1-IT was hardly able to kill PDPN-negative A549 cells (IC_{50} >1000 ng/mL) (Figure 7a), whereas PDPN-positive H226 cells were quite sensitive to NZ-1-IT treatment, with IC_{50} as low as 1.205 ng/mL (Figure 7b). These in vitro cytotoxicity results suggested that PDPN could be a robust target for antitumor therapy and NZ-1-IT specifically targets PDPN-positive SqNSCLC cells.

Anti-PDPN NZ-1-IT significantly delayed tumor growth in SqNSCLC xenografts with elevated expression of PDPN

Considering the promising in vitro results, the efficacy of anti-PDPN therapy was further investigated in nude mice bearing A549 or H226 subcutaneous xenograft tumors. Subcutaneous tumors were treated by NZ-1-IT or vehicle control. Similar to the in vitro result, anti-PDPN NZ-1-IT therapy failed to show any antitumor benefit in PDPN-negative, A549-bearing mice (Figure 7c). However, the tumor growth was significantly delayed in the NZ-1-IT-treated group in PDPN-positive H226-bearing mice ($p<0.05$) (Figure 7d). No toxicity-related adverse effects were observed. These in vivo mouse xenograft NSCLC models further confirmed that anti-PDPN therapy could be a promising targeted therapy to treat SqNSCLC tumors.

Discussion

This study revealed for the first time that elevated tumor PDPN expression on both the mRNA level and the protein level has a significant impact on clinicopathological features (pleural invasion, LVI, and lymph node involvement) along with patient survival in SqNSCLC by analyzing two separate patient cohorts. We also found that PDPN distinctly had no or mild expression in normal tissue, compared to other commonly used therapeutic targets. We further demonstrated that targeting PDPN significantly impeded tumor cell survival in vitro using an anti-PDPN immunotoxin, NZ-1-IT. Moreover, in vivo mouse models confirmed that anti-PDPN therapy significantly delayed the growth of elevated PDPN-expressing SqNSCLC xenografts, with no adverse effects observed. Taken together, these data have provided strong evidence to support that PDPN is a robust target with high potential against SqNSCLC and suggest that anti-PDPN therapy could be a promising method for SqNSCLC treatment.

PDPN has been reported to induce invasion and migration of cancer cells via the downregulation of small Rho family GTPases, providing an alternative tumor cell invasion

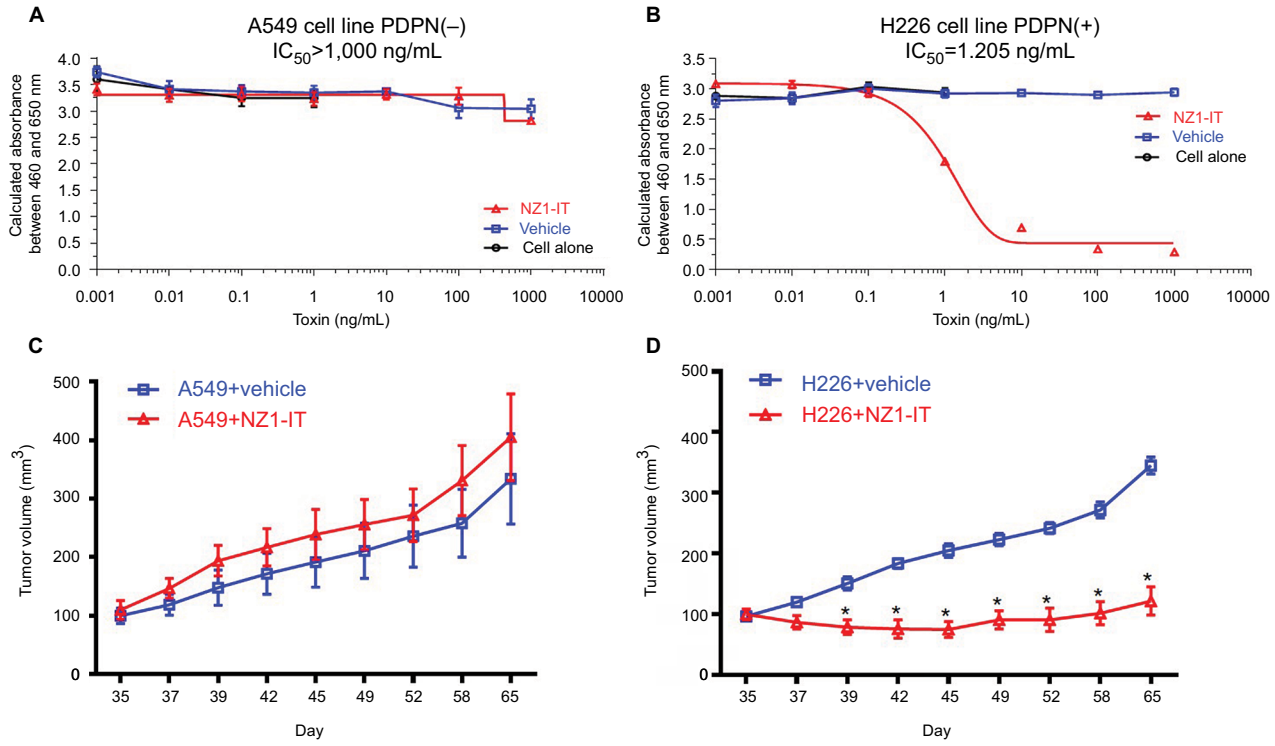

Figure 7 Preclinical therapeutic efficacy of anti-PDPN NZ-1-IT in in vitro and in vivo SqNSCLC models. In vitro cytotoxicity of NZ-1-IT against **(A)** non-squamous A549 and **(B)** squamous H226 cell lines was determined by WST-1 assay. NZ-1-IT was serially diluted to achieve final concentrations of 0.001, 0.01, 0.1, 1, 10, 100, and 1000 ng/mL in 0.2% human serum albumin–PBS. Δ, NZ-1-IT treated group; □, vehicle group; ○, cell-alone group. Effect of NZ-1-IT against A549 and H226 subcutaneous tumors was examined in xenograft mouse NSCLC models. Female athymic nude mice bearing A549 tumors **(C)** or H226 tumors **(D)** were randomized into two groups: vehicle (□) and NZ-1-IT (Δ). Once the tumors reached an average volume of 100 mm^3 on day 35, the mice were treated by intratumoral injections every other day with a total of three 300 μg/kg doses of NZ-1-IT or vehicle on days 35, 37, and 39 after the tumor implantation. (*$p<0.05$).

Abbreviations: PDPN, podoplanin; NZ-1-IT, NZ-1-immunotoxin; SqNSCLC, squamous non-small cell lung cancer; NSCLC, non-small cell lung cancer.

pathway in the absence of EMT processes (including cadherin switching).[27] It can also increase the adhesion and spreading of cancer cells on the fibronectin protein of the extracellular matrix to provide a microenvironment for further tumor cell migration and invasion, even in the presence of E-cadherin.[12,27] PDPN is also related to ezrin, which mediates the process of tumor metastasis.[28] These mechanisms may explain our finding that patients with elevated PDPN expression showed a significantly increasing rate of pleural invasion, known as pathological invasiveness in SqNSCLC. In addition, PDPN has been reported to play a critical role in promoting the metastasis of tumor cells through lymphovascular vessels to invade drainage lymph nodes in other cancers.[29,30] Although our results showed only a marginal increase in LVI and lymph node involvement in those SqNSCLC cases with elevated PDPN expression, they still strongly suggest that there may be a causal relation between PDPN level and lymph node metastasis in SqNSCLC, which needs to be further examined in more patients. Overall, as the current study revealed many unique clinicopathological features of SqNSCLC, the above factors may at least partly explain the worse prognosis in those patients with elevated PDPN expression.

During the last decade, research advances, including the development of molecular cancer genetics, targeted therapies, and large-scale global databases, have significantly shifted the paradigm of cancer treatment from solely conventional therapies to integrating targeted, precise, and personalized therapies.[7,31,32] As mentioned earlier, it is crucial for these targeted therapeutic methods to recognize specifically unique molecular abnormalities in squamous cell lung cancer instead of the normal tissues. Hence, a significant distinct expressed molecular target between tumor and normal tissue is ideal for these therapies. In our derivation cohort, we found an elevated expression of PDPN in as many as 40% of SqNSCLC cases, while PDPN showed no or only mild expression in major adult human organs compared with many other popular targets currently used in the clinic, although data from PDPN-deficient mice showed that PDPN is crucial during the development of the heart, lung, and lymphatic

systems.[33,34] In spite of the result that the HPM database did not show PDPN expression in adult lung, a previous study reported that *PDPN* transcripts had a low-level expression in human lung tissue.[35] Furthermore, with the development of modern techniques, antitumor agents can be locoregionally delivered into the lung tumor site without any serious adverse events in patients.[17] Immunotoxin therapy through locoregionally intratumoral delivery has already been used to successfully treat patients with brain cancer, to specifically target tumor cells and protect the normal tissue that may also express similar targets.[17] Overall, these findings suggested that an anti-PDPN therapy is able to target tumor cells with limited off-target effects.

In the current study, PDPN protein level in the derivation cohort was interrogated via immunohistochemistry in 70 SqNSCLC patients. In the validation cohort from TCGA data set, PDPN expression was examined on the mRNA level, with more than 100 matched pairs of cases, matched based on AJCC cancer stage. Given the relatively small scale of the FUSCC cohort and to avoid bias from the few events of death, the end point for this derivation cohort was defined by PFS instead of OS for analyzing the association of PDPN and patient prognosis. On the other hand, OS was used for the validation cohort because TCGA data set contains complete survival data with an adequate follow-up but often lacks disease progression details. Despite these caveats, a strong relationship between elevated PDPN expression and poor prognosis is suggested in survival comparisons between the two different populations.

As a subsequent investigation on the overall effect of PDPN on SqNSCLC treatment, including its obviously distinct expression in lung tumors as well as its association with poor patient prognosis, the promising findings from in vitro and in vivo experiments are believed to be the first evidence to show the therapeutic effect of anti-PDPN therapy using a targeted immunotoxin, NZ-1-IT, for SqNSCLC treatment at the preclinical level, with robust potential. Furthermore, the antitumor immunotoxin therapy can induce a secondary antitumor immunity in addition to its specific and direct killing via the toxin-induced protein synthesis inhibition. Leshem et al[36] reported that immunotoxin can synergize with an immune checkpoint inhibitor, leading to a higher rate of complete regression in mouse breast cancer models owing to an increase of tumor-infiltrating $CD8^+$ T cells, indicating that immunotoxin can promote the development of anti-tumor immunity to achieve long-term tumor elimination. Currently, two anti-PD-1 immune checkpoint inhibitors have been approved by the US Food and Drug Administration for NSCLC treatment, and are potential candidates to be combined with anti-PDPN immunotoxins to treat PDPN-positive SqNSCLC in the future.

Conclusion

Our results clearly demonstrate that there is a significantly distinct expression of PDPN in SqNSCLC and normal tissue, and its elevated expression is associated with worse clinicopathological characteristics along with poor prognosis in SqNSCLC patients. With the promising therapeutic results from both in vitro and in vivo studies, anti-PDPN therapy can thus be developed as a robust potential targeted therapy for SqNSCLC treatment in the future.

Abbreviations

NSCLC: non-small cell lung cancer
SqNSCLC: squamous non-small cell lung cancer
PDPN: podoplanin
cAMP: cyclic adenosine monophosphate
CLEC2: C-type lectin-like receptor 2
ERM: ezrin–radixin–moesin
EMT: epithelial–mesenchymal transition
NZ-1-IT: NZ-1-immunotoxin
scFv: single-chain variable-region antibody fragment
mAb: monoclonal antibody
ADC: antibody–drug conjugate
LVI: lymphovascular invasion
FUSCC: Fudan University Shanghai Cancer Center
IRB: institutional review board
AJCC: American Joint Committee on Cancer
EMR: electronic medical record
TCGA: The Cancer Genome Atlas
HPM: Human Proteome Map
ATCC: American Type Culture Collection
FBS: fetal bovine serum
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HSA: human serum albumin
OS: overall survival
PFS: progression-free survival
EGFR: epidermal growth factor receptor
FGFR1: fibroblast growth factor receptor 1
K-Ras: Kras Kirsten rat sarcoma viral oncogene homolog
MET: tyrosine-protein kinase Met
PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PD-1: programmed cell death protein 1

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Disclosure

DD Bigner owns stock in Istari Oncology and is a consultant to Genetron Health. DD Bigner and X Bao share a patent on combination therapy of immunotoxin and checkpoint inhibitor. The other authors report no conflicts of interest in this work.

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