Original Article Overexpression of B7-H3 as an opportunity for targeted therapy in head and neck cancers

Juanjuan Hu¹, Caiying Jiang², Meijun Zheng¹, Yuxin Guo⁴, Xin Tang³, Jia Ren¹, Dan Lu¹, Lingyu Yu¹, Weigang Gan^{1,5}, Shixi Liu¹, Aiping Tong², Hui Yang¹

¹Department of Otolaryngology, Head and Neck Surgery, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan, P. R. China; ²State Key Laboratory of Biotherapy, West China Medical School, Sichuan University, Chengdu, Sichuan, P. R. China; ³West China Medical School, Sichuan University, Chengdu, Sichuan, P. R. China; ⁴Department of Neurosurgery, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan, P. R. China; ⁵Department of Otolaryngology, Head and Neck Surgery, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan, P. R. China

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Abstract: Head and neck cancers (HNCs) are the sixth most common type of cancer in the world. Despite the development of refined surgical techniques and precise targeted radiation, patients with HNCs have a dismal prognosis. Here, we examine the expression profile of B7-H3 in HNCs and verify whether B7-H3 can serve as a novel therapeutic target for HNCs via anti-B7-H3×CD3 bispecific antibodies (biAbs). We analyzed the expression level of B7-H3 in 274 HNC samples and evaluated the association between B7-H3 expression and clinicopathological parameters. Anti-B7-H3×CD3 biAbs were constructed, and the efficacy of these biAbs in targeting HNCs was assessed in vitro and in vivo. As a result, high expression of B7-H3 was detected in 66.1% of clinical HNC samples and was correlated with poor survival. Specific antitumor effects of anti-B7-H3×CD3 biAbs were confirmed in vitro using HNC cell lines. In xenograft HNC mouse model, anti-B7-H3×CD3 biAbs delayed tumor growth and prolonged survival. In conclusion, B7-H3 is frequently overexpressed in HNCs and could be a promising therapeutic target for biAb therapy.

Keywords: B7-H3, bispecific antibodies, head and neck cancers, immunotherapy

Introduction

Overall, head and neck cancers (HNCs) are the sixth most common type of cancer in the world. Sixty percent of patients with HNCs exhibit anatomic damage involving speech, swallowing, and breathing problems, which impact the patients' quality of life [1] and cause more severe psychological trauma than that resulting from other cancer types [2]. Most previous studies have reported that patients with HNCs had a dismal prognosis and that their median overall survival was less than 6 months [3]. Current treatments, including surgery, radiotherapy, chemotherapy or combined therapy, are aggressive and costly. Despite refined surgical techniques, such as transoral robotic surgery, and precise targeted radiation approaches, such as intensity-modulated radiation, outcomes remain unsatisfactory [4, 5]. Thus, a safe and effective tumor-selective therapy for HNCs is urgently needed.

Tumor immunotherapy, in which the patient's own immune system is recruited to fight tumors, is a promising therapeutic strategy, and its use is increasing because monoclonal antibodies (mAbs) have revolutionized cancer therapy. With over 15 mAbs clinically approved for cancer therapy, these molecules are a standard approach for oncologic treatment, targeting various antigens overexpressed on tumor cells, such as PD-L1 [6] and EGFR [7]. Despite the predicted success, the application of pure antibodies has been limited by their overall structure, antigen specificity, target antigen affinity and "on-target, off-tumor" toxicity [8]. Fortunately, investigators continue to explore various mAb-based approaches. Currently, over 50 bispecific antibodies (biAbs) have been used in clinical trials for various malignancies, with the goal of providing treatment options for many different types of cancer [9]. Encouragingly, the anti-CD19 × anti-CD3 biAb blinatumomab has been approved by the Food and Drug Administration (FDA) and has entered the market [10]. Hence, the application of biAbs may be an attractive strategy to enhance the efficacy of targeted therapy in HNCs.

B7 homolog 3 (B7-H3), also known as CD276, is a type I transmembrane protein encoded on chromosome 9 in mice and chromosome 15 in humans, which shares up to 30% amino acid identity with other B7 family members [11]. Numerous studies have shown that B7-H3 expression was upregulated in various human malignancies, including melanoma [12] and other cancers [13-18]. Recently, some studies reported that B7-H3 was overexpressed in hypopharyngeal carcinoma and oral cancer [19, 20]; thus, it is a promising target for HNC immunotherapy. Tumor-targeted therapies with B7-H3 have garnered substantial interest. Several drugs targeting B7-H3 are in development, such as MGA271 [21], an engineered anti-B7-H3 mAb that induces potent antibody-dependent cellular cytotoxicity against a broad range of tumor cell types, and 8H9 [22], another humanized antibody that is also in Phase I clinical trials.

Our goal was to investigate the correlation of B7-H3 expression in HNCs with clinicopathological variables and to assess the efficacy of anti-B7-H3×CD3 biAbs in targeting HNCs in vitro and in vivo.

Materials and methods

Patient samples and immunohistochemical (IHC) analysis

A total of 341 samples, including 67 normal head and neck samples (non-cancerous human tissues) and 274 HNC samples, were collected from the otorhinolaryngology department of West China Hospital. All samples were fixed with 10% formalin and embedded in paraffin for staining with anti-B7-H1 rabbit mAbs (Cell Signaling Technology, 1:200), anti-B7-H3 rabbit mAbs (Cell Signaling Technology, 1:200), anti-B7-H4 rabbit mAbs (Cell Signaling Technology, 1:200), anti-B7-H5 rabbit mAbs (Cell Signaling Technology, 1:200) and anti-CD3 rat mAbs (Abcam, 1:200). IHC expression of B7-H1, B7-H3, B7-H4, B7-H5 and CD3 in these samples was interpreted by a head and neck pathologist and was blindly reviewed by another pathologist with high fidelity, thus indicating a reliable diagnosis. Separate scores were assessed for the stained area and staining intensities of B7-H1, B7-H3, B7-H4 and B7-H5. The stained area was scored as follows: 1 (≤25%), 2 (>25 to \leq 50%), or 3 (>50%). Based on the intensity of membranous expression, staining was rated as 0 (-, absent), 1 (+, weak), 2 (++, mild), or 3 (+++, strong). Then, we calculated the scores for each section by multiplying the stained area and the staining intensity scores. The sections were then divided into two groups: low expression (a final score of \leq 3) and high expression (final score of >3). Raw CD3+ T-cell counts were normalized to yield a density value as follows: 0 (≤5%), 1 (>5 to ≤25%), or 3 (>25%). The RNAseq data were downloaded from the Gene Expression Profiling Interactive Analysis (GE-PIA) (http://gepia.cancer-pku.cn/), which has access to The Cancer Genome Atlas (TCGA).

Expression and purification of anti-B7-H3×CD3 biAbs

cDNAs encoding B7-H3- and CD3-specific single-chain variable fragments (scFvs) were synthesized by Genewiz according to previously published amino acid sequences [24, 25]. The two scFvs were linked by a 5-amino-acid (G4S) linker to form a recombinant single-chain biAb. A FLAG/His tag was fused to the C-terminus of this biAb to aid in protein purification. The biAb DNA sequence was then subcloned into the vector pVAX-ßglobin between the EcoRI and Xbal sites to construct the recombinant plasmid (Figure 4B). DNA vectors were transiently transfected into HEK293FT cells in Freestyle serum-free medium (Thermo), and the mature His-tagged B7-H3×CD3-biAbs were harvested from the supernatant. Recombinant protein was initially isolated by Protein A and Ni-NTA affinity columns and subsequently subjected to size exclusion chromatography (Figure 4C).

Cell culture

Human HNC cell lines, including HNE1, CNE2, CAL27, SACC83, FADU and FDLSC, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China) and the Cell Center of the Chinese Academy of Medical Sciences (Beijing, People's Republic of China). CNE2, CAL27, FADU and FDLSC cells were cultured in dulbecco's modified eagle medium (DMEM) (HyClone), and HNE1 and SACC83 cells were cultured in roswell park memorial institute (RPMI) 1640 medium (HyClone). All media were supplemented with 10% fetal calf serum (HyClone), 2 mmol/l glutamine and 1.0 mmol/l penicillin-streptomycin (HyClone). FADU cell lines expressing luciferase (FADU-Luc cells) were established by viral transduction and puromycin selection using a lentiviral vector with double promoters (EF1 α for B7-H3 and pGK1 for luciferase-P2A-puramycin).

Immunofluorescence and western blotting

Cells were incubated in 24-well plates with coverslips for 24 hours at 37°C and were then fixed with 4% polyoxymethylene for 15 minutes before being blocked with 5% bovine serum albumin (BSA) for 30 min. The slides were incubated with the primary antibody or Fc-tagged B7-H3 scFv for 1 hour, followed by incubation with the secondary antibody for 1 h. Finally, the slides were stained sequentially with a Cy3conjugated secondary antibody (Proteintech) and 4',6-diamidino-2-phenylindole (DAPI) (Beyotime). The immunofluorescence staining was visualized by confocal microscopy, and images were acquired.

Western blotting was also used to test the B7-H3 expression level. Protein samples were collected from HNC cell lines and FADU xenograft tumor tissues. The samples were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma) for 15 minutes and were then centrifuged at 10,000 × g for 15 minutes at 4°C. Protein concentrations were determined with a BCA protein assay kit (Thermo Fisher Scientific). Equal protein concentrations were separated by 10% SDS-PAGE and subsequently transferred onto PVDF membranes (Millipore). After blocking in TBST buffer (TBS + 1% Tween) containing 5% (wt/vol) powdered milk for 1 hour at room temperature, the membranes were incubated with anti-B7-H3 (Cell Signaling Technology, 1:1000), anti-GAPDH (Abcam, 1:1000) or anti-tubulin (Abcam, 1:1000) antibodies overnight. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (Beyotime, 1:2000). The western blotting results were visualized by a ChemiScope 6000 Touch (Clinx).

Cytotoxicity assay

Chromium release assays (H-Y Biological) were used to assess cytotoxicity. Primary T-cells expanded from healthy donor peripheral blood mononuclear cells (PBMCs) were used as effector cells; HNE1, CNE2, CAL27, SACC83, FA-DU and FDLSC labeled with 51 Cr were used as target cells. To evaluate the effect of anti-B7-H3×CD3 biAbs on HNC cell proliferation in vitro, cells (1 \times 10⁴ cells) were seeded in 96well plates in triplicate with DMEM or RPMI 1640 medium for 24 hours. On the following day, T-cells with different E:T ratios and anti-B7-H3×CD3 biAbs at different concentrations were added into the wells together. After 4 hours, the supernatants were collected, and the radioactivity was examined by a gamma counter. The percentage of specific lysis was calculated by the following formula: (tested release - spontaneous release)/(maximal release - spontaneous release) × 100.

Murine model

Female NOD-SCID mice (n=5/group), originally introduced from the Charles River Company (Figure S1A), were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd (Figure S1B). Mice used for in vivo experiments were between 8 and 10 weeks old. Mice were anesthetized with 3% isoflurane (Chengdu Iverson Biotechnology Co., Ltd. China) in an induction chamber. Anesthesia on the stereotactic frame was maintained at 1% isoflurane delivered through a nose adaptor. A total of 5×10^{6} FADU-Luc cells were implanted subcutaneously in the right rear flank of female NOD/ SCID mice to establish xenograft models. Mice with established tumors were randomly divided into the PBS group and anti-B7-H3×CD3 biAb group. Tumor growth was monitored with digital calipers every second day and with bioluminescence imaging on days 7, 25 and 35. For bioluminescence imaging, mice were injected with 150 mg/kg D-luciferin (Beyotime) and imaged using an in vitro imaging system (IVIS, Caliper Life Sciences) 10 minutes after injection. Finally, mice were euthanized by cervical dislocation. Studies were carried out under protocols approved by the West China Hospital of Sichuan University Laboratory Animal Ethics Committee (Ethical approval document: 2018212A) (Figure S2).

Statistical analyses

Statistical analyses were conducted with SP-SS version 22.0 and GraphPad Prism 7.0. All image analyses were performed with ImageJ



Figure 1. B7-H1, B7-H3, B7-H4 and B7-H5 expression in various head and neck tissues. A. Box plots of B7-H1, B7-H3, B7-H4 and B7-H5 mRNA expression in head and neck tissues from TCGA (*P<0.05). B. IHC analysis of B7-H1, B7-H3, B7-H4 and B7-H5 expression was evaluated in normal head and neck tissues and HNC tissues.

and Image-Pro Plus 6.0. × 2, and Fisher's exact tests were used to compare the categorical clinicopathological factors of patients with HNCs according to low or high B7-H3 expression. For Kaplan-Meier survival analysis, a logrank test was used to compare each of the arms. All tests were two-sided, and *p* values <0.05 were considered statistically significant. For the in vitro cytolysis experiments, triplicate wells were set up, and the experiments were repeated twice.

Results

B7-H3 was aberrantly expressed in human HNC tissues

The results from the TCGA database showed that the mRNA level of B7-H3 in tumor samples was significantly higher than that in normal tissues, while there was no statistical dif-

ference of B7-H1, B7-H4 or B7-H5 mRNA expression between tumor samples and normal tissues (Figure 1A). In the IHC analysis of our 341 samples (67 normal head and neck samples and 274 HNC samples), predominant B7-H3 membranous staining was observed in tumor tissues (Figure 1B). Of the 274 HNC samples, high B7-H3 expression (defined as a score of >3 for tumor membranous staining) was observed in 181 samples (66.1%), and low B7-H3 expression (defined as a score of \leq 3 for tumor membranous staining) was observed in 93 samples (33.9%). In addition, B7-H3 staining was positive in different histological subtypes of head and neck tumor samples (Figure 2A) and was stronger in the tumor cell compartments than in the stroma (Figure 2B). In contrast, positive CD3 staining was mainly observed in the stroma instead of in the tumor cell compartments (Figure 2D).



Figure 2. Analysis of B7-H3 expression in various head and neck tissues. A. IHC analysis of B7-H3 expression in four different histological subtypes. B and D. IHC analysis was conducted in HNC tissues with different intensities of B7-H3 and densities of CD3 staining. The red arrows indicated B7-H3 positive staining, and the green triangles indicated CD3 positive staining. C. Kaplan-Meier curve for the HNC-specific survival of HNC patients with low or high B7-H3 expression. The duration of survival was measured from the first postoperative day to the time of death or at 107 months. Scale bar: 100 μm.

We further investigated the prognostic value of B7-H3 expression in HNCs. Among the 274 patients with HNC, 183 deaths occurred, with a median follow-up period of 78 months (interquartile range: 42 to 107 months). High B7-H3 expression was correlated with shorter HNCspecific survival rates of five years (**Figure 2C**). In a univariate logistic regression analysis, results showed that high B7-H3 expression was associated with an advanced stage (III-IV), lymph node metastasis, a higher smoking index, distant metastasis and a lower density value of CD3 (**Table 1**; **Figure 2B** and **2D**). Since several interrelated variables were related to high B7-H3 expression, we conducted a multivariable logistic analysis to assess the independent factors. A higher smoking index, lymph node metastasis, and a lower density value of CD3-positive staining were independently associated with high B7-H3 expression (**Table 2**).

B7-H3 expression in different HNC cell lines and the binding of anti-B7-H3 scFvs to HNC cells

We analyzed B7-H3 expression in 6 available HNC cell lines by immunofluorescence and western blotting analyses. FADU cells showed the highest level of B7-H3 expression (**Figure 3**). The binding of Fc-tagged B7-H3 scFvs to

Overexpression of B7-H3 in head and neck cancers



Figure 3. B7-H3 expression in different HNC cell lines. A and B. Immunofluorescence and the corresponding semiquantitative image analysis showed different levels of B7-H3 expression in HNC cell lines. The red fluorescence was stained with Cy3-conjugated secondary antibody (Proteintech, 1:500), and the nucleus was stained with DAPI (Beyotime, 1:10). C (original western blotting image in the Figure S3A) and D. Western blotting and the corresponding semiquantitative image analysis showed different levels of B7-H3 expression in HNC cell lines. Data was represented as the mean \pm SD. (-, negative; +, weak; ++, mild; +++, strong). (*P<0.05, **P<0.01, ***P<0.001). Scale bar: 20 µm.

FADU cells was confirmed by immunofluores-cence (Figure 4A).

Cytotoxicity of anti-B7-H3×CD3 biAbs to HNC cells

To analyze the capability of anti-B7-H3×CD3 biAbs to mediate cytotoxicity in vitro, we photographed the growth of FADU cells after 4-hour, 24-hour, 36-hour and 48-hour incubation periods. Clusters of T-cells and the lysis of FADU cells were observed after 48 hours of incubation with anti-B7-H3×CD3 biAbs. Meanwhile, in the PBS groups, T-cells distributed in the dishes without forming clusters, and the density of FADU cells was nearly 100% after a 48hour incubation period (**Figure 5A**). In summary, anti-B7-H3×CD3 biAbs induced visible lysis of FADU cells. No cytolysis was observed in the groups treated with anti-B7-H3×CD3 biAbs



Figure 4. Characterization of Fc-tagged B7-H3 scFvs and the construction of anti-B7-H3×CD3 biAbs. A. Validation of the binding of Fc-tagged B7-H3 scFvs to FADU cells was conducted by immunofluorescence, and the arrows indicated Fc-tagged B7-H3 scFvs binding to FADU cells. B. Schematic diagram of the anti-B7-H3×CD3 biAb expression vector. C. SDS-PAGE analysis of purified anti-B7-H3×CD3 biAbs. The molecular weight was approximately 65 kD. Scale bar: 50 μm.

without T-cells, anti-CD3 antibodies with T-cells and PBS with T-cells. Although anti-B7-H3 mAbs triggered cytolysis, the efficiency was not statistically significant compared to the PBS groups (Figure 5B). Thus, targeted cell lysis was strictly dependent on anti-B7-H3×CD3 biAbs and T-cells. Meanwhile, we also examined the effects of different effectors, such as the target ratio (E:T ratio) and concentration of anti-B7-H3×CD3 biAbs, on HNC cells. Specific lysis was more effective at an E:T ratio of 8:1 than at an E:T ratio of 4:1 (Figure 5C). However, picogram levels of anti-B7-H3×CD3 biAbs were therapeutically ineffective even at an E:T ratio of 8:1. To determine the cytotoxicity of different levels of B7-H3 expression in different HNC cells, cell viability was assessed in six human HNC cell lines treated with concentrations of anti-B7-H3×CD3 biAbs ranging from 1 pg/mL to 10 ug/ mL at an E:T ratio of 8:1. The results indicate that B7-H3 expression levels did affect the efficiency of anti-B7-H3×CD3 biAb activity (Figure 5D).

Taken together, these results showed that anti-B7-H3×CD3 biAbs were effective in vitro and that specific lysis was closely related to the treatment duration, E:T ratio, anti-B7-H3×CD3 biAb concentration and B7-H3 expression level on the target cells.

Cytotoxicity of anti-B7-H3×CD3 biAbs in vivo

To investigate whether the in vitro activation mediated by anti-B7-H3×CD3 biAbs would translate into in vivo activity, we used a HNC xenograft mouse model. Upon the confirmation of palpable tumors by bioluminescence imaging on day 7, mice were injected intravenously (tail vein) with in vivo expanded human T-cells (1×10⁷ per mouse). One hour later, anti-B7-H3×CD3 biAbs (40 µg in 100 µl of PBS per mouse) or PBS (100 µl per mouse) was administered i.v. (tail vein). The mice received biAbs or PBS every 4 days one hour after being injected with in vivo expanded human T-cells. On day 35, most tumors completely disappeared in the anti-B7-H3×CD3 biAb group, while in the PBS group, tumors continued growing without recession (Figure 6A and 6B). Moreover, the corresponding Kaplan-Meier survival curves showed that the anti-B7-H3×CD3 biAb group survived significantly longer than the PBS group (Figure 6C).

To further analyze B7-H3 expression and T-cell distribution after treatment, an IHC analysis of

Oliviaal		B7-H3 exp	0.4.1	
Clinical	Cases	Negative/Low	High	P value
		(scores ≤3)	(scores >3)	F value
Gender				
Male	240 (88%)	81 (87%)	159 (88%)	
Female	34 (12%)	12 (13%)	22 (12%)	0.849
Age (years)				
≤60	160 (58%)	47 (51%)	113 (62%)	
>60	114 (42%)	46 (49%)	68 (38%)	0.523
Smoking Index (SI)				
≤400	83 (30%)	40 (43%)	43 (24%)	
>400	191 (70%)	53 (57%)	138 (76%)	0.001
Lymph status				
NO	135 (49%)	54 (58%)	81 (45%)	
N+	139 (51%)	39 (42%)	100 (55%)	0.042
Clinic stage				
+	124 (45%)	51 (55%)	73 (40%)	
+ V	150 (55%)	42 (45%)	108 (60%)	0.029
Differentiation				
Well + moderately	197 (72%)	70 (75%)	127 (70%)	
Poorly	77 (28%)	23 (25%)	54 (30%)	0.398
CD3				
0 point	137 (50%)	35 (38%)	102 (56%)	
1 point	103 (38%)	40 (43%)	63 (35%)	
2 points	34 (12%)	18 (19%)	16 (9%)	0.006

Table 1. Association of B7-H3 expression levels in HNC samples with
clinicopathological parameters

Smoking index, SI = (number of cigarettes per day) × (duration in years).

Table 2.	Logistic	regression	analysis	of high	B7-H3	expression	in HNCs
	- 0	- 0		- 0			

Clinical	Croupo	Multivariable analysis		
parameters	Groups	OR (95% CI)	P value	
Smoking Index (SI)	>400 (vs ≤400)	5.26 (2.04-13.60)	0.001	
Clinic stage	III + IV (vs I + II)	8.07 (1.45-44.96)	0.017	
Lymph status	NO (vs N+)	0.99 (0.18-5.48)	0.986	
CD3	0 point (vs 1 point - 2 points)	0.10 (0.05-0.19)	0.000	

B7-H3 and CD3 expression was performed on the PBS-treated tumors and anti-B7-H3×CD3 biAb-treated tumors. We observed weak staining of B7-H3 in the tumor cell compartment and strong staining of CD3 at the periphery of the tumor cell compartment in anti-B7-H3×CD3 biAb-treated tumors. In sharp contrast, the PBS-treated tumor cells were strongly stained with B7-H3 in the tumor cell compartment, and CD3 staining was sparse in the stroma. In addition, numerous necrotic and disintegrating cells were detected in the anti-B7-H3×CD3 biAbtreated tumors, while this result was not observed in the PBS-treated tumors (**Figure 6D**). The expression level of B7-H3 decreased in the anti-B7-H3×CD3 biAb-treated tumors, as determined through a western blotting assay (**Figure 6E**).

Discussion

In this study, we confirmed the relationship between B7-H3 expression in patients with HNCs and clinical outcomes, and we report herein the effective application of anti-B7-H3×CD3 biAbs targeting HNCs in vitro and in vivo.

In the last decade, B7-H3 has been shown to be expressed in several tumor entities [26, 27]. We found B7-H3 to be highly expressed in the majority of HNC samples along with low levels or no B7-H3 staining in normal head and neck tissues. In contrast to previous studies in which B7-H3 was found to be expressed more strongly in the stroma than in the tumor compartment [28, 29], in our study, B7-H3 expression was significantly stronger in the tumor compartment than in the stroma. Different cancer types and tu-

mor heterogeneity may explain this discrepancy. Numerous studies have demonstrated that overexpression of B7-H3 on tumors is closely related to poor clinical outcomes [15, 30, 31]. Our results indicated that high expression of B7-H3 is significantly associated with a higher smoking index, lymph node metastasis, a lower density value of CD3-positive staining, and a worse survival rate. However, the role of B7-H3 in modulating the tumor microenvironment has not been fully clarified. B7-H3 has been reported to play dual roles in immunoregulation, namely, costimulatory [11, 32], or co-inhibitory



Figure 5. Cytotoxicity analysis of HNCs treated with anti-B7-H3×CD3 biAbs in vitro. A. Specific lysis of FADU cells treated with anti-B7-H3×CD3 biAbs for different durations. Obvious cytolysis was observed in the anti-B7-H3×CD3 biAb groups after 24 hours, while no cytolysis was observed in the PBS groups. B. Compared to the PBS + T-cells groups, anti-B7-H3×CD3 biAbs + T-cells groups showed statistically significant viability after a 48-hour incubation period. C. Specific lysis of FADU cells treated with different E:T ratios and anti-B7-H3×CD3 biAb concentrations. D. Cell growth inhibition curves for different HNC cell lines with different levels of B7-H3 expression. The IC₅₀ values were shown on each curve. Dotted boxes indicated the distribution and growth status of FADU cells in the wells. The red arrows indicated T-cells adhering slightly to the surface of FADU cells, and the green triangles indicated the lysis of FADU cells. Data was represented as the mean \pm SD. (n.s. not significant, *P<0.05, **P<0.01, ***P<0.001). Relative absorbance value: RAU. Scale bar: 50 µm.



Figure 6. Cytotoxicity analysis of HNC xenografts in mice treated with anti-B7-H3×CD3 biAbs in vivo. A. In vivo bioluminescence imaging of all 10 mice with luciferase-expressing FADU tumors on day 7 (before treatment), day 25 and day 34 (after treatment) was shown. B. Tumor growth curves for FADU tumors. C. Survival curves for mice engrafted with PBS-treated tumors and anti-B7-H3×CD3 biAb-treated tumors. D. Representative images of B7-H3 and CD3 IHC staining in tumor sections. The red arrows indicated positive CD3 staining, the green triangles indicated the tumor cell compartment, and the black stars indicated necrotic and disintegrating tumor cells. E. Western blotting showing expression of B7-H3 and tubulin in tumor sections (original western blotting image is in the <u>Figure S3B</u>). Scale bar: 50 μm.

regulation [33, 34]. Consistent with the results of previous studies [35-37], we observed that tumor-infiltrating lymphocytes (TILs) were abundant in tumors with low expression of B7-H3; in contrast, tumors with high B7-H3 expression had a lower count of TILs. Thus, B7-H3 expression was inversely correlated with TILs, suggesting that B7-H3 plays a crucial role in suppressing T-cell-mediated anti-tumor immunity. The possible mechanism may be that B7-H3 impairs T-cell function, inhibits T-cell-mediated antitumor immunity and then negatively regulates the T-cell-mediated tumor immune response in HNCs.

B7-H3 targeted therapies have garnered substantial interest. Consistent with the results of a previous study [38], we did not observe cytotoxicity in B7-H3-negative HNC cells, suggesting that anti-B7-H3×CD3 biAbs are specific for B7-H3-positive HNC cells. Notably, cytotoxicity mediated by anti-B7-H3×CD3 biAbs was associated with the B7-H3 expression level, anti-B7-H3×CD3 biAb concentration and E:T ratio. The number of anti-B7-H3×CD3 biAb binding sites may be a vital influencing factor for cytotoxicity. indicating that normal cells could be protected from cytotoxic effects because their B7-H3 levels are far below the threshold required for cytotoxicity. For further study, anti-B7-H3×CD3 biAbs should be appropriately evaluated in preclinical models and be subjected to extensive scrutiny before being applied to human patients.

Based on the above study, we evaluated the antitumor efficacy of anti-B7-H3×CD3 biAbs in a mouse xenograft model bearing tumors from subcutaneously implanted FADU cells. We found that the combination of anti-B7-H3×CD3 biAbs with T-cells efficiently suppressed the growth of tumors and prolonged the survival of the mice. Then, through IHC analysis and western blotting, we found that B7-H3 expression was lower in the anti-B7-H3×CD3 biAb group than in the PBS group, both indicating that B7-H3 expression was downregulated and partially illustrating the reason why some tumors did not fully recede after treatment with anti-B7-H3×CD3 biAbs. As found in previous reports [39], T-cell infiltration of tumor tissues was higher in the anti-B7-H3×CD3 biAb group than in the PBS group. These observations led to the hypothesis that B7-H3 might impair TILs and that anti-B7-H3×CD3 biAbs might increase the

population of TILs and restore the effector function of these cells. However, T-cells only infiltrated into the peritumoral region, causing finite inflammation and intratumoral necrosis. Thus, it was difficult for T-cells to mobilize at the periphery of tumors and produce a "barrier effect", explaining why some tumors recessed incompletely. Taken together, these data showed that downregulation of B7-H3 and the difficulty of the intratumoral infiltration of T-cells may be the potential mechanism of resistance. For better understanding of the precise mechanism, it is necessary to identify the receptor for B7-H3 and its downstream pathway. Furthermore, to enhance antitumor activity, we might also attempt combined therapy or alternating treatment schedule approaches, such as antibody-drug conjugates (ADCs) [29, 40], or dual blockades [41, 42]; these strategies aid in penetrating tumors, destroying stromal cells in the tumor microenvironment, and minimizing potential resistance.

We recognize the limitations of the current study. First, the molecular mechanism of differential B7-H3 expression in different parts of tumors and in different types of HNCs remains unclear. For example, Xu et al. [43] reported that a microRNA-regulatory mechanism explained the differential expression pattern of B7-H3. Second, although B7-H3 expression was negatively correlated with TILs, the immune mechanism remains controversial, partly because the B7-H3 receptor has not been identified. Thus, identifying the B7-H3 receptor and the associated downstream regulatory pathway is necessary. Third, although noticeable cytotoxicity of anti-B7-H3×CD3 biAbs was observed in the mouse xenograft models, the problem of immunotherapeutic resistance remains unsolved.

In summary, we found that the expression level of B7-H3 was higher compared with several previously described immune checkpoint ligands, including B7-H1, B7-H4 and B7-H5, in HNCs. The ability of anti-B7-H3×CD3 biAbs to suppress the growth of tumors in mouse xenografts suggests that the biAbs are valuable tools for the treatment of HNCs with high B7-H3 expression. Further studies should focus on verifying whether the therapeutic benefits of anti-B7-H3×CD3 biAbs can be replicated in patients in clinical studies.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hui Yang, Department of Otolaryngology, Head and Neck Surgery, West China Hospital, West China Medical School, Sichuan University, No. 37 Guo Xue Xiang, Chengdu 610041, Sichuan, P. R. China. Tel: +86 028854-22436; E-mail: yh8806@163.com; Dr. Aiping Tong, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, No. 37 Guo Xue Xiang, Chengdu 610041, Sichuan, P. R. China. Tel: +86 02885422439; E-mail: aipingtong@scu.edu.cn

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CERTIFICATE
This is to certify that the breed stock of the following strain has been supplied by Charles River to BeijingVital River Lab Animal Tech Co Ltd May 2018, and has been maintained in isolators at our production facilities in Wilmington, Massachusetts.
NOMENCLATURE: NOD.CB17-Prkdescid/IcrCrl ORIGIN: The SCID mutation has been transferred onto a non-obese diabetic background. Animals homozygous for the SCID mutation have impaired T and B cell lymphocyte developmentand impaired natural killer (NK) cells. To Charles River in 2005 from Frederick Cancer Research and Development Center. COAT COLOR: White (Albino). RESEARCH APPLICATION: Tumor biology and xenograft research
May 1, 2018
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Linda Doucette International Customer Service Representative

Figure S1. The original purchase record and certificate. A. The original purchase record was provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. B. The certificate of NOD-SCID mice was provided by the Charles River Company.

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Figure S2. Ethical approval document from the West China Hospital of Sichuan University Laboratory Animal Ethics Committee.

Overexpression of B7-H3 in head and neck cancers



Figure S3. The original western blotting images. A. The original western blotting images of Figure 3C. B. The original western blotting images of Figure 6E.



Figure S4. The editorial certificate.