BCMab1, A Monoclonal Antibody against Aberrantly Glycosylated Integrin α3β1, Has Potent Antitumor Activity of Bladder Cancer In Vivo

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Abstract

Purpose: To identify a novel biomarker for bladder cancer targeting therapy.

Experimental Design: The human bladder cancer cell line T24 cells were used as immunogen to generate mouse monoclonal antibodies. We screened and identified a specific antibody BCMab1 against bladder cancer. We examined BCMab1 antigen expression in the patients with bladder cancer through immunohistochemical staining and investigated the BCMab1 antigen association with clinical severity. We detected the antitumor activity of BCMab1 antibody and investigated its therapeutic efficacy by subcutaneous and orthotopic bladder cancer models.

Results: We developed a new monoclonal antibody BCMab1 against bladder cancer that specifically recognized the aberrantly glycosylated Integrin α3β1 epitope on bladder cancer cells. Expression of the BCMab1 antigen was consistent with clinical severity and prognosis of bladder cancer. The glycosyltransferase GALNT1 could contribute to aberrant glycosylation of Integrin α3. The aberrant glycosylation of integrin α3-activated integrin signaling to initiate FAK activation. BCMab1 could block Integrin engagement to inhibit its signaling leading to cell-cycle arrest. In addition, BCMab1 enhanced FcγR-dependent antitumor activity in vivo.

Conclusions: BCMab1 antigen is a new biomarker for bladder cancer. BCMab1 antibody exhibited potent antitumor activity against bladder cancer in vivo. Clin Cancer Res; 1–13. ©2014 AACR.

Introduction

Bladder cancer is a common malignancy that is more prevalent in developed countries (1, 2). Of note, 70% of bladder cancers are superficial at initial presentation, limited to the mucosa, submucosa, or lamina propria, and 30% of patients are muscle-invasive (3). Superficial tumors are usually treated by surgical resection and intravesical chemotherapy. However, approximately 70% of these patients will recur and as many as 10% to 30% will progress to invasive cancers after treatment (4). The difficulty in managing bladder cancer is inability to predict which tumors will recur or progress (5). New therapeutic approaches are likely to come from an improved understanding of the molecular basis of this cancer.

Integrins are a large family of cell membrane receptors involved in important processes such as cell proliferation, migration, and cell extracellular matrix (ECM) adhesion (6). They consist of noncovalently bound α and β subunits, in which various combinations produce polymorphisms of ligand-binding specificity (7). The Integrin α3β1 serves as a high-affinity receptor for laminin, fibronectin, and collagen (8). The Integrin α3β1-deficient mice died during the neonatal period that indicates that it has a vital role in the formation of several organ systems (9). The cell adhesion mediated by the interaction between the Integrin α3β1 and its ligands plays key roles in a variety of physiologically important processes in organogenesis and in maintenance of epithelial tissues. Altered glycosylation on Integrin α3β1 is associated with tissue invasion and metastasis in many types of cancers (10).

It was reported that Integrin α3β1 is differently glycosylated in bladder tumor and normal cell lines (11). Cell adhesion to ECM proteins is strongly modulated by their glycosylation (11). Recent studies showed that aberrant glycosylation has been implicated in tumorigenesis of some tumor types (12, 13). Here, we generated a new monoclonal antibody (mAb) BCMab1 against bladder cancer that specifically recognized the aberrantly glycosylated Integrin α3β1 epitope on bladder cancer cells. And we investigated the therapeutic activity of BCMab1 and its antitumor mechanisms for bladder cancer.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-13-3397
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Translational Relevance

BCMab1 antigen, the aberrantly glycosylated Integrin α3β1, is a new biomarker for bladder cancer that has the characteristics of high specificity so far. It is consistent with clinical severity and prognosis. GALNT1 could confer the aberrant glycosylation of Integrin α3β1 on bladder cancer. BCMab1 or BCMab1-Ra exhibits potent therapeutic efficacy for bladder cancer.

Materials and Methods

Primary antibodies

The primary antibodies were goat anti-human Integrin α3 (Santa Cruz Biotechnology; N-19) for immunoblotting, mouse anti-human Integrin α3 (Santa Cruz Biotechnology; P1B5) for fluorescence staining, mouse anti-human Integrin β1 (Santa Cruz Biotechnology; 12G10), rabbit anti-human cyclin D1 (CST; 92G2), rabbit anti-human CDK4 (CST; D9G3E), rabbit anti-human c-Jun (CST; 60A8), phospho-c-Jun (Ser63; CST; 54B3), phospho-c-Jun (Ser73; CST; D47G9), rabbit anti-human FAK (CST; D2R2E), phospho-FAK (Tyr397; CST; D20B1), Nkp46 (CD335) antibody (R&D Systems; AF2225), PE-NKp46 (CD335) antibody (eBioscience; 29A1.4), and isotype-matched IgG (Sigma). Mouse mAb BCMab1 was purified through protein A-Sepharose from ascites. Corresponding species–specific horseradish peroxidase (HRP)–biotinylated (Pierce), or fluorescein isothiocyanate (FITC)–conjugated secondary antibodies (Sigma) were used.

Cells, tissues, and animals

Bladder cancer T24 cells and normal bladder HCV29 cells were from the Peking University Health Science Center (Beijing, China). Cell lines EJ, 5637, J82, SW780, HepG2, LoVo, A375, Jurkat, PC-1, MCF-7, K562, HeLa, and other cell lines, were obtained from the Peking University Health Science Center (Beijing, China). Cell lines EJ, 5637, J82, SW780, HepG2, LoVo, A375, Jurkat, PC-1, MCF-7, K562, HeLa, and other cell lines, were obtained from the Peking University Health Science Center (Beijing, China). Cells, tissues, and animals from normal human peripheral blood mononuclear cells (PBMC). Briefly, CD14⁺ cells were purified from PBMC by the EasySep Human Monocyte Enrichment Kit without CD16 depletion (STEMCELL Technologies). Purified CD14⁺ monocytes were cultured in macrophage colony-stimulating factor (Peprotech) at 50

Silencing of Integrin α3β1 and GALNT1

The RNA sequence against Integrin α3, β1, and GALNT1 (N-acetylglucosaminyltransferase type 1) for RNAi was designed on the basis of pSUPER system instructions (Oligoengine) and cloned into pSUPER-puro that expresses 19 nt hairpin-type short hairpin RNA (shRNA) with a 9 nt loop. Integrin α3, β1, and GALNT1 shRNA-encoding sequences were as follows: For Integrin α3: 5'-GATCCCCCGTACATGATTCACGCAATTCGTAATGCGTGAATGCTGAGCGG-3' (antisense). For Integrin β1: 5'-GATCCCCCGTACATGACAGACAGAACTGGGAGTCACTTC-TGGTITTT3' (sense); 5'-AGCTTTAAAGTGCAGAAGATGGACTTCTTGAGGTGCCG-3' (antisense). For integrin β1: 5'-GATCCCCCGTACATGACAGACAGAACTGGGAGTCACTTC-TGGTITTT3' (sense); 5'-AGCTTTAAAGTGCAGAAGATGGACTTCTTGAGGTGCCG-3' (antisense). For GALNT1: 5'-GATCCCCCGTACATGACAGACAGAACTGGGAGTCACTTC-TGGTITTT3' (sense); 5'-AGCTTTAAAGTGCAGAAGATGGACTTCTTGAGGTGCCG-3' (antisense). Corresponding scrambled sequences served as controls (shCtrl). T24 cells were transfected by using Lipofectamin 2000 (Invitrogen) as described by the manufacturer's instruction. Integrin α3, β1 and GALNT1-silenced cells were selected with puromycin (Sigma). Scrambled sequences vectors transfected cells were used as controls.

Animal experiments

Female 6-week-old BALB/c nude mice with a body weight of approximately 15 g were used and kept under specific pathogen-free conditions. Xenografts of T24 cells (include wide-type cells, shIntα3- or shGALNT1-silenced cells) were produced by injecting tumor cells (1×10⁶ suspended in PBS) subcutaneously into the back of mice. When tumors reached a diameter of 3 to 5 mm, the mice transplanted wide-type cells were grouped (10 mice/group) and administered intraperitoneally BCMab1 or mIgG at a dose of 10 mg/kg, three times per week for 35 days. Tumor size was measured twice per week. For the orthotopic nude mouse model, T24 cells were labeled with luciferase and transplanted into the murine bladder cavity via 24-gauge angiocatheters (15). The mice were grouped (24 mice/group) and intravenously applied with BCMab1 or mIgG at a dose of 1 mg/kg next day. Images were obtained by the in vivo image system (IVIS) at 5, 10, 15, 20, 25, and 30 days after transplantation. The photon counts of each mouse are indicated by the pseudo-color scales.

Generation of BCMab1-Ra

The immunotoxin BCMab1-Ra was constructed by linking ricin A chain (Ra) to BCMab1 via the hetero-bifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). Indirect immunofluorescence, FACS analysis, and competition binding assay showed that the binding activity of the antibody in this immunotoxin was 95% of the original activity. Cell culture experiments had shown BCMab1-Ra to be an exceptionally effective biologic agent that was capable of killing bladder carcinoma cell lines (T24).

Antibody-dependent cell phagocytosis

Macrophages, generated from normal human peripheral blood mononuclear cells (PBMC). Briefly, CD14⁺ cells were purified from PBMC by the EasySep Human Monocyte Enrichment Kit without CD16 depletion (STEMCELL Technologies). Purified CD14⁺ monocytes were cultured in macrophage colony-stimulating factor (Peprotech) at 50
ng/mL for 10 to 14 days in a humidified incubator. Cultured macrophages were detached and plated in flat 96-well plates (50,000 cells/well) at 37°C. Carboxyfluorescein succinimidyl ester (CFSE)-stained target cells were incubated with antibodies for up to 1 hour on ice. After washing, the different cell solutions were added to the culture plates containing macrophages at a ratio of 5:1 (target cells: macrophages). Then, the culture plates were incubated for 2 hours at 37°C. The phagocytic index was calculated as the number of CFSE-positive cells per 100 macrophages.

Antibody-dependent cell cytotoxicity

Natural killer (NK) cells were isolated from purified PBMCs using positive or negative NK cell isolation kits (Miltenyi Biotec) and activated by 100 U IL2. Target cells were harvested and incubated in media with or without the indicated antibodies (2 μg/mL) for 60 minutes on ice. Then NK cells were washed, diluted in ADCC medium, and dispensed together with the antibody-coated target cells at varying effector/target cell ratios. Experiments were performed in triplicate. After incubation, TO-PRO-3 dye and counting beads (Invitrogen) were added, and cells were analyzed for membrane permeabilization using flow cytometry.

Statistical analysis

The relationship between the staining levels of BCMab1 antigen and various clinicopathologic factors was analyzed using the χ² or the Kruskal–Wallis test. Kaplan–Meier analysis was used to estimate the cumulative cause–specific survival rate, and the log-rank test was used to correlate differences in patient survival with staining intensity of BCMab1 antigen. The influence of BCMab1 on the growth of bladder cancers was analyzed by the Student t test. In all statistical analyses, P value of 0.05 or less was considered to indicate statistical significance in the two-sided test.

Study approval

All animal studies were permitted by the Institutional Animal Care and Use Committee of the Institute of Biophysics, Chinese Academy of Sciences and were conducted in compliance with its recommendations. All human studies were reviewed and approved by the IRB of Institute of Biophysics, Chinese Academy of Sciences, and written informed consent was provided according to the World Medical Association Declaration of Helsinki.

Results

Generation and specificity of the mAb BCMab1

The human bladder cancer cell line T24 cells were used as immunogen to generate mouse monoclonal antibodies. Approximately 312 hybridomas were screened for clones producing antibodies that bound to the cell surfaces of T24 cells and bladder tumor tissues, but not normal bladder tissues and other non-bladder cancer cell lines. From this screen, 11 clones were selected for further characterization. The hybridoma clone BCMab1 was detected to produce an IgG1 antibody that satisfied best the criterion. We checked several bladder tumor cell lines and found that BCMab1 stained positive on the human bladder cancer cells such as T24, EJ, 5637, J82, and SW780, but not on the nonmalignant ureter epithelium cell line HCV29 or on other tumor cells (Fig. 1A and B; Supplementary Table S1). To investigate the specificity of BCMab1 for bladder cancer, we screened 123 human tumor tissues and 56 normal tissues by immunohistochemistry. BCMab1 only positively stained the bladder cancer tissues, but not normal bladder tissues or other normal tissues, including liver, brain, adrenal gland, pancreas, stomach, colon, breast, lung, ovary, cardiac muscle, thyroid, lymph node, and bone marrow (Fig. 1C; Supplementary Table S1 and data not shown). Therefore, the BCMab1-recognized antigen was truly localized to the membrane and a tumor-specific antigen for bladder cancer.

Expression of the BCMab1 epitope is consistent with clinical severity and prognosis of bladder cancer

To further investigate whether the BCMab1 epitope is associated with clinical severity, we examined its expression in the patients with bladder cancer through immunohistochemical staining. The intensity of BCMab1 immunostaining was scored as follows: 1+; weak; 2+, moderate; and 3+, intense. Because tumors showed heterogeneous staining, the dominant pattern was used for scoring. The scores indicating the percentage of positive tumor cells and staining intensity were multiplied to produce a weighed score for each case. Cases with weighed scores <1 were defined as negative; cases with weighed scores ≥2 were defined as strongly positive; and those in between were defined as positive. We detected bladder tumor tissues from biopsies with classification of grade (G1–3) and stage (T1–4) among 69 patients with bladder cancer. Low grades G1–G2 and low stages Ta–T1 were weakly stained (Fig. 2A; Supplementary Table S2). Severe patients with high-grade G3 and high stages T3–T4 showed intense staining of BCMab1. Normal bladder tissues were not stained as a negative control. The expression levels of BCMab1 epitope correlated with pathologic grades and tumor stages of bladder cancers.

We analyzed the relationship between expression of the BCMab1 epitope and clinicopathologic features in patients with bladder cancer. The histologically high grade, deeply invasive, and lymphatic invasive bladder cancers demonstrated significantly higher expression of the BCMab1 epitope than the low-grade, superficial, and nonlymphatic invasive cancers (Supplementary Table S2). To further determine the association between the BCMab1 epitope expression and prognosis, we completed 80-month follow-up of the above 69 patients with bladder cancer with radical cystectomy. Intriguingly, the patients with high expression of the BCMab1 epitope had significantly worse prognosis than patients with low expression (P = 0.0036; Fig. 2B).

BCMab1 or BCMab1-Ra inhibits tumor growth in subcutaneous and orthotopic bladder cancer models

BCMab1-Ra was generated by conjugation of BCMab1 with the Ra chain as an immunotoxin. The Ra is a toxic agent to kill target cells. BCMab1-Ra was able to suppress
proliferation and growth of human bladder cancer cell lines \textit{in vitro} (data not shown). To determine the anticancerous effect of BCMab1 \textit{in vivo}, we generated xenografted tumor models by subcutaneously injecting T24 cells into the back of BALB/c nude mice. When tumors reached a size of 0.3 to 0.5 cm in diameter, BCMab1, BCMab1-Ra, or control mIgG was injected intraperitoneally into mice, three times per week. After treatment, the sizes of tumors were significantly reduced in BCMab1-treated groups and mice promoted their survival compared with mice treated with control mIgG (Fig. 3A). At the end of our experiments (40 days), the inhibition rates of both groups were more than 70%. Notably, BCMab1-Ra treatment almost eliminated tumors in mice bearing T24 cells (Fig. 3A), and their survival was similar to normal mice. Similar results were obtained by using other bladder cancer cell lines such as EJ, 5637, J82, and SW780 (data not shown). We repeated these experiments by using primary tumor cells from five bladder tumor samples. Expectedly, the BCMab1 and BCMab1-Ra exhibited potent therapeutic efficacy against primary bladder tumor cells (Supplementary Fig. S1).

To detect the anticancerous action of BCMab1 \textit{in situ}, we established an orthotopic bladder cancer mouse model by using luciferase-labeled T24 cells. Luciferase-labeled cells (1 × 10^6) were implanted into murine bladder cavity. Bladder cancers were apparent in all of the implanted mice by detection of high bioluminescence with an IVIS. The mice were grouped (24 mice/group) and intravesically applied with purified BCMab1, BCMab1-Ra, or control mIgG at a dose of 1 mg/kg next day (Fig. 3B). Intravesical administration of BCMab1 was able to suppress bladder tumor growth and prolong the survival rate of T24 tumor-bearing
mice compared with mice in the mIgG-treated control group \((P < 0.01; \text{Fig. 3B})\). Interestingly, BCMab1-Ra was able to prevent cancerous growth in orthotopic bladder cancer mouse models and these mice lived as long as normal mice (Fig. 3B). Similar treatment efficacy was observed by using other bladder cancer cell lines or primary tumor cells from bladder cancer samples (data not shown).

In the course of these experiments, we did not observe any toxic effect associated with administration of BCMab1 or BCMab1-Ra. No metastasis was found in the BCMab1- or BCMab1-Ra–treated group by histopathologic analysis. Therefore, BCMab1 or BCMab1-Ra is a potent cytotoxic agent against bladder cancer.

We surveyed 25 sections from each murine bladder to examine whether bladder cancers existed in the mice. Bladder cancers were apparent in all of the mice in which we observed with high bioluminescence by IVIS. At day 25 of treatment, no apparent tumors were observed in BCMab1- or BCMab1-Ra–treated murine bladders (Fig. 3C, middle). Only few cancer cells were detectable in the T24 cell implanted locations with immunohistochemical staining and hematoxyline and eosin (H&E) counterstaining. With BCMab1-Ra treatment, no tumor cells were observed by immunohistochemistry (data not shown). However, tumors were apparent in mIgG-treated mouse bladders and confirmed by histologic staining (Fig. 3C, left). The mucosal surfaces of these BCMab1- or BCMab1-Ra–treated murine bladders did not show apparent injury (Fig. 3C, right). There were no differences microscopically in the noncancerous bladder mucosa among the BCMab1- or BCMab1-Ra–treated and –untreated normal mice. Taken together, BCMab1 inhibited the growth of bladder cancers \textit{in vivo}, and some of the mice showed complete eradication of the cancer cells without any severe adverse effects. BCMab1-Ra treatment could eradicate bladder cancer cells and showed no side effect in the course of our observations.

**BCMab1 recognizes a new aberrantly glycosylated Integrin \(\alpha_3\) epitope and GALNT1 knockdown reduces glycosylation of Integrin \(\alpha_3\)**

To identify the BCMab1 antigen, T24 cell extracts were applied to affinity chromatography with BCMab1 and separated by gel filtration. Two peaks (I and II) were obtained through eluting with 500 mmol/L NaCl (Supplementary Figure 2).
Each peak fraction was concentrated and resolved by SDS–PAGE followed by silver staining. Peaks I and II showed 4 and 2 bands, respectively (Supplementary Fig. S2B). All the bands were hydrolyzed and analyzed by mass spectrometry (Supplementary Table S3). The lower two bands of peaks I and II were identified as the heavy and light chains of the BCMab1. The upper two bands of the peak I (around 130 and 100 kDa) were identified as the \( \alpha_3 \) and \( \beta_1 \) subunits of Integrin, respectively. The molecular weights of the \( \alpha_3 \) and \( \beta_1 \) subunits of Integrin bound to BCMab1 were bigger than their theoretically predicted molecular weights (118 kDa for \( \alpha_3 \) subunit and 88 kDa for \( \beta_1 \) subunit). We proposed that Integrin \( \alpha_3\beta_1 \) recognized by BCMab1 is glycosylated in bladder cancer.

To characterize the oligosaccharides of the glycosylated Integrin \( \alpha_3\beta_1 \) epitope recognized by BCMab1, we performed a glycan chip assay against BCMab1. The carbohydrate composition of oligosaccharides that reacted...
positively with BCMab1 was [3OSO3]Galβ1-4(Fucα1-3)[6OSO3]GlcNAc (Supplementary Fig. S2C and Supplementary Table S4). Furthermore, we silenced expression of the α3 or β1 subunit via pSUPER–shIntegrin α3 or pSUPER–shIntegrin β1 transfection. Integrin α3– or β1 subunit–silenced T24 cells were selected by puromycin and stable clones were established. The α3– or β1 subunit–silenced T24 cells were stained with BCMab1 antibody followed by FACS analysis. BCMab1 stained negative in the α3-silenced T24 cells (Supplementary Fig. S2D), whereas it still stained shCtrl cells. However, BCMab1 still stained the Integrin β1–silenced T24 cells (Supplementary Fig. S2D). The murine IgG (mlgG) was used as a negative staining control. These results verify that the BCMab1 epitope on Integrin α3β1 is localized on the α3 subunit.

To identify the glycosyltransferase responsible for glycosylation of the Integrin α3β1, we performed DNA microarray assay on bladder tumor tissues of patients and normal bladder tissues. The mRNA samples were extracted from 10 patients with early-stage tumor and 10 normal bladder tissues obtained from bladder biopsy. Microarray analysis revealed that GALNT1 mRNA in bladder tumor tissues was 11.2-fold higher than in normal bladder tissues (Supplementary Table S5). However, other GALNTs (GALNT2–9) and all the fucosyltransferases (FUT 1–10) had no significant changes in bladder tumor tissues compared with normal bladder tissues (Supplementary Tables S5 and S6). The mRNA expression levels of the α3 and β1 subunits were not markedly changeable in bladder tumor tissues and normal bladder tissues. To further confirm this result, we used several bladder cancer cell lines such as T24, EJ, and 5637 cells for RNAi-silencing verification. Consistently, only GALNT1 knockdown impaired BCMab1 staining on T24 cells (Supplementary Fig. S2E and S2F). Similar results were obtained in EJ or 5637 cells (data not shown).

GALNT1 expression in T24 cells was knocked down through transfection by pSUPER–shGALNT1 vector. GALNT1-silenced stable T24 cells were established by puromycin selection (Supplementary Fig. S2G). GALNT1 was silenced over 80%. GALNT1 knockdown extremely decreased the BCMab1 staining signal compared with shCtrl cells (Supplementary Fig. S2H, left). We carried out a competitive inhibition test by using the GALNT1 substrate benzyl-N-acetyl-D-galactosaminide (BG). BG treatment remarkably reduced the BCMab1 staining (Supplementary Fig. S2H, right). mlgG was used as a negative staining control. We silenced GALNT1 in EJ or 5637 cells and obtained similar results (data not shown). These results indicate that the GALNT1 might cause the aberrant glycosylation of Integrin α3β1 in bladder cancer.

**Integrin α3 or GALNT1 knockdown inhibits cancerous proliferation, migration, and tumor growth**

Integrin α3 or GALNT1 knockdown markedly reduced incorporation of [3H]-thymidine into DNA of T24 cells at all time points compared with shCtrl cells (Fig. 4A). Integrin α3 or GALNT1 knockdown significantly decreased colony formation of T24 cells compared with shCtrl cells (Fig. 4B). We examined other functional properties of Integrin α3– or GALNT1-silenced T24 cells. We found that Integrin α3 or GALNT1 silencing significantly decreased cell attachment to laminin (Fig. 4C). Wound-healing assay showed similar results (Fig. 4D). Silenced Integrin α3 or GALNT1 expression significantly inhibited subcutaneous tumor growth compared with the control groups by injection of shCtrl cells (Supplementary Fig. S3). At the end of observation course (35 days), the inhibitory rates of cancerous growth for Integrin α3 or GALNT1 knockdown were 53% and 42%, respectively. We repeated these experiments by using EJ and 5637 cells and got similar observations (data not shown).

We screened a prostatic cancer cell line LNCaP, which lacked Integrin α3β1 expression (Supplementary Fig. S4A). GALNT1-silenced LNCaP cells were established by puromycin selection (Supplementary Fig. S4B). We observed that GALNT1 knockdown had no effect on attachment, transmigration, colony formation, and wound healing (Supplementary Fig. S4C–S4F). Thus, we conclude that GALNT1 is involved in the modulation of aberrantly glycosylated integrin α3β1 in bladder cancer.

**Aberrant glycosylation of Integrin α3β1 activates Integrin signaling that is inhibited by BCMab1 treatment**

We used a transcription activator–like effector nucleases (TALEN) approach to delete Integrinα3 in T24 cells or HCV29 cells (Supplementary Fig. S5) (Ref. 40). In Integrinα3+/− T24 cells, GALNT1 knockdown extremely decreased BCMab1 staining signal compared with that of shCtrl cells (Fig. 5A, left). However, GALNT1 knockdown had no effect on the total Integrin α3 level. In contrast, Integrinα3+/− T24 cells, BCMab1 antibody stained negative, and GALNT1 knockdown still remained negative for BCMab1 staining (Fig. 5B, left). Importantly, in Integrinα3+/− HCV29 cells, GALNT1 overexpression dramatically increased BCMab1 staining signal compared with that of empty vector transfected HCV29 cells (Fig. 5C, left). However, in Integrinα3−/− HCV29 cells, GALNT1 overexpression did not increase BCMab1 antibody staining signal (Fig. 5D, left).

In T24 cells, BCMab1 could precipitate much more Talin1 and Kindin2 (Fig. 5E), which are two adaptor proteins to represent active Integrin signaling. However, GALNT1-silenced T24 cells had dramatically decreased the two protein levels. Notably, a glycosylation-insensitive antibody (Santa Cruz Biotechnology; N-19) precipitated equal levels of total Integrin α3 (Fig. 5E). We further wanted to examine the BCMab1 antitumor signaling. Surprisingly, BCMab1 treatment restrained phosphorylation of FAK and c-Jun in T24 cells (Fig. 5E). Consequently, the cyclin D1 and cyclin-dependent kinase-4 (CDK4) were also reduced after BCMab1 treatment. We examined the effect of the BCMab1 on cell cycle of bladder cancer cells. BCMab1 treatment arrested cell-cycle progression at G1/S phase (Fig. 5G). Similar results were obtained by using EJ and 5637 cells (data not shown).
BCMab1 confers Fc-FcγR–dependent antitumor activity by macrophages and NK cells

Antibody-mediated antitumor activity in vivo can be augmented by crosslinking with FcγR-expressing effector cells (16, 17). We next assessed the roles of NK cells and macrophages, two major cell types capable of conferring FcγR-dependent antitumor effects, for BCMab1 therapeutic activity. First, we determined the relative abundance of these cell types in tumor tissues harvested from BCMab1- or control mlgG–treated mice. We found that macrophages (F4/80+ cells) and NK cells (NKp46+ cells) constituted the vast majority of FcγR-expressing cells in BCMab1-treated tumors (Fig. 6A and B). These data indicate that treatment with BCMab1 significantly increased tumor macrophage and NK cell infiltration. To confirm these observations, we depleted macrophages or NK cells in nude mice bearing established T24 cells by using clodronate liposomes or anti-asialo GM1 antibody, respectively. We observed that macrophage or NK cell depletion dramatically attenuated BCMab1 antitumor activities in mice bearing T24 cells or primary bladder tumor cells from patient samples (Fig. 6C).

To further confirm the role of ADCC in BCMab1-mediated cytoreductive activity, we detected NK cell activity inside tumors with BCMab1 treatment. Expectedly, tumor-inside NK cells with BCMab1 treatment exhibited better killing activity against T24 cells than those of mlgG treatment (Supplementary Fig. S6A). Similar results were obtained by using patient with bladder cancer samples (Supplementary Fig. S6B). Moreover, tumor-inside NK cells with BCMab1 treatment significantly increased perforin expression and IFNγ production compared with those of mlgG treatment (Supplementary Fig. S6C and S6D). Collectively, macrophage-mediated ADCP and NK cell–mediated ADCC are involved in BCMab1 therapeutic activity against bladder cancer.
Figure 5. Aberrant glycosylation of integrin α₃ can activate integrin signaling and BCMab1 can counteract this activity. A, GALNT1 knockdown of integrin α₃⁺/⁺ T24 cells was stained with BCMab1 and P1B5 antibodies. P1B5 is a glycosylation-insensitive antibody against integrin α₃. B, integrin α₃ was deleted in T24 cells by TALEN approach. GALNT1 knockdown of integrin α₃⁻/⁻ T24 cells was stained by BCMab1 and P1B5 antibody. C, GALNT1 overexpression of integrin α₃⁻/⁻ HCV29 cells was stained by BCMab1 and P1B5 antibodies. D, integrin α₃ was deleted in HCV29 cells by TALEN approach. GALNT1 overexpression of integrin α₃⁻/⁻ HCV29 cells was stained by BCMab1 and P1B5 antibody. E, coimmunoprecipitation of total integrin α₃, Talin1, and Kindlin2 in shCtrl- or GALNT1-silenced cells. T24 cell lysates were precipitated by total integrin α₃ antibody (N-19). The immunoprecipitates were probed with Talin1, Kindlin2, or BCMab1 antibodies. N-19 is a glycosylation-insensitive antibody of integrin α₃ for immunoblotting. F, BCMab1 treatment represses phosphorylation of FAK and c-Jun and reduces cyclin D1 and CDK4 expression. T24 cells were preincubated with BCMab1 or control mIgG before adhesion to laminin for 30 minutes. Cell lysates were immunoblotted with antibodies against phospho-FAK (Tyr397) or phospho-c-Jun (Ser63 and Ser73). Blots were stripped and reprobed for total FAK and c-Jun. β-Actin was used as a loading control. G, BCMab1 treatment arrests cell-cycle progression. T24 cells were incubated with BCMab1 (10 μg/mL) and analyzed by flow cytometry using propidium iodide (PI) staining. mIgG was used as a negative control.

Discussion
Bladder cancer is still a major epidemiologic problem in which incidence continues to rise each year. Increasing evidence showed tumorigenesis of bladder cancer contributes to alterations in molecular pathways that modulate cellular homeostasis (18, 19). Major cellular processes are comprised of five intrinsic processes that respond to external carcinogenic signals or become internally deregulated owing to genetic changes, including cell-cycle regulation, cell death, cell growth, signal transduction, and gene regulation (19). Tumor maintenance and progression also depend on two extrinsic processes that interact with stromal elements and adjoining cells, harboring angiogenesis and tumor cell invasion. Here, we generated a new mAb BCMab1, which specifically reacted to the aberrantly glycosylated Integrin α₃β₁ on bladder cancer cells. The BCMab1 epitope was localized to the aberrantly glycosylated subunit α₃ of Integrin α₃β₁. Glycosyltransferase GALNT1 was highly expressed in bladder cancer that may contribute to the aberrant glycosylation of Integrin α₃. Silenced Integrin α₃ or GALNT1 expression suppressed cancerous proliferation, migration and tumor growth in vitro and in vivo. The
aberrantly glycosylated Integrin α3β1 expression was able to activate Integrin signaling, which might contribute to tumorigenesis of bladder cancer. BCMab1 or BCMab1-Ra treatment inhibited cancerous growth in vivo. Moreover, the BCMab1 epitope was consistent with clinical severity and prognosis of bladder cancers.

Figure 6. BCMab1 confers Fc-FcγR-dependent antitumor activity through both macrophages and NK cells. A and B, immunohistochemical quantitation of F4/80+ macrophages (A) or NKp46+ NK cells (B) in tumor tissues of mice bearing established T24 tumors treated with control mlgG or BCMab1. Graphs, mean F4/80+ and NKp46+ cells in tumor areas, respectively; **, P < 0.01. C, depletion of macrophages or NK cells attenuates antitumor activity in vivo. Tumor growth in macrophage or NK cell depleted nude mice bearing established tumors from T24 cells (left) or primary human bladder cancer cells (right) treated with BCMab1 or control mlgG; **, P < 0.01. D, BCMab1 enhances macrophage-mediated ADCP of T24 cells (left) and primary human bladder cancer cells (right) in the presence of human macrophages; ND, not detectable; **, P < 0.01. E, BCMab1 promotes NK cell-mediated ADCC of T24 cells (left) and primary human bladder cancer cells (right) in the presence of IL2-activated human NK cells. Graphs, specific lysis against target cells following treatment with BCMab1, control mlgG, or FcγR blockade; **, P < 0.01. Patient primary tumor cells represent at least four bladder cancer samples.
Integrins play central roles in controlling cell adhesion to the ECM, cell migration, growth, survival, differentiation and apoptosis (20). Integrins are heterodimers of transmembrane α and β subunits, which bind to their cognate extracellular ligands, such as fibronectin, fibrinogen and collagen (21, 22). Integrin signaling regulates adhesion-dependent growth, survival migration and invasion of tumor cells (23). Integrin α3β1 was firstly identified as a receptor for collagen, laminin-1, fibronectin and entactin (8). Mice deficient in Integrin α3β1 died during the neonatal period (9). It indicates Integrin α3β1 plays a critical role in the formation of vital organs. Ghosh and colleagues showed that decreased Integrin α3β1 expression was consistent with low invasive behavior of tumors (24). Several reports showed that Integrin α3β1 is involved in progression, cell migration, adhesion, metastasis and invasiveness in bladder cancer (25, 26, 27).

DNA microarray showed that the expression level of Integrin α3 or β1 subunit was not significantly changed in bladder cancer tissues compared with the normal bladder tissues. However, the glycosyltransferase GALNT1 in the bladder tissues was expressed 11-fold higher than the normal bladder tissues. Here, we revealed that GALNT1 might be responsible for the aberrantly glycosylated Integrin α3β1 of bladder cancer. Protein–sugar linkage between N-acetylgalactosamine (GalNAc) and Thr or Ser residues is catalyzed by members of the UDP-GalNAc polypeptide: N-acetylgalactosaminytransferase (GALNT) family (28, 29). Most of the GALNT isoforms show both unique and overlapping substrate specificities (30). Carbohydrate chip analysis showed a putative oligosaccharide for the glycosylated Integrin α3 recognized by BCMab1. A report demonstrated that the 237 mAb recognized aberrantly glycosylated OTS8 of the Ag104A fibrosarcoma cell with an altered monosaccharide as a neoepitope, which was transformed into a tumor-specific antigen (31). This glycopeptide epitope raised a high-affinity, highly specific, syngeneic mAb with antitumor activity. Qualitative and quantitative alterations in O- and N-glycosylation in Integrins are consistent features of malignancies (32). Integrins promote various adhesion-dependent effects in tumor cells, including proliferation, survival, migration, and invasion through focal adhesion kinase (FAK) signaling cascades (33–34, 35). FAK phosphorylation is considered to be one of the initial steps that allows binding of Src and Fyn for further phosphorylation leading to signaling cascade (14, 36–38). The Integrin α3β1 is differentially glycosylated in bladder cancer and control cell lines (11). Cell adhesion to ECM proteins is strongly modulated by their glycosylation. We found that silenced Integrin α3 or GALNT1 expression suppressed cancerous proliferation, migration, and tumor growth in vitro and in vivo. Blockade of Integrin α3β1 signaling by BCMab1 also inhibited cancerous proliferation and tumor growth. BCMab1 treatment mostly blocked FAK phosphorylation, consequently reduced c-Jun phosphorylation, which decreases cyclin D1 and CDK4 expression resulting in inhibition of proliferation and cell cycle.

We found that the aberrantly glycosylated Integrin α3β1 could activate Integrin signaling. In T24 cells, the aberrantly glycosylated Integrin α3β1 precipitated much more active adaptor proteins such as Talin1 and Kindlin2. However, in normal HCV29 cells, the active adaptor proteins were not detectable. GALNT1 silencing impaired the aberrant glycosylation of Integrin α3β1, leading to disassociation of the active adaptor proteins. Furthermore, Integrin α3 or GALNT1 depletion significantly inhibited FAK signaling. We propose that the aberrant glycosylation of Integrin α3β1 might change the conformation of Integrin heterodimers that initiates Integrin activation. Therefore, the aberrantly glycosylated Integrin α3β1 might aberrantly activate Integrin signaling to integrate a malignant signal for cell transformation.

Orthotopic xenografts of human bladder cancer are considered as the best model for the evaluation of new therapeutic approaches. Noninvasive bioluminescence imaging has turned out to be an excellent technique for the longitudinal surveillance of tumor development and detection of extravesical tumor spread or metastasis at an early stage (39). Using luciferase-labeled T24 cells, we established an animal model of human orthotopic bladder cancer for the noninvasive monitoring of tumor development and therapeutic efficacy. The orthotopic bladder tumor models more closely mimic the behavior of human bladder cancer. Here, we showed that the intravesical application of BCMab1 or BCMab1-Ra at different time points after instillation of bladder tumor cells was highly efficient in preventing tumor growth in the bladder. We labeled other bladder cancer cell lines and primary tumor cells from patients with bladder cancer for these therapeutic experiments and obtained similar antitumor activity. Local delivery of BCMab1 or BCMab1-Ra can avoid many problems compared with systemic therapy, such as: (i) absorption by the mononuclear phagocyte system (mainly in the liver and spleen) during antibody catabolism; (ii) only a small amount of antibodies reaching the inside tumor; (iii) localization of antibodies in normal organs and formation of human anti-mouse antibodies for clinical applications.

BCMab1 suppressed the aberrantly glycosylated integrin α3β1-mediated integrin signaling. Blockade of integrin signaling inactivated FAK signaling leading to cell-cycle arrest. Besides inhibition of cell-cycle progression, BCMab1 could enhance FcyR-dependent in vivo antitumor activity. Both macrophages and NK cells participated in eradication of tumor cells of bladder cancer. These data imply that BCMab1 will be a potent drug for clinical therapy. Notably, BCMab1-Ra was a conjugate of BCMab1 with the Ra chain as an immunotoxin. The Ra is a toxic agent to efficiently kill target cells. BCMab1-Ra could prevent cancerous growth more effectively than that of BCMab1. In the course of our experiments, we did not observe any toxic effects associated with administration of BCMab1-Ra. No metastasis was found in the BCMab1-Ra–treated group by histopathologic analysis. BCMab1-Ra treatment showed to be safe and tolerable in vivo. Taken together, BCMab1 or BCMab1-Ra may become a potent antitumor drug for treatment of...
bladder cancer. The BCMab1 epitope might serve as a novel-specific biomarker for diagnosis and prediction of prognosis as well as a new potential molecular target for treatment of bladder cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: C. Li, Z. Fan
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Li, Z. Yang, Y. Du, H. Tang, D. Hu, Z. Fan
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): C. Li, Z. Yang, Z. Fan
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Li, Y. Du, J. Chen, Z. Fan
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Acknowledgments
The authors thank R. Yan and J. Hao for their help for human bladder tumor sections. The authors thank Drs. Yangxin Fu and Andre Veillette for critical reading of this article.

Grant Support
This work was supported by the State Projects of Essential Drug Research and Development (2012ZX09103301-041), the National Natural Science Foundation of China (81130047, 81071704, 30830030, and 30972676), and 973 program (2010CB911902), and the Innovative programs of Chinese Academy of Sciences (KSCX1-YW-22 and XDA01010407).

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Received December 21, 2013; revised April 14, 2014; accepted May 11, 2014; published OnlineFirst July 7, 2014.
Clinical Cancer Research

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Clin Cancer Res Published OnlineFirst July 7, 2014.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-3397

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