A Novel Anti-sTn Monoclonal Antibody 3P9 Inhibits Human Xenografted Colorectal Carcinomas

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Summary: The aim of this study is to raise tumor-suppressing monoclonal antibodies (mAbs) against colorectal carcinomas. Here, we generated a novel mAb 3P9, targeted a cancer-associated carbohydrate antigen sialyl-Tn (sTn), which showed significant inhibitory effect on proliferation and migration of sTn^+ cells and tumor growth by inducing apoptosis. We also demonstrated that mAb 3P9 showed higher sensitivity and specificity in immunohistochemistry assay on colonic adenocarcinoma than the broadly used commercial anti-sTn antibody B72.3. These results provide the first evidence that mAb 3P9 has potential applications, not only for diagnosis but also for antibody-based tumor therapy.

Key Words: mAb 3P9, sTn, colorectal carcinoma, tumor growth

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The modification of cell surface glycosylation is a common phenotypic alteration observed in cancer. It mainly affects the outer part of the carbohydrate moiety of glycoproteins and glycolipids, leading to the expression of tumor-associated carbohydrate antigens (TACAs). Many TACAs are sialylated structures.[1] The increased expression of sialylated epitopes has been described in numerous epithelial carcinomas and these changes in glycosylation are related to grade, invasion, and metastasis.[2–4] Sialyl-Tn (sTn) antigen is one of the most common TACAs, which is expressed by most epithelial cancers such as gastric,[5] pancreatic,[6] colorectal,[7] ovarian,[8] and breast cancers[9] and is often associated with a decrease of overall survival of the patients.[10–12]

sTn (Neu5Acα2-6GalNAc-*O*-Ser/Thr) is an *O*-linked disaccharide structure and occurs on multiple secreted and surface glycoproteins and mucins. Abnormal expression of sTn is due to the premature sialylation of the core carbohydrate structure Gal-NAcα1-*O*-Ser/Thr (Tn antigen), which stops further elongation of the oligosaccharide chains.[13] It has been suggested that sTn structures play a role in cell-cell and cell-matrix interactions by mediating the recognition of lectin-like molecules, such as selectins,[14] siglecs,[15] and galectins,[16] which may contribute to cancer development. Overexpressing sTn leads to increased cell motility in breast cancer cell MDA-MB-231,[17] indicating its involvement in cell migration and invasion. In addition, previous studies have also shown that sTn antigen plays a role in cancer cell recognition by the immune system, protecting metastatic cells from degradation in the blood stream.[18] Therefore, based on close correlation between sTn expression and tumorigenesis, sTn has been used as target for cancer immunotherapy and diagnosis in preclinical and clinical studies.[19–21]

So far, many anti-sTn antibodies have been generated, such as B72.3, TKH2, and CC49. However, most of the antibodies are used in diagnosis,[22] imaging,[23] or radioimmunotherapy[24] of tumor. None of them has been reported for tumor growth inhibition. In this study, we generate a novel IgM monoclonal antibody (mAb), 3P9, against sTn. The intensive characterization study shows that mAb 3P9 specifically recognizes sTn-expressing colorectal adenocarcinoma cells. Importantly, mAb 3P9 shows marked inhibition effect for the proliferation and migration of LSC^{sTn+} cells and significant suppression effect for the growth of sTn^+ human tumor xenograft in nude mice by inducing apoptosis. In addition, most of the mAbs used as therapeutic agents in colorectal cancer are of IgG isotype,[25] mAb 3P9 reported here is IgM isotype. The value of IgM mAbs in human therapy should also be considered, due to their high efficacy in complement activation and lower level of entry into normal tissues. All of these results demonstrate that our generated novel anti-sTn mAb 3P9 can specifically recognize sTn^+ carcinoma cells and inhibit growth of human colorectal carcinoma cells. It has the potential to be a diagnostic and therapeutic agent for patients with colorectal carcinomas.

MATERIALS AND METHODS

Ethics Statement

Informed written consent and ethic approval were obtained by the institutional biomedical research ethics committee of the Institute of Biophysics, Chinese Academy of Sciences before human tissues were collected. All animal experiments were performed with the approval of the institutional biomedical research ethics committee of the

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Primary Antibodies, Cells, Tissues, and Animals

mAb 3P9 was generated by our laboratory, mAb TKH2, LSC^{sTn+} and LSB^{sTn-} cells[26] were donated by Prof Steven Itzkowitz (Mount Sinai School of Medicine, NY), mAb B72.3 (Invitrogen), mIgM (Sigma), and anti-muc1 mAb DF3 (Santa Cruz) were purchased. SW1116 was obtained from American Type Culture Collection. A total of 225 specimens including 121 patients and 104 normal tissues were obtained from Beijing Tumor Hospital, Beijing Legal Medical Institute, and the tissue bank of the Chinese PLA General Hospital. BALB/c nude mice were obtained from the Animal Center of the Chinese Academy of Medical Science, Beijing.

Generation of Monoclonal Antibody

Human colorectal adenocarcinoma SW1116 cells (5×10^6) were injected intraperitoneally with Freund complete adjuvant into 6-week-old BALB/c mice and boosted 3 times with SW1116 cells and Freund incomplete adjuvant, and then their spleens were taken for hybridoma preparation as described.[27]

Screening of Antibodies, ELISA, and Immunohistochemistry

The enzyme-linked immunosorbent assay (ELISA) was used to select antibodies binding to human colorectal adenocarcinoma SW1116 cells. Immunohistochemistry was then applied to assess the specificity of antibodies in colorectal adenocarcinoma tissues. Cell ELISA and immunohistochemistry was performed as described previously.[28] In other ELISA experiments, bovine submaxillary mucin (BSM) was coated overnight in 4°C. Subsequent steps were similar to those of cell ELISA. The pattern of sTn immunoreactivity was classified into 5 groups according to the percentage of reactive cells: 0 (no cells reactive), 1+ (< 10%), 2+ (10%– < 25%), 3+ (25%– < 50%), and 4+ (≥ 50%).

Immunofluorescence

LSC^{sTn+} and LSB^{sTn-} cells were fixed with 4% formaldehyde, blocked with 5% normal goat serum, and then incubated with mAb 3P9 for 1 hour. After washing with PBS and incubating with fluorescein isothiocyanate-conjugated anti-mouse IgM (Sigma), the fixed cells were photographed using the Olympus FV1000 confocal microscope.

Immunoprecipitation and Western Blotting

Cells were lysed and an aliquot of lysate was saved to test the total protein expression after centrifugation. The rest of lysates were precleared with protein L (Santa Cruz) or protein G PLUS-Agarose (Santa Cruz). The protein L pretreated samples were then immunoprecipitated with mIgM or mAb 3P9 and 25 μL of protein L-agarose beads and the protein G pretreated samples were immunoprecipitated with mIgG or mAb DF3 and 25 μL of protein G-agarose beads. Protein L/G-bound immunocomplexes were extensively washed and boiled in sample loading buffer. These proteins or cell extracts were electrophoresed in 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham). After blocking with 5% nonfat milk, the membrane was incubated with primary antibodies and then probed with horseradish peroxidase-conjugated secondary antibodies. All immunoblots were carried out using chemiluminescence reagent (Pierce) and exposed to x-ray films (Kodak).

Transwell Assay

Cell migration was assayed using transwell 96-well plates (8-μm pore size; Corning Costar). Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum was added to the lower chamber (0.2 mL/well) and 0.05-mL serum-free Dulbecco's Modified Eagle Media containing 2×10^4 cells treated with mAb 3P9 or control mIgM (100 μg/mL) was added to each insert (upper chamber). After incubation at 37°C for 48 hours, the invading cells migrated to the lower surface of the membranes that were fixed with ethanol and stained with Giemsa solution. The number of cells was counted under light microscope at ×40 magnification.

BrdU Incorporation Assay

Cell proliferation was determined by measuring 5-bromo-2-deoxyuridine (BrdU, Sigma) incorporation. After 24 hours incubation with different concentration of mAb 3P9, LSC^{sTn+}, and LSB^{sTn-} cells were incubated with BrdU for 1 hour. Then cells were washed, fixed with 70% ethanol, and incubated with PE-conjugated anti-BrdU antibody (Biolegend). The percentage of cells with BrdU incorporation was detected with FACSCalibur flow cytometry system (Becton Dickinson).

Cell Apoptosis Assay

Cells pretreated with mAb 3P9 or mIgM were double-stained with fluorescein isothiocyanate-conjugated Annexin V (25 μg/mL) and propidium iodide (PI, 50 μg/mL) according to the manufacturer's protocol (Biosea) and then washed and analyzed using a FACSCalibur flow cytometry system (Becton Dickinson). For tissue slides, apoptosis cells were detected using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) apoptosis kit (Keygentec). The assay was performed according to manufacture instruction.

Complement-dependent Cytotoxicity (CDC) Assay

Target cells (5×10^4) were incubated for 30 minutes at 4°C with medium alone (as negative control), mIgM as isotype control or different concentration of mAb 3P9. Cells were washed twice and incubated at 37°C for 2 hours with mouse serum (at a dilution of 1/4 in PBS) as the source of complement. Cell viability was tested by adding PI and dead cells were analyzed for PI staining by flow cytometry.

Animal Experiment

Female 3–4-week-old BALB/c nude mice were kept under specific pathogen-free conditions. Xenografts of human tumor cell lines were formed by injecting 4×10^6 tumor cells subcutaneously into the backs of mice. When tumors reached a diameter of 3 to 5 mm, the mice were grouped (6 mice per group) and injected intraperitoneally with purified mAb 3P9 at a dose of 5 μg/g/mouse, or with mIgM as control, twice per week for a month. Tumor size was measured twice per week and tumor volume was determined according to the equation: tumor size = $\text{width}^2 \times \text{length} \times (\pi/6)$. The experiment was repeated 3 times by 2 researchers independently.

Statistical Analysis

Data values were expressed as the mean + SEM and the 2-tailed Student *t* test was used for statistical analysis. Differences in proportions of patients were examined using the χ^2 test. A 2-tailed *P* value of <0.05 was considered significant.

RESULTS

Generation and Screening of mAb 3P9

Human colorectal adenocarcinoma cell line SW1116 was used as immunogen to generate mouse mAbs. Hundreds of hybridomas were obtained and subjected to antibody screen by a method of cell ELISA using fixed SW1116 cells. After several rounds of screening, 20 hybridoma clones, producing antibodies with stable and strong binding to SW1116, were selected for further screen using immunohistochemistry with tumor and normal tissues. One of these clones, named 3p9, which produced anIgM/κ specifically binding to human colorectal cancer tissues but not to normal tissues, was chosen for further characterization.

Identification of the mAb 3P9 target

On the basis of the observations in immunohistochemistry, we speculate that the antigen recognized by mAb 3P9 is probably related to mucins, which are the main secretory products of glandular epithelium. Considering mucins being highly glycosylated and usually undergoing aberrant glycosylation during the development of malignancy, we first tested if the mAb 3P9 antigen is a glycan using periodic acid-Schiff as control (Fig. 1A), which is reagent specific for glycogen. We compared the patterns of mAb 3P9 staining in colorectal cancer tissues (Fig. 1B) with that of periodic acid-Schiff and found the very similar staining patterns, suggesting that mAb 3P9 might recognize the abnormally glycosylated mucins. Furthermore, oxidation of the carbohydrate chain with 2% periodic acid severely impaired the binding of mAb 3P9 to colorectal cancer tissues (Fig. 1C), implying that the antigen recognized by mAb 3P9 was probably a carbohydrate structure on some glycoprotein. To further confirm our hypothesis, we studied the binding of mAb 3P9 to BSM, which is a widely used mucin protein. Digestion of BSM with neuraminidase, which removes sialic acid from mucoproteins, significantly impaired the binding of mAb 3P9 to BSM, whereas trypsin digestion did not influence the recognition (Fig. 1D). These biochemical data indicated that the epitope bound by mAb 3P9 was located on the carbohydrate side chain but not the peptide backbone of the glycoprotein and sialyl acid was 1 essential component of this epitope. Considering that sialyled Tn antigen was frequently expressed in many epithelial cancers, we performed competitive inhibition ELISA and found that the binding capacity of mAb 3P9 to BSM was markedly inhibited by well-known anti-sTn antibody B72.3 and TKH2 in a dose-dependent manner (Fig. 1E). Moreover, a pair of well-

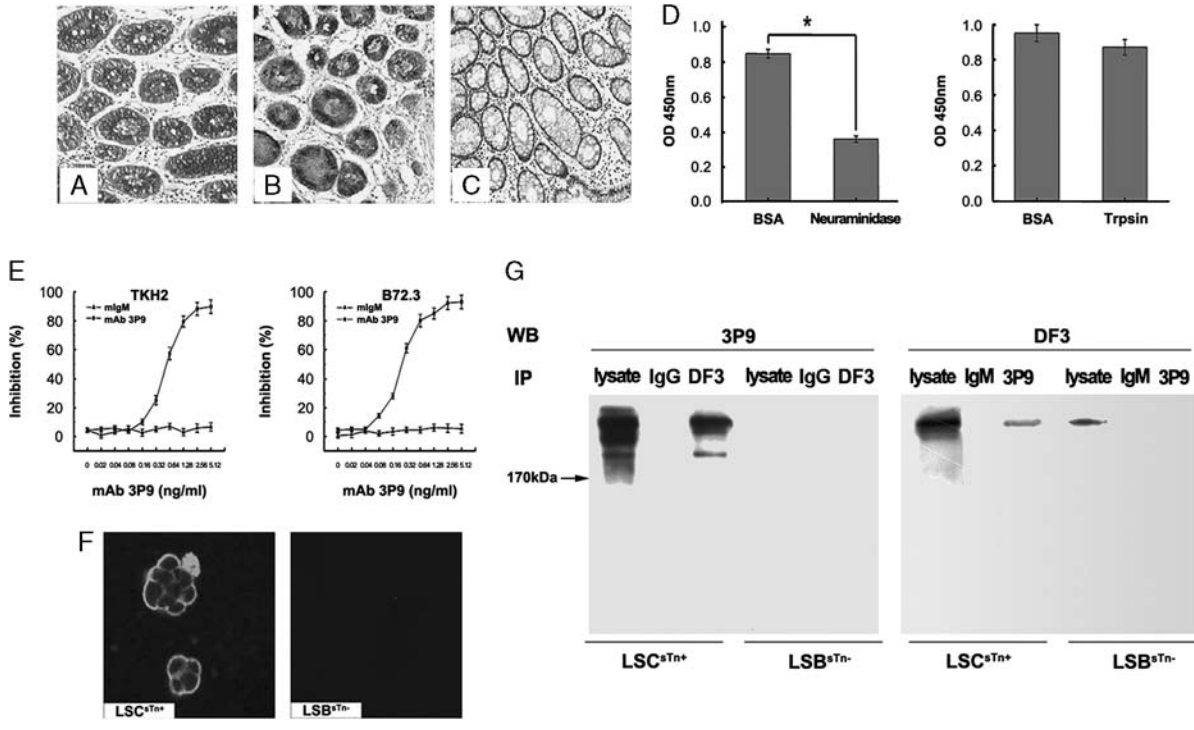

FIGURE 1. Identification the monoclonal antibody (mAb) 3P9 antigen. Paraffin sections from the same human colorectal cancer tissue were stained by immunohistochemistry with periodic acid-Schiff (A) or stained by mAb 3P9 with pretreatment with periodic acid (C) or not (B). D, Bovine submaxillary mucin (BSM) proteins coated on the plate were treated with neuraminidase (0.02 U/ng), trypsin (0.25%) or bovine serum albumin (BSA) for 30 minutes at 37°C, and then reacted with mAb 3P9 in an enzyme-linked immunosorbent assay (ELISA) assay. **P*<0.05. E, Binding of different concentrations of mAb 3P9 to BSM was tested in a competitive ELISA assay in the presence of mAb TKH2 (IgG) or mAb B72.3 (IgG). F, Immunofluorescent staining of LSC^{sTn+} or LSB^{sTn-} cells by mAb 3P9 was performed. G, Total cell extracts were immunoprecipitated with anti-muc1 mAb DF3 (lanes 2–3 and 5–6) or mAb 3P9 (lanes 8–9 and 11–12), and immunoprecipitates were submitted to Western blotting using mAb 3P9 or DF3 accordingly to determine which glycoprotein was the major carrier of sTn epitopes in LSC^{sTn+} cells.

established cell lines, namely LSC^{sTn+} and LSB^{sTn-}, was employed to determine the antigen of mAb 3P9 by immunofluorescence. LSC^{sTn+} cells express sTn and were stained by mAb 3P9, whereas LSB^{sTn-} cells, which have the exactly same genetic background as LSC^{sTn+} but do not express sTn, could not be stained by mAb 3P9 (Fig. 1F). These data finally ensured that mAb 3P9 specifically recognizes the carbohydrate structure of sTn.

To determine which glycoproteins could have their *O*-glycosylation alteration identified by 3P9, total protein extracts from LSC^{sTn+} and LSB^{sTn-} cells were submitted to Western blotting with 3P9 (Fig. 1G lanes 1 and 4). No bands were detected on protein extracts from LSB^{sTn-} cells. By contrast, a major band above 200 kDa was detected with 3P9 from LSC^{sTn+} cells, which was accordant with the bands detected with anti-muc1 antibody DF3 (Fig. 1G lanes 7 and 10). To confirm the result, cell extracts were immunoprecipitated with the antibody DF3 or 3P9 and the reactivity of immunoprecipitates with 3P9 and DF3 were detected by Western blotting correspondingly. The band >200 kDa reacting with the anti-muc1 was specifically precipitated from extracts of LSC^{sTn+} cells, indicating that indeed the sTn + glycoprotein corresponds to the muc1 (Fig. 1G lanes 3 and 9).

Specificity of mAb 3P9

The specificity of mAb 3P9 was detected by immunohistochemistry using 737 human tissues including various types of carcinomas and counterpart normal specimens. Of the 17 different types of carcinomas tested, the strongest and most sensitive staining pattern by mAb 3P9 was observed in colorectal adenocarcinoma (84%) and the specificity was relatively high (78%) (Table 1). The results in Table 1 showed that mAb 3P9-stained epithelial carcinomas but not non-epithelium–derived neoplasia, such as cerebral carcinoma, Hodgkin disease, fibrosarcoma, liposarcoma, hepatocellular carcinoma, and so on. Moreover, to demonstrate the superiority of mAb 3P9 in colorectal cancer, we compared the immumostaining pattern of mAb 3P9 in normal (n = 104) and neoplastic (n = 121) colorectal lesions with B72.3 by immunohistochemistry (Table 2). In general, the staining with 3P9 was much more sensitive and distinct than B72.3. Immunohistochemical staining of mAb 3P9 resulted in staining of 84% of colorectal cancers. In comparison, mAb B72.3 gave staining of 65% of colorectal cancers. In sections of normal individuals, 22% was identified by 3P9 and 18% was detected by B72.3 with weak sTn expression (1 + , < 10% cells stained). So the specificity of mAb 3P9 (78%) was slightly lower than B72.3 (82%). But the positive predictive value and negative predictive value of mAb 3P9 were higher than that of B72.3 (Table 2). It was further observed that the staining with 3P9 was mainly in the cytoplasm of glandular epithelial cells, and the staining sometimes spread into the inside of the glandular lumens which was indicated by arrows (Fig. 2A). In the same colorectal cancer specimen shown in Figure 2B, the staining with 3P9 (Fig. 2Ba) was very distinct, whereas staining with B72.3 (Fig. 2Bb) was weak; and in another the same colorectal cancer specimen, the staining with 3P9 (Fig. 2Bc) was obvious, whereas staining with B72.3 (Fig. 2Bd) was negative.

mAb 3P9 Inhibits Proliferation and Migration of LSC^{sTn+} Cells and Induces Apoptosis of Colorectal Cancer Cells

Since sTn is involved in cancer cell migration and invasion, we assumed that mAb 3P9 might influence cancer cell motility and growth by disturbing the function of sTn. First of all, we investigated the role of mAb 3P9 on cancer cell growth using BrdU incorporation assay. The results showed that the LSC^{sTn+} cells displayed significant DNA synthesis defect with a reduction of 10% when treated with 100 μg/mL mAb 3P9 ($P < 0.01$), compared with the corresponding mIgM control group. No obvious BrdU incorporation change was detected in the LSB^{sTn-} cells (Fig. 3A).

Subsequently, we tested whether mAb 3P9 could suppress sTn^+ cells migration. LSC^{sTn+} cells migration decreased obviously in wound healing assay when treated with 100 μg/mL mAb 3P9 (see Figure, Supplemental Digital Content 1, http://links.lww.com/JIT/A253). Similarly, in transwell assay, 100 μg/mL mAb 3P9 inhibited LSC^{sTn+} cells migration strongly in comparison with the mIgM control ($P < 0.01$), whereas it had no inhibition effect in

TABLE 1. Immunoreactive Patterns of 3P9 in Different Neoplastic and Normal Tissues

Type of Tumors	Positive*/Total	Positive (%)	Type of Tissues	Positive*/Total	Positive (%)
Colorectal adenocarcinoma	102/121	84	Colorectum	23/104	22
Pancreatic adenocarcinoma	12/18	67	Pancreas	0/7	0
Testis carcinoma	11/19	58	Testis	3/6	50
Gastric carcinoma	13/24	54	Stomach	3/6	50
Ovary carcinoma	18/34	53	Ovary	1/6	16.7
Prostate carcinoma	8/15	53	Prostate	1/9	11
Lung carcinoma	12/30	40	Lung	0/4	0
Bladder carcinoma	7/19	37	Bladder	0/6	0
Skin carcinoma	9/30	30	Skin	0/5	0
Breast carcinoma	2/31	6	Breast	1/13	3.13
Thyroid carcinoma	1/20	5	Thyroid	0/5	0
Cerebral carcinoma	0/19	0	Cerebrum	0/6	0
Hodgkin disease	0/9	0	Lymph node	0/10	0
Fibrosarcoma	0/10	0	Fibrous tissue	0/10	0
Liposarcoma	0/10	0	Fatty tissue	0/6	0
Hepatocellular carcinoma	0/19	0	Liver	0/5	0
Renal cell carcinoma	0/20	0	Kidney	0/5	0

*Pattern: 1 + , < 10%; 2 + , 10%– < 25%; 3 + , 25%– < 50%; 4 + , ≥ 50%.

TABLE 2. Comparison the Specificity, Sensitivity, PPV, NPV of 3P9, and B72.3

Anti-sTn Antibody	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
3P9	84	78	82	81
B72.3	65	82	78	70

NPV indicates negative predictive value; PPV, positive predictive value.

LSB^{sTn-} cells (Fig. 3B). These data suggested that mAb 3P9 could suppress sTn^+ cells migration.

Then we studied whether mAb 3P9 induced cell apoptosis. LSC^{sTn+} cells were treated with mAb 3P9 or control mIgM and Annexin V-PI double staining was performed to determine the percentages of apoptotic cells. The results were presented in Figure 3C showing that apoptotic cells in early stage were 20.53% and in middle stage were 26.57% in LSC^{sTn+} cells treated with 100 μg/mL mAb 3P9, which were markedly higher than that in cells treated with mIgM. However, mAb 3P9-inducedapoptosis was not apparent in LSB^{sTn-} cells. These suggested that mAb 3P9 probably triggers apoptosis through specifically binding to sTn antigens expressed on the cell surface.

mAb 3P9 Inhibits Growth of Human Xenografted Colorectal Cancer

sTn expression is associated with tumor growth, metastasis, and interaction between cancer cells and immune cells. To study whether mAb 3P9 suppresses the growth of sTn-expressing colorectal adenocarcinoma in vivo, we established a xenograft colorectal adenocarcinoma model in nude BALB/c mice and investigated the effects of treatment with mAb 3P9. Suspension containing 4×10^6 of LSC^{sTn+} cells were injected subcutaneously into nude mice at 3–4 weeks of age to develop tumor. When tumors were sufficiently established in the mice, mAb 3P9 or mIgM were injected intraperitoneally at a dose of 5 μg/g/mouse twice a week for 30 days. In sTn^+ tumors, the volume and mean weight was significantly decreased when mice were treated with mAb 3P9 antibody compared with the mIgM-treated control. Although in sTn^- tumors, there was no statistically significant differences in tumor volume and weight between the mIgM control group and the group treated with mAb 3P9 (Figs. 4A, B). These results demonstrate the strong tumor-suppressing activity of mAb 3P9 and indicate its potential for clinical application.

We also detected the apoptotic cells in tumor entities treated with mAb 3P9 or mIgM by TUNEL (Fig. 4C). Results showed that in sTn^+ tumor entities, the number of apoptotic cells in mAb 3P9-treated group was significantly higher than that in mIgM-treated control group (Fig. 4D). These data indicate that mAb 3P9 suppresses tumor growth mainly through inducing cancer cell apoptosis in vivo.

mAb 3P9 Mediates a Strong CDC Reaction in LSC^{sTn+} Cells

Fragment crystallizable (Fc)-dependent antibody functions, such as antibody-dependent cytotoxicity, complement-dependent cytotoxicity (CDC), and a long half-life, have been suggested as important clinical mechanisms of therapeutic antibodies. To study whether mAb 3P9 can inhibit the growth of human xenograft tumors in nude mice by CDC reaction, we performed this assay on LSC^{sTn+} cells and LSB^{sTn-} cells. As shown in Figure 5, mAb 3P9 induced significant LSC^{sTn+} cells lysis in the presence of mouse complement in a dose-dependent manner and a 70% lysis effect was observed when the concentration of mAb

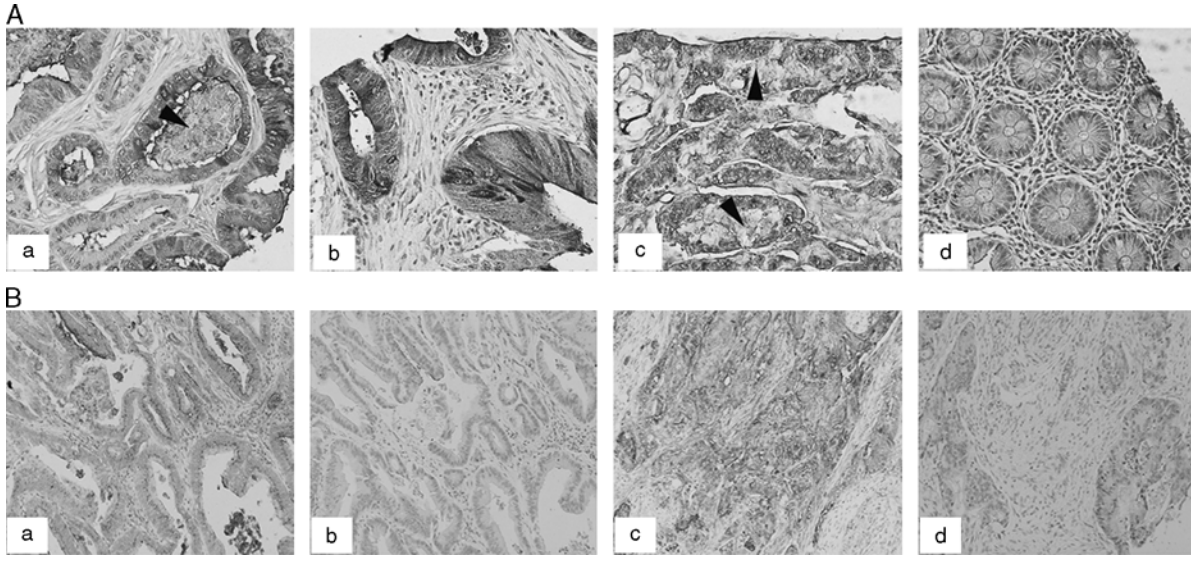

FIGURE 2. A, sTn expression in colorectal carcinomas detected by monoclonal antibody (mAb) 3P9. a, Colonic adenocarcinomal (#163); (b) colonic adenocarcinoma II (#186); (c) colonic adenocarcinoma III (#211); (d) normal colonic tissue (#239). B, Comparison the immunostaining pattern of mAb 3P9 and B72.3. a and b, Same colonic carcinoma samples, (c) and (d) were another the same colonic carcinoma samples. a and c, Immunostained by mAb 3P9, (b) and (d) were immunostained by mAb B72.3. Arrows indicated the expression inside the glandular lumens.

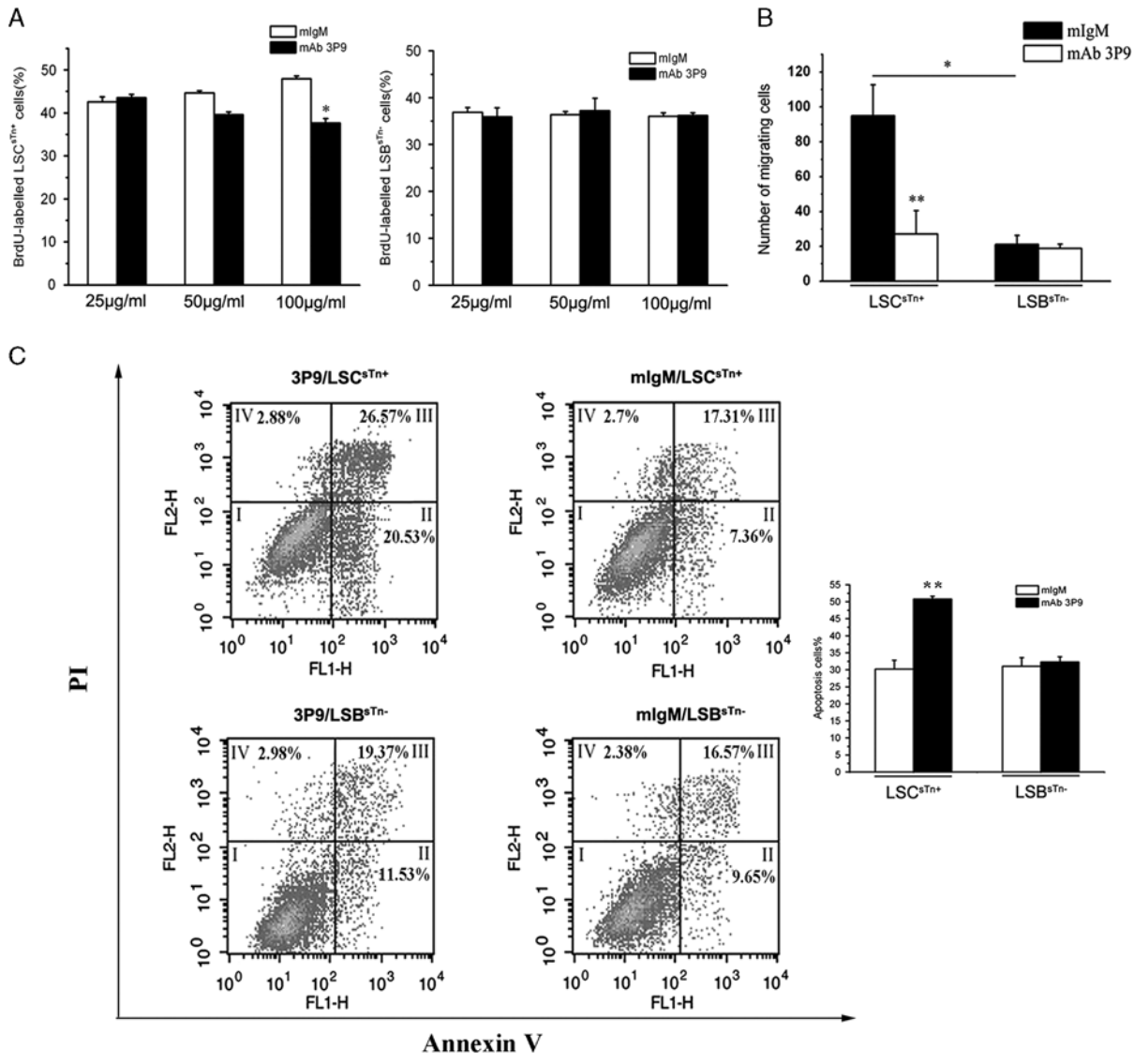

FIGURE 3. Monoclonal antibody (mAb) 3P9 inhibited proliferation, migration, and induced apoptosis of LSC^{sTn+} cells. BrdU incorporation assays were performed in the presence of different amount of mAb 3P9 or mIgM (A) and transwell assays were performed in the presence of 100 μg/mL mAb 3P9 or mIgM (B). Apoptosis assays using Annexin V-PI double staining were applied to LSC^{sTn+} cells treated with mAb 3P9 or mIgM (C); and the same assay were performed on LSB^{sTn-} cells (D). Phase II of Annexin V^+/PI^- cells represented apoptotic cells in early stage and phase IV of Annexin V^+/PI^+ cells represented apoptotic cells in late stage. $^*P<0.05$; $^{**}P<0.01$.

3P9 reached 50 μg/mL. Under the same conditions, however, mAb 3P9 did not induce cytotoxicity of LSB^{sTn-} cells (Fig. 5). These results indicate that the mAb 3P9 can activate mouse complement and induce a very efficient killing of human sTn^+ tumor cells.

DISCUSSION

In this study, we generated a novel mAb 3P9 (IgM/κ), whose epitope was a disaccharide structure, sTn, but not a part of protein. So we detected whether the SW1116 cells, as the immunogen, expressed sTn using commercial anti-sTn antibody B72.3 and found just 40% cells were sTn^+ (see Figure, Supplemental Digital Content 2, http://links.lww.com/JIT/A254). Moreover, we screened other 6 colorectal cancer cell lines, such as SW480, HT-29, HCT-15, colo320, LoVo, and LS174T, and found that none of them expressed sTn. This may be, as many report described, due to that this tumor-associated carbohydrate antigen was lost or changed in the in vitro culture after several passages, as many studies demonstrated that the carbohydrate antigens were closely related with tumor microenvironment.[29,30] Therefore, to study the effect of mAb 3P9 on cell level, we chose the established LSC^{sTn+} and LSB^{sTn-} cells that are reliable and well-known cell model for the study of sTn antigen[26,31,32] because these 2 cell lines have the same total protein electrophoretic profiles but carry markedly different oligosaccharide structures on their mucin. The mucin from LSC^{sTn+} cells has only the Tn and sTn structures, whereas LSB^{sTn-} cell mucin lacks these

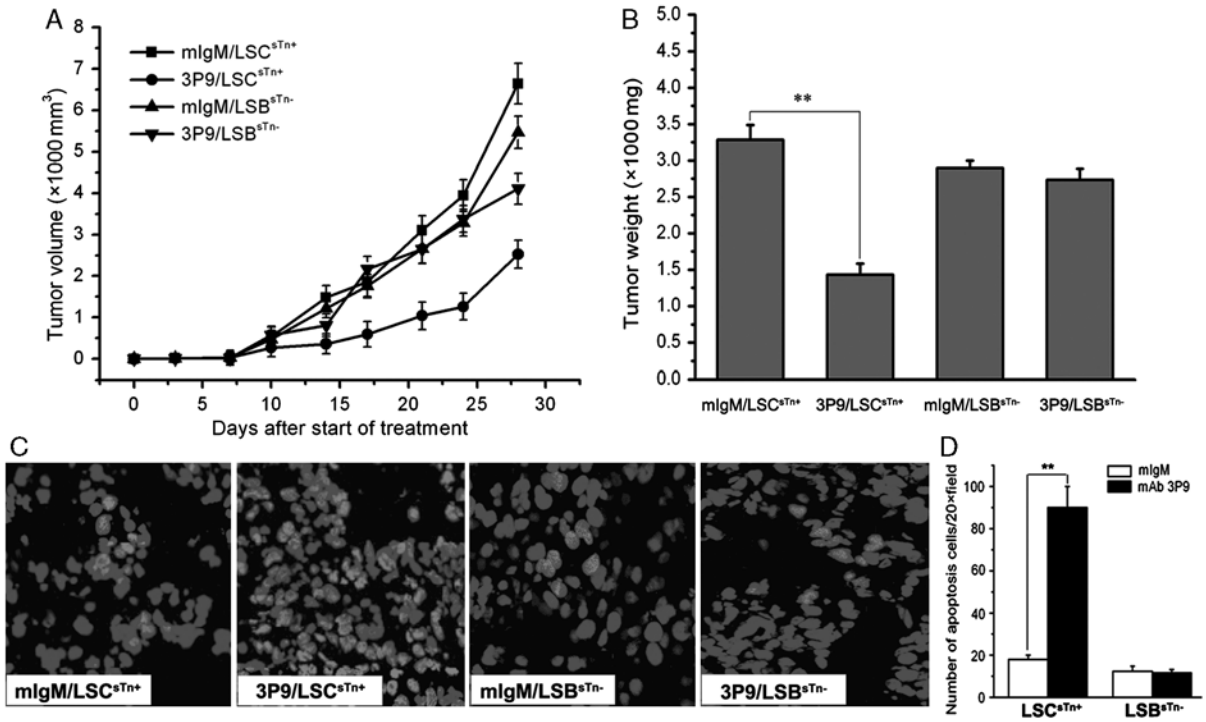

FIGURE 4. Monoclonal antibody (mAb) 3P9 inhibited tumor growth and induced apoptosis of tumor cells in vivo. LSC^{sTn+} and LSB^{sTn-} cells were injected subcutaneously into nude mice respectively. After forming tumor, mice were treated with mAb 3P9 or mIgM as a control for the times indicated. Tumors were then taken out for measuring the size, calculating the volume (A) and measuring the weight (B). Sections from a mAb 3P9-treated or mIgM-treated human colorectal adenocarcinoma were used for in situ cell apoptosis detection by TUNEL (C), in which the red dots indicated the apoptotic cells. The numbers of apoptotic cells were counted from 3 sections within ×20 fields and the average values of 5 high-power fields at ×20 fields per section were shown (D). $^{**}P<0.01$.

simple structures but carries more complex oligosaccharides.[33] We examined the LSC^{sTn+} cells using B72.3 and found they did express strong sTn (see Figure, Supplemental Digital Content 2, http://links.lww.com/JIT/A254). In addition, the expression of sTn is reported to be associated with the membrane-bound mucin MUC1 (episialin),[34] but may also be carried by a variety of *O*-glycoproteins which belong to the mucins family. Here, we proved that sTn is identified by mAb 3P9 mainly located on muc1 protein in colon cancer cells by immunoprecipitation and Western blotting.

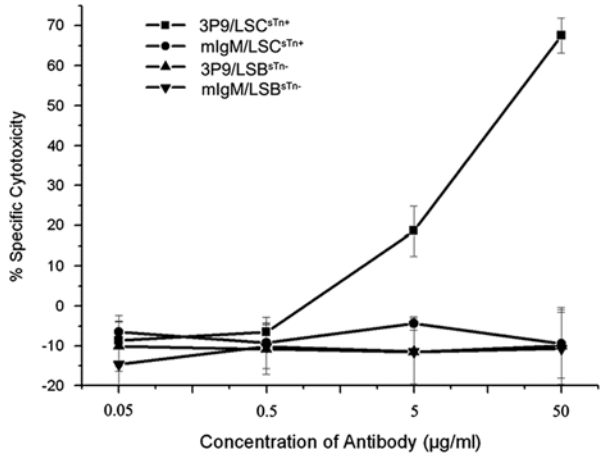

FIGURE 5. Monoclonal antibody (mAb) 3P9 raised strong complement-dependent cytotoxicity reaction in LSC^{sTn+} cells. LSC^{sTn+} cells were incubated with different amounts of mAb 3P9 or mIgM and the same assay were performed on LSB^{sTn-} cells as a control.

The sTn has been regarded as a specific and sensitive diagnostic marker for adenocarcinomas both in vivo and in vitro.[35,36] Here, to determine the potential application of the novel anti-sTn mAb 3P9 in clinical diagnosis, we assessed mAb 3P9 in immunohistochemical test using 737 tissues. The results in Table 1 showed that mAb 3P9-stained epithelial carcinomas but not non-epithelium–derived neoplasia, which was consistent with previous reports of sTn expression profiling.[37] The sensitivity of mAb 3P9 was the highest (84%) in colorectal carcinomas among 17 different tumors tested, and there was weak or almost no immunostaining in normal colon tissues (Table 1). Moreover, when compared with the immunostaining pattern of mAb 3P9 with widely used anti-sTn antibody B72.3 in colorectal cancer, mAb 3P9 had more advantages in general. The sensitivity, positive predictive value and negative predictive value of mAb 3P9 were higher than B72.3 (Table 2) and the staining of mAb 3P9 was much more pronounced (Fig. 1B). This may be due to the difference of antibody isotype, so their avidity is likely to be different. Moreover, the secondary antibody against these 2 antibodies in immunohistochemistry is different. Anyhow, these results suggested

that mAb 3P9 could be used as a diagnostic aid in clinical pathology of colorectal carcinomas. Furthermore, our results showed that mAb 3P9 can detect sTn shed from the cells into the glandular lumen, so we evaluated the use of mAb 3P9 for detecting serum sTn, which is as designated CA72-4, a widely used clinical tumor marker in serological diagnosis (data not shown). However, the specificity and sensitivity of mAb 3P9 for serological diagnosis should be examined thoroughly in future studies.

sTn level correlates with premalignant and malignant progression in the gastrointestinal tract,[38] suggesting that ectopic expression of sTn antigen may reflect the abnormal biological behavior of cancer cells. Pinho et al[39] have reported that the overexpression of sTn in gastric cell line, MKN45, was able to modulate a malignant phenotype, including giving rise to more aggressive cell behavior such as decreased cell-cell aggregation and increased extracellular matrix adhesion, migration, and invasiveness. Julien et al[17] also reported that sTn expression and concomitant changes in the overall *O*-glycan profiles induced a decrease of adhesion and an increase of migration of breast cancer cell line MDA-MB-231. Therefore, the suppression of sTn^+ colorectal cancer cell migration by mAb 3P9 shown in vitro (Fig. 3B) can be attributed to its effect of blocking sTn-mediated cell adhesion and movements.

So far, many anti-sTn antibodies such as B72.3, TKH2, CC49, and so on, have appeared. However, most of them are used in diagnosis, tumor imaging, or radioimmunotherapy and none has been reported to directly inhibit tumor growth. Here we found, for the first time, that mAb 3P9, as a novel anti-sTn antibody, could directly inhibit tumor growth. As we understand, many antibodies, although they target the same antigen, have different binding pattern and different function. This might be because of different antibodies that have different epitope and/or isotype with different effect. For instance, (1) among the numbers of anti-CD146 antibodies, only mAb AA98 showed antitumor activity, and the others just function as binder[40,41]; (2) although there are several antibodies targeting carbohydrate antigen TF, only mAbJAA-F11 has therapeutic effect.[42] In addition, recently Burchell's group showed that the mice immunized with synthetic sTn vaccine have developed a protective immune response.[43] All of these inspire us that generation of effective anti-sTn antibody is promising and it is reasonable that mAb 3P9 have different effect on tumor cells from other anti-sTn antibodies, such as B72.3 and CC49.

In this study, we demonstrate that the administration of mAb 3P9 can significantly inhibit sTn^+ tumor growth in a xenograft model. The strong CDC reaction mediated by mAb 3P9 might be an important mechanism of its efficient tumor-killing function. In this respect, mAb 3P9 has advantages over IgG because the IgM isotype is a very good complement activator. Furthermore, no allotypic differences have been described in the IgM isotype, in contrast to IgG antibodies, whose influence on antibody immunogenicity is very low. In addition, the high molecular weight of IgM reduces its ability to enter normal tissues, and therefore reduces its side effects.

We are now working on the 3P9 humanization by translated murine IgM to human IgG3 isotype that could activate complement and FcgR-mediated functions more effectively than any other subclass, such as IgG1, IgG2, and IgG4.[44–46] The function of the humanized 3P9 will be studied in the next preclinical trial.

To further explore this antitumor mechanism, we surprisingly discovered that mAb 3P9, in addition to inhibiting cell proliferation and migration, can induce the apoptosis of sTn^+ tumor cells both in vivo and in vitro. As the exact roles of sTn in cell signaling and apoptosis still remain largely unknown, it will be interesting to clarify the mechanism of how mAb 3P9 induces cellular apoptosis through binding to sTn. On the basis of the previous reports, we speculate several other possibilities of mAb 3P9-initiated tumor cell death. First, it has been demonstrated that sTn^+ mucins may inhibit the cytotoxicity of natural killer cells.[18] Moreover, siglecs family proteins, including CD33-related siglecs and CD22-related siglecs, expressed in innate immune cells, serve as inhibitory receptor and could initiate inhibitory signaling, which is triggered by the interaction with sialylated ligands.[47] Therefore, mAb 3P9 might block the sTn-mediated immune cell repression and enhance tumor immune response. Second, the binding of anti-sTn autoantibody to sTn-bearing protein could enhance vascular endothelial growth factor secretion by tumor-infiltrating macrophages, improving angiogenesis in tumor.[48] This raised the possibility that mAb 3P9 can disrupt the interaction between anti-sTn autoantibody and sTn^+ protein and thus abrogate tumor angiogenesis, leading to cancer cell apoptosis. Although these mechanisms have not been confirmed experimentally, its striking ability of inducing tumor cell death makes mAb 3P9 a promising candidate for cancer therapeutics.

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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